
**Institute for Environment and Health**

# Assessment of the feasibility of replacing current regulatory *in vivo* toxicity tests with *in vitro* tests within the framework specified in the EC White Paper 'Strategy for an EU Chemicals Policy'

**IEH Web Report W10** **DECEMBER 2001**

The Institute for Environment and Health was established by the Medical Research Council at the University of Leicester in 1993. The Institute is principally funded by UK Government Departments and Agencies by way of specific research and consultancy contracts.

This report was prepared by the Institute for Environment and Health for DEFRA and issued in December 2001.

The views expressed here do not necessarily represent those of any Government Department or Agency

Written by C Botham and P Holmes

Reviewed and edited by PTC Harrison and LS Levy

IEH will continue to make this document available at this Web Site (or by a link to a different site). Any changes to its contents will be clearly recorded, either by a note of corrigenda or the issue of a new edition, identified by an amended report reference number and date. A copy of this document is also held at the British Lending Library.

Corrigenda link

Please cite as:
IEH (2001) *Assessment of the Feasibility of Replacing Current Regulatory In Vivo Toxicity Tests with In Vitro Tests within the Framework Specified in the EU White Paper 'Strategy for an EU Chemicals Policy'* (Web Report W10), Leicester, UK, MRC Institute for Environment and Health (at http://www.le.ac.uk/ieh/ posted December 2001)



ISBN 1 899110 690

MRC Institute for Environment and Health
University of Leicester
94 Regent Road
Leicester
LE1 7DD
UK

# Contents

# Executive Summary

In February 2001 the European Commission (EC) published a White Paper *Strategy for a future chemicals policy*. One consequence of the adoption of the proposals in the White Paper would be the unification of the regulatory requirements for existing and new chemicals. In essence, this would require the testing of existing chemicals produced or marketed at volumes of 1 to 10 tonnes/annum using a restricted battery of tests that do not involve the use of intact animals, while chemicals produced or marketed at any higher volume would be assessed in a tiered testing strategy that included *in vivo* methods (i.e. using live, intact animals).

This report considers 1) whether the introduction of a first test tier (for chemicals produced or marketed at 1 to 10 tonnes/annum) using only *in vitro* methods is feasible and acceptable, and 2) the broader issue of reducing the use of animals in the toxicity testing of chemicals. In particular, the report presents an overview of the current state of development of proposed alternatives to the existing *in vivo* Base set and also Level 1 and Level 2 toxicity tests, based upon a survey of European and US organisations involved in the development of alternative tests and a search of the scientific literature, and assesses the feasibility of developing a satisfactory battery of tests within the time frames necessitated by the EC White Paper so as to allow replacement, or at least a significant reduction in the proposed use, of animals for the toxicity (hazard) assessment of chemicals.

The principal findings of the review are as follows.

- It is unlikely that, in the foreseeable future, *in vitro* methods will adequately replace the current *in vivo* tests for acute, sub-chronic and chronic toxicity or non-genotoxic carcinogenicity, or that alternative tests could be used to fully characterise reproductive toxic potential. However, progress in the development of some *in vitro* test batteries suggests that it may become possible to use such systems as an early screening tier to detect overtly toxic chemicals before progression to the definitive *in vivo* tests.

- Significant progress has already been made in applying the so-called 'three Rs' (reduction, refinement and replacement) approach to the use of animals, in particular with the regulatory acceptance of more refined acute toxicity tests that significantly reduce animal usage and suffering, and the introduction of non-animal methods to screen for dermal and ocular corrosive or severe irritancy potential. Current *in vitro* methods for assessing mutagenic potential are also sufficiently predictive as to not require *in vivo* studies in the majority of cases.

- *Ex vivo* ocular irritation and corrosion tests are now accepted as pre-screens in several EU member states; strenuous efforts should be made to extend their adoption across the EU as soon as possible.

- Development of *in vitro* models suitable for use as definitive tests for skin irritation is at an advanced stage, and it appears that *in vitro* test batteries capable of providing a reasonable level of predictivity for skin and ocular corrosion and irritation potential should become available for adoption within a relatively short time frame.

- With regard to assessing chemical sensitisation potential, the local lymph node assay (LLNA) has recently been recommended for adoption by the Organisation for Economic Co-operation and Development (OECD) and European Union (EU). The use of this test should lead to a reduction in animal usage, and it appears that an initial screening battery of *in vitro* tests might be developed within a few years to assist in the prioritisation of testing for skin sensitisation potential.

- In vitro models addressing endpoints relevant to the asthma-inducing potential of chemicals are being developed (a type of toxicity highlighted in the EC White Paper as being of concern).

- Demands to include consideration of novel areas of toxicity in the assessment process will potentially result in an increase in animal usage. For example, a number of in vivo tests for endocrine disruptive activity (a further type of toxicity specifically highlighted in the EC White Paper) are now close to completing an OECD validation process, which is likely to lead to calls for their adoption into the regulatory assessment system.

- A principal concern of a number of the stakeholder organisations surveyed is the lengthy delays that currently occur between the adoption of tests by one competent authority and the general acceptance by other national and international bodies.

With regard to the EC White Paper proposals for chemicals produced or marketed at 1 to 10 tonnes/annum, it is possible that a battery of non-animal based tests capable of addressing many aspects of chemical toxicity could be developed within the required timescales, provided sufficient funding and resources were made available. However, it is unrealistic to expect such a screen to fully supplant the need for the *in vivo* acute and 28-day toxicity studies specified for the Base set level. Further consideration therefore needs to be given to the proposal that testing of chemicals at 1 to 10 tonnes/annum using only non-animal models is acceptable for this category of chemical.

Significant replacement of the *in vivo* tests required for higher production level chemicals appears unlikely within the foreseeable future, with the possible exception of the alternative models now employed as a screen for corrosivity and severe irritancy; their use could be extended to fully replace the *in vivo* tests for dermal and ocular corrosivity/irritancy. In addition, although no suitable guidelines currently exist to permit assessment of reproductive function at Base set, *in vitro* models may soon be validated that would allow assessment of limited aspects of early embryonic/fetal development at this level.

Continued efforts to incorporate the 'three Rs' approach into regulatory test requirements should be actively encouraged. Because the market in chemicals is global, it is essential that efforts are made to improve the mechanisms for the mutual acceptance of alternative models validated by competent regulatory authorities, to avoid both the unnecessary use of animals where suitable alternative models exist and the potential needless duplication of testing to meet differing national requirements.

# 1 Introduction

## 1.1 Background

In February 2001 the European Commission (EC) published a White Paper 'Strategy for a future chemicals policy' (EC, 2001a) in response to the increasing level of concern within member states of the European Union (EU) that environmental exposure to chemicals may be a potential cause of ill-health in humans and adverse effects in wildlife, and that current legislation was proving slow and cumbersome in progressing hazard assessments.

Under current EU legislation, the use and/or release into the environment of many classes of chemical (such as pesticides, pharmaceuticals, food additives and novel industrial chemicals) are regulated through a series of EC directives. Briefly, these require novel substances to undergo varying degrees of safety testing and risk assessment before they can be authorised for release onto the open market.

The Sixth Amendment to EU Directive 67/548/EEC on the classification, packaging and labelling of dangerous substances introduced the notification system for 'new' substances (NoNS) and spawned the establishment EINECS, the European Inventory of Existing Commercial Chemical Substances, which lists all 'existing' substances that were reported to be on the market on or before 18 September 1981. The evaluation and control of risk in relation to existing substances are regulated by the Existing Substances Regulation (Council Regulation (EEC) No 793/93), which requires manufactures to provide the Commission with available information on chemicals produced in quantities of 10 tonnes/annum or more. This information has been used to form the basis of the International Uniform Chemical Information Database (IUCLID), which is the basic tool for data collection and evaluation in the European Risk Assessment Programme on Existing Substances.

Substances placed on the market for the first time after 18 September 1981 are termed 'new', and are registered on the European List of New Chemical Substances (ELINCS). In particular, the NoNS system requires that novel chemicals be subjected to toxicity tests, including studies using intact animals (*in vivo* studies). The requirements for testing are tiered, with the extent of testing being determined by the physicochemical properties of a particular chemical or chemical class, and the anticipated market volume. There are currently three levels of testing (termed Base set, Level 1 and Level 2), each consisting of a battery of tests intended to provide information on specific aspects of a chemical's hazard potential. Chemicals with production/market volumes between one and 100 tonnes/annum are tested at Base set only; those at volumes of 100–1000 tonnes/annum are tested at Base set and Level 1, and those at greater than 1000 tonnes/annum are tested at Base set, Level 1 and Level 2. For chemicals tested at all three levels, the toxic properties investigated include irritancy, sensitisation, general toxic effects, carcinogenicity and reproductive toxicity (see Annex 1). However, these requirements for the testing and assessment of industrial chemicals* do not currently extend to the previously mentioned existing chemicals on the EINECS list, which number about 100 000.

The above mentioned EC White Paper proposes a change in European policy to one in which both new and existing industrial chemicals would be assessed under a unified system. Any chemical considered to be potentially hazardous to humans or wildlife would be subjected to strict regulation or banning. A new system is proposed in the White Paper: 'Registration, Evaluation and Authorisation of Chemicals' (REACH). This requires a change in the current tiered screening system used under

* For convenience, the term 'industrial chemical' is used throughout the remainder of this report to denote all chemicals, including consumer products, that are not biocidal, pharmaceutical or veterinary products, radioactive substances, foodstuffs or waste mixtures.

NoNS, such that all new and existing chemicals produced or marketed at 10–100 tonnes/annum would undergo Base set testing, those that exceed 100 tonnes/annum would be tested at Base set and Level 1, and those in excess of 1000 tonnes/annum would be tested at all three levels. In addition, it is proposed that chemicals with production volumes of between 1 and 10 tonnes/annum (for which new substances are currently subject to Base set testing) would be subject only to tests that do not use intact animals, that is, only *in vitro* methods or structure–activity relationship (SAR) computer models. To achieve these goals, the EC proposes that all those existing chemicals for which suitable data are not already in existence should be subject to appropriate toxicity testing, and that all existing chemicals should complete assessment under the REACH system within strict time frames.

Of the 100 000 existing chemicals listed in EINECS, approximately 20 000 are believed to be produced or marketed at volumes of between 1 and 10 tonne/annum, and 10 000 at volumes greater than 10 tonne/annum (European Commission, 2001a). It has also been estimated that over 5000 chemicals are produced or marketed at 100 to 1000 tonne/annum, and over 2500 at 1000 to 10 000 tonne/annum (IEH, 2001). To achieve the EC's target, screening systems would have to be established, and testing to the proposed *in vitro*/SAR or existing Base set requirements completed, by the year 2012. In addition, testing of chemicals to Level 1 (100–1000 tonne/annum) or Level 2 (1000–10 000 tonne/annum) requirements should be completed by 2008 and 2005, respectively.

The MRC Institute for Environment and Health (IEH) has recently produced an assessment of the implications of the proposed change in testing strategy in terms of animal usage and cost, on behalf of the then Department of the Environment, Transport and the Regions (IEH, 2001). The report concluded that, assuming the tests used in the tiered structure remain unchanged, the White Paper considerably underestimates the numbers of animals required, the financial cost and the practical difficulties in undertaking the testing programme within the timescales proposed. This conclusion also holds if the proposed changes to the reduction of chemicals to be tested at Base set are taken into account.

One of the key assumptions of the EC White Paper (noted above) is that it is possible to satisfactorily, and with confidence, assess the toxicity of those chemicals with production/market volumes of between 1 and 10 tonnes/annum using study designs that do not involve the use of live animal experimentation. To date, such *in vivo* studies have constituted a key element at each of the test levels (including the Base set), and the feasibility of introducing a testing programme that replaces such studies with non-animal alternatives within the proposed time frame, whilst maintaining the same degree of protection to humans and the environment, requires careful consideration.

Subsequent to the publication of the IEH report on testing requirements, the Institute was approached by the House of Lords European Union Committee Sub-Committee D to make a contribution to its assessment of the implications of the EU chemical strategy. Following the IEH submission[*] and an oral presentation based on its findings, the Sub-Committee requested that the Institute provide further comment on the EC's assertion that the majority of existing chemicals that require toxicological assessment could be tested using a battery of *in vitro* or SAR methods. The Department for Environment, Food and Rural Affairs (DEFRA) is also interested in the general situation regarding the availability of alternatives to existing *in vivo* regulatory test designs, and therefore commissioned IEH to review the feasibility of replacing existing *in vivo* tests with validated *in vitro* or SAR methods, and to investigate if any alternatives currently undergoing development are likely to achieve international validation within the required timescales set out in the EC White Paper.

This report presents an overview of the current state of development of *in vitro* toxicity tests and SAR models, and assesses whether a satisfactory battery of tests can be developed that will allow the replacement, or at least significant reduction in the use, of animals for hazard (toxicity) assessment. The report also highlights the progress made to date in replacement, reduction and refinement of techniques using animals.

