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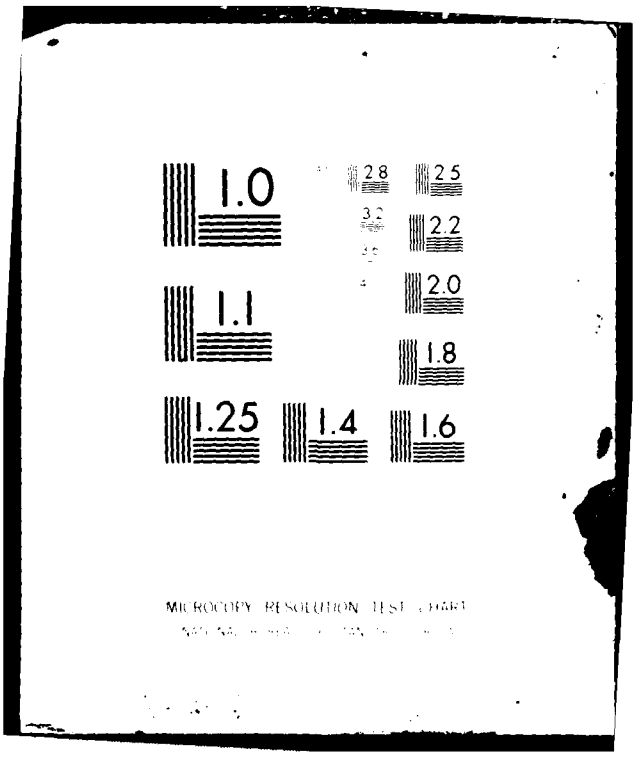
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MAMMALIAN TOXICOLOGY TESTING: PROBLEM DEFINITION STUDY

AMTR PROTOCOL/PRICING REPORT (U)

by
R. H. Reuter and J. P. Glennon

April, 1981

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

Contract DAMD17-81-C-1013

Life Systems, Inc.
Cleveland, OH 44122

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The mammalian toxicology testing needed to meet the Army's toxicology requirements are cited. Standardized protocols have been identified and pricing information summarized in this report. The pricing information used a standardized pricing procedure that included basic cost elements such as direct labor, overhead, other direct costs, general and administrative costs and profit. The approach allows a cost comparison between various performance alternatives. The prototypes were considered representative of those needed for the 19 identified potential Army toxicology tests.

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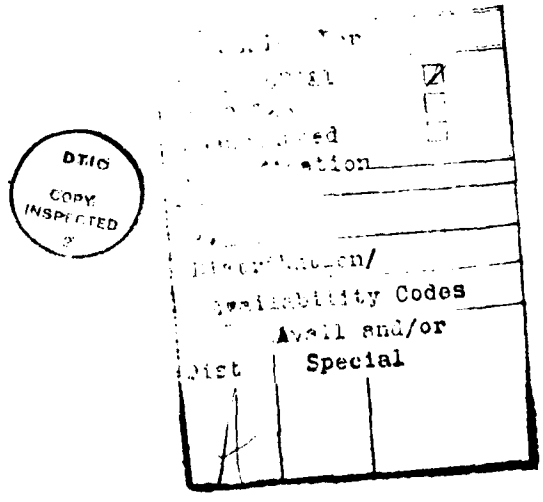
Report Subtitle	Life Systems, Inc. Report Number
Final Reports--	
Part 1. Comparative Analysis Report	LSI-TR-477-2
Part 2. Facility Installation Report	LSI-TR-477-3
Part 3. Impact of Future Changes Report	LSI-TR-477-4



FOREWORD

Reports for this Contract, DAMD17-81-C-1013, consist of three major final reports and twelve supporting documents. The Contract title, MAMMALIAN TOXICOLOGY TESTING: PROBLEM DEFINITION STUDY, is the main title for all the reports. Individual reports are subtitled and referenced with Life Systems, Inc. report numbers as detailed below. Please note that the Life Systems report numbers in text references are shortened. In the Defense Technical Information Center (DTIC) data base the reports are identified by the complete report numbers (i.e., LSI-TR-477-XXX) and complete numbers must be used for retrieval.

Report Subtitle	Life Systems, Inc. Report Number
Final Reports--	
Part 1. Comparative Analysis Report	LSI-TR-477-2
Part 2. Facility Installation Report	LSI-TR-477-3
Part 3. Impact of Future Changes Report	LSI-TR-477-4
Supporting Documents--	
Technology Changes Impact on Testing Requirements	LSI-TR-477-14
Quality Assurance Plan	LSI-TR-477-17A
Capability Modules	LSI-TR-477-19B
Technical Plan	LSI-TR-477-20A
Equipment Plan	LSI-TR-477-21A
Personnel Plan	LSI-TR-477-23A
Inhalation Chambers and Supporting Equipment Survey	LSI-TR-477-26A
Equipment List for Modules	LSI-TR-477-28B
AMTR Protocol/Pricing Report	LSI-TR-477-29A
Global Army Toxicology Requirements	LSI-TR-477-31A
Comparison Toxicology Test Costs	LSI-TR-477-36A
Annual Testing Capacity	LSI-TR-477-38A

SUMMARY

Pricing information is provided for the 19 different tests identified as being needed to satisfy the Army's requirements for applied mammalian toxicology research/testing. The prices were based on standardized protocols.

The pricing information was obtained from recent literature references. The specific tests were identified as those required by the Army.

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INTRODUCTION

A program was undertaken to study and define the Army's requirements for Applied Mammalian Toxicology Research (AMTR) and methods for meeting the requirement. Inherent in the latter is consideration of the types of toxicology testing to be performed, the numbers of tests required and the pricing of this testing.

Scope of Document

This document was conceived and prepared to accumulate, under one cover, all of the standardized Protocols that could be used for the testing projected for the Army's AMTR Program. Also, included is information on the pricing (in current year dollars) of these tests.

Objective

The objective of this document is to assemble the protocols and pricing data used for AMTR testing. This document will permit recalculations of price as a function of changes in a protocol or selection of a different protocol. Pricing information was obtained wherever possible by using a standardized pricing procedure, built on basic cost elements (direct labor, overhead, other direct costs, general and administrative costs and profit). Utilizing this cost element approach a cost comparison between various performance alternatives can be made more easily and accurately by determining the cost differences for specific cost elements. Likewise, the cost element approach provides a record of how the costs were calculated.

PROTOCOLS

Provided in Appendix 1 are the standard protocols used for the pricing of AMTR tests. These protocols were published in the Federal Register and set out the test standards for toxic substances and pesticides. They are also considered as representative of the testing protocol that would be used if the same type tests were done on other substances. There are standard protocols for 19 of the potential Army AMTR tests. Standard protocols are not available for the remaining tests of potential interest to the Army.

PRICING INFORMATION SOURCES

The pricing information sources listed in priority order used were:

1. Enviro Control, Inc. 1980. Cost analysis methodology and protocol estimates, TSCA health standards and FIFRA guidelines. Draft report. Rockville, MD: Enviro Control, Inc., U.S. Environmental Protection Agency.

2. Fribush S., Langer G., ICF, Inc. 1980. Profile of the chemical safety testing industry: an assessment of pesticide testing capacity. Final report. Washington, DC: ICF, Inc., U.S. Environmental Protection Agency contract no. WA78-B247.

3. Calculations by Life Systems, Inc.

Pricing information was available from Reference 1, for 16 tests and from Reference 2 for two tests. Price estimates were calculated by Life Systems, Inc. for all remaining tests.

PRICING DATA

Table 1 provides a summary of the pricing data for each toxicology test projected for the Army's AMTR Program.

CONCLUSIONS

This document provides the pricing data for the types of testing anticipated to be required by the Army's AMTR program and ties the pricing to standardized protocols wherever one is available. This approach allows for comparisons of costs between various performance alternatives and uses a standardized pricing approach that can be updated or recalculated, as protocols change or different protocols are selected for use by the Army.

TABLE 1

MAMMALIAN TOXICOLOGY TEST PRICE LIST (3/8/81)

Test No.	Duration	Type of Animal	Route of Exposure	Price, $(000) Per Outcome[a] — General Toxicology[c]	Special Scientific Toxicology Studies[b] — Behavioral	Onco-genic	Repro-duction	Terato-genic	Neuro-toxi-cology	Combined Protocols: Gen. Tox. + Oncog.	Combined Protocols: Repro./ Terato.
1	Acute	Rodent[d]	Oral	2.4[e]	—	—	—	—	—	—	—
2	Subchronic	Rodent[d]	Oral	56[e]	—	—	—	—	—	—	—
3	Chronic	Rodent[d]	Oral	495[e]	—	377[e]	114[e]	27[e]	—	600[f]	125[f]
4	Acute	Rodent[d]	Inhalation	5.0[e]	—	—	—	—	—	—	—
5	Subchronic	Rodent[d]	Inhalation	64[e]	100[f]	—	—	—	—	—	—
6	Chronic	Rodent[d]	Inhalation	613[e]	—	515[f]	—	—	—	1000[f]	-
7	Acute	Primate	Inhalation	39[f]	—	—	—	—	—	—	—
8	Subchronic	Primate	Inhalation	196[f]	150[f]	—	—	—	—	—	—
9	Chronic	Primate	Inhalation	518[f]	—	420[f]	—	—	—	800[f]	—
10	Subchronic	Dog	Oral	104[e]	—	—	—	—	—	—	—
11	Acute	Rabbit	Dermal	4.2[e]	—	—	—	—	—	—	—
12	Subchronic	Rabbit	Dermal	75[g]	—	—	—	—	—	—	—
13	Acute	Rabbit	Ocular	2.5[f]	—	—	—	—	—	—	—
14	Acute	Chicken	Oral	—	—	—	—	—	5.4[e]	—	—
15	Subchronic	Chicken	Oral	—	—	—	—	—	20[e]	—	—
				Irritation	Sensitization						
16	Acute	Rabbit	Dermal	0.7[e]	—						
17	Subchronic	Rabbit	Dermal	3.0[g]	—						
18	Acute	Rabbit	Ocular	0.9[e]	—						
19	Acute	Guinea Pig	Dermal	—	3.9[e]						

(a) Rounded off to nearest $1,000 for prices in excess of $5,000. Assumes one species.
(b) Special Scientific Toxicology Studies. Metabolism/Pharmacokinetics, Pharmacodynamics, and Respiratory are deleted since they are not a part of the 19 tests
(c) General Toxicology includes lethality, metabolism and pharmacokinetics/pharmocodynamics.
(d) Rodent studies price was based on use of the rat.
(e) SOURCE: Enviro Control, Inc. 1980, Cost Analysis Methodology & Protocol Estimates. TSCA Health Standards and FIFRA Guidelines. Rockville, MD. Enviro Control, Inc. U.S. Environmental Protection Agency.
(f) Price estimated by LSI since no quotable source was identified.
(g) SOURCE: ICF, Inc. 1980. Profile of the Chemical Safety Testing Industry. An Assessment of Pesticide Testing Capacity. Final Report. Washington, DC: ICF, Inc. U.S. Environmental Protection Agency.

Life Systems, Inc.

APPENDIX 1

STANDARD PROTOCOLS FOR AMTR

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PROTOCOL

Test No. 1: Acute Oral Toxicity Study (772.112-21), Rodent[a]

§ 772.112-21 Acute oral toxicity study.

(a) *Study design.* (1) *Species.* Testing must be performed with the laboratory rat.

(2) *Sex and age.* Young adult male and female animals must be used.

(3) *Number of animals and selection of dose levels.* (i) A trial test is recommended for the purpose of establishing a dosing regimen which must include one dose level higher than the expected LD_{50}. If data based on testing with at least 5 animals per sex are submitted showing that no toxicity is evident at 5g/kg, no further testing at other dose levels is necessary. If mortality is produced, the requirements of paragraph (a)(3)(ii) of this section must apply.

(ii) Enough animals per dose level and sufficient dose levels spaced appropriately must be used to produce test groups with mortality rates between 10 percent and 90 percent and to permit the calculation of the LD_{50} for males and females with a 95 percent confidence interval of 20 percent or less. At least 3 dose levels of the test substance, in addition to controls (if any), must be tested. Though the group sizes may vary for each dose level, each group must contain equal numbers of male and female animals.

(4) *Control animals.* (i) A concurrent vehicle control group is recommended if the vehicle or diluent used in administering the test substance would be expected to elicit any important acute toxicologic response, or if there are insufficient data on the acute effects of the vehicle.

(ii) A concurrent untreated control group is not required.

(5) *Dosing.* All animals must be dosed by gavage. All animals must receive the same concentration of dosing solution. They should also receive about the same volume of dosing solution, which should not exceed 4–5 ml per animal.

(6) *Duration of test.* The animals must be observed for at least 14 days after dosing, or until all signs of reversible toxicity subside, whichever occurs later.

(b) *Study Conduct.* (1) *Fasting.* Food shall be withheld from the animals the night prior to dosing.

(2) *Observation.* The animals must be observed frequently during the day of dosing and checked at least every 12 hours throughout the test period. The following must be recorded: Nature, onset, severity, and duration of all gross or visible toxic or pharmacological effects, e.g., abnormal or unusual cardiovascular, respiratory, excretory, behavioral, or other activity, as well as signs indicating an adverse effect on the central nervous system (paralysis, lack of coordination, staggering); pupillary reaction; and time of death. The weight of each animal must be determined at least semi-weekly (3–4 day intervals) throughout the test period, and at death.

(3) *Sacrifice and necropsy.* All test animals living at the termination of the observation period must be sacrificed. All test animals, whether dying by sacrifice or during the test must be subjected to a complete gross necropsy following their death, in accordance with § 772.100–2(b)(7), Subpart A. All abnormalities must be recorded.

(c) *Data reporting and evaluation.* In addition to the information required by § 772.100–2(b)(8), Subpart A, the test report must include the following information:

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

(2) Time of death after dosing.

(3) LD_{50} for each sex for each test substance calculated at the end of the observation period (with method of calculation specified);

(4) 95 percent confidence interval for the LD_{50}; and

(5) Dose-response curve and slope.

(a) Test No. 1 uses rodent as the animal, price is based on rat.

PROTOCOL

Test No. 2: Subchronic Oral Toxicity Study (772.112-31), Rodent[a]

§ 772.112-31 Subchronic oral dosing studies.

(a) *Study Design.* (1) *Species.* Testing must be performed in at least two mammalian species, preferably the same species and strain for which chronic studies are anticipated. Once species must be a generally recognized strain of laboratory rat. The second species may be a nonrodent. The nonrodent species should usually be the dog. Selection of a nonrodent species other than the dog will require full and adequate justification which should consider such factors as the comparative metabolism of the chemical and species sensitivity to the toxic effects of the test substance, as evidenced by the results of other studies.

(2) *Sex and age.* Equal numbers of males and females of each species and strain tested must be used. The tester must begin to dose as soon as possible after weaning and environmental acclimatization but no later than six weeks of age for rodents and at 4–6 months of age for dogs.

(3) *Control group.* A concurrent control group is required. This group must be an untreated control group or, if a vehicle is used in administering the test substance, a vehicle control group. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are required.

(4) *Number of animals.* Each test group and concurrent control group must contain at least 20 animals of each sex in studies with rats and at least 6 of each sex in studies with nonrodents. This number must be increased by the number, if any, scheduled to be sacrificed before completion of the study, such as, for example, rats on which hematology and blood chemistry determinations are made before and during the study.

(5) *Duration of testing.* (i) In studies with rodents, the substance being tested must be administered for at least 90 days.

(ii) In studies with nonrodents, the substance being tested must be administered daily for at least 6 months.

(6) *Number of dose levels and dose selection.* (i) At least three dose level groups (in addition to the control groups) must be tested.

(ii) The highest dosage level must result in toxicological or pharmacological effects, but not cause more than 10 percent fatalities. This level should be higher than that expected for human exposure.

(iii) The lowest dosage level must be one which does not induce any evidence of toxicity.

(7) *Route of administration.* The test substance must be administered in the animal's diet. Oral intubation may be allowed if the physical characteristics of the test substance so dictate. The chosen method must be used for all levels. If the test substance is administered by oral intubation, the amount of test substance must be adjusted weekly or biweekly to maintain a constant dose level in mg/kg (body weight). If the test substance is administered in the diet, either a constant concentration (ppm) or a constant dose level in mg/kg (body weight) must be used. The selection of dosage units of administration in the diet must be consistent with that for chronic feeding studies (Section 772.113-3 Subpart D).

(b) *Study Conduct.* (1) *Observation of animals.* All toxicological and pharmacological signs shall be recorded daily, including their time of onset, intensity, and duration. Such signs include but are not limited to: Mortality; and cardiovascular, respiratory, excretory, behavioral, and central nervous system (paralysis, ataxia, and pupillary reaction) effects. Observations must be made by an appropriately trained observer. Food consumption must be measured weekly during the test, and the animals must be weighed at least weekly. The animals must be observed as specified in Subpart A, § 772.100-2(b)(6)(ii). A complete ophthalmological examination must be conducted by a veterinarian on all nonrodents at the termination of the study.

(2) *Clinical laboratory testing.* The following determinations must be made at the time indicated below for each type of testing. For rodents, these determinations must be made on at least 10 animals of each sex in each group. For nonrodents, these determinations must be made on all animals in each group. Depending on the techniques used, it may be necessary to sacrifice animals to make the required clinical determinations. In case of said sacrifice, additional animals must be added to the study as provided by paragraph (a)(4) of this section.

(i) *Hematology.* Hematology determinations must be made as follows: For nonrodents shortly before the beginning of dosing, at least every 30 days thereafter, and at the termination of the testing period; and for rodents, shortly before the beginning of dosing.

(a) Test No. 2 uses rodent as the animal, price based on rat.

at an intermediate time, and at the termination of the testing period. The following hematology determinations must be made: Hematocrit, hemoglobin, erythrocyte count, total and differential leukocyte counts, platelet count, and, if signs of anemia are present, reticulocyte count.

(ii) *Blood chemistry.* Blood chemistry determinations must be performed as follows: For nonrodents, shortly before the beginning of dosing, at least every 30 days thereafter, and at the termination of the testing period; and for rodents, shortly before the beginning of dosing, at an intermediate time, and at the termination of the study. Nonrodents must be fasted for 1 day prior to obtaining blood samples. The following determinations must be made: Calcium, potassium, serum lactic dehydrogenase, serum glutamic pyruvic transaminase, serum glutamic oxaloacetic transaminase, glucose, blood urea nitrogen, direct and total bilirubin, serum alkaline phosphatase, total cholesterol, albumin, globulin, total protein, and such other determinations as may be necessary for adequate toxicological evaluation. The following determinations may also be useful: Chloride, uric acid, blood creatinine, and gamma-glutamyl transpeptidase.

(iii) *Cholinesterase inhibition tests.* If the test substance contains a carbamate, an organophosphate, or any chemical that produces acetyl cholinesterase inhibition, the enzyme activity for plasma and red blood cell must be monitored shortly before the beginning of dosing, at least twice during the study, and at the end of the study, and the enzyme activity for brain must be monitored at the termination of the study. In addition, when nonrodents are used, monitoring of the enzyme activity must be performed twice before the beginning of dosing. Additionally, serial determinations may be useful to provide data on time-course of development of inhibition, extent of inhibition, and recovery from inhibition (e.g., after removal from treated diet); the undertaking of such determinations should not, however, result in over-stress of the test animals.

(iv) *Urinalysis.* Urinalysis must be performed as follows: for rodents, at least once (at an intermediate time) during the testing period, and again at the termination of the testing period; and for nonrodents, shortly before the beginning of dosing, every 60 days thereafter, and at the termination of the test. Nonrodents must be fasted 1 day prior to collection of urine samples. Each animal must be evaluated individually. The urinalysis must include specific gravity or osmolarity, pH, protein, glucose, ketones, bilirubin, and urobilinogen, as well as microscopic examination of formed elements. Results of these determinations must be expressed in quantitative terms by appropriate grading scales.

(v) *Additional tests.* Depending on the known or suspected properties of the test substance, such other determinations as may be necessary for adequate toxicological evaluation must be performed.

(3) *Handling of moribund and dead animals.* (i) *Moribund animals.* Moribund animals must be sacrificed to lessen the likelihood of unobserved death and subsequent autolysis or cannibalism.

(ii) *Tissue loss and dead animals.* Requirements concerning tissue loss and the handling of dead animals are specified in Subpart A, § 772.100–2(b) (6) and (7), respectively.

(4) *Gross necropsy.* (i) The standards set for necropsy procedures in § 772.100–2(b)(7), Subpart A, must apply.

(ii) All test animals in the study must be subjected to gross necropsy, which must include examination of the external surface; all orifices; the cranial cavity; carcass; the external and cut surfaces of the brain and spinal cord; the thoracic, abdominal and pelvic cavities and their viscera; and the cervical tissues and organs.

(iii) In addition, the following organs must be weighed: Liver, kidneys, heart, gonads, and brain. Also, for nonrodents, thyroid (with parathyroid), adrenals, and pituitary must be weighed. Prior to being weighed, organs must be carefully dissected and properly trimmed to remove fat and other contiguous tissue in a uniform manner. They must be weighed as soon as possible after dissection to avoid drying.

(iv) The gross necropsy findings must be recorded and reported in accordance with paragraph (g)(3) of this section.

(v) Tissue samples must be preserved and held in accordance with § 772.110–1(j), Subpart B.

(5) *Histopathology examination.* (i) *General.* A histopathology examination shall be performed on the organs and tissues of all animals in accordance with this paragraph.

(ii) *Nonrodents.* The following organs and tissues, when present, of each test animal must be subjected to microscopic study: all gross lesions, brain (at least 3 levels from the forebrain, midbrain, and hindbrain), spinal cord (at least 2 levels), eye, pituitary, salivary gland, heart, thymus, thyroid with parathyroid, lungs with mainstem bronchi, trachea, esophagus, stomach, small and large intestines, adrenals, pancreas, liver, gall bladder, kidneys, urinary bladder, aorta, testes, prostate, ovaries, corpus and cervix uteri, spleen, a representative lymph node, bone (with marrow), skeletal muscle, skin, sciatic nerve, and mammary gland. Sites from which bone and lymph nodes are taken must be indicated.

(iii) *Rodents.* The following organs and tissues of each test animal must be subjected to microscopic study:

(A) *All animals in control and high dose groups.* All gross lesions, brain (at least 3 levels), eye, pituitary, salivary gland, heart, thymus, thyroid (with parathyroid), lungs with mainstem bronchi, trachea, esophagus, stomach, small and large intestines, adrenals, pancreas, liver, kidneys, urinary bladder, testes, prostate, ovaries, corpus and cervix uteri, spleen, bone (with marrow), and skeletal muscle. Section of bone (with marrow, when present) should be taken from sternebrae, vertebrae, or the tibio-femoral joint (the last will also include attached muscle).

(B) *All animals in intermediate and low dosage groups.* Liver, kidney, heart, any gross lesion, and any target organ either at the high dose or from laboratory tests or clinical observation at any treatment level.

(iv) Tissue and slide preparation and retention.

(A) The standards set forth in § 772.100–2(b)(7)(ii), Subpart A apply.

(B) Tissue samples, tissue blocks, and microscopic slides must be preserved and held in accordance with § 772.110–1(j), Subpart B.

(v) *Examiner.* The standards set forth in § 772.100–2(b)(1)(i), Subpart A apply.

(vi) *Records.* The histopathology findings must be recorded and reported as required by paragraph (c)(4) of this section.

(c) *Data reporting and evaluation.* In addition to the general reporting requirements of § 772.100–2(b)(8), Subpart A, a subchronic oral dosing study test report must contain the following information, presented in the format specified (unless adequate justification is supplied to present these data in another form):

(1) Animal records and clinical laboratory data. The following information must be arranged by test group (dose level and sex). All means must be accompanied by standard deviation.

(i) Significant time periods, for individual animals. In tabular form, data must be provided showing, for each animal:

(A) Its identification number;

(B) Whether it died by sacrifice, and if so, whether it was moribund before sacrifice;

(C) Its age at the beginning of the study;

(D) The week of the test when sacrifice occurred or the animal's death was noted; and

(E) Its age at death.

(ii) Variation from requirements, for individual animals. In tabular form, data must be provided showing, for each animal that was not subjected to gross necropsy and histopathology examination in accordance with requirements of this section:

(A) Its identification number;

(B) The manner of variation; and

(C) The reasons for failure to comply with the requirements of this section.

(iii) Toxic, pharmacologic, and behavioral effects for individual animals. In tabular form, data must be provided showing, for each animal:

(A) Its identification number;

(B) The date of observation of each sign of toxicity, pharmacological effect, or behavioral abnormality; and

(C) A description of the toxic sign, pharmacological effect, or behavioral abnormality. If such a response occurs repeatedly, it need be described only once and may thereafter be described by reference, with any variations noted as appropriate.

(iv) Toxic, pharmacologic, and behavioral effects for test animals. In tabular form, data must be provided showing, for each test group (dose level and sex):

(A) A list of each sign of toxicity, pharmacological effect, or behavioral abnormality affecting any animal in the test group;

(B) For each sign, effect, or abnormality, the number of animals affected;

(C) For each sign, effect, or abnormality, the median time from the beginning of the study to the first observation of such response; and

(D) The median age at death of animals not sacrificed.

(v) Food and body weight data, for individual animals. In tabular form, data must be provided showing, for each animal:

(A) Its identification number;

(B) Measured food consumption at weekly intervals throughout the test period; and

(C) Body weight measured weekly throughout the test period.

(vi) Food and body weight data, means. In tabular and graphic form, data must be provided showing, for each test group (dose level and sex):

(A) Mean measured food consumption at weekly intervals throughout the test period; and

(B) Mean body weight measured weekly during the test period.

