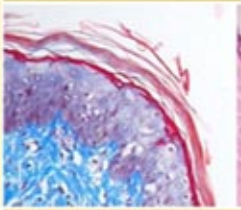
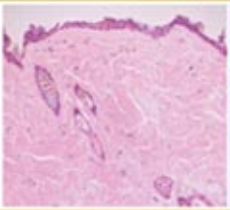
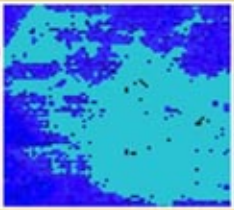
Principles and Practice of

Skin Toxicology

Editors: Robert P. Chilcott and Shirley Price

WILEY

Principles and Practice of Skin Toxicology

Editors

Robert P. Chilcott

Chemical Hazards and Poisons Division, Health Protection Agency, Chilton, UK

and

Shirley Price

School of Biomedical and Molecular Sciences, University of Surrey, UK

John Wiley & Sons, Ltd

Principles and Practice of Skin Toxicology

Principles and Practice of Skin Toxicology

Editors

Robert P. Chilcott

Chemical Hazards and Poisons Division, Health Protection Agency, Chilton, UK

and

Shirley Price

School of Biomedical and Molecular Sciences, University of Surrey, UK

John Wiley & Sons, Ltd

Copyright © 2008

John Wiley & Sons Ltd, The Atrium, Southern Gate, Chichester,
West Sussex PO19 8SQ, England

Telephone (+44) 1243 779777

Email (for orders and customer service enquiries): cs-books@wiley.co.uk
Visit our Home Page on www.wileyeurope.com or www.wiley.com

All Rights Reserved. No part of this publication may be reproduced, stored in a retrieval system or transmitted in any form or by any means, electronic, mechanical, photocopying, recording, scanning or otherwise, except under the terms of the Copyright, Designs and Patents Act 1988 or under the terms of a licence issued by the Copyright Licensing Agency Ltd, 90 Tottenham Court Road, London W1T 4LP, UK, without the permission in writing of the Publisher. Requests to the Publisher should be addressed to the Permissions Department, John Wiley & Sons Ltd, The Atrium, Southern Gate, Chichester, West Sussex PO19 8SQ, England, or emailed to permreq@wiley.co.uk, or faxed to (+44) 1243 770620.

Designations used by companies to distinguish their products are often claimed as trademarks. All brand names and product names used in this book are trade names, service marks, trademarks or registered trademarks of their respective owners. The Publisher is not associated with any product or vendor mentioned in this book.

This publication is designed to provide accurate and authoritative information in regard to the subject matter covered. It is sold on the understanding that the Publisher is not engaged in rendering professional services. If professional advice or other expert assistance is required, the services of a competent professional should be sought.

Other Wiley Editorial Offices

John Wiley & Sons Inc., 111 River Street, Hoboken, NJ 07030, USA

Jossey-Bass, 989 Market Street, San Francisco, CA 94103-1741, USA

Wiley-VCH Verlag GmbH, Boschstr. 12, D-69469 Weinheim, Germany

John Wiley & Sons Australia Ltd, 42 McDougall Street, Milton, Queensland 4064, Australia

John Wiley & Sons (Asia) Pte Ltd, 2 Clementi Loop #02-01, Jin Xing Distripark, Singapore 129809

John Wiley & Sons Canada Ltd, 6045 Freemont Blvd, Mississauga, Ontario, L5R 4J3, Canada

Wiley also publishes its books in a variety of electronic formats. Some content that appears in print may not be available in electronic books.

Library of Congress Cataloging-in-Publication Data

Principles and practice of skin toxicology / editors, Robert P. Chilcott, Shirley Price.
p. ; cm.
Includes bibliographical references and index.
ISBN 978-0-470-51172-5
1. Dermatotoxicology. I. Chilcott, Robert P. II. Price, Shirley, Dr.
[DNLM: 1. Skin Physiology. 2. Skin Absorption. 3. Skin Diseases. WR 102 P957 2008]
RL803.P75 2008
615′.778 – dc22
2008002901

British Library Cataloguing in Publication Data

A catalogue record for this book is available from the British Library

ISBN 978-0-470-51172-5

Typeset in 10/12 Minion by Laserwords Private Limited, Chennai, India
Printed and bound in Singapore by Markono Ltd
This book is printed on acid-free paper responsibly manufactured from sustainable forestry in which at least two trees are planted for each one used for paper production.

Dedications

(RC) For all my teachers, lecturers and professors. Especially the ones that were left in despair.

For Emlyn Evans and Trefor Pedrick. True gentlemen of knowledge.

For all of my family.

For the young ladies in my life:

Caroline, Florence Megan and Charlotte Rose.

(SP) For my partner in crime, Rob Chilcott, the more verbose member of the partnership, and Carolyn, for her patience

For Pete, my husband, and for Jessica and Jonathan for their patience during the editing of this document

For my mentors who taught me the essence of Toxicology – I am still learning!!

Contents

Foreword xv

Preface xvii

Acknowledgements xix

List of contributors xxi

PART I Introduction 1

1 Cutaneous anatomy and function 3
Robert P. Chilcott

1.1 Introduction and scope 3
1.2 Surface features 3
1.3 Functional histology of the epidermis and associated structures 8
1.4 Species differences 13
Summary 15
References 15

2 Biochemistry of the skin 17
Simon C. Wilkinson

2.1 Introduction and scope 17
2.2 Protein synthesis and organisation during epidermal differentiation 18
2.3 Lipid synthesis and organisation during epidermal differentiation 19
2.4 Lipid classes in the stratum corneum 20
2.5 Stratum corneum turnover 23
2.6 Biotransformations in skin 24
Summary 42
References 42

3 Skin photobiology **51**
Mark A. Birch-Machin and Simon C. Wilkinson

3.1 Introduction and scope 51
3.2 Photoprotection and melanogenesis 51
3.3 Increased environmental ultraviolet radiation exposure and its link with photoageing and skin cancer 55
3.4 Mitochondrial DNA as a biomarker of sun exposure in human skin 60
3.5 Apoptosis 61
3.6 Sun protection 63
Summary 65
References 65

PART II Skin Absorption **69**

4 Skin as a route of entry **71**
Simon C. Wilkinson

4.1 Salient anatomical features of the stratum corneum – the 'brick and mortar model' 71
4.2 Species and regional variation in skin structure 72
4.3 Species and regional variation in skin permeability 74
4.4 Intra- and inter-individual variation in percutaneous absorption 75
4.5 Effect of age on skin barrier function 76
4.6 Role of skin appendages 77
4.7 The *in vitro* skin sandwich model 78
4.8 Penetration of particles through appendages 79
Summary 80
References 80

5 Physicochemical Factors Affecting Skin Absorption **83**
Keith R. Brain and Robert P. Chilcott

5.1 Introduction 83
5.2 Physicochemical properties 84
5.3 Exposure considerations 89
Summary 91
References 91

6 Principles of Diffusion and Thermodynamics **93**
W. John Pugh and Robert P. Chilcott

6.1 Introduction and scope 93
6.2 Some definitions pertaining to skin absorption kinetics 94
6.3 Basic concepts of diffusion 97
6.4 Fick's Laws of diffusion 97
6.5 Thermodynamic activity 98
6.6 Skin absorption of a substance from two different vehicles 99
6.7 Partitioning 101
6.8 Diffusivity 102
6.9 Skin absorption data and risk assessments 105
Summary 106
References 106

7 *In vivo* measurements of skin absorption 109
James C. Wakefield and Robert P. Chilcott

7.1 Introduction and scope 109
7.2 Why conduct *in vivo* studies? 110
7.3 Ethics and legislation 110
7.4 Standard methodology: OECD Guideline 427 115
7.5 Alternative *in vivo* methods 119
Summary 126
References 126

8 *In vitro* percutaneous absorption measurements 129
Ruth U. Pendlington

8.1 Introduction and scope 129
8.2 Regulatory guidelines 129
8.3 Why assess percutaneous absorption *in vitro*? 130
8.4 Basic principle of *in vitro* percutaneous absorption measurements 131
8.5 Choice of diffusion cell 131
8.6 Skin membrane considerations 136
8.7 Integrity measurements 137
8.8 Choice of receptor fluid and sampling considerations 138
8.9 Test material considerations 139
8.10 Application of test preparation to the skin 140
8.11 Examples of results from *in vitro* skin absorption studies 142
8.12 What is considered to be absorbed? 146
8.13 Micro-autoradiography 147
Summary 147
References 147

PART III Toxicological Assessment **149**

9 Skin immunology and sensitisation **151**
David A. Basketter

9.1 Introduction 151
9.2 Definitions 151
9.3 Skin sensitisation 152
9.4 Identification of skin sensitisers 155
9.5 Risk assessment 160
9.6 Other types of allergic skin reaction 163
9.7 Future prospects 164
Summary 164
References 165

10 *In vitro* phototoxicity assays **169**
Penny Jones

10.1 Introduction and scope 169
10.2 *In vitro* strategies for phototoxicity testing 169
10.3 The UV/visible absorption spectrum as a pre-screen for phototoxicity 171
10.4 *In vitro* assays for phototoxicity using monolayer cultures 172
10.5 *In vitro* assays for photoallergenicity 174
10.6 *In vitro* assays for phototoxicity using human 3-D skin models 177
Summary 181
References 181

11 *In vitro* alternatives for irritation and corrosion assessment **185**
Penny Jones

11.1 Introduction and scope 185
11.2 Acute dermal irritation/corrosion 185
11.3 Validation/regulatory status of *in vitro* assays for skin corrosion 186
11.4 *In vitro* tests for skin corrosion 188
11.5 Validation/regulatory status of *in vitro* assays for skin irritation 194
11.6 *In vitro* tests for skin irritation 195
Summary 197
References 198

12 Instruments for measuring skin toxicity **201**
Helen Taylor

12.1 Introduction and scope 201

12.2 Skin surface pH 202
12.3 Biomechanical properties 204
12.4 Sebum 205
12.5 Skin surface contours 205
12.6 Thickness 205
12.7 Desquamation 205
12.8 Applications and measurement of transepidermal water loss 206
12.9 Guidance for TEWL measurements 208
12.10 Hydration measurement 209
12.11 Guidance for hydration measurements 212
12.12 Relationship between hydration and dermal toxicity 213
12.13 Colour measurement 213
12.14 Measurement of vascular perfusion 215
12.15 A final word of caution 216
Summary 217
References 217

PART IV Clinical Aspects 221

13 Introduction to dermatology 223
Manjunatha Kalavala and Alex Anstey

13.1 Introduction and scope 223
13.2 Clinical assessment of patient with skin disease 224
13.3 Cutaneous manifestations of disease following exposure to chemicals and pharmaceutical formulations 234
13.4 Overview of standard treatments 241
Summary 243

14 Clinical aspects of phototoxicity 245
Anthony D. Pearse and Alex Anstey

14.1 Introduction and scope 245
14.2 UV-induced skin reactions 247
14.3 Phototoxicity (photoirritancy) reactions 247
14.4 Photosensitive reactions 251
Summary 256
References 256

15 Occupational skin diseases 259
Jon Spiro

15.1 Introduction and scope 259
15.2 Dermatitis 260
15.3 Development of occupational dermatitis 263
15.4 Patterns of occupational dermatitis 264
15.5 Incidence of occupational dermatitis 265
15.6 Effects of dermatitis on work 265
15.7 The outlook in occupational dermatitis 266
15.8 Identification of occupational dermatitis 266
15.9 Other occupational skin disorders 267
15.10 Investigation of a case of dermatitis at work 270
Summary 276
References 276

16 Prevention of occupational skin disease 279
Chris Packham

16.1 Prevention of occupational skin disease 279
16.2 Defining the problem 280
16.3 Material safety data sheets 282
16.4 Chain of responsibility 283
16.5 Managing dermal exposure 284
16.6 Selection and use of personal protective equipment 289
16.7 Protective or 'barrier' creams: do they have a role? 294
16.8 The role of education and training 294
16.9 Conclusions 294
Summary 294
References 294

PART V Regulatory 297

17 Occupational skin exposures: legal aspects 299
Chris Packham

17.1 Introduction and scope 299
17.2 Brief overview of current United Kingdom legislation 300
17.3 The employer's perspective 303
17.4 Hazard identification 304
17.5 Risk assessment 306
17.6 Gloves: a note of caution 309
Summary 310
References 310

18 Safety assessment of cosmetics: an EU perspective **311**
Jo Larner

18.1 Introduction and scope 311
18.2 Overview and scope of Cosmetics Directive 76/768/EC 312
18.3 Overview of the requirements of the EU Cosmetics Directive 315
18.4 Scientific advice 316
18.5 Influence of other legislation 317
18.6 Adverse effects from cosmetics 318
18.7 Toxicity of cosmetic ingredients 320
18.8 The safety assessment 326
18.9 A final consideration 328
Summary 329
References 329
Appendix 18.1 Additional obligations for cosmetic suppliers 330

19 Regulatory dermatotoxicology and international guidelines **333**
Adam Woolley

19.1 Introduction 333
19.2 Regulatory context 334
19.3 Product groups and the human context 335
19.4 Dermal toxicology with the different product groups 336
19.5 Factors in dermal toxicity 338
19.6 Repeat dose dermal toxicology 339
19.7 Classic short-term dermal toxicity studies 341
19.8 Pragmatic considerations 344
Summary 345
References 345

20 Glossary of main terms and abbreviations **347**
James C. Wakefield

Index **358**

Foreword

Dermatologists seldom tire of telling us that the skin is a large and important organ. They are correct. The skin and the lungs are the two organ systems that are in constant and direct contact with the environment from birth to death and are thus, also, of great interest to toxicologists. The skin is susceptible to damage by a range of physical and chemical agents and responds to insult in a variety of ways. In some cases chronic exposure to chemicals leads to serious damage to the skin and to a loss of its essential protective function. Responses also include malignant changes and these, far from being protective, are sometimes lethal.

This book deals with many aspects of skin biology and skin toxicology and the editors, Dr Robert Chilcott and Dr Shirley Price, are to be congratulated on drawing together a distinguished team of authors and on producing a book that will, I think, take a leading place in the literature of his subject. The reader will find that the subject has been addressed in a systematic way beginning, appropriately, with normal structure and function and going on to consider the effects of an unusually wide range of toxic compounds. On reading this book I was struck by the truly scientific approach adopted wherever possible. This, for example in the sections dealing with the physico-chemical aspects of absorption of chemicals, has led to discussion that the beginner will find challenging. But this is appropriate in an advanced monograph and the quantitative approach developed by the authors is both very welcome and much needed in this, and other, areas of toxicology.

This book is the first from the Toxicology Unit of the Chemical Hazards and Poisons Division of the Health Protection Agency: its high standard is the best possible advertisement for our work.

Professor Robert L. Maynard CBE, FBTS
Chemical Hazards and Poisons Division,
Health Protection Agency, Chilton, UK

Preface

The idea for this book was first conceived during the planning of a module in dermal toxicology as part of the Modular Training Programme in Applied Toxicology at the University of Surrey. In preparing a background reading list for the course, it became apparent that there was a niche for a basic, introductory text on the subject. We were very fortunate in that many of the experts who lectured on the course kindly agreed to contribute chapters in their specialist area. Furthermore, considerable effort has been made to ensure that the book is not just a collection of separate monographs on discrete areas of skin toxicology but is an integrated body of general information which draws across a broad spectrum of disciplines.

We hope that this book will succeed in being a useful aid for those wishing to acquire a basic understanding of the principles and practice of skin toxicology.

Robert P. Chilcott
Shirley Price
March 2008

Acknowledgements

Firstly, we wish to thank Professor Robert Maynard for his support, advice and encouragement and for reviewing the draft manuscript.

Perhaps rather obviously, this text would have not been possible without the time and effort of the contributors to whom many thanks are due.

Finally, we would like to thank all our colleagues at the Health Protection Agency and University of Surrey for their understanding and support during times when things didn't quite go exactly to plan.

List of contributors

Alexander Anstey, Gwent Healthcare NHS Trust, Royal Gwent Hospital, Cardiff Road, Newport, Gwent NP20 2UB, UK.

David A. Basketter, St John's Institute of Dermatology, St Thomas' Hospital, London SE1 7EH, UK.

Mark Birch-Machin, Dermatological Sciences, Institute of Cellular Medicine, Newcastle-Upon-Tyne, NE2 4AA, UK.

Keith Brain, Welsh School of Pharmacy, Cardiff University, Cardiff, CF10 3XF and An-eX, Capital Business Park, Cardiff, CF3 2PX, UK.

Robert P. Chilcott, Chemical Hazards and Poisons Division, Centre for Radiation, Chemical and Environmental Hazards, Chilton, Oxfordshire OX11 0RQ, UK.

Penny Jones, Safety and Environmental Assurance Centre, Colworth Science Park, Sharnbrook, Bedford, Bedfordshire MK44 1LQ, UK.

Manjunatha Kalavala, University Hospital of Wales, Heath Park, Cardiff, CF14 4NJ, UK.

Jo Larner, ForthTox Ltd, PO Box 13550, Linlithgow, West Lothian EH49 7YU, UK.

Chris Packham, Enviroderm Services, North Littleton, Evesham, WR11 8QY, UK.

Anthony D Pearse, Cutest Systems Ltd, 214 Whitchurch Road, Cardiff, CF14 3ND, UK.

Ruth U. Pendlington, Safety & Environmental Assurance Centre, Unilever Colworth Science Park, Sharnbrook, Bedford, Bedfordshire MK44 1LQ, UK.

W. John Pugh, Welsh School of Pharmacy, Cardiff University, Redwood Building, King Edward VII Avenue, Cardiff, CF1 3XF, UK.

Jon Spiro, Capita Health Solutions, Didcot, Oxfordshire OX11 0TA, UK.

Helen Taylor, Enviroderm Services, North Littleton, Evesham, WR11 8QY, UK.

James Wakefield, Chemical Hazards and Poisons Division, Centre for Radiation, Chemical and Environmental Hazards, Chilton, Oxfordshire OX11 0RQ, UK.

Simon C. Wilkinson, Medical Toxicology Research Centre, University of Newcastle, Newcastle-Upon-Tyne, NE2 4AA, UK.

Adam Woolley, ForthTox Limited, PO Box 13550, Linlithgow, West Lothian EH49 7YU, UK.

PART I: Introduction

1 Cutaneous anatomy and function

Robert P. Chilcott

Chemical Hazards and Poisons Division, Centre for Radiation, Chemical and Environmental Hazards, Chilton, Oxfordshire OX11 0RQ, UK

Primary Learning Objectives

- *Appreciation of the highly variable morphology of the skin, particularly between anatomical regions (intra-individual variation) and between species.*
- *Basic understanding of the functional anatomy of the epidermis in relation to skin barrier properties.*

1.1 Introduction and scope

In terrestrial mammals, the integument contributes to a variety of physiological functions including thermoregulation, immune defence and the prevention of catastrophic water loss. It is the barrier property of skin that is of specific relevance to dermal toxicology (dermatotoxicology), so the purpose of this chapter is to outline the anatomical and histological features that contribute to skin barrier function. Therefore, this chapter concentrates on the outermost (epidermal) layers associated with protecting the skin from the ingress of xenobiotics. More detailed information on the structure and function of the dermis and hypodermis may be found elsewhere (Forslind *et al.* 2004; Freinkel and Woodley 2001; Montagna 1962).

1.2 Surface features

> The skin is not a homogenous covering. Its structure and function vary considerably, resulting in regional variations in permeability that may span several orders of magnitude.

The protective function of the human integument is reflected by its relatively small surface area ($\sim$2 m^2). In contrast, the lung and gastrointestinal tract have evolved to facilitate absorption and so have much higher surface areas ($\sim$150 and 200 m^2, respectively).

Principles and Practice of Skin Toxicology Edited by Robert P. Chilcott and Shirley Price
© 2008 John Wiley & Sons, Ltd

Human skin can essentially be divided into two types; glabrous (non-hairy) and non-glabrous. The former is generally thicker and less permeable than the latter and is limited to areas such as the palms of the hand, soles of the feet and lips.

Skin surface morphology varies according to anatomical region and this is particularly evident in humans where localised, functional adaptations have resulted in overt differences in appearance (Figure 1.1). Regional differences include variation in epidermal thickness and the density of hair follicles, sweat and sebaceous ducts (Table 1.1). Other differences include the presence or absence of ridges and sulci (dermatoglyphs), flexure lines, surface roughness and extent of oily (sebaceous) deposits. It is conceivable that the presence of furrows, wrinkles or dermatoglyphs may affect the skin surface distribution of liquids applied to the skin by providing a means for capillary motion away from the point of contact, thus facilitating skin surface spreading and so increasing the area of skin contamination (Figure 1.2). However, the influence of the skin surface micro-relief on skin absorption has not been thoroughly investigated. The human integument is also characterised by lines of cleavage referred to as Langer's lines, which result from the (congenital) orientation of collagen fibres within the dermis. The lines of Langer are of clinical significance in surgical procedures: incisions made

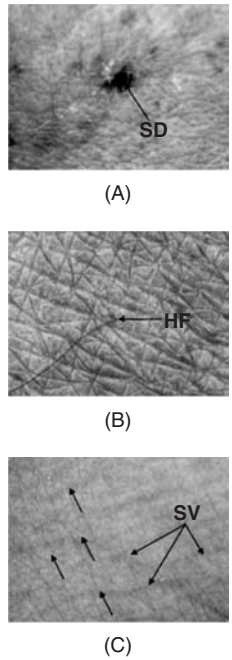


Figure 1.1 Skin surface over the inner ear (A), hand (dorsum) (B) and wrist (ventral aspect) (C). Some dermatoglyphs are discernible on the inner ear site, but the main feature of the picture is the enlarged sebaceous duct (SD) specific to this anatomical region. The duct is surrounded by fine (velous) hairs, which provide some limited protection against foreign objects. Dermatoglyphics are pronounced on the back of the hand and are occasionally punctuated by hair follicles (HF) sprouting hair of medium coarseness. The wrist area is largely free of hair but flexure lines (indicated by small arrows) can be clearly seen running in parallel. Also visible are superficial veins (SV) of this region. Photographs courtesy of Dr Helen Packham, Enviroderm Services. A full-colour version of this figure appears in the colour plate section of this book

Table 1.1 Selection of quantitative data of human skin characteristics

Anatomical Location	Thickness		Kinetics		Appendageal Density		Temperature (°C)	Surface Lipids (μg cm^{-2})
	Epidermis (μm)	Dermis (μm)	Turnover (days)	Desquamation (g m^{-2} day^{-1})	Glands (cm^{-2})	Follicles (cm^{-2})		
Finger	547	1207			0–50			
Thigh	61	1298		0.3		55	31.3	5
Forearm	53	1118	13	0.1			32.1	6
Abdomen	42	2163	9.6			70	32.6	
Thorax	51	1676				75	32.7	10
Axilla	44	1186			100	65		
Back	71	2326	15			65		7
Pubis	45	1014						
Sole	1159	1534						
Face	52	2271				700		12
Forehead	82	1500	6.3	1.7	400–900	765	34.3	24
Palms	575	1100	30	3.5				
Scalp			9.6	2.1		350		

Turnover (kinetics) refers to the average time taken for a cell in the stratum basale to reach the stratum corneum.

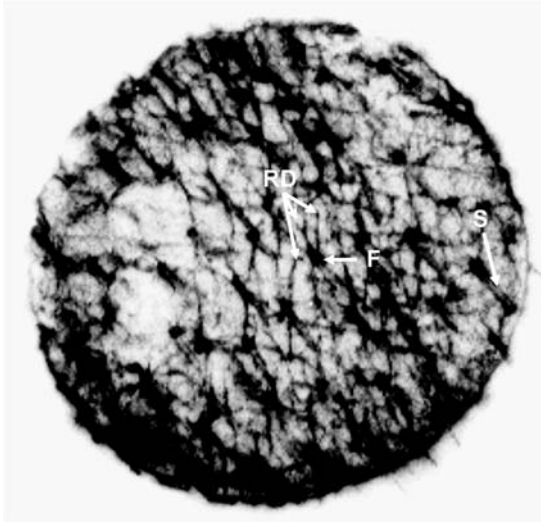


Figure 1.2 Surface autoradiograph of pig skin exposed to a single, discrete droplet (100 μl) of ^{14}C-radiolabelled benzene under unoccluded conditions. Radioactive material (indicated by the dark areas) can be seen to preferentially partition into hair follicles (F) and hair shafts (S). Dermatoglyphics can be seen radiating from (and interconnecting) adjacent hair follicles (RD), indicative of capillary movement along the sulci

parallel to Langer's lines generally heal more readily and are less likely to form scar tissue (Monaco and Grumbine 1986).

Numerous studies have demonstrated that skin permeability is also subject to anatomical variation (Feldmann and Maibach 1967, Maibach *et al.* 1971, Rougier *et al.* 1986). Whilst epidermal thickness is commonly considered to be a prime determinant of regional skin permeability, such generalisations should be interpreted with caution (for example, see Figure 1.3) as other factors such as the regional lipid content (Table 1.1) or morphology of the stratum corneum may be implicated (Rougier *et al.* 1988).

There is a superficial 'layer' of skin that is often overlooked in dermal toxicology: the 'acid mantle'. This forms a thin film on the skin surface and is comprised of sebum, corneocyte debris and residual material from sweat. This mixture of substances generally imparts a low pH on the skin surface owing to the presence of free fatty acids and, being predominantly lipophilic, may conceivably influence the partitioning of substances into the skin or act as an adsorbent matrix to trap microscopic particles such as dirt, dust or powders.

The predominant component of the acid mantle is sebum, considered by some to be vestigial (Kligman 1963). Sebum is mainly composed of triglycerides, wax esters and squalene, with the actual composition (and amount being secreted) varying according to anatomical location (Figure 1.4).

The evolutionary significance of sebum has been subject to much debate and several putative functions including anti-microbial activity, 'water-proofing' and 'sweat-sheet' formation have been proposed (Porter 2001). However, sebum may represent a significant route of excretion for lipophilic substances (Faergemann *et al.* 1993; Iida *et al.* 1999) and may be of physiological significance for the delivery of vitamin E to the skin surface where it could act as a superficial antioxidant (Thiele *et al.* 1999).

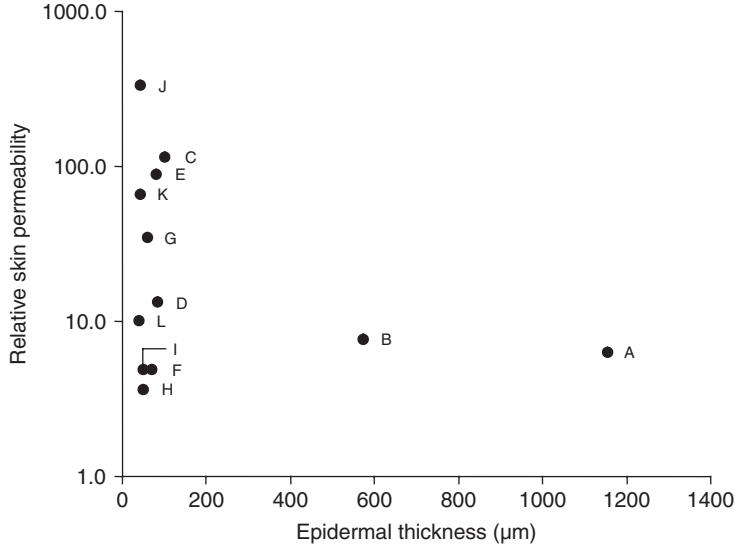


Figure 1.3 Epidermal thickness as a function of skin permeability (expressed relative to the least permeable site, the back of the hand) measured in human volunteers to the nerve agent VX (O-ethyl-S-[2(diisopropylamino)ethyl] methylphosphonothioate). Anatomical regions (in order thickest to thinnest): A = plantar; B = palmar; C = cheek; D = nape of neck; E = forehead; F = back; G = groin; H = forearm (ventral aspect); I = forearm (dorsal aspect); J = scrotum; K = axilla; L = abdomen

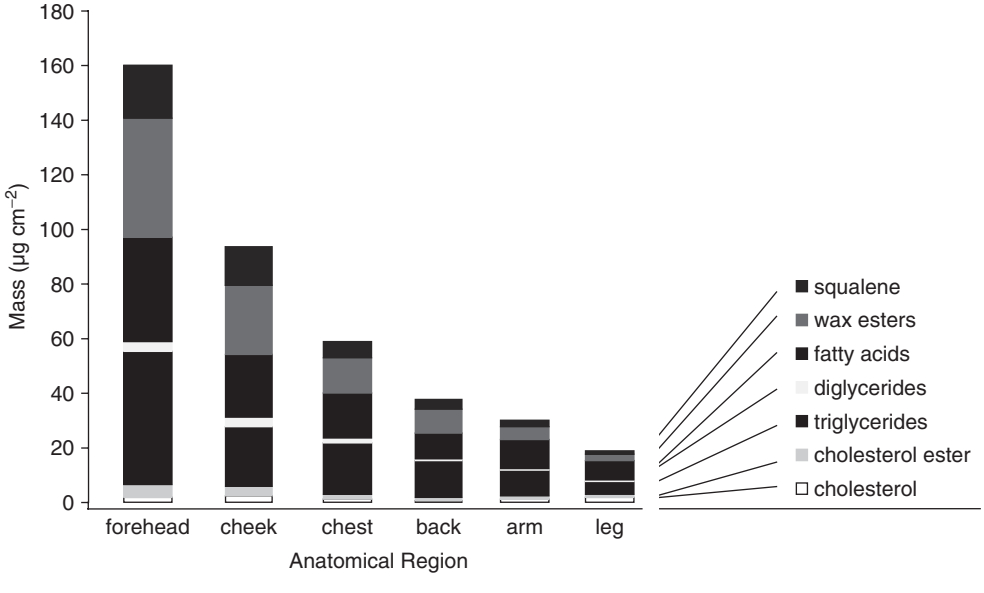


Figure 1.4 Quantity and composition of sebum, according to anatomical location (Greene *et al.* 1970, Reprinted by permission from Macmillan Publishers Ltd)

Clearly, the distribution and composition of the acid mantle will be dictated to some degree by the regional distribution of sweat and sebaceous glands. The former are found in highest abundance on palmar–plantar regions where the latter are absent. Sebaceous glands are generally associated with hair follicles, though in some areas such as the nipples, labia minora and prepuce, they open directly onto the skin surface. The highest densities of sebaceous glands are found on the scalp and face, with the forehead secreting the largest quantity of sebum per unit area of skin (Snyder *et al.* 1981).

It is possible that certain protocols involved in preparing skin tissue for *in vitro* absorption studies may alter the characteristics of the acid mantle. For example, the practice of briefly immersing skin in hot water (a standard method for the preparation of epidermal membranes) may perturb or remove the acid mantle from the skin surface. Consequently, this could affect partitioning of chemicals into the skin and so alter skin absorption kinetics.

1.3 Functional histology of the epidermis and associated structures

The upper layer of the skin (epidermis) is mainly responsible for providing protection against the ingress of chemicals and is subject to a cycle of renewal which takes 5–30 days.

The skin is a multi-layered (veneered or stratified) structure comprising three principal layers, namely, the epidermis, dermis (corium) and hypodermis (Figure 1.5). In general, the epidermis accounts for ~5% of the combined thickness of human epidermis and dermis except in regions that are exposed to physical stress such as palmar-plantar skin where the proportion of epidermis is ~60% (Table 1.1). The epidermis provides protection against xenobiotics,

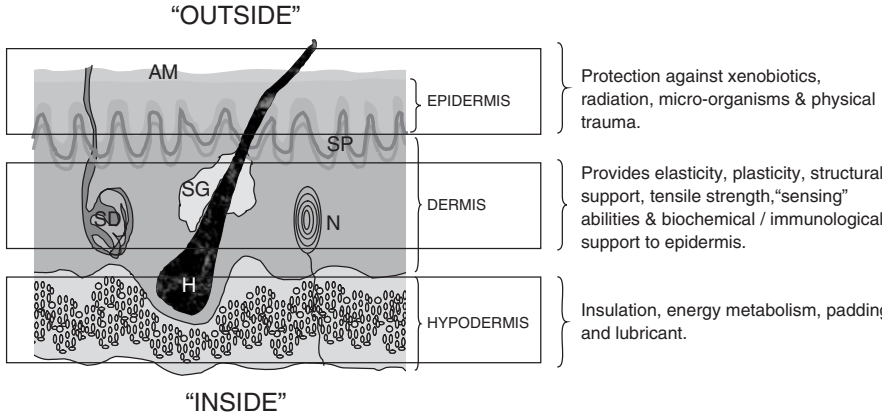


Figure 1.5 Schematic representation of skin structure and associated functions. Note that the relative thickness of each layer is not to scale (see text). Several adnexal structures are shown (SP = superficial plexus; SG = sebaceous gland; SD = sweat duct; N = Pacinian corpuscle; H = hair). In humans the skin is covered with a thin layer of lipids known as the acid mantle (AM), which comprises sebum, cell debris and sweat residua. A full-colour version of this figure appears in the colour plate section of this book

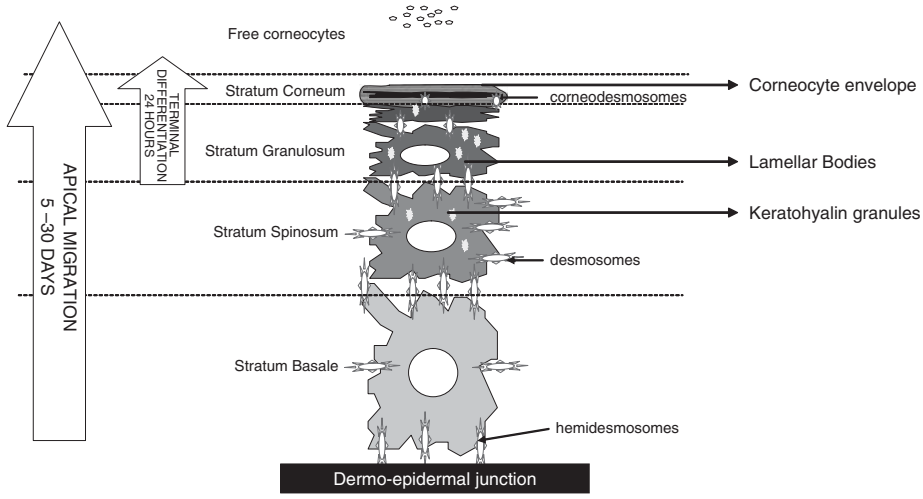


Figure 1.6 Schematic representation of individual cells of the epidermis. The basal cells (anchored to the dermo–epidermal junction via hemidesmosomes) undergo apical migration towards the skin surface whilst undergoing a process of differentiation. The first stage of differentiation results in the appearance of spinous cells (stratum spinosum) in which adjacent cells are interconnected by tight junctions (desmosomes). Keratohyaline granules, which contain profilaggrin (which facilitates the bundling of keratin in later stages of terminal differentiation) and filaggrin (the putative precursor of natural moisturising factor, NMF), begin to appear. The production of lamellar bodies is consistent with the formation of the stratum granulosum, exocytosis of which forms the lipid matrix in which corneocytes are embedded. During apical migration, cohesion of desmosomes is gradually degraded by the action of enzymes culminating the loss (sloughing) of free corneocytes thereby regulating the thickness of the stratum corneum

micro-organisms, some forms of radiation and, to a limited extent, mechanical trauma. Most of these functions are fulfilled by the stratum corneum, the outermost layer of the skin.

The epidermis is predominantly (>90%) populated by keratinocytes that continuously undergo apical migration from the stratum basale. During migration, keratinocytes undergo several stages of differentiation, which can be identified histologically as the stratum spinosum, stratum granulosum and stratum corneum (Figure 1.6). In regions where the epidermis is thicker, an additional layer (between the stratum granulosum and the stratum corneum) termed the stratum lucidum may be observed.

The nomenclature of the different epidermal layers reflects position or cellular morphology (Figure 1.6). Basal cells are sited at the base of the epidermis. Cells of the stratum spinosum radiate small spines, though this appearance is now thought to be an artefact of the light microscope rather than a definitive structural feature. Cells of the stratum granulosum have inclusion bodies (precursors of the lipid matrix of the stratum corneum) that impart a granular appearance.

Occasionally, older terminology may be found in the literature (Table 1.2). For example, the basal and spinosum layers may be referred to as the stratum Malpighii (after the Italian physician Marcello Malpighi, circa 1628–1694).

Apical migration and differentiation, from basal cell to fully formed corneocyte, takes approximately 5–30 days (Figure 1.6), depending on anatomical region (Table 1.1). In

Table 1.2 Alternative histological nomenclature of the epidermal layers, with typical thickness measurement (for human skin)

Current Nomenclature	Thickness (μm)	Alternative Nomenclature
Stratum corneum	10–20	Horny layer
Stratum granulosum		Granular layer
Stratum spinosum	50–100	Prickle cell/spinous layer, acanthocyte (refers to individual cell)
Stratum basal		Stratum germinativum, rootlets

contrast, the final stage of (terminal) differentiation may occur in less than 24 hours and enables prompt repair of superficial damage to the stratum corneum. The gradual degradation of cell–cell adhesion (mediated via desmosomes) ultimately leads to loss of corneocytes (sloughing) and can account for up to one gramme of material (the main constituent of 'house dust') per adult per day (Snyder *et al.* 1981).

Other types of cell present in the epidermis include Langerhans cells (involved with antigen presentation) and melanocytes (which synthesise the photo-protectant, melanin). The mobile nature of these two (dendritic) cell types enables them to migrate and populate the interstitial space between keratinocytes, and there is growing evidence that melanocytes, Langerhans and keratinocytes form functional units within the epidermis (Nordlund and Boissy 2001). Indeed, melanocytes interact with a predefined number of keratinocytes within the basal epidermis (the so-called melanocyte–keratinocyte unit) according to set ratios depending on constitutive (normal) skin colour (Seiberg 2001). The role of melanocytes and Langerhan's cells are considered in more detail in Chapters 3 and 10, respectively.

The outmost layer of the epidermis, the stratum corneum, is the predominant barrier layer. This property arises from the arrangement of cornified cells embedded in a lipid matrix known as the 'brick and mortar' structure.

Terminally differentiated keratinocytes of the stratum corneum are known as corneocytes and are largely devoid of normal cellular functions, being predominantly composed of protein (keratin) and a remnant of the original cell wall ('corneocyte envelope').

The ultrastructure of the stratum corneum is described by the 'brick and mortar model' (Michaels *et al.* 1975). The functional implication of this architecture is that some skin penetrants must diffuse via a long and tortuous route between adjacent corneocytes, thus reducing their rate of absorption. This is known as the intercellular route (Figure 1.7). In contrast, some chemicals may diffuse equally through both corneocytes and the lipid mortar, resulting in a transcellular route (Figure 1.7). Both inter and intracellular routes are collectively known as bulk pathways. A third, potential route of entry across the skin involves diffusion down hair follicles and into sebaceous glands or via sweat ducts (Figure 1.7). These are referred to as 'shunt pathways' and their contribution to skin absorption is currently a contentious issue. Historically, the relative role of the shunt and bulk transport pathways have been likened to an army crossing marshland that contains a few narrow bridges: whilst a small number of

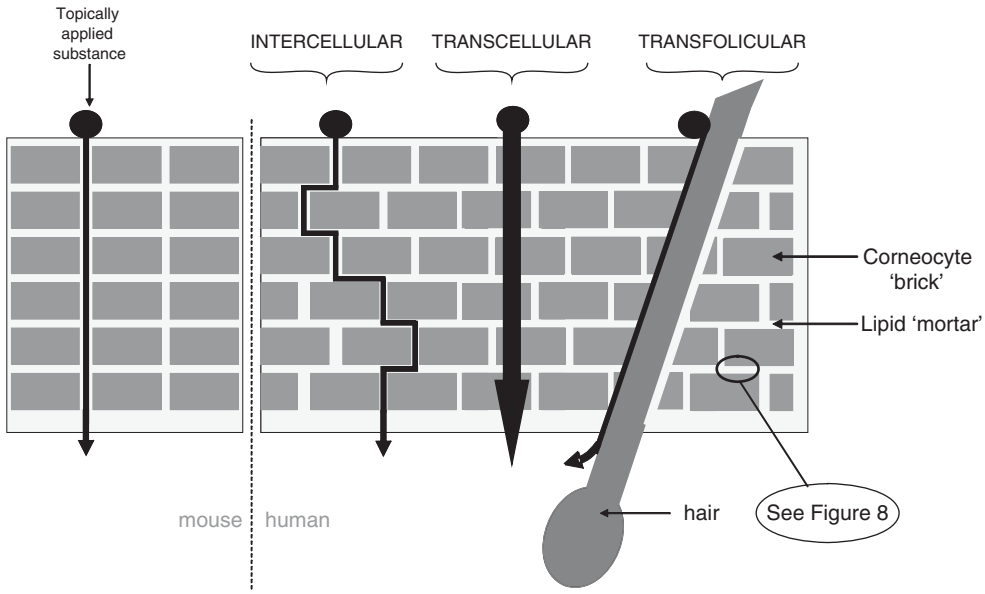


Figure 1.7 Schematic representation of arrangement of corneocytes in mouse and human stratum corneum ('brick and mortar' model). The stacked (columnar) arrangement of corneocytes in mouse stratum corneum facilitates a relatively short route for diffusion. In contrast, the oblique arrangement of corneocytes in human stratum corneum compels molecules (diffusing via the intercellular route) to take a long and tortuous route. The two other routes of entry (transcellular and transfollicular) are shown for comparison. The structure of the lipid mortar is detailed in Figure 1.8

soldiers can rapidly march across the bridges in single file, the majority have to trudge slowly through the boggy ground (Scheuplein 1976). This analogy pertains to the relatively small surface area occupied by hair follicles. For example, the average width of a scalp hair is ~50 μm and this region contains ~300 hair follicles per cm^2. Thus, the total surface area occupied by hair follicles per cm^2 of scalp skin is exceedingly small: approximately 0.007 cm^2. However, this does not take into account the fact that a hair follicle is a three dimensional structure that penetrates deep into the dermis. Assuming that an average hair follicle is 500 μm deep (and approximates in shape to a cylinder), then the total surface area of hair follicles per cm^2 can be calculated to be ~0.95 cm^2. Thus, on the scalp at least, the presence of hair follicles essentially doubles the surface area available for skin absorption.

> Skin appendages such as hair follicles provide a potential 'short-cut' for skin absorption by penetrating directly into the dermis. However, the practical relevance of such shunt pathways is of some considerable debate.

It is important to note that such shunt pathways are not the biological equivalent of intergalactic wormholes and do not provide a paranormal route of entry into the skin. Hair follicles and other appendageal structures are generally lined with cornified cells and so diffusion from the follicle into the dermis is still subject to the same barrier layer as is present on the skin surface.

