Acute Toxicity Testing *Without* Animals

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"more scientific and less of a gamble"

Report by
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Contents

Foreword

The BUAV and European Coalition to End Animal Experiments

Established in 1898, the British Union for the Abolition of Vivisection (BUAV) is the world's leading organisation campaigning peacefully to end all animal experiments. As Chair of the European Coalition to End Animal Experiments (ECEAE), the BUAV liaises with key animal groups across Europe to co-ordinate campaigning initiatives and ensure that laboratory animals are high on the European political agenda. As a founding member of the International Council for Animal Protection in OECD Programmes (ICAPO), the BUAV joins with animal protection groups across Europe, the United States and Japan to ensure that laboratory animals have an effective voice within the Organisation for Economic Co-operation and Development, as it co-ordinates international testing guidelines that affect laboratory animals around the world.

Acute Toxicity Testing Without Animals

The BUAV has led the campaign to eliminate animal testing from the European Union's REACH chemicals strategy. Whilst we fully support the aim of improving chemicals regulation to protect human health and the environment, we believe that the use of animal testing will inflict immense and needless suffering while failing to provide the reliable information essential to achieving that end.

Throughout discussions of the REACH proposals, the BUAV and ECEAE have been campaigning for the use of non-animal testing strategies, mandatory data-sharing and increased funding for the development and validation of further non-animal tests. We have contributed to expert working groups, consultations and stakeholder conferences, and thus have been instrumental in ensuring that animal suffering is not forgotten as the EU and its member countries strive for more effective chemicals regulation. This work is not only helping to guarantee that every opportunity is taken to ensure that the proposal contains the most humane and effective science available but is also shaping the wider debate about animal toxicity testing at an international level.

The BUAV has published a series of influential reports on the use of animal toxicity tests. These include *The Way Forward*, a comprehensive non-animal testing strategy, in 2001, and, in 2004, *A Regulatory Smokescreen* presenting an analysis of the systemic failings of animal toxicity testing in chemicals regulation. These are available on our website (see below).

This report, the latest in that series, targets the specific issue of acute toxicity testing.

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1. Introduction

Since the early days of the REACH proposals, it has been accepted by all parties that the numbers of animals used to gain toxicity information on chemicals should be kept to an absolute minimum.

In its ground-breaking and influential report, *The Way Forward — A Non-Animal Testing Strategy for Chemicals*[1], the BUAV and ECEAE argued that animal test data should not be part of the new chemicals policy.[2] The reasons include the suffering caused to animals by testing; the unreliability of animal (usually rodent) data when extrapolating to humans; the ways that animal data can obstruct regulatory decision-making[3]; and the cost and delays that would be caused by seeking animal data.

In this report, we argue specifically that acute toxicity data should not be sought from animal tests. The underlying principle of such tests on rats and mice is that the results can be effectively extrapolated to humans. In fact, after nearly 80 years of use of these tests, the predictivity of rodent data for human acute toxic effects has been disputed but never proven.

Tests, in which animals are poisoned to death, have no place in a 21st Century chemicals policy. There is already evidence that *in vitro* and *in silico* methods for acute toxicity are able to provide sufficient data for classification and labelling purposes, and further rapid advances in the field of non-animal research will be achieved as resources are made available.

As Erik Walum of the University of Stockholm wrote[4]:

> "The value of the prediction of acute human toxicity based on this information will depend on the similarity between the test species and man in all the events involved. ...The modelling of quantitative toxicity in vitro is more scientific and less of a gamble. It involves a multiple analysis of many

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[1] BUAV (2001). The Way Forward — A Non-Animal Testing Strategy for Chemicals. Publ. BUAV & the European Coalition to End Animal Experiments, London.

[2] In July 2003, two years after our report *The Way Forward* was published, the European Commission's DG Environment referred it to the Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE) for a detailed opinion.

The opinion [CSTEE (2004). Opinion of the Scientific Committee on Toxicity, Ecotoxicity and the Environment on the BUAV-ECEAE report on *The Way Forward – Action to End Animal Toxicity Testing.* Brussels, C7/VR/csteeop/anat/080104 D(04)] included criticisms of our report but the basis and validity of these criticisms was surprising and often unfounded. The CSTEE failed to consider the BUAV's recommendations in the proper context of the 7-point action plan we had proposed, and thus misrepresented our proposals. Criticisms that *The Way Forward* did not take dose/response relationships into account were fully refuted [BUAV response to the CSTEE criticisms of *The Way Forward.* February 2004. Publ. BUAV].

The CSTEE made extraordinary and unsubstantiated claims for the validity of animal tests, contradicting the views of many other experts, including ECVAM. We provided specific, well-referenced critiques in our original report which we expanded in our rebuttal.

[3] G Langley (2004). Chemical Safety and Animal Testing: A regulatory smokescreen? A BUAV Report, publ. BUAV/ECEAE, London.

[4] E Walum (1991). Acute Toxicity Testing. In: Animals and Alternatives in Toxicology. Eds: M Balls *et al.* Publ. FRAME, Nottingham.

> parameters, which can be studied separately or in combination — a procedure which can be designed to fit the questions asked. ...Each piece of evidence can be evaluated in terms of its relevance for the corresponding human event."

This report aims to expose the scientific weaknesses of animal tests for acute toxicity; inform readers as to the extent of animal suffering caused by these crude and outdated tests, and to propose non-animal alternative test strategies.

2. The Case Against Acute Toxicity Tests on Animals

For many decades, chemicals were routinely classified for acute toxicity by the LD50 test, which requires the poisoning of many animals per chemical. For testing by the oral route, the LD50 method has been deleted from OECD test guidelines in favour of protocols using fewer animals. These include the Fixed Dose Procedure, the Acute Toxic Class Test and the Up-and-Down Procedure, which provide an estimated LD50 value.

However, for dosing by the dermal (skin) route and by inhalation, the LD50/LC50 tests are still the norm. This means that many animals are still being poisoned to death using a highly-criticised method essentially unchanged since the late 1920s (see below). Twenty-first century science must surely be capable of greater sophistication (and humanity) than this.