(vii) Weekly survival and sacrifice data. In tabular form, data must be provided showing: the number of animals in each group which remained alive at the end of each 7-day interval, the number of animals in each group that were sacrificed or otherwise died during each 7-day interval, and the number that died by sacrifice and were moribund before sacrifice.

(viii) Clinical laboratory test protocol.

(A) The rationale for timing of the clinical laboratory tests, if different from the standards set forth in paragraph (d) of this section; and

(B) The method and rationale for selecting animals for the clinical laboratory tests.

(ix) Clinical laboratory testing, for each animal. In any appropriate form, data must be submitted showing, for each animal:

(A) Its identification number; and

(B) The results of any hematological, blood chemistry, cholinesterase inhibition, urinalysis, and other clinical laboratory tests performed.

(x) *Clinical laboratory testing, for each test group.* In any appropriate form, data must be submitted showing, for each test group (dose level and sex), the average of the results of each hematologic, blood chemical, cholinesterase inhibition, urinalysis, and other clinical laboratory test performed.

(2) *Gross necropsy.* For all means in the data required in this subparagraph, the standard deviation must be stated. The following test information, arranged by test groups (dose level and sex), must be supplied in tabular form:

(i) Data showing the identification number of any animal in which any gross abnormality or gross lesion was noted, and containing, for each such animal, a description of each gross abnormality (including measurements), and the date (if known) when it was first observed. Gross abnormalities observed repeatedly need by described only once and may thereafter be described by reference, with any variations noted, as necessary.

(ii) Data showing the number of animals in which any type of gross abnormality was observed.

(iii) Data showing, for each animal: Its identification number, weights of the organs listed under paragraph (b)(4) of this section, and corresponding organ-to-body weight ratios.

(iv) Data showing the mean weights of each type of organ listed under paragraph (b)(4) of this section, and mean organ-to-body weight ratios.

(3) *Histopathology data.* The following information must be arranged by test group (dose level and sex). All means must be accompanied by standard deviation. The number of data units on which a calculation is based must be reported for all percentages and means.

(i) *Descriptions of lesions, for each animal.* Data must be submitted in any appropriate form showing:

(A) For each animal, its identification number, and a complete description and diagnosis of every lesion in the animal. Nonneoplastic lesions which are observed frequently or which are common in both treated and control animals should be graded. (Descriptions of neoplasms may also include grading.) A commonly used scale such as ±, 1, 2, 3, and 4, for degrees ranging from very slight to extreme can be used, but other scales are acceptable. If known, the description and diagnosis should identify any lesion which caused the animal to be moribund or to die. The description and diagnosis must include the time of appearance (if any) for each lesion. Abnormalities observed repeatedly need to be described only once, and may subsequently be supplied by reference, with any individual variations noted as necessary.

(B) For each animal, a table or paragraph listing the tissues found to be normal.

(C) If a grading system is used, a description of the system.

(ii) *Counts and incidence of lesions, by test groups.* Data must be submitted in tabular form showing, for each test group:

(A) The number of animals at the start of the test, and the number of animals in which any lesion was found;

(B) The number of animals affected by each different type of lesion, the average grade of each type of lesion, the number of animals examined for each type of lesion, and the percentage of those animals examined which were affected by each type of lesion; and

(C) The number of each different type of lesion.

(iii) *Incidence of tumors.* If a tumor is observed in any animal, the report must include a complete description and diagnosis of each tumor as required in § 772.113-1(k)(2)(i)(D), Subpart D.

(4) *Evaluation of data.* An evaluation of the test results (including their statistical analysis), based on clinical findings, gross necropsy findings, and histopathology results, must be made and supplied. This submission must include an evaluation of 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, gross and histopathologic lesions, organ weight changes, effects on mortality, and any other toxic effects. The evaluation must also include dose-response curves for any toxic or pharmacologic effect which appear to be compound-related for the various groups, and a description of statistical methods.

PROTOCOL

Test No. 3: Chronic Oral Toxicity Study (772.113-3), Rodent[a]

§ 772.113-3 Non-oncogenic chronic effects test standards.

(a) *Study design.* (1) *Species and strains.* (i) The tester must use at least two mammalian species: one, a laboratory rat and the second, a nonrodent. The Agency recommends the dog as the nonrodent species. The tester may utilize other suitable nonrodent species approved by EPA.

Note.—Selection of the most appropriate nonrodent species should be predicated upon such factors as metabolism, pharmacokinetics, sensitivity or organotropism and other considerations pertinent to the study.

(ii) The sponsor or tester must select the specific strains and/or stocks of test animals to be used. Test animals must be from established strains and/or stocks. As part of the study plan submission, the sponsor must present the rationale for selection of the specific test animals along with historical data on their lifespans and disease types and incidences.

(2) *Sex.* The tester must use equal numbers of males and females at each dose level.

(3) *Age at start of test.* The tester must begin to dose as soon as possible after weaning and environmental acclimatization, but no later than six weeks of age for rodents and at ten weeks of age for dogs. For nonrodent species, other than the dog, the Agency must approve the age of initial exposure.

(4) *Group size.* Each "test group" of rodents must contain at least 50 animals (plus at least 8 additional for clinical laboratory testing). If the nonrodent species is the dog, then each group must contain at least six animals. The tester must assign animals to individual test groups by a specified randomization procedure. When the study plan calls for interim kill, the tester must increase the number of animals in each group at the start of the study by the number scheduled to be killed before completion of the study. If species other than the laboratory dog and rat are selected, EPA must approve the number of animals per group.

(5) *Control groups.* A tester must use a matched control group which is identical in every respect to the exposed groups except for exposure to the test substance.

Note (i).—If a vehicle is administered to the matched control group and if its toxic properties are not known, the tester may, at his/her discretion, use a negative or untreated control group.

Note (ii).—The EPA may require a Positive Control Group for particular chemicals when the sensitivity of the test animal to the chemical class to which the test substance belongs cannot be documented. When used, the positive control group should serve as an internal quality control to ascertain whether the test animals are sensitive to or respond in a predictable manner to known toxic agents and whether the test strain or species reacts similarly to another strain or species when exposed to the same known standard toxicant.

(6) *Route(s) of administration.* To the extent possible, route(s) of administration should be comparable to the expected or known routes of human exposure. Test rules in Part 771 will specify the route(s) to be employed for a particular chemical. For inhalation and dermal studies, Part 771 will also specify the specific conditions for administering the test substance.

(7) *Frequency of exposure.* The tester must administer test substance and vehicle, if any, by the same route and at the same frequency for the duration of the study. For gavage, the test substance must be administered daily; for feeding, *ad libitum;* for inhalation exposure, a minimum of 5 days per week, 6 hours per day; and for dermal exposure, as specified in the applicable test rule. For gavage, the tester must conduct the dosing at approximately the same time each day.

(8) *Duration of treatment and observation periods.* The tester must administer the test substance to rats for at least 30 months. In studies with nonrodents, the tester must test for at least 2 years unless the Agency authorizes specific exceptions.

(9) *Dose levels and dose selection.* (i) The tester must select doses to permit analysis of dose-response relationships and the "no observable effect level" (NOEL).

(A) A minimum of three dose levels (in addition to controls) in each sex of each species must be used.

(B) The highest dose level must demonstrate toxicologic effects. Mortality in rat groups must not exceed 50 percent before 18 months. Mortality in nonrodent groups must be kept to a minimum but significant toxicologic effect must also be demonstrated in the species.

(C) The lowest dose level must be selected to produce no observable evidence of toxicity other than tumors (NOEL).

(D) The sponsor or tester may add additional dose levels at his/her own discretion. If other dose levels are tested, the sponsor must submit the data from any such discretionary levels to the Agency along with that of the required levels.

(ii) The tester must conduct a preliminary toxicology study of at least 90 days to select the chronic dose levels which meet the requirements of this section. A preliminary toxicology study of at least 90 days that has been completed previously may be submitted for this purpose.

(iii) The sponsor must submit the rationale for dose selection including supporting data from preliminary toxicity studies as a part of the study plan submission.

(a) Test No. 3 uses rodent as the animal, price is based on rat.

(b) *Study conduct.* (1) *Clinical procedures.* A veterinarian, as specified in § 772.113–1(e)(2), must ascertain and be responsible for the health status and care of all test animals during the study. A technical employee, as specified in § 772.113–1(e)(3)(ii), must be responsible for the daily observations and care of the test animals.

(i) *Observation of animals.* (A) Each test animal must be identified by a specific identification number. The tester must account for all animals at the end of the study. The tester must establish and adhere to standard operating procedures for housing, feeding, handling, and care of test animals as specified in § 772.110–1. To further assure minimal loss of animals due to cannibalism or autolysis of tissue, technical employees, as specified above, must observe the test animals at least every 12 hours throughout the test period. EPA may consider a study to be unacceptable for purposes of satisfying a test rule requirement if losses in any test group exceed 5 percent.

(B) Technical employees must conduct routine clinical examinations on all test animals. Clinical examination must include weighing of each animal, at approximately the same time of day, at least once a week during the first 13 weeks, and every two weeks thereafter and observing all animals in relation to food and water consumption, morbidity, mortality and causes thereof, loss of animals for whatever reason, signs of toxicity, pharmacologic effects, and behavioral changes. The observer must record all data in detail at the time of observation.

(ii) *Clinical laboratory testing.* The tester must conduct the following quantitative determinations on a minimum of eight additional predesignated rats in each test group. For nonrodents, all animals in each test group must be utilized. In addition to the tests listed below, if any interim clinical observations suggest that other tests are necessary to assess the health status of test animals, the appropriate tests must be conducted.

Note.—Predesignated means that the animal has been selected to undergo these tests by a specified randomization procedure prior to initiation of the study.

(A) *Hematology.* The tester must conduct the following quantitative hematologic determinations at least at 3, 6, 12, 18, 24 months and at study termination: hematocrit, hemoglobin, erythrocyte count, total and differential leukocyte counts, platelet count, and prothrombin and clotting times. If hematologic evidence of anemia is present, reticulocyte counts must be performed within one week of the determination.

(B) *Blood chemistry.* The tester must conduct the following quantitative blood chemistry determinations at least at 3, 6, 12, 18, 24 months and at study termination: calcium, sodium, potassium, chloride, serum lactic dehydrogenase, serum glutamic pyruvic transaminase, creatinine kinase, serum glutamic oxaloacetic transaminase, glucose, blood urea nitrogen, creatinine, direct and total bilirubin, cholinesterase, total cholesterol, triglycerides, serum alkaline phosphatase, albumin, globulin, and total protein. In addition to these tests, the tester may conduct other quantitative blood chemistry determinations at his/her discretion, such as uric acid, gammaglutamyl transpeptidase, and ornithine carbamoyltransferase.

(C) *Urinalysis.* The tester must conduct the following quantitative determinations at least at 3, 6, 12, 18, 24 months and at study termination: specific gravity or osmolarity, pH, protein, glucose, ketones, bilirubin, and urobilinogen, as well as microscopic examination and analysis of formed elements. Each animal's urine must be evaluated individually.

Note.—*Additional Tests.* Based on results of concurrent or previous studies on the test substance, its metabolic or degradation products, the tester should conduct such other determinations as may be necessary for adequate toxicological evaluation.

(D) *Function tests.* (1) The tester must determine the functional capacity of the renal, hepatic, pulmonary, and cardiovascular systems.

Note.—Additional determinations must place major emphasis on organ or system function tests. Selection of the appropriate tests must be based upon the findings in the subchronic studies or observations made during the course of present study.

(2) Additional quantitative determinations may include, but not necessarily be limited to the following: water dilution and water concentration tests for renal function; total lung capacity, functional residual capacity, and residual volume for pulmonary function; bromsulphalein excretion test for liver function; electrocardiogram, blood pressure, and exercise recovery for cardiovascular function. The tester must perform these evaluations at the beginning (nonrodent only) and at least every 3, 6, 12, 18, 24 months and at study termination.

(E) *Residue analysis.* The tester must measure levels of test substance, major metabolites or other biologically significant metabolites at 3, 6, 12, 18, and 24 months ± 1 month and at the termination of the study. Tissues from the predesignated animals analyzed should include all target tissues from prechronic toxicology studies and those suggested by pharmaco-kinetic studies. These analyses must include at least plasma, 24-hour urine, feces and, at time of death or scheduled killing, liver and kidney.

(iii) *Interim kill.* The tester may kill predesignated animals (other than those predesignated for hematological tests in paragraph (b)(1)(ii)(A) of this section at any time during the study, provided that he/she increases the number of animals started in the study at least by the number scheduled or anticipated to be killed before the end of the study.

(iv) *Killing of test animals.* Animals which appear during the study as moribund, injured, or weak, and not expected to survive to the next observation, must be killed to preclude the loss of tissues from cannibalism and/or autolysis. Animals surviving to the termination of the study must also be killed. A technical employee must obtain blood samples for hematologic determinations from each animal immediately before it is killed or as it is killed. The method used for killing must be humane and the same throughout the study. The tester must select a method of killing which will not produce interfering pathologic lesions.

(2) *Pathology procedures.* A Board-Certified or Board-Eligible pathologist, as specified in § 772.113–1(e)(1)(i), must be responsible for the planning and conduct of all pathology procedures and histopathology examination, as well as for the final interpretation of all pathology data. Other doctorate pathologists, as specified in § 772.113–1(e)(1)(ii), are also acceptable for conducting procedures in their disciplines of specialization, under the direct supervision of a Board-Certified or Board-Eligible pathologist as specified in § 772.113–1(e)(1)(i).

Note.—Direct supervision means that the supervisor is immediately available for consultation, as necessary. This consultation may be done in person or by telephone.

(I) *Gross necropsy.* (A) Qualified pathologists, as specified § 772.113–1(e)(1), must perform or personaally supervise the necropsies. Other appropriately trained technical employees, as specified in § 772.113–1(e)(3)(i) may assist in the necropsy.

Note.—Personal supervision means that the supervisor is immediately available for consultation at the site.

(B) Animals must be necropsied as soon as possible after death but no later than 16 hours after death. If necropsy cannot be performed immediately after the animal is killed or found dead, a technical employee must immediately refrigerate (but not freeze) the animal at temperatures low enough to minimize tissue autolysis (4–8° C). Animals found dead upon routine clinical examination must be necropsied as soon as possible to salvage usable tissues.

(C) The gross necropsy must include an initial physical examination of the external surfaces and all orifices followed by an internal examination of tissues and organs *in situ*. The examination must include the following: external and internal portions of all hollow organs; cranial cavity and externa' surfaces of the brain and spinal cord; nasal cavity and paranasal sinuses; neck with its associated organs and tissues; thoracic, abdominal, and pelvic cavities with their associated organs and tissues; and the muscular/skeletal carcass. The urinary bladder and lungs must be inflated with a proper fixative to allow for better gross examination and preservation.

(D) The weights of the heart, liver, kidneys, testes, spleen, lung, brain, and adrenals must be recorded after careful dissection and trimming. In addition, the thyroid (with parathyroids) and pituitary must be weighed for each nonrodent. The person responsible for the gross necropsy must record all gross necreopsy findings in accordance with § 772.113–1(k)(2).

(ii) *Tissue preservation.* A technical employee must immediately preserve all tissues and organs from all test animals in 10 percent buffered formalin or another recognized and accepted fixative appropriate for the specific tissue(s). Sections from the following tissues from all test animals regardless of their time of death must be properly preserved for routine microscopic examination:

(A) All gross lesions (with a margin of normal tissue);

(B) Brain (minimum of one section each from the forebrain, midbrain, and hindbrain);

(C) Spinal cord (minimum of one section each from cervical, thoracic, and lumbar regions);

(D) Eyes and contiguous Harderian glands;

(E) Pituitary gland;

(F) Major salivary glands, thymus, thyroid with parathyroid, mammary glands, Zymbal's gland (if present);

(G) Oral mucous membrane (including random sections from tongue, buccal, and alveolar mucosa, pharynx, and nasopharynx);

(H) Heart and aorta (three sections from different locations);

(I) Trachea; lungs, with the mainstem bronchi;

(J) Esophagus, stomach, small intestines and large intestine (cecum, colon, and rectum);

(K) Adrenal glands, pancreas, liver (minimum of two lobes), gall bladder (if present), spleen;

(L) Kidneys, urinary bladder;

(M) Representative lymph nodes (including those draining any neoplasm and those with gross changes);

(N) Bone including marrow, from the sternum vertebra and/or tibiofemoral joint;

(O) Skin (sections from similar sites of all animals);

(P) Skeletal muscle;

(Q) For males: testes, prostate, and all other accessory sex organs;

(R) For females: vagina, corpus and cervix uteri, ovaries, and fallopian tubes.

(iii) *Preparation of tissue for microscopic examination.* A pathologist or a technical employee, as specified in § 772.113–1(e)(3)(i), must prepare all specimens for microscopic examination.

(A) *Tissue fixation and trimming.* The technical employee must fix tissues for the appropriate times for the fixative utilized. A pathologist must perform or directly and personally supervise tissue trimming. Routinely, tissues must be trimmed to a thickness of no more than 0.4 cm for subsequent processing. Parenchymal organs must be trimmed to allow for the largest surface areas possible for subsequent microscopic examination. Hollow organs must be trimmed to allow for a cross section mount from mucosa to serosa. Lymph nodes must be bisected through the hilus, if possible.

(B) *Slide preparation.* A technical employee must cut tissues routinely at a thickness of three to six micra (3 to 6 μ), in no case exceeding 10 μ. All tissues must be stained routinely with hematoxylin and eosin (H&E). EPA encourages the use of special stains appropriate to the specific neoplasm, lesion, or tissue. Multiple sections (step cuts) must be made on each tissue or organ that contains gross evidence of a neoplasm or lesion and on each tissue or organ in which a metastasis may be aniticipated. The tester must identify all blocks and microscopic slides by reference to the animal's specific identification number and must preserve and hold them in accordance with § 772.110–1(j)(2).

(iv) *Microscopic examination and evaluation.* (A) Qualified pathologists as described in § 772.113–1(e)(1), must perform the microscopic examination and evaluation with subsequent diagnosis. The same pathologist must examine and evaluate all microscopic slides from all test animals of a given species.

(B) Microscopic examination must be performed on all appropriate tissues described in paragraphs (b)(2)(ii), (b)(2)(v), and (b)(2)(vi) of this section. The pathologist must record, document, and report all microscopic findings including all abnormalities, lesions, neoplasms, metastatic tumors and their anatomic location in accordance with § 772.113–1(k)(2).

(v) *Additional Examinations.* All adverse health effects observed during the course of the study must be examined. When there is clinical evidence of specific toxicologic or pharmacologic effects related to specific target organs, the necropsy and microscopic examinations of the suspected target organs must be conducted in greater detail. For example, when there is clinical evidence of neurologic effects, multiple sections from brain, spinal cord, and nerves must be examined.

(vi) *Special Examinations.* (A) Additional sections must be microscopically examined from a minimum of ten rodent animals selected randomly from the long-term survivors and all nonrodent animals of each test group and in all animals in which clinical or grossly observable evidence of disease is present. If microscopic examination reveals evidence of disease in any of these tissues, then these target tissues must be examined in all test animals.

(B) The necropsy and microscopic examination must include, in addition to those tissues listed in paragraph (b)(2)(ii) of this section, the following:

(*1*) *In a feeding study:* nasal cavity; paranasal sinuses; nasopharynx.

(*2*) *In an inhalation study:* multiple sections of the upper respiratory tract: nares; nasal cavity; paranasal sinuses; hypopharynx-larynx.

(*3*) *In a dermal study:* skin (normal); skin from sites of skin painting.

PROTOCOL

Special Test No. 3: Oncogenic Effects Oral Study (772.113-2), Rodent[a]

§ 772.113-2 Oncogenic effects test standards.

(a) *Study design.* (1) *Species and Strain.* (i) The tester must use at least two rodent species, the laboratory mouse and rat. An alternative species may be used if the sponsor can provide sufficient data and/or rationale to demonstrate that it is a more appropriate species for a specific test substance. The sponsor must present such data and/or rationale for Agency approval as a part of the study plan submission.

(ii) The sponsor or tester must select the specific strains and/or stocks of test animals to be used. Established strains and/or stocks which are expected to be sensitive to the test substance must be used. As part of the study plan submission, the sponsor must present the rationale for selection of the specific test animals. This must include a summary of any prior test results with the selected species, historical data on their lifespans, spontaneous diseases and conditions (including tumors) and their incidences.

Note.—Acceptable rationale for alternate species would be results from prior oncogenicity studies which show that the alternative species is sensitive to the oncogenic effects of the chemical class to which the test substance belongs or that the alternate species has similar metabolism or pharmacokinetics to humans.

(2) *Sex.* The tester must use equal numbers of males and females at each dose level.

(3) *Age at start of test.* The tester must begin to dose rodents as soon as possible after weaning and environmental acclimatization, by no later than six (6) weeks of age. For nonrodents, the Agency must approve the age of initial exposure.

(4) *Group size.* Each "test group" of rats or mice must contain at least 50 animals. The tester must assign animals to individual test groups by a specified randomization procedure. When the study plan calls for interim kill, the tester must increase the number of animals in each group at the start of the study by the number scheduled to be killed before completion of the study. If species other than the laboratory mouse and rat are selected, EPA must approve the number of animals per group.

(5) *Control groups.* A tester must use a matched control group which is identical in every respect to the exposed groups except for exposure to the test substance.

Note (i).—*If a vehicle is administered to the matched control group and if its toxic properties are not known, the tester may, at his/her discretion, use a negative or untreated control group.*

Note (ii).—The EPA may require a Positive Control Group for particular chemicals when the sensitivity of the test animal to the chemical class to which the test substance belongs cannot be documented. When used, the positive control group should serve as an internal quality control to ascertain whether the test animals are sensitive to or respond in a predictable manner to known toxic agents and whether the test strain or species reacts similarly to another strain or species when exposed to the same known standard toxicant.

(6) *Route(s) of administration.* To the extent possible, route(s) of administration should be comparable to the expected or known routes of human exposure. The test rules in Part 771 will specify the route(s) to be employed for a particular chemical. For inhalation and dermal studies, Part 771 will also specify the specific conditions for administering the test substance.

(7) *Frequency of exposure.* The tester must administer test substance and vehicle, if any, by the same route and at the same frequency for the duration of the study. For gavage, the test substance must be administered daily; for feeding, *ad libitum;* for inhalation exposure, a minimum of 5 days per week, 6 hours per day; and for dermal exposure, as specified in the applicable test rule. The tester must conduct the dosing at approximately the same time each day.

(8) *Duration of treatment and observation periods.* The tester must administer the test substance to rodent species for a minimum of 24 months but no longer than 30 months. If a nonrodent species is used, the Agency must approve the duration of exposure.

(9) *Dose levels and dose selection.* (i) The tester must provide data from at least three dose levels (in addition to controls) in each sex of each species.

(A) The high dose level must be the maximum level that can be administered for the duration of the test period, with demonstrable but only slight toxicity, and no substantial reduction in longevity due to effects other than tumors. Signs of demonstrable, slight toxicity are a weight decrement not to exceed 10 percent compared to appropriate controls, clinical signs of toxicity, or pathologic lesions other than those related to a neoplastic response.

(B) The intermediate dose level must be some fraction (¼ to ½) of the high dose level.

(C) The lowest dose level must be some fraction (½ or less) of the intermediate dose level but not less than 10 percent of the high dose level.

(D) The sponsor or tester may add additional dose levels at his/her own discretion. If other dose levels are tested, the sponsor must submit the data from any such discretionary levels to Agency along with that of the required levels.

(ii) The tester must conduct a preliminary toxicology study of at least 90 days to select the chronic dose levels which will meet the requirements in this subsection. A preliminary toxicology study of at least 90 days that has been completed previously may be submitted for this purpose.

(iii) The sponsor must submit the rationale for dose selection including supporting data from preliminary toxicity studies as a part of the plan submission.

(b) *Study conduct.* (1) *Clinical procedures.* Veterinarians, as specified in § 772.113-1(e)(2), must ascertain and be responsible for the health status and care of all test animals prior to and during the study. Technical employees, as specified in § 772.113-1(e)(3)(ii), must be responsible for the daily observations and care of test animals.

(a) Special Test No. 3 uses rodent as the animal, price is based on rat.

(i) *Observation of Animals.* (A) Each test animal must be identified by a specific identification number. The tester must account for all animals at the end of the study. The tester must establish and adhere to standard operating procedures for housing, feeding, handling, and care of test animals as specified in § 772.110–1, Subpart B. To further assure minimal loss of animals due to cannibalism or autolysis of tissue, technical employees, as specified above, must observe the test animals every 12 hours throughout the test period. EPA may consider a study to be unacceptable for purposes of satisfying a test rule requirement if losses in any test group exceed 5 percent.

(B) Technical employees must conduct routine clinical examinations on each animal. These clinical examinations must include weighing of each animal, approximately the same time of day, at least once a week during the first 13 weeks, and every two weeks thereafter, and observing animals in relation to food and water consumption, morbidity, mortality and causes thereof, loss of animals for whatever reason, signs to toxicity, pharmacologic effects, and behavioral changes. The observer must record all data in detail at the time of observation.

(ii) *Hematology.* The tester must conduct the following quantitative hematologic determinations on a minimum of eight predesignated animals in each test group at one year (± one month) and at termination: hematocrit, hemoglobin, erythrocyte count, total and differential leukocyte counts, platelet count, and prothrombin and clotting times. If hematologic evidence of anemia is present at one year, reticulocyte counts must be performed within one week of the determination. In addition to the tests listed above, if any interim clinical observations suggest that other tests are necessary to assess the health status of test animals, the appropriate tests must be conducted. In the event that any of the predesignated animals does not survive 12 months, another animal selected by statistical randomization from the remainder of the appropriate test group can serve as a replacement.

