In special circumstances such as in inhalation studies involving aerosols or the use of an emulsifier of uncharacterised biological activity in oral studies, a concurrent negative control group should be utilised.

The negative control group is treated in the same manner as all other test animals, except that this control group should not be exposed to the test substance or any vehicle.

- Route of administration

The three main routes of administration are oral, dermal, and inhalation. The choice of the route of administration depends upon the physical and chemical characteristics of the test substance and the form typifying exposure in humans.

In general, the frequency of exposure may vary according to the route and type of administration chosen, and should be adjusted according to the toxicokinetic profile of the test substance, if available.

Oral studies

Provided it can be shown that the test substance is absorbed from the gastro-intestinal tract, the oral route of administration is preferred. The animals must receive the test substance in their diet, dissolved in drinking water, or given by gavage for the length of time specified in the duration of study, below. If the test substance is administered in the drinking water or mixed in the diet, exposure is continuous. If the test substance is mixed in the diet, the highest concentration to be tested should not exceed 5 per cent, with the exception of nutrients (see section on diet). Ideally, daily dosing on a 7-day per week basis should be used, because dosing on a 5-day per week basis may permit recovery or withdrawal toxicity in the non-dosing period and thus affect the result and subsequent evaluation. However, based primarily on practical considerations, dosing on a 5-day per week basis is considered acceptable.

Dermal studies

Cutaneous exposure may be selected to simulate a main route of human exposure and as a model for induction of skin lesions. Special studies designed for induction of skin tumours are not presented in this Guideline.

Inhalation studies

This Guideline provides some detail on inhalation studies since the technical problems are of greater complexity than for the other types of assay. It is recommended, however, that intratracheal instillation may constitute a valid alternative in specific situations.

Long-term exposures are usually patterned on projected industrial experience, giving the animals a daily exposure of 6 hours after equilibration of chamber concentrations, for 5 days a week (intermittent exposure), or on a possible environmental exposure, with 22-24 hours of exposure per day, 7 days a week (continuous exposure), with about an hour for feeding the animals and maintaining the chambers. In both cases, the animals are usually exposed to a fixed concentration of test materials. A major difference to consider between intermittent and continuous exposure is that with the former there is a 17-18 hour period in which animals may recover from the effects of each daily exposure and an even longer recovery period during weekends.

The choice of intermittent or continuous exposure depends on the objectives of the study and on the human experience that is to be simulated. However, certain technical difficulties must be considered. For example, the advantages of continuous exposure for simulating environmental conditions may be offset by the necessity of watering and feeding during exposure, and by the need for more complicated (and reliable) aerosol and vapour generation and monitoring techniques.

Exposure chambers

The animals should be tested in inhalation chambers designed to sustain a dynamic flow of 12 to 15 air changes per hour to assure an oxygen content of about 19 per cent and an evenly distributed atmosphere. Control and exposure chambers should be identical in construction and design to ensure exposure conditions comparable in all respects except for the exposures to the test substances. The chambers should minimise the crowding of test animals to maximise their exposure to the test substance. As a general rule to ensure the stability of the chamber atmosphere, the total volume of the test animals should not exceed 5 per cent of the volume of the chamber. Slight negative pressure inside the chamber is generally maintained to prevent leakage of the test substance into the surrounding areas.

Physical measurements

The following measurements should be taken with care to avoid major fluctuations in the air concentrations or major discrepancies in the operations of the chambers:

(a) Air flow: the rate of air flow through the chamber should be monitored continuously.

(b) Chamber concentrations: during the exposure period the actual concentrations of the test substance should be held as constant as practicable.

(c) Temperature and humidity: for rodents, the temperature should be maintained at 22°C (± 2°) and the humidity within the chamber at 30-70 per cent, except when water is used to suspend the test substance in the chamber's atmosphere. Preferably both should be monitored continuously.

(d) Particle size measurements: particle size distributions should be made on chamber atmospheres involving liquid or solid aerosols. The aerosol particles should be of respirable size for the test animal used. Samples of the chamber atmospheres should be taken at the breathing level of the animals. The air sample should be representative of the distribution of the particles to which the animals are exposed and should account for, on a gravimetric basis, all of the suspended aerosol, even when much of the aerosol is not respirable. The size analyses should be carried out frequently during the development of the generating system to ensure the stability of the aerosol and only as often thereafter during the exposures as necessary to determine adequately the consistency of the particle distributions to which the animals had been exposed.

- Duration of study

The satellite groups of 20 dosed animals per sex and the 10 associated control animals per sex should be retained in the study for at least 12 months. These animals should be scheduled for sacrifice for an estimation of test-substance-related pathology uncomplicated by geriatric changes.

It is necessary that the duration of the carcinogenicity portion comprises the majority of the normal life span of the animals to be used. It has been suggested that the duration of the study should be for the entire lifetime of all animals. However, a few animals may greatly exceed the average lifetime, and the duration of the study may be unnecessarily extended and

complicate the conduct and evaluation of the study. Rather, a finite period covering the majority of the expected life span of the strain is preferred since the probability is high that, for the great majority of chemicals, induced tumours will occur within such an observation period.

The following guidelines are recommended:

(a) Generally, the termination of the study should be at 18 months for mice and hamsters and 24 months for rats; however, for certain strains of animals with greater longevity and/or low spontaneous tumour rate, termination should be at 24 months for mice and hamsters and at 30 months for rats.

(b) However, termination of the study is acceptable when the number of survivors of the lower doses or control group reaches 25 per cent. For the purpose of terminating the study in which there is an apparent sex difference in response, each sex should be considered a separate study. In the case where only the high dose group dies prematurely for obvious reasons of toxicity, this should not trigger termination.

In order for a negative test to be acceptable, it should meet the following criteria:

(1) No more than 10 per cent of any group is lost due to autolysis, cannibalism, or management problems.