Furthermore, the follicles are usually full of very lipophilic material (sebum) and so effectively exclude hydrophilic substances or partition and bind very lipophilic materials. Thus, the appearance of a chemical within hair follicles in the dermal region of skin does not equate to dermal delivery: the substance is still on the outside of the body! However, it should be noted that for some chemicals (hydrophilic, charged molecules; Chapter 6), the shunt pathways may represent the predominant route of penetration, although the overall rate of absorption of such compounds is generally very low. The relative contribution of each transport pathway is discussed in more detail in Chapter 5.

Whilst corneocytes can be considered to be hydrophilic domains, they are surrounded by a lipid-rich matrix mainly comprising ceramides, free fatty acids and cholesterol (Downing *et al.* 1987). Thus, the intercellular domain is predominantly a lipophilic environment. This combination imparts a degree of 'amphiphobicity' upon the stratum corneum, providing limited protection against both lipophilic and hydrophilic penetrants. The composition and underlying metabolism of stratum corneum lipids (as opposed to the skin surface lipids discussed above) is reviewed in Chapter 2.

The molecular packing of the lipid matrix within the inter-corneocyte spaces effectively sets an upper limit on the physical size of molecules that may penetrate the stratum corneum (Figure 1.8). This is referred to as the 'rule of 500' (Bos and Meinardi 2000) since few substances with a molecular weight above 500 Da are capable of passive diffusion through the skin. However, recent studies suggest that ultra-fine particles (also termed nanoparticles)

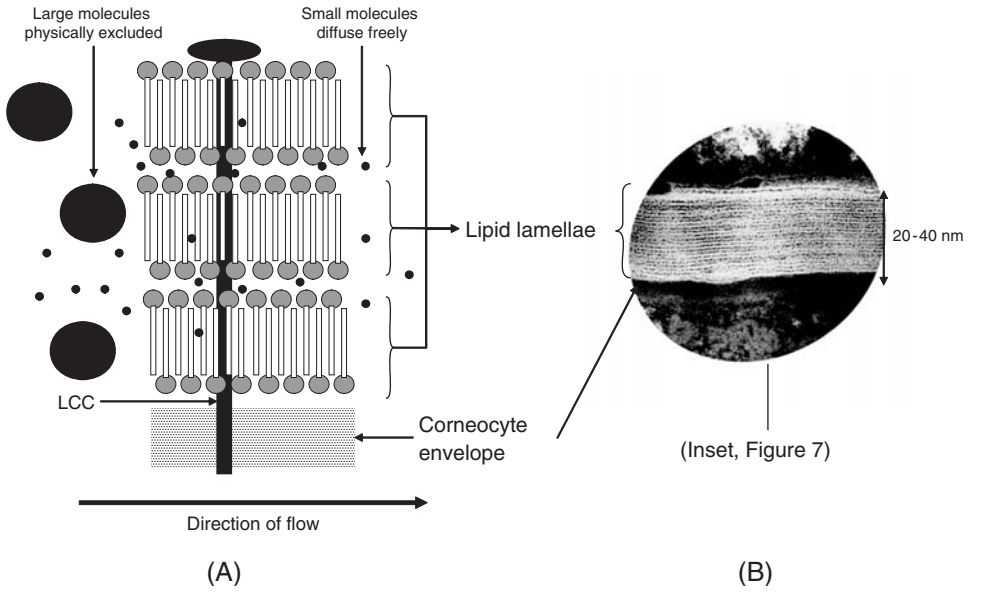


Figure 1.8 Arrangement of lipid lamellae within the inter-corneocyte space of the stratum corneum. (A) Empirical representation of adjacent lipid layers showing the physical exclusion of large molecules. The lamellae are 'riveted' to the outer corneocyte envelope by a long-chain ceramide (LCC). (B) Electron micrograph of the inter-corneocyte domain, demonstrating the lipid lamellar packing (courtesy of Professor Joke Bouwstra, University of Leiden, The Netherlands)

have the potential to penetrate the stratum corneum (Ryman-Rasmussen *et al.* 2006). Whilst this is largely unexpected in terms of molecular weight, the diameter of such particles is less than the distance that separates adjacent corneocytes and thus diffusion through the stratum corneum is plausible. Given current health concerns over the increasing use of nanoparticles in consumer products, it is likely that a great deal more research will be conducted in this relatively new area.

The epidermis is anchored to the dermis via a continuous, protein-rich region termed the dermo-epidermal junction. This structure is highly invaginated and forms characteristic ('rete') ridges on skin sections that are readily discernible under the light microscope. The underlying blood supply (superficial plexus) interdigitates with the rete ridges, thus providing a large surface area for the bi-directional transfer of nutrients, oxygen and waste products. Chemicals that are able to traverse the epidermis are generally subject to systemic absorption by the superficial (papillary) plexus at this anatomical region (Figure 1.5) and so the dermis and hypodermis are not generally relevant to the percutaneous absorption kinetics of many substances. However, if the peripheral blood supply (i.e. the superficial plexus) is reduced by vasoconstriction, systemic uptake may be diminished, resulting in accumulation of penetrant within the dermal tissue; conversely, vasodilation may increase systemic uptake from the superficial plexus (Brain *et al.* 2006, Rommen *et al.* 1999. Alternatively, the 'ground substance' of the dermis essentially represents an aqueous gel environment and this will provide an additional barrier to the ingress of strongly lipophilic substances (Flynn *et al.* 1981). Therefore, it is important when conducting *in vitro* skin absorption studies to select the most appropriate tissue preparation: epidermal membranes are arguably the most relevant model since penetration through this layer *in vivo* results in contact with the circulatory system (superficial plexus; see Figure 1.5). The presence of dermal tissue in dermatomed skin is therefore representative of an additional barrier that is not normally present *in vivo* and may lead to an underestimate of skin absorption for lipophilic substances (Chapter 9).

1.4 Species differences

Human skin is remarkable in many respects from most other mammals and this is of relevance to the interpretation of toxicological data obtained from animal models such as the rat, mouse and guinea pig.

The most obvious difference between human and animal models is pelage density (Figure 1.9): a thick coat of hair provides a substantial degree of protection against the ingress of xenobiotics and exposure to radiation. As a possible consequence of this evolutionary divergence, the stratum corneum of rodents and lagomorphs is generally more permeable and considerably more fragile than 'naked' species such as pig and human (see legend, Figure 1.9). This difference is manifest when preparing tissue samples for *in vitro* skin absorption studies: human and (to some extent) pig skin can be used to prepare strong, coherent sheets of stratum corneum or epidermis that retain their physical durability for several months at room temperature. In contrast, it is practically impossible to produce similar tissue preparations for rodent skin, although limited success can be achieved with sodium bromide separation of neonatal rat skin (Scott *et al.* 1986). This species difference in pelage density between human and rodent skin is

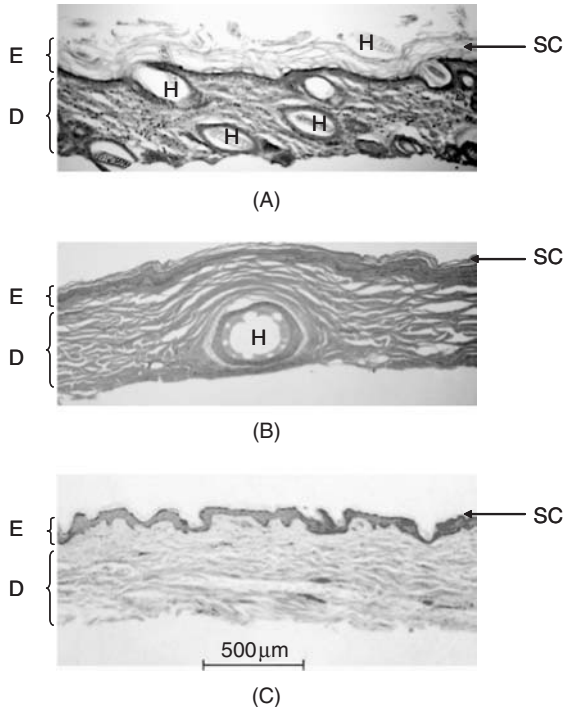


Figure 1.9 Representative sections of dermatomed guinea pig (A), pig (B) and human (C) skin. Two principal layers are discernible in each section: the epidermis (E) and dermis (D). Note that guinea pig stratum corneum (SC) appears as an incoherent, flaky layer whereas SC of pig and human retains a flatter, more compact appearance. A large number of hairs (H) are present in the guinea pig section. A full-colour version of this figure appears in the colour plate section of this book

of particular relevance when interpreting toxicological studies, especially if the test substance has demonstrable affinity for hair or associated (appendageal) structures.

Animal skin also contains a layer of muscle (*panniculus carnosus*), which is largely absent in humans with the exception of the *platysma*, situated over the ventral aspect of the neck. This is of relevance when conducting *in vitro* skin absorption studies with full thickness animal skin, as the *panniculus carnosus* represents an additional barrier layer to diffusion (although this can be avoided by the use of skin dermatomed to an appropriate thickness).

Mouse skin is generally more permeable than human and most other species. This may in part be attributable to the arrangement of corneocytes within the stratum corneum (Bergstresser and Chapman 1980). In human skin, corneocytes are normally offset between adjacent rows and this provides a tortuous route for intercellular transport. In contrast, murine corneocytes are arranged in columns (stacks) and so may offer a more direct route for the ingress of xenobiotics (Figure 1.7).

From a histological perspective, the pig (*sus scrofa*) is the species that bears most resemblance to human (Figure 1.9) and so the use of strains with reduced growth rates (such as the Göttingen minipig®) are becoming increasingly common in toxicological and pharmacological studies.

Summary

- Human skin presents a barrier to the ingress of many xenobiotics and has a correspondingly low surface area in comparison with other externalised organs such as the lung and gastrointestinal tract.
- The integument cannot be considered to be a homogeneous organ as there are substantial regional (anatomical) differences in structure and function such as permeability (which may span several orders of magnitude).
- There are three principal skin layers: epidermis, dermis and hypodermis. The former is primarily responsible for maintaining skin barrier function.
- The relative impermeability of the skin results from the structure and composition of the stratum corneum (the outermost layer of the epidermis), which is subject to a continuous cycle of regeneration through apical migration and terminal differentiation of epidermal cells (keratinocytes).
- There is considerable species variation in skin structure and function. The pig is arguably the most relevant animal model although rodents are currently the species of choice for toxicological evaluation.

References

Bergstresser, P.R. and Chapman, S.L. (1980). Maturation of normal human epidermis without an ordered structure. *Br J Dermatol* **102**(6): 641–8.

Bos, J.D. and Meinardi, M.M. (2000). The 500 Dalton rule for the skin penetration of chemical compounds and drugs. *Exp Dermatol* **9**(3): 165–9.

Brain, K.R., Green, D.M., Dykes, P.J. *et al.* (2006). The role of menthol in skin penetration from topical formulations of ibuprofen 5% *in vivo*. *Skin Pharmacol Physiol* **19**(1): 17–21.

Downing, D.T., Stewart, M.E., Wertz, P.W. *et al.* (1987). Skin lipids: an update. *J Invest Dermatol* **88**(3 Suppl): 2s–6s.

Faergemann, J., Zehender, H., Denouel, J. and Millerioux, L. (1993). Levels of terbinafine in plasma, stratum corneum, dermis–epidermis (without stratum corneum), sebum, hair and nails during and after 250 mg terbinafine orally once per day for four weeks. *Acta Derm Venereol* **73**(4): 305–9.

Feldmann, R.J. and Maibach, H.I. (1967). Regional variation in percutaneous penetration of 14C cortisol in man. *J Invest Dermatol* **48**(2): 181–3.

Flynn, G.L., Durrheim, H. and Higuchi, W.I. (1981). Permeation of hairless mouse skin II: membrane sectioning techniques and influence on alkanol permeabilities. *J Pharm Sci* **70**(1): 52–6.

Forslind, B., Lindberg, M. and Norlen, L. (eds). (2004). *Skin, hair, and nails*. Marcel Dekker Inc., New York.

Freinkel, R.K. and Woodley, D.T. (eds). (2001). *The biology of the skin*. The Parthenon Publishing Group, London.

Greene, R.S., Downing, D.T., Pochi, P.E. and Strauss, J.S. (1970). Anatomical variation in the amount and composition of human skin surface lipid. *J Invest Dermatol* **54**(3): 240.

Iida, T., Hirakawa, H., Matsueda, T., *et al.* (1999). Recent trend of polychlorinated dibenzo-p-dioxins and their related compounds in the blood and sebum of Yusho and Yu Cheng patients. *Chemosphere* **38**(5): 981–93.

Kligman, A.M. (1963). The Uses of Sebum. *Br J Dermatol* **75**(August/September): 307–319.

Maibach, H.I., Feldman, R.J., Milby, T.H. and Serat, W.F. (1971). Regional variation in percutaneous penetration in man. *Pesticides. Arch Environ Health* **23**(3): 208–211.

Michaels, A.S., Chandrasekaran, S.K. and Shaw, S.E. (1975). Drug permeation through human skin: Theory and *in vitro* experimental measurement. *AIChE Journal* **21**(5): 985–996.

Monaco, A. and Grumbine, N.A. (1986). Lines of minimal movement. *Clin Podiatr Med Surg* **3**(2): 241–247.

Montagna, W. (ed.). (1962). *The structure and function of skin*. Academic Press, New York.

Nordlund, J.J. and Boissy, R.E. (2001). The biology of melanocytes, in *The biology of the skin* (eds Freinkel, R.K. and Woodley, D.T.). The Parthenon Publishing Group., New York, pp. 113–131.

Porter, A.M. (2001). Why do we have apocrine and sebaceous glands? *J R Soc Med* **94**(5): 236–7.

Rommen, C., Leopold, C.S. and Lippold, B.C. (1999). Do local anesthetics have an influence on the percutaneous penetration of a model corticosteroid? An *in vivo* study using the vasoconstrictor assay. *Eur J Pharm Sci* **9**(2): 227–34.

Rougier, A., Dupuis, D., Lotte, C.R., *et al.* (1986). Regional variation in percutaneous absorption in man: measurement by the stripping method. *Arch Dermatol Res* **278**(6): 465–9.

Rougier, A., Lotte, C., Corcuff, T.P. and Maibach, H.I. (1988). Relationship between skin permeability and corneocyte size according to anatomic site, age and sex in man. *J Society of Cosmetic Chemists* **39**(1): 15–26.

Ryman-Rasmussen, J.P., Riviere, J.E. and Monteiro-Riviere, N.A. (2006). Penetration of intact skin by quantum dots with diverse physicochemical properties. *Toxicol Sci* **91**(1): 159–65.

Scheuplein, R.J. (1976). Percutaneous absorption after twenty-five years: or 'old wine in new wineskins'. *J Invest Dermatol* **67**(1): 31–8.

Scott, R.C., Walker, M. and Dugard, P.H. (1986). *In vitro* percutaneous absorption experiments: a technique for the production of intact epidermal membranes from rat skin. *J Society of Cosmetic Chemists* **37**(1): 35–41.

Seiberg, M. (2001). Keratinocyte–melanocyte interactions during melanosome transfer. *Pigment Cell Res* **14**(4): 236–42.

Snyder, W.S., Cook, M.J., Karhausen, L.R. *et al.* (eds). (1981). *Report of the task group on reference man*. A. Wheaton & Co Ltd, Exeter.

Thiele, J.J., Weber, S.U. and Packer, L. (1999). Sebaceous gland secretion is a major physiologic route of vitamin E delivery to skin. *J Invest Dermatol* **113**(6): 1006–10.

2 Biochemistry of the skin

Simon C. Wilkinson

Medical Toxicology Research Centre, University of Newcastle, Newcastle-Upon-Tyne, NE2 4AA, UK

Primary Learning Objectives

- *Biochemical processes occurring during maintenance and desquamation of the skin barrier, including synthesis and organisation of protein and lipid components.*
- *Biotransformations in the skin, especially those involved in metabolism of xenobiotics which may affect the toxicity of substances via the dermal route or the rate at which xenobiotics are absorbed across the skin.*

2.1 Introduction and scope

Control of the rate at which the stratum corneum is formed and lost (desquamation) is vital for optimising skin barrier function. This is achieved through interplay of various biochemical pathways (including the synthesis of specific proteins and lipids) which are linked with the terminal differentiation of keratinocytes.

The epidermis is a layered structure in which cells (keratinocytes) continuously generate by proliferation, differentiate to form the cells of the barrier and are finally lost through the process of desquamation. The cells of the stratum basale are continuously proliferating. In the stratum spinosum, synthesis of proteins (especially keratin and profilaggrin) occurs and desmosomes form to aid cell attachment (Chapter 1).

The synthesis of specialised lipids (including ceramides) and their packaging into small organelles termed lamellar bodies (or in some papers lamellar granules) occurs in the stratum granulosum. In the upper layers of the stratum granulosum, terminal differentiation occurs. The keratinocytes become enucleated, lipids are extruded into the intercellular space between the stratum granulosum and stratum corneum, and the keratinocytes are flattened to form the corneocytes of the stratum corneum. In human skin, the stratum corneum consists of 15–25 layers of corneocytes, surrounded by intercellular lipids. Each corneocyte is approximately 40 μm in diameter and 0.5 μm thick, and represents 'the end stage of keratinocyte differentiation'. The corneocytes are filled with tight bundles of intracellular keratin (70% by weight of the stratum corneum is insoluble keratin) and are surrounded by layers of highly cross-linked proteins, which are known as the corneocyte (or cornified cell)

Principles and Practice of Skin Toxicology Edited by Robert P. Chilcott and Shirley Price
© 2008 John Wiley & Sons, Ltd

envelope. Corneocytes are not metabolically active and do not respond to intercellular signals such as cytokines.

The stratum corneum is continuously renewed at a rate that is regulated by basal cell proliferation. Constant shedding of cells (desquamation) occurs in order to maintain stratum corneum thickness; this involves the degradation of cell–cell adhesion structures (corneodesmosomes). This process must be carefully regulated and matched with basal cell proliferation. If desquamation is too slow hyperkeratosis will occur; if it is too rapid barrier function will be lost. The following sections outline our current knowledge of how proteins and lipids are synthesised, the function of these proteins and lipids in forming and maintaining the skin barrier, and the putative mechanism for desquamation.

2.2 Protein synthesis and organisation during epidermal differentiation

Filaggrin is an important protein involved in formation of the stratum corneum. It is stored as a precursor (profilaggrin) in vesicles known as keratohyalin granules. Filaggrin facilitates the flattening of keratinocytes during terminal differentiation by causing collapse of the keratin cytoskeleton.

Proteins synthesised in the cytoplasm of the stratum spinosum and stratum granulosum are packaged into keratohyalin granules. One of the most important proteins involved in maintaining stratum corneum barrier function is filaggrin (filament-aggregating protein), a 37 kDa peptide named by the eminent keratinocyte biologist Peter Steinert. Filaggrin is synthesised in the cytoplasm and stored in the keratohyalin granules as profilaggrin, a 400 kDa, phosphorlyated precursor protein, consisting of 10–12 tandem repeats of filaggrin (Scott *et al.* 1982); the repeat number varies between individuals. Profilaggrin constitutes the major protein in the keratohyalin granules. Upon terminal differentiation, profilaggrin is proteolytically cleaved to multiple copies of the filaggrin, each of 37 kDa (Steinert *et al.* 1981; Dale *et al.* 1985; Gan *et al.* 1990). The transmembrane serine protease Matriptase (membrane type–serine protease 1; MT–SP1) appears to be involved in this process (List *et al.* 2003). Deimination by peptidylarginine deiminases, which are co-located with profilaggrin in the keratohyalin granules in the lower corneocytes, also appears to take place (Mechin *et al.* 2005). In the upper cells of stratum granulosum, the keratin cytoskeleton is firmly anchored to the plasma membrane by desmosomal and hemidesmosomal proteins. Adherens junctions and tight junctions are believed to be involved in attaching the cell membrane to actin filaments. Filaggrin binds the keratin causing it to bundle into highly insoluble macrofibrils (Harding and Scott, 1983). This resulting aggregation causes the collapse of the cytoskeleton, which results in compaction of the keratinocytes into corneocytes (sometime referred to as squames, Irvine and McLean, 2006). Profilaggrin (and hence filaggrin) are the products of the FLG (filaggrin) gene, part of the epidermal differentiation complex 1q21. Mutations of this gene have been functionally linked to ichthyosis vulgaris, a common keratinizing disorder (Smith *et al.* 2006), and more recently, FLG mutations were identified as strong predisposing factors for atopic dermatitis and asthma (Palmer *et al.* 2006). In the cells of the upper stratum corneum, filaggrin is proteolysed into a complex mixture of hygroscopic chemicals (mainly amino acids and derivatives), which are collectively termed skin natural moisturising factor (NMF). This has a role in determining stratum corneum hydration.

Transglutaminases catalyse the cross-linking of corneocyte membrane proteins (such as involucrin and loricrin) to lipids (ceramides) in the intercorneocyte spaces; i.e., these enzymes help glue the corneocyte bricks to the lipid mortar.

Transglutaminases are involved in the formation of the corneocyte envelope by catalysing the cross-linking of precursor proteins such as involucrin (Huber *et al.* 1995) and loricrin (Egberts *et al.* 2004). Involucrin is a soluble protein precursor of the corneocyte envelope that is synthesised in the upper cell layers of the stratum spinosum. Another protein component of the corneocyte envelope is loricrin, which is expressed at a later stage of differentiation, and is cross-linked to other epidermal proteins such as filaggrin. Mutations in the keratinocyte transglutaminase 1 gene (and hence reduced activity of this enzyme) occur in patients with lamellar ichthyosis (Yang *et al.* 2001; Egberts *et al.* 2004). Transglutaminase activity appears to be under the control of Cathepsin D (Egberts *et al.* 2004), an aspartic peptidase (Fusek and Vetvicka 2005). Transglutaminase can also catalyse the ester linkage of acyl chains of ceramides in the nearest lipid layer to the corneocyte envelope (see below).

2.3 Lipid synthesis and organisation during epidermal differentiation

The lipid components of the stratum corneum are synthesised in the stratum granulosum, where they are packaged into lamellar bodies prior to apical secretion into the intercorneocyte spaces to form the lipid mortar.

Lipid accumulates in cells of the epidermis during differentiation. Much of this is packaged into lamellar bodies. These are small ovoid organelles (0.2 μm in diameter) that appear in the stratum granulosum. They contain one or several stacks of lamellar disks (possibly flattened lipid vesicles) surrounded by a membrane. These organelles play a crucial role in stratum corneum formation. They are enriched in phospholipids, cholesterol, glucosyl ceramides and an acylglucosyl ceramide, two thirds of which is in the bounding membrane. The organelles are the precursors for stratum corneum lipids; they also contain catabolic enzymes.

In the uppermost layer of granular cells, the lamellar bodies migrate to the apical periphery of the granulocyte and fuse with the plasma membrane. The contents of the lamellar body are extruded into the stratum corneum/stratum granulosum interface, and the polar lipid precursors are enzymatically converted to the 'end product' lipids; hydrolysis of glycolipids releases ceramides, phospholipids are converted to free fatty acids, and cholesterol esters and sulphates are converted to cholesterol. The enzyme glucocerebrosidase is responsible for the hydrolysis of glucosylceramides to ceramides (Holleran *et al.* 1993). Ceramides are also believed to be liberated from sphingomyelin by the action of sphingomyelinase (Yamamura and Tezuka, 1990). In Type 2 Gaucher disease patients, the activity of beta-gluco-cerebrosidase is greatly reduced, resulting in an increased ratio of glucosylceramides to ceramides. This results in altered lipid organisation in the stratum corneum, and hence reduced barrier function (Holleran *et al.* 1994). In patients with atopic dermatitis, the activities of sphingomyelin deacylase, ceramidase and glucosylceramide deacylase are increased, resulting in reduced amounts of glucosyl ceramide in the lamellar bodies and/or reduced ceramide in the stratum

corneum (Hara *et al.* 2000; Macheleidt *et al.* 2002). Mice lacking an epidermis-specific glucosyl ceramide synthase, the enzyme which catalyses the synthesis of glucosyl ceramide from ceramide and UDP glucose, showed aberrant stratum corneum organisation, poor skin barrier function and unusual, irregularly arranged lamellar bodies (Jennemann *et al.* 2007). These findings suggest that the formation of glucosyl ceramides is essential for proper formation of skin barrier function.

2.4 Lipid classes in the stratum corneum

The main types of lipid present in the stratum corneum mortar are ceramides, free fatty acids, cholesterol and triglycerides. In contrast to other lipid structures (such as cell membranes), the stratum corneum does not contain phospholipids.

Ceramides are a class of polar lipids (Figure 2.1) and comprise over 40% of SC lipid. They consist of a polar sphingosine, phytosphingosine or 6–hydroxysphingosine moiety (Figure 2.2), covalently linked to a fatty acyl chain of varying length; the majority of ceramides have an acyl chain of 24–26 carbons (rather longer than the phospholipids normally found in

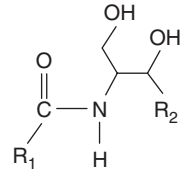


Figure 2.1 General formula for a ceramide; R_1 and R_2 represent specific side-groups (see Table 2.1)

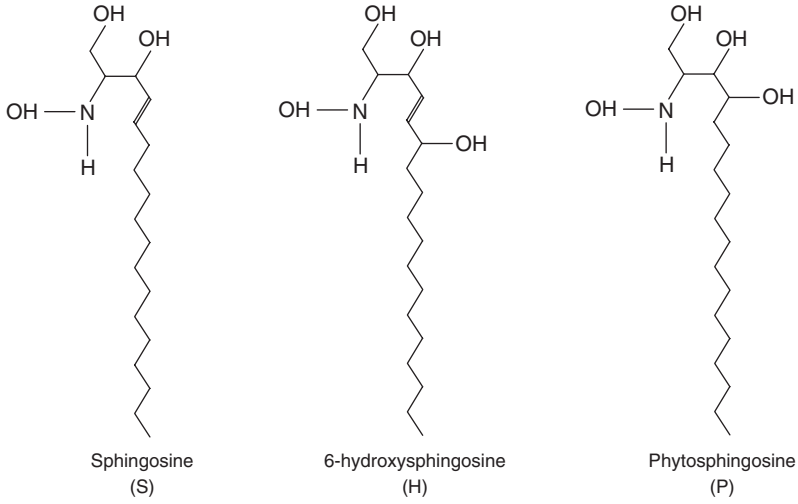


Figure 2.2 Sphingosines

Table 2.1 Ceramides found in human stratum corneum

Ceramide	New nomenclature	Acyl group (R1)	ω-hydroxy-linked linoleic acid ester	Head group
CER1	EOS	C30-C34	Yes	sphingosine
CER2	NS	C22-C24	No	sphingosine
CER3	NP	C22-C24	No	phytosphingosine
CER4	EOH	C30-C34	Yes	6-hydroxy sphingosine
CER 5	AS	C22-C24 α-hydroxy	No	sphingosine
CER 6	AP	C22-C24 α-hydroxy	No	phytosphingosine
CER 7	AH	C22-C24 α-hydroxy	No	6-hydroxy sphingosine
CER 8	NH	C22-C24	No	6-hydroxy sphingosine
CER 9	EOP	C30-C34	Yes	phytosphingosine

plasma membranes), with a smaller proportion of ceramides having a chain length of 16–18 carbons. The acyl chain is always unbranched, has no cis double bonds (except ceramides 1, 4 and 9) and may be hydroxylated at the alpha carbon (Figure 2.3). The polar head groups (Figure 2.2) of ceramides are very small and contain several functional groups that are able to form hydrogen bonds with adjacent ceramide molecules. This combination of a small polar head group and long acyl chains favours the formation of highly ordered membrane domains with gel or crystalline phases. In the case of the precursor glucosyl ceramides, the glucose is beta-glycosidically linked to the primary hydroxyl group on the sphingosine moiety.

Nine ceramides have been identified in human stratum corneum (Table 2.1) (Stewart and Downing, 1999; Ponec *et al.* 2003). In humans, ceramides 1, 4 and 9 have a linoleic acid moiety that is ester linked to a ω-hydroxy fatty acid with a chain length of 30–32 carbons (Figure 2.3). In porcine skin, only ceramide 1 has this additional structure (Wertz and Downing, 1983).

A new nomenclature has been recently introduced in which ceramides are named using a combination of the type of acyl chain (EO for ester linked omega hydroxy, N for normal or A for α-hydroxy) and the nature of the head group (S for sphingosine, P for phytosphingosine or H for 6-hydroxy sphingosine (Table 2.1).

Ceramide 1 (EOS) is believed to be derived from the acyl glucosyl ceramide in the lamellar bodies. Ceramide 1 is thought to act as a 'molecular rivet', stabilising multi-lamellar sheets. It is also thought to form covalent linkages between the nearest lipid layer to the corneocyte and the cornified envelope. The ω-hydroxyl groups in the ceramide acyl chain are believed to be ester-linked to amino acid residues in proteins of the cornified envelope, especially involucrin, a reaction catalysed by transglutaminase (Marekov and Steinert, 1998; Doering *et al.* 1999a, 1999b; Nemes *et al.* 1999; Stewart and Downing, 2001). However, other proteins may well be involved. Recent evidence from researchers cited above suggests that the linoleic acid tail is removed during the condensation reaction, though the enzyme responsible remains to be elucidated (Nemes *et al.* 1999; Kalinin *et al.* 2002).

Other lipid components in the stratum corneum include:

- Free Cholesterol (27% of SC lipids)
- Cholesterol esters (2–5%) and cholesterol sulphate (3%)
- Free fatty acids (mainly 22–24 C saturates) (9%)
- 'Others' such as triglycerides (11%).

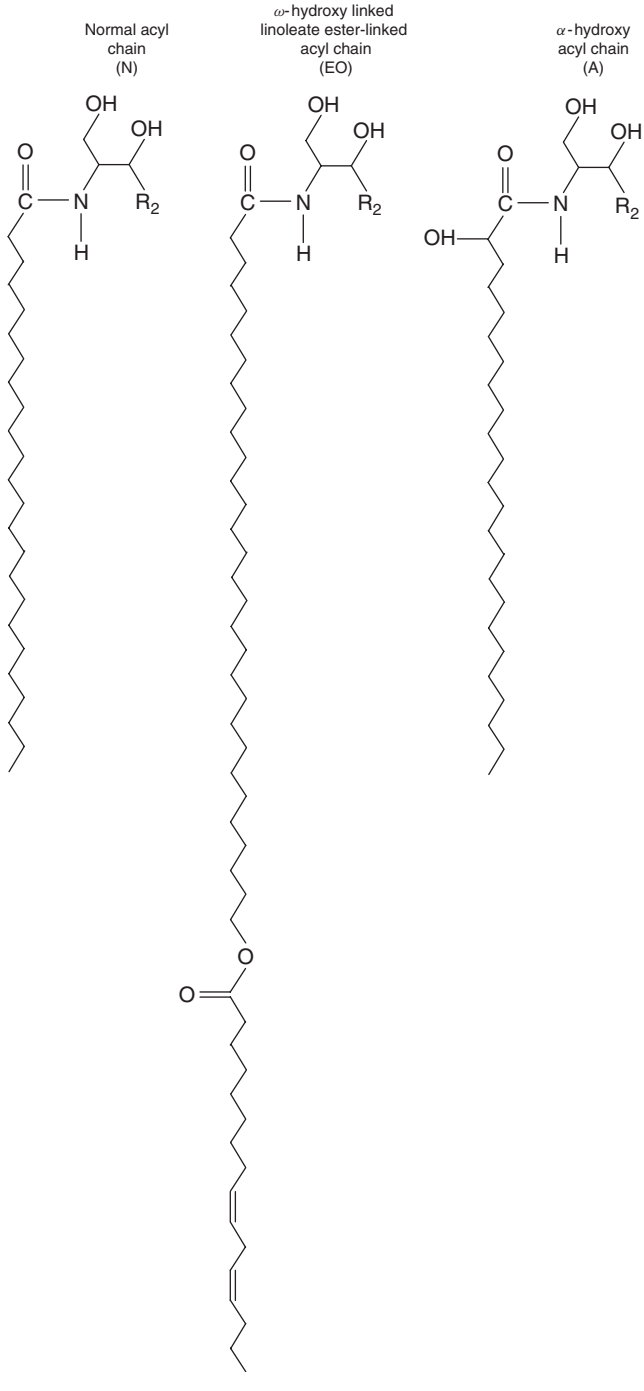


Figure 2.3 Acyl chains found in human skin ceramides

It is important to note that this *general* composition refers to lipids within the stratum corneum and not skin surface lipids associated with sebum (Chapter 1, Figure 1.4); that the relative proportions of each lipid may vary according to depth within the stratum corneum (Bonte *et al.* 1997); and that there are no phospholipids in the intercellular lamellae of the stratum corneum.

2.5 Stratum corneum turnover

The stratum corneum is a dynamic structure and is constantly assembled and then lost through the process of desquamation. Whilst lipid packing between adjacent corneocytes is predominantly a spontaneous process, both assembly and desquamation require enzymatic control. An imbalance in control mechanisms can give rise to a variety of different diseases.

2.5.1 Assembly of the stratum corneum lamellae

The lipids of the stratum corneum are assembled into lamellae (bilayers) arranged parallel to the surface of the corneocytes (Bouwstra and Ponec 2006), apparently by edge-to-edge fusion of flattened lipid vesicles. The ceramides are linked to the covalently bound lipid layer (lipid envelope) via interactions between sphingosine chains in lipid layer and fatty acid chains in ceramides (including ceramide 1). Spaces between the layers are filled with free lipids. The role of the free fatty acids in barrier function appears to be related to the lateral packing of the lipids in the lamellae. In human skin, the lipids appear to be predominately orthorhombically laterally packed (which results in low permeability), with some hexagonal lateral packing (medium permeability): the presence of free fatty acids in cholesterol–ceramide mixtures appears to be essential for phase transition to orthorhombic lateral packing (Bouwstra *et al.* 2001). In patients with lamellar ichthyosis (LI), the content of free fatty acids is markedly reduced compared to normal stratum corneum. The predominant lateral packing in the stratum corneum of LI patients was hexagonal, with very few orthorhombically packed crystals, and it is possible that the reduced fatty acid content is responsible for this (Pilgram *et al.* 2001).

2.5.2 Stratum corneum adhesion and desquamation

Strong cell–cell adhesion between corneocytes is mediated by structures termed corneodesmosomes (Serre *et al.* 1991). These are protein-containing complexes that span the inter-corneocyte space and covalently link the cell envelopes and cytoskeletons of neighbouring corneocytes. They are believed to derive from the desmosomes observed between cells of the stratum spinosum and stratum granulosum (Skerrow *et al.* 1989; Chapman *et al.* 1991), though the extracellular material is more electron-dense in corneodesmosomes. Degradation of these structures is essential for desquamation to occur and proteolytic cleavage of the extracellular portion of corneodesmosomes in the intercorneocyte space has been identified as a key step in this process (Lundstrom and Egelrud 1990; Egelrud and Lundstrom 1990;

Suzuki *et al.* 1994). The proteases responsible for this process remain to be fully characterised, though two important serine proteases from the kallikrein family have been identified, stratum corneum chymotryptic enzyme (SCCE) and the stratum corneum tryptic enzyme (SCTE) (Hanson *et al.* 1994; Suzuki *et al.*1994; Brattsand and Egelrud 1999; Ekholm *et al.* 2000). These enzymes are highly expressed in the upper stratum spinosum and stratum granulosum and are selectively located within the intercorneocyte spaces after secretion at the newly forming SG–SC interface. Evidence suggests that corneodesmosomes are progressively degraded as the corneocytes migrate towards the skin surface, resulting in a decrease in cell–cell cohesion until a threshold is reached and desquamation occurs. Regulation of this process is not well understood; it is not clear how proteolytic enzymes behave in a lipid-rich environment such as the SG–SC interface. Changes in intercellular lipid composition may also regulate desquamation.

The presence of high levels of cholesterol sulphate, such as in the skin of patients with X-linked ichthyosis (in which there is a deficiency in cholesterol sulphatase), appears to inhibit the proteolytic cleavage of corneodesmosomes (Sato *et al.* 1998). This correlates with a higher number of corneodesmosomes in superficial layers of the stratum corneum. It is suggested that cholesterol sulphate inhibits proteolytic cleavage of corneodesmosomes in the lower layers of the stratum corneum, thus limiting desquamation to the upper layers (Sato *et al.* 1998, Serizawa *et al.* 1992).

2.6 Biotransformations in skin

The skin contains a variety of enzymes that are capable of xenobiotic metabolism, including a range of Phase I and Phase II systems. This metabolic capacity is capable of modulating the toxicity of percutaneously absorbed compounds and, in some cases, can influence the rate and extent of skin absorption.

The skin is no longer regarded solely as an inert barrier capable of limiting the loss of internal moisture and entry of topical chemicals. Numerous enzyme activities have now been identified in several cutaneous tissues (including whole skin, isolated keratinocytes, appendages and cell lines); these are capable of a considerable variety of chemical transformations of both endo and xenobiotic compounds (Tables 2.2 and 2.3). These activities may modulate toxicity and in certain cases affect percutaneous absorption, and hence are potentially of great importance in the response of the skin (and the whole body) to environmental, occupational and deliberate therapeutic exposure to chemicals via the dermal route.

Xenobiotic metabolism is regarded as a multi-stage stage process (Figure 2.4). In Phase I metabolism, xenobiotics are subject to 'functionalisation', in which functional groups (especially oxygen-containing groups) are introduced as a result of oxidation, reduction or hydrolysis. In Phase II metabolism, these functionalised compounds are conjugated to compounds such as glucuronic acid, sulphate, glycine and glutathione or further metabolised by epoxyhydrases and other oxidoreductases, in order to increase their molecular weight and water solubility by the introduction of an easily ionisable group (and hence facilitate removal from the cell). Whilst Phase I metabolism can result in an increase in toxicity by

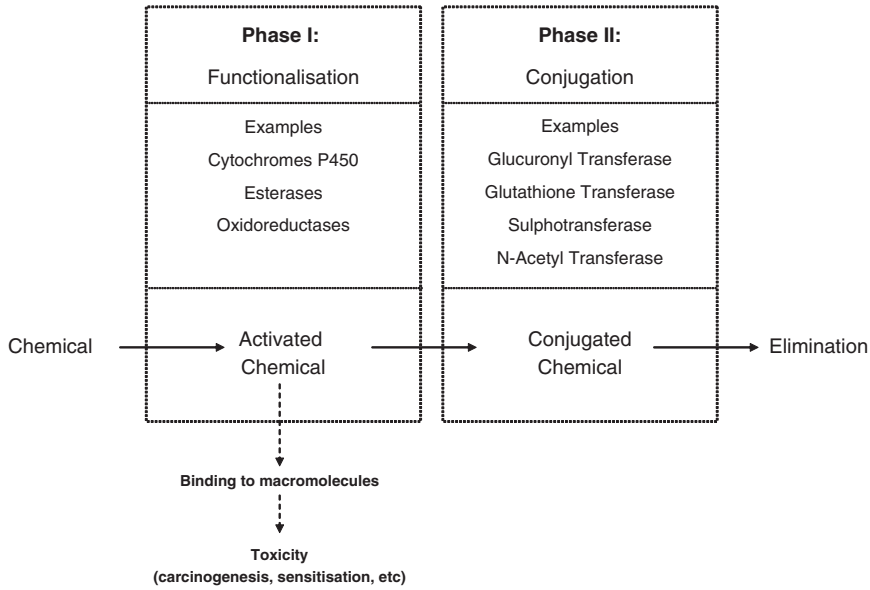
Table 2.2 Examples of cytochrome P450 isoenzymes identified in skin tissue by immunological methods

Isoform(s) reported	Species/tissue	Method of Detection	Expression	Other findings	Reference
1A1	SENCAR mouse microsomes	Monoclonal antibody	Constitutive	No 2B1 or 3A detected	Agarwal *et al.* 1994
1A1/2	Mouse skin microsomes	Protein (immunoblot-ting)	Constitutive	Lower levels of 2B1/2 and 3A	Jugert *et al.* 1994
1A1, 1B1, 2E1, 3A1	Sprague Dawley rat microsomes (neonatal to 12 weeks)	Specific antipeptide antibodies	1A1 and 3A1 present at all ages, but 3A1 lower in neonates	1B1 not present in neonates but present from 3 weeks onwards. 2E1 absent in neonatal skin, low levels at other ages. 1A2 not measured at any age.	Jameson *et al.* 1997
3A	Mouse epidermal microsomes, human keratinocytes	Western blotting (polyclonal antibodies raised versus liver isoform)	Constitutive		Jugert *et al.* 1994

(*continued overleaf*)

Table 2.2 (*continued*)

Isoform(s) reported	Species/tissue	Method of Detection	Expression	Other findings	Reference
3A, 2E1	BALB1c mouse whole skin microsomes	Specific antipeptide antibodies	Constitutive		Hotchkiss *et al.* 1996
3A	Human skin	Specific antibody against human isoform	Weak immunostaining in viable epidermis and sebaceous glands	No staining in sweat glands, hair follicles or blood vessels	Murray *et al.* 1988
Aromatase (19A)	Human sebaceous glands and hair follicles	Immunohisto-chemistry	In external root sheath of anagen terminal hair follicles and sebaceous glands	No variation between sex and body site	Sawaya and Penneys, 1992
2B12, 2C13, 2D1, 2D4, 2E1, 3A1, 3A2	Fischer 344 rat whole skin microsomes	Specific antipeptide antibodies	3A1 highest relative to liver (4.7%)	1A1, 1A2, 2C12 absent from skin	Zhu *et al.* 2002
Epoxide hydrase 2C9	Human epidermis microarrays	Immunohisto-chemistry	Comparable with pneumocytes and small and large intestine, lower than hepatocytes	2C8, 2J2 not detected	Enayehtellah *et al.* 2004



Chemicals functionalised in Phase I may be further metabolished by other enzymes such as eopoxyhydrases or NADPH quinone reductase prior to conjugation. These reactions may be important for toxicity. For example, benzo(α)pyrene is activated to 2,3-epoxide and subsequently hydrated to form the dihydrodiol which is genotoxic.

Figure 2.4 Xenobiotic metabolism – a multi-stage process

generating reactive intermediates capable of binding to macromolecules, Phase II generally results in detoxification, though an intermediate may be formed which may undergo further Phase I metabolism. In the following sections the expression, activity and localisation of enzymes in cutaneous tissues are summarised and the consequences of enzyme activity for percutaneous penetration, absorption and toxicity of topical xenobiotics are considered. The effects of species differences and the applicability of model systems such as cell lines and organotypic cell cultures are also considered.

The skin contains a range of Phase I detoxification enzymes, including a number of cytochrome P450 isoenzymes, esterases, dehydrogenases and reductases. Phase I metabolism can sometimes increase the toxicity of a substance or, in certain cases, enhance skin absorption of a pharmaceutically active or toxic metabolite.

