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Skin Irritation

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FOREWORD

The European Chemical Industry Ecology and Toxicology Centre (ECETOC) has, since its foundation in 1978, been concerned with the scientific aspects of toxicology and environmental toxicology. In its Monographs ECETOC has expressed its views on particular aspects of the toxicology of industrial chemicals to which man may be exposed. This Monograph is the fifteenth in the series.

Skin contact is the most common form of exposure to chemical substances and preparations in the home or at work. If these products are either irritant or corrosive the exposure may result in dermatitis/eczema or more persistent lesions. In order to avoid these adverse reactions it is necessary to identify the potential of a substance or preparation to be irritant or corrosive so that preventive measures can be taken to reduce the possibility or the risk of skin damage in the anticipated use conditions.

The purpose of this Monograph is to describe various aspects of the assessment of skin irritation/corrosive potential of substances and preparations including the use of alternative testing procedures. The way in which skin irritation test results can be interpreted in terms of hazard to man is also extensively discussed.

It is with pleasure that I present this Monograph to all those who are concerned with both human and animal welfare.

R.R. Knowland
Chairman of the ECETOC Board

SUMMARY

The assessment of the ability of substances and preparations to cause skin irritation, although simple in concept, has proved difficult in practice. Methods for the assessment of skin irritation have proliferated but the 1944 Draize procedure is still the method appearing in regulations worldwide. The problems of extrapolating the results of such animal studies to man have been recognised for many years.

The standard test employs the rabbit, the results of which have been used for many years for classification purposes and the assessment of hazard to man. In some cases the value of the resulting classification as an indicator of risk is questionable. A proper assessment of the hazard to man requires integration of information from a variety of tests and information on human exposure.

The problems inherent with skin irritation testing are addressed in this report. The current state of the art has been reviewed and placed in context with the regulatory requirements currently used within the European Commission. Alternative approaches have been reviewed including in vivo and in vitro tests and human studies. The latter together with the conventional tests on the rabbit have been examined to interpret the potential hazard to man.

A sequential procedure for the assessment of skin irritation potential has been proposed which is conservative in the use of animals and compatible with regulatory needs. The procedure has five optional steps: collection of information, preliminary screening tests in vitro, secondary screening tests in vivo involving single exposures, repeated exposure tests in vivo and studies in humans. This procedure is not rigid and the inclusion and design of each step should be decided on a case by case basis, ensuring that each evaluation is appropriate to the material and exposure situation.

In some cases the classification resulting from this process may differ from that of the EEC method. It is probable, however, that the result will indicate

more closely the real hazard to man; differences would have to be resolved on a case by case basis.

A. INTRODUCTION

It is necessary to predict the degree of irritation or injury likely to occur when substances or preparations come into contact with the skin. This allows precautions for safe handling to be devised for the workplace and for consumers.

The assessment of skin irritation potential is an integral part of risk assessment which has long been required for a variety of product types ranging from industrial chemicals to cosmetics and medicines. Regulations governing these product categories have invariably required skin irritation assessment.

Indications of skin irritation potential have in the past been obtained following human exposure but for the majority of chemicals, particularly new chemicals, this is not possible. New chemicals are now generally the subject of regulations which require animal testing prior to marketing.

Despite much work to improve the assessment of skin irritation potential the methods described in Regulations are still based on the 1944 Draize procedure.

In 1984, CONCAWE (The Oil Companies' European Organisation for Environmental and Health Protection) decided to examine the available information on the skin irritancy of a series of hydrocarbon solvents. The study was undertaken as a consequence of proposals made by one EEC member state for the classification and labelling of 52 solvents of which 22 were petroleum hydrocarbon solvents.

Two categories of problem were identified, those associated with the methods used to determine skin irritancy and those associated with interpretation of the results. The difficulties seemed unrelated to the hydrocarbon solvents but were more general in nature. As a consequence CONCAWE asked ECETOC to seek ways of clarifying methodology and the interpretation criteria so that industry and those responsible for regulation could proceed in a harmonised manner. A workshop, at which industry and those with a regulatory function were present, highlighted the problems associated with skin irritation testing and classification (ECETOC, 1987).

The ECETOC Scientific Committee appointed a Skin Irritation Task Force with terms of reference:

1. to identify ambiguities in the present OECD/EEC test criteria and the ensuing shortcomings and interpretation problems;

2. to assess all other experimental methods including *in vitro* alternatives;

3. to assess the relevance of the experimental methods in predicting hazard to man;

4. to recommend whether further experimental work needs to be organised to obtain a uniform approach for skin irritation testing;

5. to recommend test protocols and criteria for the interpretation of test results that ensure maximum predictability of potential hazard to man.

B. BACKGROUND

The objective of any method for the determination of skin irritation potential is the assessment of hazard to man. Direct determination cannot ethically or practically be made in man, except on rare occasions, so that skin irritation potential of the majority of chemicals is determined in animal models. The most commonly used technique has been the Draize test (Draize et al., 1944). In this test a substance is applied for 24 hour under an occluded patch to the abraded and intact skin of rabbits. Their response is often recorded only as the extent of skin reddening (erythema), eschar formation and swelling (oedema) occurring during the 72 hours following removal of the test material from the skin.

There are two main criticisms of the Draize test. Firstly, differences in the way the test has been conducted have led to considerable interlaboratory variation in results (Weil and Scala, 1971; McCreesh and Steinberg, 1977; Gelbke and Zeller, 1980). Secondly, the results in the animal model are difficult to use in predicting effects in man (Nixon et al., 1975; Murphy et al., 1984); this is not surprising as there is no fundamental reason why the hairy skin of test animals should accurately replicate the response of human skin to chemical insult.

In 1981, OECD published Test Guideline No 404 which was a modification of the Draize test. It differed from the original in three important respects. The method of patch application was changed to allow the use of occlusive and semi-occlusive patches, the patch was applied to the skin for 4 rather than 24 hours and the need to apply patches to abraded skin was removed.

This helped to standardise the method and made it more predictive of effects in man. The EEC (1984) subsequently adopted the OECD guideline for use for the classification of substances and preparations. There is flexibility in the method which allows the use of data from animals other than the rabbit and the use of data resulting from human experience.

This has led to the development of data based on the modified Draize test in the rabbit, which is widely used to classify products for labelling purposes. This has been supplemented in some cases by data from other species and man when reaching conclusions as to labelling requirements. The result of this has been some confusion and disharmony in classification and labelling.

Three questions have been addressed:

1. what is the current state of the art;
2. what approach, unrestrained by regulatory considerations, should be used to assess the skin irritation potential of materials and,
3. for regulatory purposes, how should data be interpreted?

Chapter C examines the present EEC Test Method. Alternative in vitro and in vivo methods are reviewed in Chapter D. This includes the subject of testing in man. Chapter E provides guidance on how data generated in such studies may be used most effectively.

It has not been possible to identify a single in vitro or in vivo method which will adequately assess the skin irritation potential for all materials and all exposure situations. Progressive integration of data generated in several test systems is recommended. This approach should be discretionary and used on a case by case basis, so that each evaluation is appropriate to the specific material and exposure situation. This approach is discussed in Chapter F.

Chapter G reviews the difficulties which have arisen in the classification of skin irritants and the possible ways of avoiding them. It is recognised that to translate a flexible sequential approach to the assessment of skin irritancy into a regulation will not be simple; nevertheless it is felt that the end result will be much more meaningful in that the subsequent classification will have been made on the basis of the likely effects in man.

Appendix 1 contains a glossary of terms which are used in this Monograph.

C. DESCRIPTION AND COMMENTARY OF PRESENT EEC METHODOLOGY

To define a single test method that will give results allowing a proper classification for all chemicals in all exposure situations in man is an impossible task. To overcome the difficulty, the EEC have defined a single test based on the rabbit but have allowed flexibility for individual experimentalists to adapt the method as they see appropriate. Experience from use of the EEC test method shows there is scope for its improvement, whilst still retaining the flexibility necessary to address the fact that the rabbit does not in all cases respond to skin irritants like man.

In relation to the classification of dangerous substances (EEC, 1979) and preparations (EEC, 1988), the EEC has specified a method for determining skin irritation potential (Annex V of Directive 67/548/EEC, Point B4, EEC 1984) (Appendix 3). The test is based on the OECD test guideline No 404 (OECD, 1981) (Appendix 2). The original Draize method, the OECD test guideline and the EEC test method are compared in Appendix 4.

Parts of the EEC test method are subject to different interpretations; suggestions are made below for improving the situation. The wording of the EEC test method is given in italics.

The principle of the EEC test method (Appendix 3 section 1.4) is as follows: *"The substance to be tested is applied in a single dose to the skin of several experimental animals, each animal serving as its own control. The degree of irritation is read and graded after a specific interval, and is further described to provide a complete evaluation of the effects. The duration of the observations should be sufficient to evaluate fully the reversibility of the effects observed".*

This is not sufficiently detailed to ensure the test is done in the same way by all laboratories and this will increase interlaboratory variation.

Reference substance (Appendix 3, section 1.3)

''None''. No reference substance(s) are advocated. Each animal serves as its own control and a standard scoring system should be used. Experience has shown that the scoring system does not produce uniformity in practice, particularly with mild-moderate irritants. It has been reported that the inclusion of reference irritant materials (chemically similar substances or products for similar uses) in the test can reduce interlaboratory variation (Kaestner, 1980).

Description of the test method (Appendix 3, section 1.6)
Preparations (section 1.6.1.)

(a) Animals. *''Approximately 24 hours before testing, fur should be removed by clipping or shaving from the dorsal area of the trunk of the animal. When clipping or shaving the fur, care should be taken to avoid abrading the skin. Only animals with healthy intact skin should be used''*.

Hair is removed to ensure good contact between the skin and the test material and to allow effects on the skin to be clearly observed. Removal of hair by clipping or shaving are specified; use of depilatories or of other methods which damage skin cannot be used. Hair growth in rabbits is cyclic and the state of hair growth at the site of application should be recorded. Advice to avoid testing in areas of coarse hair growth may help to reduce inconsistences between laboratories.

(b) Test materials.

i) *''When testing solids which may be pulverised if considered necessary, the test substance should be moistened sufficiently with water, or where necessary, a suitable vehicle, to ensure good contact with the skin. When vehicles are used, the influence of the vehicle on irritation of the skin by the test substance should be taken into account. Liquid test substances are generally used undiluted''*.

Effects on skin depend on the degree of contact with test material. Pulverisation of a solid material can significantly increase the degree of skin contact. The amount of water used to moisten test substances is not stated; different workers may find different amounts *"sufficient"*. Some substances that are non-irritant when applied dry under a patch are irritant when applied as pastes (Gilman et al., 1978). Many solids can absorb large volumes of water and still remain dry to the touch. While the present instructions in section 1.6.1 probably cannot be improved care is needed when interpreting the findings with powdered materials.

ii) *''Test substances which are strongly acidic or alkaline need not be tested for primary dermal irritation, owing to their predictable corrosive properties''*.

It should be noted that the pH alone does not accurately predict the potential of a material to cause severe skin irritation or corrosion (Young et al., 1988); the buffer capacity must also be considered. The sequential approach (Chapter F) makes recommendations on whether to carry out tests on substances having extreme pH.

Test Conditions (section 1.6.2)

(a) **Experimental animals (section 1.6.2.1.)**. *''Although several mammalian species may be used, the albino rabbit is the prefered species''*.

Rabbit skin is sensitive and the rabbit has been the choice of most investigators for skin irritation testing. Other species should be used only when the reaction of their skin has been shown to reflect accurately or be predictive of effects on human skin.

(b) **Number of animals (section 1.6.2.2.)**. *''At least three healthy adult animals are used. Additional animals may be required to clarify equivocal responses''*.

Where a severely irritant response might be anticipated advice should be given to use only one animal. Where this initial test shows only mild-moderate irritancy, further animals could be tested (Chapter F).

