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DEVELOPMENT, VALIDATION AND LEGAL ACCEPTANCE OF ALTERNATIVE METHODS TO ANIMAL EXPERIMENTS

(presented by the Commission)

CONTENTS

A. INTRODUCTION

In the Community internal market, cosmetic products must be able to circulate freely and meet high quality and safety standards.

The new provisions of Council Directive 93/35/EEC amending for the sixth time Directive 76/768/EEC on the approximation of the laws of the Member States relating to cosmetic products are a consistent package designed to:

- provide consumers, the supervisory authorities in the Member States and the Commission with better information, notably through compiling an inventory of ingredients used in cosmetic products, through mandatory information labelling and through provision by the national supervisory authorities of a dossier containing information on the product's formula, its physico-chemical and microbiological specifications, the method of manufacture, assessment of safety for human health, undesirable effects and proof of the claimed effect.

- assure a better level of quality and safety of products and their ingredients and hence a better level of consumer health protection.

However, in assessing safety for human health, the suffering and death of animals must be avoided wherever possible.

Hence, Article 2 of Directive 76/768/EEC laid down the very clear principle of "**safety of cosmetic products for human health**". This requirement is specified in Article 2 as amended by Directive 93/35/EEC.

*"A cosmetic product put on the market within the Community **must not cause damage to human health** when applied under normal or reasonably foreseeable conditions of use, taking account, in particular, of the product's presentation, its labelling, any instructions for its use and disposal as well as any other indication or information provided by the manufacturer or his authorised agent or by any other person responsible for placing the product on the Community market."*

Article 4, as amended by Directive 93/35/EEC, concerns **respect for the protection of animal life**.

"Without prejudice to their general obligations deriving from Article 2, Member States shall prohibit the marketing of cosmetic products containing:

i) ingredients or combinations of ingredients tested on animals after 1 January 1988 in order to meet the requirements of this Directive."

However, Directive 93/35/EEC stresses the need to offer the consumer a degree of protection equivalent to that obtained by animal experiments. It also lays down obligations for the Commission, which must present draft measures aimed at putting back the deadline for prohibiting animal experiments if alternative methods have not been

validated and legally accepted, and to present an annual report to the European Parliament and Council on trends in this domain.

"If there has been insufficient progress in developing satisfactory methods to replace animal testing, and in particular in those cases where alternative methods of testing, despite all reasonable endeavours, have not been scientifically validated as offering an equivalent level of protection for the consumer, taking into account OECD toxicity test guidelines, the Commission shall, by 1 January 1997, submit draft measures to postpone the date of implementation of this provision, for a sufficient period, and in any case for no less than two years, in accordance with the procedure laid down in Article 10. Before submitting such measures, the Commission will consult the Scientific Committee on Cosmetology."

'The Commission shall present an annual report to the European Parliament on the Council on progress in the development, validation and legal acceptance of alternative methods to those involving experiments on animals. That report shall contain precise data on the number and type of experiments relating to cosmetic products carried out on animals. *The Member States shall be obliged to collect the information in addition to collecting statistics as laid down by Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes. The Commission shall in particular ensure the development, validation and legal acceptance of experimental methods which do not use live animals."*

This report will examine in turn:

- the objectives and individual stages involved
- the safety of cosmetic ingredients, the concept of risk and scientific methods available for assessing safety
- the actors
- the initiatives
- the state of play
- statistical collection of experiments on animals
- progress achieved as of October 1994
- conclusions.

B. OBJECTIVES AND STAGES

The Commission is in the unenviable position of having to reconcile provisions relating to better protection of human health with animal welfare, the main objective being to encourage the development, scientific validation and legal acceptance of **alternative methodologies which will offer consumers a level of protection equivalent to that obtained through experiments on animal models**.

Alternative methods (AM) are defined* as all modifications to existing internationally and scientifically approved tests with a view to:

(1) *reducing* the number of animals used
(2) *refining* existing protocols, so as to reduce animal suffering
(3) *replacing* tests on animals

in the performance of toxicological tests necessary for evaluating the safety of substances and/or combinations of substances liable to imperil human health.

This concept indicates that the development of alternative methods is not confined to replacing live animals, although this is the objective of the work done in implementing Directive 93/95.

Methods which do not use live animals (case 3) include "in vitro" methods and computer simulations (mathematical models).

"In vitro" methods include simple biological systems in the form of bacteria, cultures of cells or animal or human tissues, organotypical cultures, or systems using artifical media (reconstituted skin); these methods may also be physical and chemical tests (alkaline-acid reserve, etc.).

The most widely used tissues and organs are:

- skin cultures (mouse, rabbit, man, rat, pig, chicken, etc.)
- cell cultures from bone marrow, lungs, liver, skin (keratinocytes); they may be cell lines or primary cells (rat, mouse, hamster, etc.).
- embryos (chicken, fish, frog, rat, etc.)
- eyes (cow, rabbit, mouse, etc.)

One big advantage of computer analysis programmes is the ability to manipulate the large number of variables in various experiments and to develop QSAR** studies to predict in vitro and/or in vivo toxic potential of untested substances, etc.

The replacement of toxicity tests on animal models by in vitro tests involves the following stages:

1 DEVELOPMENT OF IN VITRO TESTS

Toxic effects observed in vivo are complex and involve several mechanisms at cell level.

In the case of in vitro tests, the test system is isolated from the animal and action at cell level is simplified and more specific, but incomplete by comparison with the effects observed in vivo.

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* Russell and Burch 1959.

** Quantitative Structure Activity Relation

In vitro studies are said to constitute a "mechanistic" approach, i.e. they are based on biomechanical mechanisms at cell level with biological consequences corresponding to an in vivo toxic effect. However, certain in vitro tests are based on an observed phenomenon approach rather than on mechanisms which are not always known.

Given this mechanistic approach, the test (or battery of complementary tests) developed must cast light on the fundamental biological consequences of to a given toxic effect and it must be possible to correlate the results with toxicity data on animals in vivo and interpret them in terms of toxicity for man.

2 VALIDATION

No matter what agency is responsible for coordinating the validation of alternative methods, **validation is the complex process via which the relevance, reliability and reproducibility of a test developed for routine application and legal acceptance are assessed and monitored**.