2.6.1 Phase I enzymes

Cytochromes P450

The cytochromes P450 (CYP) are considered a key group of Phase I enzymes, especially regarding their ability to activate chemicals to toxic intermediates. They are a large group of haem-containing, mixed function oxidases, found throughout both prokaryotic and

eukaryotic domains. In eukaryotes, they are membrane bound to endoplasmic reticulum. The CYP multi-gene superfamily comprises 37 different multi-gene families, 10 of which are known in mammals, with eight being represented in humans, of which four play a role in human xenobiotic metabolism. The general equation for the chemical reaction catalysed by cytochromes P450 involves the insertion of a single oxygen atom (from molecular oxygen) in the substrate according to Equation (2.1):

$$\text{R-H} + O_2 + \text{NAD(P)H} + H_+ \longrightarrow \text{R-OH} + H_2O + \text{NAD(P)}_+ \qquad (2.1)$$

More than 300 isoenzymes have been reported; CYP enzymes have relatively low substrate specificity, so a broad range of reactions is possible. In addition to the hydroxylation reactions in Equation (2.1), these include oxidations of aliphatic or aromatic carbon double bonds to epoxides, oxidation of nitrogen and sulphur hetero-atoms, oxidative and/or reductive dehalogenation and a range of oxidative de-alkylations (Correia 2001).

Cytochrome P450 expression and activity reported in skin

Numerous experimental approaches have been used to identify and quantify expression and activity of cytochromes P450 in cutaneous tissues, ranging from spectroscopic studies through selective probe substrates and immunohistochemical studies to molecular techniques, such as reverse-transcriptase polymerase chain reaction (semi quantitative and real time) and RNAse inhibition. Tissues investigated have included skin microsomes (derived from whole skin, epidermis or dermis), keratinocytes, transformed keratinocyte cell lines and cultured human hair follicles, all in the presence and absence of classical CYP inducers such as 3-methylcholanthrene (3-MC) and phenobarbitone (PB). The amount of CYP in skin microsomes (per milligramme of protein) has been estimated to be about 6% of that in liver (Pham *et al.* 1989).

Measurements of cytochrome P450 activity using selective probe substrates

Several investigations of cutaneous cytochrome P450 activity have used classical probe substrates known to be metabolised (specifically or selectively) by different hepatic CYPs. These enzyme activities include 7-ethoxyresorufin-O-deethylase (EROD) – a marker for CYP1A1/2 – 7-ethoxycoumarin-O-deethylase (ECOD), 7-pentoxyresorufin-O-deethylase (PROD) – both markers for CYP2B – coumarin-7-hydroxylase (CYP2A6), p-nitrophenol hydroxylase (CYP2E1) and erythromycin-N-demethylase (CYP3A4). These substrates have been used to demonstrate the presence of several CYP activities in human and rodent skin, particularly CYP1A1/2 (Bickers *et al.* 1984; Rettie *et al.* 1986; Finnen 1987; Pham *et al.* 1990; Jugert *et al.* 1994). CYP2B activity has also been measured using PROD and ECOD as markers, and appears to be constitutively expressed in skin (Jugert *et al.* 1994), as have CYP2E1 and CYP3A (Beckley-Kartey *et al.* 1997), though cultured human keratinocytes appear to lack PROD and 7-benzoxyresorufin activity (Raffali *et al.* 1994). There are, however, some notable absences of CYP activity in skin. For example, CYP2A6 activity was not detected in human or rodent skin tissue (Beckley-Kartey *et al.* 1997).

Comparisons of enzyme-specific activity towards probe substrates between skin and liver vary considerably. EROD and benzo[a]pyrene epoxidase activities in skin range from 0.1–15% and 0.1–12% of hepatic activity, respectively, whilst ECOD activity ranges from 0.5–7% and PROD activity from 20–27% of hepatic activity respectively (Mukhtar and Khan 1989). 7-EROD activity in primary murine keratinocytes was approximately 2000-fold lower compared

with hepatocytes on a per cell basis (Reiners *et al.* 1990), whereas basal EROD activity in HepG2 cells was only about 2.5-fold higher than in HaCaT cells (Ledirac *et al.* 1997). These data have led to the suggestion that, since the surface area of skin is considerable (Chapter 1), then the total cutaneous activity of many CYP enzymes may approach that of the liver.

It has been suggested that CYPs have a possible role in keratinocyte differentiation. CYP1A expression in keratinocytes depends on the level of differentiation *in vitro*, with levels of EROD and ECOD activity higher in spinous cells than basal keratinocytes. Furthermore, constitutive CYP expression in murine keratinocytes can be modulated by extracellular calcium ion concentrations, known to play a key role in keratinocyte differentiation (Reiners *et al.* 1990).

Immunochemical detection of cytochrome P450 protein in cutaneous tissues

Both CYP1A1/2 and CYP2B have been detected by immunochemical methods in rat skin and both isoforms were able to catalyse aryl–hydrocarbon hydroxylation and O-deethylation of ethoxycoumarin. CYP2B has been described as 'constitutive' in rat skin, whilst CYP1A1/2 is 'very low or absent in un-induced skin' but 'preferentially expressed following induction with 3-methylcholanthrene' (Bickers and Mukhtar 1990; Khan *et al.* 1989a, 1989b). A considerable body of evidence has accumulated for the expression of several CYP isoforms from the application of immunological techniques such as Western blotting (Table 2.2). More recently, single immuno-reactive bands corresponding to CYP2B12, 2C13, 2D1, 2D4, 2E1, 3A1 and 3A2 were detected in rat skin microsomes using a panel of monospecific antibodies directed towards small, defined regions of respective cytochrome P450 enzymes whose specificity was demonstrated by immunoassay (Zhu *et al.* 2002).

Measurement of cutaneous cytochromes P450 expression using molecular biological techniques

The application of molecular biological techniques, such as reverse transcriptase polymerase chain reaction (PCR) and, especially, quantitative real-time PCR for the measurement of cutaneous CYP expression has further increased the variety of CYP isoforms detected in cutaneous tissues (Table 2.3) previously identified using immunological techniques and measurement of catalytic activity. Of particular interest is CYP2S1, which appears to be relatively highly expressed in skin compared to the liver. It must be stressed, however, that measurement of CYP mRNA and protein expression levels do not necessarily correlate with constitutive (or induced) catalytic activity.

Localisation of cytochromes P450 in cutaneous tissues

Immunohistochemical studies (such as those by Frankenberg *et al.* 1993) have repeatedly shown that cutaneous CYPs are localised in the epidermis, especially in basal keratinocytes, hair follicles and the vascular endothelium. These findings are supported by measurement of catalytic activity (AHH and EROD, Merk *et al.* 1987). CYP2E1 protein was reported by Kawakubo and co-workers (2001) to be located mainly in the upper layers in normal human epidermis (assessed using immunochemical staining), with weaker staining in the basal layer. In the dermis, vascular endothelium and eccrine sweat glands were well stained. Lee *et al.* (2001) showed that the main portion of cutaneous cytochrome P450 protein expression was in the lower epidermis, whilst the appendages were more highly stained than the other dermal tissues. CYP2A6, 2B6 and 3A4 mRNAs were expressed uniformly in the epidermis of fixed sections of adult human breast skin, as well as in sebaceous and sweat-producing glands (Janmohamed *et al.* 2001). One of the more recently discovered cutaneous CYPs, CYP2S1,

Table 2.3 Selected reports of CYP expression in cutaneous tissue based on mRNA detection

Main Isoform(s) reported	Species/tissue	Method of Detection	mRNA expression	Supporting evidence or further remarks	Reference
3A subfamily members	Human skin biopsies	RT-PCR (3A specific primers)	3A mRNA detected in all adults tested. Expression did not increase in response to clobetasol treatment.	Only 3A5 protein detected using antibodies to conserved epitopes (in all individuals tested), suggesting 3A3 and 3A4 not significantly expressed in skin.	Li *et al.* 1994
1A1, 1B1, 2B6, 2E1, 3A4, 3A5	Human proliferating epidermal keratinocytes (obtained from fresh skin)	RT-PCR	Constitutive expression of all but 3A4. 3A4 inducible by dexamethasone. 1A1 expression increased in presence of benzanthracene.	Findings confirmed by immunoreactive protein and catalytic activity.	Baron *et al.* 2001
1A1, 1B1, 2A6, 2E1, 2C, 2D6, 3A5, 3A7, 4B1	Freshly obtained cultured human Langerhans cells, keratinocytes, fibroblasts and melanocytes from six individuals	RT-PCR	1A1, 1B1, 2E1 expressed in all cell types and individuals. 2A6, 2C, 2D6, 3A5, 3A7, 4B1 expressed in some cell types or individuals. 1A2, 2A7, 2B6, 3A4 not detected.		Saeki *et al.* 2002

Table 2.3 (*continued*)

Main Isoform(s) reported	Species/tissue	Method of Detection	mRNA expression	Supporting evidence or further remarks	Reference
1B1, 2B6, 2D6, 2C18, 3A4, 2C19, 3A5	Human skin biopsies (healthy volunteers)	Real time RT PCR	Main isoforms expressed were 1B1, 2B6, 2D6, 3A4 Lower level expression of 2C18, 2C19, 3A5.	Considerable inter-individual variation in expression (some individuals apparently lacked 2B6).	Yengi *et al.* 2003
1B1, 1A1, 2E1, 2S1, 3A4, 3A5	Human skin biopsies (healthy volunteers and psoriasis patients)	Real time RT PCR	1B1 most expressed isoform in healthy volunteers, 1A1, 2S1 consistently expressed. 2E1 expressed in some individuals at levels comparable with 1B1. 3A4 levels much lower. 2S1 expression inducible by UV radiation and coal tar.	Marked individuality of expression for 1B1, 2E1 and 2S1. Lower overall expression levels in non-lesional skin in randomly selected psoriasis patients. 2E1 and 2S1 expression significantly increased in lesional versus non-lesional skin, 1B1 and 3A5 significantly decreased.	Smith *et al.* 2003a,b
1A1, 1B1, 2E1, 2C, 3A5	Commercial organotypic skin models (with and without classical inducers)	Real time RT PCR and cDNA microarrays	1A1 and 1B1 highly inducible by *liquor carbon detergens.*	Expression localised using immunofluorescent staining.	Neis *et al.* 2005

(*continued overleaf*)

Table 2.3 (*continued*)

Main Isoform(s) reported	Species/tissue	Method of Detection	mRNA expression	Supporting evidence or further remarks	Reference
2B19	Primary and differentiating mouse epidermal keratinocytes	Real-time RT PCR	Expression increased 39-fold in differentiating keratinocytes	Confirmed by protein immunoreactivity and 11, 12-EET activity.	Du *et al.* 2005
CYP1 and 2 subfamily members, 3A4 and 4B1	Human epidermal keratinocytes (proliferating and differentiating)	Real-time RT PCR	4B1, 2S1, 2J2 most abundant in pooled phenotypes. 2B6 undetectable. Significant increases in 4B1 (356-fold), 2 W1 (27-fold) and 2C18 (113-fold) in differentiating cells.	Overall range of expression covered five orders of magnitude. Expression of nearly half isoforms studied varied twofold or less between proliferating and differentiating states, majority less than ten fold different.	Du *et al.* 2006
2A6, 2B6, 3A4	Adult human breast skin primary keratinocytes HaCaT cells	RNAse protection assay	All skin samples expressed 2B6, some expressed 2A6 and 3A4. Expression of 2B6 and 3A4 was 75–100% lower in cultured primary keratinocytes. 3A4 absent. Expression of 2B6 in HaCaT more comparable to human skin than primary keratinocytes, but 2A6 and 3A4 absent.		Janmohamed *et al.* 2001

has low expression levels in liver but high and constitutive expression in many other tissues including skin (Smith *et al.* 2003b). Staining intensity for CYP2S1 using *in situ* hybridisation (ISH) and immunohistochemistry in human skin microarrays was comparable with that in the nasal cavity and bronchus/bronchiolar tissues, and greater than that of the liver. Epithelial cells in sweat glands and hair follicles also exhibited strong staining, and ISH especially indicated higher grain densities in basal cells than in upper layers of the epidermis (Saarikoski *et al.* 2005).

Effects of cytochromes P450 on percutaneous absorption and toxicity

It is generally accepted that CYP activity does not affect percutaneous absorption due to the location of these enzymes, although this does not apply to various enzymes in the stratum corneum (see below). However, activation of xenobiotics in cutaneous tissues by CYPs leading to increased toxicity has been well documented. For example, polyaromatic hydrocarbons (such as benzo(α)pyrene and dimethyl-benzanthracene) are metabolised in the skin by CYP1A1, CYP1A2 and others to epoxides, which are subsequently converted to diol epoxides by epoxide hydrolase (Rogan *et al.* 1993; Bronaugh *et al.* 1994). These diol epoxides are directly genotoxic and this phenomenon is now believed to be responsible for the high incidence of scrotal cancer identified by Sir Percival Potts in chimney sweeps in the late eighteenth century (Box 15.1). The cytochromes P450 involved in PAH metabolism (CYP1A1 and 1B1) are substrate inducible as well as being under the control of the aryl hydrocarbon receptor (AhR). This cytosolic receptor binds readily to planar PAHs such as benzo(α)pyrene, and, after dimerization with the aryl hydrocarbon receptor nuclear translocator (ARNT), enters the nucleus where the complex binds to regions of DNA known as xenobiotic response elements. This leads to upregulation or induction of genes involved in response to xenobiotics, such as CYPs (Whitlock 1999).

Several studies have been undertaken to establish the nature and toxicity of DNA adducts formed from the activation of cytochrome P450 and the contributions of the different CYP isoforms to this process in a variety of tissues (Baird *et al.* 2005). Studies with knockout mice (lacking particular genes for CYP isoforms or the aryl hydrocarbon receptor) have shown that different CYP isoforms have apparently different roles in bioactivation of PAHs to genotoxic metabolites (for example, see Kleiner *et al.* 2004 and Ide *et al.* 2004).

In contrast to activation of PAHs, reports of N-hydroxylation of amines in skin or skin models have been rare (Reilly *et al.* 2000; Vyas *et al.* 2006). The majority of reports have identified N-acetylation as the major metabolic pathway in skin for amines (see below).

Regulation of cytochrome P450 expression and activity

As mentioned earlier, cytochromes P450 are known to be regulated by the AhR and ARNT system (Table 2.4). However, this mechanism of induction of CYP can itself be influenced by other biochemical pathways. For example, in proliferating, differentiating mouse skin cells, TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) induced EROD activity in the absence of any effect on the activity of CYP1A1 and CYP1B1. Thus, it is conceivable that AhR-mediated induction of CYP may itself be subject to regulation by processes involved with keratinocyte differentiation (Jones and Reiners 1997). The induction of CYP can also be limited to enzymes within the skin. For example, Rastogi *et al.* (2006) showed that twice weekly topical application of Alfatoxin B1 to Swiss albino mice for 24 weeks resulted in a significant increase in cutaneous

Table 2.4 Examples of cytochrome P450 isoenzymes, inducers and putative mechanism(s)

CYP2B1 and CYP3A	pyridine	Agarwal *et al.* 1994
CYP 2S1	dioxin	Smith *et al.* 2003b
CYP1A1 CYP1A2 CYP1B1 CYP2C18	PAHs	Smith *et al.* 2006
CYP1B1	UVA	Smith *et al.* 2003a
1A1 and 1B1	UVB	Katiyar *et al.* 2000

CYP1A monooxygenase activity in the absence of any significant effect on hepatic CYP1A activity.

There have been several studies of the effects of UV-radiation on the expression and activity of cytochrome P450 activity, with demonstrable induction of CYP1B1, CYP1B1, CYP2E1 and CYP2S1 (following UVA exposure; Smith *et al.* 2003a) and CYP1A1 and CYP1B1 (UVB; Katiyar *et al.* 2000).

Relevance of cytochrome P450 expression and activity in model systems of percutaneous absorption

It must be stressed that, although there have been many reports of catalytic activity corresponding to CYP isoenzymes in skin microsomal fractions, there have been far fewer reports of CYP activity being measured during percutaneous absorption (Beckley-Kartey *et al.* 1997). CYP enzymes are difficult to study in *ex vivo* skin, as they are highly labile and require freshly isolated skin. Animal models are often used, as are cell culture models, though traditional rodent models appear to exhibit higher levels of certain CYP isoforms than human skin (Bickers *et al.* 1984; Storm *et al.* 1990). CYP activities in pig skin, which is considered a suitable model for human skin in terms of absorption, have so far not been fully characterised (though hepatic CYP activities have been reported). Similarly, there have been few published reports of CYP activity in cell lines and organotypic cell culture models, despite the number of reports of mRNA and protein expression in such models (see Table 2.2).

Esterases

Esterases are ubiquitously expressed in mammalian tissues including, liver, blood, kidney, intestines, testes, brain, central nervous system, skin and lung (Satoh and Hosokawa 1998). Both cytosolic and membrane bound forms have been characterised and the most significant group with respect to xenobiotic metabolism are the carboxyl esterases (formerly categorised amongst a group referred to as B esterases), or CES family, which catalyse the hydrolysis of carboxylic acid esters to the carboxylic acid and alcohol (Equation (2.2)).

$$R_1COOR_2 + H_2O \longrightarrow R_1COOH + R_2OH \tag{2.2}$$

There are four CES isoform families (categorised according to amino acid homology). Two major human isoforms of esterase have been identified from molecular studies, hCE-1 (a human CES 1 family esterase) and hCE-2 (a CES 2 family esterase). A third, brain specific esterase, hCE-3, has also been identified. Esterases in liver and gut have been most

extensively studied (Huang *et al.* 1996). hCE-1 is highly expressed in liver, and also present in macrophages, human lung epithelia and other tissues, but expression is markedly low in gastointestinal tissues. hCE-2 is highly expressed in small intestine, colon, kidney, liver, heart, brain and testis, but apparently absent from other tissues. The two isoforms differ in their substrate specificity:

- hCE-1 prefers a small alcohol group and a large acyl group, but has a wide specificity range, allowing it to act upon several structurally diverse esters. It also has high transesterification ability. Transesterification describes the exchange of one alkoxy moiety in an ester for another alcohol group (Equation (2.3)).

$$R_1COOR_2 + R_3OH \longrightarrow R_1COOR_3 + R_2OH \tag{2.3}$$

- hCE-2 utilises substrates with a large alcohol group and a small acyl group, apparently has a more restricted substrate specificity, and negligible transesterification activity (Imai 2006).

Cutaneous esterase activity

Skin has long been known to possess a considerable non-specific esterase activity that is cytosolic as well as microsomal (Clark 1992) and easily released in skin homogenates. However, esterase isoforms in cutaneous tissues remain to be fully characterised and investigations of the quantity of esterases in skin are relatively scarce.

Histochemical studies of pig, human and rat skin have localised carboxylesterase activity to the basal keratinocytes of epidermis, hair follicles and sebaceous glands (Mayer and Neurand 1976; Clark 1992), but esterase activity has also been identified in the stratum corneum (Beisson *et al.* 2001).

Inducible esterase activity is present in the cytosol and microsomal fractions of rat skin (McCracken *et al.* 1993), though activity tends to be higher in the cytosol. The activity of esterases in the liver is higher than in cutaneous tissue (Ahmed *et al.* 1997, Jewell *et al.* 2007a, Prusakiewicz *et al.* 2006).

Esterase activity in model systems

A survey of several species has demonstrated that esterase activity towards ethyl nicotinate during percutaneous absorption is higher in rodent species and rabbits than in monkey, human and snake skin (Ngawhirunpat *et al.* 2004). Furthermore, carboxylesterase activity in postmitochondrial fractions of rat skin were ten-fold higher than human (Clark *et al.* 1993), and p-hydroxybenzoic acid ester hydrolysis in rat skin was higher overall than in mini-pig or human skin (Jewell *et al.* 2007b, Prusakiewicz *et al.* 2006). In contrast, a number of other studies have found extensive esterase activity across several species, for example towards the phthalic acid esters (Mint 1995). There have been a number of reports of esterase activity in keratinocytes and living skin equivalents (Kubota *et al.* 1994; Lobemeier *et al.* 1996; Barker and Clothier 1997). However, the isoforms of carboxylesterases in keratinocyte cell lines and organotypic skin models remain to be characterised fully.

Esterase activity during percutaneous absorption

Esterases are known to be robust enzymes and, in contrast to CYPs, esterase activity has been detected in previously frozen skin. Consequently, reports of hydrolysis of esters during

percutaneous absorption studies are common (e.g. Clark *et al.* 1993; Hewitt *et al.* 2000). Skin esterases have also been shown to be stereoselective in their affinity for substrates (Ahmed *et al.* 1997). Ethyl nicotinate applied topically was converted by esterases to nicotinic acid during percutaneous absorption in a range of species (Ngawhirunpat *et al.* 2004).

It is widely understood that hydrolysis resulting from esterase activity will reduce the systemic absorption of the parent compound, thus resulting in detoxification if the parent compound is systemically or locally toxic (see for example Boogaard *et al.* 1999). It must be mentioned, however, that the products of hydrolytic cleavage of an ester are a carboxylic acid and an alcohol, either of which may be subsequently metabolised by Phase I enzymes, alcohol and/or aldehyde dehydrogenases to more reactive metabolites (see below). Esterase activity can promote percutaneous absorption by reducing the lipophilicity of topically-administered compounds in the upper layers of the epidermis, thus increasing their water solubility (and hence their ability to diffuse through the aqueous layers of the epidermis). This property has been exploited for the delivery of ester pro-drugs (Williams 1985; Higuchi and Yu 1987; Ahmed *et al.* 1997; Liederer and Borchardt 2006). Hydrophilic molecules are ester-linked to hydrophobic compounds, such as caprylic acid moieties. This increases their lipophilicity, enabling them to enter the stratum corneum. Esterases in the upper epidermal layers then hydrolyse the esters, thus releasing the more hydrophilic compound. When fluzifop butyl was applied to human and rat skin *in vitro*, only its metabolite, fluazifop, subsequently underwent skin absorption as a result of hydrolysis of the butyl ester; percutaneous absorption of fluzifop butyl itself was not detected (Clark *et al.* 1993).

Alcohol and aldehyde dehydrogenases

Alcohol dehydrogenase (ADH) oxidises alcohols to aldehydes by the removal of two hydrogen atoms, using nicotinamide adenine dinucleotide (NAD^+) as a co-substrate (Equation (2.4)). There are three classes in human liver: class I enzymes (now often referred to as ADH 1A, B and C) has a low Km (high affinity) for ethanol and are very abundant in liver; Class II (ADH2) has a higher Km for ethanol and may also contribute to alcohol elimination, but only at higher blood ethanol concentrations; Class III (ADH3) is glutathione-dependent formaldehyde dehydrogenase which has very low activity towards ethanol (Maly *et al.* 1999; Duester *et al.* 1999).

$$R_1CH_2OH + NAD^+ \longrightarrow R_1CHO + NADH + H^+ \tag{2.4}$$

Aldehyde dehydrogenase (ALDH) oxidises aldehydes to carboxylic acids in the same way (Equation (2.5)). Eight isoforms have been identified in humans, which catalyse a variety of substrates, and are located in the cytosol, mitochondria or microsomes (Yoshida *et al.* 1998). ALDH1 is a cytosolic enzyme expressed in almost all tissues with high activity towards retinal, and is involved in retinoic acid metabolism. ALDH2 is a mitochondrial enzyme, highly expressed in liver and some other tissues, which has a role in the detoxification of acetaldehyde. ALDH3 is a cytosolic enzyme, highly expressed in stomach and lung, but with very low levels of hepatic expression. It oxidises aromatic and medium-chain aliphatic aldehydes.

$$R_1CHO_2NAD^+ \longrightarrow R_1COOH + NADH + H^+ \tag{2.5}$$

The cutaneous activity of alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) has received particular attention recently, owing to the possible role of alcohol oxidation to

aldehydes in the mechanism of skin sensitisation. ADH activity may result in the formation of aldehydes from alcohols, which may subsequently react with (and covalently bind) proteins in the epidermis, potentially resulting in haptenisation. Several examples of this type of cutaneous metabolism have been reported (Garnett, 1992; Weibel and Hansen 1989; Tonge 1995; Roper *et al.* 1997; Lockley *et al.* 2002, 2004). ADH activity in skin cytosol fractions have indicated that the apparent V_{max} of this enzyme is up to 16-fold higher in liver than in skin, and about three-fold higher in mouse skin than in human skin (Cheung *et al.* 2003b). However, cutaneous ADH activity appears to be less sensitive to inhibition by 1 mM 4-methyl pyrazole than hepatic ADH.

Role of ADH/ALDH activity in percutaneous absorption

ADH and ALDH activity towards cinnamaldehyde and cinnamic alcohol during percutaneous penetration *in vitro* has been reported (Smith *et al.* 2000); when cinnamaldehyde was applied to freshly-excised human skin, the penetration rates of cinnamic alcohol and cinnamic acid exceeded that of the parent compound in the first two hours of study. Penetration of the two metabolites was maximal after 4–8 hours then decreased, whilst penetration of the parent compound was maximal after 18 hours. Pyrazole pre-treatment significantly reduced penetration of the alcohol and acid metabolites. When cinnamic alcohol was applied as the parent compound, the penetration rate of the acid metabolite was approximately half that of the parent compound; no cinnamaldehyde was detected. Pre-treatment with pyrazole significantly reduced penetration of the acid metabolite. Again, no aldehyde metabolite was detected. The authors proposed that they had observed conversion of cinnamaldehyde to cinnamic alcohol by ADH, and by ADH (acting as an aldehyde dismutase) and ALDH to cinnamic acid, as pyrazole reduced penetration of cinnamldehyde metabolites, but not the parent compound. The greater extent of metabolism to acid from the aldehyde compared to the alcohol suggested that the latter process was slower. The fact that no aldehyde metabolites were detected was not unexpected, as rapid further metabolism, conjugation or protein adduction would have occurred.

Localisation of cutaneous ADH/ALDH activity

Protein expression of ADH1 and 3 and ALDH1 and 3 have been quantified in human foreskin, breast and abdominal skin using Western blot analysis (Cheung *et al.* 1999). Densitometric analysis showed that staining intensity was significantly lower in foreskin for ADH1 and ADH2, and significantly greater in foreskin for ALDH1 and 3, than in breast or abdominal skin. Immunohistochemistry showed that ADH1 and 3 were localised mainly in the epidermis with some expression in the dermal appendages, whilst staining for ADH2 in skin sections was much less intense. ALDH1 and 3 were also localised mainly in the epidermis, with some highly localised expression in the dermal appendages.

Activity of ADH/ALDH towards glycol ethers in dermatomed skin was twice that in full thickness skin when expressed in terms of protein content, suggesting that activity was mainly located in the epidermis (Lockley *et al.* 2005).

Species differences

Species differences in expression of cutaneous ADH and ALDH classes have been demonstrated. ADH1 and ADH3, as well as ALDH1 and ALDH2, were expressed constitutively in the skin and liver of rat, mouse and guinea pig, whilst ADH2 was not expressed in any rodent skin but

was present in the liver of all rodent species (Cheung *et al.* 2003a). ALDH3 was constitutively expressed in rat and mouse skin (though not in guinea pig), and was not expressed in the liver of any rodent species tested. Immunohistochemistry showed that expression of ADH and ALDH was localised mainly in the epidermis, sebaceous glands and hair follicles in all rodent species tested and in humans. Lockley *et al.* (2005) compared the rates of ADH oxidation of a range of alcohols in rat skin cytosol with those measured in rat liver cytosol: in the latter tissue, the highest oxidation rate was measured with ethanol, followed by 2-ethoxyethanol, ethylene glycol, 2-phenoxyethanol and 2-butoxyethanol, whilst this order was reversed for skin cytosol. ADH oxidation of all alcohols tested was completely inhibited by pyrazole in rat liver cytosol, whilst pyrazole only inhibited ADH activity by 40% in skin cytosol at the same concentration, and did not inhibit oxidation of the other alcohols in skin cytosol. Furthermore, disulfiram, an ALDH inhibitor, completely inhibited metabolism of all alcohols in skin cytosol. These data suggested that different isoforms of ADH were present in skin and liver. Although skin has the capacity to metabolise 2-butoxyethanol to 2-butoxyacetic acid, the rapid percutaneous penetration of 2-butoxyethanol *in vivo* and *in vitro* prevented local metabolism; this was also the case with 2-ethoxyethanol (Lockley *et al.* 2002, 2004).

Regulation of ADH/ALDH activity

Compared to other oxidation enzymes (especially CYPs), there have been few investigations of the regulation of cutaneous ADH and ALDH activity. Gelardi *et al.* (2001) reported that aldehyde dehydrogenase activity was inducible (resulting in a four-fold increase) by 3-methylcholanthrene but not significantly influenced by phenobarbitone. Lockley *et al.* (2005) showed that multiple topical application of ethanol or 2-butoxyethanol (2-BE) to rat skin *in vivo* resulted in a preferential increase in oxidation activity of these respective substrates in skin cytosol, suggesting that the two substrates may induce different isoforms of ADH with affinities for alcohols of differing chain lengths. Topical treatment with a classical inducer (dexamethasone) resulted in enhanced activity with both ethanol and 2-BE as substrates, indicating the induction of several isoforms simultaneously. Aldehyde dehydrogenase activity is well known to be involved in the synthesis of retinoic acid; the first step in the process involves the reduction of retinol to retinaldehyde by retinol dehydrogenase/reductase. This is then further reduced to retinoic acid by ALDH. ALDH 1A3 (but not other Class 1 members, nor Class 2 and 3 members) was significantly induced by all-trans retinoic acid and TCDD in a skin equivalent model, suggesting a general detoxification role for this enzyme as well as involvement in retinoid metabolism (Ulrich *et al.* 2004).

NAD(P)H quinone reductase

NAD(P)H quinone reductase (NQR) is an important enzyme involved in the protection of tissues against oxidative stress, in particular, against the products of the quinone redox cycle. Quinones may undergo a single electron reduction to semiquinones. These compounds are unstable and oxidise spontaneously to quinones, releasing singlet oxygen. NQR reduces quinones with two electrons, to form more stable hydroquinones (Smith 1985), which are then further subjected to Phase II metabolism. Many dermal contact allergens have a quinone-type structure. NQR is an exceptional enzyme in cutaneous tissues; activity has been detected in rodent epidermal cytosol at higher levels than those measured in liver (Khan *et al.* 1987), and similar findings have been reported for human skin (Merk *et al.* 1991; Merk and Jugert 1991).

Despite the relative activity of this enzyme compared to the liver, it remains surprisingly under-researched in cutaneous tissues.

A number of important Phase II enzymes are present in dermal tissue; they contribute to the skin's tolerance of oxidative stress caused by topical exposure to chemicals and solar radiation. Phase II enzymes are not thought to generate toxic metabolites nor affect skin absorption.

2.6.2 Phase II enzymes – the transferases

The skin possesses several transferase enzymes capable of synthesising glucuronide, sulphate, acetate and glutathione conjugates of functionalised compounds generated from Phase I metabolism, or, where possible, the parent compounds themselves. This generally results in detoxification of either the parent compound or the functionalised metabolite. The capacity of skin to detoxify compounds is believed to exceed the capacity to generate functionalised compounds in Phase I, and this is corroborated by the higher relative activity of Phase II enzymes (Lilienblum *et al.* 1986). This detoxification capacity is not surprising, given the need to detoxify reactive oxygen species originating from UV exposure. Indeed, the skin maintains a battery of detoxification mechanisms designed to remove harmful reactive oxygen species such as the superoxide anion, hydroxyl radical and singlet oxygen; these mechanisms have been reviewed by Afaq and Mukhtar (2001). However, Phase II enzymes are not regarded as being modulators of percutaneous absorption, as they are mainly located in the stratum basale and appendages (though there are notable exceptions).

Glutathione transferases

Glutathione (GSH or 'reduced glutathione') is a cysteine-containing tripeptide present in all eukaryotic cells. Glutathione plays a central role in detoxification of reactive oxygen species, both directly and as a dimer (GSH disulphide or oxidised GSH, GSSG), and the skin has considerable capacity to recycle GSSG to GSH (Connor and Wheeler 1987). Glutathione-S-Tranferases (GSTs) are mainly cytosolic enzymes that catalyse the conjugation of reduced glutathione with electrophilic chemicals (Equation (2.6)). GST is a polymorphic enzyme, and there has been some discussion of the role of GST polymorphisms in susceptibility to certain skin cancers (Strange *et al.* 2001).

$$\mathrm{GSH} + \mathrm{RX} \longrightarrow \mathrm{GSR} + \mathrm{HX} \tag{2.6}$$

Five human isoforms of GST have been characterised: α (GST A), μ (GST M), π (GST P), θ (GST T) and ξ (GST Z) (Mannervik *et al.* 1992). Immunochemical studies have shown that human and rodent skins contain predominately π, with some α only in humans and μ present only in skin from rodent species. Immunhistochemical studies showed the presence of π and μ forms in sebaceous glands and the outer root sheath of hair follicles in murine skin (Raza *et al.* 1991) and π and α in the hair follicles of human skin (Campbell *et al.* 1991). Class π GST was the major isoform in cultured rat keratinocytes (Nakano *et al.* 1997). GST activity in cultured keratinocytes, reconstructed epidermis and hair follicles was greater than or equal to levels in human epidermis (Harris *et al.* 2002). GST activities are greater compared with

liver (up to 50% based on specific activity) than some Phase I enzymes (5–10%, depending on species).

Human and rodent skins have been shown to metabolise a range of substrates (2,4-dinitro chlorobenzene (DNCB), benzo[α]pyrene-4,5-oxide, styrene-7,8-oxide and others; Raza *et al.* 1991; Jewell and Williams 1996). Glutathione depletion has been reported in mouse skin during percutaneous absorption and metabolism of DNCB *in vitro* (Jewell and Williams 1996).

Glucuronyl transferases

UDP-glucuronyl transferases are a family of microsomal enzymes (in contrast to the other transferases which are cytosolic) that catalyse the glucuronidation of a range of substrates using the co-substrate uridine diphosphate glucuronic acid (UDPGA) (Equation (2.7)). Conjugation is via an ether linkage to the oxygen on position 1 of the glucuronic acid molecule (Figure 2.5) in a β-conformation. Their microsomal location is important as they are ideally positioned to carry out conjugation of the functionalised products of cytochrome P450 metabolism.

$$\mathrm{ROH} + \mathrm{UDPGA} \longrightarrow \mathrm{RGA} + \mathrm{UDP} \tag{2.7}$$

Such transformations have been reported in cutaneous tissues and activity measurements are relatively high compared to other cutaneous enzymes systems, ranging from 0.5% to 50% of hepatic activity (Lilienblum *et al.* 1986; Pham *et al.* 1989). Numerous glucuronyl transferase isoforms have been shown to be expressed in the skin (Soars *et al.* 2003; Court, 2005) but despite their role in conjugating hydroxylated metabolites and free acids, their role in the skin remains to be fully evaluated. UGT2B7, UGT2B15 and UGT2B17 isoforms have an endogenous role in the conjugation of androgens, as well as other drugs and xenobiotics, and have been shown to be expressed in the skin (Belanger *et al.* 2003).

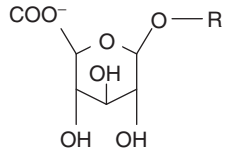


Figure 2.5 General structure of metabolite subject to enzymatic conjugation by glucoronyl transferase

Sulphotransferases

Sulphotransferases catalyse the transfer of sulphate (activated to 3'-phosphoadenosine 5'-phosphosulphate [PAPS] by ATP) to phenol (Equation (2.8)) and amines (Equation (2.9)) resulting in the structure indicated in Figure 2.6. The linkage is either via an ether linkage (phenols) or via the formation of a sulfamate (amines).

$$\mathrm{ROH} + \mathrm{PAPS} \longrightarrow \mathrm{RSO_4^-} + \text{adenosine 3', 5' bisphosphate} \tag{2.8}$$
$$\mathrm{RNH_2} + \mathrm{PAPS} \longrightarrow \mathrm{RNHSO_3^-} + \text{adenosine 3', 5' bisphosphate} \tag{2.9}$$

The general structures of these two products are given in Figure 2.6.

Sulphotransferases are mainly cytosolic enzymes and comprise at least five classes. Human sulphotransferases have been classified on their ability to catalyse sulphate transfer to particular substrates, namely phenols (class P-ST, isoforms ST1A1, 1A2 and 1A3), dopamine (class M-ST, isoform ST1A5) and oestradiol (class HST, isoforms ST1E4 [EST] and HST) (Honma *et al.* 2001, 2002). Human cutaneous sulphotransferase activity is approximately ten- to twenty-fold lower on a weight basis than in liver (Moss *et al.* 1996).

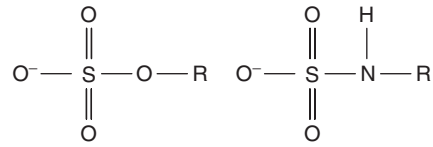


Figure 2.6 General structure of phenol (left) or amine (right) metabolites subject to enzymatic conjugation by sulphotransferases

Sulphotransferases in animals have shown less variability than in humans and similarity to human ST1A3 rather than ST1A5. The SULT2B gene encodes two isoforms of sulphotransferase, SULT2B1a and SULT2B1b. Only SUL2B1b is expressed in the skin (Kohjitani *et al.* 2006). SULT2B1b catalyses the formation of cholesterol sulphate, whose putative role in the control of desquamation is outlined above. This enzyme is expressed in normal human epidermal keratinocytes and expression is increased with differentiation (Higashi *et al.* 2004).

N-Acetyltransferases

N-acetyltransferases catalyse the N-acetylation of nitrogen-containing xenobiotics using Acetyl Coenzyme A as a co-substrate (Equation (2.10)) to produce the general structure represented in Figure 2.7.

$$\text{R-HH2} + \text{AcCoA} \longrightarrow \text{R-NHAc} + \text{CoA} \tag{2.10}$$

There are two classes, NAT-1 and NAT-2, both of which exhibit polymorphisms. NAT activity has been previously demonstrated in cutaneous tissues using a range of substrates, including azo dyes, 2-acetylaminofluorene, benzocaine, p-aminobenzoic acid and others. More recently, Kawakubo *et al.* (2000) investigated the capacity for human skin to acetylate p-phenylenediamine (PPD). Both mono and diacetylated metabolites of PPD were detected in cytosolic fractions of human skin and cultured normal human keratinocytes. The similar rates of formation were measured for the mono and diacetyl metabolites in both whole

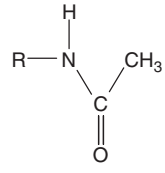


Figure 2.7 General structure of metabolite subject to enzymatic conjugation by N-acetyltransferases

human skin and keratinocytes. Formation of both products was competitively inhibited by p-aminobenzoic acid (a substrate for NAT1) but not by sulfamethazine, though the presence of both NAT1 and NAT2 mRNA was identified. There was no significant difference in the metabolic profile between human volunteers with NAT2 'slow' and 'intermediate' acetylator status after exposure to oxidative hair dyes containing radiolabelled PPD. The major urinary metabolites identified in all samples were N-mono and N, N'-diacetylated PPD, which collectively accounted for 80–95% of urinary metabolites. The data suggested that acetylation of PPD in humans following topical application was independent of NAT2 status, probably due to epidermal metabolism by NAT1 (Nohynek *et al.* 2004).

Summary

- Maintenance of skin barrier function is intimately linked to the optimal development of the stratum corneum.
  - Formation of the stratum corneum is the result of keratinocytes undergoing terminal differentiation and the role of certain proteins and lipids associated with this process are under intricate metabolic control.
  - Also under control is the balance between biosynthesis and desquamation (loss) of the stratum corneum.
  - Factors that disrupt the orchestration of such metabolic systems can lead to a variety of disease states in which there is a dysfunctional stratum corneum and/or other pathological consequences.
- The metabolic capacity of the skin provides a front-line defence against a number of toxic chemicals.
  - In some circumstances, dermal metabolism can increase the local or systemic toxicity of an absorbed compound.
  - The presence of enzymes in the stratum corneum can enhance the rate and extent to which some substances undergo percutaneous absorption.

References

Afaq, F. and Mukhtar, H. (2001). Effects of solar radiation on cutaneous detoxification pathways. *Journal of Photochemistry and Photobiology B Biology*, **63**: 61–69.

Agarwal, R., Jugert, F.K., Khan, S.G., *et al.* (1994). Evidence for multiple inducible cytochrome P450 ioszymes in Sencar mouse skin by pyridine. *Biochemical and Biophysical Research Communications,* **199**: 1400–1406.

Ahmed, S.T., Imai, T., Yoshigae, Y., *et al.* (1997). Stereospecific activity and nature of metabolizing esterases for propanolol prodrug in hairless mouse skin, liver and plasma. *Life Sciences*, **61**: 1879–87.

Baird, W.M., Hooven, L.A. and Mahadevan, B. (2005). Carcinogenic polycyclic aromatic hydrocarbon-DNA adducts and mechanism of action. *Environmental and Molecular Mutagenesis*, **45**: 106–114.

Barker, C.L. and Clothier, R.H. (1997). Human keratinocyte cultures as models of cutaneous esterase activity. *Toxicology in Vitro*, **11**: 637–640.

Baron, J.M., Holler, D., Schiffer, R., *et al.* (2001). Expression of multiple cytochrome P450 enzymes and multidrug resistance-associated transport proteins in human skin keratinocytes. *Journal of Investigative Dermatology*, **116**: 541–548.

Beckley-Kartey, S.A.J., Hotchkiss, S.A.M. and Capel, M. (1997). Comparative *in vitro* skin absorption and metabolism of coumarin (1,2-benzopyrone) in human, rat and mouse. *Toxicology and Applied Pharmacology*, **145**: 34–42.

Beisson, F., Aoubala, M., Marull, S., *et al.* (2001). Use of tape stripping technique for directly quantifying esterase activity in human stratum corneum. *Analytical Biochemistry*, **290**: 179–185.

Belanger, A., Pelletier, G., Labrie, F., *et al.* (2003). Inactivation of androgens by UDP-glucuronosyltransferase enzymes in humans. *Trends in Endocrinology and Metabolism*, **14**: 473–479.

Bickers, D.R. and Mukhtar, H. (1990). Skin as a portal for entry for systemic effect: xenobiotic metabolism, in *Skin Pharmacology and Toxicology, Recent Advances* (eds Galli, C.L., Hensby, C.N. and Marinovich, M.). Plenum Press, New York, 85–97.

Bickers, D.R., Mukhtar, H., Dutta-Choudhury, T., *et al.* (1984), Aryl hydrocarbon hydroxylase, epoxide hydrolase and benzo[a]pyrene metabolism in human epidermis: comparative studies in normal subjects and patients with psoriasis. *Journal of Investigative Dermatology*, **83**: 51–56.

