

## Chapter 12: Summary

### Introduction

This chapter brings together the summaries, conclusions and recommendations presented in this report. A distinction is made between prospects for validation on the one hand and recommendations for research and development on the other, since different organisations (and, in particular, different services of the European Commission) are responsible for funding and coordinating activities in these two areas. In addition, a number of recommendations are directed at specific organisations or expert groups, and some general recommendations are made concerning the use of alternative methods in the future REACH (Registration, Evaluation and Authorisation) system of the EU Chemicals Policy.

The prospects for prevalidation and validation are organised according to whether they are considered to be short-, medium- or long-term possibilities. This reflects an optimistic assessment based on the current state of the art, and does not take account of rate-limiting factors, such as the availability of funding and of human resources to coordinate prevalidation and validation studies. On the basis of this assessment, an indicative timetable for the availability of validated alternative methods for chemicals testing is provided in Table 12.1.

In contrast to the timetable for validation, the recommendations for research and development are not organised into a time-frame, because it is not always possible to predict the rate at which fundamental knowledge will be acquired, or to determine when a certain test will be sufficiently well-developed for entry into the validation process.

### The Current Situation

#### Acute lethal toxicity

Standardised basal cytotoxicity tests, such as the 3T3 neutral red uptake (NRU) assay, are already widely used for non-regulatory purposes, and could be used immediately for priority setting among chemicals, and for establishing the starting dose for *in vivo* acute toxicity testing.

Future activities in this area should aim to reduce and replace the use of the rodent test for determining acute toxicity values, through the development and validation of quantitative structure-activity relationships (QSARs) and cytotoxicity tests.

#### Skin corrosion

Alternative methods for skin corrosion have been validated and accepted for regulatory use in the EU, so animal testing is prohibited for this endpoint. The hazard identification (classification and labelling) of skin corrosives should be based on the use of a pH test, where appropriate, and an *in vitro* test (rat skin transcutaneous electrical resistance [TER] assay, human skin model assay or, for qualifying test chemicals, CORROSITEX®). For risk assessment (dose-response investigations), the rat skin TER or a human skin model assay are recommended.

#### Skin irritation

*In vitro* methods for skin irritation testing could be used immediately for priority setting. The human skin model assays (for example, EpiDerm™ and EPISKIN™) and the mouse skin integrity function test (SIFT) appear to be the most promising of the currently available methods.

For risk assessment purposes, there is a need to identify and evaluate the usefulness of new, mechanistically based endpoints that are more predictive of skin irritation than are simple cytotoxicity determinations. The existing *in vitro* models also need to be improved, so that they are more representative of skin *in vivo*.

#### Eye irritation

Progress in the validation of alternative tests for eye irritation has been hampered by the lack of *in vivo* data of sufficient quality. However, data from a number of tests, including the bovine corneal opacity and permeability (BCOP), hen's egg test-chorioallantoic membrane (HET-CAM), isolated rabbit eye (IRE) and isolated chicken eye (ICE) tests, are already accepted by some regulatory authorities, on a case-by-case basis, for the identification of severe eye irritants. Furthermore, the tiered testing of eye irritation is accepted by OECD Member Countries as a means of reducing and refining the use of the Draize eye test in rabbits. The use of the reference standards approach for interpreting *in vitro* data by comparison with benchmark chemicals merits further investigation.

