the filtered solar simulator used in the validation study of the *in vitro* 3T3 NRU phototoxicity test has been published (3).

26. *Dosimetry*: The intensity of light (irradiance) should be regularly checked before each phototoxicity test, by using a suitable broadband UV-meter. The UV-meter must have been calibrated to the source. The performance of the UV-meter should be checked, and for this purpose, the use of a second, reference UV-meter of the same type and identical calibration is recommended. Ideally, at greater intervals, a spectroradiometer should be used to measure the spectral irradiance of the filtered light source and to check the calibration of the broadband UV-meter, but such instruments require skilled operation by appropriately trained persons.

A dose of 5 J/cm² (UVA) was determined in the validation study to be non-cytotoxic to Balb/c 3T3 cells and sufficiently potent to excite even weak phototoxic chemicals. To achieve 5 J/cm² within a time period of 50 min, irradiance has to be adjusted to 1.666 mW/cm². If another cell line or a different light source are used, the UVA dose may have to be slightly adapted, by using the criteria of being non-deleterious to the cells and sufficient to detect standard phototoxins. The time of light exposure is calculated in the following way:

$$t(\min) = \frac{\text{irradiation dose}\ (J/cm^2) \times 1000}{\text{irradiance}\ (mW/cm^2) \times 60} \qquad (1\ J = 1\ Wsec)$$

Test conditions

Test chemical concentrations

27. The maximum concentration of a test chemical should not exceed 100 µg/mL, since all phototoxic chemicals were detected at lower concentrations, whereas at higher concentrations the incidence of false positives (overpredictions) increases (13). The pH of the highest concentration of the test chemical should be satisfactory (pH range: 6.5 - 7.8).

28. The ranges of concentrations of a chemical tested in the presence (+UVA) and in the absence (-UVA) of light should be adequately determined in preceding range-finder experiments. Range and intercept of a concentration series shall be adjusted in such a way that concentration-response curves are sufficiently supported by experimental data. Geometric concentration series (with a constant dilution factor) should be used.

Controls

29. *UVA sensitivity of the cells, historical data:* Cells should be regularly checked for sensitivity to UVA. Cells are seeded at the density used in the *in vitro* 3T3 NRU phototoxicity test, irradiated the next day with UVA doses from 1-9 J/cm², and cell viability is determined one day later using the NRU assay. Cells meet the quality criteria, if their viability after irradiation with 5 J/cm² UVA is not less than 80% of the viability of dark controls. At the highest UVA dose of 9 J/cm², viability should not be less than 50% of that of dark controls. This check should be repeated about every 10 th passage of the cells.

30. *UVA sensitivity of the negative control cells, current test:* The test meets the quality criteria if negative controls (cells in EBSS with or without 1% DMSO or 1% ETOH) in the +UVA experiment show a viability of not less than 80% of that of non-irradiated cells in the same solvent of the concurrent dark experiment (-UVA).

31. *Viability of negative controls:* The absolute optical density ($OD_{540\ NRU}$) measured in the NR extract of the negative controls indicates whether the 1×10^4 cells seeded per well have grown

with normal doubling time during the two days of the assay. A test meets the acceptance criteria if the mean $OD_{540\ NRU}$ of untreated controls is ≥ 0.2.

32. *Positive control:* A known phototoxic chemical shall be tested concurrently with each *in vitro* 3T3 NRU phototoxicity test. Chlorpromazine (CPZ) was used as positive control in the EU/COLIPA validation study and is therefore recommended. For CPZ tested with the standard protocol in the *in vitro* 3T3 NRU phototoxicity test, the following test acceptance criteria were defined: *CPZ irradiated (+UVA)*: EC_{50}= 0.1 to 2.0 µg/mL, *CPZ non-irradiated (-UVA)*: EC_{50} = 7.0 to 90.0 µg/mL. The Photo Inhibition Factor (PIF), i.e. the shift of EC_{50} should be at least 6.

33. Other known phototoxic chemicals, suitable for the chemical class or solubility characteristics of the test chemical being evaluated, may be used as the concurrent positive controls, in place of CPZ. In this case, based on historical data, the ranges of EC_{50} values and PIF or MPE should be adequately defined as acceptance criteria for the test.

<u>Test procedure</u> [details of the validated 3T3 NRU PT test(1-3, 13)]:

1st day:

34. Prepare a cell suspension of $1x10^5$/mL in culture medium and dispense 100 µL culture medium only into the peripheral wells of a 96-well tissue culture microtiter plate (= blanks). In the remaining wells, dispense 100 µL of a cell suspension of $1x10^5$ cells/mL (= $1x10^4$ cells/well). For each test chemical, prepare two plates: one for determination of cytotoxicity (-UVA), and the other for determination of photocytotoxicity (+UVA).