Bonte, F., Saunois, A., Pinguet, P. and Meybeck, A. (1997). Existence of a lipid gradient in the upper stratum corneum and its possible biological significance. *Arch Dermatol Res*, **289**(2): 78–82.

Boogaard, P.J., van Elburg, P.A., de Kloe, K.P., *et al.* (1999). Metabolic inactivation of 2-oxiranylmethyl 2-ethyl-2,5-dimethylhexanoate (C10GE) in skin, lung and liver of human, rat and mouse. *Xenobiotica*, **29**: 987–1006.

Bouwstra, J., Pilgram, G., Gooris, G., *et al.* (2001). New aspects of the skin barrier organization. *Skin Pharmacology and Applied Skin Physiology*, **14**(S1): 52–62.

Bouwstra, J.A. and Ponec, M. (2006). The skin barrier in healthy and diseased state. *Biochim Biophys Acta.* **1758**(12): 2080–95.

Brattsand, M. and Egelrud, T. (1999). Purification, molecular cloning, and expression of a human stratum corneum trypsin-like serine protease with possible function in desquamation. *Journal of Biological Chemistry*, **274**: 30033–30040.

Bronaugh, R.L., Collier, S.W., Macpherson, S.E., *et al.* (1994). Influence of metabolism in skin on dosimetry after topical exposure. *Environmental Health Perspectives*, **102**: 71–74 Suppl.

Campbell, J.A., Corrigall, A.V., Guy, A., *et al.* (1991). Immunohistologic localization of alpha, mu and pi class glutathione S-transferases in human tissues. *Cancer*, **67**: 1608–1613.

Chapman, S.J., Walsh, A., Jackson, S.M. and Friedmann, P.S. (1991). Lipids, proteins and corneocyte adhesion. *Archives of Dermatology Research*, **283**: 167–173.

Cheung, C., Smith, C.K., Hoog, J.O., *et al.* (1999). Expression and localization of human alcohol and aldehyde dehydrogenase enzymes in skin. *Biochemical and Biophysical Research Communications*, **261**: 100–107.

Cheung, C., Davies, N.G., Hoog, J.O., *et al.* (2003a). Species variations in cutaneous alcohol dehydrogenases and aldehyde dehydrogenases may impact on toxicological assessments of alcohols and aldehydes. *Toxicology*, **184**: 97–112.

Cheung, C., Hotchkiss, S.A.M. and Pease, C.K.S. (2003b). Cinnamic compound metabolism in human skin and the role metabolism may play in determining relative sensitisation potency. *Journal of Dermatological Sciences*, **31**: 9–19.

Clark, N.W.E. (1992). Cutaneous xenobiotic metabolism and its role in percutaneous absorption. PhD thesis 1992, University of Newcastle, UK.

Clark, N.W.E., Scott, R.C., Blain, P.G., *et al.* (1993). Fate of fluazifop butyl in rat and human skin *in vitro*. *Archives of Toxicology* **67**: 44–48.

Connor, M.J. and Wheeler, L.A. (1987). Depletion of cutaneous glutathione by ultraviolet radiation. *Photochemistry and Photobiology*, **46**: 239–245.

Correia, M.A. (2001). Drug biotransformation, in *Basic and Clinical Pharmacology*, 8th edn (ed. Katzung, B.G.). McGraw Hill, New York, 51–63.

Court, M.H. (2005). Isoform-selective probe substrates for *in vitro* studies of human UDP-glucuronosyltransferases. *Methods in Enzymology*, **400**: 104–116.

Dale, B.A., Resing, K.A. and Lonsdale-Eccles, J.D. (1985). Filaggrin: a keratin filament associated protein. *Annals of New York Academy of Sciences*, **455**: 330–42.

Doering, T., Holleran, W., Potratz, A., *et al.* (1999a). Sphingolipid activator proteins are required for epidermal permeability barrier formation. *Journal of Biological Chemistry*, **274**: 11038–11045.

Doering, T., Proia, R.L. and Sandhoff, K. (1999b). Accumulation of protein-bound epidermal glucosylceramides in b-glucocerebrosidase deficient type 2 Gaucher mice. *FEBS Letters*, **447**: 167–170.

Du, L.P., Yermalitsky, V., Ladd, P.A., *et al.* (2005). Evidence that cytochrome P450 CYP2B19 is the major source of epoxyeicosatrienoic acids in mouse skin. *Archives of Biochemistry and Biophysics*, **435**: 125–133.

Du, L.P., Neis, M.M., Ladd, P.A., *et al.* (2006). Effects of the differentiated keratinocyte phenotype on expression levels of CYP1-4 family genes in human skin cells. *Toxicology and Applied Pharmacology*, **213**: 135–144.

Duester, G., Farres, J., Felder, M.R., *et al.* (1999). Recommended nomenclature for the vertebrate alcohol dehydrogenase gene family. *Biochemical Pharmacology*, **58**: 389–395.

Ekholm, I.E., Brattsand, M. and Egelrud, T. (2000). Stratum corneum tryptic enzyme in normal epidermis: A missing link in the desquamation process? *Journal of Investigative Dermatology*, **114**: 56–63.

Egberts, F., Heinrich, M., Jensen, J.M., *et al.* (2004). Cathepsin D is involved in the regulation of transglutaminase 1 and epidermal differentiation. *Journal of Cell Science,* **117**: 2295–2307.

Egelrud, T. and Lundstrom, A. (1990). The dependence of detergent-induced cell dissociation in non-palmo-plantar stratum corneum on endogenous proteolysis. *Journal of Investigative Dermatology*, **95**: 456–459.

Enayetallah, A.E., French, R.A., Thibodeau,. M.S., *et al.* (2004). Distribution of soluble epoxide hydrolase and of cytochrome P4502C8, 2C9, and 2J2 in human tissues. *Journal of Histochemistry and Cytochemistry*, **52**: 447–454.

Finnen, M.J. (1987). Skin metabolism by oxidation and conjugation, in *Pharmacology and the skin. Vol 1. Skin Pharmacokinetics* (eds Shroot, B. and Schaefer, H.). Basel: Karger, 163–169.

Frankenberg, S., Jugert, F.K. and Merk, H.F. (1993). Multiple cytochrome P450 isozymes present in human hair follicle derived keratinocytes. *Journal of Investigative Dermatology*, **100**: 518.

Fusek, M. and Vetvicka, V. (2005). Dual role of cathepsin D: ligand and protease. *Biomed Papers*, **149**: 43–50.

Gan, S.Q., McBride, O.W., Idler, W.W., *et al.* (1990). Organization, structure, and polymorphisms of the human profilaggrin gene. *Biochemistry*, **29**: 9432–40.

Garnett, A. (1992) Investigation of the *in vitro* percutaneous absorption and skin metabolism of benzyl acetate and related compounds. PhD Thesis, University of London, UK.

Gelardi, A., Morini, F., Dusatti, F., *et al.* (2001). Induction by xenobiotics of phase I and phase II enzyme activities in the human keratinocyte cell line NCTC 2544. *Toxicology in Vitro*, **15**: 701–711.

Hanson, L., Stromqvist, M., Backman, A., *et al.* (1994). Cloning, expression, and characterization of stratum corneum chymotryptic enzyme. A skin-specific human serine proteinase. *Journal of Biological Chemistry*, **269**: 19420–19426.

Hara, J., Higuchi, K., Okamoto, R., *et al.* (2000). High-expression of sphingomyelin deacylase is an important determinant of ceramide deficiency leading to barrier disruption in atopic dermatitis. *Journal of Investigative Dermatology*, **115**: 406–413.

Harding, C.R. and Scott, I.R. (1983). Histidine rich proteins (filaggrins): structural and functional heterogeneity during epidermal differentiation. *Journal of Molecular Biology*, **170**: 651–673.

Harris, I.R., Siefken, W., Beck-Oldach, K., *et al.* (2002). NAD(P)H : quinone reductase activity in human epidermal keratinocytes and reconstructed epidermal models. *Skin Pharmacology and Applied Skin Physiology*, **15**(S1): 58–73.

Hewitt, P.G., Perkins, J. and Hotchkiss, S.A.M. (2000). Metabolism of fluroxypyr, fluroxypyr methyl ester, and the herbicide fluroxypyr methylheptyl ester. I: During percutaneous absorption through fresh rat and human skin *in vitro*. *Drug Metabolism and Disposition*, **28**: 748–754.

Higashi, Y., Fuda, H., Yania, H., *et al.* (2004). Expression of cholesterol sulfotransferase (SULT2B1b) in human skin and primary cultures of human epidermal keratinocytes. *Journal of Investigative Dermatology*, **122**: 1207–13.

Higuchi, W.I., Yu, C.-D. (1987). Prodrugs in transdermal delivery. in *Transdermal delivery of drugs Vol 3* (eds Kydonius, A.F., Berner, B.). CRC Press, Boca Raton, 43–83.

Holleran, W.M., Takagi, Y., Menon, G.L.K., *et al.* (1993). Processing of epidermal glucosylceramides is required for optimal mammalian cutaneous permeability barrier function. *Journal of Clinical Investigation*, **91**: 1656–1664.

Holleran, W.M., Ginns, E.I., Menon, G.K., *et al.* (1994). Consequences of betaglucocerebrosidase deficiency in epidermis. Ultra structure and permeability barrier alterations in Gaucher disease. *Journal of Clinical Investigation*, **93**: 1756–1764.

Honma, W., Kamiyama, Y., Yoshinari, K., *et al.* (2001). Enzymatic characterisoation and interspecies difference of phenol sulfotransferases, ST1A forms. *Drug Metabolism and Disposition*, **29**: 274–281.

Honma, W., Shimada, M., Sasano, H., *et al.* (2002). Phenol sulphotransferase ST1A3, as the main enzyme catalyzing the sulfation of troglitazone in human liver. *Metabolism and Disposition*, **30**: 944–949.

Hotchkiss, S.A.M., Hewitt, P.G. and Edwards, R. (1996). Immunochemical detection of specific cytochrome P450 enzymes in uninduced BALB/c mouse skin. *Proceedings of ISSX 10*, Abstract 172.

Huang, T.L., Shiotsuki, T., Uematsu, T., *et al.* (1996). Structure-activity relationships for substrates and inhibitors of mammalian liver microsomal carboxylesterases. *Pharmaceutical Research*, **13**: 1495–1500.

Huber, M., Rettler, I., Bernasconi, K., *et al.* (1995). Mutations of keratinocyte transglutaminase in lamallar ichthyosis. *Science*, **267**: 525–528.

Ide, F., Suka, N., Kitada, M., *et al.* (2004). Skin and salivary gland carcinogenicity of 7,12-dimethylbenz[a]anthracene is equivalent in the presence or absence of aryl hydrocarbon receptor. *Cancer Letters*, **214**: 35–41.

Imai, T. (2006). Human carboxylesterases isozymes: catalytic properties and rational drug design. *Drug Metabolism and Pharmacokineticsm* **21**: 174–185.

Irvine, A.D. and McLean, W.H. (2006). Breaking the (un)sound barrier: filaggrin is a major gene for atopic dermatitis. *J Invest Dermatol.*, **126**(6): 1200–2.

Jameson, H.L., Hotchkiss, S.A.M. and Edwards, R.J. (1997). Developmental changes in cytochrome P450 expression in Sprague-Dawley rat skin and liver. *Human and Experimental Toxicology*, **16**: 404.

Janmohamed, A., Dolphin, C.T., Phillips, I.R., *et al.* (2001). Quantification and cellular localization of expression in human skin of genes encoding flavin-containing monooxygenases and cytochromes P450. *Biochemical Pharmacology*, **62**: 777–786.

Jennemann, R., Sandhoff, R., Langbein, L., *et al.* (2007). Integrity and barrier function of the epidermis critically depend on glucosylceramide synthesis. *Journal of Biological Chemistry*, **282**: 3083–3094.

Jewell, C. and Williams, F. (1996). Absorption and metabolism of dinitrochlorobenzene through mouse skin *in vitro*, in *Prediction of percutaneous penetration, Vol. 4b*, (eds Brain, K.R., James, V.J., Walters, K.A.), STS Publishing, Cardiff, 218–221.

Jewell, C., Ackermann, C., Payne, N.A., *et al.* (2007a). Specificity of procaine and ester hydrolysis by human, minipig, and rat skin and liver. *Drug Metabolism and Disposition*, **35**: 2015–2022.

Jewell, C., Prusakiewicz, J.J., Ackerman, C., *et al.* (2007b). Hydrolysis of a series of paraben by skin microsomes and cytosol from human and minipigs and in whole skin in short-term culture. *Toxicology & Applied Pharmacology*, **225**: 221–228.

Jones, C.L., Reiners, J.J. (1997). Differentiation status of cultured murine keratinocytes modulates induction of genes responsive to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Archives of Biochemistry and Biophysics*, **347**: 163–173.

Jugert, F.K., Agarwal, R., Khun, A., *et al.* (1994). Multiple cytochrome P450 isozymes in murine skin: induction of P450 1A, 2B, 2E and 3A by dexamethasone. *Journal of Investigative Dermatology*, **102**: 970–975.

Kalinin, A.E., Kajava, A.V. and Steinert, P.M. (2002). Epithelial barrier function: Assembly and structural features of the cornified cell envelope. *Bioessays,* **24**: 789–800.

Katiyar, S.K., Matsui, M.S. and Mukhtar, H. (2000). Ultraviolet-B exposure of human skin induces cytochromes P450 1A1 and 1B1. *Journal of Investigative Dermatology*, **114**: 328–333.

Kawakubo, Y., Merk, H.F., Al Masaoudi, T., *et al.* (2000). N-Acetylation of paraphenylenediamine in human skin and keratinocytes. *Journal of Pharmacology and Experimental Therapeutics*, **292**: 150–155.

Kawakubo, Y., Tamiya, S., Umezawa, Y., *et al.* (2001). Distribution of cytochrome p450 (CYP) 2e1 in the skin: A novel marker for keratinocyte differentiation? *Journal of Investigative Dermatology*, **117**: 792.

Khan, W.A., Das, M., Stick, S., *et al.* (1987). Induction of epidermal NAD(P)H:quinone reductase by chemical carcinogens: a possible mechanism for detoxification. *Biochemical and Biophysical Research Communications*, **146**: 126–133.

Khan, W.A., Park, S.S., Gelboin, H.V., *et al.* (1989a). Epidermal cytochrome P-450: immunochemical characterization of isoform induced by topical application of 3-methylcholanthrene to neonatal rat. *Journal of Pharmacology and Experimental Therapeutics*, **249**: 921–927.

Khan, W.A., Park, S.S., Gelboin, H.V., *et al.* (1989b). Monoclonal antibodies directed characterization of epidermal and hepatic cytochrome P-450 isoforms induced by skin application of therapeutic crude coal tar. *Journal of Investigative Dermatology*, **93**: 40–45.

Kleiner, H.E., Vulimiri, S.V., Hatten, W.B., *et al.* (2004). Role of cytochrome p4501 family members in the metabolic activation of polycyclic aromatic hydrocarbons in mouse epidermis. *Chemical Research in Toxicology*, **17**: 1667–1674.

Kohjitani, A., Fuda, H., Hanyu, O., *et al.* (2006). Cloning, characterization and tissue expression of rat SULT2B1a and SULT2B1b steroid/sterol sulfotransferase isoforms: divergence of the rat SULT2B1 gene structure from orthologous human and mouse genes. *Gene*, **367**: 66–73.

Kubota, K., Ademola, J., Maibach, H.I. (1994). Metabolism and degradation of betamethasone 17-valerate in homogenized living skin equivalent. *Dermatology*, **188**: 13–17.

Ledirac, L., Delescluse, C., de Sousa, G., *et al.* (1997). Carbaryl induces CYP1A1 gene expression in HepG2 and HaCaT cells but is not a ligand of the human hepatic Ah receptor. *Toxicology and Applied Pharmacology*, **144**: 178–182.

Lee, A., Choi, W., Ko, D., *et al.* (2001). Constitutive expression and distribution of cytochrome P450 isozymes in normal human skin. *Journal of Investigative Dermatology*, **117**: 528.

Li, X.-Y., Duell, E.A., Qin, L., *et al.* (1994). Cytochrome P450 3A5 is the major 3A subfamily member expressed in normal human skin *in vivo*. *Journal of Investigative Dermatology*, **102**: 624.

Liederer, B.M. and Borchardt, R.T. (2006). Enzymes involved in the bioconversion of ester-based prodrugs. *Journal of Pharmceutical Sciences*, **95**: 1117–1195.

Lilienblum, W., Irmscher, G., Fusenig, N.E., *et al.* (1986). Induction of UDP-glucuronyltransferase and arylhydrocarbon hydroxylase activity in mouse skin and in normal and transformed skin cells in culture. *Biochemical Pharmacology*, **35**: 1517.

List, K., Szabo, R., Wertz, P.W., *et al.* (2003). Loss of proteolytically processed filaggrin caused by epidermal deletion of matriptase/MT-SP1. *Journal of Cell Biology*, **163**: 901–910.

Lockley, D.J., Howes, D. and Williams, F.M. (2002). Percutaneous penetration and metabolism of 2-ethoxyethanol. *Toxicology and Applied Pharmacology*, **180**: 74–82.

Lockley, D.J., Howes, D. and Williams, F.M. (2004). Percutaneous penetration and metabolism of 2-butoxyethanol. *Archives of Toxicology*, **78**: 617–628.

Lockley, D.J., Howes, D. and Williams, F.M. (2005). Cutaneous metabolism of glycol ethers. *Archives of Toxicology*, **79**: 160–168.

Lobemeier, C., Tschoetschel, C., Westie, S., *et al.* (1996) Hydrolysis of parabenes by extracts from differing layers of human skin. *Biological Chemistry*, **377**: 647–651.

Lundstrom, A. and Egelrud, T. (1990) Evidence that cell shedding from plantar stratum corneum *in vitro* involves endogenous proteolysis of the desmosomal protein desmoglein I. *Journal of Investigative Dermatology*, **94**: 216–220.

Macheleidt, O., Kaiser, H.W. and Sandhoff, K. (2002). Deficiency of epidermal protein-bound ω-hydroxyceramides in atopic dermatitis. *Journal of Investigative Dermatology*, **119**: 166–173.

Maly, I.P., Toranelli, M. and Sasse, D. (1999). Distribution of alcohol dehydrogenase isoenzymes in the human liver acinus. *Histochemical Cell Biology*, **111**: 391–397.

Mannervik, B., Awasthi, Y.C., Board, P.G., *et al.* (1992). Nomenclature for human glutathione transferases. *Biochemical Journal*, **282**: 305–306.

Marekov, L.N. and Steinert, P.M. (1998). Ceramides are bound to structural proteins of the human foreskin epidermal cornified cell envelope. *Journal of Biological Chemistry*, **273**: 17763–17770.

Mayer, W. and Neurand, K. (1976). The distribution of enzymes in the skin of the domestic pig. *Laboratory Animals*, **10**: 237–247.

McCracken, N.W., Blain, P.G. and Williams, F.M. (1993). Nature and role of xenobiotic metabolising esterases in rat liver, lung, skin and blood. *Biochemical Pharmacology*, **45**: 31–36.

Méchin, M.C., Enji, M., Nachat, R., *et al.* (2005). The peptidylarginine deiminases expressed in human epidermis differ in their substrate specificities and subcellular locations. *Cell and Molecular Life Sciences*, **62**: 1984–1995.

Merk, H.F. and Jugert, F.K. (1991). Cutaneous NAD(P)H:quinone reductase: a xenobiotic metabolizing enzyme with potential cancer and oxidative stress protecting properties. *Skin Pharmacology*, **4**: 95–100.

Merk, H.F., Mukhtar, H., Schutte, B., *et al.* (1987). Human hair follicle benzo[a]pyrene and enzo[a]pyrene-7,8-diol metabolism: effect of exposure to a coal tar-containing shampoo. *Journal of Investigative Dermatology*, **88**: 71–76.

Merk, H., Jugert, F., Bonnekoh, B., *et al.* (1991). Induction and inhibition of NAD(P)H:quinone reductase in murine and human skin. *Skin Pharmacology*, **4** Supplement 1: 183–190.

Mint, A. (1995). Investigation into the topical disposition of the phthalic acid esters, dimethyl phthalate, diethyl phthalate and dibutyl phthalate in rat and human skin. PhD Thesis, University of London, UK.

Moss, T., Howes, D. and Williams, F.M. (1996). Characteristics of sulphotransferases in human skin, in *Prediction of percutaneous penetration, Vol. 4b*, (eds Brain, K.R., James, V.J., Walters, K.A.), STS Publishing, Cardiff, 307–311.

Mukhtar, H. and Khan, W.A. (1989). Cutaneous cytochrome P-450. *Drug Metabolism Reviews*, **20**: 657–673.

Murray, G.I., Barnes, T.S., Sewell, H.F., *et al.* (1988). The immunocytochemical localisation and distribution of cytchrome P-450 in normal human hepatic and extrahepatic tissues with a monoclonal antibody to human cytochrome P-450. *British Journal of Clinical Pharmacology*, **25**: 465–475.

Nakano, H., Kimura, J., Kumano, T., *et al.* (1997). Decrease in class pi glutathione transferase mRNA levels by ultraviolet irradiation of cultured rat keratinocytes. *Japanese Journal of Cancer Research*, **88**: 1063–1069.

Nemes, Z., Marekov, L.N., Fésues, L. and Steinert, P.M. (1999). *A novel function for transglutaminase1. Attachment of long-chain ω-hydroxyceramides to involucrin by ester bond formation*. Proceedings of the National Academy of Sciences, USA 96, 8402–8407.

Ngawhirunpat, T., Opanasopit, P. and Prakongpan, S. (2004). Comparison of skin transport and metabolism of ethyl nicotinate in various species. *European Journal of Pharmaceutics and Biopharmaceutics*, **58**: 645–651.

Nohynek, G.J., Skare, J.A., Meuling, W.J.A., *et al.* (2004). Urinary acetylated metabolites and N-acetyltransferase-2 genotype in human subjects treated with a para-phenylenediamine-containing oxidative hair dye. *Food and Chemical Toxicology*, **42**: 1885–1891.

Palmer, C.N., Irvine, A.D., Terron-Kwiatkowski, A., *et al.* (2006). Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nature Genetics*, **38**(4): 441–446.

Pham, M.A., Magdalou, J., Siest, G., *et al.* (1990). Reconstituted epidermis: a novel model for the study of drug metabolism in human epidermis. *Journal of Investigative Dermatology*, **94**: 749–752.

Pham, M.A., Magdalou, J., Totis, M., *et al.* (1989). Characterization of distinct forms of cytochromes P-450, epoxide metabolising enzymes and UDP-glucuronyltransferases in rat skin. *Biochemical Pharmacology*, **38**: 2187–2194.

Pilgram, G.S.K., Vissers, D.C.J., van der Meulen, H., *et al.* (2001). Aberrant lipid organization in stratum corneum of patients with atopic dermatitis and lamellar ichthyosis. *Journal of Investigative Dermatology*, **117**: 710–717.

Ponec, M., Lankhorst, P., Weerheim, A. and Wertz, P. (2003). New acylceramide in native and reconstructed epidermis. *Journal of Investigative Dermatology*, **120**: 581–588.

Prusakiewicz, J.J., Ackermann, C. and Voorman, R. (2006) Comparison of skin esterase from different species. *Pharmaceutical Research*, **23**: 1517–1524.

Raffali, F., Rougier, A. and Rouget, R. (1994). Measurement and modulation of cytochrome P450 dependent enzyme activity in cultured human keratinocytes. *Skin Pharmacology*, **7**: 345–354.

Rastogi, S., Dogra, R.K.S., Khanna, S.K., *et al.* (2006). Skin tumorigenic potential of aflatoxin B1 in mice. *Food and Chemical Toxicology*, **44**: 670–677.

Raza, H., Awasthi, Y.C., Zaim, M.T., *et al.* (1991). Glutathione S-trasnferases in human and rodent skin: multiple forms and species specific expression. *Journal of Investigative Dermatology*, **96**: 463–467.

Reiners, J.J., Amador, R.C., Pavone, A. (1990). Modulation of constitutive cytochrome P450 expression *in vivo* and *in vitro* in murine keratinocytes as a function of differentiation and extracellular Ca2+ concentration. *Proceedings of the National Academy of Sciences*, **87**: 1825–1829.

Rettie, A.E., Williams, F.M., Rawlins, M.D., *et al.* (1986) Major differences between lung, skin and liver in the microsomal metabolism of homologous series of resorufin and coumarin ethers. *Biochemical Pharmacology*, **35**: 3495–3500.

Reilly, T.P., Lash, L.H., Doll, M.A., *et al.* (2000). A role for bioactivation and covalent binding within epidermal keratinocytes in sulfonamide-induced cutaneous drug reactions. *Journal of Investigative Dermatology*, **114**: 1164–1173.

Rogan, E.G., Devanesan, P.D., Ramakrishna, N.V.S., *et al.* (1993). Identification and quantitation of benzo[a]pyrene dna adducts formed in mouse skin. *Chemical Research in Toxicology*, **6**: 356–363.

Roper, C.S., Howes, D., Blain, P.G., *et al.* (1997). Percutaneous penetration of 2-phenoxyethanol through rat and human skin. *Food and Chemical Toxicology*, **35**: 1009–1016.

Saarikoski, S.T., Wikman, H.A.L,. Smith, G., *et al.* (2005). Localization of cytochrome P450CYP2S1 expression in human tissues by *in situ* hybridization and immunohistochemistry. *Journal of Histochemistry and Cytochemistry*, **53**: 549–556.

Saeki, M., Saito, Y., Nagano, M., *et al.* (2002). mRNA expression of multiple cytochrome P450 isozymes in four types of cultured skin cells. *International Archives of Allergy and Immunology*, **127**: 333–336.

Sato, J., Denda, M., Nakanishi, J., *et al.* (1998). Cholesterol sulfate inhibits proteases that are involved in desquamation of stratum corneum. *Journal of Investigative Dermatology*, **111**: 189–193.

Satoh, T. and Hosokawa, M. (1998). The mammalian carboxylesterases: from molecules to function. *Annual Review of Pharmacology and Toxicology*, **38**: 257–288.

Sawaya, M.E. and Penneys, N.S. (1992). Immunohistochemical distribution of aromatase and 3B-hydroxysteroid dehydrogenase in human hair follicle and sebaceous gland. *Journal of Cutaneous Pathology*, **19**: 309–314.

Scott, I.R., Harding, C.R. and Barrett, J.G. (1982). Histidine-rich protein of the keratohyalin granules. Source of the free amino acids, urocanic acid and pyrrolidone carboxylic acid in the stratum corneum. *Biochimica et Biophysica Acta*, **719**: 110–117.

Serizawa, S., Osawa, K., Togashi, K., *et al.* (1992). Relationship between cholesterol sulfate and intercellular cohesion of the stratum corneum: demonstration using a push-pull meter and an improved high-performance thin-layer chromatographic separation system of all major stratum corneum lipids. *Journal of Investigative Dermatology*, **99**(2): 232–6.

Serre, G., Mils, V., Haftek, M., *et al.* (1991). Identification of late differentiation antigens of human cornified epithelia, expressed in re-organized desmosomes and bound to cross-linked envelope. *Journal of Investigative Dermatology*, **97**: 1061–1072.

Skerrow, C.J., Clelland, D.G. and Skerrow, D. (1989). Changes to desmosomal antigens and lectin-binding sites during differentiation in normal human epidermis: A quantitative ultrastructural study. *Journal of Cell Science*, **92**: 667–677.

Smith, M. (1985). Quinones as mutagens, carcinogens, and anticancer agents: Introduction and overview. *Journal of Toxicology and Environmental Health*, **16**: 665–672.

Smith, C.K., Moore, C.A., Elahi, E.N., *et al.* (2000). Human skin absorption and metabolism of the contact allergens, cinnamic aldehyde, and cinnamic alcohol, *Toxicology and Applied Pharmacology*, **168**: 188–199.

Smith, G., Dawe, R.S., Clark, C., *et al.* (2003a). Cytochrome P450 Quantitative real-time reverse transcription-polymerase chain reaction analysis of drug metabolizing and cytoprotective genes in psoriasis and regulation by ultraviolet radiation. *Journal of Investigative Dermatology*, **121**: 390–398.

Smith, G., Deeni, Y.Y., Dawe, R.S., *et al.* (2003b). Cytochrome P450CYP2S1 expression in human skin: individuality in regulation by therapeutic agents for psoriasis and other skin diseases. *British Journal of Dermatology*, **148**: 853.

Smith, G., Ibbotson, S.H., Comrie, M.M., *et al.* (2006). Regulation of cutaneous drug-metabolizing enzymes and cytoprotective gene expression by topical drugs in human skin *in vivo*. *British Journal of Dermatology*, **155**: 275–281.

Soars, M.G., Ring, B.J. and Wrighton, S.A. (2003). The effect of incubation conditions on the enzyme kinetics of UDP-glucuronosyltransferases. *Drug Metabolism and Disposition*, **31**: 762–767.

Steinert, P.M., Cantieri, J.S., Teller, D.C., *et al.* (1981). *Characterization of a class of cationic proteins that specifically interact with intermediate filaments*. Proceedings of the National Academy of Sciences, USA 78, 4097–101.

Stewart, M.E. and Downing, D.T. (1999). 6-Hydroxy-4-sphingenine in human epidermal ceramides. *Journal of Lipid Research*, **40**: 1434–1439.

Stewart, M.E. and Downing, D.T. (2001) The ω-hydroxyceramides of pig epidermis are attached to corneocytes solely through ω-hydroxyl groups. *Journal of Lipid Research*, **42**: 1105–1110.

Storm, J.E., Collier, S.W., Stewart, R.F., *et al.* (1990). Metabolism of xenobiotics during percutaneous absorption: role of absorption rate and cutaneous enzyme activity. *Fundamental and Applied Toxicology*, **15**: 132–141.

Strange, R.C., Spiteri, M.A., Ramachandran, S., *et al.* (2001). Glutathione-S-transferase family of enzymes. *Mutation Research Fundamentals and Molecular Mechanisms of Mutagenesis*, **482**(special issue): 21–26.

Suzuki, Y., Nomura, J., Koyama, J. and Horii, I. (1994). The role of proteases in stratum corneum. Involvement in stratum corneum desquamation. *Archives of Dermatology Research*, **286**: 249–253.

Tonge, R.P. (1995). The cutaneous disposition of the sensitizing chemicals hydroxycitronellal and dinitrochlorobenzene. PhD Thesis, University of London, UK.

Ulrich, K., Amatschek, S., Uthman, A., *et al.* (2004). Treatment of human skin with retinoic acid strongly induces aldehyde dehydrogenase 1A3. *Journal of Investigative Dermatology*, **122**: A87.

Vyas, P.M., Roychowdhury, S., Khan, F.D., *et al.* (2006). Enzyme-mediated protein haptenation of dapsone and sulfamethoxazole in human keratinocytes: I. expression and role of cytochromes p450. *Journal of Pharmacology and Experimental Therapeutics*, **319**: 488–496.

Weibe, I.H., Hansen, J. (1989) Interaction of cinnamaldehyde (a sensitiser in fragrance) with protein. *Contact Dermatitis*, **20**: 161–166.

Wertz, P.W. and Downing, D.T. (1983). Acylglucosylceramides of pig epidermis: structure determination. *Journal of Lipid Research*, **24**: 753–758.

Whitlock, J.P. (1999). Induction of cytochrome P4501A1. *Annual Review of Pharmacology and Toxicology*, **39**: 103–125.

Williams, F.M. (1985). Clinical significance of esterases in man. *Clinical Pharmacokinetics*, **10**: 392–403.

Yamamura, T. and Tezuka, T. (1990). Change in sphingomyelinase activity in human epidermis during aging. *Journal of Dermatological Sciences*, **1**: 79–84.

Yang, J.M., Ahn, K.S., Cho, K., *et al.* (2001). Novel mutations of the transglutaminase 1 gene in lamellar ichthyosis. *Journal of Investigative Dermatology*, **117**: 214–218.

Yengi, L.G., Xiang, Q., Pan, J.M., *et al.* (2003). Quantitation of cytochrome P450 mRNA levels in human skin. *Analytical Biochemistry*, **316**: 103–110.

Yoshida, A., Rzhetsky, A., Hsu, L.C. and Chang, C. (1998). Human aldehyde dehydrogenase gene family. *European Journal of Biochemistry*, **251**: 549–557.

Zhu, Z.Y., Hotchkiss, S.A., Boobis, A.R., *et al.* (2002). Expression of P450 enzymes in rat whole skin and cultured epidermal keratinocytes. *Biochemical and Biophysical Research Communications*, **297**: 65–70.

3 Skin photobiology

Mark A. Birch-Machin[1] and **Simon C. Wilkinson**[2]

[1]*Dermatological Sciences, Institute of Cellular Medicine, Newcastle-Upon-Tyne, NE2 4AA, UK*
[2]*Medical Toxicology Research Centre, University of Newcastle, Newcastle-Upon-Tyne, NE2 4AA, UK*

Primary Learning Objectives

- *Overview of cutaneous photoprotection systems.*
- *Understanding of the link between 1. ultraviolet radiation exposure and 2. photoageing and skin cancer.*
- *Mechanisms of oxidative stress.*
- *Mitochondrial DNA as a biomarker of sun exposure in human skin.*
- *Role of apoptosis.*
- *Mitochondria and cancer.*
- *Physical sun protection.*

3.1 Introduction and scope

The solar spectrum consists of ultraviolet, visible and infrared rays with ultraviolet radiation (UVR) comprising 5% of the total spectrum (Diffey 2002a, 2002b; Figure 3.1). There are three wavelength regions of UVR; these are defined as UVA (315–400 nm), UVB (280–315 nm) and short wave UVC (100–280 nm) (Diffey 2002a, 2002b). UVB contributes approximately 6% of the total UVR reaching the earth's surface and UVA makes up the remaining 94%. UVC has little biological importance as wavelengths <290 nm are absorbed by the stratospheric ozone layer (Sander *et al.* 2004).

3.2 Photoprotection and melanogenesis

Melanin can provide limited photoprotection by absorbing and dissipating the energy contained in ultraviolet (solar) radiation. In the absence of exposure to sunlight, the baseline amount of pigment in the skin is genetically controlled but solar exposure can stimulate the production of additional melanin through the process of 'tanning'. Melanin can also bind certain drugs and so modulate efficacy/toxicity.

Principles and Practice of Skin Toxicology Edited by Robert P. Chilcott and Shirley Price
© 2008 John Wiley & Sons, Ltd

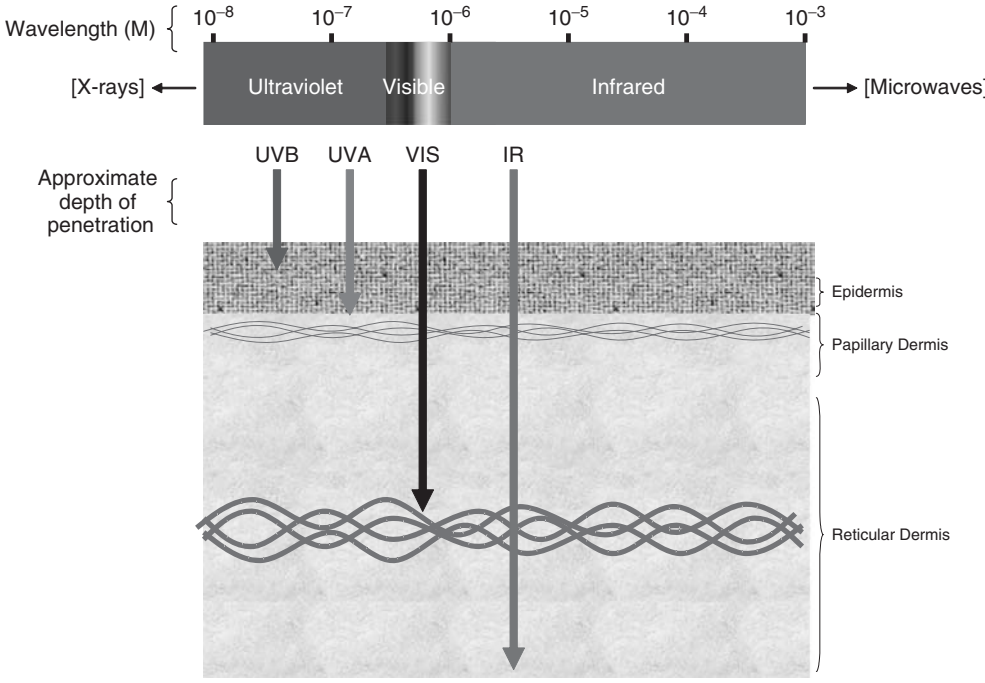


Figure 3.1 Representation of the solar spectrum and approximation of the relative depth to which UVB, UVA, visible and IR radiation penetrates cutaneous tissue. Note that the representation of skin tissue is not to scale: the epidermis is actually much thinner in comparison with dermal tissue. A full-colour version of this figure appears in the colour plate section of this book

Table 3.1 Fitzpatrick skin colour scale

Phototype	Description	Burns	Tans
I	Very fair skin tone, blonde or redhead, freckles.	Always	Never
II	Light skin tone.	Easily	Minimally
III	White to olive skin tone, auburn to light brown hair.	Moderately	Gradually to light brown
IV	Medium brown skin tone	Minimally	Well to moderately brown
V	Dark brown skin tone, dark eyes, dark hair.	Rarely	Profusely to dark
VI	Black skin tone, very dark eyes and dark hair.	Never	Deeply pigmented

Skin colour is commonly defined by the Fitzpatrick classification (Table 3.1) on the basis of pigmentation and the ability to tan or burn. While skin colour and ethnicity are associated, ethnicity alone does not necessarily predict susceptibility to UV damage.

Skin colour mainly depends on relative amounts of melanins, namely eumelanin (brown/black) and pheomelanin (red/yellow). All skin and hair colour is derived from melanins. Some individuals are unable to synthesise eumelanin and have very fair skin and red hair.

3.2.1 Photoprotective role of melanins

Melanins in skin protect sensitive biological materials from damage by dissipating ultraviolet radiation (UVR) as heat or in a chemical reaction.

Eumelanins are polymorphous, nitrogenous polymers that are black to brown in colour, highly insoluble in most liquid media and tightly bound to proteins via covalent bonds. They contain semiquinone moieties with both oxidising and reducing capabilities towards oxygen radicals and other reactive species. Eumelanins also exhibit polyanionic behaviour and hence can sequester charged compounds such as metals and drugs.

In contrast, pheomelanins have a benzothiazine backbone, are red to brown in colour and are photolabile; photolysis products include potentially damaging superoxide and hydroxyl radicals and hydrogen peroxide.

3.2.2 Melanin and melanocytes

Melanins are polymorphous, multifunctional polymers of indolecarboxylic acid derivatives (eumelanin) or glutathinoyl and cysteinyl conjugates of L-dihydroxyphenylalanine (l-DOPA). They are synthesised from L-phenylalanine and/or L-tyrosine through a phylogenetically ancient pathway (melanogenesis). This process occurs in specialised, highly dentritic, neural crest-derived cells known as melanocytes. Melanin is synthesised and packaged, along with the melanogenic enzymes detailed below, into organelles termed melanosomes, which originate from rough endoplasmic reticulum (Boissy 2003). Melanosome formation and maturation is a complex process (Slominski *et al.* 2004). It is believed that melanogenic enzymes are present as inactive precursors in early stage melanocytes, and that melanogenesis does not normally commence until the melanosomes have reached maturity, when the intraorganelle pH falls, resulting in cleavage and release of the active enzymes. This process is under complex genetic control and aberrations in this process are thought to be involved in melanoma. Melanogenesis occurs continuously in the epidermis.

3.2.3 Melanogenesis

The biochemical pathway for synthesis of both eumelanins and pheomelanins begins with tyrosine (Figure 3.2), though phenylalanine may be converted to tyrosine by hydroxylation at the para position on the benzene ring. The next two steps are both catalysed by tyrosinase, a copper-containing enzyme which accepts a wide range of substrates and is present in animals, plants and fungi. The first step (catalysed by tyrosinase) is hydroxylation of tyrosine on the benzene ring to L-DOPA; this is then oxidised to dopaquinone, the last biosynthetic intermediate common to both eu- and phaeomelanins. These steps are believed to be the rate-limiting steps for melanogenesis. In eumelanogenesis, dopaquinone is further metabolised to leukodopachrome (in which dopachrome tautomerase [tyrosine related protein 2] is thought to play a role), followed by a series of oxidation/reduction reactions to form dihydroxyindole and dihydroxyindole carboxylic acid. These latter two intermediates undergo polymerisation to form eumelanin. In phaeomelanogenesis, dopaquinone is conjugated to glutathione or cysteine to form glutathionyldopa and cysteinyldopa respectively. These are further metabolised by hydrolysis and oxidation, resulting in intramolecular cyclization to form the benzathiazinemonomers of pheomelanin (Prota 1995). The type of melanin

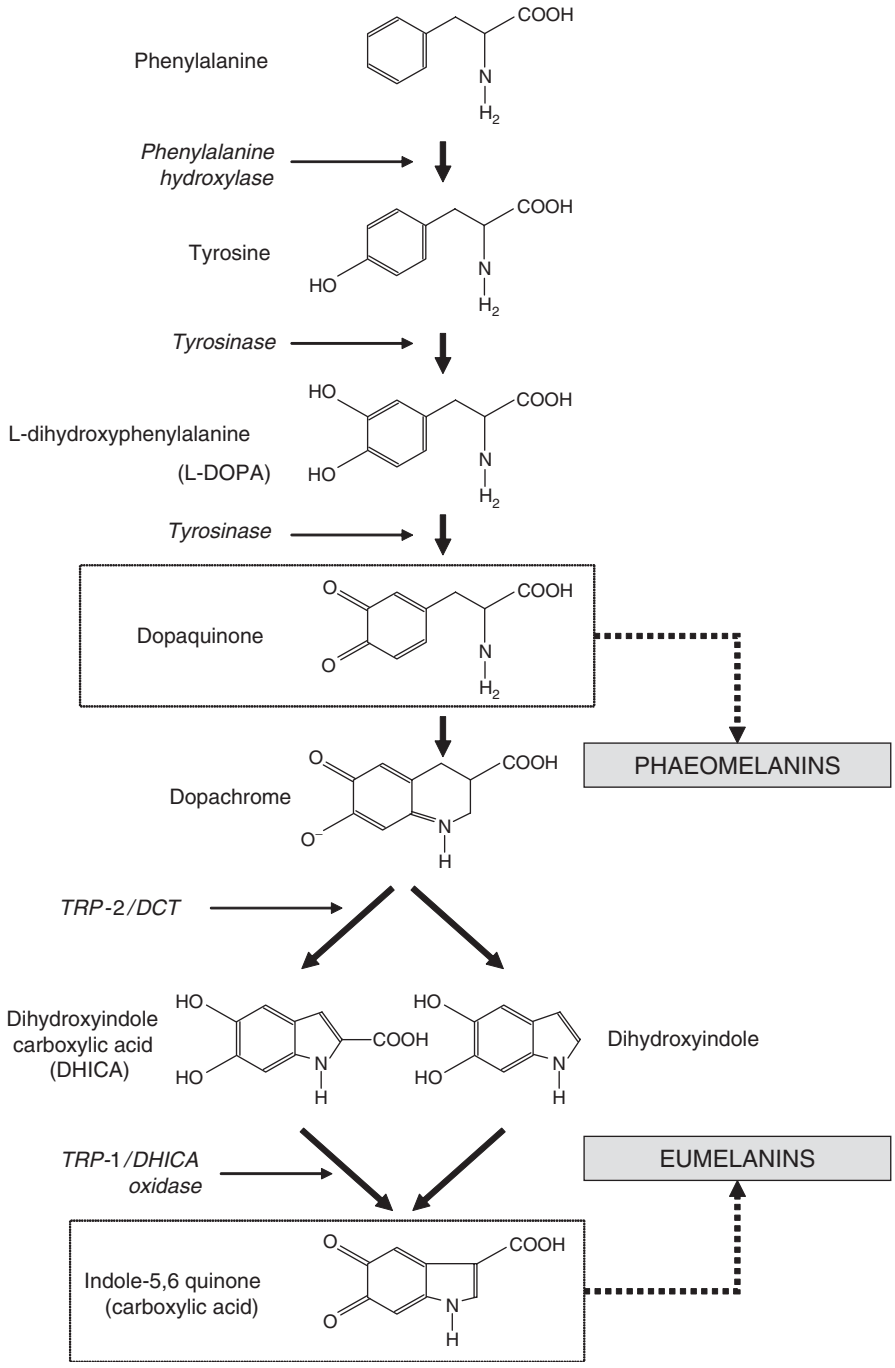


Figure 3.2 Empirical overview of melanogenesis (Abbreviations: TRP-1 & TRP-2 – tyrosine related protein 1 & 2; DCT – dopachrome tautomerase)

synthesised appears to be under the control of two genes, which code for tyrosine related protein-1 and -2 respectively.

