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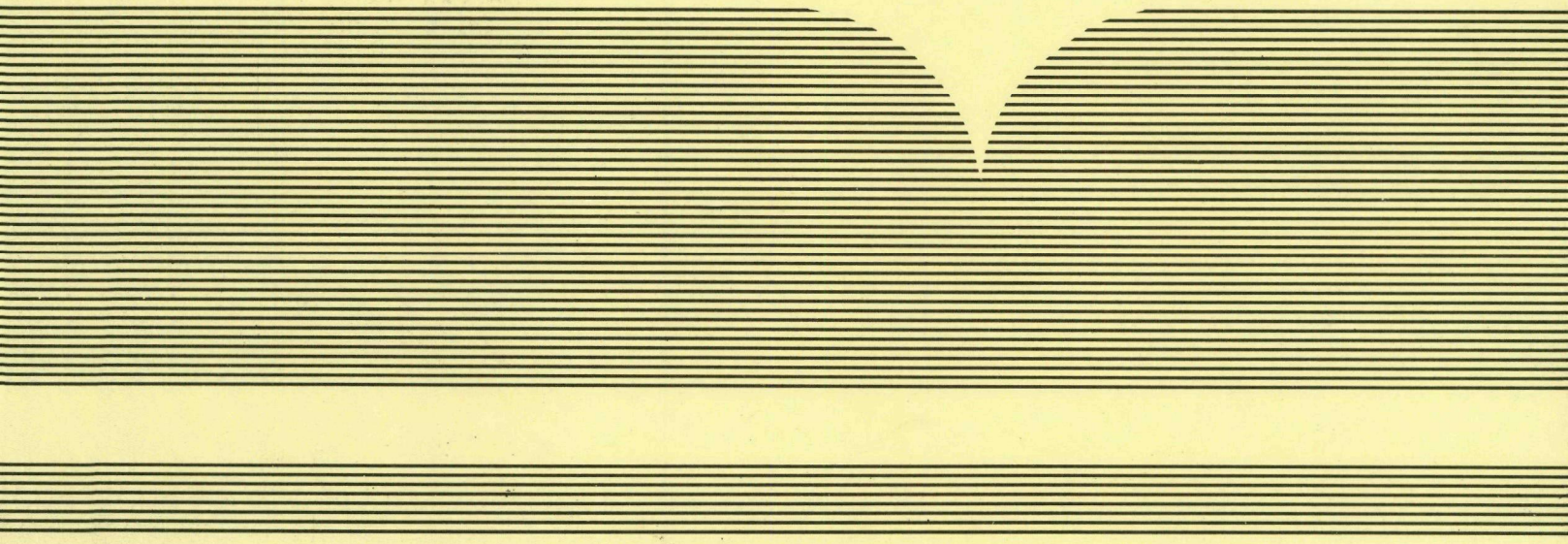

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ACUTE EXPOSURE
DERMAL TOXICITY

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

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These documents constitute a set of 2 new health effects test guidelines as well as 3 existing health effects test guidelines that had been subjected to major revision. These will be added to (new guidelines) and serve as substitutes for (revised guidelines) the health effects test guidelines published by NTIS in October, 1982.

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I. PURPOSE

In the assessment and evaluation of the toxic characteristics of a substance, determination of acute dermal toxicity is usually an initial step. It provides information on health hazards likely to arise from short-term exposure by the dermal route. Data from an acute study may serve as a basis for classification and labeling. It is traditionally a step in establishing a dosage regimen in subchronic and other studies and may provide initial information on dermal absorption and the mode of toxic action of a substance. An evaluation of acute toxicity data should include the relationship, if any, between the animals' exposure to the test substance and the incidence and severity of all abnormalities, including behavioral and clinical abnormalities, the reversibility of observed abnormalities, gross lesions, body weight changes, effects on mortality, and any other toxic effects.

II. DEFINITIONS

A. Acute dermal toxicity is the adverse effects occurring within a short time of dermal application of a single dose of a substance or multiple doses given within 24 hours.

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

C. Dose-response is the relationship between the dose and the proportion of a population sample showing a defined effect.

III. APPROACHES TO THE DETERMINATION OF ACUTE TOXICITY

At present, the evaluation of chemicals for acute toxicity is necessary for the protection of public health and the environment. When animal testing is required for this purpose, this testing should be done in ways that minimize numbers of animals used and that take full account of their welfare.

EPA recommends the following means to reduce the number of animals used to evaluate acute effects of chemical exposure while preserving its ability to make reasonable judgments about safety:

- Attempt the use of existing data on structurally related chemicals.

- If data for calculating an LD_{50} are needed, perform an acute toxicity study whereby the value of the data derived from the investment of animal lives is enhanced. EPA does not encourage the use of animals solely for the calculation of an LD_{50}.
- Use methods that minimize the numbers of animals in the test.

The following provides an expanded discussion of these principles and their application to the evaluation of acute toxicity of chemicals.

Using Data From Structurally Related Chemicals. In order to minimize the need for animal testing, the Agency encourages the review of existing acute toxicity information on chemical substances that are structurally related to the agent under investigation. In certain cases, one may be able to glean enough information from these surrogate chemicals to make preliminary safety evaluations that may obviate the need for further animal testing.

"Limit" Test. When acute lethality data are desirable, EPA's test guideline encourages the use of methods that minimize the requirement for animals, sometimes by a factor of 90% as compared to the more traditional LD_{50} test. In the "limit" test, a single group of animals receives a large dose (2 g/kg body weight) of the agent by the dermal route. If no lethality is demonstrated, no further testing for acute dermal toxicity is pursued.

Estimation of Lethal Dose. For those substances demonstrating lethality in a "limit" test or for substances for which there are data on structurally related chemicals that indicate potential acute toxicity below 2 g/kg, the Agency can use estimates of the dose associated with some level of acute lethality that are derived from a study comprising three doses as described in this guideline. With such an approach, use of greater numbers of animals or increased numbers of dose levels are not necessary.

Multiple Endpoint Evaluation. The Agency stresses the simultaneous monitoring of several endpoints of toxicity in animals in a single acute study including sublethal effects as well as lethality. Dosed animals are observed for abnormal behavioral manifestations such as increased salivation or muscular incoordination, in addition to the recovery from these effects during the observation period. Both dead and surviving animals are autopsied to evaluate gross anatomical evidence of organ toxicity. In selected cases, additional testing may be justified to characterize better the kinds of abnormalities that have been found in the organs of the autopsied animals.

These sound, scientific practices represent some of the means which maximize the utility of the data obtained from a limited number of test animals to achieve a balance between protecting humans and the environment, and the welfare and utilization of laboratory animals. When animal testing is, nonetheless, determined to be necessary to achieve this balance, the following test method incorporates the principles discussed above.

IV. PRINCIPLE OF THE TEST METHOD

When conducting acute toxicity testing, exposure by dermal application is recommended for chemicals where exposure of humans by the dermal route is likely. A single exposure and a 14-day observation period are used. The test substance is applied dermally in graduated doses to several groups of experimental animals, one dose being used per group. For the limit test, however, only one group is tested at a single (high) dose. Subsequent to exposure, systematic daily observations of effects and deaths are made. Based on the results of cage-side observations or gross necropsy, the tester may decide to initiate histopathological review of certain organs, and/or additional clinical laboratory tests. Animals that die during the test are necropsied, and at the conclusion of the observation period, the surviving animals are sacrificed and are necropsied.

