erythrocytes or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(3) *Test evaluation.* (i) Positive results in the micronucleus test provide information on the ability of a chemical to induce micronuclei in erythrocytes of the test species under the conditions of the test. This damage may have been the result of chromosomal damage or damage to the mitotic apparatus.

(ii) Negative results indicate that under the test conditions the test substance does not produce micronuclei in the bone marrow of the test species.

(f) *Test report.* In addition to the reporting recommendations as specified under §79.60, the following specific information shall be reported:

(1) Test atmosphere concentration(s) used and rationale for concentration selection.

(2) Rationale for and description of treatment and sampling schedules, toxicity data, negative and positive controls.

(3) Historical control data (negative and positive), if available.

(4) Details of the protocol used for slide preparation.

(5) Criteria for identifying micronucleated erythrocytes.

(6) Micronucleus analysis by animal and by group for each concentration (sexes analyzed separately).

(i) Ratio of polychromatic to normochromatic erythrocytes.

(ii) Number of polychromatic erythrocytes with micronuclei.

(iii) Number of polychromatic erythrocytes scored.

(7) Statistical methodology chosen for test analysis.

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

(1) 40 CFR 798.5395, *In Vivo*, Mammalian Bone Marrow Cytogenetics Tests: Micronucleus Assay.

(2) Cihak, R. ''Evaluation of Benzidine by the Micronucleus Test.'' Mutation Research, 67: 383–384 (1979).

(3) Evans, H.J. ''Cytological Methods for Detecting Chemical Mutagens.'' Chemical Mutagens: Principles and Methods for Their Detection, Vol. 4. Ed. A. Hollaender (New York and London: Plenum Press, 1976) pp. 1–29.

(4) Heddle, J.A., *et al.* ''The Induction of Micronuclei as a Measure of Genotoxicity. A Report of the U.S. Environmental Protection Agency Gene-Tox Program.'' Mutation Research, 123:61–118 (1983).

(5) Preston, J.R. *et al.* ''Mammalian *In Vivo* and *In Vitro* Cytogenetics Assays: Report of the Gene-Tox Program.'' Mutation Research, 87:143–188 (1981).

(6) Schmid, W. ''The micronucleus test for cytogenetic analysis'', Chemical Mutagens, Principles and Methods for their Detection. Vol. 4 Hollaender A, (Ed. A ed. (New York and London: Plenum Press, (1976) pp. 31–53.

(7) Tice, R.E., and Al Pellom ''User's guide: Micronucleus assay data management and analysis system'', NTIS Order no. PB–90–212–598AS.

§79.65 *In vivo* sister chromatid exchange assay.

(a) *Purpose.* The *in vivo* sister chromatid exchange (SCE) assay detects the ability of a chemical to enhance the exchange of DNA between two sister chromatids of a duplicating chromosome. The most commonly used assays employ mammalian bone marrow cells or peripheral blood lymphocytes, often from rodent species.

(b) *Definitions.* For the purposes of this section, the following definitions apply:

C-metaphase means a state of arrested cell growth typically seen after treatment with a spindle inhibitor, *i.e.*, colchicine.

Sister chromatid exchange means a reciprocal interchange of the two chromatid arms within a single chromosome. This exchange is visualized during the metaphase portion of the cell cycle and presumably requires the enzymatic incision, translocation and ligation of at least two DNA helices.

(c) *Test method*—(1) *Principle of the test method.* (i) Groups of rodents are exposed by the inhalation route for a minimum of 6 hours/day over a period of not less than 28 days to three or more concentrations of a test substance in air. Groups of animals are sacrificed at the end of the exposure

period and blood lymphocyte cell cultures are prepared from study animals. Cell growth is suspended after a time and cells are harvested, fixed and stained before scoring for SCEs. Researchers may need to run a trial at the highest tolerated concentration of the test atmosphere to optimize the sample collection time for second division metaphase cells.

(ii) This assay may be done separately or in combination with the subchronic toxicity study, pursuant to the provisions in §79.62.

(2) *Description.* (i) The method described here employs peripheral blood lymphocytes (PBL) of laboratory rodents exposed to the test atmosphere.

(ii) Within twenty-four hours of the last exposure, test animal lymphocytes are obtained by heart puncture and duplicate cell cultures are started for each animal. Cultures are grown in bromo-deoxyuridine (BrdU), and then a spindle inhibitor (e.g., colchicine) is added to arrest cell growth. Cells are harvested, fixed, and stained and their chromosomes are scored for SCEs.

(3) *Species and strain.* The rat is the recommended test animal. Other rodent species may be used in this assay, but use of that species will be justified by the tester.

(4) *Animal number and sex.* At least five female and five male animals per experimental and control group shall be used. The use of a single sex or different number of animals shall be justified.

(5) *Positive control group.* A single concentration of a compound known to produce SCEs *in vivo* is adequate as a positive control if it shows a significant response at any one time point; additional concentration levels may be used. To select an appropriate concentration level, a pilot or trial study may be advisable. Initially, one concentration of the test substance may be used, the maximum tolerated dose or that producing some indication of toxicity as evidenced by animal morbidity (including death) or target cell toxicity. Intraperitoneal injection of 1,2-dimethyl-benz-anthracene or benzene are examples of positive control exposures. A concentration of 50–80 percent of an LD50 would also be a suitable guide.