3.2.4 Immediate and delayed tanning

The melanogenic response to radiation manifests itself in two phases: immediate and delayed tanning.

In immediate tanning, characterised by hyperpigmentation after 5–10 minutes exposure to UVR, there is no new synthesis of melanin – melanosomes in basal cells are redistributed to form a 'nuclear cap' which shields the nucleus from UV radiation, thus limiting the potential for direct and/or indirect DNA damage. Immediate tanning fades quickly after withdrawal from irradiation (within three to four hours).

Delayed tanning, which appears slowly after several days UVR exposure, requires the production of new melanosomes. Some 48 to 72 hours following UVR exposure, an increase in tyrosinase activities (tyrosine hydroxylation and DOPA oxidation) can be measured. If UVR exposure is repeated, the number of melanocytes is increased by cell division, and new melanosomes are recruited. The melanosomes are translocated along the dendritic processes of the melanocytes and captured by cytoskeletal elements at the dendritic tip. They are then transferred from melanocytes to keratinocytes in close proximity to the dendritic processes. This results in an increase in the number of melanin granules in the epidermis.

The mechanism of melanosome transfer is still the subject of debate and may involve endocytosis of melanosomes, phagocytosis of portions of melanocytes, active transfer or the formation of a continuous pore (Boissy 2003). After transfer to the keratinocytes, the melanosomes are translocated to the apical pole of the keratinocyte where they can protect the nucleus from damage. Melanosomes undergo degradation during terminal differentiation of the keratinocytes, such that none or very few are present in the stratum corneum.

3.3 Increased environmental ultraviolet radiation exposure and its link with photoageing and skin cancer

Skin exposure to sunlight (i.e. ultraviolet radiation) can culminate in a number of chronic dermal effects such as premature ageing and cancer. These are generally ascribed to oxidative stress arising from the generation of free radicals that have a spectrum of detrimental cellular effects.

3.3.1 Pathological effects of UVR on the skin

In general, the shorter the UV wavelength the greater the biological effect; therefore, UVB is thought to be more harmful to skin that UVA. This is highlighted in the case of erythema (or reddening of the skin). which is more commonly known as sunburn. UVB is 1000 times more effective in inducing erythema than UVA (Hill *et al.* 2004). In addition, UVB is known to be a potent carcinogen and mutagen, and has been suggested to be the major component of sunlight responsible for DNA mutations leading to human skin cancer (Ikehata *et al.* 2004). Until recently it has been generally thought that UVB is more detrimental than UVA.

However, UVA is able to penetrate deeper into the skin when compared to UVB (Figure 3.1). For example, 20–50% of UVA radiation is able to reach the basal layer of dividing keratinocytes compared to less than 10% of UVB. These facts, coupled with the increased proportion of UVA present in sunlight, thereby suggest that a greater dose of UVA than UVB would be expected to penetrate into the dividing basal/stem cell layer of the skin causing cellular and DNA damage. Furthermore, it has been previously assumed that UVB played the pivotal role in the development of skin cancer owing to its ability to directly damage DNA. Recently, however, Agar *et al.* (2004) demonstrated a greater frequency of UVA fingerprint mutations compared to UVB fingerprint mutations in human non-melanoma skin cancer (NMSC) samples, thereby suggesting an important role for UVA in human skin carcinogenesis.

3.3.2 Ageing

UVR is known to produce cellular changes in the skin that are characteristic of photodamage and photoageing. It is generally thought that UVA is the main determinant for these changes. Chronic exposure to UVR gives rise to the phenotypic signs of skin ageing which are generally characterised by impaired wound healing, the leathery appearance of skin, wrinkles, increased fragility and blister formation (Wenk *et al.* 2001). In one study, daily exposure of human skin to sub-erythemal doses of UVA for one month induced epidermal hyperplasia, thickening of the stratum corneum and several other cellular changes associated with ageing which are thought to result from UVA-induced oxidative stress (Matsumura and Ananthaswamy 2004).

Photoageing can vary between individuals, depending mostly on factors such as skin type, sun habits, hairstyle, dress and also individual repair capacity (Berneburg *et al.* 2000). Unlike normal aged skin, photoaged skin occurs strictly on sun-exposed areas of the body such as the face and hands. Photoageing affects various layers of the skin, with the majority of the damage seen in the connective tissues of the dermis. The most prominent epidermal changes are pigmentary alterations such as lentigines and diffuse hyperpigmentation (Gilchrest and Rogers 1993).

Histologically, photoaged skin is characterised by a loss of mature dermal collagen and can lead to destablisation of the epidermal–dermal junction through the reduction in collagen type VII-containing anchoring fibrils. Keratinocytes and melanocytes appear irregular with areas of increased and decreased numbers of melanocytes. Langerhans cells are diminished in actinic skin (Pinnell 2003). Collagen type I, which constitutes the major structural component of the dermal connective tissue, has also been found to be reduced in photoaged human and murine skin (Griffiths and Voorhees 1993). Interestingly, UVR-induced effects on collagen fibres can be identified and quantified using non-invasive techniques such as reflectance spectroscopy (Gillies *et al.* 2000; Kollias *et al.* 1998; Tian *et al.* 2001).

3.3.3 Skin cancer

There is a continual debate as to the relative contributions of global warming, climate change and ozone depletion in relation to recreational exposure to UV through foreign holidays and sun beds (Sander *et al.* 2003). The cause of the great majority of skin cancers (including malignant melanoma) in pale skinned individuals is a combination of the DNA damaging

effects of UVR in sunlight and the characteristics that determine the response of the body to that radiation.

Skin carcinogenesis is a slow, multi-step process that may involve DNA damage, epidermal hyperplasia, cellular proliferation, impaired signal transduction pathways, depletion of antioxidant enzymes and an increase in prostaglandin synthesis. The skin responds to increased levels of reactive oxygen species (ROS) by increasing levels of antioxidant enzymes, although this may insufficient to prevent the development of skin cancer (F'guyer *et al.* 2003).

Skin cancer is the most commonly diagnosed human cancer throughout the world (Boukamp 2005), accounting for 40% of all cancers diagnosed in the USA (Bowden 2004). There are broadly two categories of skin cancer: malignant melanoma and non-melanoma skin cancer (NMSC), with the latter representing 85–90% of the incidence of skin cancer (Diffey 2004). According to figures from Cancer Research UK, 65 000 new cases of non-melanoma skin cancer (NMSC) were diagnosed in the United Kingdom in 2002. This number is thought to be vastly under-reported and it is suggested that the incidence rate is closer to 100 000 cases annually and continually increasing (Diffey 2005, 2006).

Non-melanoma skin cancer

NMSCs occur mainly on sun-exposed areas and as such UVR is known to be a major determinant in its development. UVR has the ability to induce skin cancer in the absence of further carcinogens and it is known to be a complete carcinogen (Matsumura and Ananthaswamy 2004). UVR can cause DNA mutations and increased cellular proliferation, therefore acting as both initiator and promoter in carcinogenesis. A high frequency of mutations in the p53 gene has been associated with human NMSC (de Gruijl 2002). p53 is the guardian of the genome and induces apoptosis in cells with severely damaged DNA. However, mutations in the p53 gene allow the damage to be maintained and passed on to progeny, which leads to the development of a malignant cell (Ichihashi *et al.* 2003). Mutations in proto-oncogenes and tumour suppressor genes have been associated with ROS generation within cells, therefore suggesting a link between NMSC and oxidative stress within keratinocytes.

There are two types of NMSC: squamous cell carcinoma (SCC) and basal cell carcinoma (BCC), with BCC being the most common type of skin cancer among white Caucasians.

BCCs arise predominantly from the basal keratinocytes of the epidermis but also from cells in hair follicles and sebaceous glands. They are locally invasive but rarely metastasise. It is thought that BCC formation is associated with sun-exposure during childhood and adolescence (Diffey 2004). Conversely, SCCs have a stronger association with occupational sun-exposure (Armstrong and Kricker 2001). SCCs arise from moderately differentiated basal keratinocytes within precursory lesions such as actinic keratoses.

Unlike BCCs, SCCs are highly invasive and can metastasise (Bowden 2004). They are mainly found in sun-exposed areas, such as the face, neck and arms, and have a strong association with cumulative, lifetime sun-exposure (Armstrong and Kricker 2001).

Melanoma

Cutaneous malignant melanoma is a malignant tumour of the melanocytes usually arising in melanocytes of the epidermis and is the most lethal of the skin cancers. Human cutaneous

malignant melanoma is the most aggressive form of skin cancer and is one of the most difficult forms of human cancer to treat, as highlighted by the 1800 deaths from 8000 new cases each year in the United Kingdom. Over the last twenty-five years, the incidence of malignant melanoma has increased more than any other major cancer in the United Kingdom. This trend is also reflected worldwide, with an approximate doubling of melanoma rates every 10–20 years in countries with caucasian populations (Lens and Dawes 2004).

Epidemiological observations suggest that cutaneous melanoma arises from a complex interplay between environmental and phenotypical features, the strongest of which are intermittent exposure to UVR and fair or lightly pigmented skin coupled with atypical naevi respectively (Gandini 2005a, 2005b). Improvements in early detection and the surgical management of early disease have resulted in an improved prognosis for most patients who present with primary melanoma. However, advanced melanoma evolves with an extensive repertoire of molecular defences against immunological and cytotoxic attack, rendering it largely untreatable, with disproportionate mortality in those individuals of young and middle age (Thompson *et al.* 2005). Genetic, functional and biochemical studies suggest that metastatic tumours become 'bullet proof' against chemotherapeutic drugs owing to an intrinsic resistance to apoptosis.

Oxidative stress

The skin is continuously exposed to UVR, which is known to stimulate the intracellular production of reactive oxygen species (ROS, e.g. superoxide and hydrogen peroxide) and reactive nitrogen species (RNS, e.g. nitric oxide). This has implications in mutagenesis, carcinogenesis and photoaging. An imbalance in the production of ROS/RNS and the antioxidant defence systems leads to oxidative stress. UVB can be directly absorbed by DNA bases, therefore inducing damage within cells. The most common UVB-induced DNA modulations are dimeric photoproducts between adjacent pyrimidines on the same strand of DNA. However, UVB has also been shown to cause oxidation of guanine residues in a process mediated by ROS (Ichihashi *et al.* 2003). In addition, UVA irradiation of spontaneously immortalised HaCaT keratinocyte cell line has been shown to induce the generation of a similar oxidative product (Riemschneider *et al.* 2002). The products are 8-oxo-2'-deoxyguanosine (8-oxodGs), which are specific ROS/RNS-induced DNA products. 8-oxodGs are known to be produced by the reaction of $ONOO^-$ with guanine residues in DNA (Kawanishi *et al.* 2001). In addition to the DNA products, it has been reported that UVB irradiation alters the intracellular antioxidant enzyme levels of, for example, SOD (superoxide dismutase), GPx (glutathione peroxidase) and catalase. Following UVB irradiation the activity of SOD, GPx and catalase diminishes with increasing dose, which is possibly due to an increase in ROS production (Takahashi *et al.* 2004). The harmful effects of UVA are thought to be mainly mediated through the generation of ROS and RNS rather than direct DNA damage. Following UVA irradiation the intracellular accumulation of hydrogen peroxide (H_2O_2) has been reported (Gniadecki *et al.* 2000); this has shown a correlation with UVA-induced DNA damage (Petersen *et al.* 2000). These latter studies have particularly focussed on the role of mitochondria in UV-induced oxidative stress. Before describing this scenario in more detail it is worth providing a little background on mitochondria and mitochondrial DNA.

3.3.4 Mitochondria and mitochondrial DNA (mtDNA) rearrangements

Collectively, mitochondria generate approximately 90% of cellular energy by the process of oxidative phosphorylation using a multi-subunit pathway (Birch-Machin and Turnbull 2001) that results from the complementation of both the nuclear and the mitochondrial genome. Alongside the three billion base pair nuclear genome, each human cell contains hundreds to several thousand copies of the 16.5 kb human mitochondrial genome, which incidentally exhibits a maternal pattern of inheritance (Parr *et al.* 2006). This closed circular genome encodes 13 polypeptides of the respiratory chain complexes, as well as 22 transfer RNAs and two ribosomal RNAs used in mitochondrial protein synthesis. The complete mitochondrial DNA (mtDNA) sequence was determined in 1981 and re-sequenced in 1999 (Parr *et al.* 2006). A growing collection of reported mtDNA mutations and rearrangements has been associated with muscle and neurodegenerative diseases, a proportion of which exhibit skin manifestations (Birch-Machin 2000). MtDNA has the capacity to form a mixture of both wild type and mutant mtDNA genotypes within a cell, a phenomenon known as heteroplasmy. This is important because cellular dysfunction usually occurs when the ratio of mutated:wild type mtDNA exceeds a threshold level (Birch-Machin 2000).

Approximately 90% of the oxygen consumed within a eukaryote is used in mitochondrial respiration and, therefore, the metabolic rate of a cell and indeed tissue is related to mitochondrial function. Incomplete oxygen reduction within the mitochondrial respiratory chain can lead to the formation of the superoxide radical, the first molecule in the pathway responsible for the production of reactive oxygen species (Raha and Robinson 2000). Mitochondrial ROS formation is mainly due to electron leakage naturally occurring at complexes I and III of the respiratory chain but recent evidence in human skin cells has postulated the additional contribution by complex II (Aitken *et al.* 2007).

Growing evidence suggests that cancer cells exhibit increased ROS stress (due in part to oncogenic stimulation), increased metabolic activity and mitochondrial malfunction. Since the mitochondrial respiratory chain is a major source of ROS generation and the naked mtDNA molecule is in close proximity to the source of ROS, the vulnerability of the mtDNA to ROS-mediated damage appears to be a mechanism to amplify ROS stressing cancer cells (Pelicano *et al.* 2004) (Section 3.5.2).

3.3.5 Anti-oxidant treatment

As mitochondrial ROS are thought to play an important pathogenic role in an increasing number of degenerative diseases, this has led to efforts to develop and use antioxidant compounds. Interestingly, there is a range anti-oxidants based around the ubiquinone structure, which has been designed to specifically target to the mitochondria (e.g. 'MitoQ'; Murphy 2004). In addition, resveratrol is a non-toxic natural plant compound particularly well known for its occurrence in the skin of grapes and has been shown to be a useful naturally occurring anti-oxidant. Studies on the effects of resveratrol have shown that not only can it oppose the production of ROS from mitochondria but that it can also scavenge them and thereby fulfil some of the requirements of an anti-oxidant (Zini *et al.* 1999) as well as extending lifespan and impacting mitochondrial function (Lagouge *et al.* 2006).

3.4 Mitochondrial DNA as a biomarker of sun exposure in human skin

An examination of cellular effects can provide an indication of the extent of dermal exposure to solar radiation. Assessment of mitochondrial DNA is one such method for quantifying exposure to ultraviolet radiation.

Although the major determinant of non-melanoma skin cancer (NMSC) is the UVR in sunlight, which induces the DNA damage, it is both the pattern and the cumulative amount of sun exposure which influences the development of NMSC (Armstrong and Kricker 2001). A major limitation of current studies relating genotype to phenotype of human skin cancer is the absence of reliable markers of exposure to UVR and this is compounded by inter-individual differences in the ability to repair photoproducts in nuclear DNA. To determine a reliable marker of cumulative UVR exposure in human skin, several research groups have pioneered the novel idea of using mtDNA, rather than nuclear DNA, as a biomarker of UV-induced DNA damage (Birch-Machin *et al.* 1998; Birch-Machin 2006). Compared to mutation screening of candidate nuclear DNA genes such as p53, there are certain advantages in studying mtDNA damage in sun-exposed skin (Birch-Machin 2000):

- The literature agrees that mitochondria are deficient in nuclear excision repair pathways and cannot repair UV-induced photoproducts such as pyrimidine dimers. However, mitochondria do show repair of a variety of other types of DNA damage, confirming that mitochondria possess base excision repair pathways, although repair involving both recombination and mismatch repair remains a very controversial area.
- MtDNA has a 10-fold higher mutation rate than nuclear DNA. MtDNA is highly susceptible to damage because it is not associated with protective histones. In addition, mtDNA is located in the matrix, which is in close proximity to the inner membrane where reactive oxygen species (ROS) are continually produced in the electron transport chain.
- There are many mitochondrial genomes (2–10 copies) per mitochondria and many mitochondria per cell (a mammalian cell typically contains 200–2000 mitochondria). Consequently, mitochondrial genomes can tolerate very high levels (up to 90%) of damaged DNA through complementation by the remaining wild type mtDNA.

Therefore, cells are able to accumulate photodamage in mtDNA without compromising cell function, a necessary requirement for a reliable and sensitive biosensor of UV exposure. There has been a spectrum of mtDNA deletions associated with UV exposure (Ray *et al.* 2000). Of the spectrum of mtDNA deletions identified in sun-exposed human skin the major species have been the 4977bp common deletion and a 3895bp deletion (Krishnan *et al.* 2004; Krishnan and Birch-Machin 2006). These mtDNA deletions can be also be induced in human skin and cultured skin cells by sub-lethal repetitive doses of UVR (Berneburg *et al.* 2004; Krishnan *et al.* 2004). Apart from deletions, a higher frequency of tandem mtDNA duplications has been observed in sun-exposed human skin (Krishnan and Birch-Machin 2006). These deletions and tandem duplications occur more frequently in usually sun-exposed skin as opposed to occasionally sun-exposed skin (Harbottle *et al.* 2004; Krishnan *et al.* 2004). This is important because the relative density of NMSC is highest on body sites 'usually' exposed to the sun

when outdoors (such as scalp, face, neck and ears) compared to occasionally sun-exposed sites (such as shoulders, back and chest). This data shows that mtDNA damage in human skin provides a potential biomarker for cumulative UV exposure in human skin. In addition, it may provide a method of monitoring long-term safety of clinical UV phototherapy regimes and possibly an early warning system for development of skin cancer (Section 3.5.2).

3.5 Apoptosis

An understanding of the fundamental mechanisms involved with the skin's response to sunlight may allow the identification of effective therapies for skin cancer.

Apoptosis is controlled by one or more of three mechanisms: ligation of death receptors; intracellular stress/damage involving mitochondria; and endoplasmic reticulum (ER) stress (reviewed in Szegezdi *et al.* 2006). Drug resistance resulting from dysregulation of apoptosis has been described in numerous studies of melanoma and may result from defective signalling mediated through death receptor ligation or the upstream events of mitochondrial-mediated apoptosis.

3.5.1 Link between mitochondria and apoptosis

One important development has been the recognition that mitochondria play a central role in the regulation of programmed cell death or apoptosis. A number of apoptotic signals converge on mitochondria and lead to increased permeability of the outer membrane with the subsequent release of cytochrome c, apoptosis inducing factors and Smac/DIABLO into the cytoplasm. Cytochrome c binds to adaptor molecules (including apoptosis protease-activating factor 1 and initiator pro-caspase proteins), forming an 'apoptosome' which leads to cleavage of pro-caspase-9 to active caspase-9; this can then activate downstream effector caspases (e.g. caspase-3), resulting in apoptosis (Vermeulen *et al.* 2005). In certain cell types there is also cross-talk between the death receptor apoptotic pathway and mitochondria. It is now well established that apoptosis is an important mechanism in the therapeutic action of most anticancer drugs (Lowe and Lin 2000) and that mitochondria play a key role in this process (Box 3.1).

Box 3.1 Therapeutic mechanisms – psoriasis and the induction of mitochondrial apoptosis

Many therapeutic drugs are derived from compounds found in nature, and elucidating their mechanism of action has provided important insights into diverse areas of biology. Anthralin [1,8-dihydroxy-9(10H)-anthracenone, dithranol] was first synthesised as a derivative of chrysarobin, prepared from the araroba tree, and is an established, safe and effective topical treatment for psoriasis, a hyperproliferative skin disease affecting ~2% of the population in Western countries. Studies have shown that anthralin disrupts mitochondrial membrane potential and causes endogenous cytochrome *c* release, resulting in the activation of caspase-3 and characteristic morphological changes of apoptosis (McGill *et al.* 2005). Human Rho zero cells (which lack mitochondrial DNA) were resistant to anthralin-induced cell death, disruption of mitochondrial membrane potential and

cytochrome *c* release compared with the isogenic Rho positive cell line. These data also suggest that anthralin may be modulating the redox status of the endogenous ubiquinone pool and this may lead to increased generation of ROS and apoptosis induction (Pelicano *et al.* 2004), thereby resulting in the preferential death of highly proliferative psoriatic keratinocytes, which demand a high energetic requirement from mitochondria.

3.5.2 Mitochondria and cancer

Growing evidence suggests that cancer cells exhibit increased intrinsic ROS stress, due in part to oncogenic stimulation, increased metabolic activity and mitochondrial malfunction. Since the mitochondrial respiratory chain (electron transport complexes) is a major source of ROS generation in the cells, the vulnerability of the mitochondrial DNA to ROS-mediated damage appears to be a mechanism to amplify ROS stress in cancer cells. Mitochondria have therefore been implicated in the carcinogenic process because of their role in apoptosis and other aspects of tumour biology but also due to their role as a generator of ROS (Jacupciak *et al.* 2005). Many types of human malignancy such as colorectal, liver, breast, pancreatic, lung, prostate and bladder as well as skin cancer have been shown to harbour somatic mtDNA mutations (Pelicano *et al.* 2004; Birch-Machin 2005; Jacupciak *et al.* 2005). Moreover, sequence variations of mtDNA have been observed in pre-neoplastic lesions, which suggest mutations occur early in tumour progression (Parr *et al.* 2006). Durham *et al.* (2003) provided the first detailed study of multiple forms of mtDNA damage (including deletions, tandem duplications and point mutations) in NMSC. Somatic heteroplasmic point mutations were identified in addition to clear differences in the distribution of deletions in the tumours compared to perilesional skin. There are three recent studies that have identified somatic mtDNA mutations in cutaneous malignant melanoma (Diechmann *et al.* 2004; Takeuchi *et al.* 2004; Poetsch *et al.* 2004). One cautionary note provided by the Durham study and confirmed by subsequent studies (Eshaghian *et al.* 2006) is the use of appropriate control tissue, as perilesional skin may also harbour UV-induced mtDNA damage.

It is currently unknown whether the observed mtDNA damage has a primary and causative link to the process of cancer development or whether it may simply represent a secondary 'bystander effect' which reflects an underlying nuclear DNA instability. The interplay between nuclear and mitochondrial genes requires careful investigation and may hold the final understanding of the mitochondrial role in tumourigenesis. The escalated ROS generation in cancer cells serves as an endogenous source of DNA-damaging agents that promote genetic instability and development of drug resistance. Malfunction of mitochondria also alters cellular apoptotic response to anticancer agents. Despite the negative impacts of increased ROS in cancer cells, it is possible to exploit this biochemical feature and develop novel therapeutic strategies to preferentially kill cancer cells through ROS-mediated mechanisms.

3.5.3 Modulation of mitochondrial function as a drug treatment

Disruption of mitochondrial function by drugs can result in cell death by necrosis or can signal cell death by apoptosis. The drugs that damage mitochondria usually do so by inhibiting the respiratory complexes of the electron chain, inhibiting/uncoupling oxidative phosphorylation, inducing mitochondrial oxidative stress, or inhibiting DNA replication,

transcription or translation. It is therefore key to test for mitochondrial toxicity (such as screening for mitochondrial function by the methyl thiazol tetrazolium (MTT) assay) early in drug development as impairment of mitochondrial function can induce various pathological conditions. On the other hand, mitochondrial toxicity induced by drugs could be part of therapeutic action, for example tumour mitochondrial toxicity.

3.6 Sun protection

Clearly, many individuals cannot rely on endogenous photoprotective mechanisms to prevent diseases arising from acute or chronic sun exposure. Whilst topical products can be applied to help limit this damage, it is important to consider the potential hazards of such an action.

Epidemiological observations suggest that skin cancer arises from a complex interplay between environmental and phenotypical features, the strongest of which are intermittent exposure to ultraviolet radiation (intermittent versus frequent exposure) and fair or lightly pigmented skin (Elwood 2004; de Snoo and Hayward 2005). As mentioned earlier, pigmentation results from the synthesis (Figure 3.2) and subsequent distribution of melanin, which itself is a major determinant of sensitivity to UVR and risk of skin cancer in caucasian populations. Variations in the melanocortin 1 receptor (MC1R) gene result in a shift in the balance of skin pigmentation from the brown/black pigment eumelanin to the red/yellow and potentially mutagenic pheomelanin (Healy *et al.* 1999; Flanagan *et al.* 2000; Rees 2004). Several studies have demonstrated that germline variants in the MC1R gene – which encodes the melanocortin 1 receptor – are associated with increased risk for melanoma and NMSC in Caucasians (Stratigos *et al.* 2006; Rees 2004; Healy *et al.* 1999; Flanagan *et al.* 2000). The MC1R protein is a G-protein coupled receptor on melanocytes that responds to alpha melanocyte stimulating hormone (α-MSH) secreted in response to UVR, resulting in the synthesis of the black photoprotective pigment eumelanin. MC1R is highly polymorphic in Caucasians and numerous variants have been demonstrated to lead to a loss of function and associated with skin cancer risk phenotypes, such as fair skin, freckling and red hair (Rees 2004).

The term sunscreen describes any material that protects the skin from UV radiation. As a reminder, there are three types of wavelengths in UVR (Figure 3.1). UVB (280–320 nm range) can cause sunburn and is partially blocked by the earth's ozone layer. UVA (320–400 nm) penetrates the skin deeper than UVB causing more long-term damage, such as premature ageing and skin cancer. UVA can even penetrate windows and some clothes. It is not absorbed by the ozone layer. Finally there is UV-C, which is totally absorbed by the earth's atmosphere and so under normal circumstances is not a hazard, although several industrial applications such as water sterilisation require intense UV-C sources.

Most sunscreens carry a SPF, or sun protection factor, which indicates how long a user is protected from UVB rays. A user can determine how long the sunscreen is effective simply by multiplying the SPF by the length of time it takes for him or her to suffer a burn without sunscreen. The protection offered by a sunscreen, defined by the SPF, is assessed at an application thickness of 2 mg cm^{-2}. Several scientific studies have shown that the general public applies much less than this amount – typically from about 0.5–1.3 mg cm^{-2}. Most users of sunscreens therefore only achieve 20–50% SPF of that expected from the product label.

However, the SPF system does not provide an objective measure of protection against UVA radiation. Some sunscreens also carry a star rating, which is defined as the UVA:UVB absorbance ratio per unit wavelength. According to one high street pharmacist the star rating system was designed to reflect the broadness of UVA protection compared to that afforded by UVB or, put another way, was a measure of the balance of UV protection. What this means in real English is that the star rating is a ratio of UVB to UVA protection. This is where a lot of confusion can arise. Most people believe that the higher the star rating the more protection is recieved. Unfortunately, this is not always the case. Take, for example, a SPF sunscreen of 8 with a star rating of 4. Now compare this to a sunscreen with SPF of 15 and a star rating of 3. Which sunscreen provides the greatest UVA protection? The answer is that the SPF 15 sunscreen with star rating of 3 provides the greatest UVA protection.

Active sunscreen components are based on organic molecules, inorganic nanoparticles or a combination of the two (Nelson 2005). Organic, or chemical, sunscreens are made up of relatively complex molecules that are absorbed into the skin, but mostly block UVB only. They work primarily by absorbing UV light and dissipating it as heat. They include para amino benzoic acid, octyl methoxycinnamate, 4-methylbenzylidene camphor, avobenzone, oxybenzone and homosalate. About half of the chemical sunscreens sold are put into cosmetics such as foundation and lipstick.

Inorganic, or physical, sunscreens are mainly zinc oxide or titanium dioxide, formulated as ultrafine (20–50 nm) particles. They work primarily by reflecting and scattering UV light. However, titanium dioxide only protects against UVB and short wave UVA, not long wave UVA. Zinc oxide, however, protects against UVB and short and long-wave UVA, making it a 'broad spectrum' sunscreen.

For the next generation of products, the industry is mainly focusing on developing broad spectrum active ingredients that provide consistent protection across all wavelengths, particularly in the UVA range. Another important goal is to develop simple *in vitro* tests to show the efficacy of UVA and UVB actives. While UVB chemical sunscreens have been used for years, UVA chemical sunscreens are a relatively recent occurrence.

Most UV absorbers used in sunscreens can form free radicals that are implicated in skin ageing and cell damage, and so manufacturers add anti-oxidants to try and neutralise them. For example, doping titanium dioxide lattices with manganese atoms can prevent electrons from migrating to form free radicals (Wakefield *et al.* 2004).

As our understanding of the science behind sunburn, skin cancer and skin ageing advances, it looks likely that sunscreens will move more into the realm of healthcare. The hope is that future sunscreens will contain materials, whether synthetic or natural, that will protect against the whole range of UV wavelengths. They will work on several levels, preventing superficial sunburn whilst also protecting the DNA in our cells. This is important because sunscreens are currently developed and tested in relation to erythema only. This approach is severely limited as it is a well accepted fact within international academic dermatology research that there is a significant degree of sun-induced DNA damage well before erythema occurs (Cooke *et al.* 2003; Burren *et al.* 1998). This sub-erythemal genetic damage can be caused by both UVA and UVB. This is important because it is the DNA damage in skin cells rather than skin erythema that is the key step in the cancer and pre-mature ageing process. Therefore, allowing an individual to spend longer in the sun without burning their skin actually leads to increased sun-induced genetic damage, which in turn may be a contributory factor to the increasing incidence of skin cancer world wide.

Summary

- The skin has a variety of protective mechanisms to limit the effects of exposure to ultraviolet radiation. However, such photoprotective systems are limited in nature.
- The primary mechanism of skin damage resulting from exposure to ultraviolet radiation is the generation of free radicals, leading to oxidative stress.
- The pathological consequences of oxidative stress include premature ageing and cancer.
- An understanding of these mechanisms has identified a number of potential pharmaceutical strategies.
- Whilst it is possible to augment the natural defences of the skin through the use of topical sunscreens, this may paradoxically increase the risk of developing chronic health effects by prolonging an individual's ability to remain in the sun!

References

Agar, N.S., Halliday, G.M., Barnetson, R.S. *et al.* (2004). The basal layer in human squamous tumors harbors more UVA than UVB fingerprint mutations: a role for UVA in human skin carcinogenesis. *Proc Natl Acad Sci USA.* **101**: 4954–9.

Aitken, G., Henderson, J.R., Seung-Cheol, C. *et al.* (2007). Direct Monitoring of UV-Induced Free Radical Generation in HaCaT Keratinocytes. *Clin Exp Dermatol* **32**: 722–727.

Armstrong, B.K. and Kricker, A. (2001). The epidemiology of UV induced skin cancer. *J Photochem Photobiol B* **63**(1–3): 8–18.

Boukamp, P. (2005). UV-induced skin cancer: similarities–variations. *JDDG* **3**: 493–503.

Berneburg, M., Plettenberg, H. and Krutmann, J. (2000). Photoaging of human skin. *Photodermatology Photoimmunology and Photomedicine* **16**: 239–44.

Berneburg, M., Plettenberg, H., Medve-Konig, *et al.* (2004). Induction of the photoaging-associated mitochondrial common deletion *in vivo* in normal human skin. *J Invest Dermatol*, **122**: 1277–83.

Birch-Machin, M.A., Tindall, M., Turner, R. *et al.* (1998). Mitochondrial DNA deletions in human skin reflect photo rather than chronologic aging. *J Invest Dermatol*, **110**: 149–52.

Birch-Machin, M.A. (2000). Mitochondria and Skin Disease. *Clin. Exp. Dermatol.* **25**(2): 141–146.

Birch-Machin, M.A. and Turnbull, D.M. (2001). Assaying mitochondrial respiratory complex activity-in mitochondria isolated from human cells and tissues, in *Mitochondria* (eds Pon, LA and Schon, EA), *Methods in Cell Biology* Volume 65, Chapter 5, pp 97–117.

Birch-Machin, M.A. (2005). Using Mitochondrial DNA as a Biosensor of Early Cancer Development. *Brit J Cancer* **93**: 271–272.

Birch-Machin, M.A. (2006). The role of mitochondria in ageing and carcinogenesis. *Clin Exp Dermatol.* **31**(4): 548–52.

Boissy, R.E. (2003). Melanosome transfer to and translocation in the keratinocyte. *Experimental Dermatology* **12**(Suppl 2): 5–12.

Bowden, G. (2004). Prevention of non-melanoma skin cancer by targetting ultraviolet-B-light signalling. *Nature Reviews Cancer* **4**: 23–34.

Boukamp, P. (2005). UV-induced skin cancer: similarities – variations. *J Dtsch Dermatol Ges*, **3**: 493–503.

Burren, R., Scaletta, C., Frenk, E. *et al.* (1998). Sunlight and carcinogenesis: expression of p53 and pyrimidine dimers in human skin following UVA I, UVA I + II and solar simulating radiations. *Int J Cancer.* **76**(2): 201–6.

Cooke, M.S., Podmore, I.D., Mistry, N. *et al.* (2003). Immunochemical detection of UV-induced DNA damage and repair. *J Immunol Methods.* **280**(1–2): 125–33.

de Snoo, F.A. and Hayward, N.K. (2005). Cutaneous melanoma susceptibility and progression genes. *Cancer Letts*, **230**: 153–186.

Deichmann, M., Kahle, B., Benner, A. *et al.* (2004). Somatic mitochondrial mutations in melanoma resection specimens. *Int J Oncol* **24**: 137–141.

Diffey, B. (2002a). Source and measurement of ultraviolet radiation. *Methods* **28**: 4–13.

Diffey, B. (2002b). What is light? *Photodermatol Photoimmunol Photomed* **18**: 68–74.

Diffey, B. (2004). Climate change, ozone depletion and the impact on ultraviolet exposure of human skin. *Phys Med Biol*, **49**: R1–11.

Diffey, B. (2005). Skin cancer incidence and the ageing population. *Brit. J Dermatol.*, **153**: 679–70.

Diffey, B. (2006). Do we need a revised public health policy on sun exposure? *Br J Dermatol.*, **154**(6): 1046–1049.

Durham, S.E., Krishnan, K.J., Betts, J. and Birch-Machin, M.A. (2003). Mitochondrial DNA damage in non-melanoma skin cancer. *Br J Cancer* **88**: 90–95.

Elwood, M. (2004). In *Prevention of Skin Cancer* Vol. 3, Kluwer Acedemic Publishers, The Netherlands, pp 3–20.

Eshaghian, A., Vleugels, R.A,. Canter, J.A. *et al.* (2006). Mitochondrial DNA deletions serve as biomarkers of aging in the skin, but are typically absent in NMSC. *J Invest Dermatol.*, **126**: 336–344.

F'guyer, S., Afaq, F. and Mukhtar, H. (2003). Photochemoprovention of skin cancer by botanical agents. *Photodermatol Photoimmunol Photomed*, **19**: 56–72.

Flanagan, N., Healy, E., Ray, A. *et al.* (2000). Pleiotropic effects of the melanocortin 1 receptor (MC1R) gene on human pigmentation. *Hum Mol Genet*, **9**(17): 2531–2537.

Gandini, S., Sera, F., Cattaruzza, M.S. *et al.* (2005a). Meta-analysis of risk factors for cutaneous melanoma: I. Common and atypical naevi. *Eur J Cancer*, **41**(1): 28–44.

Gandini, S., Sera, F., Cattaruzza, M.S. *et al.* (2005b). Meta-analysis of risk factors for cutaneous melanoma: II. Sun exposure. *Eur J Cancer*, **41**(1): 45–60.

Gilchrest, B.A. and Rogers, G. (1993). Photoaging, in *Clinical Photomedicine* (eds Lim, H. and Soter, N.), Marcel Dekker, New York, 95–111.

Gillies, R., Zonios, G., Anderson, R.R. and Kollias, N. (2000). Fluorescence excitation spectroscopy provides information about human skin *in vivo*. *J Invest Dermatol*, **115**(4): 704–7.

Gniadecki, R., Thorn, T., Vicanova, G. *et al.* (2000). Role of mitochondria in ultraviolet-induced oxidative stress. *J Cell Biochem.* **80**(2): 216–22.

Griffiths, C.E. and Voorhees, J.J. (1993). Topical retinoic acid for photoaging: clinical response and underlying mechanisms. *Skin Pharmacology*, **6**(Suppl 1): 70–7.

de Gruijl, F.R. (2002). Mutations as a marker of skin cancer risk: comparison of UVA and UVB effects. *Experimental Dermatology*, **11**(1): 37–39.

Harbottle, A., Krishnan, K.J. and Birch-Machin, M.A. (2004). Implications of using the ND1 gene as a control region for real-time PCR analysis of mitochondrial DNA deletions in human skin. *J Invest Dermatol*, **122**(6): 1518–21.

Healy, E., Birch-Machin, M.A. and Rees, J.L. (1999). The Human Melanocortin1-Receptor Gene, in *The Melanocortin Receptors* (ed. Cone, RD). Humana Press Inc., New Jersey, USA.

Hill, D., Elwood, J. and English, D. (2004). *Prevention of skin cancer*, Vol 3. Dordrecht: Kluwer Academic Publishers, The Netherlands.

Ichihashi, M., Ueda, M., Budiyanto, A. *et al.* (2003). UV-induced skin damage. *Toxicology*, **189**: 21–39

Ikehata, H., Nakamura, S., Asamura, T. and Ono, T. (2004). Mutation spectrum in sunlight exposed mouse skin epidermis: small but appreciable contribution of oxidative stress-mediated mutagenesis. *Mutation Research*, **556**: 11–24.

Jakupciak, J.P., Wang, W., Markowitz, *et al.* (2005). Mitochondrial DNA as a cancer biomarker. *J Mol Diagnostics*, **7**: 258–267.

Kawanishi, S., Hiraku, Y. and Oikawa, S. (2001). Mechanism of guanine-specific DNA damage by oxidative stress and its role in carcinogenisis and aging. *Mutation Research*, **488**: 65–76.

Krishnan, K., Harbottle, A. and Birch-Machin, M.A. (2004). The use of a 3895 bp Mitochondrial DNA Deletion as a Marker for Sunlight Exposure in Human Skin. *J Invest Dermatol*, **123**: 1020–1024.

Krishnan, K.J. and Birch-Machin, M.A. (2006). The incidence of both tandem duplications and the common deletion in mtDNA from three distinct categories of sun-exposed human skin and in prolonged culture of fibroblasts. *J Invest Dermatol*, **126**(2): 408–15.

Kollias, N., Gillies, R., Moran, M. *et al.* (1998). Endogenous skin fluorescence includes bands that may serve as quantitative markers of aging and photoaging. *J Invest Dermatol*, **111**(5): 776–80.

Lagouge, M., Argmann, C., Gerhart-Hines, Z. *et al.* (2006). Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. *Cell*, **127**(6): 1109–22.

Lens, M.B. and Dawes, M. (2004). Global perspectives of contemporary epidemiological trends of cutaneous malignant melanoma. *Br J Dermatol*, **150**(2): 179–85.

Lowe, S.W. and Lin, A.W. (2000). Apoptosis in Cancer. *Carcinogenesis*, **21**: 485–495.

Matsumura, Y. and Ananthaswamy, H.N. (2004). Toxic effects of ultraviolet radiation on the skin. *Toxicol Appl Pharmacol*, **195**: 298–308.

McGill, A., Frank, A., Emmett, N. *et al.* (2005). The antipsoriatic drug anthralin accumulates in keratinocyte mitochondria, dissipates mitochondrial membrane potential, and induces apoptosis through a pathway dependent on respiratory competent mitochondria. *FASEB J.*, **19**(8): 1012–4.

Murphy, M.P. (2004). Investigating mitochondrial radical production using targeted probes. *Biochem. Soc. Transact*, **32**: 1011–1014.

Nelson, C.G. (2005) Photoprotection, in *Sunscreens*, 3rd edn (ed. Shaath, N.A.), Taylor and Francis, Boca Raton.

Parr, R.L., Dakubo, G.D., Thayer, R.E. *et al.* (2006). Mitochondrial DNA as a potential tool for early cancer detection. *Hum Genomics*, **2**(4): 252–257.

Pelicano, H., Carney, D. and Huang, P. (2004). ROS stress in cancer cells and therapeutic implications. *Drug Resistance Updates*, **7**: 97–110.

Petersen, A.B., Gniadecki, R., Vicanova, J. *et al.* (2000). Hydrogen peroxide is responsible for UVA-induced DNA damage measured by alkaline comet assay in HaCaT keratinocytes. *J Photochem Photobiol B*, **59**: 123–31.

Pinnell, S.R. (2003). Cutaneous photodamage, oxidative stress, and topical antioxidant protection. *Journal of the American Academy of Dermatology*, **48**: 1–22.