(c) Dose level (section 1.6.2.3). *"Unless there are contra indications, 0.5 ml of liquid or 0.5 g of solid or semi-solid is applied to the test area. Separate animals are not required for an untreated control group. Adjacent areas of untreated skin of each animal serve as controls for the test".*

The selection of this volume and weight is to ensure that an area of 6 cm^2 and no more is exposed to the test material (section 1.6.3). In some cases, e.g. with low viscosity organic solvents, other procedures (e.g. Finn chamber, see below) might be required to prevent excessive spreading.

(d) Observation period (section 1.6.2.4). *''The duration of the observation period should not be fixed rigidly. It should be sufficient to evaluate fully the reversibility or irreversibility of the effects observed, but need not normally exceed 14 days after application''.*

The observation period needs not be extended beyond 72 hours if all signs of irritation have regressed completely. If not the time required to study reversibility of effects should be decided on a case by case basis but should be extended beyond 14 days if complete healing has not taken place.

Procedure (cf Appendix 3, section 1.6.3)

(a) Application of test substance. *''The test substance should be applied to a small area (approximately 6 cm^2) of skin and covered with a gauze patch, which is held in place with a non-irritating tape. The patch should be loosely held in contact with the skin by means of a suitable semi-occlusive dressing for the duration of the exposure period. However, the use of an occlusive dressing may be considered appropriate in certain cases. Access by the animal to the patch and resultant ingestion or inhalation of the test substance should be prevented''.*

A number of factors could lead to interlaboratory variation in test results.

- The composition of the gauze. This could affect the rate of evaporation of vehicle and hence the amount of test substance and vehicle contacting the skin.

- The composition of tape and the method for taping the gauze to the skin are not specified. As well as influencing the rate of evaporation interactions with or solubilisation of adhesive constituents could take place.

- The sensitivity of various areas of skin may differ (Vinegar, 1979). The most appropriate site to locate patches on the test animal is not stipulated, although the back is almost invariably used.

- There are ways to stop animals interfering with patches. Various types of holding cover are available (e.g. rubberised sheet to canvas sleeves) which vary in degree of occlusivity. The type of occlusive or semi-occlusive dressing used can affect skin irritancy significantly (Lansdown, 1978; Gilman et al., 1978; Walker, 1988). The nature of a *"suitable semi-occlusive dressing"* and the circumstances under which an occlusive dressing is appropriate are not specified.

- Excessive pressure applied to an occlusive dressing can spread the test substance beyond the test area.

This section of the Test Method might be redrafted to take into account the above factors and in particular to define more clearly a suitable semi-occlusive dressing. Where liquids may remain in contact with human skin testing using Finn chambers or similar devices may prove to be of value. The interpretation of the results in terms of effects in man may prove difficult where human exposure is different from that of the conditions of the test. Such testing may give an indication of ultimate skin irritation potential but may bear little relation to the actual hazard to man.

b) Exposure duration.

''If an exposure period shorter than four hours is used, and a serious skin reaction is observed, the experiment need not be repeated using a four hour exposure period''.

It would help if the term *"serious skin reaction"* was defined. It may also be possible to avoid further testing if the results from of a validated in vitro test are available.

''Longer exposures may be indicated under certain conditions, e.g. expected pattern of human use and exposure''.

There is a need for criteria to ensure consistent use of data from such studies for classification purposes (see chapter G).

''At the end of the exposure period, residual test substance should be removed, where practicable, using water or an appropriate solvent, without altering the existing response or the integrity of the epidermis''.

This must be done with care as the response can be effected significantly by pressure, friction during wiping off or drying, test material left on the skin, or the use of solvents which can both enhance and retard the penetration of test chemical.

It is impossible to give advice that is applicable to all chemicals in all situations and it is recommended that more attention is drawn to the problems of removing test substance from the skin and that advice is given to select a suitable technique on a case by case basis.

Observation and grading (section 1.6.3.1). *''Animals should be observed for signs of erythema and oedema and the response graded at 30 and 60 minutes, and then at 24, 48 and 72 hrs after patch removal. Dermal irritation is graded and recorded according to the system in Table 1. Further observations may be needed, as necessary, to establish reversibility. In addition to the*

observation of irritation, any serious lesions such as corrosion (irreversible destruction of skin tissue) and other toxic effects should be fully described''.

Although there is a standard system for the numerical scoring of erythema and oedema marked intra- and interlaboratory variation can arise in scores allotted, particularly in relation to materials of low to moderate irritancy. For some substances the onset of the response may be delayed (Jacobs and Martens, 1987) and it may, in some cases, increase in intensity. Attention should be drawn to such possibilities and provision made for their inclusion in the grading system.

No guidance is given on the recording of signs of irritation other than erythema or oedema (e.g. thickening (hyperplasia), cracking (surface fissuring), bleeding, desquamation (scaling) and hair-loss (alopecia), cellular oedema, inflammatory cell infiltration, etc.). Any serious lesion such as corrosion and other toxic effects have to be fully described but no guidance is given on how to use this information in classification. Formal guidance in the EEC method on the additional signs which characterise the degree of irritation and the extent of recovery is required if they are to be used in a standard way for classification.

Skin corrosion is characterised by tissue destruction (necrosis), whether immediate, as with acid or alkalis, or delayed with substances toxic to skin, or by irreversible skin change, i.e. scarring (FDA, 1972).

The EEC criteria for corrosion (Annex VI, Point B of EEC 67/548/EEC, EEC, 1984) states that *"a substance or preparation is considered to be corrosive if, when applied to healthy and intact animal skin, it produces full thickness destruction of skin tissue on at least one animal during the test for skin irritation"*.

Necrosis which is less extensive than *"full thickness"* can cause scarring. If tissue destruction is observed, the observations should be continued (where the well-being of animals allows) to enable reversibility to be assessed. Eschar (scab) will prevent this. Observations should be continued until the eschar

has sloughed off and the state of the skin can be assessed. If this procedure is not followed an inappropriate classification may result.

Although the problems have been identified that arise when a single test method is used to classify the hazard of all chemicals in all exposure situations, no attempt has been made to lay down a method which corrects the deficiencies as this would impose too severe a restriction on toxicologists wishing to identify the skin irritation potential of chemicals and to define the risk they pose to man. Flexibility is required to cover the wide range of chemicals that needs testing and this automatically introduces the possibility of major variations in test results between laboratories. This issue has led the Task Force to develop an approach to skin irritancy testing based on a sequential procedure covering data collection, in vitro tests, animal in vivo tests and human studies (see Chapter F).

D. ALTERNATIVE TEST APPROACHES

1. *NEW IN VIVO EXPERIMENTAL METHODS*

Since the publication of the Draize Primary Skin Irritation Test in 1944, many modifications have been introduced to remove subjectivity from the test, to increase its sensitivity, to make the test more predictive of effects in man and to reduce the number of animals used. Some of the modifications aim to improve on the quantification of the endpoints measured, whilst others represent alternative approaches to investigation of the inflammatory processes. A review of the tests is given in Appendix 5; only the key issues are highlighted here.

1.1. Use of Alternative Species

Interspecies variability in the response of the skin to irritant chemicals is to be expected. In addition to the rabbit, the guinea pig, mouse, rat, monkey, beagle dog and miniature swine have been studied, but validation of the tests has been attempted with only a small number of chemicals. In some studies comparisons have been made with results of tests carried out in man.

Since the majority of published data has been generated in the rabbit, it must remain the *"preferred species"* at present. Other species such as the rat, mouse and guinea pig show promise but further validation with a wider range of chemicals is required before they can be considered as alternatives to or replacements for the rabbit in classifying chemicals.

1.2. Methods to reduce the Subjective Nature of Observation

The subjective nature of the evaluation of erythema and oedema leads to considerable inter- and intra-laboratory variation. Some investigators have developed methods to quantify a wide range of parameters such as cutaneous blood flow by laser Doppler flowmetry, temperature of the skin by infrared detectors, changes in skin thickness using calipers and the

number of wrinkles formed upon reefing of the skin. Some authors have quantified the area of damage in excised skin by image analysis whilst others have estimated the degree of irritancy by the accumulation of vital dye previously injected at the site of exposure.

While these approaches are interesting, extensive evaluation would be needed before the techniques could be used routinely for hazard assessment or classification.

1.3. Alternatives to Patch Tests Techniques

Various exposure techniques have been investigated including the application of test compounds by means of a chamber, open application, skin painting and intradermal injection. Application of a single chemical for different contact times or several different chemicals for the same contact time on a single animal has also been used.

Multiple site exposure would reduce significantly the number of animals used, provided the species of laboratory animals has a large dermal surface. Care would have to be taken that none of the chemicals applied is corrosive or systemically toxic. Chamber techniques may provide uniform application of test compound within discrete areas, a particular problem when testing liquids, but their routine use may provide results that are not relevant to human exposure.

1.4. Other Indicators of Irritant Response

In an attempt to quantify the mechanisms involved in an irritant (inflammatory) reaction, parameters other than erythema and oedema have been assessed. For example leucocytes have been counted in fluid removed from treated skin, thymidine uptake and cell mitosis measured, transepidermal water loss or enzyme leakage measured or the histological or cellular response evaluated by light- or electronmicroscopy.
Whereas these parameters are useful for research purposes none is yet sufficiently validated to be recommended for routine use.

2. *ALTERNATIVE IN VITRO APPROACHES*

2.1. Introduction

One aim of *in vitro* tests is to reduce the number of or replace experimental animals for the assessment of skin irritation. The numerous approaches can be classified broadly into those that use established cell lines of human or animal origin, those that use skin tissue and those which do not use biological procedures. These tests are reviewed in Appendix 6; the highlights are discussed here.

2.2. Techniques using Cells (Appendix 6, 6.1)

Skin is a complex tissue consisting of many morphologically and functionally different cell types (Parish, 1985). Two experimental approaches have been developed. In the first, tissue specific cell lines such as keratinocytes, mast cells, macrophages and polymorphonuclear leucocytes are used; these retain functional properties such as keratinisation and degranulation which are fundamental to the response of the tissue to irritants. The second uses cells derived from other tissues in which the influence of irritants is evaluated from effects on general biochemical processes.

The endpoints tend to be crude measures of cytotoxicity such as cell death and leakage of cellular enzymes and other endogenous substances. Some investigators have observed morphological or functional changes such as keratin formation in an attempt to increase the sensitivity of the test systems.

Cells in suspension culture or as mono/multilayers do not retain the selective barrier to chemicals of natural skin tissue so they tend to be sensitive to chemicals which are of a low skin irritancy *in vivo*. Furthermore, these techniques do not allow investigation of differences in species sensitivity resulting from variations in percutaneous penetration.

The results of some of the tests show a good correlation with in vivo animal findings, particularly within a structurally related chemical series. They are therefore potentially suitable for the screening of chemically related substances before in vivo assessment.

2.3. Techniques using Tissues (Appendix 6, 6.2 and 6.3)

Techniques using skin tissue retain the natural species-specific permeability characteristics of intact skin. In addition they allow the examination of the wide range of physical forms of test chemical to which man might be exposed.

Short-term maintenance of freshly prepared slices or discs of animal skin by nutrient medium has been studied by many investigators. While such skin is avascular and cannot show some aspects of the inflammatory process it does allow measurement of tissue responses to chemical exposure, for example:

- morphological change, e.g. tissue and cellular oedema,
- biochemical change, e.g. cellular ATP,
- respiration and enzyme inhibition,
- compromised membrane integrity e.g. enzyme release,
- tissue proliferative response to injury e.g. thymidine incorporation,
- induction of enzyme activity (ornithine decarboxylase),
- cell replication.

The techniques using viable tissues have so far proved to be of limited use in predicting skin irritation potential.

Non-viable animal and human skin tissue also has been used to observe the physico-chemical action of irritant and corrosive substances on tissue integrity, in particular integrity of the stratum corneum which is the natural barrier to the penetration of substances and ions. In contrast to the viable tissues the techniques using non-viable isolated skin tissue have shown promise in the identifying substances corrosive to the skin (see Appendix 6).