35. Incubate the cells for 24 h (7.5% CO_2, 37°C) until they form a half-confluent monolayer. This incubation period allows for cell recovery and adherence, and for exponential growth.

2nd day:

36. After incubation, decant the culture medium from the cells and wash twice with 150 µL EBSS/PBS per well. Add 100 µL of EBSS/PBS containing the appropriate concentration of test chemical or just solvent (negative control). Apply 8 different concentrations of the test chemical. Incubate cells with the test chemical in the dark for 60 minutes (7.5% CO_2, 37°C).

37. To perform the +UVA part of the assay, irradiate the cells at room temperature for 50 minutes through the lid of the 96-well plate with 1.7 mW/cm^2 UVA (= 5 J/cm^2). Ventilate with a fan to prevent H_2O condensation under the lid. Keep duplicate plates (-UVA) at room temperature in a dark box for 50 min (= UVA exposure time).

38. Decant test solution and wash twice with 150 µL EBSS/PBS. Replace EBSS/PBS with culture medium and incubate (7.5% CO_2, 37 °C) overnight (18-22 h).

3rd day:

Microscopic evaluation

39. Examine the cells under a phase-contrast microscope. Record changes in morphology of the cells due to cytotoxic effects of the test chemical. This check is recommended, to exclude experimental errors, but these records are not used for evaluation of cytotoxicity or phototoxicity.

Neutral Red Uptake test

40. Wash the cells with 150 µL prewarmed EBSS/PBS. Remove the washing solution by gentle tapping. Add 100 µl NR medium and incubate at 37 °C, in a humidified atmosphere of 7.5% CO_2, for 3 h.

41. After incubation, remove the NR medium, and wash the cells with 150 µL EBSS/PBS. Decant and blot EBSS/PBS totally.(*Optionally*: centrifuge reversed plate.)

42. Add exactly 150 µL NR desorb solution (freshly prepared ethanol/acetic acid)

43. Shake microtiter plate rapidly on a microtiter plate shaker for 10 min, until the NR has been extracted from the cells and has formed a homogeneous solution.

44. Measure the optical density of NR extract at 540 nm in a spectrophotometer, using blanks as a reference. Save the data in appropriate file format (e.g. ASCII) for subsequent analysis.

DATA AND REPORTING:

Quality and quantity of data

45. The data should permit a meaningful analysis of the concentration-response obtained in the presence and in the absence of UVA/vis irradiation. If cytotoxicity is found, both the concentration range and the intercept of individual concentrations should be set in a such way as to allow the fit of a curve to experimental data. Due to the fact, that a test chemical might not be cytotoxic up to the defined limit concentration of 100 µg/mL in the dark experiment (-UVA), but highly cytotoxic when irradiated (+UVA), the concentration ranges to be tested in both parts of the experiment may need to differ by orders of magnitude to fulfil the requirement of adequate data quality. If no cytotoxicity is found in both parts of the experiment (-UVA and +UVA), testing with a great intercept between single doses up to the highest concentration is sufficient.

46. There is no requirement for verification of a clear positive result by performing a repeat experiment. In addition, clear negative results need not to be verified, provided the test chemical was tested at sufficiently high concentrations. In such cases, one main experiment, supported by one or more range-finding preliminary experiments, is sufficient.

47. Tests with borderline results near to the cut-off line of the prediction model should be repeated for verification.

48. If repeat testing is considered necessary, then variation of the experimental conditions may be important to achieve a clear result. A key variable in this test is preparation of solutions of the test chemical. Hence, variation of these conditions (co-solvent, trituration, sonication) may be most relevant in the repetition of a test. Alternatively, variation of the pre-irradiation incubation time may be considered. A shorter time can be relevant for water-unstable chemicals.

Data analysis: EC_{50}

49. Where possible, the concentration of a test chemical reflecting a 50% inhibition of the cellular NRU (EC_{50}) is determined. This can be done by applying any appropriate non-linear regression procedure (preferably a Hill function or logistic regression) to the concentration-response data, or by using other fitting procedures (14). Before using an EC_{50} for further calculations, the quality of the fit should be appropriately checked. Alternatively, graphical fitting methods can be used to calculate the EC_{50}. In this case, the use of probability paper is recommended (x-scale: log, y-scale: probit), as in many cases the concentration response function will become almost linear after this transformation.