Poetsch, M., Petersmann, A., Lignitz, E. and Kleist, B. (2004). Relationship between mitochondrial DNA instability, mitochondrial DNA large deletions, and nuclear microsatellite instability in head and neck squamous cell carcinomas. *Diagn Mol Pathol*, **13**: 26–32.

Prota, G. (2000). Melanins, melanogenesis and melanocytes: looking at their functional significance from the chemist's viewpoint. *Pigment Cell Res.*, **13**(4): 283–93.

Raha, S. and Robinson, B.H. (2000). Mitochondria, oxygen free radicals, disease and ageing. *Trends in Biochemistry*, **25**: 502–508.

Ray, A.J., Turner, R., Rees, J.L. and Birch-Machin, M.A. (2000). The spectrum of mitochondrial DNA deletions is a ubiquitous marker of ultraviolet radiation exposure in human skin. *J Invest Dermatol*, **115**: 674–679.

Rees, J.L. (2004). The genetics of sun sensitivity in humans. *Am J Hum Genet.*, **75**: 739–751.

Riemschneider, S., Podhaisky, H.P., Klapperstuck, T. and Wohlrab, W. (2002). Relevance of reactive oxygen species in the induction of 8-oxo-2'-deoxyguanosine in HaCaT keratinocytes. *Acta Derm Venereol*, **82**: 325–8.

Sander, C.S., Hamm, F., Elsner, P. and Thiele, J.J. (2003). Oxidative stress in malignant melanoma and non-melanoma skin cancer. *Brit. J of Dermatol*, **148**: 913–922.

Sander, C.S., Chang, H., Hamm, F. *et al.* (2004). Role of oxidative stress and the antioxidant network in cutaneous carcinogenesis. *Int J Dermatol*, **43**(5): 326–35.

Slominski, A., Tobin, D.J., Shibahara, S. *et al.* (2004) Melanin pigmentation in mammalian skin and its hormonal regulation. *Physiological Reviews*, **84**: 1155–1228.

Stratigos, A.J., Dimisianos, G., Nikolaou, V. *et al.* (2006). Melanocortin receptor-1 gene polymorphisms and the risk of cutaneous melanoma in a low-risk southern European population. *J Invest Dermatol*, **126**(8): 1842–9.

Szegezdi, E., Logue, S.E., Gorman, A.M. and Samali, A. (2006). Mediators of endoplasmic reticulum stress-induced apoptosis. *EMBO Rep*, **7**(9): 880–5.

Takahashi, H., Suzuki, Y., Miyauchi, Y. *et al.* (2004). Roxithomycin decreases ultraviolet B irradiation-induced reactive oxygen intermediates produced and apoptosis of keratinocytes. *J Dermatol Sci*, **34**: 25–33.

Takeuchi, H., Fujimoto, A., Hoon, D.S. (2004). Detection of mitochondrial DNA alterations in plasma of malignant melanoma patients. *Ann NY Acad Sci*, **1022**: 50–54.

Thompson, J.F., Scolyer, R.A. and Kefford, R.F. (2005). Cutaneous Melanoma, *Lancet*, **365**: 687–701.

Tian, W.D., Gillies, R., Brancaleon, L. and Kollias, N. (2001). Aging and effects of ultraviolet A exposure may be quantified by fluorescence excitation spectroscopy *in vivo*. *J Invest Dermatol*, **116**(6): 840–5.

Vermeulen, K., Van Bockstaele, D.R. and Berneman, Z.N. (2005). Apoptosis: mechanisms and relevance in cancer. *Ann Hematol.*, **84**(10): 627–39.

Wakefield, G., Lipscomb, S., Holland, E. and Knowland, J. (2004). The effects of manganese doping on UVA absorption and free radical generation of micronised titanium dioxide and its consequences for the photostability of UVA absorbing organic sunscreen components. *Photochem Photobiol Sci*, **3**(7): 648–52.

Wenk, J., Brenneisen, P., Meewes, C. *et al.* (2001). UV-Induced Oxidative Stress and Photoaging. *Curr Problems in Dermatol*, **29**: 83–94.

Zini, R., Morin, C., Bertelli, A. *et al.* (1999). Effects of resveratrol on the rat brain respiratory chain. *Drugs Exp Clin Res*, **25**(2–3): 87–97.

PART II:
Skin Absorption

4 Skin as a route of entry

Simon C. Wilkinson

Medical Toxicology Research Centre, University of Newcastle, Newcastle-Upon-Tyne, NE2 4AA, UK

Primary Learning Objectives

- *Anatomical features of the stratum corneum relevant to the passage of chemicals through this barrier and the contribution from shunt pathways (appendages).*
- *Differences in chemical absorption between anatomical sites (usually termed regional variation).*
- *Differences in percutaneous absorption between species used in animal models and humans.*
- *Inter- and intra-individual variation in percutaneous absorption in humans, in vivo and in vitro.*
- *The influence of age on percutaneous absorption.*
- *Contribution of appendages to percutaneous absorption.*

4.1 Salient anatomical features of the stratum corneum – the 'brick and mortar model'

The principal barrier to chemical entry through the skin is generally accepted to be the stratum corneum. This uppermost layer of the epidermis consists of non-viable cells termed corneocytes, which are filled with highly cross-linked, insoluble keratin bundles surrounded by a lipid matrix. These two elements can be regarded as resembling bricks and mortar.

The gross structure of the skin has been covered in Chapter 1. It is generally agreed that the principal barrier to chemical entry (and exit) in the skin is the stratum corneum. This is the outermost layer of the epidermis and consists of several layers of completely keratinised, dead cells, termed corneocytes, surrounded by a lipid-rich extracellular matrix. These two elements have been described by Elias (1983) as resembling 'bricks and mortar'.

There are two possible routes through the stratum corneum: intercellular and transcellular (intracellular). It is widely believed that the majority of chemical compounds traverse the stratum corneum via the intercellular route.

Principles and Practice of Skin Toxicology Edited by Robert P. Chilcott and Shirley Price
© 2008 John Wiley & Sons, Ltd

Corneocytes represent the endpoint of epidermal differentiation. The corneocytes are flattened, stacked hexagonal cells, each approximately 40 micrometre in diameter and 0.5 micrometer thick (though corneocytes are ten-fold thicker on palmar and plantar surfaces). They are non-viable cells, have no nucleus or organelles, and are highly protein rich, consisting of 70% by weight of insoluble bundled keratin. They have a thicker plasmalemma than other epidermal cells, a highly cross-linked structure known as the cornified cell envelope. They are constantly being shed, whilst being replaced by the products of basal cell proliferation. They form the 'bricks' in the Brick and Mortar model described in Chapter 1.

Surrounding the corneocytes is the extracellular lipid matrix, which represents the 'mortar'. This consists of several multi-lamellar sheets of polar lipids, known as ceramides, as well as other important lipids, namely cholesterol (free, sulphate and esters), free fatty acids and others. However, the corneocytes are not 'floating in a sea of lipid'. The layer of lipid adjacent to the corneocytes is bound to the cornified cell envelope of corneocytes by numerous covalent linkages to amino acids in the proteins of the envelope. A more detailed description of how the stratum corneum comes into being is provided in Chapter 2.

The stratum corneum is 3–20 microns in thickness, and there are 15 to 25 layers of corneocytes, depending on species and site; it provides an effective barrier against transcutaneous water loss. The stratum corneum is usually a much drier structure (15–20% water by weight) than the rest of the body (70%). If the appendages are ignored for the moment, there are two theoretical routes through which chemicals can cross the stratum corneum barrier:

- the intercellular (or extracellular) route, in which chemicals pass exclusively through the lipid matrix;
- the intracellular or transcellular route, in which chemicals pass through both the lipid matrix and through the corneocytes themselves.

Note that chemicals must pass through the extracellular matrix, regardless of which of these theoretical routes they take (Figure 1.7). The general consensus of current opinion is that it is predominantly intercellular lipids that contribute to barrier function and that the route taken through the stratum corneum by all molecules, even polar compounds, is through the lipid layers (Elias *et al.* 1981; Cornwell and Barry 1993; Sznitowska *et al.* 1998), though it appears that passive diffusion of ions may occur via the appendegeal shunt route (see below) as well as through the intercellular regions of the stratum corneum (Cornwell and Barry 1993; Tanojo *et al.* 2001).

Logically, the rate of diffusion through the stratum corneum should relate to the length of the pathway through the intercellular lipids. This theoretically depends on four parameters: the number of cell layers; the overall thickness of the stratum corneum; the size of the corneocytes; and the tortuosity of the path (which is essentially a function of how the corneocytes are 'stacked').

4.2 Species and regional variation in skin structure

Alterations in lipid composition, as well as in stratum corneum thickness, are thought to play a role in regional and interspecies variation in percutaneous absorption and in inter- and intra-individual variation in skin permeability in humans.

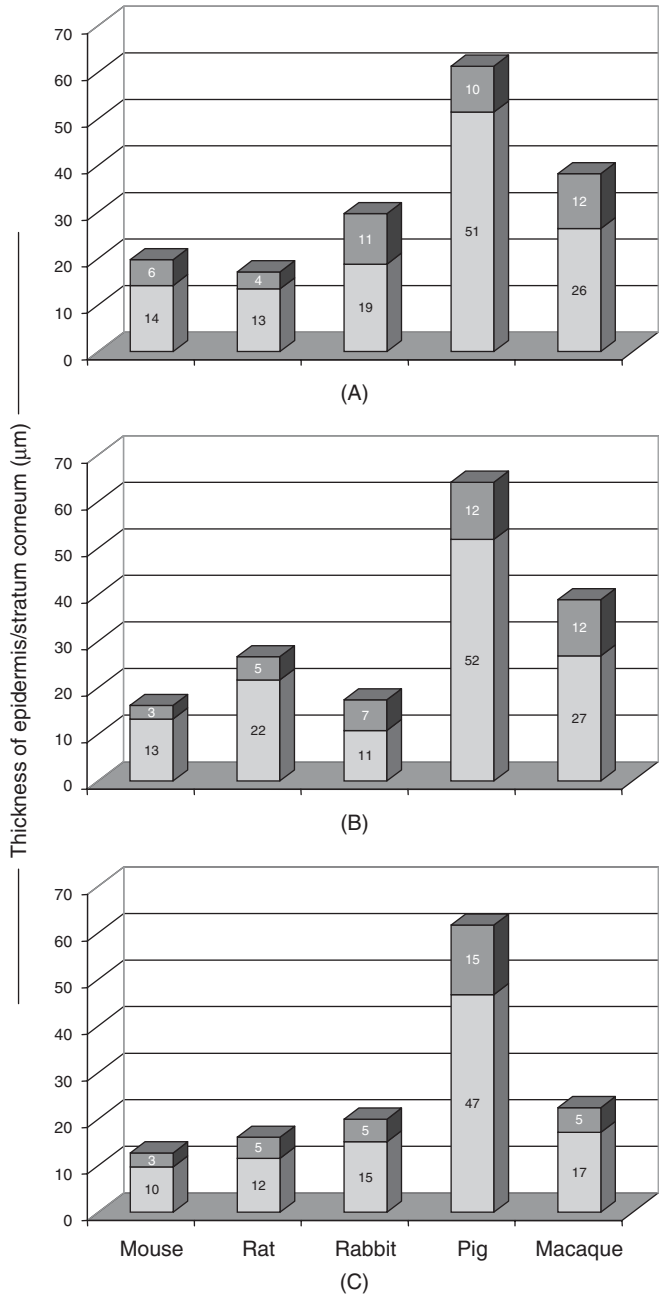


Figure 4.1 Regional and species variations in stratum corneum and epidermal thickness. Values indicate the mean thickness of the epidermis (lower column) and stratum corneum (upper column) of mouse (Blb/CBys), rat (Sprague Dawley), rabbit (New Zealand White), pig (Yorkshire) and macaque skin from the ear (A), thoracolumbar (B) and ventroabdominal (C) regions. (From Monteiro-Riviere *et al*. 1990, Reprinted by permission from Macmillan Publishers Ltd)

The thickness of the stratum corneum varies considerably between species and anatomical site. Monteiro-Riviere *et al.* (1990) measured stratum corneum and epidermal thickness (as well as the number of cell layers in the stratum corneum) in sections of skin from several species and anatomical sites. A selection of their data is presented in Figure 4.1. Monteiro-Riviere *et al.* demonstrated that there were considerable differences in both stratum corneum and epidermal thickness not only between species but also between different regions in the same species. For example, the stratum corneum from commonly used rodent model animals is generally thinner than from pigs and macaques, but stratum corneum from the ventroabdominal (front) skin of a macaque is significantly thinner than on the ear or thoracolumbar (back) skin, and is comparable in thickness with the stratum corneum of rat and rabbit.

Much less is known about regional variation in stratum corneum thickness in humans, as only a few areas of the human body have been systematically studied. Holbrook and Odland (1974) showed that there were differences in stratum corneum thickness and in the number of cell layers in the stratum corneum between back, abdomen, thigh and flexor forearm, despite considerable inter-individual variation. In particular, the stratum corneum was thicker and consisted of more cell layers on the flexor forearm than on the abdomen or back. The authors concluded that the thickness of the stratum corneum resulted from differences in the number of cell layers, rather than from differences in the thickness of the layers themselves. More recently, Schwindt *et al.* (1998) estimated stratum corneum thickness from *in vivo* measurements of transepidermal water loss (TEWL) in six women following successive tape strips to remove the stratum corneum. They concluded that the stratum corneum of all women was significantly thicker at the extremities ($13 \pm 4\,\mu m$) than on the abdomen ($8 \pm 2\,\mu m$, mean $\pm$ standard deviation in each case). In a larger study of 71 human volunteers, Sandby-Moller *et al.* (2003) reported that the mean ($\pm$ standard deviation) thickness of the stratum corneum was $18 \pm 5\,\mu m$ at the dorsal aspect of the forearm, $11 \pm 2\,\mu m$ at the shoulder and $15 \pm 3\,\mu m$ at the buttock.

4.3 Species and regional variation in skin permeability

In contrast to regional differences in stratum corneum morphology, anatomical (regional) differences in percutaneous penetration of chemicals are well known. For example, Marzulli (1962) measured the *in vitro* permeation of various solutes through excised skin. Permeability was highest through postauricular skin, followed by scrotum, scalp/ventral thigh, instep, anterior forearm and plantar surface. Maibach *et al.* (1971) showed marked variation in absorption of organophosphorus insecticides in human volunteers. For parathion, the most permeable region was the scrotum, followed by the scalp, forehead, jaw, dorsal hand, abdomen, ball of foot and palm, with the forearm being least permeable. For malathion, the order of permeability was similar: forehead > dorsal hand > abdomen > ball of foot = forearm > palm. A similar order of permeability was reported for hydrocortisone by Feldmann and Maibach (1967). Lotte *et al.* (1987) measured TEWL and the permeation of benzoic acid, caffeine and aspirin *in vivo*, and reported the following order of permeability (from highest to lowest): forehead > postauricular > abdomen = arm (upper outer). Differences in stratum corneum thickness are not sufficient in themselves to explain these differences, and it is now believed that differences may originate in differences in lipid content and lipid composition

between anatomical sites. For example, sphingolipids and cholesterol are present at higher concentrations in palmar and plantar surfaces than in extensor surfaces of extremities.

Differences in percutaneous absorption between species, especially between traditional laboratory animal models (rodent and rabbits) and primates and humans, are well documented and it has been suggested that follicular density (Scott *et al.* 1991) and stratum corneum lipid composition (Sato *et al.* 1991) may be major contributing factors. It is generally accepted that mouse, rat and guinea pig skin is generally more permeable to a range of chemicals than human and primate skin. For example, the *in vivo* percutaneous absorption of both propoxur (van de Sandt *et al.* 2000) and orthophenyl phenol (Cnubben *et al.* 2002) is more extensive in rats than human volunteers under the same conditions. However, some studies have shown a more complex picture. For example, Scott *et al.* (1991) showed that the permeability of rat skin was significantly higher than human skin for highly hydrophilic chemicals (paraquat and mannitol) but not for water and ethanol. In contrast, an *in vitro* study found no clear difference in the percutaneous absorption of benzoic acid and testosterone between human and rat skin; only the penetration of caffeine differed significantly between the two species (van de Sandt *et al.* 2004).

The general consensus is that best models for human skin are the domestic pig and non-human primates (from both *in vivo* and *in vitro* data), though some would argue that the relatively high density of hair follicles in some non-human primates makes them an unsuitable model for human skin. In particular, pig skin has recently been gaining popularity as a suitable model for human skin because of its similar permeability characteristics. For example, Dalton *et al.* (2006) demonstrated no significant difference in the *in vitro* absorption kinetics of the nerve agent VX through human and pig skin under several test conditions and a recent, thorough examination of the structure of pig skin concluded that porcine skin was indeed a suitable model for human (Jacobi *et al.* 2007).

Several studies have compared *in vivo–in vitro* correlations between species, though these studies are often confounded by differences in study design (for example, use of occluded versus unoccluded conditions). Ross *et al.* (2005) reviewed data for permeation of 2,4-diphenoxyacetic acid *in vivo* in several species. Data obtained from rat, mouse and rabbit each reflected higher permeation than human skin, whilst data obtained with rhesus monkeys was in the same range as the human volunteer data. The authors also noted that there was 'far less uncertainty in human data than in extrapolating from inbred lab animals.' Similarly, Wester *et al.* (2004) concluded that percutaneous absorption values in monkey were 'slightly higher' than human counterpart data across a wide range of chemicals, whilst rat, rabbit and pig data were 'not nearly as close to human data as rhesus monkey'.

4.4 Intra- and inter-individual variation in percutaneous absorption

Both intra- and inter-individual variation in the rate and extent of percutaneous absorption of a variety of chemicals have been demonstrated *in vitro* as well as *in vivo* (Oestmann *et al.* 1993; Fullerton *et al.* 1994). This variation may well originate from differences in lipid composition of the intercellular regions of the stratum corneum within and between individuals, but there is an increasing recognition of the role of genetic factors to inter-individual variation in skin

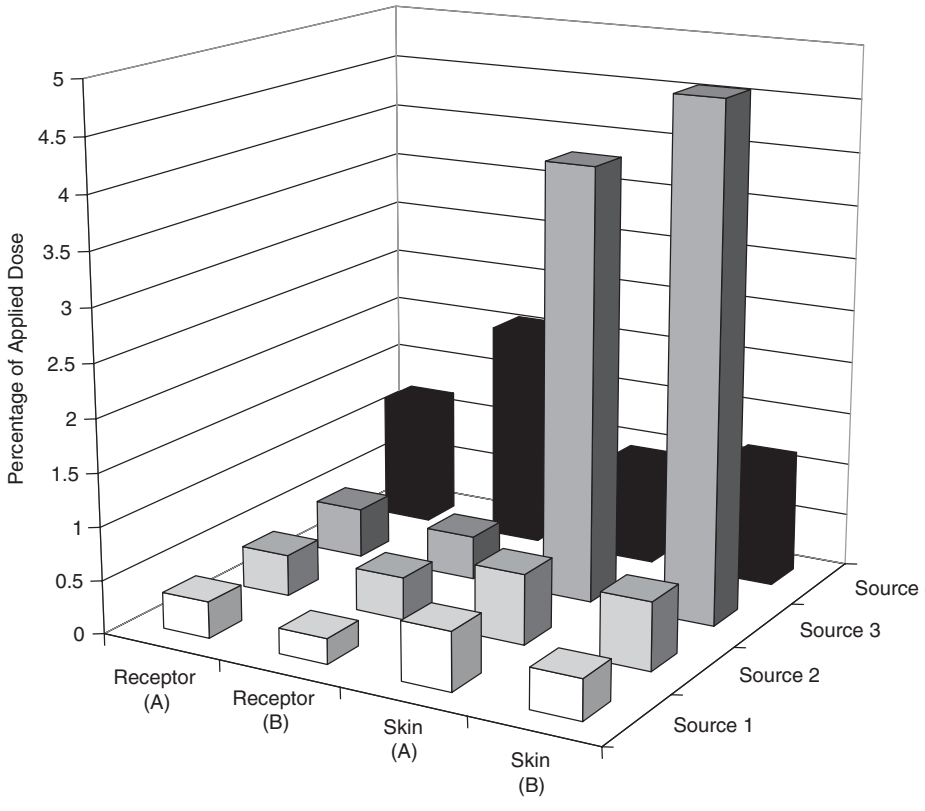


Figure 4.2 Inter-individual variation in absorption of Vitamin E acetate from two formulations (A and B) in human volunteers (from Wester and Maibach 2004), expressed as percentage of applied dose recovered from receptor chamber fluid ('Receptor') or within skin ('Skin') from four individuals (sources 1–4). Note the variation between individuals (along the z-axis). Copyright © 2004, CRC press

permeability, especially the filaggrin allele. (A more detailed consideration of the importance of filaggrin is given in Chapter 3.)

There have been few systematic studies of inter- and intra-individual variation in percutaneous absorption and the available information is inconsistent. For example, Wester and Maibach (2004) studied the *in vitro* human percutaneous absorption of vitamin E acetate from two formulations (which differed in pH) and demonstrated that inter-individual variation was relatively consistent whereas intra-individual variation was significant (Figure 4.2). In contrast, Larsen *et al.* (2003) reported limited intra-individual variation but extensive inter-individual variation in the *in vitro* human skin absorption of an analgesic drug (fentanyl) from two formulations.

4.5 Effect of age on skin barrier function

The influence of the age of an individual on skin permeability to chemicals, either *in vivo* or *in vitro*, is a controversial area. It is difficult to isolate effects of age itself from other age-related

effects on skin physiology, such as sun exposure, chemical exposure, physical trauma, maturation and hormonal changes. Furthermore, numerous physiological changes take place as skin ages, e.g. dermo-epidermal junction flattening, thinning, reduction in number and output of sweat glands, and a more heterogenous basal cell population (Monteiro-Riviere 2004; Waller and Maibach 2005).

Numerous ultrastructural changes occur with age, such as a reduction in amount and quality of collagen. The current consensus is that, in the case of human skin, the stratum corneum thickness and general morphology do not change with age. Sandby-Moller *et al.* (2003) found no correlation between either stratum corneum thickness or epidermal thickness with age in a study of 71 human volunteers, though stratum corneum thickness was positively correlated with pigmentation and inversely proportional to the number of years an individual had smoked tobacco products.

Although lipid content, intercellular cohesion and cholesterol synthesis are known to decrease with age, any effects on percutaneous absorption are marginal. In fact, some human volunteer studies have shown a trend for decreased absorption with increasing age (Roskos *et al.* 1986; 1990; Marzulli 1962). Age-related changes in experimental animals have been documented; these have been explained by physiological alterations affecting cutaneous blood flow and/or stratum corneum thickness (e.g. Hall *et al.* 1989). It should be noted that age-related changes in percutaneous penetration in rat skin *in vitro* can be confounded by changes in the depth of hair follicle penetration with age.

4.6 Role of skin appendages

The contribution of the appendages (hair follicles, sebaceous glands and sweat glands) to percutaneous absorption remains controversial. Although percutaneous absorption is higher in areas with high follicular density, the stratum corneum is also thinner in these areas.

There remains considerable interest in the skin appendages (hair follicles, sebaceous glands, sweat glands) as a possible 'shunt' pathway for topically applied chemicals. In theory, this route might enable the stratum corneum barrier to be circumvented, both for reasons of therapeutic benefit (transdermal drug delivery) and toxicology (especially nanoparticles, which do not generally appear to penetrate undamaged stratum corneum).

Hair follicles penetrate through the epidermis into the lower dermis. The associated sebaceous glands lie closer to the skin surface. It is known that some chemicals can easily penetrate these conduits: whilst the hair follicle is surrounded by stratum corneum, there is no stratum corneum around the sebaceous gland duct and only one epidermal cell layer between the sebaceous gland duct and dermis. This cell layer separates a highly hydrophobic environment (sebum) from a hydrophilic environment (dermis). Hence, if a molecule can partition between these two phases it will cross the epidermal barrier layer, thus facilitating diffusion directly into the deeper, dermal tissue.

The contribution of the appendageal pathways to percutaneous penetration has remained controversial. However, an *in vitro* model for quantifying the contribution of hair follicles to percutaneous absorption has been recently described; the *in vitro* 'skin sandwich' system (Barry 2002; Meidan *et al.* 2005).

4.7 The *in vitro* skin sandwich model

The *in vitro* skin sandwich model may provide a quantitative method for determining the contribution of the follicles to percutaneous absorption. The appendageal route remains a possible shunt route for the delivery of topical chemicals.

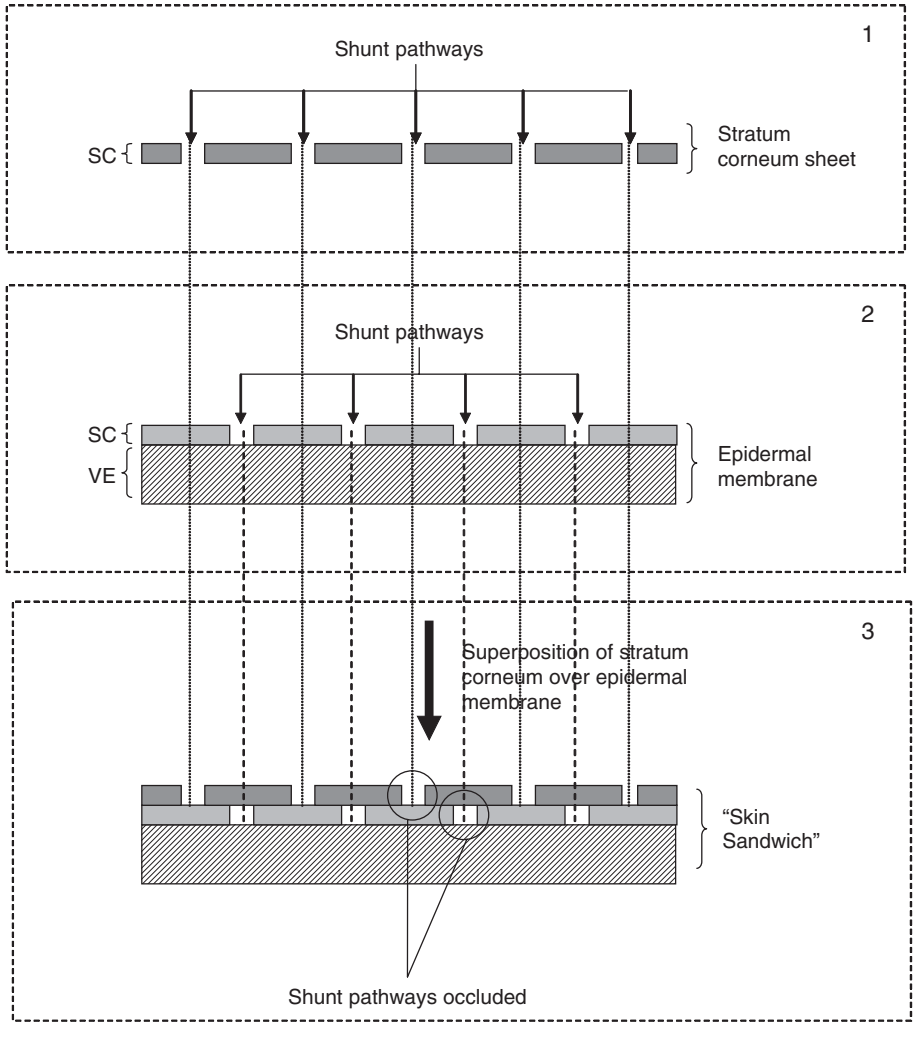


Figure 4.3 Schematic of the *in vitro* 'skin sandwich' system to study follicular contribution to percutaneous absorption. A sheet of stratum corneum, comprising integral shunt pathways arising from hair follicles (1) is placed over an epidermal membrane (2) derived from the same skin donor source. The resulting 'skin sandwich' effectively occludes the majority of shunt pathways. The epidermal membrane is composed of a layer of stratum corneum (sc) and viable epidermis (VE). In this model, the contribution of the viable epidermis to diffusional resistance is assumed to be negligible. (From Meidan *et al*. 2005, Reprinted with permission from Elsevier)

In this system, a sheet of stratum corneum is prepared from excised skin. This is then superimposed on an intact epidermal membrane (comprising stratum corneum and epidermis) from same skin donor. The probability of two hair follicles aligning between the epidermal membrane and the sheet of stratum corneum is virtually zero and so all these shunt pathways become obstructed (Figure 4.3).

The theory behind the skin sandwich model is relatively straight forward: the rate at which a chemical diffuses through the skin (flux) is inversely proportional to the thickness of the stratum corneum as described by Fick's first law of diffusion (Chapter 6); a two-fold increase in the thickness of the stratum corneum should lead to a two-fold decrease in flux. If the shunt pathways make no contribution to the penetration of a chemical, then the flux through the skin sandwich (which comprises two layers of stratum corneum) should be half that of the epidermal membrane (which has just one layer of stratum corneum). Conversely, if the chemical penetrates solely via the shunt pathways, then the flux will be zero, as no shunt pathways are in alignment and so diffusion through the sandwich will be prevented. If the shunt pathways make a partial contribution to the penetration of a chemical, then the flux through the sandwich will be less than half the flux through the skin sandwich.

There are several assumptions that must be made when using the model and there are limitations to its applicability:

- the shunts represent hair follicles as it is believed that the sweat ducts orifices, with their much smaller dimensions, play a smaller role in drug absorption;
- the small resistance of the nucleated epidermis to permeation is ignored for the sake of simplicity;
- no new pores are created during the permeation process;
- the model cannot be used with highly lipophilic chemicals, as the solubility of these chemicals in the aqueous epidermis is a limiting factor.

To date, this model has indicated that highly hydrophilic substances tend to favour the follicular route, whereas the transport of more lipophilic compounds is almost exclusively non-follicular (Essa *et al.* 2002). For compounds of intermediate and low lipophilicity (i.e., log P of 1.60 to −1.05), the follicular contribution ranges from 34% to 60%, respectively (Frum *et al.* 2007).

4.8 Penetration of particles through appendages

The appendageal route as a possible shunt pathway for the ingress of particles has attracted renewed interest recently with the increased application of nanoparticles in products such as sunscreens. The influence of particle size on follicular penetration was investigated by Rolland *et al.* (1993) using fluorescent microspheres. Particles greater than 10 μm in diameter were excluded from follicles, 5 μm diameter particles entered the follicles but could not penetrate the stratum corneum, whilst particles less than 2 μm in diameter entered both follicles and the outer two to three layers of stratum corneum. Particles deposited within the inner lumen of follicles did not penetrate surrounding tissue.

The available evidence for the absorption and penetration of nanoparticles (including insoluble nanoparticles and nanoemulsions containing vesicles in the range 50–5000 nm in

diameter) was recently reviewed by Nohynek *et al.* (2007). They concluded that insoluble nanoparticles do not penetrate into or through normal human skin and that vesicle material may penetrate into the stratum corneum but not into viable skin. However, there remains some concern over the safety of nanoparticles used for industrial purposes (Ryman-Rasmussen *et al.* 2006), i.e., those which are not designed for intentional application to the skin. In addition, current *in vitro* test systems lack an important physical characteristic that may conceivably affect the absorption of nanoparticles *in vivo* – normal stretching and flexing of the skin. Therefore, it is likely that the dermal penetration of nanoparticles will remain the subject of further investigation.

Summary

- The principle barrier layer of the skin is the stratum corneum, which has evolved a structure that reflects this role.
- Diffusion of a substance through the stratum corneum can occur via one (or a combination) of three routes, termed intracellular, transcellular and/or transfolicular (transappendageal).
- There are a range of biological factors that can influence the rate and extent to which a chemical can be absorbed across the skin. These include:
  - Anatomical site (regional variation), age and species of animal.

  The primary features underpinning these factors include:
  - Folicular (pelage) density and stratum corneum morphology (stacking of corneocytes, thickness and lipid composition).

References

Barry, B.W. (2002). Drug delivery routes in skin: a novel approach. *Adv Drug Deliv Rev*, **54**: S31–S40.

Cnubben, N.H.P., Elliott, G.R., Hakkert, B.C. *et al.* (2002). Comparative *in vitro–in vivo* percutaneous penetration of the fungicide ortho-phenylphenol. *Reg Toxicol Pharmacol*, **35**: 198–208.

Cornwell, P.A. and Barry, B.W. (1993). The routes of penetration of ions and 5-fluorouracil across human skin and the mechanisms of action of terpene skin penetration enhancers. *Int J Pharm*, **94**: 189–194.

Dalton, C.H., Hattersley, I.J., Rutter, S.J. and Chilcott, R.P. (2006). Absorption of the nerve agent VX (O-ethyl-S-[2(di-isopropylamino)ethyl]methyl phosphonothioate) through pig, human and guinea pig skin *in vitro*. *Toxicol In vitro*, **20**: 1532–1536.

Elias, P.M. (1983). Epidermal lipids, barrier function and desquamation. *J Invest Dermatol*, **80**: 44–49.

Elias, P.M., Cooper, E.R., Korc, A. and Brown, B.E. (1981). Percutaneous transport in relation to stratum corneum structure and lipid composition. *J Invest Dermatol*, **76**: 297–301.

Essa, E.A., Bonner, M.C. and Barry, B.W. (2002). Human skin sandwich for assessing shunt route penetration during passive and iontophoretic drug and liposome delivery. *J Pharm Pharmacol*, **54**: 1481–1490.

Feldmann, R.J. and Maibach, H.I. (1967). Regional variation in percutaneous penetration of ^{14}C cortisol in man. *J Invest Dermatol*, **48**: 181–183.

Frum, Y., Bonner, M.C., Eccleston, G.M. and Meidan, V.M. (2007). The influence of drug partition coefficient on follicular penetration: *In vitro* human skin studies. *Eur J Pharm Sci*, **30**: 280–287.

Fullerton, A., Brobyjohansen, U. and Agner, T. (1994). Sodium lauryl sulfate penetration in an in-vitro model using human skin. *Contact Dermatitis*, **30**: 222–225.

Hall, L.L., Fisher, H.L., Sumler, M.R. *et al.* (1989). Dose response of skin absorption in young and adult rats, in *Performance of protective clothing: second symposium* (eds Mansdorf, S.Z., Sager, R. and Nielsen, A.P.) Philadelphia. American Society for Testing and Materials, pp 177–194.

Holbrook, K.A. and Odland, G.F. (1974). Regional differences in thickness (cell layers) of human stratum corneum – ultrastructural analysis. *J Invest Dermatol*, **62**: 415–422.

Jacobi, U., Kaiser, M., Toll R. *et al.* (2007). Porcine ear skin: an *in vitro* model for human skin. *Skin Res Technol*, **13**: 19–24.

Larsen, R.H., Nielsen, F., Sorenson, J.A. and Nielsen, J.B. (2003). Dermal penetration of fentanyl: inter- and intraindividual variations. *Pharmacol Toxicol*, **93**: 244–248.

Lotte, C., Rougier, A., Wilson, D.R. and Maibach, H.I. (1987). *In vivo* relationship between trans-epidermal water-loss and percutaneous penetration of some organic-compounds in man – effect of anatomic site. *Arch Dermatol Res*, **279**: 351–356.

Maibach, H.I., Feldmann, R.J., Mitby, T.H. and Serat, W.F. (1971). Regional variation in percutaneous penetration in man: pesticides. *Arch Environ Health*, **23**(2): 08–211.

Marzulli, F.N. (1962). Barriers to skin penetration. *J Invest Dermatol*, **39**: 387–390.

Meidan, V.M., Bonner, M.C. and Michniak, B.B. (2005). Transfollicular drug delivery – is it a reality? *Int J Pharm*, **306**: 1–14.

Monteiro-Riviere, N.A. (2004). Anatomical Factors Affecting Barrier Function, in *Dermatotoxicology*, 6th Edn (eds Zhai, H. and Maibach, H.I.), CRC Press, Washington, DC pp 42–70.

Monteiro-Riviere, N.A., Bristol, D.G., Manning, T.O. *et al.* (1990). Interspecies and interregional analysis of the comparative histologic thickness and laser Doppler blood flow measurements at five cutaneous sites in nine species. *J Invest Dermatol*, **95**: 582–586.

Nohynek, G.J., Lademann, J., Ribaud, C. and Roberts, M.S. (2007). Grey goo on the skin? Nanotechnology, cosmetic and sunscreen safety. *Crit Rev Toxicol*, **37**: 251–277.

Oestmann, E., Lavrijsen, A.P.M., Hermans, J. and Ponec, M. (1993). Skin barrier function in healthy-volunteers as assessed by transepidermal water-loss and vascular-response to hexyl nicotinate-intraindividual and interindividual variability. *Brit J Dermatol*, **128**: 130–136.

Rolland, A., Wagner, N., Chaletus, A. *et al.* (1993). Polymeric microspheres as a novel topical site-specific drug delivery system for targeting a naphthoic acid-derivative, adapalene, to the pilo-sebaceous unit. *J Invest Dermatol*, **100**: 218.

Roskos, K.V., Guy, R.H. and Maibach, H.I. (1986). Percutaneous absorption in the aged. *Dermatol Clin*, **4**: 455–465.

Roskos, K.V., Bircher, A.J., Maibach, H.I. and Guy, R.H. (1990). Pharmacodynamic measurements of methyl nicotinate percutaneous absorption. *Brit J Dermatol*, **122**: 165–171.

Ross, J.H., Driver, J.H., Harris, S.A. and Maibach, H.I. (2005). Dermal absorption of 2,4-D: a review of species differences. *Reg Toxicol Pharmacol*, **41**: 82–91.

Ryman-Rasmussen, J.P., Riviere, J.E. and Monteiro-Riviere, N.A. (2006). Penetration of intact skin by quantum dots with diverse physicochemical properties. *Toxicol Sci*, **91**(1): 159–65.

Sandby-Moller, J., Poulsen, T. and Wulf, H.C. (2003). Epidermal thickness at different body sites: relationship to age, gender, pigmentation, blood content, skin type and smoking habits. *Acta Derm-Venereol*, **83**: 410–413.

Sato, K., Sugibayashi, K. and Morimoto, Y. (1991). Species-differences in percutaneous-absorption of nicorandil. *J Pharm Sci*, **80**: 104–107.

Schwindt, D.A., Wilhelm, K.P. and Maibach, H.I. (1998). Water diffusion characteristics of human stratum corneum at different anatomical sites *in vivo*. *J Invest Dermatol*, **111**: 385–389.

Scott, R.C., Corrigan, M.A., Smith, F. and Mason, H. (1991). The influence of skin structure on permeability – an intersite and interspecies comparison with hydrophilic penetrants. *J Invest Dermatol*, **96**: 921–925.

Sznitowska, M., Janicki, S. and Williams, A.C. (1998). Intracellular or intercellular localization of the polar pathway of penetration across stratum corneum. *J Pharm Sci*, **87**: 1109–1114.

Tanojo, H., Hostynek, J.J., Mountford, H.S. and Maibach, H.I. (2001). *In vitro* permeation of nickel salts through human stratum corneum. *Acta Derm-Venereol*, **S212**: 19–23.

van de Sandt, J.J.M., Meuling, W.J.A., Elliott, G.R. *et al.* (2000). Comparative *in vitro–in vivo* percutaneous absorption of the pesticide propoxur. *Toxicol Sci*, **58**: 15–22.

van de Sandt, J.J.M., van Burgsteden, J.A., Cage, S. *et al.* (2004). *In vitro* predictions of skin absorption of caffeine, testosterone, and benzoic acid: a multi-centre comparison study. *Reg Toxicol Pharmacol*, **39**: 271–281.

Waller, J.M. and Maibach, H.I. (2005). Age and skin structure and function, a quantitative approach (I): blood flow, pH, thickness, and ultrasound echogenicity. *Skin Res Technol*, **11**: 221–35.

Wester, R.C. and Maibach, H.I. (2004). Percutaneous absorption: short term exposure, lag time, multiple exposures, model variations and absorption from clothing, in *Dermatotoxicology*, 6th Edn (eds Zhai, H. and Maibach, H.I.), CRC Press, Washington, DC pp 83–103.

Wester, R.C., Hui, X.Y., Barbadillo, S. *et al.* (2004). *In vivo* percutaneous absorption of arsenic from water and CCA-treated wood residue. *Toxicol Sci*, **79**: 287–295.

5 Physicochemical Factors Affecting Skin Absorption

Keith R. Brain[1] and **Robert P. Chilcott**[2]

[1]*Welsh School of Pharmacy, Cardiff University, CF10 3XF and An-eX, Capital Business Park, Cardiff, CF3 2PX, UK*
[2]*Chemical Hazards and Poisons Division, Centre for Radiation, Chemical and Environmental Hazards, Chilton, Oxfordshire OX11 0RQ, UK*

Primary Learning Objectives

- *Relating the concept of 'dose' to skin absorption.*
- *Identifying the major physicochemical determinants of skin absorption and a consideration of how other modulating factors (principally exposure conditions) can affect dermal absorption.*

5.1 Introduction

The primary factor that dictates the percutaneous toxicity of a chemical is its ability to penetrate the skin. This dictum, whilst relatively straightforward, is complicated by the fact that there is a myriad of factors which can affect skin absorption.

The central dogma of toxicology was formulated by Paracelsus (a.k.a. Theophrastus Phillippus Aureolus Bombastus von Hohenheim; Box 5.1), who understood that it is the dose of a chemical which ultimately dictates its toxicity; 'sola dosis facit venenum' (Oser 1987). Thus, factors which influence skin absorption (and thus 'dose') are necessarily factors which affect percutaneous toxicity: a toxic chemical which is unable to traverse normal skin will not be toxic via the percutaneous route. For example, botulinum toxin is one of the most poisonous substances known. However, it does not penetrate healthy skin and so is essentially non-toxic following skin contact.

Skin absorption of chemicals is a passive process. Unfortunately, this does not mean that the process of dermal absorption is simple and highly predictable, as there are a diverse range of factors that can affect the rate and extent to which a chemical is absorbed. These include (amongst others) vehicle effects (Hilton *et al.* 1994), ageing (Roskos and Maibach 1992), race (Kompaore *et al.* 1993), gender (Bronaugh *et al.* 1983), disease (Moon and Maibach 1991),

Principles and Practice of Skin Toxicology Edited by Robert P. Chilcott and Shirley Price
© 2008 John Wiley & Sons, Ltd

Box 5.1 Paracelsus

Paracelsus, also known as Theophrastus Phillippus Aureolus Bombastus von Hohenheim (circa 1493 – 1541), was a largely self-taught polymath who recognised the dose-response relationship which is an underpinning principal of modern toxicology.

A somewhat interesting character, he roamed Europe, north Africa and parts of Asia in his pursuit of alternative medical knowledge. His published works, personal activities and outspoken criticism of contemporaneous medical practices did not particularly endear him to his peers!