Acute toxicity refers to the effects on the whole body of a single dose of a chemical (or several doses within a 24-hour period), usually manifested over a period of 14 days.

Acute toxicity data are used mainly to:

i] Identify lethal/toxic doses of chemicals for humans (primarily for the regulatory purposes of classification and labelling);
ii] Indicate the mode of toxicity in humans, including the susceptibility of key target organs; and
iii] Provide a rough guide for dose selection in repeat-dose tests in animals.

From the regulatory point of view, acute toxicity data are mainly required for classifying and labelling chemicals according to their intrinsic toxicity[5], with the aim of allowing their safe transport and the protection of people handling and using them. This aim is frustrated by species differences, and can be achieved by non-animal methods (see Section 6). Further, worker and consumer exposures are most commonly at a low-dose, repetitive level rather than by a single, massive dose.

For identifying target organ susceptibility, acute toxicity tests on animals are of limited relevance to people, partly because of variations between species in the way they deal with and react to chemicals. Autopsy of animals may highlight major affected target

[5] L Gribaldo *et al* (2004). Report for establishing the timetable for phasing out animal testing for the purpose of the cosmetics directive. Acute Toxicity Chapter. European Commission DG Enterprise website: http://pharmacos.eudra.org/F3/cosmetic/AnimalTest.htm

organs, but these are usually liver and/or kidney because they receive the highest chemical exposure[6]. Similar information could be obtained from *in vitro* studies.

Zbinden and Flury-Roversi[7] compared lethal dose values from animal tests with those discovered in cases of accidental human poisoning, and concluded:

> "For the recognition of symptomatology of acute poisoning in man, and for the determination of the human lethal dose, the LD50 in animals is of very little value."

For dose selection for repeat-dose studies, acute toxicity data can provide a rough guide. However non-animal methods, for example human cell culture approaches, would allow *in vitro* repeat-dose studies to be conducted rapidly and without the problems of species extrapolation, cost and delay incurred by animal tests.

For example, under the EU Framework Programme 6 a project called PREDICTOMICS is being funded to develop short-term *in vitro* assays for long-term toxicity[8]. This will be achieved using co-cultures of resident cell types, target cell transformation, stem cell technology and new developments in organotypic cell culture. The project aims to identify specific early mechanistic markers of toxin-induced cell alterations by using integrated genomic, proteomic and cytomic analysis; and to establish and prevalidate a screening platform (cell systems together with analysis tools) which is unambiguously predictive of toxin-induced chronic renal and hepatic disease.

2.1 Human relevance of acute toxicity tests on animals

The most important limitations of animal tests are the wide variations in responses caused by species and strain differences. These variations occur in sensitivities to toxic chemicals as well as in rates and routes of metabolism and in absorption, distribution and excretion.

All animal studies conducted in the hopes of protecting human health have this critical weakness. As Garattini wrote, concerning the difficulties of extrapolating from animals to humans[9]:

> "A third problem concerns the difference in various animal species in the biological substrates on which chemicals exert their toxic effects. Equal concentrations of chemicals and their metabolites do not mean equal toxic effects across animal species because endogenous metabolic processes, cell permeability, enzymes, and receptors are not necessarily the same in animals and man."

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[6] SM Barlow *et al* (2002). Hazard identification by animal-based methods of toxicology. Food & Chem. Toxicol.
[7] G Zbinden & M Flury-Roversi (1981). Significance of the LD50 test for the toxicological evaluation of chemical substances. Archives Toxicol. 47:77-99.
[8] http://fp6.cordis.lu/fp6/home.cfm
[9] S Garattini (1985). Toxic effects of chemicals: difficulties in extrapolating data from animals to man. Crit. Rev. Toxicol. 16:1-29.

Acute toxicity tests on animals, whether using the LD50 or other protocols, have never undergone formal validation to modern standards[10] to establish their relevance for humans. As Ekwall and colleagues said of the LD50 test[11],

> "The test has never been formally validated. The widespread use of the test has therefore not been based on a documented good performance, but on the lack of better tests."

The LD50 test itself was designed in 1927 for the purpose of standardising biological preparations, and then adapted for testing the acute toxicity of chemical substances. Concern about species and strain variations was first expressed many decades ago and continues to the present day.

For historical reasons, many of the criticisms were made of the original LD50 test, but those cited here are equally valid for modified oral toxicity tests including the Fixed Dose Procedure, the Acute Toxic Class and the Up-and-Down Procedure, as well as the LD50 (dermal) and LC50 (inhalation) methods, which are still in use today.

The Physicians Committee for Responsible Medicine[12] illustrate the problem by means of a table comparing LD50 values for rats and mice for 10 substances:

Comparison of LD50 values in rats and mice
(NIOSH/Registry of Toxic Effects of Chemical Substances)

Chemical	**Rat LD50 (mg/kg)**	**Mouse LD50 (mg/kg)**	**Ratio (rat:mouse)**
Carbon tetrachloride	2,350	8,260	0.28
Dextropropoxyphene HCl	84	225	0.37
Dichloromethane	1,600	873	1.8
Diphenylhydantion	1,640	150	10.9
Ethanol	7,060	3,450	2.0
Mercury (II) Chloride	1	6	0.17
Nicotine	50	3	16.7
Paracetamol	2,400	340	7.0
Sodium oxalate	11,200	5,100	2.2
Thioridazine HCl	995	385	2.6

There are many other examples: acetaminophen is fatal to mice at 250-400 mg/kg causing death through liver necrosis. In rats the LD50 value is about 1,000 mg/kg but there is little sign of liver damage[13]. Mice and rats show significant species differences in

[10] OECD (1996). Final report of the Solna Workshop on validation and regulatory acceptance criteria for alternative tests. OECD report ENV/MC/CHEM/TG(96)9.
[11] B Ekwall *et al* (1998). MEIC evaluation of acute systemic toxicity. Part VI. The prediction of human toxicity by rodent LD50 values and results from 61 *in vitro* methods. ATLA 26:617-658.
[12] PCRM (1999). Website: www.pcrm.org/resch/anexp/LD50.html [visited December 2004].
[13] DJ Jollow *et al* (1974). Acetaminophen-induced hepatic necrosis. VI. Metabolic disposition of toxic and non-toxic doses of acetaminophen. Pharmacol. 12:251-271.

response to naphthalene[14]. The LD50 value for thiourea in the Hopkins strain of rat is 4 mg/kg, but in the wild Norway rat is 1,830 mg/kg[15].