**Table 12.1: An indicative time-table for the prevalidation and validation of alternative tests for chemicals testing**

Effect	EU test methods	Alternative methods	Status	Prospects for completion of validation
Acute lethal toxicity	B.1bis	<ol style="list-style-type: none"> <li>Basal cytotoxicity tests, including the neutral red uptake assay in Balb/c 3T3 cells and normal human keratinocytes</li> <li>QSARs for basal cytotoxicity</li> </ol>	<ol style="list-style-type: none"> <li>NICEATM-ECVAM validation study in progress</li> <li>R&amp;D</li> </ol>	<ol style="list-style-type: none"> <li>Short-term: expected completion of validation study by June 2003</li> <li>Medium-term</li> </ol>
	B.2			
	B.3			
	B.40			
Skin corrosion	B.40	<ol style="list-style-type: none"> <li>Rat skin TER</li> <li>Human skin models (EPISKIN, EpiDerm)</li> <li>CORROSITEX</li> <li>QSARs and expert system rulebases</li> </ol>	<ol style="list-style-type: none"> <li>Validated by ECVAM and accepted at the EU level</li> <li>Validated by ICCVAM and endorsed by ECVAM/ESAC</li> <li>R&amp;D</li> </ol>	<ol style="list-style-type: none"> <li>No further validation of these models is required; however, new human-skin models could be validated</li> </ol>
	B.4	<ol style="list-style-type: none"> <li>Human skin models (e.g. EPISKIN, EpiDerm)</li> <li>Skin integrity function test</li> <li>Pig ear test</li> <li>QSARs and expert system rulebases</li> </ol>	<ol style="list-style-type: none"> <li>ECVAM prevalidation study completed</li> </ol>	<ol style="list-style-type: none"> <li>Medium-term</li> </ol>
	B.5	<ol style="list-style-type: none"> <li>BCOP test</li> <li>HET-CAM</li> <li>Isolated rabbit eye test</li> <li>Isolated chicken eye test</li> <li>Fluorescein leakage assay</li> <li>Neutral red uptake assay</li> <li>Neutral red release assay</li> <li>Red blood cell haemolysis test</li> <li>EpiOcular assay</li> <li>EYTEX</li> <li>QSARs and expert system rulebases</li> </ol>	<p>Several validation studies have been conducted, but no method is regarded as validated</p> <p>Data obtained with some of these tests are accepted by regulatory authorities on a case-by-case basis</p>	<ol style="list-style-type: none"> <li>Medium-term: provided that several problems are overcome, including provision of high-quality <i>in vivo</i> data, and further definition of the reference standards approach</li> <li>Not recommended</li> <li>Short-term</li> </ol>
	B.6	<ol style="list-style-type: none"> <li>Protein-binding assays</li> <li>Numerous cell-based systems, including dendritic cells, Langerhans cells and human skin models</li> <li>QSARs and expert system rulebases</li> </ol>	<ol style="list-style-type: none"> <li>R&amp;D</li> </ol>	<ol style="list-style-type: none"> <li>Medium-term</li> <li>Short-term</li> </ol>
Respiratory sensitisation	No Annex V method	No well-characterised system available	R&D	Long-term

Table 12.1: continued

Effect	EU test methods	Alternative methods	Status	Prospects for completion of validation
Biokinetics	B.36	Numerous alternative methods are available, including: 1. <i>In vitro</i> and QSAR models for barrier function 2. <i>In vitro</i> models for metabolism 3. QSARs and expert system rulebases for metabolism 4. Mathematical models for biokinetic processes	R&D Data obtained with <i>in vitro</i> models of the skin barrier are accepted by regulatory authorities on a case-by-case basis	1. Medium-term for QSAR models; long-term for <i>in vitro</i> models 2. Long-term 3-4. Long-term
Target organ/system toxicity	B.7 B.8 B.9	Numerous <i>in vitro</i> systems are being developed for cell-specific toxicity, especially for: 1. Hepatotoxicity 2. Nephrotoxicity 3. Neurotoxicity 4. Endocrine system toxicity	R&D	1. Medium-term 2-4. Long-term
Chronic toxicity	B.26 B.27 B.28 B.29 B.30	Numerous <i>in vitro</i> systems are being developed, especially for: 1. Hepatotoxicity 2. Nephrotoxicity 3. Neurotoxicity	R&D	1-3. Long-term
Genotoxicity	B.10-B.25, B.39	Tests for gene mutation, including: 1. Bacterial reverse-mutation assay (Ames) 2. Mammalian cell mutation assay (for example, mouse lymphoma assay) Tests for clastogenicity, including: 3. <i>In vitro</i> cytogenetic assay using metaphase analysis 4. <i>In vitro</i> micronucleus assay 5. QSARs and expert system rulebases	Data obtained with tests 1-4 are accepted by regulatory authorities on a case-by-case basis  4. Ongoing validation 5. R&D	5. Medium-term
Carcinogenicity	B.21 B.32 B.33	1. Syrian hamster embryo cell transformation assay 2. Balb/c 3T3 cell transformation assay 3. 3D invasiveness assays	1-3. R&D	1-3. Long-term
Reproductive toxicity	B.31 B.34 B.35	1. Whole-embryo culture assay 2. Embryonic stem cell test 3. Micromass assay 4. Sertoli cell lines 5. Leydig cell lines 6. Sperm motility and morphology test 7. FETAX	1-3. Validated by ECVAM	1-3. Ready for regulatory consideration; further assessment of these models should focus on improvement of their prediction models 4-6. Long-term 7. Not recommended