Evaluation of results: Prediction Model Version 1 (PIF)

50. If both, in the presence (+UVA) and in the absence (-UVA) of light, complete concentration response curves are obtained, a Photo-Irritation-Factor (PIF) is calculated by means of the following formula:

$$PIF = \frac{EC_{50}(-UV)}{EC_{50}(+UV)} \quad (1)$$

A PIF < 5, predicts no phototoxic potential, whereas a PIF ≥ 5 predicts phototoxic potential.

51. If a chemical is only cytotoxic +UVA and is not cytotoxic when tested -UVA, the PIF cannot be calculated, although this is a result which indicates phototoxic potential. In such cases, a "> PIF" can be calculated if the (-UV) cytotoxicity test is performed up to the highest test concentration (C_{max}) and this value is used for calculation of the "> PIF":

$$> PIF = \frac{C_{max}(-UV)}{EC_{50}(+UV)} \quad (2)$$

If only a "> PIF" can be obtained, then any value >1 predicts phototoxic potential.

52. If both EC_{50} (-UV) and EC_{50} (+UV) cannot be calculated due to the fact that a chemical does not show any cytotoxicity up to the highest test concentration, this indicates no phototoxic potential. In such cases, a formal "PIF = *1" is used to characterise the result

$$PIF = *1 = \frac{C_{max}(-UV)}{C_{max}(-UV)} \quad (3)$$

If only a "PIF = *1" can be obtained, this predicts no phototoxic potential.

53. In cases (2) and (3), concentrations achieved in the *in vitro* 3T3 NRU phototoxicity test should be carefully taken into consideration when predicting phototoxic potential.

Evaluation of results: Prediction Model Version 2 (MPE)

54. Alternatively, a novel version of the model for predicting phototoxic potential can be applied, which has been developed by using data of the EU/COLIPA validation study (15) and tested under blind conditions in a subsequent study on the *in vitro* phototoxicity of UV filter chemicals (13). This model overcomes the limitation of the PIF model in cases where an EC_{50} cannot be obtained. The model uses the "Mean Photo Effect" (MPE), a measure which is based on comparison of the complete concentration response curves. For application of the MPE model, a special computer software was developed at the Humboldt University (Berlin, D), which can be obtained free of charge.

Interpretation of data

55. A positive result in the *in vitro* 3T3 NRU phototoxicity test (PIF ≥ 5 or MPE ≥ 0.1) indicates that the test substance has phototoxic potential. If this result is obtained at concentrations below 10 µg/mL, the test chemical is also likely to act as phototoxin also under various exposure conditions *in vivo*. If a positive result is obtained only at the highest test concentration of 100 µg/mL, further considerations may be necessary for the assessment of hazard or phototoxic potency. These may include data on penetration, absorption and possible accumulation of the chemical in the skin, or testing of the chemical in a confirmatory alternative test, e.g. using a human *in vitro* skin model.

56. A negative result from the *in vitro* 3T3 NRU phototoxicity test (PIF < 5 or MPE < 0.1) indicates that the test substance was not phototoxic to the cultured mammalian cells under the conditions used. In cases where the chemical could be tested up to the highest concentration of 100 µg/mL, a negative result indicates that the chemical has no phototoxic potential, and phototoxicity *in vivo* may be considered unlikely. In cases where identical concentration-toxicity responses (EC_{50}+UV and EC_{50}-UV) were obtained at lower concentrations, the interpretation of data would be the same. In contrast, if no toxicity was demonstrated (+UV and -UV) and if aqueous solubility limited concentrations to values less than 100 µg/mL, then compatibility of the test substance with the assay may be questioned and confirmatory testing should be considered (e.g. using an *in vitro* skin model, or an *ex vivo* skin model or an *in vivo* model).

Test Report

57. The test report must include the following information:

Test chemical:
- identification data and CAS no., if known
- physical nature and purity
- physicochemical properties relevant to conduct of the study
- stability and photostability, if known

Solvent:
- justification for choice of solvent
- solubility of the test chemical in this solvent
- percentage of solvent present in treatment medium (EBSS or PBS)

Cells:
- type and source of cells
- absence of mycoplasma
- number of cell passages, if known
- UVA sensitivity of cells, determined with the irradiation equipment used in the *in vitro* 3T3 NRU phototoxicity test

Test conditions (1); *incubation before and after treatment:*
- type and composition of culture medium
- incubation conditions (CO_2 concentration, temperature, humidity)
- duration of incubation (pre-treatment, post-treatment)

Test conditions (2); *treatment with the chemical:*
- rationale for selection of concentrations of the test chemical used both in the presence and in the absence of UV/vis irradiation
- in case of limited solubility of the test chemical and absence of cytotoxicity, rationale for the highest concentration tested
- type and composition of treatment medium (buffered salt solution)
- duration of the chemical treatment