Genomic research has revealed that, in evolutionary terms, rats and mice diverged as separate species between 18-24 million years ago. They are much more similar, genotypically and phenotypically, than are rodents and humans — who diverged 80 million years ago. Differences in reactions to chemicals between closely related rats and mice demonstrate why acute toxicity tests on rodents have even more dubious predictivity for humans.

A 1948 report found that the sensitivity of humans to chemicals was occasionally the same as measured in animals, but in general was higher. Large differences (up to 2,000-fold) between humans and animals were typical[16]. As Gerhard Zbinden commented[17]:

> "...with such enormous variations, it is clear that the knowledge of the LD50 in a mouse or a rat does not provide much support for the prognosis in a human case of acute poisoning."

The 1981 study by Zbinden and Flury-Roversi[18], comparing animal lethal dose values with human poisoning data, revealed many discrepancies. For example, humans were 1000 times more susceptible to atropine than predicted by animal tests, while laboratory animals were 15 times more sensitive to barbital.

Lorke[19] added in 1983 that:

> "...even if the LD50 could be measured exactly and reproducibly, the knowledge of its precise numerical value would barely be of practical importance, because an extrapolation from the experimental animals to man is hardly possible."

The Multicentre Evaluation of *In vitro* Cytotoxicity tests (MEIC) programme found that for 50 reference chemicals, the rat LD50 values predicted values in mice well (correlation coefficient 0.88), but rat and mouse LD50 values predicted human acute lethal doses rather poorly (correlation coefficients 0.61 and 0.65 respectively)[20].

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[14] DJ Quick & ML Shuler (1999). Use of *in vitro* data for construction of a physiologically based pharmacokinetic model for naphthalene in rats and mice to probe species differences. Biotechnol. Prog. 15:540-555.
[15] SH Dieke & CP Richter (1945). Acute toxicity to rats in relation to age, diet, strain, and species variation. J. Pharmacol. Exp. Ther. 83:195-202.
[16] R Müller (1948). Vergleich der im Tierexperiment und beim Menschen tödlichen Dosen wichtiger Pharmaka. Diss. Univ. Frankfurt/Main.
[17] G Zbinden (1984). Acute toxicity testing, purpose. In: Acute Toxicity Testing; Alternative Approaches. Ed. AM Goldberg. Publ. Mary Ann Liebert Inc., New York.
[18] G Zbinden & M Flury-Roversi (1981). Significance of the LD50 test for the toxicological evaluation of chemical substances. Arch. Toxicol. 47:77-99.
[19] D Lorke (1983). A new approach to practical acute toxicity testing. Arch. Toxicol. 54:275-287.
[20] B Ekwall *et al* (1998). MEIC evaluation of acute systemic toxicity. Part VI. The prediction of human toxicity by rodent LD50 values and results from 61 *in vitro* methods. ATLA 26 (Suppl. 2):617-658.

The Olson report, published in 2000[21], reviewed animal toxicity test data and human side effects for 150 drugs. Comparisons were made between a wide range of toxic effects in animals and the significant toxicities actually found when humans were given the drug during clinical trials. The report therefore excluded side effects seen in animals but not in humans; and long-term side effects such as carcinogenicity or effects on reproduction (since these human toxicities cannot be assessed in relatively short-term clinical trials).

Olson and colleagues found that results from rodent tests (mainly acute studies) *only predicted 43 per cent of human effects*. This appalling performance would have been even worse if carcinogenicity and toxicity to reproduction had been included in the comparison. If a non-animal method undergoing validation yielded results of such low predictivity, it would be considered totally unsuitable.

It is hardly surprising that there are significant species differences in acute toxicity results, given the variations that exist in critical determinants of toxicity such as metabolism and bloodstream protein binding[22]. The spectrum and activities of crucial liver enzymes that detoxify chemicals, such as cytochrome P450 enzymes, vary from species to species[23]. For example, metabolism largely accounts for the difference in butadiene toxicity seen between rats and mice. In mice (compared to rats), peak concentrations of the chemical are 4-8 times higher in the blood, 13-15 times higher in the lungs and 5-8 times higher in the liver[24].

Finally, most cases of acute poisoning involve children under five years of age. Their susceptibility is very different to that of adults, yet a study comparing newborn and adult animals found large variations in toxic effects related to developmental patterns that are species-specific and cannot be easily extrapolated to human infants[25].

For nearly 80 years an unproven assumption — that tests on rodents accurately predict toxicity in humans — has formed the mainstay of acute toxicity testing. Retrospective assessments have continued to cast serious doubts on the validity of the tests, especially their relevance to humans. It is unbelievable that unproven methods of toxicity testing that are eight decades old should be considered suitable to include in a 21st-century chemical regulatory system.

2.2 Reliability of acute toxicity tests on animals

A 1964 study found the LD50 test to have unacceptably poor reproducibility[26]. Lethal dose tests using the inhalational and dermal routes, as well as other acute toxicity animal tests, also suffer from variability.

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[21] H Olson *et al* (2000). Concordance of the toxicity of pharmaceuticals in humans and in animals. Reg. Toxicol. Pharmacol. 32:56-67.
[22] T Marrs (1988). In: Perspectives in basic and applied toxicology. Ed. B Ballantyne. Publ. John Wight & Co., Bristol.
[23] JW Paxton (1995). The allometric approach for interspecies scaling of pharmacokinetics and toxicity of anti-cancer drugs. Clin. Exp. Pharmacol. Physiol. 22:851-854.
[24] MW Himmelstein *et al* (1996). Metabolism of 1,3-butadiene: inhalation pharmacokinetics and tissue dosimetry of butadiene epoxides in rats and mice. Toxicol. 113:306-309.
[25] E Goldenthal (1971). A compilation of LD50 values in newborn and adult animals. Toxicol. Appl. Pharmacol. 18:185-207.
[26] JF Griffith (1964) Toxicol. & Applied Pharmacol. 6:726-730.