Note.—Predesignated means that the animal has been selected to undergo this test by a specified randomization procedure prior to initiation of the study.

(iii) *Interim kill.* The tester may kill predesignated animals (other than those predesignated for hematological test in paragraph (b)(1)(ii) of this section at any time during the study, provided that he (she) increased the number of animals started in the study at least by the number scheduled or anticipated to be killed before the end of the study.

(iv) *Killing of test animals.* Animals which appear during the study as moribund, injured, or weak, and not expected to survive to the next observation, must be killed to preclude the loss of tissues from cannibalism and/or autolysis. Animals surviving to the termination of the study must also be killed. A technical employee must obtain blood samples for hemotologic determinations from each animal immediately before it is killed or as it is killed. The method used for killing must be humane and the same throughout the study. The tester must select a method of killing which will not produce interfering pathologic lesions.

(2) *Pathology procedures.* A Board-certified or Board-eligible pathologist, as specified in § 772.113–1(e)(1)(i), must be responsible for the planning and conduct of all pathology procedures and histopathology examinations, as well as for the final interpretation of all pathology data. Other doctorate pathologists, as specified in § 772.113–1(e)(1)(ii), are also acceptable for conducting procedures in their disciplines of specialization, under the direct supervision of a Board-Certified or Board-Eligible pathologist as specified in § 772.113–1(e)(1)(i).

Note.—Direct supervision means that the supervisor is immediately available for consultation, as necessary. This consultation may be done in person or by telephone.

(i) *Gross necropsy.* (A) Qualified pathologists, as specified in § 772.113–1(e)(2)(1), must perform or personally supervise the necropsies. Other appropriately trained technical employees, as specified in § 772.113–1(e)(3)(i), may assist in the necropsy.

Note.—Personal supervision means that the supervisor is immediately available for consultation at the site.

(B) Animals must be necropsied as soon as possible after death but no later than 15 hours after death. If necropsy cannot be performed immediately after the animal is killed or found dead, a technical employee must immediately

refrigerate (but not freeze) the animal at temperatures low enough to minimize tissue autolysis (4–8° C). Animals found dead upon routine clinical examination must be necropsied as soon as possible to salvage useable tissues.

(C) The gross necropsy must include an initial physical examination of the external surfaces and all orifices followed by an internal examination of tissues and organs *in situ*. The examination must include the following: external and internal portions of all hollow organs; cranial cavity and external surfaces of the brain and spinal cord; nasal cavity and paranasal sinuses; neck with its associated organs and tissues; thoracic, abdominal, and pelvic cavities with their associate organs and tissues; and the muscular/skeletal carcass. The urinary bladder and lungs must be inflated with a proper fixative to allow for better gross examination and preservation.

(D) The person responsible for the gross necropsy must record all gross necropsy findings in accordance with § 772.113–1 (k)(2).

(ii) *Tissue preservation.* A technical employee must immediately preserve all tissues and organs from all test animals in ten percent (10%) buffered formalin or another recognized and accepted fixative appropriate for the specific tissue(s). Sections from the following tissues from all test animals regardless of their time of death must be properly preserved for routine microscopic examination:

(A) All gross lesions (with a margin of normal tissue);

(B) Brain (minimum of one section each from the forebrain, midbrain, and hindbrain);

(C) Eyes and contiguous Harderian glands;

(D) Pituitary gland;

(E) Major salivary glands, thymus, thyroid with parathyroid, mammary glands; Zymbal's gland (if present);

(F) Oral mucous membrane (including random sections from tongue, buccal, and alveolar mucosa, pharynx, and nasopharynx);

(G) Heart and aorta;

(H) Trachea; lungs with the mainstem bronchi;

(I) Esophagus, stomach, small intestines and large intestine (cecum, colon, and rectum);

(J) Adrenal glands, pancreas, liver (minimum of two lobes), gall bladder (if present), spleen;

(K) Kidneys, urinary bladder;

(L) Representative lumph nodes (including those draining any neoplasm and those with gross changes);

(M) Bone including marrow from the sternum, vertebra and/or tibiofemoral joint;

(N) Skeletal muscle;

(O) For males: testes, prostate, and all other accessory sex organs;

(P) For females: vagina, corpus and cervix uteri, ovaries, and fallopian tubes.

(iii) *Preparation of tissue for microscopic examination.* A pathologist or a technical employee, as specified in § 772.113–1(e)(3)(i), must prepare all specimens for microscopic examination.

(A) *Tissue fixation and trimming.* A technical employee must fix tissues for the appropriate time for the fixative utilized. A pathologist must perform or directly and personally supervise tissue trimming. Routinely, tissues must be trimmed to a thickness of no more than 0.4 cm for subsequent processing. Parenchymal organs must be trimmed to allow for the largest surface area possible for subequent microscopic examination. Hollow organs must be trimmed to allow for a cross section mount from mucosa to serosa. Lymph nodes must be bisected through the hilus, if possible.

(B) *Slide preparation.* A technical employee must cut tissues routinely at a thickness of three to six micra (3 to 6 μ), in no case exceeding 10μ. All tissues must be stained routinely with hematoxylin and eosin (H&E). EPA encourages the use of special stains appropriate to the specific neoplasm, lesion, or tissue. Multiple sections (step cuts) must be made on each tissue or organ that contains gross evidence of a neoplasm or lesion and on each tissue or organ in which a metastasis may be anticipated. The tester must identify all blocks and microscopic slides by references to the animal's specific identification number and must preserve and hold them in accordance with § 772.110–1(j)(2).

(iv) *Microscopic examination and evaluation.* (A) Qualified pathologists as described in § 772.113–1(e)(1), must perform the microscopic examination and evaluation with subsequent diagnosis. The same pathologist must examine and evaluate all microscopic slides from all test animals of a given species.

(B) Microscopic examination must be performed on all tissues described in paragraphs (b)(2)(ii), (b)(2)(v), and (b)(2)(vi) of this section. The pathologist must record, document, and report all microscopic findings including all abnormalities, lesions, neoplasms, metastatic tumors and their anatomic locations in accordance with § 772.113–1(k)(2).

(v) *Additional examination.* All adverse health effects, including non-oncogenic effects, observed during the course of the study must be examined. When there is clinical evidence suggesting specific toxicologic or pharmacologic effects, including non-oncogenic effects, the necropsy and microscopic examinations of the suspected target organs must be conducted in greater detail. For example, when there is clinical evidence of neurologic effects, multiple sections from brain, spinal cord, and nerves must be examined.

(vi) *Special examinations.* (A) Additional sections, as specified below, must be microscopically examined from a minimum of ten animals selected randomly from the long-term survivors of each test group and in all animals in which clinical or grossly observable evidence of disease is present. If microscopic examination reveals evidence of disease in any of the tissues, then these target tissues must be examined in all test animals.

(B) The necropsy and microscopic examinations must include, in addition to those tissues listed in paragraph (b)(2)(ii) of this section, the following:

(1) *In a feeding study:* nasal cavity; paranasal sinuses; nasopharynx.

(2) *In an inhalation study:* multiple sections of the upper respiratory tract: nares; nasal cavity; paranasal sinuses; hypopharynx-larynx.

(3) *In a dermal study:* skin (normal); skin from sites of skin painting.

PROTOCOL

Special Test No. 3: Reproductive Effects Study (772.116-3), Rodent[a]

§ 772.116-3 Reproductive effects test standards.

(a) *Study design.* (1) *Species.* Testing must be performed in at least one mammalian species which may be the same as one of the two species used in the teratogenic effects study pursuant to § 772.116-2 of this subpart. The rat is preferred.

(2) *Number and sex of animals.* In testing with rodents, each dose and control group must contain enough females to produce approximately 20 litters (20 sampling units) at each breeding, assuming typical mating and fertility for the strain. At least 10 fertile males per dose in the first mating of the F generation must be used. Subsequently at least 10 males per dose level are required.

(3) *Number of doses and dose selection.* (i) At least three dose level groups, in addition to the control group, must be tested.

(ii) The highest dose level must produce an observable toxicological or pharmacological effect in the test animals, but not cause more than 10 percent fatalities. This level must be higher than that expected for human exposure.

(iii) The lowest dose level must produce no observable adverse effects.

(4) *Control group.* Concurrent control groups are required as follows:

(i) A vehicle control group is required if a vehicle is used in administering the test substance. If there are insufficient data on the toxic properties of the vehicle used in administering the test substance, a separate control group receiving no chemical treatment is also required.

(ii) If no vehicle is used in administering the test substance, a separate control group receiving a sham treatment (e.g., physiological saline) is required.

(5) *Route of administration.* To the extent possible, route(s) of administration should be comparable to the expected or known routes of human exposure. The test rules in Part 771 will specify the route(s) to be employed for a particular chemical.

(6) *Duration of testing.* (i) The test substance must be administered to two generations of animals, F_0 and F_1. A third generation of animals, F_2, will be exposed to the test substance *in utero* and through nursing.

(ii) Dosing of animals in the F_0 generation must begin as soon as possible after weaning and acclimatization, and in any case before the animals are 6 weeks old. The test substance must be administered daily to the F_0 generation. Dosing must continue until all F_1 generation animals have been weaned.

(iii) Dosing of the animals selected from the F_1 generation for breeding must begin as soon as the animals are weaned (approximately 30 days after birth). The test substance must be administered daily to these animals, dosing must continue until 30 days after all F_2 animals have been weaned. (Dosing of animals from F_1 generation is not required if they have not been selected for breeding).

(b) *Study conduct.* (1) *Breeding.* After the F_0 generation animals such as rodents and lagomorphs have received the test substance for at least 100 days, they must be bred to produce the F_1 generation. Appropriate numbers of males and females must be selected at random from different litters of the F_1 generation for breeding. After the test substance has been administered to these animals for at least 120 days, they must be bred to produce the F_2 generation. Figure 1 of the Appendix indicates an acceptable breeding and dosing schedule.

(2) *Animal care.* Pregnant females must be caged separately and furnished with nesting materials.

(3) *Observations.* The requirements for observation of animals as specified in Subpart A, § 772.100-2(b)(6) apply to Subpart F.

(i) *Frequency.* Each animal must be observed for effects as long as it is being exposed to the test substance. Animals must be observed as frequently as necessary to obtain the data required by paragraph (c) of this section and Subpart A Section 772.100-2 (b)(6).

(ii) *Growth and delivery data.* The weight of each weanling must be recorded weekly to weight maturity and monthly thereafter. The dates of delivery must be recorded.

(iii) *Maternal data.* Observation must be made of the general condition and behavior of mothers, including nesting and nursing. Any abnormalities must be recorded.

(iv) *Paternal data.* Measurments must be made of spermatogenesis of all males in the F_0, F_1, and F_2 generations used to produce the subsequent generations. Such measurements should be undertaken with.1 one week after breeding. In addition, or as an alternative, histopathology examinations of the tests, as indicated in paragraph (c)(11) of this section, must be undertaken. Additional useful information may be obtained by histopathology examinations of the tests of males in the F_0 and F_2 generations, particularly those males used for producing the subsequent generations. If *spermatogenesis or histopathology of* tests is evaluated in males in the F_0 and F_2 generations, such males should be of the same approximate age and should have been dosed for the same approximate length of time as males used in the F_1 generation (at the time the F_2 generation males were examined).

(v) *Litter data.* All litters must be examined as soon as possible after delivery. Where possible, effort should be made to prevent cannibalism of young. The following must be recorded: Litter size: number of stillborn; and number of live births. Viability counts and pup weight must be recorded at birth, four days after birth, and weaning. Additional viability counts between the fourth day and weaning are required for non-rodents. Any physical or behavioral abnormalities must be recorded.

(4) *Gross necropsy and histopathology.* (i) *F_1 generation.* Ten males and 25 females from each dose level and the control group must be subjected to a complete gross necropsy

(a) Special Test No. 3 uses rodent as the animal, price is based on rat.

and histopathology examination. The animals must be chosen from the F_1 generation animals used to produce the F_2 generation. The animals must be sacrificed at the end of the required period of dosing. The necropsy and histopathology examination must include examination of the reproductive organs.

(ii) *F_1 and F_2 generation.* A complete gross necropsy and histopathology examination must be conducted on five randomly selected weanlings of each sex from each test group (dose level and sex) in each generation (F_1 and F_2).

(iii) *Conduct of examinations.* All examinations must be conducted by or under the supervision of a qualified pathologist. The standards set forth in § 772.100–2(b)(1) and (7), Subpart A Apply.

(c) *Data reporting and evaluation.* The tester must submit to EPA the following reports:

"Study Plan" as required in § 772.100–2(b)(2), Subpart A;

"Interim Quarterly Summary Reports" outlining the current status of the study including any significant findings; and a "Final Test Report".

In addition to the basic information required by § 772.100–2(b)(8), Subpart A, the "Final Test Report" must include the following information, presented in the format specified:

(1) *Test protocol.* (i) The rationale for species and strain selection; and

(ii) The rationale for selection for the dosage levels; dosage levels must be reported as mg/kg/day as well as ppm.

(2) *Animal data.* For all means in the data required in this subparagraph, such means must be accompanied by the standard deviation.

(i) *Female data.* The following information relating to the reproduction of each female must be supplied in tables, with footnotes and description where appropriate:

(A) For each animal: Date of delivery; and unusual or abnormal behavior during estrous, gestation, or delivery; and fertility.

(B) Cumulative data showing means for controls and each dose level group in the F_0 and F_1 generation: the gestation index; approximate duration of gestation; and number and percent of animals showing behavioral abnormalities in connection with reproductive activity.

(C) For each mother: Its identification number; any abnormalities in nesting or nursing; total number of offspring per litter; number and percent of live and dead offspring; and general condition of offspring and mother through weaning.

(D) For each dose level and control group in the F_0 and F_1 generation: The fertility index; average size of litter; average number of dead and live offspring per litter; and number and percent of mothers showing behavioral abnormalities in nesting and nursing.

(ii) *Male data.* For each male evaluated for spermatogenesis in accordance with paragraph (b)(3)(iv) of this section: identification number and the results of the evaluation.

(iii) *Litter data on preweanling animals.* The following litter data on preweanling animals must be supplied in tables, with footnotes and descriptions where appropriate:

(A) For each litter arranged by dose level and generation: Total litter size; number and percent of stillborn; number and percent of live births; viability index; lactation index; weekly viability counts and weekly weight of each pup from day 4 of life to weaning; and number and nature of physical abnormalities observed.

(B) For each dose level and generation: Mean weekly weight of all pups from day 4 of life to weaning; number and percent of pups with physical or behavioral abnormalities; number and percent of pups surviving at birth, 1 week, and 3 weeks; and mean viability and lactation indices.

(iv) *Litter data on postweanling and mature animals.* the following informaiton, arranged by test group (dose level and sex), must be supplied in tabular form (unless adequate justification is supplied to present these data in another form):

(A) For each animal: Its identification number; its age at the beginning of the study; its age at death and manner of death; and its weight, as measured weekly through 1 month of age and monthly thereafter.

(B) Cumulative data showing means for each control and test group: The weekly or monthly weights; and the number and percent of animals with behavioral abnormalities.

(3) *Gross necropsy data.* The following test information, arranged by test groups (dose level and sex) must be reported:

(i) Data showing the identification of any animal for which any gross abnormality or lesion was observed, and containing for each such animal a description of each abnormality or lesion. Gross abnormalities or lesions observed repeatedly in gross necropsies need be described only once and thereafter may be described by reference.

(ii) Data showing the number of animals affeced by each type of abnormality or lesion; and the number of animals in which any abnormality or lesion was observed.

(4) *Evaluation.* (i) Evaluation of the results with respect to all toxic or pharmacological effects, including:

(A) An evaluation of the relationships, if any, between exposures to the test substance and the incidence and severity of effects (including effects on reproduction, behavior, tumors and lesions, and mortality).

(B) An indication of the dosage level at which no toxic effects attributable to the test substance would appear.

(ii) Statistical analyses must be performed to assist in the reporting and evaluation of data. All statistical methods used must be identified by reference and/or fully described.

PROTOCOL

Special Test No. 3: Teratogenic Effects Study (772.116-2), Rodent[(a)]

§ 772.116-2 Teratogenic effects test standards.

(a) *Study design.* (1) *Species and strain.* Testing must be performed in at least two mammalian species. The rat, mouse, hamster, or rabbit are acceptable. Other species may be used if adequate justification is supplied. One species must be the same as the species used in the reproductive study. Strains with low fecundity must not be used. Historical teratogenic data for the specific strain tested must be submitted.

(2) *Sex and age.* All test and control animals must be young, mature, pregnant females of uniform age, size, and parity. Prima gravida females are preferred.

(3) *Control groups.* Concurrent control group(s) are required as follows:

(i) A positive control group is required, unless historical data from the laboratory performing the test are submitted which demonstrate that the strains of animals being used are sensitive to known teratogenic agents.

(ii) A vehicle control group is required if a vehicle is used in administering the test substance. In addition, if there are insufficient data on toxic properties of the vehicle used in administering the test substance, an untreated (negative) control group receiving a sham treatment (e.g., physiological saline) is also required.

(iii) If no vehicle is used in administering the test substance, a separate control group receiving a sham treatment (e.g., physiological saline) is required.

(4) *Number of animals.* Each test and control group must include 20 or more pregnant females for rat, mouse, and hamster, and at least 12 pregnant females for rabbit.

(5) *Duration of test and time of delivery.* (i) The test subtance must be administered daily beginning at, or before, the time of implantation and continuing through the period of major organogenesis. Exposure of each species must encompass the gestation period up to the day before term.

(ii) Fetuses must be delivered by cesarean section approximately 1 day prior to term.

(iii) Females (parents) must ordinarily be sacrificed at time of cesarean section unless conditions indicate earlier sacrifice as required by paragraph (ii) of this section.

(6) *Dosage.* (i) At least three dosage levels must be tested in addition to the control(s).

(ii) The highest dosage level must induce some fetal or maternal toxicity, as demonstrated by body weight reduction or other toxic signs, but not cause more than 10 percent maternal fatalities. This level must be higher than that expected for human exposure.

(iii) The intermediate dose(s) must be spaced logarithmically (or at some approximate comparable point) between the high and low dosage level and must induce some observable fetal effects attributable to the test substance, when possible.

(iv) The low dosage level must induce no observable adverse effects attributable to the test substance.

(v) The dose administered to each animal must be based on the individual animal's body weight on the first day of test substance administration.

(vi) Dosing must be scheduled at approximately the same time during the day.

(7) *Route of administration.* To the extent possible, route(s) of administration should be comparable to the expected or known routes of human exposure. The test rules in Part 771 will specify the route(s) to be employed for a particular chemical.

(b) *Study conduct.* (1) *Animal care.* Food and water must be provided *ad libitum.* Pregnant females must be provided nesting materials or justification for not providing such material must be submitted. Animals may be individually caged or group caged.

(2) *Observation.* (i) The requirements for observation of animals as specified in Subpart A, § 772.100-2(b)(6) apply to Subpart F. Each such observation must be made by an appropriately-trained observer, who must note and record behavioral abnormalities, and all clinical signs of toxicity, including mortality.

(ii) Any female showing signs of abortion or premature delivery must be sacrificed on the day such evidence is observed. These animals must be analyzed, and all observations reported separately.

(iii) Females must be weighed at the first day of test substance administration and at sacrifice.

(3) *Necropsy.* (i) Immediately after the female is sacrificed, the uterus must be excised and weighed, then examined for fetal resorption, number of live fetuses and number of dead or resorbed fetuses. The litter weight of live fetuses must be determined.

(ii) One-half of each litter must be examined for skeletal anomalies, and the remaining one-half of each litter must be examined for soft tissue anomalies.

(a) Special Test No. 3 must be performed in two rodents, price is based on a single species rat study.

(iii) External and soft tissue examination of the fetuses must be performed by or under the supervision of an individual experienced and suitably trained interatogenic studies. The sex of each fetus must be determined, if possible. Gross observations of the skeleton and external and internal organs must be made with the aid of a dissecting microscope (or other instrument providing similar magnification). The internal gross morphology must be examined by sectioning through soft tissues (using razor blade sectioning or comparable techniques).

(iv) The necropsy data must be recorded and reported in accordance with paragraph (3) of this section.

(v) Entire fetuses must be preserved and held in accordance with § 772.110–1(j), Subpart B.

(c) *Data reporting and evaluation.* In addition to the basic information required by § 772.100–2(b)(8), Subpart A, the final test report must include the following information, presented in the format specified:

(1) *Test protocol.* Rationale for selection of the species and strain used.

(i) Dose levels (expressed as mg/kg of body weight per day) administered, and the rationale for their selection; and the number of days of tests substance administration.

(ii) Route and method of administration utilized and the rationale for selection if other than oral intubation.

(iii) Positive control data or historical data from the laboratory performing the test which demonstrate the sensitivity of the strains being used.

(iv) Justification statement for not providing nesting materials for pregnanat females, if such materials were not provided.

(2) *Maternal data.* (i) The following information, arranged by test groups, must be supplied in tabular form:

(A) Data showing, for each animal:

(*1*) Its identification number;

(*2*) Its age (or approximate age) at the start of the test;

(*3*) Data of caesarian section and sacrifice;

(*4*) Body weight on first day of dosing;

(*5*) Its body weight at sacrifice (actual, and corrected (by subtracting gravid uterus));

(*6*) The body weight change based on the foregoing weight measurements; and

(*7*) Any signs of abortion or premature delivery.

(B) Data showing, for each dose level:

(*1*) The number of animals initially on study;

(*2*) The number and percent that died;

(*3*) The number and percent that were pregnant; and

(*4*) The average maternal body weight change.

(ii) The following test information must be supplied in any appropriate form: A description of all observed signs of toxicity accompanied by the animal's identification number, test group, and date(s) of observation.

(3) *Fetal data.* When an anomaly is difficult to describe, a photograph of it may be submitted. All means must be accompanied by standard deviation. The following information arranged by test group must be supplied in tabular form:

(i) Numerical data showing, for each litter:

(A) Identification numbers;

(B) Number and percent of live fetuses;

(C) Average live fetal weight;

(D) Number of each sex, if determined;

(E) Number and percent of dead and resorbed fetuses;

(F) Number of implantations; and

(G) Number and percent of fetuses with any soft tissue of skeletal abnormality.

(ii) Anomaly data showing, for each litter:

(A) Identification number(s);

(B) Number of fetuses examined by necropsy;

(C) Number and percent of fetuses having soft tissue anomalies;

(D) Number of fetuses examined for skeletal anomalies;

(E) Number and percent of fetuses having skeletal anomalies; and

(F) Incidence and a full description of each type of anomaly.

(iii) Cumulative data showing, for each dose level:

(A) Identification of the dose level group;

(B) Number of litters examined;

(C) Number of implantations per litter;

(D) Average number of live fetuses per litter;

(E) Average of live fetal weights;

(F) Percent of dead and resorbed fetuses per litter;

(G) Number and percent of fetuses bearing anomalies of each kind observed;

(H) Number and percent of fetuses bearing any anomaly;

(I) Number and percent of abnormal fetuses per litter; and

(J) Number and percent of litters having anomalous fetuses.

(4) *Evaluation.* The litter or dam is an accepted unit for evaluation. Data on individual fetuses with anomalies should also be considered.

(i) Evaluation of the results with respect to observed effects, must include:

(A) An evaluation of the relationship, if any, between exposure to the test substance and the anomalies and all other toxic signs observed; and

(B) An indication of the dosage level at which no toxic effects attributable to the test substance would appear.

(ii) Statistical analyses must be performed to assist in the reporting and evaluation of data. All statistical methods used should be identified by reference and/or fully described.

PROTOCOL

Special Test No. 3: Combined Chronic Toxicity and Oncogenic Effects Oral Study, Rodent[a]

(No standard protocol available)

(a) Special Test No. 3 uses rodent as the animal, price is based on rat.

PROTOCOL

Special Test No. 3: Combined Reproduction/Teratogenic Effects Study, Rodent[a]

(No standard protocol available)

(a) Special Test No. 3 uses rodent as animal, price is based on rat.

PROTOCOL

Test No. 4: Acute Inhalation Toxicity Study (772.112-23), Rodent[(a)]

§ 772.112-23 Acute inhalation toxicity study.

(a) *Study design.* (1) *Species, sex, and age.* Testing must be performed with the laboratory rat. Young adult male and female animals must be used.

(2) *Number of animals and selection of dose levels.* (i) A trial test is recommended for the purpose of establishing a dosing regimen which must include one dose level higher than the expected LC_{50} and at least one dose level below the expected LC_{50}. If data based on testing with at least 5 animals per sex are submitted showing that no toxicity is evident at 5 mg/1. no further testing at other dose levels is necessary. If mortality is produced. the requirements of paragraph (a)(2)(ii) of this section apply.

(ii) The number of animals per dose level. and the number and the spacing of dose levles must be chosen to produce test groups with mortality rates between 10 percent and 90 percent. and to permit calculation of the LC_{50} with a 95 percent confidence limit of 20 percent or less. At least 4 dose levels of the test substance. in addition to controls. must be tested. Though the group sizes may vary for each dose level. the group must contain an equal number of male and female animals.

(3) *Duration of test.* In selecting the exposure period. allowance must be made for changed concentration equilibration time. Where there is no difficulty in maintaining a steady concentration of the test substance in the chamber(s). the exposure period must be at least 1 hour. Where there is some difficulty in maintaining a study concentration the exposure period must last up to 4 hours. The animals must be observed for 14 days. or until all signs of reversible toxicity subside. whichever occurs later.

