II

Acts whose publication is not obligatory

COMMISSION

COMMISSION DIRECTIVE

of 18 November 1987

adapting to technical progress for the ninth time Council Directive 67/548/EEC on the approximation of laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances

(87/302/EEC)

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Economic Community,

Having regard to Council Directive 67/548/EEC of 27 June 1967 on the approximation of laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances (1), as amended for the sixth time by Directive 79/831/EEC (2), and in particular Article 19 thereof,

Whereas Article 3 (1) of Directive 67/548/EEC provides that the physico-chemical properties, toxicity and ecotoxicity of the substances and preparations shall be determined according to the methods specified in Annex V;

Whereas Article 3 (2) of Directive 67/548/EEC provides that the real or potential environmental hazard of a substance or preparation shall be assessed according to the characteristics set out in Annexes VII and VIII;

Whereas Annex V to the version introduced by Commission Directive 84/449/EEC (3) presently contains only those test methods corresponding to the characteristics detailed in Annex VII and it is necessary also to make available test methods corresponding to the characteristics detailed in Annex VIII;

Whereas the provisions of this Directive are in accordance with the opinion of the Committee for the Adaptation to Technical Progress of the Directives on the Removal of Technical Barriers to Trade in Dangerous Substances and Preparations,

HAS ADOPTED THIS DIRECTIVE:

Article 1

The text of the Annex to this Directive is added to Annex V to Directive 67/548/EEC.

Article 2

Member States shall adopt and publish before 31 December 1988 the measures needed to comply with this Directive and shall immediately inform the Commission thereof. They shall apply those measures by 30 June 1989 at the latest.

Article 3

This Directive is addressed to the Member States.

Done at Brussels, 18 November 1987.

For the Commission
Stanley CLINTON DAVIS
Member of the Commission

(1) OJ No 196, 16. 8. 1967, p. 1/67.
(2) OJ No L 259, 15. 10. 1979, p. 10.
(3) OJ No L 251, 19. 9. 1984, p. 1.

ANNEX

The test methods described herein are for the determination of some of the toxicological and ecotoxicological properties listed in Annex VIII to Council Directive 79/831/EEC. Test methods appropriate to level 1 and level 2 of Annex VIII are described, but the tests are not subdivided as a function of the different levels.

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PART B: METHODS FOR THE DETERMINATION OF TOXICITY

GENERAL INTRODUCTION: PART B

LONG-TERM STUDIES

Sub-chronic, chronic and carcinogenicity studies

Characterization of the test substance and treatment mixture

The composition of the test substance, including major impurities, and its relevant physico-chemical properties, including stability, should be known prior to the initiation of any toxicity study.

The physico-chemical properties of the test substance provide important information for the selection of the route of administration, the design of sub-chronic, chronic or carcinogenicity studies and the handling and storage of the test substance.

Information on chemical structure and physico-chemical properties may also provide and indication of the absorption characteristics by the intended route of administration and the metabolic and tissue distribution possibilities. There may also be information on toxicokinetic parameters from preceding toxicity and toxicokinetic studies.

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 the study.

Experimental animals: selection of species and strain

Since it is necessary to treat animals for a major portion of their lifespan, the studies tend to be limited to easily maintained and relatively short-lived test species. It is highly desirable that the incidence of spontaneous disease and tumours in the strain of animal used, when kept under similar conditions, be known.

Strains should be well characterized and free from interfering congenital defects. The use of inbred strains, of F1 hybrids, has some advantages in this respect, but where sufficient background data are available on outbred strains, with animals derived from closed stocks, these are acceptable.

Animal care, diet and water supply

Animal tests and studies shall be conducted in accordance with national regulations and shall take into account humane principles and international developments in the field of animal welfare.

Stringent control of environmental conditions and proper animal care techniques are mandatory for meaningful results. 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 repeated dose studies. In general, the effect of chemical sterilants on the study should be known.

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. Rodents should be fed and watered *ad libitum*, with food replaced at least weekly. At present, three types of diet are utilized: conventional, synthetic, and various open formula diets.

Whichever diet is chosen, suppliers must ascertain by periodic monitoring the nutritive value and the level of contaminants in the basal diet and provide this information to the laboratory with each batch of feed. It is highly desirable that the effects of the dietary regimen on metabolism, as well as on the development of tumours and animal longevity, are known.

In addition, check analyses of the basal diet may be carried out by the testing laboratory for both food components and unintentional contaminants, including carcinogens. If this is done, the results of analyses should be retained and included in the final report on each test substance.

Common dietary constituents which are known to influence carcinogenesis (e.g. anti-oxidants, unsaturated fatty acids, selenium) should not be present in interfering concentrations. The potential impact of several common dietary contaminants upon carcinogenicity assessment requires that special attention is paid to the presence in the diet of pesticide residues, organochlorine compounds, polycyclic aromatic hydrocarbons, oestrogens, heavy metals, nitrosamines and mycotoxins.

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

When diets are sterilized, the effects of such procedures on the test substance and dietary constituents should be know. Any appropriate adjustments should be carried out.

During carcinogenicity tests, investigators should be aware of potential contaminants in the water used. Water approved for human consumption is generally satisfactory and information on its composition should be available.

The concentration of a test substance in the diet may need to be adjusted as the animals grow in order to maintain a reasonably constant intake of test substance relative to body weight.

The nutritive value of control and test diets should be made as similar as possible. Thus the nutritive value of a test substance mixed in the diet needs to be considered. Experience suggests that up to 5 % of non-nutritive test substances in the diet is unlikely to interfere significantly with the nutritional value of the diet.

1. Inhalation studies

No limit test is specified because it has not been found possible to define a single inhalation exposure limit value.

2. Teratogenicity study

The test method is primarily directed to administration by the oral route. Alternatively, other routes may be used depending on the physical properties of the test substance or likely route of human exposure. In such cases, the test method should be suitably adapted taking into consideration the appropriate elements of the 28-day test methods.

3. Toxicokinetics

Toxicokinetic studies help in the interpretation and evaluation of toxicity data. These studies are intended to elucidate particular aspects of the toxicity of the chemical under test and the results may assist in the design of further toxicity studies. It is not envisaged that in every case all parameters will need to be determined. Only in rare cases will the whole sequence of toxicokinetic studies (absorption, excretion, distribution and metabolism) be necessary. For certain compounds, changes in this sequence may be advisable or a single-dose study may be sufficient.

Definitions

Toxicokinetics:	the study of the absorption, distribution, metabolism and excretion of test substances;
Absorption:	the process(es) by which an administered substance enters the body;
Excretion:	the process(es) by which the administered substance and/or its metabolites are removed from the body;
Distribution:	the process(es) by which the absorbed substance and/or its metabolites partition within the body;
Metabolism:	the process(es) by which the administered substances is structurally changed in the body by either enzymatic or non-enzymatic reactions.

4. Acute and sub-acute study in a second species

The purpose of a study in a second species is to complement the conclusions drawn from the first.

In the case of a study in a second species, the test method already described may be used or adapted for a smaller number of animals.

5. Fertility studies

Where a three-generation reproduction test is required, the described method for the two-generation reproduction test can be extended to cover a third generation.

6. Mutagenicity studies

Additional mutagenicity tests including screening tests for carcinogenicity

In Annex VIII to the Directive, additional studies to investigate mutagenicity further or the pre-screen for carcinogenicity are mentioned. The studies outlined in this section may in general be used to investigate either aspects.

Introduction

The initial assessment of the mutagenic activity of a substance consists of assays for gene (point) mutations in bacteria and for cytogenetic damage in mammalian cells (*in vitro* or *in vivo*); suitable methods for these 'base set' studies were described previously. This section is concerned with supplementary studies which are suitable for the verification and/or extension of results obtained in the base set and which can be used for a number of purposes:

1. to confirm results obtained in the base set;
2. to investigate end points not studied in the base set;
3. to initiate or extend *in vivo* studies.

For these purposes the range of tests described include both *in vitro* and *in vivo* eukaryotic systems and an extended range of biological end-points. The tests provide information on point mutations in organisms more complex than the bacteria used in the base set, and extend the information on the ability of a substance to induce chromosomal aberrations.

Tests for end-points other than point mutations and chromosomal aberrations are also described. These provide complementary information and can be used as appropriate in testing schemes.

As a general principle, when a programme of further mutagenicity studies is considered, it should be designed so as to provide relevant additional information on the mutagenic and/or carcinogenic potential of that substance.

The actual studies which may be appropriate in a specific instance will depend on numerous factors, including the chemical and physical characteristics of the substance, the results of the initial bacterial and cytogenetic assays, the metabolic profile of the substance, the results of other toxicity studies, and the known uses of the substance. A rigid schedule for selection of tests is therefore inappropriate in view of the variety of factors which may require consideration. However, some general principles may give guidance. If a test was positive in the base set, the supplementary studies should include at least one test able to detect the same genetic end-point. If both tests in the base set were negative, a test for gene mutations as well as a test for chromosome aberrations should normally be performed as supplementary studies. It may also be appropriate to obtain additional data from the indicator tests (as listed below).

Methods for such investigations are grouped below on the basis of their principal genetic end-point.

Studies to investigate gene (point) mutations

To investigate further the potential of a substance to produce gene (point) mutations, one or other of the following tests may be appropriate:

(a) Forward or reverse mutation studies using eukaryotic micro-organisms *(Saccharomyces cerevisiae).*

(b) *In vitro* studies to investigate forward mutation in mammalian cells.

(c) The sex-linked recessive lethal assay in *Drosophila melanogaster.*

(d) *In vivo* somatic cell mutation assay: the mouse spot test.

Studies to investigate chromosome aberrations

To investigate further the potential of a substance to produce chromosome aberrations one or other of the following tests may be appropriate:

(a) *In vivo* cytogenetic studies in mammals;

In vivo metaphase analysis of bone marrow cells should be considered if it has not been included in the initial assessment ('base-set' studies). In addition *in vivo* germ cell cytogenetics may be investigated;

(b) *In vitro* cytogenetic studies in mammalian cells, if this has not been included in the initial assesment;

(c) Dominant lethal studies in rodents;

(d) Mouse heritable translocation test.

Indicator tests for effects on DNA

Methods are available which provide an indication of some effects on DNA but which do not have a mutagenic event as the 'end point'. These studies may provide information complementary to that obtained from the mutagenicity studies, and which may be useful in the interpretation of such studies. When there is a need for these investigations one or other of the following methods using eukaryotic microorganisms or mammalian cells, may be appropriate:

(a) Mitotic recombination in *Saccharomyces cerevisiae*;

(b) DNA damage and repair — unscheduled DNA synthesis — mammalian cells *(in vitro)*;

(c) Sister chromatid exchange in mammalian cells *(in vitro)*.

Other indicator tests for carcinogenic potential

Mammalian cell-transformation assays are available which measure the ability of a substance to induce morphological and behavioral changes in cell culture which are thought to be associated with malignant transformation *in vivo*. A number of different cell types and criteria for transformation may be used.

Risk assessment for heritable effects in mammals

There are methods available to measure heritable effects in whole mammals produced by gene (point) mutations (the mouse specific locus test [1]) or for chromosome aberrations (the mouse heritable translocation test). Such methods may be used when estimating the possible genetic risk of a substance to man. However, in view of the complexities involved in those tests and the very large number of animals necessary, particularly for the specific locus test, a strong justification is needed before undertaking these studies.

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[1] The mouse specific locus test (which is not described in this document) can be used to measure germ-cell mutation in the first generation after exposure to a mutagenic substance. Genetic alterations leading to changes in gene products producing visible phenotypes, can be detected and quantified.

SUB-CHRONIC ORAL TOXICITY TEST

90-DAY REPEATED ORAL DOSE USING RODENT SPECIES

1. METHOD

1.1. Introduction

See General Introduction Part B.

1.2. Definitions

See General Introduction Part B.

1.3. Reference substances

None.

1.4. Principle of the test method

The test substance is administered daily, orally, in graduated doses to several groups of experimental animals, one dose per group for a period of 90 days. During the period of administration the animals are observed daily to detect signs of toxicity. Animals which die during the test are necropsied, and at the conclusion of the test surviving animals are necropsied.

1.5. Quality criteria

None.

1.6. Description of the test method

Preparations

The animals are kept under the experimental housing an feeding conditions for at least five days prior to the test. Before the test healthy young animals are randomized and assigned to the treatment and control groups.

The test substance may be administered in the diet, by gavage, in capsules, or in the drinking water. All animals should be dosed by the same method during the entire experiment period. If a vehicle or other additives are used to facilitate dosing, they should be known not to produce toxic effects. Historical data can be used if appropriate.

Test conditions

Experimental animals

Unless there are contra-indications, the preferred species is the rat. Commonly used laboratory strains of young healthy animals should be employed and dosing should begin ideally before the rats are six and in any case not more than eight weeks old. At the commencement of the study the weight variation in the animals used should not exceed ± 20 % of the mean value. Where a sub-chronic oral study is conducted as a preliminary to a long-term study, the same species and strain should be used in both studies.

Number and sex

At least 20 animals (10 female and 10 male) should be used at each dose level. The females should be nulliparous and non-pregnant. If interim sacrifices are planned, the number should be increased by the number of animals scheduled to be sacrificed before the completion of the study. In addition, a satellite group of 20 animals (10 animals per sex) may be treated with the high-dose level for 90 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for 28 days post treatment.

Dose levels

At least three dose levels and a control should be used. Except for treatment with the test substance, animals in the control group should be handled in an identical manner to the test group subjects. Where a vehicle is used to facilitate dosing, the controls should be dosed with the vehicle in the same way as the treated groups, and receive the same amount of vehicle as that received by the highest dose level group. The highest dose level should result in toxic effects but produce no, or few, fatalities. The lowest dose level should not produce any evidence of toxicity. Where there is a usable estimation of human exposure the lowest level should exceed this. Ideally, the intermediate dose level should produce minimal observable toxic effects. If more than one intermediate dose is used the dose levels should be spaced to produce a gradation of toxic effects.

In the low and intermediate groups, and in the controls, the incidence of any fatalities should be low to permit a meaningful evaluation of results.

When the test substance is administered in the diet either a constant dietary concentration (ppm or mg/kilogram of food) or a constant dose level in terms of the animals' body weight may be used; the alternative used must be specified. For a substance administered by gavage, the dose should be given at similar times each day. Dose levels should be adjusted at intervals (weekly or bi-weekly) to maintain a constant dose level in terms of animal body weight.

Limit test

If a 90-day test conducted in accordance with the method detailed below, at one dose level of 1 000 mg/kilogram body weight/day, or a higher dose level related to possible human exposure where this is known, produces no evidence of toxic effects, further testing may not be considered necessary. For substances of low toxicity it is important to ensure that, when administered in the diet, the quantities and other properties of the test substance involved do not interfere with normal nutritional requirements.

Observation period

All the animals should be observed daily and signs of toxicity recorded including their time of onset, degree and duration. The time of death and the time at which signs of toxicity appear and disappear should be recorded.

Procedure

The animals are dosed with the test substance ideally on seven days per week for a period of 90 days. Animals in any satellite group scheduled for follow-up observations should be kept for a further 28 days without treatment to detect recovery from, or persistence of, toxic effects.

Cageside observations should include changes in skin and fur, eyes and mucous membranes as well as respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behaviour pattern. Measurements should be made of food consumption (and water consumption when the test substance is administered in the drinking water) weekly and the animals weighed weekly.

Regular observation of the animals is necessary to ensure that as far as possible animals are not lost from the test due to causes such as cannibalism, autolysis of tissues or misplacement. At the end of the test period all survivors in the non-satellite treatment group are necropsied. Moribund animals should be removed and necropsied when noticed.

The following examinations are customarily made for all animals including the controls:

(a) Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, should be made prior to the administration of the test substance and at the termination of the study, preferably in all animals but at least in the high dose and control groups. If changes in the eyes are detected all animals should be examined.

(b) Haematology, including haematocrit, haemoglobin concentration, erythrocyte count, total and differential leucocyte count, and a measure of clotting potential such as clotting time, prothrombin time, thromboplastin time, or platelet count should be investigated at the end of the test period.

(c) Clinical biochemistry determination on blood should be carried out at the end of the test period. Test areas which are considered appropriate to all studies are electrolyte balance, carbohydrate metabolism, liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the

substance. Suggested determinations are: calcim, phosphorus, chloride, sodium, potassium, fasting glucose (with period of fasting appropriate to the species), serum glutamic pyruvic transaminase (1), serum glutamic oxaloacetic transaminase (2), ornithine decarboxylase, gamme glutamyl transpeptidase, urea nitrogen, albumin, blood creatinine total bilirubin and total serum protein measurements. Other determinations which may be necessary for an adequate toxicological evaluation include analyses of lipids, hormones, acid/base balance, methaemoglobin and cholinesterase activity. Additional clinical biochemistry may be employed where necessary to extend the investigation of observed effects.

(d) Urinalysis is not required on a routine basis but only when there is an indication based on expected or observed toxicity.

If historical baseline data are inadequate, consideration should be given to determination of haematological and clinical biochemistry parameters before dosing commences.

Gross necropsy

All animals should be subjected to a full gross necropsy which includes examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. The liver, kidneys, adrenals and testes should be weighed wet as soon as possible after dissection to avoid drying.

The following organs and tissues should be preserved in a suitable medium for possible future histopathological examination: all gross lesions, brain — including sections of medulla/pons, cerebellar cortex and cerebral cortex, pituitary, thyroid/parathyroid, any thymic tissue, trachea and lungs, heart, aorta, (salivary glands), liver, spleen, kidneys, adrenals, pancreas, gonads, uterus (accessory genital organs), (skin), oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, urinary bladder, representative lymph node, (female mammary gland), (thigh musculature), peripheral nerve, sternum with bone marrow, (eyes), (femur, including articular surface), (spinal cord at three levels — cervical, mid-thoracic and lumbar), and (exorbital lachrymal glands). The tissues mentioned between brackets need only be examined if indicated by signs of toxicity or target-organ involvement.

Histopathological examination

(a) Full histopathology should be carried out on the organs and tissues of animals in the control and high-dose groups.

(b) All gross lesions should be examined.

(c) Target organs in other dose groups should be exmined.

(d) The lungs of animals in the low- and intermediate-dose groups should be subjected to histopathological examination for evidence of infection, since this provides a convenient assessment of the state of health of the animals. Consideration should also be given to histopathological examination of the liver and kidneys in these groups. Further histopathological examination may not be required routinely on the animals in these groups but must always be carried out in organs which showed evidence of lesions in the high-dose group.

(e) When a satellite group is used, histopathology should be performed on tissues and organs identified as showing effects in the treated groups.

2. DATA

Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, and the percentage of animals displaying each type of lesion. Results should be evaluated by an appropriate statistical method. Any recognized statistical method may be used.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following information:

— species, strain, source, environmental conditions, diet,

— test conditions,

— dose levels (including vehicle, if used) and concentrations,

— toxic response data by sex and dose,

(1) Now known as serum alanine aminotransferase.
(2) Now known as serum aspartate aminotransferase.

— no-effect level when possible,

— time of death during the study or whether animals survived to termination,

— description of toxic or other effects,

— the time of observation of each abnormal sign and its subsequent course,

— food and bodyweight data,

— ophthalmological findings,

— haematological tests employed and all results,

— clinical biochemistry tests employed and all results (including results of any urinalysis),

— necropsy findings,

— a detailed description of all histopathological findings,

— statistical treatment of results, where possible,

— discussion of the results,

— interpretation of the results.

3.2. **Evaluation and interpretation**

See General Introduction Part B.

4. **REFERENCES**

See General Introduction Part B.

SUB-CHRONIC ORAL TOXICITY TEST

90-DAYS REPEATED ORAL DOSE STUDY USING NON-RODENT SPECIES

1. METHOD

1.1. Introduction

See General Introduction Part B.

1.2. Definitions

See General Introduction Part B.

1.3. Reference substances

None.

1.4. Principle of the test method

The test substance is administered daily, orally in graduated doses to several groups of experimental animals (non-rodent), one dose per group for a period of 90 days. During the period of administration the animals are observed daily to detect signs of toxicity. Animals which die during the test are necropsied, and at the conclusion of the test surviving animals are necropsied.

1.5. Quality criteria

None.

1.6. Description of the test method

Preparations

The animals are kept under the experimental housing and feeding conditions for at least five days prior to the test. Befor the test, healthy young animals are randomized and assigned to the treated and control groups.

The test substance may be administered in the diet, or administration in capsules may be found more convenient. Other means of oral administration may be used. All animals should be dosed by the same method during the entire experimental period. If a vehicle or other additives are used to facilitate dosing, they should be known not to produce toxic effects. Historical data can be used if appropriate.

Test conditions

Experimental animals

The commonly used non-rodent species is the dog, preferably of a defined breed. Other non-rodent species may be used. Young healthy animals should be employed and, in the case of the dog, dosing should be commenced preferably at four to six months and not later than nine months of age. Where a sub-chronic oral test is conducted as a preliminary to a long-term test, the same species/breed should be used in both tests.

Number and sex

At least eight animals (four female and four male) should be used at each dose level. The number of animals at the termination of the study must be adequate for a meaningful evaluation of toxic effects.

Dose levels

At least three dose levels and a control should be used. Except for treatment with the test substance, animals in the control group should be handled in an identical manner to the test group subjects. The highest dose level should result in toxic effects but not produce fatalities. The lowest dose level should not produce any evidence of toxicity.

Where there is a usable estimation of human exposure the lowest level should exceed this. Ideally, the intermediate dose level should produce minimal observable toxic effects. If more than one intermediate dose is used, the dose levels should be spaced to produce a gradation of toxic effects.

In the low and intermediate groups, and in the controls, there should also be no fatalities.

For substances of low toxicity it is important to ensure that, when administered in the diet, the quantities of the test substance involved do not interfere with normal nutrition.

When the test substance is administered in the diet either a constant dietary concentration (ppm or mg/kilogram of food) or a constant dose level in terms of animal body weight may be used; the alternative used must be specified. When the dose is administered directly, for example by capsule, the dose should be given at similar times each day, and adjusted as necessary at weekly intervals to maintain a constant dose level in terms of animal body weight. Where a sub-chronic test is used as a preliminary to a long-term test, a similar diet should usually be used in both studies.

Limit test

If a 90-day study conducted in accordance with the method detailed below, at one dose level of 1 000 mg/kilogram body weight/day, or a higher dose level related to possible human exposure where this is known, produces no evidence of toxic effects, further testing may not be considered necessary. For substances of low toxicity it is important to ensure that, when administered in the diet, the quantities and other properties of the test substance involved do not interfere with normal nutritional requirements.

Observation period

All the animals should be observed daily and signs of toxicity recorded including their time of onset, degree and duration. The time of death and the time at which signs of toxicity appear and disappear should be recorded.

Procedure

The animals are dosed with the test substance ideally on seven days per week over a period of 90 days. However, based primarily on practical considerations, when the substance is administered other than in the diet, dosing on a five-day per week basis is considered to be acceptable.

Observation should include, but not be limited to, changes in skin and fur, eyes and mucous membranes, as well as respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behaviour pattern. Measurements should be made of food consumption (and water consumption when the test substance is administered in the drinking water) weekly, and the animals weighed weekly.

A careful clinical examination of the animals should be made daily with appropriate actions taken to minimize loss of animals to the test. At the end of the exposure period all surviving animals are necropsied. Moribund animals should be removed and necropsied when noticed.

The following examinations are customarily made on all animals including the controls:

(a) Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, should be made prior to the administration of the test substance and at the termination of the study, preferably in all animals but at least in the high-dose and control groups. If changes in the eyes are detected all animals should be examined.

(b) Haematology, including haematocrit, haemoglobin concentration, erythrocyte count, total and differential leucocyte count, and a measure of clotting potential such as clotting time, prothrombin time, thromboplastin time, or platelet count should be investigated at the beginning, then either at monthly intervals or midway through the test period, and finally at the end of the test period.

(c) Clinical biochemistry determination on blood should be carried out at the beginning, then either at monthly intervals or midway through the test and finally at the end of the test period. Test areas which are considered appropriate to all studies are electrolyte balance, carbohydrate metabolism, liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance. Suggested determinations are calcium, phosphorus, chloride, sodium, potassium, fasting glucose (with period of fasting appropriate to the species/breed), serum glutamic pyruvic transaminase (1), serum glutamic oxaloacetic transaminase (2), ornithine decarboxylase, gamma glutamyl transpeptidase, urea nitrogen, albumin, blood

(1) Now known as serum alanine aminotransferase.
(2) Now known as serum aspartate aminotransferase.

creatinine, total bilirubin and total serum protein measurements. Other determinations which may be necessary for an adequate toxicological evaluation include analyses of lipids, hormones, acid/base balance, methaemoglobin and cholinesterase actitivty. Additional clinical biochemistry may be employed where necessary to extend the investigation of observed effects. Non-rodents should be fasted for a period (not more than 24 hours) before taking blood samples.

(d) Urinalysis is not required on a routine basis but only when there is an indication based on expected or observed toxicity.

Gross necropsy

All animals should be subjected to a full gross necropsy which includes examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. The liver, kidneys, adrenals, thyroid (with parathyroids), and testes should be weighed wet as soon as possible after dissection to avoid drying.

The following organs and tissues should be preserved in a suitable medium for possible future histopathological examination: all gross lesions, brain — including sections of medulla/pons, cerebellar cortex and cerebral cortex, pituitary, thyroid/parathyroid, any thymic tissue, (trachea), lungs, heart, aorta, salivary glands, liver, spleen, kidneys, adrenals, pancreas, gonads, uterus (accessory genital organs), (skin), gall bladder, oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, urinary bladder, representative lymph node, (female mammary gland), (thigh musculature), peripheral nerve, (eyes), sternum with bone marrow, (femur, including articular surface), and (spinal cord at three levels — cervical, mid-thoracic and lumbar). The tissues mentioned between brackets need only be examined if indicated by signs of toxicity, or target-organ involvement.

Histopathological examination

Full histopathology should be carried out on the organs and tissues of animals in the control and high-dose groups. Further histopathology in other dose groups should be carried out on organs which show lesions in the high-dose group, or for which clinical observations indicate such a need.

2. DATA

Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the types of lesions and the percentage of animals displaying each type of lesion. Results should be evaluated by an appropriate statistical method. Any recognized statistical method may be used.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following information:

— species breed or strain, source, environmental conditions, diet,

— test conditions,

— dose levels (including vehicle, if used) and concentrations,

— toxic response data by sex and dose,

— no-effect level when possible,

— time of death during the study or whether animals survived to termination,

— description of toxic or other effects (with particular attention to clinical findings),

— the time of observation of each abnormal sign and its subsequent course,

— food and bodyweight data,

— ophthalmological findings,

— haematological tests employed and all results,

— clinical biochemistry tests employed and all results (including results of any urinalysis),

— necropsy findings,

— a detailed description of all histopathological findings,

— statistical treatment of results where appropriate,

— discussion of the results,

— interpretation of the results.

3.2. **Evaluation and interpretation**

See General Introduction Part B.

4. **REFERENCES**

See General Introduction Part B.

SUB-CHRONIC DERMAL TOXICITY STUDY

90-DAY REPEATED DERMAL DOSE STUDY USING RODENT SPECIES

1. METHOD

1.1. Introduction

See General Introduction Part B.

1.2. Definitions

See General Introduction Part B.

1.3. Reference substances

None.

1.4. Principle of the test method

The test substance is applied daily to the skin in graduated doses to several groups of experimental animals, one dose per group for a period of 90 days. During the period of application the animals are observed daily to detect signs of toxicity. Animals which die during the test are necropsied, and at the conclusion of the test surviving animals are necropsied.

1.5. Quality criteria

None.

1.6. Description of the test method

Preparations

The animals are kept under the experimental housing and feeding conditions for at least five days prior to the test. Before the test healthy young animals are randomized and assigned to the treated and control groups. Shortly before testing fur is clipped from the dorsal area of the trunk of the test animals. Shaving may be employed but it should be carried out approximately 24 hours before the test. Repeat clipping or shaving is usually needed at approximately weekly intervals. When clipping or shaving the fur, care must be taken to avoid abrading the skin. Not less than 10 % of the body surface area should be clear for the application of the test substance. The weight of the animal should be taken into account when deciding on the area to be cleared and on the dimensions of the covering. When testing solids, which may be pulverized if appropriate, the test substance should be moistened sufficiently with water or, where necessary, a suitable vehicle to ensure good contact with the skin. Liquid test substances are generally used undiluted. Daily application on a five to seven-day per week basis is used.

Test conditions

Experimental animals

The adult rat, rabbit or guinea pig may be used. Other species may be used but their use would require justification. At the commencement of the test the range of the weight variation should be ± 20 % of the mean weight. Where a sub-chronic dermal study is conducted as a preliminary to a long-term study, the same species and strain should be used in both studies.

Number and sex

At least 20 animals (10 female and 10 male) with healthy skin should be used at each dose level. The females should be nulliparous and non-pregnant. If interim sacrifices are planned the number should be increased by the number of animals scheduled to be sacrificed before the completion of the study. In addition, a satellite group of 20 animals (10 animals per sex) may be treated with the high-dose level for 90 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for 28 days post-treatment.

Dose levels

At least three dose levels are required with a controle or a vehicle control if a vehicle is used. The exposure period should be at least six hours per day. The application of the test substance should be made at similar times each day, and the amount of substance applied adjusted at intervals (weekly or bi-weekly) to maintain a constant dose level in terms of animal body weight. Except for treatment with the test substance, animals in the control group should be handled in an identical manner to the test group subjects. Where a vehicle is used to facilitate dosing, the vehicle control group should be dosed in the same way as the treated groups, and receive the same amount as that received by the highest dose level group. The highest dose level should result in toxic effects but produce no, or few, fatalities. The lowest dose level should not produce any evidence of toxicity. Where there is a usable estimation of human exposure the lowest level should exceed this. Ideally, the intermediate dose level should produce minimal observable toxic effects. If more than one intermediate dose is used, the dose levels should be spaced to produce a gradation of toxic effects. In the low and intermediate groups, and in the controls, the incidence of fatalities should be low, in order to permit a meaningful evaluation of the results.

If application of the test substance produces severe skin irritation the concentrations should be reduced and this may result in a reduction in, or absence of, other toxic effects at the high-dose level. If the skin has been badly damaged it may be necessary to terminate the study and undertake a new study at lower concentrations.

Limit test

If a preliminary study at a dose level of 1 000 mg/kilograms, or a higher dose level related to possible human exposure where this is known, produces no toxic effects, further testing may not be considered necessary.

Observation period

The experimental animals should be observed daily for signs of toxicity. The time of death and the time at which signs of toxicity appear and disappear should be recorded.

Procedure

Animals should be caged individually. The animals are treated with the test substance, ideally on seven days per week, for a period of 90 days.

Animals in any satellite groups scheduled for follow-up observations should be kept for a further 28 days without treatment to detect recovery from, or persistence of, toxic effects. Exposure time should be six hours per day.

The test substance should be applied uniformly over an area which is approximately 10 % of the total body surface area. With highly toxic substances, the surface area covered may be less but as much of the area should be covered with as thin and uniform a film as possible.

During exposure the test substance is held in contact with the skin with a porous gauze dressing and non-irritating tape. The test site should be further covered in a suitable manner to retain the gauze dressing and test substance and ensure that the animals cannot ingest the test substance. Restrainers may be used to prevent the ingestion of the test substance but complete immobilization is not a recommended method.

At the end of the exposure period, residual test substance should be removed where practicable using water or some other appropriate method of cleansing the skin.