In 1979 a European Community multi-laboratory trial of the test's reproducibility showed that LD50 values *for the same chemicals* varied by 3-fold to 11-fold between different laboratories[27].

A non-animal test with reproducibility this poor would never be accepted as valid, and this performance is quite unacceptable as a means of protecting human health.

LD50 values for five chemicals from a European Community multi-laboratory study[28]

Substance	LD50 (mg/kg)	Ratio largest:smallest value
I	46 – 522	11.3
II	800 – 4,150	5.2
III	350 – 1,280	3.7
IV	805 – 5,420	6.7
V	70 – 513	7.3

There are many explanations for the poor reproducibility of acute toxicity tests on rodents. If different species (see above), strains and ages of animals[29] are used by different laboratories, the results will be significantly affected. This is also true of differences in the weight[30] and diet of animals. Other factors may include differences in technical ability of those conducting the tests, as well as variations in ambient temperature, the housing conditions of animals, humidity, noise and the light/dark cycle.

2.3 Practicalities of acute toxicity testing on animals

All chemical testing programmes relying on animal tests have proved dramatically more costly and time-consuming than envisaged.

For example, the dedicated programme of chemical testing on animals solely for long-term toxicity and carcinogenicity, conducted jointly by the US National Toxicology Program and the US National Cancer Institute, took 36 years to achieve results for only 500 chemicals[31].

The OECD's Screening Information Data Set (SIDS) programme requires limited information that includes acute and repeat dose tests, and reproductive and genetic toxicity tests, conducted mainly on animals. In ten years of international co-operation

[27] WJ Hunter *et al* (1979). Intercomparison study on the determination of single administration toxicity in rats. J. Assoc. Official Analyt. Chemist. 62:864-873.
[28] D Lorke (1983). A new approach to practical acute toxicity testing. Arch. Toxciol. 54:275-287.
[29] SH Dieke & CP Richter (1945). Acute toxicity to rats in relation to age, diet, strain, and species variation. J. Pharmacol. Exp. Ther. 83:195-207.
[30] T Balazs *et al* (1972). Protection against the cardiotoxic effect of isoproterenol HCl by restricted food intake in rats. Toxicol. Appl. Pharmacol. 21:237.
[31] International Life Sciences Institute (1998). Assessing the toxicity of exposure to mixtures of disinfection by-products — research recommendations. ILSI:USA.

under the SIDS programme, only 60 chemicals had been evaluated[32].

Thus, even though acute toxicity data may be lacking for only a small percentage of phase-in substances under REACH (see Section 5), relying on animal tests will delay the timely regulation which human health protection demands.

2.4 Animal suffering in acute toxicity tests

Acute toxicity tests cause many animals to experience distress and serious illness, and even the Fixed Dose Procedure causes deaths. Lethality is still the endpoint in LD50/LC50 tests (i.e. dermal and inhalational routes), the Acute Toxic Class method and the Up-and-Down Procedure[33].

Symptoms experienced by test animals may include:

> Tremors, unsteady gait, breathlessness, lethargy, weight loss, loss of balance, convulsions, excessive salivation, intestinal distension, diarrhoea, lethargy, nasal or anal bleeding or discharge, coma and death.

Anaesthetics and analgesics are never used and the tests last several days, so only euthanasia brings an end to this suffering. The OECD test guidelines state that animals "...that are moribund or suffering severe pain *and* distress must be humanely killed" (our emphasis). However, euthanasia would not be applied until animals are literally poisoned to the point of death, as humanely killed animals count as animals that died on test.

It is inconceivable that techniques causing this level of suffering to sentient animals should be tolerated and even advocated. The success of REACH is partly dependent on its acceptability to the public, whose opposition to tests causing pain and distress is clear[34].

3. Classification and Labelling

Acute toxicity tests are used to generate information on which classification and labelling decisions are based. Labels used vary from Category 1 (danger/fatal if swallowed/in contact with skin/inhaled) to Category 5 (warning/may be harmful if swallowed/in contact with skin/inhaled). The symbols used are familiar: skull and crossbones for categories 1 – 3, with exclamation mark for category 4, and no symbol for category 5. Because labels must give clear messages in a simple form, detailed information about precise effects is not needed for this purpose.

Traditionally, the 'LD50' value of chemicals has given information on degrees of toxicity. (LD50 stands for Lethal Dose 50%, meaning the dose at which half of the number of test animals die.) A chemical with a low LD50 value is highly toxic because a low dose will

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[32] AM Goldberg & H Spielmann (2000). High production volume (HPV) testing. Developments in Animal & Veterinary Sciences 31B:1639.

[33] SM Barlow *et al* (2002). Hazard identification by animal-based methods of toxicology. Food & Chem. Toxicol.

[34] Royal Commission on Environmental Pollution (2003). Chemicals in Products — Safeguarding the environment and human health. 24th report. Cm 5827.

kill an animal. Category 1 substances have an oral LD50 value of (less than or equal to) 5 mg/kg, while category 5 substances have an oral LD50 value of (less than or equal to 5000 mg/kg.

Classification and labelling based on LD50 values has been in use for decades. Now, implementation of the Globally Harmonised System for Classification and Labelling (GHS) is well underway. This international system means that all participating countries will interpret test results in the same manner. In the area of acute toxicity, the GHS currently relies on data from animal tests but need not be test-specific. The system could be adapted to use non-animal data to inform classification and labelling decisions.

In vitro and *in silico* tests for acute toxicity, as proposed in this report, will provide quantitative data that can be used to classify and label chemicals in the usual categories according to the GHS.

