Special Contributions

ECVAM's Progress in Implementing the 3Rs in Europe

Thomas Hartung
ECVAM – European Centre for the Validation of Alternative Methods, Ispra, Italy

Summary
Starting with the animal welfare directive of 1986 and continuing up to the most recent chemicals and cosmetics legislation, Europe has laid the groundwork for the implementation of alternative methods. In order to meet these political expectations, certain technical and strategic developments became necessary:
- *Analysis of current in vivo test performance to set benchmarks for alternatives*
- *Analysis of the frequency (prevalence) of toxic health effects in different areas of test application*
- *Inventory and database of available alternative methods*
- *Coached development of lacking tests, also making use of novel technologies*
- *Acceleration and international harmonisation of the validation process and regulatory implementation*
- *Development of quality assurance systems for in vitro methods such as Good Laboratory Practice and Good Cell Culture Practice*
- *Transition from single tests as stand-alone replacements to the composition of test strategies and their validation*

The European Centre for the Validation of Alternative Methods (ECVAM) has played a proactive role in all these processes, coordinating many stakeholder activities. A review of the state of these developments shall be given in order to demonstrate how a new type of evidence-based toxicology is emerging, based on validated and quality controlled test strategies.

Keywords: ECVAM, validation, alternatives, political environment

Origin of ECVAM, legal basis, short history

ECVAM is an international reference centre for the development and validation of alternative testing methods aimed at the replacement, reduction or refinement of the use of laboratory animals in the biomedical sciences, with emphasis on toxicological assessments. ECVAM was established by a communication of the European Commission (SEC 91/1794) to the European Parliament and Council referring to Article 7.2 and Article 23 of Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes. The Directive requires that the Commission and Member States should encourage research into the development and validation of alternative methods that could provide the same level of information as that obtained in experiments using animals, but which involve fewer animals or which entail less painful procedures. ECVAM became operational as a Unit within the EU Joint Research Centre in 1992. ECVAM's work is focused on the development and evaluation of *in vitro* methods (e.g. cell and tissue cultures), of computer modelling based on structure-activity relationships and of physiological and biokinetic modelling. Current political needs for ECVAM's core activities are created by REACH and the 7th amendment to the Cosmetics Directive (Hartung et al., 2003).

ECVAM objectives and strategy

ECVAM pursues its objective to pioneer the process of quality assurance in the life sciences and regulatory testing by:

- communicating regulatory needs to test developers
- coaching the development and optimisation of methods
- tailoring the validation process and good practices
- participating actively in R&D and validation projects
- developing test strategies
- promoting successfully validated tests.

From a strategic point of view ECVAM has worked out a business plan for the next ten years. Overall costs of about € 150 million over these ten years were estimated. These costs will have to be shared with industry, which now urgently needs validated alternatives. Structurally, a dense network of stakeholders is in the process of being established for the development of strategies, the provision of unpublished *in vivo* data and reference chemicals as well as in-house test methods from industry. Candidate tests for validation are selected either in taskforces/ workshops or submitted by test developers, usually via the ECVAM website, and then prioritised by taskforces.

Procedure for validation and regulatory acceptance

ECVAM operates as a coordinator of international validation studies, as a focal point for the exchange of information, as the provider of a central database on alternative methods, as a centre of public dialogue and as a pre-normative research facility of the JRC. Participation in validation studies requires a running infrastructure and active research maintains a practical and realistic view on science and technology. Furthermore, high-quality research ensures credibility in the scientific community.

Due to the political sensitivity of its duties, ECVAM has its own Scientific Advisory Committee (ESAC) composed of members from all 25 European Member States, from relevant industrial associations, from academic toxicology, from the animal welfare movement, as well as from other Commission services with an interest in the area of alternative methods.

ECVAM has established a wide international network with OECD, with its American counterpart ICCVAM and with other Commission services, such as Directorate General (DG) Environment, DG Enterprise, DG Research and Development and DG Health and Consumer Protection. This network is used to reach international expert consent, test implementation and emission of opinions.

A typical validation lasts 3 years (costs per test are about € 300,000) and the subsequent regulatory implementation takes between 2 and 7 years. Main constraints in the process are the availability of reference substances and animal test data, the need for further optimisation of test methods, the duration of financial/administrative procedures and the long-lasting consensus process of regulatory implementation.

Changes in the political environment

The new European legislation on chemicals

The European Commission has proposed to harmonise the testing requirements for existing chemicals, for which there is a lack of safety assessment data (i.e. chemicals marketed before 1981) and new chemicals, by developing a new system for the Registration Evaluation and Authorisation of CHemicals (REACH). The new system will have less stringent testing requirements compared to those imposed by current legislation on new chemicals and will apply to approximately 30,000 substances that are currently marketed in volumes greater than 1 tonne per year. The extent of data requirements will depend on the tonnage of chemical produced in or imported into the EU. Consequently, this will result in a substantial increase of animal use for the safety assessment of chemicals. Several estimates of the number of laboratory animals required for these assessments and the costs for performing the tests have been made. They indicate that several million animals will be required, that testing costs will range in billions of Euros and that the availability of animal testing facilities will be a limiting factor. Beside the ethical aspects and the public concern, economic considerations also call for the timely development and validation of *in vitro* alternatives.

The Seventh Amendment to the Cosmetics Directive

Much of the scientific work on alternatives conducted, coordinated and sponsored in the EU was strongly pushed by the animal protection community and by public opinion, which broadly does not support animal testing for cosmetic products. In the EU, the safety of cosmetic products is regulated by Council Directive 76/768/EEC (EC, 1976). Its 7th amendment was finally approved by the European Parliament and the Council in March 2003. It foresees an immediate ban on animal testing for finished products and a complete ban on animal testing for cosmetic ingredients no later than six years after the implementation of the Directive. Moreover, it requires an immediate marketing ban for new cosmetics (finished products and ingredients) tested on animals where alternative methods validated by ECVAM and accepted by the Community exist. It also foresees a complete marketing ban on cosmetics for some targeted human health effects tested on animals within six years and ten years for repeated-dose toxicities, reproductive toxicity and toxicokinetics. This latter date can be postponed if by that date no validated alternative methods are available.

