Theme 5
Safety testing, validation and risk assess

Session 5.01
Strategies for using non-animal methods in relation to chemicals legislation (HPV, REACH, ECVAM-Session)

REACH and CEFIC's Conception of a Feasible, Information and Priority Based Approach

Wolf-Rüdiger Bias
BASF Aktiengesellschaft, Ludwigshafen, Germany

Summary
The European Commission's proposal for a new Chemicals Legislation, REACH, presented on 29 October 2003, is currently under strong discussion in the processes of the first readings in the EU-Council and in parallel in the EU-Parliament. The legislative proposal is highly complex and sets out formal, volume dependent information requirements for the registration of substances. It calls for submission of extensive documentation of all information underlying a safety assessment. CEFIC proposes a leaner, more flexible approach. The prerequisites for a more efficient system shall be explored.

Keywords: REACH, chemicals assessment, pre-registration, prioritisation, exposure-related information

Introduction

REACH, the new European chemicals legislation, is in its fifth consecutive year of construction. The discussion of its main features has not yet reached a state that gives confidence that the act can enter into force by 2007, as currently envisaged by Council, Commission and Parliament of the European Union. What are the reasons for this controversial discussion? The most conflicting views seem to arise from matching the objectives of protecting man and the environment with the objective of competitiveness. All goals set out in the White Paper on 13 February 2001 on the Strategy of a future Chemicals Policy were welcomed by industry, especially since the environmental objectives were balanced with the economic one of "maintaining and improving the competitiveness of the chemical industry of the EU". This was also in line with the Lisbon Goal on competitiveness of 24 March 2000, namely to "make the EU the most competitive, dynamic and knowledge-based economic region of the world". However, if both goals, as formulated, are supported, a different approach must evidently be taken.

The new chemicals legislation is supposed to consolidate current chemicals legislation, adding consistency and efficiency in working with it, according to the conclusions of the Informal Environment Council held at Chester under the UK Presidency in 1998: "Environment Ministers in June 1999 called on the Commission to consider, *inter alia*, measures that provide an efficient and integrated design of the various legal instruments for chemicals; place the main responsibility on industry for generating and assessing data; provide a more flexible approach to risk assessment with the aim of targeting assessments; and establish effective risk management strategies for certain chemicals that may cause threats of serious or irreversible damage to human health or the environment as a result of their inherent properties by giving appropriate weight to their use pattern and the possibility of exposure" (Chester, 1999). In pursuit of its assignment, the Commission chose to focus on the following major pieces of European chemicals legislation:

(i) Directive 67/548 on classification, packaging and labelling of dangerous substances; (ii) Directive 88/379 on classification, packaging and labelling of dangerous preparations; (iii)

Directive 76/769 on the marketing and use of certain dangerous substances and preparations; and (iv) Regulation 793/93, the Existing Substances Regulation.

Outline and assessment of the legislative draft REACH

While the quoted laws that REACH is to absorb do not cover the entire area of EU chemicals legislation, they nevertheless encompass the universe of commercially handled substances. As proposed in the REACH draft, the steps evaluation and authorisation could be applied to any chemical, either on its own or as part of a preparation or an article manufactured in or imported into the EU. The registration is foreseen for all industrial chemicals exceeding a level of one ton per year per manufacturer or importer (fig. 1).

The registration would, despite some specific requirements and exemptions for certain classes of chemicals and certain uses, have to be performed for roughly 30,000 chemicals. It is therefore crucial to define the scope, the tasks, and the processes so precisely as to leave no doubt with the registrants about their duties and their freedom to act. There are two reasons to do so. First, because the entire registration has to be completed within 11 years (timeline, s. fig. 2), and second, because work that is not necessary for deciding on risk management measures, and, especially, duplication of vertebrate studies, must be avoided.

Due to the fact that only the four major pieces of European chemicals legislation shall become obsolete when REACH enters into force, the scope of REACH cannot be as clear cut as requested by the council. It has to take into consideration the obligations tied to many remaining laws regulating chemicals for specific applications. This requires excellent knowledge of the adjacent legislation on behalf of the registrant as well as on the part of the competent authorities. It also adds to complexity when having to observe a multitude of different laws in interfacing areas and therefore calls for clear legibility of REACH.