Test conditions (3); *irradiation*:
- rationale for selection of the light source used
- spectral irradiance characteristics of the light source
- transmission / absorption characteristics of the filter(s) used
- characteristics of the radiometer and details on its calibration
- distance of the light source from the test system
- UVA irradiance at this distance, expressed in mW/cm^2
- duration of the UV/vis light exposure
- UVA dose (irradiance × time), expressed in J/cm^2
- temperature employed to cell cultures during irradiation and for cell cultures concurrently kept in the dark

Test conditions (4); *NRU test*
- composition of NR medium
- duration of NR incubation
- incubation conditions (CO_2 concentration, temperature, humidity)
- NR extraction conditions (extractant, duration)
- wavelength used for spectrophotometric reading of NR optical density
- second wavelength (reference), if used
- content of spectrophotometer blank, if used

Results
- cell viability obtained at each concentration of the test chemical, expressed in percent mean viability of controls
- concentration - response curves (test chemical concentration vs. relative cell viability), obtained in concurrent +UVA and -UVA experiments
- data analysis of the concentration response curves: if possible, computation / calculation of EC_{50} (+UVA) and EC_{50} (-UVA)
- comparison of the two concentration response curves obtained in the presence and in the absence of UVA/vis irradiation, either by calculation of the Photo Inhibition Factor (PIF), or by calculation of the Mean Photo Effect (MPE)
- classification of phototoxic potential
- test acceptance criteria (1), *concurrent* negative control:
  - absolute viability (optical density of NR extract) of irradiated and non irradiated cells
  - historical data of negative control, mean and standard deviation
- test acceptance criteria (2), *concurrent* positive control:
  - EC_{50}(+UVA) and EC_{50}(-UVA) and PIF of positive control chemical
  - historical data of positive control chemical: EC_{50}(+UVA) and EC_{50}(-UVA) and PIF, mean and standard deviation

Discussion of the results

Conclusion

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ANNEX 1

DEFINITIONS

Irradiance. the intensity of ultraviolet (UV) or visible light incident on a surface, measured in W/m^2 or mW/cm^2.

Dose of light. the quantity (= intensity × time) of ultraviolet (UV) or visible radiation incident on a surface, expressed in Joules (= W × s) per surface area, e.g. J/m^2 or J/cm^2.

UV light wavebands. The designations recommended by the CIE (Commission Internationale de L'Eclairage) are: UVA (315-400nm), UVB (280-315nm) and UVC (100-280nm). Other designations are also used: the division between UVB and UVA is often placed at 320nm, and the UVA may be divided into UV-A1 and UV-A2 with a division made at about 340nm.

Cell viability. parameter measuring total activity of a cell population (e.g. uptake of the vital dye Neutral Red into cellular lysosomes) which, depending on the endpoint measured and the test design used, correlates with the total number and / or vitality of the cells.

Relative cell viability. cell viability expressed in relation to negative (solvent) controls which have been taken through the whole test procedure (either +UV or -UV), but not treated with a test chemical.

Prediction model. an algorithm used to transform the results of a toxicity test into a prediction of toxic potential. In the present test guideline, PIF and MPE can be used for transformation of the results of the *in vitro* 3T3 NRU phototoxicity test into a prediction of phototoxic potential.

PIF (Photo Irritation Factor). a factor generated by comparing two equally effective cytotoxic concentrations (EC_{50}) of the test chemical obtained in the absence (-UV) and in the presence (+UV) of a non-cytotoxic irradiation with UVA/vis light.

MPE (Mean Photo Effect). a novel measure derived from mathematical analysis of the complete shape of two concentration response curves obtained in the absence (-UV) and in the presence (+UV) of a non-cytotoxic irradiation with UVA/vis light.

Phototoxicity. an acute toxic response that is elicited after the first exposure of skin to certain chemicals and subsequent exposure to light, or that is induced similarly by skin irradiation after the systemic administration of a chemical.

Photoirritation. a sub-species of the term 'phototoxicity', which is used to describe only those phototoxic reactions which are produced with topically applied chemicals.

Photoallergy. an acquired immunological reactivity, which does not occur on first treatment with chemical and light, and needs an induction period of one or two weeks before skin reactivity can be demonstrated.

Photogenotoxicity. a genotoxic response observed with a genetic endpoint, which is elicited after the exposure of cells to a non-genotoxic dose of UV/visible light and a non-genotoxic chemical.

Photocarcinogenicity. carcinogenicity induced by repeated application of light and a chemical. The term 'photo co-carcinogenesis', is used if UV induced tumorigenesis is enhanced by a chemical.