ECVAM's Strategic Vision

From its inception, ECVAM has been more than the administrator of alternative methods and their formal validation: The field of alternatives requires a proactive contribution (fig. 1), where ECVAM:

a) communicates the regulatory needs to putative developers of new tests
b) surveys opportunities for new technologies
c) steers a strategic debate between stakeholders
d) coaches the development and optimisation of methods
e) develops and tailors the validation process
f) participates in R&D as well as the validation process with its laboratories
g) collects, compiles and provides the information on relevant methods

h) integrates tests into test strategies
i) promotes tests after validation
j) pioneers the process of quality assurance in the life sciences

These roles have to be regularly revisited in light of the political needs. At this moment, two very obvious areas of concern are REACH and the 7th amendment to the Cosmetics Directive. Beside these, even more impressive with regard to animal consumption (fig. 2), are biologicals (16%) when compared to chemicals (1%) and cosmetics (0.025%). Areas like pharmaceutical articles (Hartung, 2002) and basic research (Gruber and Hartung, 2005) also deserve further attention.

Highlights of some recent activities related to the ECVAM strategies include:

a) Communication of needs of the regulatory area to putative developers of new tests
The ECVAM workshop series has just celebrated the 50th workshop report (Gennari et al., 2004) published in the journal Alternatives to Laboratory Animals (*ATLA*). Acknowledging ECVAM's collaboration with FRAME, the publisher of *ATLA*, a joint workshop on "Invalidation of test methods" was carried out in September 2005.

b) Surveying opportunities for new technologies
Following a joint workshop with ICCVAM on validation of toxicogenomics in 2003, a taskforce was established in 2004 and a pilot study was carried out with Affymetrix and Bayer. A workshop on metabolomics in toxicology is in preparation. Very extensive efforts are spent on (Q)SAR (Worth et al., 2004 a & b). This activity – a close collaboration between the European Chemical Bureau (ECB) and ECVAM – was put under the umbrella of OECD, where agreement on the principles of (Q)SAR validation and regulatory use are sought. In parallel, the first formal validation studies were initiated in 2004, addressing skin sensitisation, skin penetration, acute fish toxicity and endocrine disruption.

c) Strategic debate between stakeholders
ECVAM has established a network of about 400 experts regularly working in taskforces and workshops. A broad variety of collaborations with all relevant institutions in the field, aiming to bundle stakeholder activities, also exists. For the first time, a European opinion leader meeting was organised in 2004, convening the organisations acting on the European scale in order to discuss the perception of alternative methods by the (scientific) public and how to improve their image. In close partnership, all ECVAM activities have been opened to the American counterpart ICCVAM. With a view to extend this partnership, the establishment of an International Council of Validation Bodies is under discussion.

d) Coaching of the development and optimisation of methods
The EU has already invested more than € 200 million into the development of alternative methods by funding respective research. Lately, by installing three large Integrated Projects, a new dimension of tailored development of alternatives was reached (fig. 3). These projects, which involve more than 90 institutions and a funding of about € 40 million aim to make available batteries of tests plus the respective test strategies within five years each.

ReProTect (Hareng et al., 2005), started in July 2004, and deals with the field of reproductive toxicology including endocrine disruption. Noteworthy, the different tests included in the project for optimisation and integration into a test strategy originate not only from the field of alternatives, but also from areas such as mechanistic biomedicine, especially reproductive medicine, pharmaceutical agent discovery, clinical diagnostics, breeding of farm animals, etc. These models were never suggested as alternative methods, since they have different purposes and reflect only partial aspects of the reproductive cycle, but, put together in a conceptual framework, they might allow building a predictive test strategy.

A-Cute-Tox was based on an ECVAM workshop in 2003 (Gennari et al., 2004). It started in January 2005 and aims to establish an animal-free classification of acute toxicity, substituting for the classical LD_{50} test. Several studies have shown a good correlation of *in vitro* cytoxicity studies with the animal experiment. The project aims to improve this correlation to an acceptable level by outlier reduction.

Sens-it-iv was started in November 2005 with a view to complete the development of animal-free test strategies for skin and

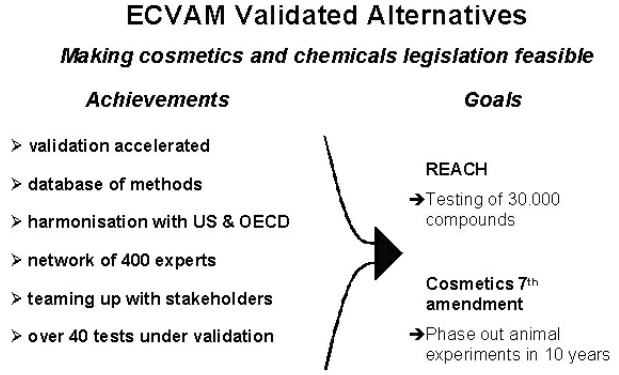

Fig. 1: ECVAM validated alternatives

Purposes of animal experiments in Europe in 2002

Total number	**10,700,000**	**100%**
Safety evaluations	**1,060,000**	**10 %**
Agricultural chemicals	**123, 000**	**1 %**
Industrial chemicals	**136,000**	**1 %**
Cosmetics	**2,700**	**0.025%**

Fig. 2: Purposes of animal experiments in Europe in 2002

lung sensitisation. It was based on an ECVAM workshop (Casati et al., 2005).

e) Development and tailoring of the validation process
On the one hand just recently for the first time international consensus on the role and procedure of the validation process was reached by the creation of OECD guidance document 34 on the validation and international acceptance of new or updated test methods for hazard assessment. The guidance document mainly reflects the ECVAM principles. On the other hand, various challenges to the validation process as such exist. For example, the enormous investments involved for the validation of a single test with regard to laboratory work and costs have often been questioned. In light of the need to validate a very high number of tests for the purposes of the chemicals and cosmetics legislations this aspect had to be reviewed.