All the animals should be observed daily and signs of toxicity recorded, including the time of onset, their degree and duration. Cageside observations should include changes in skin and fur, eyes and mucous membranes, as well as respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavior pattern. Measurements should be made of food consumption weekly and the animals weighed weekly. Regular observations of the animals is necessary to ensure that animals are not lost from the study due to causes such as cannibalism, autolysis of tissues or misplacement. At the end of the study period all survivors in the non-satellite treatment groups are necropsied. Moribund animals should be removed and necropsied when noticed.

The following examinations are customarily made on all animals including the controls:

(a) Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, should be made prior to exposure to the test substance and at the termination of the study, preferably in all animals but at least in the high-dose and control groups. If changes in the eyes are detected all animals should be examined.

(b) Haematology, including haematocrit, haemoglobin concentration, erythrocyte count, total and differential leucocyte count, and a measure of clotting potential, such as clotting time, prothrombin time, thromboplastin time, or platelet count, should be investigated at the end of the test period.

(c) Clinical biochemistry determination on blood should be carried out at the end of the test period. Test areas which are considered appropriate to all studies are electrolyte balance, carbohydrate metabolism, liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance. Suggested determinations are calcium, phosphorus, chloride, sodium, potassium, fasting glucose (with period of fasting appropriate to the species), sercum glutamic pyruvic transaminase (1), serum glutamic oxaloacetic transaminase (2), ornithine decarboxylase, gamma glutamyl transpeptidase, urea nitrogen, albumin, blood creatinine, total bilirubin and total serum protein measurements.

Other determinations which may be necessary for an adequate toxicological evaluation include analyses of lipids, hormones, acid/base balance, methaemoglobin and cholinesterase activity. Additional clinical biochemistry may be employed, where necessary, to extend the investigation of observed effects.

(d) Urinalysis is not required on a routine basis but only when there is an indication based on expected or observed toxicity.

If historical baseline data are inadequate, consideration should be given to determination of haematological and clinical biochemistry parameters before dosing commences.

Gross necropsy

All animals should be subjected to a full gross necropsy which includes examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. The liver, kidneys, adrenals and testes must be weighed wet as soon as possible after dissection to avoid drying. The following organs and tissues should be preserved in a suitable medium for possible future histopathological examination: all gross lesions, brain — including sections of medulla/pons, cerebellar cortex and cerebral cortex, pituitary, thyroid/parathyroid, any thymic tissue, (trachea), lungs, heart, aorta, salivary glands, liver, spleen, kidneys, adrenals, pancreas, gonads, uterus, accessory genital organs, gall bladder (if present), oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, urinary bladder, representative lymph node, (female mammary gland), (thigh musculature), peripheral nerve, (eyes), (sternum with bone marrow), (femur — including articular surface), (spinal cord at three levels — cervical, mid-thoracic and lumbar), and (exorbital lachrymal glands). The tissues mentioned between brackets need only be examined if indicated by signs of toxicity or target organ involvement.

Histopathological examination

(a) Full histopathology should be carried out on normal and treated skin and on organs and tissues of animals in the control and high-dose groups.

(b) All gross lesions should be examined.

(c) Target organs in other dose groups should be examined.

(d) Where rats are used, lungs of animals in the low- and intermediate-dose groups should be subjected to histopathological examination for evidence of infection, since this provides a convenient assessment of the state of health of the animals. Further histopathological examination may not be required routinely on the animals in these groups, but must always be carried out in organs which show evidence of lesions in the high-dose group.

(e) When a satellite group is used, histopathology should be performed on tissues and organs identified as showing effects in the other treated groups.

2. DATA

Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the type of lesions and the percentage of animals displaying each type of lesion. Results should be evaluated by an appropriate statistical method. Any recognized statistical method may be used.

(1) Now known as serum alanine aminotransferase.
(2) Now known as serum aspartate aminotransferase.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following information:

— species, strain, source, environmental conditions, diet,

— test conditions,

— dose levels (including vehicle, if used) and concentrations,

— toxic response data by sex and dose,

— no-effect level, where possible,

— time of death during the study or whether animals survived to termination,

— description of toxic or other effects,

— the time of observation of each abnormal sign and its subsequent course,

— food and bodyweight data,

— ophthalmological findings,

— haematological tests employed and all results,

— clinical biochemistry tests employed and all results (including results of any urinalysis),

— necropsy findings,

— a detailed description of all histopathological findings,

— statistical treatment of results where possible,

— discussion of the results,

— interpretation of the results.

3.2. Evaluation and interpretation

See General Introduction Part B.

4. REFERENCES

See General Introduction Part B.

SUB-CHRONIC INHALATION TOXICITY STUDY

90-DAY REPEATED INHALATION DOSE STUDY USING RODENT SPECIES

1. METHOD

1.1. Introduction

See General Introduction Part B.

1.2. Definitions

See General Introduction Part B.

1.3. Reference substances

None.

1.4. Principle of the test method

Several groups of experimental animals are exponed daily for a defined period to the test substance in graduated concentrations, one concentration being used per group, for a period of 90 days. Where a vehicle is used to help generate an appropriate concentration of the test substance in the atmosphere, a vehicle control group should be used. During the period of administration the animals are observed daily to detect signs of toxicity. Animals which die during the test are necropsied and at the conclusion of the test surviving animals are necropsied.

1.5. Quality criteria

None.

1.6. Description of the test method

Preparations

The animals are kept under the experimental housing and feeding conditions for at least five days prior to the experiment. Before the test, healthy young aimals are randomized and assigned to the treatment and control groups. Where necessary, a suitable vehicle may be added to the test substance to help generate an appropriate concentration of the substance in the atmosphere. If a vehicle or other additives are used to facilitate dosing, they should be known not to produce toxic effects. Historical data can be used if appropriate.

Test conditions

Experimental animals

Unless there are contra-indications, the rat is the preferred species. Commonly used laboratory strains of young healthy animals should be employed. At the commencement of the study the range of weight variation of animals used should not exceed ± 20 % of the appropriate mean value. Where a subchronic inhalation study is conducted as a preliminary to a long-term study, the same species and strain should be used in both studies.

Number and sex

At least 20 animals (10 female and 10 male) should be used for each exposure concentration. The females should be nulliparous and non-pregnant. If interim sacrifices are planned the number should be increased by the number of animals scheduled to be sacrified before the completion of the study. In addition, a satellite group of 20 animals (10 animals per sex) may be treated with the high concentration level for 90 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for 28 days post treatment.

Exposure concentrations

At least three concentrations are required, with a control or a vehicle control (corresponding to the concentration of vehicle at the highest level) if a vehicle is used. Except for treatment with the test substance, animals in the control group should be handled in an identical manner to the test group subjects. The highest concentration should result in toxic effects but no, or few, fatalities. Where there is a usable estimation of human exposure the lowest level should exceed this. Ideally, the intermediate concentration should produce minimal observable toxic effects. If more than one intermediate concentration is used the concentrations should be spaced to produce a gradation of toxic effects. In the low and intermediate groups, and in the controls, the incidence of fatalities should be low to permit a meaningful evaluation of the results.

Exposure time

The duration of daily exposure should be six hours after equilibration of the chamber concentrations. Other durations may be used to meet specific requirements.

Equipment

The animals should be tested in inhalation equipment designed to sustain a dynamic air flow of at least 12 air changes per hour to ensure an adequate oxygen content and an evenly distributed exposure atmosphere. Where a chamber is used its design should minimize crowding of the test animals and maximize their exposure by inhalation to the test substance. As a general rule, to ensure stability of a chamber atmosphere the total volume of the test animals should not exceed 5 % of the volume of the test chamber. Oro-nasal, head only, or whole body individual chamber exposure may be used; the first two will minimize uptake by other routes.

Observation period

The experimental animals should be observed daily for signs of toxicity during the entire treatment and recovery period. The time of death and the time at which signs of toxicity appear and disappear should be recorded.

Procedure

The animals are exposed to the test substance daily, five to seven days per week, for a period of 90 days. Animals in any satellite groups scheduled for follow-up observations should be kept for a further 28 days without treatment to detect recovery from, or persistence of, toxic effects. The temperature at which the test is performed should be maintained at 22 ± 3 °C. Ideally, the relative humidity should be maintained between 30 % and 70 %, but in certain instances (e.g. tests of aerosols) this may not be practicable. Food and water should be withheld during exposure.

A dynamic inhalation system with a suitable analytical concentration control system should be used. To establish suitable exposure concentrations a trial test is recommended. The air flow should be adjusted to ensure that conditions throughout the exposure chamber are homogeneous. The system should ensure that stable exposure conditions are achieved as rapidly as possible.

Measurements or monitoring should be made of:

(a) the rate of air flow (continuously);

(b) the actual concentration of the test substance measured in the breathing zone. During the daily exposure period the concentration should not vary by more than ± 15 % of the mean value. However, in the case of dusts and aerosols, this level of control may not be achievable and a wider range would then be acceptable. During the total duration of the study, the day-to-day concentrations should be held as constant as practicable. During the development of the generating system, particle-size analysis should be performed to establish the stability of aerosol concentrations. During exposure, analysis should be conducted as often as necessary to determine the consistency of particle-size distribution;

(c) temperature and humidity;

(d) during and following exposure, observations are made and recorded systematically; individual records should be maintained for each animal. All the animals should be observed daily and signs of toxicity recorded including the time of onset, their degree and duration. Cageside observations should include: changes in the skin and fur, eyes, mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behaviour pattern. Measurements should be made of food consumption weekly and the animals weighed weekly. Regular observation of the animals is necessary to ensure that animals are

not lost from the study due to causes such as cannibalism, autolysis of tissues or misplacement. At the end of the exposure period all surviving animals are necropsied. Moribund animals should be removed and necropsied when noticed.

The following examinations are customarily made on all animals including the controls:

(a) ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, should be made prior to the exposure to the test substance and at the termination of the study, preferably in all animals but at least in the high-dose and control groups. If changes in the eyes are detected all animals should be examined;

(b) haematology, including haematocrit, haemoglobin concentration, erythrocyte count, total and differential leucocyte count, and a measure of clotting potential, such as clotting time, prothrombin time, thromboplastin time, or platelet count, should be investigated at the end of the test period;

(c) clinical biochemistry determination on blood should be carried out at the end of the test period. Test areas which are considered appropriate to all studies are electrolyte balance, carbohydrate metabolism, liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance. Suggested determinations are calcium, phosphorus, chloride, sodium, potassium, fasting glucose (with period of fasting appropriate to the species), serum glutamic pyruvic transaminase (1), serum glutamic oxaloacetic transaminase (2), ornithine decarboxylase, gamma glutamyl transpeptidase, urea nitrogen, albumin, blood creatinine, total bilirubin and total serum protein measurements. Other determinations which may be necessary for an adequate toxicological evaluation include analyses of lipids, hormones, acid/base balance, methaemoglobin and cholinesterase activity. Additional clinical biochemistry may be employed where necessary to extend the investigation of observed effects;

(d) urinalysis is not required on a routine basis but only when there is an indication based on expected or observed toxicity.

If historical baseline data are inadequate, consideration should be given to determination of haematological and clinical biochemistry parameters before dosing commences.

Gross necropsy

All animals should be subjected to a full gross necropsy which includes examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. The liver, kidneys, adrenals and testes should be weighed wet as soon as possible after dissection to avoid drying. The following organs and tissues should be preserved in a suitable medium for possible future histopathological examination: all gross lesions, lungs — which should be removed intact, weighed and treated with a suitable fixative to ensure that lung structure is maintained (perfusion with the fixative is considered to be an effective procedure), nasopharyngeal tissues, brain — including sections of medulla/pons, cerebellar cortex and cerebral cortex, pituitary, thyroid/parathyroid, any thymic tissue, trachea, lungs, heart, aorta, salivary glands, liver, spleen, kidneys, adrenals, pancreas, gonads, uterus (accessory genital organs), (skin), gall bladder (if present), oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, urinary bladder, representative lymph node, (female mammary gland), (thigh musculature), peripheral nerve, (eyes), sternum with bone marrow, (femur, including articular surface), and (spinal cord at three levels — cervical, mid-thoracic and lumbar). The tissues mentioned between brackets need only be examined if indicated by signs of toxicity, or target organ involvement.

Histopathological examination

(a) Full histopathology should be carried out on the respiratory tract and other organs and tissues of all animals in the control and high-dose groups.

(b) All gross lesions should be examined.

(c) Target organs in other dose groups should be examined.

(d) Lungs of animals in the low- and intermediate-dose groups should also be subjected to histopathological examination; since this can provide a convenient assessment of the state of health of the animals. Further histopathological examination may not be required routinely on the animals in these groups but must always be carried out on organs which show evidence of lesions in the high-dose group.

(e) When a satellite group is used, histopathology should be performed on tissues and organs identified as showing effects in other treated groups.

(1) Now known as serum alanine aminotransferase.
(2) Now known as serum aspartate aminotransferase.

2. DATA

Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the types of lesions and the percentage of animals displaying each type of lesion. Results should be evaluated by an appropriate statistical method. Any recognized statistical method may be used.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following information:

— species, strain, source, environmental conditions, diet,

— test conditions:

Description of exposure apparatus: including design, type, dimensions, source of air, system for generating particulates and aerosols, method of conditioning air, treatment of exhaust air and the method of housing animals in a test chamber when this is used. The equipment for measuring temperature, humidity and, where appropriate, stability of aerosol concentrations or particle size, should be described.

Exposure data: these should be tabulated and presented with mean values and a measure of variability (e.g. standard deviation) and should include:

(a) air flow rates through the inhalation equipment;

(b) temperature and humidity of air;

(c) nominal concentrations (total amount of test substance fed into the inhalation equipment divided by the volume of air);

(d) nature of vehicle, if used;

(e) actual concentrations in test breathing zone;

(f) median particle sizes (where appropriate),

— toxic response data by sex and concentration,

— no-effect level when possible,

— time of death during the study or whether animals surived to termination,

— description of toxic or other effects,

— the time of observation of each abnormal sign and its subsequent course,

— food and bodyweight data,

— ophthalmological findings,

— haematological tests employed and results,

— clinical biochemistry tests employed and results (including results of any urinalysis),

— necropsy findings,

— a detailed description of all histopathological findings,

— statistical treatment of results where appropriate,

— discussion of the results,

— interpretation of the results.

3.2. Evaluation and interpretation

See General Introduction Part B.

4. REFERENCES

See General Introduction Part B.

TERATOGENICITY STUDY — RODENT AND NON-RODENT

1. METHOD

1.1. Introduction

See General Introduction Part B.

1.2. Definitions

See General Introduction Part B.

1.3. Reference substances

None.

1.4. Principle of the test method

The test substance is administered in graduated doses, or concentrations, for at least that part of the pregnancy covering the period of organogenesis, to several groups of pregnant experimental animals, one dose being used per group. Shortly before the expected date of delivery, the dam is sacrificed, the uterus removed and the contents examined. This test method covers embryo- and fetotoxicity.

1.5. Quality criteria

None.

1.6. Description of the test method

Preparations

Healthy young adult virgin females of comparable age and size are acclimatized to the laboratory conditions for at least five days prior to the test and are then mated with males of established fertility. Inseminated females are randomized and assigned to treatment groups.

Mating may be accomplished naturally or by artificial insemination. The test substance is administered daily to the females beginning soon after implantation and continuing through the period of organogenesis. One day prior to term, foetuses are delivered by hysterectomy and examined for visceral or skeletal abnormalities, including growth retardation, delayed ossification and intestinal haemorrhages.

Test conditions

Experimental animals

Species commonly used are the rat, mouse, hamster and rabbit. The preferred species are the rat and the rabbit. Commonly used laboratory strains should be employed. The strain should not have low fecundity and should be characterized for its response to teratogens. Animals should be caged individually.

Number and sex

At least 20 pregnant rats, mice or hamsters or 12 pregnant rabbits are required at each dose level. The objective is to ensure that sufficient litters and pups are produced to permit an evaluation of the teratogenic potential of the substance.

Dose levels

At least three dose levels and a control should be used. When the test substance is administered in a vehicle, a vehicle control group should also be used. If a vehicle is used its toxicological properties should be understood; it should not be teratogenic or have effects on reproduction. Except for treatment with the test substance, animals in the control group(s) should be handled in an identical manner to the test group subjects. Unless limited by the

physical/chemical nature or biological properties of the substance, the highest dose level should ideally induce some overt maternal toxicity such as slight weight loss, but not more than 10 % maternal deaths. The low-dose level should not induce observable effects attributable to the test substance. The intermediate dose(s) should be spaced geometrically between the high- and low-dose levels.

Limit test

In the case of substances of low toxicity, if a dose level of at least 1000 mg/kilogram produces no evidence of embryotoxicity or teratogenicity, studies at other dose levels may not be considered necessary.

Exposure time

Day 0 in the test is the day on which vaginal plug and/or sperm are observed (where feasible). The dose period should cover the period of major organogenesis. This may be taken as days 6 to 15 for the rat and mouse, 6 to 14 for hamster, or 6 to 18 for rabbit. If day 0 is based on observation of mating or artificial insemination, the times stated should be adjusted by adding one day. Alternatively, the period of dosing may be extended to approximately one day before the expected delivery date.

Observation period

A careful clinical examination should be made at least once each day. Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study.

Procedure

The test substance is administered orally by gavage. The test substance should be administered at approximately the same time each day.

The female test animals are treated with the test substance daily throughout the appropriate treatment period. The dose may be based on the weight of the females at the start of the substance administration, or, alternatively, in view of the rapid weight gain which takes place during pregnancy, the animals may be weighed periodically and the dose based on the most recent weight determination. Signs of toxicity should be recorded as they are observed, including the time of onset, the degree and duration. Females showing signs of abortion or premature delivery should be sacrificed and subjected to thorough macroscopic examination. The post-treatment observation period should continue until approximately one day prior to term; the objective is to cover most of the pregnancy period but to avoid complicating the interpretation of results which could arise following natural birth. Cageside observations should include, but not be limited to, changes in skin and fur, eyes and mucous membranes, as well as respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behaviour pattern. Measurements should be made of food consumption weekly. Animals should be weighed weekly.

Necropsy

At death during, or at the end of, the study the dam should be examined macroscopically for any structural abnormalities or pathological changes which may have influenced the pregnancy. Immediately after death, the uterus should be removed and the contents examined for embryonic or foetal deaths and the number of live foetuses. It is usually possible to estimate the time of death *in utero* where this has occurred. In rats and rabbits the number of *corpora lutea* may be determined. The sex of the foetuses should be determined and they should be weighed individually, the weights recorded, and the mean foetal weight derived. Following removal each foetus should be examined externally. For rats, mice and hamsters, one-third to one-half of each litter should be prepared and examined for skeletal anomalies and the remaining part of each litter should be prepared and examined for soft tissue anomalies using appropriate methods. For rabbits, each foetus should be examined by careful dissection for visceral abnormalities and then examined for skeletal anomalies.

2. DATA

Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number which became pregnant, the number and percentages of live foetuses and foetuses with any soft tissue or skeletal abnormalities and their relation to specific litters. Results should be evaluated by an appropriate statistical method. Any recognized statistical method may be used.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following information:

— species, strain, source, environmental conditions, diet,

— test conditions,

— dose levels (including vehicle, if used) and concentrations,

— toxic response data by dose,

— no-effect level (where possible),

— time of death during the study or whether animals survived to termination,

— description of toxic or other effects,

— the time of observation of each abnormal sign and its subsequent course,

— food and bodyweight data,

— duration of pregnancy and litter data (including historical data),

— foetal data (live/dead, sex, soft tissue and skeletal defects),

— litter data (live/dead, sex, soft tissue and skeletal defects for each litter),

— statistical treatment of results,

— discussion of the results,

— interpretation of the results.

3.2. Evaluation and interpretation

See General Introduction Part B.

4. REFERENCES

See General Introduction Part B.

CHRONIC TOXICITY TEST

1. METHOD

1.1. Introduction

See General Introduction Part B.

1.2. Definitions

See General Introduction Part B.

1.3. Reference substances

None.

1.4. Principle of the test method

The test substance is administered normally seven days per week, by an appropriate route, to several groups of experimental animals, one dose per group, for a major portion of their life span. During and after exposure to the test substance, the experimental animals are observed daily to detect signs of toxicity.

1.5. Quality criteria

None.

1.6. Description of the test method

Preparations

The animals are kept under the experimental housing and feeding conditions for at least five days prior to the test. Before the test healthy young animals are randomized and assigned to the treated and control groups.

Test conditions

Experimental animals

The preferred species is the rat. Based upon the results of previously conducted studies other species (rodent or non-rodent) may be used. Commonly used laboratory strains of young healthy animals should be employed and dosing should begin as soon as possible after weaning.

At the commencement of the study the weight variation in the animals used should not exceed ± 20 % of the mean value. Where a sub-chronic oral study is conducted as a preliminary to a long-term study, the same species/breed and strain should be used in both studies.

Number and sex

For rodents at least 40 animals (20 female and 20 male) should be used at each dose level and concurrent control group. The females should be nulliparous and non-pregnant. If interim sacrifices are planned, the number should be increased by the number of animals scheduled to be sacrificed before the completion of the study.

For non-rodents a smaller number of animals, but at least four per sex per group, is acceptable.

Dose levels and frequency of exposure

At least three dose levels should be used in addition to the concurrent control group. The highest dose level should elicit definite signs of toxicity without causing excessive lethality. The lowest dose level should not produce and evidence of toxicity.

The intermediate dose(s) should be established in a mid-range between the high and low doses.

The selection of dose levels should take into account data from preceding toxicity tests and studies.

Frequency of exposure is normally daily. If the chemical is administered in the drinking water or mixed in the diet it should be continuously available.

Controls

A concurrent control group which is identical in every respect to the treated 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 uncharacterized biological activity in oral studies, a concurrent negative control group should also be used. The negative control group is treated in the same manner as the test groups except that the animals are not exposed to the test substance or any vehicle.

Route of administration

The two main routes of administration are oral and inhalation. The choice of the route of administration depends upon the physical and chemical characteristics of the test substance and the likely route of exposure in humans.

The use of the dermal route presents considerable practical problems. Chronic systemic toxicity resulting from percutaneous absorption can normally be inferred form the results of another oral test and a knowledge of the extent of percutaneous absorption derived from preceding percutaneous toxicity tests.

Oral studies

Where the test substance is absorbed from the gastrointestinal tract, and if the ingestion route is one by which humans may be exposed, the oral route of administration is preferred unless there are contra-indications.

The animals may receive the test substance in the diet, dissolved in drinking water or given by capsule.

Ideally, daily dosing on a seven-day per week basis should be used because dosing on a five-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 five-day per week basis is considered to be acceptable.

Inhalation studies

Because inhalation studies present technical problems of greater complexity than the other routes of administration, more detailed guidance on this mode of administration is given here. It should also be noted that intratracheal instillation may constitute a valid alternative in specific situations.

Long-term exposures are usually patterned on projected human exposure, giving the animals either a daily exposure of six hours after equilibration of chamber concentrations, for five days a week (intermittent exposure), or, relevant to possible environmental exposure, 22 to 24 hours of exposure per day for seven days a week (continuous exposure), with about an hour for feeding the animals daily at a similar time and maintaining the chamber. In both cases, the animals are usually exposed to fixed concentrations of test substance. A major difference between intermittent and continuous exposure is that with the former there is a 17 to 18 hour period in which animals may recover from the effects of each daily exposure, with 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 exposure 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 for 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 at least 12 air changes per hour to assure adequate oxygen content and an evenly distributed exposure atmosphere. Control and exposure chambers should be identical in construction and design to ensure exposure conditions comparable in all respects except for exposure to the test substances. Slight negative pressure inside the chamber is generally maintained to prevent leakage of the test substance into the surrounding area. The chambers should minimize the crowding of test animals. As a general rule, to ensure the stability of the chamber atmosphere, the total volume of the test animals should not exceed 5 % of the volume of the chamber.

Measurements or monitoring should be made of:

(i) Air flow: the rate of air flow through the chamber should preferably be monitored continuously;

(ii) Concentration: during the daily exposure period the concentration of the test substance should not vary more than ± 15 % of the mean value;

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

(iv) Particle size measurements: particle-size distribution should be determined in 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 in the breathing zone of the animals. The air sample should be representative of the distribution of the particles to which the animals are exposed and should account, on a gravimetric basis, for all of the suspended aerosol even when much of the aerosol is not respirable. Particle size analyses should be carried out frequently during the development of the generating system to ensure the stability of the aerosol and thereafter as often as necessary during the exposures to determine adequately the consistency of the particle distribution to which the animals have been exposed.

Duration of study

The duration of the period of administration should be at least 12 months.

Procedure

Observations

A careful clinical examination should be made at least once each day. Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study, for example necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals. Careful observations should be made to detect onset and progression of all toxic effects as well as to minimize loss due to disease, 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.

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

Haematological examination

Haematological examination (e.g. haemoglobin content, packet cell volume, total red blood cells, total white blood cells, platelets or other measures of clotting potential) should be performed at three months, six months, and thereafter at approximately six-month intervals and at termination on blood samples collected form all non-rodents and from 10 rats/sex of all groups. If possible, samples should be from the same rats at each interval. In addition, a pre-test sample should be collected from non-rodents.

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

A differential blood count is performed on samples from the animals in the highest dose 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 by pathological findings.

Urinalysis

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

— appearance: volume and density for individual animals,

— protein, glucose, ketones, occult blood (semi-quantitatively),

— microscopy of sediment (semi-quantitatively).

Clinical chemistry

At approximately six-monthly intervals and at termination, blood samples are drawn for clinical chemistry measurements from all non-rodents and 10 rats/sex of all groups, if possible, from the same rats at each interval. In addition, a pre-test sample should be collected from non-rodents. Plasma is prepared from these samples and the following determinations are made:

— total protein concentration,

— albumin concentration,

— liver function tests (such as alkaline phosphatase activity, glutamic pyruvic transaminase (1) activity and glutamic oxaloacetic transaminase (2) activity), gamma glutamyl transpeptidase, ornithine decarboxylase,

— carbohydrate metabolism such as fasting blood glucose,

— kidney function tests such as blood urea nitrogen.

Gross necropsy

Full gross necropsy should be performed on all animals, including those which died during the experiment or were killed having been found in a moribund condition. Prior to sacrifice, 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 histopathological examination. This usually concerns the following organs and tissues: brain (3) (medulla/pons, cerebellar cortex, cerebral cortex), pituitary, thyroid (including parathyroid), thymus, lungs (including trachea), heart, aorta, salivary glands, liver (3), spleen, kidneys (3), adrenals (3), oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, uterus, urinary bladder, lymph nodes, pancreas, gonads (3), 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. Inflation of lungs and urinary bladder with a fixative is the optimal way to preserve these tissues; inflation of the lungs in inhalation studies is essential for appropriate histopathological 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 visible changes, particularly tumours and other lesions occurring in any organ should be examined microscopically. In addition, the following procedures are recommended:

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

1. all animals that died or were killed during the study;

2. all of the high-dose group and controls;

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

(c) Where the result of the test gives evidence of substantial reduction of the animals' normal lifespan or the induction of effects that might affect a toxic response, the next-lower dose level should be examined as described above;

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

(1) Now known as serum alanine aminotransferase.
(2) Now known as serum aspartate aminotransferase.
(3) These organs, from ten animals per sex per group for rodents and all non-rodents, plus thyroid (with parathyroids) for all non-rodents, should be weighed.

2. DATA

Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions and the percentage of animals displaying each type of lesion. Results should be evaluated by an appropriate statistical method. Any recognized statistical method may be used.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following information:

— species, strain, source, environmental conditions, diet,

— test conditions:

Description of exposure apparatus:

including design, type, dimensions, source of air, system for generating particulates and aerosols, method of conditioning air, treatment of exhaust air and the method of housing animals in a test chamber when this is used. The equipment for measuring temperature, humidity and, where appropriate, stability of aerosol concentration or particle size, should be described.

Exposure data:

these should be tabulated and presented with mean values and a measure of variability (e.g. standard deviation) and should include:

(a) air flow rates through the inhalation equipment;

(b) temperature and humidity of air;

(c) nominal concentrations (total amount of test substance fed into the inhalation equipment divided by the volume of air);

(d) nature of vehicle, if used;

(e) actual concentrations in test breathing zone;

(f) median particle sizes (where appropriate),

— dose levels (including vehicle, if used) and concentrations,

— toxic response data by sex and dose,

— no-effect level,

— time of death during the study or whether animals survived to termination,

— description of toxic and other effects,

— the time of observation of each abnormal sign and its subsequent course,

— food and bodyweight data,

— ophthalmological findings,

— haematological tests employed and all results,

— clinical biochemistry tests employed and all results (including results of any urinalysis),

— necropsy findings,

— a detailed description of all histopathological findings,

— statistical treatment of results where possible,

— discussion of the results,

— interpretation of the results.

3.2. Evaluation and interpretation

See General Introduction Part B.

4. REFERENCES

See General Introduction Part B.

CARCINOGENICITY TEST

1. METHOD

1.1. Introduction

See General Introduction Part B.

1.2. Definitions

See General Introduction Part B.

1.3. Reference substances

None.

1.4. Principle of the test method

The test substance is administered normally seven days per week, by an appropriate route, to several groups of experimental animals, one dose per group, for a major portion of their lifespan. During and after exposure to the test substance, the experimental animals are observed daily to detect signs of toxicity, particularly the development of tumours.

1.5. Quality criteria

None.

1.6. Description of the test method

The animals are kept under the experimental housing and feeding conditions for at least five days prior to the test. Before the test healthy young animals are randomized and assigned to the treated and control groups.

Experimental animals

Based upon the results of previously conducted studies other species (rodent or non-rodent) may be used. Commonly used laboratory strains of young healthy animals should be employed and dosing should begin as soon as possible after weaning.

At the commencement of the study the weight variation in the animals used should not exceed ± 20 % of the mean value. Where a sub-chronic oral study is conducted as a preliminary to a long-term study, the same species/breed and strain should be used in both studies.

Number and sex

For rodents at least 100 animals (50 female and 50 male) should be used at each dose level and concurrent control group. The females should be nulliparous and non-pregnant. If interim sacrifices are planned the number should be increased by the number of animals scheduled to be sacrificed before the completion of the study.

Dose levels and frequency of exposure

At least three dose levels should be used in addition to the concurrent control group. The highest dose level should elicit signs of minimal toxicity, such as a slight depression of bodyweight gain (less than 10 %), without substantially altering the normal lifespan due to effects other than tumours.

The lowest dose level should not interfere with normal growth, development and longevity of the animal or produce any indication of toxicity. In general, this should not be lower than 10 % of the high dose.

The intermediate dose(s) should be established in a mid-range between the high and low doses.

The selection of dose levels should take into account data from preceding toxicity tests and studies.

Frequency of exposure is normally daily. If the chemical is administered in the drinking water or mixed in the diet it should be continuously available.

Controls

A concurrent control group which is identical in every respect to the treated 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 uncharacterized biological activity in oral studies, an additional control group which is not exposed to the vehicle should be used.

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 likely route of exposure in humans.

Oral studies

Where the test substance is absorbed from the gastro-intestinal tract, and if the ingestion route is one by which humans may be exposed, the oral route of administration is preferred, unless there are contra-indications. The animals may receive the test substance in their diet, dissolved in drinking water or given by capsule.

Ideally, daily dosing on a seven-day per week basis should be used because dosing on a five-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 five-day per week basis is considered to be acceptable.

Dermal studies

Cutaneous exposure by skin painting may be selected to simulate a main route of human exposure and as a model system for induction of skin lesions.

Inhalation studies

Because inhalation studies present technical problems of greater complexity than the other routes of administration, more detailed guidance on this mode of administration is given here. It should be noted that intratracheal instillation may constitute a valid alternative in specific situations.

Long-term exposures are usually patterned on projected human exposure, giving the animals either a daily exposure of six hours after equilibration of chamber concentrations, for five days a week (intermittent exposure), or, relevant to possible environmental exposure, 22 to 24 hours of exposure per day for seven days a week (continuous exposure), with about an hour for feeding the animals daily at a similar time and maintaining the chambers. In both cases, the animals are usually exposed to fixed concentrations of test substance.