2.4. Conclusions

Many *in vitro* tests are being developed but before they can be accepted as alternative methods for evaluation of the irritancy of substances and preparations to man or for classification they require adequate validation.

This validation process is not clearly defined (Goldberg, 1988) but should include intra- and inter-laboratory testing of a wide range of substances under blind conditions with comparisons being made to known human responses to exposure or, in the absence of this, responses in animals.

Two techniques have been so validated, the skin corrosivity test (Oliver and Pemberton, 1985, 1986; Oliver et al., 1986, 1988) and the classification model for alkaline and acidic preparations (Young et al., 1988).

Numerous *in vitro* tests have shown some correlations with *in vivo* observations (see Appendix 6) and it is understood that some are at present being validated in 'ring' tests in the US and Europe.

In vitro tests have a place in the preliminary assessment of chemicals but care should be taken to ensure that tests with a predictive value are used strictly in those circumstances in which they are known to be applicable. Guidance to the applicability of these tests can be found in the relevant publications (Appendix 6).

3. *HUMAN STUDIES*

Provided ethical principles are followed and there is evidence from studies in animals that exposure of volunteers is unlikely to present a serious hazard, studies may be carried out in man. Various forms of patch testing have been used, the irritant response, as judged from the gross appearance of the treated skin, being given a numerical value.

A variety of protocols have been used and a wide range of responses have been reported by investigators using identical irritants. More details about human testing are given in Appendix 7.

Only one set of internationally approved guidelines exists for human patch testing (Fregert, 1986). Although this was developed for the assessment of contact sensitisation the method described for the application of the test substance is equally valid for the assessment of skin irritation.

To minimise the subjective nature of the human skin irritation test, a number of techniques are currently under development to measure more precisely the complex responses of skin to irritants. To date most methods have measured blood flow in the skin area exposed to the chemical, with or without an assessment of erythema and oedema. Other techniques are intended to measure tissue damage by, for example, determining transepidermal water loss, carbon dioxide loss or electrical impedance.

Test protocols are available in member companies for the assessment of skin irritation, the results of which have been correlated with human experience. It is strongly recommended that these methods be published.

4. *OTHER APPROACHES (Appendix 6 - 6.4)*

A number of methods have been used to assess skin irritation potential of chemicals by measuring their physico-chemical properties directly (e.g. acidity/alkalinity and chelating properties) or indirectly (e.g. by measuring chemical denaturation of collagen or other protein). For substances that cause corrosion and severe skin irritancy, results from physico-chemical measurements predict well the in vivo response.

Structure activity relationships (SAR) have been developed with the aim of predicting skin irritation potential by analogy to chemicals whose irritation potential is known. This approach should be restricted to substances from well defined chemical families for which a data base of in vivo results exists and which show consistent responses.

One of these relationships, the Topkat computerised structure activity relationships model, has received much attention (Enslein et al., 1987) but experience shows it cannot be relied on to give reliable predictions with individual chemicals.

E. ASSESSMENT OF HAZARD TO MAN FROM EXPERIMENTAL SKIN IRRITATION TEST RESULTS

There have been many attempts to correlate results of irritation studies carried out in animals and man (Brown, 1970; Phillips et al., 1972; MacMillan et al., 1975; Nixon et al., 1975; Campbell and Bruce, 1981). Although techniques used on animals have differed somewhat from those used on man, making it difficult to correlate the results obtained, it is clear that rabbit skin is significantly more sensitive than human skin to irritants (Motoyoshi et al., 1979).

Attempts have also been made to relate effects found in animal tests with those occurring in man following accidental exposure, giving rise to customer complaints and occurring in volunteer studies.

Information arising from accidents is of limited value in assessing the relevance of animal test results since reports following single exposure are usually only associated with severely irritant or corrosive materials. Such materials are reasonably easy to identify because the response to them is usually immediate and the causative agent is therefore obvious. Moderate or slight irritation on the other hand is rarely reported as an accident but is often reported in the form of worker or consumer complaints following chronic exposure. When it is reported, the causative agent is often not immediately obvious and exposure to other materials may have occurred. Identification of chronic irritants is further complicated by the fact that the observed response may have been due to exposure to an allergen. To identify the cause and to distinguish between an allergic or irritant reaction can be extremely difficult even with well conducted patch testing.

Information from volunteer studies can be used to identify slight or moderate irritants. Since such studies are normally designed to determine threshold concentrations for irritation or as preliminary studies to skin sensitisation testing many of the materials tested do not prove irritant and in any case the information is generally unpublished.

On the basis of the limited comparative data available two general conclusions may be drawn:

- animal irritation studies can readily identify strongly irritant or corrosive materials;

- animal irritation studies are unreliable for accurate prediction of low to moderate skin irritants to man.

In the absence of other information it is prudent to assume that slightly irritant materials (as shown by animal studies) may also cause irritation in man if regular or repeated skin contact occurs.

There is a need to establish a sound base of comparative human and animal data which could in time be used to make predictions from animal data of effects in man. There is thus a need for the collection and review of human data relating to acute and chronic skin irritancy due to chemical exposure. Without this it will not be possible to relate the results of animal studies to effects in man. Although it is appreciated that chronic irritation contributes significantly to the prevalence of dermatitis it is believed that data on chronic skin irritation in man cannot directly be used for the prediction of acute effects.

F. ASSESSMENT OF SKIN IRRITATION

As explained previously the EEC test method gives information for use in making assessments of the skin irritation potential of substances and preparations and in their classification. The flexible elements of the EEC test method can be used in a constructive way for these purposes, whilst retaining it as one of the steps in a sequential series.

For some chemicals, the EEC test method will not give a true indication of the potential hazard to man. For such chemicals further work is needed to allow a proper assessment to be made. This assessment may, in some cases, produce a classification which differs from that based upon the single test method. These situations will have to be resolved on a case by case basis, as occurs at present in an informal way.

Many schemes can be envisaged but one approach, to be outlined below, encourages the inclusion of information that may help to eliminate the need for animal tests. It is emphasised that it would not always be necessary to take all of the steps outlined. The number of steps will depend on the type of material being evaluated, on its intended use pattern and on legislatory requirements.

In the scheme subsequently described steps 1 - 3 are concerned with collecting basic information which would eliminate unnecessary testing. Step 3 allows a prediction of skin irritation likely to occur with a single exposure to a material. Such data are useful for predicting the skin irritation which may occur following accidental exposures which are likely to be infrequent and of short duration. Step 4 is intended primarily for materials to which regular or repeated skin contact may occur during their anticipated use. Finally, for some chemicals, studies in humans may be necessary (Step 5).

STEP 1 - Collection of Information

Information on the identity and nature of the substance or preparation should be assembled. Data on physico-chemical properties, occurrence and level of impurities, solubility, toxicity data and previous experience in man or animals for similar or chemically related materials and preparations are particularly useful. Chemicals of extreme pH (≤ 2 or ≥ 11.5) and large alkaline or acid reserve should not be tested in animals as their effects may be reliably predicted.

This may provide sufficient information for an evaluation, in which case no further action is required to examine the skin irritation potential of the material.

STEP 2 - Preliminary Screen (in vitro)

A preliminary *in vitro* screen may consist of one or more studies. These should be the most appropriate for the laboratory involved and the particular material to be evaluated. Some *in vitro* studies will be reliable with chemicals of a particular type or with products intended for a particular usage. Each laboratory should choose those techniques which it feels are the most relevant and which have been validated for the family of chemicals investigated (see Chapter D). For these reasons it is not possible to prescribe a list of techniques to be used for every evaluation. Results of such *in vitro* studies may eliminate the need for *in vivo* studies.

STEP 3 - Secondary Screen (in vivo) involving Single Exposures

Depending on the outcome of Steps 1 and 2 *in vivo* studies may be required to provide an understanding of the irritant nature of the test material. The results of such studies in Steps 1 and 2 may show materials to have a high or a low potential to cause skin irritation.

If the results of Steps 1 and 2 testing indicate that a material may cause corrosion it may be necessary to conduct the EEC method using only one rabbit but with three test patches. The first patch should be removed after three

minutes exposures and if corrosion has occurred the study should be terminated. If corrosion has not occurred the second patch should be removed following 1 hour exposure. The same principle applies when deciding whether to terminate or continue the study beyond 1 hour. Although for some chemicals the development of a corrosive lesion may be delayed beyond the exposure period the procedure would, for many chemicals, allow corrosive materials to be identified without the need for further testing. If the material is found to be non- or only moderately irritant, two extra rabbits could be used to increase the total number to 3 as required for EEC classification purposes and to check for inter-animal variability in response.

STEP 4 - Repeated Exposures in vivo

Man comes into repeated and prolonged skin contact with many chemicals, which are not overtly skin corrosive or severely irritant, either as a consequence of their normal use or of poor hygiene. The results of repeated exposure studies in animals may be useful in predicting the hazard to man of repeated exposure to substances or preparations.

In designing repeat exposure studies, particular attention should be paid to the concentration of the material and the frequency of likely exposure in man. Meaningful results cannot be obtained in such studies if the skin at the test site is severely damaged. Thus, graded concentrations of test material which are more comparable to those to which man will be exposed, may need to be applied. This will allow a dose-response curve to be constructed. The need to avoid unnecessary stress to the test animal also precludes the induction of severe damage to skin, thus emphasising the need to examine the effects of dilute solutions of test material initially. To obtain as much information as possible about local and systemic effects histological studies may be needed in addition to the conventional gross observations. It is not possible to specify a single protocol which is universally applicable; the protocol should be that which is most appropriate to the material being tested and the exposure expected.

STEP 5 - Studies in Man

For products which are to be widely used or where human exposure is inevitable, test results in animals may need confirmation in human volunteer tests prior to release into the market. In some cases these may be followed by further clinical trials or consumer trials. Studies in man should always be carried out in a stepwise manner, gradually building up comprehensive experience of human exposure but allowing exposure to be terminated at any time if adverse effects come to light (Schmidt, 1983).

Even if a new material has proved to be non-irritant in such trials there is still a need to monitor those people exposed when a product is marketed in order to detect possible unforeseen effects or to confirm the original assessment.

A stepwise evaluation of the kind outlined above is a logical way to evaluate skin irritation potential and has long been the practice of some companies. The approach allows irritant materials to be identified at an early stage thereby eliminating the need to carry out unnecessary animal experiments. The stepwise approach should be modified as necessary to suit the material being evaluated and each evaluation should be judged on a case by case basis.

G. CLASSIFICATION OF SKIN IRRITANTS

This chapter is concerned only with classification of substances as corrosive and irritant to the skin in relation to the EEC Dangerous Substances Directive (EEC, 1979). Other classification schemes for e.g. transport and other national requirements are not considered.

Since the introduction of the Sixth Amendment (EEC, 1979) there has been several years' experience in testing new substances for skin irritancy and their subsequent classification. The foreword to the Dangerous Substances Directive allows for both flexibility in the interpretation of the test method for the assessment of skin irritation in laboratory animals and the acceptance of data on effects in man. In practice many laboratories have used protocols based closely upon the EEC test method. Many new chemicals have as a consequence been classified as to their presence or absence of inherent irritancy from results of tests in the rabbit, and have taken no account of expected exposure in man.

Even though the EEC has defined criteria for the classification of substances as irritant to the skin, based upon test data in rabbits, there has been considerable difficulty in classifying substances. The difficulties appear to have arisen either because of ambiguities of wording in the criteria and the lack of adequate guidance on how to use data generated in the EEC and other test methods for assessing skin irritancy.

The purpose of this chapter is to highlight some of the difficulties, explain their cause and suggest possible ways of avoiding them in the future.

1. DEFINITION OF CORROSIVE

Article 2.2.i. of the Sixth Amendment (EEC, 1979) defines corrosives as: *"substances and preparations which may, on contact with living tissues destroy them."*

Part II (B) (a) of Annexe VI defines the criteria by which substances should be judged as either corrosive or irritant (EEC, 1983). The criteria are:

"A substance or preparation is considered to be corrosive if, when applied to healthy intact animal skin it produces full thickness destruction of skin tissue on at least one animal during the test for skin irritation cited in Annex V or an equivalent method or if the result can be predicted, for example from strongly acid or alkaline reactions."

