Alternatives to Animal Testing in the Safety Evaluation of Products

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Summary — The conventional method for assessing the safety of products, ranging from pharmaceuticals to agrochemicals, biocides and industrial and household chemicals — including cosmetics — involves determining their toxicological properties by using experimental animals. The aim is to identify any possible adverse effects in humans by using these animal models. Providing safe products is undoubtedly of the utmost importance but, over the last decade or so, this aim has come into conflict with strong public opinion, especially in Europe, against animal testing. Industry, academia and the regulators have worked in partnership to find other ways of evaluating the safety of products, by non-animal testing, or at least by reducing the numbers of animals required and the severity of the tests in which they are used. There is a long way to go before products can be evaluated without any animal studies, and it may be that this laudable aim is an impossible dream. Nevertheless, considerable progress has been made by using a combination of *in vitro* tests and the prediction of properties based on chemical structure. The aim of this review is to describe these important and worthwhile developments in various areas of toxicological testing, with a focus on the European regulatory framework for general industrial and household chemicals.

Key words: *chemical safety assessment,* in vitro *testing, structural prediction of properties*

Introduction

The Three Rs

In their 1959 book, *The Principles of Humane Experimental Technique* (1), Russell & Burch defined a strategy for minimising animal use without compromising the quality of scientific work. This was to be achieved through the use of three different categories of alternative testing, which they termed the Three Rs; namely *reduction, refinement* and *replacement*. These proposals are still used as a benchmark today.

The purpose of alternative tests

In 1997, the British Toxicology Society Working Group on *in vitro* toxicology produced a report on the value of currently available *in vitro* studies in all aspects of safety evaluation, and which made recommendations for future work (2). Such studies are used in various contexts, from excluding substances from further development on the basis of their potential toxicity, to regulatory safety evaluation.

There are great incentives to develop *in vitro* alternatives to the existing animal toxicology methods. Safety assessment can be improved by the better prediction of effects in humans, as the scientific understanding of the toxicological effect is improved. There are commercial benefits in reducing the cost and in increasing the speed of product development. The key consideration, though, is animal welfare (the Three Rs), underpinned in the European Union (EU) by *Directive 86/609/EEC* (3), which requires that animal experiments should not be performed, if another scientifically satisfactory way of obtaining the results sought is reasonably and practicably available.

Validation of Alternative Toxicology Methods

Before alternative tests, and *in vitro* tests in particular, can be used, they have to be validated scientifically, and established as acceptable by industry and the regulators. With this in mind, a number of international bodies have been set up that concentrate all their efforts and resources on making this process as efficient and effective as possible.

European Centre for the Validation of Alternative Methods (ECVAM)

The European Commission strongly promotes reducing the numbers of animals used in research, replacing animal experiments and refining alternative techniques and, since 1985, there have been a number of research activities aimed at making progress toward the validation and regulatory acceptance of alternative tests. The Commission of the European Communities (EC) set up ECVAM in 1991, to implement article 23 of *Directive 86/609/EEC* on the protection of animals for use in

experimental and other scientific procedures. ECVAM is located at the Joint Research Centre (JRC) at Ispra in Italy, and its main aim is to coordinate the validation of alternative methods that comply with the Three Rs of Russell & Burch, while providing useful results and information for the biosciences. As part of its work, ECVAM holds workshops and reports their recommendations (for example, 4).

In addition, in 1993, the European Commission was active in setting up the Industrial Platform on *In Vitro* Testing (IVTIP), which involves European companies with activities in the pharmaceutical, chemical and cosmetic sectors, with the main objective of maximising technology transfer from academia to industry.

The US Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)

The US organisation comparable to ECVAM is ICCVAM, which undertakes scientific peer review on the validation of alternative test methods. The various US agencies then decide whether to adopt the methods recommended as scientifically validated by ICCVAM, and appropriate for their regulatory purposes. The Johns Hopkins Center for Alternatives to Animal Testing (CAAT) also contributes to the US validation process. Until non-animal test methods are validated and achieve regulatory acceptance, they cannot be relied on as alternatives to established test-guideline studies for the purposes of the High Production Volume (HPV) Challenge Program or any associated test rules. The Environmental Protection Agency (EPA) is a member of ICCVAM, however, and is working with other federal agencies to identify, validate, and peer-review potential alternative protocols, and to ensure the scientific and regulatory acceptability of the tests.

Validation procedures

It is particularly important to obtain international agreement on the principles of validation, and the Organization for Economic Cooperation and Development (OECD) has reported on recommendations for harmonising the scientific validation and subsequent regulatory acceptance criteria for alternative test methods (5). They adopted the definition of validation as "the process by which the reliability and the relevance of a procedure are established for a particular purpose." Fielder *et al.* (2) consider that there is a pragmatic division between two types of validation, depending on the use of the studies. Validation of alternative procedures for use in non-regulatory studies or for mechanistic studies, perhaps to refine the safety evaluation, normally require only "internal" validation to ensure that the laboratory or company involved has confidence in the results. This type of informal validation can often be based on "in-house" data on chemicals of a similar class, or products of similar formulation. In contrast, studies to be used as alternatives to existing test guidelines for routine safety evaluation require more-extensive formal validation. Convincing evidence must be obtained from published validation studies (usually undertaken internationally) to support the wider, worldwide acceptance of the alternative method. The regulatory acceptance of new alternative methods follows the scientific validation process, and in practice is greatly facilitated if the regulatory authorities are involved in the validation process and the design of the validation studies.

ECVAM has an independent scientific advisory committee (the ESAC) that considers the outcomes of validation studies. When the ESAC publicly endorses a method as scientifically validated, ECVAM communicates this within the European Commission and to ICCVAM and other agencies, including the national Competent Authorities of the EU countries. The various services of the European Commission then consider the applicability of the scientifically validated method in relation to their specific legal responsibilities, in consultation with their own advisers and the appropriate national Competent Authorities. Meanwhile, international discussions can lead to harmonisation of the new test method as an OECD guideline.

Development of *In Vitro* Tests to Evaluate Toxicological Properties

The current status of alternative tests for evaluating the systemic toxicological properties of chemicals is summarised briefly in this section. For a more comprehensive discussion of the background to this area, see the review by Fielder *et al.* (2), the report of the 13th meeting of the Scientific Group on Methodologies for the Safety Evaluation of Chemicals (6), and the recommendations from the May 1998 joint UK Government/ECVAM meeting (7). Progress in the validation of alternative tests for systemic toxicity has been restricted by the limitations of current *in vitro* tests to replicate the complexity of the *in vivo* system. It is unlikely that a single *in vitro* test will ever be capable of predicting toxicity. Rather, a battery of *in vitro* tests, each focusing on a different aspect of the toxic response, will probably prove to be most effective in the prediction of systemic toxicity. The increasing use of computer-based prediction systems will also aid in this. These battery systems may never have a higher status than adoption for screening purposes, and it is unlikely that they will fully replace animal tests.

