PART B: METHODS FOR THE DETERMINATION OF TOXICITY AND OTHER HEALTH EFFECTS

GENERAL INTRODUCTION: PART B

A. EXPLANATORY NOTE

For the purpose of this General Introduction the following nymbering applies:

B.15 Gene Mutation – *Saccharomyces Cerevisae*

B.16 Mitotic Recombination - *Saccharomyces Cerevisae*

B.17 In Vitro Mammalian Cell Gene Mutation Test

B.18 Dna Damage And Repair – Unscheduled Dna Synthesis – Mammalian Cells *In Vitro*

B.19 Sister Chromatid Exchange Assay *In Vitro*

B.20 Sex-Linked Recessive Lethal Test In *Drosophila Melanogaster*

B.21 *In Vitro* Mammalian Cell Transformation Test

B.22 Rodent Dominant Lethal Test

B.23 Mammalian Spermatogonial Chromosome Aberration Test

B.24 Mouse Spot Test

B.25 Mouse Heritable Translocation

B.26 Sub-Chronic Oral Toxicity Test: 90-Day Repeated Oral Dose Study Using Rodent Species

B.27 Sub-Chronic Oral Toxicity Test: 90-Day Repeated Oral Dose Study Using Non-Rodent Species

B.28 Sub-Chronic Dermal Toxicity Test: 90-Day Repeated Dermal Dose Study Using Rodent Species

B.29 Sub-Chronic Inhalation Toxicity Test: 90-Day Repeated Inhalation Dose Study Using Rodent Species

B.30 Chronic Toxicity Test

B.31 Teratogenicity Test – Rodent And Non-Rodent

B.32 Carcinogenicity Test

B.33 Combined Chronic Toxicity/Carcinogenicity Test

B.34 One-Generation Reproduction Toxicity Test

B.35 Two Generation Reproduction Toxicity Test

B.36 Toxicokinetics

B. GENERAL DEFINITIONS FOR TERMS USED IN THE TEST METHODS IN THIS ANNEX

(i) **Acute toxicity** comprises the adverse effects occurring within a given time (usually 14 days), after administration of a single dose of a substance.

(ii) **Evident toxicity** is a general term describing clear signs of toxicity following administration of test substance. These should be sufficient for hazard assessment and should be such that an increase in the dose administered can be expected to result in the development of severe toxic signs and probable mortality.

(iii) **Dose** is the amount of test substance administered. Dose is expressed as weight (grams or milligrams) or as weight of test substance per unit weight of test animal (e.g. milligrams per kilogram body weight), or as constant dietary concentrations (parts per million or milligrams per kilogram of food).

(iv) **Discriminating dose** is the highest out of the four fixed dose levels which can be administered without causing compound-related mortality (including human kills).

(v) **Dosage** is a general term comprising of dose, its frequency and the duration of the dosing.

(vi) **LD_{50}** (median lethal dose) is a statistically derived single dose of a substance that can be expected to cause death in 50 % of dosed animals. The LD_{50} value is expressed in terms of weight of test substance per unit weight of test animal (milligrams per kilogram).

(vii) **LC_{50}** (median lethal concentration) is a statistically derived concentration of a substance that can be expected to cause death during exposure or within a fixed time after exposure in 50 % of animals exposed for a specified time. The LC_{50} value is expressed as weight of test substance per standard volume of air (milligrams per litre).

(viii) **NOAEL** is the abbreviation for no observed adverse effect level and is the highest dose or exposure level where no adverse treatment-related findings are observed.

(ix) **Repeated dose/Sub-chronic toxicity** comprises the adverse effects occurring in experimental animals as a result of repeated daily dosing with, or exposure to, a chemical for a short part of their expected life-span.

(x) **Maximum Tolerated Dose** (MTD) is the highest dose level eliciting signs of toxicity in animals without having major effects on survival relative to the test in which it is used.

(xi) **Skin irritation** is the production of inflammatory changes in the skin following the application of a test substance.

(xii) **Eye irritation** is the production of changes in the eye following the application of a test substance to the anterior surface of the eye.

(xiii) **Skin sensitisation** (allergic contact dermatitis) is an immunologically mediated cutaneous reaction to a substance.

(xiv) **Dermal corrosion** is the production of irreversible tissue damage in the skin following the application of a test substance for the duration period of 3 minutes up to 4 hours.