Although the criteria for corrosion may have been intended to amplify the definition, the statements remain ambiguous. The definition states that tissues are destroyed, while the criteria mentions "*full thickness destruction of skin tissue*". Full thickness destruction means loss of both dermis and epidermis and this normally leads to scarring. In practice, less than full depth destruction may result in scarring.

It is difficult to define the degree of tissue destruction which will result in scarring. Where tissue destruction is insufficient in extent or intensity to classify it immediately as corrosive the animals should be observed until it is clear whether complete healing or scar formation will take place. The present ambiguity would be eliminated if the definition and criteria statements were harmonised to make scar formation the major determinant factor in classifying substances as corrosive.

2. *DEFINITION OF IRRITANT*

Article 2.2.j. of the Sixth Amendment EEC (1979) defines an irritant as: *"non-corrosive substances and preparations which, through immediate, prolonged or repeated contact with skin or mucous membranes, can cause inflammation."*

Part II (B) (b) of Annex VI (EEC, 1983) defines an irritant substance as one which causes inflammation of the skin of a certain degree. The degree of inflammation is given as:

"Inflammation of the skin which persists for at least 24 hours after an exposure period of up to four hours and corresponds to the following values determined on the rabbit according to the cutaneous irritation test method cited in Annex V.

- *The mean value of the scores for either erythema and eschar formation or oedema formation, calculated over all the animals tested, is 2 or more.*
- *Or, in the case where the Annex V test has been completed using three animals, either erythema and eschar formation or oedema formation equivalent to a mean value of 2 or more calculated for each animal separately has been observed in two or more animals.*

In both cases all scores at each of the reading times (24, 48 and 72 hours) for an effect should be used in calculating the respective mean values."

The statements in the "Definition" and "Criteria" quoted above are ambiguous. It is not clear whether a mean score of at least 2, lasting 24 hours is required for a substance to be classified as irritant, or whether the mean score has to be calculated over the three observation periods (24, 48 and 72 hours).

The latter was probably intended but it would be helpful if the criteria were reworded to avoid this ambiguity, for example as follows:

- when more than three animals are used the mean value of the scores for either erythema and eschar formation or oedema formation calculated for all animals and the three observation times (24, 48, 72 hrs) is 2 or more;
- or, in the case where the Annex V test has been completed using three animals, when the individual animal mean scores for either erythema and eschar formation or oedema formation calculated over the three observation times (24, 48, 72 hrs) is 2 or more in at least two animals.

The present Annex VI (EEC, 1983) does not define Erythema, Oedema or Eschar. It may be helpful if a clear definition for "Eschar" is provided

since it is interpreted by some investigators as desquamation or scaling/flaking of stratum corneum, rather than as scab formation.

Definitions which could be given in the method are in Appendix 1.

3. *THE USE OF OTHER DATA*

The EEC test method requires data other than erythema and eschar formation and oedema formation to be recorded. It is not stated how this information should be used in classification so that it is left to individuals to judge their significance from their own experience. This will inevitably lead to some differences in interpretation but while observations should be as detailed as possible in order to assess the hazards it is doubtful if all such data need to be used for classification.

However, the classification of the inflammatory response is based upon key scores for erythema and eschar formation or oedema calculated over the first three days and for some substances the inflammatory response may be prolonged beyond the first three days (Jacobs and Martens, 1987). ECETOC considers that when this is so, the scores given to these reactions should be taken into account in classification.

3.1. Use of Data from Other in vivo Methods

Skin irritancy data have been produced since 1944 using the 24 hour Draize test and some countries (e.g. USA) still accept data generated using this method. As a consequence much data at present available cannot be used directly for classifying substances and preparations according to the EEC system which uses a 4 hour exposure period (EEC, 1983). For non-irritant materials this is not a problem since a substance non-irritant in a 24 hour occluded patch test will be non-irritant in the EEC test. The degree of irritancy occurring in a 4 hour semi-occluded test cannot be estimated by simple extrapolation from the degree of irritancy occurring in an occluded 24 hour test; to retest these materials would lead to unnecessary use of animals.

A possible solution would be to accept the scores for erythema and eschar formation or oedema in the 24 hours Draize test on intact skin for classification purposes. Although not ideal, this would ensure that such data are dealt with in a harmonised way.

To reduce the use of animals, use of dermal irritation information derived from other types of studies (e.g. dermal toxicity studies) has been proposed. Such data are often obtained with species other than the rabbit using different exposure periods, vehicles and dose rates. For example a dose proportional to body weight is applied to the skin in dermal toxicity studies whereas a small fixed dose is applied in skin irritation studies.

While such data can be of value in hazard evaluation, their use for classification purposes would require definition by the regulatory authority of the circumstances under which data can be derived and used.

The Directive gives no guidance on how data from prolonged or repeated contact studies should be used. It is not clear whether inflammation resulting from repeated and/or prolonged contact in experimental studies should require a substance to be classified as irritant, since the only criteria provided relate to inflammation occurring as a consequence of a 4 hours exposure.

It should be recognised that prolonged contact with some substances and preparations may cause irritation. There is a need for these to be labelled consistently appropriately. To accomplish this there is a requirement for a defined test method and criteria for suitable R and S phrases.

3.2. Use of Data from in vitro Methods

There has been much emphasis on reducing animal use by use of in vitro methods. If such techniques are to find a place in the assessment of irritant responses criteria will need to be identified which allow a harmonised interpretation of test results. The sequential approach

advocated here employs *in vitro* methods as screening methods for identifying materials which are likely to be strongly irritant or corrosive.

It would be helpful if a data base could be established to allow the correlation between EEC test method results and *in vitro* data to be studied for strongly irritant and corrosive substances.

3.3. Use of Data from Human Experience

The EEC gives precedence to use of human data over the results obtained in animal tests but no criteria are provided as to how these data are to be generated and applied to classification. Anecdotal evidence on human exposure has been used on occasions to alter labelling derived from acute animal studies. This may lead to disharmony; if human data are to be used particularly for reducing the warnings given in labelling they should be generated in well controlled studies (cf Chapter D -3).

H. CONCLUSIONS

There are two primary objectives of skin irritation testing; the classification of substances and preparations for regulatory purposes and the assessment of the hazard to man. The former requires a defined test method to minimise inter- and intra-laboratory variability, thereby producing a more uniform classification of chemicals throughout the community. An assessment of the EEC test method and experience gained since its introduction has led to a conclusion that the test method as described in Annex V of the 6th Amendment has scope for improvement without loss of flexibility.

The assessment of the hazard to man is not a clearly defined process because it integrates information from a variety of sources, such as in vivo or in vitro testing and human exposure. No single animal test method can ever hope to define the skin irritation potential for all chemicals and preparations and for all possible exposure situations. While satisfactory in vivo and in vitro methods exist to elucidate skin irritation, they require further evaluation and validation.

It has been concluded that the proper way forward is to use a sequential approach with information gathering, use of in vitro methods, single exposure tests in vivo, repeated exposure tests in vivo and studies in man providing the basis for the future development of the assessment of skin irritation potential. It is emphasised that it will not be necessary always to follow all the steps in this sequential approach; the extent to which it is followed will depend on the type of material being evaluated, its intended use pattern and regulatory requirements.

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APPENDICES

APPENDIX 1

1. GLOSSARY OF TERMS

Erythema: redness of the skin produced by vascular congestion or increased perfusion.

Dermal irritation: the production of reversible inflammatory changes in the skin following the application of a substance.

Dermal corrosion: the production of scarring usually as a result of tissue destruction (necrosis) following the application of a substance.

Eschar (scab): a superficial dry slough at the site of a heat or caustic burn which contains cell debris and dried tissue exudate and occludes the healing skin.

Hazard assessment: the evaluation of a hazard; it involves the integration of the potential of a chemical to harm man or the environment and the potential for exposure to a chemical.

Oedema: the presence of abnormally large amounts of fluid in the intercellular tissue spaces of the epidermis, dermis or subcutaneous tissues.

Risk: the probability that a hazard will occur under specific exposure conditions.

Scar (cicatrix): fibrous tissue replacing normal tissues which have been destroyed by injury or disease.

APPENDIX 2

OECD - TEST - GUIDELINE 404

ACUTE DERMAL IRRITATION/CORROSION

1. INTRODUCTORY INFORMATION

° Prerequisites

- Solid or liquid test substance
- Chemical identification of test substance
- Purity (impurities) of test substance
- Solubility characteristics
- pH (where appropriate)
- Melting point/boiling point

° Standard documents

There are no relevant international standards.

2. METHOD

A. Introduction, Purpose, Scope, Relevance, Application and Limits of Tests

In the assessment and evaluation of the toxic characteristics of a substance, determination of the irritant or corrosive effects on skin of mammals is an important initial step. Information derived from this test serves to indicate the existence of possible hazards likely to arise from exposure of the skin to the test substance.

° Definitions

Dermal irritation is the production of reversible inflammatory changes in the skin following the application of a test substance.

Dermal corrosion is the production of reversible tissue damage in the skin following the application of a test substance.

° Principle of the test method

The substance to be tested is applied in a single dose to the skin of several experimental animals, each animal serving as its own control. The degree of irritation is read and scored at specified intervals and is further described to provide a complete evaluation of the effects. The duration of the study should be sufficient to evaluate fully the reversibility or irreversibility of the effects observed.

B. Description of the Test Procedure

° Preparations

Approximately 24 hours before the test, fur should be removed by clipping or shaving from the dorsal area of the trunk of the animals. Care should be taken to avoid abrading the skin. Only animals with healthy intact skin should be used.

When testing solids (which may be pulverised if considered necessary) the test substance should be moistened sufficiently with water, or, where necessary, a suitable vehicle, to ensure good contact with the skin. When vehicles are used, the influence of the vehicle on irritation of skin by the tests substance should be taken into account. Liquid test substances are generally used undiluted.

Strongly acidic or alkaline substances, for example with a demonstrated pH of 2 or less or 11.5 or greater, need not be tested for primary dermal irritation, owing to their predictable corrosive properties. The testing of materials which have been shown to be highly toxic by the dermal route is unnecessary.

° Experimental animals

Selection of species

Although several mammalian species may be used, the albino rabbit is recommended as the preferred species.

Number of animals

At least 3 healthy adult animals should be used. Additional animals may be required to clarify equivocal responses.

Housing and feeding conditions

Animals should be individually housed. The temperature of the experimental animal room should be 22°C (+/- 3°) for rodents, 20°C (+/- 3°) for rabbits, and the relative humidity 30 to 70 per cent. Where the lighting is artificial, the sequence should be 12 hours light, 12 hours dark. Conventional laboratory diets are suitable for feeding and an unrestricted supply of drinking water should be available.

° Test conditions

Dose level

A dose of 0.5 ml of liquid or 0.5 g of solid or semi-solid is applied to the test site. Separate animals are not required for an untreated control group. Adjacent areas of untreated skin of each animal serve as control for the test.

Observation period

The duration of the observation period should not be fixed rigidly but should be sufficient to evaluate fully the reversibility or irreversibility of the effects observed. It need not normally exceed 14 days after application.

° Procedure

The test substance should be applied to a small area (approximately 6 cm^2) of skin and covered with a gauze patch, which is held in place with non-irritating tape. In the case of liquids or some pastes it may be necessary to apply the test substance to the gauze patch and then apply that to the skin. The patch should be loosely held in contact with the skin by means of a suitable semi-occlusive dressing for the duration of the exposure period. However, the use of occlusive dressing may be considered appropriate in some cases. Access by the animal to the patch and resultant ingestion/inhalation of the tests substance should be prevented.

Exposure duration is four hours. Longer exposures may be indicated under certain conditions, e.g. expected pattern of human use and exposure. At the end of the exposure period, residual test substance should be removed, where practicable, using water or an appropriate solvent, without altering the existing response or the integrity of the epidermis.