A major difference between intermittent and continuous exposure is that with the former there is a 17 to 18 hour period in which animals may recover from the effects of each daily exposure with 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 exposure 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 for 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 at least 12 air changes per hour to assure adequate oxygen content and an evenly distributed exposure atmosphere. Control and exposure chambers should be identical in construction and design to ensure exposure conditions comparable in all respects except for exposure to the test substances. Slight negative pressure inside the chamber is generally maintained to

prevent leakage of the test substance into the surrounding area. The chambers should minimize the crowding of test animals. As a general rule, to ensure the stability of the chamber atmosphere, the total volume of the test animals should not exceed 5 % of the volume of the chamber.

Measurements or monitoring should be made of:

(i) Air flow: the rate of air flow through the chamber should preferably be monitored continuously;

(ii) Concentration: during the daily exposure period the concentration of the test substance should not vary more than ± 15 % of the mean value. During the total duration of this study, the day-to-day concentrations should be held as constant as practicable;

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

(iv) Particle size measurements: particle size distribution should be determined in 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 in the breathing zone of the animals. The air sample should be representative of the distribution of the particles to which the animals are exposed and should account, on a gravimetric basis, for all of the suspended aerosol even when much of the aerosol is not respirable. Particle size analyses should be carried out frequently during the development of the generating system to ensure the stability of the aerosol and thereafter as often as necessary during the exposures to determine adequately the consistency of the particle distribution to which the animals have been exposed.

Duration of study

The duration of a carcinogenicity test comprises the major portion of the normal lifespan of the test animals. The termination of the test 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. Alternatively, termination of such an extended study is acceptable when the number of survivors in the lowest dose or control group reaches 25 %. When terminating a test in which there is an apparent sex difference in response, each sex should be considered separately. Where only the high-dose group dies prematurely for obvious reasons of toxicity, this need not trigger termination providing toxic manifestations are not causing problems in the other groups. For a negative test result to be acceptable, not more than 10 % of any group may be lost from the experiment due to autolysis, cannibalism or management problems and the survival of all groups is not less than 50 % at 18 months for mice and hamsters and at 24 months for rats.

Procedure

Observations

Daily cageside observations should include changes in skin and fur, eyes and mucous membranes as well as respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behaviour pattern.

Regular observations of the animals is necessary to ensure that, as far as possible, animals are not lost from the study due to causes such as cannibalism, autolysis of tissues or misplacement. Moribund animals should be removed and necropsied when noticed.

Clinical signs and mortality should be recorded for all animals. Special attention must be paid to tumour development: the time of onset, location, dimensions, appearance and progression of each grossly visible or palpable tumour should be recorded.

Mesurements should be made of food consumption (and water consumption when the test substance is administered in the drinking water) 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.

Bodyweights should be recorded individually for all animals once a week during the first 13 weeks of the test period and at least once every four weeks thereafter.

Clinical examinations

Haematology

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

At 12 months, 18 months, and prior to sacrifice, a blood smear is obtained from the animals. A differential blood count is performed on samples from the animals in the high-dose group and the controls. If these data, particularly those obtained prior to sacrifice, or data from the pathological examination indicate a need, differential blood counts should be performed on the next-lower group(s) as well.

Gross necropsy

Full gross necropsy should be performed on all animals, including those which died during the experiment or were sacrificed having been found in a moribund condition. All grossly visible tumours or lesions, or lesions suspected of being tumours, should be preserved.

The following organs and tissues should be preserved in suitable media for possible future histopathological examination: brain (including sections of medulla/pons, cerebellar cortex, cerebral cortex), pituitary, thyroid/parathyroid, any thymic tissue, trachea and lungs, heart, aorta, salivary glands, liver, spleen, kidneys, adrenals, pancreas, gonads, uterus, accessory genital organs, skin, oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, urinary bladder, representative lymph node, female mammary gland, thigh musculature, peripheral nerve, sternum with bone marrow, femur (including joint), spinal cord at three levels (cervical, mid-thoracic and lumbar) and eyes.

Inflation of lungs and urinary bladder with a fixative is the optimal way to preserve these tissues; inflation of the lungs in inhalation studies is essential for appropriate histopathological examination. In inhalation studies, the entire respiratory tract should be preserved, including nasal cavity, pharynx and larynx.

Histopathology

(a) Full histopathology should be carried out on the organs and tissues of all animals that died or were sacrificed during the test and all animals in the control and high-dose groups.

(b) All grossly visible tumours or lesions suspected of being tumours should be examined microscopically in all groups.

(c) If there is a significant difference in the incidence of neoplastic lesions in the high-dose and control groups, histopathology should be carried out on that particular organ or tissue in the other groups.

(d) If the survival of the high-dose group is substantially less than the control then the next-lower dose group should be examined fully.

(e) If there is evidence in the high-dose group of the induction of toxic or other effects that might affect a neoplastic response, the next-lower dose level should be examined fully.

2. DATA

Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing tumours detected during the test, the time of detection and the number of animals found to have tumours following sacrifice. Results should be evaluated by an appropriate statistical method. Any recognized statistical method may be used.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following information:

— species, strain, source, environmental conditions, diet,

— test conditions:

Description of exposure apparatus:

including design, type, dimensions, source of air, system for generating particulates and aerosols, method of conditioning air, treatment of exhaust air and the method of housing animals in a test chamber when this is used. The equipment for measuring temperature, humidity and, where appropriate, stability of aerosol concentration or particle size, should be described.

Exposure data:

these should be tabulated and presented with mean values and a measure of variability (e.g. standard deviation) and should include:

(a) air flow rates through the inhalation equipment;

(b) temperature and humidity of air;

(c) nominal concentrations (total amount of test substance fed into the inhalation equipment divided by the volume of air);

(d) nature of vehicle, if used;

(e) actual concentrations in test breathing zone;

(f) median particle sizes (where appropriate),

— dose levels (including vehicle, if used) and concentrations,

— tumour incidence data by sex, dose and tumour type,

— time of death during the study or whether animals survived to termination,

— toxic response data by sex and dose,

— description of toxic or other effects,

— the time of observation of each abnormal sign and its subsequent course,

— food and bodyweight data,

— haematological tests employed and all results,

— necropsy findings,

— a detailed description of all histopathological findings,

— statistical treatment of results with a description of the methods used,

— discussion of the results,

— interpretation of the result.

3.2. **Evaluation and interpretation**

See General Introduction Part B.

4. **REFERENCES**

See General Introduction Part B.

COMBINED CHRONIC TOXICITY/CARCINOGENICITY TEST

1. METHOD

1.1. Introduction

See General Introduction Part B.

1.2. Definitions

See General Introduction Part B.

1.3. Reference substances

None.

1.4. Principle of the test method

The objective of a combined chronic toxicity/carcinogenicity test is to determine the chronic and carcinogenic effects of a substance in a mammalian species following prolonged exposure.

To this end a carcinogenicity test is supplemented with a least one treated satellite group and a control satellite group. The dose used for the high-dose satellite group may be higher than that used for the high-dose group in the carcinogenicity test. The animals in the carcinogenicity test are examined for general toxicity as well as for carcinogenic response. The animals in the treated satellite group are examined for general toxicity.

The test substance is administered normally seven days per week, by an appropriate route, to several groups of experimental animals, one dose per group, for a major portion of their lifespan. During and after exposure to the test substance, the experimental animals are observed daily to detect signs of toxicity and the development of tumours.

1.5. Quality criteria

None.

1.6. Description of the test method

The animals are kept under the experimental housing and feeding conditions for at least five days prior to the test. Before the test healthy young animals are randomized and assigned to the treated and control groups.

Experimental animals

The preferred species is the rat. Based upon the results of previously conducted tests other species (rodent or non-rodent) may be used. Commonly used laboratory strains of young healthy animals should be employed and dosing should begin as soon as possible after weaning.

At the commencment of the test the weight variation in the animals used should not exceed ± 20 % of the mean value. Where a sub-chronic oral test is conducted as a preliminary to a long-term test, the same species and breed/strain should be used in both studies.

Number and sex

For rodents, at least 100 animals (50 female and 50 male) should be used at each dose level and concurrent control group. The females should be nulliparous and non-pregnant. If interim sacrifices are planned, the number should be increased by the number of animals scheduled to be sacrificed before the completion of the study.

The treated satellite group(s) for the evaluation of pathology other than tumours should contain 20 animals of each sex, while the satellite control group should contain 10 animals of each sex.

Dose levels and frequency of exposure

For carcinogenicity testing purposes, at least three dose levels should be used in addition to the concurrent control group. The highest dose level should elicit signs of minimal toxicity, such as a slight depression of body weight gain (less than 10 %), without substantially altering the normal lifespan due to effects other than tumours.

The lowest dose level should not interfere with normal growth, development and longevity of the animal or produce any indication of toxicity. In general, this should not be lower than 10 % of the high dose.

The intermediate dose(s) should be established in a mid-range between the high and low doses.

The selection of dose levels should take into account data from preceding toxicity tests and studies.

For chronic toxicity testing purposes, additional treated groups and a concurrent control satellite group are included in the test. The high dose for treated satellite animals should produce definite signs of toxicity.

Frequency of exposure is normally daily. If the chemical is administered in the drinking water or mixed in the diet, it should be continuously available.

Controls

A concurrent group which is identical in every respect to the treated 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 uncharacterized biological activity in oral studies, an additional control group which is not exposed to the vehicle should be utilized.

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 likely route of exposure in humans.

Oral tests

Where the test substance is absorbed from the gastro-intestinal tract and the ingestion route is one by which humans may be exposed, the oral route of administration is preferred, unless there are contra-indications. The animals may receive the test substance in their diet, dissolved in drinking water or given by capsule. Ideally, daily dosing on a seven-day per week basis should be used because dosing on a five-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 five-day per week basis is considered to be acceptable.

Dermal tests

Cutaneous exposure by skin painting may be selected to simulate a main route of human exposure and as a model system for induction of skin lesions.

Inhalation tests

Because inhalation tests present technical problems of greater complexity than the other routes of administration, more detailed guidance on this mode of administration is given here. It should be noted that intratracheal instillation may constitute a valid alternative in specific situations.

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

The choice of intermittent or continuous exposure depends on the objectives of the test and on the human exposure 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 for 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 at least 12 air changes per hour to assure adequate oxygen content and an evenly distributed exposure atmosphere. Control and exposure chambers should be identical in construction and design to ensure exposure conditions comparable in all respects except for exposure to the test substances. Slight negative pressure inside the chamber is generally maintained to prevent leakage of the test substance into the surrounding area. The chambers should minimize the crowding of test animals. As a general rule, to ensure the stability of the chamber atmosphere, the total volume of the test animals should not exceed 5 % of the volume of the chamber.

Measurements or monitoring should be made of:

(i) Air flow: the rate of air flow through the chamber should perferably be monitored continuously;

(ii) Concentration: during the daily exposure period the concentration should not vary more than ± 15 % of the mean value. During the total duration of this study, the day-to-day concentrations should be held as constant as practicable;

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

(iv) Particle size measurements: particle size distribution should be determined in 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 in the breathing zone of the animals. The air sample should be representative of the distribution of the particles to which the animals are exposed and should account, on a gravimetric basis, for all of the suspended aerosol even when much of the aerosol is not respirable. Particle size analyses should be carried out frequently during the development of the generating system to ensure the stability of the aerosol and thereafter as often as necessary during the exposures to determine adequately the consistency of the particle distribution to which the animals have been exposed.

Duration of test

The duration of the carcinogenicity part of the test comprises the major portion of the normal life span of the test animals. The termination of the test 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. Alternatively, termination of such an extended test is acceptable when the number of survivors in the lowest dose or control group reaches 25 %. When terminating a test in which there is an apparent sex difference in response, each sex should be considered separately. Where only the high-dose group dies prematurely for obvious reasons of toxicity, this need not trigger termination providing toxic manifestations are not causing problems in the other groups. For a negative test result to be acceptable not more than 10 % of any group may be lost from the experiment due to autolysis, cannibalism or management problems, and the survival of all groups is not less than 50 % at 18 months for mice an hamsters and at 24 months for rats.

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

Procedure

Observations

Daily cageside observations should be made and should include changes in skin and fur, eyes and mucous membranes as well as respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behaviour pattern.

Clinical examination should be performed at appropriate intervals on animals in the treated satellite group(s).

Regular observations of the animals is necessary to ensure, as far as possible, that animals are not lost from the test due to causes such as cannibalism, autolysis of tissues or misplacment. Moribund animals should be removed and necropsied when noticed.

Clinical signs, including neurological and ocular changes as well as mortality should be recorded for all animals. Special attention must be paid to tumour development: the time of onset, location, dimensions, appearance and progression of each grossly visible or palpable tumour should be recorded; the time of onset and progression of toxic conditions should be recorded.

Measurements should be made of food consumption (and water consumption when the test substance is administered in the drinking water) 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.

Bodyweights should be recorded individually for all animals once a week during the first 13 weeks of the test period and at least once every four weeks thereafter.

Clinical examinations

Haematology

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 three months, six months and at approximately six-month intervals thereafter, and at termination on blood samples collected from 10 rats/sex of all groups. If possible, samples should be from the same rats at each interval.

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

A differential blood count is performed on samples of those animals in the highest dose 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 by pathological findings.

Urinalysis

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

— appearance: volume and density for individual animals,

— protein, glucose, ketones, occult blood (semi-quantitatively),

— microscopy of sediment (semi-quantitatively).

Clinical chemistry

At approximately six-monthly intervals, and at termination, blood samples are drawn for clinical chemistry measurements from all non-rodents and 10 rats/sex of all groups, if possible, from the same rats at each interval. In addition, a pre-test sample should be collected from non-rodents. Plasma is prepared from these samples and the following determinations are made:

— total protein concentration,

— albumin concentration,

— liver function tests (such as alkaline phosphatase activity, glutamic pyruvic transaminase (1) activity and glutamic oxaloacetic transaminase (2) activity), gamma glutamyl transpeptidase, ornithine decarboxylase,

— carbohydrate metabolism such as fasting blood glucose,

— kidney function tests such as blood urea nitrogen.

(1) Now knowm as serum alanine aminotransferase.
(2) Now known as serum aspartate aminotransferase.

Gross necropsy

Full gross necropsy should be performed in all animals, including those which died during the experiment or were sacrificed having been found in a moribund condition. Prior to sacrifice, samples of blood should be collected from all animals for differential blood counts. All grossly visible tumours or lesions suspected of being tumours should be preserved. An attempt should be made to correlate gross observations with the microsopic findings.

All organs and tissues should be preserved for histopathological examination. This usually concerns the following organs and tissues: brain (1) (medulla/pons, cerebellar cortex, cerebral cortex), pituitary, thyroid (including parathyroid), thymus, lungs (including trachea), heart, aorta, salivary glands, liver (1), spleen, kidneys (1), adrenals (1), oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, urinary bladder, lymph nodes, pancreas, gonads (1), 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, inflation of the lungs in inhalation studies is a necessary requirement for appropriate histopathological 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 microsopic examination, because it may give significant guidance to the pathologist.

Histopathology

For the chronic toxicity testing portion:

Detailed examination should be made of all preserved organs of all animals of the satellite high-dose and control groups. Where test-substance-related pathology is found in the high-dose satellite group, target organs of all other animals in any other treated satellite group should be subjected to full and detailed histological examination as well as those of the treated groups in the carcinogenicity testing portion of the study at its termination.

For the carcinogenicity testing portion:

(a) Full histopathology should be carried out on the organs and tissues of all animals that died or were sacrificed during the test, and of all animals in the control and high-dose groups;

(b) All grossly visible tumours or lesions suspected of being tumours in all groups occurring in any organ should be examined microscopically;

(c) If there is a significant difference in the incidence of neoplastic lesions in the high-dose and control groups, histopathology should be carried out on that particular organ or tissue in the orther groups;

(d) If the survival of the high-dose group is substantially less than the control then the next-lower dose group should be examined fully;

(e) If there is evidence in the high-dose group of the induction of toxic or other effects that might affect a neoplastic response, the next-lower dose level should be examined fully.

2. DATA

Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing tumours or toxic effects detected during the test, the time of detection and the number of animals found to have tumours following sacrifice. Results should be evaluated by an appropriate statistical method. Any recognized statistical method may be used.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following information:

— species, strain source, environmental conditions, diet,

(1) These organs, from 10 animals per sex per group for rodents, should be weighed.

— test conditions:

Description of exposure apparatus:

including design, type, dimensions, source of air, system for generating particulates and aerosols, method of conditioning air, treatment of exhaust air and the method of housing animals in a test chamber when this is used. The equipment for measuring temperature, humidity and, where appropriate, stability of aerosol concentration or particle size, should be described.

Exposure data:

These should be tabulated and presented with mean values and a measure of variability (e.g. standard deviation), and should include:

(a) air flow rates through the inhalation equipment;

(b) temperature and humidity of air;

(c) nominal concentrations (total amount of test substance fed into the inhalation equipment divided by the volume of air);

(d) nature of vehicle, if used;

(e) actual concentrations in test breathing zone;

(f) median particle sizes (where appropriate),

— dose levels (including vehicle, if used) and concentrations,

— tumour incidence data by sex, dose and tumour type,

— time of death during the study or whether animals survived to termination, including satellite group,

— toxic response data by sex and dose,

— description of toxic or other effects,

— the time of observation of each abnormal sign and its subsequent course,

— ophthalmological findings,

— food and bodyweight data,

— haematological tests employed and all results,

— clinical biochemistry test employed and all results (including any urinalysis),

— necropsy findings,

— a detailed description of all histopathological findings,

— statistical treatment of results with a description of the methods used,

— discussion of the results,

— interpretation of the results.

3.2. **Evaluation and interpretation**

See General Introduction Part B.

4. **REFERENCES**

See General Introduction Part B.

ONE-GENERATION REPRODUCTION TOXICITY TEST

1. METHOD

1.1. Introduction

See General Introduction Part B.

1.2. Definitions

See General Introduction Part B.

1.3. Reference substances

None.

1.4. Principle of the test method

The test substance is administered in graduated doses to several groups of males and females. Males should be dosed during growth and for at least one complete spermatogenic cycle (approximately 56 days in the mouse and 70 days in the rat) in order to elicit any adverse effects on spermatogenesis by the test substance.

Females of the parental (P) generation should be dosed for at least two complete oestrous cycles in order to elicit any adverse effects on oestrus by the test substance. The animals are then mated. The test substance is administered to both sexes during the mating period and thereafter only to females during pregnancy and for the duration of the nursing period.

For administration by inhalation the method will require modification.

1.5. Quality criteria

None.

1.6. Description of the test method

Preparations

Before the test, healthy young adult animals are randomized and assigned to the treated and control groups. The animals are kept under the experimental housing and feeding conditions for at least five days prior to the test.

It is recommended that the test substance be administered in the diet or drinking water. Other routes of administration are also acceptable. All animals should be dosed by the same method during the appropriate experimental period. If a vehicle or other additives are used to facilitate dosing, they should be known not to produce toxic effects.

Dosing should be on a seven-day per week basis.

Experimental animals

Selection of species

The rat or mouse are the preferred species. Healthy animals, not subjected to previous experimental procedures, should be used. Strains with low fecundity should not be used. The test animals should be characterized as to species, strain, sex, weight and/or age.

For an adequate assessment of fertility, both males and females should be studied. All test and control animals should be weaned before dosing begins.

Number and sex

Each treated and control group should contain a sufficient number of animals to yield about 20 pregnant females at or near term.

The objective is to produce enough pregnancies and offspring to assure a meaningful evaluation of the potential of the substance to affect fertility, pregnancy and maternal behaviour in P generation animals and suckling, growth and development of the F_1 offspring from conception to weaning.

Test conditions

Food and water should be provided *ad libitum*. Near parturition, pregnant females should be caged separately in delivery or maternity cages and may be provided with nesting materials.

Dose levels

At least three treated groups and a control group should be used. If a vehicle is used in administering the test substance, the control group should receive the vehicle in the highest volume used. If a test substance causes reduced dietary intake or utilization, then the use of a paired fed control group may be considered necessary. Ideally, unless limited by the physical/chemical nature or biological effects of the test substance, the highest dose level should induce toxicity but not mortality in the parental (P) animals. The intermediate dose(s) should induce minimal toxic effects attributable to the test substance, and the low dose should not induce any observable adverse effects on the parents or offspring. When administered by gavage or capsule the dosage given to each animal should be based on the individual animal's body weight and adjusted weekly for changes in body weight. For females during pregnancy, dosages may be based on the body weight at day 0 or 6 of the pregnancy, if desired.

Limit test

In the case of substances of low toxicity, if a dose level of at least 1 000 mg/kilogram produces no evidence of interference with reproductive performance, studies at other dose levels may not be considered necessary. If a preliminary study at the high-dose level, with definite evidence of maternal toxicity, shows no adverse effects on fertility, studies at other dose levels may not be considered necessary.

Performance of the test

Experimental schedules

Daily dosing of the parental (P) males should begin when they are about five to nine weeks of age, after they have been weaned and acclimatized for at least five days. In rats, dosing is continued for 10 weeks prior to the mating period (for mice, eight weeks). Males should be killed and examined either at the end of the mating period or, alternatively, males may be retained on the test diet for the possible production of a second litter and should be killed and examined at some time before the end of the study. For parental (P) females dosing should begin after at least five days of acclimatization and continue for at least two weeks prior to mating. Daily dosing of the P females should continue throughout the three-week mating period, pregnancy and up to the weaning of the F_1 offspring. Consideration should be given to modification of the dosing schedule based on other available information on the test substance, such as induction of metabolism or bioaccumulation.

Mating procedure

Either 1:1 (one male to one female) or 1:2 (one male to two females) mating may be used in reproduction toxicity studies.

Based on 1:1 mating, one female should be placed with the same male until pregnancy occurs or three weeks have elapsed. Each morning the females should be examined for presence of sperm or vaginal plugs. Day 0 of pregnancy is defined as the day a vaginal plug or sperm is found.

Those pairs that fail to mate should be evaluated to determine the cause of the apparent infertility. This may involve such procedures as providing additional opportunities to mate with other proven sires or dams, microscopic examination of the reproductive organs, and examination of the oestrous cycle or spermatogenesis.

Litter sizes

Animals dosed during the fertility study are allowed to litter normally and rear their progency to the stage of weaning without standardization of litters.

Where standardization is done, the following procedure is suggested. Between day 1 and day 4 after birth, the size of each litter may be adjusted by eliminating extra pups by selection to yield, as nearly as possible, four males and four females per litter. Whenever the number of male or female pups prevents having four of each sex per litter, partial adjustment (for example, five males and three females) is acceptable. Adjustments are not applicable for litters of less than eight pups.

Observations

Throughout the test period, each animal should be observed at least once daily. Pertinent behavioural changes, signs of difficult or prolonged parturition, and all signs of toxicity, including mortality, should be recorded. During pre-mating and mating periods, food consumption may be measured daily. After parturition and during lactation, food consumption measurements (and water consumption measurements when the test substance is administered in the drinking water) should be made on the same day as the weighing of the litter. P males and females should be weighed on the first day of dosing and weekly thereafter. These observations should be reported individually for each adult animal.

The duration of gestation should be calculated from day 0 of pregnancy. Each litter should be examined as soon as possible after delivery to establish the number and sex of pups, still births, live births and the presence of gross anomalies.

Dead pups and pups sacrificed at day 4 should be preserved and studied for possible defects. Live pups should be counted and litters weighed on the morning after birth and on days 4 and 7 and weekly thereafter until the termination of the study, when animals should be weighed individually. Physical or behavioural abnormalities observed in the dams or offspring should be recorded.

Pathology

Necropsy

At the time of sacrifice or death during the study the animals of the P generation should be examined macroscopically for any structural abnormalities or pathological changes, with special attention being paid to the organs of the reproductive system. Dead or moribund pups should be examined for defects.

Histopathology

The ovaries, uterus, cervix, vagina, testes, epididymes, seminal vesicles, prostate, coagulating gland, pituitary gland and target organ(s) of all P animals should be preserved for microscopic examination. In the event that these organs have not been examined in other multiple-dose studies, they should be microscopically examined in all high-dose and control animals and animals which die during the study where practicable.

Organs showing abnormalities in these animals should then be examined in all other P animals. In these instances, microscopic examination should be made of all tissues showing gross pathological changes. As suggested under mating procedures, reproductive organs of animals suspected of infertility may be subjected to microscopic examination.

2. DATA

Data may be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of fertile males, the number of pregnant females, the types of changes and the percentage of animals displaying each type of change.

When possible, numerical results should be evaluated by an appropriate statistical method. Any generally accepted statistical method may be used.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following information:

— species/strain used,

— toxic response data by sex and dose, including fertility, gestation and viability,

— time of death during the study or whether animals survived to time of scheduled sacrifice or to termination of the study,

— table presenting the weights of each litter, the mean pup weights and the individual weights of the pups at termination,

— toxic or other effects on reproduction, offspring and postnatal growth,

— the day of observation of each abnormal sign and its subsequent course,

— bodyweight data for P animals,

— necropsy findings,

— a detailed description of all microscopic findings,

— statistical treatment of results, where appropriate,

— discussion of the results,

— interpretation of the results.

3.2. **Evaluation and interpretation**

See General Introduction Part B.

4. **REFERENCES**

See General Introduction Part B.

TWO-GENERATION REPRODUCTION TOXICITY TEST

1. METHOD

1.1. Introduction

See General Introduction Part B.

1.2. Definitions

See General Introduction Part B.

1.3. Reference substances

None.

1.4. Principle of the test method

The test substance is administered in graduated doses to several groups of males and females. Males of the parental (P) generation should be dosed during growth and for at least one complete spermatogenic cycle (approximately 56 days in the mouse and 70 days in the rat) in order to elicit any adverse effects on spermatogenesis by the test substance.

Females of the parental (P) generation should be dosed for at least two complete oestrous cycles in order to elicit any adverse effects on oestrus by the test substance. The animals are then mated. The test substance is administered to both sexes during the mating period and thereafter only to females during pregnancy and for the duration of the nursing period. At weaning the administration of the substance is continued to F_1 offspring during their growth into adulthood, mating and production of an F_2 generation, until the F_2 generation is weaned. For administration by inhalation the method will require modification.

1.5. Qualitiy criteria

None.

1.6. Description of the test method

Preparations

Before the test, healthy animals are randomized and assigned to the treated and control groups. The parental (P) animals are kept under the experimental housing and feeding conditions for at least five days prior to the test. It is recommended that the test substance be administered in the diet or drinking water. Other routes of administration are also acceptable. All animals should be dosed by the same method during the entire experimental period. If a vehicle or other additive is used to facilitate dosing, they should be known not to produce toxic effects. Dosing should be on a seven-day per week basis.

Experimental animals: selection of species

The rat or mouse are the preferred species.

Healthy P animals, not subjected to previous experimental procedures, should be used. Strains with low fecundity should not be used. The test animals should be characterized as to species, strain, sex, weight and/or age.

For an adequate assessment of fertility, both males and females should be studied. All test and control animals should be weaned before dosing begins.

Number and sex

Each treated and control group should contain a sufficient number of animals to yield about 20 pregnant females at or near term. The objective is to produce enough pregnancies and offspring to assure a meaningful evaluation of the

potential of the subtance to affect fertility, pregnancy and maternal behaviour and suckling, growth and development of the F_1 offspring from conception to maturity, and the development of their offspring (F_2) to weaning.

Test conditions

Food and water should be provided *ad libitum*. Near parturition, pregnant females should be caged separately in delivery or maternity cages and may be provided with nesting materials.

Dose levels

At least three treatment groups and a control group should be used. If a vehicle is used in administering the test substance, the control group should receive the vehicle in the highest volume used. If a test substance causes reduced dietary intake or utilization, then the use of a paired fed control group may be considered necessary. Ideally, unless limited by the physical/chemical nature or biological effects of the test substance, the highest dose level should induce toxicity but not mortality in the parental (P) animals. The intermediate dose(s) should induce minimal toxic effects attributable to the test substance, and the low dose should not induce any observable adverse effects on the parents or offspring. When administered by gavage or capsule the dosage given to each animal should be based on the individual animal's body weight and adjusted weekly. For females during pregnancy, the dosage may be based on the body weight at day 0 or 6 of the pregnancy, if desired.

Limit test

In the case of substances of low toxicity, if a dose level of a least 1 000 mg/kilogram produces no evidence of interference with reproductive performance, studies at other dose levels may not be considered necessary. If a preliminary study at the high-dose level, with definite evidence of maternal toxicity, shows no adverse effects on fertility, studies at other dose levels may not be considered necessary.

Performance of the test

Experimental schedules

Daily dosing of the parental (P) males should begin when they are about five to nine weeks old, after they have been weaned and acclimatized for at least five days. In rats dosing is continued for 10 weeks prior to the mating period (for mice, eight weeks). Males should be killed and examined either at the end of the mating period or, alternatively, males may be retained on the test diet for the possible production of a second litter and should be killed and examined at some time before the end of the experiment.

For parental (P) females, dosing should begin after at least five days of acclimatization and continue for at least two weeks prior to mating. Daily dosing of the P females should continue throughout the three-week mating period, pregnancy and up to the weaning of the F_1 offspring. Consideration should be given to modifications of the the dosing schedule based on other available information on the test substance, such as induction of metabolism or bioaccumulation.

Dosing of the F_1 animals begins at weaning and ends when they are sacrificed.

Mating procedure

Either 1:1 (one male to one female) or 1:2 (one male to two females) matings may be used in reproduction toxicity studies.

Based on 1:1 mating, one female should be placed with the same male until pregnancy occurs or three weeks have elapsed. Each morning the females should be examined for presence of sperm or vaginal plugs. Day 0 of pregnancy is defined as the day a vaginal plug or sperm is found. Taking into account spermiogenesis the F_1 offspring should not be mated until they are aged at least 11 weeks for mice, 13 weeks for rats. For mating the F_1 offspring, one male and one female are randomly selected from each litter for cross-mating with a pup of another litter of the same dose group to produce the F_2 generation. F_1 males and females not selcted for mating are killed at weaning.

Those pairs that fail to mate should be evaluated to determine the cause of the apparent infertility. This may involve such procedures as additional opportunities to mate with other proven sires or dams, microscopic examination of the reproductive organs, and examination of the oestrous cycles or spermatogenesis.

Litter sizes

Animals dosed during the fertility study are allowed to litter normally and rear their progeny to the stage of weaning without standardization of litters.

Where standardization is done, the following procedure is suggested. Between days 1 and 4 after birth, the size of each litter may be adjusted by eliminating extra pups by selection to yield, as nearly as possible, four males and four females per litter. Whenever the number of male or female pups prevents having four of each sex per litter, partial adjustment (for example, five males and three females) is acceptable. Adjustments are not applicable for litters of less than eight pups. Adjustments of the F_2 litters are conducted in the same manner.

Observations

Throughout the test period, each animal should be observed at least once daily. Pertinent behavioural changes, signs of difficult or prolonged parturition and all signs of toxicity, including mortality, should be recorded. During pre-mating and mating periods, food consumption may be measured weekly. Optionally, during pregnancy, food consumption may be measured daily. After parturition and during lactation, food consumption measurements should be made on the same day as the weighing of litters. Parental animals (P and F_1) should be weighed on the first day of dosing and weekly thereafter. These observations should be reported individually for each adult animal.

The duration of gestation should be calculated from day 0 of pregnancy. Each litter should be examined as soon as possible after delivery to establish the number and sex of pups, still births, live births and the presence of gross anomalies.