Mutagenicity and carcinogenicity

It is now widely accepted by regulatory authorities that screening for mutagenic potential can be carried out by using *in vitro* methods. Studies in animals are required to assess whether activity can be expressed *in vivo*, but these are not used in routine screening. In view of the clear relationship between mutagenicity and carcinogenicity, chemicals that induce mutations in somatic cells *in vivo* can be regarded as potential carcinogens, i.e. they can be identified as potential "genotoxic" carcinogens. However, some chemicals that are carcinogenic in animals do not have mutagenic potential, and screening for such "non-genotoxic" carcinogens presents great difficulty because of the wide range of mechanisms apparently involved. The development of *in vitro* carcinogenicity screens would therefore significantly reduce the numbers of animals that are used. Syrian hamster embryo (SHE) cells have been used to study transformation *in vitro* since the early 1960s (8). Transformation is a multistage phenomenon in SHE cells, requiring multiple mutational events to occur. Endpoints such as chromosomal number and morphology have also been used to study the carcinogenic capability of a wide variety of chemical and physical agents (9).

Toxicity to reproduction

Reproductive toxicity presents another difficulty for complete replacement of animal studies, because of the complexity of the interacting systems related to reproduction, which involve a wide range of direct and indirect effects (including behavioural effects on mating). Developmental toxicants are likely to act through various mechanisms. A single *in vitro* assay cannot therefore be used, since a false-negative assessment may result.

Although there is little likelihood of the adoption of alternatives to completely replace animal testing for regulatory purposes, *in vitro* studies are of value in mechanistic studies for refining assessment of hazards to humans, and there is an opportunity for developing *in vitro* partial-replacement studies in specific areas. One such area could be effects on male fertility, for example, by the use of immortalised testicular somatic and germ cells with specific markers for toxicological damage. Another, already much studied, area is screening for developmental toxicity. This is a highly complex area of reproductive toxicology, for which several *in vitro* screening tests have been introduced. However, when sub-mammalian species (for example, *Hydra*) are used, the prediction of human risk is difficult. Furthermore, mammalian systems such as whole embryo culture (WEC; 10) and rodent limb bud culture (micromass [MM] test; 11) still require a considerable number of experimental animals. The use of permanent mammalian cell lines, such as embryonic stem cell lines (embryonic stem cell test [EST]), is a promising way of establishing an assay for teratogenicity testing *in vitro,* with improved predictability for humans (12). An ECVAM workshop on the screening of chemicals for reproductive toxicity (13) recommended that existing embryotoxicity tests should be improved and validated, if possible, and that further effort and resources should go into the development and introduction of new ones. Following these recommendations, a study was set up in 1997 on the prevalidation and validation of the EST, the MM test and the WEC test. An ESAC statement on this validation study, which is now completed (14), is imminent. In view of the many potential mechanisms involved in developmental toxicity, including "indirect" effects relating to maternal toxicity, no method would be a complete replacement, but a method for detecting direct effects would be of value in identifying the most important teratogens, i.e. those that present a risk at maternally non-toxic doses.

There is a growing belief among scientists that a number of chemicals that are being released into the environment are having a disruptive effect on the endocrine systems of animals, leading to reproductive disorders and other abnormalities. There are also signs that sperm counts in men have been falling, in tandem with an increase in the incidence of reproductive tract abnormalities (15, 16). Many of the screening tests being developed to investigate endocrine-disrupting effects of chemicals are conducted on *in vitro* systems. Such *in vitro* assays include simple competitive binding assays, which rely on the ability of the chemical to bind to the oestrogen receptor, as well as more-sophisticated systems, in which the receptor is activated following binding of the chemical. Examples of the latter type of assay include assays based on yeasts that express either rainbow trout (17) or human (18) oestrogen receptors, vitellogenin gene expression in hepatocyte cultures (19), and proliferation of MCF-7 (a human cancer cell line; 20). However, a problem with these assays is that they would fail to detect a chemical acting independently of a receptor. Also, it has been found that impurities in chemicals can lead to false-positive results (21). There are a number of other problems with the existing *in vitro* assays, and these have been extensively discussed elsewhere (22–24). It is generally accepted that a battery of *in vitro* assays, along with information from quantitative structure–activity relationship (QSAR — a computerised prediction system) studies, should form the basis of a tiered testing strategy. The need to standardise and to validate these assays is vital, as inconsistencies between results obtained with the various assays abound in the literature.

Neurotoxicity

In view of the complexity of the nervous system, particularly the central nervous system (CNS), *in vitro* models do have a role in elucidating the mechanisms of neurotoxic effects. However, no single *in vitro* neurotoxicity test or package of tests has been adequately evaluated and validated, although progress has been made in devising potential prescreening strategies. One of the main problems is the great cellular diversity of the nervous system. Another issue is the interdependence needed to maintain the normal function of the nervous system. In addition, there is the need to consider separately the CNS and peripheral nervous system (PNS), as well as bioavailability considerations, such as the blood–brain barrier.

Absorption, distribution, metabolism and excretion (ADME), and general toxicology

One of the biggest problems for *in vitro* approaches is the evaluation of systemic toxicity. Once potential target organs have been defined, the susceptibility of the relevant cells in culture to the test substance can be investigated. Likely metabolites can be predicted from studies with hepatocytes and other cell cultures, and then their effects on target organs can be investigated. Considerable progress has been made in developing *in vitro* systems to detect toxic effects in some such specific target tissues. These "target organ" studies are of value in investigating mechanisms of action, but in view of the many potential target tissues, it is not practical to expect such studies to be used to predict general systemic toxicity.

If *in vitro* data are to be used to predict systemic toxicity, extrapolation of *in vitro* concentrations to those that would occur at the target site *in vivo* is necessary. Hence, predictions are needed of absorption via different routes from a knowledge of physicochemical properties and *in vitro* data (for example, on skin absorption). There are also physiologically based pharmacokinetic (PBPK) models that are capable of assessing *in vivo* concentrations from *in vitro* studies.