(4) *Use of solvent.* A solvent may be added to the test substance. if necessary. to help generate an exposure atmosphere. If a product's labeling instructions specify the use of a particular solvent. that solvent is preferred. If no solvent is specified in the product's labeling instructions. the solvent. if any. which is used to formulate the product should be used.

(5) *Control groups.* (i) A concurrent untreated control group is required.

(ii) If any solvent. other than water. is used in generating the exposure atmosphere. a vehicle control group must be tested. The vehicle control group must be exposed to an atmosphere containing the greatest concentration of solvent present in any test system.

(b) *Study conduct.* (1) *Exposure chamber design and operation.*

(i) Inhalation exposure techniques described in this section are based on the use of whole-body inhalation chambers which allow the experimental animals to receive whole-body dermal exposure and possible large oral exposure. as well as the exposure by inhalation. In some cases. the investigators will want to use other inhalation exposure techniques involving face masks. head-only exposure. intratracheal instillation. or other similar techniques which reduce or preclude added dermal and oral exposures. Some alternative techniques are described by Phalen. 1976. When alternative techniques are used. the procedures and results must be reported in a manner similar to that required with the use of whole-body inhalation chambers.

(ii) Animals must be tested in a dynamic air flow exposure chamber. The chamber design must be chosen to enable production of an evenly distributed exposure atmosphere throughout the chamber. The chamber design also should minimize crowding of the test animals and maximize their exposure to the test substance.

(2) *Operation measurements.* The following measurements must be taken with care to avoid major fluctuations in the air concentrations or major discrepancies in the operation of the chambers.

(i) *Air flow.* The rate of air flow through the chamber must be measured continuously.

(ii) *Chamber concentrations.* (A) Nominal concentrations must be calculated for each run by dividing the amount of the test substance used for the generating system by the air flowing through the chamber during the

(a) Test No. 4 uses rodent as the animal, price is based on rat.

(B) Actual chamber concentrations must be determined by samples of chamber air taken near to the breathing zone of the animals as frequently as necessary to obtain an averaged integrated external exposure which is representative of the entire exposure period. The system used to generate the vapor, gas, or aerosol should be such that the chamber concentrations and particle size distributions are controlled under stable conditions, reflecting the current state-of-the-art, and should not vary in a range greater than 30 percent of the average (range/mean equal to or less than 30 percent).

(iii) *Temperature and Humidity.* The temperature must be maintained at 24 ± 2° C. and the humidity within the chamber at 40–60 percent. Both must be monitored continuously.

(iv) *Oxygen.* The rate of air flow through the chamber must be adjusted to insure that the oxygen content of exposure atmosphere is at least 19 percent.

(v) *Particle Size Measurement.* (A) *General.* In the case of gases and vapors, particulate sampling should be carried out at intervals to insure the animals are not being exposed to unknown and unexpected particulate materials. Aerosol particle size measurements should be made on samples taken at the breathing level of the animals. These analyses should be carried out using techniques and equipment reflective of the state-of-the-art. All of the suspended aerosol (on a gravimetric basis) should be accounted for, even when most of the aerosol is not respirable.

(B) *Sizing Analysis.* The sizing analysis should be in terms of equivalent aerodynamic diameters and should be represented as geometric mean (median) diameters and their geometric standard deviations (see NIOSH syllabus in the Appendix to this section), as calculated from log probability graphs or computer programs. The size analyses should be carried out frequently during the development of the generating system to insure proper stability of aerosol particles, and only as often thereafter during the exposure as is necessary to determine adequately the consistency of particle distributions to which the animals are exposed, maintaining at least 20 percent of the particles at 10 microns or less. At a minimum, these analyses should be carried out once per hour for each level of exposure for gaseous test substances, twice per hour for liquid test substances, and 4 times per hour for dusts and powders.

(3) *Observation.* The animals must be observed frequently during the day of dosing and checked at least every 12 hours throughout the test period, for at least 14 days after dosing or until all signs of reversible toxicity subside, whichever occurs later. The following must be recorded: Nature, onset, severity, and duration of all gross or visible toxic or pharmacologic effects, i.e., abnormal or unusual cardiovascular, respiratory, excretory, behavioral, or other activity, as well as signs indicating an adverse effect on the central nervous system (paralysis, lack of coordination, staggering); pupillary reactions; and time of death. The weight of each animal must be determined on the day of dosing, 2, 3, 4, 7, and 14 days after dosing, weekly thereafter, and at death.

(4) *Sacrifice and Necropsy.* All animals living at the termination of the observation period must be sacrificed. All test animals, whether dying by sacrifice or during the test, must be subjected to a complete gross necropsy following their death, in accordance with § 772.100–2(b)(7), Subpart A. Examination must include nasal passages, trachea, bronchi, and lungs, and any other tissues known to be affected by the test substance. All abnormalities must be recorded.

(5) *Preservation of tissues and histopathology examination.* The following are required:

(i) Those tissues designated in paragraph (b)(5)(ii) of this section must be placed in suitable fixative as soon as possible. Tissues and microscopic slides must be prepared according to the standards set forth in § 772.100–2(b)(7)(ii) and (iii), Subpart A. Tissue samples, tissue blocks, and microscopic slides must be preserved and held in accordance with § 772.110–1(j).

(ii) The following tissues must be examined microscopically:

(A) Lungs, liver, and kidneys at all dose levels.

(B) Any tissue or organ that appears abnormal, at any dosage level, as determined in the necropsy examination.

(iii) The histopathology findings must be recorded and reported as required by paragraph (c)(10) of this section.

(c) *Data reporting evaluation.* In addition to information required by § 772.100–2(b)(8), Subpart A, and paragraphs (b)(3) and (b)(4) of this section, the test report must include the following:

(1) Vapor pressure and particulate size (median size with geometric standard deviation).

(2) Description of the chamber design and operation, including type of chamber, its dimensions, the source of makeup air and its conditioning (heating or cooling) for use in the chamber, the treatment of exhausted air, the housing and maintenance of the animals in the chambers, and similar related information. Equipment for measuring temperatures and humidity, the generating system, and the methods of analyzing airborne concentrations and particle sizing must be described:

(3) The following operation data must be tabulated both individually and in summary form, using means and standard deviations (with or without ranges) in tabular form. The data summaries must be grouped according to experimental groups, and nonexpected differences (such as in temperature and airflow) and must be tested for statistical significance.

(i) Airflow rates through the chamber;

(ii) Chamber temperature and humidity;

(iii) Nominal concentrations;

(iv) Actual concentrations; and

(v) Median particle sizes and their geometric standard deviations and percent of particles 10 microns or less.

(4) Tabulation of the response data (number of animals dying per number of animals showing signs of toxicity per number of animals exposed) at each exposure level by sex, and time of death after dosing;

(5) Tabulation of the body weights on the day of dosing, 2, 3, 4, 7, and 14 days after dosing, weekly thereafter, and at death.

(6) The LC_{50} (calculated on an exposure of one hour) for each sex for each test substance;

(7) Specification of the method used for LC_{50} calculation;

(8) The 95 percent confidence interval for the LC_{50};

(9) The dose-response curve and slope (with confidence limits); and

(10) The histopathology findings including a complete record of lesions and abnormalities observed, and the histological diagnosis and characterization of each kind of lesion or abnormality observed, naming those which apparently caused death or morbidity.

PROTOCOL

Test No. 5: Subchronic Inhalation Toxicity Study (772.112-33), Rodent[a]

§ 772.112-33 Subchronic inhalation toxicity study.

(a) *Study design.* (1) *Species and Age.* Testing must be performed on young adult laboratory rats.

(2) *Number and Sex of Test Animals.* A minimum of 10 animals per sex per exposure level must be used. This number must be increased by the number, if any, scheduled to be sacrificed before completion of the study, such as, for example, rats on which hematology and blood chemistry determinations are made before and during the study.

(3) *Number and selection of exposure concentration levels.* (i) At least three exposure concentration levels, in addition to the control(s), must be used.

(ii) The lowest atmospheric concentration must not show toxic effects.

(iii) The highest atmospheric concentration must demonstrate some toxicological effects, but not cause more than 10 percent fatalities. This level should be higher than that expected for human exposure.

(iv) All exposure levels and control(s) must be performed concurrently.

(4) *Duration of testing.* Animals must be exposed to the test substance at least 6 hours per day for at least 5 days per week over a 90-day period. Longer or more continuous exposures may be selected, depending on the test substance and the expected use pattern of the test substance. If shorter or less continuous exposures seem appropriate, the tester must consult with the Agency concerning the exposure times.

(5) *Use of vehicle.* A vehicle may be added to the test substance, if necessary, to help generate an exposure atmosphere. If the product's labeling instructions specify the use of a vehicle, that vehicle is preferred. If no vehicle is specified in the product's labeling instructions, the vehicle, if any, that has been used to formulate the product should be used, if possible.

(6) *Controls.* (i) *Vehicle control.* If any vehicle other than water is used in generating the exposure atmosphere, a concurrent solvent control group is required.

(ii) *Negative control.* A concurrent negative control group is required. These control animals must be treated in the same manner as all other test animals (including placement in exposure chambers), except that this control group must not be exposed to an atmosphere containing the test substance or any solvent.

(b) *Study conduct.* (1) *Exposure chamber design and operation.* Inhalation exposure techniques described in this section are based on the use of whole-body inhalation chambers. In such chambers, the experimental animals receive whole-body dermal exposure and possibly large oral exposure, as well as exposure by inhalation. In some cases, the tester may want to use other inhalation exposure techniques involving face masks, head-only exposures, intratracheal instillation, and other similar techniques which reduce or preclude dermal and oral exposures. Some alternative techniques are described by Phalen, 1976. When alternative techniques are used, the procedures and results must be reported in a manner similar to that required with the use of whole-body inhalation chambers.

(2) *Operational measurements.* The following measurements must be taken, with care to avoid major fluctuations in the air concentrations or major discrepancies in the operation of the chambers:

(a) Test No. 5 uses rodent as the animal, price is based on rat.

(i) *Air flow.* The rates of air flow through the chamber must be measured continuously.

(ii) *Chamber concentrations.* (A) Nominal concentrations must be calculated for each test exposure by dividing the amount of the agent used for the generating system by the air flow through the chamber during the exposure.

(B) Actual concentrations must be determined by samples of chamber air taken near the breathing zone of the animals as frequently as necessary to obtain an averaged integrated external exposure which is representative of the entire exposure period. The system used to generate the vapor, gas, aerosol must be such that the chamber concentrations are controlled under stable conditions, reflecting the current state-of-the-art, and must not vary in a range greater than 30 percent of the average (range/mean equal to or less than 30 percent).

(iii) *Temperature and Humidity.* The temperature must be maintained at 24±2° C and the humidity within the chamber at 40–60 percent. Both must be monitored continuously.

(iv) *Oxygen.* The rate of air flow through the chamber must be adjusted to insure that the oxygen content of the exposure atmosphere is at least 19 percent.

(v) *Particle size measurements.*

(A) *General.* In the case of gases and vapors, particle size measurements must be carried out at intervals to insure the animals are not being exposed to unknown and unexpected materials. Aerosol particle size measurements must be made on samples taken at the breathing level of the animals. These analyses must be carried out using techniques and equipment reflective of the state-of-the-art. All of the suspended aerosol (on a gravimetric basis) must be accounted for, even when most of the aerosol is not respirable.

(b) *Sizing analysis.* The sizing analysis must be in terms of equivalent aerodynamic diameters and must be represented as geometric mean (median) diameters and their geometric standard deviation (see NIOSH syllabus for reference) as calculated from log-probability graphs or computer programs. The size analyses must be carried out frequently during the development of the generating system to insure proper stability of aerosol particles and only as often thereafter during the exposure as is necessary to determine adequately the consistency of particle distributions to which the animals are exposed. At a minimum, these analyses must be carried out a daily basis.

(3) *Observation of animals.* All toxicological and pharmacological signs must be recorded daily, including their time of onset, intensity, and duration. Observations must be made at least 12 hours throughout the test period and, in particular, at the times the animals are exposed to the test substance. (Also see Subpart A, § 772.100–2(b)(6).) Such signs include, but are not limited to: Mortality; and cardiovascular, respiratory, excretory, behavioral, and central nervous system (paralysis, ataxia, and pupillary reaction) effects. Observations must be made by an appropriately trained observer. Food consumption must be measured weekly during the test, and the animals must be weighed at least weekly. Surveillance of animals must be made according to the requirements stated in Subpart A, § 772.100–2(b)(6).

(4) *Clincial laboratory testing.* The following determinations must be made at the times indicated below for each type of testing. These determinations must be made on at least five animals of each sex in each group. Depending on the technique used, it may be necessary to sacrifice animals to make the required clinical determinations. In case of such sacrifice, the number of animals started in the study must be increased by the number scheduled or anticipated to be killed before the end of the study.

(i) *Hematology.* The following hematology determinations must be made on at least five animals of each sex in each group at the beginning (before dosing), at an intermediate time, and at the termination of the testing period: Hematocrit, hemoglobin, erythrocyte count, total and differential leukocyte counts, platelet count, and if signs of anemia are present, reticulocyte count.

(ii) *Blood Chemistry.* Blood chemistry determinations must be performed at the beginning (before dosing), at an intermediate time, and at the termination of the study. The following determinations must be made: Calcium, potassium, serum lactic dehydrogenase, serum glutamic pyruvic transaminases, serum glutamic oxaloacetic transaminase, glucose, blood urea nitrogen, direct and total bilirubin, total cholesterol, serum alkaline phosphatase, albumin globulin, total protein, and such other determinations as may be necessary for adequate toxicological evaluation. The following additional determinations may also be useful: Chloride, uric acid, blood creatinine; and gammaglutamyl transpeptidase.

(iii) *Cholinesterase inhibition tests.* If the test substance contains a carbamate, an organophosphate, or any chemical that produces acetyl cholinesterase inhibition, the enzyme activity for plasma and red blood cell must be monitored twice before treatment, twice during treatment, and at the termination of the study, and the enzyme activity for brain at the termination of the study.

(iv) *Additional tests.* Additional tests such a blood pH, blood CO_2, specific enzyme analyses, and pulmonary function tests should be carried out in order to conform the diagnosis of suspected disease states or to help to follow the development of disease states known to occur with exposure to the test substance(s).

(5) *Handling of moribund and dead animals.* (i) *Moribund animals.* Moribund animals must be sacrificed to lessen the likelihood of unobserved death and subsequent autolysis or cannibalism.

(ii) *Tissue loss and dead animals.* Requirements concerning tissue loss and the handling of dead animals are specified in Subpart A, § 772.100–2(b) (6) and (7), respectively.

(6) *Gross necropsy.* (i) the standards set forth for necropsy procedures in § 772.100–2(b)(7)(i) must apply.

(ii) All animals in this study must be subjected to gross necropsy, which must include examination of the external surface; all orifices; the cranial cavity, carcass; the external and cut surfaces of the brain; spinal cord; the thoracic, abdominal, and pelvic cavities, and their viscera; and the cervical tissues and organs. The following organs and tissues must be examined for gross lesions: Adrenals, heart, lungs, trachea, bronchi, nasal passages and paranasal sinuses, spleen, liver, kidneys, stomach, small and large intestines, pancreas, ovary and uterus, testes with epididymis, prostate, urinary bladder, eye, bone (with marrow), and skin. Tissues in which any gross lesions are seen must be preserved for microscopic study.

(iii) Special treatment of the lung must be undertaken for morphological evaluation of the development of emphysema. Thus, the lungs must be removed *in toto*, weighed, and perfused intratracheally or intrabronchially (depending on the species) with an amount of 10 percent neutral buffered formalin that is equal to approximately 75 percent of the total lung capacity for that species. A maximum of 25 cc of water should be used for perfusion.

(iv) In addition, the organs which must be weighed include the brain, liver, kidneys, and heart. Prior to being weighed, organs must be carefully dissected and properly trimmed to remove fat and other contiguous tissue in a uniform manner. They must be

weighed as soon as possible after dissection to avoid drying.

(v) The gross necropsy findings must be recorded and reported in accordance with paragraph (c)(4) of this Section.

(vi) Tissue samples must be preserved and held in accordance with § 772.110–1(j).

(7) *Histopathology examination.* (i) To the extent indicated below in paragraphs (A) and (B), the following tissues must be examined microscopically:

(A) In the control and highest dose-level animals: Brain (at least 3 levels from the forebrain, midbrain, and hindbrain), eye, pituitary, salivary gland, thymus, heart, esophagus, lungs (with mainstem bronchi), trachea, nasal passages and paranasal sinuses, liver, stomach, small and large intestines, spleen, kidneys, thyroid (with parathyroid), adrenals, pancreas, urinary bladder, aorta, testes, ovaries, corpus and cervix uteri, bone (with marrow), skeletal muscle, skin, and all other tissues in which lesions were observed at necropsy; and

(B) In all other animals, the lungs, trachea, nasal passages and paranasal sinuses, liver, kidneys, and all tissues in which lesions were seen at necropsy and in which abnormalities were observed during the histopathology examination described in paragraph (b)(7)(i)(A) of this section.

(ii) Tissues and microscopic slides must be prepared according to the standards set forth in § 772.100–2(b)(7)(ii) and (iii), Subpart A. Tissue samples, tissue blocks, and microscopic slides must be preserved and held in accordance with § 772.110–1(j). A qualified pathologist must have final responsibility for the histopathology examination. The standards set forth in § 772.100–2(b)(1)(i), Subpart A, must apply.

(iv) The histopathology findings must be recorded and reported as required by paragraph (c)(5) of this section.

(c) *Data reporting and evaluation.* In addition to information meeting the general reporting requirements of § 772.100–2(b)(8), Subpart A, the test report must contain the following information, presented in the format specified:

(1) *Test conditions.* (i) *Chamber and generating system.* Description of the chamber design and operation including type of chamber, its dimensions, the source of make-up air and its conditioning (heating or cooling) for use in the chamber, the treatment of exhausted air, the housing and maintenance of the animals in the chambers, and similar related information. Equipment for measuring of temperature and humidity, the generating system, and the methods of analyzing airborne concentrations and particle sizing must be described.

(ii) *Exposure data.* The following chamber operational data must be tabulated individually and in summary form using means and standard deviations (with or without ranges) in tabular format. The data summaries must be grouped according to experimental groups, and the non-expected differences (such as temperature or airflow) tested for statistical significance.

(A) Airflow rates through the chamber;

(B) Chamber temperature and humidity;

(C) Nominal concentrations;

(D) Actual concentrations; and

(E) Median particle sizes and their geometric standard deviations.

(2) *Animal Records and Clinical Laboratory Data.* The following information must be arranged by test group (dose level and sex). All means must be accompanied by standard deviation.

(i) Significant Time Periods, for Individual Animals. In tabular form, data must be provided showing, for each animal:

(A) Its identification number;

(B) Whether it died by sacrifice, and if so, whether it was moribund before sacrifice;

(C) Its age at the beginning of study;

(D) The week of the test when sacrifice occurred or the animal's death was noted; and

(E) Its age at death.

(ii) Variation from Requirements, for Individual Animals. In tabular form, data must be provided showing, for each animal that was not subjected to gross necropsy and histopathology examination in accordance with requirements of this section:

(A) Its identification number;

(B) The manner of variation; and

(C) The reasons for failure to comply with the requirements of this section.

(iii) Toxic, Pharmacologic, and Behavioral Effects, for Individual Animals. In tabular form, data must be provided showing, for each animal:

(A) Its identification number;

(B) The date of observation of each sign of toxicity, pharmacological effect, or behavioral abnormality; and

(C) A description of the toxic sign, pharmacological effect, or behavioral abnormality. If such a response occurs repeatedly, it need be described only once and may thereafter be described by reference.

(iv) Toxic, Pharmacologic, and Behavioral Effects, for test Groups. In tabular form, data must be provided showing, for each test group (dose level and sex):

(A) A list of each sign of toxicity, pharmacological effect, or behavioral abnormality affecting any animal in the test group;

(B) For each sign, effect, or abnormality, the number of animals showing such effect, sign, or abnormality;

(C) For each sign, effect, or abnormality, the median time from the beginning of the study to when such response was first observed; and

(D) The median age at death of animals not sacrificed.

(v) Food and Body Weight Data, for Individual Animals. In tabular form, data must be provided showing, for each animal:

(A) Its identification number;

(B) Measured food consumption weekly throughout the test period;

(C) Body weight measured weekly throughout the test period.

(vi) *Food and body weight data, averages.* In tabular and graphic form, data must be provided showing, for each test group (dose level and sex):

(A) Mean measured food consumption weekly throughout the test period; and

(B) Mean body weight measured weekly throughout the test period.

(vii) *Weekly survival and sacrifice data.* In tabular form, data must be provided showing: The number of animals in each group which remained alive at the end of each 7-day interval, the number of animals in each group that were sacrificed or otherwise died during each 7-day interval, and the number that died by sacrifice and were moribund before sacrifice.

(viii) *Clinical laboratory test protocol.*

(A) The rationale for the timing of clinical laboratory test, if different from the standards set forth in paragraph (b)(4) of this section; and

(B) The method and rationale for selecting animals for the clinical laboratory tests.

(ix) *Clinical laboratory testing for each animal.* In any appropriate form, data must be submitted showing, for each animal:

(A) Its identification number; and

(B) The results of any hematological, blood chemistry, cholinesterase inhibition, and other clinical laboratory tests performed.

(x) *Clinical laboratory testing, for each test group.* In any appropriate form, data must be submitted showing, for each test group (dose level and sex), the average of the results of each

hematologic, blood chemical, cholinesterase inhibition, and other clinical laboratory test performed.

(3) *Gross Necropsy data.* For all averages in the data required in this subparagraph, the standard deviation must be stated. The following test information, arranged by test groups (dose level and sex), must be supplied in tabular form:

(i) Data showing the identification number of any animal in which any gross abnormality was noted, and containing, for each such animal, a description of each gross abnormality (including measurements), and the date (if known) when it was first observed. Cross abnormalities observed repeatedly need be described only once and may thereafter be described by reference, with any variations noted, as necessary.

(ii) Data showing the number of animals in which any type of gross abnormality was observed.

(iii) Data showing, for each animal: Its identification number, weights of its organs listed under paragraph (b)(6)(ii) of this section and corresponding organ-to-body weight ratios.

(iv) Data showing the mean weights of each type of organ listed under paragraph (b)(6)(ii) of this section, and mean organ-to-body weight ratios.

(4) *Histopathology data.* The following information must be arranged by test group (dose level and sex). All means must be accompanied by standard deviation. The number of data units on which a calculation is based must be reported for all percentages and means.

(i) Description of Lesions, for each Animal. Data must be submitted in an appropriate form showing:

(A) For each animal, its identification number, and a complete description and diagnosis of every lesion in the animal. Non-neoplastic lesions which are observed frequently or which are common in both treated and control animals must be graded. (Descriptions of neoplasms may also include grading.) A commonly-used scale such as ±1, 2, 3, and 4 for degrees ranging from very slight to extreme can be used, but other scales are also acceptable. If known, the description and diagnosis must identify any lesion which caused the animal to be moribund or to die. The description and diagnosis must include the time of appearance (if known) for each lesion. Abnormalities observed repeatedly need be described only once, and may subsequently be supplied by reference, with any individual variations noted as necessary.

(B) For each animal, a paragraph listing the tissues examined and designation by check mark of those tissues found to be normal.

(C) If a grading system is used, a description of the system.

(ii) Counts and Incidence of Lesions, by Test Groups. Data must be submitted in tabular from showing, for each test group:

(A) The number of animals at the start of the test, the number of animals surviving to the termination of the test, and the number of animals in which any lesion was found;

(B) The number of animals affected by each different type of lesion, the average grade of each type of lesion, the numbers examined for each type of lesion, and the percentage of those animals examined which were affected by each type of lesion; and

(C) The number of each different type of lesion.

(iii) Incidence of Tumors. If a tumor is observed in any animal, the report must include a complete description and diagnosis of each tumor as required in Section 772.113-1(k)(2).

(5) *Evaluation of Data.* An evaluation of the test results (including their statistical analysis), based on clinical findings, gross necropsy findings, and histopathology results, must be made and supplied. This submission must include an evaluation of 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, gross and histopathologic lesions, organ weight changes, effects on mortality, and any other toxic effects. The evaluation must also include dose-response curves for any toxic or pharmacological effect which appear to be compound-related for the various groups, and a description of statistical methods.

PROTOCOL

Special Test No. 5: Subchronic Behavioral Effects Inhalation Study, Rodent[a]

(No standard protocol available)

(a) Special Test No. 5 uses rodent as the animal, price is based on rat.

PROTOCOL

Test No. 6: Chronic Inhalation Toxicity Study (772.113-3), Rodent[(a)]

§ 772.113-3 Non-oncogenic chronic effects test standards.

(a) *Study design.* (1) *Species and strains.* (i) The tester must use at least two mammalian species: one, a laboratory rat and the second, a nonrodent. The Agency recommends the dog as the nonrodent species. The tester may utilize other suitable nonrodent species approved by EPA.

Note.—Selection of the most appropriate nonrodent species should be predicated upon such factors as metabolism, pharmacokinetics, sensitivity or organotropism and other considerations pertinent to the study.

(ii) The sponsor or tester must select the specific strains and/or stocks of test animals to be used. Test animals must be from established strains and/or stocks. As part of the study plan submission, the sponsor must present the rationale for selection of the specific test animals along with historical data on their lifespans and disease types and incidences.

(2) *Sex.* The tester must use equal numbers of males and females at each dose level.