ANNEX 2

Role of the 3T3 NRU PT in a Sequential Approach to Phototoxicity Testing of Chemicals

§ = based on (13)

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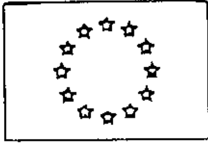
ここから5ページ分は雑誌/図書等に掲載された論文となりますので
下記の「研究成果の刊行に関する一覧表」をご参照ください。

研究成果の刊行に関する一覧表

Report on the COLIPA Workshop on Mechanisms of Eye Irritation
Leon H.Bruner, Odile de Silva, Lesley K.Earl…
ATLA 26 P.811-820 1998



EUROPEAN COMMISSION

添付資料 7

1997 COMMISSION REPORT ON THE DEVELOPMENT, VALIDATION AND LEGAL ACCEPTANCE OF ALTERNATIVE METHODS TO ANIMAL EXPERIMENTS IN THE FIELD OF COSMETICS

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A. SUMMARY

The 1997 report is the fourth annual report of the Commission on the development and validation of alternative methods to animal testing in cosmetics. The previous year represented a landmark in the field of alternative test methods in cosmetics.

Following the efforts that had been made in method development, results from the various programmes were a little disappointing in some fields. Therefore, with regret, it was necessary to postpone the implementation of the prohibition of animal testing on cosmetics from 1 January 1998 to 30 June 2000. This was due to the fact that scientifically validated alternative methods to animal testing of cosmetics were not available.

Competence for the Cosmetics Directive was transferred from DG XXIV to DG III, and the scientific committee that advises the Commission on the safety evaluation of cosmetics underwent a fundamental restructuring.

In scientific terms, some very good progress was made. The second phase of an EU/COLIPA (The European Cosmetic, Toiletry and Perfumery Association) international validation study was successfully completed, and the scientific validity of the 3T3 neutral red uptake phototoxicity (NRU PT) test was endorsed by the Commission's expert services. Also, an international validation study on skin corrosivity co-ordinated by ECVAM was successfully completed, and two test methods were judged to have been scientifically validated.

New research proposals are now under development, to continue the efforts made to date. The cosmetics industry continues to be one of the main focus points of alternative method development. The best available estimates demonstrate that approximately 35,000 animals are used in the EU each year, for the specific purposes of testing cosmetics. This is less than one percent of the estimated ten million animals used in the EU each year in experimentation. Not all of these animals are necessarily used for tests conducted to meet the requirements of the EU Cosmetics Directive 76/768/EEC; tests may also be conducted in order to satisfy the requirements of different sectoral legislation.

In spite of the relatively low number of animals used for the testing of cosmetic products, the industry has often been at the forefront of research activities in recent years. Further, the Commission services have devoted significant resources to the replacement and reduction of animal testing in the cosmetics industry.

B. INTRODUCTION

The 1997 Annual report on the development, validation and legal acceptance of alternative methods to animal experiments in the field of cosmetics is the fourth report presented by the Commission. It presents the scientific and regulatory situation in the development and validation of alternative methods in cosmetics as of December 1997.

The report is produced in order to comply with Article 4 (1) (I) of the EU Cosmetics Directive 76/768/EEC which states that,

"The Commission shall present an annual report to the European Parliament and 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 that information in addition to collecting statistics as laid down by Directive 86/609/EEC on the protection of animals used for experimental and scientific purposes. The Commission shall in particular ensure the development, validation and legal acceptance of experimental methods which do not use live animals."

The 1997 report therefore outlines the status of alternative methods at the end of the year, a year in which it was necessary for the Commission to postpone the proposed ban on the marketing of cosmetics containing ingredients and combinations of ingredients that had been tested on animals, based on expert scientific advice. The context of the postponement is discussed, and recent progress in the development and validation of alternative methods is reviewed.

Data on animal usage for the testing of cosmetic materials is presented, for the past three years. An examination of the problems associated with compilation of these data is also given. Lastly, conclusions on the current status of the development of alternative methods are given.

C. COMPETENCE FOR COSMETICS IN 1997

1997 was an important year for cosmetics legislation within the European Commission, both from an administrative and technical viewpoint. In recent years, responsibility for the cosmetics dossier resided under the competence of DG XXIV. However, early in 1997 the Commission reviewed procedures within its administration and a reorganisation in the responsibilities of its services was initiated. This was primarily to meet the needs of the Commission in differentiating those services responsible for the provision of scientific advice from those services responsible for legislation.