In 1990 the OECD published a general report on the scientific criteria for validating in vitro toxicity tests designed to contribute to the discussions on the validation process:

The major stages of validation are:

- *an intra-laboratory evaluation* designed to standardise the test protocol, to establish feasibility in regard to the objective in mind and the reproducibility of the results for a given substances within a given laboratory

- *an inter-laboratory evaluation* designed to verify reproducibility in several laboratories

- *development of test databases*, which are a scientific tool for analysing and categorising the findings and which are mainly used to compare data derived from various studies of cell toxicity, to establish in vitro/in vivo correlations, to study the applicability of the method to substances belonging to different chemical classes, to determine sensitivity and predictability in regard to an in vivo biological consequence.

  evaluation of the results.

The agencies responsible for validation work observe the principles developed in the Amden workshop report reproduced in Annex 1.* A second workshop has been held at Amden under the auspices of the ECVAM. The report on the second workshop will be published in ATLA in January 1995.

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* Balls M., Blaauber B., Frazier J., Lamb D., Reinhardt C., Roberfroid M., Schmid B., Spielmann H., Stammati A.L. and Walum E. (1990).
Amden Report.: Report and recommendations of the CAAT/ERGATT workshop on the validation of toxicity test procedures. ATLA 18, p. 313-337.

A validation exercise is considered complete when these stages have been successfully terminated and when the results correspond exactly to the objectives laid down at the beginning of the exercise.

3 LEGAL ACCEPTANCE

A proposal for a new guideline or for revising an existing guideline from the OECD, must undergo a critical evaluation of its scientific justification, its sensitivity and its reproducibility.

The OECD procedure for adopting alternative methods is subject to the same criteria. This is a multi-stage procedure which generally takes between one and three years. It requires a consensus within the scientific community of the 25 Member States, and acceptance, by the regulatory agencies in Northern America, Europe and Japan, of alternative methods as substitutes to animal experiments in evaluating the safety of substances belonging to different classes.

For example, the OECD secretariat began revising the genetic toxicology methods at the request of the European Commission in 1991. Three meetings have been held (London, 30 November - 2 December 1992, Ottowa, 31 January - 2 February 1994 and Rome 19 - 23 September 1994) with a view to completing the review, which should be finalised in 1995.

C. SAFETY OF COSMETIC INGREDIENTS AND THE CONCEPT OF RISK

To get a better picture of the state of play, it is useful to recall the legal backdrop to the assessment of the safety of ingredients used in cosmetic products, the different aspects of toxicity to be examined, the scientific methods available for evaluating safety, the definition of cosmetic product within the meaning of Directive 76/768/EEC and the concept of risk.

1 EVALUATION OF COSMETIC INGREDIENTS

1.1 Cosmetic ingredients in annexes to Directive 76/768/EEC

The Cosmetic Products Directive includes annexes with a list of prohibited substances and a list of substances that are authorised under certain conditions.

Substances which are not included on these lists are authorised a priori with the exception of three classes of substances to which the human skin is considered to be particularly sensitive, and for which positive lists exist: colouring agents, preservatives and UV filters.

Before being entered in one of these annexes, which are regularly updated in a procedure involving a Committee on Adaptation to Technical Progress, the Scientific Committee on Cosmetology (SCC), created by Commission Decision of 19 December 1977, must be consulted and deliver an opinion on the assessment of safety for human health of these substances. The SCC's evaluation takes into account experimental toxicity data, the type and level of exposure in cosmetic usage, applying its own guidelines.

Council Directive 93/35/EEC amending for the sixth time Directive 76/768/EEC contains a supplementary instrument designed to make the finished product better and safer in the form of provisions mandating the national supervisory authorities to make available an information dossier, which must inter alia contain a safety evaluation of the finished product. However this is largely based on evaluation of toxicity and safety data relating to the ingredients. The information dossier will be essential after 1 January 1997, the date on which cosmetic products which do not conform to the provisions of Directive 93/35/EEC will no longer be allowed to be marketed.

The basic toxicity data provided for in the guidelines for evaluating the safety of cosmetic products are described below:

The test procedures are described in OECD monographs and in Directives 87/302/EEC and 92/69/EEC, except for the photomutagenicity tests, photoirritation tests, photosensitisation tests and percutaneous absorption tests.

1. Acute toxicity, oral route [or inhalation] - OECD 401 -

   All adverse effects produced by oral administration (or via inhalation) of a single dose or multiple doses of substances, administered within 24 hours.
   Selected species: rat

   LD_{50} is the dose of the substance administered by oral route (LC_{50} by inhalation) which causes the death of 50% of the animals.

   Fixed dose method - OECD 420
   The in vivo fixed dose method was accepted by the OECD. It has been approved with the preliminary screening test which provides information on the dose to be applied in the main study.

2. Percutaneous absorption - Draft -

   Percutaneous absorption is the process by which a substance applied to the skin penetrates through the stratum corneum and enters the bloodstream.
   Selected species: rat

   A substance's percutaneous absorption factor is the quantity of the substance which is biologically available at systemic level after epicutaneous application.

   Because of the decisive role of data on percutaneous absorption, the SCC has prepared a document that critically analyses existing test methods.

3. Skin irritation - OECD 404 -
   Reversible inflammatory changes to the skin following epicutaneous application of the substance to be tested.
   Selected species: albino rabbit

4. Eye irritation - OECD 405 -

Reversible changes to the eye following application of a substance to be tested to the anterior surface of the latter.
Selected species: albino rabbit

5. Skin sensitisation - OECD 406 -

Secondary immunological cutaneous reaction on contact with the substance (allergic contact eczema)
Selected species: guinea-pig

6. Sub-chronic toxicity, oral route (or inhalation) - OECD 408 - (413)

Complex of adverse effects resulting from repeated and daily administration of a substance by oral route (or by inhalation) during part of the lifetime of the test animals (not exceeding 10%).
Selected species: rodents, in particular the rat

The 90-day sub-chronic toxicity tests provide information on the likely effects of repeated exposure over a limited period of time, the target organs, the possibility of cumulative effects and the no toxic effect level.

No observed adverse effect level (NOAEL) is the maximum dose of the substance which can be used in a test without producing harmful effects.

7. Mutagenicity (photomutagenicity of UV filters) -OECD 471 - 472 - 473 - 476

Modifications of the information content of the genetic material (DNA, i.e. desoxyribonucleic acid) transmitted to following generations of cells or individuals.

Photomutagenicity refers to all modifications to the information content of the genetic material in the presence of UV radiation.