(3) *Age at start of test.* The tester must begin to dose as soon as possible after weaning and environmental acclimatization, but no later than six weeks of age for rodents and at ten weeks of age for dogs. For nonrodent species, other than the dog, the Agency must approve the age of initial exposure.

(4) *Group size.* Each "test group" of rodents must contain at least 50 animals (plus at least 8 additional for clinical laboratory testing). If the nonrodent species is the dog, then each group must contain at least six animals. The tester must assign animals to individual test groups by a specified randomization procedure. When the study plan calls for interim kill, the tester must increase the number of animals in each group at the start of the study by the number scheduled to be killed before completion of the study. If species other than the laboratory dog and rat are selected, EPA must approve the number of animals per group.

(5) *Control groups.* A tester must use a matched control group which is identical in every respect to the exposed groups except for exposure to the test substance.

Note (i).—If a vehicle is administered to the matched control group and if its toxic properties are not known, the tester may, at his/her discretion, use a negative or untreated control group.

Note (ii).—The EPA may require a Positive Control Group for particular chemicals when the sensitivity of the test animal to the chemical class to which the test substance belongs cannot be documented. When used, the positive control group should serve as an internal quality control to ascertain whether the test animals are sensitive to or respond in a predictable manner to known toxic agents and whether the test strain or species reacts similarly to another strain or species when exposed to the same known standard toxicant.

(6) *Route(s) of administration.* To the extent possible, route(s) of administration should be comparable to the expected or known routes of human exposure. Test rules in Part 771 will specify the route(s) to be employed for a particular chemical. For inhalation and dermal studies, Part 771 will also specify the specific conditions for administering the test substance.

(7) *Frequency of exposure.* The tester must administer test substance and vehicle, if any, by the same route and at the same frequency for the duration of the study. For gavage, the test substance must be administered daily; for feeding, *ad libitum;* for inhalation exposure, a minimum of 5 days per week, 6 hours per day; and for dermal exposure, as specified in the applicable test rule. For gavage, the tester must conduct the dosing at approximately the same time each day.

(8) *Duration of treatment and observation periods.* The tester must administer the test substance to rats for at least 30 months. In studies with nonrodents, the tester must test for at least 2 years unless the Agency authorizes specific exceptions.

(9) *Dose levels and dose selection.* (i) The tester must select doses to permit analysis of dose-response relationships and the "no observable effect level" (NOEL).

(A) A minimum of three dose levels (in addition to controls) in each sex of each species must be used.

(B) The highest dose level must demonstrate toxicologic effects. Mortality in rat groups must not exceed 50 percent before 18 months. Mortality in nonrodent groups must be kept to a minimum but significant toxicologic effect must also be demonstrated in the species.

(C) The lowest dose level must be selected to produce no observable evidence of toxicity other than tumors (NOEL).

(D) The sponsor or tester may add additional dose levels at his/her own discretion. If other dose levels are tested, the sponsor must submit the data from any such discretionary levels to the Agency along with that of the required levels.

(ii) The tester must conduct a preliminary toxicology study of at least 90 days to select the chronic dose levels which meet the requirements of this section. A preliminary toxicology study of at least 90 days that has been completed previously may be submitted for this purpose.

(iii) The sponsor must submit the rationale for dose selection including supporting data from preliminary toxicity studies as a part of the study plan submission.

(a) Test No. 6 uses rodent as the animal, price is based on rat.

(b) *Study conduct.* (1) *Clinical procedures.* A veterinarian, as specified in § 772.113–1(e)(2), must ascertain and be responsible for the health status and care of all test animals during the study. A technical employee, as specified in § 772.113–1(e)(3)(ii), must be responsible for the daily observations and care of the test animals.

(i) *Observation of animals.* (A) Each test animal must be identified by a specific identification number. The tester must account for all animals at the end of the study. The tester must establish and adhere to standard operating procedures for housing, feeding, handling, and care of test animals as specified in § 772.110–1. To further assure minimal loss of animals due to cannibalism or autolysis of tissue, technical employees, as specified above, must observe the test animals at least every 12 hours throughout the test period. EPA may consider a study to be unacceptable for purposes of satisfying a test rule requirement if losses in any test group exceed 5 percent.

(B) Technical employees must conduct routine clinical examinations on all test animals. *Clinical examination* must include weighing of each animal, at approximately the same time of day, at least once a week during the first 13 weeks, and every two weeks thereafter and observing all animals in relation to food and water consumption, morbidity, mortality and causes thereof, loss of animals for whatever reason, signs of toxicity, pharmacologic effects, and behavioral changes. The observer must record all data in detail at the time of observation.

(ii) *Clinical laboratory testing.* The tester must conduct the following quantitative determinations on a minimum of eight additional predesignated rats in each test group. For nonrodents, all animals in each test group must be utilized. In addition to the tests listed below, if any interim clinical observations suggest that other tests are necessary to assess the health status of test animals, the appropriate tests must be conducted.

Note.—Predesignated means that the animal has been selected to undergo these tests by a specified randomization procedure prior to initiation of the study.

(A) *Hematology.* The tester must conduct the following quantitative hematologic determinations at least at 3, 6, 12, 18, 24 months and at study termination: hematocrit, hemoglobin, erythrocyte count, total and differential leukocyte counts, platelet count, and prothrombin and clotting times. If hematologic evidence of anemia is present, reticulocyte counts must be performed within one week of the determination.

(B) *Blood chemistry.* The tester must conduct the following quantitative blood chemistry determinations at least at 3, 6, 12, 18, 24 months and at study termination: calcium, sodium, potassium, chloride, serum lactic dehydrogenase, serum glutamic pyruvic transaminase, creatinine kinase, serum glutamic oxaloacetic transaminase, glucose, blood urea nitrogen, creatinine, direct and total bilirubin, cholinesterase, total cholesterol, triglycerides, serum alkaline phosphatase, albumin, globulin, and total protein. In addition to these tests, the tester may conduct other quantitative blood chemistry determinations at his/her discretion, such as uric acid, gammaglutamyl transpeptidase, and ornithine carbamoyltransferase.

(C) *Urinalysis.* The tester must conduct the following quantitative determinations at least at 3, 6, 12, 18, 24 months and at study termination: specific gravity or osmolarity, pH, protein, glucose, ketones, bilirubin, and urobilinogen, as well as microscopic examination and analysis of formed elements. Each animal's urine must be evaluated individually.

Note.—*Additional Tests.* Based on results of concurrent or previous studies on the test substance, its metabolic or degradation products, the tester should conduct such other determinations as may be necessary for adequate toxicological evaluation.

(D) *Function tests.* (1) The tester must determine the functional capacity of the renal, hepatic, pulmonary, and cardiovascular systems.

Note.—Additional determinations must place major emphasis on organ or system function tests. Selection of the appropriate tests must be based upon the findings in the subchronic studies or observations made during the course of present study.

(2) Additional quantitative determinations may include, but not necessarily be limited to the following: water dilution and water concentration tests for renal function; total lung capacity, functional residual capacity, and residual volume for pulmonary function; bromsulphalein excretion test for liver function; electrocardiogram, blood pressure, and exercise recovery for cardiovascular function. The tester must perform these evaluations at the beginning (nonrodent only) and at least every 3, 6, 12, 18, 24 months and at study termination.

(E) *Residue analysis.* The tester must measure levels of test substance, major metabolites or other biologically significant metabolites at 3, 6, 12, 18, and 24 months ± 1 month and at the termination of the study. Tissues from the predesignated animals analyzed should include all target tissues from prechronic toxicology studies and those suggested by pharmaco-kinetic studies. These analyses must include at least plasma, 24-hour urine, feces and, at time of death or scheduled killing, liver and kidney.

(iii) *Interim kill.* The tester may kill predesignated animals (other than those predesignated for hematological tests in paragraph (b)(1)(ii)(A) of this section at any time during the study, provided that he/she increases the number of animals started in the study at least by the number scheduled or anticipated to be killed before the end of the study.

(iv) *Killing of test animals.* Animals which appear during the study as moribund, injured, or weak, and not expected to survive to the next observation, must be killed to preclude the loss of tissues from cannibalism and/or autolysis. Animals surviving to the termination of the study must also be killed. A technical employee must obtain blood samples for hematologic determinations from each animal immediately before it is killed or as it is killed. The method used for killing must be humane and the same throughout the study. The tester must select a method of killing which will not produce interfering pathologic lesions.

(2) *Pathology procedures.* A Board-Certified or Board-Eligible pathologist, as specified in § 772.113–1(e)(1)(i), must be responsible for the planning and conduct of all pathology procedures and histopathology examination, as well as for the final interpretation of all pathology data. Other doctorate pathologists, as specified in § 772.113–1(e)(1)(ii), are also acceptable for conducting procedures in their disciplines of specialization, under the direct supervision of a Board-Certified or Board-Eligible pathologist as specified in § 772.113–1(e)(1)(i).

Note.—Direct supervision means that the supervisor is immediately available for consultation, as necessary. This consultation may be done in person or by telephone.

(i) *Gross necropsy.* (A) Qualified pathologists, as specified § 772.113–1(e)(1), must perform or personaally supervise the necropsies. Other appropriately trained technical employees, as specified in § 772.113–1(e)(3)(i) may assist in the necropsy.

Note.—Personal supervision means that the supervisor is immediately available for consultation at the site.

(B) Animals must be necropsied as soon as possible after death but no later than 16 hours after death. If necropsy cannot be performed immediately after the animal is killed or found dead, a technical employee must immediately refrigerate (but not freeze) the animal at temperatures low enough to minimize tissue autolysis (4–8° C). Animals found dead upon routine clinical examination must be necropsied as soon as possible to salvage usable tissues.

(C) The gross necropsy must include an initial physical examination of the external surfaces and all orifices followed by an internal examination of tissues and organs *in situ. The* examination must include the following: external and internal portions of all hollow organs; cranial cavity and external surfaces of the brain and spinal cord; nasal cavity and paranasal sinuses; neck with its associated organs and tissues; thoracic, abdominal, and pelvic cavities with their associated organs and tissues; and the muscular/skeletal carcass. The urinary bladder and lungs must be inflated with a proper fixative to allow for better gross examination and preservation.

(D) The weights of the heart, liver, kidneys, testes, spleen, lung, brain, and adrenals must be recorded *after careful* dissection and trimming. In addition, the thyroid (with parathyroids) and pituitary must be weighed for each nonrodent. The person responsible for the gross necropsy must record all gross necreopsy findings in accordance with § 772.113–1(k)(2).

(ii) *Tissue preservation.* A technical employee must immediately preserve all tissues and organs from all test animals in 10 percent buffered formalin or *another recognized and accepted* fixative appropriate for the specific tissue(s). Sections from the following tissues from all test animals regardless of their time of death must be properly preserved for routine microscopic examination:

(A) All gross lesions (with a margin of normal tissue);

(B) Brain (minimum of one section each from the forebrain, midbrain, and hindbrain);

(C) Spinal cord (minimum of one section each from cervical, thoracic, and lumbar regions);

(D) Eyes and contiguous Harderian glands;

(E) Pituitary gland;

(F) Major salivary glands, thymus, thyroid with parathyroid, mammary glands, Zymbal's gland (if present);

(G) Oral mucous membrane (including random sections from tongue, buccal, and alveolar mucosa, pharynx, and nasopharynx);

(H) Heart and aorta (three sections from different locations);

(I) Trachea; lungs, with the mainstem bronchi;

(J) Esophagus, stomach, small intestines and large intestine (cecum, colon, and rectum);

(K) Adrenal glands, pancreas, liver (*minimum of two lobes*), gall bladder (if present), spleen;

(L) Kidneys, urinary bladder;

(M) Representative lymph nodes (including those draining any neoplasm and those with gross changes);

(N) Bone including marrow, from the sternum vertebra and/or tibiofemoral joint;

(O) Skin (sections from similar sites of all animals);

(P) Skeletal muscle;

(Q) For males: testes, prostate, and all other accessory sex organs;

(R) For females: vagina, corpus and cervix uteri, ovaries, and fallopian tubes.

(iii) *Preparation of tissue for microscopic examination.* A pathologist or a technical employee, as specified in § 772.113–1(e)(3)(i), must prepare all specimens for microscopic examination.

(A) *Tissue fixation and trimming.* The technical employee must fix tissues for the appropriate times for the fixative utilized. A pathologist must perform or directly and personally supervise tissue trimming. Routinely, tissues must be trimmed to a thickness of no more than 0.4 cm for subsequent processing. Parenchymal organs must be trimmed to allow for the largest surface areas possible for subsequent microscopic examination. Hollow organs must be trimmed to allow for a cross section mount from mucosa to serosa. Lymph nodes must be bisected through the hilus, if possible.

(B) *Slide preparation.* A technical employee must cut tissues routinely at a thickness of three to six micra (3 to 6 μ), in no case exceeding 10 μ. All tissues must be stained routinely with hematoxylin and eosin (H&E). EPA encourages the use of special stains appropriate to the specific neoplasm, lesion, or tissue. Multiple sections (step cuts) must be made on each tissue or organ that contains gross evidence of a neoplasm or lesion and on each tissue or organ in which a metastasis may be aniticipated. The tester must identify all blocks and microscopic slides by reference to the animal's specific identification number and must preserve and hold them in accordance with § 772.110–1(j)(2).

(iv) *Microscopic examination and evaluation.* (A) Qualified pathologists as described in § 772.113–1(e)(1), must perform the microscopic examination and evaluation with subsequent diagnosis. The same pathologist must examine and evaluate all microscopic slides from all test animals of a given species.

(B) Microscopic examination must be performed on all appropriate tissues described in paragraphs (b)(2)(ii), (b)(2)(v), and (b)(2)(vi) of this section. The pathologist must record, document, and report all microscopic findings including all abnormalities, lesions, neoplasms, metastatic tumors and their anatomic location in accordance with § 772.113–1(k)(2).

(v) *Additional Examinations.* All adverse health effects observed during the course of the study must be examined. When there is clinical evidence of specific toxicologic or pharmacologic effects related to specific target organs, the necropsy and microscopic examinations of the suspected target organs must be conducted in greater detail. For example, when there is clinical evidence of neurologic effects, multiple sections from brain, spinal cord, and nerves must be examined.

(vi) *Special Examinations.* (A) Additional sections must be microscopically examined from a minimum of ten rodent animals selected randomly from the long-term survivors and all nonrodent animals of each test group and in all animals in which clinical or grossly observable evidence of disease is present. If microscopic examination reveals evidence of disease in any of these tissues, then these target tissues must be examined in all test animals.

(B) The necropsy and microscopic examination must include, in addition to those tissues listed in paragraph (b)(2)(ii) of this section, the following:

(*1*) *In a feeding study:* nasal cavity; paranasal sinuses; nasopharynx.

(*2*) *In an inhalation study:* multiple sections of the upper respiratory tract: nares; nasal cavity; paranasal sinuses; hypopharynx-larynx.

(*3*) *In a dermal study:* skin (normal); skin from sites of skin painting.

PROTOCOL

Special Test No. 6: Oncogenic Effects Inhalation Study (772.113-2), Rodent[a]

§ 772.113-2 Oncogenic effects test standards.

(a) *Study design.* (1) *Species and Strain.* (i) The tester must use at least two rodent species, the laboratory mouse and rat. An alternative species may be used if the sponsor can provide sufficient data and/or rationale to demonstrate that it is a more appropriate species for a specific test substance. The sponsor must present such data and/or rationale for Agency approval as a part of the study plan submission.

(ii) The sponsor or tester must select the specific strains and/or stocks of test animals to be used. Established strains and/or stocks which are expected to be sensitive to the test substance must be used. As part of the study plan submission, the sponsor must present the rationale for selection of the specific test animals. This must include a summary of any prior test results with the selected species, historical data on their lifespans, spontaneous diseases and conditions (including tumors) and their incidences.

Note.—Acceptable rationale for alternate species would be results from prior oncogenicity studies which show that the alternative species is sensitive to the oncogenic effects of the chemical class to which the test substance belongs or that the alternate species has similar metabolism or pharmacokinetics to humans.

(2) *Sex.* The tester must use equal numbers of males and females at each dose level.

(3) *Age at start of test.* The tester must begin to dose rodents as soon as possible after weaning and environmental acclimatization, by no later than six (6) weeks of age. For nonrodents, the Agency must approve the age of initial exposure.

(4) *Group size.* Each "test group" of rats or mice must contain at least 50 animals. The tester must assign animals to individual test groups by a specified randomization procedure. When the study plan calls for interim kill, the tester must increase the number of animals in each group at the start of the study by the number scheduled to be killed before completion of the study. For species other than the laboratory mouse and rat are selected, EPA must approve the number of animals per group.

(5) *Control groups.* A tester must use a matched control group which is identical in every respect to the exposed groups except for exposure to the test substance.

Note (i).—If a vehicle is administered to the matched control group and if its toxic properties are not known, the tester may, at his/her discretion, use a negative or untreated control group.

Note (ii).—The EPA may require a Positive Control Group for particular chemicals when the sensitivity of the test animal to the chemical class to which the test substance belongs cannot be documented. When used, the positive control group should serve as an internal quality control to ascertain whether the test animals are sensitive to or respond in a predictable manner to known toxic agents and whether the test strain or species reacts similarly to another strain or species when exposed to the same known standard toxicant.

(6) *Route(s) of administration.* To the extent possible, route(s) of administration should be comparable to the expected or known routes of human exposure. The test rules in Part 771 will specify the route(s) to be employed for a particular chemical. For inhalation and dermal studies, Part 771 will also specify the specific conditions for administering the test substance.

(7) *Frequency of exposure.* The tester must administer test substance and vehicle, if any, by the same route and at the same frequency for the duration of the study. For gavage, the test substance must be administered daily; for feeding, *ad libitum;* for inhalation exposure, a minimum of 5 days per week, 6 hours per day; and for dermal exposure, as specified in the applicable test rule. The tester must conduct the dosing at approximately the same time each day.

(8) *Duration of treatment and observation periods.* The tester must administer the test substance to rodent species for a minimum of 24 months but no longer than 30 months. If a nonrodent species is used, the Agency must approve the duration of exposure.

(9) *Dose levels and dose selection.* (i) The tester must provide data from at least three dose levels (in addition to controls) in each sex of each species.

(A) The high dose level must be the maximum level that can be administered for the duration of the test period, with demonstrable but only slight toxicity, and no substantial reduction in longevity due to effects other than tumors. Signs of demonstrable, slight toxicity are a weight decrement not to exceed 10 percent compared to appropriate controls, clinical signs of toxicity, or pathologic lesions other than those related to a neoplastic response.

(B) The intermediate dose level must be some fraction (¼ to ½) of the high dose level.

(C) The lowest dose level must be some fraction (½ or less) of the intermediate dose level but not less than 10 percent of the high dose level.

(D) The sponsor or tester may add additional dose levels at his/her own discretion. If other dose levels are tested, the sponsor must submit the data from any such discretionary levels to Agency along with that of the required levels.

(ii) The tester must conduct a preliminary toxicology study of at least 90 days to select the chronic dose levels which will meet the requirements in this subsection. A preliminary toxicology study of at least 90 days that has been completed previously may be submitted for this purpose.

(iii) The sponsor must submit the rationale for dose selection including supporting data from preliminary toxicity studies as a part of the plan submission.

(b) *Study conduct.* (1) *Clinical procedures.* Veterinarians, as specified in § 772.113-1(e)(2), must ascertain and be responsible for the health status and care of all test animals prior to and during the study. Technical employees, as specified in § 772.113-1(e)(3)(ii), must be responsible for the daily observations and care of test animals.

(a) Special Test No. 6 uses rodent as the animal, price is based on rat.

(i) *Observation of Animals.* (A) Each test animal must be identified by a specific identification number. The tester must account for all animals at the end of the study. The tester must establish and adhere to standard operating procedures for housing, feeding, handling, and care of test animals as specified in § 772.110–1, Subpart B. To further assure minimal loss of animals due to cannibalism or autolysis of tissue, technical employees, as specified above, must observe the test animals every 12 hours throughout the test period. EPA may consider a study to be unacceptable for purposes of satisfying a test rule requirement if losses in any test group exceed 5 percent.

(B) Technical employees must conduct routine clinical examinations on each animal. These clinical examinations must include weighing of each animal, approximately the same time of day, at least once a week during the first 13 weeks, and every two weeks thereafter, and observing animals in relation to food and water consumption, morbidity, mortality and causes thereof, loss of animals for whatever reason, signs to toxicity, pharmacologic effects, and behavioral changes. The observer must record all data in detail at the time of observation.

(ii) *Hematology.* The tester must conduct the following quantitative hematologic determinations on a minimum of eight predesignated animals in each test group at one year (± one month) and at termination: hematocrit, hemoglobin, erythrocyte count, total and differential leukocyte counts, platelet count, and prothrombin and clotting times. If hematologic evidence of anemia is present at one year, reticulocyte counts must be performed within one week of the determination. In addition to the tests listed above, if any interim clinical observations suggest that other tests are necessary to assess the health status of test animals, the appropriate tests must be conducted. In the event that any of the predesignated animals does not survive 12 months, another animal selected by statistical randomization from the remainder of the appropriate test group can serve as a replacement.

Note.—Predesignated means that the animal has been selected to undergo this test by a specified randomization procedure prior to initiation of the study.

(iii) *Interim kill.* The tester may kill predesignated animals (other than those predesignated for hematological test in paragraph (b)(1)(ii) of this section at any time during the study, provided that he (she) increased the number of animals started in the study at least by the number scheduled or anticipated to be killed before the end of the study.

(iv) *Killing of test animals.* Animals which appear during the study as moribund, injured, or weak, and not expected to survive to the next observation, must be killed to preclude the loss of tissues from cannibalism and/or autolysis. Animals surviving to the termination of the study must also be killed. A technical employee must obtain blood samples for hemotologic determinations from each animal immediately before it is killed or as it is killed. The method used for killing must be humane and the same throughout the study. The tester must select a method of killing which will not produce interfering pathologic lesions.

(2) *Pathology procedures.* A Board-certified or Board-eligible pathologist, as specified in § 772.113–1(e)(1)(i), must be responsible for the planning and conduct of all pathology procedures and histopathology examinations, as well as for the final interpretation of all pathology data. Other doctorate pathologists, as specified in § 772.113–1(e)(1)(ii), are also acceptable for conducting procedures in their disciplines of specialization, under the direct supervision of a Board-Certified or Board-Eligible pathologist as specified in § 772.113–1(e)(1)(i).

Note.—Direct supervision means that the supervisor is immediately available for consultation, as necessary. This consultation may be done in person or by telephone.

(i) *Gross necropsy.* (A) Qualified pathologists, as specified in § 772.113–1(e)(2)(1), must perform or personally supervise the necropsies. Other appropriately trained technical employees, as specified in § 772.113–1(e)(3)(i), may assist in the necropsy.

Note.—Personal supervision means that the supervisor is immediately available for consultation at the site.

(B) Animals must be necropsied as soon as possible after death but no later than 16 hours after death. If necropsy cannot be performed immediately after the animal is killed or found dead, a technical employee must immediately

refrigerate (but not freeze) the animal at temperatures low enough to minimize tissue autolysis (4–8° C). Animals found dead upon routine clinical examination must be necropsied as soon as possible to salvage useable tissues.

(C) The gross necropsy must include an initial physical examination of the external surfaces and all orifices followed by an internal examination of tissues and organis *in situ*. The examination must include the following: external and internal portions of all hollow organs; cranial cavity and external surfaces of the brain and spinal cord; nasal cavity and paranasal sinuses; neck with its associated organs and tissues; thoracic, abdominal, and pelvic cavities with their associate organis and tissues; and the muscular/skeletal carcass. The urinary bladder and lungs must be inflated with a proper fixative to allow for better gross examination and preservation.

(D) The person responsible for the gross necropsy must record all gross necropsy findings in accordance with § 772.113–1 (k)(2).

(ii) *Tissue preservation.* A technical employee must immediately preserve all tissues and organs from all test animals in ten percent (10%) buffered formalin or another recognized and accepted fixative appropriate for the specific tissue(s). Sections from the following tissues from all test animals regardless of their time of death must be properly preserved for routine microscopic examination:

(A) All gross lesions (with a margin of normal tissue);

(B) Brain (minimum of one section each from the forebrain, midbrain, and hindbrain);

(C) Eyes and contiguous Harderian glands;

(D) Pituitary gland;

(E) Major salivary glands, thymus, thyroid with parathyroid, mammary glands; Zymbal's gland (if present);

(F) Oral mucous membrane (including random sections from tongue, buccal, and alveolar mucosa, pharynx, and nasopharynx);

(G) Heart and aorta;

(H) Trachea; lungs with the mainstem bronchi;

(I) Esophagus, stomach, small intestines and large intestine (cecum, colon, and rectum);

(J) Adrenal glands, pancreas, liver (minimum of two lobes), gall bladder (if present), spleen;

(K) Kidneys, urinary bladder;

(L) Representative lumph nodes (including those draining any neoplasm and those with gross changes);

(M) Bone including marrow from the sternum, vertebra and/or tibiofemoral joint;

(N) Skeletal muscle;

(O) For males: testes, prostate, and all other accessory sex organs;

(P) For females: vagina, corpus and cervix uteri, ovaries, and fallopian tubes.

(iii) *Preparation of tissue for microscopic examination.* A pathologist or a technical employee, as specified in § 772.113–1(e)(3)(i), must prepare all specimens for microscopic examination.

(A) *Tissue fixation and trimming.* A technical employee must fix tissues for the appropriate time for the fixative utilized. A pathologist must perform or directly and personally supervise tissue trimming. Routinely, tissues must be trimmed to a thickness of no more than 0.4 cm for subsequent processing. Parenchymal organs must be trimmed to allow for the largest surface area possible for subequent microscopic examination. Hollow organs must be trimmed to allow for a cross section mount from mucosa to serosa. Lymph nodes must be bisected through the hilus, if possible.