4. High-throughput Prioritisation – Not Mass Animal Testing

Since we published our non-animal testing strategy for chemicals, *The Way Forward,* in 2001 support for chemical prioritisation on the basis of rapid screening has come from several organisations. The Danish Environmental Protection Agency has used (Q)SARs to list the hazardous properties, including acute oral toxicity, of some 47,000 chemicals[35] . They recommend a wider application of this approach.

The British Royal Commission on Environmental Pollution, in its 2003 report[36] on chemical regulation, also advocated a mass sorting approach based on existing data and computational techniques, together with some *in vitro* studies. The Royal Commission also argued that:

> "To avoid undue delay and unnecessary use of animal tests, the sorting process should not be delayed while experimental toxicity information in generated *de novo*. Indicators [of] toxicity and ecotoxicity can be derived from knowledge of chemical structure using various computational approaches. The US EPA's procedure for assessing pre-market notifications is heavily reliant on the use of structure activity relationships, which are used qualitatively to estimate human acute and chronic toxicity... Estimates of the probable human pharmacokinetics of the chemical are made, evaluating absorption, distribution and redistribution, metabolism and excretion of the substance."

In 2004 a Dutch proposal described how chemical prioritisation under REACH could additionally incorporate PBT and vPvB properties[37]. This would mean that after consideration of CMR and HPV chemicals, each tonnage category would be sub-divided for prioritisation according to PBT and vPvB properties. Chemicals that have these toxic

[35] Danish Environmental Protection Agency (2003). Advisory list for self-classification of dangerous substances.
[36] Royal Commission on Environmental Pollution (2003). Chemicals in Products — Safeguarding the environment and human health. 24th report. Cm 5827.
[37] Note from the Netherlands delegation on "Registration Prioritisation" (2004). Council of the European Union Working document 22/04 (ad hoc working party on chemicals).

properties could be regulated on the precautionary principle without the need for acute toxicity data relating to human health hazards.

5. Existing Data, Read-across and (Q)SARs for Acute Toxicity

The strategy we advocated in our report *The Way Forward* is highly suited to assessing chemicals for acute toxicity, based on:

i. *Existing data*;
ii. *Read-across* and *QSARs*;
iii. New data from selected *in vitro tests* and *related computational techniques*.

Existing data include human health hazard data from all sources, environmental data (including PBT and vPvB status), physico-chemical properties and human exposure information.

Many high-priority chemicals will be identified by step [i] alone. For example, a PBT chemical would be subject to restriction on the precautionary principle, even in the absence of acute toxicity data.

Chemicals without existing acute toxicity or other relevant information would be analysed using the approaches in [ii] where appropriate; and/or in [iii] i.e. cell-based and other non-animal tests, providing qualitative and quantitative data, as we explain in Section 6.

In 2001[38], the Environment Council of the European Commission called upon the Commission to:

> "...explore ways in which chemicals of concern can be identified to allow prioritisation for taking action, developing clear and transparent screening criteria, essential information requirements, and exploring the use of chemical grouping and modelling techniques..." (Council conclusion 37).

The European Commission published a report in November 2004 showing how total animal use under REACH could be reduced by the intelligent application and wider acceptance of chemical grouping, read-across, (Q)SARs and similar non-animal approaches[39]. The document calculated that REACH would require tests on between 1.7 – 2.4 million animals (mean 2.1 million) for phase-in substances, if regulatory acceptance of chemical grouping, read-across, (Q)SARs and similar estimates was optimal. If acceptance of these approaches was minimal, 3.2 – 4.6 million animals (mean 3.9 million) would be required. Thus maximum use of these 'intelligent' techniques could, at the most, save up to 3.1 million animals during the 11-year implementation of REACH.

In the specific case of acute toxicity data, the report calculated that these measures would reduce the need for new acute toxicity data to two per cent of all phase-in

[38] Council of Ministers (2001). Report of the 2355th session of the Council "Environment," Luxembourg, 7 June 2001. Luxembourg: Council of Ministers.
[39] K van der Jagt *et al* (2004). Alternative approaches can reduce the use of test animals under REACH. Publ. European Commission DG JRC, Brussels.

substances[40].

The information obtained from these procedures is thus a key step in prioritising and classifying chemicals for acute toxicity.

5.1 Existing data

An analysis by the US Environmental Protection Agency (EPA) indicated that acute toxicity data are available for 75 per cent of the 2,700 high-production volume chemicals[41]. In fact the US Challenge Program for HPV chemicals has demonstrated that 50 per cent of data for *all* human health endpoints already existed in previously unpublished studies, later submitted by industry[42].

For many lower tonnage chemicals there will also be acute toxicity results in the confidential databases of commercial companies. In fact, it has been estimated that acute oral toxicity data (from animals) are already available for most substances in each volume band[43].

Because of the problems of species differences, existing data from animal studies have unproven relevance to humans (see Section 2, above). Therefore it is also critical to seek existing *human* acute toxicity data which provide 'gold standard' information. Acute lethal concentrations for humans are already known for some chemicals. Additionally, records from hospitals, workplaces and poison information centres must be searched for further details of human acute toxicity data, including lethal doses.

5.2 Read-across

For some substances, data are available for structurally related analogues. Where it can be done, 'reading across' information to predict the toxicity of the untested chemical is desirable from practical (cost, time) and animal-welfare points of view.

Read-across is applicable to physico-chemical properties (such as physical form, molecular weight, water solubility, partition coefficient, vapour pressure); as well as to manufacturing processes; uses; and positive findings from tests that may no longer meet modern requirements.

In the UK, more than 10 per cent of new substance notifications have included some use of read-across. The biggest impact on animal numbers was on 28-day oral test data, but even in the case of acute toxicity, animals were saved. As read-across is more extensively practised, the numbers of animals saved will increase.

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[40] This estimate is based on the current version of Annex V which excludes acute toxicity data requirements for chemicals in the 1-10 tonnage band. However, see section 5.1.

[41] E-mail from Charles M Auer, 25.6.2003, cited in F Pedersen *et al* (2003). Assessment of additional testing needs under REACH. Publ. European Commission DG JRC.