° Clinical observations and scoring

Animals should be examined for signs of erythema and oedema and the responses scored at 30-60 minutes, and then at 24, 48 and 72 hours after patch removal.

Dermal irritation is scored and recorded according to the grades in Table 1, below. Further observations may be needed, as necessary, to establish reversibility. In addition to the observation of irritation, any serious lesions and other toxic effects should be fully described.

TABLE 1 : EVALUATION OF SKIN REACTION

Erythema and Eschar Formation	Value
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4

maximum possible - 4

Oedema Formation

No oedema	0
Very slight oedema (barely perceptible)	1
Slight oedema (edges of area well defined by definite raising)	2
Moderate oedema (raised approximately 1 millimetre)	3
Severe oedema (raised more than 1 millimetre and extending beyond area of exposure).	4

maximum possible - 4

3. DATA AND REPORTING

° Treatment of results

Data may be summarised in tabular form, showing for each individual animal the irritation scores for erythema and oedema at 30 - 60 minutes, 24, 48 and 72 hours after patch removal, any serious lesions, a description of the degree and nature of irritation, corrosion or reversibility, and any other toxic effects observed.

° Evaluation of results

The dermal irritation scores should be evaluated in connection with the nature and reversibility or otherwise of the responses observed. The individual scores do not represent an absolute standard for the irritant properties of a material, and they should be viewed as reference values which are only meaningful when supported by a full description and evaluation of the observation(s). The use of an occlusive dressing is a severe test and the results are relevant to very few likely human exposure conditions.

° Test report

The test report must include the following information :

- species/strain used;

- physical nature and, where appropriate, concentration and pH value for the test substance;

- tabulation of irritation response data for each individual animal for each observation time period (e.g. 30 - 60 minutes, 24, 48 and 72 hours after patch removal);

- description of any serious lesions observed;

- narrative description of the degree and nature of irritation observed; and

- description of any toxic effects other than dermal irritation.

Interpretation of the results

Extrapolation of the results of dermal irritancy/corrosivity studies in animals to man is valid only to a limited degree. The albino rabbit is more sensitive than man to irritant substances in most cases. The finding of similar results in tests on other animal species may give more weight to extrapolation from animal studies to man.

4. LITERATURE

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APPENDIX 3

EEC TEST METHOD B4 ACUTE TOXICITY - SKIN IRRITATION

1. METHOD

1.1. **Introduction**

See General Introduction Part B (A).

1.2. **Definition**

See General Introduction Part B (B).

1.3. **Reference substances**

None.

1.4. **Principle of the test method**

The substance to be tested is applied in a single dose to the skin of several experimental animals, each animal serving as its own control. The degree of irritation is read and graded after a specific interval, and is further described to provide a complete evaluation of the effects. The duration of the observations should be sufficient to evaluate fully the reversibility of the effects observed.

1.5. **Quality criteria**

None.

1.6. **Description of the test method**

1.6.1. *Preparations*

Approximately 24 hours before testing, fur should be removed, by clipping or shaving, from the dorsal area of the trunk of the animal.

When clipping or shaving the fur, care should be taken to avoid abrading the skin. Only animals with healthy intact skin should be used.

When testing solids (which may be pulverized if considered necessary) the test substance should be moistened sufficiently with water or, where necessary, a suitable vehicle, to ensure good contact with the skin. When vehicles are used, the influence of the vehicle on irritation of skin by the test substance should be taken into account. Liquid test substances are generally used undiluted.

Test substances which are strongly acidic or alkaline need not be tested for primary dermal irritation, owing to their predictable corrosive properties. The testing of materials which have been shown to be very toxic by the dermal route may be unnecessary.

1.6.2. *Test conditions*

1.6.2.1. Experimental animals

Although several mammalian species may be used, the albino rabbit is the preferred species.

1.6.2.2. Number of animals

At least three healthy adult animals are used. Additional animals may be required to clarify equivocal responses.

1.6.2.3. Dose level

Unless there are contra-indications 0,5 ml of liquid or 0,5 g of solid or semi-solid is applied to the test site. Separate animals are not required for an untreated control group. Adjacent areas of untreated skin of each animal serve as controls for the test.

1.6.2.4. Observation period

The duration of the observation period should not be fixed rigidly. It should be sufficient to evaluate fully the reversibility or irreversibility of the effects observed, but need not normally exceed 14 days after application.

1.6.3. *Procedure*

Animals should be caged individually. The test substance should be applied to a small area (approximately 6 cm^2) of skin and covered with a gauze patch, which is held in place with non-irritating tape. In the case of liquids or some pastes it may be necessary to apply the test substance to the gauze patch and then apply that to the skin. The patch should be loosely held in contact with the skin by means of a suitable semi-occlusive dressing for the duration of the exposure period. However, the use of an occlusive dressing may be considered appropriate in certain cases. Access by the animal to the patch and resultant ingestion/inhalation of the test substance should be prevented.

Exposure duration is four hours. If it is suspected that the substance might produce a severe skin reaction, (i.e. be corrosive), the duration of exposure should be reduced (e.g. to one hour or three minutes).

If an exposure period shorter than four hours is used, and a serious skin reaction is observed, the experiment need not be repeated using a four hour exposure period. Longer exposures may be indicated under certain conditions, e.g. expected pattern of human use and exposure. At the end of the exposure period, residual test substance should be removed, where practicable, using water or an appropriate solvent, without altering the existing response or the integrity of the epidermis.

1.6.3.1. Observation and grading

Animals should be observed for signs of erythema and oedema and the response graded at 30 to 60 minutes, and then at 24, 48 and 72 hours after patch removal. Dermal irritation is graded and recorded according to the system in table 1. Further observations may be needed, as necessary, to establish reversibility. In addition to the observation of irritation, any serious lesions such as corrosion (irreversible destruction of skin tissue) and other toxic effects should be fully described.

2. DATA

Data should be summarized in tabular form, showing for each individual animal the irritation gradings for erythema and oedema throughout the observation period. Any serious lesions, a description of the degree and nature of irritation, reversibility or corrosion and any other toxic effect observed should be recorded.

3. REPORTING

3.1. Test report

The test report shall, if possible, include the following information:

— species, strain, source, environmental conditions, diet, etc.,

— test conditions (including the relevant physicochemical properties of chemical, and the technique of skin preparation and cleansing),

— tabulation of irritation response data for each individual animal for each observation time period (e.g. 1, 24, 48 and 72 hours, etc., after patch removal),

— description of any serious lesions observed, including corrosivity,

— description of the degree and nature of irritation observed and any histopathological findings,

— description of any toxic effects other than dermal irritation,

— discussion of the results,

— interpretation of the results.

3.2. Evaluation and interpretation

See General Introduction Part B (C).

4. REFERENCES

See General Introduction Part B (D).

Appendix

TABLE: GRADING OF SKIN REACTION

Erythema and eschar formation

	Value
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to servere erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4

Oedema formation

No oedema	0
Very slight oedema (barely perceptible)	1
Slight oedema (edges of area well defined by definite raising)	2
Moderate oedema (edges raised approximately 1 mm)	3
Severe oedema (raised more than 1 mm and extending beyond the area of exposure)	4

APPENDIX 4

COMPARISON OF TEST METHODS FOR ASSESSING SKIN IRRITATION

	OECD (1981)	EEC (1984)	DRAIZE (1965)
Species	Several species may be used, but albino rabbit is preferred	Similar to OECD	Similar to OECD
Sex	NS*	Similar to OECD	Similar to OECD
Weight	NS	Similar to OECD	Similar to OECD
Number	3 minimum	Similar to OECD	6
Number of Patches per Compound	NS	Similar to OECD	4
Dose	0.5 ml of liquid or	Similar to OECD	Similar to OECD
	0.5 g solid or semi-solid	Similar to OECD	solids dissolved in appropriate solvent
Patch Size	6 cm2	Similar to OECD	1 in2
Patch Material	Gauze	Similar to OECD	2 layers
Patch tape and cover	non-irritating tape, loosely held by semi-occlusive dressing;	Similar to OECD	adhesive tape, entire trunk wrapped with impervious material
	occlusive dressing may be used;	Similar to OECD	NS
	access to patch to be prevented	Similar to OECD	NS
Exposure	4 hrs	Similar to OECD; may be reduced if severe skin reaction is suspected	24 hrs
	may be extended under certain conditions	Similar to OECD	NS
Washing	using water or appropriate solvent	Similar to OECD	NS

APPENDIX 4 (cont.)

	OECD (1981)	EEC (1984)	DRAIZE (1965)
Abrasion	NS	Similar to OECD	abrasion + normal skin for each animal
Examination	1/2 to 1, 24, 45 and 72 hrs after patch removal	Similar to OECD	after 24 hrs exposure and 48 hrs later
Scoring	Draize scoring	Similar to OECD	Similar to OECD
Corrosion	note if corrosive	Similar to OECD	NS
Various	record any serious lesions, reversibility, any other toxic effects	Similar to OECD Similar to OECD Similar to OECD	NS NS NS

* NS = not specified

APPENDIX 5

ALTERNATIVE IN VIVO ASSAYS

1. *INTRODUCTION*

To date a modified Draize patch test in the rabbit has generally been the preferred in vivo experimental method for assessment of skin irritation potential. The irritant response is given a numerical value derived from the gross appearance of the skin (redness and swelling). A critique of the procedure is given in Chapter D. New approaches are under investigation to overcome the subjective nature of this test, to improve its reproducibility with substances which are mild to irritants and to enable more reliable predictions to be made of likely effects in man.

2. *USE OF ALTERNATIVE SPECIES*

The use of the rabbit as a model for skin irritancy has been widely criticised mainly because of anatomical differences between rabbit and human skin and the high sensitivity of the rabbit to irritant compounds, leading to exaggerated reactions compared to humans.

Use of alternative species has been investigated. Most investigators have focused on smaller laboratory animals such as guinea pigs (Roudabush et al., 1965; Steele and Wilhelm, 1966; Nixon et al., 1975; Imokawa, 1979; Stenn, 1979; Andersen and Maibach, 1980), mice (Gloxhuber and Kaestner, 1985; Helman et al., 1986; Patrick et al., 1985, Patrick et al., 1987; Walz, 1984, 1985) and rats (Gray et al., 1985; Yarom et al., 1987). Some studies have been carried out with dogs, pigs and monkeys (Davies et al., 1972; MacMillan et al., 1975; Motoyoshi et al., 1979) but their size and cost limit their acceptability for routine test purposes. Most published data come from a wide variety of application techniques and/or different evaluations of the responses.

Reactions of the guinea pig and the rabbit to various household products were compared by Roudabush et al. (1965) using the conventional Draize technique. Intact guinea pig skin was found to be as sensitive as, or more sensitive than intact rabbit skin.

Only a limited amount of published data, mainly on cosmetic products and ingredients, compares the irritancy potential in these various species and man. Several household products or their components were compared in rabbits, guinea pigs and man by a 4 hour patch test on intact and abraded skin (Nixon et al., 1975). Human beings were found to be the most sensitive to some of the compounds tested. Rabbits and guinea pigs were similarly predictive for screening household products. Testing on intact animal skin provided more reliable results than testing on abraded skin.

MacMillan et al. (1975) compared the irritant response to cosmetic products in rabbits and guinea pigs, beagle dogs and man. Results in rabbits and guinea pigs showed some correlation with those in man, whereas the beagle dog was found to be an unsuitable model.

Davies et al. (1972) assessed the primary skin irritant effects of a range of common cosmetic ingregients on mice, guinea pigs, rabbits, miniature pigs, piglets, dogs, baboons and man and found considerable variability in the irritant response of these species. The rabbit was the only species that elicited reactions similar to or more severe than man. The mouse, guinea pig and beagle dog were suggested to be of similar value, but there was insufficient experience with a wide enough range of chemicals to demonstrate this. Under the particular experimental conditions the baboon and miniature pig did not prove suitable for predictive patch testing.