(B) *Slide preparation.* A technical employee must cut tissues routinely at a thickness of three to six micra (3 to 6 μ), in no case exceeding 10μ. All tissues must be stained routinely with hematoxylin and eosin (H&E). EPA encourages the use of special stains appropriate to the specific neoplasm, lesion, or tissue. Multiple sections (step cuts) must be made on each tissue or organ that contains gross evidence of a neoplasm or lesion and on each tissue or organ in which a metastasis may be anticipated. The tester must identify all blocks and microscopic slides by references to the animal's specific identification number and must preserve and hold them in accordance with § 772.110–1(j)(2).

(iv) *Microscopic examination and evaluation.* (A) Qualified pathologists as described in § 772.113–1(e)(1), must perform the microscopic examination and evaluation with subsequent diagnosis. The same pathologist must examine and evaluate all microscopic slides from all test animals of a given species.

(B) Microscopic examination must be performed on all tissues described in paragraphs (b)(2)(ii), (b)(2)(v), and (b)(2)(vi) of this section. The pathologist must record, document, and report all microscopic findings including all abnormalities, lesions, neoplasms, metastatic tumors and their anatomic locations in accordance with § 772.113–1(k)(2).

(v) *Additional examination.* All adverse health effects, including non-oncogenic effects, observed during the course of the study must be examined. When there is clinical evidence suggesting specific toxicologic or pharmacologic effects, including non-oncogenic effects, the necropsy and microscopic examinations of the suspected target organs must be conducted in greater detail. For example, when there is clinical evidence of neurologic effects, multiple sections from brain, spinal cord, and nerves must be examined.

(vi) *Special examinations.* (A) Additional sections, as specified below, must be microscopically examined from a minimum of ten animals selected randomly from the long-term survivors of each test group and in all animals in which clinical or grossly observable evidence of disease is present. If microscopic examination reveals evidence of disease in any of the tissues, then these target tissues must be examined in all test animals.

(B) The necropsy and microscopic examinations must include, in addition to those tissues listed in paragraph (b)(2)(ii) of this section, the following:

(*1*) *In a feeding study:* nasal cavity; paranasal sinuses; nasopharynx.

(*2*) *In an inhalation study:* multiple sections of the upper respiratory tract; nares; nasal cavity; paranasal sinuses; hypopharynx-larynx.

(*3*) *In a dermal study:* skin (normal); skin from sites of skin painting.

PROTOCOL

Special Test No. 6: Combined Chronic Toxicity and Oncogenic Effects Inhalation Study, Rodent[a]

(No standard protocol available)

(a) Special Test No. 6 uses rodent as the animal, price is based on rat.

PROTOCOL

Test No. 7: Acute Inhalation Toxicity Study, Primate[a]

(No standard protocol available)

(a) Test No. 7 uses primate as the animal, price is based on monkey.

PROTOCOL

Test No. 8: Subchronic Inhalation Toxicity Study, Primate[a]

(No standard protocol available)

(a) Test No. 8 uses primate as the animal, price is based on monkey.

PROTOCOL

Special Test No. 8: Subchronic Behavioral Effects Inhalation Study, Primate[a]

(No standard protocol available)

(a) Special Test No. 8 uses primate as the animal, price is based on monkey.

PROTOCOL

Test No. 9: Chronic Inhalation Toxicity Study (772.113-3), Primate[(a)]

§ 772.113-3 Non-oncogenic chronic effects test standards.

(a) *Study design.* (1) *Species and strains.* (i) The tester must use at least two mammalian species: one, a laboratory rat and the second, a nonrodent. The Agency recommends the dog as the nonrodent species. The tester may utilize other suitable nonrodent species approved by EPA.

Note.—Selection of the most appropriate nonrodent species should be predicated upon such factors as metabolism, pharmacokinetics, sensitivity or organotropism and other considerations pertinent to the study.

(ii) The sponsor or tester must select the specific strains and/or stocks of test animals to be used. Test animals must be from established strains and/or stocks. As part of the study plan submission, the sponsor must present the rationale for selection of the specific test animals along with historical data on their lifespans and disease types and incidences.

(2) *Sex.* The tester must use equal numbers of males and females at each dose level.

(3) *Age at start of test.* The tester must begin to dose as soon as possible after weaning and environmental acclimatization, but no later than six weeks of age for rodents and at ten weeks of age for dogs. For nonrodent species, other than the dog, the Agency must approve the age of initial exposure.

(4) *Group size.* Each "test group" of rodents must contain at least 50 animals (plus at least 8 additional for clinical laboratory testing). If the nonrodent species is the dog, then each group must contain at least six animals. The tester must assign animals to individual test groups by a specified randomization procedure. When the study plan calls for interim kill, the tester must increase the number of animals in each group at the start of the study by the number scheduled to be killed before completion of the study. If species other than the laboratory dog and rat are selected, EPA must approve the number of animals per group.

(5) *Control groups.* A tester must use a matched control group which is identical in every respect to the exposed groups except for exposure to the test substance.

Note (i).—If a vehicle is administered to the matched control group and if its toxic properties are not known, the tester may, at his/her discretion, use a negative or untreated control group.

Note (ii).—The EPA may require a Positive Control Group for particular chemicals when the sensitivity of the test animal to the chemical class to which the test substance belongs cannot be documented. When used, the positive control group should serve as an internal quality control to ascertain whether the test animals are sensitive to or respond in a predictable manner to known toxic agents and whether the test strain or species reacts similarly to another strain or species when exposed to the same known standard toxicant.

(6) *Route(s) of administration.* To the extent possible, route(s) of administration should be comparable to the expected or known routes of human exposure. Test rules in Part 771 will specify the route(s) to be employed for a particular chemical. For inhalation and dermal studies, Part 771 will also specify the specific conditions for administering the test substance.

(7) *Frequency of exposure.* The tester must administer test substance and vehicle, if any, by the same route and at the same frequency for the duration of the study. For gavage, the test substance must be administered daily; for feeding, *ad libitum;* for inhalation exposure, a minimum of 5 days per week, 6 hours per day; and for dermal exposure, as specified in the applicable test rule. For gavage, the tester must conduct the dosing at approximately the same time each day.

(8) *Duration of treatment and observation periods.* The tester must administer the test substance to rats for at least 30 months. In studies with nonrodents, the tester must test for at least 2 years unless the Agency authorizes specific exceptions.

(9) *Dose levels and dose selection.* (i) The tester must select doses to permit analysis of dose-response relationships and the "no observable effect level" (NOEL).

(A) A minimum of three dose levels (in addition to controls) in each sex of each species must be used.

(B) The highest dose level must demonstrate toxicologic effects. Mortality in rat groups must not exceed 50 percent before 18 months. Mortality in nonrodent groups must be kept to a minimum but significant toxicologic effect must also be demonstrated in the species.

(C) The lowest dose level must be selected to produce no observable evidence of toxicity other than tumors (NOEL).

(D) The sponsor or tester may add additional dose levels at his/her own discretion. If other dose levels are tested, the sponsor must submit the data from any such discretionary levels to the Agency along with that of the required levels.

(ii) The tester must conduct a preliminary toxicology study of at least 90 days to select the chronic dose levels which meet the requirements of this section. A preliminary toxicology study of at least 90 days that has been completed previously may be submitted for this purpose.

(iii) The sponsor must submit the rationale for dose selection including supporting data from preliminary toxicity studies as a part of the study plan submission.

(a) Test No. 9 uses primate as the animal, price is based on monkey.

(b) *Study conduct.* (1) *Clinical procedures.* A veterinarian, as specified in § 772.113–1(e)(2), must ascertain and be responsible for the health status and care of all test animals during the study. A technical employee, as specified in § 772.113–1(e)(3)(ii), must be responsible for the daily observations and care of the test animals.

(i) *Observation of animals.* (A) Each test animal must be identified by a specific identification number. The tester must account for all animals at the end of the study. The tester must establish and adhere to standard operating procedures for housing, feeding, handling, and care of test animals as specified in § 772.110–1. To further assure minimal loss of animals due to cannibalism or autolysis of tissue, technical employees, as specified above, must observe the test animals at least every 12 hours throughout the test period. EPA may consider a study to be unacceptable for purposes of satisfying a test rule requirement if losses in any test group exceed 5 percent.

(B) Technical employees must conduct routine clinical examinations on all test animals. Clinical examination must include weighing of each animal, at approximately the same time of day, at least once a week during the first 13 weeks, and every two weeks thereafter and observing all animals in relation to food and water consumption, morbidity, mortality and causes thereof, loss of animals for whatever reason, signs of toxicity, pharmacologic effects, and behavioral changes. The observer must record all data in detail at the time of observation.

(ii) *Clinical laboratory testing.* The tester must conduct the following quantitative determinations on a minimum of eight additional predesignated rats in each test group. For nonrodents, all animals in each test group must be utilized. In addition to the tests listed below, if any interim clinical observations suggest that other tests are necessary to assess the health status of test animals, the appropriate tests must be conducted.

Note.—Predesignated means that the animal has been selected to undergo these tests by a specified randomization procedure prior to initiation of the study.

(A) *Hematology.* The tester must conduct the following quantitative hematologic determinations at least at 3, 6, 12, 18, 24 months and at study termination: hematocrit, hemoglobin, erythrocyte count, total and differential leukocyte counts, platelet count, and prothrombin and clotting times. If hematologic evidence of anemia is present, reticulocyte counts must be performed within one week of the determination.

(B) *Blood chemistry.* The tester must conduct the following quantitative blood chemistry determinations at least at 3, 6, 12, 18, 24 months and at study termination: calcium, sodium, potassium, chloride, serum lactic dehydrogenase, serum glutamic pyruvic transaminase, creatinine kinase, serum glutamic oxaloacetic transaminase, glucose, blood urea nitrogen, creatinine, direct and total bilirubin, cholinesterase, total cholesterol, triglycerides, serum alkaline phosphatase, albumin, globulin, and total protein. In addition to these tests, the tester may conduct other quantitative blood chemistry determinations at his/her discretion, such as uric acid, gammaglutamyl transpeptidase, and ornithine carbamoyltransferase.

(C) *Urinalysis.* The tester must conduct the following quantitative determinations at least at 3, 6, 12, 18, 24 months and at study termination: specific gravity or osmolarity, pH, protein, glucose, ketones, bilirubin, and urobilinogen, as well as microscopic examination and analysis of formed elements. Each animal's urine must be evaluated individually.

Note.—*Additional Tests.* Based on results of concurrent or previous studies on the test substance, its metabolic or degradation products, the tester should conduct such other determinations as may be necessary for adequate toxicological evaluation.

(D) *Function tests.* (1) The tester must determine the functional capacity of the renal, hepatic, pulmonary, and cardiovascular systems.

Note.—Additional determinations must place major emphasis on organ or system function tests. Selection of the appropriate tests must be based upon the findings in the subchronic studies or observations made during the course of present study.

(2) Additional quantitative determinations may include, but not necessarily be limited to the following: water dilution and water concentration tests for renal function; total lung capacity, functional residual capacity, and residual volume for pulmonary function; bromsulphalein excretion test for liver function; electrocardiogram, blood pressure, and exercise recovery for cardiovascular function. The tester must perform these evaluations at the beginning (nonrodent only) and at least every 3, 6, 12, 18, 24 months and at study termination.

(E) *Residue analysis.* The tester must measure levels of test substance, major metabolites or other biologically significant metabolites at 3, 6, 12, 18, and 24 months ± 1 month and at the termination of the study. Tissues from the predesignated animals analyzed should include all target tissues from prechronic toxicology studies and those suggested by pharmaco-kinetic studies. These analyses must include at least plasma, 24-hour urine, feces and, at time of death or scheduled killing, liver and kidney.

(iii) *Interim kill.* The tester may kill predesignated animals (other than those predesignated for hematological tests in paragraph (b)(1)(ii)(A) of this section at any time during the study, provided that he/she increases the number of animals started in the study at least by the number scheduled or anticipated to be killed before the end of the study.

(iv) *Killing of test animals.* Animals which appear during the study as moribund, injured, or weak, and not expected to survive to the next observation, must be killed to preclude the loss of tissues from cannibalism and/or autolysis. Animals surviving to the termination of the study must also be killed. A technical employee must obtain blood samples for hematologic determinations from each animal immediately before it is killed or as it is killed. The method used for killing must be humane and the same throughout the study. The tester must select a method of killing which will not produce interfering pathologic lesions.

(2) *Pathology procedures.* A Board-Certified or Board-Eligible pathologist, as specified in § 772.113–1(e)(1)(i), must be responsible for the planning and conduct of all pathology procedures and histopathology examination, as well as for the final interpretation of all pathology data. Other doctorate pathologists, as specified in § 772.113–1(e)(1)(ii), are also acceptable for conducting procedures in their disciplines of specialization, under the direct supervision of a Board-Certified or Board-Eligible pathologist as specified in § 772.113–1(e)(1)(i).

Note.—Direct supervision means that the supervisor is immediately available for consultation, as necessary. This consultation may be done in person or by telephone.

(i) *Gross necropsy.* (A) Qualified pathologists, as specified § 772.113–1(e)(1), must perform or personaally supervise the necropsies. Other appropriately trained technical employees, as specified in § 772.113–1(e)(3)(i) may assist in the necropsy.

Note.—Personal supervision means that the supervisor is immediately available for consultation at the site.

(B) Animals must be necropsied as soon as possible after death but no later than 16 hours after death. If necropsy cannot be performed immediately after the animal is killed or found dead, a technical employee must immediately refrigerate (but not freeze) the animal at temperatures low enough to minimize tissue autolysis (4–6° C). Animals found dead upon routine clinical examination must be necropsied as soon as possible to salvage usable tissues.

(C) The gross necropsy must include an initial physical examination of the external surfaces and all orifices followed by an internal examination of tissues and organs *in situ.* The examination must include the following: external and internal portions of all hollow organs; cranial cavity and external surfaces of the brain and spinal cord; nasal cavity and paranasal sinuses; neck with its associated organs and tissues; thoracic, abdominal, and pelvic cavities with their associated organs and tissues; and the muscular/skeletal carcass. The urinary bladder and lungs must be inflated with a proper fixative to allow for better gross examination and preservation.

(D) The weights of the heart, liver, kidneys, testes, spleen, lung, brain, and adrenals must be recorded after careful dissection and trimming. In addition, the thyroid (with parathyroids) and pituitary must be weighed for each nonrodent. The person responsible for the gross necropsy must record all gross necreopsy findings in accordance with § 772.113–1(k)(2).

(ii) *Tissue preservation.* A technical employee must immediately preserve all tissues and organs from all test animals in 10 percent buffered formalin or another recognized and accepted fixative appropriate for the specific tissue(s). Sections from the following tissues from all test animals regardless of their time of death must be properly preserved for routine microscopic examination:

(A) All gross lesions (with a margin of normal tissue);

(B) Brain (minimum of one section each from the forebrain, midbrain, and hindbrain);

(C) Spinal cord (minimum of one section each from cervical, thoracic, and lumbar regions);

(D) Eyes and contiguous Harderian glands;

(E) Pituitary gland;

(F) Major salivary glands, thymus, thyroid with parathyroid, mammary glands, Zymbal's gland (if present);

(G) Oral mucous membrane (including random sections from tongue, buccal, and alveolar mucosa, pharynx, and nasopharynx);

(H) Heart and aorta (three sections from different locations);

(I) Trachea; lungs, with the mainstem bronchi;

(J) Esophagus, stomach, small intestines and large intestine (cecum, colon, and rectum);

(K) Adrenal glands, pancreas, liver (minimum of two lobes), gall bladder (if present), spleen;

(L) Kidneys, urinary bladder;

(M) Representative lymph nodes (including those draining any neoplasm and those with gross changes);

(N) Bone including marrow, from the sternum vertebra and/or tibiofemoral joint;

(O) Skin (sections from similar sites of all animals);

(P) Skeletal muscle;

(Q) For males: testes, prostate, and all other accessory sex organs;

(R) For females: vagina, corpus and cervix uteri, ovaries, and fallopian tubes.

(iii) *Preparation of tissue for microscopic examination.* A pathologist or a technical employee, as specified in § 772.113–1(e)(3)(i), must prepare all specimens for microscopic examination.

(A) *Tissue fixation and trimming.* The technical employee must fix tissues for the appropriate times for the fixative utilized. A pathologist must perform or directly and personally supervise tissue trimming. Routinely, tissues must be trimmed to a thickness of no more than 0.4 cm for subsequent processing. Parenchymal organs must be trimmed to allow for the largest surface areas possible for subsequent microscopic examination. Hollow organs must be trimmed to allow for a cross section mount from mucosa to serosa. Lymph nodes must be bisected through the hilus, if possible.

(B) *Slide preparation.* A technical employee must cut tissues routinely at a thickness of three to six micra (3 to 6 μ), in no case exceeding 10 μ. All tissues must be stained routinely with hematoxylin and eosin (H&E). EPA encourages the use of special stains appropriate to the specific neoplasm, lesion, or tissue. Multiple sections (step cuts) must be made on each tissue or organ that contains gross evidence of a neoplasm or lesion and on each tissue or organ in which a metastasis may be aniticipated. The tester must identify all blocks and microscopic slides by reference to the animal's specific identification number and must preserve and hold them in accordance with § 772.110–1(j)(2).

(iv) *Microscopic examination and evaluation.* (A) Qualified pathologists as described in § 772.113–1(e)(1), must perform the microscopic examination and evaluation with subsequent diagnosis. The same pathologist must examine and evaluate all microscopic slides from all test animals of a given species.

(B) Microscopic examination must be performed on all appropriate tissues described in paragraphs (b)(2)(ii), (b)(2)(v), and (b)(2)(vi) of this section. The pathologist must record, document, and report all microscopic findings including all abnormalities, lesions, neoplasms, metastatic tumors and their anatomic location in accordance with § 772.113–1(k)(2).

(v) *Additional Examinations.* All adverse health effects observed during the course of the study must be examined. When there is clinical evidence of specific toxicologic or pharmacologic effects related to specific target organs, the necropsy and microscopic examinations of the suspected target organs must be conducted in greater detail. For example, when there is clinical evidence of neurologic effects, multiple sections from brain, spinal cord, and nerves must be examined.

(vi) *Special Examinations.* (A) Additional sections must be microscopically examined from a minimum of ten rodent animals selected randomly from the long-term survivors and all nonrodent animals of each test group and in all animals in which clinical or grossly observable evidence of disease is present. If microscopic examination reveals evidence of disease in any of these tissues, then these target tissues must be examined in all test animals.

(B) The necropsy and microscopic examination must include, in addition to those tissues listed in paragraph (b)(2)(ii) of this section, the following:

(*1*) *In a feeding study:* nasal cavity; paranasal sinuses; nasopharynx.

(*2*) *In an inhalation study:* multiple sections of the upper respiratory tract: nares; nasal cavity; paranasal sinuses; hypopharynx-larynx.

(*3*) *In a dermal study:* skin (normal); skin from sites of skin painting.

PROTOCOL

Special Test No. 9: Oncogenic Effects Inhalation Study (772.113-2), Primate[a]

§ 772.113-2 Oncogenic effects test standards.

(a) *Study design.* (1) *Species and Strain.* (i) The tester must use at least two rodent species, the laboratory mouse and rat. An alternative species may be used if the sponsor can provide sufficient data and/or rationale to demonstrate that it is a more appropriate species for a specific test substance. The sponsor must present such data and/or rationale for Agency approval as a part of the study plan submission.

(ii) The sponsor or tester must select the specific strains and/or stocks of test animals to be used. Established strains and/or stocks which are expected to be sensitive to the test substance must be used. As part of the study plan submission, the sponsor must present the rationale for selection of the specific test animals. This must include a summary of any prior test results with the selected species, historical data on their lifespans, spontaneous diseases and conditions (including tumors) and their incidences.

Note.—Acceptable rationale for alternate species would be results from prior oncogenicity studies which show that the alternative species is sensitive to the oncogenic effects of the chemical class to which the test substance belongs or that the alternate species has similar metabolism or pharmacokinetics to humans.

(2) *Sex.* The tester must use equal numbers of males and females at each dose level.

(3) *Age at start of test.* The tester must begin to dose rodents as soon as possible after weaning and environmental acclimatization, by no later than six (6) weeks of age. For nonrodents, the Agency must approve the age of initial exposure.

(4) *Group size.* Each "test group" of rats or mice must contain at least 50 animals. The tester must assign animals to individual test groups by a specified randomization procedure. When the study plan calls for interim kill, the tester must increase the number of animals in each group at the start of the study by the number scheduled to be killed before completion of the study. If species other than the laboratory mouse and rat are selected, EPA must approve the number of animals per group.

(5) *Control groups.* A tester must use a matched control group which is identical in every respect to the exposed groups except for exposure to the test substance.

Note (i).—If a vehicle is administered to the matched control group and if its toxic properties are not known, the tester may, at his/her discretion, use a negative or untreated control group.

Note (ii).—The EPA may require a Positive Control Group for particular chemicals when the sensitivity of the test animal to the chemical class to which the test substance belongs cannot be documented. When used, the positive control group should serve as an internal quality control to ascertain whether the test animals are sensitive to or respond in a predictable manner to known toxic agents and whether the test strain or species reacts similarly to another strain or species when exposed to the same known standard toxicant.

(6) *Route(s) of administration.* To the extent possible, route(s) of administration should be comparable to the expected or known routes of human exposure. The test rules in Part 771 will specify the route(s) to be employed for a particular chemical. For inhalation and dermal studies, Part 771 will also specify the specific conditions for administering the test substance.

(7) *Frequency of exposure.* The tester must administer test substance and vehicle, if any, by the same route and at the same frequency for the duration of the study. For gavage, the test substance must be administered daily; for feeding, *ad libitum;* for inhalation exposure, a minimum of 5 days per week, 6 hours per day; and for dermal exposure, as specified in the applicable test rule. The tester must conduct the dosing at approximately the same time each day.

(8) *Duration of treatment and observation periods.* The tester must administer the test substance to rodent species for a minimum of 24 months but no longer than 30 months. If a nonrodent species is used, the Agency must approve the duration of exposure.

(9) *Dose levels and dose selection.* (i) The tester must provide data from at least three dose levels (in addition to controls) in each sex of each species.

(A) The high dose level must be the maximum level that can be administered for the duration of the test period, with demonstrable but only slight toxicity, and no substantial reduction in longevity due to effects other than tumors. Signs of demonstrable, slight toxicity are a weight decrement not to exceed 10 percent compared to appropriate controls, clinical signs of toxicity, or pathologic lesions other than those related to a neoplastic response.

(B) The intermediate dose level must be some fraction (¼ to ½) of the high dose level.

(C) The lowest dose level must be some fraction (½ or less) of the intermediate dose level but not less than 10 percent of the high dose level.

(D) The sponsor or tester may add additional dose levels at his/her own discretion. If other dose levels are tested, the sponsor must submit the data from any such discretionary levels to Agency along with that of the required levels.

(ii) The tester must conduct a preliminary toxicology study of at least 90 days to select the chronic dose levels which will meet the requirements in this subsection. A preliminary toxicology study of at least 90 days that has been completed previously may be submitted for this purpose.

(iii) The sponsor must submit the rationale for dose selection including supporting data from preliminary toxicity studies as a part of the plan submission.

(b) *Study conduct.* (1) *Clinical procedures.* Veterinarians, as specified in § 772.113-1(e)(2), must ascertain and be responsible for the health status and care of all test animals prior to and during the study. Technical employees, as specified in § 772.113-1(e)(3)(ii), must be responsible for the daily observations and care of test animals.

(a) Special Test No. 9 uses primate as the animal, price is based on monkey.

(i) *Observation of Animals.* (A) Each test animal must be identified by a specific identification number. The tester must account for all animals at the end of the study. The tester must establish and adhere to standard operating procedures for housing, feeding, handling, and care of test animals as specified in § 772.110–1, Subpart B. To further assure minimal loss of animals due to cannibalism or autolysis of tissue, technical employees, as specified above, must observe the test animals every 12 hours throughout the test period. EPA may consider a study to be unacceptable for purposes of satisfying a test rule requirement if losses in any test group exceed 5 percent.

(B) Technical employees must conduct routine clinical examinations on each animal. These clinical examinations must include weighing of each animal, approximately the same time of day, at least once a week during the first 13 weeks, and every two weeks thereafter, and observing animals in relation to food and water consumption, morbidity, mortality and causes thereof, loss of animals for whatever reason, signs to toxicity, pharmacologic effects, and behavioral changes. The observer must record all data in detail at the time of observation.

(ii) *Hematology.* The tester must conduct the following quantitative hematologic determinations on a minimum of eight predesignated animals in each test group at one year (± one month) and at termination: hematocrit, hemoglobin, erythrocyte count, total and differential leukocyte counts, platelet count, and prothrombin and clotting times. If hematologic evidence of anemia is present at one year, reticulocyte counts must be performed within one week of the determination. In addition to the tests listed above, if any interim clinical observations suggest that other tests are necessary to assess the health status of test animals, the appropriate tests must be conducted. In the event that any of the predesignated animals does not survive 12 months, another animal selected by statistical randomization from the remainder of the appropriate test group can serve as a replacement.

Note.—Predesignated means that the animal has been selected to undergo this test by a specified randomization procedure prior to initiation of the study.

(iii) *Interim kill.* The tester may kill predesignated animals (other than those predesignated for hematological test in paragraph (b)(1)(ii) of this section at any time during the study, provided that he (she) increased the number of animals started in the study at least by the number scheduled or anticipated to be killed before the end of the study.