[42] C Auer (2004). Experience from the US HPV Challenge Program: presented by C Auer (US EPA) at the EU-US Transatlantic Conference on Chemicals, April 26-28 2004, Charlottesville, USA.

[43] F Ackerman & R Massey (2004). The True Costs of REACH. Publ. The Nordic Council of Ministers.

5.3 (Q)SARs

According to a detailed and recent review of (Q)SARs[44]:

> "Given the limitations in the testing capacity of EU industry, it seems likely that the envisaged deadline for obtaining the required information will only be met if QSAR approaches are used wherever it is scientifically feasible to do so. For example, QSAR models could be used to prioritize chemicals for further testing, to identify certain types of toxic hazard (possibly in order to derogate from further testing), or to provide estimates of toxic potency for use in risk assessments."

There are several software packages for predicting acute toxicity from chemical structure. One example is TOPKAT, a statistically based package comprising several QSAR models, including TOPKAT Model Rat Oral LD50. This has been developed using experimentally derived LD50 values for about 4,000 chemicals, and assesses acute toxicity for a range of chemical classes. There is also a TOPKAT Model for Rat Inhalation LC50 that is applicable to five classes of chemicals. The TOPKAT packages are used by various European regulatory bodies[45].

The Danish Environmental Protection Agency has used (Q)SARs to screen 47,000 chemicals and identify 9,538 as likely to be acutely toxic by the oral route[46]. Their analysis did not attempt to differentiate between different levels of acute toxicity (e.g. very toxic, toxic, harmful), but such data can be gained from *in vitro* assays (see Section 6).

The US Environmental Protection Agency and the US International Testing Committee (comprising 16 US government organisations, many with regulatory responsibilities) are already using SARs to predict acute toxicity for regulatory purposes[47]. They are also using SARs to predict dermal absorption for regulatory applications, and the Danish Environmental Protection Agency is using QSARs for the same purpose.

6. Non-Animal Tests for Acute Toxicity

For those chemicals with no available acute toxicity data; and where read-across and (Q)SARs are not possible, new testing is required to rapidly identify highly hazardous chemicals and to permit classification and labelling.

Non-animal tests — *in vitro* and other methods — are ideal for this purpose, as explained in previous BUAV publications[48,49]. They allow the prioritisation of the most

[44] MTD Cronin *et al* (2003). Use of QSARs in international decision-making frameworks to predict health effects of chemical substances. Environ. Health Perspectives 111:1391-1401.
[45] L Gribaldo *et al* (2004). Report for establishing the timetable for phasing out animal testing for the purpose of the cosmetics directive. Acute Toxicity Chapter. European Commission DG Enterprise website: http://pharmacos.eudra.org/F3/cosmetic/AnimalTest.htm
[46] Danish Environmental Protection Agency (2003). Advisory list for self-classification of dangerous substances.
[47] MTD Cronin *et al* (2003). Use of QSARs in international decision-making frameworks to predict health effects of chemical substances. Environ. Health Perspectives 111:1391-1401.
[48] BUAV (2001). The Way Forward — Strategy for a future chemicals policy. Publ. BUAV & the European Coalition to End Animal Experiments, London.

directly toxic chemicals for precautionary regulation in the short term, which is essential if human health is to be protected effectively. In the meantime, further and more sophisticated non-animal methods must be finalised, as a matter of urgency, for more in-depth testing of acute toxicity.

The US HPV Challenge Program has shown that for high-production volume chemicals[50], only six per cent of all data required under REACH had to be obtained by new testing.

In the particular case of acute toxicity, the European Commission report estimated that only two per cent of all phase-in chemicals would need new tests to be conducted[51]. Acute toxicity data will be required for novel chemicals too. We argue that these data can and should be filled by computational methods such as (Q)SARs and by *in vitro* cell-based techniques, as explained below.

6.1 Rapid screening for acute toxicity

Computational techniques such as (Q)SARs provide the first screening step for acutely toxic chemicals. Packages are available to predict acute toxicity and dermal absorption, and these will be sufficient for some chemicals (see Section 5, above). The second prioritisation step, if needed, comprises simple tests measuring cytotoxicity in human cells.

The Multicentre Evaluation of *In vitro* Cytotoxicity (MEIC) programme organised by Swedish scientists involved dozens of laboratories around the world using 61 cytotoxicity methods. Each laboratory used the same reference chemicals for which human acute lethal concentration data were available[52]. They showed that a combination of four human cell culture tests[53] were practical, rapid, cost-effective and highly predictive of human acute toxicity as measured by acute lethal peak bloodstream concentrations.

The four in vitro tests predicted the human data (R^2 = 0.77 for 50 chemicals) *better than rodent LD50 tests predicted lethal doses in humans* (R^2 = 0.64)*.* These *in vitro* methods have been prevalidated by the MEIC programme[54] and would provide a quick and informative way of identifying chemicals that are highly and directly toxic.

Additionally, a validation study of two other cytotoxicity protocols for acute toxicity is nearly complete. This joint study, undertaken by the US NTP Interagency Center for the

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[49] BUAV (2004). Endocrine disrupting chemicals — A non-animal testing approach. Publ. BUAV, London.

[50] C Auer (2004). Experience from the US HPV Challenge Program: presented by C Auer (US EPA) at the EU-US Transatlantic Conference on Chemicals, April 26-28 2004, Charlottesville, USA.

[51] K van der Jagt *et al* (2004). Alternative approaches can reduce the use of test animals under REACH. Publ. European Commission DG JRC, Brussels.

[52] C Clemedson *et al* & B Ekwall *et al* (2000). MEIC evaluation of acute systemic toxicity parts VII & VIII. ATLA 28(Suppl. 1).

[53] The four assay endpoints are protein content, levels of the energy compound ATP, cell shape (morphology) and acidity/alkalinity (pH) changes.