Dead pups and pups killed at day 4 should be preserved and studied for possible defects. Live pups should be counted and litters weighed on the morning after birth and on days 4 and 7 and weekly thereafter until the termination of the study, when animals should be weighed individually. Physical or behavioural abnormalities observed in the dams or offspring should be recorded.

Pathology

Necropsy

All P and F_1 adult animals should be killed when they are no longer necessary to assess reproductive effects. F_1 offspring not selected for mating and all F_2 offspring should be killed when weaned.

At the time of sacrifice or death during the study all parental animals (P and F_1) should be examined microscopically for any structural abnormalities or pathological changes, with special attention paid to the organs of the reproductive system. Dead or moribund pups should be examined for defects.

Histopathology

The ovaries, uterus, cervix, vagina, testes, epididymes, seminal vesicles, coagulating gland, prostate, pituitary gland and target organ(s) of all P and F_1 animals should be preserved for microscopic examination. In the event that these organs have not been examined in other multiple-dose studies, they should be examined microscopically in all high-dose and control P and F_1 animals selected for mating and, where practicable, in animals which die during the study. Organs showing abnormalities in these animals should then be examined in animals from the other dose groups. In these instances, mircoscopic examination should be made of all tissues showing gross pathological changes. As suggested under mating procedures, reproductive organs of animals suspected of infertility may be subjected to microsocpic examination.

2. DATA

Treatment of results

Data may be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of pregnant animals, the type of changes and the percentage of animals displaying each type of change.

When possible, numerical results should be evaluated by an appropriate statistical method. Any generally accepted statistical method may be used.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following information:

— species/strain used,

— toxic response data by sex and dose, including fertility, gestation and viability indices,

— time of death during the study or whether animals survived to termination of the study,

— table presenting the weights of each litter, the mean pup weight and the individual weight of the pups at termination,

— toxic or other effects on reproduction, offspring and postnatal growth,

— the day of observation of each abnormal sign and its subsequent course,

— bodyweight data for P and F_1 animals selected for mating,

— necropsy findings,

— a detailed description of all microscopic findings,

— statistical treatment of results, where appropriate,

— discussion of the results,

— interpretation of the results.

3.2. Evalutation and interpretation

See General Introduction Part B.

4. REFERENCES

See General Introdution Part B.

TOXICOKINETICS

1. METHOD

1.1. Introduction

See General Introduction Part B.

1.2. Definitions

See General Introduction Part B.

1.3. Reference substances

None.

1.4. Principle of the test method

The test substance is administered by an appropriate route, Depending on the purpose of the study, the substance may be administered in single or repeated doses over defined periods to one or several groups of experimental animals. Subsequently, depending on the type of study, the substance and/or metabolites are determined in body fluids, tissues and/or excreta.

Studies may be done with 'unlabelled' or 'labelled' forms of the test substance. Where a label is used it should be positioned in the substance in such a way to provide the most information about the fate of the compound.

1.5. Quality criteria

None.

1.6. Description of the test method

Preparations

Healthy young adult animals are acclimatized to the laboratory conditions for at least five days prior to the test. Before the test, animals are randomized and assigned to the treatment groups. In special situations, very young, pregnant or pre-treated animals may be used.

Test conditions

Experimental animals

Toxicokinetic studies may be carried out in one or more appropriate animal species and should take account of the species used or intended to be used in other toxicological studies on the same test substance. Where rodents are used in a test the weight variation should not exceed ± 20 % of the mean weight.

Number and sex

For absorption and excretion studies, there should be four animals in each dose group initially. Sex preference is not mandatory, but under some circumstances both sexes may need to be studied. If there are sex differences in response, then four animals of each sex should be tested. In the case of studies with non-rodents fewer animals may be used.

When tissue distribution is being studied, the initial group size should take into account both the number of animals to be sacrificed at each time point and the number of time points to be examined.

When metabolism is being studied, the group size is related to the needs of the study.

For multiple-dose and multiple-time-point studies, the group size should take into account the number of time points and planned sacrifice(s), but may not be smaller than two animals. The group size should be sufficient to provide an acceptable characterization of uptake, plateau and depletion (as appropriate) of the test substance and/or metabolites.

Dose levels

In the case of single-dose administration, at least two dose levels should be used. There should be a low dose at which no toxic effects are observed and a high dose at which there might be changes in toxicokinetic parameters or at which toxic effects occur.

In the case of repeated-dose administration the low dose is usually sufficient, but under certain circumstances a high dose may also be necessary.

Route of administration

Toxicokinetic studies should be performed using the same route and, where appropriate, the same vehicle as that used or intended to be used in the other toxicity studies. The test substance is usually administered orally by gavage or in the diet, applied to the skin, or administered by inhalation for defined periods to groups of experimental animals. Intravenous administration of the test substance may be useful in determining relative absorption by other routes. In addition, useful information may be provided on the pattern of distribution soon after the intravenous administration of a substance.

The possibility of interference of the vehicle with the test substance should be taken into consideration. Attention should be given to differences in absorption between the administration of the test substances by gavage and in the diet and the need for an accurate determination of dose particularly when the test substance is given in the diet.

Observation period

All the animals should be observed daily and signs of toxicity and other relevant clinical features recorded, including time of onset, degree and duration.

Procedure

After weighing test animals, the test substance is administered by an appropriate route. If considered relevant, animals may be fasted before the test substance is administered.

Absorption

The rate and extent of absorption of the administered substance can be evaluated using various methods, with and without reference groups (1), for example by:

— determination of the amount of test substance and/or metabolites in excreta, such as urine, bile, faeces, exhaled air and that remaining in the carcase,

— comparison of the biological response (e.g. acute toxicity studies) between test and control and/or reference groups,

— comparison of the amount of renally excreted substance and/or metabolite in test and reference groups,

— determination of the area under the plasma-level/time curve of the test substance and/or metabolites and comparison with data from a reference group.

(1) In this method a reference group is one in which the test substance is administered by another route that ensures complete bioavailability of the dose.

Distribution

Two approaches are available at present, one or both of which may be used for analysis of distribution patterns:

— useful qualitative information is obtained using whole body autoradiographic techniques,

— quantitative information is obtained by sacrificing animals at different times after exposure and determining the concentration and amount of the test substance and/or metabolites in tissues and organs.

Excretion

In excretion studies, urine, faeces and expired air and, in certain circumstances, bile are collected. The amount of test substance and/or metabolites in these excreta should be measured several times after exposure, either until about 95 % of the administered dose has been excreted or for seven days, whichever comes first.

In special cases, the excretion of the test substance in the milk of lactating test animals may need to be considered.

Metabolism

To determine the extent and pattern of metabolism, biological samples should be analysed by suitable techniques. Structures of metabolites should be elucidated and appropriate metabolic pathways proposed where there is a need to answer questions arising from previous toxicological studies. It may be helpful to perform studies *in vitro* to obtain information on metabolic pathways.

Further information on the relationship of metabolism to toxicity may be obtained from biochemical studies, such as the determination of effects on metabolizing enzyme systems, depletion of endogenous non-protein sulphydryl compounds and binding of the substance with macromolecules.

2. DATA

According to the type of study performed, data should be summarized in tabular form supported by graphical presentation whenever appropriate. For each test group, mean and statistical variations of measurements in relation to time, dosage, tissues and organs should be shown when appropriate. The extent of absorption and the amount and rates of excretion should be determined by appropriate methods. When metabolism studies are performed, the structure of identified metabolites should be given and possible metabolic pathways presented.

3. REPORTING

3.1. Test report

According to the type of study performed, the test report shall, if possible, contain the following information:

— species, strain, source, environmental conditions, diet,

— characterization of labelled materials, when used,

— dosage levels and intervals used,

— route(s) of administration and any vehicles used,

— toxic and other effects observed,

— methods for determination of test substance and/or metabolites in biological samples, including expired air,

— tabulation of measurements by sex, dose, regimen, time, tissues and organs,

— presentation of the extent of absorption and excretion with time,

— methods for the characterization and identification of metabolites in biological samples,

— methods for biochemical measurements related to metabolism,

— proposed pathways for metabolism,

— discussion of the results,

— interpretation of the results.

3.2. **Evaluation and interpretation**

See General Introduction Part B.

4. **REFERENCES**

See General Introduction Part B.

MUTAGENICITY TESTING AND SCREENING FOR CARCINOGENICITY

GENE MUTATION — *SACCHAROMYCES CEREVISIAE*

1. METHOD

1.1. Introduction

See General Introduction Part B.

1.2. Definition

See General Introduction Part B.

1.3. Reference substances

None.

1.4. Principle of the test method

A variety of haploid and diploid strains of the yeast *Saccharomyces cerevisiae* can be used to measure the production of gene mutations induced by chemical agents with and without metabolic activation.

Forward mutation systems in haploid strains, such as the measurement of mutation from red, adenine-requiring mutants (*ade*-1, *ade*-2) to double adenine-requiring white mutants and selective systems such as the induction of resistance to canavnaine and cycloheximide, have been utilized.

The most extensively validated reverse mutation system involves the use of the haploid strain XV 185-14C which carries the ochre nonsense mutations *ade* 2-1, *arg* 4-17, *lys* 1-1 and *trp* 5-48, which are reversible by base substitution mutagens that induce site specific mutations or ochre suppressor mutations. XV 185-14C also carries the *his* 1-7 marker, a missense mutation reverted mainly by second site mutations, and the marker *hom* 3-10 which is reverted by frameshift mutagens.

In diploid strains of *S. cerevisiae* the only extensively used strain is D_7 which is homozygous for *ilv* 1-92.

1.5. Quality criteria

None.

1.6. Description of the test method

Preparations

Solutions of test chemicals and control should be prepared just prior to testing, using an appropriate vehicle. In the case of organic compounds which are not water soluble, not more than a 2 % solution v/v of organic solvents such as ethanol, acetone or dimethylsulphoxide (DMSO) should be used. The final concentration of the vehicle should not significantly affect cell viability and growth characertistics.

Metabolic activation

Cells should be exposed to test chemicals both in the presence and absence of an appropriate exogenous metabolic activation system.

The most commonly used system is a co-factor supplemented post-mitochondrial fraction from the livers of rodents pre-treated with enzyme inducing agents. The use of other species, tissues, post-mitochondrial fractions, or procedures may also be appropriate for metabolic activation.

Test conditions

Tester strains

The haploid strain XV 185-14C and the diploid strain D_7 are the most used in gene mutation studies. Other strains may also be appropriate.

Media

Appropriate culture media are used for the determination of survival and mutant numbers.

Use of negative and positive controls

Positive, untreated and solvent controls should be performed concurrently. Appropriate positive control chemicals should be used for each specific mutational endpoint.

Exposure concentration

At least five adequately spaced concentrations of the test substance should be used. For toxic substances, the highest concentration tested should not reduce survival below 5 to 10 %. Relatively water-insoluble substances should be tested up to their limit of solubility, using appropriate procedures. For freely water-soluble non-toxic substances, the upper concentration should be determined on a case by case basis.

Incubation conditions

The plates are incubated four to seven days at 28 to 30 °C in the dark.

Spontaneous mutation frequencies

Sub-cultures should be used with spontaneous mutation frequencies within the accepted normal range.

Number of replicates

At least three replicate plates should be used per concentration for the assay of prototrophs produced by gene mutation and for cell viability. In the case of experiments using markers such as *hom* 3-10 with a low mutation rate, the number of plates used must be increased to provide statistically relevant data.

Procedure

Treatment of *S. cerevisiae* strains is usually performed in a liquid test procedure involving either stationary or growing cells. Initial experiments should be carried out on growing cells: $1 - 5 \times 10^7$ cells/ml are exposed to the test chemical for up to 18 hours at 28 to 37 °C with shaking; an adequate amount of metabolic activation system is added during treatment when appropriate. At the end of the treatment, cells are centrifuged, washed and seeded upon an appropriate culture medium. After incubation, plates are scored for survival and the induction of gene mutation.

If the first experiment is negative, then a second experiment should be carried out using stationary phase cells. If the first experiment is positive it is confirmed in an appropriate independent experiment.

2. **DATA**

Data should be presented in tabular form indicating the number of colonies counted, number of mutants, survival and mutant frequency. All results should be confirmed in an independent experiment. The data should be evaluated using appropriate statistical methods.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following information:

— strain used,

— test conditions: stationary phase or growing cells, compositions of media, incubation temperature and duration, metabolic activation system,

— treatment conditions: exposure levels, procedure and duration of treatment, treatment temperature, positive and negative controls,

— number of colonies counted, number of mutants, survival and mutant frequency, dose/response relationship if applicable, statistical evaluation of data,

— discussion of results,

— interpretation of results.

3.2. Evaluation and interpretation

See General Introduction Part B.

4. REFERENCES

See General Introduction Part B.

MITOTIC RECOMBINATION — *SACCHAROMYCES CEREVISIAE*

1. METHOD

1.1. Introduction

See General Introduction Part B.

1.2. Definition

See General Introduction Part B.

1.3. Reference substances

None.

1.4. Principle of the test method

Mitotic recombination in *Saccharomyces cerevisiae* can be detected between genes (or more generally between a gene and its centromere) and within genes. The former event is called mitotic crossing-over and generates reciprocal products whereas the latter event is most frequently non-reciprocal and is called gene conversion. Crossing-over is generally assayed by the production of recessive homozygous colonies or sectors produced in a heterozygous strain, whereas gene conversion is assayed by the production of prototrophic revertants produced in an auxotrophic heteroallelic strain carrying two different defective alleles of the same gene. The most commonly used strains for the detection of mitotic gene conversion are D_4 (heteroallelic at *ade* 2 and *trp* 5) D_7 (heteroallelic at *trp* 5) BZ_{34} (heteroallelic at *arg* 4) and JD1 (heteroallelic at *his* 4 and *trp* 5). Mitotic crossing-over producing red and pink homozygous sectors can be assayed in D_5 or in D_7 (which also measures mitotic gene conversion and reverse mutation at *ilv* 1-92) both strains being heteroallelic for complementing alleles of *ade* 2.

1.5. Quality criteria

None.

1.6. Description of the test method

Preparations

Solutions of test chemicals and control or reference compounds should be prepared just prior to testing, using an appropriate vehicle. With organic compounds that are water insoluble not more than a 2 % solution v/v of organic solvents such as ethanol, acetone or dimethylsulphoxide (DMSO) should be used. The final concentration of the vehicle should not significantly affect cell viability and growth characteristics.

Metabolic activation

Cells should be exposed to test chemicals both in the presence and absence of an appropriate exogenous metabolic activation system. The system most commonly used is a co-factor supplemented post-mitochondrial fraction from the livers of rodents pre-treated with enzyme inducing agents. The use of other species, tissues, post-mitochondrial fractions, or procedures may also be appropriate for metabolic activation.

Test conditions

Tester strains

The most frequently used strains are the diploids D_4, D_5, D_7 and JD1. The use of other strains may be appropriate.

Media

Appropriate culture media are used for the determination of survival and the frequency of mitotic recombination.

Use of negative and positive controls

Positive, untreated and solvent controls should be performed concurrently. Appropriate positive control chemicals should be used for each specific recombination endpoint.

Exposure concentrations

At least five adequately spaced concentrations of the test substance should be used. Among the factors to be taken into consideration are cytotoxicity and solubility. The lowest concentration must have no effect on cell viability. For toxic chemicals, the highest concentration tested should not reduce survival below 5 to 10 %. Relatively water-insoluble chemicals should be tested up to the limit of solubility using appropriate procedures. For freely water-soluble non-toxic substances the upper concentration should be determined on a case by case basis.

Cells may be exposed to test chemicals in either the stationary phase or during growth for periods of up to 18 hours. However, for long treatment times cultures should be microscopically inspected for spore formation, the presence of which invalidates the test.

Incubation conditions

The plates are incubated in the dark for four to seven days at 28 to 30 °C. Plates used for the assay of red and pink homozygous sectors produced by mitotic crossing-over should be kept in a refrigerator (about 4 °C) for a further one to two days before scoring to allow for the development of the appropriate pigmented colonies.

Spontaneous mitotic recombination frequencies

Sub-cultures should be used with spontaneous mitotic recombination mutation frequencies within the accepted normal range.

Number of replicates

A minimum of three replicate plates should be used per concentration for the assay of prototrophs produced by mitotic gene conversion and for viability. In the case of the assay of recessive homozygosis produced by mitotic corssing-over, the plate number should be increased to provide an adequate number of colonies.

Procedures

Treatment of *S. cerevisiae* strains is usually performed in a liquid test procedure involving either stationary or growing cells. Initial experiments should be done on growing cells. $1 - 5 \times 10^7$ celles/ml are exposed to the test chemical for up to 18 hours at 28 to 37 °C with shaking; an adequate amount of metabolic activation system is added during treatment when appropriate.

At the end of the treatment, cells are centrifuged, washed and seeded upon appropriate culutre medium. After incubation plates are scored for survival and the induction of mitotic recombination.

If the first experiment is negative, then a second experiment should be carried out using stationary phase cells. If the first experiment is positive it is confirmed in an independent experiment.

2. **DATA**

Data should be presented in tabular form indicating the number of colonies counted, the number of recombinants, survival and the frequency of recombinants.

Results should be confirmed in an independent experiment.

The data should be evaluated using appropriate statistical methods.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following information:

— strain used,

— test conditions: stationary phase or growing cells, composition of media, incubation temperature and duration, metabolic activation system,

— treatment conditions: exposure concentration, procedure and duration of treatment, treatment temperature, positive and negative controls,

— number of colonies counted, number of recombinants; survival and recombination frequency, dose/response relationship if applicable, statistical evaluation of data,

— discussion of the results,

— interpretation of the results.

3.2. Evaluation and interpretation

See General Introduction Part B.

4. REFERENCES

See General Introduction Part B.

IN VITRO MAMMALIAN CELL GENE MUTATION TEST

1. METHOD

1.1. Introduction

See General Introduction Part B.

1.2. Definition

See General Introduction Part B.

1.3. Reference substances

None.

1.4. Principle of the test method

Mammalian cell culture systems may be used to detect mutations induced by chemical substances. Widely used cell lines include L5178Y mouse lymphoma cells and the CHO and V-79 lines of Chinese hamster cells. In these cell lines the most commonly used systems measure mutations at the thymidine kinase (TK), hypoxanthine guanine phosphoribosyl transferase (HPRT) (1) and Na^+/K^+ ATPase loci. The TK and HPRT mutational systems detect base-pair mutations, frameshift mutations and small deletions; the Na^+/K^+ system detects base-pair mutations only.

Cells deficient in thymidine kinase (TK), due to the forward mutation $TK^+ \rightarrow TK^-$, are resistant to bromodeoxyuridine (BrdU), fluorodeoxyuridine (FdU) or trifluorothymidine (TFT) since these antimetabolites are not incorporated into cellular nucleotides by the 'salvage' enzyme system thymidine kinase; the nucleotides needed for cellular metabolism are obtained solely from *de novo* synthesis. However, in the presence of thymidine kinase BrdU, FdU or TFT are incorporated into the nucleotides, resulting in inhibition of cellular metabolism and cytotoxicity. Thus, the mutant cells are able to proliferate in the presence of BrdU, FdU or TFT whereas the normal cells, which contain thymidine kinase, are not. Similarly, cells deficient in HPRT are selected by resistance to 8-azaguanine (AG) or 6-thioguanine (TG). Cells with altered Na^+/K^+ ATPase are selected by resistance to ouabain.

Cytotoxicity is determined by measuring the effect of the test material on colony-forming abilities (cloning efficiency) or growth rates of the cultures. Mutant frequency is determined by seeding known numbers of cells in medium containing the selective agent to detect mutant cells, and in medium without selective agent to determine the surviving cells. After a suitable incubation period, colonies are counted. Mutant frequencies are calculated from the number of mutant colonies corrected for cell survival.

1.5. Quality criteria

None.

1.6. Description of the test method

Preparations

Cells

A variety of cell lines are available for use in this assay. These include subclones of L5178Y, CHO cells or V-79 cells with a demonstrated sensitivity to chemical mutagens, a high cloning efficiency and a low spontaneous mutation frequency. Cells may be periodically checked for karyotype stability and should be checked for *Mycoplasma* contamination. Other cell types may be used providing their validity as an assay for chemically induced gene mutations can be fully documented.

(1) Formerly HGPRT.

Medium

Appropriate culture media and incubation conditions (e.g. temperature, cultre vessels used, CO_2 concentrations and humidity) should be used. Media and sera should be chosen according to the selective systems and cell type used in the assay.

Test substance

Test substances my be prepared in culture media or dissolved or suspended in appropriate vehicles prior to treatment of the cells. The final concentration of the vehicle in the culture system should not affect cell viability or growth rate.

Metabolic activation

Cells should be exposed to the test substance both in the presence and absence of an appropriate exogenous mammalian metabolic activation system. Alternatively, where cell types with intrinsic metabolic activity are used, the rate and nature of the activity should be known to be appropriate to the chemical class being tested.

Test conditions

Use of negative and positive controls

Positive controls, using both a direct acting compound and a compound requiring metabolic activation, should be included in each experiment; a negative (vehicle) control should also be used.

The following are examples of substances which might be used as positive controls:

- direct acting compounds:
  - ethylmethanesulphonate,
  - hycanthone,
- indirect acting compounds:
  - 2-acetylaminofluorene,
  - 7,12-dimethylbenzanthracence,
  - N-nitrosodimethylamine.

When appropriate, an additional positive control of the same chemical class as the chemical under test could be included.

Exposure concentrations

Several concentrations of the test substance should be used. These concentrations should yield a concentration-related toxic effect, the highest concentration producing a low level of survival and the survival in the lowest concentration being approximately the same as that in the negative control.

Relatively water-insoluble substances should be tested up to the limit of solubility using appropriate procedures. For freely water-soluble non-toxic substances, the upper test substance concentration should be determined on a case-by-case basis.

Procedure

The number of cells used per culture should be related to the spontaneous mutation frequency; a general guide is to use a number of viable cells which is 10 times the inverse of the spontaneous mutation frequency.

Cells should be exposed for a suitable period of time, in most cases two to five hours is effective. Cells without sufficient intrinsic metabolic activity should be exposed to the test substance in the presence and absence of an appropriate metabolic activation system. At the end of the exposure period, cells are washed free of test substance and cultured to determine viability and to allow for expression of the mutant phenotype.

At the end of the expression period, which should be sufficient to allow near optimal phenotypic expression of induced mutants, the cells are grown in medium with and without selective agents for the determination of numbers of mutants and viability.

All results are confirmed in an independent experiment.

2. DATA

Data should be presented in tabular form. Individual plate counts for the test substance and controls should be presented for both mutation induction and survival. The mean number of colonies per plate and the standard deviation should also be given. Mutation frequency should be expressed as number of mutants per number of surviving cells. Survival and cloning efficiencies are expressed as a percentage of the control level.

Data should be evaluated using appropriate statistical methods.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following information:

— cell line used, number of cell cultures, methods for maintenance of cell cultures,

— test conditions: composition of media, CO_2 concentration, concentration of test substance, vehicle used, incubation temperature, incubation time, length of expression period (including number of cells seeded and subcultures and feeding schedules, if appropriate), duration of treatment, cell density during treatment, type of mammalian metabolic activation system used, positive and negative controls, selective agent used,

— rationale for dose selection,

— method used to enumerate numbers of viable and mutant cells,

— statistical evaluation,

— discussion of results,

— interpretation of results.

3.2. Evaluation and interpretation

See General Introduction Part B.

4. REFERENCES

See General Introduction Part B.

DNA DAMAGE AND REPAIR — UNSCHEDULED DNA SYNTHESIS — MAMMALIAN CELLS *IN VITRO*

1. METHOD

1.1. Introduction

See General Introduction Part B.

1.2. Definition

See General Introduction Part B.

1.3. Reference substances

None.

1.4. Principle of the test method

The Unscheduled DNA Synthesis (UDS) test measures the DNA repair synthesis after excision and removal of a stretch of DNA containing the region of damage induced by chemical and physical agents. The test is based on the incorporation of tritium labelled thymidine (^{3}H-TdR) into the DNA of mammalian cells which are not in the S phase of the cell cycle. The uptake of ^{3}H-TdR may be determined by autoradiography or by liquid scintillation counting (LSC) of DNA from the treated cells. Mammalian cells in culture, unless primary rat hepatocytes are used, are treated with the test agent with and without an exogenous metabolic activation system. UDS may also be measured in *in vivo* systems.

1.5. Quality criteria

None.

1.6. Description of the test method

Preparations

Test chemicals and control or reference substances should be prepared in growth medium or dissolved or suspended in appropriate vehicles and then further diluted in growth medium for use in the assay. The final concentration of the vehicle should not affect cell viability.

Primary cultures of rat hepatocytes, human lymphocytes or established cell lines (e.g. human diploid fibroblasts) may be used in the assay.

Cells should be exposed to the test chemical both in the presence and absence of an appropriate metabolic activation system.

Test conditions

Number of cultures

At least two cell cultures for autoradiography and six cultures (or less if scientifically justified) for LSC UDS determinations are necessary for each experimental point.

Use of negative and positive controls

Concurrent positive and negative (untreated and/or vehicle) controls with and without metabolic activation should be included in each experiment.

Examples of positive controls for the rat hepatocyte assay include 7,12-dimethylbenzathracene (7,12-DMBA) or 2-acetylaminofluorene (2-AAF). In the case of established cell lines 4-nitroquinoline-N-oxide (4-NQO) is an example of a positive control for both the autoradiographic and LSC assays performed without metabolic activation; N-dimethylnitrosamine is an example of a positive control compound when metabolic activation systems are used.

Exposure concentrations

Multiple concentrations of the test substance over a range adequate to define the response should be used. The highest concentration should elicit some cytotoxic effects. Relatively water-insoluble compounds should be tested up to the limit of solubility. For freely water-soluble non-toxic chemicals, the upper test chemical concentration should be determined on a case-by-case basis.

Cells

Appropriate growth media, CO_2 concentration, temperature and humidity should be used in maintaining cultures. Established cell lines should be periodically checked for *Mycoplasma* contamination.

Metabolic activation

A metabolic activation system is not used with primary hepatocyte cultures. Established cell lines and lymphocytes are exposed to test substance both in the presence and absence of an appropriate metabolic activation system.

Procedure

Preparation of cultures

Established cell lines are generated from stock cultures (e.g. by trypsinization or by shaking off), seeded in culture vessels at appropriate density, and incubated at 37 °C.

Short-term cultures of rat hepatocytes are established by allowing freshly dissociated hepatocytes in an appropriate medium to attach themselves to the growing surface.

Human lymphocyte cultures are set up using appropriate techniques.

Treatment of the cultures with the test substance

Primary rat hepatocytes

Freshly isolated rat hepatocytes are treated with the test substance in a medium containing ^{3}H-TdR for an appropriate length of time. At the end of the treatment period, medium should be drained off the cells, which are then rinsed, fixed and dried. Slides should be dipped in autoradiographic emulsion (alternative stripping film may be used), exposed, developed, stained and counted.

Established cell lines and lymphocytes

Autoradiographic techniques: Cell cultures are exposed to the test substance for appropriate durations followed by treatment with ^{3}H-TdR. The times will be governed by the nature of the substance, the activity of metabolising systems and the type of cells. To detect the peak of UDS, ^{3}H-TdR should be added either simultaneously with the test substance or within a few minutes after exposure to the test substance. The choice between these two procedures will be influenced by possible interactions between test substance and ^{3}H-TdR. In order to discriminate between UDS and semi-conservative DNA replication, the latter can be inhibited, for example, by the use of an arginine-deficient medium, low serum content or by hydroxyurea in the culture medium.

LSC measurements of UDS: Prior to treatment with test substance, entry of cells into S-phase should be blocked as described above; cells should then be exposed to test chemical as described for autoradiography. At the end of the incubation period, DNA should be extracted from the cells and the total DNA content, and the extent of ^{3}H-TdR incorporation determined.

It should be noted that, where human lymphocytes are used in the above techniques, the suppression of semi-conservative DNA replication is unnecessary in unstimulated cultures.

Analysis

Autoradiographic determinations

In determining UDS in cells in culture, S-phase nuclei are not counted. At least 50 cells per concentration should be counted. Slides should be coded before counting. Several widely separated random fields should be counted on each slide. The amount of ^{3}H-TdR incorporation in the cytoplasm should be determined by counting three nucleus-sized areas in the cyptoplasm of each cell counted.

LSC determinations

An adequate number of cultures should be used at each concentration and in the controls in LSC UDS determinations.

All results should be confirmed in an independent experiment.

2. DATA

Data should be presented in tabular form.

2.1. Autoradiographic determinations

The extent of ^{3}H-TdR incorporation in the cytoplasm and the number of grains found over the cell nucleus should be recorded separately.

Mean, median and mode may be used to describe the distribution of the extent of ^{3}H-TdR incorporation in the cytoplasm and the number of grains per nucleus.

2.2. LSC determinations

For LSC determinations, ^{3}H-TdR incorporation should be reported as dpm/μg DNA. The mean dpm/μg DNA with standard deviation may be used to describe the distribution of incorporation.

Data should be evaluated using appropriate statistical methods.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following information:

— cells used, density and passage number at time of treatment, number of cell cultures,

— methods used for maintenance of cell cultures including medium, temperature and CO_2 concentration,

— test substance, vehicle, concentrations and rationale for selection of concentrations used in the assay,

— details of metabolic activation systems,

— treatment schedule,

— positive and negative controls,

— autoradiographic technique used,

— procedures used to block entry of cells into S-phase,

— procedures used for DNA extraction and determination of total DNA content in LSC determination,

— dose/response relationship, where possible,

— statistical evaluation,

— discussion of results,

— interpretation of results.

3.2. **Evaluation and interpretation**

See General Introduction Part B.

4. **REFERENCES**

See General Introduction Part B.

SISTER CHROMATID EXCHANGE ASSAY *IN VITRO*

1. METHOD

1.1. Introduction

See General Introduction Part B.

1.2. Definition

See General Introduction Part B.

1.3. Reference substances

None.

1.4. Principle of the test method

The Sister Chromatid Exchange (SCE) assay is a short-term test for the detection of reciprocal exchanges of DNA between two sister chromatids of a duplicating chromosome. SCEs represent the interchange of DNA replication products at apparently homologous loci. The exchange process presumably involves DNA breakage and reunion, although little is known about its molecular basis. Detection of SCEs requires some means of differentially labelling sister chromatids and this can be achieved by incorporation of bromodeoxyuridine (BrdU) into chromosomal DNA for two cell cycles.

Mammalian cells *in vitro* are exposed to the test chemical with and without a mammalian exogenous metabolic activation system, if appropriate, and cultured for two rounds of replication in BrdU-containing medium. After treatment with a spindle inhibitor (e.g. colchicine) to accumulate cells in a metaphase-like stage of mitosis (c-metaphase), cells are harvested and chromosome preparations are made.

1.5. Quality criteria

None.

1.6. Description of the test method

Preparations

— Primary cultures, (human lymphocytes) or established cell lines (e.g. Chinese hamster ovary cells) may be used in the assay. Cell lines should be checked for *Mycoplasma* contamination,

— Appropriate culture media and incubation conditions (e.g. temperature, culture vessels, CO_2 concentration and humidity) should be used,

— Test substances may be prepared in culture media or dissolved or suspended in appropriate vehicles prior to treatment of the cells. The final concentration of a vehicle in the culture system should not significantly affect cell viability or growth rate and effects on SCE frequency should be monitored by a solvent control,

— Cells should be exposed to the test substance both in the presence and absence of an exogenous mammalian metabolic activation system. Alternatively, where cell types with instrinsic metabolic activity are used, the rate and nature of the activity should be appropriate to the chemical class being tested.

Test conditions

Number of cultures

At least duplicate cultures should be used for each experimental point.

Use of negative and positive controls

Positive controls, using both a direct acting compound and a compound requiring metabolic activation should be included in each experiment; a vehicle control should also be used.