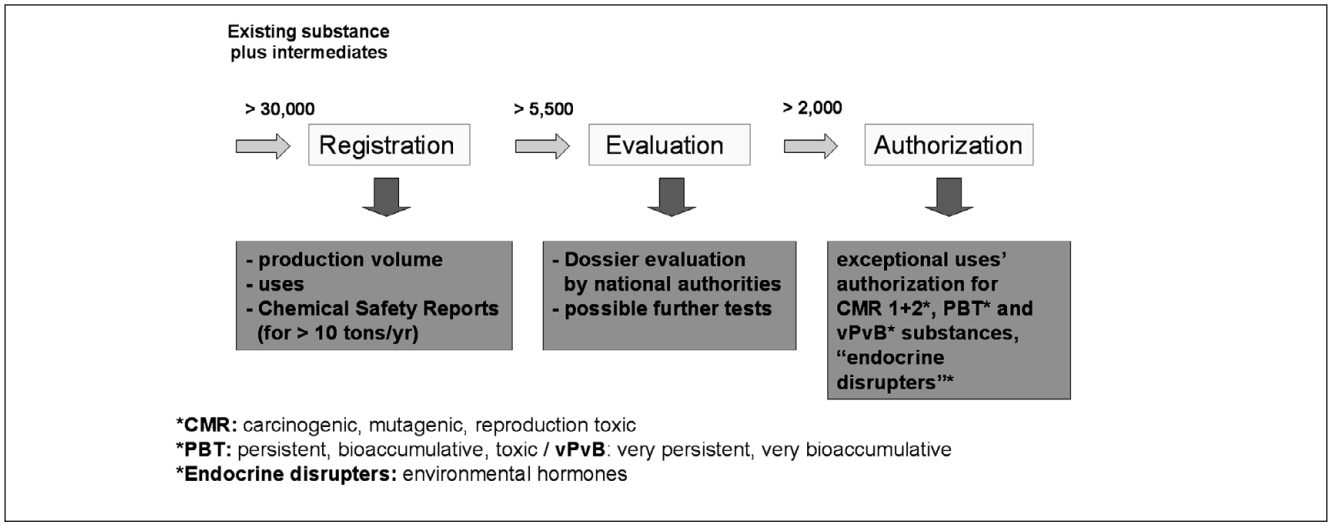

Fig. 1: Elements of REACH

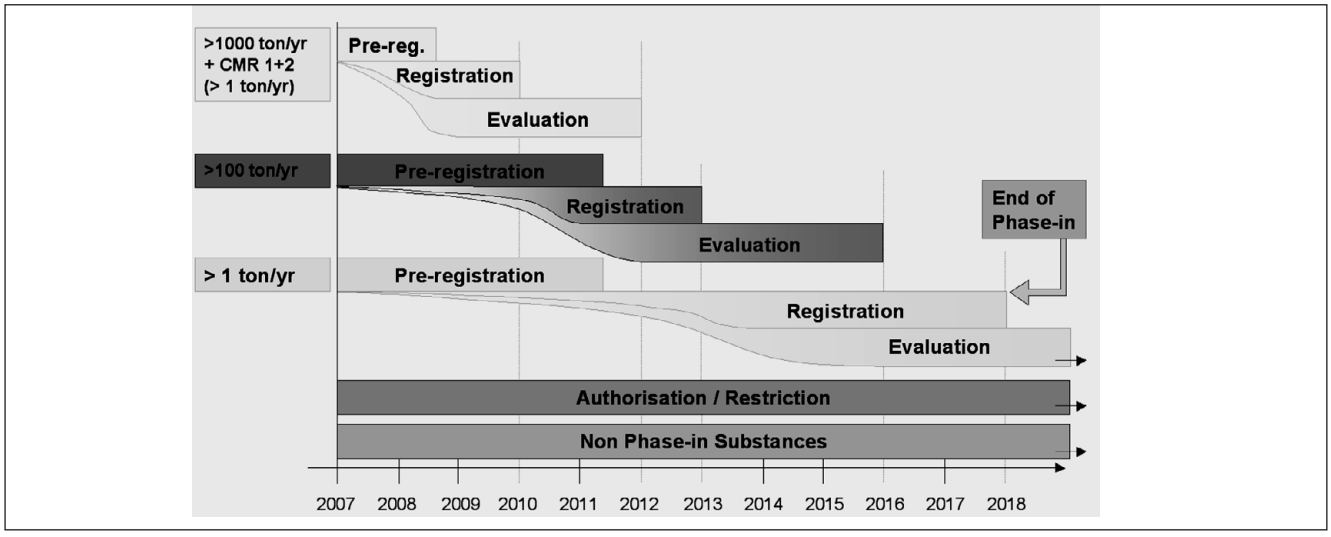

Fig. 2: REACH timeline – a process overview

Industry would favour legislation which, instead of offering workarounds for existing laws, would build upon a more systematic clean-out of the current chemicals legislation. For example, the usefulness of the Biocidal Products Directive 98/8/EC in the presence of REACH is questioned as much as those pieces of various legislation setting out information requirements on substances as a basis for an assessment. A clear, exposure-related approach, triggering the information needed for any safety assessment in REACH, could be the basis to many a legislation once generally accepted strategies on information requirements have been established.

The lack of a distinct tier for exposure-related information requirements is one of the major shortcomings of REACH. Instead, in overestimating the influence that the volume of a substance may exert on risk, a tonnage-related trigger has been established. Volume usually is a minor cofactor in determining exposure-regimes. Adhering to the volume-trigger has not only prompted numerous proposals by Industry to find compromises with the Commission. In effect, it leads to furnishing substances with uniform sets of information generated according to a formal standard as required under annexes V-VIII, rather than focusing on uses that lead to crucial exposure-regimes and which call for more information for their assessment. This way, not only resources are spread and spent evenly, but work on substances may also be allocated to the wrong point in time.

On the whole, it is also by far underestimated how difficult volume tracking within one company actually can be. Such figures need to be established by diligent tracing of constituents in products, their transfer to other business units, and adding up their total quantities while, at the same time, different points of entry within one legal entity have to be observed.

Regarding the registration of chemicals, the Commission has proposed a staggered approach for pre-registration and the submission of registration documents according to the volume manufactured or imported per registrant. While the timeline is highly ambitious, the current scheme with two deadlines for pre-registration has another disadvantage. It leads to uncertainty among the market participants as to whether substances are intended to be registered and available in the future. Moreover, it would lead to registration of one and the same substance at different points in time if one manufacturer/importer has to register early because of the large volume he manufactures/imports and another, who only deals with small volumes of the same substance, submits his vertebrate data at a later stage.

For the purpose of registering a substance, the registrant submits the required documents in the form of a technical dossier and additionally, for all substances above 10 tons/year, a Chemical Safety Report (CSR) to the Agency. The Agency will check the documents for completeness but not for quality, ask for supplements if parts are missing and pass on the dossiers to the competent authority of the relevant member state within 30 days. It is the task of the member state's competent authority then to examine any test proposals made by the registrant and to check for any dossier, whether it complies with the rules as set out, or to decide whether additional information is needed for the dossier to comply with the relevant information requirements. The registrants of a substance can either jointly submit a dossier or choose to register individually. As seen from work under the Existing Substances Regulation, a considerable effort is needed to coordinate cooperation. For low volume substances it is therefore expected that the latter will most likely be the prevailing situation. This will entail that each relevant member state competent authority will separately evaluate the dossiers of the manufacturer or importer resident to his state (fig. 3).

Such a process calls for improvement. Not only that the workflow between the central Agency and the member state's competent authorities is complicated. Evaluation by several competent authorities of different member states would be a duplication of work. There is also the likelihood that different competent authorities come to different conclusions on the proposals, justifications and assessments taken.

company A manufactures substance X in Germany → Evaluation by German authorities

company B manufactures same substance X in France → evaluation by French authorities

company C imports same substance X into Spain → evaluation by Spanish authorities

Consequence:
- double work with authorities*
- no build-up of competence
- avoidable costs

*In case companies A,B & C form a consortium only the authority of the resident registrant submitting will initially have to act

Fig. 3: Dossier-Evaluation: doubling the work in Europe

- one pre-registration step only
- maintain tonnage trigger for administration, but introduce more flexibility through:
  - prioritisation and
  - exposure-related information needs
- central agency responsible for evaluation and all subsequent steps

Fig. 4: Key elements of the CEFIC proposal

- one-step obligatory pre-registration, within 18 months to be sent to the Agency with the following information:
  - company name
  - volume band
  - substance name and CAS n°
  - availability of vertebrate studies
  - interest in joining consortia and sharing of own data

Fig. 5: Pre-registration

- Prioritisation procedure
  - prepare a Core Information Set for all substances above 10 tons and assemble appropriate available information for substances 1 – 10 tons/yr
  - identification of substances of potential high risk
  - Core Information Set for substances above 10 tons/yr together with the prioritisation to be sent to the Agency within 5 years after REACH has entered into force

Fig. 6: Prioritisation

Physico-chemical data	• Physical form (particle size) • Melting point • Boiling point • Vapour pressure • Partition coefficient octanol / water • Relative density • Explosiveness • Flammability • Water solubility • Flash point
Ecotoxicity	• Biodegradation • Acute toxicity (daphnia)
Toxicity	• Acute toxicity – relevant route • Skin irritation • Eye irritation • Skin sensitization (if no clues available) • Genetic toxicity bacterial test (Ames)
⇩ ⇩	• Classification & Labelling • Generic Exposure Information

Fig. 7: Core Information Set

In order to succeed, a series of further stipulations of REACH call for very carefully designed processes, which the proposal currently does not provide. The unsolved issues of maintaining the protection of confidential business information, of unrestricted ownership rights in studies, contractual antitrust agreements for consortia formation, and information flow through the supply chain give a perspective to which extent bureaucratic rules might be needed to cope with these issues, making REACH theoretically safe but non-sustainable by practical means.

Remedies for selected shortcomings of the current REACH proposal

CEFIC, the European Chemical Industry Council, has accompanied the development of REACH with numerous constructive proposals made in order to obtain a viable legislation. While drafting the proposal, the Commission took only few of them into consideration. If the present structure of REACH is to be maintained, CEFIC advocates substantial changes, some of which shall be outlined below (fig. 4).