So far, validation studies did not make use of existing data. However, in some instances, such retrospective validation might be an appropriate shortcut to the assessment of validity. Further challenges to the current validation scheme originate from new technologies (pattern-based or "-omics" approaches, transgenic animals, *in silico* methods such as (Q)SAR or computer modelling). In response, ECVAM has proposed a modular approach (Hartung et al., 2004), which opens up ways to accommodate these needs. The discussion as to the optimisation of the validation procedure, however, continues.

f) Participation of ECVAM in R&D as well as in the validation process with its laboratories
As part of the EU Joint Research Centre, ECVAM is also a place of research and education. Repeatedly, ECVAM has made contributions to the development and optimisation of alternative methods. ECVAM's laboratories also allow active participation in validation studies. Details are given in a chapter below.

g) Collection, compilation and provision of information on relevant methods
ECVAM hosts a database on alternative methods (dbAlm), which provides high-quality information related to alternative methods, including protocols for relevant *in vitro* tests. The major part of this scientific information service will be available online by the end of the year. A valuable resource of documents, such as the ECVAM workshop reports, is provided free of charge via the ECVAM website (www.ecvam.jrc.it).

Another major contribution to the field has been the compilation of inventories of alternative methods that are currently available. An inventory of 280 pages compiled with 75 experts in the context of the cosmetics legislation was published as an *ATLA supplement*.

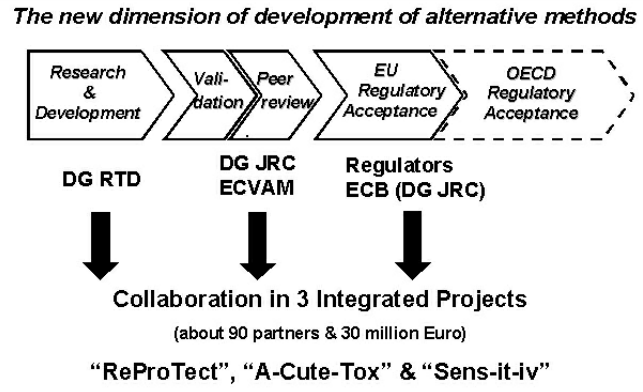

Fig. 3: The new dimension of development of alternative methods

h) Integration of tests into test strategies
Many of the more complex toxicological endpoints will not be replaced by single alternative methods. Instead, it will be necessary to develop testing strategies based on batteries of tests and their intelligent combination in test strategies. An important element of this is the concept of prevalence of health effects of chemicals (Hoffmann and Hartung, 2005), i.e. the actual proportion of chemicals showing a certain toxic property. Furthermore, it is necessary in order to develop such strategies, to analyse the performance of the animal experiment, an effort which has just started for example in the field of skin irritation (Hoffmann et al., 2005). Methods adapted from decision theory and evidence-based medicine will be employed in order to compose and validate final testing strategies.

i) Promotion of tests after validation
Today the regulatory implementation of validated tests often lasts longer than the validation process itself. This obvious bottleneck can only be overcome by collaboration with regulators, such as the National Coordinators of the Chemical Test Guideline Program in Europe, or on the level of the OECD. However, for this purpose, validation has to take into account the needs of regulators, i.e. a validity statement is less a scientific judgement but more a proof that the method is fit for its (regulatory) purpose.

j) Pioneering the process of quality assurance in the life sciences
Further to an ECVAM workshop in 1999, an OECD guidance document on Good Laboratory Practice (GLP) for *in vitro* toxicological studies was accepted in 2004 (fig. 4). In parallel, a Good Cell Culture Practice (GCCP) guidance document (Hartung et al., 2003) was completed recently (Coecke et al. 2005). It sets the minimal standards for quality control of *in vitro* work and will enable an international discussion over the next year. In the context of quality control, ECVAM is aiming for further adoption of principles on evidence-based medicine to the field of toxicology. The establishment of a taskforce on evidence-based toxicology was agreed in 2004.

Validated and accepted alternative methods

Eight alternative methods for chemicals/cosmetics have been endorsed by ECVAM including tests for skin corrosivity, skin sensitisation, phototoxicity as well as embryotoxicity. Seven alternative methods reached scientific acceptance for e.g. the safety evaluation of biologicals such as vaccines. Eleven alternative methods for myelotoxicity, pyrogen testing, acute fish

toxicity, mutagenicity and chronic toxicity in dogs are currently under peer-review by ESAC.

Relevant methods were accepted by both the European Commission (Annex V to Council Directive 67/548/EEC on the classification, labelling and packaging of dangerous substances) as well as by the Test Guideline Programme of the OECD. Three potency tests of biologicals have been accepted by the European Directorate for the Quality of Medicines (European Pharmacopoeia).

Estimated impact of current activities

The impact of alternative methods can be very substantial: The most successful replacement method, the Limulus test for pyrogens, reaches an annual turnover of € 200 million and saves more than one million rabbits per year. The remaining 200,000 rabbits used per year in Europe can most probably be fully replaced by the five methods currently being peer-reviewed (Hoffmann et al., 2005). For the time being, a reduction method saving one third of animals was proposed (Hoffmann et al., 2005).