The following are examples of substances which might be used as positive controls:

— direct acting compound:
  — ethylmethanesulphonate,
— indirect acting compound:
  — cyclophosphamide.

When appropriate, an additional positive control of the same chemical class as the chemical under test may be included.

Exposure concentrations

At least three adequately spaced concentrations of the test substance should be used. The highest concentration should give rise to a significant toxic effect but must still allow adequate cell replication to occur. Relatively water-insoluble substances should be tested up to the limit of solubility using appropriate procedures. For freely water-soluble non-toxic substances the upper test substance concentration should be determined on a case-by-case basis.

Procedure

Preparation of cultures

Established cell lines are generated from stock cultures (e.g. by trypsinization or by shaking off), seeded in culture vessels at appropriate density and incubated at 37 °C. For monolayer cultures, the number of cells per culture vessel should be adjusted so that the cultures are not much more than 50 % confluent at the time of harvest. Alternatively, cells may be used in suspension culture. Human lymphocyte cultures are set up from heparinized blood, using appropriate techniques, and incubated at 37 °C.

Treatment

Cells in an exponential stage of growth are exposed to the test substance for a suitable period of time; in most cases one to two hours may be effective, but the treatment time may be extended up to two complete cell cycles in certain cases. Cells without sufficient instrinsic metabolic activity should be exposed to the test chemical in the presence and absence of an appropriate metabolic activation system. At the end of the exposure period, cells are washed free of test substance and cultured for two rounds of replication in the presence of BrdU. As an alternative procedure cells may be exposed simultaneously to the test chemical and BrdU for the complete culture time of two cell cycles.

Human lymphocyte cultures are treated while they are in a semisynchronous condition.

Cells are analysed in their second post-treatment division, ensuring that the most sensitive cell cycle stages have been exposed to the chemical. All cultures to which BrdU is added should be handled in darkness or in dim light from incandescent lamps up to the harvesting of cells in order to minimize photolysis of BrdU-containing DNA.

Harvesting of cells

Cell cultures are treated with a spindle inhibitor (e.g. colchicine) one to four hours prior to harvesting. Each culture is harvested and processed separately for the preparation of chromosomes.

Chromosome preparation and staining

Chromosome preparations are made by standard cytogenetic techniques. Staining of slides to show SCEs can be performed by several techniques, (e.g. the fluorescence plus Giemsa method).

Analysis

The number of cells analysed should be based on the spontaneous control frequency of SCE. Usually, at least 25 well-spread metaphases per culture are analysed for SCEs. Slides are coded before analysis. In human lymphocytes only metaphases containing 46 centromeres are analysed. In established cell lines only metaphases containing ± 2 centromeres of the modal number are analysed. It should be stated whether or not centromeric switch of label is scored as an SCE. The results should be confirmed in an independent experiment.

2. DATA

Data should be presented in tabular form. The number of SCEs for each metaphase and the number of SCEs per chromosome for each metaphase should be listed separately for all treated and control cultures.

The data should be evaluated using appropriate statistical methods.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following information:

— cells used, methods of maintenance of cell culture,

— test conditions: composition of media, CO_2 concentration, concentration of test substance, vehicle used, incubation temperature, treatment time, spindle inhibitor used, its concentration and the duration of treatment with it, type of mammalian activation system used, positive and negative controls,

— number of cell cultures per experimental point,

— details of the technique used for slide preparation,

— number of metaphases analysed (data given separately for each culture),

— mean number of SCE per cell and per chromosome (data given separately for each culture),

— criteria for scoring SCE,

— rationale for dose selection,

— dose-response relationship, if applicable,

— statistical evaluation,

— discussion of results,

— interpretation of results.

3.2. Evaluation and interpretation

See General Introduction Part B.

4. REFERENCES

See General Introduction Part B.

SEX-LINKED RECESSIVE LETHAL TEST IN *DROSOPHILA MELANOGASTER*

1. METHOD

1.1. Introduction

See General Introduction Part B.

1.2. Definition

See General Introduction Part B.

1.3. Reference substances

None.

1.4. Principles of the test method

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

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

1.5. Quality criteria

None.

1.6. Description of the test method

Preparations

Stocks

Males of a well-defined wild-type stock and females of the Muller-5 stock may be used. Other appropriately marked female stocks with multiple inverted X-chromosomes may also be used.

Test substance

Test substances should be dissolved in water. Substances which are insoluble in water may be dissolved or suspended in appropriate vehicles (e.g. a mixture of ethanol and Tween-60 or 80), then diluted in water or saline prior to administration. Dimethylsulphoxide (DMSO) should be avoided as a vehicle.

Number of animals

The test should be designed with a predetermined sensitivity and power. The spontaneous mutant frequency observed in the appropriate control will influence strongly the number of treated chromosomes that must be analysed.

Route of administration

Exposure may be oral, by injection or by exposure to gases or vapours. Feeding of the test substance may be done in sugar solution. When necessary, substances may be dissolved in a 0,7 % NaCl solution and injected into the thorax or abdomen.

Use of negative and positive controls

Negative (vehicle) and positive controls should be included. However, if appropriate laboratory historical control data are available, no concurrent controls are needed.

Exposure levels

Three exposure levels should be used. For a preliminary assessment one exposure level of the test substance may be used, that exposure level being either the maximum tolerated concentration or that producing some indication of toxicity. For non-toxic substances exposure to the maximum practicable concentration should be used.

Procedure

Wild-type males (three to five days old) are treated with the test substance and mated individually to an excess of virgin females from the Muller-5 stock or from another appropriately marked (with multiple inverted X-chromosomes) stock. The females are replaced with fresh virgins every two to three days to cover the entire germ cell cycle. The offspring of these females are scored for lethal effects corresponding to the effects on mature sperm, mid or late-stage spermatids, early spermatids, spermatocytes and spermatogonia at the time of treatment.

Heterozygous F_1 females from the above crosses are allowed to mate individually (i.e. one female per vial) with their brothers. In the F_2 generation, each culture is scored for the absence of wild-type males. If a culture appears to have arisen from an F_1 female carrying a lethal in the parental X-chromosome (i.e. no males with the treated chromosome are observed) daughters of that female with the same genotype should be tested to ascertain whether the lethality is repeated in the next generation.

2. DATA

Data should be tabulated to show the number of X-chromosomes tested, the number of non-fertile males and the number of lethal chromosomes at each exposure concentration and for each mating period for each male treated. Numbers of clusters of different sizes per male should be reported. These results should be confirmed in a separate experiment.

Appropriate statistical methods should be used in evaluation sex-linked recessive lethal tests. Clustering of recessive lethals originating from one male should be considered and evaluated in an appropriate statistical manner.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following information:

— stock: *Drosophila* stocks or strains used, age of insects, number of males treated, number of sterile males, number of F_2 cultures established, number of F_2 cultures without progeny, number of chromosomes carrying a lethal detected at each germ cell stage,

— criteria for establishing the size of treated groups,

— test conditions: detailed description of treatment and sampling schedule, exposure levels, toxicity data, negative (solvent) and positive controls, if appropriate,

— criteria for scoring lethal mutations,

— exposure/effect relationship where possible,

— evaluation of data,

— discussion of results,

— interpretation of results.

3.2. Evaluation and interpretation

See General Introduction Part B.

4. REFERENCES

See General Introduction Part B.

IN VITRO MAMMALIAN CELL TRANSFORMATION TESTS

1. METHOD

1.1. Introduction

See General Introduction Part B.

1.2. Definition

See General Introduction Part B.

1.3. Reference substances

None.

1.4. Principle of the test method

Mammalian cell culture systems may be used to detect phenotypic changes *in vitro* induced by chemical substances associated with malignant transformation *in vivo*. Widely used cells include C3H10T$^{1}/_{2}$, 3T3, SHE, Fischer rat and the tests rely on changes in cell morphology, focus formation or changes in anchorage dependence in semi-solid agar. Less widely used systems exist which detect other physiological or morphological changes in cells following exposure to carcinogenic chemicals. None of the *in vitro* test endpoints has an established mechanistic link with cancer. Some of the test systems are capable of detecting tumour promotors. Cytotoxicity may be determined by measuring the effect of the test material on colony-forming abilities (cloning efficiency) or growth rates of the cultures. The measurement of cytotoxicity is to establish that exposure to the test chemical has been toxicologically relevant but cannot be used to calculate transformation frequency in all assays since some may involve prolonged incubation and/or replating.

1.5. Quality criteria

None.

1.6. Description of the test method

Preparations

Cells

A variety of cell lines or primary cells are available depending on the transformation test being used. The investigator must ensure that the cells in the test being performed exhibit the appropriate phenotypic change after exposure to known carcinogens and that the test, in the investigator's laboratory, is of proven and documented validity and reliability.

Medium

Media and experimental conditions should be used that are appropriate to the transformation assay in use.

Test substance

Test substances may be prepared in culture media or dissolved or suspended in appropriate vehicles prior to treatment of the cells. The final concentration of the vehicle in the culture system should not affect cell viability, growth rate or transformation incidence.

Metabolic activation

Cells should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system. Alternatively, when cell types are used that possess intrinsic metabolic activity, the nature of the activity should be known to be appropriate to the chemical class being tested.

Test conditions

Use of negative and positive controls

Possitive controls, using both a direct-acting compound and a compound requiring metabolic activation should be included in each experiment; a negative (vehicle) control should also be used.

The following are examples of substances which might be used as positive controls:

- Direct-acting chemicals:
  - ethylmethanesulphonate,
  - β-propiolactone,
- Compounds requiring metabolic activation:
  - 2-acetylaminofluorene,
  - 4-dimethylaminoazobenzene,
  - 7,12-dimethylbenzanthracene.

When appropriate, an additional positive control of the same chemical class as the compound under test should be included.

Exposure concentrations

Several concentrations of the test substance should be used. These concentrations should yield a concentration-related toxic effect, the highest concentration producing a low level of survival and the survival in the lowest concentration being approximately the same as that in the negative control. Relatively water-insoluble substances should be tested up to the limit of solubility using appropriate procedures. For freely water-soluble non-toxic substances the upper test substance concentration should be determined on a case-by-case basis.

Procedure

Cells should be exposed for a suitable period of time depending on the test system in use, and this may involve re-dosing accompanied by a change of medium (and if necessary, fresh metabolic activation mixture) if exposure is prolonged. Cells without sufficient intrinsic metabolic activity should be exposed to the test substance in the presence and absence of an appropriate metabolic activation system. At the end of the exposure period, cells are washed free of test substance and cultured under conditions appropriate for the appearance of the transformed phenotype being monitored and the incidence of transformation determined. All results are confirmed in an independent experiment.

2. DATA

Data should be presented in tabular form and may take a variety of forms according to the assay being used e.g. plate counts, positive plates or numbers of transformed cells. Where appropriate, survival should be expressed as a percentage of control levels and transformation frequency expressed as the number of transformants per number of survivors. Data should be evaluated using appropriate statistical methods.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following information:

- cell type used, number of cell cultures, methods for maintenance of cell cultures,
- test conditions: concentration of test substance, vehicle used, incubation time, duration and frequency of treatment, cell density during treatment, type of exogenous metabolic activation system used, positive and negative controls, specification of phenotype being monitored, selective system used (if appropriate), rational for dose selection,

— method used to enumerate viable and transformed cells,

— statistical evaluation,

— discussion of results,

— interpretation of results.

3.2. **Evaluation and interpretation**

See General Introduction Part B.

4. **REFERENCES**

See General Introduction Part B.

RODENT DOMINANT LETHAL TEST

1. METHOD

1.1. Introduction

See General Introduction Part B.

1.2. Definition

See General Introduction Part B.

1.3. Reference substances

None.

1.4. Principle of the test method

Dominant lethal effects cause embryonic or foetal death. Induction of dominant lethals by exposure to a chemical substance indicates that the substance has affected germinal tissue of the test species. It is generally accepted that dominant lethals are due to chromosomal damage (structural and numerical anomalies). Embryonic death if females are treated may also be the result of toxic effects.

Generally, male animals are exposed to the test compound and mated to untreated virgin females. The various germ cell stages can be tested separately by the use of sequential mating intervals. The increase of dead implants per female in the treated group over the dead implants per female in the control group reflects the post-implantational loss. Pre-implantational loss can be estimated based on *corpora lutea* counts or by comparing the total implants per female in treated and control groups. The total dominant lethal effect is the sum of pre- and post-implantational loss. The calculation of the total dominant lethal effect is based on comparison of the live implants per female in the test group to the live implants per female in the control group. A reduction in the number of implants at certain intervals may be the result of cell killing (i.e. of spermatocytes and/or spermatogonia).

1.5. Quality criteria

None.

1.6. Description of the test method

Preparations

When possible, test substances should be dissolved or suspended in isotonic saline. Chemicals insoluble in water may be dissolved or suspended in appropriate vehicles. The vehicle used should neither interfere with the test chemical nor produce toxic effects. Fresh preparations of the test chemical should be employed.

Test conditions

Route of administration

The test compound should generally be administered only once. Based on toxicological information a repeated treatment schedule can be employed. The usual routes of administration are oral intubation or intraperitoneal injection. Other routes of administration may be appropriate.

Experimental animals

Rats or mice are recommended as the test species. Healthy fully sexually mature animals are randomized and assigned to treatment and control groups.

Number and sex

An adequate number of treated males should be used, taking into account the spontaneous variation of the biological character being evaluated. The number chosen should be based on the pre-determined sensitivity of detection and power of significance. For example in a typical test, the number of males in each dose group should be sufficient to provide between 30 and 50 pregnant females per mating interval.

Use of negative and positive controls

Generally concurrent positive and negative (vehicle) controls should be included in each experiment. When acceptable positive control results are available from experiments conducted recently in the same laboratory these results can be used instead of a concurrent positive control. Positive control substances should be used at an appropriate low dose (e.g. MMS, intraperitoneally, at 10 mg/kilogram) to demonstrate the test sensitivity.

Dose levels

Normally, three dose levels should be used. The high dose should produce signs of toxicity or reduced fertility in the treated animals. In certain cases a single high dose level may be sufficient.

Limit test

Non-toxic substances should be tested at 5 g/kilogram on a single administration or at 1 g/kilogram/day on repeated administration.

Procedure

Several treatment schedules are available. Single administration of the test substance is the most widely used. Other treatment schedules may be used.

Individual males are mated sequentially to one or two untreated virgin females at appropriate intervals after treatment. Females should be left with the males for at least the duration of one oestrous cycle or until mating has occurred as determined by the presence of sperm in the vagina or by the presence of a vaginal plug.

The number of matings following treatment is governed by the treatment schedule and should ensure that all germ cell stages are sampled after treatment.

Females are sacrificed in the second half of pregnancy and uterine contents are examined to determine the number of dead and live implants. The ovaries may be examined to determine the number of *corpora lutea*.

2. **DATA**

Data should be tabulated to show the number of males, the number of pregnant females, and the number of non-pregnant females. Results of each mating, including the identity of each male and female, should be reported individually. For each female, week of mating, dose level received by the males, the frequencies of live implants and of dead implants should be recorded.

The calculation of the total dominant lethal effect is based on comparison of the live implants per female in the test group to the live implants per female in the control group. The ratio of dead to live implants from the treated group compared to the same ratio from the control group is analysed to indicate the post-implantation loss.

If the data are recorded as early and late deaths, the tables should make that clear. If pre-implantation loss is estimated, it should be reported. Pre-implantation loss can be calculated as a discrepancy between the number of *corpora lutea* and the number of implants or as a reduction in the average number of implants per uterus in comparison with control matings.

Data are evaluated using appropriate statistical methods.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following information:

— species, strain, age and weights of animals used, number of animals of each sex in experimental and control groups,

— test substance, vehicle, dose levels tested and rationale for dose selection, negative and positive controls, toxicity data,

— route and treatment schedule,

— mating schedule,

— method used to determine that mating has occurred,

— time of sacrifice,

— criteria for scoring dominant lethals,

— dose/response relationship, if applicable,

— statistical evaluation,

— discussion of results,

— interpretation of results.

3.2. Evaluation and interpretation

See General Introduction Part B.

4. REFERENCES

See General Introduction Part B.

IN VIVO MAMMALIAN GERM-CELL CYTOGENETICS

1. METHOD

1.1. Introduction

See General Introduction Part B.

1.2. Definition

See General Introduction Part B.

1.3. Reference substances

None.

1.4. Principle of the test method

This *in vivo* cytogenetic test detects structural chromosome aberrations in spermatogonia. It consists of analysis of spermatogonial mitoses for chromatid and chromosome type aberrations.

The method employs testes preparations of mammals which have been exposed to the test chemicals by appropriate routes and sacrificed at varying intervals. Animals are further treated, prior to sacrifice, with spindle inhibitors such as colchicine to accumulate cells in a metaphase-like stage of mitosis (c-metaphase). Air-dried chromosome preparations are made, stained and microscopically analysed.

Analysis of spermatocytes at diakinesis-metaphase I for translocation multivalents after treatment of spermatogonial stem cells may provide useful additional information.

1.5. Quality criteria

None.

1.6. Description of the test method

Preparations

The test chemicals are dissolved in isotonic saline. If insoluble, they are dissolved or suspended in appropriate vehicle. Freshly prepared solutions of the test compound are employed. If a vehicle is used to facilitate dosing, it must not interfere with the test chemical or produce toxic effects.

Route of administration

Test compounds should generally be administered only once. Based on toxicological information a repeated treatment schedule may be employed. However, the repeated treatment can only be applied if the test compound does not exhibit major cytotoxic effects in differentiating spermatogonia.

The usual routes of administration are oral and intraperitoneal injections. Other routes of administration may be appropriate.

Experimental animals

Most commonly, mice and Chinese hamsters are used. Any other mammalian species may be employed.

Sexually mature males are used and randomly assigned to treatment and control groups.

Number of animals

At least five males per experimental and control group are employed.

Use of negative and positive controls

Concurrent positive and negative (vehicle) controls should be included in each experiment.

Positive control substances should be used at an appropriate low dose (e.g. mitomycin C, intraperitoneally, at 0,3 mg/kilogram) to demonstrate the test sensitivity.

Dose levels

One dose of the test compound is used, the dose being the maximum tolerated dose or that producing some indication of cytotoxicity. If this dose produces high cell killing then an additional lower dose showing cytotoxicity should be used. When it is necessary to establish a dose/response relationship at least three doses are required (e.g. to confirm a weak positive response). Non-toxic substances should be tested at the highest practicable dose for both single or repeated administration.

Procedure

Animals are generally treated with the test compound only once. In the highest dose group three sampling intervals after treatment are used. The central sampling interval is 24 hours. Since cell cycle kinetics can be influenced by the test compound, one earlier and one later sampling interval adequately spaced within the range of 6 to 48 hours are applied. For additional dose levels samples should be taken at the particularly sensitive interval, or, if that is not known, 24 hours after treatment.

Alternatively, a repeated treatment schedule may be employed and the animals should be sacrificed 24 hours after the last treatment. Additional sampling times within the range of 6 to 24 hours may be used.

Testis preparation

For analysis of spermatogonial mitoses animals are injected intraperitoneally with an adequate dose of a spindle inhibitor such as colchicine. Animals are sacrificed at an appropriate interval thereafter. For mice this interval varies from three to five hours, for Chinese hamsters more than five hours may be required.

The air-drying techniquer is used. Different species may require modifications of the standard procedure. Cell suspensions are obtained, treated with hypotonic solution and fixed. Cells are spread on slides and stained. Slides are coded before microscopic analysis.

Analysis

At least 100 well-spread mitotic metaphases with the complete number of centromeres are analysed for structural chromosome aberrations. Additionally, the ratio of spermatogonial mitoses to first and second meiotic metaphases may be determined in a total sample of 100 dividing cells per animal to establish a possible cytotoxic effect.

2. **DATA**

Data should be presented in tabular form. For each control and treated animal all types of aberrations are listed separately. The total number of cells analysed and the total number of aberrant cells per group are included. Means and standard deviation are given for all parameters. If determined the mean ratio of spermatogonial mitoses to first and second meiotic metaphases is tabulated for each experimental and control group.

The data are evaluated using appropriate statistical methods.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following information:

— species and strain of males, age and weights of males,

— number of animals for each experimental and control group,

— test conditions, detailed description of treatment, dose levels, solvents, spindle inhibitor used,

— number of cells analysed per animal in each group,

— for each treated and control animal: types and numbers of aberrations,

— statistical evaluation,

— discussion of results,

— interpretation of results.

3.2. Evaluation and interpretation

See General Introduction Part B.

4. REFERENCES

See General Introduction Part B.

MOUSE SPOT TEST

1. METHOD

1.1. Introduction

See General Introduction Part B.

1.2. Definition

See General Introduction Part B.

1.3. Reference substances

None.

1.4. Principle of the test method

This is an *in vivo* test in mice in which developing embryos are exposed to the chemicals. The target cells in the developing embryos are melanoblasts, and the target genes are those which control the pigmentation of the coat hairs. The developing embryos are heterozygous for a number of these coat colour genes. A mutation in, or loss of (by a variety of genetic events), the dominant allele of such a gene in a melanoblast results in the expression of the recessive phenotype in its descendant cells, constituting a spot of changed colour in the coat of the resulting mouse. The number of offspring with these spots, mutations, are scored and their frequency is compared with that among offspring resulting from embryos treated with the solvent only. The mouse spot test detects presumed somatic mutations in foetal cells.

1.5. Quality criteria

None.

1.6. Description of the test method

Preparations

When possible, test substances are dissolved or suspended in isotonic saline. Chemicals insoluble in water are dissolved or suspended in appropriate vehicles. The vehicle used should neither interfere with the test chemical nor produce toxic effects. Fresh preparations of the test chemical should be used.

Experimental animals

Mice of the T strain (nonagouti, a/a; chinchilla, pink eye, $c^{ch}p/c^{ch}p$; brown, b/b; dilute, short ear, d se/d se; piebald spotting, s/s) are mated either with the HT strain (pallid, nonagouti, brachypody, pa a bp/pa a bp; leaden fuzzy, ln fz/ln fz; pearl pe/pe) or with C57 BL (nonagouti, a/a). Other appropriate crosses such as between NMRI (nonagouti, a/a; albino, c/c) and DBA (nonagouti, a/a; brown, b/b; dilute d/d) may be used provided they produce nonagouti offspring.

Number and sex

Sufficient pregnant females are treated to provide an appropriate number of surviving offspring for each dose level used. The appropriate sample size in governed by the number of spots observed in the treated mice and the scale of the control data. A negative result is acceptable only when at least 300 offspring from females treated with the highest dose have been scored.

Use of negative and positive controls

Concurrent control data from mice treated with the vehicle only (negative controls) should be available. Historical control data from the same laboratory may be pooled to increase the sensitivity of the test provided they are

homogeneous. Positive control data recently obtained in the same laboratory from treatment with a chemical known to show mutagenicity by this test should be available if no mutagenicity of the test chemical is detected.

Route of administration

The usual routes of administration are oral intubation or intraperitoneal injection of the pregnant females. Treatment by inhalation or other routes of administration are used when appropriate.

Dose levels

At least two dose levels are used including one showing signs of toxicity or reduced litter size. For non-toxic chemicals exposure to the maximum practicable dose should be used.

Procedure

A single treatment is normally given on day 8, 9 or 10 of pregnancy, counting as day 1 the day on which the vaginal plug is first observed. These days correspond to 7,25, 8,25 and 9,25 days after conception. Successive treatments over these days may be used.

Analysis

The offspring are coded and scored for spots between three and four weeks after birth. Three classes of spots are distinguished:

(a) white spots within 5 mm of the mid-ventral line which are presumed to result from cell killing (WMVS);

(b) yellow, agouti-like, spots associated with mammae, genitalia, throat, axillary and inguinal areas and on the mid-forehead, which are presumed to result from misdifferentiation (MDS); and

(c) pigmented and white spots randomly distributed on the coat which are presumed to result from somatic mutations (RS).

All three classes are scored but only the last, RS, is of genetic relevance. Problems of distinguishing between MDS and RS may be solved by fluorescence microscopy of sample hairs.

Obvious gross morphological abnormalities of the offspring should be noted.

2. DATA

The data are presented as the total number of offspring scored and the number having one or more presumed somatic mutation spots. Treatment and negative control data are compared by appropriate methods. Data are also presented on a per-litter basis.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following information:

— the strains used in the cross,

— the number of pregnant females in the experimental and control groups,

— the average litter size in the experimental and control groups at birth and at weaning,

— the dose level(s) of the test chemical,

— the solvent used,

— the day of pregnancy on which treatment was given,

— the route of treatment,

— the total number of offspring scored, and the number with WMVS, MDS and RS in the experimental and control groups,

— gross morphological abnormalities,

— Dose/response relationship of RS when possible,

— statistical evaluation,

— discussion of results,

— interpretation of results.

3.2. **Evaluation and interpretation**

See General Introduction Part B.

4. **REFERENCES**

See General Introduction Part B.

MOUSE HERITABLE TRANSLOCATION

1. METHOD

1.1. Introduction

See General Introduction Part B.

1.2. Definition

See General Introduction Part B.

1.3. Reference substances

None.

1.4. Principle of the test method

The mouse heritable translocation test detects structural and numerical chromosome changes in mammalian germ cells as recovered in first generation progeny. The types of chromosome changes detected are reciprocal translocations and, if female progeny are included, X-chromosome loss. Carriers of translocations and XO-females show reduced fertility which is used to select F_1 progeny for cytogenetic analysis. Complete sterility is caused by certain types of translocations (X-autosome and c-t type). Translocations are cytogenetically observed in meiotic cells at diakinesis- metaphase I of male individuals, either F_1 males or male offspring of F_1 females. The XO-females are cytogenetically identified by the presence of only 39 chromosomes in bone marrow mitoses.

1.5. Quality criteria

None.

1.6. Description of the test method

Preparations

The test chemicals are dissolved in isotonic saline. If insoluble they are dissolved or suspended in appropriate vehicles. Freshly prepared solutions of the test compound are employed. If a vehicle is used to facilitate dosing, it must not interfere with the test compound or produce toxic effects.

Route of administration

Routes of administration are usually oral intubation or intraperitoneal injection. Other routes of administration may be appropriate.

Experimental animals

For the ease of breeding and cytological verification these experiments are performed with mice. No specific mouse strain is required. However, the average litter-size of the strain should be greater than eight and be relatively constant. Healthy sexually mature animals are used.

Number of animals

The number of animals necessary depends upon the spontaneous translocation frequency and the minimal rate of induction required for a positive result.

The test is usually performed by analyses of male F_1 progeny. At least 500 male F_1 progeny should be tested per dose group. If female F_1 progeny are included, 300 males and 300 females are required.

Use of negative and positive controls

Adequate control data, derived from concurrent and historic control should be available. When acceptable positive control results are available from experiments conducted recently in the same laboratory these results can be used instead of a concurrent positive control.

Dose levels

One dose level is tested, usually the highest dose associated with the production of minimal toxic effects, but without affecting reproductive behaviour or survival. To establish a dose/response relationship two additional lower doses are required. For non-toxic chemicals exposure to the maximum practicable dose should be used.

Procedure

Treatment and mating

Two treatment schedules are available. Single administration of the test substanace is most widely used. Administration of the test substance on seven days per week for 35 days may also be used. The number of matings following treatment is governed by the treatment schedule and should ensure that all treated germ cell stages are sampled. At the end of the mating period females are caged individually. When females give birth, the date, litter size and sex of progeny are recorded. All male progeny are weaned and all female progeny are discarded unless they are included in the experiment.

Testing for translocation heterozygosity

One of two possible methods is used:

— Fertility testing of F_1 progeny and subsequent verification of possible translocation carriers by cytogenetic analysis,

— Cytogenetic analysis of all male F_1 progeny without prior selection by fertility testing.

(a) Fertility testing

Reduced fertility of an F_1 individual can be established by litter size observation and/or analysis of uterine contents of female mates.

Criteria for determining normal and reduced fertility must be established for the mouse strain used.

Litter size observation: F_1 males to be tested are caged individually with females either from the same experiment or from the colony. Cages are inspected daily beginning 18 days after mating. Litter size and sex of the F_2 progeny are recorded at birth and litters are discarded thereafter. If female F_1 progeny are tested the F_2 progeny of small litters are kept for further testing. Female translocation carriers are verified by cytogenetic analysis of a translocation in any of their male offspring. XO-females are recognized by the change in sex ratio among their progeny from 1:1 to 1:2 males *vs.* females. In a sequential procedure, normal F_1 animals are eliminated from further testing if the first F_2 litter reaches or exceeds a predetermined normal value, otherwise a second or third F_2 litter is observed.

F_1 animals that cannot be classified as normal after observation of up to three F_2 litters are either tested further by analysis of uterine contents of female mates or directly subjected to cytogenetic analysis.

Analysis of uterine contents: The reduction in litter size of translocation carriers is due to embryonic death so that a high number of dead implants is indicative of the presence of a translocation in the animal under test. F_1 males to be tested are mated to two to three females each. Conception is established by daily inspection for vaginal plugs in the morning. Females are sacrified 14 to 16 days later and living and dead implants in their uteri are recorded.

(b) Cytogenetic analysis

Testes preparations are made by the air-drying technique. Translocation carriers are identified by the presence of multivalent configurations at diakinesis-metaphase I in primary spermatocytes. Observation of at least two cells with multivalent association constitutes the required evidence that the tested animal is a translocation carrier.

If no breeding selection has been performed all F_1 males are inspected cytogenetically. A minimum of 25 diakinesis-metaphase I cells per male must be scored microscopically. Examination of mitotic metaphases, in spermatogonia or bone-marrow, is required in F_1 males with small testes and meiotic breakdown before diakinesis or from F_1 female XO suspects. The presence of an unusually long and/or short chromosome in each of 10 cells is evidence for a particular male sterile translocation (c-t type). Some X-autosome translocations that cause male sterility may only be identified by banding analysis of mitotic chromosomes. The presence of 39 chromosomes in all of 10 mitoses is evidence for an XO condition in a female.

2. DATA

Data are presented in tabular form.

The mean litter size and sex ratio from parental matings at birth and weaning are reported for each mating interval.

For fertility assessment of F_1 animals, the mean litter size of all normal matings and the individual litter sizes of F_1 translocation carriers are presented. For analysis of uterine contents, the mean number of living and dead implants of normal matings and the individual numbers of living and dead implants for each mating of F_1 translocation carriers are reported.

For cytogenetic analysis of diakinesis-metaphase I, the numbers of types of multivalent configurations and the total number of cells are listed for each translocation carrier.

For sterile F_1 individuals, the total number of matings and the duration of the mating period are reported. Testes weights and cytogenetic analysis details are given.

For XO females, the mean litter size, sex ratio of F_2 progeny and cytogenetic analysis results are reported.

Where possible F_1 translocation carriers are preselected by fertility tests, the tables have to include information on how many of these were confirmed translocation heterozygotes.

Data from negative controls and the positive control experiments are reported.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following information:

— strain of mice, age of animals, weights of treated animals,

— numbers of parental animals of each sex in experimental and control groups,

— test conditions, detailed description of treatment, dose levels, solvents, mating schedule,

— number and sex of offspring per female, number and sex of offspring raised for translocation analysis,

— time and criteria of translocation analysis,

— number and detailed description of translocation carriers, including breeding data and uterine content data, if applicable,

— cytogenetic procedures and details of microscopic analysis, preferably with pictures,

— statistical evaluation,

— discussion of results,

— interpretation of results.

3.2. Evaluation and interpretation

See General Introduction Part B.

4. REFERENCES

See General Introduction Part B.