Motoyoshi et al. (1979) compared the skin irritancy of oils and synthetic perfumes in the rabbit, guinea pig, rat, miniature swine and man. The reddening of the skin, dilatation of blood vessels, swelling and blueing of the skin on Evans blue injection were taken into account for the evaluation of a primary irritant index. In man only reddening of the skin was examined. Except for hydrocarbons, no correlation between animal and human tests were obtained among the oils tested. Skin sensitivity was found to decrease in the following order: rabbit, guinea pig, rat, man and miniature pig.

Kaestner (1977) compared the topical irritancy potential of fatty or fat-derived cosmetic ingredients on the rabbit, guinea pig, hairless mouse and man using a 24 hour patch test. He found the hairless mouse to be the most suitable model for predicting human skin reactions. The rabbit and guinea pig displayed exaggerated reactions.

3. *METHODS TO REDUCE THE SUBJECTIVE NATURE OF OBSERVATION*

The major criticism of the Draize type test is the subjective evaluation of the inflammatory repsonse, particularly erythema and oedema.

Erythema, produced by an increased cutaneous blood flow has been measured in guinea pigs using laser Doppler flowmetry (Froedin and Anderson, 1987). An increase of skin fold thickness upon repeated intradermal injection of various concentrations of sodium laurylsulphate and nonanoic acid was measured by Wahlberg (1983) in guinea pigs, rabbits and one human. In all cases the rabbit was the most "reactive" species. The guinea pig was either less sensitive than man or equally so, depending on the concentration of the applied chemical. Walz (1984, 1985) assessed the number of wrinkles formed on reefing the skin after intradermal application of test solutions in mice. The measurement of damaged skin area by image analysis of excised and dried skin has been performed by Gloxhuber and Kaestner (1985).

Finkelstein et al. (1963) established an animal formalin-trypan blue test procedure for screening substances of low irritancy. Rats, rabbits and guinea pigs were used. After pretreatment of the skin with formaldehyde the test substance was applied for 16 hours by means of a pad. At the same time trypan blue was injected. The degree of irritancy was estimated by the accumulation of dye at the treated site. The authors reported an excellent agreement with human repetitive occlusive patch testing. The technique of vital dye injection was also used by Steele

and Wilhelm (1966) and Patrick et al. (1985) mainly to assess changes of vascular permeability. Infrared detectors to measure the thermal radiation resulting from irritation have been evaluated by Collins and Ring (1972).

4. *ALTERNATIVES TO PATCH TEST TECHNIQUES*

The conventional patches used in animal tests, although effective in many circumstances are crude and do not provide uniform exposure for some substances such as liquid solvents. Jacobs and Martens (1986) exposed rabbits to various solvents by means of a chamber to avoid evaporation from the skin.

Kaminsky et al. (1986) tested two commercial bar soaps on rabbits using a chamber (Hill Top Chamber) and the standard gauze patch method of application. Although no important differences in irritancy scores were observed, the authors concluded that the validity of the chamber technique as a means of applying test materials remains to be determined.

Open application or "skin painting" has been used mainly to assess the irritancy of cosmetics and cosmetic ingredients. Uttley and van Abbe (1973) proposed repeated application on mouse ear skin as a screening procedure prior to human studies. A 4-week open cumulative irritancy test in guinea pigs was described by Andersen and Maibach (1980) as a model for testing and discriminating between low grade irritants. Intradermal injection of the irritant Sudan dye has been used by Stenn (1979) to study epidermal mechanisms in guinea pigs. Although useful for special purposes neither procedure optimises exposure.

Application of different substances on multiple sites on one animal (Van Beek and Vulpen, 1987) or simultaneous application of one substance on three pairs of intact and abraded skin sites of one rabbit, leaving one pair occluded for 4 hours, one for 24 hours and one unoccluded for 24 hours (Cruzan et al., 1986) are other examples of methods of application that differ from those of the classical test.

5. *ASSESSMENT OF ENDPOINTS OTHER THAN FROM ERYTHEMA AND OEDEMA*

Redness (erythema) and swelling (oedema) are the conventional endpoints evaluated when testing for skin irritancy. To achieve a better understanding of the complex inflammatory process more sophisticated techniques are required.

Light- or electronmicroscopic investigation of the morphological or cellular responses such as cell infiltration, cell mitosis, appearance of blood vessels and condition of epidermis (Patrick et al., 1985, 1987; Helman et al., 1986; Yarom et al., 1987), investigation of leucocyte migration (Gray et al., 1985), enzyme inhibition or leakage (Imokawa, 1979, Helman et al., 1986), thymidine uptake and cell mitosis (Stenn, 1979) have all been used to examine the reaction of the skin to irritant substances.

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Appendix 6

Alternative in vitro Methods

6.1: Description and Assessment of in-vitro Methods - cell cultures

Test system	Testing procedure and evaluation	Tested substances	Correlation to in vivo data (Draize Test rabbit skin) or other test results	References
1. 3T3 Swiss mouse fibroblasts 2. XB-2 cell line	1. Cytotoxicity: Effects on cell proliferation, cell metabolism, cell membrane integrity (leakage of enzymes). 2. Keratinocyte differentiation: Examination of test compound to impair or induce the stratification and keratinization characteristics.	Eight dermal irritants and 11 non-irritants, SLS as a reference substance.	Inhibition of stratification gave a high number (27 %) of false positives. In combination with an effect on at least one other parameter a good correlation appeared.	Duffy et al. (1986)
C3H-10 T½ cells, mouse embryo fibroblast cell line, prelabelled with ^{3}H-arachidonic acid (AA) or ^{3}H-choline (Ch)	Day 1: Cells seeded and allowed to attach and grow to sub-confluency. Day 2: Cells labelled with AA or Ch. Day 3: Labelling media removed, cells washed with assay media. Assay media alone or with surfactant added incubation at 37° C for 2 hrs, removing the media and assaying for radioactivity by liquid scintillation counting. Results in terms of total release of label into media.	3 surfactants (LAS, AEOS, Tween 20).	The rank order response (release of arachidonic acid and choline) correlates directly with the rank order irritancy in animals skin. LAS > AEOS > Tween 20	De Leo et al. (1985)

Appendix 6: (continued 2)

6.1: Description and Assessment of in-vitro Methods - cell cultures

Test system	Testing procedure and evaluation	Tested substances	Correlation to in vivo data (Draize Test rabbit skin) or other test results	References
Human keratinocytes	Day 1: Cells seeded and allowed to attach and grow to sub-confluency. Day 4 - 9: Cells labelled with AA. After 24 hrs labelling period labelling media removed, cells washed with assay media. Assay media alone or with surfactant added incubation at 37° C for 2 hrs, removing the media and assaying for radioactivity by liquid scintillation counting. Results in terms of total release of label into media.	3 surfactants (LAS, AEOS, Tween 20)	Same rank order as in vivo skin irritancy. LAS > AEOS > Tween 20	De Leo et al. (1985)
Differentiating keratinocytes	Multilayered differentiating keratinocytes derived from rat sublingual epithelium. Evaluation of total protein content, acid phosphatase release, prolinase activity and light microscopy.	3,3',4,4'tetrachloro biphenyl	Considered suitable for screening compounds of low toxicity.	Hopley et al. (1985)
Human keratinocytes	Cultured human keratinocytes grown as multilayers on 3T3 mouse fibroblasts. Evaluation of lactate dehydrogenase N-acetyl-β-glucosaminidase and K^+ concentration.	Tributyltin, sodium dodecyl sulphate, cyclohexamide, diacetoxyscirpenol and T-2 fusarium toxin. Other test chemicals inferred but not listed.	Correlation with cutaneous toxicity but no data as to correlation with skin irritancy potential.	Mol et al. (1986)

APPENDIX 6: (continued 3)

6.1: Description and Assessment of in-vitro Methods - cell cultures

Test system	Testing procedure and evaluation	Tested substances	Correlation to in vivo data (Draize Test rabbit skin) or other test results	References
1. Guinea-pig kidney fibroblasts	1. Capacity to inhibit DNA-synthesis ([6-^{3}H]-thymidine). 2. Cytolytic release of radioactively-labelled ^{51}Cr-chromate cytoplasmatic proteins.	Surfactants: A homologous series of carbolytic acid sodium soaps (C_8-C_{16}), a series of surfactants of C_{12} chain length but with different chemical head groups.	The effects have been related to the chemical structure of the tested surfactants. Sodium laurate was more potent than either the higher or lower homologues. Exchange of head group and introduction of 3 or 6 ethoxy groups increased the potency to effect DNA synthesis.	Ferguson and Prottey (1976)
2. Rat peritoneal mast cells	Release of histamine			
Rat peritoneal mast cells	Release of histamine: Mast cells were incubated (3 hr, 37° C) in buffered salt solution containing surfactants. Histamine was determined spectrofluorimetrically in the supernatant after centrifugation.	Surfactants: C_8 - C_{18}-alkyl carboxylates, isothionates, sulfates, mono-, tri- and hexaethoxylates and mono- and triethoxylate sulfates.	C_{12} (lauryl) moiety was the most potent at releasing histamine. Higher and lower homologues required a higher concentration. Alkyl sulfates were more potent than isothionates and carboxylates. Ethoxylate sulfates were uniformly similar to their parent alkyl sulfates. Non-ionic ethoxylates were unexpectedly effective (as effective as corresponding alkyl sulfates), at releasing histamine.	Prottey and Ferguson (1976)
HEp-2 cells (continuous cell line derived from human carcinoma of the larynx)	Cell Growth Assay (cell toxicity) Determination of the total protein content of the cells after addition of serial dilutions of the test substances and 24 hr incubation. Results: IC_{50}-values.	A series of n-alkyl benzoates.	Good correlation between IC_{50} and toxicity ranking in vivo so skin irritancy was not carried out.	De Angelis et al. (1986)

Appendix 6: (continued 4)

6.1: Description and Assessment of in-vitro Methods - cell cultures (I. Neutral Red Uptake, II. Uridine Uptake Inhibition)

Test system	Testing procedure and evaluation	Tested substances	Correlation to in vivo data (Draize Test rabbit skin) or other test results	References
I. Permanent cell line: Balb/c-3T3 (mouse fibroblasts)	Neutral Red (NR) Uptake Assay: 1. Cells exposed to various concentrations of test samples (24 hrs); 2. After removing the test samples NR added; 3. Determination of NR-Uptake. NR-concentration in extraction medium is determined spectrophotometrically, result expressed in terms of percent of the control cultures.	14 different chemicals (alcohols, surfactants, aldehyds, salts, amines etc.)	Good correlation (comparative testing with the same classes). Used as a screening test.	Kuenstler et al. (1986a) Kuenstler et al. (1986b)
II. Permanent cell line: Balb/c-3T3 (mouse fibroblasts)	Uridine Uptake Inhibition Assay 1. Cells exposed to various concentrations of test samples (24 hrs) 2. ^{3}H-Uridine added 3. Determination of uridine uptake Results expressed in terms of the control cultures			
KB cells (cell line derived from an oral epidermoid carcinoma)	Uridine Uptake Inhibition Assay. Determination of the UI_{50} (concentration required to induce a 50 % inhibition of Uridine Uptake).	11 different chemicals (n-hexane, chloroform, 1-butanol, tributyltin chloride, benzalkonium chloride, etc.).	An acceptable correlation between UI_{50}-values and skin irritation was found (N = 10 r = 0.92; N = 11 r = 0.80)	Berlin and van der Venne (1989)

Appendix 6: (continued 5)