Referring to the assessment of the current proposal above:

- Pre-registration should be a one-step obligatory affair, in order to allow simultaneous registration of the same substance regardless of the quantities manufactured/imported at one point in time (fig. 5) and to provide certainty about the availability of substances in the future. This would be the initial step to establish a new chemicals inventory within 18 months time.
- The tonnage trigger shall only be maintained as an administrative tool to divide the workload and to define the maximum in proportionate information requirements. However, two features are added to achieve a much more flexible approach: They consist of prioritisation (fig. 6) and exposure-driven information needs. For all substances above 10 tons/year a Core Information Set (fig. 7) shall be elaborated within a period of 5 years. On the basis of a potential for risk, as suggested by inherent properties like vapour pressure, dustiness and high toxicity as well as the use domain, substances will be prioritised by very simple arithmetic (fig. 8). An exposure analysis could not be established within such a time frame, which, together with hazard information, would allow addressing the risk. Therefore only the potential for risk shall be utilised.

The prioritisation shall also help to spread the workload more evenly. CEFIC proposes to adhere to registering high volume and cmr substances with the first lot, but with a time frame of 5 years. The next lot would encompass substances of 100-1000 tons/year as well as those of lower volume per year with a potential for elevated risk (fig. 9).

Finally, the remaining substances with a potential for lower risk shall be registered.

- For all substances, based on the available information and above 10 tons/year based on the Core Information Set, the use-related exposure should determine whether additional information is needed to the maximum of volume-relating annexes.

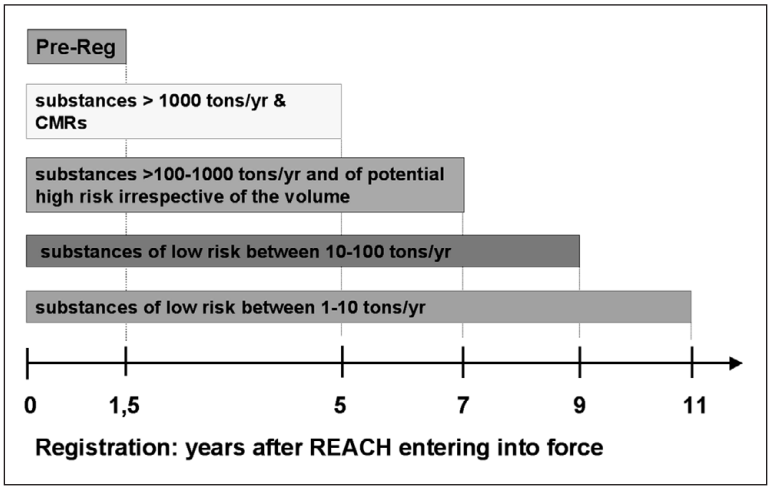

Fig. 8: Registration schedule

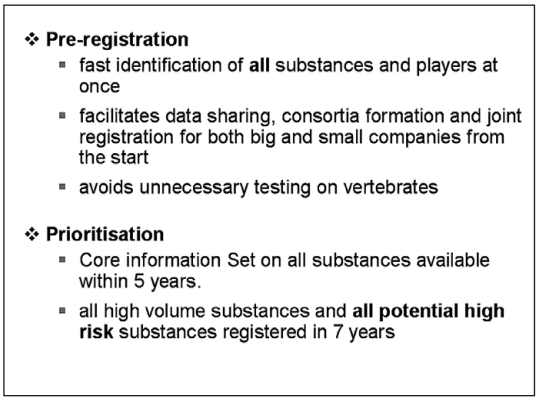

Fig. 9: Benefits: procedural

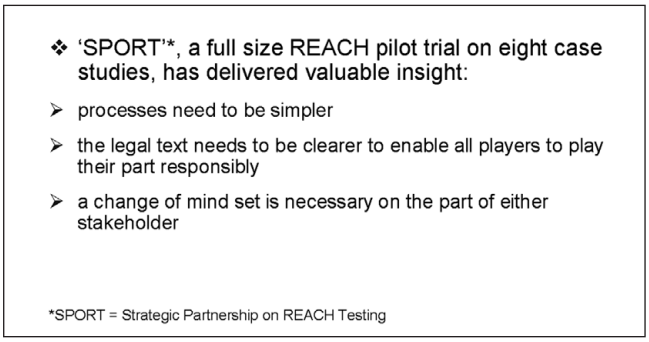

Fig. 10: Recommendations from 'Field Studies'

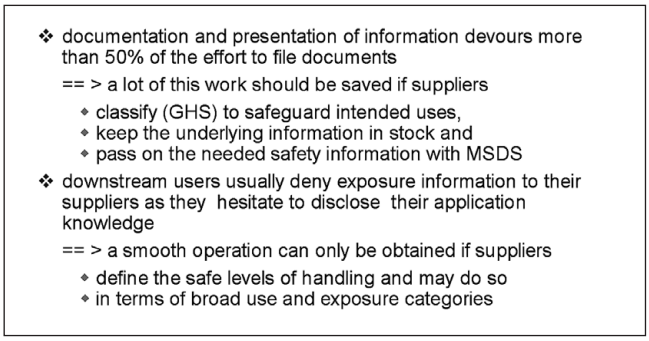

Fig. 11: Less complexity for better performance

- To warrant that evaluation is conducted following the same principles and rules throughout Europe, the responsibility for the evaluation shall reside with the Agency. This would entail an alleviated information flow, improved consistency, and would help avoid duplication of evaluation.

It is obvious that the proposed steps are not the only prerequisites for a viable REACH. Many more proposals have been made by CEFIC, which cannot be presented here.

- The recent pilot study on REACH called SPORT just completed in the middle of this year (fig. 10). SPORT, i.e. "Strategic Partnership on REACH Testing" was an Industry initiative jointly supported by the EU Commission, Member States and Industry. More than 150 recommendations were distilled from 8 projects conducted under the conditions of the current REACH proposal. The joint recommendations by the three partners called for simplification of processes to enable every stakeholder to contribute according to his skills and capacity. They furthermore set out that REACH needs to be understood by everyone so that everyone can live up to his/her obligations. Also, a uniform view was reached that exchange of data on structurally related compounds should be facilitated in order to enable grouping of substances and to maximise the use of information to avoid duplication of vertebrate studies.

Reduced complexity is the only way to avoid running into a high implementation deficit very quickly. A way forward to improve the performance further is outlined in figure 11 as a personal view. I sincerely hope that Commission, Council and Parliament now demonstrate their flexibility to set the controls for a lean but efficient REACH.

Correspondence to
Dr. Wolf-Rüdiger Bias
BASF Aktiengesellschaft
D-67056 Ludwigshafen
Germany
e-mail: ruediger.bias@basf-ag.de

Strategy for Minimising the Use of Animal Testing as Part of REACH

Uwe Lahl
Federal Ministry for the Environment, Nature Conservation and Reactor Safety, Bonn, Germany

Summary
This paper outlines the current state of decision-making in the reform of EU chemicals law. REACH will mean that we will acquire extensive data on the properties of chemicals and existing exposures. This will make it possible to reduce the negative effects they have on humans and the environment. Although consumer protection as well as occupational health and safety are at the centre of the public discussion, the implications for environmental protection should not be neglected. Many dangerous environmental chemicals (e.g. endocrine-disrupting substances) contribute to the fact that individual animal populations are in decline regionally and even globally. REACH is thus also needed to better protect wildlife from environmental chemicals.
The aim to protect wildlife is at odds with the fact that it is not always possible to ascertain the risks associated with these chemicals without carrying out tests on animals. However, the Commission's proposal for a regulation identifies all the endeavours being made to limit the number of animal tests. OSOR will prevent duplicate or even multiple tests on animals. The use of historical data will also be permitted, provided that they are still valid. Alternative methods to replace animal testing (particularly in vitro methods) will also be used wherever available. Opening up risk analysis to methods that analyse structure/activity relationships (in silico methods, SAR, QSAR) creates further prospects for reducing the extent of animal testing in the medium term.
Finally, the time between now and the date REACH enters into force is a clearly defined time frame within which current research into alternative methods can be advanced to a state that will allow additional possibilities for replacing animal testing to be developed.