PART C: METHODS FOR THE DETERMINATION OF ECOTOXICITY

GENERAL INTRODUCTION: PART C

The test methods described below are for the determination of some of the ecotoxicological properties listed in Annex VIII to Directive 79/831/EEC. Notifiers should be aware that methods for the determination of the following properties foreseen in Level 1 of Annex VIII are not included in the text:

— Prolonged toxicity study with *Daphnia magna*,

— Test on a higher plant,

— Prolonged toxicity study with fish,

— Test for species accumulation.

When appropriate test methods for the determination of these properties are finalized they will be published in the form of a further adaptation to technical progress. In the interim, notifiers should use suitable, internationally recognized methods which should be identified to the competent authority.

ALGAL INHIBITION TEST

1. METHOD

1.1. Introduction

The purpose of this test is to determine the effects of a substance on the growth of a unicellular green algal species. Relatively brief tests can assess effects over several generations. This method can be adapted for use with several unicellular algal species, in which case a description of the method used must be provided with the test report.

This method is most easily applied to water-soluble substances which, under the conditions of the test, are likely to remain in the water.

For substances with limited solubility in the test medium, it may not be possible to determine the EC_{50} quantitatively (see Definitions, below).

The method can be used for substances that do not interfere directly with the measurement of algal growth.

The following information could be helpful when performing this test:

— Water solubility,

— Vapour pressure,

— Structural formula,

— Purity of the substance,

— Chemical stability in water and light,

— Methods of analysis for quantification of the substance in water,

— pK_a value,

— n-Octanol/water partition coefficient,

— Results of a ready biodegradability test.

1.2. Definitions and units

Cell concentration: the number of cells per millilitre;

Growth: the increase in cell concentration over the test period;

Growth rate: the increase in cell concentration per unit time;

EC_{50}: in this method, that concentration of test substance which results in a 50 % reduction in either growth or growth rate relative to the control;

NOEC (no observed effect concentration): in this method, the highest concentration tested at which the measured parameter(s) show(s) no significant inhibition of growth relative to control values.

1.3. Reference substances

A reference substance may be tested as a means of detecting unsatisfactory test conditions. If a reference substance is used, the results should be given in the test report. Potassium dichromate can be used as a reference substance.

1.4. Principle of the test method

Exponentially-growing cultures of selected green algae are exposed to various concentrations of the test substance over several generations under defined conditions. The inhibition of growth in relation to a control culture is determined over a fixed period.

1.5. **Quality criteria**

1.5.1. *Conditions for the validity of the test*

The cell concentration in the control cultures should have increased by a factor of at least 16 within three days.

Disappearance of the test substance from the water into the biomass does not necessarily invalidate the test.

1.6. **Description of the test procedure**

1.6.1. *Preparation*

1.6.1.1. Equipment and materials

— Normal laboratory equipment,

— Test flasks of suitable volume (e.g. 250 ml conical flasks are suitable when the volume of the test solution is 100 ml),

— Culturing apparatus: cabinet or chamber in which a temperature in the range 21 to 25 ± 2 °C can be maintained, and continuous uniform illumination provided in the spectral range 400 to 700 nm. (A quantum flux of $0{,}72 \times 10^{20}$ photons/m^2s within ± 20 % is recommended. This quantum flux equals 120 µE/m^2s and can be obtained with universal white-type fluorescent lamps (light-temperature of approximately 4 200 K) yielding approximately 8 000 lux measured with a spherical collector.)

— Apparatus to determine cell concentrations, e.g. electronic particle counter, microscope with counting chamber, fluorimeter, spectrophotometer, colorimeter (*Note:* in order to provide useful measurements at low cell concentrations when using a spectrophotometer, it may be necessary to use cuvettes with a light path of at least 4 cm).

1.6.1.2. Algal medium

The following medium is recommended:

NH_4Cl:	15	mg/l,
$MgCl_2.6H_2O$:	12	mg/l,
$CaCl_2.2H_2O$:	18	mg/l,
$MgSO_4.7H_2O$:	15	mg/l,
KH_2PO_4:	1,6	mg/l,
$FeCl_3.6H_2O$:	0,08	mg/l,
$Na_2EDTA.2H_2O$:	0,1	mg/l,
H_3BO_3	0,185	mg/l,
$MnCl_2.4H_2O$:	0,415	mg/l,
$ZnCl_2$:	3×10^{-3}	mg/l,
$CoCl_2.6H_2O$:	$1{,}5 \times 10^{-3}$	mg/l,
$CuCl_2.2H_2O$:	10^{-5}	mg/l,
$Na_2MoO_4.2H_2O$:	7×10^{-3}	mg/l,
$NaHCO_3$:	50	mg/l.

The pH of this medium after equilibration with air is approximately 8.

The use of other media is not precluded by the above recommendation provided however, that the following limits of essential constituents are respected:

P:	⩽ 0,7	mg/l,
N:	⩽ 10	mg/l,
chelators:	⩽ 10^{-3}	mmol/l,
hardness (Ca + Mg):	⩽ 0,6	mmol/l.

The recommended medium and the medium given in reference (1) meet this requirement.

1.6.1.3. Experimental organisms

Selection of species

It is suggested that the species of green algae used be a fast-growing species that is convenient for culturing and testing. The following species are considered suitable:

— *Selenastrum capricornutum* ATCC 22662,

— *Scenedesmus subspicatus* 86.81 SAG,

— *Chlorella vulgaris* CCAP 211/11b.

If other species are used, the strain should be reported.

1.6.1.4. Test design

Initial cell concentration

It is recommended that the initial cell concentration in the test cultures be approximately 10^4 cells/ml for *Selenastrum capricornutum* and *Scenedesmus subspicatus*. When other species are used the biomass should be comparable.

Concentrations of test substance

The concentration range in which effects are likely to occur is determined on the basis of results from range-finding tests. For the test, at least five concentrations arranged in a geometric series should be selected. The lowest concentration tested should have no observed effect on the growth of the algae. The highest concentration tested should inhibit growth by at least 50 % relative to the control and, preferably, stop growth completely.

Replicates and controls

The test design should include preferably three replicates at each test concentration and ideally twice that number of controls. If justified, the test design may be altered to increase the number of concentrations and reduce the number of replicates per concentration.

When a vehicle is used to solubilize the test substance additional controls containing the vehicle at the highest concentrations used in the test cultures should be included in the test design.

1.6.2. *Performance of the test*

This section contains guidance for the testing of readily soluble and poorly soluble substances and of volatile substances.

1.6.2.1. Testing readily water-soluble substances

Test cultures containing the desired concentrations of test substance and the desired quantity of algal inoculum are prepared by diluting with filtered algal medium, aliquots of stock solutions of the test substance and of algal suspension.

The culture flasks are shaken and placed in the culturing apparatus. During the test it is necessary to keep the algae in suspension and to facilitate transfer of CO_2. To this end shaking, stirring or aeration may be used. The cultures should be maintained at a temperature in the range of 21 to 25 °C, controlled at ± 2 °C.

The cell concentration in each flask is determined at least at 24, 48 and 72 hours after the start of the test. Filtered algal medium is used to determine the background when using particle counters or as a blank when using spectrophotometers.

The pH is measured at the beginning of the test and at 72 hours.

The pH of the solutions should not normally deviate by more than one unit during the test.

1.6.2.2. Testing substances with limited water solubility

When the solubility of the test substance is of the order of the highest concentration used in the test, only slight deviations from the above procedure are necessary to make up the test solutions. A saturated solution may serve as the stock solutions of the test substance. Another approach can be to dissolve the test substance at the desired concentration in the algal medium prior to the introduction of algal suspension.

Stock solutions of substances of low water-solubility may be prepared by mechanical dispersion or by the use of vehicles of low toxicity to algae, such as organic solvents, emulsifiers or dispersants. When a vehicle is used the concentration should not exceed 100 mg/litre, and additional controls, in which the vehicle is incorporated at the highest concentration present in the test solutions, must be included in the test design.

1.6.2.3. Testing volatile substances

There is to date no generally accepted way to test volatile substances. When a substance is known to have a tendency to vaporize, closed test flasks with increased head-space may be used. Variations to this method have been proposed (see reference (1)).

Attempts should be made to determine the amount of the substance which remains in solution, and extreme caution is advised when interpreting results of tests with volatile chemicals using closed systems.

2. DATA AND EVALUATION

The measured cell concentrations in the test cultures and controls are tabulated together with the concentrations of the test substance and the times of measurements. The mean value of the cell concentration for each test substance concentration and for the controls is plotted against time to produce growth curves.

To determine the concentration/effect relationship, the two following approaches should be used.

2.1. **Comparison of areas under the growth curves**

The area below the growth curves may be calculated according to the formula:

$$A = \frac{N_1 - N_0}{2} \times t_1 + \frac{N_1 + N_2 - 2N_0}{2} \times (t_2 - t_1) + \frac{N_{n-1} + N_n - 2N_0}{2} \times (t_n - t_{n-1})$$

where:

A = area,
N_0 = nominal number of cells/ml at time t_0,
N_1 = measured number of cells/ml at t_1,
N_n = measured number of cells/ml at time t_n,
t_1 = time of first measurement after beginning of test,
t_n = time of n^{th} measurement after beginning of test.

The percentage inhibition of the cell growth at each test substance concentration (I_A) is calculated as the difference between the area under the control growth curve (A_c) and the area under the growth curve at each test substance concentration (A_t) as:

$$I_A = \frac{A_c - A_t}{A_c} \times 100$$

I_A values are plotted on semilogarithmic paper or on semilogarithmic probit paper against the corresponding concentrations. If plotted on probit paper, the points are fitted by a straight line be eye, or, when a log-normal distribution of values can be assumed, a computed regression line may be drawn.

An EC_{50} value results from the intercept of the (regression) line with the parallel drawn to the abscissa at I_A = 50 %. To denote this value unambiguously in relation to this method of calculation it is proposed to use the symbol E_bC_{50}. In relation to this method which specifies measurements at 24, 48 and 72 hours, the symbol becomes E_bC_{50} (0—72 h).

Other EC values, like E_bC_{10}, can also be derived from the plot of I_A *versus* log concentration.

2.2. **Comparison of growth rates**

The average specific growth rate (μ) for exponentially growing cultures can be calculated as

$$\mu = \frac{\ln N_n - \ln N_1}{t_n - t_1}$$

Alternatively, the average specific growth rate may be derived from the slope of the regression line in a plot of ln N *versus* time.

The percentage reduction in average specific growth rate at each test substance concentration compared to the control value is plotted against the logarithm of the concentration. Their EC_{50} may be read from the resulting graph. To denote unambiguously the EC_{50} derived by this method it is proposed to use the symbol E_rC_{50}. The times of measurment must be indicated, e.g. if the value relates to observation times 24 and 48 hours, the symbol becomes E_rC_{50} (24—48 h).

Note: specific growth rate is a logarithmic term, and small changes in growth rate may lead to great changes in biomass. E_bC and E_rC values are therefore not numerically comparable.

3. **REPORTING**

The test report shall, if possible, contain the following:

— Test substance: chemical identification data,

— Test organisms: origin, laboratory culture, strain number, method of cultivation,

— Test conditions:
  — date of the start and the end of the test and its duration,
  — temperature,

— composition of medium,
— culturing apparatus,
— pH of solutions at the start and end of the test (an explanation should be provided if pH deviations of more than one unit are observed),
— vehicle and method used for solubilizing the test substance and concentration of the vehicle in the test solutions,
— light intensity and quality,
— concentrations tested (measured or nominal).

— Results:
— cell concentration for each flask at each measuring point and method for measuring cell concentration,
— mean values of cell concentrations,
— growth curves,
— graphical presentation of the concentration effect relationship,
— EC values and method of calculation,
— NOEC,
— other observed effects.

4. **REFERENCES**

(1) OECD, Paris, 1981, *Test Guideline 201,* Decision of the Council C(81) 30 Final.

(2) Umweltbundesamt, Berlin, 1984, Verfahrensvorschlag 'Hemmung der Zellvermehrung bei der Grünalge *Scenedesmus subspicatus',* in: Rudolph/Boje: *Ökotoxikologie,* ecomed, Landsberg, 1986.

Appendix

EXAMPLE OF A PROCEDURE FOR THE CULTURING OF ALGAE

General observations

The purpose of culturing on the basis of the following procedure is to obtain algal cultures for toxicity tests.

Suitable methods should be used to ensure that the algal cultures are not infected with bacteria (ISO 4833). Axenic cultures may be desirable but unialgal cultures are essential.

All operations may be carried out under sterile conditions in order to avoid contamination with bacteria and other algae.

Equipment and materials

See under 1.6.1: Preparations and experimental organisms.

Procedures for obtaining algal cultures

Preparation of nutrient solutions (media)

All nutrient salts of the medium are prepared as concentrated stock solutions and stored in the dark and cold. These solutions are sterilized by filtration or by autoclaving.

The medium is prepared by adding the correct amounts of stock solutions to sterile distilled water, taking care that no infections occur. For solid medium 0,8 % of agar is added.

Stock culture

The stock cultures are small algal cultures that are regularly transferred to fresh medium to act as initial test material. If the cultures are not used regularly they are streaked out on sloped agar tubes. These are transferred to fresh medium at least once every two months.

The stock cultures are grown in conical flasks containing the appropriate medium (volume about 100 ml). When the algae are incubated at 20 °C with continuous illumination, a weekly transfer is required.

During transfer an amount of 'old' culture is transferred with sterile pipettes into a flask of fresh medium, so that with the fast-growing species the initial concentration is about 100 times smaller than in the old culture.

The growth rate of a species can be determined from the growth curve. If this is known, it is possible to estimate the density at which the culture should be transferred to new medium. This must be done before the culture reaches the death phase.

Pre-culture

The pre-culture is intended to give an amount of algae suitable for the inoculation of test cultures. The pre-culture is incubated under the conditions of the test and used when still exponentially growing, normally after an incubation period of about three days. When the algal cultures contain deformed or abnormal cells, they must be discarded.

TOXICITY FOR EARTHWORMS

ARTIFICIAL SOIL TEST

1. METHOD

1.1. Introduction

In this laboratory test, the test substance is added to an artificial soil in which worms are placed for 14 days. After this period (and optionally after seven days) the lethal effect of the substance on the earthworms is examined. The test provides a method for relatively short-term screening of the effect of chemicals on earthworms, by dermal and alimentary uptake.

1.2. Definition and unit

LC_{50}: The concentration of a substance estimated as killing 50 % of the test animals during the test period.

1.3. Reference substance

A reference substance is used periodically as a means of demonstration that the sensitivity of the test system has not changed significantly.

Analytical grade chloroacetamide is recommended as the reference substance.

1.4. Principle of the test

Soil is a variable medium, so for this test a carefully defined artificial loam soil is used. Adult earthworms of the species *Eisenia foetida* (see note in Appendix) are kept in a defined artificial soil treated with different concentrations of the test substance. The content of the containers is spread on a tray 14 days (and optionally seven days) after the beginning of the test, and the earthworms surviving at each concentration counted.

1.5. Quality criteria

The test is designed to be as reproducible as possible with respect to the test substrate and organism. Mortality in the controls must not exceed 10 % at the end of the test, or the test is invalid.

1.6. Description of the test method

1.6.1. *Materials*

1.6.1.1. Test substrate

A defined artificial soil is used as a basic test substrate.

(a) Basic substrate (percentages are in terms of dry weight)

— 10 % sphagnum peat (as close to pH 5,5 to 6,0 as possible with no visible plant remains and finely ground),

— 20 % kaolinite clay with preferably more than 50 % kaolinite,

— About 69 % industrial quartz sand (dominant fine sand with more than 50 % of particle size 0,05 to 0,2 mm). If the substance is not sufficiently dispersible in water, 10 g per test container should be kept available for mixing with the test substance later on,

— About 1 % calcium carbonate ($CaCO_3$), pulverized, chemically pure, added to bring the pH to 6,0 ± 0,5.

(b) Test substrate

The test substrate contains the basic substrate, the test substance and deionized water.

Water content is about 25 to 42 % of the dry weight of the basic substrate. The water content of the substrate is determined by drying a sample to constant weight at 105 °C. The key criterion is that the artificial soil must be wetted to a point where there is no standing water. Care should be taken in mixing to obtain an even distribution of the test substance and the substrate. The way of introducing the test substance to the substrate has to be reported.

(c) Control substrate

The control substrate contains the basic substrate and water. If an additive agent is used, an additional control should contain the same quantity of the additive agent.

1.6.1.2. Test containers

Glass containers of about one litre capacity (adequately covered with plastic lids, dishes or plastic film with ventilation holes) filled with an amount of wet test or control substrate equivalent to 500 g dry weight of substrate.

1.6.2. *Test conditions*

Containers should be kept in climatic chambers at a temperature of 20 ± 2 °C with continuous light. Light intensity should be 400 to 800 lux.

The test period is 14 days, but mortality can be assessed optionally seven days after starting the test.

1.6.3. *Test procedure*

Test concentrations

Concentrations of the test substance are expressed as weight of substance per dry weight of basic substrate (mg/kg).

Range finding test

The range of concentrations just causing mortalities of 0 to 100 % may be determined in a range-finding test to provide information on the range of concentrations to be used in the definitive test.

The substance should be tested at the following concentrations: 1 000; 100; 10; 1; 0,1 mg substance/kilogram test substrate (dry weight).

If a full definitive test is to be carried out, one test batch per concentration and one for the untreated control, each with 10 worms, could be sufficient for the range-finding test.

Definitive test

The results of the range-finding test are used to choose at least five concentrations in a geometric series just spanning the range 0 to 100 % mortality and differing by a constant factor not exceeding 1,8.

Tests using these series of concentration should allow the LC_{50} value and its confidence limits to be estimated as precisely as possible.

In the definitive test at least four test batches per concentration and four untreated controls, each with 10 worms, are used. The results of these replicate batches are given as a mean and standard deviation.

When two consecutive concentrations, at a ratio of 1,8, give only 0 % and 100 % mortality, these two values are sufficient to indicate the range within which the LC_{50} falls.

Mixture of the basic test substrate and the test substance

The test substrate should, whenever possible, be made up without any additional agents other than water. Immediately before the start of the test, an emulsion or dispersion of the test substance in deionized water or other solvent is mixed with the basic test substrate, or sprayed evenly over it with a fine chromatographic or similar spray.

If insoluble in water, the test substance can be dissolved in as small a volume as possible of suitable organic solvent (e.g. hexane, acetone or chloroform).

Only agents which volatilize readily may be used to solubilize, disperse or emulsify the test substance. The test substrate must be ventilated before use. The amount of water evaporated must be replaced. The control should contain the same quantity of any additive agent.

If the test substance is not soluble, dispersible or emulsifiable in organic solvents, 10 g of a mixture of fine ground quartz sand and a quantity of test substance necessary to treat 500 g dry weight of artificial soil are mixed with 490 g of dry weight of test substrate.

For each test batch, an amount of wet test substrate equivalent to 500 g dry weight is placed in each glass container and 10 earthworms, which have been conditioned for 24 hours in a similar wet basic substrate and then washed quickly and surplus water absorbed on filter paper before use, are placed on the test substrate surface.

The containers are covered with perforated plastic lids, dishes or film to prevent the substrate drying and they are kept under the test conditions for 14 days.

The assessments should be made 14 days (and optionally seven days) after setting up the test. The substrate is spread on a plate made of glass or stainless steel. The earthworms are examined and the numbers of surviving earthworms determined. Earthworms are considered dead if they do not respond to a gentle mechanical stimulus to the front end.

When the examination is made at seven days, the container is refilled with the substrate and the surviving earthworms are replaced on the same test substrate surface.

1.6.4. *Test organisms*

Test organisms should be adult *Eisenia foetida* (see note in Appendix) (at least two months old with clitellum) wet weight 300 to 600 mg. (For breeding method see Appendix.)

2. DATA

2.1. Treatment and evaluation of results

The concentrations of the substance tested are reported with reference to the corresponding percentages of dead earthworms.

When the data are adequate the LC_{50} value and the confidence limits ($p = 0,05$) should be determined using standard methods (Litchfield and Wilcoxon, 1949, for equivalent method). The LC_{50} should be given as mg of test substance per kilogram of the test substrate (dry weight).

In those cases where the slope of the concentration curve is too steep to permit calculation of the LC_{50}, a graphical estimate of this value is sufficient.

When two consecutive concentrations at a ratio of 1,8 give only 0 % and 100 % mortality, the two values are sufficient to indicate the range within which the LC_{50} falls.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following:

— statement that the test has been carried out in accordance with the abovementioned quality criteria,

— test carried out (range finding test and/or definitive test),

— exact description of the test conditions or statement that the test has been carried out in accordance with the method; any deviations have to be reported,

— exact description of how the test substance has been mixed into the basic test substrate,

— information about test organisms (species, age, mean and range in weight, keeping and breeding conditions, supplier),

— method used for determination of LC_{50},

— test results including all data used,

— description of observed symptoms or changes in behaviour of test organisms,

— mortality in the controls,

— LC_{50} or highest tested concentration without mortality and lowest tested concentration with a mortality of 100 %, 14 days (and optionally seven days) after setting up the test,

— plotting of the concentration/response curve,

— results obtained with the reference substance, whether in association with the present test or from previous quality control exercises.

4. REFERENCES

(1) OECD, Paris, 1981, *Test Guideline 207,* Decision of the Council C(81) 30 final.

(2) Edwards, C. A. and Lofty, J. R., 1977, *Biology of Earthworms,* Chapman and Hall, London, 331 pp.

(3) Bouche, M. B., 1972, *Lombriciens de France, Écologie et Systématique,* Institut National de la Recherche Agronomique, 671 pp.

(4) Litchfield, J. T. and Wilcoxon, F., A simplified method of evaluation dose effect experiments. *J. Pharm. Exp. Therap.*, vol. 96, 1949, p. 99.

(5) Commission of the European Communities, *Development of a standardized laboratory method for assessing the toxicity of chemical substances to earthworms,* Report EUR 8714 EN, 1983.

(6) Umweltbundesamt/Biologische Bundesanstalt für Land- und Forstwirtschaft, Berlin, 1984, Verfahrensvorschlag 'Toxizitätstest am Regenwurm *Eisenia foetida* in künstlichem Boden', in: Rudolph/Boje, *Ökotoxikologie,* ecomed, Landsberg, 1986.

Appendix

Breeding and keeping of the worms before testing

For breeding the animals, 30 to 50 adult worms, are put in a breeding box with fresh substrate and removed after 14 days. These animals may be used for further breeding batches. The earthworms hatched from the cocoons are used for testing when mature (under the prescribed conditions after two to three months).

Keeping and breeding conditions

Climatic chamber: temperature 20 ± 2 °C preferably with continuous light (intensity 400 to 800 lux).

Breeding boxes: suitable shallow containers of 10 to 20 l volume.

Substrate: *Eisenia foetida* may be bred in various animal excrements. It is recommended to use as breeding medium a mixture of 50 % by volume peat and 50 % cow or horse dung. The medium should have a pH value of about 6 to 7 (regulated with calcium carbonate) and a low ionic conductivity (less than 6 mmhos or 0,5 % salt concentration).

The substrate should be moist but not too wet.

Other successful procedures may be used besides the method given above.

Note: Eisenia foetida exists in two races which some taxonomists have separated into species (Bouche, 1972). These are morphologically similar but one, *Eisenia foetida foetida,* has typically transverse striping or banding on the segments and the other, *Eisenia foetida andrei,* lacks this and has a variegated reddish colour. Where possible *Eisenia foetida andrei* should be used. Other species may be used if the necessary methodology is available.

BIODEGRADATION

ZAHN — WELLENS TEST

1. METHOD

1.1. Introduction

The purpose of the method is the evaluation of the potential ultimate biodegradability of water-soluble, non-volatile organic substances when exposed to relatively high concentrations of micro-organisms in a static test.

Physico-chemical adsorption on the suspended solids may take place and this must be taken into account when interpreting results (see 3.2).

The substances to be studied are used in concentrations corresponding to DOC-values in the range of 50 to 400 mg/litre or COD-values in the range of 100 to 1 000 mg/litre (DOC = dissolved organic carbon; COD = chemical oxygen demand). These relatively high concentrations have the advantage of analytical reliability. Compounds with toxic properties may delay or inhibit the degradation process.

In this method, the measure of the concentration of dissolved organic carbon or the chemical oxygen demand is used to assess the ultimate biodegradability of the test substance.

A simultaneous use of a specific analytical method may allow the assessment of the primary biodegradation of the substance (disappearance of the parent chemical structure).

The method is applicable only to those organic test substances which, at the concentration used in the test:

— are soluble in water under the test conditions,

— have negligible vapour pressure under the test conditions,

— are not inhibitory to bacteria,

— are adsorbed within the test system only to a limited extent,

— are not lost by foaming from the test solution.

Information on the relative proportions of the major components of the test material will be useful in interpreting the results obtained, particularly in those cases where the results are low or marginal.

Information on the toxicity of the substance to micro-organisms is desirable for the interpretation of low results and in the selection of appropriate test concentrations.

1.2. Definitions and units

The amount of degradation attained at the end of the test is reported as the 'Biodegradability in the Zahn — Wellens test':

$$D_T(\%) = \left[1 - \frac{(C_T - C_B)}{(C_A - C_{BA})}\right] \times 100$$

where:

D_T = biodegradation (%) at time T,

C_A = DOC (or COD) values in the test mixture measured three hours after the beginning of the test (mg/l) (DOC = Dissolved Organic Carbon, COD = Chemical Oxygen Demand),

C_T = DOC or COD values in the test mixture at time of sampling (mg/l),

C_B = DOC or COD value of the blank at time of sampling (mg/l),

C_{BA} = DOC or COD value of the blank, measured three hours after the beginning of the test (mg/l).

The extent of degradation is rounded to the nearest full percent.

Percentage degradation is stated as the percentage DOC (or COD) removal of the tested substance.

The difference between the measured value after three hours and the calculated or preferably measured initial value may provide useful information on the elimination of the substance (see 3.2, Interpretation of results).

1.3. **Reference substances**

In some cases when investigating new substances reference substances may be useful; however, specific reference substances cannot yet be recommended.

1.4. **Principle of the test method**

Activated sludge, mineral nutrients and the test material as the sole carbon source in an aqueous solution are placed together in a one to four litre glass vessel equipped with an agitator and an aerator. The mixture is agitated and aerated at 20 to 25 °C under diffuse illumination or in a dark room for up to 28 days. The degradation process is monitored by determination of the DOC (or COD) values in the filtered solution at daily or other appropriate regular time intervals. The ratio of eliminated DOC (or COD) after each interval to the value three hours after the start is expressed as percentage biodegradation and serves as the measure of the extent of degradation at this time. The result is plotted *versus* time to give the biodegradation curve.

When a specific analytical method is used, changes in the concentration of the parent molecule due to biodegradation can be measured (primary biodegradability).

1.5. **Quality criteria**

The reproducibility of this test has been proven to be satisfactory in a ring test.

The sensitivity of the method is largely determined by the variability of the blank and, to a lesser extent, by the precision of the determination of dissolved organic carbon and the level of test compound in the liquor.

1.6. **Description of the test procedure**

1.6.1. *Preparations*

1.6.1.1. Reagents

Test water: drinking water with an organic-carbon content < 5 mg/litre. The concentration of calcium and magnesium ions together must not exceed 2,7 mmole/litre; otherwise adequate dilution with deionized or distilled water is required.

Sulphuric acid, analytical reagent (A.R.):	50 g/l.
Sodium hydroxide solution A.R.:	40 g/l.
Mineral nutrient solution: dissolve in one litre deionized water:	
ammonium chloride, NH_4Cl, A.R.:	38,5 g,
sodium dihydrogenphosphate, $NaH_2PO_4.2H_2O$, A.R.:	33,4 g,
potassium dihydrogenphosphate, KH_2PO_4, A.R.:	8,5 g,
di-potassium mono-hydrogenphosphate, K_2HPO_4, A.R.:	21,75 g.

The mixture serves both as a nutrient and as buffering system.

1.6.1.2. Apparatus

Glass vessels with a volume of one to four litres (e.g. cylindrical vessels).

Agitator with a glass or metal stirrer on a suitable shaft (the stirrer should rotate about 5 to 10 cm above the bottom of the vessel). A magnetic stirrer with a 7 to 10 cm long rod can be used instead.

Glass tube of 2 to 4 mm inner diameter to introduce air. The opening of the tube should be about 1 cm above the bottom of the vessel.

Centrifuge (about 3 550 g).

pH-meter.

Dissolved-oxygen meter.

Paper filters.

Membrane filtration apparatus.

Membrane filters, pore size 0,45 µm. Membrane filters are suitable if it is assured that they neither release carbon nor absorb the substance in the filtration step.

Analytical equipment for determining organic carbon content and chemical oxygen demand.

1.6.1.3. Preparation of the inoculum

Activated sludge from a biological treatment plant is washed by (repeatedly) centrifuging or settling with test water (above).

The activated sludge must be in an appropriate condition. Such sludge is available from a properly working waste-water treatment plant. To get as many different species or strains of bacteria as possible, it may be preferred to mix inocula from different sources (e.g. different treatment plants, soil extracts, river waters, etc.). The mixture is to be treated as described above.

For checking the activity of the activated sludge see 'Functional control', below.

1.6.1.4. Preparation of the test solutions

To the test vessel add 500 ml of test water, 2,5 ml/litre mineral nutrient solution and activated sludge in an amount corresponding to 0,2 to 1,0 g/litre dry matter in the final mixture. Add sufficient stock solution of the substance to be tested so that a DOC concentration of 50 to 400 mg/litre results in the final mixture. The corresponding COD-values are 100 to 1 000 mg/litre. Make up with test water to a total volume of one to four litres. The total volume to be chosen is dependent on the number of samples to be taken for DOC or COD determinations and the volumes necessary for the analytical procedure.

Normally a volume of two litres can be regarded as satisfactory. At least one control vessel (blank) is set up to run in parallel with each test series; it contains only activated sludge and mineral nutrient solution made up with test water to the same total volume as in the test vessels.

1.6.2. *Performance of the test*

The test vessels are agitated with magnetic stirrers or screw propellers under diffuse illumination or in a dark room at 20 to 25 °C. Aeration is accomplished by compressed air cleaned by a cotton-wool strainer and a wash bottle if necessary. It must be ensured that the sludge does not settle and the oxygen concentration does not fall below 2 mg/litre.

The pH-value must be checked at regular intervals (e.g. daily) and adjusted to pH 7 to 8, if necessary.

Losses from evaporation are made up just before each sampling with deionized or distilled water in the required amounts. A good procedure is to mark the liquid level on the vessel before starting the test. New marks are made after each sampling (without aeration and stirring). The first samples are always taken three hours after the start of the test in order to detect adsorption of test material by the activated sludge.

The elimination of the test material is followed by DOC or COD determinations made daily or at some other regular interval. The samples from the test vessel and the blank are filtered through a carefully washed paper filter. The first 5 ml of test solution filtrate are discarded. Sludges difficult to filter may be removed previously by centrifugation for 10 minutes. DOC and COD determinations are made at least in duplicate. The test is run for up to 28 days.

Note: Samples remaining turbid are filtered through membrane filters. The membrane filters must not release or adsorb any organic material.

Functional control of activated sludge

A vessel containing a known substance should be run in parallel with each test series in order to check the functional capacity of the activated sludge. Diethyleneglycol has been found useful for this purpose.

Adaptation

If analyses are carried out at relatively short intervals (e.g. daily), adaptation can be clearly recognized from the degradation curve (see Figure 2). The test should therefore not be started immediately before the weekend.

If the adaptation occurs in the end of the period, the test can be prolonged until the degradation is finished.

Note: If a broader knowledge of the behaviour of the adapted sludge is needed, the same activated sludge is exposed once again to the same test material in accordance with the following procedure:

Switch of the agitator and the aerator and allow the activated sludge to settle. Draw off the supernatant liquid, fill up to two litres with test water, stir for 15 minutes and allow to settle again. After the supernatant liquid is drawn off again, use the remaining sludge to repeat the test with the same material in accordance with 1.6.1.4 and 1.6.2, above. The activated sludge can also be isolated by centrifuging instead of settling.