V. LIMIT TEST

If a test at a dose level of at least 2 g/kg body weight produces no compound-related mortality, then a study using three dose levels will not be necessary.

VI. TEST PROCEDURES

A. Animal selection

1. Species and strain

The rat, rabbit or guinea pig may be used. The albino rabbit is preferred because of its size, skin permeability and extensive data base. Commonly used laboratory strains should be employed. If a species other than the three indicated above is used, the tester should provide justification and reasoning for its selection.

2. Age

Young adult animals should be used. The following weight ranges are suggested 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.

3. Sex

a. Equal numbers of animals of each sex with healthy intact skin should be used for each dose level.

b. The females should be nulliparous and non-pregnant.

4. Numbers Per Dose Group

At least 5 animals of each sex should be used at each dose level.

B. Control groups

A concurrent untreated control is not necessary. A vehicle control group should be run concurrently except when historical data are available to determine the acute toxicity of the vehicle.

C. Dose levels and dose selection

1. Three dose levels should be used and spaced appropriately to produce test groups with a range of toxic effects and mortality rates. The data should be sufficient to produce a dose- response curve and permit an acceptable estimation of the median lethal dose. Range finding studies using single animals may help to estimate the positioning of the dose groups so that no more than three dose levels will be necessary.

2. Vehicle

a. Where necessary, the test substance is dissolved or suspended in a suitable vehicle. It is recommended that wherever possible the use of an aqueous solution be considered first, followed by consideration of a solution in oil (e.g. corn oil) and then by possible solution in other vehicles. For non-aqueous vehicles the toxic characteristics of the vehicle should be known, and if not known should be determined before the test.

b. 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 skin. When a vehicle is used, the influence of the vehicle on penetration of skin by the test substance should be taken into account.

D. Exposure duration

The test substance should be administered over a period not exceeding 24 hours.

E. Observation period

The observation period should be at least 14 days. Although 14 days is recommended as a minimum period, the duration of observation should not be fixed rigidly. It should be determined by the toxic reactions, rate of onset and length of recovery period, and may thus be extended when considered necessary. The time at which signs of toxicity appear and disappear, their duration and the time to death are important, especially if there is a tendency for deaths to be delayed.

F. Preparation of animal skin

1. Shortly before testing, fur should 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. Care must be taken to avoid abrading the skin, which could alter its permeability.

2. 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.

G. Application of test substance

1. The test substance should 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 should be covered with as thin and uniform a film as possible.

2. The test substance should be held in contact with the skin with a porous gauze dressing and non-irritating tape throughout a 24-hour exposure period. 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. Restrainers may be used to prevent the ingestion of the test substance, but complete immobilization is not a recommended method.

3. At the end of the exposure period, residual test substance should be removed where practicable using water or an appropriate solvent.

H. Observation of animals

1. A careful clinical examination should be made at least once each day.

2. Additional observations should be made daily, especially in the early days of the study. Appropriate actions should be taken to minimize loss of animals to the study (e.g. necropsy or refrigeration of those animals found dead and isolation of weak or moribund animals).

3. Cage-side observations should include, at the least, evaluations of the skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavior pattern. Particular attention should be directed to observation of tremors, convulsions, lethargy, other signs of central nervous system depression, salivation and diarrhea.

4. Individual weights of animals should be determined shortly before the test substance is applied, weekly thereafter, and at death. Changes in weights should be calculated and recorded when survival exceeds one day.

5. The time of death should be recorded as precisely as possible.

6. At the end of the test, surviving animals should be weighed and sacrificed.

I. Gross pathology

A gross necropsy should be performed on all animals under test. All gross pathology changes should be recorded.

J. Additional evaluations

In animals surviving 24 hours or more, clinical chemistry tests or microscopic examination of organs showing evidence of gross pathology should be considered because they may yield additional useful information on the induced toxic effects.

VII. DATA AND REPORTING

A. Treatment of results

Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, body weights, time of death of individual animals at different dose levels, number of animals displaying other signs of toxicity, description of toxic effects and necropsy findings.

B. Evaluation of results

An evaluation of results should include the relationship, if any, between the dose of the test substance and the incidence, severity and reversibility of all abnormalities, including behavioral and clinical effects, gross lesions, body weight changes, effects on mortality, and any other toxicological effects.

C. Test report

In addition to the reporting requirements as specified in the EPA Good Laboratory Practice Standards [Subpart J, Part 792, Chapter I of Title 40, Code of Federal Regulations] the following specific information should be reported:

1. Tabulation of response data by sex and dose level (i.e. number of animals exposed; number of animals showing signs of toxicity; number of animals dying);

2. Dose-response curves for mortality and other toxic effects (when permitted by the method of determination);

3. Description of toxic effects including their time of onset, duration, reversibility, and relationship to dose;

4. Time of death after dosing;

5. Body weight data;

6. Gross pathology findings; and

7. Histopathology findings and any additional clinical chemistry evaluations, if performed.

VIII. REFERENCES

The following references may be helpful in developing acceptable protocols, and provide a background of information on which this section is based. This should not be considered the only source of information on test performance, however.

1. Bliss, C.I. 1938. The determination of the dosage mortality curve from small numbers. Quarterly Journal Pharm. Pharmacology. 11:192-216.

2. Finney, D.G. 1971. Probit Analysis. Chapter 3--Estimation of the median effective dose, Chapter 4--Maximum likelihood estimation. 3rd Edition. London: Cambridge University Press. 60 pp.

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5. NAS. 1977. National Academy of Sciences. Principles and procedures for evaluating the toxicity of household substances. Washington, D.C.: 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. 130 pp.

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7. Weil, C.S. 1952. Tables for convenient calculation of median effective dose and instructions in their use. Biometrics. 8:249-263.

8. WHO. 1978. World Health Organization. Principles and Methods for Evaluating the Toxicity of Chemicals. Part I. Environmental Health Criteria 6. Geneva: World Health Organization. 272 pp.

ACUTE EXPOSURE
INHALATION TOXICITY

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

I. PURPOSE

In the assessment and evaluation of the toxic characteristics of a substance that may be inhaled, determination of acute toxicity is usually an initial step. It provides information on health hazards likely to arise from short-term exposure by the inhalation route. Data from an acute study may serve as a basis for classification and labeling. It is traditionally a step in establishing a dosage regimen in subchronic and other studies and may provide initial information on the mode of toxic action of a substance. An evaluation of acute toxicity data should include the relationship, if any, between the animals' exposure to the test substance and the incidence and severity of all abnormalities, including behavioral and clinical abnormalities, the reversibility of observed abnormalities, gross lesions, body weight changes, effects on mortality, and any other toxic effects.

II. DEFINITIONS

A. Acute inhalation toxicity is the adverse effects caused by a substance following a single uninterrupted exposure by inhalation over a short period of time (24 hours or less) to a substance capable of being inhaled.