For chemicals and cosmetic ingredients the following animal experiments (% animal use in Europe in 1999) have to be considered:

- acute toxicity (35%): OECD-accepted alternative methods in 2000 reduced the number of animals used from 45 to 8 per chemical; alternative methods which have been currently validated (to be completed 2005) reduce this number again to an estimated 3-6 animals per chemical. ECVAM set up the Integrated Project A-Cute-Tox (2005-2009, 9 million € funding, 37 partners) which aims for full replacement of the animal tests based on an ECVAM workshop (Gennari et al., 2004).
- Skin sensitisation (5%): The OECD-accepted refinement method has reduced the number of animals used per chemical as well as their suffering. ECVAM set up an application for an Integrated Project Sens-it-iv (2006-2010, € 11 million funding, 31 partners) following an ECVAM workshop (Casati et al., 2005).
- Chronic toxicity (27%): a strategy for chronic toxicity was developed at an ECVAM workshop held in 2004; currently a consortium and a work programme are being set up.
- Toxicokinetics (2%): a strategy for toxicokinetics was developed at an ECVAM workshop in 2004. The OECD accepted a test for skin penetration in 2004. First validations on barrier models (blood brain barrier and gut absorption) and work on physiology-based pharmacokinetic modelling as well as metabolism start in 2005.
- Mutagenicity and carcinogenicity (8%, costs of an animal cancer study are as high as 800,000 Euro per substance): two validation studies started in 2005 in which variants of the cell transformation assay and the *in vitro* micronucleus test are being evaluated.
- Reproductive toxicity (13% of animal use in toxicology in 1999; up to 55% of the testing costs of REACH): Three embryotoxicity tests were validated in 2002; ECVAM set up the Integrated Project ReProTect (2004-2008, 9 million €, 27 partners) to develop an alternative test strategy.
- Endocrine disrupters (2%): Validation studies with the US were agreed on for 2006 and will be part of ReProTect.
- Skin-eye corrosion (3%): OECD accepted tests in 2004.
- Phototoxicity (3%): OECD accepted test in 2004.

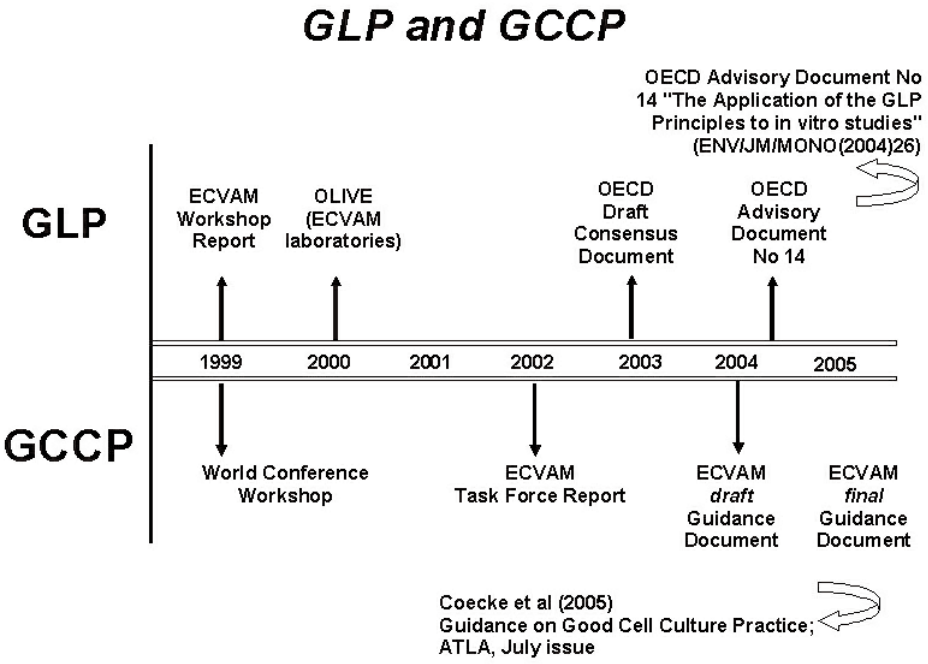

Fig. 4: GLP and GCCP

- Skin irritation (1%): a validation study is currently underway (2004-2005).
- Eye irritation (1%): Joint review of existing data with US ICCVAM from 2004-2005. Depending on the outcome, joint validation studies will be organised in 2006-2007.
- Acute fish toxicity (up to 40% of animals used for REACH): The reduction alternative developed by ECVAM will reduce the number of fish used by 60%. The method will be peer-reviewed in 2005. The fish egg test, which is a full replacement of the current animal test, will be validated in 2006-2007.

The impact of QSAR (*in silico* methods) cannot yet be anticipated, since no experience with validation exists. Regulatory use so far was mainly in priority setting and not in substitution of the current animal tests. Furthermore, it is not clear which percentage of chemicals can be subjected to QSAR (problems with lack of purity, mixtures, salts, metal compounds, etc.). Calculations by the ECB suggest an up to 60% reduction. Similarly, possible use of toxicogenomics is encouraged.

Beside the aspect of animal reduction, cost savings which, in some cases, can reach more than 90%, and a higher throughput, which will be extremely relevant for the testing of 30,000 chemicals under REACH, will have to be considered.

ECVAM's own research activities

Participation in validation studies requires a running infrastructure. The active participation of ECVAM in validation studies, which ranges from checking SOPs and transferability to full participation in blinded ring trials under GLP, helps identify problems of methods and allows flexible filling of gaps in studies. It also ensures neutrality of the study. Since GLP is increasingly becoming standard for validation studies, ECVAM is currently establishing this quality assurance regimen.

Active research maintains a practical and realistic view on science and technology. ECVAM must not become a purely administrative body, because the validation process is always interlinked with developmental aspects. The pipeline of methods to be validated has to be filled actively in interaction with the basic and applied research community. ECVAM's own research safeguards that this dialogue remains realistic and effective.

High-quality research also ensures credibility in the scientific community. This requires visibility and, most importantly, publications, favourably in higher impact factor journals. Currently, ECVAM researchers publish about 40 scientific papers per year, also in prestigious journals such as Nature and the Proceedings of the National Academy of Science.

ECVAM's laboratories have high standards with regard to infrastructure, space and resources. A very unusual combination of technologies and expertise (e.g. various *in vitro* technologies, metal toxicology, stem cells) is further amplified by the links to neighbouring units and the enormous network of external collaborators. This provides an excellent environment for about 20 Ph.D. students, post-docs and visiting scientists. Altogether, a unique integrated approach spanning from basic to applied research and the regulatory view is possible. ECVAM's research is uniquely positioned between basic research and validation.