Keywords: REACH, EU chemicals legislation, animal testing, three Rs concept, minimisation strategy

Introduction

The discussion on animal testing has been running for many years and is highly controversial[1]. For example, people question the fundamental ethical and moral right to carry out experiments that (may) cause suffering, detriment and even death to animals[2]. This stance was adopted particularly with regard to animal testing in the area of pharmaceutical research, cosmetics and basic research. These are the areas in which the highest numbers of animal tests are conducted (tab. 1).

Fundamental arguments and ethical reasons are cited in standpoints that justify animal testing[3]. For example, it is said that animal testing leads to extremely important additional knowledge. Furthermore, it is seen as an ethically acceptable consideration that the suffering of animals should be tolerated in order to prevent human suffering. In recent years the discussion has split into different camps. However, all the groupings agree that animal testing should be reduced to the absolute minimum. For that reason, animal tests are subject to a permit or notification procedure under which the justification for the test has to be submitted[4]. The competent authority bases its decision on a prescribed set of criteria[5].

For regulatory practice, the ethical consideration presents itself in a very particular way. Here, the benefit of laboratory tests on animals is not simply that humans are spared suffering and damage by the way chemicals are marketed today. Animal tests are also used to identify the harmful effects of substances on the environment. Avoiding or reducing this impact is the ultimate aim of REACH. It is to be achieved by testing and by management measures following the evaluation of the substances. Only in this way will it be possible to avoid or reduce suffering and harm to animals in the wild, which can otherwise, in extreme cases, go so far as to cause the extinction of individual species.

[1] On this topic see: http://www.verbraucherschutzministerium.de/ and http://www.tierschutz.de/ and http://www.bmt-tierschutz.dsn.de/ and http://www.tierschutzbund.de/ and http://www.ihep.jrc.it/ and http://www.ebra.org/ and http://www.bfr.bund.de/cd/1433
[2] Wolf U.: Tierversuche und angebliche moralische Konflikte. http://www.tierversuchsgegener.org/Tierversuche/ursula.wolf.html und Wolf J.-C.: Warum Tierversuche moralisch unzulässig sind. http://www.tierversuchsgegner.org/Tierversuche/jean-claude.wolf.html and http://www.tierversuchsgegner.org/Tierversuche/hartinger.html
[3] German Research Foundation: Tierversuche in der Forschung. VCH-Verlagsgesellschaft, 1993
[4] Animal Welfare Act. BGBl. I (Official Gazette) p. 1105, 1818 and Allgemeine Verwaltungsvorschrift zur Durchführung des Tierschutzgesetzes of 9 February 2000 (Secondary legislation implementing the Animal Welfare Act)
[5] Under the Animal Welfare Act, animal tests that are required by law (e.g. under pharmaceutical or chemicals legislation) are merely notifiable.

Tab. 1: Number of animals used in Germany in 2002 by purpose of test*

Basic biological research	Development & quality assurance of pharmaceuticals for human and veterinary use	Toxicological studies, safety testing	Diagnosing illnesses	Other purposes
826,729	854,078	207,511	50,700	273,358

*Federal Ministry of Consumer Protection, Food and Agriculture: Tierschutzbericht 2003

REACH

REACH stands for the Registration, Evaluation and Authorisation of Chemicals[6]. Without going into the details of the EU Commission's proposed regulation, we shall describe just those aspects that are of greatest importance for the topic under consideration here (animal testing). For more extensive treatment, please refer to the relevant literature[7,8,9,10].

Approximately 30,000 existing chemicals with sales volumes in excess of one tonne per annum are on the market in Europe. Under REACH, these substances will be subject to registration with a central agency. In connection with the registration, the applicant will have to submit a set of data describing, amongst other things, the intrinsic effects of the substance. The effect data are supplied by the manufacturer of the substance. If the manufacturer or the government agencies involved identify any "risks" on the basis of the hazards already ascertained, the substances will undergo more intensive evaluation. For particularly critical substances, the manufacturer will have to provide evidence of their safe use and apply for their authorisation. He will only receive authorisation if the governmental agency is convinced by the evidence provided and approves the use.

The proposal for REACH is currently being discussed in the European Parliament and Council. The EU Parliament is scheduled to complete the first reading in autumn this year and it is envisaged that a common position will be agreed in the Council under the British presidency. It is to be expected that a process of mutual consultation between the Commission, Council and Parliament will take place at the beginning of 2006. According to this timetable, REACH will then enter into force in 2007. Setting up the working structures needed and implementing the necessary secondary legislation will occupy the whole of 2008, so that REACH will necessitate data acquisition on a significant scale in 2010 at the earliest (*in other words, in over five years time*).

[6] http://europa.eu.int/eur-lex/de/com/pdf/2003/com2003_0644de.html
[7] http://www.umweltbundesamt.de/reach/
[8] http://www.bmu.de/chemikalien/doc/6486.php
[9] http://www.bmu.de/chemikalien/doc/6073.php
[10] http://www.bmu.de/chemikalien/aktuell/doc/35399.php

Tab. 2: Testing requirements under REACH as set out in Annexes V to VIII of the EU Commission's proposed regulation

> 1 t/a	> 10 t/a	> 100 t/a	> 1,000 t/a
Skin irritation	*In vivo* skin irritation test		
Eye irritation	*In vivo* eye irritation test		
Skin sensitisation			
Mutagenicity *In vitro* Ames-Test	*In vitro* mammalian gene mutation *In vitro* cytogenetics test		
	Acute toxicity		
	28-day test		Possibly long-term toxicity > 12 months
	Screening for toxicity related to reproduction	Toxicity related to reproduction for one animal species	Two generation reprotoxicity
Short-term toxicity for daphnia		Long-term test for daphnia	
	Growth-inhibitor test on algae		
	Short-term toxicity for fish	Long-term toxicity for fish	
	Absorption/desorption screening test	Further absorption/ desorption tests	
		Accumulation in fish	
		Short-term test on earthworms	Long-term test on earthworms
			Long-term toxicity for invertebrate animals
			Long-term toxicity for birds
		Short-term toxicity for plants	Long-term toxicity for plants

The data will be collected over a 10 to 15 year period in a tiered procedure based on quantity thresholds and risk criteria.

REACH will entail animal testing

The tests to be conducted under REACH are laid down in Annexes V to VIII. If the Commission's proposal is accepted, no tests on animals will be necessary for the basic data set in the volume range of 1 to 10 tonnes per year, as shown in table 2.