B. 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.

C. 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 persent 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 geometric standard deviation is used to statistically describe the particle size distribution of any aerosol based on the weight and size of the particles.

D. 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, the inhalable diameter is considered as 15 micrometers or less.

E. Dose response is the relationship between the dose (or concentration) and the proportion of a population sample showing a defined effect.

III. APPROACHES TO THE DETERMINATION OF ACUTE TOXICITY

At present, the evaluation of chemicals for acute toxicity is necessary for the protection of public health and the environment. When animal testing is required for this purpose, this testing should be done in ways that minimize numbers of animals used and that take full account of their welfare.

EPA recommends the following means to reduce the number of animals used to evaluate acute effects of chemicals exposure while preserving its ability to make reasonable judgements about safety:

- Attempt the use of existing data on structurally related chemicals.
- If data for calculating an LC_{50} are needed, perform an acute toxicity study whereby the value of the data derived from the investment of animal lives is enhanced. EPA does not encourage the use of animals solely for the calculation of an LC_{50}.
- Use methods that minimize the numbers of animals in the test.

The following provides an expanded discussion of these principles and their application to the evaluation of acute toxicity of chemicals.

Using Data From Structurally Related Chemicals. In order to minimize the need for animal testing, the Agency encourages the review of existing acute toxicity information on chemical substances that are structurally related to the agent under investigation. In certain cases one may be able to glean enough information from these surrogate chemicals to make preliminary safety evaluations that may obviate the need for further animal testing.

"Limit" Test. If a test at an exposure of 5 mg/l (actual concentration of respirable substances) for 4 hours or, where this is not possible due to physical or chemical properties of the test substance, the maximum attainable concentration, using the procedures described for this study, produces no compound-related mortality, then a full study using three dose levels will not be necessary.

Estimation of Lethal Dose. For those substances demonstrating lethality in a "limit" test or for substances for which there are data on structurally related chemicals that indicate potential acute toxicity below 5 mg/l, the Agency can use estimates of the dose associated with some level of acute lethality that are derived from a study comprising three doses as described in this guideline. With such an approach, use of greater numbers of animals or increased numbers of dose levels are not necessary.

Multiple Endpoint Evaluation. The Agency stresses the simultaneous monitoring of several endpoints of toxicity in animals in a single acute study including sublethal effects as well as lethality. Dosed animals are observed for abnormal behavioral manifestations such as increased salivation or muscular incoordination, in addition to the recovery from these effects during the observation period. Both dead and surviving animals are autopsied to evaluate gross anatomical evidence of organ toxicity. In selected cases, additional testing may be justified to characterize better the kinds of abnormalities that have been found in the organs of the autopsied animals.

These sound, scientific practices represent some of the means which maximize the utility of the data obtained from a limited number of test animals to achieve a balance between protecting humans and the environment, and the welfare and utilization of laboratory animals. When animal testing is, nonetheless, determined to be necessary to achieve this balance, the following test method incorporates the principles discussed above.

IV. PRINCIPLE OF THE TEST METHOD

When conducting acute toxicity testing, exposure by inhalation is recommended for chemicals where exposure of humans by inhalation is likely. A single exposure and a 14-day observation period are used. The test substance is administered in graduated doses to several groups of experimental animals, one dose being used per group. For the limit test, however, only one group is tested at a single (high) dose. Subsequent to exposure, systematic daily observations of effects and deaths are made. Based on the results of cage-side observations or gross necropsy, the tester may decide to initiate histopathological review of certain organs, and/or additional clinical laboratory tests. Animals that die during the test are necropsied, and at the conclusion of the observation period, the surviving animals are sacrificed and are necropsied.

V. LIMIT TEST

If a test at a dose level of at least 5 mg/l (actual concentration of respirable substances) for 4 hours or, where this is not possible due to physical or chemical properties of the test substance, the maximum attainable concentration, produces no compound-related mortality, then a full study using three dose levels will not be necessary.

VI. TEST PROCEDURES

A. Animal selection

1. Species and strain

Although several mammalian test species may be used, the rat is the preferred species. Commonly used laboratory strains should be employed. If another mammalian species is employed, the tester should provide justification and reasoning for its selection.

2. Age

Young adult animals should be used. The weight variation of animals used in a test should not exceed ± 20 percent of the mean weight for each sex.

3. Sex

a. Equal numbers of animals of each sex should be used for each dose level.

b. The females should be nulliparous and non-pregnant.

4. Numbers Per Dose Group

At least 5 animals of each sex should be used at each dose level.

B. Control groups

A concurrent untreated control is not necessary. Where a vehicle is used to help generate an appropriate concentration of the substance in the atmosphere, a vehicle control group should be used when historical data are not available or adequate to determine the acute toxicity of the vehicle.

C. Dose levels and dose selection

1. Three exposure concentrations should be used and spaced appropriately to produce test groups with a range of toxic effects and mortality rates. The data should be sufficient to produce a dose-response curve and permit an acceptable estimation of the median lethal concentration. Range finding studies using single animals may help to estimate the positioning of the test groups so that no more than three doses will be necessary.

2. Where necessary, a suitable vehicle may be added to the test substance to help generate an appropriate concentration of the test substance in the atmosphere. If a vehicle or diluent is needed, ideally it should not elicit important toxic effects itself or substantially alter the chemical or toxicological properties of the test substance.

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

4. To establish suitable exposure concentrations, a trial test is recommended.

D. Exposure duration

The duration of exposure should be at least 4 hours after equilibration of the chamber concentrations.

E. Observation period

The observation period should be at least 14 days. Although 14 days is recommended as a minimum period, the duration of observation should not be fixed rigidly. It should be determined by the toxic reactions, rate of onset and length of recovery period, and may thus be extended when considered necessary. The time at which signs of toxicity appear and disappear, their duration and the time to death are important, especially if there is a tendency for deaths to be delayed.

F. Inhalation Exposure

1. 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.

2. 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 a slight negative pressure inside the chamber will prevent leakage of the test substance into the surrounding area.

3. 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 on aerosols, use of water vehicle) this may not be practicable.

G. Physical measurements

Measurements or monitoring should be made of the following:

1. The rate of air flow should be monitored continuously, but should be recorded at least every 30 minutes.

2. The actual concentrations of the test substance should be measured in the breathing zone. During the exposure period the actual concentration of the test substance should be held as constant as practicable. Continuous monitoring is desirable. Measurement of actual concentrations should be recorded near the beginning, middle, and end of the exposure period.

3. 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 made as often as necessary to determine the consistency of particle size distribution and homogeneity of the exposure stream.

4. Temperaturee and humidity should be monitored continuously but should be recorded at least every 30 minutes.

H. Food and water during exposure period

Food should be withheld during exposure. Water may also be withheld in certain cases.

I. Observation of animals

1. A careful clinical examination should be made at least once each day.

2. Additional observations should be made daily, especially in the early days of the study. Appropriate actions should be taken to minimize loss of animals to the study (e.g., necropsy or refrigeration of those animals found dead and isolation of weak or moribund animals).

3. Cage-side observations should include, at the least, evaluations of the skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavior pattern. Particular attention should be directed to observation of tremors, convulsions, lethargy, other signs of central nervous system depression, salivation and diarrhea.