Given the difficult access to animal primary cells at ECVAM and the limitations of cell lines, clear priority is given to human (primary) cells. The emerging stem cell technologies (embryonic and adult) (Bremer and Hartung, 2004; Pellizzer et al., 2005) offer new opportunities as do the classic accessible human cell sources blood and bone marrow. Cryopreserved human blood (Schindler et al., 2004) represents a very promising source of standardised cell material without the problem of blood donations. Among the animal cell lines, Balb 3T3 deserve special attention, since they are used both for the cell transformation assay and for the currently validated basic cytotoxicity test.

Several projects have led to the identification of prototypic toxins, e.g. metal compounds or test substances from validation studies. In some instances, ECVAM possesses high-quality *in vivo* and human data. Many of these substances have already been characterised in several standardised *in vitro* systems. This allows synergy linking between projects by testing the same set of reference substances. This will be further increased by the planned establishment of repositories of substances. Currently, a high-throughput testing facility is established jointly as a JRC exploratory research project, which offers opportunities for several projects, such as those on acute toxicity (A-Cute-Tox), acute fish toxicity, neurotoxicity and immunotoxicity.

Work on human (stem) cells will always be limited by the number of cells available. Furthermore, organotypic (co)cultures require analysing individual cells in mixtures, which is also the case for most stem cell derived differentiated cells. This calls for the establishment of technologies allowing single cell analysis, such as confocal microscopy, FACS, cell sorting (all existing at ECVAM), laser scanning microscopy, *in situ* PCR, laser microdissection, cell chips, etc. Setting up an array of cutting-edge methods should keep ECVAM attractive for visiting scientists.

As another aspect, the fate of test substances *in vitro*, e.g. their solubility, binding to plastic or serum albumin, etc., is routinely not considered. Addressing this might improve the predictive capacity by reducing an uncertainty factor. Similarly, the effect of exposure patterns to test substances *in vitro* has hardly been studied. Several current projects aim to understand the effective exposure of cells *in vitro* and the consequences for toxic responses.

Signature-based ("-omics") and computational models promise new approaches in several fields. The full integration in all key areas will be important to leverage these technologies. Current projects make use of toxicogenomics and metabolomics (Nuclear Magnetic Resonance, NMR, and Mass Spectroscopy, MS).

Co-operation between ECVAM and USA

Interactions between ECVAM and governmental bodies in the USA started as early as 1993, shortly after the creation of ECVAM. Since 1995, ECVAM has had a bilateral co-operation with the US Interagency Co-ordinating Committee on the Validation of Alternative Methods (ICCVAM). Its aim is to

ensure an early exchange of information on the validation of test methods to facilitate mutual recognition, acceptance and implementation of scientifically validated testing methods. Secondly, this co-operation serves to facilitate the OECD process in providing harmonised protocols to the scientific community and promoting international adoption of validated alternative methods.

The existing collaboration between ECVAM and ICCVAM in the field of alternative testing methods has been strengthened during the last three years and comprises the following activities: ICCVAM has an observer status on the ECVAM Scientific Advisory Committee. The Head of ECVAM became member of SACATM, the US Scientific Advisory Committee for Alternative Toxicological Methods. Both ESAC and ICCVAM have agreed on parallel peer-review and arbitration of results for the upcoming peer-reviews (pyrogen tests, haematotoxicity, chronic toxicity in dogs, micronucleus test). ICCVAM and ECVAM have agreed on creating an International Council of Validation Bodies to coordinate validation studies at the level of OECD. Discussion about formal collaboration with OECD has been initiated. About 20 visits of ICCVAM members or ICCVAM-nominated experts to ECVAM taskforces, workshops and validation management groups take place per year. The FDA has allotted a specific budget for parts of these travel costs. A sabbatical programme to exchange ECVAM and ICCVAM personnel was agreed upon. In 2003, the Head of ECVAM also became member of the Scientific Advisory Committee of CAAT, the Center for Alternatives to Animal Testing at Johns Hopkins University, Baltimore, which has pioneered the field of alternative methods in the US for about 25 years. At the same time, he became member of the Scientific Advisory Committee of the Institute for In-Vitro Sciences (IIVS), Gaithersburg. Furthermore, a senior American manager from The Procter and Gamble Company is on secondment for two years at ECVAM.

Good Laboratory Practice – Good Cell Culture Practice

The requirement for carrying out validation studies under standardised conditions, i.e. GLP and GCCP rules, has been recognised by national and international validation bodies. ECVAM plays a leading role in this process and actively contributes to the drafting of advisory and guidance documents. ECVAM, DG Enterprise and ICCVAM were part of a GLP working group which drafted an OECD Guidance document on GLP and *in vitro* toxicology that was finalised in May 2004.

ECVAM is also playing a leading role in drafting a new Guidance Document on Good Cell Culture Practice (GCCP). The aim of this GCCP document is to reduce uncertainty in the development and application of animal and human cell and tissue culture procedures and products by encouraging greater international harmonisation, rationalisation and standardisation of laboratory practices, quality control systems, safety procedures, recording, reporting and compliance with regulations and ethical principles. In order to give this document an international dimension, ECVAM invited ICCVAM to be part of the steering group that drafted this document (Coecke et al., 2005).

Databases

As an outcome of a project of the ECVAM Task Force on Alternatives Databases in collaboration with the Head of the thesaurus section of the US National Library of Medicine (NLM), a first version of a thesaurus on animal alternatives has been developed using the novel "bottom-up" approach. The thesaurus was generated in a semi-automatic manner, by selecting actual phrases that occurred in 2000 documents, and should therefore reflect the preferred terminology used by the authors of the articles. This first version focuses on toxicity testing and the first 11 main sectors identified. Following two consultation rounds, the thesaurus will be made available throughout the new Internet version of the ECVAM Database for Alternative Methods (formerly ECVAM Scientific Information Service) with practical application for end-users expected in fall 2005.