The adapted sludge may be mixed with fresh sludge to a concentration of 0,2 to 1 g dry weight/litre.

Analytical means

Normally samples are filtered through a carefully washed paper filter (for washing use deionized water).

Samples which remain turbid are filtered through membrane filters (0,45 μm).

The DOC concentration is determined in duplicate in the sample filtrates (the first 5 ml are discarded) by means of the TOC instrument. If the filtrate cannot be analysed on the same day, it must be stored in the refrigerator until the next day. Longer storage cannot be recommended.

The COD concentration is determined in the sample filtrates with a COD analytical set-up by the procedure described in reference (2), below.

2. DATA AND EVALUATION

DOC and/or COD concentrations are determined at least in duplicate in the samples according to 1.6.2, above. The degradation at time T is calculated according to the formula (with definitions) given unter 1.2, above.

The extent of degradation is rounded to the nearest full percent. The amount of degradation attained at the end of the test is reported as the 'Biodegradability in the Zahn — Wellens test'.

Note: If complete degradation is attained before the test time is over and this result is confirmed by a second analysis on the next day, the test can be concluded.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following:

— the initial concentration of the substance,

— all other information and the experimental results concerning the tested substance, the reference substance if used, and the blank,

— the concentration after three hours,

— biodegradation curve with description,

— date and location where test organisms were sampled, status of adaptation, concentration used, etc.,

— scientific reasons for any changes of test procedure.

3.2. Interpretation of results

Removal of DOC (COD) which takes place gradually over days or weeks indicates that the test substance is being biodegraded.

However, physico-chemical adsorption can, in some cases, play a role and this is indicated when there is complete or partial removal from the outset, within the first three hours, and the difference between control and test supernatant liquors remains at an unexpectedly low level.

Futher tests are necessary if a distinction is to be drawn between biodegradation (or partial biodegradation) and adsorption.

This can be done in a number of ways, but the most convincing is to use the supernatant or sludge as inoculum in a base-set test (preferably a respirometric test).

Test substances giving high, non-adsorptive removal of DOC (COD) in this test should be regarded as potentially biodegradable. Partial, non-adsorptive removal indicates that the chemical is at least subject to some biodegradation. Low, or zero removals of DOC (COD) may be due to inhibition of microorganisms by the test substance and this may also be revealed by lysis and loss of sludge, giving turbid supernatants. The test should be repeated using a lower concentration of test substance.

The use of a compound-specific analytical method or of ^{14}C-labelled test substance may allow greater sensitivity. In the case of ^{14}C test compound, the recovery of the $^{14}CO_2$ will confirm that biodegradation has occurred.

When results are given in terms of primary biodegradation, an explanation should, if possible, be given on the chemical structure change that leads to the loss of response of the parent test substance.

The validation of the analytical method must be given together with the response found on the blank test medium.

4. REFERENCES

(1) OECD, Paris, 1981, *Test Guideline 302 B,* Decision of the Council C(81) 30 final.

(2) Annex V C.9 Degradation: Chemical Oxygen Demand, Commission Directive 84/449/EEC, *Official Journal of the European Communities,* No L 251, 19. 9. 1984.

Appendix

EVALUATION EXAMPLE

Organic compound: 4-Ethoxybenzoic acid
Theoretical test concentration: 600 mg/l
Theoretical DOC: 390 mg/l

Inoculum Sewage Treatment plant of . . .
Concentration: 1 gram dry material/litre
Adaptation status: not adapted

Analysis: DOC-determination

Amount of sample: 3 ml

Control substance: Diethyleneglycol

Toxicity of compound: No toxic effects below 1 000 mg/l
Test used: Fermentation tubes test

Test time	Control substance				Test substance		
	Blank DOC (1) mg/l	DOC (1) mg/l	DOC net mg/l	Degradation %	DOC (1) mg/l	DOC net mg/l	Degradation %
0	—	—	300,0	—	—	390,0	—
3 hours	4,0	298,0	294,0	2	371,6	367,6	6
1 day	6,1	288,3	282,2	6	373,3	367,2	6
2 days	5,0	281,2	276,2	8	360,0	355,0	9
5 days	6,3	270,5	264,2	12	193,8	187,5	52
6 days	7,4	253,3	245,9	18	143,9	136,5	65
7 days	11,3	212,5	201,2	33	104,5	93,2	76
8 days	7,8	142,5	134,7	55	58,9	51,1	87
9 days	7,0	35,0	28,0	91	18,1	11,1	97
10 days	18,0	37,0	19,0	94	20,0	2,0	99

(1) Mean values of triplicate determinations.

Figure 1

Examples of biodegradation curves

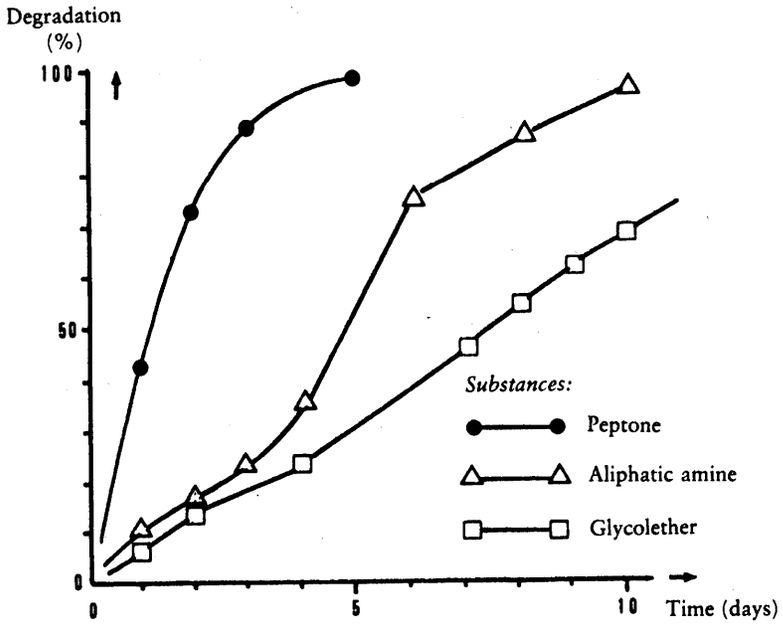
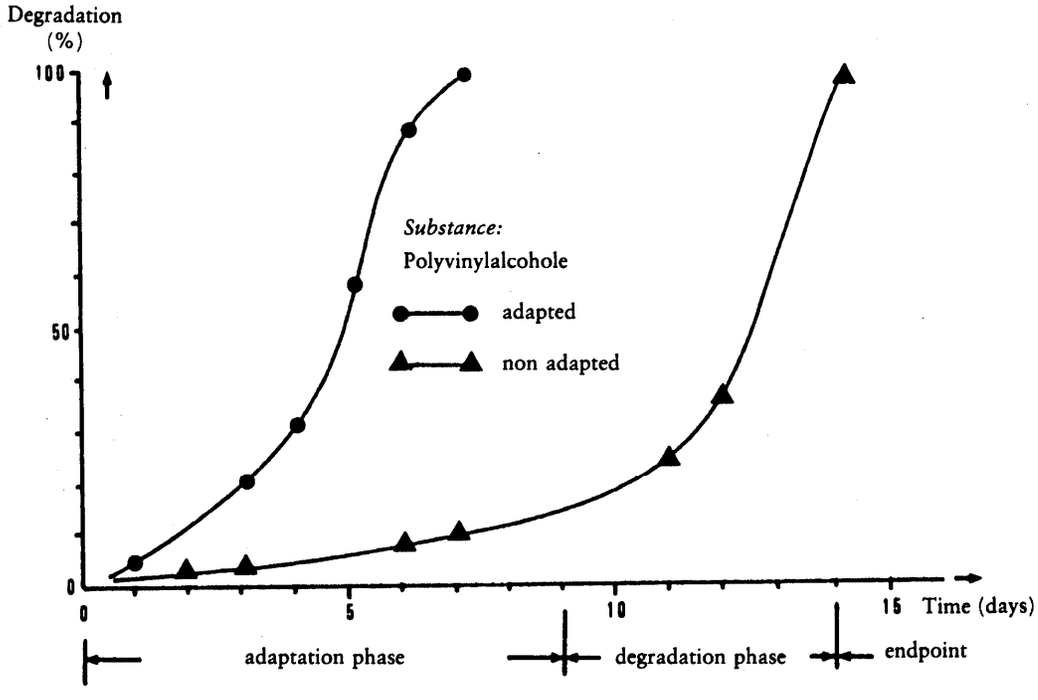


Figure 2

Examples of sludge adaptation



BIODEGRADATION

ACTIVATED SLUDGE SIMULATION TESTS

1. METHOD

1.1. Introduction

1.1.1. *General remarks*

The method is applicable only to those organic substances which, at the concentration used in the test:

— are soluble in water to the extent necessary for the preparation of the test solutions,

— have negligible vapour pressure under the test conditions,

— are not inhibitory to bacteria.

Information on the relative proportions of the major components of the test material will be useful in interpreting the results obtained, particularly in those cases where the results are low or marginal.

Information on the toxicity of the substance to micro-organisms is desirable for the interpretation of the low results and in the selection of appropriate test concentrations.

1.1.2. *Determination of ultimate biodegradability (DOC/COD analysis)*

The purpose of the method is to determine the ultimate biodegradability by the measurement of the removal of the substance and any metabolites in an activated sludge plant model at a concentration corresponding to > 12 mg DOC/litre (or approximately 40 mg COD/litre); 20 mg DOC/litre seem to be optimal. (DOC = Dissolved Organic Carbon; COD = Chemical Oxygen Demand).

The organic carbon content (or the chemical oxygen demand) of the test material must be established.

1.1.3. *Determination of primary biodegradability (specific analysis)*

The purpose of the method is the determination of the primary biodegradability of a substance in an activated sludge plant model, at a concentration of about 20 mg/litre, using a specific analytical method (lower or higher concentration can be used if analytical method and consideration of toxicity permits). This allows the assessment of the primary biodegradability of the substance (disappearance of the parent chemical structure).

The purpose of this method is *not* the determination of the mineralization of the tested substance.

An adequate analytical method for the determination of the tested substance must be available.

1.2. Definitions and units

1.2.1. *DOC/COD analysis*

The degree of removal of the substance is given by:

$$DR = \frac{T - (E - E_o)}{T} \times 100\% \qquad [1\ (a)]$$

where:

DR = degree of removal in percent DOC (or COD) within the given mean retention time with respect to the test material,

T = concentration of the test material in the influent in mg DOC/litre (or mg COD/litre),

E = DOC (or COD) concentration in the effluent of the test unit in mg DOC/litre (or mg COD/litre),

E_o = DOC (or COD) concentration in the effluent of the blank unit in mg DOC/litre (or mg COD/litre).

The degradation is stated as the percentage DOC (or COD) removal within the given retention time with respect to the test material.

1.2.2. *Specific analysis*

The percentage elimination of the tested substance from the aqueous phase (R_W) within the given mean retention time is given by

$$R_W = \frac{C_I - C_O}{C_I} \times 100\% \qquad [1\ (b)]$$

where:

C_I = concentration of the substance in the influent of the test unit (mg substance/litre, determined by specific analysis),

C_O = concentration of the substance in the effluent of the test unit (mg substance/litre, determined by specific analysis).

1.3. **Reference substances**

In some cases when investigating a new substance, reference substances may be useful; however, specific reference substances cannot yet be recommended.

1.4. **Principle of the test methods**

For the determination of ultimate biodegradability, two activated sludge pilot units (OECD confirmatory test or porous pot units) are run in parallel. The test substance is added to the influent (synthetic or domestic sewage) of one of the units, while the other receives the sewage alone. For the determination of primary biodegradation with specific analysis in the influent and effluent, only one unit is used.

The DOC (or COD) concentrations are measured in the effluents, or the substance concentrations are determined by specific analysis.

The DOC due to test material is not measured but simply stated.

When DOC (or COD) measurements are performed, the difference in mean concentrations between the test and the control effluents is assumed to be due to undegraded test material.

When specific analyses are preformed, change in the concentration of the parent molecule can be measured (primary biodegradation).

The units may be operated following the 'coupled units mode', by a transinoculation procedure.

1.5. **Quality criteria**

The starting concentration of the substance depends on the type of analysis performed and its limitation.

1.6. **Description of the test method**

1.6.1. *Preparation*

1.6.1.1. Apparatus

A pair of units of the same type are needed except when specific analyses are performed. Two types of device may be used:

OECD confirmatory test

The equipment (Appendix 1) consists of a storage vessel (A) for synthetic sewage, dosing pump (B), aeration vessel (C), separator (D), air-lift pump (E), to recycle activated sludge, and vessel (F) for collecting the treated effluent.

Vessels (A) and (F) must be of glass or suitable plastic and hold at least 24 litres. Pump (B) must provide a constant flow of synthetic sewage to the aeration vessel; any suitable system may be used, providing that input flow and concentration are assured. During normal operation the height of separator (D) is so fixed that the volume

contained in the aeration vessel is three litres of mixed liquor. A sintered aeration cube (G) is suspended in vessel (C) at the apex of the cone. The quantity of air blown through the aerator may be monitored by means of a flow meter.

Air-lift pump (E) is set so that the activated sludge from the separator is continually and regularly recycled to aeration vessel (C).

'Porous pot'

The porous pot is constructed from sheets of porous polyethylene (2 mm thick, maximum pore size 95 µm), which are made into cylinders 14 cm in diameter with a conical base at 45° (Figures 1 and 2 of Appendix 2). The porous pot is contained in an impervious vessel of suitable plastic 15 cm in diameter with an outlet at a height of 17,2 cm on the cylindrical part, which determines the volume (3 litres) in the pot. There is a rigid supporting ring made of suitable plastic around the top of the inner vessel, so that there is an effluent space of 0,5 cm between the inner and outer vessels.

The porous pots may be mounted in the base of a thermostatically controlled water-bath. There is an air supply to the base of the inner vessel on which are placed suitable diffusers.

Vessels (A) and (E) must be of glass or suitable plastic and hold at least 24 litres. Pump (B) must provide a constant flow of synthetic sewage to the aeration vessel; any suitable system may be used, providing that input flow and concentration are assured.

Spare inner porous pots are required to replace any which may block in use; blocked pots are cleaned by 24-hour immersion in hypochlorite solution followed by thorough washing in tap water.

1.6.1.2. Filtration

Membrane filtration apparatus and membrane filters with a pore size of 0,45 µm. Membrane filters are suitable if it is assured that they neither release carbon nor adsorb the substance in the filtration step.

1.6.1.3. Sewage

Either suitable synthetic feed or domestic sewage may be used.

Example of synthetic feed

Dissolve in each litre of tap water:

Peptone:	160 mg,
Meat extract:	110 mg,
Urea:	30 mg,
NaCl:	7 mg,
$CaCl_2 . 2H_2O$:	4 mg,
$MgSO_4 . 7H_2O$:	2 mg,
K_2HPO_4:	28 mg.

Domestic sewage

This should be collected freshly each day from the overflow of the primary settlement tank of a treatment plant treating predominantly domestic sewage.

1.6.1.4. Stock solution of test material

A solution of test material, e.g. 1%, should be prepared for addition to the test unit. The concentration of the material must be determined, so that the appropriate volume to be added to the sewage or directly to the unit via a second pump to give the required test concentration is known.

1.6.1.5. Inoculum

Remark: When domestic sewage is used, there would be no point in using an inoculum of low bacterial concentration, but activated sludge may be used.

A variety of inocula may be used.

Three examples of suitable inoculum are given:

(a) Inoculum from secondary effluent

The inoculum should be obtained from a secondary effluent of good quality collected from a treatment plant dealing with predominantly domestic sewage. The effluent must be kept under aerobic conditions in the period between sampling and use. To prepare the inoculum, the sample is filtered through a coarse filter, the first 200 ml being discarded. The filtrate is kept aerobic until used. The inoculum must be used on the day of collection. At least 3 ml are to be used for inoculation.

(b) Composite inoculum

Inoculum from secondary effluent:

See description above.

Inoculum from soil:

100 g of garden soil (fertile, not sterile) are suspended in 1 000 ml chlorine-free drinking water. (Soils with an extremely large fraction of clay, sand or humus are unsuitable.) After stirring, the suspension is allowed to settle for 30 minutes. The supernatant is filtered through a coarse filter paper, the first 200 ml being discarded. The filtrate is aerated immediately and until use. The inoculum must be used on the day of collection.

Inoculum from a surface water:

A further partial inoculum is drawn from a mesosaprobic surface water. The sample is filtered through a coarse paper, the first 200 ml being discarded. The filtrate is kept aerobic until used. The inoculum must be used on the day of collection.

Equal volumes of the three partial inoculum samples are united, mixed well, and the final inoculum drawn from this mixture. At least 3 ml are to be used for inoculation.

(c) Inoculum from activated sludge

A volume (not more than 3 litres) of activated sludge (suspended solids content of up to 2,5 g/litre) taken from the aeration tank of a plant treating predominantly domestic sewage may be used as an inoculum.

1.6.2. *Procedure*

The test is performed at room temperature; this should be kept between 18 and 25 °C.

If it is appropriate, the test may be performed at a lower temperature (down to 10 °C); if the substance is degraded then no further work is normally required. If, however, the substance is not degraded, the test must be conducted at a steady temperature between 18 and 25 °C.

1.6.2.1. Running-in period: Sludge formation/stabilization of the units

The sludge growth/stabilization period is the period during which the concentration of the activated sludge suspended solids and the performance of the units progress to a steady state under the operating conditions used.

The running-in period is the period which lasts from the time the test substance is first added to the time when its removal reaches a plateau (relatively constant value). This period must not exceed six weeks.

The evaluation period is a three weeks period, three weeks from the time that the removal of the test substance reaches a relatively constant, and ususally high, value. For those substances which show little or no degradation in the first six weeks, the evaluation period is taken as the following three weeks.

Initially, fill the unit(s) needed for one test with the inoculum mixed with influent.

The aerator (and air lift (E) in the case of the OECD confirmatory test units) and dosing device (B) are then set in operation.

Influent without substance to be tested must pass through the aeration vessel (C) either at the rate of one litre per hour or a rate of one-half litre per hour; this gives a mean retention time of either three or six hours.

The rate of aeration should be regulated so that the content of vessel (C) is kept constantly in suspension while the dissolved oxygen content is at least 2 mg/litre.

Foaming must be prevented by appropriate means. Anti-foaming agents which inhibit the activated sludge must not be used.

The sludge which has accumulated around the top of the aeration vessel (C) (and, in the case of the OECD confirmatory test units, in the base of the settling vessel (D), and in the circulation circuit) must be returned to the mixed liquor at least once each day by brushing or some other appropriate means.

When sludge fails to settle, its density may be increased by addition of 2 ml portions of a 5 % solution of ferric chloride, repeated as necessary.

The effluent is collected in vessel (E or F) for 20 to 24 hours, and a sample is taken after thorough mixing. Vessel (E or F) must be carefully cleaned.

In order to monitor and control the efficiency of the process, the chemical oxygen demand (COD) or the dissolved organic carbon (DOC) of the filtrate of the accumulated effluent is measured at least twice weekly, as well as that of the filtered influent (using a membrane of pore size 0,45 μm, the first 20 ml (approximately) of the filtrate are discarded).

The reduction in COD or DOC should level off when a roughly regular daily degradation is obtained.

The dry matter content of the activated sludge in the aeration tank should be determined twice a week (in g/litre). The units may be operated in one of two ways: either the content of dry matter in the activated sludge should be determined twice a week, and, if it is more than 2,5 g/litre, the excess activated sludge must be discarded, or 500 ml of mixed liquor is wasted from each pot daily to give a mean sludge retention time of six days.

When the measured and estimated parameters (efficiency of the process (in COD or DOC removal), sludge concentration, sludge settleability, turbidity of the effluents, etc.) of the two units are sufficiently steady, the test substance may be introduced in the influent of one of the units, following 1.6.2.2.

Alternatively, the test substance may be added at the beginning of the sludge growth period (1.6.2.1), especially when sludge is added as the inoculum.

1.6.2.2. Test procedure

The operating conditions of the running-in period are maintained and sufficient stock solution (approximately 1 %) of the test material is added to the influent of the test unit so that the desired concentration of test material (approximately 10 to 20 mg DOC/litre or 40 mg COD/litre) in the sewage is obtained. This can be done by mixing the stock solution to the sewage daily or by means of a separate pumping system. This concentration may be reached progressively. If there are no toxic effects of the test substance on the activated sludge, higher concentrations can also be tested.

The blank unit is fed only with influent without added substances. Adequate volumes of the effluents are taken for analysis and filtered through membrane filters (0,45 μm) the first 20 ml (approximately) of filtrate being discarded.

The filtered samples have to be analysed on the same day, otherwise they must be preserved by any suitable method, for example, by using 0,05 ml of a 1 % mercuric chloride ($HgCl_2$) solution for each 10 ml of filtrate or by storing them at 2 to 4 °C up to 24 hours, or below – 18 °C for longer periods.

The running-in time, with addition of test substance, should not exceed six weeks and the evaluation period should not be shorter than three weeks, i.e. about 14 to 20 determinations should be available for calculation of the final result.

Coupled units mode

The coupling of the units is achieved by interchanging 1,5 litres of mixed liquor (including sludge) from the activated sludge aeration vessels between the two units once a day. In the case of strongly absorbing test materials, 1,5 litres of supernatant liquid only are drawn from the settling vessels and poured into the activated sludge vessel of the other unit.

1.6.2.3. Analysis

Two kinds of analyses may be performed in order to follow the behaviour of the substance:

DOC and COD

The DOC concentrations are performed in duplicate with the carbon analyser and/or the COD values according to reference (2).

Specific analysis

The concentrations of the tested substance are determined by a suitable analytical method. When possible, specific determination of the substance absorbed on sludge should be performed.

2. DATA AND EVALUATION

2.1. Coupled units mode

When using 'coupled units mode', the daily degrees of removal, DR are calculated according to 1.2.1.

These daily degrees of removal DR are corrected to DRc for the material transfer due to the transinoculation procedure with equation [2] for a three-hour or equation [3] for a six-hour mean retention time.

$$DRc = \frac{8}{7} DR - \frac{100}{7} \qquad [2]$$

$$DRc = \frac{4}{3} DR - \frac{100}{3} \qquad [3]$$

The mean of the series of DRc values is calculated and in addition the standard deviation according to equation [4]

$$s_{DRc} = \sqrt{\frac{\sum_{i=1}^{n} (\overline{DRc} - DRc_i)^2}{n - 1}} \qquad [4]$$

where:

s_{DRc} = standard deviation of the series of DRc values,

$\overline{DRc}$ = mean of DRc value,

n = number of determinations.

Outliers of the DRc series are eliminated according to a suitable statistical procedure, e.g. Nalimov (6), at the 95 % probability level and the mean and the standard deviation of the outlier-free DRc data set are recalculated.

The final result is then calculated with equation [5] as

$$DRc = \overline{DRc} \pm \frac{t_{n-1};\alpha \; s_{DRc}}{\sqrt{n}} \qquad [5]$$

where:

$t_{n-1};\alpha$ = table value of t for n value pairs of E and E_O and statistical confidence P (P = 1 – α) whereby P is at 95 % (1).

The result is stated as the mean with tolerance limits at the 95 % probability level, the respective standard deviation and the number of data of the outlier-free DRc data set, and the number of outliers, e.g.

DRc = 98,6 ± 2,3 % DOC removal,

s = 4,65 % DOC removal,

n = 18,

x = number of outliers.

2.2. **Non-coupled units mode**

The performance of the units may be checked as follows:

$$\text{percentage removal of COD or DOC} = \frac{\text{COD or DOC of sewage — COD or DOC of effluent}}{\text{COD or DOC sewage}} \times 100$$

These daily removals may be plotted graphically to reveal any trends, e.g. to acclimatization.

2.2.1. *Using COD/DOC determinations*

The daily degree of removal DR is calculated according to 1.2.1.

The mean of the series of DR values is calculated; in addition, its standard deviation is calculated according to:

$$s_{DR} = \sqrt{\frac{\sum_{i=1}^{n} (\overline{DR} - DR_i)^2}{n - 1}} \qquad [6]$$

where:

s_{DR} = standard deviation of the series of DR_i values,

$\overline{DR}$ = mean of DR_i values,

n = number of determinations.

Outliers of the DR series are eliminated according to a suitable statistical procedure, e.g. Nalimov (6), at the 95 % probability level, and the mean and the standard deviation of the outliers-free DR set are recalculated.

The final result is then calculated with equation [7] as

$$DR = \overline{DR} \pm \frac{t_{n-1;\alpha}}{\sqrt{n}} s_{DR} \qquad [7]$$

where:

$t_{n-1;\alpha}$ = table value of t for n value pairs of E and E_O and statistical confidence P ($P = 1 - \alpha$) whereby P is set at 95 % (1).

The result is stated as the mean with tolerance limits at the 95 % probability level, the respective standard deviation and the number of data of the outlier free DR data set, and the number of outliers, e.g.

DR = (98,6 ± 2,3) % DOC removal,

s = 4,65 % DOC removal,

n = 18,

x = number of outliers.

2.2.2. *Using specific analysis*

The percentage of elimination of the tested substance from the aqueous phase (R_W) is calculated according to 1.2.2.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following:

— the formsheet given in Appendix 3, showing the operating conditions for the test,

— which apparatus was chosen (OECD confirmatory test or porous pot),

— which operating mode was chosen: coupled units mode or not,

— which sewage: synthetic or domestic — in the case of domestic sewage, date and location of sample,

— which inoculum, with date and location of sample,

— a statement with description of the analytical method if specific analyses were performed,

— plot of COD or DOC removal *versus* time, including running-in and evaluation period,

— analytical recovery of the test substance as COD of DOC in the stock solution,

— if specific analyes were performed, plot of the percentage removal of the tested substance from the aqueous phase *versus* time (running-in and evaluation period),

— the mean removal of DOC or COD of test substance and standard deviation are calculated from the results of the evaluation period, i.e. when there is a steady removal of test material or period of steady operation,

— plot of activated sludge concentration *versus* time,

— any remark concerning the activated sludge (discard of excess sludge, presence of bulking, $FeCl_3$, etc.),

— concentration of the substance used in the test,

— any results concerning analysis done on the sludge,

— all information and experimental results concerning the test substance and the reference substance if used,

— scientific reasons for any changes of the procedure.

3.2. **Interpretation of results**

Low removal of the tested substance from the aqueous phase may be due to inhibition of micro-organisms by the test substance. This may also be revealed by lysis and loss of sludge, giving a turbid supernatant, and by a decrease of the COD (or DOC) removal efficiency of the pilot plant.

Physico-chemical adsorption can sometimes play a role. Differences between biological action on the molecule and phyisico-chemical adsorption may be revealed by analysis performed on the sludge after an adequate desorption.

Further tests are necessary if a distinction is to be drawn between biodegradation (or partial biogradation) and adsorption.

This can be done in a number of ways, but the most convincing is to use the supernatant as inoculum in a base-set test (respirometric test preferably).

If high DOC or COD removals are observed, then this is due to biodegradation while, at low removals, biodegradation is indistinguishable from elimination. For example, if a soluble compound exhibits a high adsorption constant of 98 % and the surplus sludge wastage rate is 10 % per day, an elimination of up to 40 % is possible; at a surplus sludge wastage rate of 30 % elimination due to adsorption on and removal with surplus sludge may amount to up to 65 % (4).

When using specific analysis, attention should be paid to the relationship between the structure of the substance and the specific analysis used. In this case, the phenomenon observed cannot be interpreted as a mineralization of the substance.

4. **REFERENCES**

(1) OECD, Paris, 1981, *Test Guideline 303 A,* Decision of the Council C(81) 30 final.

(2) Annex V C9 Degradation Test — Chemical Oxygen Demand, Commission Directive 84/449/EEC, *Official Journal of the European Communities,* No L 251, 19. 9. 1984.

(3) Painter, H. A., King, E. F., *WRC Porous-Pot method for assessing biodegradability,* Technical Report TR70, June 1978, Water Research Centre, United Kingdom.

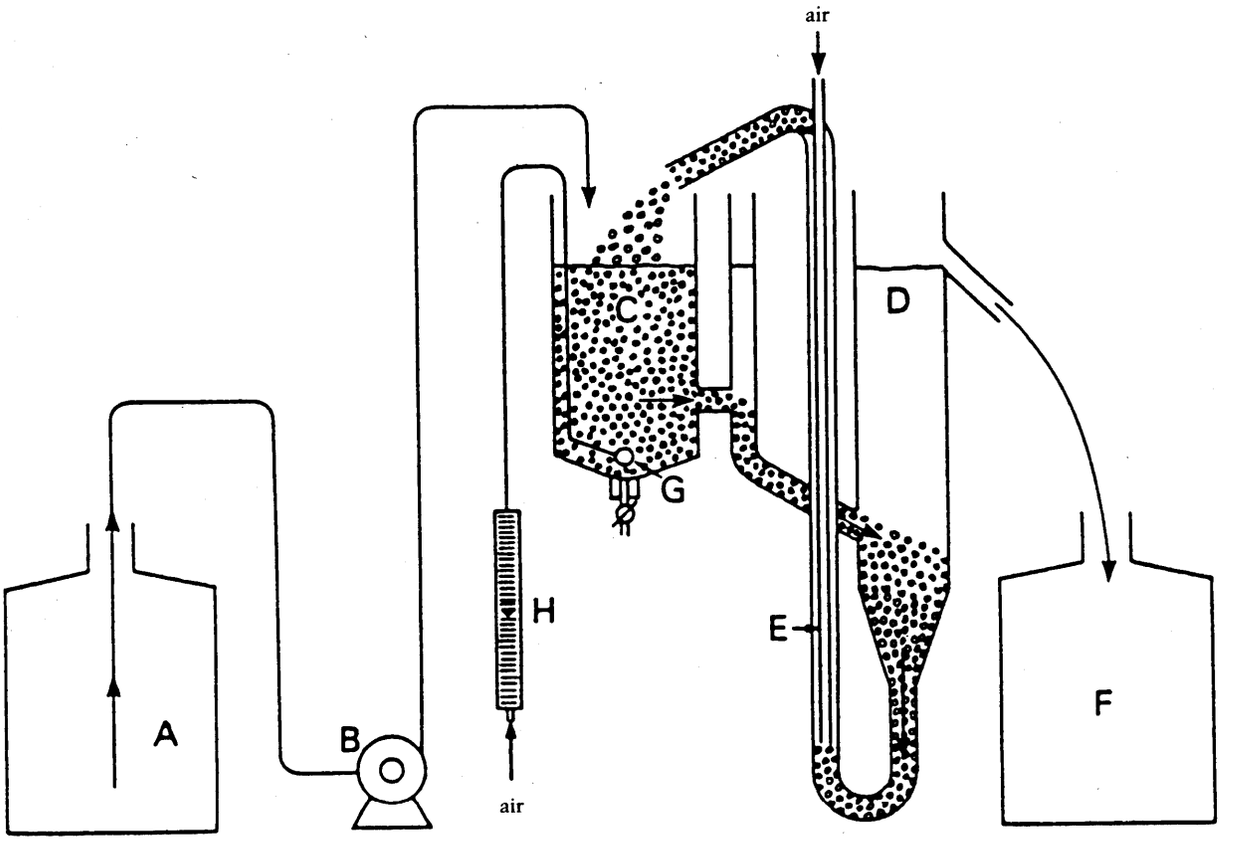
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(5) Council Directives 82/242/EEC and 82/243/EEC, *Official Journal of the European Communities,* No L 109, 22. 4. 1982, amending Council Directives 73/404/EEC and 73/405/EEC on biodegradability of detergents, *Official Journal of the European Communities,* No L 347, 17. 12. 1973.