Above the 10 tonne per year threshold, *in vivo* tests for skin and eye irritation and toxicity in fish must be performed, although only if prior *in vitro* tests suggest that the test animal's skin will *not* be damaged. The purpose of the tests is thus merely to confirm negative results. Toxicologists consider this additional back-up necessary. Furthermore, the results of animal tests from what is known as the "28-day test" must also be submitted. This test provides initial indications of the effect of repeated exposure to a substance. This test is essential to carry out risk assessment for health and safety at the workplace, for example. A screening test for reprotoxicity gives initial indications of the possible risk to reproduction.

For the purpose of human toxicology, additional animal tests are required above the 100 tonne per year mark. They include a sub-chronic toxicity test (90 days), a two-generation test and a teratogenicity test to investigate toxicity related to reproduction. In the area of ecotoxicology, long-term toxicity to fish is one of the areas studied.

Ultimately, for production volumes in excess of 1,000 tonnes per year, further toxicological tests are only necessary in isolated and justified cases, e.g. to clarify suspected carcinogenicity. The tests for ecotoxicity include a test on birds.

Above and beyond the animal tests listed in table 2, the competent authority can demand further tests, if appropriate, as part of the process of evaluating a chemical. This will be the case when the set of data submitted shows grounds to suspect harmful effects that need further clarification. This evaluation is carried out for chemicals that, on the basis of the data submitted, are classified as high-risk or suspect chemicals and that therefore had to be placed on a rolling plan.

Animal testing only where absolutely essential

Of course, it is important to analyse critically whether the above-mentioned animal tests are necessary in cases where industry does not have the required data already. The people with the political responsibility for this decision are therefore asking scientists whether it is possible to evaluate the risk of chemicals in terms of chronic eco- and human toxicity without animal testing – using existing alternative methods, for example. The panel of experts appointed by the Commission (CSTEE, now Scher) gave its opinion on this in an extensive report published last year[11]. The background to this report was a proposal by the British anti-vivisection organisation to end animal testing under REACH and use alternative methods instead[12,13]. The panel of experts came to the conclusion that the animal tests mentioned cannot currently be replaced and that not carrying them out would significantly diminish today's level of health and environmental protection from hazardous chemicals.

This verdict nevertheless does not release us from the obligation to make every conceivable effort to reduce the number of animal tests required. However, it would also not be appropriate for politicians to disregard the verdict.

The 3R principle

As early as 1959, scientists Russel and Burch proposed a way of consistently reducing the number of animal tests. The 3Rs principle – replacement, reduction, refinement – is an integral part of the Commission's proposed REACH regulation.

Reduction

It is possible to reduce the number of animals used for testing without diminishing the informative value of the test or its validity. Table 3 shows the successes that have been achieved by reducing the number of test animals, illustrated by the example of the LD_{50} test used to determine acute toxicity.

A further aspect in reducing the number of test animals is the non-performance of duplicate or even multiple tests. It was by no means unusual in the past to see the duplication of tests. One reason for this was that national agencies did not reciprocally recognise each other's tests, but another frequent reason was that tests of that kind are expensive and companies regarded them as their personal property, so that sharing data with other companies did not happen automatically.

[11] European Commission, Health & Consumer Protection Directorate: Opinion of the Scientific Committee on Toxicity, Ecotoxicity and Environment (CSTEE) on the BUAV-EDEA Report. Brussels 8 Jan. 2004; http://66.249.93.104/search?q=cache:vYwulqWfyXwJ:europa.eu.int/comm/health/ph_risk/committees/sct/documents/out217_en.pdf+BUAV-ECEAE&hl=de

[12] BUAV-ECEAE Report: "The way forward – Action to end animal toxicity testing" http://66.249.93.104/search?q=cache:m3sUmnMOu5QJ:www.ne.jp/asahi/kagaku/pico/eu/reach/eu/REACH_Part_3.pdf+BUAV-ECEAE&hl=de

[13] See also: http://www.reachnonanimaltests.org/

Tab. 3: Success in reducing the number of test animals using the example of one test

In the area of toxicology, 37% of all animals are currently used for LD_{50} tests	
1970's	150 animals per substance
1980's, following harmonisation by the OECD	45 animals
1990's, tiered testing strategy	15 animals
Current ECVAM/ICCVAM test	5 animals ??
Future: *in vitro* test	0 (no animal)

In recent years, the OECD in particular has rendered outstanding service in this area[14]. The principle of MAD (Mutual Acceptance of Data) has been achieved through standard test methods and GLP (Good Laboratory Practice). This has led to a significant reduction in the number of animal tests, because the system is used worldwide (i.e. also by non-OECD countries). REACH is based on GLP and MAD and has developed this set of regulations further. Thus, every possibility of reducing the number of animal tests is being exploited.

"Officially," i.e. according to statements issued by the Commission, the Commission's proposal for REACH contains an obligation to use existing data from previous animal tests. However, there are in fact some gaps in the regulations here. This means that under the Commission's proposal duplication of tests is theoretically possible. Germany has thus submitted suggestions for corresponding text amendments to close these gaps.

One way in which the number of tests carried out will be reduced is the fact that multiple registrations of a single substance will be avoided. The avoidance of duplicated animal tests will be achieved under OSOR (*O*ne *S*ubstance, *O*ne *R*egistration) by the obligation to submit one joint set of core data per tonnage band. For subsequent tonnage bands, use should be made of the substances already registered (through a Substance Information Exchange Forum (SIEF) per tonnage band). Obliging companies to jointly submit a core data set also facilitates a better overview of existing (test) data. Under the Commission's REACH proposal, all the animal tests listed in table 2 for the 100 tonne per year category are subject to a proviso. They are not to be carried out automatically but only if the data is required for risk assessment. To this end, the manufacturer of a particular chemical has to submit a proposal for the further tests. He also has the possibility at this point to make a case for why the data are not necessary. Only when the competent authority has reviewed this proposal as part of its obligatory evaluation of the dossier and has come to a conclusion, are the tests listed in table 2 carried out.

Under REACH there are three possibilities for waiving tests:
1. the possibility that is fundamentally always there of not carrying out tests if they are not scientifically necessary or technically feasible;
2. the general stipulations in Annex IX of the REACH Regulation for deviations from the standard testing programmes set out under Annexes V to VIII;
3. special conditions for waiving individual tests (e.g. as stipulated in Annex VI.6.6.1: the 28-day test does not need to be conducted if relevant human exposure can be ruled out).