In-vitro test systems:

gene mutations:	bacteria
	mammal cells
chromosome aberrations:	mammal cells

8. Cutaneous photosensitivity (for UV filters)

Photoirritation or phototoxicity is the reaction of the skin after epicutaneous application of a chemical substance and exposure to light.

Cutaneous photoallergy or photosensitisation is a skin reaction after percutaneous application of a chemical substance and exposure to light, involving the immune system.
Selected species: guinea-pig, rabbit, mouse.

9. Clinical data

Data on man when available, for example data on allergic sensitivity.

In the case of potential oral absorption or significant cutaneous absorption, and depending on the results of in-vitro mutagenicity tests, supplementary information may be necessary.

10. Toxicokinetics - OECD 417 -
Study of the absorption, distribution, excretion and metabolism of a substance.
Selected species: rodents, in particular the rat.

11. Teratogenesis, reproduction, carcinogenesis and additional genetic toxicity

Teratogenesis - OECD 414

Permanent functional or structural anomalies caused by administration of a substance during gestation.
Selected species: rat, mouse, hamster, rabbit

Reproduction over one generation (OECD 415) and over two generations (OECD 416)
Effects of the test substance on reproductive functioning in males and females

Reproduction tests provide preliminary information on the toxic effects of the test substance, on development, neonatal morbidity, mortality, behaviour and teratogenesis.
Selected species: rat, mouse

Carcinogenesis - OECD 451 -

Observation of test animals during most of their lives to monitor the possible development of neoplastic lesions after or during exposure to different doses of a test substance to be administered via the appropriate route.
Selected species: at least two species, rat and mouse.

Additional in vivo genetic toxicity - OECD 474-475-478-483-484-485-

Harmful (direct and indirect) effects on genetic material which are not necessarily associated with mutagenicity.
Selected species: hamster, mouse, guinea-pig

Most chemical carcinogens cause DNA lesions as well as gene and chromosome mutations.
The transformation of mutated cells into a cancerous state depends on other stages (promotion, progression) whose mechanisms are less well-known.

A **safety margin** is calculated for hair dyes, preservatives and sun filters, taking into account the no adverse effect level dose obtained on the basis of toxicity studies at appropriate repeated doses, percutaneous absorption data and exposure data resulting from normal and intensive use communicated by COLIPA.

1.2 Cosmetic ingredients subject to notification in accordance with the 7th amendment to Dangerous Substances Directive 92/32/EEC

Although the Dangerous Substances Directive does not apply to cosmetic products in their finished state, any "substance" placed on the market after 18.9.1981 intended (notably) to be included in a cosmetic product, must be notified to the competent national authority of the Member State in which it is produced or imported into the EEC, in line with the provisions of Directive 92/32/EEC.
The toxicity data required for notification depend on annual tonnage in the EC.
The test procedures are described in Directives 87/302/EEC and 92/69/EEC and in the OECD monographs.

2 EUROPEAN DEFINITION OF COSMETIC PRODUCTS AND CONCEPT OF "RISK"

It is useful to remember why cosmetic products which seem harmless may imperil human health.

Cosmetic products are defined in Article 1 of Directive 93/35/EEC:

"A "cosmetic product" shall mean any substance or preparation intended to be placed in contact with the various external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance and/or correcting body odours and/or protecting them or keeping them in good condition."

This definition of cosmetic products covers a very wide range of products, including:

- classical make-up products
- perfumes
- products for use on the hair
- hygiene and toilet products (including soap, toothpaste, pre-shave and after-shave products)
- "natural" cosmetic products.

and various products which protect the skin or mucous membrane and keep them in good condition, such as sun creams, baby creams, certain anti-dandruff shampoos, wart removers, moisteners, deodorants, cariostatic toothpastes, etc.

The concept of risk to human health is associated with intimate (skin, mucous membrane) and continued contact with these products and with chronic exposure (consequence of

frequent and usually daily usage, extending over a major part of a person's life) of all categories of consumers.

Consequently, apart from the immediate and invisible adverse effects of cutaneous intolerance, **we cannot a priori rule out systemic effects involving action via the blood stream after percutaneous absorption or long-term systemic or cutaneous effects**.

The potential systemic risk justifies the performance of prospective sub-chronic toxicity and mutagenicity tests and, possibly, tests of toxicokinetics, teratogenesis/reproduction, additional genetic toxicology and carcinogenesis tests for certain ingredients, as well as calculating a safety margin for human use.

D. THE ACTORS

The actors responsible for promoting research, development and validation of alternative methods are active at Community and international level, viz. the Commission, European industry and international relations.

1 THE COMMISSION

Several Commission departments deal with animal tests and work together towards realising the three Rs – replacement, reduction, refinement.

The Commission subsidises all or part of several "ad hoc" studies conducted by university and industrial laboratories and by scientific companies.

1.1 The Consumer Policy Service (CPS)

The Consumer Policy Service, whose remit includes cosmetic products, has close and regular links with the Directorates-General for Industry (DG III), the Environment (DG XI), Agriculture (DG VI), Employment, Industrial Relations and Social Affairs (DG V) and the Joint Research Centre (DG XII).

The CPS manages the Cosmetic Products Directive and is advised by the Scientific Committee on Cosmetology (SCC). The SCC is one of the Commission's advisory committees and was created by Commission decision of 19 December 1977*. Members include experts in the field of toxicology, pharmacology, biology and dermatology, and it is responsible for evaluating the safety of cosmetic products for human use and notably evaluating the toxicity of cosmetic ingredients listed in the annexes to Directive 76/768/EEC.

The CPS has requested the SCC to issue, before 1 January 1997, the final date for a possible report on the application of the provisions of Directive 93/35/EEC, **a scientific opinion on the applicability of validated alternative methods in the process of evaluating the safety of cosmetic products**.

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* OJ 13, 17.1.1978, page 24

In the context of this mandate, a subgroup on guidelines and alternative methods was set up by the SCC and is responsible for:

- actively monitoring the validation studies coordinated by ECVAM and other centres
- adopting methods validated by the OECD
- liaising with experts from industry involving dialogue at the highest scientific level in order to learn more about the basic biochemical mechanisms of in vitro methods and obtaining a handle to interpret the results, with a view to preparing an objective and solidly reasoned opinion addressed to the Commission
- assessing the applicability of validated alternative methods to evaluating the safety of cosmetic ingredients.
- updating the guidelines for evaluating the safety of cosmetic ingredients.