(2) Survival in each group is no less than 50 per cent at 18 months for mice and hamsters and at 24 months for rats.

3. DATA AND REPORTING

- Observations

A careful clinical examination should be made at least once each day. Additional observations should be made daily with appropriate actions taken to minimise loss of animals to the study, e.g. necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals. Careful observations should be performed to detect onset and progression of toxic effects as well as to minimise loss due to diseases, autolysis or cannibalism.

Clinical signs including neurological and ocular changes as well as mortality should be recorded for all animals. Time of onset and progression of toxic conditions, including suspected tumours, should be recorded.

Body weight should be recorded individually on all animals once a week during the first 13 weeks of the test period and at least once every 4 weeks thereafter. Food intake should be determined weekly during the first 13 weeks of the study and then at approximately three-month intervals unless health status or body weight changes dictate otherwise.

Haematological examination

Haematological examination (e.g. haemoglobin content, packed cell volume, total red blood cells, total white blood cells, platelets, or other measures of clotting potential) should be performed at 3 months, 6 months, and at approximately 6-month intervals thereafter and at termination on blood samples collected from 20 rats/sex of all groups. If possible, these collections should be from the same rats at each interval. Differential white blood cell counts of control and highest dose rats, and only if necessary for the intermediate dose rats, should be determined at the same intervals.

If clinical observations suggest a deterioration in health of the animals during the study, a differential blood count on the affected animals should be performed.

A differential blood count is performed on samples from those animals in the highest dosage group and the controls. Differential blood counts are performed for the next lower group(s) only if there is a major discrepancy between the highest group and the controls, or if indicated from the pathological examination.

Urinalysis

Urine samples from 10 rats/sex of all groups, if possible from the same rats at the same intervals as haematological examination above, should be collected for analysis. The following determinations should be made from either individual animals or on a pooled sample/sex/group:

- appearance: volume and density for individual animals

- protein, glucose, ketones, occult blood (semi-quantitatively)
- microscopy of sediment (semi-quantitatively)

Clinical chemistry

At approximately 6-month intervals, and at termination, blood samples are drawn for clinical chemistry measurements from 10 rats/sex of all groups, if possible, from the same rats at each interval. Plasma is prepared from these samples and the following determinations are suggested:

- total protein concentration
- albumin concentration
- liver function tests (such as alkaline phosphatase activity, glutamic-pyruvic transaminase* activity and glutamic oxalacetic transaminase** activity), gamma glutamyl transpeptidase, ornithine decarboxylase
- carbohydrate metabolism such as fasting blood glucose
- kidney function tests such as blood urea nitrogen

• Pathology

The pathological examination, macroscopy as well as microscopy, is often the cornerstone of the chronic toxicity/carcinogenicity study. These aspects should therefore get all necessary attention and should be described and reported in detail, including diagnosis.

Necropsy procedures

A well-performed gross necropsy may provide optimal information for microscopic examination and may in certain cases facilitate more restrictive microscopic examination. An inadequate gross necropsy cannot be replaced by microscopic examination no matter how well-performed. Gross necropsy should be carried out under the guidance of a trained laboratory animal pathologist.

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* Now known as serum alanine aminotransferase.

** Now known as serum aspartate aminotransferase.

Complete gross examination should be done on all animals including those which died during the experiment or were killed in moribund conditions. Prior to sacrifice of all animals, samples of blood should be collected from all animals for differential blood counts. All grossly visible lesions, tumours or lesions suspected of being tumours should be preserved. An attempt should be made to correlate gross observations with the microscopic findings.

All organs and tissues should be preserved for microscopic examination. This usually concerns the following organs and tissues: brain* (medulla/pons, cerebellar cortex, cerebral cortex), pituitary, thyroid (including parathyroid), thymus, lungs (including trachea), heart, salivary glands, liver*, spleen, kidneys*, adrenals*, oesophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, urinary bladder, lymph nodes, pancreas, gonads*, uterus, accessory genital organs, female mammary gland, skin, musculature, peripheral nerve, spinal cord (cervical, thoracic, lumbar), sternum with bone marrow and femur (including joint) and eyes. Although inflation of lungs and urinary bladder with a fixative is the optimal way to preserve these tissues, the inflation of lungs in inhalation studies is essential for appropriae histological examination. In special studies, such as inhalation studies, the entire respiratory tract should be studied including nose, pharynx, and larynx.

If other clinical examinations are carried out, the information obtained from these procedures should be available before microscopic examination, because it may give significant guidance to the pathologist.

Histopathology

All grossly visible tumours and other lesions should be examined microscopically. In addition, the following procedures are recommended:

(a) Microscopic examination with complete description of all lesions found in all preserved organs and tissues of

(1) all animals that died or were killed during the study, and

(2) all of the highest dose groups(s) and controls.

* These organs from 10 animals per sex per group for rodents and all non-rodents, plus thyroid (with parathyroid) for all non-rodents, should be weighed.

(b) Organs or tissues showing abnormalities caused, or possibly caused, by the test substance are also examined in the lower dose groups.

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

(d) The incidence of lesions normally occurring in the strain of animals used (under the same laboratory conditions, i.e. historical control) is indispensible for correctly assessing the significance of changes observed in exposed animals.

• Test report

Each test report must identify:

– the laboratory where the test was performed by name and address;

– the inclusive dates of the test; and

– the individual responsible for the conduct and report of the study.

The test report must include all information necessary to provide a complete and accurate description of the test procedures and an evaluation of the results. It should contain a summary of the data, an analysis of the data, and a statement of the conclusions drawn from the analysis. The summary must highlight data or observations and any deviations from control data which may be indicative of toxic effects including hyperplasia, pre-neoplasia, or neoplasia.