4. Individual weights of animals should be determined shortly before the test substance is administered, weekly thereafter, and at death. Changes in weights should be calculated and recorded when survival exceeds one day.

5. The time of death should be recorded as precisely as possible.

6. At the end of the test, surviving animals should be weighed and sacrificed.

J. Gross pathology

A gross necropsy should be performed on all animals under test, with particular reference to any changes in the respiratory tract. Where there are significant signs of toxicity indicating the possible involvement of other organs, these should be examined. All gross pathology changes should be recorded.

K. Additional evaluations

In animals surviving 24 hours or more, clinical chemistry tests or microscopic examination of organs showing evidence of gross pathology should be considered because they may yield additional useful information on the nature of the induced toxic effects.

VII. DATA AND REPORTING

A. Treatment of results

Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, body weights, time of death of individual animals at different dose levels, number of animals displaying other signs of toxicity, description of toxic effects and necropsy findings.

B. Evaluation of Results

An evaluation of results should include the relationship, if any, between the concentration of the test substance and the incidence, severity and reversibility of all abnormalities, including behavioral and clinical effects, gross lesions, body weight changes, effects on mortality, and any other toxicological effects.

C. Test report

In addition to the reporting requirements as specified in the EPA Good Laboratory Practice Standards [Subpart J, Part 792, Chapter I of Title 40, Code of Federal Regulations] the following specific information should be reported:

1. 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 the animals in a test chamber.

   b. The equipment of measuring temperature, humidity, and particulate aerosol concentrations and size be described.

2. Exposure data

   These should be tabulated and presented with mean values and a measure of variability (e.g. standard deviation) and should 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; and

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

3. Animal data

   a. Tabulation of response data by sex and exposure level (i.e. number of animals exposed, number of animals showing signs of toxicity, number of animals dying);

   b. Dose-response curves for mortality and other toxic effects (when permitted by the method of determination);

   c. Description of toxic effects including their time of onset, duration, reversibility, and relationship to dose;

d. Time of death during or following exposure;

e. Body weight data;

f. Gross pathology findings; and

g. Histopathology findings and any additional clinical chemistry evaluation, if performed.

VIII. REFERENCES

The following references may be helpful in developing acceptable protocols, and provide a background of information on which this section is based. This should not be considered the only source of information on test performance, however.

1. Bliss, C.I. 1938. The determination of the dosage mortality curve from small numbers. Quarterly Journal Pharm. Pharmacology. 11:192-216.

2. Finney, D.G. 1971. Probit Analysis. Chapter 3--Estimation of the median effective dose, Chapter 4--Maximum likelihood estimation. 3rd Edition. London: Cambridge University Press. 60 pp.

3. Litchfield, J.T., Jr., Wilcoxon, F. 1949. A simplified method of evaluating dose-effect experiments. Journal of Pharmacology and Experimental Therapeutics. 96:99-115.

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7. Thompson, W.R. 1947. Use of moving averages and interpolation to estimate median effective dose. Bacteriological Review. 11:115-145.

8. Weil, C.S. 1952. Tables for convenient calculation of median effective dose and instructions in their use. Biometrics. 8:249-263.

9. WHO. 1979. World Health Organization. Principles and Methods for Evaluating the Toxicity of Chemicals. Part I. Environment Health Criteria 6. Geneva: World Health Organization. 272 pp.

ACUTE EXPOSURE
ORAL TOXICITY

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

I. PURPOSE

In the assessment and evaluation of the toxic characteristics of a substance, determination of acute oral toxicity is usually an initial step. It provides information on health hazards likely to arise from short-term exposure by the oral route. Data from an acute study may serve as a basis for classification and labeling. It is traditionally a step in establishing a dosage regimen in subchronic and other studies and may provide initial information on the mode of toxic action of a substance. An evaluation of acute toxicity data should include the relationship, if any, between the animals' exposure to the test substance and the incidence and severity of all abnormalities, including behavioral and clinical abnormalities, the reversibility of observed abnormalities, gross lesions, body weight changes, effects on mortality, and any other toxic effects.

II. DEFINITIONS

A. Acute oral toxicity is the adverse effects occurring within a short time of oral administration of a single dose of a substance or multiple doses given within 24 hours.

B. 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).

C. Dose-response is the relationship between the dose and the proportion of a population sample showing a defined effect.

III. APPROACHES TO THE DETERMINATION OF ACUTE TOXICITY

At present, the evaluation of chemicals for acute toxicity is necessary for the protection of public health and the environment. When animal testing is required for this purpose, this testing should be done in ways that minimize numbers of animals used and that take full account of their welfare.

EPA recommends the following means to reduce the number of animals used to evaluate acute effects of chemical exposure while preserving its ability to make reasonable judgments about safety:

- Attempt the use of existing data on structurally related chemicals.

- If data for calculating an LD_{50} are needed, perform an acute toxicity study whereby the value of the data derived from the investment of animal lives is enhanced. EPA does not encourage the use of animals solely for the calculation of an LD_{50}.
- Use methods that minimize the numbers of animals in the test.

The following provides an expanded discussion of these principles and their application to the evaluation of acute toxicity of chemicals.

Using Data From Structurally Related Chemicals. In order to minimize the need for animal testing, the Agency encourages the review of existing acute toxicity information on chemical substances that are structurally related to the agent under investigation. In certain cases one may be able to glean enough information from these surrogate chemicals to make preliminary safety evaluations that may obviate the need for further animal testing.

"Limit" Test. When acute lethality data are desirable, EPA's test guideline encourages the use of methods that minimize the requirement for animals, sometimes by a factor of 90% as compared to the more traditional LD_{50} test. In the "limit" test, a single group of animals is given a large dose (5 g/kg body weight) of the agent. If no lethality is demonstrated, no further testing for acute oral toxicity is pursued.

Estimation of Lethal Dose. For those substances demonstrating lethality in a "limit" test or for substances for which there are data on structurally related chemicals that indicate potential acute toxicity below 5 g/kg the Agency can use estimates of the dose associated with some level of acute lethality that are derived from a study comprised of three doses as described in this guideline. With such an approach, use of greater numbers of animals or increased numbers of dose levels are not necessary.

Multiple Endpoint Evaluation. The Agency stresses the simultaneous monitoring of several endpoints of toxicity in animals in a single acute study including sublethal effects as well as lethality. Dosed animals are observed for abnormal behavioral manifestations such as increased salivation or muscular incoordination, in addition to the recovery from these effects during the observation period. Both dead and surviving animals are autopsied to evaluate gross anatomical evidence of organ toxicity. In selected cases, additional testing may be justified to characterize better the kinds of abnormalities that have been found in the organs of the autopsied animals.

These sound, scientific practices represent some of the means which maximize the utility of the data obtained from a limited number of test animals to achieve a balance between protecting humans and the environment, and the welfare and utilization of laboratory animals. When animal testing is, nonetheless, determined to be necessary to achieve this balance, the following test method incorporates the principles discussed above.