Refinement

Refinement in this context means carrying out the testing in a way in which additional information on toxic properties is acquired without the use of additional animals. This in turn means that further studies are not required. In recent years it has, for example, become possible to replace acute tests on fish by a test on fish eggs or embryos[15]. By incorporating modern molecular biological methods ("-omics" techniques, "toxicogenomics") into classic tests, it is already possible to classify chemicals in a particular category and thus predict their charac-

[14] http://www.oecd.org/searchResult/0,2665,en_2649_201185_1_1_1_1_1,00.html

[15] Gies A. et. al.: Replacing vertebrate testing in regulatory ecotoxicology, Umweltbundesamt, Workshop Ecotoxicity – Applying the 3 Rs, www.bmu.de/files/chemikalien/downloads/application/pdf/reach_minimierung_tierversuche.pdf

Tab. 4: *In vitro* testing as an alternative to animal testing (according to the Federal Institute for Risk Assessment – BfR)

OECD test method	Validated and accepted	Under development	Planned
Skin absorption, *In vitro* method **OECD TG 428**, Accepted 13.04.2004	X		
Skin corrosion, *in vitro*, Rat skin model **OECD TG 430,** Accepted 13.04.2004	X		
Skin corrosion, *in vitro*, Human skin model **OECD TG 431,** Accepted 13.04.2004	X		
***In vitro* phototoxicity test,** 3T3 NRU phototoxicity test **OECD TG 432,** Accepted 13.04.2004	X		
Acute inhalation toxicity Fixed Concentration Procedure – FCP **OECD TG 433,** 2nd draft 08.12.2004		x	
Acute dermal toxicity, Fixed Dose Procedure – FDP **OECD TG 434,** 1st draft 08.12.2004			x
In vitro micronucleus test **OECD TG 487.** 1st draft 14.06.2004			x
***In vitro* skin irritation,** Skin irritation *in vitro* ECVAM validation study 2003-2005		x	
Eye irritation, Acute eye irritation/corrosion **OECD TG 405,** ECVAM/ICCVAM co-operation project		x	
Chemical carcinogenicity , SHE cell transformation assay **OECD Draft TG,** ECVAM validation proposed		x	

teristic toxicological profile or a significant property, such as carcinogenicity. The use of these molecular biological methods has great potential. Their development should therefore be promoted more intensively in the future.

Another important aspect is making use of prior information (e.g. from screening assays, information about probable effect mechanisms) to carry out any necessary tests on vertebrates in such a way that the maximum amount of information for the relevant regulatory issue is obtained using the minimum number of animals (see toxicogenomics above and *in vitro* methods below).

This requires clear criteria and rules that have been agreed by all parties involved that set out the conditions under which tests are essential or can be waived. During the implementation phase of REACH[16], intensive discussions that incorporate a broad range of scientific and regulatory expertise will be necessary.

Replacement

When it comes to replacement, two different fields of action can be distinguished:

- the development of *in vitro* methods and models,
- the development of *in silico* methods and models.

From the regulatory point of view, it must be stressed that the "intelligent combination" of *in vitro* methods can help to minimise the number of definitive tests necessary (which as a rule are animal tests) and to maximise the information yield from those animal tests that are necessary. The successful implementation of these two aspects (1: marked reduction in the number of animal tests required and 2: the prevention, as far as possible, of false negative results) will probably always require the combination of several *in vitro* methods. Here it must be said that the cost involved in "intelligent" combinations of testing strategies should not be underestimated.

One obstacle to these methods becoming more widespread is the question of recognition (validation). This recognition often takes place in international bodies such as the OECD. The following table shows the current state of progress and a prognosis for the period up to 2010.

Table 4 shows that *in vitro* tests for many relevant endpoints still have to be developed or validated. It follows therefore that the results achieved are not yet satisfactory. Unfortunately, currently available *in vitro* test methods (that do not use animals) cannot determine to a fully satisfactory level how dangerous chemicals are. This also emerges from a report published by ECVAM[17]. It seems therefore that efforts in this direction need to be intensified. In my opinion, the availability of research funds should not be a problem here.

"*In silico* toxicology" (computer-aided analysis of effects and their interactions) is a relatively young discipline, but it already has a broad range of applications and methods[18]. It searches for connections between effects and chemical structures in order to make prognoses.

The models can to some extent be used for different effect endpoints. Table 5 shows a selection of the *in silico* models available today for endpoints that are significant for human toxicology and ecotoxicology. The decisive point for the issue we are looking at here (animal testing) is whether the methods mentioned can also produce sound results. To try and determine this, the European Centre for Ecotoxicology and Toxicology of Chemicals carried out a study, the results of which are summarised in table 5 (32).

The study shows that validated models are available for particular endpoints, especially in the area of ecotoxicology, and that they provide sound results. But this is not the case for the issue under discussion here, i.e. the long-term test for chronic toxicity to humans. However, the available models do already provide important additional information that can be used to back-up evaluations.

The applicability of an *in silico* model is highly dependent on the chemical structure of a substance. In other words, all models are not equally well suited for all chemicals. To improve the applicability of *in silico* methods we must therefore establish which methods are suitable for which chemicals. That is the only way to establish whether an *in silico* method is applicable and what level of uncertainty is associated with the prediction.

It is not yet possible to significantly reduce the number of animal tests on the basis of the knowledge described above. The majority of toxic effects of substances are currently still difficult to predict. It would therefore seem essential to step up efforts in the area of *in silico* methods, too. In my opinion, the availability of research funds should similarly not be a problem here. It

[16] REACH Implementing Process (RIPs): http://ecb.jrc.it/REACH/RIP_PROJECTS/

[17] Worth A.P., Balls M. (Eds.): Alternative (Non-animal) Methods for Chemical Testing. A Report prepared by EVCAM, ATLA 30, 2002

[18] http://www.ndsu.nodak.edu/qsar_soc/resource/software.htm

[19] http://www.bgvv.de/cm/232/alternative_testverfahren_und_intelligente_teststrategien_position_aus_sicht_der_wissenschaft.pdf

Tab. 5: Applicability of *in silico* models for endpoints relevant to ecotoxicology and human toxicology*

Acute toxicity to fish	Good
Bioaccumulation	Good
Biodegradability	Good
Mutagenicity	Limited to good
Acute oral toxicity	Limited
Acute toxicity to algae	Limited
Acute toxicity to bacteria	Limited
Acute toxicity to mammals	Limited
Eye irritation	Limited
Hydrolisation	Limited
Photo-degradability	Limited
Skin irritation	Limited
Skin sensitisation	Limited
Carcinogenicity	Very limited
Chronic toxicity	Very limited
Teratogenicity	Very limited

*ecetoc: (Q)SARs: Evaluation of the commercially available software for human health and environmental endpoints with respect to chemical management applications. Technical Report No. 89

therefore seems possible that, within the next few years, we will be able to make predictions for some endpoints that are clearly defined in terms of mechanisms (such as acute toxicity, corrosive effects, irritant effect), and that they will be able to replace animal testing as part of an intelligent, tiered procedure[19].

Conclusion

It has become clear that neither the replacement methods nor methods based on molecular biology are advanced enough to make it possible to dispense completely with animal testing. On the other hand, it is impressive to see in concrete terms how selected examples illustrate just what progress has already been made in developing alternative methods to replace animal testing.

Annex IX of the Commission's REACH proposal explicitly provides for the possibility of data on toxicity to be acquired using replacement methods, on condition that these methods have been validated.

The German federal government explained in detail its position on the problem of animal testing under REACH back in spring 2004 in its response to Question 14 of the CDU/CSU parliamentary party's Major Interpellation – "The economic impact of the EU's chemicals policy". In this context it advocated:

- more, far-reaching regulation on data acquisition within the Community and on the use of alternative methods along the lines of the regulations already in place in Germany,
- the rapid validation of a number of other alternative methods that have already been developed and that could be used as part of the programme to phase-in existing chemicals, and
- encouraging systematic use of the data acquired in the initial phases for the further development of structure-activity analyses.

In line with these statements, the federal government has already fed into the Council's deliberations in Brussels on the Regulation extensive proposals for wording the text in a way that would close up regulatory gaps and consistently avoid multiple testing. With regard to the explanations of the data requirement annexes, it has also pushed for greater importance to be attached to alternative methods in the context of testing strategies for certain toxicological endpoints, for which this is possible.