(xv) **Toxicokinetics** is the study of the absorption, distribution, metabolism and excretion of test substances.

(xvi) **Absorption** is the process(es) by which an administered substance enters the body.

(xvii) **Excretion** is the process(es) by which the administered substance and/or its metabolites are removed from the body.

(xviii) **Distribution** is the process(es) by which the absorbed substance and/or its metabolites partition within the body.

(xix) **Metabolism** is the process(es) by which the administered substances are structurally changed in the body by either enzymatic or non enzymatic reactions.

B.I **Acute - repeated dose / subchronic and chronic toxicity**

The acute toxic effects and organ or system toxicity of a substance may be evaluated using a variety of toxicity tests (Methods B.1 -B.5) from which, following a single dose, a preliminary indication of toxicity may be obtained.

Dependant on the toxicity of the substance, a limit test approach or a full LD50 may be considered, although no limit test is specified in inhalation studies, because it has not been possible to define a single inhalation exposure limit value.

Consideration should be given to methods which use as few animals as possible and minimise animal suffering, for example the fixed dose method (Method B.1 bis) and acute toxic class (Method B.1 tris). In level 1 testing, a study in a second species may complement the conclusions drawn from the first study. In this case, a standard test method may be used or the method may be adapted for a smaller number of animals.

The repeated dose toxicity test (Methods B.7, B.8 and B.9) includes assessment of toxic effects arising from repeated exposure. The need for careful clinical observations of the animals is stressed, so as to obtain as much information as possible. These tests should help to identify the target organs of toxicity and the toxic and toxic doses. Further in-depth investigation of these aspects may be required in long term studies (Methods B.26 - B.30 and B.33).

B.II **Mutagenicity - Genotoxicity**

Mutagenicity refers to the induction of permanent transmissible changes in the amount or structure of the genetic material of cells or organisms. These changes, 'mutations', may involve a single gene or gene segments, a block of genes, or whole chromosomes. Effects on whole chromosomes may be structural and / or numerical.

The mutagenic activity of a substance, for the base set information, is assessed by *in vitro* assays for gene (point) mutations in bacteria (Method B.13/14) and for structural chromosome aberrations in mammalian cells, (Method B.10).

Acceptable are also *in vivo* procedures, e.g. the micronucleus test (Method B.12) or the metaphase analysis of bone marrow cells, (Method B.11). However, in the absence of any contraindication the *in vitro* methods are strongly preferred.

Additional studies to investigate mutagenicity further or to pre-screen for carcinogenicity are required for higher production volumes and/or to conduct or follow-up a risk assessment, and these can be used for a number of purposes: to confirm results obtained in the base set; to investigate end-points not studied in the base set; to initiate or extend *in vivo* studies.

For these purposes, methods B.15 to B.25 include both *in vivo* and *in vitro* eukariotic systems and an extended range of biological end-points. These tests provide information on point mutations and other end-points in organisms more complex than the bacteria used for the base set.

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.

Some general principles for the testing strategy are laid down by Dir. 93/67/EEC, but clear testing strategies may be found in the technical guidance document for Risk Assessment, which nevertheless is flexible and can be adapted as appropriate to specific circumstances.

Methods for further investigation are however grouped below, on the basis of their principal genetic end-point:

Studies to investigate gene (point) mutations

a) Forward or reverse mutation studies using eukaryotic micro-organisms (*Saccharomyces cerevisiae*) (Method B.15)

b) *In vitro* studies to investigate forward mutation in mammalian cells, (Method B.17)

c) The sex-linked recessive lethal assay in *Drosophila melanogaster*, (Method B.20)

d) *In vivo* somatic cell mutation assay, the mouse spot test, (Method B.24)

Studies to investigate chromosome aberrations

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 (Method B.11). In addition, *in vivo* germ cell cytogenetics may be investigated, (Method B.23)

b) *In vitro* cytogenetic studies in mammalian cells, if this has not been included in the initial assessment, (Method B.10)

c) Dominant lethal studies in rodents, (Method B.22)

d) Mouse heritable translocation test, (Method B.25)

Genotoxic effects - effects on DNA

Genotoxicity, identified as harmful effects on genetic material not necessarily associated with mutagenicity, may be indicated by induced damage to DNA without direct evidence of mutation. The following methods using eukaryotic micro-organisms or mammalian cells may be appropriate for such investigation:

a) Mitotic recombination in *Saccharomyces cerevisiae*, (Methods B.16)

b) DNA damage and repair - unscheduled DNA synthesis - mammalian cells - *in vitro*, (Method B.18)

c) Sister chromatid exchange in mammalian cells - *in vitro*, (Method B.19)