**Table 12.1: continued**

*The EU Test Methods are described in Table 1.4.*

*BCOP = bovine corneal opacity and permeability; ECVAM = European Centre for the Validation of Alternative Methods; ESAC = ECVAM Scientific Advisory Committee; FETAX = ; HET-CAM = hen's egg test-chorioallantoic membrane; ICCVAM = Interagency Coordinating Committee for the Validation of Alternative Methods; NICEATM = National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods; QSAR = quantitative structure–activity relationship; R&D = research and development; TER = transcutaneous electrical resistance;*

*Short-term = by end of 2003; Medium-term = by end of 2006; Long-term = by end of 2010. The time-frame represents an optimistic assessment based on the current state of the art. In practice, the achievement of progress in line with the time frame will depend on the availability of sufficient human and financial resources, and on the coordination at the EU level of complementary activities. The realisation of the medium-term and long-term prospects will depend on adequate test development in the short-term and medium-term. The time frame takes into account the need to perform prevalidation prior to formal validation, where appropriate.*

## Skin sensitisation

A number of methods, including QSAR models and the Deductive Estimation of Risk from Existing Knowledge (DEREK) skin sensitisation rulebase, reconstructed epidermis models, and dendritic cell cultures are available, and could be used for priority setting. In cases where animal testing is required, the local lymph node assay (LLNA) should be used in preference to the conventional guinea-pig tests, except for those classes of chemicals for which the LLNA is not considered to be appropriate. In addition to hazard identification, the LLNA can be used for potency testing. Further fundamental work and development are needed before *in vitro* systems for skin sensitisation could be validated and used for regulatory purposes (classification and labelling, and dose-response assessment).

## Respiratory sensitisation

Respiratory sensitisation is an important endpoint in the context of occupational exposure to allergenic chemicals. There is no method for respiratory sensitisation in Annex V of *Directive 67/548/EEC*, and at present no *in vitro* test is sufficiently well-developed for prevalidation. Further research and development are required to provide alternative methods in this area.

## Biokinetics (barrier function)

Some QSARs for membrane permeability, such as those based on log P and molecular mass, may be sufficiently predictive to identify chemicals that are likely to cross biological barriers by passive diffusion. These QSARs could be used for priority setting, but chemicals that are not predicted to undergo passive diffusion should not be neglected, as non-passive modes of membrane transport are also important.

A variety of *in vitro* methods for percutaneous absorption are available and could be used for priority setting. For a complete assessment of ADME (absorption, distribution, metabolism and excretion), *in vivo* methods can be used. For the purposes of toxicity testing, *in vitro* methods may be more appropriate than *in vivo* ones.

For gastrointestinal absorption, the Caco-2 culture model is considered to be sufficiently reproducible for use as a high-throughput screening system in the priority setting of chemicals. However, further work is needed to assess the predictive capacity of this system.

To assess the passage of chemicals across the blood-brain barrier (BBB), a number of *in vitro* systems are under development. Further work is needed before any of these systems will be ready for prevalidation.