6.1: Description and Assessment of in-vitro Methods - cell cultures

Test system	Testing procedure and evaluation	Tested substances	Correlation to in vivo data (Draize Test rabbit skin) or other test results	References
KB cells (cell line, derived from an oral epidermoid carcinoma)	1. Incubation (4 hr) in the medium with the test substance 2. Incubation (15 min) together with ^{3}H-Uridine for uridine uptake Quantification of uridine in the cells by liquid scintillation counting Results expressed in percentage compared to control cultures - determination of UI_{50} values (concentration, which caused 50 % inhibition)	2-Butoxyethyl-acetate, methoxy ethanol, n-butanol, acetaldehyde, toluene, hexane, chloroform, benzalkonium chloride, SDS, tributyltin chloride etc.	Linear relationship between the test compound concentration and uridine uptake inhibition. With exception of toluene and 2-butoxyethyl acetate a good correlation was found between UI_{50} and skin irritation.	Dierickx (1987)
3T3-L cells (fibroblast cell line derived from mouse embryos)	Measurement of total cell protein (kenacid blue method for protein), Neutral red uptake (ID_{50}-values), morphological effects (HTD = highest tolerated dose)	30 chemicals	Close correlation between the 3 test methods, no comparison to in vivo data	Riddel et al. (1986)
Human endothelial cell cultures from umbilical cords	Cells in suspension culture. Chemical induced production and release of prostanoids into the media.	Acrolein and N-ethylmaleimide	Induced prostanoid release with two irritant chemicals	Newcombe in Goldberg (1985)

Appendix 6: (continued 6)

6.1: Description and Assessment of in-vitro Methods - cell cultures

Test system	Testing procedure and evaluation	Tested substances	Correlation to in vivo data (Draize Test rabbit skin) or other test results	References
Mouse connective tissue cells and mouse liver cells	Established cell lines in culture. Evaluation of cytotoxicity by total purine and pyrimidine.	16 local anaesthetics and closely related compounds.	Good correlation found between threshold irritant concentration (I.D. rabbit) and toxicity to the cell cultures.	Schmidt et al. (1959)
HEp-2 cells (derived from human carcinoma of larynx)	1) Dose that gave 50 % growth inhibition (GI_{50}), measured as total protein content. 2) Exposing confluent cultures for 24 and 48 hrs to the substances and measuring the total protein (ID_{50}) and acid phosphatase (AcP) content and LDH release in the medium.	10 different chemicals (n-hexane, chloroform, 1-butanol, tributyltin chloride, benzalkonium chloride etc.)	A good agreement with in vivo data ID_{50} (2.5 - 50 mM) corresponded to in vivo mild and moderates irritants (r = 0.84).	Berlin and van der Venne (1989)
KB cells	Uridine uptake inhibition assay - determination of UI_{50} (concentration required to induce a 50 % inhibition of uridine uptake)	2-Methoxyethanol, 2-butoxyethyl acetate, toluene, hexane, n-butanol.	A bad correlation between in vitro and in vivo results.	Jacobs et al. (1989)

Appendix 6: (continued 7)

6.2: Description and Assessment of in-vitro Methods - organ cultures (Skin culture assay)

Test system	Testing procedure and evaluation	Tested substances	Correlation to in vivo data (Draize Test rabbit skin) or other test results	References
Donor animals: hairless mice	Study of irritation in microscale skin cultures (50 mm^2): 1. Preparation of the skin; 2. Pre-incubation (16 hrs, 37° C); 3. Treatment with test product; 4. Incubation (24 hrs, 37° C); 5. Measurements of enzyme activity (GOT, MDH, LDH and glucose) in culture medium.	Different chemicals, alcohols, surfactants	Tests for validation still not finished. More or less good correlation depending on the chemical class of substance tested.	Kuenstler et al. (1986a)
Mouse skin	48 hrs organ culture system. The degree of cellular injury (physical and chemical insults) was reflected by inhibition of in vitro incorporation of ^{3}H-thymidine and ^{14}C-leucine into epidermal DNA and protein and the leakage of intracellular enzymes (LDH, GOT, GPT, MDH) into the culture medium in a dose and time related manner.	Topical application of tributyltin chloride (10 - 100 mole/cm^2 - chemical insult) freeze-thaw treatment (physical insult)	In vitro morphological and biochemical changes were similar to those reported from in vivo topical treatment of rat skin.	Kao et al. (1983)

Appendix 6: (continued 8)

6.2: Description and Assessment of in-vitro Methods - organ cultures (Skin culture assay)

Test system	Testing procedure and evaluation	Tested substances	Correlation to in vivo data (Draize Test rabbit skin) or other test results	References
Mouse skin	Comparison of the histo-pathological changes in the skin of mice. 1. In vivo application: after 20 hrs contact with the skin fixation and staining of the treated skin area. 2. Skin culture incubated 20 hrs with the test substance. Estimation of LDH in the medium and fixation of the skin disc with formalin and staining with haematoxylin and eosin.	DNCB, Croton oil, ethanolamine, epoxyethylbenzene, isopropylmyristate	Good correlation between the magnitude of the skin lesions and the levels of enzyme activity in the culture medium. Morphologic responses of skin maintained in organ culture are an indicator of in vitro skin toxicity.	Helman et al. (1986)
Full thickness, clipped skin of young rabbits	24 - 48 hrs incubation at 32° C in medium. Energy status: (Tissue ATP-concentration), tissue respiration (lactate production) and leakage of selected enzymes (AST, MDH, GLDH) into the medium of the untreated and chemically treated skin during incubation were measured.	3 severe and 3 moderate irritant chemicals, mild, slight and non-irritant chemicals.	Good correlation between reducing lactate production, increasing leakage of AST, MDH and GLDH and irritant potential of the tested chemicals	Pemberton and Oliver (1986)
Hairless mouse and human skin - biochemical changes in vitro	Evaluation of histochemical change and oxygen uptake in hairless mouse skin; amino-acid extraction, measurement of SH groups, K^+ ion permeability and test chemical permeability in human skin.	Nine surfactant based materials	Lack of correlation with a series of in vivo tests using human, rabbit, guinea pig, rat and hairless mouse skin.	Brown (1971)

Appendix 6: (continued 9)

6.2: Description and Assessment of in-vitro Methods - organ cultures (Skin culture assay)

Test system	Testing procedure and evaluation	Tested substances	Correlation to in vivo data (Draize Test rabbit skin) or other test results	References
I. Human skin epithelium	Fragments 2 mm² were explanted together with cockerel plasma, penicillin, embryonic extract with increasing concentrations of test substances. Incubation at 37° C for 8 days. Determination of least injurious dose (LID = smallest quantity to effect the first evidence of injury) and the minimum inhibitory dose (MID = smallest quantity for total inhibition of outgrowth).	18 drugs or chemicals (nickel and barium chloride, antibiotics etc. The results of 4 drugs and 4 antibiotics were compared with clinical data.	Significant differences between the results on human skin and chick spleen results. Comparison with results of closed "cup" patch-test (minimum average irritation for intact human skin) = in general there was a gross correlation.	Livingood and Hu (1954)
II. Embryotic chick spleen explants	Explants of spleen from 18 day chicken embryos. Destination of LID and MID in the same manner as in the human skin, but after 7 days of incubation.			
Calf and human skin disc of 8 mm diameter	Thickness measurements (swelling or contraction of collagen) after 24 hrs exposure to unbuffered or buffered sodium alkyl sulfates (C_8 - C_{18}) solutions.	Sodium alkyl sulfates (C_8 - C_{18})	Good agreement of swelling between the results of calf and human skin, C_{12} chain caused the greatest effect on skin tissue.	Choman (1961)

Appendix 6: (continued 10)

6.2: Description and Assessment of in-vitro Methods - organ cultures (Skin culture assay)

Test system	Testing procedure and evaluation	Tested substances	Correlation to *in vivo* data (Draize Test rabbit skin) or other test results	References
Rat skin slices	Skin slices mounted in glass chambers. Evaluation of leakage of acid phosphatase, lactate dehydrogenase and N-acetyl glucosaminidase into media.	Sodium lauryl-sulphate, sodium laurate and sodium lauryl isothionate (non-irritant)	Leakage at 24 hrs was elevated for irritant chemicals. Further work (unpublished) showed no reliable correlation.	Gibson and Teall (1983)
Human and rabbit skin keratome slices	Skin slices mounted on wire mesh and millipore membrane in a 'Boyden' like diffusion chamber. Evaluation of histology, histochemistry, radioisotope utilisation and enzyme release.	Sodium hydroxide, acetic acid, sodium lauryl sulphate, hydrochloric acid, mercuric chloride, chromium trioxide and formaldehyde.	Some correlation with strong irritants and corrosives but exceptions were observed.	Parish (1985)

Appendix 6: (continued 11)

6.3: Description and Assessment of in-vitro Methods - isolated skin (non-viable epidermal slices)

Test system	Testing procedure and evaluation	Tested substances	Correlation to *in vivo* data (Draize Test rabbit skin) or other test results	References
Epidermal slices (0.4 mm thick) of rat skin	Lysis of stratum corneum measured as a lowering of electrical resistance of the skin slice. 24 hrs or 4 hr contact time with a 20 hrs delay in resistance measurement.	63 chemicals with corrosive (44) or irritant (19) properties (alkylamines, alkylamine salts, ethoxylates, N-oxides, QAV, sodium silicate, inorganic and organic chemicals, organic solvents).	Moderate (64 %) to high (97 %) sensitivity in relation to contact time (4 or 24 hrs) or time of measurement regarding the classification corrosive, irritant and non-irritant chemicals	Oliver et al. (1986)
Epidermal slices of rat and human skin	Lysis of stratum corneum measured as a lowering of electrical resistance of the skin slice, after 1, 4 or 24 hrs contact time with test chemical.	59 chemicals with corrosive (43) and irritant (16) properties (alkylamine, alkyl amine salts and N-oxides, QAV, sodium silicates, formulated organic chemicals)	Human skin response was comparable with that of animal skin *in vitro* and *in vivo*. Animal skin was more susceptible than human skin. High sensivity and good specifity for *in vivo* corrosive chemicals, with human skin 33 % fewer chemicals were positive.	Oliver and Pemberton (1986)

Appendix 6: (continued 12)

6.3: Description and Assessment of in-vitro Methods - isolated skin (non-viable Epidermal slices)

Test system	Testing procedure and evaluation	Tested substances	Correlation to in vivo data (Draize Test rabbit skin) or other test results	References
Rat epidermal slices	Lysis of stratum corneum measured as a lowering of electrical resistance of the skin slice.	63 chemicals with corrosive (41) and irritant (22) properties (alkylamines, alkylamine salts, ethoxylates, N-oxides, QAV, sodium silicate, formulated organic chemicals).	A comparison of in vitro and in vivo classification has shown that this in vitro technique identifies corrosive agents with high precision.	Oliver and Pemberton (1985)
Pig-Epidermis-Test	Swelling behaviour of the isolated epidermis of pigs: Strips of epidermis (1 x 6 cm) are incubated in surfactant solutions (2 % AS) 30 min (39° C, pH 6.5), after removal of the solution, the strips are weighed and after 24 hr drying they are reweighed again.	Surfactants: Alkyl sulfates, alkyl ether sulfates and others.	Good correlation between swelling values and skin compatibility or irritancy (animal and human skin tests). Spearman's rank correlation r = 84 between swelling values and human skin Duhring-Chamber-Test.	Zeidler and Reese (1983)
Epidermal permeability on isolated human epidermis	Radioactive labelled soap solutions were applied with chambers on an area (0.5 cm^2) of isolated epidermis for 7 days.	K-Laurate, K-Octanoate, K-Palmitate, K-Oleate.	Strong correlation between skin irritation (48 hrs patch-test on human) and penetration rate.	Bettley (1963)

Appendix 6: (continued 13)