Acute systemic toxicity can arise by various mechanisms. Such potentially complex effects in animals effectively preclude the development of a single *in vitro* assay to predict acute toxicity *in vivo*. However, the individual toxic mechanisms of a substance might be modelled in a battery of *in vitro* assays, whose endpoints of toxicity indicate specific adverse effects. An *in vitro* test battery has been developed following the Multicentre Evaluation of *In Vitro* Cytotoxicity (MEIC) study (25) to improve both the predictive and analytical abilities of existing animal tests. The study also led to the suggestion that a number of supplementary tests were missing from the existing battery, and that an indication of systemic toxicity in humans may be facilitated by the coupling of a battery approach to QSAR-derived toxicokinetic parameters (26). The use of HEp-G2 transformants that express a series of human cytochrome P450 subtypes has recently been proposed as a means of investigating the metabolism and toxicology of chemicals (27).

A recently published *in vitro* long-term cytotoxicity study (28), in which 27 of the chemicals from the original MEIC study were tested on HEp-G2 cells, found that the results obtained correlated quite well with acute human toxicity data ($r^2 = 0.709$), when linear-regression analysis was used. There were no long-term human toxicity data available for comparison. These findings underline the need for long-term *in vitro* cytotoxicity tests, which should be validated against human chronic toxicity and toxicokinetics. This has been addressed in an ECVAM workshop devoted to assessing the current status of *in vitro* methods for long-term toxicity testing, resulting in a number of recommendations for future development (29).

Validated Alternative Toxicology Tests

Use in the EU

More progress has been made in developing alternative tests for evaluating local effects than for evaluating general systemic effects. Some of these new alternative tests can already be used within the context of EU regulation of chemical products.

Once test methods are incorporated into Annex V of the EU Dangerous Substances Directive (DSD; 30), *Directive 67/548/EEC*, they are applicable in the various EU schemes (such as chemical notification [31], the Existing Chemicals Regulation [32], the Plant Protection Products Directive [33], the new Biocidal Products Directive [34], and food-contact plastics [35]). After validation, and endorsement by ECVAM and the ESAC, and acceptance by the EU National Coordinators for *Directive 67/548/EEC*, alternative tests become part of Annex V of the DSD. The first alternatives methods to go through this process appeared in the 27th Adaptation to Technical Progress (ATP) of the DSD (30), which officially came into force on 1 October 2001. Method B.40 is for evaluating skin corrosivity (the transcutaneous electrical resistance [TER] assay, and a reconstituted human skin model), and Method B.41 is for phototoxicity [3T3 neutral red uptake (3T3 NRU) phototoxicity test].

Other alternative tests are being validated by ECVAM, and alternatives not yet in Annex V may still, in principle, be used for EU notifications on a case-by-case basis. The main issue is whether there

is adequate information for definitive "dangerous" classification, and whether the information is adequate for risk assessment. In practice, this usually means the test must be positive (i.e. resulting in classification). If it is negative, the standard Annex V animal test is normally needed, but there is room for negotiation with the relevant Competent Authority, taking into account a "weight-of-evidence" approach (i.e. considering the chemical structure and any data on analogous substances). The Annex V methods encourage prior evaluation by alternative tests. The use of the local lymph node assay (LLNA; 36), in preference to the conventional guinea-pig test, to assess the skin-sensitisation potential of chemicals has recently been endorsed by ICCVAM and the ESAC (37). The choice of alternative tests for use in evaluating skin irritation and eye irritation depends on the structure and other properties of the substance.

Corrosivity

Skin corrosivity testing is a relatively simple procedure in biological terms. The endpoint is severe tissue destruction, not a subtle biological change, and the application route is topical, with no problems of dilution or distribution. These two factors made the development of non-animal methods for the prediction of skin corrosion easier than for other toxic effects. Over about 4 years, ECVAM successfully coordinated the validation of two new *in vitro* corrosivity tests. Following a pre-validation study (38), an international validation study (39) was conducted during 1996 and 1997. The main objectives of the study were to: a) identify tests capable of discriminating corrosives from non-corrosives for selected types of chemicals and/or for all chemicals; and b) determine whether these tests could correctly identify chemicals already established as R35 (UN packing group I) and R34 (UN packing groups II and III).

The tests evaluated were the TER assay, CORROSITEX™, the SKIN^{2}™ ZK1350 corrosivity test and EPISKIN™. Sixty coded chemicals were used to evaluate each of these tests, in a blind trial conducted in three independent laboratories. The interlaboratory and intralaboratory reproducibilities were acceptable for all the tests. In addition, both the TER and SKIN^{2} tests were capable of testing a diverse group of chemicals with differing physical forms. However, only the TER assay and EPISKIN were successfully validated, because they alone met the agreed criteria concerning acceptable underprediction and overprediction rates. These two assays can now be used as alternatives to the animal tests used to distinguish between corrosive and non-corrosive chemicals. The corrosive potentials of about 40% of the test chemicals could not be assessed with CORROSITEX, and the assay did not meet all the criteria for it to be considered acceptable as a replacement test. However, CORROSITEX may be valid for testing specific classes of chemicals, such as organic bases and inorganic acids. The SKIN^{2} assay did not meet the criteria for it to be considered scientifically validated.

The 27th ATP of the DSD adopted two skin-corrosivity tests as Method B.40 of Annex V and, as noted, the method must now be used (30). The B.40 corrosivity test is mainly for UN transportation classification, but it can be used as a screen before skin-irritation testing with rabbits, for notification of new substances. The rat skin TER test does not distinguish between UN packing groups or between the EU classifications R35 and R34, whereas the human-skin model does. If the corrosivity test is negative, a normal Method B.4 skin irritation test would be needed for notification.

The TER test is a replacement test that still requires the use of laboratory animals. The test material is applied for up to 24 hours to the epidermal surfaces of skin discs taken from the pelts of humanely killed young rats. Corrosive materials are identified by their ability to produce a loss of normal stratum corneum integrity and barrier function, which is measured as a reduction in the inherent TER below a threshold level. Irritant and non-irritant materials do not reduce the TER below this threshold level. A dye-binding step can be incorporated into the test procedure for surfactants and neutral organics, to reduce the number of false-positive results obtained specifically with these chemical types.

The second EU Method B.40 test uses no animals. It is referred to in Annex V under the non-proprietary name of the "human skin model assay". This is the generic version of the ECVAM-validated EPISKIN test. The Epiderm™ skin corrosivity test has been subjected to a "catch-up" study (40), and is now accepted by the ESAC as a valid and usable B.40 test (41). In both assays, the test material is applied topically for a defined period (depending on the model used) to the model skin, which comprises a reconstructed epidermis with a functional stratum corneum. Corrosive materials are identified by their ability to produce a decrease in cell viability below defined threshold levels, following specified exposure periods. The principle of the test is based on the hypothesis that chemicals which are corrosive are those that can penetrate the stratum corneum (by diffusion or erosion), and are sufficiently cytotoxic to cause cell death in the underlying cell layers.