Alternative methods for investigating carcinogenic potential

Mammalian cell-transformation assays are available which measure the ability of a substance to induce morphological and behavioural changes in cell cultures, which are thought to be associated with malignant transformation - *in vivo*, (Method B.21). 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, e.g. the mouse specific locus test, to measure germ-cell mutation in the first generation, (not included in this Annex), or for chromosome aberrations, e.g. the mouse heritable translocation test, (Method B.25). Such methods may be used when estimating the possible genetic risk of a substance to man. However, in view of the complexities involved in these tests and the very large number of animals necessary, particularly for the specific locus test, a strong justification is needed before undertaking these studies.

B.III **Carcinogenicity**

Chemicals may be described as genotoxic or non-genotoxic carcinogens, dependant on the presumed mechanism of action.

Pre-screening information for genotoxic carcinogenic potential of a substance may be obtained from the mutagenicity/genotoxicity studies. Additional information may be obtained from the repeated dose, subchronic or chronic toxicity tests. The repeated dose toxicity test, Method B.7 and longer repeated dose studies include assessment on histopathological changes observed in repeated dose toxicity tests, e.g. hyperplasia in certain tissues which could be of concern. These studies and toxicokinetic information may help to identify chemicals with carcinogenic potential, which may require further in-depth investigation of this aspect, in a carcinogenicity test (Method B.32) or often in a combined chronic toxicity/carcinogenicity study (Method B.33)

B.IV **Reproductive Toxicity**

Reproductive toxicity may be detected in different ways e.g. impairment of male and female reproductive functions or capacity, identified as 'effects on fertility', or induction of non-inheritable harmful effects on the progeny, identified as 'developmental toxicity' where teratogenicity and effects during lactation are also included.

For teratogenicity studies, as part of the developmental toxicity testing, the test method (Method B.31), 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.

Where a three-generation reproduction (fertility) test is required, the described method for the two-generation reproduction test (Method B.35), can be extended to cover a third generation.

B.V **Neurotoxicity**

Neurotoxicity may be detected in different ways e.g. functional changes and/or structural and biochemical changes in the central or peripheral nervous system. A preliminary indication of neurotoxicity may be obtained from acute toxicity tests. The repeated dose toxicity test, Method B.7, includes assessment of neurotoxicological effects, and the need for careful clinical observations of the animals is stressed, so as to obtain as much information as possible. The method should help to identify chemicals with neurotoxic potential, which may require further in-depth investigation of this aspect. Additionally, it is important to consider the potential of substances to cause specific neurotoxic effects that may not be detected in other toxicity studies. For example, certain organophosphorous substances have been observed to cause delayed neurotoxicity and can be evaluated in methods B.37 and B.38, following single or repeated-dose exposure.

B.VI **Immunotoxicity**

Immunotoxicity may be detected in different ways e.g. immunosupression and/or enhancement of the responsiveness of the immune system resulting in either hypersensitivity or induced autoimmunity. The repeated dose toxicity test, Method B.7, includes assessment of immunotoxic effects. The method should help to identify chemicals with immunotoxic potential, which may require further in-depth investigation of this aspect.

B.VII **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 (Method B.36).

Information on chemical structure (SAR) and physico-chemical properties may also provide an 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.

C. CHARACTERISATION OF THE TEST SUBSTANCE

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 each particular study and the handling and storage of the test substance.

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 the biological material should precede the initiation of the study.

All information relating to the identification, the physico-chemical properties, the purity, and behaviour of the test substance should be included in the test report.

D. ANIMAL CARE

Stringent control of environmental conditions and proper animal care techniques are essential in toxicity testing.

(i) *Housing conditions*

The environmental conditions in the experimental animal rooms or enclosures should be appropriate to the test species. For rats, mice and guinea pigs, suitable conditions are a room temperature of 22°C ± 3°C with a relative humidity of 30 to 70 % ; for rabbits the temperature should be 20 ± 3°C with a relative humidity of 30 to 70 %.

Some experimental techniques are particularly sensitive to temperature effects and, in these cases, details of appropriate conditions are included in the description of the test method. In all investigations of toxic effects, the temperature and humidity should be monitored, recorded, and included in the final report of the study.

Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. Details of the lighting pattern should be recorded and included in the final report of the study.

Unless otherwise specified in the method, animals may be housed individually, or be caged in small groups of the same sex; for group caging, no more than five animals should be housed per cage.

In reports of animal experiments, it is important to indicate the type of caging used and the number of animals housed in each cage both during exposure to the chemical and any subsequent observation period.

(ii) *Feeding conditions*

Diets should meet all the nutritional requirements of the species under test. Where test substances are administered to animals in their diet the nutritional value may be reduced by interaction between the substance and a dietary constituent. The possibility of such a reaction should be considered when interpreting the results of tests. Conventional laboratory diets may be used with an unlimited supply of drinking water. The choice of the diet may be influenced by the need to ensure a suitable admixture of a test substance when administered by this method.

Dietary contaminants which are known to influence the toxicity should not be present in interfering concentrations.

E. ANIMAL WELFARE

When elaborating the test methods due consideration was given to animal welfare. Some examples are briefly given below, but this list is not exhaustive. The exact wording and/or conditions should be read in the text of the methods :

- For the determination of acute oral toxicity, two alternative methods, the 'Fixed Dose Procedure' and the 'Acute Toxic Class method' should be considered. The 'Fixed Dose Procedure' does not utilise death as specific endpoint and it uses fewer animals. The 'Acute Toxic Class method' uses on average 70% less animals than Method B.1 for Acute Oral toxicity. Both these alternative methods result in less pain and distress than the classical methodology.

- The number of animals used is reduced to the scientifically acceptable minimum : only 5 animals of the same sex are tested per dose level for methods B.1 and B.3; only 10 animals (and only 5 for the negative control group) are used for the determination of the skin sensitisation by the guinea pig maximisation test (method B.6); the number of animals needed for the positive control when testing mutagenicity *in vivo* is also lowered (methods B.11 and B.12)

- Pain and distress of animals during the tests are minimised : animals showing severe and enduring signs of distress and pain may need to be humanely killed; dosing test substances in a way known to cause marked pain and distress due to corrosive or irritating properties need not to be carried out (methods B.1, B.2 and B.3).

- Testing with irrelevantly high doses is avoided by the introduction of limit tests, not only in the acute toxicity tests (methods B.1, B.2 and B.3) but also in the *in vivo* tests for mutagenicity (methods B.11 and B.12).

- A strategy of testing for irritancy now allows the non-performance of a test, or its reduction to a single animal study, when sufficient scientific evidence can be provided.

Such scientific evidence can be based on the physico-chemical properties of the substance, the results of other tests already performed, or the results of well validated *in vitro* tests. For example, if an acute toxicity study by the dermal route has been conducted at the limit test dose with the substance (method B.3), and no skin irritation was observed, further testing for skin irritation (method B.4) may be unnecessary; materials which have demonstrated definite corrosion or severe skin irritancy in a dermal irritation study (method B.4) should not be further tested for eye irritancy (method B.5).

F. ALTERNATIVE TESTING

A scientific objective for the European Union is the development and validation of alternative techniques which can provide the same level of information as current animal tests, but which use fewer animals, cause less suffering or avoid the use of animals completely.

Such methods, as they become available, must be considered wherever possible for hazard characterisation and consequent classification and labelling for intrinsic hazards.

G. EVALUATION AND INTERPRETATION

When tests are evaluated and interpreted, limitations in the extend to which the results of animal and *in vitro* studies can be extrapolated directly to man must be considered and therefore, evidence of adverse effects in humans, where available, may be used for confirmation of testing results.

These results, can be used for the classification and labelling of the new and the existing chemicals for human health effects, on the basis of their intrinsic properties, identified and quantified by these methods. Corresponding Annex VI criteria for classification and labelling relate also to the end-points of the testing protocols included in these testing methods.

These results can also be used for risk assessment studies, for new and existing chemicals, and appropriate testing strategies for these purposes are indicated in the corresponding guidance documents.

H. LITERATURE REFERENCES

Most of these methods are developed within the framework of the OECD programme for Testing Guidelines, and should be performed in conformity with the principles of Good Laboratory Practice, in order to ensure as wide as possible 'mutual acceptance of data'.

Additional information may be found in the references found in the OECD guidelines and the relevant literature published elsewhere.