## Biokinetics (xenobiotic metabolism)

A wide variety of *in vitro* tests (referred to as Tier 1 tests in this report) are available for identifying metabolic pathways, metabolism-mediated toxic effects, metabolic stability and enzyme inhibition. These tests could be used immediately to obtain mechanistic information. Other tests are available for assessing enzyme induction (Tier 2) and polymorphic effects (Tier 3). An important question for the implementation of the EU Chemicals Policy will be whether Tier 2 and Tier 3 tests are necessary, and if so, at what tonnage level of production/importation.

## Biokinetics (physiologically based biokinetic modelling)

Physiologically based biokinetic (PBBK) models describe the processes of ADME by integrating physicochemical, physiological and *in vitro* data. They can be used to determine target organ/system doses and to extrapolate between routes of exposure and between species. To date, most of the models have been developed for application to specific chemicals, so there is a need to develop and validate generic models, applicable to broad groups of chemicals.

## Repeat-dose toxicity

A wide range of endpoints are investigated in *in vivo* chronic toxicity studies, so an integrated approach to chronic toxicity testing, based on the use of alternative methods with complementary endpoints, will need to be developed, in order to reduce the current reliance on chronic animal tests. At present, a variety of *in vitro* systems — derived mainly from the liver, kidney and brain — are being developed. Considerable investment at the research level is needed to maintain progress in this area.

Because of the nature of long-term toxicity testing and the need to maintain *in vitro* systems with physiological characteristics similar to those in the *in vivo* situation, it should be recognised that *in vitro* systems for chronic toxicity will be more complicated than *in vitro* systems for acute toxicity. This should not preclude the development of long-term *in vitro* systems, and their development should not be focused on their ability to permit high-throughput screening.

## Target organ and system toxicity

For the assessment of target organ and system toxicity, attention should be focused primarily on

potential effects on the liver, the kidneys, the nervous system and the endocrine system.

Liver function can be affected by metabolism-mediated toxicity (see Chapter 7), whereas kidney function can also be adversely affected by loss of barrier function. Renal transepithelial resistance and paracellular permeability are reproducible endpoints for assessing the function of the kidney barrier.

A large number of *in vitro* systems are being developed for investigating the wide variety of endpoints associated with neurotoxicity. This is an area where a reduction in animal use is likely to result from the integrated use of alternative tests with complementary endpoints. An important question in relation to the chemicals policy is the extent to which all mechanisms need to be assessed, because, from a strategic viewpoint, it is possible that an assessment of barrier function, combined with assessments of basal cytotoxicity and energy metabolism, might be sufficient to identify substances of concern.

### Genotoxicity and carcinogenicity

QSAR, expert system and *in vitro* approaches could be used immediately to prioritise chemicals for further testing on the basis of their potential genotoxicity and carcinogenicity. For the definitive assessment of genotoxicity, negative results in a bacterial assay for gene mutation and in a mammalian assay for clastogenicity/aneugenicity are normally regarded as sufficient evidence of lack of genotoxic potential.

For carcinogenicity testing, the rodent bioassay is not suitable for the testing of large numbers of chemicals, not only for scientific reasons, but also because of the considerable time and cost involved, as well as the large numbers of animals required. Therefore, it is important that alternative methods for the definitive assessment of carcinogenicity are further developed and then subjected to validation.

### Reproductive toxicity

Since it is not possible to model the whole of the reproductive system *in vitro*, the main components need to be studied individually, and then integrated in the form of a testing strategy.

For the assessment of embryotoxic potential, the embryonic stem cell test (EST), the whole-embryo culture (WEC) test and the micromass (MM) test can be used immediately. However, the EST is the only one of the three which could have a relatively high throughput, and which would not involve the killing of large numbers of pregnant animals.

Considerable effort is required to develop and validate alternative tests for assessing the adverse effects of chemicals on fertility.

It will take time to develop and validate a battery of alternative tests that cover other important aspects of the reproductive cycle, so animal tests will continue to be required, at least for certain aspects of reproductive toxicity testing. Therefore, a short-term priority should be to review the existing animal tests for reproductive toxicity, in order to reduce the numbers of animals required and to minimise animal suffering.