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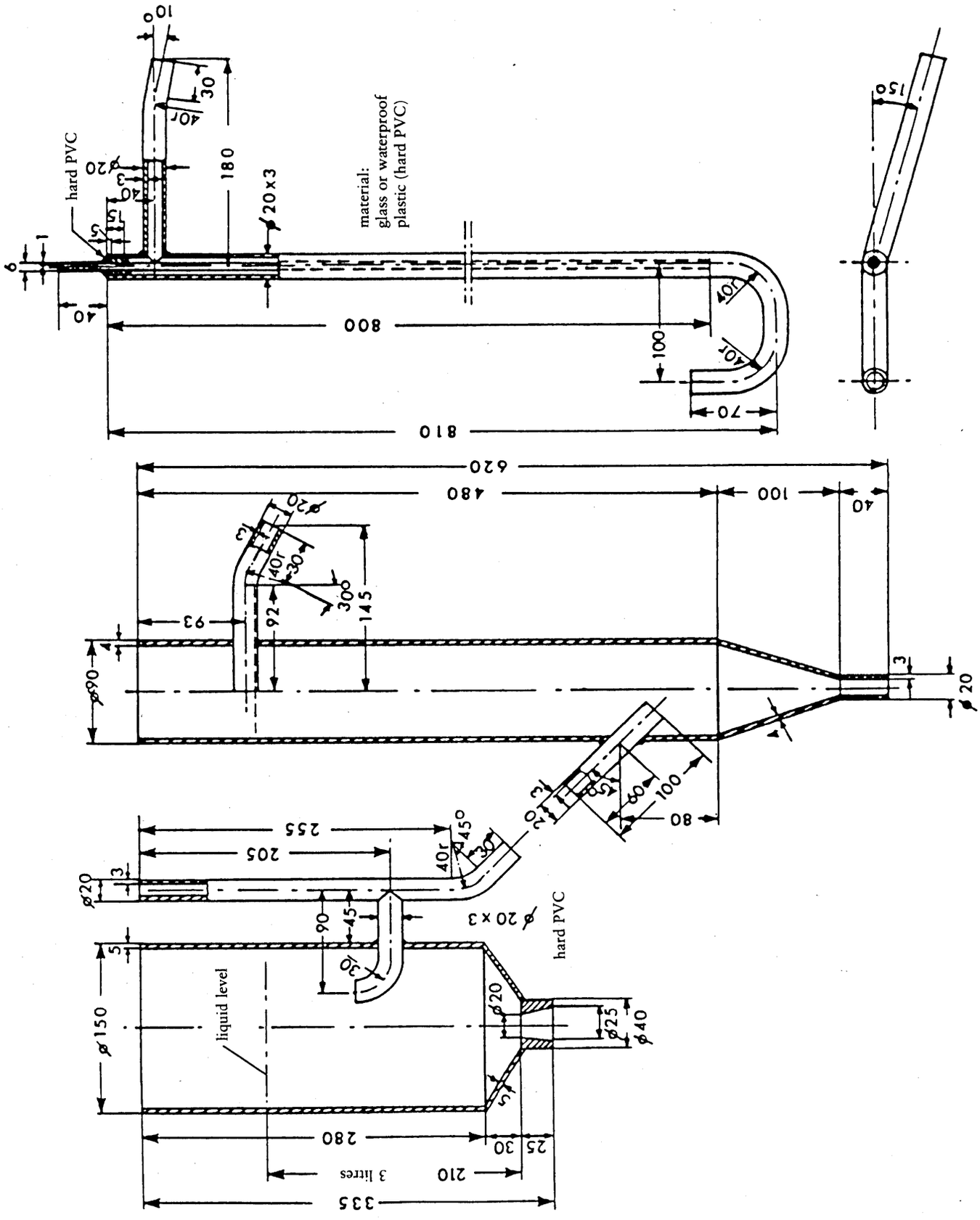
APPENDIX 1

Figure 1



Key: A = storage vessel;
B = dosing device;
C = aeration chamber (3 l capacity);
D = settling vessel;
E = air lift;
F = collector;
G = aerator;
H = air flow meter (optional).

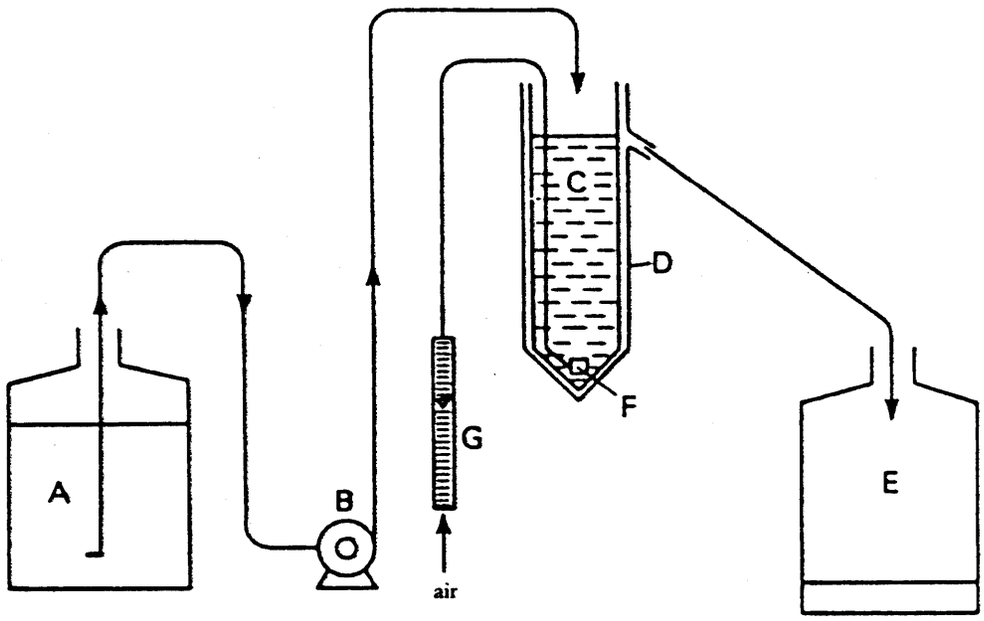
Figure 2


material:
glass or waterproof
plastic (hard PVC)
hard PVC
liquid level
3 litres

APPENDIX 2

Figure 1

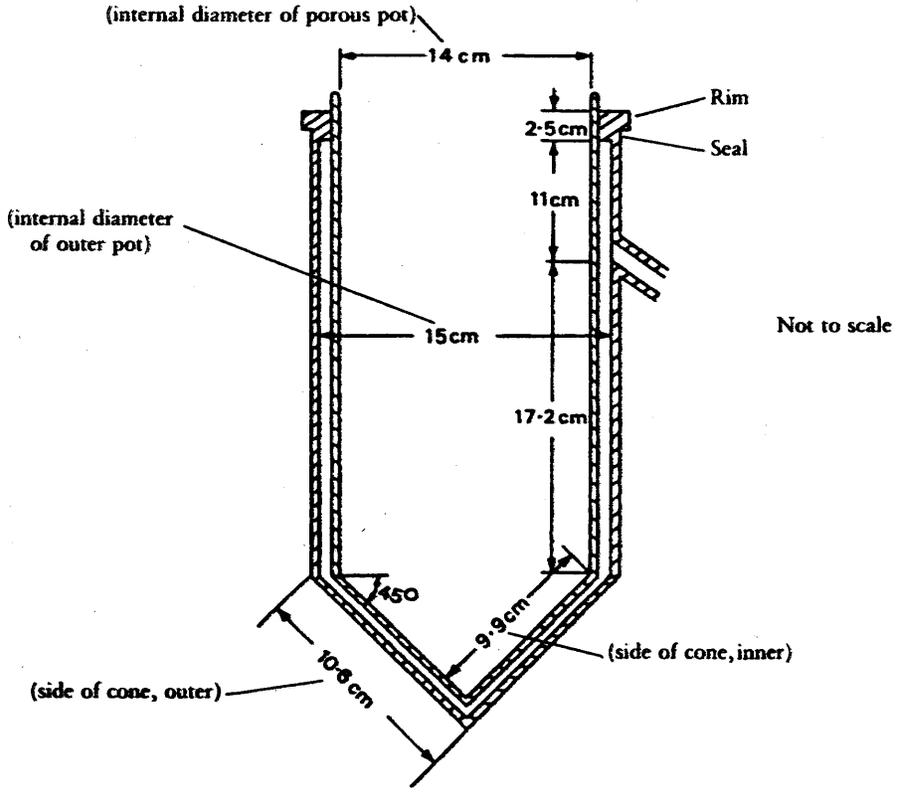
Equipment used for assessing biodegradability



Key: A = storage vessel;
B = dosing pump;
C = porous aeration vessel;
D = outer impermeable vessel;
E = effluent collection vessel;
F = diffuser-stone aerator;
G = rotameter (optional).

Figure 2

Details of three-litre porous-pot aeration vessel



Not to scale

Appendix 3

Operating conditions for the Activated Sludge Simulation Test

Check in each group

Apparatus

OECD confirmatory ☐
Porous pot ☐

Mode of operation

Single unit ☐
Coupled units ☐
Non-coupled units ☐

Transinoculation

None ☐
Activated sludge ☐
Supernatant ☐

Mean retention time

Three hours ☐
Six hours ☐

Base nutrient

Domestic sewage ☐
Synthetic sewage ☐

Inoculum

Secondary effluent ☐
Composite ☐
Activated sludge ☐

Test material addition

From the start ☐
Stepwise increase ☐
After sludge has formed ☐

Analysis

Specific ☐
COD ☐
DOC ☐

BIODEGRADATION

ACTIVATED SLUDGE RESPIRATION INHIBITION TEST

1. METHOD

1.1. Introduction

The method described assesses the effect of a test substance on micro-organisms by measuring the respiration rate under defined conditions in the presence of different concentrations of the test substance.

The purpose of this method is to provide a rapid screening method whereby substances which may adversely affect aerobic microbial treatment plants can be identified, and to indicate suitable non-inhibitory concentrations of test substances to be used in biodegradability tests.

A range-finding test may precede a definitive test. It provides information about the range of concentrations to be used in the main test.

Two controls without test substance are included in the test design, one at the start and the other at the end of the test series. Each batch of activated sludge should also be checked using a reference substance.

This method is most readily applied to substances which, due to their water solubility and low volatility, are likely to remain in water.

For substances with limited solubility in the test media, it may not be possible to determine the EC_{50}.

Results based on oxygen uptake may lead to erroneous conclusions when the test substance has the propensity to uncouple oxidative phosphorylation.

It is useful to have the following information to perform the test:

— water solubility,

— vapour pressure,

— structural formula,

— purity of the test substance.

Recommendation

Activated sludge may contain potentially pathogenic organisms and should be handled with care.

1.2. Definitions and units

The respiration rate is the oxygen consumption of waste-water micro-organisms in aerobic sludge, expressed generally as mg O_2 per mg of sludge per hour.

In order to calculate the inhibitory effect of a test substance at a particular concentration, the respiration rate is expressed as a percentage of the mean of the two control respiration rates:

$$\left(1 - \frac{2R_s}{Rc_1 + Rc_2}\right) \times 100 = \text{per cent inhibition}$$

where:

R_s = oxygen-consumption rate at tested concentration of test substance,

Rc_1 = oxygen-consumption rate, control 1,

Rc_2 = oxygen-consumption rate, control 2.

EC_{50} in this method is the concentration of the test substance at which the respiration rate is 50 % of that shown by the control under conditions described in this method.

1.3. **Reference substances**

It is recommended that 3,5-dichlorophenol, as a known inhibitor of respiration, be used as a reference substance and tested for EC_{50} on each batch of activated sludge as a means of checking that the sensitivity of the sludge is not abnormal.

1.4. **Principle of the test method**

The respiration rate of an activated sludge fed with a standard amount of synthetic sewage feed is measured after a contact time of 30 minutes or three hours, or both. The respiration rate of the same activated sludge in the presence of various concentrations of the test substance under otherwise identical conditions is also measured. The inhibitory effect of the test substance at a particular concentration is expressed as a percentage of the mean respiration rates of two controls. An EC_{50} value is calculated from determinations at different concentrations.

1.5. **Quality criteria**

The test results are valid if:

— the two control respiration rates are within 15 % of each other,

— the EC_{50} (30 minutes and/or three hours) of 3,5-dichlorophenol is in the accepted range 5 to 30 mg/litre.

1.6. **Description of the test method**

1.6.1. *Reagents*

1.6.1.1. Solutions of the test substance

Solutions of the test substance are freshly prepared at the start of the study using a stock solution. A stock solution concentration of 0,5 g/litre is appropriate if the procedure recommended below is followed.

1.6.1.2. Solution of control substance

A solution of 3,5-dichlorophenol can for example be prepared by dissolving 0,5 g 3,5-dichlorophenol in 10 ml of 1M NaOH, diluting to approximately 30 ml with distilled water, adding under stirring 0,5M H_2SO_4 to the point of incipient precipitation — approximately 8 ml of 0,5M H_2SO_4 will be required — and finally diluting the mixture to one litre with distilled water. The pH should then be in the range 7 to 8.

1.6.1.3. Synthetic sewage

A synthetic sewage feed is made by dissolving the following amounts of substances in one litre of water:

— 16 g peptone,
— 11 g meat extract,
— 3 g urea,
— 0,7 g NaCl,
— 0,4 g $CaCl_2.2H_2O$,
— 0,2 g $MgSO_4.7H_2O$,
— 2,8 g K_2HPO_4.

Note 1: This synthetic sewage is a 100-fold concentrate of that described in the OECD Technical Report 'Proposed method for the determination of the biodegradability of surfactants used in synthetic detergents' (June 11, 1976), with the addition of dipotassium hydrogen phosphate.

Note 2: If the prepared medium is not used immediately, it shall be stored in the dark at 0 to 4 °C, for no longer than one week, in conditions which do not produce any change in its composition. The medium may also be sterilized prior to storage, or the peptone and meat extract may be added shortly before carrying out the test. Before use, it shall be mixed thoroughly and the pH adjusted.

1.6.2. *Apparatus*

Measuring apparatus: The precise design is not critical. However, there should be head space and the probe should fit tightly in the neck of the measuring flask.

Normal laboratory equipment and especially the following is necessary:

— measuring apparatus,

— aeration device,

— pH-electrode and measuring equipment,

— O_2-electrode.

1.6.3. *Preparation of the inoculum*

Activated sludge from a sewage treatment plant treating predominantly domestic sewage is used as the mircobial inoculum for the test.

If necessary, on return to the larboratory, coarse particles may be removed by settling for a short period, e.g. 15 minutes, and decanting the upper layer of finer solids for use. Alternatively, the sludge may be mixed using a blender for a few seconds.

In addition, if it is thought that inhibitory material is present, the sludge should be washed with tap water or an isotonic solution. After centrifuging, the supernatant is decanted (this procedure is repeated three times).

A small amount of the sludge is weighed and dried. From this result, the amount of wet sludge can be calculated which must be suspended in water in order to obtain an activated sludge with a mixed liquor suspended solids range between 2 and 4 g/litre. This level gives a concentration between 0,8 and 1,6 g/litre in the test medium if the procedure recommended below is followed.

If the sludge cannot be used on the day of collection, 50 ml of synthetic sewage is added to each litre of the activated sludge prepared as described above; this is then aerated overnight at 20 ± 2 °C. It is then kept aerated for use during the day. Before use the pH is checked and adjusted, if necessary, to pH 6 to 8. The mixed liquor suspended solids should be determined as described in the preceding paragraph.

If the same batch of sludge is required to be used on subsequent days (maximum four days), a further 50 ml of synthetic sewage feed is added per litre of sludge at the end of each working day.

1.6.4. *Performance of the test*

Duration/contact time:	30 minutes and/or three hours, during which aeration takes place
Water:	Drinking water (dechlorinated if necessary)
Air supply:	Clean, oil-free air. Air flow 0,5 to 1 litre/minute
Measuring apparatus:	Flat bottom flask such as a BOD-flask
Oxygen meter:	Suitable oxygen electrode, with a recorder
Nutrient solution:	Synthetic sewage (see above)
Test substance:	The test solution is freshly prepared at the start of the test
Reference substance:	e.g. 3,5-dichlorophenol (at least three concentrations)
Controls:	Inoculated sample without test substance
Temperature:	20 ± 2 °C.

A suggested experimental procedure which may be followed for both the test and reference substance for the three-hour contact period is given below:

Several vessels (e.g. one-litre beakers) are used.

At least five concentrations, spaced by a constant factor preferably not exceeding 3,2, should be used.

At time '0', 16 ml of the synthetic sewage feed are made up to 300 ml with water. 200 ml of microbial inoculum are added and the total mixture (500 ml) poured into a first vessel (first control C_1).

The test vessels should be aerated continuously such as to ensure that the dissolved O_2 does not fall below 2,5 mg/litre and that, immediately before the measurement of the respiration rate, the O_2 concentration is about 6,5 mg/litre.

At time '15 minutes' (15 minutes is an arbitrary, but convenient, interval) the above is repeated, except that 100 ml of the test substance stock solution are added to the 16 ml of synthetic sewage before adding water to 300 ml and microbial inoculum to make a volume of 500 ml. This mixture is then poured into a second vessel and aerated as above. This process is repeated at 15-minute intervals with different volumes of the test substance stock solution to give a series of vessels containing different concentrations of the test substance. Finally, a second control is prepared (C_2).

After three hours the pH is recorded, and a well-mixed sample of the contents of the first vessel is poured into the measuring apparatus and the respiration rate is measured over a period of up to 10 minutes.

This determination is repeated on the contents of each vessel at 15-minute intervals, in such a way that the contact time in each vessel is three hours.

The reference substance is tested on each batch of microbial inoculum in the same way.

A different regime (e.g. more than one oxygen meter) will be necessary when measurements are to be made after 30 minutes of contact.

If mesurement of the chemical oxygen consumption is required, further vessels are prepared containing test substance, synthetic sewage feed and water, but no activated sludge. Oxygen consumption is measured and recorded after an aeration time of 30 minutes and/or three hours (contact time).

2. DATA AND EVALUATION

The respiration rate is calculated from the recorder trace between approximately 6,5 mg O_2/litre and 2,5 mg O_2/litre, or over a 10-minute period when the respiration rate is low. The portion of the respiration curve over which the respiration rate is measured should be linear.

If the respiration rates of the two controls are not within 15 % of each other, or the EC_{50} (30 minutes and/or three hours) of the reference substance is not in the accepted range (5 to 30 mg/litre for 3,5-dichlorophenol), the test is invalid and must be repeated.

The per cent inhibition is calculated at each test concentration (see 1.2). The per cent inhibition is plotted against concentration on log-normal (or log-probability) paper, and an EC_{50} value derived.

95 % confidence limits for the EC_{50} values can be determined using standard procedures.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following:

- test substance: chemical identification data,
- test system: source, concentration and any pre-treatment of the activated sludge,
- test conditions:
  - pH of the reaction mixture before the respiration measurement,
  - test temperature,
  - test duration,
  - reference substance and its measured EC_{50},
  - abiotic oxygen uptake (if any).
- results:
  - all measured data,
  - inhibition curve and method for calculation of EC_{50},
  - EC_{50} and, if possible, 95 % confidence limits, EC_{20} and EC_{80},
  - all observations and any deviations from this test method which could have influenced the result.

3.2. Interpretation of data

The EC_{50} value should be regarded merely as a guide to the likely toxicity of the test substance either to activated sludge sewage treatment or to waste-water microorganisms, since the complex interactions occurring in the environment cannot be accurately simulated in a laboratory test. In addition, test substances which may have an inhibitory effect on ammonia oxidation may also produce atypical inhibition curves. Accordingly, such curves should be interpreted with caution.

4. REFERENCES

(1) International Standard ISO 8192-1986.

(2) Broecker, B., Zahn, R., *Water Research* 11, 1977, p. 165.

(3) Brown, D., Hitz, H. R., Schaefer, L., *Chemosphere* 10, 1981, p. 245.

(4) ETAD (Ecological and Toxicological Association of Dyestuffs Manufacturing Industries), *Recommended Method No 103,* also described by:

(5) Robra, B., *Wasser/Abwasser* 117, 1976, p. 80.

(6) Schefer, W., *Textilveredlung* 6, 1977, p. 247.

(7) OECD, Paris, 1981, *Test Guideline 209,* Decision of the Council C(81) 30 final.

BIODEGRADATION

MODIFIED SCAS TEST

1. METHOD

1.1. Introduction

The purpose of the method is the evaluation of the potential ultimate biodegradability of water-soluble, non-volatile organic substances when exposed to relatively high concentrations of micro-organisms over a long time period. The viability of the microorganisms is maintained over this period by daily addition of a settled sewage feed. (For weekend requirements, the sewage may be stored at 4 °C. Alternatively, the synthetic sewage of the OECD confirmatory test may be used.)

Physico-chemical adsorption on the suspended solids may take place and this must be taken into account when interpreting results (see 3.2).

Because of the long detention period of the liquid phase (36 hours), and the intermittent addition of nutrients, the test does not simulate those conditions experienced in a sewage treatment plant. The results obtained with various test substances indicate that the test has a high biodegradation potential.

The conditions provided by the test are highly favourable to the selection and/or adaptation of micro-organisms capable of degrading the test compound. (The procedure may also be used to produce acclimatized inocula for use in other tests.)

In this method, the measure of the concentration of dissolved organic carbon is used to assess the ultimate biodegradability of the test substances. It is preferable to determine DOC after acidification and purging rather than as the difference of $C_{total} - C_{inorganic}$.

The simultaneous use of a specific analytical method may allow the assessment of the primary degradation of the substance (disappearance of the parent chemical structure).

The method is applicable only to those organic test substances which, at the concentration used in the test:

— are soluble in water (at least 20 mg dissolved organic carbon/litre),

— have negligible vapour pressure,

— are not inhibitory to bacteria,

— do not significantly adsorb within the test system,

— are not lost by foaming from the test solution.

The organic carbon content of the test material must be established.

Information on the relative proportions of the major components of the test material will be useful in interpreting the results obtained, particularly in those cases where the results are low or marginal.

Information on the toxicity to microorganisms of the substance may be useful to the interpretation of low results and in the selection of an appropriate test concentration.

1.2. Definitions and units

C_T = concentration of test compound as organic carbon as present in or added to the settled sewage at the start of the aeration period (mg/litre),

C_t = concentration of dissolved organic carbon found in the supernatant liquor of the test at the end of the aeration period (mg/litre),

C_c = concentration of dissolved organic carbon found in the supernatant liquor of the control at the end of the aeration period (mg/litre).

The biodegradation is defined in this method as the disappearance of the organic carbon. The biodegradation can be expressed as:

1. The percentage removal D_{da} of the amount of substance added daily:

$$D_{da} = \frac{C_T - (C_t - C_c)}{C_T} \times 100 \qquad [1]$$

where D_{da} = degradation/daily addition.

2. The percentage removal D_{ssd} of the amount of substance present at the start of each day:

$$D_{ssd} = \frac{2C_T + C_{ti} - C_{ci} - 3C_{t(i+1)} + 3C_{c(i+1)}}{2C_T + C_{ti} - C_{ci}} \times 100 \qquad [2\,(a)]$$

$$\simeq \frac{2C_T - 2(C_t - C_c)}{2C_T + (C_t - C_c)} \times 100 \qquad [2\,(b)]$$

where D_{ssd} = degradation/substance start of day;

the indices i and (i + 1) refer to the day of measurement.

Equation 2(a) is recommended if effluent DOC varies from day to day, while equation 2(b) may be used when effluent DOC remains relatively constant from day to day.

1.3. **Reference substances**

In some cases, when investigating a new substance, reference substances may be useful; however, no specific reference substance is recommended here.

Data on several compounds evaluated in ring tests are provided (see Appendix 1) primarily so that calibration of the method may be performed from time to time and to permit comparison of results when another method is employed.

1.4. **Principle of the test method**

Activated sludge from a sewage treatment plant is placed in a semi-continuous activated sludge (SCAS) unit. The test compound and settled domestic sewage are added, and the mixture is aerated for 23 hours. The aeration is then stopped, the sludge allowed to settle and the supernatant liquor is removed.

The sludge remaining in the aeration chamber is then mixed with a further aliquot of test compound and sewage and the cycle is repeated.

Biodegradation is established by determination of the dissolved organic carbon content of the supernatant liquor. This value is compared with that found for the liquor obtained from a control tube dosed with settled sewage only.

When a specific analytical method is used, changes in the concentration of the parent molecule due to biodegradation can be measured (primary biodegradability).

1.5. **Quality criteria**

The reproducibility of this method based on removal of dissolved organic carbon has not yet been established. (When primary biodegradation is considered, very precise data are obtained for materials that are extensively degraded.)

The sensitivity of the method is largely determined by the variability of the blank and to a lesser extent by the precision of the determination of dissolved organic carbon and the level of test compound in the liquor at the start of each cycle.

1.6. **Description of the test procedure**

1.6.1. *Preparations*

A sufficient number of clean aeration units, alternatively, the original 1,5 litre SCAS test unit may be used, and air inlet tubes (Figure 1) for each test substance and controls are assembled. Compressed air supplied to the test units, cleaned by a cotton wool strainer, should be free of organic carbon and pre-saturated with water to reduce evaporation losses.

A sample of mixed liquor, containing 1 to 4 g suspended solids/litre, is obtained from an activated sludge plant treating predominantly domestic sewage. Approximately 150 ml of the mixed liquor are required for each aeration unit.

Stock solutions of the test substance are prepared in distilled water; the concentration normally required is 400 mg/litre as organic carbon which gives a test compound concentration of 20 mg/litre carbon at the start of each aeration cycle if no biodegradation is occurring.

Higher concentrations are allowed if the toxicity to microorganisms permits it.

The organic carbon content of the stock solutions is measured.

1.6.2. *Test conditions*

The test should be performed at 20 to 25 °C.

A high concentration of aerobic microorganisms is used (from 1 to 4 g/litre suspended solids), and the effective detention period is 36 hours. The carbonaceous material in the sewage feed is oxidized extensively, normally within eight hours after the start of each aeration cycle. Thereafter, the sludge respires endogenously for the remainder of the aeration period, during which time the only available substrate is the test compound unless this is also readily metabolized. These features, combined with daily re-inoculation of the test when domestic sewage is used as the medium, provide highly favourable conditions for both acclimatization and high degress of biodegradation.

1.6.3. *Performance of the test*

A sample of mixed liquor from a suitable predominantly domestic activated-sludge plant or laboratory unit is obtained and kept aerobic until used in the laboratory. Each aeration unit as well as the control unit are filled with 150 ml of mixed liquor (if the original SCAS test unit is used, multiply the given volumes by 10) and the aeration is started. After 23 hours, aeration is stopped and the sludge is allowed to settle for 45 minutes. The tap of each vessel is opened in turn, and 100 ml portions of the supernatant liquor are withdrawn. A sample of settled domestic sewage is obtained immediately before use, and 100 ml are added to the sludge remaining in each aeration unit. Aeration is started anew. At this stage no test materials are added, and the units are fed daily with domestic sewage only until a clear supernatant liquor is obtained on settling. This usually takes up to two weeks, by which time the dissolved organic carbon in the supernatant liquor at the end of each aeration cycle approaches a constant value.

At the end of this period, the individual settled sludges are mixed, and 50 ml of the resulting composite sludge are added to each unit.

95 ml of settled sewage and 5 ml of water are added to the control units, and 95 ml of the settled sewage plus 5 ml of the appropriate test compound stock solution (400 mg/litre) are added to the test units. Aeration is started again and continued for 23 hours. The sludge is then allowed to settle for 45 minutes and the supernatant drawn off and analysed for dissolved organic carbon content.

The above fill-and-draw procedure is repeated daily throughout the test.

Before settling, it may be necessary to clean the walls of the units to prevent the accumulation of solids above the level of the liquid. A separate scraper or brush is used for each unit to prevent cross contamination.

Ideally, the dissolved organic carbon in the supernatant liquors is determined daily, although less frequent analyses are permissible. Before analysis the liquors are filtered through washed 0,45 µm membrane filters or centrifuged. Membrane filters are suitable if it is assured that they neither release carbon nor absorb the substance in the filtration step. The temperature of the sample must not exceed 40 °C while it is in the centrifuge.

The length of the test for compounds showing little or no biodegradation is indeterminate, but experience suggests that this should be at least 12 weeks in general, but not longer than 26 weeks.

2. DATA AND EVALUATION

The dissolved organic carbon values in the supernatant liquors of the test units and the control units are plotted against time.

As biodegradation is achieved, the level found in the test will approach that found in the control. Once the difference between the two levels is found to be constant over three consecutive measurements, such number of further measurements as are sufficient to allow statistical treatment of the data are made and the percentage biodegradation of the test compound is calculated (D_{da} or D_{ssd}, see 1.2).

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following:

— all information on the kind of sewage, the type of unit used and the experimental results concerning the tested substance, the reference substance if used, and the blank,

— the temperature,

— removal curve with description, mode of calculation (see 1.2),

— date and location where the activated sludge and the sewage were sampled, status of adaptation, concentration, etc.,

— scientific reasons for any changes of test procedure,

— signature and date.

3.2. Interpretation of results

Since the substance to be tested by this method will not be readily biodegradable, any removal of DOC due solely to biodegradation will normally be gradual over days or weeks, except in such cases where acclimatization is sudden as indicated by an abrupt disappearance occurring after some weeks.

However, physico-chemical adsorption can sometimes play an important role; this is indicated when there is complete or partial removal of the added DOC at the outset. What happens subsequently depends on factors such as the degrees of adsorption and the concentration of suspended solids in the discarded effluent. Usually the difference between the concentration of DOC in the control and test supernatant liquors gradually increases from the initial low value and this difference then remains at the new value for the remainder of the experiment, unless acclimatization takes place.

If a distinction is to be drawn between biodegradation (or partial biodegradation) and adsorption, further tests are necessary. This can be done in a number of ways, but the most convincing is to use the supernatant liquor, or sludge, as inoculum in a base-set test (preferably a respirometric test).

Test substances giving high, non-adsorptive removal of DOC in this test should be regarded as potentially biodegradable. Partial, non-adsorptive removal indicates that the chemical is at least subject to some biodegradation.

Low, or zero removals of DOC may be due to inhibition of microorganisms by the test substance and this may also be revealed by lysis and loss of sludge, giving turbid supernatants. The test should be repeated using a lower concentration of test substance.

The use of a specific analytical method or of ^{14}C-labelled test substance may allow greater sensitivity. In the case of ^{14}C test compound, the recovery of the $^{14}CO_2$ will confirm that biodegradation has occurred.

When results are also given in terms of primary biodegradation, an explanation should, if possible, be given on the chemical structure change that leads to the loss of response of the parent test substance.

The validation of the analytical method must be given together with the response found on the blank test medium.

4. REFERENCES

(1) OECD, Paris, 1981, *Test Guideline 302 A*, Decision of the Council C(81) 30 final.

Appendix 1

SCAS test: example of results

Substance	C_T (mg/l)	$C_t - C_c$ (mg/l)	Percentage biodegradition, D_{da}	Test duration (days)
4-acetyl aminobenzene sulphonate	17,2	2,0	85	40
Tetra propylene benzene sulphonate	17,3	8,4	51,4	40
4-nitrophenol	16,9	0,8	95,3	40
Diethylene glycol	16,5	0,2	98,8	40
Aniline	16,9	1,7	95,9	40
Cyclopentane tetra carboxylate	17,9	3,2	81,1	120

APPENDIX 2

Example of test apparatus

Figure 1