IV. PRINCIPLE OF THE TEST METHOD

When conducting acute toxicity testing, exposure by gavage is recommended for chemicals where exposure of humans by the oral route is likely. A single exposure and a 14-day observation period are used. The test substance is administered orally in graduated doses to several groups of experimental animals, one dose being used per group. For the limit test, however, only one group is tested at a single (high) dose. Subsequent to exposure, systematic daily observations of effects and deaths are made. Based on the results of cage-side observations or gross necropsy, the tester may decide to initiate histopathological review of certain organs, and/or additional clinical laboratory tests. Animals that die during the test are necropsied, and at the conclusion of the observation period, the surviving animals are sacrificed and are necropsied.

V. LIMIT TEST

If a test at a dose level of at least 5 g/kg body weight produces no compound-related mortality, then a study using three dose levels will not be necessary.

VI. TEST PROCEDURES

A. Animal selection

1. Species and strain

Although several mammalian test species may be used, the rat is the preferred species. Commonly used laboratory strains should be employed. If another species is used, the tester should provide justification and reasoning for its selection.

2. Age

Young adult animals should be used. The weight variation of animals used in a test should not exceed ± 20 percent of the mean weight for each sex.

3. Sex

a. Equal numbers of animals of each sex should be used for each dose level.

b. The females should be nulliparous and non-pregnant.

4. Numbers Per Dose Group

At least 5 animals of each sex should be used at each dose level.

B. Control groups

A concurrent untreated control is not necessary. A vehicle control group should be run concurrently except when historical data are available[4] to determine the acute toxicity of the vehicle.

C. Dose levels and dose selection

1. Three dose levels should be used and spaced appropriately to produce test groups with a range of toxic effects and mortality rates. The data should be sufficient to produce a dose- response curve and permit an acceptable estimation of the median lethal dose. Range finding studies using single animals may help to estimate the positioning of the dose groups so that no more than three dose levels will be necessary.

2. Vehicle

Where necessary, the test substance is dissolved or suspended in a suitable vehicle. It is recommended that wherever possible the use of an aqueous solution be considered first, followed by consideration of a solution in oil (e.g. corn oil) and then by possible solution in other vehicles. For non-aqueous vehicles the toxic characteristics of the vehicle should be known, and if not known should be determined before the test.

3. Volume

The maximum volume of liquid that can be administered at one time depends on the size of the test animal. In rodents, the volume should not exceed 1 ml/100 g body weight, except when an aqueous solution is used where 2 ml/100 g may be administered. Variability in test volume should be minimized by adjusting the concentration to ensure a constant volume at all dose levels.

D. Exposure duration

The test substance should be administered over a period not exceeding 24 hours.

E. Observation period

The observation period should be at least 14 days. Although 14 days is recommended as a minimum period, the duration of observation should not be fixed rigidly. It should be determined by the toxic reactions, rate of onset and length of recovery period, and may thus be extended when considered necessary. The time at which signs of toxicity appear and disappear, their duration and the time to death are important, especially if there is a tendency for deaths to be delayed.

F. Exposure

1. The test substance should be administered in a single dose by gavage, using a stomach tube or suitable intubation cannula.

2. Animals should be fasted prior to test substance administration. For the rat, food should be withheld overnight; for other rodents with higher metabolic rates a shorter period of fasting is appropriate.

3. After the substance has been administered, food may be withheld for an additional 3-4 hours.

4. If a single dose is not possible, the dose may be given in smaller fractions over a period not exceeding 24 hours. Where a dose is administered in fractions, it may be necessary to provide the animals with food and water depending on the length of the dosing period.

G. Observation of animals

1. A careful clinical examination should be made at least once each day.

2. Additional observations should be made daily, especially in the early days of the study. Appropriate actions should be taken to minimize loss of animals to the study (e.g. necropsy or refrigeration of those animals found dead and isolation of weak or moribund animals).

3. Cage-side observations should include, at the least, evaluation of the skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavior pattern. Particular attention should be directed to observation of tremors, convulsions, lethargy, other signs of central nervous system depression, salivation and diarrhea.

4. Individual weights of animals should be determined shortly before the test substance is administered, weekly thereafter, and at death. Changes in weights should be calculated and recorded when survival exceeds one day.

5. The time of death should be recorded as precisely as possible.

6. At the end of the test, surviving animals should be weighed and sacrificed.

H. Gross pathology

A gross necropsy should be performed on all animals under test. All gross pathology changes should be recorded.

I. Additional evaluations

In animals surviving 24 hours or more, clinical chemistry tests or microscopic examination of organs showing evidence of gross pathology should be considered because they may yield additional useful information on the nature of the induced toxic effects.

VII. DATA AND REPORTING

A. Treatment of results

Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, body weights, time of death of individual animals at different dose levels, number of animals displaying other signs of toxicity, description of toxic effects and necropsy findings.

B. Evaluation of results

An evaluation of results should include the relationship, if any, between the dose of the test substance and the incidence, severity and reversibility of all abnormalities, including behavioral and clinical effects, gross lesions, body weight changes, effects on mortality, and any other toxicological effects.

C. Test report

In addition to the reporting requirements as specified in the EPA Good Laboratory Practice Standards [Subpart J, Part 792, Chapter I of Title 40, Code of Federal Regulations] the following specific information should be reported:

1. Tabulation of response data by sex and dose level (i.e. number of animals exposed; number of animals showing signs of toxicity; number of animals dying);

2. Dose-response curves for mortality and other toxic effects (when permitted by the method of determination);

3. Description of toxic effects, including their time of onset, duration, reversibility, and relationship to dose;

4. Time of death after dosing;

5. Body weight data;

6. Gross pathology findings; and

7. Histopathology findings and any additional clinical chemistry evaluations, if performed.

VIII. REFERENCES

The following references may be helpful in developing acceptable protocols, and provide a background of information on which this section is based. This should not be considered the only source of information on test performance, however.

1. Balazs, T. 1970. "Measurement of acute toxicity," in "Methods in Toxicology." Edited by G.E. Paget. Philadelphia: F.A. Davis Co. PP. 49-82.

2. Bliss, C.I. 1938. The determination of the dosage mortality curve from small numbers. Quarterly Journal Pharm. Pharmacology. 11:192-216.

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5. Litchfield, J.T., Jr., Wilcoxon, F. 1949. A simplified method of evaluating dose-effect experiments, Journal of Pharmacology and Experimental Therapeutics. 96:99-115.

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9. Weil, C.S. 1952. Tables for convenient calculation of median effective dose and instructions in their use. Biometrics. 8:249-263.

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October 1984

DEVELOPMENTAL TOXICITY STUDY

Office of Toxic Substances
Office of Pesticides and Toxic Substances
United States Environmental Protection Agency
Washington, D.C. 20460

I. 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.

II. DEFINITIONS

A. Developmental toxicity is the induction of adverse effects on the developing organism as a result of *in utero* exposure to an agent. It is a generic term which includes endpoints such as resorptions, structural abnormalities, growth retardation as well as functional and behavioral deficits.

B. 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).

C. 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).

III. PRINCIPLE OF THE TEST METHOD

The test substance is administered in graduated doses for at least that 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.

