(f) *Data and report*—(1) *Treatment of results.* Data shall be presented in tabular form and shall permit independent analysis of cell stage specific effects and dose dependent phenomena. The data shall be recorded and analyzed in such a way that clusters of identical mutations are clearly identified. The individual mutants detected shall be thoroughly described. In addition, concurrent positive and negative control data, if they are available, shall be tabulated so that it is possible to differentiate between concurrent (when available) and long-term accumulated mutation frequencies.

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

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

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

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

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

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

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

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

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

(iii) Route and duration of exposure.

(iv) Mating schedule.

(v) Time of examination for mutant progeny.

(vi) Criteria for scoring mutants.

(vii) Use of concurrent or negative controls.

(viii) Dose response relationship, if applicable.

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

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

(2) [Reserved]

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

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

§ 798.5265 The salmonella typhimurium reverse mutation assay.

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

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

(2) Base pair mutagens are agents which cause a base change in the DNA. In a reversion assay, this change may

occur at the site of the original mutation or at a second site in the chromosome.

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

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

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

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

(i) The direct plate incorporation method.

(ii) The preincubation method.

(iii) The azo-reduction method.

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

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

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

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

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

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

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

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

(B) TA 98, 2-nitrofluorene.

(C) TA 1537, 9-aminoacridine.

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

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

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

(ii) *Exposure concentrations.* (A) The test should initially be performed over a broad range of concentrations. Among the criteria to be taken into consideration for determining the upper limits of test chemical concentration are cytotoxicity and solubility. Cytotoxicity of the test chemical may be altered in the presence of metabolic activation systems. Toxicity may be evidenced by a reduction in the number of spontaneous revertants, a clearing of the background lawn or by the degree of survival of treated cultures. Relatively insoluble compounds should be tested up to the limits of solubility. For freely soluble nontoxic chemicals, the upper test chemical concentration should be determined on a case by case basis.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

(4) *Test evaluation.* (i) Positive results from the *S. typhimurium* reverse mutation assay indicate that, under the test conditions, the test substance induces point mutations by base changes or frameshifts in the genome of this organism.

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

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

(i) Bacterial strain used.

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

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

(iv) Positive and negative controls.

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

(vi) Dose-response relationship, if applicable.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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