I. INTRODUCTORY INFORMATION

• Prerequisites for studies by oral route
  - Solid or liquid test substance
  - Chemical identification of test substance
  - Purity and impurities of test substance
  - Solubility characteristics
  - Stability, including stability in feed or water when so administered
  - Hydrolysis in relation to pH
  - Ability to form complexes
  - Melting point/boiling point

• Prerequisites for studies by inhalation route
  - Gas, volatile material or aerosol/particulate test substance
  - Chemical identification of test substance
  - Purity and impurities of test substance
  - Liquid: vapour pressure, boiling point
  - Aerosol/particulate: particle size, shape and density distribution
  - Flash point
  - Explosivity

• Standard documents

A number of documents have been published providing guidelines for toxicological/safety evaluation. A review of these documents shows both common elements as well as variations in approach and experimental design. As the development of these Guidelines progressed, the use of draft guidelines from a number of nations was made available for our consideration. In addition to the efforts of the many national experts in the group, acknowledgement is given to the World Health Organization and the International Agency for Research on Cancer for providing documents as well as the expertise of representatives of these international bodies.
2. **METHOD**

**A. INTRODUCTION, PURPOSE, SCOPE, RELEVANCE, APPLICATION AND LIMITS OF TEST**

The objective of a combined chronic toxicity/carcinogenicity study is to determine the effects of a substance in a mammalian species following prolonged and repeated exposure. The application of these Guidelines should generate data on which to identify the majority of chronic and carcinogenic effects and to determine dose-response relationships.

Ideally, the design and conduct should allow for the detection of neoplastic effects and a determination of carcinogenic potential as well as general toxicity, including neurological, physiological, biochemical, and hematological effects and exposure-related morphological (pathology) effects.

**B. DESCRIPTION OF THE TEST PROCEDURE**

- **Characterisation of the test substance and treatment mixture**

Prior to the initiation of toxicological studies, there should be a characterisation of the test substance. The information on chemical identity and structure can sometimes be used in an analysis based on structure-activity relationships to indicate possible biological or toxicological activity. The physical-chemical properties of the test substance provide important information for the selection of the route of administration, the design of the studies and the handling and storage of the test substance.

The composition of the test substance, including major impurities, should be known prior to initiating the study.

The relevant physical-chemical properties, including stability of the test substance, should be known prior to the initiation of a combined chronic toxicity/carcinogenicity study.

The development of an analytical method for qualitative and quantitative determination of the test substance (including major impurities when possible) in the dosing medium and biological material should precede the initiation of long-term studies.
Experimental animals

Selection of species and strain

Preliminary studies providing data on acute, subchronic, and toxicokinetic responses should have been carried out to permit an appropriate choice of animals (species and strain). As discussed in other Guidelines, the mouse and rat have been most widely used for assessment of carcinogenic potential, while the rat and dog have been most often studied for chronic toxicity.

Typically the rat has been used for a combined chronic toxicity/carcinogenicity assessment. However, this does not mitigate against using other species. Where available, the strain selected should be susceptible to the carcinogenic or toxic effect of the class of substances being tested, provided it does not have a spontaneous background too high for meaningful assessment.

Sex and age at start of experiment

Both sexes should be used. Long-term bioassays for chronic toxicity and carcinogenicity have been initiated most commonly in weanling or post-weanling animals. This procedure has allowed the greater part of the animals' life span for tumour development to occur coincident with the exposure to the test substance.

Interest in the possible increased susceptibility of the neonate arose with the evidence which established the influence of host age on viral carcinogenesis. More recently, prenatal exposure has been the subject of considerable experimentation. It has been demonstrated that some tissues, particularly tissues from the nervous system, are more susceptible during foetal life than later. This greater susceptibility is predicated on the active organogenesis and cellular proliferation that occurs in the foetus.

An adequate assessment of the extent of specific foetal sensitivity has not yet been accomplished.

It has been shown that the neonate is usually, but not always, more sensitive than the adult. At present, there is only limited evidence that prenatal exposure may reveal the carcinogenic potential of a chemical that would not have been revealed had the treatment started at a later age.
Foetal, neonatal and weanling animals differ from adults in several additional respects, including anatomical, biochemical and physiological characteristics, viral susceptibility, hormonal status and immunologic competence. The exact role of these factors in carcinogenesis development is in need of further definition.

Dosing of the rodents should begin as soon as possible after weaning and acclimatisation, and preferably before the animals are 6 weeks old. Studies using prenatal or neonatal animals may be recommended under special conditions.

Size of experimental groups

Statistical advice should be obtained to assure maximum reliability of the study and results amenable to statistical evaluation. The use of adequate randomisation procedures for the proper allocation of animals to test and control groups is of particular importance.

A sufficient number of animals should be used so that at the end of the study enough animals in every group are available for thorough biological and statistical evaluation.

For such reasons, each dose group and concurrent control group not intended for earlier sacrifice should contain at least 50 animals of each sex. A high dose satellite group for evaluation of pathology other than neoplasia should contain 20 animals of each sex, while the satellite control group would contain 10 animals of each sex. A moderate increase in group size will provide relatively little increase in statistical power of the test. If interim sacrifice(s) are included in the study plan, the initial number should be increased by the number of animals scheduled for the interim sacrifice(s).