1.2 ECVAM

The European Centre for the Validation of Alternative Methods (ECVAM) was set up by the Commission under Decision EC/90/67. It is attached to the Joint Research Centre at Ispra and uses its technical and scientific infrastructure. Its task is:

- to coordinate the validation of alternative methods at Community level
- to pool information
- to develop and manage a database on AM
- to encourage dialogue between all operators concerned.

In implementing the sixth amendment to the Cosmetic Products Directive, ECVAM works together with the CPS, the European cosmetic industry and research laboratories specialised in the field of alternative methods.

The CPS and ECVAM organised a joint workshop at Ispra (11-13 April 1994) on the use of AM in implementing the sixth amendment to Directive 76/768/EEC. This workshop made it possible to focus more clearly on the problems and to identify areas in which action was desirable.

2 EUROPEAN INDUSTRY

European industry devoted ECU 25 million during 1993 to meet the challenge presented by the sixth amendment to the Cosmetics Directive. It played a major role in the research and development of in vitro methods which made it possible to reduce or avoid the use of animals.

In 1992 the Liaison Committee of European Associations of the Perfume, Cosmetics Products and Toiletries Industries (COLIPA) set up the Steering Committee on Alternatives to Animal Testing (SCAAT).

SCAAT members include heads of R&D at several European cosmetics firms. It provides guidance to the cosmetics industry with a view to replacing animal tests by validated and approved alternative methods. In this connection it supervises and reviews COLIPA

validation studies and coordinates the validation work of the cosmetics industry throughout the world. SCAAT works together with all scientific, industrial and regulatory agencies concerned with the development, validation and implementation of alternative methods.

3 INTERNATIONAL RELATIONS

Europe, America and Japan have been working together for some time as a result of initiatives by COLIPA.

Since 1981 the CFTA (Cosmetics, Toiletry and Fragrance Association) has supported the establishment of a Centre for Alternatives to Animal Tests (CAAT) at the John Hopkins University (USA).

COLIPA works together with CTFA, ECVAM and JCLA (Japanese Cosmetic Industry Association), notably in regard to programmes to validate alternative methods to the Draize eye irritation test.

E. THE INITIATIVES

Several initiatives have been launched in the European Union by CPS/SCC, JRC/ECVAM, COLIPA/SCAAT as well as in the United States and Japan, and instruments to develop and validate alternative methods have been implemented.

1 CPS/SCC

The purpose of this report, drawn up by the Consumer Policy Service, is to inform the European Parliament and Council, of the state of progress of alternative methods and to stimulate research with a view to achieving the objectives enshrined in Directive 93/35/EEC.

The SCC subgroup on alternative methods/guidelines organised two working meetings in July and October 1994 concerning the progress of the validation studies coordinated by SCAAT/COLIPA.

2 JRC/ECVAM

ECVAM draws on the two databases GALILEO DATA BANK and INVITTOX.

GALILEO DATA BANK (GDB), a database subsidised by CPS/DG XI and JRC/ECVAM, has been set up at the University of Pisa to collect and evaluate the results of in vitro toxicity tests.

GALILEO has already evaluated 21 000 toxicity tests results conducted using alternative methods and other methods.

INVITTOX, financed by FRAME (Fund for the Replacement of Animals in Medical Experiments) and the EC, is located at Nottingham and its main function is to produce

protocols of in vitro tests. Up to now INVITTOX has collected approximately 100 protocols. An exhaustive list of these protocols is provided in annex 2.

Of the six ongoing prevalidation/validation studies involving ECVAM, those of direct interest to evaluating cosmetic products safety are:

- an EC/UK study on in vitro/eye irritation
- a COLIPA/EC study on in vitro photoirritation/phototoxicity
- a European/US study on in vitro skin corrosion.

Among the subjects of the 15 working meetings scheduled by ECVAM in 1993/94, those of direct relevance to evaluating the safety of cosmetic products are:

- in vitro phototoxicity
- in vitro teratogenicity
- in vitro acute toxicity
- in vitro cutaneous penetration
- the challenge of the sixth amendment to the Cosmetics Directive (11-13 April 1994).

The specific responsibilities of ECVAM (coordinating the validation of AM) and the Scientific Committee on Cosmetology (evaluating the safety of cosmetic products) were defined at the CPS/ECVAM workshop of 11-13 April 1994.

However, no definitive agreement was reached as regards the use of alternative methods in implementing the sixth amendment to Directive 76/768/EEC.

3 COLIPA/SCAAT

SCAAT liaises directly with the Commission and Member States.

The European cosmetics industry has been involved for a number of years in numerous programmes for developing and optimising alternative methods. Such methods have already been applied in industrial research during pilot phases in connection with the development of new substances and are also used to evaluate finished products.

Two validation programmes have been initiated by SCAAT (photoirritation and eye irritation) and working parties have been given the task of developing methods in the fields of skin tolerance in man and percutaneous absorption.

4 USA/JAPAN

CFTA, in the context of its programme for evaluating analytical methodologies, and JCLA, in cooperation with the Japanese government, are participating in the new programme for validating alternative methods to the Draize eye irritation test, coordinated by COLIPA.

The Food and Drug Administration encourages the development of alternative methods **but points out that no alternative test has yet been accepted by the entire scientific community to replace the Draize test** and concludes that it is unlikely that animal tests can be completely dispensed with in the near future.

F. STATE OF PLAY

The state of play may be summarised as follows:

1 IN VITRO METHODS CURRENTLY BEING DEVELOPED AND VALIDATED

Photoirritation/phototoxicity: validation (COLIPA/DG XI/ECVAM)

There are no in vivo phototoxicity tests in the OECD guidelines.

The first experimental phase, focusing on biochemical tests and a standardised cytotoxicity test, has been concluded.

The results make it possible to identify a limiting factor distinguishing phototoxic substances from non-phototoxic substances.

Correlation with in vivo data in the literature (man/animal) is excellent.

The second phase comprises a validation study of two cell tests and a prevalidation study of eight other tests. The results of the validation involving a blind study coordinated by ECVAM are due in late 1994.

Percutaneous absorption: prevalidation (COLIPA/ECVAM)

A standardised Franz cell diffusion method using the freshly excised, shaved and thinned skin of the pig is currently being practised in numerous laboratories on cosmetic ingredients and on finished products (particulary on hair dyes).