IV. 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.

V. TEST PROCEDURES

A. Animal selection

1. Species and strain

Testing should 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 should provide

justification/reasoning for their selection. Commonly used laboratory strains should be employed. The strain should not have low fecundity and should preferably be characterized for its sensitivity to developmental toxins.

2. Age

Young adult animals (nulliparous females) should be used.

3. Sex

Pregnant female animals should be used at each dose level.

4. Number of animals

At least 20 pregnant rats, mice or hamsters or 12 pregnant rabbits are recommended 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.

B. Control group

A concurrent control group should be used. 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. Except for treatment with the test substance, animals in the control group(s) should be handled in an identical manner to test group animals.

C. Dose levels and dose selection

1. At least 3 dose levels with a control and, where appropriate, a vehicle control, should be used.

2. The vehicle should neither be developmentally toxic nor have effects on reproduction.

3. 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 should be determined.

4. Unless limited by the physical/chemical nature or biological properties of the substance, the highest dose level should

induce some overt maternal toxicity such as weight loss or decrease in weight gain, but not more than 10 percent maternal deaths.

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

6. 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.

D. Observation period

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

E. 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 or administration. The test substance should be administered at the same time each day.

F. 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.

G. Observation of animals

1. A gross examination should be made at least once each day.

2. 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).

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

4. During the treatment and observation periods, cage-side observations should 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.

5. Measurements should be made weekly of food consumption for all animals in the study.

6. Animals should be weighed at least weekly.

7. Females showing signs of abortion or premature delivery should be sacrificed and subjected to a thorough macroscopic examination.

J. Gross necropsy

1. At the time of sacrifice or death during the study, the dam should be examined macroscopically for any structural abnormalities or pathological changes which may have influenced the pregnancy.

2. Immediately after sacrifice or death, the uterus should be removed, weighed and the contents examined for embryonic or fetal deaths and the number of viable fetuses. The degree of resorption should be described in order to help estimate the relative time of death.

3. The number of corpora lutea should be determined for all species except mice.

4. The sex of the fetuses should be determined and they should be weighed individually, the weights recorded, and the mean fetal weight per litter derived.

5. Following removal, each fetus should be examined externally.

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

7. For rabbits, each fetus should be examined by careful dissection for visceral anomalies and then examined for skeletal anomalies.

VI. DATA AND REPORTING

A. Treatment of results

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 pregnant animals, the number and percentages of live fetuses per litter and the number of fetuses per litter with any soft tissue or skeletal abnormalities.

B. Evaluation of results

The findings of a developmental toxicity study should 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.

C. Test report

In addition to the reporting requirements as specified in the EPA Good Laboratory Practice Standards [Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations] the following specific information should be reported:

1. Toxic response data by dose;

2. Species and strain;

3. Time of death during the study or whether animals survived to termination;

4. Time of onset and duration of each abnormal sign and its subsequent course;

5. Food, body weight, weight gain and uterine weight data;

6. Pregnancy and litter data; and

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

VII. REFERENCES

The following references may be helpful in developing acceptable procotols, and provide a background of information on which this section is based. They should not be considered the only source of information on test performance, however.

1. Health Protection Branch. 1975. Ministry of Health and Welfare. The Testing of Chemicals for Carcinogenicity, Mutagenicity and Teratogenicity. Canada: The Honorable Marc Lalonde, Minister of Health and Welfare, Ministry of Health and Welfare. 183 pp.

2. NAS. 1977. National Academy of Sciences. Principles and Procedures for Evaluating the Toxicity of Household Substances. Washington, D.C.: 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. 130 pp.

3. WHO. 1967. World Health Organization. Principles for the Testing of Drugs for Teratogenicity. WHO Technical Report Series No. 364. Geneva: World Health Organization. 18 pp.

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October 1984

INHALATION DEVELOPMENTAL TOXICITY STUDY

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, DC 20460

I. 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.

II. DEFINITIONS

A. Developmental toxicity is the induction of adverse effects on the developing organism as a result of in utero exposure to an agent. It is a generic term which includes endpoints such as resorptions, structural abnormalities, growth retardation as well as functional and behavioral deficits.

B. "Aerodynamic diameter" 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 and in themselves cannot be related to deposition within the respiratory tract.

C. "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 geometric standard deviation are used to statistically describe the particle size distribution of any aerosol based on the weight and size of the particles.

D. "Inhalable diameter" refers to that aerodynamic diameter of a particle which is considered to be inhalable by an organism. Particles which are capable of being inhaled may be deposited anywhere within the respiratory tract from the trachea to the deep lung (the alveoli).

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

F. 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

III. 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.

IV. LIMIT TEST

If a test at an exposure of 5 mg/l (actual concentration of inhalable 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.

V. TEST PROCEDURES

A. Animal selection

1. Species and strain

Testing should 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 should provide justification/reasoning for their

selection. Commonly used laboratory strains should be employed. The strain should not have low fecundity and should preferably be characterized for its sensitivity to developmental toxins.

2. Age

Young adult animals (nulliparous females) should be used.

3. Sex

Pregnant female animals should be used at each exposure level.

4. Number of animals

At least 20 pregnant rats, mice or hamsters or 12 pregnant rabbits are recommended 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.

B. Control group

A concurrent control group should be used. This group should 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.

C. Dose levels and dose selection

1. At least 3 dose levels with a control and, where appropriate, a vehicle control, should be used.

2. The vehicle should neither be developmentally toxic nor have effects on reproduction.

3. To select the appropriate dose levels, a pilot or trial study may be advisable. Since pregnant animals have an increased minute ventilation compared to non-pregnant animals, it is recommended that the trial study be conducted in pregnant animals. Similarly, since presumeably 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.

4. Unless limited by the physical/chemical nature or biological properties of the substance, the highest concentration level should induce some overt maternal toxicity such as weight loss or decrease in weight gain, but not more than 10 percent maternal deaths.

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

6. Ideally, the intermediate concentration 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.

D. Exposure duration

The duration of exposure should be at least six hours daily allowing appropriate additional time for chamber equilibrium.

E. Observation period

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

F. Inhalation exposure

1. The animals should be tested in 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.

Pregnant animals should be subjected to the minimum amount of stress. Since whole-body exposure appears to be the least stressful mode of exposure it is the preferred method. In general, oro-nasal or head-only exposure, which is sometimes used to avoid excessive 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 should provide justification/reasoning for its selection.

2. A dynamic inhalation system with a suitable concentration control/monitoring system should be used. The system should 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 the surrounding area.

3. 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.

G. Physical measurements

Measurements or monitoring of the following should be conducted:

1. The rate of air flow should be monitored continuously and should be recorded at least every 30 minutes.

2. The actual concentrations of the test substance should be measured in the breathing zone. During the exposure period the actual concentrations of the test substance should

be held as constant as practicable, monitored continuously and recorded at least at the beginning, at an intermediate time and at the end of the exposure period.

3. The behavior of the particle generating system should be established for each substance under study. In effect, particle size analysis should be performed to establish the stability of the particle size distribution of the aerosol. During exposure, analysis should be conducted as often as necessary to determine the consistency of the particle size distribution. Likewise, the particle concentration in the atmosphere should be established and monitored.