[*] posted on the web at http://www.le.ac.uk/ieh/pdf/resp_ecwp.pdf

# 1.2 Structure of the report

The following section (1.3) describes the processes used to survey the opinion of various key organisations with regard to their work on, and views with regard to, the development of alternative test models. In addition, details of the literature searches conducted to identify relevant published literature are described. Section 1.4 discusses the achievements made to date in reducing animal usage and suffering.

Sections 2 and 3 briefly describe the test methods used in the current tiered screening system, and review progress on developing non-animal alternatives. Section 4 summarises the responses received from the various organisations contacted. The feasibility of introducing alternative methods is discussed in Section 5, and conclusions are presented in Section 6.

# 1.3 Review and assessment process

## 1.3.1 Internet and database searches

An extensive literature search of relevant on-line databases (Medline and BIOSIS) was undertaken. References were selected which related to the current situation regarding animal replacement, refinement and reduction policies in regulatory testing. In addition, papers that considered alternative *in vitro* tests for specific areas of toxicological testing (e.g. reproductive toxicology) were identified and obtained. A detailed search of the Internet was also undertaken to identify any documents of relevance that may have been posted by organisations interested in the subject of alternatives to animal testing.

## 1.3.2 Survey of organisations in Europe and the USA involved in alternative test method development

In order to present as wide a picture as possible of the research efforts currently underway to develop alternatives to existing *in vivo* test systems, a number of organisations identified as working on, or supporting, the development of alternative (non-animal) tests were requested to provide an update on their activities, and to indicate their opinion on this subject. The selection of organisations to be contacted was based upon an inventory of groups conducting *in vitro* research on behalf of the European Commission, Internet searches, and through personal contacts within relevant organisations. Ten European and three USA-based groups were identified (see Annex 2).

Appropriate contacts within each organisation were sent a questionnaire letter (see Annex 3) requesting information on the following aspects: current activities in the development/ validation of *in vitro* toxicity methods relevant to Base set, Level 1 and Level 2 testing requirements; expected timescales for full validation and incorporation into Organisation for Economic Co-operation and Development (OECD) guidelines; anticipated impact on resource capacities of testing laboratories; and a more general opinion or comment on the feasibility of replacing animal testing using alternative methods.

Overall, eight organisations responded to the questionnaire within the given timeframe (see Section 4).

# 1.4 Current replacement, refinement and reduction strategies used in regulatory toxicology

One of the stated objectives of the proposed EC chemicals strategy is the 'promotion of non-animal testing'. To achieve this, the EC proposes to provide funds for the development and validation of *in vitro* or other non-animal test methods. The basic philosophy underlying this approach was first formulated in the 1950s, for example in Russell and Burch's book *The Principles of Humane Experimental Technique* (Russell & Burch, 1959), and has become known as the 'three Rs' (reduction, refinement and replacement). The 'three Rs' encompasses any technique or approach that replaces the use of animals, reduces the need for animals in a particular test, or refines a technique in order to reduce the amount of suffering endured by the animal. The 'three Rs' is not infrequently associated with the term 'alternative', to indicate test methods that do not use intact, living animals. In 1986, the EC incorporated the 'three Rs' into Council Directive 86/609/EEC, which addresses the laws, regulations and administrative provisions in member states relating to the protection of animals used for experimental or other scientific purposes.

It is of note that significant reductions in the number of animals required for certain regulatory toxicity tests have already been achieved, for example through the redesign of studies in the light of statistical considerations, or the re-examination of test objectives (e.g. see Section 2.1 on acute toxicity testing). In recent years, the increase in international harmonisation of test guidelines has further helped reduce studies where duplication occurred in order to meet slightly differing national regulatory requirements. The increased acceptance of published 'non-regulatory' toxicity study findings by regulators, and improved exchange of information (thereby avoiding repetition of toxicity testing), has also helped to reduce animal usage (Gad, 1990). Many methods offering an alternative to the use of higher animals have also been developed, including the use of invertebrates, cultured cells or tissues and, for groups of chemicals with similar chemical structures, mathematical models such as SAR computer models (Fentam & Balls, 1997). The majority of such methods have found application in screening novel substances (e.g. pharmaceuticals) prior to taking a decision to progress a candidate chemical to formal registration, rather than for regulatory purposes. Nonetheless, such methods have significantly contributed to a reduction in animal suffering by avoiding the progression of compounds with overt toxicity to the regulatory testing stage. Given the increasingly global nature of the trade in chemicals, continued progress towards international harmonisation of test requirements will be of particular importance if further reductions in overall animal usage and suffering are to be achieved. Adoption of an *in vitro* replacement for an existing *in vivo* test model by one nation, or even a multinational body such as the EC, will not result in a reduction in overall animal usage if the *in vivo* test remains a regulatory requirement of another country in which the chemical is to be marketed. This emphasises the important role played by the OECD in developing internationally accepted guidelines, in line with the OECD Mutual Acceptance of Data Agreement (OECD, 1981). In particular, the OECD has, in the so-called 'Solna Principles' (OECD, 1996), clearly defined the necessary requirements for the validation and adoption of new test guidelines.

A number of alternatives to *in vivo* methods are recognised as regulatory tests (for example by the OECD). Although these mostly relate to the study of various aspects of genetic toxicology, the OECD now accepts that, where a chemical has certain physicochemical properties (such as a pH of 2 or less, or greater than 11.5), or is established as a definitive corrosive or a severe skin irritant by either *in vivo* skin irritation tests or well validated *in vitro* tests, then there is no need to perform the controversial *in vivo* ocular irritation/corrosion test. In line with this provision, a number of EU member states, including the UK, no longer require *in vivo* ocular testing for chemicals that have been shown to have corrosive or severe dermal irritancy potential using identified *in vitro* models (rat skin transcutaneous electrical resistance (TER), human skin (reconstructed skin) and severe eye irritation (bovine enucleated eye) methods). Development and validation of a number of other *in vitro* test systems is also underway (see Table 1). It must, however, be stressed that each of the *in vitro* tests currently available can only examine one, or a few, specific toxic endpoints (e.g. mutagenicity),

whereas the use of intact, live animals as the biological test system permits the detection of unexpected or novel toxicity, and provides information on a wide range of toxic endpoints, including organ-specific toxicity, and behavioural, physiological and biochemical systems and processes, as well as informing on a chemical's pharmacokinetic, pharmacodynamic and metabolic profile. Information from these profiles is invaluable in assessing the significance to humans, or other species of concern, of changes seen in a test animal.

Other action has focused on the reduction of pain and suffering of individual animals used in testing through the careful control and limitation of procedures that may be performed on the animal, and through addressing housing conditions and the provision of environmental enrichment. A recent OECD publication *'Guidance Document On The Recognition, Assessment, And Use Of Clinical Signs As Humane Endpoints For Experimental Animals Used In Safety Evaluation'* (OECD, 2000a) has addressed the question of pain and distress arising from the performance of experimental procedures. This document provides clear guidance on identifying clinical signs and behaviour of animals indicative of various degrees of pain and distress, with the intention that, once identified, appropriate action can be taken to minimise or eliminate the distress either by humanely killing the animal or, where feasible within the study design, temporarily stopping or reducing the treatment to which the animal is exposed. However, it should be noted that the refinement of experimental procedures, while reducing an individual animal's level of distress or discomfort, could in some cases result in an overall increase in the number of animals used on a study. For example, restrictions on the number, volume and frequency of blood sampling to which an animal can be subjected may result in a dramatic increase in the number of animals required to complete a study to determine, for example, the pharmacokinetic profile of a chemical.

# 2 Current Status and Purpose of *In Vivo* and *In Vitro* Tests Required for Base Set and Alternatives under Development

## 2.1 Current toxicity test methods

The *in vivo* tests on vertebrates undertaken to meet current Base set requirements are listed in Annex 1. Together, these tests are intended to provide the information necessary to establish the acute toxicity profile of a chemical, its irritant and sensitisation potential in mammals, and its mutagenic potential, and also to provide limited information on the general toxicity of the chemical following sub-acute exposures of up to 28 days. An acute toxicity test in fish is incorporated to provide some guidance to the potential environmental hazard posed by the chemical.

### 2.1.1 Acute systemic toxicity

As noted above, a number of tests are conducted to assess the potential for a chemical to cause acute systemic toxicity, up to and including death. In these studies, animals are exposed through oral, dermal or inhalation routes. Substances other than gases (which are only administered by inhalation) are studied using a minimum of two routes, one of which is usually oral. The second route chosen depends upon the nature of chemical and the anticipated route of human exposure.

Examination of acute toxicity is required to assess the likely degree of toxic hazard posed by short-term, high-level exposure to a chemical, and to identify adverse effects and possible target organs. The information thus generated is used to establish the potential for injury or death to humans from an intentional or accidental single or short-term chemical exposure, and is also used to derive 'hazardous property' information and determine labelling, under EU Directive 67/548/EEC, as well as health and safety information on handling and use. Multiple routes of exposure are used to inform the human risk assessment, and both male and female animals of each species are usually studied to identify if there is any sex-specific toxicity or sex-related differences in sensitivity.

Traditionally, much testing on mammals has been undertaken using an acute oral toxicity test design developed almost 50 years ago that generally required the use of at least 20 to 40 animals per study. In this test, graduated single doses are given to animals assigned to several groups, one dosage level being given to each group, at concentrations expected to cause death of a number of individuals. The animals are then examined over a 14-day period for death or signs of toxicity; severely incapacitated animals are killed to prevent further suffering. An $LD_{50}$ value (the dose that kills 50% of the animals within the 14-day period) is then calculated. These studies have long been considered to involve a high degree of suffering, and this design has been superseded by three other methods (see below) that require significantly fewer individuals (generally one-third or fewer) and tend to use much lower dosages, thereby minimising the likelihood of severe suffering of the animals.

Under Organisation for Economic Co-operation and Development (OECD) regulatory guidelines, alternatives to the classic $LD_{50}$ test (OECD Guideline 401) are: Fixed Dose procedure method (OECD Test Guideline 420); Acute Toxic Class method (OECD Test Guideline 423); and the Up and Down procedure (OECD Test Guideline 425)[*]. It should be noted that the fixed dose method does not use death as an endpoint; for the other two tests no more than three deaths are typically expected. The

[*] Full titles of all OECD tests referred to in this report are listed as an extension to the Bibliography.

design of these alternative tests not only markedly reduces suffering and death but, importantly, also provides more detailed toxicological information through the assessment of a wider range of more sensitive and informative endpoints, thus meeting at least two of the objectives of the 'three Rs', namely reduction and refinement.

The fixed dose procedure and the acute toxic class methods have been accepted into Annex V of EU Directive 67/548/EEC. Indeed, the EC and all EU member states have now banned the use of OECD Guideline 401 (the classical $LD_{50}$ test).

## 2.1.2 Skin irritancy/corrosion

A much-used method for testing skin irritancy was first proposed by Draize in 1944. It involves the application of the test substance directly onto the shaved skin of albino rabbits under an occlusive dressing for up to 24 hours. The skin is then examined over a 72-hour period for signs of graded damage, such as erythema, oedema or necrosis. The OECD adopted this approach in 1981, with the albino rabbit being identified as the animal of choice. Subsequent revisions, adopted in 1992, incorporated a number of refinements, including allowing use of data derived from suitable non-animal tests as a basis for not proceeding to *in vivo* testing. Thus, under the current guideline no *in vivo* testing is necessary where a chemical's physiochemical properties indicate a high corrosive potential, or where corrosivity is predicted by suitable (as yet unspecified) *in vitro* models. Testing is also not considered necessary where the chemical has previously been demonstrated to be highly toxic via the dermal route, or when the acute dermal toxicity test (OECD 402) has demonstrated no irritation at the limit dose of 2000 mg/kg bodyweight. Further, where *in vivo* testing is considered necessary but there is a suspicion that severe irritancy or corrosion may occur, testing is initially restricted to a single animal, with the testing of an additional two animals only undertaken if no severe effects are observed. The OECD is currently at a late stage of the review procedure to define a suitable test guideline for *in vitro* skin corrosion tests. A draft was produced in November 1999 (OECD, 1999), which has been commented on by member countries. Based on the comments submitted by the national coordinators, a number of scientific and technical issues were highlighted that required further consideration. Primary concerns included the lack of guidance on interpreting borderline results, lack of sufficient detail on the generic *in vitro* human skin model assay, and lack of consistency with the Globally Harmonised Classification System (GHS) with respect to the treatment of negative results in the *in vitro* skin corrosion tests (OECD, 2001a). It was concluded at the OECD's 13th Working Group of National Coordinators of the Test Guidelines Programme (May 30–June 1, 2001 in Paris, France) that an Extended Expert Consultation Meeting would be held on 1–2 November, 2001 in Berlin, Germany, with the intention of achieving a consensus on these outstanding technical issues. The results of this meeting have yet to be published. Notwithstanding the OECD deliberations, the EU has made progress on this issue, having already included two *in vitro* models of dermal corrosivity into Annex V of EU Directive 67/548/EEC, namely the TER assay, and a Human Skin Model Assay (European Commission 2000b). The US Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) also recently reviewed available tests, and has recommended models for use within the US regulatory guidelines as part of an integrated testing system for dermal corrosion potential (NICEATM, 2001). One aspect highlighted during the reviews by the various organisations is that generic rather than proprietary methods are preferred; in particular, the OECD has a policy not to recommend test guidelines for methods requiring equipment or material from a unique (proprietary) source.