### Endocrine disruption

The mechanisms whereby endocrine disruptors (EDs) act are complex and diverse, and this has hindered efforts to develop reliable and relevant animal and non-animal tests for their detection. The emergence of relevant and reliable animal tests has also been hindered by the fact that few, if any, of the available animal test methods have been designed with the specific purpose of detecting EDs. They are, in fact, modifications of existing animal tests. The use of animal tests for EDs poses several important welfare problems, and there are complications due to the possible existence of low-dose effects.

The validation of currently available non-animal approaches should be made a priority, so that they can be used for compound prioritisation, and incorporated into tiered testing strategies in such a way that they reduce or replace further testing in animals. In addition, there is much research to be undertaken to assess the need for non-animal assays to cover the full spectrum of possible modes of action of EDs, and to provide definitive evidence of whether a substance does or does not interact with the endocrine systems of humans and animals.

### Prospects for Prevalidation and Validation

#### Short-term prospects

1. The validation of basal cytotoxicity assays for predicting: a) rat oral and/or mouse i.p. LD50 values; and b) human lethal blood concentrations. Such a study has been initiated under the auspices of ICCVAM and ECVAM.
2. The validation of QSARs and/or expert system rulebases for skin corrosion, skin sensitisation and eye irritation.
3. The validation of modified test protocols for the human skin models, EPISKIN and EpiDerm, and for SIFT, to determine whether the valid-

ity of any of these methods can adequately distinguish acute skin irritants from non-irritants.

4. The prevalidation of *in vitro* tests for identifying metabolic pathways, metabolism-mediated toxic effects, metabolic stability and enzyme inhibition.
5. The prevalidation of *in vitro* tests for enzyme induction, based on human hepatocyte cultures.
6. The prevalidation of re-aggregating brain cell cultures and the human neuroblastoma SHSY5Y cell line as complementary methods for assessing neurotoxicity.
7. The prevalidation of the complementary use of primary glial and neuronal cell cultures for assessing neurotoxicity.
8. The validation of (Q)SAR models and cell culture systems for predicting receptor binding of potential endocrine-disrupting chemicals.

#### Medium-term prospects

1. The validation of QSAR models for barrier function (skin barrier, gastrointestinal barrier and BBB), following an assessment of their mechanistic relevance.
2. The validation of QSAR models for skin irritation.
3. The prevalidation of *in vitro* models of the gastrointestinal barrier and the BBB.
4. The validation of *in vitro* tests for identifying metabolic pathways, metabolism-mediated toxic effects, metabolic stability and enzyme inhibition.
5. The validation of *in vitro* tests for induction, based on human hepatocyte cultures.
6. The prevalidation of models for evaluating the effects of enzyme polymorphism on metabolism.
7. The validation of algorithms for predicting biokinetic processes, such as *in vivo* metabolic clearance.
8. The validation of receptor-binding assays for non-genotoxic carcinogens.

9. The validation of QSAR models and other computer-based approaches for predicting genotoxicity and carcinogenicity.
10. The validation of a modified EST, refined with target cell-specific endpoints and human embryonic stem cells.
11. The prevalidation of methods for metal-induced infertility and spermotoxicity.
12. The prevalidation of a Leydig cell line test for assessing adverse effects on male fertility.

#### Long-term prospects

1. The validation of new *in vitro* methods for skin sensitisation.
2. The validation of new *in vitro* methods for respiratory sensitisation.
3. The validation of models for evaluating the effects of enzyme polymorphism on metabolism.
4. The validation of computer-based systems for predicting metabolism from chemical structure.
5. The prevalidation of suitably developed models for assessing long-term toxicity.