4. Temperature and humidity should be monitored continuously and recorded at least every 30 minutes.

H. 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.

I. Observation of animals

1. A gross examination should be made at least once each day.

2. 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).

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

4. During the treatment and observation periods, cage-side observations should 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.

5. Measurements should be made weekly of food consumption for all animals in the study.

6. Animals should be weighed at least weekly.

7. Females showing signs of abortion or premature delivery should be sacrificed and subjected to a thorough macroscopic examination.

J. Gross necropsy

1. At the time of sacrifice or death during the study, the dam should be examined macroscopically for any structural abnormalities or pathological changes which may have influenced the pregnancy.

2. Immediately after sacrifice or death, the uterus should be removed, weighed and the contents examined for embryonic or fetal deaths and the number of viable fetuses. The degree of resorption should be described in order to help estimate the relative time of death.

3. The number of corpora lutea should be determined for all species except mice.

4. The sex of the fetuses should be determined and they should be weighed individually, the weights recorded, and the mean fetal weight per litter derived.

5. Following removal, each fetus should be examined externally.

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

7. For rabbits, each fetus should be examined by careful dissection for visceral anomalies and then examined for skeletal anomalies.

VI. DATA AND REPORTING

A. Treatment of results

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 pregnant animals, the number and percentages of live fetuses per litter and the number of fetuses per litter with any soft tissue or skeletal abnormalities.

B. Evaluation of results

The findings of a developmental toxicity study should 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.

C. Test report

In addition to the reporting requirements as specified in the EPA Good Laboratory Practice Standards [Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations] the following specific information should be reported:

1. Test conditions

(a) Description of the exposure apparatus including design, type, dimensions, source of air, system for generating particulates/ aerosols, gas or volatile substance, methods of conditioning air, and the method of housing the animals in the chamber.

(b) Description of the equipment for measuring temperature, humidity, and particulate/aerosol concentrations and size or concentration of the gas or volatile substance.

2. Exposure data

The following data shall be tabulated and presented with mean values and a measure of variability (eg., standard deviation):

a. Airflow rate through the test chamber;

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 concentration of particulates/aerosols/gaseous phases in the test atmosphere at breathing zone level; and

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

3. Animal data

a. Toxic response data by dose;

b. Species and strain;

c. Time of death during the study or whether animals survived to termination;

d. Time of onset and duration of each abnormal sign and its subsequent course;

e. Food, body weight, weight gain and uterine weight data;

f. Pregnancy and litter data; and

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

VII. REFERENCES

The following references may be helpful in developing acceptable procotols, and provide a background of information on which this section is based. They should not be considered the only source of information on test performance, however.

1. Cage JC. 1970. Experimental inhalation toxicology. In: Methods in toxicology. Paget GE, ed. Philadelphia: F.A. Davis Company, pp. 258-277.

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HG-Organ/Tissue-Dev Tox Screen
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PRELIMINARY DEVELOPMENTAL TOXICITY SCREEN

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I. PURPOSE

The *in vivo* developmental toxicity assay is designed to assess the potential of agents to induce toxic effects in the conceptus after administration to the mother during pregnancy. This test should be used only to prioritize environmental agents for testing by more rigorous standard protocols (see TSCA Guidelines: Developmental Toxicity Study).

II. DEFINITIONS

A. Developmental toxicity is the induction of adverse effects on the developing organism as a result of *in utero* exposure to an agent. It is a generic term which includes endpoints such as resorption, structural abnormalities, growth retardation, as well as functional deficits.

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

C. 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).

III. PRINCIPLE OF THE TEST METHOD

The test substance is administered to pregnant animals during a significant portion of the period of major organogenesis. A single dose level is administered. This dose level should be high enough to elicit overt maternal toxicity. This toxicity should not exceed a mortality level of 10% or a reduction of overall maternal weight gain of more than 30% of the control value during the treatment period. The dams are allowed to give birth and the neonates are counted and weighed on days 1 and 3 postpartum (day 1 is the day of birth). The underlying hypothesis for this assay is that a wide spectrum of developmentally toxic effects will be expressed as intrauterine death or impaired neonatal growth or survival.

IV. TEST PROCEDURES

A. Animal selection

1. Species and strain

Testing must be performed in a mammalian species and strain which will allow human handling of newborn pups without cannibalization or abandonment. The preferred species is either rat or mouse. The strain should be commonly used and should not have low fecundity.

2. Age

Young adult animals should be used.

3. Sex

Pregnant (primigravid) female animals should be used.

4. Number of animals

Sufficient animals should be used to produce a sample size of at least 20 pregnant animals at term. The objective is to ensure that sufficient litters are produced to permit meaningful evaluation of the potential developmental toxicity of the test substance.

B. Control group

A concurrent control group large enough to produce at least 30 pregnant females at term should be used. 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. Except for treatment with the test substance, animals in the control group(s) should be handled in an identical manner to test group animals.

C. Dose levels and dose selection

1. A single dose level with a concurrent control should be used. It may be advisable to use a second dose level which is some fraction (e.g., 30-50%) of the first dose level. This will facilitate the identification of studies with potentially false positive results.

2. The vehicle should be neither developmentally toxic nor have effects on reproduction in the dams. If there is uncertainty as to the developmental toxicity potential of the vehicle, a sham control group should be used also.

3. To select the appropriate dose levels, a pilot or trial study should be conducted. If a pilot study is not done, a rationale or justification should be provided.

4. Unless limited by the physical/chemical nature or biological properties of the substance, the dose level used should be:

(a) high enough to cause overt maternal toxicity as evidenced by death, significant weight loss or reduced weight gain, or

(b) 1000 mg/kg, if lower dose levels fail to induce maternal toxicity.

D. Observation period

Day 0 in the test is the day in which a vaginal plug and/or sperm are observed. The dose period should encompass a significant portion of the period of major organogenesis. This may be taken as days 6-15 for the rat and mouse.

E. Administration of test substance

The test substance or vehicle is usually administered orally, by 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 should be administered at the same time each day.

F. Exposure conditions

Female test animals should be housed individually and provided with bedding. They should be treated with the test substance daily throughout the appropriate treatment period. When given by gavage, in view of the

rapid weight gain which takes place during pregancy, the animals should be weighed frequently and the dosage based on the most recent weight determination.

G. Observation of pregnant animals

1. Pregnant animals should be observed at least once daily throughout the study, or until they die or are sacrificed.

2. During the treatment and observation periods, cage-side observations should 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.

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

4. During the dosing period females that die or are sacrificed because they are moribund should be exmained for dosing errors and signs of pregnancy; details of the conditions of the uterus and/or its

contents should be recorded. Animals that have not delivered three days after the expected date of parturition should be sacrificed and similar examinations made. Uteri that do not show gross evidence of pregnancy may be chemically treated (e.g. 10% ammonium sulfide) to improve detection of early termination of pregnancy.

H. Observation of dams after birth

Dams should be observed for signs of overt toxicity during the postpartum period at the same time neonatal examinations are being made.

I. Neonatal examinations

1. Dams are allowed to give birth and the litters are examined for gross anomalies and presence of milk, counted, and weighed on postpartum days 1 and 3.