## 2.1.3 Skin sensitisation

Allergic contact dermatitis (ACD) is a significant occupational health problem, with symptoms in humans characterised by the appearance of pruritis, erythema, oedema, papules, vesicles and/or bullae. Skin sensitisation tests are used to determine whether a chemical can elicit an immune response of the type that could lead to ACD.

The OECD first adopted skin sensitisation guidelines in 1981, and the tests originally comprised adjuvant and non-adjuvant studies in the guinea pig. These are the guinea pig maximisation test (GPMT) of Magnusson and Kligman, and the occluded patch test developed by Buehler. Guidelines were revised in 1992 to include two pre-screening assays using mice; the mouse ear swelling test (MEST), and the local lymph node assay (LLNA). The MEST assay measures the challenge-induced increase in ear thickness of previously sensitised mice, and is based upon the analysis of challenge-induced dermal reactions in previously sensitised animals. One of the advantages of this test over the guinea pig tests is that the MEST uses a quantitative measurement of response. In the LLNA, the induction phase of the immune reaction (i.e. cellular proliferation in lymph nodes) is investigated. This test requires a smaller number of animals, involves a lower level of suffering, uses objective endpoints, and is more sensitive than either of the guinea pig tests. The LLNA has now been validated by ICCVAM and by the European Centre for the Validation of Alternative Methods (ECVAM). It has been recommended to the OECD for formal adoption as a replacement for the Magnussen–Kligman test by national co-ordinators, and is being progressed for inclusion under Annex V of EU Directive 67/548/EEC (ECVAM, 2000).

## 2.1.4 Ocular toxicity

For reasons similar to those that necessitate the dermal testing of a chemical, there is a requirement to establish the potential hazard posed by a chemical to the eye. The acute eye irritation/corrosion test determines whether a chemical can cause reversible inflammatory changes or irreversible tissue damage to the conjunctiva, cornea or iris.

The OECD first adopted a study design in 1981 (OECD 405), which was revised in 1987 to reduce the number of animals used (6 to 3) and to exclude from testing substances with established severe dermal irritancy/corrosive properties. In effect, the changes introduced an hierarchical approach, with a requirement to initially consider, on a weight-of-evidence basis, the chemical's physicochemical properties, the results from dermal irritation/corrosivity studies, and data from 'well-validated alternative studies' (although acceptable alternative methods were not specifically identified), to identify if a chemical may potentially cause a severe response. Evidence of such a potential obviates the need to proceed to *in vivo* testing. If such a response is not considered likely, but there remains a possibility of a marked response, the guideline requires that application of the chemical is initially restricted to only one animal, with the remaining animals being dosed only if no severe effects are elicited. In 1996, the OECD drafted a more formal statement of this hierarchical approach that was submitted for approval in May 2001; this has now been agreed but not yet formally adopted.

Within Europe, four EU member states currently accept several *ex vivo* tests for assessing eye corrosion. These have not yet been adopted into Annex V of Directive 67/548/EEC but are under review by ECVAM. They are the Hen's Egg Test–Chorioallantoic Membrane (HET-CAM) assay in embryonated chicken eggs on day 9 of incubation, the Isolated Rabbit Eye (IRE) test on eyes of rabbits that have been sacrificed for other purposes, the Bovine Cornea Opacity and Permeability (BCOP) test on the cornea of freshly isolated bovine eyes from the slaughterhouse, and the Isolated Chicken Eye (ICE) test on chicken eyes freshly obtained from the slaughterhouse. If a positive result is obtained in any of these *in vitro* tests, further testing in animals is not required. However, where all tests show negative results a confirmatory *in vivo* test using one to three rabbits is still required.

## 2.1.5 Short-term repeat-dose toxicity

Short duration (14 to 28 days), repeat-dose studies are a key element in chemical hazard assessment, being used to define the likely adverse effects (which may sometimes be delayed) of repeated exposure to non-acutely lethal levels of a chemical, over a relatively short time frame. These studies are generally used to investigate a wider range of endpoints, and may include pathological examination of direct tissue or cellular effects in over 50 target tissues, each composed of multiple cell types. They are also used to examine more subtle toxic effects (including behavioural modifications and neurotoxic or clinical pathological changes) than those addressed in the acute

studies. The test designs also allow for the possibility of investigating the reversibility of any effects detected. Dosage is selected to enable the detection of a 'no observed effect level', which is of value to the risk assessment process. The OECD guideline for this test using the oral route (OECD 407) was originally adopted in 1981 and later revised in 1995 to give more emphasis to toxicological endpoints such as neurotoxicity, immunotoxicity and reproductive organ effects. There are also versions that relate to the assessment of dermal and inhalation toxicity (OCED, 410 & 412).

The guideline for short-term toxicity studies as stated in Annex V of the EU Directive 67/548/EEC is closely related to the OECD Test Guideline.

## 2.1.6 Reproductive toxicology

Reflecting the high level of societal concern about adverse effects on reproductive capacity and possible harm to future generations, the Base set includes a requirement to screen a chemical for effects on male and female reproductive performance. Although this is a requirement under Annex V of the EU Directive 67/548/EEC, there are no adopted guidelines for either *in vivo* or *in vitro* tests and hence, in practice, testing for this important aspect of toxicity is not conducted at the level of the Base set.

While it is anticipated that *in vitro* test designs may soon be available to address this gap in testing (see Section 2.2), it should be noted that in 1995 the OECD adopted a reproduction/developmental toxicity screening test (OECD 421) intended to provide an initial evaluation of reproductive toxicity. In this design, male and female rodents (generally rats) are dosed prior to mating and, in the case of females, until the fourth day following parturition. Aspects considered include gonad function, mating behaviour, conception, embryo/fetal development, and parturition. However the design is not intended to provide definitive information on all aspects of reproductive performance, and does not replace the various reproductive test designs included under Levels 1 and 2. In particular, effects attributable to early postnatal exposure or possible adverse effects that can only be detected later during the development and maturation of offspring cannot be addressed using this study design.

## 2.1.7 Genetic toxicology

*In vivo* studies to assess genotoxicity are not routinely required at Base set. Chemicals are, however, screened for their potential to cause genetic damage using *in vitro* methods. Indeed, such testing forms an essential part of the hazard identification process, and has important implications for any subsequent risk assessment. Mutations are classified as: point or gene mutations (changes in the nucleotide sequence of DNA); chromosomal mutations (changes in chromosomal morphology); or genomic mutations (alterations in the number (ploidy) of chromosomes). Mutations have the potential to induce cancerous changes in the cells of an organ or to lead to hereditable changes that can affect the offspring.

Under Annex V of EU Directive 67/548/EEC, industrial chemicals tested at Base set are studied using two test types. One is a bacteriological (reverse mutation) test, with and without metabolic activation (to provide a degree of confidence regarding the possibility that a metabolite of a chemical may be active whilst the parent compound itself is not). The second type is a non-bacteriological model to detect chromosome aberrations or damage. This test is normally conducted using an *in vitro* design, again with and without metabolic activation. Substances producing negative findings in the *in vitro* studies are considered to have no significant mutagenic potential. In the event of a positive result in either test, further testing to verify the results would normally be undertaken which could, on occasion, extend to the use of *in vivo* assays to determine whether the mutagenic potential observed *in vitro* actually extends to the *in vivo* situation. The requirements for determining mutagenic potential and the interpretation of test results have been discussed in detail by the UK's Committee on Mutagenicity (COM, 2000).

## 2.2 Alternative toxicity tests currently under development or validation

At present, *in vitro* techniques validated for use under OECD guidelines play a significant role only in relation to assessing genotoxicity. Although *in vitro* methods have also been developed to address a number of other toxic endpoints (e.g. to identify severe skin and ocular corrosives/irritants), these have generally not yet been formally adopted into OECD regulatory guidelines. As discussed above, some such tests are, however, already accepted for use within the EU. Relevant *in vitro* test designs that are either under development, validation or review, or that have been adopted by the regulatory bodies of the EU, USA and OECD are summarised in Table 1. The *in vitro* test designs included are those considered by ECVAM, ICCVAM or other authoritative bodies to provide, or to be most likely to show, a high level of correlation with *in vivo* data, and hence to be those most likely to achieve acceptance and adoption by the competent regulatory authorities.

### 2.2.1 Acute systemic toxicity

There are several *in vitro* methods under development as possible screening steps or replacements for *in vivo* acute toxicity testing.

The Neutral Red Cytotoxicity assay investigates the cytotoxic effect of chemicals on cultured mammalian cells, and allows the determination of a highest tolerated dose. In this test, cell viability is assessed by measuring the uptake of neutral red dye, and total cell protein using Coomassie blue dye. This method has been extensively studied by organisations within the EU, and is under consideration by ECVAM for inclusion in a major European validation exercise to determine the effectiveness of replacement methods for acute systemic toxicity.

A multi-centre evaluation of an *in vitro* cytotoxicity programme (MEIC) was organised by the Scandinavian Society for Cell Toxicology in 1989. This was a voluntary effort involving 96 international laboratories to assess the relevance and reliability of several *in vitro* cytotoxicity tests that had originally been developed as possible alternative or screening methods for acute systemic toxicity, chronic systemic toxicity, organ toxicity, skin irritancy, or other forms of toxicity. The programme's aims were to investigate the predictivity of results from such *in vitro* tests for acute toxic activity in humans, and to establish a battery of *in vitro* tests that could be utilised as a replacement for *in vivo* testing. The results demonstrated that tests using human cells to assess basal cytotoxicity were of relevance to acute human toxicity but, at the same time, showed that other important toxic mechanisms existed, which could possibly only be identified by additional types of *in vitro* tests. It was also suggested that the modelling of human toxicity using these tests could be improved by additional toxicokinetic data, which would require new *in vitro* kinetic test designs. The findings from the MEIC programme provided the scientific basis for a six-year programme, which started in 1998, 'The evaluation guided development of new in vitro tests' (EDIT). The main goal of this project is the development of a battery of approximately six *in vitro* tests that are capable of an at least 90% predictivity of human acute toxicity for any (i.e. non-pre-selected) group of chemicals. A second goal is to attempt to develop a battery of *in vitro* tests to predict effects of repeated dosing (i.e. to determine minimal steady state blood concentrations and daily dosages that would lead to significant toxic effects). A third goal is to discover whether chemical carcinogens can be identified solely through the use of *in vitro* tests.

Other tests currently under scrutiny include modelled cell cultures to predict specific toxic mechanisms. These include testing of epithelial cell barrier functions to determine whether organs protected by epithelial barriers are likely target organs, measurement of cellular energy metabolism to determine whether the nervous and cardiovascular systems (both of which have high metabolic demands) are target organs, and tissue/organ specific effects. Attempts are also being made to develop simple models to assess toxicokinetic activity, in order to determine whether metabolites of chemicals under test would have a greater or lesser systemic toxicity compared with their parent compound.

The outcome of these development and validation exercises is keenly awaited, and it is to be hoped that a screening battery may be developed that will be capable of detecting at least highly toxic chemicals, potentially even to the extent of obviating the need to progress to *in vivo* testing for such chemicals (for example, as part of the selection process for candidate chemicals). However, it remains open to question if such a battery can be developed sufficiently within the foreseeable future to address all the potential endpoints and interactions that are currently investigated by an acute *in vivo* test, or to provide sufficient information to allow the development of guidance on the clinical treatment of acute poisonings. It is uncertain whether sufficient dose–response information could be derived by the *in vitro* models to confidently inform dosage selection for subsequent *in vivo* repeat-dose studies.

## 2.2.2 Skin corrosion

As previously noted, replacement *in vitro* tests have been validated for the study of skin corrosion, and have been incorporated into Annex V of EU Directive 67/548/EEC (see Section 2.1). No additional designs are being considered at this time.

CORROSITEX, a test system based upon a biomembrane and chemical detection system, has been validated by ICCVAM for certain chemical groups. However this test was found to be predictive only for specific classes of chemical and hence has not been recommended to the EU.

## 2.2.3 Skin irritancy

There are several *in vitro* assays and one *ex-vivo* assay currently under investigation as possible replacements for *in vivo* skin irritancy testing.

ECVAM is currently considering the possible extension of human skin model assays, such as the EPISKIN™ and EpiDerm™ systems, to the prediction of irritancy in addition to their accepted use in predicting corrosivity. The Skin Integrity Function Test (SIFT), which uses isolated mouse skin and measures transepidermal water loss and electrical resistance to assess skin integrity and, hence, irritation potential, is shortly to undergo validation in contract research laboratories, and may be considered for use as a pre-screen or a useful addition to a tiered testing battery.