The problem with tissue cultures such as EPISKIN is that they have a limited life-span, and a high level of technical skill is needed to set up and maintain them. Simpler tests such as CORROSITEX can be used as screens for acids, bases and their derivatives. This assay is based on the time it takes a substance to break through a biobarrier.

The assay has recently been accepted by the ESAC as being scientifically validated for use with those chemicals that meet the technical requirements of the assay (42). ICCVAM also recommends CORROSITEX as a non-animal test for skin corrosivity in the USA, and the US Department of Transport now accepts the test for transportation classification.

Guidelines similar to those adopted in Annex V of the DSD were sent to the OECD Secretariat, and are now under consideration by OECD Member Countries. Therefore, it is hoped that the new EU methods will soon achieve worldwide acceptance through the OECD. However, there is an important difference between EU Annex V test methods and OECD test guidelines, even though they are now almost totally harmonised, because the former are mandatory in the EU, whereas OECD guidelines are merely recommendations.

Phototoxicity

As mentioned earlier, the 27th ATP of the DSD also contains, as Method B.41, an *in vitro* phototoxicity assay, the 3T3 neutral red uptake (NRU) phototoxicity test (30). This assay is used for the evaluation of the phototoxic potential of a wide range of chemicals, including cosmetic ingredients, and it is linked to the EU initiative to stop all animal testing on new cosmetic ingredients and formulated products in the near future. Its applicability is not restricted to cosmetic ingredients, however, and it is important that any chemical capable of reaching the skin through systemic distribution following ingestion, or by topical application should be tested. This test is also expected, in due course, to become an OECD Guideline. The validation work, which took about 7 years, was jointly undertaken by ECVAM and COLIPA, the European cosmetics industry association (43, 44).

Phototoxicity is a toxic response that is elicited after exposure of skin to certain chemicals and subsequent exposure to light, or which is similarly induced by skin irradiation after systemic administration of a chemical. No standardised international guidance for testing chemicals for phototoxic potential had previously been accepted for regulatory purposes. The first phase was designed as a prevalidation study, to identify *in vitro* test procedures and test protocols for a formal validation trial under blind conditions, according to the recommendations of ECVAM for the prevalidation and validation of toxicity test procedures (45, 46). In the second phase, the formal validation study, the most promising *in vitro* phototoxicity tests were validated with 30 carefully selected test chemicals in 11 laboratories in a blind trial. The 3T3 NRU phototoxicity test was performed as a core test in nine laboratories, since it provided the best results in Phase I of the study. The purpose of Phase II was to confirm the reliability and relevance of the *in vitro* tests for predicting phototoxic effects and for identifying phototoxic chemicals. Results obtained with two approaches to evaluating phototoxic effects in the 3T3 NRU phototoxicity test in Phase II were reproducible in the nine laboratories, and the correlation between *in vitro* and *in vivo* data was very high.

The 3T3 NRU phototoxicity test is a type of replacement alternative test. The test is based on a comparison of the cytotoxicity of a substance when tested in the presence and absence of a non-cytotoxic dose of UV light. Cytotoxicity in the test is expressed as a concentration-dependent reduction in the uptake of the vital dye neutral red, 24 hours after treatment with the test material and UV irradiation. Because the toxicological endpoint of this test is determination of photocytotoxicity induced by the combined action of a chemical and light, substances that are phototoxic *in vivo* after systemic application and distribution to the skin, as well as those that act as photoirritants after topical application to the skin, can be identified by the test. The test is not designed to predict other adverse effects that may arise from the combined action of a chemical and light (for example, photogenotoxicity, photoallergy and photocarcinogenicity), although many chemicals that show these specific properties will react positively in the *in vitro* 3T3 NRU phototoxicity test. In addition, the test is not designed to permit an assessment of phototoxic potency (47).

The European Commission consulted the Scientific Committee on Cosmetics and Non-food Products (SCCNFP) of its Directorate General for Health & Consumer Protection about the suitability of the method for cosmetic testing purposes. The SCCNFP recommended further evaluation of the use of the 3T3 NRU phototoxicity test with sunscreen filter chemicals from Annex VII of EU *Directive 76/768/EEC*, as they felt that cosmetics ingredients were insufficiently represented in the formal validation project. ECVAM commissioned a special study, the outcome of which was successful (48). Hence, in 1998, the ESAC formally accepted the study on UV filter chemicals, and confirmed that the 3T3 NRU phototoxicity test was suitable for use in EU Member States to assess the phototoxic potential of chemicals (49). By the end of 1998, the SCCNFP had also concluded, from the formal validation study and from the study on UV filter chemicals, that the 3T3 NRU phototoxicity test is a well-validated *in vitro* phototoxicity test that can be used for the safety testing of cosmetic ingredients and of finished cosmetic products (50).

Skin sensitisation

The current standard “benchmark” animal study to evaluate skin sensitisation potential is the

Magnusson & Kligman (M & K) guinea-pig maximisation test of EU Method B6 (51). This test is normally used for EU notification and classification. Non-adjuvant tests, such as the Buehler method (52), are used only if the M & K test is not technically feasible (i.e. the substance has a physical form that prevents injection). Nevertheless, such non-adjuvant tests are generally considered useful for risk assessment, because they are more relevant to normal dermal exposure. There are various theoretical problems with the M & K test: it is generally considered to over-predict skin sensitisers (i.e. giving false-positives, with chemicals classified as "sensitising" which are not human sensitisers); it gives no indication of potency; it is not a realistic model of human exposure; and there are various technical issues in interpreting skin reactions and dealing with equivocal results.

The local lymph node assay (LLNA), first conceived in the mid-1980s, identifies contact allergens as a function of their ability to provoke T lymphocyte proliferative responses in draining lymph nodes. This is not an *in vitro* method, relying as it does on the measurement of lymph node cell responses stimulated by repeated topical exposure of mice to the test chemical. Nevertheless, the LLNA does provide significant animal welfare benefits: fewer animals are required and, importantly, the trauma to which animals are potentially subjected is significantly reduced. An LLNA test usually includes four or five control mice and three to five groups of test mice treated with various concentrations of test substance. The scientific basis for the test is the measurement of the incorporation of ^{3}H-*methyl*-thymidine into lymphocytes in draining lymph nodes of animals topically exposed to the test substance as a measurement of sensitisation. The endpoint of interest is a stimulation index giving the ratio of thymidine incorporation into lymph nodes from dosed animals compared with the incorporation into lymph nodes from control animals. The test is positive when the stimulation index exceeds 3. It is possible to investigate potency, because, as noted, the stimulation within draining lymph nodes of T lymphocyte responses is a requirement for the successful induction of skin sensitisation. There is therefore a strong correlation between the strength of that proliferative response and the degree to which sensitisation will develop.