2. Dead pups may be preserved and subsequently necropsied and abnormalities noted.

3. For those compounds that induce only neonatal growth reduction, litters should be normalized on postpartum day 3 (to approximately four females and four males) and left with the dam through weaning. This procedure will determine if the growth reduction is transient or if it represents a permanent functional alteration.

V. DATA AND REPORTING

A. Treatment of results

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 pregnant animals, the average duration of pregnancy, the maternal weight gain during the treatment period, the number of dams with a viable

litter (at least one live neonate), the average number of live neonates per litter on days 1 and 3, the average neonatal weight on days 1 and 3, the average neonatal weight gained during that period, and data on necropsied pups.

B. Evaluation of results

The findings of this bioassay should be evaluated in terms of the types of effects noted. All data analyses should compare treatment groups with their concurrent controls. Statistical treatment of the results should involve analysis of variance, and the number of live pups on days 1 and 3 should be used as a covariate in the analyses of postnatal body weight so as to correct for differences in pup weights due to litter size. Fully resorbed females should be considered to have zero line on days 1 and 3. The number of animals going to term must be sufficiently large to allow for a reasonable detection of compound-induced deficiences.

Conditions which significantly reduce the number of dams going to term (e.g. lack of pregnancy or compound-induced maternal death) should lead to a repeat of the study. Lack of both overt maternal toxicity and reproductive effects also should lead to a repeat of the study if the dose level used was less than 1000 mg/kg.

C. Test report

In addition to the reporting requirements as specified in the EPA Good Laboratory Practice Standards (Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations) the following specific information should be reported:

1. Toxic response data;

2. Species and strain;

3. Time of maternal death during the study or whether animals survived to termination;

4. Time of onset and duration of each abnormal sign and its subsequent course;

5. Pregnancy data;

6. Litter data including number live and dead; and average litter weight on days 1 and 3 postpartum; and

7. Necropsy data on dead pups, if such were generated.

VI. REFERENCES

The following references may be helpful in developing acceptable protocols, and provide a background of information on which this section is based. They should not be considered the only source of information on test performance, however.

1. Chernoff N and Kavlock R. 1982. An in vivo teratology screen utilizing pregnant mice. J. Toxicol. Environ. Health 10: 541-550.

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THE SALMONELLA TYPHIMURIUM REVERSE MUTATION ASSAY

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I. PURPOSE

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

II. DEFINITIONS

A. 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.

B. Base pair mutagens are agents which cause a base change in the DNA. This change may occur at the site of the original mutation or at a second site in the DNA molecule.

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

III. REFERENCE SUBSTANCES

These may include, but need not be limited to, sodium azide, nitroturantoin, 4-nitro-o-phenylene-diamine and 2-aminoanthracene for the plate incorporation method and direct blue 6 for the azo-reduction method.

IV. TEST METHOD

A. 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 plates.

B. Description

Several methods for performing the test have been described. Among those used are:

1. the direct plate incorporation method,
2. the azo-reduction method,
3. the preincubation method,
4. the dessicator method, and
5. the suspension method

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

C. Strain selection

1. 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.

2. Preparation and storage

Scientifically accepted 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. The extent of spontaneous mutation should be in the range expected either as reported in the literature or as established in the laboratory by historical control values.

3. 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).

D. 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.

E. Control groups

1. Concurrent controls

Concurrent positive and negative (untreated and/or vehicle) controls should be included in each experiment. Positive controls should insure both strain responsiveness and efficacy of the metabolic activation system.

2. Strain specific positive controls

Strain specific positive controls should be included in the assay. Examples of such controls are as follows:

a. Strain TA 1535, sodium azide;
b. Strain TA 100, nitroturantoin
c. TA 98, TA 1537, 4-nitro-o-phenylene-diamine

3. 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. Direct Blue 6 is an example of a positive control compound in the azo-reduction method. Other positive control reference substances may be used.

4. Class-specific positive controls

The azo-reduction method should include positive controls from the same class of compounds as the test agent wherever possible. For example, benzidine based dyes with known mutagenic potential should be used as controls in experiments with benzidine based dyes of unknown mutagenic potential.

F. Test chemicals

1. Vehicle

Test chemicals and positive control reference substances should be dissolved in an appropriate vehicle and then further diluted in vehicle for use in the assay.

2. 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 appearance of pinpoint nonrevertant colonies. 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 should be tested with adequate intervals between test points.

c. A suspected positive response not showing a clear dose-related response should be confirmed by testing over a narrow range of concentrations.

V. TEST PERFORMANCE

A. Direct plate incorporation method

For this test without metabolic activation, test chemical and 0.1 ml 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 should be mixed and poured over the surface of a selective agar plate. Overlay agar should be allowed to solidify before incubation. At the end of the incubation period, revertant colonies per plate should be counted.

B. Azo-reduction method

For this test, 0.5 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. Chemical should be added and the tubes should be incubated without 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 should be allowed to solidify before incubation. At the end of the incubation period, revertant colonies per plate should be counted.

It is recommended that all azo dyes be tested in either the plate incorporation or preincubation method and in the azo-reduction method.

C. Other methods

Other methods may also be appropriate.

D. Media

An appropriate selective medium with an adequate overlay agar should be used.

E. Incubation conditions

All plates within a given experiment should be incubated for the same time period. This incubation period should be for 48-72 hours at 37 C.

F. Number of cultures

In general, all plating should be done at least in triplicate. If scientifically justified, the use of duplicates may be acceptable. All results should be confirmed in an independent experiment.

VI. DATA AND REPORT

A. Treatment of results

Data should 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 should also be presented. Individual plate counts, the mean number of revertant colonies per plate and standard deviation should be presented for test chemical and positive and negative (untreated and/or vehicle) controls.

B. Statistical evaluation

Data should be evaluated by appropriate statistical methods.

C. Interpretation of results

1. 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.

2. 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 non-mutagenic in this system.

3. Both biological and statistical significance should be considered together in the evaluation.

D. Test evaluation

1. 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.

2. Negative results indicate that under the test conditions the test substance is not mutagenic in S. typhimurium.

E. Test report

In addition to the reporting recommendations as specified in the EPA Good Laboratory Practices Standards (Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations) the following specific information should be reported:

1. bacterial strains used;

2. metabolic activation system used and its preparation for use in the assay; for S-9 preparations this should include cofactor cocktail (contents, storage conditions and amount used); source and amount of S-9 and details of preparation and storage of S-9 mix;

3. dose levels and rationale for selection of dose;

4. positive and negative controls;

5. individual plate counts, mean number of revertant colonies per plate, standard deviation;

6. dose-response relationship, if applicable.

VII. REFERENCES

The following references may be helpful in developing acceptable protocols, and provide a background of information on which this section is based. They should not be considered the only source of information on test performance, however.

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4. Prival MJ, Mitchell VD. 1982. Analysis of a method for testing azo dyes for mutagenic activity in Salmonella typhimurium in the presence of flavin mononculeotide and hamster liver S-9. Mutation Res 97:103-116.

5. Vogel HJ, Bonner DM. 1956. Acetylornithinase of E. coli: partial purification and some properties. J Biol Chem 218:97-106.

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