A full-colour version of this figure appears in the colour plate section of this book

chemical damage (Wahlberg 1972), lipid content (Elias 1981), hydration (Behl *et al.* 1980), pH (Allenby *et al.* 1969), stress (Denda *et al.* 2000) and physicochemical properties of the penetrant (Lien *et al.* 1973); one could even imagine that the services of an astrologer may be a useful adjunct to predicting skin absorption! However, the problem can be simplified by considering just a relatively small number of factors which exert the greatest influence over skin absorption. The most pertinent to dermal toxicology include the physicochemical properties of the penetrant and the exposure conditions.

Other important factors influencing percutaneous absorption (e.g. effects of metabolism, species and regional variation, thermodynamic factors and methodological considerations) are considered in Chapters 2, 4, 6 and 8, respectively. A number of authoritative texts can also be consulted (Bronaugh and Maibach 1999; Schaefer and Redelmeier 1996; Wester and Maibach 1983).

5.2 Physicochemical properties

A major determinant of skin absorption relates to the physicochemical properties of the applied chemical, in particular, size, solubility, charge and hydrogen bonding capacity. An understanding of these factors can allow an approximation of the extent to which a given chemical will cross the stratum corneum.

The primary factors affecting skin absorption are concerned with the physicochemical properties of the penetrant. The most important physicochemical parameters are arguably molecular weight, solubility, charge and hydrogen bonding capacity. A basic understanding of these relatively simple factors will enable even the least experienced toxicologist to be able to make a reasonable judgement as to the dermal bioavailability of a given chemical.

5.2.1 Molecular weight

As a general rule of thumb, chemicals with a molecular weight greater than ~500 Da do not penetrate the skin. This is known as the 'rule of 500' (Bos and Meinardi 2000). This upper limit on molecular size mainly results from the physical arrangement of lipids between adjacent corneocytes of the stratum corneum (see Chapters 1 and 2). However, there is some evidence to suggest that large, linear, 'wiggly' molecules (such as heparin and DNA) may be able to traverse the stratum corneum, albeit in relatively small quantities.

5.2.2 Solubility

The solubility of a chemical is commonly quantified in terms of how it partitions between two immiscible liquids, such as water and ether. The more common measure of solubility is the octanol–water partition coefficient (Log P, also known as Kow). The Log P value can be experimentally derived (Figure 5.1) or estimated using commercially available computer software[1]. Clearly, an experimentally derived value represents the gold standard. The value of Log P is calculated using Equation (5.1).

$$K_{ow} = \text{Log}\left[\frac{[\text{octanol}]}{[\text{water}]}\right] \tag{5.1}$$

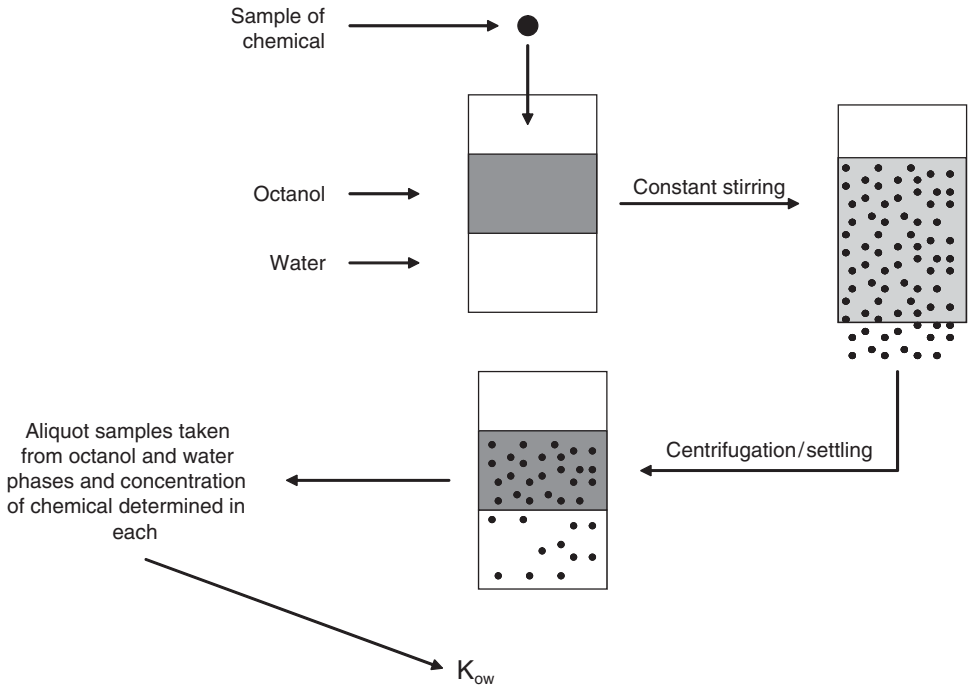
Where [octanol] and [water] represent the concentration of a chemical in octanol and water, respectively.

Being hydrophobic, octanol represents a lipophilic environment. In contrast, water is (perhaps rather obviously!) a hydrophilic environment. Thus, the tendency of a chemical to partition into octanol rather than water is reflected in a positive Log P value, whereas preferential partitioning into the water phase results in a negative value (Table 5.1). An equally amphiphilic compound would have a Log P of 0.

The relationship between solubility and the rate of skin absorption stems primarily from the ability of a chemical to partition into the stratum corneum. If a chemical is excessively hydrophilic, it will not partition into the predominantly lipid environment of the stratum corneum (Chapters 1 and 4). In contrast, if a chemical is too strongly lipophilic, it will readily partition into the stratum corneum but will not partition out into the predominantly hydrophilic environment of the underlying epidermal tissue. Put simply, it will remain stuck within stratum corneum. Thus, in order to penetrate the skin, the solubility of a chemical requires a balance between these two extremes. In general, a Log P of between 1 and 3 is considered to be optimal for skin absorption (Figure 5.2).

The importance of lipophilicity and molecular size on skin permeation has been well established and incorporated into a series of models, the best known of which is the 'Potts and Guy' equation (Potts and Guy 1992); Equation (5.2). In recent years, considerable efforts have been put into refinement of these models, driven both by innate scientific curiosity and an increased requirement for cost-effective methods of generating dermal safety data on a large range of existing chemicals. However, it is important to appreciate that these models

[1] It should be noted that Log P can only be used to describe the solubility of uncharged species. Log D (distribution coefficient) should be used for charged molecules. However, as most charged molecules do not readily penetrate the skin Log P is the most commonly used parameter.



The chemical is introduced into a vial containing octanol and water (which are immiscible and so separate on standing into to distinct layers). The mixture is then stirred for a period before centrifugation (to separate out the octanol and water layers). Samples of the upper (octanol) and lower (water) phases are then carefully obtained and the concentration of chemical in each phase determined using an appropriate analytical method. The K_{ow} is then calculated by dividing the concentration of the chemical in the octanol phase by the concentration in the water (Equation (5.1)).

Figure 5.1 Summary of method to measure Log P (K_{ow}) of a test compound

are based on a limited data set on permeation from saturated aqueous solutions and neither formulation effects nor physiological factors are considered. Obvious anomalies, such as differential permeation of stereo-isomers with identical MW and log P values, demonstrate that predictions generated by such models should be used with caution.

$$\text{Log Kp} = 0.71 \text{ Log } K_{ow} - 0.006.\text{mw} - 2.74 \tag{5.2}$$

Where Kp = permeability coefficient (see Chapter 6), K_{ow} is the octanol–water partition coefficient (Log P) and mw is molecular weight.

5.2.3 Charge

The presence of proteins (such as keratin) endows the stratum corneum with both positively and negatively charged groups. This characteristic, in combination with the lipophilic nature of the stratum corneum provides an effective barrier against charged molecules (ions). Thus, in general, ions are (at best) poorly absorbed across the stratum corneum. Indeed, there is

Table 5.1 A small selection of chemicals and their associated Log P values obtained from the EDETOX database[2] and Flynn list (Reproduced from Flynn, G.L. (1990). Physicochemical determinants of skin absorption, in *Principles of route to route extrapolation for risk assessment* (eds Gerrity, T.R. and Henry, C.J.), Copyright © 1990, Elsevier Science)

Chemical	Log P
glucose	−3.24
sucrose	−2.25
water	−1.38
butanediol	−0.92
ethanol	−0.31
scopolamine	0.98
Nicotine	1.17
nicotine	1.17
paroxon	1.98
diethyltoluamide	2.18
oestradiol	2.69
meperidine	2.72
testosterone	3.32
fentanyl	4.05
chlorpyrifos	4.96

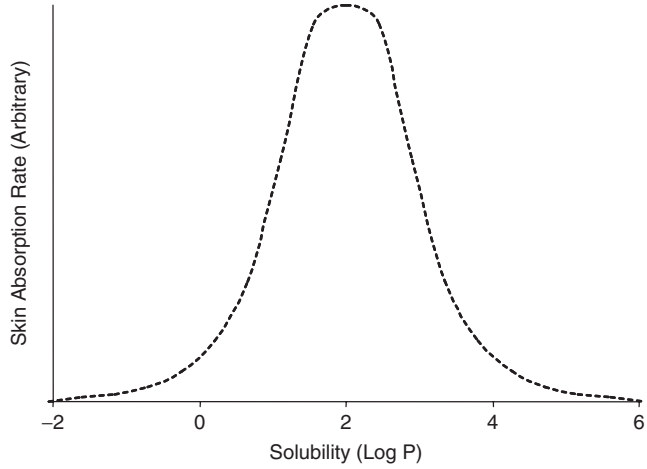


Figure 5.2 Representation of the theoretical effect of solubility (expressed as Log P) on the rate of skin absorption through skin (Note that this is an empirical generalisation! In reality, the actual relationship will vary according to the particular group of chemicals being studied (e.g. alkanols, phenols, esters etc))

evidence to suggest that appendageal routes (Chapter 4) may be the predominant pathway for diffusion for charged molecules, especially hydrophilic ions.

The presence of negatively charged groups outnumbers those that are positive and so the stratum corneum carries a net negative charge. For this reason, the penetration of positively

[2]http://edetox.ncl.ac.uk/index.html

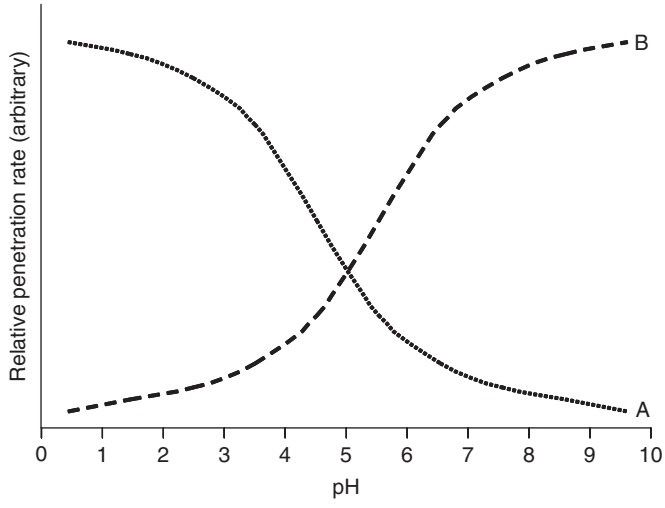


Figure 5.3 Theoretical effect of vehicle pH on the skin absorption of a weak acid (A) and a weak base (B) (This illustration assumes a pKa or pKb of 5 for the acid and base, respectively. The fraction of non-ionised acid increases at low pH, resulting in an increase in skin absorption. Conversely, the fraction of unionised base decreases at lower pH, leading to reduced penetration)

charged molecules (cations) is generally faster than negatively charged molecules (anions). In other words, the stratum corneum is 'cation selective', which has implications for transdermal delivery of drugs (Walters 2002).

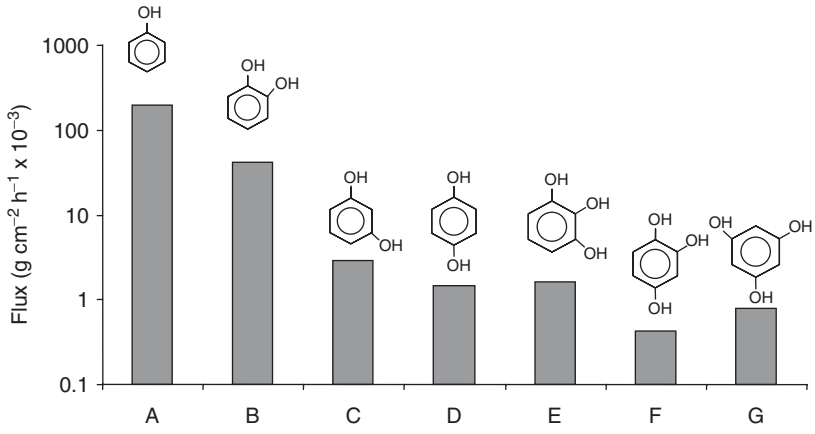
The case is slightly more complicated for chemicals whose ionisation state is pH-dependent, such as weak acids, bases and zwitterions (molecules which have both acid and base groups). In general, unionised moieties penetrate better than ionised (Figure 5.3) and so a vehicle pH that favours the formation of non-ionised molecules will result in more extensive skin absorption. Furthermore, unionised molecules tend to be more lipophilic than ionised forms and so a pH that favours the formation of non-ionised moieties may also promote skin absorption through a change in solubility.

Given that the pH of the stratum corneum ranges from around 4 to 6, then molecules which are predominantly non-ionised will tend to be absorbed more extensively than chemicals which are predominantly ionised within this pH range.

5.2.4 Hydrogen bonding

The stratum corneum contains a wealth of hydrogen bonding groups arising from its lipid and protein composition. These can form reversible bonds with chemicals as they diffuse through the stratum corneum, provided that the penetrant has complementary hydrogen bonding groups. Diffusion of a chemical through the stratum corneum can be retarded if it undergoes hydrogen bonding within the stratum corneum. Put simply, hydrogen bonds between a penetrant and the components of the stratum corneum can be thought of as brief molecular handshakes.

There are essentially two factors that affect the extent to which hydrogen bonding will slow down diffusion of a molecule through the stratum corneum. The first is the potential strength



A: phenol; B: catechol; C: resorcinol; D: hydroquinone; E: pyrogallol; F: benzenetriol; G: phloroglucinol.

Figure 5.4 The effect of the number and position of hydrogen bonding groups for a range of phenol derivatives on penetration through a surrogate biological membrane (Du Plessis *et al*. 2001, page 7, Copyright 2001, reprinted with permission from Elsevier)

of the hydrogen bond; some groups interact more strongly than others – the handshaking is firmer and longer. For example, hydrogen bonding between a nitrogen atom and an alcohol (OH) group is roughly twice as strong as that between a nitrogen atom and an amine (NH_2) group. Secondly, the number of hydrogen bonding groups (and their relative position on the penetrating molecule) is also important: more hands mean more handshakes! This is illustrated by the differential skin absorption of phenol derivatives (Figure 5.4). A quantitative consideration of the effects of hydrogen bonding on skin absorption is given in Chapter 6.

5.3 Exposure considerations

The way in which a chemical is presented to the skin can have a substantial impact on the subsequent rate of absorption and this must be accounted for in the experimental design of skin absorption studies.

Whilst the physicochemical properties of a molecule can strongly influence skin absorption, the way in which skin exposure occurs is also important. Of relevance to toxicological studies are such considerations as the solvent (vehicle) in which the chemical is dissolved, whether the exposure site is covered (occluded) or left open to the environment (unoccluded) and the general condition of the skin.

5.3.1 Vehicle effects

The influence of a vehicle on the skin absorption of a chemical cannot be overstated. Even apparently small changes to a topical formulation can have a profound influence on the

rate and extent of skin absorption; this is reflected by international toxicological or cosmetic testing guidelines, which generally require that a topical formulation being assessed should be as close as possible to that intended to be marketed (see Chapters 7, 8 and 19). It is pointless, for example, to develop a margin of safety factor for a compound that is formulated for use in an aqueous based gel, based on skin permeation data of the test compound applied in an ethanolic solution. Cumulative permeation of the compound will probably be totally different over set exposure periods and the margin of safety may be completely over or underestimated.

Vehicle effects are also of relevance to the pharmaceutical industry (transdermal drug delivery); whilst outside the remit of this book, a number of good texts are available on this subject (Barry 2003; Delgado-Charro and Guy 2001; Walters 2002). One reason why vehicles can so profoundly affect skin absorption is that they can alter the thermodynamic activity or fugacity of a penetrant. A more detailed overview of vehicle effects on the thermodynamic activity of a penetrant is given in Chapter 6.

5.3.2 Volatility

Volatility can affect the duration over which a chemical remains in contact with the skin and this can have a considerable influence on the rate and extent of skin absorption (and thus percutaneous toxicity). This is particularly apparent for highly toxic substances, such as chemical warfare agents, where systemic toxicity is directly proportional to the volatility of a given substance within a chemical series (Chilcott 2007).

5.3.3 Occlusion

Occlusion can have two effects. The first (rather obvious) consequence of occlusion relates to volatile chemicals: preventing evaporative loss from the skin surface can enhance skin absorption and thus increase percutaneous toxicity. For example, contamination of skin with benzene results in very little systemic absorption as the vast majority of the applied dose (>99.9%) is lost through evaporation under normal circumstances. However, occlusion (for example, with a plastic film) can significantly reduce vapour loss and consequently increases skin absorption (Figure 5.5), potentially resulting in greater local or systemic toxicity.

A second effect of occlusion is to increase skin hydration by preventing the normal loss of water from the skin surface from sweating or transepidermal water loss (TEWL; see Chapter 12). Water is essential for the maintenance of skin barrier function and, in normal skin, accounts for around 10% of the weight of the stratum corneum. Occlusion can increase the amount of water to 50% (w/w) and this excess hydration is generally associated with disruption of the normal structure of the stratum corneum with a corresponding loss of skin barrier function. However, the general rule that increased hydration leads to increased skin absorption has some notable exceptions, especially hydrophilic chemicals, which do not appear to be affected by occlusion-induced hydration (Zhai and Maibach 2001).

5.3.4 Skin treatments

Many guidelines for dermal toxicity assessment prescribe clipping, shaving or depilation as steps for preparing skin exposure sites (see Chapters 7 and 19). Such measures are necessary

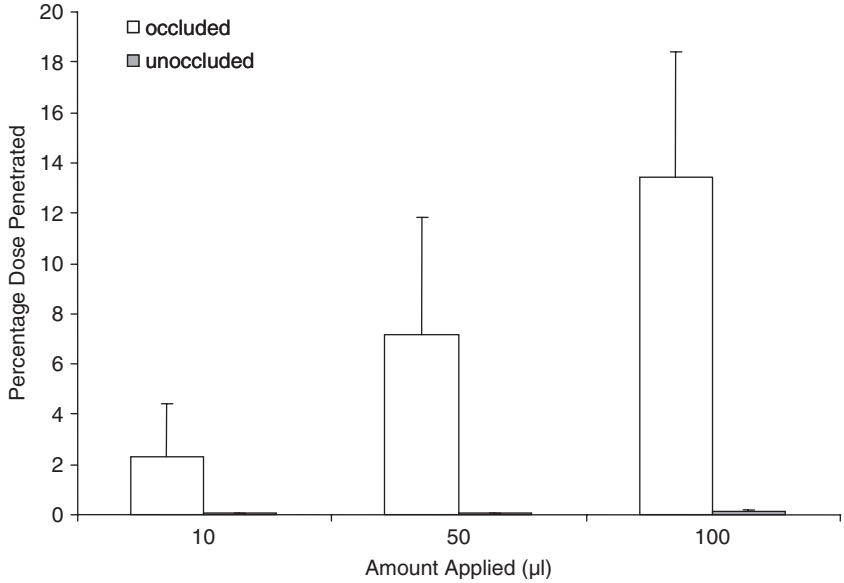


Figure 5.5 Effect of occlusion on the evaporative loss of benzene from pig skin; the amount penetrating occluded skin is consistently greater than unoccluded skin, regardless of the amount originally applied to the skin surface (Hattersley 2002)

to provide an even site for application of a test substance and application chamber (where applicable). It should be noted that depilatories and shaving (wet and dry) both cause considerable damage to the stratum corneum (Marti *et al.* 2003; Wahlberg 1972) and so their use should be avoided or accounted for with suitable controls where appropriate.

Summary

- The 'dose makes the poison' and so the extent to which a chemical can penetrate the skin will largely dictate its percutaneous toxicity.
- Skin absorption is largely influenced by the physicochemical properties of the penetrant:
  - Whilst biological factors can be a source of significant variation, physicochemical properties generally control the magnitude of skin absorption.
- The way in which a chemical is applied to the skin surface can also have a substantial effect on the rate or extent of dermal absorption (and hence percutaneous toxicity).

References

Allenby, A.C., Fletcher, J., Schock, C. and Tees, T.F.S. (1969). The effect of heat, pH and organic solvents on the electrical impedance and permeability of excised human skin. *Br J Dermatol*, **81**(S4): 31–39.

Barry, B.W. (2003). Transdermal drug delivery, in *Pharmaceutics: the science of dosage form design* (ed. Autlon, M.E.), Churchill Livingstone, London, pp 499–533.

Behl, C.R., Flynn, G.L., Kurihara, T. *et al.* (1980). Hydration and percutaneous absorption: I. Influence of hydration on alkanol permeation through hairless mouse skin. *J Invest Dermatol*, **75**(4): 346–352.

Bos, J.D. and Meinardi, M.M. (2000). The 500 Dalton rule for the skin penetration of chemical compounds and drugs. *Exp Dermatol*, **9**(3): 165–169.

Bronaugh, R.L. and Maibach, H.I. (eds) (1999). *Percutaneous absorption: drugs – cosmetics – mechanisms – methodology*. Marcel Dekker Inc, New York.

Bronaugh, R.L., Stewart, R.F. and Congdon, E.R. (1983). Differences in permeability of rat skin related to sex and body sites. *J Soc Cosmet Chem*, **34**: 127–135.

Chilcott, R.P. (2007). Dermal aspects of chemical warfare agents, in *Chemical warfare agents: toxicology and treatment* (eds Marrs, T.C. Maynard, R.L. and Sidell, F.R.), John Wiley and Sons Ltd, Chichester, pp 409–422.

Delgado-Charro, M.B. and Guy, R.H. (2001). Transdermal drug delivery, in *Drug delivery and targetting* (eds Hillery, A.M. Lloyd, A.W. and Swarbrick, J.), Taylor and Francis, London, pp. 207–236.

Denda, M., Tsuchiya, T., Elias, P.M. and Feingold, K.R. (2000). Stress alters cutaneous permeability barrier homeostasis. *Am J Physiol Regul Integr Comp Physiol*, **278**(2): R367–72.

Du Plessis, J., Pugh, W.J., Judefeind, A. and Hadgraft, J. (2001). The effect of hydrogen bonding on diffusion across model membranes: consideration of the number of H-bonding groups. *Eur J Pharm Sci*, **13**(2): 135–141.

Elias, P.M. (1981). Lipids and the epidermal permeability barrier. *Arch Dermatol Res.* **270**(1): 95–117.

Flynn, G.L. (1990). Physicochemical determinants of skin absorption, in *Principles of route to route extrapolation for risk assessment* (eds Gerrity, T.R. and Henry, C.J.), Elsevier Science, New York, pp. 93–127.

Hattersley, I.J. (2002). Skin absorption of benzene. MSc Toxicology Thesis, University of Birmingham, UK.

Hilton, J., Woollen, B.H., Scott, R.C. *et al.* (1994). Vehicle effects on *in vitro* percutaneous absorption through rat and human skin. *Pharm Res* **11**(10): 1396–400.

Kompaore, F., Marty, J.P. and Dupont, C. (1993). *In vivo* evaluation of the stratum corneum barrier function in blacks, Caucasians and Asians with two noninvasive methods. *Skin Pharmacol*, **6**(3): 200–207.

Lien, E.H.J., Marty, J.P. and Dupont, C.H. (1973). Physicochemical properties and percutaneous absorption of drugs. *J Soc Cosmet Chem*, **24**: 371–384.

Marti, V.P.I., Lee, R.S., Moore, A.E. *et al.* (2003). Effect of shaving on axillary stratum corneum. *Int J Pharm*, **25**(4): 193–198.

Moon, K.C. and Maibach, H.I. (1991). Percutaneous absorption in diseased skin: relationship to exogenous dermatoses, in *Exogenous dermatoses: environmental dermatoses*, (eds Menne,T. and Maibach,H.I.), CRC Press, Florida, pp. 221–238.

Oser, B.L. (1987). Toxicology then and now. *Regul Toxicol Pharmacol*, **7**(4): 427–443.

Potts, R.O. and Guy, R.H. (1992). Predicting skin permeability. *Pharm Res*, **9**(5): 663–669.

Roskos, K.V. and Maibach, H.I. (1992). Percutaneous absorption and age. Implications for therapy. *Drugs Aging*, **2**(5): 432–449.

Schaefer, H. and Redelmeier, T.E. (1996). *Skin barrier: principles of percutaneous absorption*. Karger, Basel.

Wahlberg, J.E. (1972). Impairment of skin barrier function by depilatories. *J Invest Dermatol*, **59**(2): 160–162.

Walters, K.A. (ed). (2002). *Dermatological and transdermal formulations*. Marcel Dekker Inc., New York.

Wester, R.C. and Maibach, H.I. (1983). Cutaneous pharmacokinetics: 10 steps to percutaneous absorption. *Drug Metab Rev*, **14**(2): 169–205.

Zhai, H. and Maibach, H.I. (2001). Effects of skin occlusion on percutaneous absorption: an overview. *Skin Pharmacol Appl Skin Physiol*, **14**(1): 1–10.

6 Principles of Diffusion and Thermodynamics

W. John Pugh[1] and **Robert P. Chilcott**[2]

[1]*Welsh School of Pharmacy, Cardiff University, Redwood Building, King Edward VII Avenue, Cardiff, CF1 3XF, UK*

[2]*Chemical Hazards and Poisons Division, Centre for Radiation, Chemical and Environmental Hazards, Chilton, Oxfordshire OX11 0RQ, UK*

Primary Learning Objectives

- *Basic understanding of the physics of diffusion.*
- *Identification of important criteria which affect the applicability or interpretation of skin absorption kinetics.*

6.1 Introduction and scope

$$\begin{aligned} A &= B \\ A^2 &= AB \\ A^2 - B^2 &= AB - B^2 \\ (A + B)(A - B) &= B(A - B) \\ A + B &= B \\ 2A &= A \\ 2 &= 1 \end{aligned}$$

Don't worry – this is not a chapter on mathematics. The little puzzle is meant to illustrate how easy it is to go wrong if the validity of our arguments is not continually assessed. Many workers in the field of skin absorption don't like (or understand) thermodynamics, but a consideration of this fundamental principle applied to the passage of a permeant molecule from the vehicle across the stratum corneum barrier layer can help keep us on the right track and, furthermore, prevent us from making some very basic errors in interpreting skin absorption data.

In skin permeation *two* processes have to be considered. Firstly, transfer of a penetrant from a vehicle *into* the stratum corneum. This partitioning depends upon a thermodynamic *equilibrium*. Secondly, diffusion across the stratum corneum, which depends on thermodynamic *gradient*. Eventually a *steady state* is reached, where the flux is the same at any depth in the membrane.

Principles and Practice of Skin Toxicology Edited by Robert P. Chilcott and Shirley Price
© 2008 John Wiley & Sons, Ltd

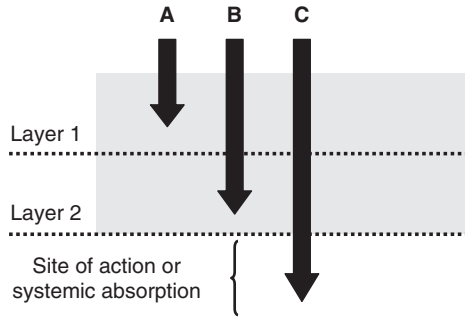


Figure 6.1 Representation of permeation (A), penetration (B) and absorption (C). Permeation is diffusion of a penetrant into a certain skin layer. Subsequent diffusion through that layer represents penetration (in this example, the substance has 'penetrated' layer 1). Penetration through one or more layers of skin to either the site of action or systemic circulation represents absorption. Local absorption is required for topical therapies such as steroid creams for skin conditions such as dermatitis whereas systemic absorption is usually the objective for transdermal patches

6.2 Some definitions pertaining to skin absorption kinetics

As with most specialist subjects, skin absorption has its own 'jargon' and familiarisation with some of the more common terms is very useful.

It is useful here to establish some definitions to describe the various aspects of skin absorption. Diffusion of a penetrant into a certain layer of skin such as the stratum corneum is termed permeation. If the penetrant diffuses through a skin layer, it has penetrated that layer. Skin absorption can be defined as the diffusion of a penetrant from the skin surface to a region within the skin where a local effect or systemic absorption will occur (Figure 6.1). Historically, this has also been referred to as percutaneous, cutaneous, dermal or transdermal penetration.

The next set of definitions arise from the two main types of dosing regimes which are generally used in skin absorption studies, particularly those performed *in vitro*: 'infinite' and 'finite' dose. The former refers to the condition where the amount (or concentration) of chemical present on the skin surface effectively remains constant (even though small amounts are absorbed by the underlying skin tissue). This is not a realistic scenario for most skin exposure to chemicals, as it is effectively the equivalent of being immersed in a swimming pool of a chemical solution but, as will be seen in subsequent sections, this largely artificial condition is frequently used to determine important kinetic parameters such as flux (J), permeability coefficient (Kp) and diffusion coefficient (D). A finite dose condition is more representative of a real world dermal exposure and describes the situation where a discrete quantity of chemical is applied to the skin surface and is gradually depleted with time (as skin absorption occurs). Most *in vivo* studies are based on finite dosing regimes although, in some cases, repeated application of a finite dose of test substance may essentially result in infinite dose conditions.

The 'absorption profile' of a substance is a graph of the cumulative amount of substance penetrating the skin with time. Such plots are most commonly derived from *in vitro* experiments,

although similar graphs can be obtained *in vivo*. Both infinite and finite dose conditions yield characteristic penetration profiles. In the case of an infinite dose, a 'steady state' is achieved, where the amount of substance penetrating the skin per unit time becomes constant – at this point, the profile is linear (Figure 6.2). A penetration rate (flux) derived from such a study is correspondingly called a steady-state flux (Jss). In contrast, a finite dose condition may result in a 'pseudo steady state' condition, where the profile may be transiently linear but then plateaus and becomes flat due to depletion of the penetrant from the skin surface (Figure 6.3). An alternative way of presenting finite dose data is to plot the amount penetrated between time points against time (Figure 6.4). In this method of analysis, the peak of the graph gives the maximum penetration rate (Jmax).

The time taken to achieve steady-state conditions under infinite dose conditions is referred to as the lag time (τ) and the preceding period is the lag-phase. Lag time is often calculated from linear extrapolation of the steady-state portion of the penetration profile back to the x-axis (Figure 6.2). However, this is incorrect (see Equation (6.7)).

Finally, there is the concept of the 'infinite sink' condition. This is where any penetrant which diffuses through a rate limiting barrier (such as the stratum corneum) is instantly removed to effectively maintain a zero concentration of penetrant directly beneath the barrier layer. *In vivo*, removal of penetrant can occur when the compound is absorbed into the systemic circulation (Chapter 1). *In vitro*, flow-through diffusion cells maintain infinite sink conditions by constant replenishment of the receptor chamber fluid (see Chapter 8). In the case of static diffusion cells, it is commonly stated that the receptor chamber fluid should contain no more than 10% of the saturated concentration of penetrant for infinite sink conditions to be assumed.

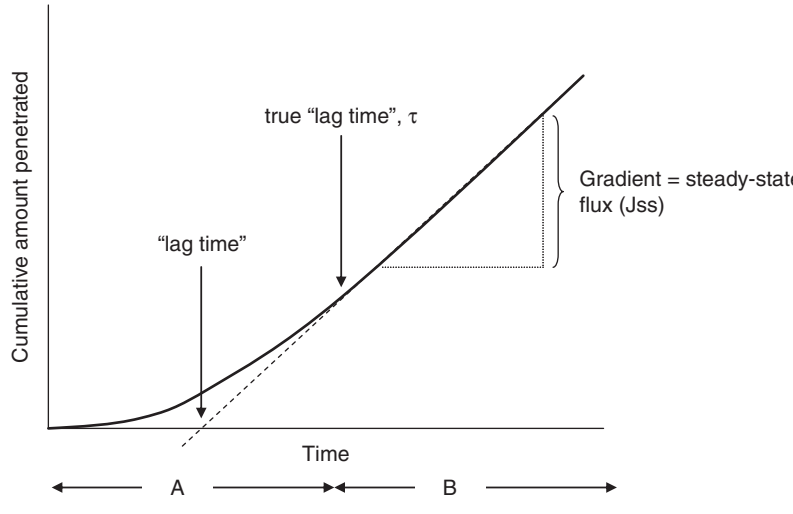


Figure 6.2 Example of 'penetration profile' obtained under infinite dose conditions. Following a lag phase (A); the amount penetrating the skin attains a steady state (B) where the amount penetrating per unit time is constant; the gradient of this line can be used to calculate the steady-state flux (Jss; often expressed as g cm^{-2} h^{-1}). Erroneously, lag time is often calculated by linear extrapolation of the steady state back to the x-axis. In fact, the true lag time (τ) is calculated from Equation (6.7). This condition is described by Fick's first law of diffusion, Equation (6.3)

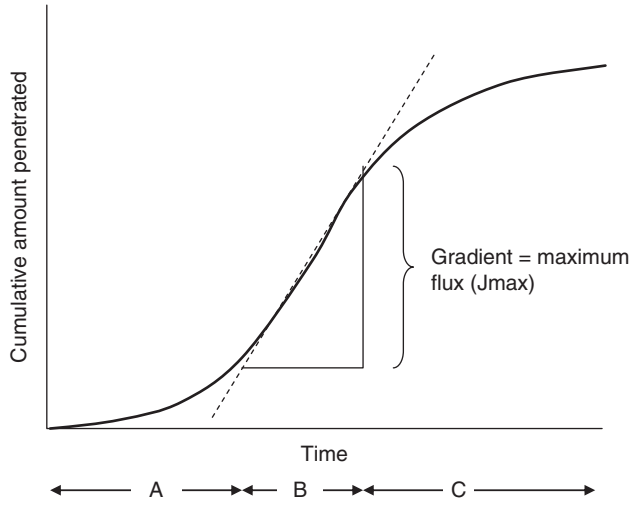


Figure 6.3 Example of 'penetration profile' obtained under finite dose conditions. Following a lag phase (A); the amount penetrating the skin attains an apparently linear, pseudo-steady-state (B), the gradient of which can be used to estimate the maximum flux (Jmax). Eventually, the amount of penetrant available on the skin surface becomes depleted and the profile eventually plateaus (C), at which point the penetration rate is zero. This condition is described by Fick's second law of diffusion

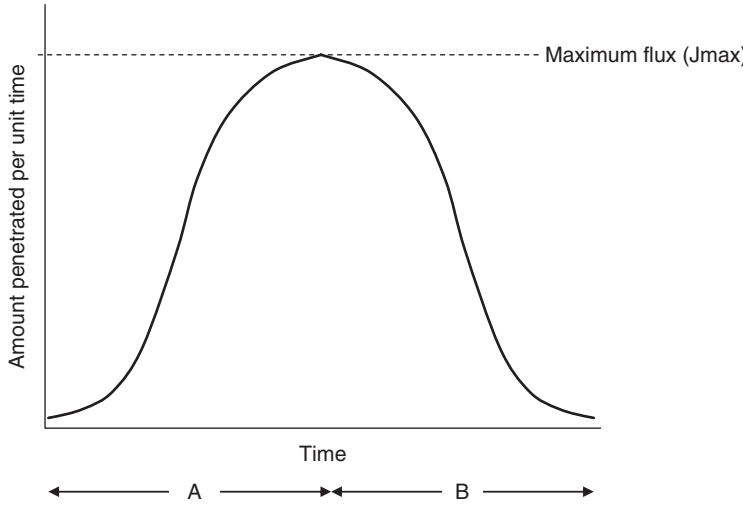


Figure 6.4 Example of an alternative penetration profile for a finite dose experiment. The amount penetrating per unit time is plotted on the Y axis instead of the cumulative amount (technically, this can be defined as the first differential coefficient of cumulative amount penetrated). Extrapolation of the peak of the curve to the Y axis gives the maximum flux (Jmax). This method is particularly useful when the pseudo-steady-state portion of the graph is absent, i.e., only the lag phase (A) and donor depletion phase (B) are discernible

6.3 Basic concepts of diffusion

The random movement of a molecule in a solution is termed diffusion and this passive process is responsible for the transport of chemicals across the stratum corneum.

Diffusion can be defined as the random movement of molecules from an area of high concentration to an area of low concentration. This can be simply demonstrated by carefully adding ink to a beaker of water: At first, the ink remains within a well defined, concentrated region but if left unstirred it slowly spreads out until eventually attaining an even distribution throughout the water. At this point, the ink has reached equilibrium, where no further movement can be observed. Looking at diffusion in this 'macroscopic' way can provide very useful approximations for mathematical models. However, it would be wrong to assume that within this equilibrated system there is no further diffusion. The ink molecules still move through the water. But, as the process of diffusion is random and there are literally billions of ink molecules present, when one molecule moves to a different location it is rapidly replaced by another. Thus overall, no further diffusion is visibly apparent. However, if the ink solution were analysed under an immensely high power microscope, it would no longer appear to be evenly dispersed and instead would appear to transiently contain concentrated areas of ink similar to the patterns of 'snow' seen on the screen of an untuned television set.

Now, if the direction and velocity of each diffusing molecule were known as well as each individual interaction between all the molecules of ink and water, a 'microscopic' mathematical description of diffusion could be produced. Whilst this would represent an extremely accurate method of analysis, it would obviously be practically impossible, especially when trying to characterise the diffusion of billions of penetrant molecules through such a complex membrane as the skin. Therefore, theorists have developed a macroscopic model: the non-equilibrium thermodynamic model, or NET (Strutt 1873; Onsager 1931), that allows the skin to be considered as a homogenous medium in respect to its interaction with a diffusing molecule (Ogston and Michel 1978). The interactions of each diffusing molecule with individual skin structures are accounted for in the NET model by the use of 'phenomenological equations' (Kedem and Katchalsky 1961; Katchalsky and Curran 1967). The NET model is approximated by Fick's law of diffusion (Crank 1975).

6.4 Fick's Laws of diffusion

Assuming that a molecule behaves in an 'ideal' manner, Fick's laws of diffusion can be used to characterise skin absorption.

Paraphrasing the definition given above, diffusion can be described as the 'process by which matter is transported from one part of a system to another as a result of random molecular motions' (Crank 1975). The earliest mathematical model of diffusion was derived by Fourier (Fourier 1822; Whelan and Hodgson 1985) who characterised the transfer of heat by conduction (Equation (6.1)):

$$J = -k \frac{\delta T}{\delta x} \tag{6.1}$$

where J (the energy current density, i.e. flux) is proportional to the change of temperature (δT) with the distance travelled (δx). The relationship is linear with respect to the constant, k (Whelan and Hodgson 1985). The analogy between conduction of heat and diffusion of molecules was recognised by Fick who formulated this into his first law of diffusion (Equation (6.2)), which is specific to an infinite dose condition:

$$J = -D \frac{\delta C}{\delta x} \tag{6.2}$$

where J is the rate of transfer per unit area (flux), δC is the concentration gradient, δx is the linear distance travelled and D is the diffusion coefficient. (The negative sign indicates that the transfer of molecules is occurring in the opposite direction to the concentration gradient.) Fick's second law of diffusion relates to finite dose conditions (which are not considered here). The more conventional form of Fick's first law is given in Equation (6.3):

$$Jss = Kp.Co \tag{6.3}$$

where Jss is the steady state flux per unit area, Kp is the permeability coefficient for a given solute in a given vehicle and Co is the concentration of the solute in the vehicle. (It is important to remember that Kp can only be used to predict the penetration rate of a chemical at a given concentration from the same vehicle.)

Equation (6.3) relies on the assumptions that (i) the solute exhibits ideal behaviour (Bransom 1961), (ii) that the concentration gradient is equal to the original concentration of the solute (i.e., maintenance of infinite dose and infinite sink conditions) and (iii) that the concentration of solute is low[1] (Dugard 1977). The reason for the latter assumption is explained later (see Section 6.6).

6.5 Thermodynamic activity

> It is very important to understand that diffusion across the stratum corneum is driven by a thermodynamic gradient, not a concentration gradient.

The phrase 'random molecular motions' (as used in the above definition of diffusion) is important because it emphasises that it is the physical (kinetic) movement of molecules that drives diffusion. From a simple perspective, the dependence of diffusion on concentration (Equation (6.3)) seems rather obvious, as the more molecules there are in a system then the greater the total number of movements being made at any one time, and so the higher the probability that molecules will diffuse with time to a region of lower concentration. However, this apparent concentration-dependence does not take into account the interaction of each molecule with other, surrounding molecules. This is of particular relevance where a molecule is diffusing from one environment into another (such as between a vehicle and the stratum corneum). An important consequence of such molecular interactions is termed 'fugacity'.

Derived from the Latin word 'Fugax' meaning 'to flee' or 'escape', fugacity can be defined as the tendency of a molecule to leave a particular environment. For example, a person with

[1]Technically, the concentration should be an 'infinite dilution'.

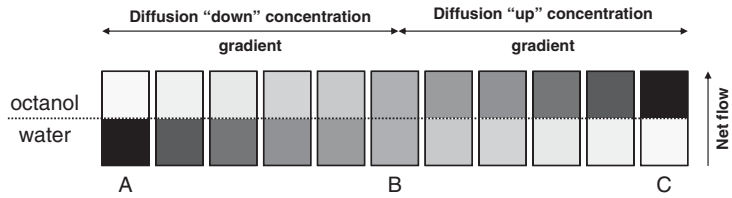


Figure 6.5 Example of diffusion 'up' a concentration gradient. Such behaviour can occur during the experimental determination of the octanol:water partition coefficient (Log P). In this example, hexachlorobenzene (HCB; Log P = 5.3) is first dissolved in the water phase. Although it has a high Log P value, HCB is fairly soluble in water (5 g L^{-1} at room temperature). The Log P value means that, at equilibrium, there will be approximately 204 000 molecules of HCB in the octanol phase for each molecule of HCB in the water phase. At time 'A', the octanol is carefully placed on top of the water phase (the two are immiscible). At this point, all of the molecules of HCB are in the water phase and diffusion occurs 'down' the concentration gradient, which results in the net transfer of HCB into the octanol phase. After a period, the concentration of HCB in the water and octanol phases will be the same ('B'). However, whilst there is now no concentration gradient, there remains a thermodynamic gradient; the fugacity of HCB molecules in the water phase is greater than that of the octanol phase and so diffusion of HCB continues from the water to the octanol 'up' a concentration gradient. The net movement of HCB will cease when a thermodynamic gradient no longer exists, that is, when a 204 000:1 ratio of HCB in octanol to water is attained (time 'C')

claustrophobia will exhibit greater fugacity when placed in a small room than an agoraphobic. Similarly, a lipophilic molecule dissolved in an aqueous vehicle will exhibit a greater fugacity than a hydrophilic molecule when it is brought into contact with the lipophilic environment of the stratum corneum. A molecule's fugacity within a given environment can be quantified in terms of its thermodynamic activity (α) and this is the driving force for diffusion, not concentration. In some circumstances, the thermodynamic gradient can be in the opposite direction to a concentration gradient and so diffusion will occur against the concentration gradient (for example, see Figure 6.5). This has very important practical consequences and a misunderstanding of this basic concept can lead to an erroneous interpretation of skin absorption data.