## 2.2.4 Skin sensitisation

No *in vitro* tests have yet been validated in Europe, the US or at the OECD level. The local lymph node assay, which represents a refinement and reduction strategy, has recently been incorporated into Annex V of EU Directive 67/548/EEC (see Section 2.1). Of potential use is the *Deductive Estimation of Risk from Existing Knowledge* (DEREK) computer model. DEREK is a system that brings together structural information and a toxicity database, so that qualitative predictions can be made and chemicals with potentially toxic properties identified. DEREK is still under development and its eventual performance level is as yet unknown, but the DEREK Skin Sensitisation Rule base, combined with an assessment of likely skin permeability of the chemical, could potentially constitute an initial step in a tiered testing strategy. Other potentially useful techniques include the use of dendritic cell lines that synthesise and release the cytokine 1L-1beta (a precursor to allergic reaction), and protein binding assays (de Silva *et al.*, 1996) although there is as yet no consensus on the utility of this latter approach.

## 2.2.5 Ocular toxicity

As previously noted, there are several *ex vivo* tests accepted for use by some EU member states as screens for ocular corrosion or severe dermal irritation before proceeding to an *in vivo* test, but these have yet to be fully adopted as screens at either the OECD or EU level. To date, none of the tests have been accepted as a complete replacement for *in vivo* testing. A number of additional *in vitro* methods are under development or validation, including:

- Fluorescein leakage assay: a cell culture assay that determines damage caused by the test compound to the junctions in cultured canine renal epithelial cell monolayers, by measuring fluorescein leakage through the cell layer. Validation within the EU has been undertaken (INVITTOX, 2001) and the test is being considered by ECVAM as a potential *in vitro* replacement for ocular irritancy.

- EpiOcular™: a corneal model consisting of normal, human-derived epidermal keratinocytes that have been cultured to form a stratified, squamous epithelium similar to that found in the cornea. This model provides a predictive, morphologically relevant *in vitro* means to assess ocular irritancy and is currently being considered by ECVAM as a potential *in vitro* replacement for *in vivo* ocular irritancy tests.

- Neutral Red Release assay: this assay is intended to determine the short-term toxicity and irritancy of a test compound by measuring the release of vital dye from cultured cells. This test is accepted by the French regulatory authorities for the testing of cosmetic products, and is under consideration by ECVAM as a potential alternative to determine eye irritation.

- Irritection (also known as Eytex) system: measures the reduction in light transmission resulting from the precipitation caused by interaction of the test compound with a protein matrix, to determine potential eye irritancy. This system has poor performance compared with *in vivo* data, and has been rejected by ECVAM (Horst Spielmann, personal communication*).

## 2.2.6 Short-term (28-day) repeat-dose toxicity

Historically, various *in vitro* models (e.g. primary cell cultures, organotypic cultures, tissue slices and organ cultures) have been extensively used as tools to investigate underlying mechanisms of toxic action once a target tissue(s) has been identified by *in vivo* studies. *In vitro* and, to some extent, structure–activity relationship (SAR) models also have an established but limited role in screening candidate chemicals within well-defined chemical series for which the mechanism of action is firmly established. However, as previously noted, the short-term *in vivo* toxicity test is a key element in hazard assessment, which investigates numerous wide-ranging endpoints. Any replacement or reduction strategy would, therefore, require a very extensive battery of *in vitro* assays capable of detecting toxicity in a wide range of tissues and for a very wide range of chemicals. At present there are no systems available that have a wide degree of acceptance for use in such a role.

Research is currently being undertaken by ECVAM to assess the feasibility of long-term exposure of *in vitro* cell lines (e.g. the EDIT programme) that might be applicable to both short-term and chronic toxicity. This work includes consideration of cells from the reproductive, renal, haematopoietic, cardiovascular, respiratory and autonomic nervous systems. It is, however, considered unlikely that such a test battery could be adequately developed in the foreseeable future to provide acceptable predictivity for a sufficiently diverse range of chemicals and the wide range of cellular and tissue interactions, and toxic endpoints, that are currently covered by the repeat-dose *in vivo* model. In addition, such *in vitro* models appear unlikely to be capable of predicting higher level effects (such as disturbance of physiological homeostasis or central nervous functions, as may be seen on occasion using *in vivo* models even in the absence of pathologically-observable cellular or tissue change). It appears more likely that such a battery of assays could provide an initial screening system, and thereby reduce the need to expose animals to overtly toxic chemicals.

## 2.2.7 Genetic toxicology

As noted in Section 2.1, only *in vitro* assays are routinely employed to meet genotoxicity testing requirements at the Base set, and there is only an occasional need to undertake *in vivo* studies to clarify equivocal findings.

* by letter to the author, 26.9.01

## 2.2.8 Reproductive and developmental screening

As noted in Section 2.1, there are no current *in vitro* models that have gained regulatory approval. A number of *in vitro* methods exist, and are discussed in detail in relation to their possible use as alternatives to the reproductive studies required at the higher test levels (Section 3.2).

## 2.2.9 Acute fish toxicity

There are currently no accepted alternatives to the acute *in vivo* fish test under the EU, US or OECD test guidelines. The predictivity of five alternative tests has been compared with existing acute toxicity data for the fathead minnow, as part of a workshop held by the Centre for Alternatives to Animal Tests (CAAT, 1999). The alternatives comprised two SAR computer models and three methods using alternative organisms, the Tetratox, Microtox and FETAX assays.

SAR was shown to be capable of predicting the toxicity of acyclic compounds to fathead minnow using data derived from tests on Daphnia. However, there was insufficient data relating to Daphnia available for other classes of compound to enable a fuller evaluation of the usefulness of the model.

The Ecological Structure Activity Relationship (ECOSAR) system is being investigated by the US Environmental Protection Agency (EPA) as a possible alternative to the fish toxicity test. This computer model uses chemical structures or log $K_{ow}$ values to predict $LC_{50}$ (concentration expected to kill 50% of the animals over a specified period) in fish. In a recent collaborative study involving the EU and the EPA, ECOSAR was demonstrated to give a high degree of correlation.

The Teratox assay employs the protozoan *Tetrahymen*. A high correlation has been reported with fathead minnow data for neutral organic compounds. However, there are some serious limitations relating to the interspecies differences in bioreactivity mechanisms and basic physiology.

Details of the FETAX method are given in Section 3.2.

The Microtox assay uses bioluminescence produced by the bacterium *Vibrio fischeri* as an indicator of toxicity. A good correlation has been established for non-polar narcosis and also for esters. CAAT considers that this assay might be developed into a screening tool, although it is probably not a good candidate as a complete replacement.

# 3 Regulatory Toxicology: Level 1 and Level 2 Tests

## 3.1 Current toxicity test methods

The tests required for Level 1 and 2 testing are presented in Annex 1. At Level 1, the majority of tests relate to the detailed study of the effects of sub-chronic (up to 13 weeks) exposure to the test chemical or the provision of more detailed information on the chemical's potential reproductive toxicity. Additionally at this level, potential adverse effects on the environment are studied through the inclusion of a 14-day (sub-acute) toxicity test in fish, while evidence on the potential of the chemical to accumulate within biota is provided by the bioaccumulation study in fish. At Level 2, the focus of general toxicology studies is the investigation of the chronic toxicity of a chemical, and its potential to cause cancer in animals when administered throughout the life span. Similarly, additional reproductive tests (including the use of non-rodent species, generally the rabbit) are included at Level 2. Aspects of the reproduction process that are investigated by the various studies performed at Levels 1 and 2 include: hormonal homeostasis; gamete production and viability; mating behaviour and performance; fertilisation; implantation and subsequent embryonic and fetal development (including the potential for teratogenic effects); parturition; maternal behaviour and the development of offspring; and the subsequent maturation and reproductive ability of such offspring.

Particularly at Level 2, there is an emphasis on understanding the toxicokinetic [*] profile of a chemical and its underlying mechanisms of toxicity, in order to provide a greater confidence in extrapolating the significance of any findings from the animal species tested to the human situation and the wider ecosystem. Currently, most aspects of such investigations are conducted on intact animals, with the design being developed on a case-by-case basis for individual chemicals. Data from this type of study aid the evaluation of test results from the toxicity studies and in the extrapolation from animals to humans or, potentially, other species. These studies may also assist in the design of *in vitro* experiments by identifying the amount and rate of absorption of a chemical, the pattern of distribution among tissues, organs and fluid compartments, and the possible reversible binding of the test compound to tissue sites or plasma proteins. In addition, the rates of metabolism and excretion of the chemical, and biochemical parameters such as irreversible binding to macromolecules, can be used as indicators of potential toxicity.

As a consequence of the more rigorous nature of investigations conducted at Level 1 and, particularly, Level 2, study designs (whilst conforming to minimum Organisation for Economic Co-operation and Development (OECD) requirements) tend to be constructed on a case-by-case basis, in terms of species or strains used, dosages and routes of administration, the inclusion of reversibility phases and other particular investigations, so as to elucidate the effects of the particular chemical under scrutiny. Understanding toxic mechanisms and dose–response is essential to inform subsequent risk assessment and management decisions, in particular in relation to establishing appropriate exposure limits necessary to safeguard human and environmental health.

[*] Toxicokinetics is the study of the rates of absorption, distribution, metabolism and excretion of test compounds.

## 3.2 Alternative toxicity tests currently under development or validation

Neither the development of alternative methods, nor the reduction of numbers of animal and refinement of study design, is specifically addressed in the EC White Paper with respect to Level 1 and 2 requirements.

Although there are as yet no accepted alternatives capable of replacing any of the repeat dose tests currently performed (including carcinogenicity and reproductive toxicity studies), there is considerable research (in progress or planned) to develop *in vitro* models. Attention is focused on constructing batteries of tests that could act as screens for the many aspects of toxicity currently assessed *in vivo*.

### 3.2.1 Carcinogenicity

Several *in vitro* assays have been suggested as possible tools for establishing carcinogenicity. These include the Syrian hamster embryo (SHE) cell transformation assay and a number of transformed mouse and human cell lines (such as Balb/c 3T3 and C3H/10T½ mouse cells and HaCaT and MSU-1 human cells; see Table 1). Development of such a battery is, however, at an early stage, and the value of the SHE assay has already been questioned since the test is complex, lengthy and too expensive for use in routine testing. A particular outstanding problem faced in developing *in vitro* alternatives to *in vivo* testing for carcinogenicity is that chemicals may act as carcinogens through a variety of mechanisms, both genotoxic and non-genotoxic. It should be noted that potential genotoxic carcinogens will generally have already been highlighted by the findings from the mutagenicity assessment (Section 2.1).

### 3.2.2 Reproductive toxicology

As can be seen from Annex 1, a series of *in vivo* study types is currently required to investigate the various potential effects of a chemical on different stages of the reproductive cycle.

The US Environmental Protection Agency (EPA), in response to the OECD's call for the screening of high production volume chemicals (i.e. those produced in excess of 1 million pounds/annum, thought to number about 2800) has proposed a number of refinement and reduction strategies, including the possible replacement of the teratogenicity test in rats, the one-generation reproductive test and the 28-day repeat dose study by the reproductive/developmental toxicity screening test (OECD 422). While it has been suggested that such refinements could potentially result in a significant saving in overall animal usage (Green *et al.*, 2001), it has to be appreciated that this OECD screening design does not provide complete information on reproduction and development. In particular, only very limited information is obtained on the post-natal manifestations of effects arising from pre-natal or lactational exposure. In addition, the combining of test designs, which of necessity involves an increase in endpoints studied and procedures performed on the test animals, may potentially result in significant increases in the level of stress and suffering of individual animals.

Numerous *in vitro* tests would be needed to attempt to address the multitude of endpoints that are currently studied by the existing *in vivo* reproductive tests. A number of alternative designs that address specific aspects of developmental toxicity have been subject to validation by the European Centre for the Validation of Alternative Methods (ECVAM; see Table 1):

- Embryonic stem cell test (EST): intended to predict teratogenic potential of chemicals using 3T3 and ES cells, a permanent cell line derived from mouse embryonic stem cells;
- Micromass test: investigates effects, in the presence or absence of metabolic activation (S-9 mix), on the differentiation and growth of rat limb bud and central nervous system cells; and

- Whole embryo culture: post-implantation rodent embryos are exposed to a chemical *in vitro* for a limited period, in the presence or absence of a metabolic activation system, and abnormalities in development and growth are assessed.

Although the results are not yet formally published, it appears that the Micromass test only detected strongly embryotoxic compounds. In contrast, the EST and whole embryo tests were found to be predictive of embryotoxic chemicals (Department of Health, personal communication[a]).

In addition, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) has undertaken a validation exercise on a *Xenopus* embryo teratogenesis assay (FETAX), although they identified a need to revise the methodology to achieve a close replication of *in vivo* findings. Computer models have also been developed, which attempt to predict teratogenic potential on the basis of a chemical's structure.

Further tests to provide information on other aspects of reproductive function are in development. These include the use of Sertoli cell lines and cultured primary follicles, sperm motility, morphology and penetration assays, and various receptor binding assays (Spielmann, personal communication[b]). Other suggested systems include rodent limb bud cultures, gene-chip technologies, chick embryo neural retina cell assays and the chick embryo toxicity screening test (CHEST; Prati *et al.*, 1993; Jelinek *et al.*, 1985).