(iv) *Killing of test animals.* Animals which appear during the study as moribund, injured, or weak, and not expected to survive to the next observation, must be killed to preclude the loss of tissues from cannibalism and/or autolysis. Animals surviving to the termination of the study must also be killed. A technical employee must obtain blood samples for hemotologic determinations from each animal immediately before it is killed or as it is killed. The method used for killing must be humane and the same throughout the study. The tester must select a method of killing which will not produce interfering pathologic lesions.

(2) *Pathology procedures.* A Board-certified or Board-eligible pathologist, as specified in § 772.113–1(e)(1)(i), must be responsible for the planning and conduct of all pathology procedures and histopathology examinations, as well as for the final interpretation of all pathology data. Other doctorate pathologists, as specified in § 772.113–1(e)(1)(ii), are also acceptable for conducting procedures in their disciplines of specialization, under the direct supervision of a Board-Certified or Board-Eligible pathologist as specified in § 772.113–1(e)(1)(i).

Note.—Direct supervision means that the supervisor is immediately available for consultation, as necessary. This consultation may be done in person or by telephone.

(i) *Gross necropsy.* (A) Qualified pathologists, as specified in § 772.113–1(e)(2)(1), must perform or personally supervise the necropsies. Other appropriately trained technical employees, as specified in § 772.113–1(e)(3)(i), may assist in the necropsy.

Note.—Personal supervision means that the supervisor is immediately available for consultation at the site.

(B) Animals must be necropsied as soon as possible after death but no later than 18 hours after death. If necropsy cannot be performed immediately after the animal is killed or found dead, a technical employee must immediately

refrigerate (but not freeze) the animal at temperatures low enough to minimize tissue autolysis (4–8° C). Animals found dead upon routine clinical examination must be necropsied as soon as possible to salvage useable tissues.

(C) The gross necropsy must include an initial physical examination of the external surfaces and all orifices followed by an internal examination of tissues and organis *in situ*. The examination must include the following: external and internal portions of all hollow organs; cranial cavity and external surfaces of the brain and spinal cord; nasal cavity and paranasal sinuses; neck with its associated organs and tissues; thoracic, abdominal, and pelvic cavities with their associate organis and tissues; and the muscular/skeletal carcass. The urinary bladder and lungs must be inflated with a proper fixative to allow for better gross examination and preservation.

(D) The person responsible for the gross necropsy must record all gross necropsy findings in accordance with § 772.113–1 (k)(2).

(ii) *Tissue preservation.* A technical employee must immediately preserve all tissues and organs from all test animals in ten percent (10%) buffered formalin or another recognized and accepted fixative appropriate for the specific tissue(s). Sections from the following tissues from all test animals regardless of their time of death must be properly preserved for routine microscopic examination:

(A) All gross lesions (with a margin of normal tissue);

(B) Brain (minimum of one section each from the forebrain, midbrain, and hindbrain);

(C) Eyes and contiguous Harderian glands;

(D) Pituitary gland;

(E) Major salivary glands, thymus, thyroid with parathyroid, mammary glands; Zymbal's gland (if present);

(F) Oral mucous membrane (including random sections from tongue, buccal, and alveolar mucosa, pharynx, and nasopharynx);

(G) Heart and aorta;

(H) Trachea; lungs with the mainstem bronchi;

(I) Esophagus, stomach, small intestines and large intestine (cecum, colon, and rectum);

(J) Adrenal glands, pancreas, liver (minimum of two lobes), gall bladder (if present), spleen;

(K) Kidneys, urinary bladder;

(L) Representative lumph nodes (including those draining any neoplasm and those with gross changes);

(M) Bone including marrow from the sternum, vertebra and/or tibiofemoral joint;

(N) Skeletal muscle;

(O) For males: testes, prostate, and all other accessory sex organs;

(P) For females: vagina, corpus and cervix uteri, ovaries, and fallopian tubes.

(iii) *Preparation of tissue for microscopic examination.* A pathologist or a technical employee, as specified in § 772.113–1(e)(3)(i), must prepare all specimens for microscopic examination.

(A) *Tissue fixation and trimming.* A technical employee must fix tissues for the appropriate time for the fixative utilized. A pathologist must perform or directly and personally supervise tissue trimming. Routinely, tissues must be trimmed to a thickness of no more than 0.4 cm for subsequent processing. Parenchymal organs must be trimmed to allow for the largest surface area possible for subequent microscopic examination. Hollow organs must be trimmed to allow for a cross section mount from mucosa to serosa. Lymph nodes must be bisected through the hilus, if possible.

(B) *Slide preparation.* A technical employee must cut tissues routinely at a thickness of three to six micra (3 to 6 μ), in no case exceeding 10μ. All tissues must be stained routinely with hematoxylin and eosin (H&E). EPA encourages the use of special stains appropriate to the specific neoplasm, lesion, or tissue. Multiple sections (step cuts) must be made on each tissue or organ that contains gross evidence of a neoplasm or lesion and on each tissue or organ in which a metastasis may be anticipated. The tester must identify all blocks and microscopic slides by references to the animal's specific identification number and must preserve and hold them in accordance with § 772.110–1(j)(2).

(iv) *Microscopic examination and evaluation.* (A) Qualified pathologists as described in § 772.113–1(e)(1), must perform the microscopic examination and evaluation with subsequent diagnosis. The same pathologist must examine and evaluate all microscopic slides from all test animals of a given species.

(B) Microscopic examination must be performed on all tissues described in paragraphs (b)(2)(ii), (b)(2)(v), and (b)(2)(vi) of this section. The pathologist must record, document, and report all microscopic findings including all abnormalities, lesions, neoplasms, metastatic tumors and their anatomic locations in accordance with § 772.113–1(k)(2).

(v) *Additional examination.* All adverse health effects, including non-oncogenic effects, observed during the course of the study must be examined. When there is clinical evidence suggesting specific toxicologic or pharmacologic effects, including non-oncogenic effects, the necropsy and microscopic examinations of the suspected target organs must be conducted in greater detail. For example, when there is clinical evidence of neurologic effects, multiple sections from brain, spinal cord, and nerves must be examined.

(vi) *Special examinations.* (A) Additional sections, as specified below, must be microscopically examined from a minimum of ten animals selected randomly from the long-term survivors of each test group and in all animals in which clinical or grossly observable evidence of disease is present. If microscopic examination reveals evidence of disease in any of the tissues, then these target tissues must be examined in all test animals.

(B) The necropsy and microscopic examinations must include, in addition to those tissues listed in paragraph (b)(2)(ii) of this section, the following:

(*1*) *In a feeding study:* nasal cavity; paranasal sinuses; nasopharynx.

(*2*) *In an inhalation study:* multiple sections of the upper respiratory tract; nares; nasal cavity; paranasal sinuses; hypopharynx-larynx.

(*3*) *In a dermal study:* skin (normal); skin from sites of skin painting.

PROTOCOL

Special Test No. 9: Combined Chronic Toxicity and Oncogenic Effects Inhalation Study, Primate [a]

(No standard protocol available)

(a) Special Test No. 9 uses primate as the animal, price is based on monkey.

PROTOCOL

Test No. 10: Subchronic Oral Toxicity Study (772.112-31), Dog

§ 772.112-31 Subchronic oral dosing studies.

(a) *Study Design.* (1) *Species.* Testing must be performed in at least two mammalian species, preferably the same species and strain for which chronic studies are anticipated. Once species must be a generally recognized strain of laboratory rat. The second species may be a nonrodent. The nonrodent species should usually be the dog. Selection of a nonrodent species other than the dog will require full and adequate justification which should consider such factors as the comparative metabolism of the chemical and species sensitivity to the toxic effects of the test substance, as evidenced by the results of other studies.

(2) *Sex and age.* Equal numbers of males and females of each species and strain tested must be used. The tester must begin to dose as soon as possible after weaning and environmental acclimatization but no later than six weeks of age for rodents and at 4–6 months of age for dogs.

(3) *Control group.* A concurrent control group is required. This group must be an untreated control group or, if a vehicle is used in administering the test substance, a vehicle control group. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are required.

(4) *Number of animals.* Each test group and concurrent control group must contain at least 20 animals of each sex in studies with rats and at least 6 of each sex in studies with nonrodents. This number must be increased by the number, if any, scheduled to be sacrificed before completion of the study, such as, for example, rats on which hematology and blood chemistry determinations are made before and during the study.

(5) *Duration of testing.* (i) In studies with rodents, the substance being tested must be administered for at least 90 days.

(ii) In studies with nonrodents, the substance being tested must be administered daily for at least 6 months.

(6) *Number of dose levels and dose selection.* (i) At least three dose level groups (in addition to the control groups) must be tested.

(ii) The highest dosage level must result in toxicological or pharmacological effects, but not cause more than 10 percent fatalities. This level should be higher than that expected for human exposure.

(iii) The lowest dosage level must be one which does not induce any evidence of toxicity.

(7) *Route of administration.* The test substance must be administered in the animal's diet. Oral intubation may be allowed if the physical characteristics of the test substance so dictate. The chosen method must be used for all levels. If the test substance is administered by oral intubation, the amount of test substance must be adjusted weekly or biweekly to maintain a constant dose level in mg/kg (body weight). If the test substance is administered in the diet, either a constant concentration (ppm) or a constant dose level in mg/kg (body weight) must be used. The selection of dosage units of administration in the diet must be consistent with that for chronic feeding studies (Section 772.113–3 Subpart D).

(b) *Study Conduct.* (1) *Observation of animals.* All toxicological and pharmacological signs shall be recorded daily, including their time of onset, intensity, and duration. Such signs include but are not limited to: Mortality; and cardiovascular, respiratory, excretory, behavioral, and central nervous system (paralysis, ataxia, and pupillary reaction) effects. Observations must be made by an appropriately trained observer. Food consumption must be measured weekly during the test, and the animals must be weighed at least weekly. The animals must be observed as specified in Subpart A, § 772.100–2(b)(6)(ii). A complete ophthalmological examination must be conducted by a veterinarian on all nonrodents at the termination of the study.

(2) *Clinical laboratory testing.* The following determinations must be made at the time indicated below for each type of testing. For rodents, these determinations must be made on at least 10 animals of each sex in each group. For nonrodents, these determinations must be made on all animals in each group. Depending on the techniques used, it may be necessary to sacrifice animals to make the required clinical determinations. In case of said sacrifice, additional animals must be added to the study as provided by paragraph (a)(4) of this section.

(i) *Hematology.* Hematology determinations must be made as follows: For nonrodents shortly before the beginning of dosing, at least every 30 days thereafter, and at the termination of the testing period; and for rodents, shortly before the beginning of dosing.

at an intermediate time, and at the termination of the testing period. The following hematology determinations must be made: Hematocrit, hemoglobin, erythrocyte count, total and differential leukocyte counts, platelet count, and, if signs of anemia are present, reticulocyte count.

(ii) *Blood chemistry.* Blood chemistry determinations must be performed as follows: For nonrodents, shortly before the beginning of dosing, at least every 30 days thereafter, and at the termination of the testing period; and for rodents, shortly before the beginning of dosing, at an intermediate time, and at the termination of the study. Nonrodents must be fasted for 1 day prior to obtaining blood samples. The following determinations must be made: Calcium, potassium, serum lactic dehydrogenase, serum glutamic pyruvic transaminase, serum glutamic oxaloacetic transaminase, glucose, blood urea nitrogen, direct and total bilirubin, serum alkaline phosphatase, total cholesterol, albumin, globulin, total protein, and such other determinations as may be necessary for adequate toxicological evaluation. The following determinations may also be useful: Chloride, uric acid, blood creatinine, and gamma-glutamyl transpeptidase.

(iii) *Cholinesterase inhibition tests.* If the test substance contains a carbamate, an organophosphate, or any chemical that produces acetyl cholinesterase inhibition, the enzyme activity for plasma and red blood cell must be monitored shortly before the beginning of dosing, at least twice during the study, and at the end of the study, and the enzyme activity for brain must be monitored at the termination of the study. In addition, when nonrodents are used, monitoring of the enzyme activity must be performed twice before the beginning of dosing. Additionally, serial determinations may be useful to provide data on time-course of development of inhibition, extent of inhibition, and recovery from inhibition (e.g., after removal from treated diet); the undertaking of such determinations should not, however, result in overstress of the test animals.

(iv) *Urinalysis.* Urinalysis must be performed as follows: for rodents, at least once (at an intermediate time) during the testing period, and again at the termination of the testing period; and for nonrodents, shortly before the beginning of dosing, every 60 days thereafter, and at the termination of the test. Nonrodents must be fasted 1 day prior to collection of urine samples. Each animal must be evaluated individually. The urinalysis must include specific gravity or osmolarity, pH, protein, glucose, ketones, bilirubin, and urobilinogen, as well as microscopic examination of formed elements. Results of these determinations must be expressed in quantitative terms by appropriate grading scales.

(v) *Additional tests.* Depending on the known or suspected properties of the test substance, such other determinations as may be necessary for adequate toxicological evaluation must be performed.

(3) *Handling of moribund and dead animals.* (i) *Moribund animals.* Moribund animals must be sacrificed to lessen the likelihood of unobserved death and subsequent autolysis or cannibalism.

(ii) *Tissue loss and dead animals.* Requirements concerning tissue loss and the handling of dead animals are specified in Subpart A, § 772.100–2(b) (6) and (7), respectively.

(4) *Gross necropsy.* (i) The standards set for necropsy procedures in § 772.100–2(b)(7), Subpart A, must apply.

(ii) All test animals in the study must be subjected to gross necropsy, which must include examination of the external surface; all orifices; the cranial cavity; carcass; the external and cut surfaces of the brain and spinal cord; the thoracic, abdominal and pelvic cavities and their viscera; and the cervical tissues and organs.

(iii) In addition, the following organs must be weighed: Liver, kidneys, heart, gonads, and brain. Also, for nonrodents, thyroid (with parathyroid), adrenals, and pituitary must be weighed. Prior to being weighed, organs must be carefully dissected and properly trimmed to remove fat and other contiguous tissue in a uniform manner. They must be weighed as soon as possible after dissection to avoid drying.

(iv) The gross necropsy findings must be recorded and reported in accordance with paragraph (g)(3) of this section.

(v) Tissue samples must be preserved and held in accordance with § 772.110–1(j), Subpart B.

(5) *Histopathology examination.* (i) *General.* A histopathology examination shall be performed on the organs and tissues of all animals in accordance with this paragraph.

(ii) *Nonrodents.* The following organs and tissues, when present, of each test animal must be subjected to microscopic study: all gross lesions, brain (at least 3 levels from the forebrain, midbrain, and hindbrain), spinal cord (at least 2 levels), eye, pituitary, salivary gland, heart, thymus, thyroid with parathyroid, lungs with mainstem bronchi, trachea, esophagus, stomach, small and large intestines, adrenals, pancreas, liver, gall bladder, kidneys, urinary bladder, aorta, testes, prostate, ovaries, corpus and cervix uteri, spleen, a representative lymph node, bone (with marrow), skeletal muscle, skin, sciatic nerve, and mammary gland. Sites from which bone and lymph nodes are taken must be indicated.

(iii) *Rodents.* The following organs and tissues of each test animal must be subjected to microscopic study:

(A) *All animals in control and high dose groups.* All gross lesions, brain (at least 3 levels), eye, pituitary, salivary gland, heart, thymus, thyroid (with parathyroid), lungs with mainstem bronchi, trachea, esophagus, stomach, small and large intestines, adrenals, pancreas, liver, kidneys, urinary bladder, testes, prostate, ovaries, corpus and cervix uteri, spleen, bone (with marrow), and skeletal muscle. Section of bone (with marrow, when present) should be taken from sternebrae, vertebrae, or the tibio-femoral joint (the last will also include attached muscle).

(B) *All animals in intermediate and low dosage groups.* Liver, kidney, heart, any gross lesion, and any target organ either at the high dose or from laboratory tests or clinical observation at any treatment level.

(iv) Tissue and slide preparation and retention.

(A) The standards set forth in § 772.100–2(b)(7)(ii), Subpart A apply.

(B) Tissue samples, tissue blocks, and microscopic slides must be preserved and held in accordance with § 772.110–1(j), Subpart B.

(v) *Examiner.* The standards set forth in § 772.100–2(b)(1)(i), Subpart A apply.

(vi) *Records.* The histopathology findings must be recorded and reported as required by paragraph (c)(4) of this section.

(c) *Data reporting and evaluation.* In addition to the general reporting requirements of § 772.100–2(b)(8), Subpart A, a subchronic oral dosing study test report must contain the following information, presented in the format specified (unless adequate justification is supplied to present these data in another form):

(1) Animal records and clinical laboratory data. The following information must be arranged by test group (dose level and sex). All means must be accompanied by standard deviation.

(i) Significant time periods, for individual animals. In tabular form, data must be provided showing, for each animal:

(A) Its identification number;

(B) Whether it died by sacrifice, and if so, whether it was moribund before sacrifice;

(C) Its age at the beginning of the study;

(D) The week of the test when sacrifice occurred or the animal's death was noted; and

(E) Its age at death.

(ii) Variation from requirements, for individual animals. In tabular form, data must be provided showing, for each animal that was not subjected to gross necropsy and histopathology examination in accordance with requirements of this section:

(A) Its identification number;

(B) The manner of variation; and

(C) The reasons for failure to comply with the requirements of this section.

(iii) Toxic, pharmacologic, and behavioral effects for individual animals. In tabular form, data must be provided showing, for each animal:

(A) Its identification number;

(B) The date of observation of each sign of toxicity, pharmacological effect, or behavioral abnormality; and

(C) A description of the toxic sign, pharmacological effect, or behavioral abnormality. If such a response occurs repeatedly, it need be described only once and may thereafter be described by reference, with any variations noted as appropriate.

(iv) Toxic, pharmacologic, and behavioral effects for test animals. In tabular form, data must be provided showing, for each test group (dose level and sex):

(A) A list of each sign of toxicity, pharmacological effect, or behavioral abnormality affecting any animal in the test group;

(B) For each sign, effect, or abnormality, the number of animals affected;

(C) For each sign, effect, or abnormality, the median time from the beginning of the study to the first observation of such response; and

(D) The median age at death of animals not sacrificed.

(v) Food and body weight data, for individual animals. In tabular form, data must be provided showing, for each animal:

(A) Its identification number;

(B) Measured food consumption at weekly intervals throughout the test period; and

(C) Body weight measured weekly throughout the test period.

(vi) Food and body weight data, means. In tabular and graphic form, data must be provided showing, for each test group (dose level and sex):

(A) Mean measured food consumption at weekly intervals throughout the test period; and

(B) Mean body weight measured weekly during the test period.

(vii) Weekly survival and sacrifice data. In tabular form, data must be provided showing: the number of animals in each group which remained alive at the end of each 7-day interval, the number of animals in each group that were sacrificed or otherwise died during each 7-day interval, and the number that died by sacrifice and were moribund before sacrifice.

(viii) Clinical laboratory test protocol.

(A) The rationale for timing of the clinical laboratory tests, if different from the standards set forth in paragraph (d) of this section; and

(B) The method and rationale for selecting animals for the clinical laboratory tests.

(ix) Clinical laboratory testing, for each animal. In any appropriate form, data must be submitted showing, for each animal:

(A) Its identification number; and

(B) The results of any hematological, blood chemistry, cholinesterase inhibition, urinalysis, and other clinical laboratory tests performed.

(x) *Clinical laboratory testing, for each test group.* In any appropriate form, data must be submitted showing, for each test group (dose level and sex), the average of the results of each hematologic, blood chemical, cholinesterase inhibition, urinalysis, and other clinical laboratory test performed.

(2) *Gross necropsy.* For all means in the data required in this subparagraph, the standard deviation must be stated. The following test information, arranged by test groups (dose level and sex), must be supplied in tabular form:

(i) Data showing the identification number of any animal in which any gross abnormality or gross lesion was noted, and containing, for each such animal, a description of each gross abnormality (including measurements), and the date (if known) when it was first observed. Gross abnormalities observed repeatedly need by described only once and may thereafter be described by reference, with any variations noted, as necessary.

(ii) Data showing the number of animals in which any type of gross abnormality was observed.

(iii) Data showing, for each animal: Its identification number, weights of the organs listed under paragraph (b)(4) of this section, and corresponding organ-to-body weight ratios.

(iv) Data showing the mean weights of each type of organ listed under paragraph (b)(4) of this section, and mean organ-to-body weight ratios.

(3) *Histopathology data.* The following information must be arranged by test group (dose level and sex). All means must be accompanied by standard deviation. The number of data units on which a calculation is based must be reported for all percentages and means.

(i) *Descriptions of lesions, for each animal.* Data must be submitted in any appropriate form showing:

(A) For each animal, its identification number, and a complete description and diagnosis of every lesion in the animal. Nonneoplastic lesions which are observed frequently or which are common in both treated and control animals should be graded. (Descriptions of neoplasms may also include grading.) A commonly used scale such as ±, 1, 2, 3, and 4, for degrees ranging from very slight to extreme can be used, but other scales are acceptable. If known, the description and diagnosis should identify any lesion which caused the animal to be moribund or to die. The description and diagnosis must include the time of appearance (if any) for each lesion. Abnormalities observed repeatedly need to be described only once, and may subsequently be supplied by reference, with any individual variations noted as necessary.

(B) For each animal, a table or paragraph listing the tissues found to be normal.

(C) If a grading system is used, a description of the system.

(ii) *Counts and incidence of lesions, by test groups.* Data must be submitted in tabular form showing, for each test group:

(A) The number of animals at the start of the test, and the number of animals in which any lesion was found;

(B) The number of animals affected by each different type of lesion, the average grade of each type of lesion, the number of animals examined for each type of lesion, and the percentage of those animals examined which were affected by each type of lesion; and

(C) The number of each different type of lesion.

(iii) *Incidence of tumors.* If a tumor is observed in any animal, the report must include a complete description and diagnosis of each tumor as required in § 772.113-1(k)(2)(i)(D), Subpart D.

(4) *Evaluation of data.* An evaluation of the test results (including their statistical analysis), based on clinical findings, gross necropsy findings, and histopathology results, must be made and supplied. This submission must include an evaluation of 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, gross and histopathologic lesions, organ weight changes, effects on mortality, and any other toxic effects. The evaluation must also include dose-response curves for any toxic or pharmacologic effect which appear to be compound-related for the various groups, and a description of statistical methods.

PROTOCOL

Test No. 11: Acute Dermal Toxicity Study (772.112-22), Rabbit

§ 772.112-22 Acute dermal toxicity study.

(a) *Study design.* (1) *Condition of test substance.* If the test substance is a liquid, it must be applied as a liquid. If the test substance is a solid, it must be slightly moistened (made pasty) with physiological saline before application.

(2) *Species.* Testing must be performed with at least one mammalian species, preferably albino rabbits. An alternative species may be used if the sponsor can provide sufficient data and/or rationale to demonstrate that it is a more appropriate species for a specific test substance.

(3) *Age.* Young adult male and female animals must be used.

(4) *Number of animals and selection of dose levels.* (i) A trial test is recommended for the purpose of establishing a dosing regimen which must include one dose level higher than the expected LD_{50} and at least one dose level below the expected LD_{50}. If data based on testing with at least 5 animals per sex with abraded skin are submitted showing that no toxicity is evident at 2 g/kg, no further testing at other dose levels is necessary. If mortality is produced, the requirements of paragraph (a)(4)(ii) of this section apply.

(ii) The number of animals per dose level, and the number and spacing of dose levels must be chosen to produce test groups with mortality rates between 10 percent and 90 percent, and permit calculation of the LD_{50} (abraded skin and intact skin) of males and females with a 95 percent confidence interval of 20 percent or less. At least 3 dose levels of the test substance, in addition to controls, must be tested. Though the group sizes may vary for each dose level, the groups must contain equal numbers of male and female animals.

(5) *Control animals.* A concurrent untreated control group of animals is required. A concurrent vehicle control group is required if a vehicle or diluent used in administering the test substance would be expected to elicit any important acute toxicologic response, or if there are insufficient data on the acute effects of the vehicle.

(b) *Study conduct.* (1) *Application.* In all animals, the application site must be free of hair. In addition, the application sites in abraded-skin groups must be abraded in such a way as to penetrate the stratum corneum but not the dermis. The test substance must be kept in contact with the skin of at least 10 percent of the body surface (for rabbits) for at least 24 hours. (See Draize (1944) for equivalent sq. cm of body surface.) The preferred application site is a band around the trunk of the test animal. A wrapping material such as gauze covered by impervious, nonreactive rubberized or plastic material should be used to retard evaporation and keep the test substance in contact with the skin. At the end of the exposure period, the wrapping should be removed and the skin wiped (but not washed) to remove any test substance still remaining.

(2) *Duration of observation.* Animals must be observed for at least 14 days after dosing or until all signs of reversible toxicity in survivors subside, whichever occurs later.

(3) *Observation.* Animals must be observed frequently during the day of administration of the test and checked at least every 12 hours throughout the test period. The following must be recorded: Nature, onset, severity, and duration of each toxic and pharmacologic sign, such as abnormal or unusual cardiovascular, respiratory, excretory, behavioral, or other activity, as well as signs indicating an adverse effect on the central nervous system (paralysis, lack of coordination, staggering); pupillary reaction; and time of death. The weight of each animal must be determined at least semi-weekly (3–4 day intervals) throughout the test period, and at death.

(4) *Sacrifice and necropsy.* All animals living at the termination of the observation period must be sacrificed. All test animals, whether dying by sacrifice or during the test, must be subjected to a complete gross necropsy following their death, in accordance with § 772.100-2(b)(7), Subpart A. All abnormalities must be recorded.