(6) *Inhalation exposure.* (i) All data developed within this study shall be in accordance with good laboratory practice provisions under §79.60.

(ii) The general conduct of this study shall be in accordance with the vehicle emissions inhalation exposure guideline in §79.61.

(d) *Test performance*—(1) *Treatment.* At the conclusion of the exposure period, all test animals are anaesthetized and heart punctures are performed. Lymphocytes are isolated over a Ficoll gradient and replicate cell cultures are started for each animal. After some 21 hours, the cells are treated with BrdU and returned to incubation. The following day, a spindle inhibitor (e.g., colchicine) is added to arrest cell growth in c-metaphase. Cells are harvested 4 hours later and second-division metaphase cells are washed and fixed in methanol:acetic acid, stained, and chromosome preparations are scored for SCEs.

(2) *Staining method.* Staining of slides to reveal SCEs can be performed according to any of several protocols. However, the fluorescence plus Giemsa method is recommended.

(3) *Number of cells scored.* (i) A minimum of 25 well-stained, second-division metaphase cells shall be scored for each animal for each cell type.

(ii) At least 100 consecutive metaphase cells shall be scored for the number of first, second, and third division metaphases for each animal for each cell type.

(iii) At least 1000 consecutive PBL's shall be scored for the number of metaphase cells present.

(iv) The number of cells to be analyzed per animal shall be based upon the number of animals used, the negative control frequency, the pre-determined sensitivity and the power chosen for the test. Slides shall be coded before microscopic analysis.

(e) *Data and report*—(1) *Treatment of results.* In addition to the reporting requirements specified under §§79.60 and 61, data shall be presented in tabular form, providing scores for both the number of SCE for each metaphase. Differences among animals within each group shall be considered before making comparisons between treated and control groups.

(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 SCE. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test concentrations.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of SCE or a statistically significant and reproducible positive response at any one of the test concentrations is considered not to induce rearrangements of DNA segments in this system.

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

(4) *Test evaluation.* (i) A positive result in the *in vivo* SCE assay for either, or both, the lung or lymphocyte cultures indicates that under the test conditions the test substance induces reciprocal interchanges of DNA in duplicating chromosomes from lung or lymphocyte cells of the test species.

(ii) Negative results indicate that under the test conditions the test substance does not induce reciprocal interchanges in lung or lymphocyte cells of the test species.

(5) *Test report.* In addition to the reporting recommendations as specified under §§ 79.60 and 79.61, the following specific information shall be reported:

(i) Test concentrations used, rationale for concentration selection, negative and positive controls;

(ii) Toxic response data by concentration;

(iii) Schedule of administration of test atmosphere, BrdU, and spindle inhibitor;

(iv) Time of harvest after administration of BrdU;

(v) Identity of spindle inhibitor, its concentration and timing of treatment;

(vi) Details of the protocol used for cell culture and slide preparation;

(vii) Criteria for scoring SCE;

(viii) Replicative index, *i.e.,* [percent 1st division+(2×percent 2nd division) + (3×percent 3rd division) metaphases]/100; and

(ix) Mitotic activity, *i.e.,* # of metaphases/1000 cells.

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

(1) 40 CFR 798.5915, *In vivo* Sister Chromatid Exchange Assay.

(2) Kato, H. ''Spontaneous Sister Chromatid Exchanges Detected by a BudR-Labeling Method.'' Nature, 251:70–72 (1974).

(4) Kligerman, A. D., *et al.* ''Sister Chromatid Exchange Analysis in Lung and Peripheral Blood Lymphocytes of Mice Exposed to Methyl Isocyanate by Inhalation.'' Environmental Mutagenesis 9:29–36 (1987).

(5) Kligerman, A.D., *et al.*, ''Cytogenetic Studies of Rodents Exposed to Styrene by Inhalation'', IARC Monographs no. 127 ''Butadiene and Styrene: Assesment of Health Hazards'' (Sorsa, *et al.*, eds), pp 217–224, 1993.

(6) Kligerman, A., *et al.*, ''Cytogenetic Studies of Mice Exposed to Styrene by Inhalation.'', Mutation Research, 280:35–43, 1992.

(7) Wolff, S., and P. Perry. ''Differential Giemsa Staining of Sister Chromatids and the Study of Sister Chromatid Exchanges Without Autoradiography.'' Chromosoma 48: 341–53 (1974).

§ 79.66 Neuropathology assessment.

(a) *Purpose.* (1) The histopathological and biochemical techniques in this guideline are designed to develop data in animals on morphologic changes in the nervous system associated with repeated inhalation exposures to motor vehicle emissions. These tests are not intended to provide a detailed evaluation of neurotoxicity. Neuropathological evaluation should be complemented by other neurotoxicity studies, e.g. behavioral and neurophysiological studies and/or general toxicity testing, to more completely assess the neurotoxic potential of an exposure.

(2) [Reserved]

(b) *Definition.* Neurotoxicity (NTX) or a neurotoxic effect is an adverse change in the structure or function of the nervous system following exposure to a chemical substance.