The method was proposed to the OECD. It has been validated through reproducible intra- and interlaboratory results showing a good in vitro/in vivo correlation for several substances. It can replace in vivo cutaneous penetration tests but tells us nothing about the metabolism and toxicokinetics.

This autumn SCAAT will examine ongoing experimental work on the use of standard protocols for specific groups of substances tested.

Eye irritation: validation (COLIPA)

This programme supplements the international validation exercise on alternative methods to the Draize eye irritation test for classifying and labelling chemical substances (EC DG XI/UK Home Office), illustrating the difficulties of validation studies.

The purpose is to validate 10 in vitro tests on a sample of 23 cosmetic ingredients and 32 formulations chosen to cover a range of potential irritants.
The experimental phase ends in July 1995. The results of the final report will be available in September 1995.

Although sampling is not enough to cover the entire cosmetic universe (in particular, ingredients covered by Directive 76/768/EEC), the study should cast light on certain classes of products.

Skin sensitisation: development (DG XII - BRIDGE)

It is too early to develop a validation programme, because of the complex mechanisms of the sensitisation process. A project for developing an in vitro test for detecting sensitising substances was launched in 1991 (DG XII).

Acute toxicity: development (EC/FRAME)

The methods developed on cell cultures are useful for determining test doses for tests on the entire animal and contribute to reducing the number of animals tested. Most of the biological consequences are quantitative for a given group of substances and make it possible to posit a lowest effective dose (LED). No conclusive results have been obtained from the validation programmes developed to date.

Skin irritation: development (UK/France/Germany/USA/EVCAM)

In vitro approaches, using cell lines of skin tissue and abiotic systems, have been developed with mixed success. Adequate evaluation depends on the availability of an extensive in vitro database and a database on in vivo irritation.

In view of the current state of research, and the difficulty of validation exercises, in vitro methods cannot yet replace tests on the live animal.

Adequate validation of the methods is the key to their legal acceptance.

Difficulties involved in validation studies

Validation studies are costly and time-consuming.

As regards alternative in vitro tests, it is difficult to draw conclusions on the performance of the individual tests. The performance of the test systems varies with the substances and the groups of products tested and it is impossible to extrapolate the results of a validation exercise to all types of substances and products.

The mechanistic nature of in vitro tests effectively makes it essential to identify and validate an optimised battery of supplementary tests to mimic an in vivo toxic effect.

The numerous validation exercises associated with in vitro eye irritation tests illustrate these problems.

A survey has been conducted of a total of 31 studies using 30 protocols with 41 biological consequences in tests involving between 10 and 465 substances and formulations.

Of these studies, the most recent was developed by EC – DG XI/UK – Home Office and involves 37 laboratories, 60 test substances and nine protocols at a total cost of 1 361 000 ecus.

The nine tests were selected because they satisfied the following criteria:

- specific, well-defined and supplementary objectives
- developed, standardised protocols, appropriately documented
- promising results in one or several interlaboratory studies
- the possibility of being used on a routine basis.

The substances to be tested were selected to represent different ranges of chemical functions, irritant potentials, physical states and physico-chemical properties.

The 37 laboratories selected on the basis of their experience are located in nine countries including the USA and Japan.

An independent agency, BIBRA, has been commissioned to collect and distribute the substances to be tested and to receive the results.

The tests will be evaluated to determine their performance, individually and in combination.

The results will be published in January 1995 and ECVAM will organise a meeting with a view to a final discussion in September 1995.

2 LEGAL ACCEPTANCE: OECD

Legal acceptance of alternative methods using a limited number of animals

Acute toxicity, oral route

Two methods contribute to reducing the number of test animals and the sufferings inflicted on them as compared with the conventional method described in the OECD guidelines 401 (see page 5).

Fixed dose method – OECD 420

The in vivo fixed dose method proposed by the British Toxicology Society restricting the number of test animals and reducing animal suffering was accepted by the OECD [3] after an international validation study showed that it was scientifically valid for the values LD < 25 mg/kg, 25 to 200 mg/kg, 200 to 2000 mg/kg and > 2000 mg/kg. It has been approved with the preliminary screening test which provides information on the dose to be applied in the main study (OECD, 7.1.1992).

Acute toxic class method (ACT)

ACT is based on a step by step procedure, using the minimum number of animals, and provides sufficient information on the acute toxicity of the test substance for it to be classified in accordance with the current schedules. Although the animal's death is the main biological consequence, this procedure reduces the number of test animals and alleviates suffering. ACT is in the final acceptance stage after international validation under the patronage of the OECD.

Skin sensitisation

LLNA and MEST tests

The local lymphatic test (LLNA) and the mouse ear oedema test (MEST) can identify moderate to strong sensitising potential.

OECD recommends using these tests as a first stage in evaluating a sensitising potential. If a positive result is obtained in one of these tests, it is not necessary to continue with in vivo tests.

Legal acceptance of in vitro methods

The domains in which in vitro methods have been legally accepted are essentially restricted to mutagenicity/genotoxicity data (basic tests) and screening tests with a view to studying severe irritation of the skin and eyes.

Mutagenicity/genotoxicity

On 26 May 1994 the OECD distributed a draft review of eight short-term mutagenicity/genotoxicity tests, of which three in vitro:

- **back mutation test on bacteria**
- **chromosome aberration test on in vitro mammal cells**
- **gene mutation test on in vitro mammal cells.**

The combination of these three tests makes it possible to identify mutagenic potential and/or carcinogenic potential via a genotoxic mechanism.

Use of in vivo tests can be limited to certain cases, to verify whether an activity observed in vitro is also expressed in vivo.

Skin and eye irritation

The OECD guidelines, revised in 1992, recommend adoption of a hierarchical approach to eliminate severe irritants to the skin and eyes by considering the physico-chemical properties, pH values and data obtained from in vitro tests. The latter are not defined.

Since then animals have only been used to prove the absence of irritation or to define light to moderate potential.

G. ANIMAL TESTS: STATISTICS

1. **It is not yet possible to provide a realistic estimate of the number of animals used to test the toxicity of cosmetic ingredients**, as required by Article 4 of **Directive 93/35/EEC.**

   The data available through Member States mainly concern finished cosmetic products, including body hygiene products.