Reflecting the position that exists with repeat-dose toxicity testing, it is doubtful whether *in vitro* tests will, in the foreseeable future, be able to address all the areas covered by the Level 1 and 2 *in vivo* tests, in particular with regard to changes in reproductive behaviour, the overall process of fetal development, and the detection of subsequent subtle (but important) behavioural or learning effects. Nonetheless, *in vitro* tests such as those validated by ECVAM (although only capable of addressing certain aspects of fetal development), together with some of the other models addressing toxicity of chemicals to key reproductive cell types, hold out great promise as components of an initial screening battery that could be incorporated into the Base set to address the as-yet unanswered requirement to include screening for reproductive toxicity at this initial test level.

## 3.2.3 Toxicokinetics

*In vitro* models are already widely used in the study of comparative metabolism, particularly to assist in the identification of a chemical's likely metabolic profile in humans so as to facilitate comparison with those of the various animal species used in the toxicity studies. The information so derived is used to inform the risk assessment process. In the context of the types of chemicals considered in this report, such studies would only be expected for some of those tested at Level 2.

Attempts are underway to develop a range of *in vitro* systems capable of predicting the toxicokinetics of a wide range of chemicals, with the intention of using such a battery of tests to reduce or replace the use of animals in toxicokinetic profiling. ECVAM currently favours the use of a battery of cell lines transfected with five of the major cytochrome $P_{450}$ enzymes, and intends to assess their relevance and reliability using a range of chemicals. Other possible approaches include computer expert systems designed to intelligently predict the Phase I and II metabolism of chemicals (e.g. METEOR), while physiological-based biokinetic models (PBBKs) may be capable of predicting the kinetics of chemicals. However, the systems developed to date are very chemical specific and require a high level of modelling expertise.

## 3.2.4 Long-term studies

Owing to the wide range of endpoints investigated using long-term *in vivo* toxicological studies, a substantial battery of *in vitro* tests would be required to achieve a reduction in animal use at test

[a] by email to the author, 5.11.01
[b] by letter to the author, 26.9.01

Levels 1 and 2. A recent ECVAM workshop described numerous *in vitro* methods for long-term or chronic toxicity testing that are currently under development or are used in scientific research (Pfaller *et al.*, 2001). The majority of these tests use either primary or transformed cell lines, derived from the liver, kidneys or the brain, to predict organ-specific toxicity. Other assays described use isolated organ slices, derived from the brain or liver. Some of these systems were reported as promising, although further refinement of the methodologies was recommended.

# 3.3 Testing for additional aspects of toxicity

## 3.3.1 Endocrine disruption

As highlighted by the EU White Paper, one of the emerging areas of concern in Europe is that some chemicals present in the environment (termed endocrine disrupting chemicals, EDCs) may be capable of adversely affecting the normal functioning of the endocrine system, with consequent adverse effects on various physiological processes. Although no international agreement has yet been reached on the battery of tests required to identify whether or not a chemical has endocrine disrupting potential, the OECD Endocrine Disrupter Testing and Assessment Task Force, established in 1998, has proposed a conceptual framework and has begun work on developing new tests and refining existing guidelines to better address endpoints of relevance to endocrine disruption. Currently, work is progressing on the validation of guidelines for *in vivo* uterotrophic and Herschberger assays, together with enhancements to the existing short-term repeat dose rodent test (OECD 407). Where necessary, longer-term effects would be identified using modifications to existing designs, such as a revised version of the two-generation reproduction toxicity study (OECD 416). In addition, progress is being made on the development and validation of fish screening assay(s), a developmental assay based on the fish early life stage toxicity test (OECD 210), a reproduction or partial life cycle test in fish and a fish full life cycle. There is also development of one and/or two generation avian reproductive tests in birds (quail), and a frog (*Xenopus*) metamorphosis assay. A role for *in vitro* assays within the OECD strategy for testing for endocrine disrupting activity has yet to be fully defined, but such assays are likely to form part of any future screening or prioritisation system. The EU actively participates in the OECD Task Force, and it has been estimated that the first of the agreed test methods relevant to human health assessment will be available during 2002, while tests for environmental effects are expected to be authorised between 2003 and 2005 (European Commission, 2001b).

It should be noted that, in response to legal requirements incorporated in the Food Quality Protection Act and the amended Safe Drinking Water Act of 1996, a tiered testing strategy with *in vitro* and *in vivo* elements has been proposed by the US EPA (EDSTAC, 1998; EPA, 2000). The EPA intends that this should be used to screen new and existing chemicals.

## 3.2.2 Phototoxicity

Although not routinely required under Directive 67/548/EEC, study of phototoxic activity is sometimes required for specific chemicals as a result of a risk assessment based upon exposure and/or physicochemical criteria.

*In vitro* models for assessing phototoxicity are available for use; for example the 3T3 NRU photoxicity test has been validated and adopted by the EU.

## 3.3.3 Dermal absorption

In 2001, the OECD drafted new guidelines for an *in vitro* test employing human skin from donors undergoing surgery (OECD, 2000b) as an alternative method to an *in vivo* test in the rat for assessing percutaneous absorption or skin penetration. The assessment of dermal absorption is mandatory in international regulations for pesticides and biocides, cosmetic ingredients and finished products. Although not routinely required under Directive 67/548/EEC, investigation into dermal absorption is

sometimes required for specific chemicals as a result of questions arising during the risk assessment process in relation to exposure and/or physicochemical issues.

### 3.3.4 Asthma

Particular attention was drawn in the EC White Paper to the need to identify chemicals capable of inducing asthma, with the intention of introducing controls in the hope of reversing the increase in levels of asthma seen in many populations. Paradoxically, this effect is not examined in any of the tiered levels outlined in the White Paper, nor is any mention made of the need to develop such a test.

To our knowledge, there are at present no accepted *in vivo* or *in vitro* tests to examine endpoints relevant to asthma, although *in vitro* models, such those using human lung adenocarcinoma (A549) and bronchial epithelial (BEAS-2B) cell lines, are currently at a developmental stage.

**Table 1** Current status of alternative toxicity tests specified in Annex V of the EU Directive 67/548/EEC

| Current test required under Directive 67/548/EEC | Alternative test methods | Research and development stage (EU and/or USA) | Current status of tests | | |
|---|---|---|---|---|---|
| | | | EU | OECD | USA |
| Acute systemic toxicity | Neutral red cytotoxicity test | + | - | - | - |
| | MEIC/EDIT | + | - | - | - |
| | Modelled cell cultures | + | - | - | - |
| Skin corrosion | CORROSITEX™ | + | R | - | A |
| | Transcutaneous Electrical Resistance (TER) assay | + | A | U | V |
| | Episkin™ | + | A | U | V |
| | EpiDerm™ | + | A | U | V |
| Skin irritancy | Episkin™ | + | U | - | V |
| | EpiDerm™ | + | U | - | V |
| | Skin integrity function test (SIFT) | + | ? | - | ? |
| Skin sensitivity | DEREK computer model | + | U | - | - |
| | Protein binding assay | + | U | - | - |
| | Dendritic cell line | + | U | - | - |
| Ocular toxicity | EYTEX | + | R | ? | ? |
| | EpiOcular assay | + | - | ? | U |
| | Fluorescein leakage assay | + | - | ? | ? |
| | Neutral red release assay | + | U | - | - |
| | Isolated rabbit eye test | + | U | - | - |
| | Isolated chicken eye test | + | U | - | - |
| | HET-CAM test | + | U | - | - |
| | BCOP test | | U | - | - |
| Genotoxicity | *In vitro* Syrian hamster embryo (SHE) cell transformation assay | + | R | - | + |
| | *In vitro* micronucleus assay | + | U | - | ? |
| | Transformed human cell lines | + | U | - | ? |
| | Transformed murine cell lines | + | U | - | ? |
| Repeated dose (28 & 90 day) studies | *In vitro* cell lines: (reproductive, kidney, haematopoietic, cardiovascular, respiratory, autonomic nervous system) | + | - | - | - |

Taken from Prof. Spielmann (personal communication); Prati *et al.*, 1993; Jelinek *et al.*, 1985; Robinson *et al.*, 2000; Green *et al.*, 2001 and Calvin, 1992.

Key: +, ongoing activity; -, no ongoing activity; ?, no available information; A, accepted after validation; R, rejected after validation; U, under review; V, validated; EDIT, Evaluation guided development of new *in vitro* tests; DEREK, Deductive Estimation of Risk from Existing Knowledge; MEIC, Multicenter evaluation of *in vitro* cytotoxicity

**Table 1** continued

| Current test required under Directive 67/548/EEC | Alternative test methods | Research and development stage (EU and/or USA) | Current status of tests | | |
|---|---|---|---|---|---|
| | | | EU | OECD | USA |
| Reproductive/ development screening | FETAX | + | R | - | A |
| | Whole embryo culture | + | V | - | - |
| | Embryonic stem cells | + | V | - | - |
| | Rodent limb bud culture | + | - | - | - |
| | Micromass cultures | + | V | - | - |
| | Sertoli cell lines | + | - | - | - |
| | Sperm motility and morphology | + | - | - | - |
| | Sperm penetration | + | - | - | - |
| | Cultured primary follicles | + | - | - | - |
| | CHEST test | + | - | - | - |
| | Chick embryo neural retina cells | + | - | - | - |
| Acute fish toxicity | TETRATOX | + | - | - | U |
| | MICROTOX | + | - | - | U |
| | FETAX | + | - | - | U |
| | ECOSAR | + | - | - | U |
| | SAR | + | | | U |

Taken from Prof. Spielmann (personal communication); Prati *et al.*, 1993; Jelinek *et al.*, 1985; Robinson *et al.*, 2000; Green *et al.*, 2001 and Calvin, 1992.

Key: +, ongoing activity; -, no ongoing activity; ?, no available information; A, accepted after validation; R, rejected after validation; U, under review; V, validated

# 4 Positions, Views and Current Research Programmes of Organisations Involved in Developing Alternative Tests

For each organisation contacted in the questionnaire survey exercise (see Section 1.3.2), a review of their Web site was undertaken to supplement any information they provided, and to obtain available information on those organisations unable to respond within the available time frame.

The following organisations responded to the survey:

British Union for the Abolition of Vivisection (BUAV)

Centre for Documentation and Evaluation of Alternative Methods to Animal Experiments (ZEBET)

European Centre for the Validation of Alternative Methods (ECVAM)

European Research Group for Alternatives in Toxicity Testing (ERGATT)

Fund for the Replacement of Animals in Medical Experiments (FRAME)

John Hopkins Centre for Alternatives to Animal Testing (CAAT)

Nordic Information Centre for Alternative Methods (NICA)

Royal Society for the Prevention of Cruelty to Animals (RSPCA)

The views of the organisations surveyed are summarised below.

## Belgium Platform for Alternative Methods (BPAM)

This group was officially established in 1997 in Brussels, Belgium. Represented in this group are individuals from academia, industry, animal welfare groups and government. The main aims of BPAM are to fund research into the development and validation of alternative methods at the national level, to provide information, and to promote *in vitro* research and methodology. The main focus is on developing *in vitro* cultures, xenobiotic metabolic systems and novel *in vitro* toxicity tests.

BPAM achieves these goals through the organisation of meetings and provision of funding for related research. BPAM did not respond to the questionnaire.

## British Union for the Abolition of Vivisection (BUAV)

BUAV is co-ordinating an EU-wide campaign to challenge the continued use of animals in chemical testing within the EC's proposed chemical strategy. As part of this campaign, BUAV has concentrated on the potential of in vitro methodologies to replace traditional in vivo experiments. It has published a report *The Way Forward — a non-animal testing strategy for chemicals* (BUAV, 2001) containing an analysis of the current status of in vitro methods, and has made the key recommendation that the Commission must ensure sufficient funding and resources for the targeted and fast-track validation of

in vitro methods in order for them to replace in vivo methods in the timescales required by the proposed EU chemicals strategy.

BUAV considers the research industry to be largely to blame for the lack of progress towards *in vitro* testing to date, and that it is therefore unnecessary to consider the implications for the contract research community of the proposed changes. BUAV believes that financial, administrative and technical hurdles are too easily used by industry as an excuse for inaction, and that there should be a change in the scope of thinking, not just by industry but by national governments and EU institutions. BUAV considers nonetheless that, in the UK at least, most major contract testing companies already possess the whole range of *in vitro* test systems.

# Centre for Alternative and Complementary Methods in Animal Experiments (ZET)

ZET was established in 1996 in Linz, Austria. The Centre aims to develop, improve and validate alternative methods using the 'three Rs' approach, and seeks to participate in discussions at the national and international level, as well as providing information to interested parties.

The centre has produced several publications, plus a congress series 'Austria International Congress' on alternative and complementary methods to animal experiments. ZET did not respond to the questionnaire.