ECVAM has been invited to participate in a newly formed ad hoc group of the NLM to address the following main subjects:

- Journals on Alternatives and MEDLINE
- Keywords on alternatives in MEDLINE
- Adding Search Filters to PubMed

The following significant outcomes have been reached: Based on the advice given by the participants during the expert meeting, the Directorate of the NLM decided to add 9 journals on alternatives to MEDLINE, change the index terms for MeSH to better identify papers related to the alternatives concept and to sponsor a study on the feasibility of adding query filters for animal alternatives searches.

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Correspondence to

Prof. Thomas Hartung, MD, PhD
European Commission, JRC – Joint Research Centre
Institute for Health and Consumer Protection
ECVAM – European Centre for the Validation of Alternative Methods
Via E. Fermi 1, TP 580
21020 Ispra (VA)
Italy
e-mail: Thomas.HARTUNG@cec.eu.int

The Three Rs: Looking Back ... and Forward

Michael Balls
FRAME, Nottingham, UK

Summary
Personal experiences at FRAME and ECVAM are recalled, alongside the evolutionary progress of the Three Rs (reduction, refinement, replacement) approach to animal experimentation, described in great decades analogous to the Great Ages of Western Civilisation.

Keywords: animal experimentation, ECVAM, FRAME, reduction, refinement, replacement

Introduction

When I was asked to prepare this talk for a session entitled *Looking Back – The Voices of Experience* at the Berlin Congress, I decided to revisit the great ages of the Three Rs, originally presented in my plenary lecture at the 1996 Utrecht Congress as my interpretation of the development of alternatives over decades in analogy to the Great Ages in the evolution of Western civilisation over centuries (Balls, 1997), but this time placing greater emphasis on my own experiences during the last 50 years.

The 1950s: The age of renaissance

The 1950s saw the birth of the Three Rs (*reduction, refinement, replacement*) concept, as a result of the vision of Charles Hume of UFAW and the penetrative thinking and skilful analysis of William Russell and Rex Burch, culminating in the publication of *The Principles of Humane Experimental Technique* (Russell and Burch, 1959).

I was at high school at the time, majoring in biology, where I was better at botany than zoology, but could never remember the names of the many different plants. As a result, apparently being sufficiently familiar with the comparative anatomy of the rat, frog and dogfish, as well as knowing enough Latin, I found myself reading zoology at Oxford.

The 1960s: The age of darkness

Despite the warm welcome given by the Scientific Establishment to the Three Rs concept, little seemed to happen during the 1960s, and Russell and Burch had gone off to further their careers in other ways. However, at the end of the decade, Dorothy Hegarty and Charles Foister, impatient with the squabbling between the pro- and anti-vivisectionists, founded an organisation with a more positive goal, the Fund for the Replacement of Animals in Medical Experiments – FRAME (Annett, 1995). I have searched through all the FRAME archives, and I can find no evidence that the two founders of FRAME had ever heard of the Three Rs, so I truly think that this was a case of what we zoologists would call convergent evolution, i.e. ending up at the same place via different routes.

When preparing for my finals in 1960, my ambition was to be a zoologist with the British whaling fleet in the Antarctic, or, if I had to do compulsory military service, an officer on the bridge of a destroyer carving its way through the Atlantic. Instead, Michael Fischberg asked me to join his experimental embryology group at Oxford, where my project was to transplant cancer cell nuclei into *Xenopus laevis* eggs, to see whether the egg cytoplasm could reprogramme the errant genome to produce a normal embryo.

To do this exciting work (which turned out to be technically impossible, by the way), I had to work on amphibian tumours, and this meant having a vivisection licence. These were the days when it was politically acceptable to use words like "vivisection" – now we have to talk about "animal procedures". Getting a licence under the *Cruelty to Animals Act 1876* required the signatures of the head of a Royal college and of a medical professor. I therefore went to see Professor Sir Lyndor Brown, Professor of Physiology at Oxford, with a carefully worded draft of what I proposed to put on the licence application form. "No", he said, taking a small piece of paper from a drawer in his desk, "that won't do at all. Use these words and you'll be able to do whatever you like." I did, and got my licence.

Shortly afterwards, Fischberg was offered chairs at Edmonton, Geneva and Yale, and asked me to go with him, whichever choice he made. As a result, I had three very happy years in Geneva, before going as a post-doc to the University of California at Berkeley, where I learned cell culture with Harry Rubin, in the research group where Howard Temin discovered reverse transcriptase, later being awarded a Nobel Prize. I then went on to Reed College in Portland, Oregon, to join Larry Ruben, with whom I have continued to study various aspects of the biology of amphibians, ever since we first met in Geneva in 1962.

By 1966, I was back in the UK as a lecturer in developmental biology at the University of East Anglia. In 1967, a young man came into my office and said that, since he had just become a biology teacher at a local high school, he wondered if he could

do some part-time research. This was Richard Clothier, and our friendship and partnership continue to this day.

The 1970s: The age of reason

Interest in the issues raised by animal experimentation increased during the 1970s, especially in Britain, where it focused on the centenary of the *Cruelty to Animals Act 1876* and the need to reform it. Meanwhile, Andrew Rowan was Scientific Director at FRAME, and in 1978, he organised a meeting at the Royal Society on *The Use of Alternatives in Drug Research* (Rowan and Stratman, 1980), one of the first meetings on the application of replacement, at which I was one of the speakers.

Richard Clothier and I had been trying to work with our students on the cell cycle and the control of cell division in amphibian tissues *in vitro*. Along the way, we discovered that tissues such as liver, kidney and pancreas from *Amphiuma means*, the Congo eel (not from the Congo, but from the Southern USA, and not an eel, but a newt-like amphibian), could survive as organotypic cultures for several weeks, whereas their mammalian equivalents could only last for a few hours. Unfortunately, there was little sign of any cell division, so, desperate to help our students to get the results they needed for their Ph.D. theses, we started working on tissue functions instead, along the way becoming comparative endocrinologists, pathologists, pharmacologists, physiologists and toxicologists, as well as comparative anatomists.