(5) *Histopathology.* Examination of skin must include histological examination of treated tissue in accordance with § 772.100-2(b)(7), Subpart A.

(c) *Data reporting and evaluation.* In addition to the information required by § 772.100-2(b)(8) and paragraphs (b) (3), (4), and (5) of this section, the test report must include the following information.

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

(2) Time of death after treatment;

(3) Time of recovery for fully recovered animals;

(4) LD_{50} for each sex for each test substance for animals with abraded skin and for animals with intact skin, calculated at the end of the observation period (with method of calculation specified);

(5) 95 percent confidence interval for each LD_{50}; and

(6) Dose-response curve, and slope (with confidence limits).

PROTOCOL

Test No. 12: Subchronic Dermal Toxicity Study, Rabbit

(No standard protocol available)

PROTOCOL

Test No. 13: Acute Ocular Toxicity Study, Rabbit

(No standard protocol available)

PROTOCOL

Test No. 14: Acute Delayed Neurotoxicity Study (163.81-7), Chicken

§ 163.81-7 Acute delayed neurotoxicity study.

Data from this study will identify compounds which induce the specific neuropathy commonly associated with organophosphates, and referred to as delayed neurotoxicity. Additional data from a subchronic neurotoxicity study would be required on any compound which produced positive results in a study performed according to this section (see § 163.82-5(a)(1)). Other kinds of central nervous system neuropathies are evaluated in subchronic testing required by §§ 163.82-1 through 163.82-4, and in the subchronic neurotoxicity study (§ 163.82-5).

(a) *When required.* An acute delayed neurotoxic evaluation is required to support the registration of each manufacturing use product and formulated product if the active ingredient(s) or any of its (their) metabolites, degradation products, or impurities cause acetyl cholinesterase depression or are structurally related to a substance that induces delayed neurotoxicity. Organophosphorous pesticides and carbamates (other than methylcarbamates and demethylcarbamates) are examples of substances that require this test.

(b) *Standards.* In addition to the general standards set forth in § 163.80-3, an acute delayed neurotoxicity study shall meet the following standards:

(1) *Substance tested.* The technical grade of each active ingredient in the product shall be tested.

(2) *Species and sex.* Testing shall be performed in the female domestic chicken (hen), standard size breeds or strains.

(3) *Number and age of animals.* Normally, a minimum of 10 animals shall be tested. Additional animals may be required when atropine and/or PAM do not protect the animal sufficiently to insure that at least 10 survive. Adult hens, at least 8-14 months of age (2-3 kg each) at the beginning of the study, shall be used.

(4) *Selection of dose level.* Each animal, when protected by atropine alone or atropine in combination with a suitable reactivator such as PAM, shall receive a dose greater than the unprotected LD_{50} dose. A repeat dosing may be required according to paragraph (b)(7) of this section.

(5) *Control groups.* (i) If a vehicle is used, a concurrent vehicle control group is required. The vehicle control group shall be exposed only to the vehicle used in administering the test substance.

(ii) A positive control is required. Triorthocresylphosphate (TOCP) is recommended.

(iii) A concurrent untreated control group is not required.

(6) *Route of administration.* The test substance shall be administered in known quantities by gavage (for example, in gelatin capsule) or by a comparable method.

(7) *Observation of animals.* Animals shall be observed daily for at least 21 days. Each observation shall be made by an appropriately trained observer, who shall note (and record where pertinent) food consumption (every 3-4 days), and behavioral abnormality, moribundity, locomotor ataxia, paralysis, and any other clinical signs of toxicity or pharmacological effect. If symptoms or signs of marginal value or questionable significance are observed, the dose shall be administered again and animals observed for an additional 21 days. The weight of each animal shall be recorded every 3-4 days.

(8) *Handling of moribund animals.* Moribund animals shall be sacrificed to lessen the likelihood of unobserved death and subsequent autolysis. All test and control animals shall be subjected to gross necropsy at their death. The standards for necropsy set forth in § 163.80-3(b)(10) shall apply.

(9) *Histopathology examination.*—(i) *General.* A histopathology examination shall be performed on all test and control animals in accordance with this paragraph.

(ii) *Examination.* The examination shall include multiple longitudinal and cross sections of the spinal cord (cervical, thoracic, and lumbar regions) and a peripheral nerve (preferably the sciatic nerve).

(iii) *Slide preparation.* Microscopic slides shall be made from tissues fixed in situ with systemic perfusion (e.g., 4 percent paraformaldehyde followed by 5 percent glutaradehyde). These sections shall be stained with hematoxylin and eosin, as well as or in combination with an appropriate myelin- and axon-specific stain. Tissue samples and microscopic slides shall be preserved and held in accordance with § 163.40-5.

(iv) *Examiner.* The standards set forth in § 163.80-3(b)(1)(iii) shall apply.

(v) *Recording.* The histopathology findings shall be recorded and reported by paragraph (c)(3) of this section.

(c) *Data reporting and evaluation.* In addition to information meeting the general reporting requirements of § 163.80-4, the test report shall contain the following information:

(1) *Test protocol.* A description and rationale of the dosage level(s) used.

(2) *Observations and animal records.* All means in the data required in this subparagraph shall be accompanied by the standard deviation.

(i) The following test information, arranged by test group, shall be supplied in tabular form (unless adequate justification is supplied to present these data in another form) and with means, for appropriate measurements:

(A) Data showing, for each animal: Its identification number; whether it died by sacrifice, and if so, whether it was moribund before sacrifice; the week of the test when the sacrifice occurred or the animal's death was noted;

(B) Data showing, for each animal: Its identification number; its weight in grams at the beginning of the test period, at 3- to 4-day intervals throughout the test period, and at death; and its estimated food consumption at 3- to 4-day intervals throughout the test period; and

(C) Data showing the identification number of each animal not subjected to histopathology examination in accordance with the requirements of this section. Whenever the required histopathology examination was not performed, or the manner of performance varied from that required, the reasons and circumstances shall be fully described.

(ii) The following test information shall be supplied in any appropriate form: A description of all observed signs of toxicity of pharmacological effects, or behavioral abnormalities including locomotor ataxia and paralysis, accompanied by the animal's identification number, test group, and date(s) of observation;

(3) *Histopathology data.* The following information shall be presented in the format specified (unless adequate justification is supplied to present these data in another form). All means shall be accompanied by standard deviation. The number of data units on which a calculation is based shall be reported for all percentages and averages.

(i) *Descriptions of lesions, for each animal.* Data shall be submitted in an appropriate form showing:

(A) For each animal, its identification number, and a complete description and diagnosis of every lesion in the animal. Lesions should be graded. A commonly used scale such as ±1, ±2, ±3, and ±4 degrees ranging from very slight to extreme can be used, but other scales are acceptable. Abnormalities observed repeatedly need to be described only once and may subse-

quently be supplied by reference, with any individual variations noted as necessary.

(B) For each animal, a paragraph listing the tissues examined and designated by check mark of those tissues found to be normal.

(C) If a grading system is used, a description of the system.

(ii) *Counts and incidence of lesions.* Data shall be submitted in tabular form, showing:

(A) The number of animals at the start of the test, the number of animals surviving to the termination of the test, and the number of animals in which any lesion was found;

(B) The number of animals affected by each different type of lesion, the average grade of each type of lesion, the number of animals for each type of lesion, and the percentage of those animals examined which are affected by each type of lesion; and

(C) The number of each different type of lesion.

(4) *Evaluation of data.* An evaluation of the test results (including their statistical analysis), based on observations and histopathology results, shall be made and supplied. This submission shall include an evaluation of the relationship, if any, between the animals' exposure to the test substance and the incidence and severity of all abnormalities, including behavioral abnormalities, effects on mortality, and any other toxic effects. The evaluation shall also include a description of statistical methods.

PROTOCOL

Test No. 15: Subchronic Neurotoxicity Study (163.82-5), Chicken

§ 163.82-5 Subchronic Neurotoxicity Studies.

This section contains requirements governing the performance of studies to evaluate delayed neurotoxicity and studies to evaluate other kinds of central nervous system neuropathies. Paragraph (c) of this section establishes standards for performing a subchronic test with hens to assess the potential of a compound to induce delayed neurotoxicity. (See also § 163.81-7). The standards in paragraph (c), as modified in paragraph (b) of this section, apply to studies with other different species (mammals) to evaluate other kinds of central nervous system neuropathies.

(a) *When required.* Data from a subchronic neurotoxicity evaluation are required to support the registration of each manufacturing-use product and formulated product if the active ingredient(s) or any of its (their) metabolites, degradation products, or impurities:

(1) Induces neuropathy or delayed neurotoxicity, as evidenced by the results of an acute test; or

(2) Has a molecular structure closely related to a compound, other than organophosphates or carbamates, known to induce neuropathy or delayed neurotoxicity.

(b) *Standards for tests with mammals.* In addition to the standards set forth in § 163.80-3, the following standards apply if the acute oral (§ 163.81-1) acute dermal (§ 163.81-2), or acute inhalation (§ 163.81-3) studies with the product showed neuropathy or neurotoxicity:

(1) *Conduct of test.* The test methodology shall conform to the standards contained in paragraph (c) of this section for testing of hens, provided, however, that:

(i) Ten animals of each sex of the species which showed the neuropathy or neurotoxicity shall be tested at each dose level;

(ii) The route of exposure shall be the same as the route used in the acute study in which neuropathy or neurotoxicity (if observed) was observed; and

(iii) A compound which is known to induce neuropathy or neurotoxicity in the test species shall be used as the positive control, and if possible, should be structurally related to the test substance.

(2) *Combined protocol.* A subchronic neurotoxicity test conducted in accordance with this paragraph may be combined with a subchronic toxicity test using the same route of exposure required by § 163.82-1 (subchronic oral), § 163.82-3 (subchronic 90-day dermal), or § 163.82-4 (subchronic inhalation) if the standards for both types of testing are met.

(c) *Standards for tests with hens.* In addition to the general standards set forth in § 163.80-3, the following standards apply if the acute delayed neurotoxicity test performed in accordance with § 163.81-7 showed neuropathy or neurotoxicity:

(1) *Substance tested.* The technical grade of the active ingredient which induced the neuropathy or neurotoxicity shall be tested.

(2) *Species and sex.* Testing shall be performed in the female domestic chicken (hen), standard size breeds or strains.

(3) *Number and age of animals.* A minimum of 10 animals per dose shall be tested. Adult hens, preferably between 8-14 months of age (2-3 kg each) at the beginning of the study, shall be used.

(4) *Number and selection of dose levels.* Test data may be rejected by the Agency if the following standards are not satisfied.

(i) A minimum of 3 dose levels, in addition to any control(s), shall be tested.

(ii) The highest dose level shall be one which induces some toxic effects.

(iii) In selection of the high and median dose levels, consideration shall be given to the anticipated slope of the dose response curve.

(iv) The lowest dosage level should be one which does not induce any deleterious effects.

(5) *Control groups.* (i) A concurrent untreated control group is required.

(ii) A concurrent vehicle control group is required. This group shall be exposed only to the diet and to the vehicle used in administering the test substance.

(iii) A positive control is required. Triorthocresylphosphate (TOCP) is recommended.

(6) *Route of administration.* The test substance shall be administered in known quantities by gavage (for example, in gelatin capsule) or by a comparable method, or in the diet.

(7) *Duration of dosing.* All animals shall receive the test substance daily for at least 90 days.

(8) *Observation of animals.* Throughout the test period, each test animal shall be observed daily. Estimates shall be made of food consumption weekly during the test period, and body weight shall be recorded weekly. Each observation shall be made by an appropriately-trained observer, who shall note (and record where pertinent) and behavioral abnormality, locomotor ataxia, and paralysis. Sufficient surveillance of animals shall be made to insure that not more than 10 percent of the animals in any test group are lost from the test due to cannibalism, autolysis of tissues, misplacement, or similar management problems.

(9) *Handling of moribund animals.* Moribund animals shall be sacrificed to lessen the likelihood of unobserved death and subsequent autolysis or cannibalism. All test and control animals shall be subjected to gross necropsy at their death. The standards for necropsy set forth in § 163.80-3(b)(10) shall apply.

(10) *Histopathology examination*—(i) *General.* A histopathology examination (microscopic study) shall be performed on all test and control animals in accordance with this paragraph.

(ii) *Examination.* The examination shall include multiple longitudinal and cross sections of the spinal cord (cervical, thoracic, and lumbo-sacral regions) and ganglia; of the medulla and pons, cerebral and cerebellar cortex, basal ganglia, and hippocampus; of the optic and other cranial nerves; and of the proximal, middle, and distal peripheral nerves (including the sciatic nerve).

(iii) *Tissue and slide preparation and retention.* Microscopic slides shall be made from tissues fixed in situ with systemic perfusion (*e.g.*, 4% paraformaldehyde followed by 5% glutaraldehyde). These sections shall be stained with hematoxylin and eosin, as well as or in combination with an appropriate myelin- and axon-specific stain. Tissue samples, tissue blocks, and microscopic slides shall be preserved and held in accordance with § 163.40-5.

(iv) *Examiner.* The standards set forth in § 163.80-3(b)(1)(iii) shall apply.

(v) *Recording.* The histopathology findings shall be recorded and reported as required by paragraph (d)(3) of this section.

(d) *Data reporting and evaluation.* In addition to information meeting the general reporting requirements of § 163.80-4, the test report shall contain the following information, presented in the format specified (unless adequate justification is submitted to present the data in another form):

(1) *Test protocol.* (i) A description of the test dosage levels used; and

(ii) The rationale for selection of dosage levels.

(2) *Animal data.* For all means in the data required in this subpara-

graph, such means shall be accompanied by the standard deviation.

(i) The following test information, arranged by test groups (sex and dose level), shall be supplied in tabular form:

(A) Data showing, for each animal: Its identification number; whether it died by sacrifice, and, if so, whether it was moribund before sacrifice; and the week of the test when the sacrifice occurred or the animal's death was noted;

(B) Data showing, for each animal: Its identification number, its weight in grams at the beginning and at weekly intervals throughout the test period, and estimated food consumption weekly throughout the test period; and

(C) Data showing the identification number of each animal that was not subjected to histopathology examination in accordance with the requirements of this section. Whenever the required histopathology examination was not performed, or the manner of performance varied from that required, the reasons and circumstances shall be fully described.

(ii) The following test information shall be supplied in any appropriate form: a description of all observed signs of toxicity, pharmacological effect, or behavioral abnormality (including locomotor ataxia and paralysis) accompanied by the animal's identification number, test group (sex and dose level), and date(s) of observation;

(iii) Statistical analyses shall be performed, where appropriate, to assist in the reporting and evaluation of data. All statistical methods used should be identified by reference or fully described.

(3) *Histopathology data.* The following information shall be arranged by test group (dose level and sex) and presented in the format specified. All means shall be accompanied by standard deviation. The number of data units on which a calculation is based shall be reported for all percentages and means.

(i) *Description of lesions, for each animal.* Data shall be submitted in an appropriate form showing:

(A) For each animal, its identification, and a complete description and diagnosis of every lesion in the animal. Non-neoplastic lesions which are observed frequently or which are common in both treated and control animals should be graded. A commonly-used scale such ±, 1, 2, 3, and 4 for degrees ranging from very slight to extreme can be used, but other scales are acceptable. If known, the description and diagnosis should identify any lesion which caused the animal to be moribund or to die. The description and diagnosis shall include the time of appearance (if known) for each lesion. Abnormalities observed repeatedly need to be described only once, and may subsequently be supplied by reference, with any individual variations noted as necessary.

(B) For each animal, a paragraph listing the tissues examined and designation by check mark of those tissues found to be normal.

(C) If a grading system is used, a description of the system.

(ii) *Counts and incidence of lesions, by test group.* Data shall be submitted in tabular form showing for each test group:

(A) The number of animals at the start of the test, the number of animals surviving to the termination of the test, and the number of animals in which any lesion was found;

(B) The number of animals affected by each different type of lesion, the average grade of each type of lesion, the number of animals examined for each type of lesion, and the percentage of those animals examined which were affected by each type of lesion; and

(C) The number of each different type of lesion.

(4) *Evaluation of data.* An evaluation of the test results (including their statistical analysis), based on gross necropsy findings, and histopathology results, shall be made and supplied. This submission shall include an evaluation of the relationship, if any, between the animals' exposure to the test substance and the incidence and severity of all abnormalities, including behavioral abnormalities, histopathologic lesions, effects on mortality, and any other toxic effects. The evaluation shall also include dose-response curves for the various groups, and a description of statistical methods.

PROTOCOL

Test No. 16: Acute Dermal Irritation Study (772.112-25), Rabbit

§ 772.112-25 Primary dermal irritation study.

(a) *Study Design.* (1) *Condition of test substance.* (i) If the substance is a liquid, it must be applied undiluted.

(ii) If the test substance is a solid, it must be slightly moistened with physiological saline before application.

(2) *Species.* Testing must be performed in at least one mammalian species, preferably the albino rabbit. Selection of other species and strains may be acceptable, but must be justified.

(3) *Age.* Young adult animals must be used.

(4) *Number of Animals.* At least six animals must be used.

(5) *Number and selection of dose levels.* A dose of 0.5 ml of liquid or 0.5 g of solid or semisolid is to be applied to each application site.

(6) *Control groups.* (i) A vehicle control group is required if the vehicle is known to cause any toxic dermal reactions or if there is insufficient information about the dermal effects of the vehicle.

(ii) Separate animals are not required for an untreated control group. Each animal serves as its own control.

(b) *Study Conduct.* The test substance must be introduced under 1-inch square gauze patches. The patches must be applied to two intact and two abraded skin sites on each animal. In all animals, the application sites must be clipped free of hair. In addition, the abrasion must penetrate the stratum corneum, but not the dermis. A wrapping material such as gauze covered by an impervious, nonreactive rubberized or plastic material should be used to retard evaporation and keep the test substance in contact with the skin. The animals should be restrained. The test substance must be kept in contact with the skin for 24 hours. At the end of the exposure period, the wrapping should be removed and the skin wiped (but not washed) to remove any test substance still remaining. It may be necessary to rinse off the material if colored test substances are used.

(c) *Observation and scoring.* Animals must be observed and signs of erthyema and edema must be scored at 24 hours and 72 hours after application of the test substance. The irritation is to be scored according to the technique of Draize, J. H. (1959). Observation for irritation and scoring of any irritation must continue daily until all irritation subsides or is obviously irreversible.

(d) *Data reporting and evaluation.* In addition to the information required by § 772.100-2(b)(8), Subpart A, the test report must include the following information:

(1) pH value of each test substance.

(2) In tabular form, the following data for each individual animal and averages and ranges for each test group:

(i) Scores for erythema and edema at 24 hours, at 72 hours, and at any subsequent observations; and

(ii) Primary skin irritation scores according to the technique of Draize.

PROTOCOL

Test No. 17: Subchronic Dermal Irritation Study, Rabbit

(No standard protocol available)

PROTOCOL

Test No. 18: Primary Eye Irritation Study (772.112-24), Rabbit

§ 772.112-24 Primary eye irritation study.

(a) *Study Design.* (1) *Condition of test substance.*

(i) If the test substance is a liquid, it must be placed in the eye undiluted, in accordance with paragraph (b) of this section.

(ii) If the test substance is a solid or granular product, it must be ground into a fine dust or powder. The test substance must not be moistened before it is placed in the eye in accordance with paragraph (b) of this section.

(2) *Species.* Testing must be performed with the albino rabbit.

(3) *Age and condition of animals.* Young adult animals should be used. The eyes must be examined using fluorescein dye procedures at least 24 hours before application of the test substance. Animals showing preexisting corneal injury are to be eliminated.

(4) *Number of animals.* At least nine animals must be used.

(5) *Number and selection of dose.* A dose of 0.1 ml of liquid or 100 mg of solid must normally be applied to each test eye. Smaller quantities may be used when the standard quantities would be lethal, or when 100 mg of the solid cannot feasibly be administered to the eye.

(6) *Caging.* Caging must be designed to minimize exposure to sawdust, wood chips, and other extraneous materials that might enter the eye.

(b) *Study Conduct.* The test substance must be placed on the everted lower lid of one eye; the upper and lower lids are then to be gently held together for 1 second before releasing to prevent loss of material. The other eye, remaining untreated, serves as a control. The treated eyes of six rabbits must remain unwashed. The remaining three rabbits receive test material, and then the treated eye is flushed for one minute with lukewarm water starting no sooner than 20–30 seconds after instillation. A local anaesthetic to reduce pain in test animals may be used prior to administration of the test substance, provided that evidence can be presented indicating no significant difference in toxic reaction to the test substance will result from use of the anaesthetic.

(c) *Observation and scoring.* (i) *Observation.* Readings of ocular lesions must be made at 24, 48, and 72 hours after treatment and at 4 and 7 days after treatment. Readings must be made every 3 days thereafter, if injury persists, for at least 13 days after treatment or until all signs of reversible toxicity subside. Grading and scoring of irritation are to be performed in accordance with the following tables (from Draize, J. H., *et al.* (1965)). The most serious effects, such as pannus or blistering of the conjunctivae and other effects indicative of corrosive action must be reported separately.

(ii) Table of scale of weight scores for grading the severity of ocular lesions.

I. Cornea

(A) Opacity—Degree of Density (Area Taken for Reading) Scattered or diffuse area—details of iris clearly visible—1.

Easily discernible translucent areas, details of iris slightly obscured—2.

Opalescent areas, no details of iris visible, size of pupil barely discernible—3.

Opaque, iris invisible—4.

(B) Area of Cornea Involved.

One quarter (or less) but not zero—1.

Greater than one quarter—less than one-half—2.

Greater than one-half—less than three quarters—3.

Greater than three quarters up to whole area—4.

Score equals AXBX5 Total maximum = 80.

II. Iris

(A) *Values.*—Folds above normal, congestion, swelling, circumcorneal injection (any one or all of these or combination of any thereof), iris still reacting to light (sluggish reaction is positive)—1.

No reaction to light, hemorrhage; gross destruction (any one or all of these)—2.

Score AX5 total possible maximum = 10.

III. Conjunctivae

(A) Redness (Refers to Palpebral Conjunctivae Only).

Vessels definitely injected above normal—1. More diffuse, deeper crimson red, individual vessels not easily discernible—2.

Diffuse beefy red—3.

(B) *Chemosis.*—Any swelling above normal (includes nictitation membrane—1).

Obvious swelling with partial eversion of the lids—2.

Swelling with lids about half closed—3.

Swelling with lids about half closed to completely closed—4.

(C) *Discharge.*—Any amount different from normal (does not include small amount observed in inner canthus of normal animals)—1.

Discharge with moistening of the lids and hairs just adjacent to the lids—2.

Discharge with moistening of the lids and considerable area around the eye—3.

Score (A + B + C) × 2 Total maximum = 20.

The maximum total score is the sum of all scores obtained for the cornea, iris, and conjunctivae.

(d) *Data reporting and evaluation.* In addition to the information required by § 772.100-2(b)(8), Subpart A, the test report must include the following information:

(1) pH value of each test substance.

(2) In tabular form, the following data for each individual animal and the averages and range for each test group (eyes washed and unwashed):

(i) The primary eye irritation score at 24, 48, and 72 hours and 4 and 7 days and any other readings; and

(ii) Description of any serious lesions.

PROTOCOL

Test No. 19: Dermal Sensitization Study (772.112-26), Guinea Pig

§ 772.112-26 Dermal sensitization study.

(a) *Study Design.* (1) *Condition of test substance.* The test substance must be applied undiluted. If the test substance causes marked irritation, it must be diluted with physiological saline until a concentration is found which produces only slight irritation. If the test substance is a solid to be injected intradermally, it should be dissolved in a minimum amount of physiological saline.

(2) *Species.* The test must be performed in at least one mammalian species. The albino guinea pig is the preferred species.

(3) *Age and sex.* Young adult males should be used when albino guinea pigs are tested. Young adults of either sex may be used when albino rabbits are tested.

(4) *Number of animals.* At least 10 animals must be used.

(5) *Number and selection of dose levels.* (i) An initial dose of 0.05 ml must be injected intradermally. This dose must be followed by injection of 0.1 ml three times weekly on alternate days for 3 weeks, so that a total of 10 treatments is administered. Following the 10th sensitizing treatment, the animals should be set aside for 2 weeks after which they should be challenged by a final injection (Landsteiner and Jacobs, 1935).

(ii) If the intradermal injection is impractical because the substance is highly irritating or cannot be dissolved or suspended in a form allowing injection, topical patch application can be substituted using the same schedule but 0.5 ml per application. For patch applications, other materials such as water or alcohol can be used to moisten the test substance (see Buehler, E. V., 1965).

(6) *Controls.* (i) A positive control using a known sensitizing agent is recommended.

(ii) A concurrent vehicle control group is not required.

(b) *Study conduct.* (i) *Preparation of test animals.* Hair must be removed first by clipping and then by shaving from a strip running from flank to trunk along each side of each animal. This procedure must be repeated as necessary.

(ii) *Intradermal injection.* After preparation of the test animal, the test substance must be injected intradermally. The first sensitizing injection must be made by starting at one end of one strip. The succeeding injections must be made by moving along the shaved strip choosing a new location for each treatment.

(c) *Observation and scoring.* Erythema, edema, and other lesions must be scored at 24 hours and 48 hours after each application, according to the standard method (Draize, 1959).

(d) *Data reporting and evaluation.* In addition to the basic information required by § 772.100-2(b)(8), Subpart A, the following information must be reported:

(1) Tabular data for each animal on scores for erythema and edema at 24 and 48 hours postapplication or injection.

(2) Tabular data for the average score from all sensitizing treatments and the score of the challenge treatment.

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