   On the basis of partial information communicated by the Member States under Articles 13 and 26 of Directive 86/609/EEC on the number of animals used for experimental purposes or other scientific purposes during 1991, no monkeys, cats or dogs were used to test cosmetics, and the numbers of rodents and rabbits used to test cosmetics and body hygiene products, compared with the total number of animals of the same species used in toxicity tests in the field of human, animal and environmental protection, are:

   248 to 25 994 in the Netherlands
   2 028 to 19 468 in Spain
   22 880 to 89 620 in France
   3 082 to 171 530 in the United Kingdom.

   However, these data may not be as revealing as one might think, since:

   - toxicity tests for American and Japanese companies are mainly conducted in France, the United Kingdom and Germany
   - most of the tests were performed on finished products or for internal monitoring purposes
   - the statistical data furnished by the Member States are incomplete.

2. **On the other hand, an estimated 38 900 laboratory animals were used to test the 43 cosmetic ingredients evaluated in 1993 by the Scientific Committee on Cosmetology; these tests last on average ten years; this number corresponds to approximately 900 animals per cosmetic ingredient or less than 100 animals per ingredient per year.**

Number of laboratory animals used to evaluate the safety of cosmetic ingredients, calculated on the basis of the dossiers examined by the Scientific Committee on Cosmetology in 1993						
43 cosmetic ingredients	Rats	Mice	Guinea pigs	Rabbits	Hamsters	Total
5 preservatives 8 UV filters 30 hair dyes	21 000	14 500	2 600	700	100	38 900

The studies were conducted in Europe, except for most of the long-term carcinogenicity studies (which require a greater number of animals) developed in the USA under the National Toxicology Program.

The figure of 38 900 relates to the number of rodents and rabbits used to test cosmetic ingredients and is extremely low by comparison with the total number of animals used in all sectors (11 790 485) (Directive 86/609/EEC).

H. PROGRESS: STATUS – OCTOBER 1994

CLASSICAL IN VIVO METHODS		ALTERNATIVE METHODS: toxic effect	Coordination/ Support/ Cooperation	End of Study	Screening and strategy of the three Rs
OECD 406 (1992)	DEVELOPMENT	**– Cutaneous sensitisation** Predicative in vitro test to detect sensitising components	DG XII (BRIDGE)	1994	Screening
OECD 404 (1992)		**– Skin irritation** In vitro prevalidation tests of cutaneous agressiveness (3 tests, 7 laboratories, 50 test materials)	UK / France / Germany USA / ECVAM	1994	Screening / Reduction
OECD 401 (1987)		**– Acute toxicity** Utilisation of a battery of in vitro tests to predict acute lethal potential	EC / FRAME		Screening / Reduction
OECD (draft 1994)		**– Percutaneous absorption** Prevalidation of standardised in vitro protocols for specific groups of substances	COLIPA / ECVAM		Replacement
		– Photomutagenicity	COLIPA		Screening
OECD 405 (1987)	VALIDATION	**– Eye irritation**			
		1) Alternatives of AM to the Draize test with a view to classifying and labelling chemical substances (9 tests, 37 laboratories, 60 test materials)	EC DG XI / UK Home Office	September 1994	Replacement / Reduction
		2) Validation of AM to Draize tests on a sample of cosmetic products (10 tests, 55 test materials)	COLIPA	July 1995	Replacement / Reduction
		– Phototoxicity/photoirritation Validation by blind test (second phase), (2 tests, 32 test materials)	EC DG XI / COLIPA / ECVAM	end 1994	Replacement
LEGAL ACCEPTANCE – OECD		**– Acute toxicity** Fixed dose OECD 420 (1992) ATC OECD (draft 1994)			Reduction / Refining
		– Mutagenicity/genotoxicity OECD 471. 472, 473, 476 (draft 1994)			Screening / Refining

I. CONCLUSIONS

Since the adoption of Directive 93/35/EEC, an enormous amount of work has been put in by the Commission and the European cosmetics industry, which had already been working towards this end for a number of years, and close cooperation has been established with the USA and Japan with an eye to improving animal welfare.

The state of play in October 1994 is summarised in Table H. It should be noted, however, that:

A. Up to now, **no** conclusions can be drawn from the numerous validation studies conducted in 1993 which would have **an immediate influence on the implementation of the sixth amendment to Directive 76/768/EEC**.

To ensure consumer protection, Directive 76/768/EEC and its annexes prohibits the use of toxic substances in finished cosmetic products, lays down specific limits for the use of certain ingredients and provides for a system of positive lists for certain categories of ingredients particularly liable to imperil human health; in the context of positive lists, only those ingredients listed may be used. Before being included in the annexes to the Directive, the substances and ingredients considered must first be analysed by the SCC in regard to their potential toxic effects.

The results obtained in the development and validation of batteries of in vitro eye irritation tests concern specific chemical families and certain groups of finished products; there is no conclusive evidence that these results can be extrapolated to the evaluation of ingredients belonging to different structural classes and functional groups – notably to the ingredients likely to be included in the positive lists, viz. preservatives, colouring agents and UV filters (hair dyes).

Barring some exceptions, the cosmetic ingredients belonging to these categories and certain cosmetologically "active" substances have not been included in the validation studies. The 57 studies covered include only 15 of the 723 cosmetic ingredients so far listed in the annexes to Directive 76/768/EEC.

B. **The interesting but limited findings to date mean that animal models cannot be replaced, though they can contribute to reducing the number of animals used in subsequent studies.**

C. **COLIPA envisages using clinical data** rather than animal tests to test skin irritation potential in finished products after elimination of the severe irritants on the basis of an initial and suitable in vitro approach.

D. Application of in vitro methods to evaluating the safety of "house" products carried out by leading European firms clearly shows the different approaches in regard to ingredients and finished products.

The findings to date shows that it is too early to replace animal methods by alternative methods in testing the ingredients, although the finished products can be generally tested in vitro thanks to existing knowledge of toxicity data concerning the ingredients.

It is interesting to note that the STOA report[1] (Scientific and Technological Options Assessment) drawn up on the basis of a proposal by the Environment Committee of the European Parliament comes to practically the same conclusions. This study, intended to assess the possibility of totally eliminating the use of laboratory animals in cosmetic product toxicity tests, describes the situation clearly. It is divided into two sections: the first, based on a questionnaire drawn up by STOA, analyses industry practice in Europe, the USA and Japan, while the second reviews the literature relating to animal tests used for cosmetic products and on recently developed alternative methods.