The LLNA has undergone extensive validation. It is now accepted, if positive, instead of an M & K test for EU notifications. Hence it is often conducted first, to screen out sensitisers. The LLNA has recently been endorsed by ICCVAM (53) as a standalone method for assessing skin sensitising activity, provided that various technical details are adhered to in conducting the test, notably that only female mice should be used until there has been a systematic comparison of results with male and with female mice. The LLNA has also been endorsed by the ESAC (37).

When modelling the *in vivo* skin sensitisation reaction, the critical steps are considered to be: skin permeation by the allergen; reaction of the hapten with a skin protein; processing haptenated proteins by epidermal Langerhans cells; migration of Langerhans cells to draining lymph nodes and interaction with T-cells; and recognition of hapten by specific T-cells. The use of computerised expert systems, based on the knowledge of the reactivity of chemicals with proteins, together with consideration of skin absorption, offers promise as a first step in a hierarchical approach to identifying skin sensitisation, and should be developed further. It should also be possible to use biological systems to provide data on such initial aspects. However, the development of *in vitro* assays to cover the later stages is dependent on the elucidation of the critical rate-limiting steps in the sensitisation process. In summary, the only existing method for making a prediction of skin sensitisation hazard is on the basis of chemistry, by using data on skin permeation coupled with information of the presence of structural alerts. Nevertheless, predictions based on structural alerts for groups within a molecule that are often associated with skin sensitisaton can be useful as a screening tool before animal testing. DEREK (Deductive Estimation of Risk from Existing Knowledge) is an example of a system that can be used for this purpose. Skin sensitisation is only one of several toxicological endpoints included in this system, which was first developed by Sanderson & Earnshaw (54), and has since been updated (55).

Skin and eye irritation

The field of skin and eye irritancy is one that has attracted a particularly large amount of effort. Many non-animal methods for predicting skin and eye irritation have been developed, and several have been subjected to prevalidation and full validation studies. However, none has so far proved acceptable as a complete replacement for a regulatory guideline. The major reason for this relates to the complexity of the endpoints in both tissues, with a lack of knowledge of the mechanisms involved. Consequently, most of the *in vivo* models are not based on knowledge of relevant mechanisms in humans. Another complicating factor is the lack of reliable data from humans and animals against which to judge the predictive performance of new tests.

The ECVAM Skin Irritation Task Force was established in November 1996 to address the need for progress in the validation of suitable *in vitro* tests for acute skin irritation (56). It recommended that ECVAM should support a prevalidation study

on four promising *in vitro* tests, by subjecting them to the prevalidation scheme defined by ECVAM (45), to assess their readiness to progress to a formal validation study (57). These tests were Epiderm, EPISKIN, PREDISKIN™ and the non-perfused pig-ear method. A fifth test, the mouse skin integrity function test (SIFT), was added later. All five tests were sufficiently promising in Phase I (protocol refinement) to allow them to progress directly to Phase II (protocol transfer). The intralaboratory reproducibilities of Epiderm, EPISKIN and the SIFT were sufficiently acceptable for these tests to progress to Phase III (initial assessment of protocol performance). The PREDISKIN protocol was overly sensitive, whereas the pig-ear test showed an unacceptably large variability in the results provided. When their protocols were assessed in Phase III, neither Epiderm nor EPISKIN met the criteria required for progress to a formal validation project. The performance of the SIFT in Phase III is now being evaluated.

The Management Team concluded that each of the test protocols and prediction models needs to be reviewed, and all arising issues must be addressed, before any of these tests can be included in a formal validation study. In particular, they suggested that additional endpoints, other than cytotoxicity, could increase the predictive ability of the human skin models. These skin models reflect to varying extents the dermal penetration of chemicals, and their subsequent cytotoxicity. However, the precise roles of inflammatory mediators and other cellular signalling molecules in the skin irritation response are still insufficiently understood to develop an *in vitro* model for accurately predicting human skin irritation potential. The human skin models are promising test systems for safety evaluation studies, but their value will only be realised if a small battery of key endpoints can be identified, and simple assays can be developed to enable these endpoints to be measured accurately and precisely.

However, progress has been made in obviating the need to use animals in assessing severe effects, by "screening out" severe irritants by using a hierarchial approach. This screening process is likely to improve with further use of SAR and mathematical modelling, and when better *in vitro* methods are developed for detecting severe irritants.

Much work has been done in attempts to assess ocular irritation with non-animal methods. The goal is to be able to predict eye irritation potential over the whole range of the response (from slight to severe) for any chemical substance. The information is needed to assess hazard (i.e. the intrinsic toxicity of the chemical), in order to be able to protect users through the classification and labelling of substances. The other issue is that, for finished products which may come into contact with the eye (for example, ophthalmologic or cosmetic products), it is necessary to ensure that they are as safe as possible, i.e. non-irritant.

The results were published in 1995 of a systematic evaluation of the then existing *in vitro* eye irritation tests, undertaken by the UK Home Office and the European Commission, with participation from industry, academia and ECETOC (58). It was concluded that, with the possible exception of predicting the irritancy of surfactants, none of the nine tests met any of the performance targets. Subsequently, COLIPA undertook an international validation study on alternatives to the rabbit eye irritation test (59). The principal goal of this study was to determine whether the results from a set of alternative methods used by cosmetics companies are valid for predicting the eye irritation potential of cosmetics formulations and ingredients. For the first time in a validation study, prediction models (PMs) that convert the *in vitro* data from an assay to a prediction of eye irritation were developed for each alternative method before the study began. In this study, ten alternative methods were evaluated by using 55 test chemicals selected as representative of substances commonly used in the cosmetics industry (23 ingredients and 32 formulations). Twenty of the single ingredients were common to the European Commission/Home Office (EC/HO) eye irritation validation study (58). Based on the criteria of reliability and relevance, as defined in the study, none of the alternative methods evaluated could be confirmed as a valid replacement for the Draize eye irritation test across the full irritation scale. However, three alternative methods (the fluorescein leakage test, the red blood cell assay, and the tissue equivalent assay) each satisfied one criterion of reliability or relevance.

On ethical grounds, only non-animal tests are now used to assess formulated cosmetic products. Consequently, alternative methods have been chosen for their capacity to predict the ocular irritating potential of cosmetic products, whatever their formulation type (powder, emulsion or solution). Cosmetic companies develop their specific batteries of *in vitro* tests according to their product categories, some examples of which are listed below.