In order to reduce the number of animal tests to be carried out, it will be decisive to what extent it proves possible in the next five years to bring to a successful and prompt conclusion the research efforts already begun on the development and validation of *in vitro* and *in silico* replacement methods.

However, we do not expect that complete replacement of animal testing will be achieved, particularly in the area of long-term effects of chemicals.

Correspondence to
Dr. rer. nat. Uwe Lahl
Director General
Federal Ministry for the Environment
Nature Conservation and Reactor Safety
Robert-Schumann-Platz 3
53175 Bonn, Germany
e-mail: Uwe.Lahl@bmu.bund.de

ICCVAM's Role in Validating *In Vitro* Test Methods for Endocrine Disruptor Screening*

Marilyn Wind[1], Bradley Blackard[2], Raymond Tice[2], William Stokes[3], David Hattan[4] and Leonard Schechtman[4]

[1]US Consumer Product Safety Commission, Bethesda, Maryland, USA; [2]Integrated Laboratory Systems, Inc./NICEATM, Research Triangle Park, North Carolina, USA; [3]National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)/National Institute of Environmental Health Sciences Research, Research Triangle Park, North Carolina, USA; [4]US Food and Drug Administration, Rockville, Maryland, USA

Summary

Estrogen receptor (ER) and androgen receptor (AR) binding and transcriptional activation (TA) assays have been proposed as part of Tier 1 of the two-tiered endocrine disruptor screening battery the U.S. Environmental Protection Agency (EPA) is developing. ICCVAM comprehensively reviewed all in vitro ER and AR binding and TA assays and concluded that none were adequately validated. Minimum procedural standards such as dose selection criteria, number of replicates per test, appropriate positive and negative controls and criteria for an acceptable test were proposed that should be incorporated into standardised protocols for each of the four types of assays evaluated.

Keywords: endocrine disruptor screening, ER and AR binding tests, ER and AR transcriptional activation tests

Background

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) is an interagency group consisting of both U.S. Federal regulatory and non-regulatory agencies. Table 1 lists the 15 U.S. Federal agencies participating in ICCVAM.

ICCVAM was formed as an *ad hoc* committee in 1994 and was officially established by Congress in 1997. Its purpose is to: 1) Increase the efficiency and effectiveness of the U.S. Federal agency test method review; 2) Eliminate unnecessary duplicative efforts and share experience between U.S. Federal regulatory agencies; 3) Optimise utilisation of scientific expertise outside the U.S. Federal government; 4) Ensure that new and revised test methods are validated to meet the needs of U.S. Federal agencies and 5) Replace, reduce, or refine the use of animals in testing, where feasible.

Under the ICCVAM Authorization Act of 2000, ICCVAM's duties are defined as follows: 1) Review and evaluate new, revised or alternative test methods; 2) Facilitate interagency and international harmonisation of test methods; 3) Facilitate and provide guidance on test method development, validation criteria, and validation processes; 4) Facilitate acceptance of scientifically valid test methods; 5) Submit test recommendations to U.S. Federal agencies and 6) Consider petitions from the public for review and evaluation of validated test methods.

The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) is located at the National Institute of Environmental Health

*This paper reflects the views of the authors, has not been reviewed or approved by, and may not necessarily reflect the views of the Consumer Product Safety Commission or other agencies. This work was supported in part by NIEHS Contract N01-ES 35504. Since this paper was written as part of the official duties of the authors, it can be freely copied.

Tab. 1: ICCVAM participating U.S. Federal Agencies

Regulatory/Research	Non-Regulatory/Research
Consumer Product Safety Commission	Department of Defense
Department of Agriculture	Department of Energy
Department of Interior	National Cancer Institute
Department of Transportation	National Institute of Environmental Health Sciences
Environmental Protection Agency	National Institute for Occupational Safety and Health
Food and Drug Administration	National Library of Medicine
Occupational Safety and Health Administration	National Institutes of Health, OD
Agency for Toxic Substances and Disease Registry	

Sciences (NIEHS) in Research Triangle Park, NC. It functions as ICCVAM's operational arm, providing technical support. NICEATM conducts test method peer reviews and workshops and communicates and forms partnerships with stakeholders. All the background review documents referenced, the report of the peer review panel and documents on all the alternative test methods ICCVAM has reviewed can be found on our website (http://iccvam.niehs.nih.gov).

This paper focuses on the work ICCVAM has done on endocrine disruptor assays. In order to understand ICCVAM's role in validating *in vitro* test methods for endocrine disruptor screening, it is necessary to understand some of the history and why this is an area of interest. During the last two decades, there have been an increasing number of observations of reproductive and developmental abnormalities in animal populations exposed to high levels of certain persistent pollutants in the environment. There has also been an increased incidence of birth defects, cancers in hormonally receptive tissues and decreased fertility, which have been attributed to exposure of humans to endocrine disruptors. As a result of concern about the observations in both animals and humans, the U.S. Congress enacted The Food Quality Protection Act of 1996 and Amendments to the Safe Drinking Water Act in 1996. The U.S. Congress required the EPA to develop and validate a screening and testing programme to identify substances with endocrine disrupting (ED) activity. In 1998, EPA proposed their Endocrine Disruptor Screening Program (EDSP), which consists of a Tier 1 screening battery of *in vitro* and *in vivo* assays designed to identify substances capable of interacting with the endocrine system. Tier 2 of the EDSP is a battery of *in vivo* assays that provides detailed information on concentration response relationships and specific abnormal effects. Based on a weight-of-evidence evaluation of the results from the Tier 1 screening battery, Tier 2 *in vivo* tests are conducted. Included among the proposed Tier 1 *in vitro* assays are ER and AR binding and TA assays.

In April 2000, EPA requested ICCVAM to evaluate the validation status of *in vitro* ER and AR binding and TA assays. Background Review Documents were prepared for each type of assay. These documents can be found on the ICCVAM website. On May 21-22, 2002 (http://iccvam.niehs.nih.gov/docs/docs.htm#endocrine), an Expert Panel met in public session. In October 2002, a final report of the Expert Panel and the Endocrine Disrupter Working Group's (EDWG's) list of proposed substances for validation were made public for comment.

The Expert Panel was charged with the following:

- Review Background Review Documents and provide conclusions and recommendations on the following:
  - What assays should be considered for further evaluation in validation studies, and what is their relative priority
  - The adequacy of the proposed minimum procedural standards for each of the 4 types of assays
  - The adequacy of available test method protocols for assays recommended for validation studies
  - The adequacy and appropriateness of the substances/chemicals recommended for validation studies

Table 2 summarises what the databases for the ER and AR binding assays looked like.