To get closer to medical matters, we moved to the University of Nottingham Medical School in 1975, shortly after which we were visited by David Smyth, who was conducting a survey for the Research Defence Society, which was to lead to the publication of *Alternatives to Animal Experiments* (Smyth, 1978). He asked us if we knew that we were working on alternatives, to which we replied in the negative, and he suggested that we should try to get research support from animal welfare organisations. We took up this suggestion and were delighted to get some significant help from the Humane Research Trust.

Meanwhile, Andrew Rowan persuaded Dorothy Hegarty that I might make a good FRAME Trustee, so, in 1979, I was invited to join FRAME. He had also suggested that FRAME should set up an independent committee to look at the application of the Three Rs in toxicology and toxicity testing. The Trustees put me on the committee to watch over FRAME's interests, whereupon I was made Chairman, since I was the only member who had no right to call himself a toxicologist.

The 1980s: The age of reformation

At the end of the 1970s, Merlyn Rees MP, Home Secretary in the then Labour Government, who was responsible for the regulation of animal experimentation and who had come under great pressure to reform the 1876 Act, said that he would not meet lots of different groups of campaigners separately, but would be prepared to consider an agreed joint submission. This led to the formation of an alliance between the British Veterinary Association (BVA), the Committee for the Reform of Animal Experimentation (CRAE) and FRAME, which produced its proposals in 1983 (Anon, 1983). By this time, there had been a change to a Conservative Government, and David Mellor MP was the Home Office minister given the task of preparing and introducing the new legislation.

The BVA/CRAE/FRAME proposals were used as the basis for what was to become the *Animals (Scientific Procedures)* Act 1986, and representatives of the Triple Alliance advised the Government at every stage, from the drafting of the Bill to the Royal Assent. We succeeded in securing a number of major inclusions, such as the establishment of an independent Animal Procedures Committee (APC), and the requirement that the Home Secretary must weigh the balance between likely (animal) suffering and likely (human) benefit, before granting a project licence.

It was while serving on the APC that I first came across Russell and Burch's book. There was no copy at FRAME, so Clive Hollands lent me his, which I photocopied. Nearly 15 years later, my friend, Rodger Curren, presented me with a first edition of *The Principles* during the Bologna Congress, which Bill Russell signed there and then.

Changes were also taking pace in Europe, which saw the introduction of Three Rs legislation in the form of *Directive 86/609/EEC* and Council of Europe *Convention ETS123*, and in the USA, with the passing of the *Animal Welfare Act*.

These were also exciting times at FRAME. I had become Chairman of the Trustees on the retirement of Mrs Hegarty, and moved the FRAME headquarters to Nottingham. *ATLA Abstracts* was re-launched as a typeset journal, now called *ATLA (Alternatives to Laboratory Animals)* and with an international editorial board, and the report of the Report Toxicity Committee was published and discussed at a conference held at the Royal Society. Thanks to David Mellor, long a friend of FRAME and now a patron, we received the first grant ever given by British Government specifically to support alternatives. We used it to establish the INVITTOX database (which is still in use, but is now owned and run by ECVAM), and the FRAME International Alternatives Validation Scheme (which established principles which were later to be the basis of the ECVAM/ICCVAM/OECD principles that are in force today). We also began collaborative research with the University of Nottingham and others, with the support of various industrial companies, which led to the establishment of a FRAME Alternatives Laboratory (FAL) in the Medical School, as well as to the development of the kenacid blue cytotoxicity test.

The 1990s: The age of revolution?

My plenary lecture was given in 1996, and was aimed at challenging the participants of the Utrecht Congress to join me as revolutionaries – hence the question mark. I will return to that in due course.

There had already been a very successful congress at Baltimore in 1993, and the CAAT/ERGATT and ERGATT/EC workshops on the validation and regulatory acceptance of alter-

native toxicity test methods had established sound foundations on which a peaceful revolution could be built in that field.

My own life had changed considerably. First, I had become Professor of Medical Cell Biology at the University of Nottingham, and the Vice-Chancellor had given me his explicit support in my role as *de facto* honorary Director of FRAME, which meant that I spent almost all my time at the FRAME Office, while Richard Clothier ran the FAL.

I had expected to spend the rest of my professional and personal life in Nottingham, but I was persuaded to accept an invitation to apply to become the first Head of the European Centre for the Validation of Alternative Methods (ECVAM), which was being set up as part of the Environment Institute at the European Commission's Joint Research Centre, at Ispra, near Lago Maggiore, in north-western Italy. This came about because, having (I thought anonymously) advised a London agency during the preparation of a report for the Commission's Environment Directorate General (then DGXI), I had been rewarded by being asked to do various other small jobs for DGXI, including advising on the possibility of establishing a validation centre (I advised against it!).

Thus, offered the position on 15 March 1993, by 2 April I was already at ECVAM. Remarkably, I somehow managed to retain my roles as Chairman of the FRAME Trustees and Editor of *ATLA* throughout my stay in Italy, and, in the days before e-mail, I was able to keep contact with Nottingham via the fax machine in our villa, overlooking Lago di Monate. I take great pleasure from the fact that FRAME was going from strength to strength following the appointment of a real Director, Robert Combes, while the FAL continued to flourish under Richard Clothier.

Reminiscences of ECVAM (1993 – 2002)

Becoming a civil servant in a rigid hierarchical system was a great shock to me, as I had never before had to receive or obey orders from my superiors. There was betting in Brussels that I wouldn't survive for more than six months, and I was told, on more than one occasion, that I was to be sacked and that my successor had already been appointed. However, my immediate boss, Fritz Geiss, Director of the Environment Institute, advised me to ignore all that, decide what needed to be done, and get on and do it well. I took this advice, and stayed in place while three Commissioners, four Director-Generals and five Directors came and went. I have many amazing stories to tell, but they will have to wait until I publish my memoirs, if I ever summon up the courage to do so.