#### Recommendations for Research and Development Activities

##### Computer modelling

1. The further development of QSAR models and/or expert system rulebases for predicting *in vitro* cytotoxicity, skin corrosion, skin irritation, eye irritation, genotoxicity and carcinogenicity.
2. The further development of QSAR models and/or expert system rulebases for barrier function (skin barrier, gastrointestinal barrier, BBB and the blood-testis barrier) and for predicting chemical effects on metabolism.
3. The investigation of computer-based methods for the clustering of chemicals according to their physicochemical characteristics, to produce an appropriate clustering method for prioritising existing substances for assessment.
4. The development of a user-friendly software package for the prediction of target organ/

tissue distribution. The aim should be to make it possible for the user to enter a SMILES code or a CAS Registry number for a given chemical, and to obtain a qualitative indication of the major target organs and tissues. This information could then be used to indicate the *in vitro* tests that could be performed for a chemical known to enter the body by the dermal route of exposure.

5. The further development of algorithms for predicting metabolic clearance *in vivo* from *in vitro* data.

#### **Acute dermal and ocular toxicity**

6. The further investigation of the applicability of the reference standards approach to the validation of *in vitro* tests for eye irritation.
7. The identification of new, mechanistically based endpoints for skin and eye irritation, through the application of genomics and proteomics.

#### **Sensitisation**

8. The development of systems for skin sensitisation testing, based on an enhanced understanding of underlying biological mechanisms, including protein binding assays, human reconstructed epidermis models, and human dendritic cell cultures, which represent promising cell-based approaches.
9. The further development of *in vitro* methods for respiratory sensitisation.

#### **Biokinetics (barrier function)**

10. The further development of reconstituted human skin models for percutaneous absorption testing, to make their barrier properties similar to those found *in vivo*.
11. An evaluation of the feasibility of predicting bioavailability from *in vitro* data, and not just the fraction absorbed.
12. The further investigation of the expression of transport/efflux proteins in cell lines derived from the human gastrointestinal tract, and the influence of such transporters on absorption.
13. Research on the effects of anti-transport mechanisms (mdr, P-gp) and gut wall metabolism (CYP3A4) on bioavailability.

14. The further investigation of co-cultures consisting of cell lines with enterocytic markers and cell lines with mucus secretory functions, to understand the effects of mucus on the absorption rate.
15. The further optimisation of a test protocol for the BBB which involves primary endothelial cells co-cultured with primary astrocytes.
16. The design and evaluation of a battery of *in vitro* assays for predicting the distribution of compounds to the brain. Such a battery could include, for example, measurements of protein binding and clearance, and the use of MDCK cells transfected with transporter proteins (such as mdr-1).
17. The improvement and/or establishment of new cell models of the BBB that have characteristics more consistent with the *in vivo* situation, with an emphasis on the use of human cell lines.

#### **Biokinetics (xenobiotic metabolism)**

18. The further improvement of techniques for the cryopreservation of human hepatocytes.
19. The further development of *in vitro* models for evaluating the effects of enzyme polymorphism on metabolism.

#### **Target organ and system toxicity**

20. The identification of relevant biomarkers of exposure and effect for target organ/system toxicity testing. The endpoints selected should cover general cytotoxic mechanisms and cell-type-specific mechanisms of toxicity. There is a need to develop non-invasive methods for determining such endpoints.
21. An assessment of the immunological basis of target-organ toxicity.
22. The further investigations of the use of tissue renal slices as a model for studying the mechanisms of nephrotoxicity.
23. The immortalisation of primary proximal tubular cell cultures, and further characterisation of the new cell lines, to establish whether they retain the characteristics of their *in vivo* precursor cells through several passages.
24. The further development of the use of HK-2 cells (human proximal tubular epithelial cell line) and LLC-PK1 cells (porcine proximal