— The hen's egg test–chorioallantoic membrane (HET–CAM) method (60), which can reflect specific eye irritation effects, such as corneal opacity or vascular effects. This is one of the few *in vitro* methods that allows the blood vessels to be examined. Also, the eggs examined morphologically in the HET–CAM test can also be used to provide more-objective and more-quantitative information, by performing chorioallantoic membrane–trypan blue staining (CAM–TB; 61).

— The neutral red release assay (Predisafe™ assay), which is a short-term monolayer culture system able to predict the cytotoxicity of cosmetic products (62).

— Tests using reconstituted human epithelial cultures (for example, the SkinEthic™, EpiOcular™, EPISKIN and Epiderm models).

A recent feasibility study (63) on the use of a battery of cell-based toxicity assays as predictors of eye irritation, found that such an approach had distinct potential, provided that the assays included in the battery were fully validated. The most important aspects of this battery approach are that the requirements for both reliability and predictability are met. Therefore, the choice of assays that are used in combination is vital. Tests that are mechanistically similar would fulfil a confirmatory role, increasing overall reliability, whereas the inclusion of tests that differ in their mechanistic basis would provide a broader screen, and thus increase the predictive ability of the battery.

The rabbit enucleated eye test (REET), also called the isolated rabbit eye (IRE) test, involves the use of eyes removed from recently killed rabbits (64). It has been found to provide accurate predictions of ocular irritancy, based on *in vivo* classifications of 21 chemicals tested in a collaborative study sponsored by the Commission of the European Communities (65). The test has the advantage that the animals used are not bred exclusively for this purpose, and have previously been used in dermal irritation studies. The eyes are mounted in a chamber, and perfused with warm saline. Under these conditions, the eyes remain viable for the duration of the experiment. The test material is placed on the corneal surface of the test eyes for 10 seconds, and then washed off. At set intervals, corneal opacity and thickness, as well as fluoroscein dye uptake are assessed.

The bovine corneal opacity and permeability (BCOP) assay (66) is similar to the REET, in that it is based on changes in opacity and epithelial permeability of isolated eyes, following exposure to the test material. It has been subjected to a multinational international study, where it was found to correctly classify 44 of the 52 compounds tested (67). However, the EC/HO study on the REET and the BCOP showed that both assays were lacking in predictive ability when results were compared with *in vivo* data for 59 chemicals (58). It is clear that considerable refinement and evaluation are required for both assays, before they can be considered for regulatory purposes. The use of additional endpoints, such as those provided by histological examination, can provide supplementary information that may improve the predictive ability of both assays. The importance of investigating non-specific damage to the corneal endothelium has recently been shown by Bruner *et al*. (68), who detected chemical-specific and time-dependent damage to the endothelium by using trypan blue staining (69). However, they also discovered that conventional corneal holders can cause significant damage to the endothelium, which can complicate the interpretation of chemically induced damage. Therefore, modification of these holders would be required before endothelial morphology could be used as an endpoint in the BCOP assay. The use of prolonged exposure times, and the measurement of corneal swelling, as well as the incorporation of the additional endpoint of histological evaluation, have all been proposed by Cooper *et al*. (70) as a means of achieving greater correlation between the BCOP assay and *in vivo* data.

An additional *ex vivo* model, employing rabbit corneas, has recently been proposed as a possible future replacement assay for ocular irritation (71). It is based on previous *in vivo* findings that the irritation potential of surfactants is largely determined by the extent of the initial injury. An initial assessment of the assay provided a favourable correlation with results of animal studies. The authors, however, acknowledged that further model development would be needed before this mechanistically based assay could be considered as a valid animal replacement.

Finally, the fluorescein leakage assay is a replacement test that uses Madin-Darby canine kidney (MDCK) cell monolayers for the assessment of both ocular and dermal irritancy potential. It was first proposed by Tchao (72), and further developed by Shaw *et al*. (73). The cells are grown to confluency on a porous filter, forming tight junctions similar to those found in the corneal epithelium. Chemically induced loss of impermeability of the barrier is determined through the measurement of the leakage of a non-toxic dye (fluorescein) through the cellular layer.

Structure-activity Relationships and Prediction of Properties

Conventionally, the hazardous properties of chemicals are determined by testing according to standardised procedures, normally in compliance with Good Laboratory Practice (GLP). These laboratory studies are, in effect, a model for the effect that the chemical may have on the systems of primary concern, namely, humans and the environment. There are, however, circumstances when adequate predictions of hazardous properties can be made without testing. In particular, in some cases, the so-called "read-across" approach can be used to predict the properties of new substances, by interpreting results for close analogues with similar physicochemical properties and impurity profiles. The principle is that, because the toxicokinetics (especially absorption and metabolism) of the new and known substance would be expected to be similar, similar biological properties would also be expected. Only negative general toxicity can be read across, whereas read-across of both positive and negative

mutagenicity is normally applicable. It is preferable to see as many matching toxicological properties as possible, to provide evidence in support of read-across for missing endpoints. The use of "read-across", or other surrogate data, has to be negotiated in advance with the national Competent Authority.

A typical physicochemical properties testing programme to justify "read-across" for a notification involves determining the melting point, boiling point, density, surface tension, water solubility, *n*-octanol–water partition coefficient and particle size distribution. Assuming that a full set of standard GLP data is available for a first substance, it may be that only those tests that are considered to be pivotal in deciding the feasibility of read-across may have to be conducted according to GLP, in particular, the determination of water solubility, *n*-octanol–water partition coefficient, and particle-size distribution. The study most likely to be acceptable for "read-across" is the 28-day repeat-dose oral rat toxicity study, as this uses many animals and, fortuitously, also offers a considerable cost saving. A basic (eco)toxicological package to confirm "read-across" would include acute oral toxicity, skin sensitisation (occasionally omitted), and an Ames test, with perhaps skin irritation and biodegradation tests. It may also be necessary to confirm that there is a similar acute toxicity to aquatic organisms, by using the basic ecotoxicological package of one acute study in fish, *Daphnia* or algae, whichever species is most sensitive to the fully tested substance.

The computerised determination of QSARs has been used for many years in the design of potentially useful chemical agents, but it is only in recent years that these techniques have been used in toxicology. QSAR evaluations aim to use the known biological activity of a set of chemicals to establish mathematical relationships between their activities and their structures. An alternative approach, using structure–activity relationships (SARs), is to use computerised methods to identify fragments of molecules that are known to be associated with particular biological properties.