# Centre for Documentation and Evaluation of Alternative Methods to Animal Experiments (ZEBET)

ZEBET was established in Berlin, Germany in 1989. Its main areas of activity are the promotion of the use of alternative methods as replacements for animal testing and the development and validation of these methods. ZEBET provides an information service (ZIS), a Web-based database on alternative tests. Since 1990, ZEBET has funded numerous research projects on alternative test methods. In addition, ZEBET undertakes validation studies of *in vitro* methods in co-operation with ECVAM and the European Cosmetic, Toiletry and Perfumery Industry (COLIPA); this has included the validation of an alternative to *in vivo* eye tests, the HET-CAM test, and of *in vitro* models of phototoxicity. ZEBET also managed the validation of *in vitro* embryo toxicity tests on behalf of ECVAM.

This organisation has developed strategies to incorporate *in vitro* methods into the regulatory testing of chemicals. ZEBET is collaborating with ECVAM, and two scientists from ZEBET are active on an ECVAM working group relating to the EU Chemicals Policy.

# The Doctor Hadwen Trust*

The Dr Hadwen Trust funds non-animal research into major health problems such as cancer, heart disease, meningitis and Alzheimer's disease. None of the Trust's research uses animals or animal tissues, and all of it contributes to the replacement of animals whilst furthering research into human medical problems.

The respondent to the survey questionnaire commented that the possibility and timescales for developing and validating alternative test methods depended largely on the political will to achieve

*Contacted through BUAV

those goals. The Trust believes that there are many specific areas where development and validation are required, but estimates that a focused, fully funded, high priority programme could achieve functional tests in most areas within five years. They are also of the opinion that the EU test requirements should not be based on OECD test guidelines development programme, since the OECD, with more than 30 member states, operates by consensus and they consider that new method acceptance is cumbersome, unwieldy and totally irrelevant to the EU's proposed timescales. For example, the Trust believes that a method which has been accepted as formally validated in one member state or even within Europe as a whole, may take two to three years to be adopted by the OECD, while a method with considerable scientific backing but no formal validation could take up to seven years. They also expressed the concern that the OECD process was not dealing even-handedly with new animal tests versus new non-animal tests. They pointed out that the EU has given regulatory approval to some *in vitro* methods (e.g. the 3T3 photo-irritancy test, the human skin equivalent corrosivity method, the TER corrosivity assay) and should therefore, under Directive 86/609 and the mutual acceptance of data between member states, no longer permit the animal equivalents to be performed. Hence, for the EU chemicals policy, the Trust believes that the standard of what is valid and approved should be based on EU-definitions rather than those of the OECD.

# European Centre for the Validation of Alternatives (ECVAM)

ECVAM was established in 1991 at the Joint Research Centre, Ispra, Italy, and its main area of interest is the development and validation of alternative methods and *in vitro* tests, and the development of related strategies for testing industrial chemicals, cosmetics and vaccines. In particular, ECVAM seeks to co-ordinate the validation of test methodology at the EU level, to facilitate exchange of information through workshops, publications and databases, and to promote dialogue between industry, legislators and animal welfare groups. To date, ECVAM's activities have resulted in the validation and acceptance of several *in vitro* tests for regulatory purposes, 45 published works from organised workshops and numerous task forces, collaborations and development projects covering a multitude of toxicity tests.

ECVAM has responded to the EC White Paper by setting up the ECVAM Scientific Advisory Committee (ESAC). This group is addressing the immediate, medium-term and long-term possibilities for replacing animal testing at the Base set level, and has produced a proposal for a tiered *in vitro*/*in vivo* approach to testing. Although ESAC is not currently considering the possibilities for reduction and refinement of the animal tests used at Levels 1 and 2, ECVAM considers that this should be given serious consideration within the context of the White Paper.

# European Research Group for Alternatives in Toxicity Testing (ERGATT)

This group, based at Utrecht University, The Netherlands, was established in 1985. Its membership comprises experts in the field of *in vitro* toxicity testing. The principal objective is to stimulate innovative concepts and new practical approaches to toxicity testing, in the belief that greater understanding of the underlying toxic mechanisms will improve the quality of safety evaluation. The main areas of activity are the development, evaluation and validation of *in vit*ro methods. The group seeks to promote dialogue with groups and organisations with relevant interests, offers consultancy on *in vitro* methodologies, publishes reviews and test guidelines and also organises workshops. A Web-based database, INVITTOX, has been developed.

The group is represented on the ECVAM advisory committee and is involved with ECVAM and ICCVAM in discussions on the validation and implementation of non-animal based tests into

regulatory toxicity testing. ERGATT has also been involved in developing risk assessment methodology. In a collaboration sponsored by the EU, the ECVAM/CFN (Swedish Board for Laboratory Animals) Integrated Toxicity Test Scheme (ECITTS), an integrated approach to predicting systemic toxicity by use of computer-based biokinetic models and biological *in vitro* test methods, was developed in order to further develop the prediction of toxicity of chemicals by integrating the knowledge obtained from non-animal studies (e.g. *in vitro* assays) with physiologically-based biokinetic models derived from *in vitro* or other non-animal experiments.

ERGATT believes that there is considerable opportunity to improve the evaluation of chemical hazard and risk assessment by the introduction of a more structured and sophisticated process than that using current EU strategies and regulatory guidelines. Although accepting that OECD guidelines have served an important goal in harmonising toxicity testing over the last decades, ERGATT believes that the opportunity has now arisen to base risk assessment on a structure that incorporates advancements made in toxicology in recent years. These proposals have been published and are under discussion within the EU.

# Fund for the Replacement of Animals in Medical Experiments (FRAME)

FRAME was established in 1969 in Nottingham, UK. The aim of this group is to promote the replacement of *in vivo* experiments through the gradual introduction of scientifically valid and acceptable alternative methods, whilst promoting progress in reduction and refinement until replacement is achievable. As such, the group's main areas of interest are the development, assessment, validation and promotion of new *in vitro* techniques through the funding of research, organisation of workshops, and production of a bimonthly journal, and input into the Web-based database INVITTOX. In this manner, FRAME has developed several guidelines now used for *in vitro* toxicity testing and has participated in several successful validation exercises. FRAME's current activities include laboratory-based studies investigating alternative *in vitro* methods for all the major aspects of mammalian toxicity, as well as desk-based work investigating methods of refining test methods so as to allow the reduction of numbers and species used in toxicity testing. FRAME also actively seeks legislative and regulatory reform.

In response to our survey, the Director of FRAME declared his concern that the lack of a strategic framework for undertaking the testing of existing chemicals would prevent the EU completing the proposals in the White Paper. In particular, he remarked that the current assessment process, which relies on *in vivo* testing, would take too long. However, he believes that an alternative prioritising/screening approach employing alternative techniques might well allow testing to be completed by the 2012 target.

# Institute for *In Vitro* Sciences (IIVS)

Previously the '*In Vitro* Toxicology Division' of Microbiological Associates, IIVS was established in 1997 in Gaithersburgh, US and has approximately 10 staff. The Institute is a non-profit organisation promoting the development of *in vitro* testing methods and their acceptance and use. Its main areas of interest are testing for skin and ocular toxicology, bioassay development, and the pre-validation and validation of methodologies. The Institute offers testing to industry and government and organises workshops and provision of information for interested parties. The IIVA did not respond to the questionnaire.

# Interagency Co-ordinating Committee on the Validation of Alternative Methods (ICCVAM)

ICCVAM was established in the USA in 1997 to develop and validate new test methods, and to establish criteria and processes for the regulatory acceptance of toxicological testing methods within the US. The committee comprises representatives from 15 federal regulatory and research agencies and seeks to generate, use, or provide information from toxicity test methods for risk assessment purposes, as well as to co-ordinate cross-agency issues relating to the development, validation, acceptance, and national/international harmonisation of toxicological test methods. The National Toxicology Program (NTP) Interagency Centre for the Evaluation of Alternative Toxicological Methods (NICEATM) was established in 1998 to provide operational support for ICCVAM, and to undertake related activities such as peer review and the hosting of workshops. NICEATM and ICCVAM thus co-ordinate the scientific review of validations of proposed methods, and make recommendations on their usefulness. The ultimate goal of this work is the validation and regulatory acceptance of methods that are most predictive of adverse human and ecological effects.

Recent activities include the validation of the mouse LLNA for skin sensitisation (validated in 1998, accepted into regulatory guidelines 1999), validation of CORROSITEX for skin corrosion (accepted into regulatory guidelines in 2000), and, in 2000, a review of FETAX (for which further improvement was recommended). The in vivo Up and Down procedure for acute systemic toxicity was also reviewed in 2000, and incorporated into US regulatory guidelines. A battery of in vitro tests of relevance to assessing acute systemic toxicity (MEIC) was evaluated in 2001 resulting in a recommendation for further development. ICCVAM has also recently recommended adoption of the three in vitro skin corrosivity test methods that are accepted by the EU. Currently ICCVAM and NICEATM are reviewing in vitro screening methods for EDCs. ICCVAM did not respond to the questionnaire.

# John Hopkins Centre for Alternatives to Animal Testing (CAAT)

CAAT was founded in 1981 to develop the basic scientific knowledge needed to develop *in vitro* methods for the safety evaluation of commercial products. Its main areas of interest and activity are the funding of projects that follow the 'three Rs' strategy and assisting in the exchange of information through publications, workshops and teaching. The centre also interacts with ICCVAM, OECD, ECVAM and EDA advisory committees.

CAAT has made a major contribution to the 'alternatives' movement through partial responsibility for the development of several *in vitro* methods now in use and as a leading source of funding for alternatives research in the United States. It has regularly brought together academic and industrial scientists, animal welfare organisations and the government regulatory community for discussion of common ground, through workshops and symposia, and has also developed a Web site, Altweb, that is devoted to alternative testing methodologies. The site differs from almost all others in its scope and audience, providing information for both scientists and schoolchildren, and the Centre has participated in every US government-related activity addressing alternatives, including the FDA's Sub-Committee on Toxicology, the Interagency Coordinating Committee for the Validation of Alternative Methods (ICCVAM), and the National Academy of Science's Institute on Animals.

CAAT staff focus on three critical activities: funding new research, disseminating information on alternative methods, and creating a forum in which industry, regulatory agencies and the animal welfare community can work together. The centre supports the combining of studies that use large numbers of animals in ways in which the total number of animals used on a test can be reduced.

Vision 20/20 is CAAT's vehicle for promoting such developments across society; it brings together leaders from industry, government, academia, and the animal welfare movement, as well as the alternatives field, to identify areas in which CAAT and others may increase the rate of progress. In 1999, the Vision 20/20 Program introduced TestSmart, a new approach to risk assessment intended to provide a new model for toxicology that aims to be both more humane and more predictive. To achieve this, the Centre has worked with both the US regulatory community and industry to identify novel, improved testing methods. The short-term goal is to reduce the number of animals required for testing, while the long-term goal is to develop novel systems to improve the protection of people and the environment. CAAT is currently developing TestSmart-Pharmaceuticals and TestSmart-Endocrine Disruptors.

# Netherlands Centre for Alternatives to Animal Use (NCA)

NCA is a non-profit organisation established in 1994 in Utrecht, The Netherlands. The aims of the centre are to promote the development and acceptance of alternative toxicity tests at the national level within the Netherlands. This has involved the organisation of symposia and workshops, publications in peer-reviewed journals, and the development of alternative tests. In addition, the organisation has developed a database of alternative methods. The NCA did not respond to the questionnaire.

# Nordic Information Centre for Alternative Methods (NICA)

NICA was established in Sweden in 1997 to promote the implementation of *in vitro* tests into regulatory toxicity, and to provide information on alternative methods. Its main activities have been the evaluation and collation of data from a multicentre evaluation of *in vitro* cytotoxicity, and the development of have also designed Web-based databases. NICA's director is also the co-ordinator of the EDIT project, initiated in 1998 as a six-year effort by international laboratories to develop new *in vitro* replacement tests for toxicity and toxicokinetics. The intention is that such tests are used to optimise the batteries used to assess acute and chronic systemic toxicity (see Section 2.2 and 3.2).

# Royal Society for the Prevention of Cruelty to Animals (RSPCA)

The RSPCA is not directly involved in the development or validation of alternative methods, although it does support research into developing *in vitro* models of potential relevance to regulatory testing. Close links exist with organisations such as FRAME and ECVAM, and the Society has representation on a number of their committees. The RSPCA is a member of the group *Focus on Alternatives*, which also includes FRAME, the Dr Hadwen Trust, the Humane Research Trust, The Lord Dowding Fund and the St Andrew Animal Fund, all organisations that fund *in vitro* research.

The RSPCA has been very concerned about the implications of the proposed new European chemicals strategy and has expressed its concerns to the House of Lords Committee on this issue. The main thrust of the Society's policy is the deployment of what can be called 'strategic alternatives', rather than seeking test-by-test replacement of the individual Base set, Level 1 and 2 tests, with the intention of achieving full use of validated alternatives and ensuring a substantial commitment to further research and validation of non-animal tests. In particular, the RSPCA is totally opposed to a "mindless, box-ticking approach" such as they consider implicit in the proposed REACH system. Instead, they would want a thoughtful approach, taking account of all existing information, which

would eliminate the need to undertake a large proportion of the animal testing, and a clear statement of risk management policy to discourage industry from pursuing 'lost causes' (e.g. chemicals that are persistent, bioaccumulative and toxic (PBT)).