From 1st April 1997 the Cosmetics Directive was moved from DG XXIV to DG III (Industry), which is headed by Commissioner Bangemann. The Directive is now under the responsibility of Unit DG III/E/3 which is also the competent group for pharmaceutical legislation in Europe. DG III is the Commission service that holds competence for all legislative measures relating to the EU Cosmetics Directive 76/768/EEC including provisions relating to the use of animals in the testing of cosmetic products and ingredients. In this context, DG III is also responsible for the Annual Report on the development, validation and legal acceptance of alternative methods to animal experiments with cosmetics.

The services of DG XXIV retained responsibility for the management of the scientific committee that advises the Commission on scientific matters relating to cosmetics safety, namely the Scientific Committee on Cosmetology (SCC). Therefore, DG XXIV is now responsible for providing scientific advice on the safety of cosmetic ingredients to the Commission.

The SCC also underwent a fundamental restructuring in 1997. The Committee was originally created to meet the provisions of Commission Decision 78/45/EEC establishing a Scientific Committee on Cosmetology. The mandate of the SCC expired in October 1996, although the Committee continued to fulfil its role during the Commission's re-organisation of its scientific services.

Commission Decision 97/579/EC outlined the requirements for setting up the Scientific Committee on Cosmetic Products and Non-Food Products (SCCNFP) intended for Consumers, as one of eight new committees. The membership of the new committee, SCCNFP was published in the Official Journal on 12 November 1997. In its first Plenary meeting of 14 November 1997, the specific working party on alternatives to animal testing was established.

The SCCNFP shall, through the advice of the working group on alternatives to animal testing, act as an expert resource to the European Commission in the development and applicability of alternative methods to animal testing in cosmetics. Upon the request of the Commission services they will review data submitted on alternative methods to animal testing that have been assessed and validated by the services of the European Commission, or could be considered appropriate for the replacement of test methods using animals.

One of the most important aspects of the responsibilities of this work will be to advise the European Commission on the status of alternatives to animal testing in cosmetics on an on-going basis and in particularly, in accordance with Article 4 (1) (I) of the EU Cosmetics Directive 76/768/EEC.

D. POSTPONEMENT OF THE DATE FOR PROHIBITION OF ANIMAL TESTING FOR COSMETICS

As concluded in the 1996 report, a Directive was drawn up by the Commission which postponed the deadline of 1 January 1998 for the prohibition of animal testing of cosmetics. This Directive was proposed after extensive consultations within the services of the Commission and was required to meet the following considerations:

- The main objective of the Cosmetics Directive 76/768/EEC is to protect public health and it is therefore indispensable to carry out certain toxicological tests to evaluate the safety for human health of ingredients and combinations of ingredients used in cosmetic product formulations.
- The development, validation and acceptance of alternative methods proved to be an extremely complex scientific challenge. In particular, the timetable for the various stages of the development and validation process had previously been underestimated, as exemplified by the need for pre-validation studies.
- Progress had been made in research into alternative methods to animal testing, particularly in the end-points of percutaneous absorption, phototoxicity and local risks to the eyes and skin. However, no alternative testing methods had been scientifically validated and the OECD had not adopted guidelines for any toxicity tests using non-animal methods.

Whilst it was not possible to foresee the date by which alternative methods for testing ingredients and combinations of ingredients for risk to human health would become available for all toxic end-points, it was equally important not to excessively delay the timings for scientific reassessment of the situation. Therefore, Commission Directive 97/18, published on 1st May 1997, postponed the ban on animal testing of cosmetics and their combinations until 30 June 2000. Most importantly, the publication of this Decision in no way prejudiced the objective of reducing the number of test animals and their suffering. In this respect, the Commission committed itself to the promotion of research and validation of alternative methods.

The revised wording of Article 4 of the EU Cosmetics Directive 76/768/EEC states that Member States shall prohibit the marketing of cosmetic products containing:

"ingredients or combinations of ingredients tested on animals after 30 June 2000 in order to meet the requirements of the Directive.

If there has been insufficient progress in developing satisfactory methods to replace animal testing, and in particular to 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 2000, submit draft measures to postpone the date of implementation of this provision, for those test methods in respect of which there has been insufficient progress in developing alternative methods, in Article 10. Before submitting such measures, the Commission will consult the Scientific Committee on Cosmetology."

The situation regarding the availability of alternative methods to animal testing did not change in the meantime, indicating that the postponement, as proposed by the Commission and agreed by the Council of Ministers, was appropriate.

Following this postponement, there was a clear need for new initiatives in the field of animal testing for cosmetics.

As the experimental phases of many validation studies have drawn to a close, experts have examined the extensive databases generated in order to determine the most promising next steps. Also, a number of expert workshops have been convened in order to assess progress to date and identify promising leads from recent research programmes.