QSARs can be used in certain circumstances for the notification of new substances in the EU, and, in particular, to refine the risk assessment, rather than to provide surrogate data to replace the base set testing. It is the responsibility of the notifier to make the QSAR predictions, and to establish that they are valid, although Competent Authorities may advise and offer expert scientific input. Chapter 4 of the technical guidance on risk assessment (74) gives extensive regulatory and scientific information on the use of QSARs in the EU process for risk assessment of chemical substances. The emphasis, though, is on the use by Competent Authorities of QSARs to support the risk assessment of the priority HPV chemicals selected for detailed review under the EU Existing Chemicals Regulation.

When carrying out the risk assessment for humans and the environment, the exposure of humans and of environmental compartments to the substance is estimated. These exposure assessments will be based on available monitoring data and/or modelling. For modelling exposure, several physicochemical and environmental fate parameters are used. In the absence of experimental data, these parameters may be derived from QSARs. Depending on the exposure–effect ratio from the risk assessment, a decision is taken as to whether the substance presents a risk to humans, the environment or both, or whether further data are necessary to clarify a concern. When the Competent Authority considers that there is potential need for further data, QSARs may serve as a supporting tool in making this decision. Furthermore, if further testing is needed, QSARs may also be used to optimise the testing strategies.

Validated QSARs are not currently available for toxicity endpoints. Instead, expert judgement is used, based on knowledge of close structural analogues and/or the presence of "structural alerts" (i.e. structural fragments associated with effects). Nevertheless, such predictions may be useful for the risk assessment for human health, especially for endpoints without test results, and they may be of value in indicating a potential hazard, the probable toxicokinetic properties, or the need for further testing. Since biological activity depends on both partition and reactivity, a QSAR model must be capable of modelling both of these, so that a high correlation with the *in vivo* response can be expected. Furthermore, all the chemicals covered by the relationship must have a dependent property that is elicited by a mechanism that is common to the set of chemicals and the dependent property (75).

Reliable QSAR estimates for fish, *Daphnia* and algal toxicity are available for chemicals with a non-specific mode of action. These estimates can be used to assist in data evaluation or to contribute to making a decision on whether further testing is necessary in order to clarify an endpoint of concern and, if so, to optimise the testing strategy. Also, QSARs can help in assessing long-term aquatic toxicity of very hydrophobic organic chemicals, for which tests are difficult to perform.

Another use of QSAR is in the selection of a set of chemicals for use in the validation of *in vitro* tests. In this context, QSAR can be used to select chemicals that differ greatly in the *in vivo* responses that they elicit. In the recent ECVAM international validation study for skin corrosivity (38), Principle Components Analysis was used to select chemicals spanning the full range of corrosivity potential.

Strategies to Minimise Use of Animals in Evaluating Safety

Over many years, the UK Health and Safety Executive (HSE) has done its best to encourage the reduction of animal use during chemicals testing. Although its actions are constrained to a degree by the EU and by international regulatory systems under which the UK operates, the HSE seeks to minimise animal testing wherever possible. For notification of new substances, the HSE makes particular efforts to bring together two or more intending notifiers of the same substance, so that they can share data and avoid duplication of animal testing. The HSE also encourages the use of methods (such as the fixed-dose procedure for acute oral toxicity) that offer reduced animal use or reduced severity of the testing procedures. In general, the philosophy is to encourage thoughtful toxicology, rather than routine testing. Hence, the aim is to promote a tiered approach to testing, with non-animal information being used in the initial assessments. The HSE is keen to use *in vitro* alternatives for notifications, but these tests must be scientifically validated and, preferably, form an OECD guideline, or at least be in development for the OECD. Only positive *in vitro* test results are acceptable and, if the result is negative, the standard animal test would normally be required.

To minimise testing for notification purposes, the German Competent Authority (76, 77) has developed stepwise assessment procedures, including structure–activity considerations, alternative methods (*in vitro* tests) and computerised SAR models. An electronic database has been developed, which contains physicochemical and toxicological data on approximately 1300 chemical substances. It is used for regulatory structure–property relationship (SPR) and SAR considerations, and for the development of rules for a decision-support system (DSS) for the introduction of alternative methods into local irritancy/corrosivity testing strategies. The information stored in the database is derived from proprietary data, so it is not possible to publish it directly. Therefore, the database is evaluated by regulators, and the information derived from the data is used for the development of scientific information about SARs. This information can be published by means of tables correlating measured physicochemical values and specific toxic effects caused by a chemical. This information is introduced to the public by means of a DSS that predicts the local irritant/corrosive potential of a chemical by listing so-called exception rules. The DSS can predict whether a chemical produces: a) corrosive effects (i.e. no testing is necessary); b) might have corrosive effects (i.e. no animal testing, and instead *in vitro* tests are suitable); and c) will produce no effects or only marginal effects (i.e. animal tests are necessary). In addition, the DSS provides reliable data for classification and labelling based on a specific result.

Broadhead *et al.* (78) have suggested a tiered strategy for testing food additives. The initial stages of the strategy involve the use of PBPK modelling to identify the metabolites produced and their potential target organs. PBPK modelling would also assess the concentrations likely to be encountered in humans. This is particularly important, as the demonstration of the effects of a substance at high concentrations in an *in vitro* assay might not necessarily imply that the same effects would be observed *in vivo*. After identification of the potential target organs, a battery of cell lines could be used to assess the genotoxicity and tissue-specific toxicity of the substance. Short-term and long-term genotoxicity and non-genotoxicity studies would be carried out concurrently. Should any undesirable results be obtained, the substance could be rejected. If no cytotoxic or genotoxic effects were observed at relevant concentrations, further testing would continue for neurotoxicity, immunotoxicity, reproductive toxicity and acute toxicity. The acceptable daily intake (ADI) for food additives is a key result in safety assessment. It is usually derived from the no-observed adverse effect level (NOAEL) in long-term animal *in vivo* studies, and includes a safety factor (usually 100) to allow for interspecies and inter-individual variability. The application of *in vitro* data to derivation of the ADI is discussed elsewhere (79), where the appropriate future role of *in vitro* tests was suggested as a supplement to the *in vivo* tests, which would still be required for mechanistic information.

Future Developments and Conclusions

There is considerable weight of evidence that, of the potential number of gene expression patterns for the entire human genome ($10^{30,000}$), the actual number that are toxicologically relevant is exceedingly low. This number has been calculated to be about 317 (80). When the 256 human cell types are accounted for, this leaves about 60 other states for potential responses to environmental stress and toxicant exposure. The genes involved in such responses can be characterised according to the type of alteration that they attempt to revert. This has given rise to four broad categories: those that respond to the presence of a compound; those that respond to damage caused by a compound; those that respond to altered levels of crucial metabolites, such as ATP and NADP(H); and those that respond to changes in the cellular redox status or pH (80).