In addition to their response to the House of Lords Committee, the RSPCA has produced a number of relevant documents including contributions to position papers from the Eurogroup for Animal Welfare and Focus on Alternatives.

# Spanish Speciality Group on Alternative Methods (GTEMA)

GTEMA was formally established in 1995 and comprises approximately 70 active members drawn from Spanish research groups. The major focus is on cellular and organ toxicity, toxicokinetics and the development of model systems. The main objectives are to seek to develop alternative models in line with the 'three Rs' approach and to promote co-operation between the various member groups. The Group also promotes the participation of Spanish scientists in pre-validation and validation programmes and the regulatory acceptance of alternative methods. GTEMA did not respond to the questionnaire.

# 5 Discussion

The limited scope of this review must be stressed. It is clearly impractical in an overview report of this nature to fully discuss the rationale behind the currently established risk assessment processes or the background to, scientific basis of, or the objectives, methodology and predictivity of each of the existing *in vivo* or *vitro* toxicological tests required by EU legislation for the assessment of the hazard potential of industrial chemicals. However, it is apparent that, particularly for those chemicals tested at the higher levels, the existing scheme addresses a very wide range of potential endpoints in some detail, and provides a high level of reassurance as to the toxic potential of a chemical with regard to both human health and wildlife effects. Similarly, it was impractical to attempt to assess the scope, practicality, predictivity, validity and limitations of each of the multitude of individual computer models or *in vitro* test systems that have been proposed as possible replacements for the existing *in vivo* test methods, much less to establish the relative value of the various test batteries that have or are being developed. Nonetheless, on the basis of the available published literature, and in the light of the information received from various organisations with an interest in the field of alternative test development, a number of general observations can be made with some confidence.

There now appears to be general acceptance of, and active support for, the 'three Rs' approach to developing improved or alternative test methods across the range of stakeholders, including regulators, non-governmental organisations and industry. However, differences of opinion still exist between stakeholders as to the extent to which animal testing can, if ever, be completely replaced by alternative methodologies. To date, significant progress towards the objectives of the 'three Rs' approach has been made in a number of areas of toxicity testing, particularly with regard to the regulatory acceptance of more refined acute toxicity tests (that have significantly reduce animal usage and suffering) and the availability of validated non-animal methods to act as a screen for assessing dermal and ocular corrosive or severe irritancy potential. Also, it is now accepted that the available *in vitro* methods for assessing mutagenic potential are in themselves sufficiently predictive as to preclude the use of *in vivo* studies in the majority of cases. This latter aspect, in turn, may have consequences for the study of genotoxic carcinogenic potential. However, despite some claims to the contrary, there appear to be no reliable methods yet available that can assess non-genotoxic carcinogenic potential, nor are there *in vitro* methods which can adequately address all the toxic endpoints considered by *in vivo* acute or repeat-dose toxicity studies, or that allow a full characterisation of reproductive toxic potential. It thus appears that, for the foreseeable future, it will continue to be necessary to assess acute, sub-chronic and chronic toxicity, non-genotoxic carcinogenicity and, particularly, the reproductive toxicity of individual chemicals principally through the use of *in vivo* tests. Nonetheless, there is certainly scope for refinement and reduction strategies to be applied to the existing test designs, to optimise methods to reduce animal usage and limit the degree of suffering. There are also promising developments in the design of *in vitro* test batteries, which suggest that it may be possible to utilise such test systems as an early screening tier to detect overtly toxic chemicals before they reach the definitive *in vivo* testing stage.

Focusing specifically on the tests required at Base set, alternative tests (including replacement and/or refinement techniques) are now available for use in relation to genotoxicity and as screens for corrosion or severe irritation of the skin. Work is also at an advanced stage to determine whether *in vitro* models are also suitable for use as definitive tests for skin irritation. *Ex vivo* ocular irritation and corrosion tests are now accepted as pre-screening tests in several EU member states, with *in vivo* testing only being required in the event of negative findings in these alternative tests, and strenuous efforts should continue to be made to incorporate them into the EU-wide regulatory process as soon as possible. In addition, the LLNA, a refinement/reduction test, has been recommended for adoption by the OECD and EU to assess sensitisation potential; *in vitro* replacement models are yet to be validated for use. *In vitro* alternatives for assessing acute systemic toxicity are currently only at a very early

stage of development, although promising progress is being made, suggesting that a battery of tests capable of acting at least as a screen to detect the most toxic chemicals may become available within the timescale defined in the White Paper. Screens for the 28-day repeat dose rodent study and the acute fish toxicity test are still under development.

The length of time it would take to complete validation and adoption of the alternative tests currently at the development or validation stage, or to design further novel systems, is uncertain, being to a large extent dependent on the level of resource made available. Historically, proposed reduction, refinement or replacement changes have taken 10 to 20 years from the date of first publication to regulatory adoption (for example, the LLNA and the Ames Test). More recently, human skin model assays, such as the EpiDerm™ and Episkin™ assays, first reported as potential alternatives to *in vivo* skin corrosivity testing by Calvin in 1992, were adopted into EU regulatory guidelines in 2001.

Overall, it appears that it should be possible to validate *in vitro* test batteries capable of providing a reasonable level of reassurance with regard to skin/ocular corrosivity and irritation potential, and to apply these tests as a modified Base set package for chemicals produced at 1 to 10 tonnes/annum, within the timescale envisaged in the EC White Paper. It may also be possible to develop an initial screening system using *in vitro* models for skin sensitisation. However, on the basis of existing knowledge, it is not possible to define a realistic timescale in which the complete replacement of *in vivo* tests for acute, repeat-dose toxicity at this level could be achieved, although it may be possible to develop a screening system capable of identifying at least the most toxic chemicals before recourse to whole animal models. Potentially, therefore, the proposed restriction of testing of the low production volume chemicals to solely an *in vitro* test battery will necessitate the acceptance that, for such chemicals, these latter important aspects of toxicity would not be addressed.

For those chemicals with volumes of 10 to 100 tonnes/annum that will be tested for the full range of toxicities assessed in the existing Base set, it seems unlikely that replacement methods will be developed in time to allow the adoption of a non-animal based approach. In addition, at Levels 1 and 2, little progress appears to have yet been made in revising the existing test guidelines to incorporate reduction or refinement strategies into the assessment of repeat dose or reproductive toxicity and carcinogenicity, and it seems unlikely that significant 'three Rs' developments will take place within the timescales envisaged in the EC White Paper to significantly reduce the level of animal use for the testing of chemicals produced at Level 1 or 2 tonnages.

It should be noted that a number of tests relating to some aspects of endocrine disruption are close to validation, even though the development programmes were initiated less than five years ago. This is a reflection of the intense activity at the national level and the massive international funding, co-operation and effort by agencies such as the EU and US authorities and the OECD. While highlighting what can potentially be achieved with a suitable level of commitment, it should also be noted that these are *in vivo* models and hence will probably lead to an increase in animal usage (see Section 3.3).

One aspect that was considered of particular concern by a number of the organisations consulted was the lengthy delay experienced between the adoption of a particular test by one competent authority and its general acceptance by other national, international or global bodies. For example, a number of the *in vitro* tests now adopted under Annex V of the EU Directive 67/548/EEC have still to be accepted by the wider international community. Continued delays in adoption of new tests will potentially lead to situations in which there is a duplication of testing of chemicals to different guidelines, thus potentially leading to an increase in the number of animals used and the cost of assessment, rather than the intended reduction in animal usage and, potentially, costs. It is therefore important that mechanisms be developed to ensure the rapid review and acceptance of the 'three Rs' tests on a global basis if such waste is to be prevented. Clearly, the activities of the OECD are likely to play a key role in achieving this objective.

Finally, it is important to note that this review has focused specifically on the testing of industrial chemicals and should not be taken to be of relevance to the testing regimen that is appropriate or

necessary for the regulatory control of other classes of chemical, such as pharmaceutical and agrochemical products, where the uses, biological activities, and intended or accidental exposures are quite different.

# 6 Conclusions

Following the general acceptance of the 'three Rs' approach to test development, significant progress has been made in the refinement and reduction of animal usage, and in the development of replacement non-animal alternatives, in a number of important areas of toxicology. However, there remain a number of key areas (particularly the identification of non-genotoxic carcinogens and the assessment of repeat dose and reproductive toxicity) where it is unlikely that suitable alternatives to replace the use of animals will be developed within the foreseeable future. It should, however, be possible to reduce levels of animal usage through the development of initial screening batteries, thereby allowing the identification of overtly toxic chemicals at an early stage, and by refinement of existing *in vivo* test designs.

With specific regard to the proposals on the regulation of chemicals in the EC White Paper, it appears that, for those produced at 1 to 10 tonnes/annum, it may be possible to develop a package of non-animal based tests to assess a range of toxic endpoints within the timescale envisaged by the White Paper, provided sufficient funding and resources are made available. In addition, the possibility that *in vitro* models may become available that can address aspects of embryo/fetal development is to be welcomed, particularly since there are no *in vivo* or *in vitro* tests currently available to address any aspect of reproductive toxicity at the Base set level. However, there appears little realistic prospect of any screen being developed that could adequately assess all aspects currently covered by the *in vivo* acute and 28-day toxicity studies. There is thus a need to consider if this reduction in scope of the testing strategy, compared with the current Base set requirements, is acceptable for this category of chemical.

It is considered unlikely that significant replacement of Level 1 and 2 tests will be possible within the foreseeable future, and certainly not within the timescales proposed for completion of testing of the higher production volume chemicals within the White Paper. Nonetheless, reduction and refinement strategies should be actively encouraged, particularly since *in vivo* tests for some aspects of endocrine disruption are now nearing the end of the validation and adoption process, and will result in a consequent increase in animal usage.

In order to prevent the avoidable use of animals, it is important to develop mechanisms whereby validated alternative test models are accepted by all competent regulatory authorities as quickly as possible, to prevent the use of animals where suitable *in vitro* methods exist and to prevent needless duplication of tests to meet differing international requirements.

# Bibliography


BUAV (2001) The way forward – Action to end animal toxicity testing, London, UK, The British Union for the Abolition of Vivisection

Calvin G (1992) New approaches to the assessment of eye and skin irritation. *Toxicol Letts*, *64/65*, 157–164

CAAT (1999) *TestSmart – a humane and efficient approach to Screening Information Data Sets (SIDS) Data.* US, Center For Alternatives To Animal Testing, Johns Hopkins University. Available [4.12.01] at caat.jhsph.edu/programs/workshops/testsmart/hpv-intro.htm

COM (2000) Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment *Guidance on a Strategy for Testing of Chemicals for Mutagenicity.* UK, Department of Health. Available [04/12/01] at: http://www.doh.gov.uk/com/guidance.pdf

European Commission (1993) *Council Regulation (EEC) No 793/93 of 23 March 1993 on the evaluation and control of the risks of existing substances.* Available [04/12/01] at: http://ecb.ei.jrc.it/Directives/793_93.htm

European Commission (1967) *EU Directive 67/548/EEC on the classification, packaging and labelling of dangerous substances.* Brussels, Belgium, EU DG Environment

European Commission (1986) *EU Directive 86/609/EEC 86/609/EWG on the use of experimental animals.* Brussels, Belgium, EU DG Environment

European Commission (2000a) *Annex V of the EU Directive 67/458/EEC for classification and labelling of hazardous chemicals*. Brussels, Belgium,. EU DG Environment

European Commission (2000b) EU Directive 2000/33/EU for the 21st Amendment of Annex V of the EU Directive 67/458/EEC for classification and labelling of hazardous chemicals: Test guideline B.40 'Skin Corrosion'. *O. J. European Communities, June 8 2000, L136*, 98–107

European Commission (2000c) EU Directive 2000/33/EU for the 21st Amendment of Annex V of the EU Directive 67/458/EEC for classification and labelling of hazardous chemicals: Test guideline B.41 'Phototoxicity - *in vitro* 3T3 NRU phototoxicity test'. *O. J. European Communities*, *L136*, 98–107

European Commission (2001a) *White Paper: Strategy for a future chemicals policy.* Brussels, Belgium, Commission of the European Communities, 27.2.2001 COM (2001) 88 final. Available [09/03/01] at: http://www.europa.eu.int/comm/environment/chemicals/0188_en.pdf

European Commission (2001b) *Implementation of the Community Strategy for Endocrine Disrupters – a Range of Substances Suspected of Interfering with the Hormone Systems of Humans and Wildlife*. Brussels, Belgium, Commission of the European Communities. COM (2001) 262. Available [26/10/01] at: http://www.europa.eu.int/comm/environment/docum/01262_en.htm

ECVAM (2000) Statement on the scientific validity of the local lymph node assay for skin sensitisation testing. *ATLA, 28*, 365–367

EDSTAC (1998) *Endocrine disruptor screening and testing advisory committee Final Report*. US Environmental Protection Agency. Available [04/12/01] at: http://www.epa.gov/scipoly/oscpendo/history/coverv14.pdf