The Commission will now contribute to and closely monitor the development and regulatory acceptance of alternative methods over the next 18 months in order to determine whether the requirements of Article 4 can be met in line with the timings given i.e. whether scientifically validated alternative methods will be available by January 2000. This guidance will come from the Commission services responsible for the developments and validation of such methods, and on the basis of the advice of the SCCNFP regarding the acceptability of alternative methods to animal testing in the safety evaluation of cosmetics.

E. INITIATIVES TAKEN IN 1997

ECVAM

ECVAM continued to promote the scientific and regulatory acceptance of alternative methods which are of importance to the biosciences and which reduce, refine or replace the use of laboratory animals throughout 1997. ECVAM's major achievements during 1997 related to the following, in accordance with its duties as a source of scientific and technical support to other Commission services:

ECVAM's main validation activities in 1997 involved two international studies. The first was the completion of the formal validation phase of an EU/COLIPA study on an *in vitro* phototoxicity test which is now considered by the Commission to be a scientifically validated test which is ready to be considered for regulatory acceptance. The second study was a validation study on *in vitro* tests for skin corrosivity from which it was concluded by the ECVAM Management Team that two of the methods can be considered as scientifically validated for use as replacements for the animal test for distinguishing between corrosive and non-corrosive chemicals.

In addition, a validation study is ongoing in the endpoint of embryotoxicity testing. A further prevalidation study on haematotoxicity testing has been initiated which will assess the granulocyte-macrophage-colony forming unit (GM-CFU) test for acute neutropenia.

The validation of new test methods, in terms of assessing their relevance and reliability, requires the application of biostatistical methods and close collaboration between biostatisticians and experimental scientists. ECVAM's biostatistician played a key role during the successful validation study on *in vitro* tests for skin corrosivity, having input into the study design and being responsible for the data collection and analysis stages. The ECVAM Biostatistics Task Force continues to develop and evaluate new ideas for improving the analysis of data obtained from alternative tests during validation studies, through the proper application of biostatistical techniques. In particular, the importance of developing and assessing prediction models, for interpreting the data obtained with alternative methods in relation to known *in vivo* effects, was demonstrated during 1997.

One of ECVAM's priorities is to ensure that it is well informed about the state of the art of non-animal test development and validation. ECVAM workshops are therefore held to

review the current status of various types of alternative tests and their potential uses, and to identify the best ways forward. The reports and recommendations of ECVAM workshops are published in international scientific journals. During 1997, five workshops were held, on:

- The Use of Transgenic Animals in the European Union;
- Issues Relating to the Release of Proprietary Information and Data for Use in the Validation of Alternative Methods;
- Non-animal (Alternative) Tests for Evaluating the Toxicity of Solid Xenobiotics;
- The Use of Human Keratinocytes and Human Skin Models for Predicting Skin Irritation; and
- Validation of Alternative Methods for the Potency Testing of Immunobiologicals.

ECVAM Task Forces have been established on topics of importance to ECVAM (for example, on biostatistics, prevalidation, skin irritation, and developmental toxicity), to focus on more specific issues, such as the actual design of prevalidation or validation studies.

ECVAM's Scientific Information Service (SIS) has developed a database on alternative methods. This database contains information on their uses, their state of development and validation encompassing details of the methods, test chemicals and results as well as literature references. A second database has been developed on validation studies (*dbVas-online*), which provides support for ECVAM's validation studies and includes information on participating organisations, test protocols and prediction models, test chemicals and results. Access to *dbVas-online* will be via the Internet (general access) and Intranet (access restricted to participants in on-going validation studies). A third database on *in vitro* pharmacotoxicology laboratories is at the planning stage.

Collaborative experimental studies with groups in the EU Member States, focusing on the evaluation and prevalidation of new *in vitro* tests, are also being undertaken. A study on embryotoxicity testing *in vitro* with embryonal stem cell lines aims to characterise native and engineered embryonal stem cell lines for the development of more specific and more sensitive endpoints for embryotoxicity.

There is also a study on the characterisation and use of genetically engineered cell lines in research into metabolism-mediated toxicity. The study will characterise and evaluate the applicability of various genetically engineered mammalian cell lines that express human cytochrome P450 isoforms.

Lastly, a study is on-going on the identification and evaluation of new endpoints for use in an *in vitro* nephrotoxicity screening test. In this programme, the integrity of renal epithelial cells grown on microporous supports following exposure to chemicals is being assessed by measuring several markers of epithelial barrier function.