Microarrays provide a method for surveying variation in both RNA and DNA. The modern microarray is a natural progression from the classic Southern blot technique (81). This technique, con-

ceived by Edwin Southern in the early 1970s, is based on the idea that immobilised DNA molecules can be identified through their ability to bind to labelled nucleic acids. In the Southern blot technique, nitrocellulose or nylon provides the membrane support for the tethered probes, whereas, in microarrays, the DNA is bound to a more solid support, such as glass or a silicone. Once the microarray has been created, it can then be used to analyse the global gene expression of a target cell. This invariably involves hybridisation of the sample to the DNA present in the array. The subsequent detection strategies can be quite varied. Autoradiography of radiolabelled samples is a conventional approach, but other options are available, including electronic signal transduction.

These advances in gene expression analysis have huge implications for the field of toxicology. It has been found that, in most cases examined, gene expression is altered, either directly or indirectly, following exposure to a toxicant (82). The mechanisms of chemical toxicity could, one day, be elicited from this altered gene expression. There is a close association between the function of a gene product and its expression pattern. In general, a gene is expressed in specific cells and under specific conditions in which its product will contribute to overall fitness. The stringent regulation that governs when, where and in what quantity a gene is expressed, is a result of natural selection in the evolutionary process (83). Different gene expression patterns are seen following exposure to different chemicals at varying doses. These data can then be used in predictive assessments of the potential toxicity of new chemicals or chemical mixtures.

Microarrays may also provide for more-accurate dose–response assays than those now used. Many risk assessments assume that the risk for toxicity is linear at low doses, but this is not necessarily true. Some receptor-mediated chemicals, for example, may require a minimum threshold dose before a response is achieved. Likewise, at low doses, DNA-damaging chemicals may show nonlinear responses because of the DNA-repair mechanisms of the cell (82). Microarrays can be used to circumvent this problem in both receptor and non-receptor systems, because of their ability to determine a cell's response to a chemical at the genetic level. Changes in gene expression over a wide range of doses can be used to create a curve that accurately depicts the linearity or nonlinearity of the dose–response relationship.

Chronic toxicity tests are normally conducted at high doses, close to the maximum tolerated dose (MTD). Such assays are not always useful in the prediction of the effects of low doses of chemicals, which would often be a more accurate and more realistic representation of the exposure levels that humans would expect to encounter. Also, these chronic bioassays do not give much information on the type of mechanisms that are involved in secondary effects such as cytotoxicity and regenerative cell proliferation, and on the role of dose in these mechanisms. Again, as with acute assays, microarrays may be able to identify differences in gene expression at various doses, and enable the prediction of the potential mode of action of a compound before a chronic bioassay is conducted.

Microarray technology can also be used to predict the potential toxicity of a chemical mixture. Chemicals can interact with each other to produce synergistic, additive or antagonistic effects, depending on their modes of action. These interactions can be evaluated in a laboratory in defined animal systems where the gene-expression profiles of each chemical are already well known. The additive effect of these individual chemicals can be compared with the effect of the mixture, to determine the nature of the interaction. This can be used to assess the toxicity of the combined assortment of chemicals that humans are exposed to on a routine basis in various environmental settings.

This mechanistic approach can provide information that is more relevant to the human situation than can currently be gained from animal experiments. For example, the mechanism of action of peroxisome proliferators is being investigated by using this approach (84). The liver tumours caused by these chemicals in rodents are thought to be as a result of a different mechanism to that which occurs in humans, and so it is important that investigators know more about how liver metabolism is affected. Similarly, gene arrays can be used to study the effect of chemicals such as endocrine disruptors on various tissues and organs. This can lead to the generation of hypotheses on the possible biochemical pathways involved, and these hypotheses could, in turn, be tested on transgenic mice.

Human exposure to various chemicals can also be calculated, based on fingerprinting techniques, such as those described in the gene-expression analysis of animals exposed to chemicals. However, there are two main problems with this. Firstly, obtaining human volunteers might prove difficult. Secondly, it is very difficult to obtain a satisfactory baseline for expression levels, as this will vary widely because of differences in diet, inter-individual variability, and differences in the standard of health among individuals. A national human gene expression database may provide a solution to this difficulty (85).

At the moment, gene expression data are being generated faster than they can be interpreted, and the field of bioinformatics will be of critical importance in this respect. Collaboration between scientists from several disciplines, including engineering, statistics, molecular biology, chemistry and bioinformatics will be necessary, if significant progress is to be made (86). Databases must be easily accessible (the issues of intellectual-property rights and patenting may be sig-

nificant here), and continually updated, so that reproducible and comparable results can be obtained from separate studies. A national gene-expression database for humans has been suggested as a means of dealing with the issue of background noise of gene expression variability (85, 87). This is particularly important when assessing low-dose exposure, where the changes induced may not be greater than this variability. The specificity and sensitivity of various methods of obtaining gene expression data may differ slightly. This must also be standardised to facilitate accurate comparisons of results.

The current gene-array technology remains too cumbersome and too expensive to be used routinely to perform the large-scale gene expression experiments required to generate EC50 averages, which, in turn, could be used to rank compounds. This issue will be resolved by forthcoming technological advances.

Although this area is only in its infancy, it has already greatly enhanced our understanding of the molecular mechanisms that underlie many toxic responses, and scientists have been able to use this information to successfully engineer and improve the sensitivity and selectivity of the current testing models. In the future, the impact of this technology can only increase as its potential applications are fully realised.

An extremely important use of alternative tests is as a screen before animal testing, to eliminate obviously hazardous chemicals from further study. Alternative tests are also useful in the less-prescriptive safety-evaluation processes. Such studies, on their own, or combined with property predictions based on chemical structure (by formal "read-across" to tested analogues, QSAR or prediction by professional judgement), can be used for evaluation of the safety of workers, and for the production of safety data sheets. The other use of such studies is in risk assessments, when there is no obligation to do new studies, i.e. to refine uncertain outcomes of risk assessment for some notifications and for Existing Chemical Regulation priority chemicals, and also for the less-formal risk assessments for other types of product (for example, cosmetics).

Other regulatory agencies outside the EU also accept alternative tests, but there are cultural and regulatory differences. For example, in the USA, there is normally no obligation to do studies on chemicals for the *Toxic Substances Control Act* or for the Occupational Safety and Health Administration, so available alternative tests can be used, whereas in Japan the testing for notification is highly prescriptive and stylised, with no opportunity to use non-standard tests.

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