- tubular epithelial cell line), grown under long-term conditions, and an assessment of their usefulness for studies on transport across the kidney barrier.
25. An assessment of the extent to which loss of barrier function can account for kidney damage.
  26. An evaluation of the usefulness of genetically modified PC12 cell lines as a component in a testing strategy for neurotoxicity.
  27. The further development of *in vitro* models for evaluating the mechanisms of neurotoxicity, such as genetically engineered cell lines, and re-aggregating cultures of human embryonic stem cell lines.
  28. The evaluation of genomics, proteomics and new electrophysiological and biochemical profiling methods for neurotoxicity testing
  29. The development and evaluation of a tiered testing strategy for neurotoxicity, taking into account the results obtained in previous studies.
  30. The development of systems for predicting toxicotolerance.
  31. The identification of reference compounds suitable for the development of relevant and reliable *in vitro* procedures for long-term toxicity testing.
  32. The development of non-invasive imaging techniques for detecting long-term effects *in vivo*.
  33. The development of methods for long-term toxicity testing, employing human-based hepatic, renal and neuronal cell lines expressing a wide range of drug- and xenobiotic-metabolising enzymes and transport molecules, as an alternative to primary cultures.
  34. The further development of long-term culture methods (several weeks to months) for hepatic and renal epithelial and endothelial cells, as well as for neurons and glial cells.
  35. The further development of co-culture systems for long-term toxicity testing: a) neurons with glial cells; b) hepatocytes with monocytic, Ito cells and/or endothelial cells; c) renal epithelial cells with renal microvascular endothelial cells; and d) renal glomerular mesangial cells with glomerular endothelial and/or glomerular epithelial cells.
  36. The refinement of perfusion culture systems for chronic toxicity testing, with an emphasis on miniaturisation and practicability, to provide effective, technically simple and sensitive systems for assessing the effects of test compounds with biologically relevant endpoints.
  37. The further development of metabolically competent, genetically engineered cell lines, grown on microporous supports and continuously perfused with conventional culture medium, as models for evaluating the effects of continuous low doses and long-term exposure in the liver, kidney and neuronal tissue.
  38. The further development of the available human renal co-culture systems (for example, with metabolically competent cells), since epithelial and endothelial cells can be successfully grown in combination under static culture conditions and under continuous medium perfusion.
- ### Genotoxicity and carcinogenicity
39. A review of the current validation and regulatory status of aneuploidy and micronucleus assays in mammalian cells, to include an assessment of the range of aneugens detected by both methods, with a view to eliminating redundancy.
  40. A review of the current validation and regulatory status of rodent cell transformation assays (especially the SHE cell and Balb/c systems).
  41. A review of the need to conduct *in vivo* genotoxicity testing, especially for chemicals that are not intended for human consumption or direct exposure, and those for which indirect exposure is expected to be negligible.
  42. The development of receptor-binding assays for non-genotoxic carcinogens.
  43. The development of human cell-based cell transformation assays for carcinogenicity testing.
  44. Further research concerning: a) the known modes of action of non-genotoxic carcinogens; b) their relevance to human hazard; and c) the available and required non-animal testing strategies for detecting and characterising those carcinogens that are relevant.
- ### Reproductive toxicity
45. The identification of the most predictive toxicological endpoints for use in fertility testing with semen analysis.

46. An evaluation of the use of Sertoli cell lines and Sertoli cell co-cultures for assessing adverse effects on male fertility.
47. The development of a testing strategy for developmental toxicity, which covers not only malformations, but also other manifestations of developmental toxicity, such as growth retardation and embryolethality.
48. An evaluation of the use of primary cultures of spermatogonia and oocytes for the assessment of adverse effects on fertility.
49. The further development of granulosa cells and theca cells as toxicological screening systems.
50. Research on the use of primordial germ cells (PGCs) and/or PGC lines for identifying germ cell mutagens.
51. The development of a testing strategy covering all essential aspects of the male and female reproductive cycles.

### Endocrine disruption

52. Investigations on the use of microarrays for screening for receptor binding and gene induction, coupled with the search for further relevant receptors.
53. Research on the use of *in vitro* receptor-binding assays in conjunction with biokinetic modelling.
54. The assessment of the potential use of biomarkers of exposure and effect for EDs.
55. Investigations on the suitability of *in vitro* systems for yielding consistent data regarding the potential interactive effects of chemical mixtures of EDs.
56. Further research on the basic mechanisms of ED action, especially by way of non-receptor pathways, and the development of *in vitro* models for such processes.
57. The development of appropriate *in vitro* methods for measuring steroidogenesis in males and females, and for investigating endocrine function.
58. The development of approaches for detecting ED activity by using exogenous metabolising systems and cell culture systems, comprising transgenic cell lines with relevant hormone-receptor response elements, reporter gene

sequences, and genes for phase I and phase II metabolism.