With regard to the future, the situation may be summarised as follows:

1 THE OUTLOOK

One can reasonably hope that:

1.1 **In vitro alternative methods will be able to replace animal models in the near future, while affording consumers a level of protection equal to that associated with animal tests, in the fields of:**

- **eye irritation**
- **percutaneous absorption**
- **mutagenicity (basic tests)**
- **phototoxicity/photoirritation.**

With the exception of the mutagenicity tests already adopted, **their legal acceptance cannot be assured until the difficulties and unknowns in the validation and evaluation exercises referred to earlier on have been removed.**

1.2 Finished products can rapidly be tested exclusively in vitro thanks to existing knowledge on the toxicity of ingredients.

2 FORESEEABLE DIFFICULTIES

Research on alternative methodologies is considerably limited in toxicity studies, which are indispensable for evaluating the systemic risk. This is because of the difficulty of integrating in vivo or ex vivo the numerous complex interfering biochemical mechanisms which characterise life processes in the higher animal and in man.

Given the current state of the art, it is unlikely that animal tests can be totally replaced in:

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[1] "An assessment of current scientific developments in the field of non-animal testing for cosmetic products" Final report, 98 pp. Ed. V. Zuang, 1994, PE 164-906.

- studies of **acute lethal toxicity**
- studies of **chronic and subchronic toxicity**, whose objective is to determine the toxicological profile of the substance studied and the no adverse effect level
- **toxicokinetics** studies
- **carcinogenesis** studies
- **reproduction/teratogenesis** studies.
- **sensitisation** studies

However, in vitro studies developed in these domains give us a better picture of the action mechanisms relating to a metabolic process in man or in the animal, or a toxic effect identified in animals, and **contribute to reducing the number of test animals**.

3 PROPOSALS FOR THE FUTURE

In the context of implementing Directive 93/35/EEC it would be important to undertake the following future measures, the need for which was stressed at the joint ECVAM/CPS workshop:

- Verify whether studies currently being developed and validated can be applied to a greater number of different substances and, notably, ingredients regulated by the Cosmetics Products Directive
- Select, from among the cosmetic ingredients whose safety has been evaluated by the SCC, a group of substances whose in vivo toxicity data are pertinent to an in vitro/in vivo correlation exercise
- Optimise exploitation of databases of tests and implement an adequate and coordinated system for providing information by creating a cosmetic products database.

The Commission is convinced that research into alternative methodologies intended to replace live animals will continue in parallel with methods to reduce animal suffering and/or contributing to reducing the number of test animals and that ECVAM will be able to expedite the process of legal adoption of methods validated by the OECD.

It hopes that this report will stimulate all operators concerned and encourage the adoption of alternative methods in a number of areas.

Annexe I

Validation: the Amden principles

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1. The purpose of a validation study should be fully defined, particularly in relation to the level of assessment (toxic potential, toxic potency, hazard or risk), and in relation to the type of test required (screening, adjunct or replacement), the type of toxicity to be evaluated, and the chemical spectrum of interest.
2. Tests should only be considered for inclusion in validation studies, if the specific purposes for which they have been developed are well defined and are consistent with the overall objectives of the validation study.
3. Tests must have been adequately developed, standardised and documented and a need for them in relation to the availability of other tests must exist, before they should be considered eligible for validation.
4. Various sets of reference chemicals are required for the validation process, namely:
   Reference set 1: a calibration set for use in test development and interlaboratory assessment;
   Reference set 2: an interlaboratory reference set for use in the blind trial phase of interlaboratory assessment;
   Reference set 3: an interlaboratory reference set for use in the definitive phase of interlaboratory assessment, and
   Reference set 4: a database reference set for use in test database development.
5. The four sets of chemicals selected for validation studies should form the basis of a Chemical Reference bank, to facilitate the provision of chemicals and reference data for the validation of tests internationally. The establishment of an International Chemicals Reference Bank is a matter of urgency. The bank should provide open-access listings of scientifically-selected chemicals, backed by toxicological data reviews, safety advice and a source of chemicals of known purity and stability.
6. Any particular test should be validated against the most appropriate collection of reference chemicals, bearing in mind the specific purpose for which the test is proposed.
7. The toxicological classification of reference chemicals in terms of their toxic properties should be carried out by a panel of expert toxicologists, taking into account all the available relevant data. Final classifications should be fully documented with respect to both acceptable data and criteria, and the statistical evaluation procedure used.
8. When classifying reference chemicals for use in validation studies, consideration should be given to the numerous factors which affect the generation and quality of reference data and the expression and evaluation of toxicity in the animal investigated.
9. It is highly desirable that industry should play an active role in validation, specifically by supplying data not generally available to the scientific community at this time.
10. Schemes currently being developed for assessing human toxicology data and making them more readily available to validation studies, should be welcomed and supported.
11. Information toxicologists should be encouraged to investigate ways of integrating the information obtained in experimental toxicology, human toxicology and veterinary toxicology, and incorporating this data into the validation process for reference classification of chemicals, whenever this feasible.
12. Wherever it is desirable and practicable, methods for the collection, collation, evaluation and expression of experimental, human and veterinary toxicological data should be standardised in ways which have been agreed upon by, and are acceptable to, the toxicological community as a whole.