Animal care, diet and water supply

Stringent control of environmental conditions and proper animal care techniques are mandatory for meaningful results. As part of such control, access to animal facilities should be monitored to prevent excessive traffic.

Factors such as housing conditions, intercurrent disease, drug therapy, impurities in diet, air, water, and bedding, and general animal care facilities can significantly influence the outcome of animal experiments.
The control of intercurrent infectious diseases or parasites is facilitated if rodents are bred and maintained in conditions free from specific pathogenic organisms. Bedding to be used in long-term studies should be sterilised. Animals should be housed in quiet, well-ventilated rooms having controlled lighting, temperature and humidity. Experiments should not be initiated until animals have been allowed a period of acclimatisation to environmental conditions, nor should animals from outside sources be placed on test without an adequate period of quarantine.

Housing of more than one animal species in a room should be avoided. Unless there is little chance for the inadvertant cross exposure of animals to the test substance, only one chemical should be tested in each room. The same consideration should be given to the housing of control animals in the same room as test animals. If control animals are housed in different quarters, additional problems in data evaluation may arise.

Cages, racks, and other equipment must be capable of regular and easy cleaning. The use of disinfectants and pesticides should be avoided, particularly where they may come in contact with the animal, since such biologically active compounds may affect the results of animal tests. More detailed animal husbandry procedures can be found in the scientific literature, animal welfare publications and official documents of Good Laboratory Practices, including those of the OECD.

The diet should meet all the nutritional requirements of the species tested and should be free of impurities that might influence the outcome of the test, because dietary contaminants and the level of various nutrients have been shown to alter physiological processes of experimental animals. Rodents should be fed and watered *ad libitum* with food replaced at least weekly. At present, three types of diet are utilised: conventional (standard), synthetic, and various open formula diets. Of these, the first two are the most widely used in carcinogenicity bioassays. Whichever diet is chosen, suppliers must ascertain by periodic monitoring the nutrient quality and the level of contaminants in the basal diet. It is highly desirable to known the effect of the dietary regimen on metabolism and animal longevity as well as tumour development.

Special attention should be paid to the dietary composition when the test material itself is a nutrient (e.g. an industrially treated protein or starch; single cell protein; irradiated food...
product), because such a product usually has to be incorporated into the diet at levels as high as 20 per cent to 60 per cent at the expense of a corresponding nutrient (e.g. modified versus unmodified starch; single cell protein versus soybean meal).

Variations in the use patterns of industrial and agricultural chemicals throughout the world preclude harmonization by OECD on one list of dietary contaminants. Notwithstanding this fact, common dietary constituents which are known to influence carcinogenesis (e.g. antioxidants, unsaturated fatty acids, selenium) should not be present in interfering concentrations. The potential impact of several common dietary contaminants upon carcinogenicity assessment necessitates that special attention be given to their presence. In this respect, substances of concern include pesticide residues, chlorinated and polycyclic aromatic hydrocarbons, oestrogens, heavy metals, nitrosamines and mycotoxins.

In addition, periodic analysis of the basal diet may be carried out by the testing laboratory for both nutrients and unintentional contaminants, including carcinogens. The results from such analyses should be retained and included in the final report on each test substance.

When the test substance is administered in the water or food, stability tests are essential. Properly conducted stability and homogeneity tests prior to the chronic study should be used to establish the frequency of diet preparation and monitoring required.

When diets are sterilised, the effects of such procedures on the test substance and dietary constituents should be known. Appropriate adjustments to nutrient levels should be carried out. The effect of chemical sterilants (e.g. ethylene oxide) on the bioassay should be ascertained.

During carcinogenicity tests, investigators should be aware of potential contaminants in the water used. Although water approved for human consumption is generally satisfactory, the investigator should ascertain the data available on the components in the water supply.
• **Test conditions**

*Dose levels and frequency of exposure*

For carcinogenicity risk assessment purposes, at least three dose levels should be used, in addition to the concurrent control group. The highest dose level should be sufficiently high to elicit signs of minimal toxicity without substantially altering the normal life span due to effects other than tumours. Signs of toxicity are those that may be indicated by alterations in serum enzyme levels or slight depression of body weight gain (less than 10 per cent). For a diet-mixture, the highest concentration should not exceed 5 per cent with the exception of nutrients (see section on diet).

The lowest dose should not interfere with the normal growth, development, and longevity of the animal; and it must not otherwise cause any indication of toxicity. In general, this should not be lower than 10 per cent of the high dose.

The intermediate dose(s) should be established in a mid-range between the high and low doses, depending upon the toxicokinetic properties of the chemical, if known.

For chronic toxicological assessment, an additional treated and a concurrent control satellite group are included in the study. The highest dose for satellite animals should be chosen so as to produce frank toxicity in order to elucidate a toxicological profile of the test substance.

The selection of these dosage levels should be based on existing data, preferably on the results of a subchronic study.

Frequency of exposure normally is daily but may vary according to the route chosen. If the chemical is administered in the drinking water or mixed in the diet, it should be continuously available. The frequency of administration may be adjusted according to the toxicokinetic profile of the test substance.

*Controls*

A concurrent control group which is identical in every respect to the exposed groups, except for exposure to the test substance, should be used.
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.
"Combined Chronic Toxicity/Carcinogenicity Studies"

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°C) 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.

* 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 appropriate 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 indispensable 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.