[54] L Gribaldo *et al* (2004). Report for establishing the timetable for phasing out animal testing for the purpose of the cosmetics directive. Acute Toxicity Chapter. European Commission DG Enterprise website: http://pharmacos.eudra.org/F3/cosmetic/AnimalTest.htm

Evaluation of Alternative Toxicological Methods (NICEATM) and ECVAM, has a number of aims including[55]:

> "To assess the accuracy of two standardised *in vitro* cytotoxicity assays for estimating rodent oral LD50 values and human lethal concentrations across the five Globally Harmonised System categories of acute oral toxicity as well as unclassified toxicities."

The tests involved are neutral red uptake by normal human keratinocytes and by a mouse cell line (BALB/c 3T3 fibroblasts), methods already used widely for non-regulatory purposes. Phases I and II were finished by November 2003 and resulted in standardised protocols of sufficient reproducibility to proceed to the final validation stage, due to complete at the end of 2004.

Importantly, it was the view of ECVAM even in 2002, prior to formal validation, that these tests could *immediately* be used for priority setting among chemicals[56]. Therefore, for identifying chemicals with a high acute toxicity hazard, several rapid cytotoxicity tests are already available for use.

EU funding has been obtained for a collaborative project aimed at the high-throughput screening of chemicals within a few years. The new concept is toxicity screening using nanodrops of cells, from a range of organs, on microchips. The TOXDROP project aims to test 2,600 chemicals before the end of 2005, 2,900 chemicals by 2008, and a total 24,600 chemicals by 2012.

6.2 Fuller assessment for acute toxicity

The most toxic chemicals can thus be identified and controlled on the basis of rapidly acquired *in vitro* hazard data, using the precautionary principle. Further development/validation of more sophisticated non-animal methods, many already available, will permit a fuller assessment, incorporating dose/response information as well as absorption and metabolism data.

In vitro methods should use human rather than animal cells or tissues, so that species variations — in cell permeabilities, enzymes, metabolic rates and routes, and receptors — are eliminated.

A full assessment of a chemical's acute toxicity requires knowledge of its toxicokinetics. Toxicokinetics aims to answer several questions, including: Is the chemical absorbed (e.g. from the gut, through the skin or via the airways) into the bloodstream? How long is it in the circulation? Is it metabolised by the liver or other organs? Does it accumulate selectively in certain organs?

In 1996 an ECVAM expert workshop recommended a stepwise strategy for acute toxicity testing based primarily on non-animal tests. It combined basal cytotoxicity tests (Section 6.1) with physico-chemical data; a second tier comprised metabolism of chemicals using

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[55] *In Vitro* Cytotoxicity Validation Study – ICCVAM website [visited December 2004]: http://iccvam.niehs.nih.gov/methods/ivcytoval.htm

[56] A Worth & M Balls (Eds.) (2002). Alternative (non-animal) methods for chemicals testing: Current status and future prospects. ATLA 30(Suppl. 1):1-125.

liver cells in the test tube. A third tier involved *in vitro* studies on specialised target cells such as kidney, brain, vascular cells or heart. *In vitro* data, including metabolism studies, were integrated with computer simulations of absorption, metabolism, distribution round the body and excretion. Even in 1996 the ECVAM experts stated that the entirely *in vitro* steps in the strategy were sufficient for the classification of chemicals for acute toxicity[57] . Today, these studies are further advanced.

For example, a new project called A-Cute-Tox is being part-funded by the European Union. The project involves more than 30 participants from industry, academia and the Commission itself. The aim of A-Cute-Tox is to develop a simple and robust *in vitro* testing strategy for prediction of human acute systemic toxicity, which could replace the animal acute toxicity tests used today for regulatory purposes. This will include the development of new cellular and *in silico* systems as well as prediction models that better relate cell culture concentrations of chemicals to *in vivo* doses.

The PREDICTOMICS project is intended to develop short-term *in vitro* assays for long-term toxicity[58]. This will be achieved using co-cultures of different cell types, target cell transformation, stem cell technology and organotypic cell culture. The project aims to identify early mechanistic markers of toxin-induced cell alterations by using integrated genomic, proteomic and cytomic analysis; and to establish and prevalidate a screening platform (cell systems together with analysis tools) which is unambiguously predictive of toxin-induced chronic renal and hepatic disease.

These ambitious and integrated approaches are essential, and promise to revolutionise the testing of chemicals and drugs for systemic toxicity. However, in the meantime, there are many robust non-animal methods for predicting acute toxic effects, already validated or widely in use. Some of these techniques are described below.

6.2.1 Absorption in vitro and in silico

The absorption of a chemical across epithelial barriers (e.g. skin, gut, and lung) can be estimated very quickly and cost-effectively on the basis of the octanol/water partition coefficient and molecular size[59].

There are integrated computational models (e.g. physiologically-based biokinetic models) that predict the absorption of chemicals via skin, lung and intestine[60]. The availability of more and better quality data will allow these models to be continually improved.

Better predictivity is obtained using human gut cells in culture such as Caco-2 or TC-7 cells, as a model of the intestinal barrier. The techniques are fast, simple and flexible

[57] H Seibert *et al* (1996). Acute toxicity testing *in vitro* and the classification and labelling of chemicals. ATLA 24:499-510.
[58] http://fp6.cordis.lu/fp6/home.cfm
[59] R Combes *et al* (2003). An overall strategy for the testing of chemicals for human hazard and risk assessment under the EU REACH system. ATLA 31:7-19.
[60] S Coecke *et al* (2004). Report for establishing the timetable for phasing out animal testing for the purpose of the cosmetics directive. Metabolism and Toxicokinetics Chapter. European Commission DG Enterprise website: http://pharmacos.eudra.org/F3/cosmetic/AnimalTest.htm

and already well established. According to an EU expert group, they provide qualitative absorption data without problems of species differences[61].

For skin absorption, methods using isolated skin biopsy fragments are already fully validated and listed in the OECD test guidelines. Cultures of human lung and airways cells (e.g. Calu-3 and 16HBE14o cell lines) have been used in chemical absorption studies. If a chemical does not penetrate to the bloodstream it cannot cause systemic toxicity and the tests below are not required.