I now look back on this period with much pleasure and with great gratitude – living in Italy (the scenery, the culture, the food and wine, the people) was an absolute joy, leading an international team of gifted young people was a great privilege, and developing ECVAM's vast international network of collaborators was vitally important. Chairing the ECVAM Scientific Advisory Committee (ESAC) was both stressful and enjoyable, and I can trace most of what ECVAM did under my leadership to what was discussed with the members of the ESAC.

When I went to ECVAM, I said that there would be only one true measure of our success (or failure) – the number of alternative methods validated as reliable and relevant for their particular purposes. We had agreed with DG Environment that our target would be 15 methods by the end of the 5th Framework Programme in 2003, and I am proud of the fact that 16 methods had been endorsed by the ESAC when I retired (at a time of my own choosing, by the way) in 2002 (Balls, 2002). In addition, ECVAM assisted in securing the regulatory acceptance of *in vitro* methods for percutaneous absorption, and also of three refinement acute *in vivo* toxicity tests, which permitted the deletion of the OECD LD_{50} test guideline.

I feel that I must list just a few of many other ECVAM highlights, such as:

1. The ECVAM workshop series, based on the successful CAAT/ERGATT/EC model – a series set up soon after I arrived at Ispra, because ECVAM had to spend a lot of money quickly, or lose it (Combes, 2002). Many of these workshops were held at the Hotel Lido, Angera, where much of the controversy and bitterness of the day dissipated as we ate and drank excellent Italian food and wine as the sun set on the other side of Lago Maggiore. In September 2005, an ECVAM/FRAME workshop was held, partly to celebrate the success of the series of more than 50 workshops held so far.

2. *The Three Rs: The Way Forward* workshop, held in Norfolk about 1 km from where we now live, and organised by Bill Annett of FRAME, and jointly chaired by Alan Goldberg for CAAT, and me for ECVAM (Balls et al., 1995). This was the first meeting that Russell and Burch had attended together since 1959, and sadly, it was to be the last, as Rex Burch, already very ill, died a few months later. The meeting was held in Norfolk because of his illness, beginning with an opening ceremony in Sheringham Town Hall, where he had his laboratories. A frequent visitor to the town, I had passed within a few metres of him on many occasions over about 30 years, but I hadn't noticed the name plaque on the wall!

3. In addition to the workshop series, ECVAM also had a number of task forces, i.e. small groups of individuals asked to do specific tasks. One of the most important of these was the prevalidation task force, which in a few days produced an historic document by putting the meat on the bones of a prevalidation scheme which Rodger Curren and I had roughed out while having a coffee overlooking Baltimore harbour (Curren et al., 1995).

4. Having me as Editor of *ATLA* also had advantages for ECVAM, since I was able to get things published within weeks. One significant occasion was when this ensured that the ECVAM validation principles (Balls and Karcher, 1995) could be published just before the ICCVAM and OECD held meetings on validation, which eventually led to what are now known as the ECVAM/ICCVAM/OECD principles.

5. One of the most memorable events of the 1990s was the *3rd World Congress on Alternatives and Animal Use in the Life*

Sciences, held in Bologna in 1999. The Congress was organised by ECVAM, with Marlies Halder playing the anchor role, and with invaluable support from the Public Relations Unit of the Joint Research Centre and FRAME.

The 2000s: The age of achievement ...

The current decade is one of great challenge and great opportunity for alternatives, not least in Europe, because of the EU REACH system for new and existing chemicals and the 7th Amendment to the EU Cosmetics Directive, and the resultant unparalleled support for ECVAM from the European Commission and the European Parliament.

I retired from ECVAM in June 2002, but I am continuing to support ECVAM and my friend and successor, Thomas Hartung, mainly through editing and publishing ECVAM reports of various kinds, as well as participating in workshops. Meanwhile, I am privileged to be able to continue to support FRAME, especially through working with Bob Combes, Gerard Duvé and our gifted and energetic young colleagues.

That the 5th Congress in Berlin has more than 850 participants is an indication that much is being achieved, but the question is, "Is it enough, and will the revolution that didn't occur in the 1990s, now take place in the 2000s?" Sadly, the question mark I used in 1996 was entirely justified.

or of disappointment?

I see a number of disturbing trends, which cause me great concern.

The progressive *reduction* in the numbers of animal experiments, which had been foreseen when the new legislation was passed in the 1980s, seems to have come to an end, especially as more and more mice are sacrificed on the altar of genetic exploitation. Also, far from working together toward the zero option of the use of non-human primates, there is pressure to build more and more primate research centres. *Refinement*, claimed by many to be the poor relation among the Three Rs, seems to be becoming increasingly fashionable, especially as it can be linked with the indefinite continuation of the reliance of research and testing on animal models. However, for me at least, refinement cannot answer many of the fundamental questions. Giving a monkey a tennis ball to play with, and hiding its food so it must search for it, may be better than long confinement in a barren cage, but that's not enough for me. This is in line with the thinking of Russell and Burch, who said: *Refinement is never enough, and we should always seek further reduction and if possible replacement. … Replacement is always a satisfactory answer*. (Russell and Burch, 1959, p. 66).

Therefore, our goal must be *replacement*, as Jane Goodall and Andrew Rowan emphasised at the opening lecture in this Congress. Achieving it will require skill, dedication and commitment – and the energy of young people trained in modern approaches to medical problems and toxicity testing. We have begun the journey, but there is still a very long way to go.

So, my message as I look to the future is the same as that I gave at the end of the Bologna Congress, when I wore a tie given to me by my friend, Klaus Cussler, of the Paul Ehrlich Institute, Langen, Germany (Balls, 2000). It has about 100 tortoises on it, all moving slowly in the same direction. But one of them is saying to the others, "GET A MOVE ON!"

Please, dear readers, don't disappoint me.

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Correspondence to

Prof. Michael Balls
FRAME
Russell & Burch House
96-98 North Sherwood Street
Nottingham NG1 4EE
UK
e-mail: Michael.Balls@btopenworld.com