EPA (2000) *Endocrine Disruptor Screening Programme Report to Congress August 2000*. US Environmental Protection Agency. Available [04/12/01] at: http://www.epa.gov/scipoly/oscpendo/reporttocongress0800.pdf

Fentem JH & Balls M (1997) Application of the 'three Rs' in toxicology. *Comments Toxicology*, *6*, 5–20

Gad SC (1990) Recent developments in replacing, reducing and refining animal use in toxicological research and testing. *Fundam Appl Toxicol, 15*, 8–16

Green S, Goldberg A & Zurlo J (2001) TestSmart-high production chemicals: an approach to implementing alternatives into regulatory toxicology. *Toxicol Sci, 63*, 6–14

IEH (2001) *Testing Requirements for Proposals under the EC White Paper 'Strategy for a Future Chemicals Policy'* (Web Report W6). Leicester, UK, Institute for Environment and Health, Available [04/12/01] at: http://www.le.ac.uk/ieh/webpub/webpub.html

INVITTOX protocols (2001) Nottingham, UK, FRAME. Available [10/10/01] at: http://www.frame-uk.demon.co.uk/invittox.htm

Jelinek R, Peterka M & Rychter Z (1985) Chick embryotoxicity screening test – 130 substances. *Indian J Exper Biol, 23*, 588–595

NICEATM (2001) *Episkin™, EpiDerm™, and Rat Skin Transcutaneous Electrical Resistance (TER).* In Vitro *Test Methods for Assessing the Dermal Corrosivity Potential of Chemicals: Background Review Document.* Research Triangle Park, NC, 27709, US, The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods

OECD (1981) *Decision of the Council concerning the Mutual Acceptance of Data in the Assessment of Chemicals* [C(81)30(Final)]. Organisation for Economic Co-operation and Development. Available [12.12.01] at: http://www1.oecd.org/ehs/glplcal.htm

OECD (1996) *Final report of the OECD workshop on harmonization of validation and acceptance criteria for alternative toxicological test methods*, ENV/MC/CHEM/TG(96)9. Organisation for Economic Co-operation and Development

OECD (1999) In Vitro *Skin Corrosion Tests (Draft Proposal for New Guideline, November, 1999).* Organisation for Economic Co-operation and Development. Available [04.12.01] at: http://www1.oecd.org/ehs/test/skincorrosion.pdf

OECD (2000a) *Guidance document on the recognition, assessment and use of clinical signs as human endpoints for experimental animals used in safety evaluation.* ENV/JM/MONO(2000)7. Organisation for Economic Co-operation and Development. Available [04/12/01] at: http://www1.oecd.org/ehs/test/Mono7.pdf

OECD (2000b) *Draft New Guideline 428: Skin Absorption: In Vitro Method (Draft Document, December 2000).* Organisation for Economic Co-operation and Development. Available [04.12.01] at: http://www1.oecd.org/ehs/test/health.htm [04/12/01]

OECD (2001a) *Episkin™, EpiDerm™ and Rat Skin Transcutaneous Electrical Resistance. In vitro methods for assessing the dermal corrosivity potential of chemicals. Background review document, ENV/JM/TG(2001)7.* Organisation for Economic Co-operation and Development. Website page: http://iccvam.niehs.nih.gov/methods/epiddocs/epis_brd.pdf [04/12/01]

OECD (2001b) *Guidelines for Testing of Chemicals*. Paris, France, OECD Publication Office. *[see appended list of individual test guidelines]*

Pfaller W, Balls M, Clothier R, Coeck S, Dierickx P, Ekwall B, Hanley BA, Hartun T, Prieto P, Ryan MP, Schmuck G, Sladowski D, Vericat J-A, Wendel A, Wo A & Zimmer J (2001) Novel advanced in vitro methods for longer toxicity testing. The report and recommendations of ECVAM Workshop 45. *ATLA, 29*, 393–426

Prati M, Giavini E & Menegola E (1993) Alternatives to *in vivo* tests for teratologic screening. *Annali Dell'Instituto Superiore Di Sanita, 29*, 41–46

Robinson MK, Osborne R & Perkins MA (2000) *In vitro* and human testing strategies for skin irritation. *Ann N Y Acad Sci, 919*, 192–204

Russell WMS & Burch RL (1959) The Principles of Humane Experimental Technique. London, UK, Methuen

de Silva O, Basketter DA, Barratt MD, Corsini E, Cronin MTD, Das PK, Degwert J, Enk A, Garrigue JL, Hauser C, Kimber I, Lepoittevin JP, Peguet J & Ponec M (1996) Alternative methods for skin sensitisation testing. The report and recommendations of ECVAM Workshop 19. *ATLA, 24*, 683–705

# OECD Test Guidelines

| Test | Title | Status |
|---|---|---|
| 210 | Fish, Early-Life Stage Toxicity Test | Original Guideline, adopted 17th July 1992 |
| 401 | Acute Oral Toxicity | Updated Guideline, adopted 24th February 1987 |
| 402 | Acute Dermal Toxicity | Updated Guideline, adopted 24th February 1987 |
| 405 | Acute Eye Irritation/Corrosion | Updated Guideline, adopted 24th February 1987 |
| 407 | Repeated Dose 28-day Oral Toxicity Study | Updated Guideline, in Rodents adopted 27th July 1995 |
| 410 | Repeated Dose Dermal Toxicity: 21/28-day Study | Original Guideline, adopted 12th May 1981 |
| 412 | Repeated Dose Inhalation Toxicity: 28-day or 14-day Study | Original Guideline, adopted 12th May 1981 |
| 416 | Two-Generation Reproduction Toxicity Study | Updated Guideline, adopted 22nd January 2001 |

| | | |
|---|---|---|
| 420 | Acute Oral Toxicity - Fixed Dose Method | Original Guideline, adopted 17th July 1992 |
| 421 | Reproduction/Developmental Toxicity Screening Test | Original Guideline, adopted 27th July 1995 |
| 422 | Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test | Original Guideline, adopted 22nd March 1996 |
| 423 | Acute Oral toxicity - Acute Toxic Class Method | Original Guideline, adopted 22nd March 1996 |
| 425 | Acute Oral Toxicity: Up-and-Down Procedure | Original Guideline, adopted 21st September 1998 |

# Annex 1

## Animal (vertebrate) usage in Base set, Level 1 and Level 2 studies

| | Number of test groups (minimum) | Number of animals (minimum) |
|---|---|---|
| **Base set (for 1-100 tonnes pa)** | | |
| Acute oral toxicity – fixed dose procedure | | 12 per chemical |
| Acute dermal irritation/corrosion | | 3 per chemical |
| Acute eye irritation/corrosion | | 3 per chemical |
| Skin sensitisation | | 30 per chemical |
| Repeated dose 28-day oral toxicity study in rodents | 3 + control | 10 per group |
| Reproduction/developmental toxicity screening test | 3 + control | 20 per group |
| Acute toxicity in fish | 5 + control | 7 per group |
| **Level 1 (for 100–1000 tonnes pa)** | | |
| Fertility and general reproduction toxicity study | 3 + control | 20 per group |
| Teratogenicity study (rodent) | 3 + control | 20 per group |
| Repeated dose 90-day oral toxicity study | 3 + control | 20 per group |
| Bioaccumulation in fish | Approx. 4 | 80 per group |
| 14-day prolonged fish toxicity | 5 + control | 7 per group |
| **Level 2 (for 1000+ tonnes pa)** | | |
| Chronic toxicity studies | 3 + control | 40 per group |
| Carcinogenicity studies | 3 + control | 100 per group |
| Peri- & postnatal reproduction toxicity study | 3 + control | 20 per group |
| Teratogenicity study (non-rodent species) | 3 + control | 12 per group |
| Toxicokenetics (biotransformation & pharmacokenetics) | 2 + control | 20 per group |
| Organ or system toxicological study (additional) | Not definable | Not definable |
| Early life stage fish toxicity test | 5 + 2 control | 60 eggs |

Base set, Level 1 and Level 2 tests were cited from OECD test assessment guidelines (OECD, 2001b)

# Annex 2

## Organisations contacted

Belgium Platform for Alternative Methods
VUB Laarbeeklaan 103
B1090
Brussels
Belgium

ZET
Universitat Graz und Zet
Institut fur Medizinische, Physik und Biophysik
Harrachgasse 21
A-8010 Graz
Austria

ZEBET at the BgVV
Diedersodorfer Weg 1
D-12277 Berlin
Germany

European Centre for the Validation of Alternatives
JRC Environment Institute
21020 Ispra (VA)
Italy

European Research Group for Alternatives in Toxicity Testing
RITOX
Utrecht University
P O Box 80.178
3508 TD Utrecht
The Netherlands

Fund for the Replacement of Animals in Medical Experiments
Russell and Burch House
96-98 North Sherwood Street
Nottingham, UK

Institute for *In Vitro* Sciences
21 Firstfield Road
Suite 220
Gaithersburg
MD 20878
USA

Interagency Co-ordinating Committee on the Validation of Alternative Methods
National Institute of Environmental Health Sciences
P O Box 12233
MD EC-17
Research Triangle Park
NC 27709
USA

The John Hopkins Centre for alternatives to Animal Testing
111 Market Place
Suite 840
Baltimore
MD 21202-6709
USA

Netherlands Centre for Alternatives to Animal Use
Yalelaan 17
NL-3584 CL Utrecht
The Netherlands

Nordic Information Centre for Alternative Methods
Vintervagen 17
SE-182 74 Stocksund
Sweden

Royal Society for the Prevention of Cruelty to Animals
Wilberforce Way
Southwater
Horsham
West Sussex RH13 7WN

Spanish Specialty Group on Alternative Methods
National Institute of Toxicology
P O Box 863
41080 Sevilla,
Spain

# Annex 3

## Questionnaire facsimile

# Fax Transmission

| | |
|---|---|
| **TO:** | **FROM:** |
| **AT:** | **DATE:** |
| **FAX NO:** | **TIME:** |
| **PAGES:** 3 **(INCLUDING THIS ONE)** | **REF:** IEH3/13/5E |

**MESSAGE**

Dear ,

### Alternative *in vitro* toxicity tests for regulatory purposes

In February this year the European Commission (EC) published a White Paper[a] proposing a significant revision to policy on chemical regulation within Europe. As you are no doubt aware, this has led to a vigorous debate within the European scientific, regulatory and political communities as to the costs, benefits and practicality of the proposed approach. As a contribution to the discussion, our Institute made a submission to the House of Lords Select Committee's inquiry into the implications of the EC's Strategy for a future chemicals policy, and has also conducted a review on behalf of the (then) UK Department of the Environment, Transport and the Regions into the potential implications in terms of financial cost and animal usage of the proposed changes to test requirements[b].

Following this work, we have been asked, by both the UK House of Lords Committee and the UK Department of the Environment, Food and Rural Affairs (DEFRA), to consider and comment on the possibility and timescales for developing and validating alternative (*in vitro* or SAR model) systems to replace existing *in vivo* toxicity testing guidelines. Given the background to this work, we intend to focus primarily on those *in vivo* tests currently required in EU Base Set and Level 1 and 2 lists, but

[a] Strategy for a Future Chemicals Policy, http://europa.eu.int/comm/environment/chemicals/0188_en.pdf

[b] Testing requirements for proposals under the EC White Paper 'Strategy for a Future Chemicals Policy': http://www.le.ac.uk/ieh/pdf/w6.pdf

will also consider wider aspects of testing (e.g. non-industrial chemicals). In addition, we wish to take this opportunity to assess the scope for increasing the efficiency of animal usage where no valid alternatives are available or achievable within a realistic timescale; that is to determine whether it is possible to refine/replace existing animal tests with designs that either require less animals or which involve a lower level of suffering.

We are contacting you to enquire about your involvement in, or awareness of, current efforts to develop and validate *in vitro* toxicity methods that could replace regulatory *in vivo* tests. As was noted above, we are particularly interested in the replacement of *in vivo* toxicity tests currently used for the assessment of industrial chemicals (e.g. Base set, Level 1 and Level 2), details of which are attached. We are specifically interested in the types of *in vitro* tests under development and what is the expected timescale for these tests to be fully validated and able to be incorporated into the OECD regulatory guidelines. We would also be interested in receiving details of any relevant research programmes that your organisation is currently involved with, together with your views and opinions concerning the feasibility of replacement of animal tests with *in vitro* tests. In addition, we wish to address the implications for the contract research industry if they are required to assemble *in vitro* test systems within their laboratories (e.g. the cost of setting up sterile culture conditions and relevant training for staff).

In addition, we invite you to make any other general comments that you feel appropriate. Since we are operating within a tight time frame to meet the schedule of the Lords Committee, we would ask for any reply to reach us by Friday 26$^{th}$ September 2001. Since it is intended that the output from this work will include an internal report to DEFRA and a submission to the Lords Committee, both of which can be expected to be publicly available and possibly published on our website, please advise us if any aspect of your reply should be treated 'in confidence'.

If you require confirmation of the objectives of our project or our remit, please feel free to contact either me or Dr Bill Parish at DEFRA (+ 44 (0) 207 944 5237) who commissioned the Institute to undertake this work.

Yours sincerely,