6.4: Description and Assessment of in-vitro Methods - other methods

Test system	Testing procedure and evaluation	Tested substances	Correlation to *in vivo* data (Draize Test rabbit skin) or other test results	References
Zein-Test	Solubilisation of the protein Zein (Zea mais) by anionic surfactants is measured.	16 anionic surfactants (alkyl sulfates, alkyl ether sulfates and others)	Good correlation with *in vivo* data (animals and humans)	Kästner and Frosch (1981)
Hydrophobicity	Hydrophobicity measured by reverse phase HPLC and TLC	N-Lauroyl (C_{12} fatty acid acyl) amino acids	Primary skin irritancy is inversely proportional to the hydrophobicity, except for glutamic acid.	Sakamoto (1985)
Collagen swelling	Not detailed	Alkyl sulphate and ethoxylated alcohol sulphates	Collagen swelling correlate with *in vivo* clinical irritation assessments.	Blake-Haskins et al. (1985)
Haemolytic Test (rabbit erythrocytes)	Dose response curves were constructed for each surfactant enabling calculation of the concentration required to produce 50 % haemolysis (EC_{50}-haemolysis).	16 anionic surfactants (alkyl sulfates, alkyl ether sulfates and others)	No correlation to *in vivo* skin irritancy	Kästner and Frosch (1981)
Skintex™ Dermal Irritation Assay	The absorption through a permeability barrier (a keratin/collagen membrane on a cellolosic membrane support = synthetic matrices) and the direct reactivity with a protein matrix are quantitated spectrophotometrically.	42 diverse chemicals and formulations (solvents, industrial chemicals, household products, surfactants, cosmetic and pharmaceutical products, acids and bases).	94 % equivalence 37 samples with full equivalence 5 samples with partial equivalence 0 samples with no equivalence	Gordon et al. (1989)

Appendix 6: (continued 14)

6.4: Description and Assessment of in-vitro Methods - other methods

Test system	Testing procedure and evaluation	Tested substances	Correlation to in vivo data (Draize Test rabbit skin) or other test results	References
Lysis of rabbit polymorphonuclear leucocyte (PMNL) granules.	The granules were obtained from a suspension of heterophil PMNL by homogenization, filtering and centrifugation. Lysis of granules by surfactants was measured by a spectrophotometer at 400 nm before and 20 sec after addition of the surfactant solution to the granules suspension. Results in percentage of maximal decrease in optical density (OD_{400})	Anionic surfactants: Dodecyl benzene sulfonate, sodium lauryl sulfate, dodecyl ethoxy sulfate (3 EO), alcohol ethoxylate (C_{12} - C_{15}, 11 EO) sulfate. Cationic surfactants: Cetyl trimethyl ammonium bromide, dodecyl dimethyl ammonium chloride, alkyldimethyl benzyl ammonium chloride. Nonionic surfactants: Alcohol ethoxylate (C_{16}, C_{18}, C_{20}; 10 EO), alcohol ethoxylate (C_{12} - C_{15}; 11 EO)	1. In vivo tests for comparison: Open epicutaneous application 0.5 - 1 % active detergent (AD) once or twice daily for 4 days to the clipped dorsal skin of rats. On day 5 animals were killed. Irritation reactions were assessed and graded macroscopically during and also microscopically at the end of the test. 2. Intradermal injections at concentrations of 0.02, 0.05, 0.1 and 0.5 % AD on the clipped dorsal skin of New Zealand rabbits. The size and the appearance of reactions were assessed at 24 and 48 hr after injection. Comparison of the results of in vitro and in vivo methods: some positive correlations and some important non-correlations were observed, 2 cationic detergents were similar or less effective than sodium lauryl sulfate in causing granule lysis, but were more irritant than this standard, when tested on skin.	Gibson (1980)

Appendix 6: (continued 15)

6.4: Description and Assessment of in-vitro Methods - other methods

Test system	Testing procedure and evaluation	Tested substances	Correlation to in vivo data (Draize Test rabbit skin) or other test results	References
Alkali/acid reserve	Calculations are made based upon the measured pH of a preparation and an assessment of the acid/alkali reserve. Threshold limits to skin corrosive, irritant and non-irritant properties	34 test substances were used in the validation	Good correlations were found between substances, whose skin irritant properties were due to the acidity/ alkalinity of the substance, and patch test results in rabbits.	Young et al. (1988)
"In vitro" electrical resistance measurements (ERM) in rat skin	1. Measure with the inductance/capacitance/resistance (LCR) meter, by placing electrodes on either side of the skin disc. 2. The penetration of sulphorhodamine B (SRB) through chemically treated rat skin "in vitro" SRB content measured at 565 nm on a spectrophotometer	12 different chemicals (n-hexane, chloroform, 1-butanol, tributyltin chloride, benzalkonium chloride etc.)	Good prediction for classification of "corrosive" or "not corrosive", but otherwise a poor correlation (r = 0.60)	Berlin and van der Venne (1989)
Structure activity relationships TOPKAT	Structure activity relationships (SAR) based upon molecular connectivity indexes, sub-structural keys and molecular length parameters.	Equations based upon 526 compounds containing rings and 260 without rings	Prediction rate of chemicals in the data set ranges between 90 and 95 % accuracy.	Enslein et al. (1987)

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APPENDIX 7

HUMAN TESTING

As a preliminary to any human studies it is essential to conduct screening in laboratory animals to gain an initial indication of the materials' irritancy potential and thus to preclude any significant risk to volunteers. Any test subsequently carried out should conform with generally accepted ethical principles (WMA, 1983; Declaration of Helsinki, 1983).

Some form of repeat insult patch testing is used in most instances. The patch test as described by Draize et al. (1944) for use in human studies is essentially similar to that used in rabbits. Occlusive patches are applied to the forearm or back and reactions scored after a 24 or 48 h exposure period. The process is repeated a number of times, the exact number depending on the investigator but commonly between 5 and 10 (Marks, 1983). The number of subjects employed also depends on the investigator; Kligman considers 10 adequate, others consider as many as 60 are required (Marks and Kingston, 1985).

The conventional patch test is crude and suffers from a mechanical disadvantage in that it is difficult to obtain uniform exposure. The patch, for example, frequently slides or wrinkles and the test material often escapes to surrounding skin. Many efforts have been made to overcome these limitations, with varying degrees of success. Pirilä (1975) introduced the Finn Chamber, an aluminium chamber designed to prevent leakage by having an elevated flange round the rim. To overcome the disadvantage of its small capacity (20 µl) Frosch and Kligman (1979-a) subsequently developed the Duhring Chamber, also an aluminium chamber but considered more useful than the Finn Chamber, mainly because of increased capacity (50 - 200 µl). As the concentration and volume of the test material did not change appreciably, the method allowed some quantification and yielded more reproducible results than the conventional patch test. Six or eight Chambers could be applied to the mid-volar forearm (chambers were more difficult to secure on the back).

Occluded patch testing on human skin tends to exaggerate response and can cause severe reactions with some materials (for example volatile materials and soap solutions) which would be virtually non-irritant on normal "open" usage. Various adaptations - such as the use of a semi-occlusive patch (Holland et al., 1950), have been proposed to overcome this. Finkelstein et al. (1963) described a method of conducting human patch tests to assess primary irritancy using the intermittent application of patches and precautions to avoid excessive skin damage.

The conventional methods of irritancy testing are generally time-consuming, burdensome for subjects and costly. Various techniques have thus been proposed to enhance the sensitivity for assessing the irritancy of topically-applied materials. One such procedure is first to compromise the skin by either repeat adhesive taped stripping (Kligman, 1982) or by slight scarification (Frosch and Kligman, 1976).

Other types of tests include those designed to attempt to simulate the intended use of the product in man. These have a particular value for materials such as

soaps or detergents which are difficult to test by the usual repeat insult method. One such is a technique in which the hands and arms of the volunteers are immersed daily for periods of a few days to several weeks in suitable solutions of the test substance. The exposed area is examined daily and scores allocated for any reactions present (Kooyman and Snyder, 1942). More recently a Soap Chamber technique has been proposed which requires only a small number of test subjects and provides comparative data in a short period of time (Frosch and Kligman, 1979-b).

In many instances the wide variations in response reported by investigators employing identical irritants may be explained by differences in the testing procedures. There are many variables which may influence the irritant response of a chemical. These include the concentrations used, the vehicles used, the total amount applied to the skin, the length and method of exposure to the irritant and the site of the patch/chamber (Lansdown, 1972; Mathias and Maibach, 1978). None of these factors has been standardised. In addition the outcome of human patch testing can be influenced by variations in the sensitivity of the individual (e.g. sex, ethnic origin, health, age) and by environmental factors such as temperature and humidity (Lansdown, 1972; Mathias and Maibach, 1978; Marks and Kingston, 1985).

Various methods for evaluating irritant responses have been developed. Kligman and Wooding (1967) suggested that a minimal erythema threshold be used as a visual index of irritation. Thus for strong irritants the concentration/dose which produced perceptible erythema in 50 % of subjects (ID_{50}) might be calculated while for weak irritants a threshold reaction in 50 % of the population (IT_{50}) would be appropriate.

A disadvantage of the patch test is that assessments are subjective and rely entirely on assessment of the appearance of the test site. No objective measurements are made nor is any microscopic examination of the skin carried out to detect changes not visible to the naked eye.

Various techniques are under investigation to measure accurately some of the various parameters affected by irritants. Most of these to date relate to erythema and oedema.

There have been many attempts to measure vasodilatation by increase in skin temperature. A potentially useful non-invasive technique is the Laser-Doppler effect which relies on the fact that a laser light undergoes the phenomenon of Doppler shift when scattered by moving erythrocytes in the cutaneous micro-vessels (Nilsson et al., 1982; Bernardesca and Maibach, 1988). The validity of its use in animals to predict skin irritation in man has not yet been established.

Babulak et al. (1986) claim significant correlation between skin redness measured by the Minolta Chroma Reflectance meter and visually assessed erythema.

Skinfold thickness has been measured by calipers and a high degree of correlation found with results from x-ray techniques (Wahlberg, 1983; Dykes et al., 1976). The use of an ultrasound device is claimed to give a simple, accurate and reproducible measure of the degree of oedema present.

Although most investigators have concentrated on erythema and oedema as indices of irritancy, other workers are developing techniques capable of detecting subtle degrees of skin damage. Thiele (1974) has defined and standardised techniques for assessing skin barrier function by measuring transepidermal water loss, carbon dioxide loss and electrical impedance.

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APPENDIX 8

MEMBERS OF THE TASK FORCE

G. CALVIN (Chairman)	PROCTER AND GAMBLE B - Brussels
M.S. BUTLER	BP UK - Guildford
G. HOLLAND	UNILEVER UK - Sharnbrook
W. KAESTNER	HENKEL D - Dxsseldorf
M. MARTENS *	INSTITUUT VOOR HYGIENE EN EPIDEMIOLOGIE B - Brussel
M.A. PEMBERTON	ICI UK - Macclesfield
M. SCHNEIDER	CIBA GEIGY CH - Stein
B. SIMPSON	SHELL (1) NL - Den Haag
W.J. BONTINCK (Secretary)	ECETOC B - Brussels

* Now at MONSANTO-EUROPE in Brussels

APPENDIX 9

MEMBERS OF THE SCIENTIFIC COMMITTEE

I.F.H. PURCHASE (Chairman), Director, Central Toxicology Laboratory	ICI UK - Alderley Park
M. SHARRATT *, (Vice-Chairman), Group Toxicology Advisor	BP UK - Guildford
H. DE HENAU, European Technical Centre Professional and Regulatory Services	PROCTER AND GAMBLE B - Brussels
H.O. ESSER, Vice-Director Central Function - Product Safety	CIBA-GEIGY CH - Basel
P.A. GILBERT, Head, Environmental Relations	UNILEVER UK - Port Sunlight
I.J. GRAHAM-BRYCE, Head of Environmental Affairs	SHELL NL - Den Haag
B. HILDEBRAND, Head, Department of Toxicology	BASF AG D - Ludwigshafen
J.R. JACKSON, Director Medicine and Health Science	MONSANTO EUROPE B - Brussels
K. KUENSTLER, Head of Toxicology Department	HENKEL D - Duesseldorf
E. LOESER, Head of Institute of Industrial Toxicology	BAYER D - Wuppertal
R. MILLISCHER *, Chief Toxicologist	ATOCHEM F - Paris
W.F. TORDOIR, Head of Occupational Health and Toxicology Division	SHELL NL - Den Haag
H. VERSCHUUREN, Head of Toxicology Department	DOW CHEMICAL CH - Horgen

* Steward responsibility