6.2.2 Plasma levels and target organ effects in vitro

On reaching the bloodstream, the pharmacokinetic and pharmacodynamic properties of substances are related to their reversible binding to plasma proteins. To estimate likely plasma levels of chemicals, protein-binding can be measured rapidly *in vitro* in multiwell plates using human plasma. Pharmaceutical companies use this method routinely in their drug discovery and development programmes[62].

Predictions of tissue levels of chemicals and their volumes of distribution can be made by means of blood-tissue partitioning *in vitro*. The techniques are familiar to contract research organisations and industrial laboratories and have been applied to industrial chemicals[63] as well as pharmaceuticals.

If necessary, studies of selective effects of chemicals on particular target cells (such as kidney, brain, vascular cells or heart) can be conducted *in vitro.* However, the International Workshop on *in vitro* methods for assessing acute systemic toxicity, held in 2000, pointed out that it is probably unnecessary to routinely test for all specific organ effects *in vitro*[64]. Non-animal tests of energy metabolism and to assess a chemical's ability to disrupt epithelial barriers may be sufficient[65].

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[61] S Coecke *et al* (2004). Report for establishing the timetable for phasing out animal testing for the purpose of the cosmetics directive. Metabolism and Toxicokinetics Chapter. European Commission DG Enterprise website: http://pharmacos.eudra.org/F3/cosmetic/AnimalTest.htm

[62] S Coecke *et al* (2004). Metabolism in the context of the follow-up of the 7th amendment of the cosmetics directive. Chapter by the sub-group of experts on toxicokinetics and metabolism. European Commission DG Enterprise website: http://pharmacos.eudra.org/F3/cosmetic/AnimalTest.htm

[63] E Artola-Garicano *et al* (2000). Validation of negligible depletion solid-phase microextraction as a tool to determine tissue/blood partition coefficients for semivolatile and nonvolatile organic chemicals. Toxicol. Appl. Pharmacol. 166:138-144.

[64] Report of the International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity (2001). Results of an International Workshop organized by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM). NIH publication 01-4499. Website: http://iccvam.niehs.nih.gov/methods/invidocs/finalrpt/finalcov.htm

[65] L Gribaldo *et al* (2004). Report for establishing the timetable for phasing out animal testing for the purpose of the cosmetics directive. Acute Toxicity Chapter. European Commission DG Enterprise website: http://pharmacos.eudra.org/F3/cosmetic/AnimalTest.htm

6.2.3 Metabolism in vitro

Likely metabolism by liver enzymes can be measured in the test tube using pure enzymes, isolated (preferably human) liver cells or liver slices, or subcellular fractions. Pharmaceutical companies have been conducting these tests in-house for drug development, for many years. The sources of metabolic activity can be varied by gender, age and tissue, if necessary, offering potential to assess sub-groups of the human population (e.g. children or women).

As another practical approach, FRAME has recommended the development of established cell lines genetically engineered to express human metabolic enzymes, and states that one laboratory can screen some 200 chemicals per month by means of such cell lines[66].

6.2.4 Toxicokinetic modelling

Computer models can be constructed to interpret *in vitro* data and provide answers to the toxicokinetic questions. For example, physiologically-based kinetic models[67,68] estimate chemical absorption, distribution, metabolism and excretion, as well as dose/response effects.

Other computer models include those such as Symcyp™, developed to simulate human pharmacokinetics from simple and commonly available *in vitro* data[69]. These include *in vitro* metabolic stability information obtained from recombinantly expressed human cytochrome P450s, or characterised human liver microsomes.

7. Summary and Conclusions

Assessing the acute toxicity of chemicals should not involve outdated animal poisoning tests.

It is expected that acute toxicity data from animal studies are already available for many existing chemicals. These data should be published, although regulators should be aware that species differences-hinder extrapolation to humans.

Existing data on acute toxicity in humans, for example from records of accidental poisoning, should take precedence over animal data and should be sought from all possible sources. Human data, and data obtained from *in vitro* studies, should be used to classify and label chemicals according to the Globally Harmonised System for Classification and Labelling.

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[66] R Combes *et al* (2003). An overall strategy for the testing of chemicals for human hazard and risk assessment under the EU REACH system. ATLA 31:7-19.
[67] ME Andersen (2003). Toxicokinetic modeling and its applications in chemical risk assessment. Toxicol. Lett. 138:9-27.
[68] P Poulin & FP Theil (2002). Prediction of pharmacokinetics prior to *in vivo* studies. II. Generic physiologically based pharmacokinetic models of drug disposition. J. Pharm. Sci. 91:1358-1370.
[69] NJ Proctor *et al* (2003). ISEF: an intersystem extrapolation factor for use with recombinant cytochrome P450 expression systems. Br. J. Clin. Pharmacol. 55:437-438.

In screening large numbers of chemicals to prioritise those in need of further testing, chemicals without existing acute toxicity information should first be assessed for potential to use read-across techniques from structurally related analogues. (Q)SAR models and *in vitro* cytotoxicity tests (currently under validation) would be applied for the identification of highly toxic substances

A fuller assessment of acute toxicity, if needed in some cases, would be based on a combination of absorption/penetration assays, test-tube measurements of plasma levels and likely target organ distribution; plus *in vitro* metabolism studies, using toxicokinetic modelling.

Not all these techniques have yet been fully validated to regulatory standards but, in all cases, validation studies are either underway or are planned. Moreover, there are many years of experience with most of them. Their utility is well accepted, especially within the drug industry — where their predictions can be confirmed or contradicted in subsequent clinical trials.

When considering the current status of new non-animal tests, it is also essential to remember that the animal tests for acute toxicity have never been validated to modern standards. Their reproducibility between laboratories is so poor that if a validation study were to be undertaken, the tests would fail[69].

The research required to finalise the necessary non-animal tests should now be immediately prioritised by the European Commission, and workshops should be planned to disseminate the knowledge required to use data from non-animal methods for assessing acute toxicity in chemicals.

[69] WJ Hunter *et al* (1979). Intercomparison study on the determination of single administration toxicity in rats. J. Assoc. Official Analyt. Chemist. 62:864-873.