ER binding was measured in fourteen different *in vitro* assays. These assays used ER derived from uterine cytosol from the mouse, rat and rabbit, from MCF-7 cells and MCF-7 cytosol and

Tab. 2: Estrogen and androgen receptor binding assays

	ER Binding Assay	AR Binding Assay
# of Assays	14	11
# Substances	635	108
% Substances Tested ≥ 2/Assay	37	31
# Chemical Classes Tested	17	5
Most Frequent Chemical Class	Polychlorinated Biphenyls	Nonphenolic Steroids
# Product Classes Tested	7	3
Most Frequent Product Class	Pharmaceuticals	Pharmaceuticals

Tab. 3: Transcriptional activation assays

	ER TA Assay	AR TA Assay
# of Assays	95	17
Total # of Substances Tested	703	146
# Tested for Agonism	634	109
% Agonists ≥ 2 Assays	36	45
# Tested for Antagonism	255	87
% Antagonists ≥ 2 Assays	37	26
# Chemical Classes Tested	15	7
Most Frequent Chemical Class	Polychlorinated Biphenyls	Nonphenolic Steroids
# Product Classes Tested	11	5
Most Frequent Product Class	Pesticides and Metabolites	Pharmaceuticals

from human cDNA clones of two human ER isoforms, ERα and ERβ (hERα and hERβ). Fusion proteins, in which glutathione (GST) was fused with the *def* domains of the human ERα (GST-hERα) and the ER from mice (GST-mER), chicken (GST-cER), anole (GST-aER) and rainbow trout (GST-rtER) were the basis for five assays. None of these assays had been validated and standard protocols were not used, even when the same assay was used in multiple laboratories. Although 635 chemicals had been tested in these assays, few were tested more than once in the same assay or in multiple assays. Only 8% of the substances were tested in seven or more assays. Thirty-seven percent of the substances were tested in two or more assays.

AR binding was measured in eleven different *in vitro* assays. These assays used AR derived from cytosol from rat prostate, rat epididymis and calf uteri, human cell lines (MCF-7, LnCaP) with endogenous AR, and a mammalian cell line (COS-1) transfected with human (h) AR. In addition, primary human genital fibroblasts (HGF) with endogenous AR, mammalian cell lines (COS-1) transfected with either hAR or rainbow trout ARα, and recombinant hAR Sf9 insect cells were also used in the assays. A majority (61%) of the 108 substances tested were only tested in one test. Since so few substances had been tested more than once in the same *in vitro* AR binding assay or in multiple assays using the same reference androgen, no quantitative or qualitative analyses of the comparative performance or the reliability of these assays was possible.

Table 3 summarises the database for the ER and AR TA assays. There are currently no generally accepted standardised methods for these assays. The *in vitro* TA assays used to identify ER agonists and antagonists fall into three broad groups: reporter gene assays using yeast cells; reporter gene assays using mammalian cells and cell proliferation assays using mammalian cells. Most of the mammalian cell lines and all the yeast cells lack an endogenous ER. The yeast strains and mammalian cell lines used in the various studies are listed in tables 2-1 to 2-3 of the Background Review Document for the ER TA assays. The ER used in the majority of the *in vitro* ER TA studies was human in origin. A few studies used ER derived from mouse or rainbow trout. A total of 703 substances had been tested in 95 *in vitro* ER TA assays. 634 substances were tested for agonism, with only 36% of the substances tested in two or more assays and only 8% tested in five or more assays. 255 substances were tested for antagonism; 37% percent of the substances were tested in two or more assays and only 3% were tested in five or more assays.

There are no standardised methods for performing AR TA assays. Studies were conducted using yeast (*Saccharomyces cerevisiae*), nine different mammalian cell lines, and one fish (carp) cell line. Of the mammalian cell lines used, six were human, two monkey and one was from Chinese hamster. The majority of studies used cells that were transiently transfected with AR. In other studies, cells were stably transfected with a plasmid containing the gene coding for the AR or contained an endogenous AR. The human AR was used in all but two of the studies. The remaining two studies used cells transfected with trout and mouse AR. One hundred forty-six substances were tested in 17 AR TA assays. Of the 109 substances tested for agonism, 45% were tested in two or more assays and 16% were tested in four or more assays. Of the 87 substances tested for antagonism, 26% were tested in two or more assays and 7% were tested in four or more assays.

Based upon a review of the background review documents, the expert panel concluded and ICCVAM concurred that:

1. There are no adequately validated *in vitro* ER- or AR-based assays.
2. No assays could serve as the basis for establishing performance standards.
3. There was little consistency among available protocols.
4. No test method protocol was adequately detailed and standardised.

In order to validate any of these test methods, the expert panel recommended minimum standards for all the assays; ICCVAM agreed with the recommendations. They are as follows:

- The limit concentration is 1 millimolar (mM); solubility characteristics must be taken into consideration.
- The concentration range should span at least 7 orders of magnitude and include at least 7 different concentrations.
- Triplicate measurements should be made at each concentration.
- For TA assays that use transient transfection methods, a constitutive reporter gene assay is needed to assess the efficiency of transfection.
- For TA assays, stability of cell lines with a stably transfected reporter should be monitored.
- For TA assays, cytotoxicity to define the upper limit for test substance concentrations is required.
- Reference estrogen/androgen and/or positive control responses must be consistent with historical data.
- For binding assays, substances that bind but do not bring about a 50% reduction in ER/AR binding should be classified as “equivocal”.
- For TA assays, a nonlinear regression model such as the Hill equation should be used to estimate the potency (EC_{50} or IC_{50} values) and slope of the concentration-response curve.
- Classification of a test substance as 'positive' should be based on statistical models pertinent to the characteristics of the assay.
- Replicate studies are not essential, but questionable data should be confirmed by re-testing.
- All studies requiring animals as tissue sources should be approved by an IACUC.
- The assays should be conducted following Good Laboratory Practice guidelines.

The following recommendations were made by ICCVAM for each of the four types of assays: For ER binding assays:

- Recombinant rat or human ER’s should be given highest priority for further test method standardisation, prevalidation and validation.
- An effort should be made to optimise a fluorescence-based method.
- Protocols should be standardised to incorporate minimum procedural standards.
- A minimum of 53 reference substances should be used in validation studies.

For AR binding assays, the following were recommended:

- Use recombinant protein as the source of AR.
- Standardise the protocol to incorporate the recommended minimum procedural standards.
- Conduct validation studies using, at a minimum, the 44 recommended reference substances.

For ER TA assays, the following were recommended:

- A comparative study to determine whether transiently or stably transfected lines are more appropriate should be conducted.
- Protocols should be standardised to incorporate minimum procedural standards.
- At minimum, assays should be validated using the designated 53 reference substances.

For AR TA assays the following were recommended:

- Develop a cell line containing an endogenous AR that is transduced with an adenovirus containing a reporter vector that shows high specificity for the AR.
- Standardise the assay protocol to include the recommended minimum procedural standards.
- Validate the assay using the same 44 reference substances recommended for the AR binding assays.

In addition to the recommendations for the specific types of assays, ICCVAM also recommended:

- No metabolic activation system should be recommended at this time.
- Prevalidation studies should be conducted to generate data necessary for biostatisticians to develop statistical methods for analysing data.
- The predictive value of these *in vitro* assays for estimating *in vivo* responses should be determined.
- A central repository of 78 substances should be organised.

Acknowledgments

All the expert panel members and especially those who chaired the individual panels (George Daston, John Stegman, Terry Brown and Elizabeth Wilson). The ICCVAM Endocrine Disruptor Working Group, especially the co-chair, David Hattan. The entire ICCVAM committee, especially Leonard Schechtman, the Chair, and William Stokes, the Executive Director. NICEATM staff without whose support we would not have had the background review documents, the expert panel meeting and all the documents on our website.

Correspondence to

Marilyn Wind, Ph.D.
Directorate for Health Sciences
US Consumer Product Safety Commission
4330 East West Highway
Bethesda, MD
USA 20814
e-mail: MWind@cpsc.gov