59. The development and application of immortalised fetal and pre-pubertal cell lines.
60. The development of an integrated testing strategy for EDs, based on the maximal use of non-animal approaches.

### General

61. An assessment of the usefulness of genomics and proteomics in toxicity testing.
62. An assessment of the replacement, reduction and refinement possibilities in relation to the EU REACH system.
63. The development of a strategy for assessing ecotoxicological endpoints, based on the use of alternative methods.

### Recommendations to Specific Organisations

1. National regulatory authorities should consider harmonising their positions on the acceptance of the BCOP, HET-CAM, IRE, ICE and other non-animal tests for eye irritation.
2. The DEREK User Group should be encouraged to make more information publicly available on the DEREK rulebase for skin sensitisation.
3. The ECVAM Scientific Advisory Committee (ESAC) should consider making an endorsement of a statement on the applicability of *in vitro* methods for percutaneous absorption, assessed on the basis of a weight-of-evidence approach.
4. ECVAM should organise workshops on:
  - a) non-genotoxic carcinogens, in conjunction with the FRAME Toxicity Committee and the International Agency for Research on Cancer, to discuss and make recommendations for: i) the known modes of action of non-genotoxic carcinogens; ii) their relevance to human hazard; and iii) the available and required non-animal testing strategies for detecting and characterising those carcinogens that are relevant;
  - b) the use of genetically engineered cell lines for predicting metabolism-mediated genotoxicity;

- c) embryotoxicity, to define the areas of application of the scientifically validated EST;
  - d) the use of hormone-producing cells for predicting the adverse effects of chemicals on fertility; and
  - e) the use of *in vitro* systems for screening for EDs, to define the roles of such tests for this purpose; the workshop should include experts on endocrine disruption, *in vitro* toxicologists, clinical endocrinologists, ecologists, chemists, experts in (Q)SAR, and regulators; the workshop should focus on the limitations of *in vitro* approaches, and the need for them in relation to *in vivo* tests, bearing in mind that, so far, no uniquely *in vivo*-positive chemicals have been identified.
5. The appropriate regulatory authorities should consider the scientific merits of a test battery for EDs, either to supplement the existing reproductive toxicity test package, or to be applied as a separate set of tests specifically for ED activity.

### **General Recommendations for the Use of Alternatives in the EU REACH System**

1. To maximise the number of alternative tests available and suitable for use in the REACH system, emphasis should be placed on the development and validation of alternative tests that are already considered to be promising, because validated and accepted tests are likely to be required by 2008, in order to meet the proposed deadline of 2012 for the testing of the 20,000 existing chemicals produced in the range of 1–10 tonnes/year.
2. For the assessment of endpoints where replacement alternatives are unlikely to be available in the near future (for example, chronic toxicity testing), attempts should be made to reduce and refine the currently required animal tests to be used until suitable alternative methods, or batteries of such methods, have been developed and validated. To make progress in this respect, a comprehensive and independent review should be undertaken to assess the relevance and reliability of all the animal tests that will eventually be required for chemicals testing under the REACH system, and the outcome of this review should be published in the peer-review literature. This should also include an assessment of refinement and reduction possibilities in relation to the new legislation.
3. Testing strategies for the assessment of toxicological endpoints should be designed with respect to the known uses of chemicals and the exposure to them, at the beginning of the strategy.
4. Testing strategies should be based on a consideration of all of the Three Rs, including the use of alternative methods and the use of existing information, wherever possible.
5. Human volunteers should be used only to confirm safety. In particular, biokinetic evaluations of absorption, distribution, metabolism and excretion, and assessments of corrosivity, sensitisation and mutagenicity, should be undertaken before any human studies are conducted.