LIST OF INVITTOX METHODS

- RABBIT ARTICULAR CHONDROCYTE FUNCTIONAL TOXICITY TEST
- SIRC CYTOTOXICITY TEST
- V79 CYTOTOXICITY TEST FOR MEMBRANE DAMAGE
- LS-L929 CYTOTOXICITY TEST
- RED BLOOD CELL TEST SYSTEM
- AUTOMATED IN VITRO DERMAL ABSORPTION (AIDA) PROCEDURE
- QUANTITATIVE VIDEO MICROSCOPY OF INTRACELLULAR MOTION AND MITOCHONDRIA - SPECIFIC FLUORESCENCE
- LLC-RK_1 CELL SCREENING TEST FOR NEPHROTOXICITY
- HEp-2 CYTOTOXICITY TEST FOR IMPLANT MATERIALS
- H-4-II-E RAT HEPATOMA CELL BIOASSAY
- LUNG CELL ASSAY
- HET-CAM TEST
- THE FRAME MODIFIED PHOTOTOXICITY ASSAY USING HUMAN KERATINOCYTE MONOLAYER CULTURES
- FIXED DOSE PROCEDURE FOR THE FLUORESCEIN LEAKAGE TEST
- THE FRAME CYTOTOXICITY TEST (KENACID BLUE)
- THE POLLEN TUBE GROWTH TEST (PTG-TEST)
- HEN'S EGG TEST - YOLK-SAC BLOOD VESSEL ASSAY
- HUMAN AND BOVINE LENS EPITHELIAL CULTURE
- UV ABSORPTION AS AN APPROXIMATION FOR CELL NUMBER
- EYE LENS ORGAN CULTURE
- AN IN VITRO MODEL FOR STUDIES OF PROSTAGLANDIN H SYNTHASE (PHS)-MEDIATED
- GENOTOXICITY OF XENOBIOTICS
- SCREENING SYSTEM OF PROMOTERS USING RAS TRANSFECTED BALB 3T3 CLONE (BHAS 42)
- THE NEUTRAL RED CYTOTOXICITY ASSAY
- LUCIFER YELLOW INTERCELLULAR EXCHANGE ASSAY FOR TUMOUR PROMOTERS
- SERUM-FREE LIVER MITOGEN TEST
- EMBRYOTOXICITY TESTING USING A WHOLE-EMBRYO CULTURE (W.E.C.) PROCEDURE
- PRIMARY HUMAN HEPATOCYTE CULTURES FROM SMALL SURGICAL BIOPSIES
- THE ISOLATED PIG-EAR SKIN PERMEATION MODEL
- THE FLUORESCEIN LEAKAGE TEST
- RAT WHOLE EMBRYO CULTURE
- TWO-COMPARTMENT HUMAN TISSUE CYTOTOXICITY TEST
- TETRAHYMENA THERMOPHILA CHEMOSENSORY RESPONSE
- TETRAHYMENA PROLIFERATION RATE AND MAXIMAL DENSITY
- NEUTRAL RED BIOASSAY USING BALB/c3T3 CELLS
- BLIND TRIAL PROTOCOL
- GAP JUNCTIONAL INTERCELLULAR COMMUNICATION ASSAY
- TETRAHYMENA ASSAY FOR MEMBRANE-STABILISING ACTIVITY
- THE COMPLEMENT PHOTOACTIVATION ASSAY
- 3T3 NRU PHOTOTOXICITY ASSAY
- ATS $SKIN^2$ ZK 1350 PHOTOTOXICITY ASSAY
- SOLATEX-PI PHOTOTOXICITY TEST
- CHICKEN ENUCLEATED EYE TEST (CEET)
- THE RABBIT ENUCLEATED EYE TEST
- TRANS-EPITHELIAL PERMEABILITY (TEP) ASSAY

- ARACHIDONIC ACID RELEASE AS A MEASURE OF MEMBRANE TOXICITY
- PHOTOBINDING TO PROTEIN
- PHOTOSENSITIZED OXIDATION OF HISTIDINE
- SPONTANEAOUSLY CONTRACTING CULTURED RAT SKELETAL MUSCLE CELLS FOR TESTING TOXIC EFFECTS ON EXCITABLE TISSUES
- CULTURE OF HUMAN CUMULUS GRANULOSA CELLS
- METHYL GLUCOSE UPTAKE IN PRIMARY CULTURES OF PROXIMAL TUBULAR CELLS
- METHYL GLUCOSE UPTAKE IN ISOLATED PROXIMAL TUBULAR CELLS
- ISOLATION OF RAT TYPE II ALVEOLAR EPITHELIAL CELLS
- POLYMORPHONUCLEAR LEUKOCYTES LOCOMOTION
- CYTOSKELETAL ALTERATIONS AS A PARAMETER FOR ASSESSMENT OF TOXICITY
- THE AMES TEST
- MODEL CAVITY METHOD
- DNA BINDING STUDIES FOR ALKYLATING COMPOUNDS USING ISOLATED PERFUSED RAT LIVER
- DNA BINDING IN BACTERIA
- RED BLOOD CELL LYSIS AND PROTEIN DENATURATION
- THE BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY
- THE SILICON MICROPHYSIOMETER TOXICITY TEST
- THE HEN'S EGG TEST ON THE CHORIOALLANTOIC MEMBRANE (HET-CAM) BIS
- THE HEN'S EGG TEST ON THE CHORIOALLANTOIC MEMBRANE (HET-CAM)
- BOVINE ISOLATED CORNEA TEST
- RABBIT ISOLATED TERMINAL ILEUM
- REACTIVE METABOLITE FORMATION BY FORTIFIED LIVER MICROSOMES
- WHOLE RAT BRAIN REAGGREGATE CULTURE
- HEPATOMA CELL CULTURES AS IN VITRO MODELS FOR HEPATOTOXICITY
- HEL 30 CYTOTOXICITY TEST
- CYTOTOXICITY AND GENOTOXICITY IN PRIMARY CULTURES OF HUMAN HEPATOCYTES
- MTT ASSAY
- UNSCHEDULED DNA SYNTHESIS IN HEPATOCYTE CULTURES
- ASSESSED BY THE NUCLEI PROCEDURE
- ALKALINE UNWINDING GENOTIXICITY TEST
- ISOLATION OF RAT HEPATOCYTES
- BOVINE SPERMATOZOA CYTOTOXICITY TEST
- TETRAHYMENA THERMOPHILA OCULAR IRRITANCY TEST
- RAT HEPATOCYTE FLOW CYTOMETRIC CYTOTOXICITY TEST
- LASER DIFFRACTION MEASUREMENT OF TUMOR SPHEROIDS
- THE ZEIN TEST
- HUMAN SKIN FIBROBLAST/COLLAGEN LATTICE CYTOTOXICITY TEST
- HUMAN OESOPHAGEAL CULTURE
- HUMAN THYROID CULTURE
- AGAROSE OVERLAY ASSAY
- DUST TOXICITY IN RAT ALVEOLAR MACROPHAGE CULTURES
- YEAST GROWTH RATE CYTOTOXICITY TEST
- YEAST PLASMA MEMBRANE H+-ATPASE TOXICITY TEST
- CHINESE HAMSTER OVARY CELL NA+/K+ - ATPASE TEST
- CHINESE HAMSTER OVARY (CHO) CELL PROLIFERATION TEST
- ISOLATED RAT GLOMERULI AND PROXIMAL TUBULES
- HUMAN LYMPHOCYTE CYTOTOXICITY ASSAY
- THE ISOLATION AND CULTURE OF RAT HEPATIC CELLS
- THE USE OF MEMBRANE PERMEABILITY AS A MEASURE OF CYTOTOXICITY IN PERFUSED CELL CULTURES