In addition to these laboratory studies, other projects are being undertaken in collaboration with scientists in the EU Member States such as a review on the scientific, ethical and legal aspects of the production, breeding and use of transgenic animals. Also, projects are underway looking at the use of human volunteers in assessing the efficacy and safety of cosmetic products and at the use of mathematical models in the development and validation of non-animal tests and testing strategies.

DG III

DG III took over responsibility for the Cosmetics sector in 1997. Since then, it has become active in the Commission's work on the development and acceptance of alternative methods. DG III is represented on ESAC and has been proposed for participation in the ECVAM Management Board; DG III will also participate in relevant ECVAM workshops. Also, it collaborates with the other Commission services that work in the field of alternatives such as DG XXIV, DG XI and DG XII.

DG III now holds responsibility for the preparation of the annual report and consequently for the compilation of data on animal experimentation for cosmetics within the EU. Further to several communications reminding Member States of their obligations in this respect, DG III is now considering infringement procedures against some Member States that have failed to meet these obligations to date.

In terms of future initiatives from DG III, international discussions in the field of animal testing of cosmetics have been identified as a high priority. An integral part of DG III's future work programme will be the discussion of the issue of animal testing of cosmetics at an international regulatory level. The intention of such discussions will be to raise awareness of the on-going efforts in Europe and facilitate the international regulatory acceptance of alternative methods.

DG XI

DG XI holds responsibility for Council Directive 86/609/EEC on the approximation of laws, regulations and administrative provisions of Member States regarding the protection of animals used for experimental and other scientific purposes. Pursuant to Articles 13 and 26 of this Directive, DG XI presents to the Council and the European Parliament statistical data on animals used for experimentation in the EU. The second in this series of reports is currently in preparation on the basis of data submitted by EU Member States.

In the latter half of 1997, the Member States agreed to a uniform format for presentation of these data allowing for a harmonised compilation method throughout the EU. This method will be applied in the third Commission report foreseen for the year 2000.

In addition, DG XI takes a lead role along with ECVAM, in advising the Commission on the validation of alternative methods. To this end, DG XI and ECVAM produced a joint endorsement statement on the *in vitro* test for phototoxic potential, stating that this test is now scientifically validated and ready for regulatory acceptance.

In 1997, DG XI took the initiative on several occasions at OECD in relation to alternative test methods. These initiatives include the forwarding of the above-mentioned endorsement to the OECD secretariat, the pressing for a deletion of OECD Test Guideline 401 for the determination of LD 50 and the continued promotion of the acceptance of the *in vitro* method for percutaneous absorption.

DG XII

In 1997, DG XII continued to promote research in the field of "Prenormative research: *In vitro* alternatives to animal experiments in pharmaco-toxicology" under the BIOTECH and BIOMED programmes.

BIOTECH

The following projects are currently funded across the 4 topics contained in the sector "*In vitro*" alternatives to animal experiments, for a total of 13,006,400 ECUs.

"*In vitro*" tests for developmental pharmaco-toxicology:

- Development of *in vitro* mammalian germ cell culture systems and genetic markers for reproductive pharmaco-toxicology
- Development and evaluation of Leydig cell lines as *in vitro* models for toxicological testing

"*In vitro*" tests for neuro-pharmaco-toxicology:

-New immuno-pharmaco-toxicological model: human reconstructed epidermis containing Langerhans cells
- Development of *in vitro* tests for drug allergenicity and B cell switching to IgE synthesis
- Development of *in vitro* systems using human immortalised cell lines for testing skin irritancy
- Establishment of stable immortal differentiated cell lines for the development of *in vitro* tests

Immuno-pharmaco-toxicology

- *In vitro* neurotoxicology tests based on the coupling of brain slices to silicon microelectrode arrays
- Novel human renal and hepatic co-culture *in vitro* test systems for the evaluation of biotechnology-derived cytokines
- Development of 3D *in vitro* models of human tissues for pharmaco-toxicological applications

Cell cultures for the development of *in vitro* tests

- Development of a yeast-based model system for expression of higher eukaryotic K^+ channels and their pharmacological analysis
- New developments of cultured precision-cut tissue slices for studies of organ pharmaco-toxicology

Three new projects successfully evaluated during the fourth and last call for proposals are now undergoing negotiation.

Four projects are directly contributing to increasing scientific knowledge which may lead to the implementation in the Cosmetic Directive 76/768/EEC.

In order to ensure the exploitation and industrial relevance, close interactions with IVTIP are encouraged. The "*In vitro* Testing Industrial Platform" was funded in 1993 by 19 European companies with activities in the pharmaceutical, chemical and cosmetic sectors. Industrial platforms are technology-driven industrial groupings established on the initiative of industry around Biotechnology RTD contracts, that monitor EU-funded