Thermodynamic activity represents the effective concentration of a molecule in a solution and is equal to the product of concentration and the ideality coefficient (γ). For ideal solutions (where $\gamma = 1$), then thermodynamic activity is equal to concentration.

6.6 Skin absorption of a substance from two different vehicles

> Under most circumstances, concentration and thermodynamic activity are not equivalent parameters. This misunderstanding is a common source of error when planning and interpreting skin absorption experiments (particularly those comparing drug delivery rates from different formulations).

The maximum thermodynamic activity of a molecule occurs when it is dissolved to saturation in a vehicle. Under ideal conditions, the thermodynamic activity (α) of a given molecule at

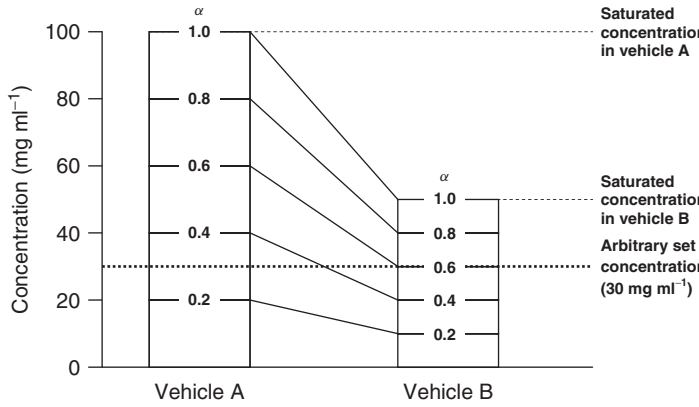


Figure 6.6 Empirical relationship between concentration and thermodynamic activity (α) for a lipophilic compound 'X' dissolved in an oil (A) or water (B) vehicle. At saturation, the thermodynamic activity of X is the same, even though the concentrations are different. If the concentration of X is gradually diluted in both vehicles, the difference in thermodynamic of X within the two vehicles diminishes until, at very high dilutions (low concentration), the thermodynamic activity approximates to the concentration

saturation in any vehicle will be the same (conventionally, α is numerically equal to one at saturation). However, the thermodynamic activity of a molecule at the same concentration in two different vehicles will be different. To explain this, the following example can be worked through.

Consider the situation where moderately lipophilic molecules of 'X' are dissolved in two different vehicles, 'A' (oil) and 'B' (water). Figure 6.6 illustrates that, at saturation, the concentration of X in vehicle A (100 mg ml^{-1}) is twice that of B (50 mg ml^{-1}). If it is erroneously assumed that concentration was the driving force for diffusion it would be assumed that the flux of X through the skin from vehicle A would be twice that from vehicle B. However, the thermodynamic activity of X is the same in both saturated solutions ($\alpha = 1$ in both solutions; Figure 6.5). Thus, the flux of X when delivered from vehicles A and B at saturation will be the same.

If the concentration of X is gradually decreased, there is a corresponding decrease in thermodynamic activity and it can be seen from Figure 6.5 that the *difference* in thermodynamic activity of X between the two vehicles also decreases (as indicated by the slope of the lines connecting the same α values). Thus, at very low dilutions, the thermodynamic activity of X in vehicle A will approach that of X in vehicle B. At this point, concentration becomes a good approximation of thermodynamic activity and this is why Fick's law is applicable at low solute concentrations (technically, an 'infinite dilution').

One error that is commonly seen in the design of skin absorption studies is the practice of using a set concentration of solute to assess skin absorption from different vehicles. Using the above example, if an arbitrary concentration of 30 mg ml^{-1} was used for compound X in both vehicles, then there would be a corresponding two-fold difference in thermodynamic activity (Figure 6.5); this experimental design is flawed if the purpose is to compare the effects of the two vehicles on skin absorption of compound X. The correct method would be to use either saturated solutions ($\alpha = 1$ in each vehicle) or very dilute solutions ($\alpha_A \cong \alpha_B$).

6.7 Partitioning

The first stage of percutaneous absorption requires partitioning of the penetrant into the stratum corneum.

Skin absorption is essentially a three-stage process. Firstly, the penetrant has to partition from the vehicle into the stratum corneum. The main limiting factor here is the thermodynamic activity (fugacity) of the penetrant in the vehicle relative to the stratum corneum. The second stage involves diffusion of the penetrant through the stratum corneum (which is driven by a thermodynamic gradient). Finally, the penetrant must partition from the stratum corneum into the underlying tissue. The process of partitioning is now examined in more detail and linked to Fick's laws of diffusion.

Following topical application, an equilibrium forms between the concentrations of penetrant in the vehicle and stratum corneum (C_V and C_{SC}, respectively). These are not equal, but are related by the partition coefficient K_{SC} (often referred to as Km; membrane partition coefficient) between the stratum corneum and vehicle (Equation (6.4)):

$$\mathrm{Ksc} = \frac{C_{sc}}{C_C} \tag{6.4}$$

An important point to remember is that the concentration of penetrant within the stratum corneum is not constant as commonly assumed until steady state conditions have been attained; it changes with time (Figure 6.7).

Partitioning is an important parameter of skin absorption because it affects the amount of penetrant in the stratum corneum which, in turn, affects the thermodynamic gradient across the stratum corneum. It is related to the permeability coefficient (Kp) by the equation:

$$\mathrm{Kp} = \frac{K_{sc}.D}{x} \tag{6.5}$$

where D is called the diffusion coefficient (see diffusivity, below) and x is the thickness of the stratum corneum. Inserting the terms of Equation (6.3) into Equation (6.5) gives Equation (6.6), which shows the relationship between the main factors that affect skin absorption:

$$\mathrm{Jss} = \frac{K_{sc}.C_v.D}{x} \tag{6.6}$$

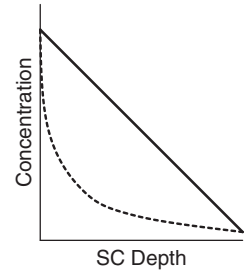


Figure 6.7 Concentration profile of a penetrant across the stratum corneum during the lag phase (dotted line) and at steady state (solid line)

6.8 Diffusivity

The mobility of a penetrant within the stratum corneum is quantified by the diffusion coefficient (D), which can be used to investigate a range of factors that affect skin absorption (such as hydrogen bonding).

The diffusion coefficient (D) is essentially a measure of the mobility of a penetrant within the stratum corneum and depends on the size, shape and 'stickiness' of the penetrant to components of the stratum corneum. The relation between the diffusion coefficient and other parameters of skin absorption is shown in Equations (6.2), (6.5), (6.6) and (6.7):

$$\tau = \frac{x^2}{6D} \tag{6.7}$$

The diffusion coefficient is a useful parameter as it can provide information on intermolecular interactions between a penetrant and the components of the stratum corneum.

The effect of intermolecular binding between a penetrant and the constituents of the stratum corneum can be simply demonstrated by plotting D (expressed as D/x) against the number of hydrogen bonds. It can be seen that introduction of three hydrogen-bonding groups has a saturation effect (Figure 6.8).

Hydrogen bonds are intermolecular interactions between a hydrogen atom (attached to oxygen, nitrogen or sulphur group) on a donor molecule and an electronegative functional group (such as oxygen, nitrogen or sulphur) on the receptor molecule. The ability with which a molecule or functional group can participate in hydrogen bonding can be quantified in terms of donor capacity (α) or acceptor capacity (ß) (see Table 6.1). The diffusion coefficient can be related to these and to size (molecular weight; mw) according to Equation (6.8).

$$\mathrm{Log}\left[\frac{D}{x}\right] = -1.50 - 0.911\,\alpha - 1.58\,\beta - 0.0037\,\mathrm{mw} \tag{6.8}$$

Note the negative signs for all predictors; each term slows diffusion. The relative effects of acceptor and donor binding can be estimated using Equation (6.9), showing that hydrogen-bond acceptor groups are more effective than donors in binding to the stratum corneum[2]:

$$\frac{\beta}{\alpha} \cong 4.7 \tag{6.9}$$

The relative effects of functional groups on D can be investigated by examining data for a variety of mono-functional penetrants to elicit the overall contribution of each group.

[2]The value of 4.7 comes from subtracting the antilog of 0.911 (the value from the α term in Equation (6.8)) from the antilog of 1.58 (value of the ß term) and indicates that a molecule is nearly five times more likely to undergo hydrogen bonding with the stratum corneum if it contains an acceptor, rather than donor group.

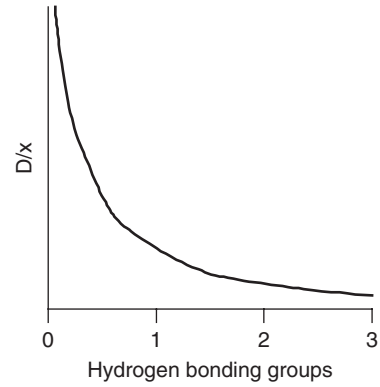


Figure 6.8 Relation between diffusion coefficient (normalised to membrane thickness; D/x) and number of hydrogen bonding groups present on the diffusing molecule

Table 6.1 Scaled hydrogen bonding donor capacity (α) and scaled hydrogen bonding acceptor capacity (β) of some common molecules/functional groups (Abraham 1993, Reproduced by permission of the Royal Society of Chemistry)

Functional Group	α	β
Alkane	0.00	0.00
Alkene	0.00	0.07
Ether	0.00	0.45
Aldehyde	0.00	0.45
Ketone	0.00	0.51
Amine (1°)	0.16	0.61
Alcohol (1°)	0.37	0.48
Amide (1°)	0.55	0.68
Carboxylic acid	0.61	0.44
Phenol	0.60	0.38

For example, Equation (6.10) is derived from such an analysis of 31 substances and yields a correlation coefficient (r^2) of 87%:

$$\text{Log}\left[\frac{D}{x}\right] = -1.36 - 1.67\,A - 1.41\,B - 1.17\,C - 0.986\,D - 0.759\,E - 0.0502\,F \qquad (6.10)$$

where the upper case letters indicate the number of acid (A), alcohol (B), phenol (C), ketone (D) and ether (E) groups, and F is the number of carbon atoms not attached to an oxygen atom. The small effect of F (number of carbon atoms) is due to the effect of increasing the molecular bulk on diffusion. Equation (6.10) allows the effect of each hydrogen-bonding group to be quantified in terms of its 'retardation coefficient' relative to ether (Figure 6.9).

It is important to note that such an empirical assessment does not take into account the position of the hydrogen-bonding groups on a molecule. This has been well illustrated by

a study of phenol derivatives (du Plessis *et al.* 2002). From the discussion above it might be expected that phenols containing three hydroxyl (OH) groups (trihydric phenols) would all have approximately equal diffusion coefficients, which would be significantly lower than that of phenol. Benzenetriol does, indeed, have a much lower diffusion coefficient, but the values for the other two phenol derivatives studied are much higher than expected (Figure 6.10). This can be explained by the effect of symmetry in the permeant molecules (Table 6.2). An increase in the number of planes of symmetry enables a greater degree of intra-species bonding, and

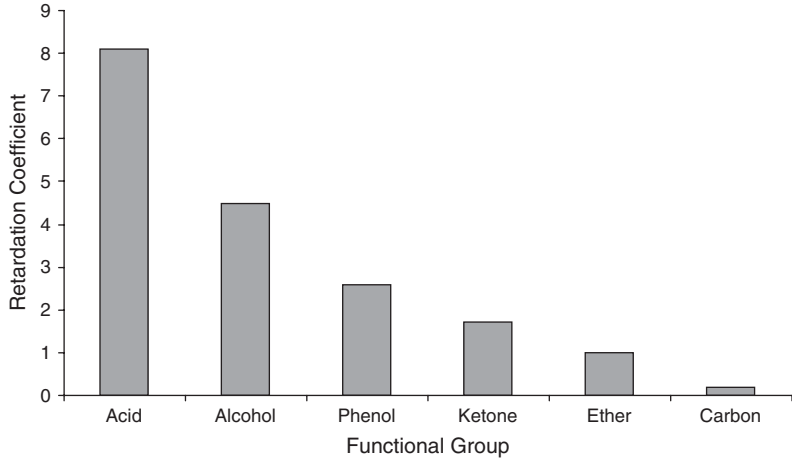


Figure 6.9 Retardation coefficients of various functional groups (derived from Equation (6.10)) relative to ether

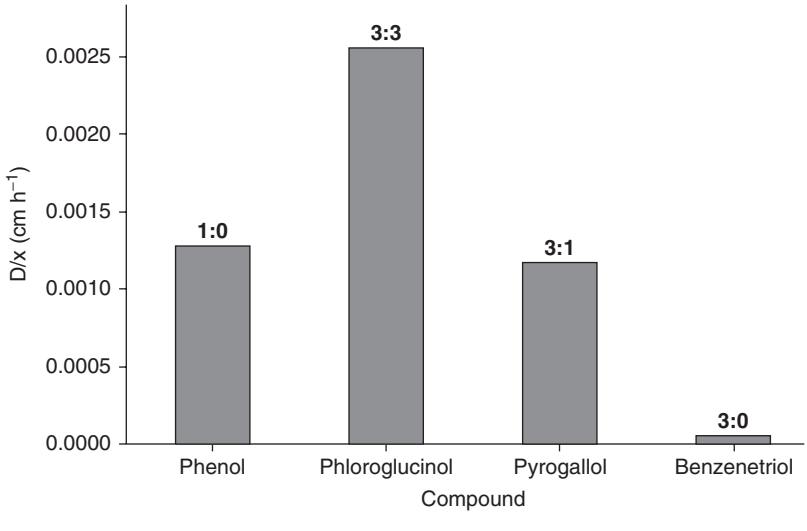
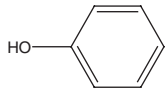
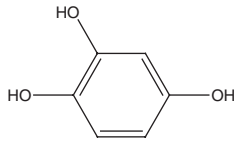
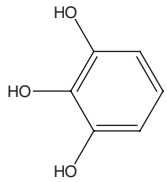
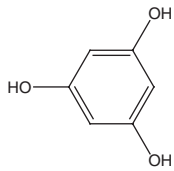


Figure 6.10 Combined effect of the number of hydrogen-bonding groups and planes of symmetry on diffusion of four phenolic compounds within the stratum corneum (the numbers above the bars indicate the number of hydrogen-bonds:planes of symmetry)

Table 6.2 Planes of symmetry for some phenolic compounds

Structure	Compound	Planes of Symmetry
	Phenol	0
	Benzenetriol	0
	Pyrogallol	1
	Phloroglucinol	3

a concomitant decrease in inter-species bonding (van Krevelen and Hoftyzer 1976). This also accounts for the very low solubility of phloroglucinol (0.084 M) in water relative to benzenetriol (0.97M) or phenol (0.88 M), since the phloroglucinol molecules are incapable of hydrogen-bonding with water molecules. In the case of diffusion across the stratum corneum, the intra-specific bonding that occurs as a result of increased planes of symmetry means than little bonding capacity is left for the skin components, so the molecules diffuse freely.

6.9 Skin absorption data and risk assessments

Kinetic data must be used carefully when formulating risk assessments. There are a number of potential pitfalls, which include regional variation and hydration-induced changes in skin thickness.

Experimental determinations of various skin absorption kinetic parameters (such as Kp, Jss and D) are only useful as tools for risk assessment if the data are used appropriately. It should be remembered that most kinetic studies are performed by exposing a small area of skin (relative to the whole organism). Estimating absorbed dose from kinetic studies is fairly simple: multiply the flux by the surface area of skin being exposed and divide this by the duration of exposure[3]. For humans, whole body surface area (SA) can be calculated using

[3]This basic calculation assumes a negligible lag-phase.

standard equations (for example, Equation (6.11) or nomograms (Snyder *et.al.* 1981):

$$SA = 71.48 \times W^{0.425} + h^{0.725} \tag{6.11}$$

where W is weight (kg) and h is height (cm).

However, it should not be assumed that the permeability of the exposure site is representative of the whole body (see Chapter 4 for an overview of biological factors affecting skin absorption).

A further consideration is that many kinetic parameters are dependent on the thickness of the stratum corneum (e.g. Equations (6.2) and (6.5)–(6.8)). The application of a vehicle to the skin may alter the hydration status of the underlying tissue and cause changes in the thickness of the stratum corneum. For example, an increase in hydration may lead to a five-fold increase in thickness due to swelling of corneocytes. Clearly, this may have a profound effect of the measurement of diffusion parameters!

Summary

- **A grasp of the basic terminology provides a useful foundation for understanding skin absorption kinetics.**
- **Concentration gradient is not the driving force for diffusion of a substance across the stratum corneum; it is controlled by the thermodynamic gradient. The two cannot be assumed to be equivalent unless certain criteria are met.**
- **There is a range of kinetic parameters that can be measured experimentally but there are also a variety of factors that must be carefully considered to prevent their inappropriate interpretation or application.**

References

Abraham, M.H. (1993). Scales of solute hydrogen-bonding: their construction and application to physicochemical and biochemical processes. *Chemical Society Reviews*, **22**: 73–83.

Bransom, S.H. (1961). *Applied Thermodynamics*, Van Nostrand Company Ltd, London, pp. 161–178.

Crank, J. (1975). The Diffusion Equations, in *The Mathematics of Diffusion*, 2nd edn (Crank, J.), Clarendon Press, Oxford, pp. 1–10.

Dugard, P.H. (1977). Skin Permeability Theory in Relation to Measurements of Percutaneous Absorption in Toxicology, in *Advances in Modern Toxicology, Dermatotoxicity and Pharmacology* (eds Marzulli, F.N. and Maibach, H.I.), Halstead Press, Oxford, pp. 525–548.

du Plessis, J. Pugh, W.J. Judefeind, A. and Hadgraft, J. (2002). Physico-chemical determinants of dermal drug delivery: effects of the number and substitution pattern of polar groups. *European Journal of Pharmaceutical Sciences*, **16**: 107–112.

Fourier, J.B. (1822). *Théorie analytique de la chaleur* (Translation by Freeman, A.), Dover Publications, New York, 1953.

Katchalsky, A. and Curran P.F. (1967). The Phenomenological Equations Relating Flows and Forces; Onsagers's Law, in *Non-equilibrium Thermodynamics in Biophysics* (eds Katchalsky A. and Curran P.F.), Harvard University Press, Harvard, pp. 85–97.

Kedem, O. and Katchalsky, A. (1961). A Physical Interpretation of the Phenomenological Coefficients of Membrane Permeability, *J Gen Physiol*, **45**: 143–179.

Ogston, A.G. and Michel, C.C. (1978). General Descriptions of Passive Transport of Neutral Solute and Solvent Through Membranes, *Prog Biophys Mol Biol*, **34**: 197–217.

Onsager, L. (1931). Reciprocal Relations in Irreversible Processes. II., *Phys Rev*, **38**: 2265–2279.

Snyder, W.S. Cook, M.J. Karhausen, L.R. *et al.* (eds). (1981). *Report of the task group on reference man.* A. Wheaton & Co Ltd, Exeter.

Strutt, J.W. [aka Lord Rayleigh] (1873). Some General Theorems Relating to Vibrations, *Proc Math Soc (London)*, **4**: 357–369.

Van Krevelen, D.W. and Hoftyzer, P.J. (1976). Cohesive Properties and Solubility, in *Properties of Polymers: their estimation and Correlation with Chemical Structure; their numerical estimation and prediction from additive group contribution* (ed. Van Krevelen D.W.), Elsevier, New York, pp. 129–159.

Whelan, P.M. and Hodgson, M.J. (1985). Ideal Gases: Kinetic Theory in *Essential Principles of Physics* (eds Whelan P.M. and Hodgson M.J.), John Murray, London, pp. 177–184.

7 *In vivo* measurements of skin absorption

James C. Wakefield and **Robert P. Chilcott**

Chemical Hazards and Poisons Division, Centre for Radiation, Chemical and Environmental Hazards, Chilton, Oxfordshire OX11 0RQ, UK

Primary Learning Objectives

- *Background to the regulatory and physiological reasons for conducting studies of skin absorption in vivo.*
- *Ethical considerations underpinning human and animal experiments.*
- *Salient features of the international guideline for the conduct and interpretation of in vivo skin absorption studies: OECD 427.*
- *An awareness of alternative methodologies.*

7.1 Introduction and scope

There are various reasons for conducting skin absorption studies *in vivo*. From a toxicological point of view, quantification of the rate and extent of skin absorption may be of fundamental importance in the risk assessment of compounds that are active via the dermal route of entry.

In vivo skin absorption studies may be conducted in support of investigative research, optimisation of topical formulations, risk assessments, regulatory submissions and for the further development of *in vitro* or *in silico* models. For investigative research, it is necessary to perform *in vivo* skin absorption studies to further our fundamental understanding of dermal absorption. Risk assessments and regulatory submissions require data from *in vivo* skin absorption studies to provide substantive evidence to support a lack of toxic effect at a dose range commensurate with the perceived dose. *In vivo* skin absorption studies are also necessary for the development, evaluation and validation of *in vitro* and *in silico* models. Thus, somewhat paradoxically, *in vivo* studies make a long-term contribution to the '3 Rs' of reduction, refinement and replacement of animals in scientific research.

So, why is skin absorption relevant to dermal toxicology? The answer to this can be related to the central dogma of toxicology: 'the dose makes the poison' (Chapter 5). Take the hypothetical situation where two equitoxic substances (A and B) are applied to the

Principles and Practice of Skin Toxicology Edited by Robert P. Chilcott and Shirley Price
© 2008 John Wiley & Sons, Ltd

skin. Substance A penetrates the skin whereas B does not. Clearly, only substance A will be considered toxic via dermal exposure. This very simple example illustrates the point that the extent of skin absorption (and thus 'dose') is necessarily a factor which will influence percutaneous toxicity.

7.2 Why conduct *in vivo* studies?

Where possible, *in vivo* studies should be avoided by the judicious use of suitable *in vitro* alternatives. However, *in vitro* systems lack a variety of biochemical, immunological and physiological systems which are critical to determining local or systemic toxicity.

It is not generally acceptable (either ethically or economically) to conduct *in vivo* studies where suitable *in vitro* techniques are readily available. There is a wealth of evidence to suggest that *in vitro* absorption systems can predict percutaneous absorption *in vivo* (Dick *et al.* 1997, Scott *et al.* 1992, van de Sandt *et al.* 2000). Therefore, is there really a need to conduct *in vivo* studies if an appropriate *in vitro* study can be performed?

There are a number of known limitations to the predictive accuracy and application of *in vitro* models:

- The solubility of the test compound in the receptor phase (Chapter 8).
- The absence of systemic uptake and distribution to maintain infinite sink conditions (Chapter 5).
- A limited metabolic response or lack of systemic metabolism, which may be required for the formation of a toxicologically relevant species (Chapter 2).
- The absence of an inflammatory-mediated response, which can dictate local toxicity (Chapter 9).

Owing to these limitations, it is not currently feasible to rely solely on data from *in vitro* or *in silico* models to accurately determine percutaneous absorption *in vivo*. Thus, *in vivo* studies are a necessity, as current *in vitro* techniques lack vital biological elements, whilst *in silico* techniques are still essentially in their infancy.

7.3 Ethics and legislation

The conduct of *in vivo* studies involving either human volunteers or laboratory animals is governed by legislation and guidelines to ensure that the experiments are ethical and produce reliable information.

The use of animal or human subjects for experimental purposes is subject to a variety of ethical and legal requirements. These are generally country-specific and must be fully consulted and acted upon prior to conducting a study. Any experiment, whether it is with animals or humans, must be fully justifiable on scientific, moral and ethical grounds.

In vivo methods for determining percutaneous absorption can involve the use of relevant laboratory species or human volunteers. There is currently increasing pressure to reduce the

number of animals used for *in vivo* experiments. In particular, there are certain restrictions on the use of animals for the assessment of percutaneous absorption of cosmetics in the European Union (Chapter 18).

The order of relevance of species for *in vivo* studies for predicting dermal absorption in humans is generally perceived as being human > primate > pig > guinea pig > rat > rabbit > mouse (Howes *et al.* 1996). This ranking should be considered if animals are to be used to predict percutaneous absorption in humans. Obviously, human studies are the 'gold standard' for the prediction of percutaneous absorption in humans. However, it is unethical to expose volunteers to substances that may cause adverse health effects. Thus, animal studies are used to identify toxic effects and demonstrate safety of test compounds prior to assessment with human volunteers.

7.3.1 Human studies

The conduct of *in vivo* studies using human volunteers is governed by international treaties to ensure that studies are carried out ethically. Before 1948, there were no formal guidelines on the conduct of human studies, with the experimenter being responsible for establishing the ethics of the study. Such studies were generally conducted subject to the ideology of the Hippocratic Oath, some key points of which include: 'to practice and prescribe. . . . for the good of my patients'; 'to never deliberately do harm for anyone else's interest'; 'to avoid violating the morals of my community'; 'to keep the good of the patient as the highest priority'; and 'to keep confidential all private patient information'.

Following the atrocities conducted in the name of science during World War II, the Nuremberg Code was introduced in 1948. The code is a ten point guideline, which established the four main elements of:

- Informed consent.
- Absence of coercion.
- Scientifically valid design.
- Beneficence for the participant.

In 1964, the World Medical Association proposed the Declaration of Helsinki, which governs international research ethics and has since been subject to further revision (1975, 1983, 1989, 1996 and 2000). The declaration forms the basis of 'good clinical practice' and expanded upon the guidelines set out by the Nuremberg Code. The Declaration of Helsinki advocates the use of preliminary laboratory and animal studies, review and approval of the study by an independent committee, conduct by suitably qualified individuals and states that the benefit derived from a study must outweigh the risk to the volunteer. Publication of human volunteer studies is not generally possible in mainstream scientific and medical journals unless compliance with the Declaration of Helsinki and appropriate national legislation can be demonstrated.

7.3.2 Animal studies

The conduct of animal studies is governed primarily by national legislation and guidelines, which vary widely in scope and enforcement between countries (Table 7.1). Legislation

Table 7.1 Summary of animal research legislation from selected countries

Country	Principal Act(s) and Responsible Department	Salient features
EU	Directive 86/609/EEC; Protection of animals used for experimental and other scientific purposes (1986).	• Requires competent person to conduct study under national authority. • Prohibits use of endangered species (subject to certain exclusions) and requires suppliers to be registered or approved. • Sets minimum standards of care. • Promotes 3Rs. • Actual processes (notification, approval, etc) matter for individual country. • Requests statistical information. • Essentially sets a baseline level for countries to build upon. • Inspection not required, but individual countries must submit statistical information under 'article 13'.
France	'Grammot' law (1850). Decree of 19th October, 1987 and three ministerial orders (10th July 1988): Ministry of Agriculture and ministries with responsibility for the activities of the particular institute or state-bodies funding the research.	• Apply to living vertebrates only. • Subject to inspection by nominated veterinary officers. • Meets salient features of EU directive 609. • Requires a personal licence (subject to applicant demonstrating appropriate educational qualifications). • Statistical returns required. • Facilities must be approved. • Ministries advised by the Commission nationale de l'experimentation Animale.

Germany	Animal Protection Laws (1934, 1972, 1986, 1993). Responsibility delegated to regional states (Lander) and their regional authorities.	• Application to conduct research submitted to regional authority. • Regional authorities advised by local commission (comprising at least one-third pro-welfare individuals). • Institute must appoint Animal Welfare Officer. • Permission usually granted only to medical/veterinary doctors and zoologists. Special permission required by scientists. • Ad hoc inspections.
Japan	Humane Treatment and Management of Animals (2000).	• Self regulatory. • No inspections. • Not specific to animal experimentation. • Sets minimal standards of welfare only: Statistical returns and inspections not legally required.
Switzerland	Animal Welfare Act (1978), Animal Protection Orders (1981, 1991). There are also additional guidelines (legally) regulate licensing, caging requirements, statistical returns, etc: Delegated to regional (Cantonal) authorities.	• Scientific procedures must be licensed by Cantonal authority (Canton Veterinary Officer). • Licence must be renewed every two years. • Persons conducting research must demonstrate competency, limited to those with relevant higher education. • Broadly similar to UK regulations. • Animal welfare groups may have right to appeal against licenses in some Cantons.

(*continued overleaf*)

Table 7.2 (*continued*)

Country	Principal Act(s) and Responsible Department	Salient features
United Kingdom	Cruelty to Animals Act (1876) subsequently replaced by Animal (Scientific Procedures) Act (1986; amended 1993): Home Office.	• Strictly regulated/enforced. • 'No-notice' inspections. • Individuals must demonstrate competence through accredited training schemes (e.g. Institute of Biology Modules 1–5) and must reapply for licence every five years. • Statistical returns must be made annually. • Individual and project licences required, • Institute and suppliers must be registered (certified). • Project licence must be time limited (maximum of 5 years). • Experimental protocols must be passed by local ethical committee and are subject to inspection by Animal Welfare Advisory Committee (AWAC).
USA	Animal Welfare Act (1966): US Department of Agriculture (USDA).	• Sets out minimal level of care. • Bi-annual inspections. • Requires institute conducting research to set up an Institutional Animal Care and Use Committee (IACUC) to approve protocols. • Excludes rats, mice and birds.
	Public Health Service Policy on Humane Care and Use of laboratory Animals (1985). [Health Research Extension Act]: Office of Laboratory Animal Welfare (OLAW).	• Specific to institutes/individuals receiving federal funding. • Includes all vertebrates. • Inspections only following suspicion of malpractice.

in the United Kingdom is considered to be among the strictest in the world, with the Animals (Scientific Procedures) Act 1986 requiring experiments to be regulated by a project licence, a certificate for the institute to ensure adequate facilities and personal licences for individuals performing the procedure (House of Commons Stationary Office 1986). The act also states that procedures should involve the minimum number of animals that are likely to produce statistically valid results. However, in several countries (e.g. Japan) there are no formal requirements regarding the use of animals for research, with the experimenter being responsible for self-regulation.

Whilst legislation and guidelines may vary between countries, the welfare of the animals is widely acknowledged to be paramount to the conduct of good science. Good science can only be practised using healthy animals that are subject to good husbandry and welfare practices. As such, most reputable journals do not accept manuscripts unless they are accompanied by an animal welfare statement.

7.4 Standard methodology: OECD Guideline 427

A guideline outlining the standard conduct of *in vivo* percutaneous absorption studies using experimental animals was published by the Organisation for Economic and Co-operative Development (OECD) and formally adopted in April 2004.

The standard methodology for conducting *in vivo* skin absorption studies using animals is outlined in OECD 427 (OECD guideline for the testing of chemicals), which was formally adopted in April 2004 (OECD 2004). The guideline outlines the method for the determination of the penetration of a test substance through the skin into the systemic compartment.

The main advantage of using *in vivo* measurements of skin absorption is the presence of systemic features (e.g. cardiovascular system, metabolism, immune system etc.) that cannot currently be replicated using an *in vitro* system. However, a disadvantage of *in vivo* techniques are that they may require the test compound to be radio-labelled to allow detection of the absorbed compound at low concentrations in all tissues and compartments; the custom synthesis of radio-labelled products may be expensive and time consuming. There is also difficulty in determining the early phase of absorption, due to the time taken for the test compound to be present in the excreta. A further disadvantage of *in vivo* techniques is that there may be significant differences in the skin permeability of different species. Therefore, the use of rats (for example) may not provide an accurate determination of the absorption of the test compound in humans.

In vivo skin absorption measurements are prohibited in the European Union if a substance is known (or reasonably anticipated) to be caustic or corrosive, as this would lead to unnecessary suffering to the animal.

The salient features of OECD 427 guide are (i) the selection of animal species, (ii) number and sex of animals, (iii) housing and feeding conditions, (iv) preparation of animals, (v) test substance, (vi) skin application, (vii) duration of exposure and sampling, (viii) terminal procedures, (ix) sample analysis and (x) data analysis and reporting. These points ensure that the study is conducted to a suitable standard and allow uniformity and compatibility between other centres or laboratories also conducting *in vivo* percutaneous absorption studies.

7.4.1 Selection of animal species

The species most commonly used for the assessment of skin absorption *in vivo* is the rat. However, animal species that have rates of dermal absorption that more closely match those seen in humans may also be used. The animals should be healthy young adults of a common laboratory strain.

7.4.2 Number and sex of animals

Each treatment group should consist of a minimum of four animals, which should all be of the same sex. The animals used may be of either gender, unless there is available data demonstrating differences in dermal toxicity between males and females. In such case, the sex which is more susceptible should be used.

7.4.3 Housing and feeding conditions

The animals should be housed at a temperature of $22 \pm 3^{\circ}$C and a relative humidity of approximately 50–60%. The room should be lit artificially, with a cycle of 12 hours of light followed by 12 hours of dark. An unlimited supply of drinking water and food (conventional laboratory diet) should be readily available. During the study and for an acclimatisation period, the animals should be housed individually in metabolism cages.

7.4.4 Preparation of animals

The animals are individually marked, to allow identification throughout the duration of the study and should be placed in cages for five days to allow for a period of acclimatisation prior to the start of the study. At the end of the acclimatisation period and approximately 24 hours before application of the test compound, the skin on the back of the animal is clipped to remove fur, which would otherwise result in the test compound not being applied directly to the skin and may influence absorption kinetics. During clipping, care should be taken not to damage or abrade the skin, as this may also affect the rate of absorption. The area of skin exposed by clipping should ideally be greater than 10 cm^2 (Figure 7.1).

7.4.5 Test substance

The test compound should preferably be radio-labelled in a metabolically stable position for ease of analysis of all the samples by liquid scintillation counting. The formulation of the test substance should be the same or as similar as possible to the preparation with which humans may be exposed. The test compound should be dissolved or suspended in a suitable vehicle where necessary. If the vehicle selected is one other than water, then the influence of the vehicle upon the absorption characteristics and any potential interactions between the test compound and the vehicle should be characterised.

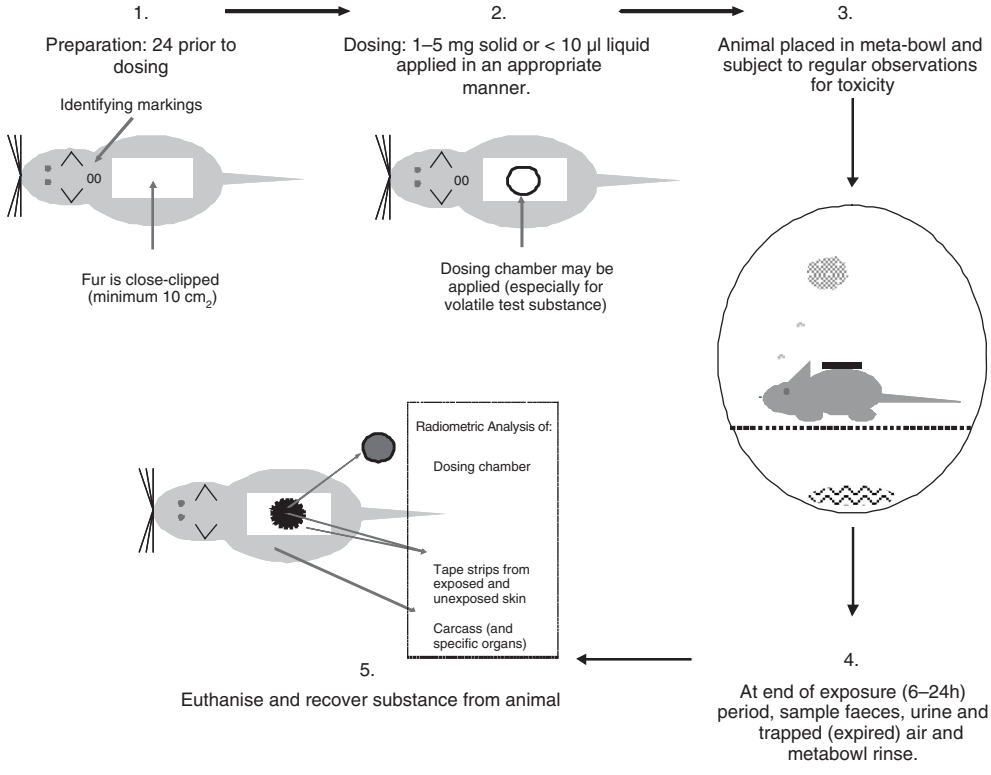


Figure 7.1 Salient features of OECD 427. Following preparation (close-clipping of test site and application of identifying markings), each animal is exposed to the test material which may be applied using an exposure chamber (Figure 7.2). A collar is often placed around the neck of the animal (to avoid ingestion or damage to the exposure site) and a meta-bowl can be used to collect samples of urine, faeces and expired air. The animal is then euthanized and samples of the dosing chamber, exposure site, internal organs and/or whole carcass subject to analysis

7.4.6 Application to the skin

The test preparation should be applied to a defined application site on the skin surface with a specific surface area. This commonly involves the adhesion of a ring formed from an inert material to the area of clipped skin using cyanoacrylate glue (Figure 7.2). A known amount of the test substance should be applied and evenly spread over the application site. The amount applied should mirror the potential exposure to humans and is commonly between $1–5\,mg\,cm^{-2}$ for a solid and up to $10\,\mu l\,cm^{-2}$ for a liquid (Figure 7.1). The application site must be protected from grooming as any oral ingestion of the test compound would invalidate the assessment of dermal absorption. The cover for the application site is normally non-occlusive, an example being permeable nylon gauze. However, the occlusion conditions should mimic the normal exposure conditions and therefore occlusion of the application site may be necessary. If the test preparation is semi-volatile, the rate of recovery of the test compound may be reduced by evaporation. In such cases, any evaporated material should

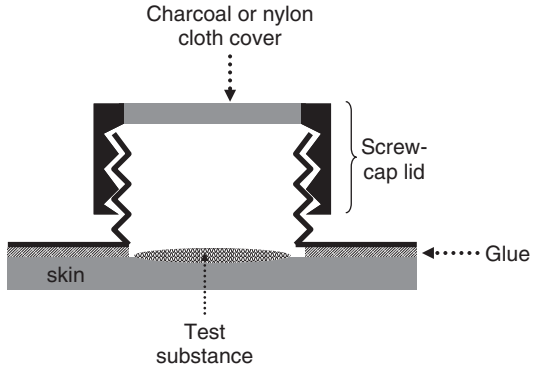


Figure 7.2 Representation of a standard dosing chamber for OECD 427

be trapped in a charcoal filter covering the application site. The animal should be fitted with a collar to prevent dislocation or any interference with the application chamber during the study. The animals are then returned to their individual metabolism cages, for collection of urine and faeces.

7.4.7 Duration of exposure and sampling

The duration of exposure is the time between application of the test compound and its removal by washing of the skin. The exposure duration should be relevant to the exposure expected in humans and is typically 6–24 hours. After the period of exposure, the animals remain in their individual metabolism cages until the scheduled termination. The animals are observed for signs of irritation at the site of application and for evidence of toxicity or abnormal reactions at regular intervals.

The excreta are collected throughout the exposure period and for up to 24 hours after the initial application. Separate collection of the first three excreta are usually adequate although the purpose of the study or need to acquire kinetic data may necessitate additional time points. After this period, the excreta are collected daily until the end of the experiment. The metabolism cages should allow the urine and faeces to be collected separately throughout the study to enable reliable estimates of the route of excretion. If the compound used is volatile, or any volatile products are formed (such as $^{14}CO_2$), then this material should be collected through the use of appropriate vapour traps. If there is adequate evidence that no volatile metabolites are formed, open cages may be used.

At the end of the exposure period, the device protecting the application site is removed from each animal and retained for separate analysis. The site of application on each of the animals should be washed at least three times with a suitable cleansing agent (such as an aqueous soap solution) and swabs, taking care to avoid contamination of other parts of the body. The skin should be dried and all swabs and washings should be retained for analysis. For animals in groups investigated at time intervals following removal of the treatment, a fresh cover should be applied to the treated site to avoid interference by the animal prior to being returned to the metabolism cage.

7.4.8 Terminal procedures

For each treatment group the individual animals should be killed at the scheduled time and the blood collected for analysis. The protective cover over the application site should be removed for analysis. The skin of the application site along with a ring of untreated skin is excised from each animal and pinned to a board. A similar section of non-treated, clipped skin should also be removed from each animal for separate analysis. The stratum corneum can be separated from the underlying dermis to obtain more information on the deposition and fate of the test compound within the application site. This can be achieved by applying a strip of adhesive tape to the skin surface. The tape is then removed along with corneocytes from the outermost layer of stratum corneum. The stratum corneum may be completely removed by successive tape strips until the tape no longer adheres to the skin surface. For each animal, the tape strips may be combined into one container to which a tissue digestant is added to solubilise the stratum corneum. Any tissues that may be potential targets for the test compound should be removed for separate analysis before determining the absorbed dose remaining within the carcass. Any urine remaining in the bladder after termination should be added to the final urine collection. The excreta should be collected and removed from the metabolism cage. The cage and traps should be washed with a suitable solvent and any residue should be analysed.

7.4.9 Sample analysis

The analysis of the amount of administered dose present in each sample should be carried out by a method that has been suitably validated. For all studies, a total recovery in the range of $100 \pm 10\%$ of the applied dose should be achieved.

7.4.10 Data analysis and reporting

The measurements for each animal at each time point for the test compound or metabolites should include the amounts present (i) on the protective covering, (ii) on the skin surface that can be removed by washing, (iii) in the skin that can not be removed by washing, (iv) in sampled blood, (v) present in the excreta (and expired air, if appropriate) and (vi) in the carcass and any organs removed for individual analysis.

The quantity of the test compound or metabolites remaining in the excreta, expired air, blood and carcass enable determination of the total amount absorbed at each time point. The amount of test compound absorbed per cm^2 of exposed skin over the exposure duration may also be calculated from these observations.

7.5 Alternative *in vivo* methods

Alternative methods to OECD Guidelines 427 are available and may be used for investigative studies of skin absorption, although not all of these methods are suitable for regulatory submissions.

Essentially, *in vivo* skin absorption can be measured by invasive or non-invasive methods, both of which monitor the appearance or disappearance of the penetrant within a specific tissue

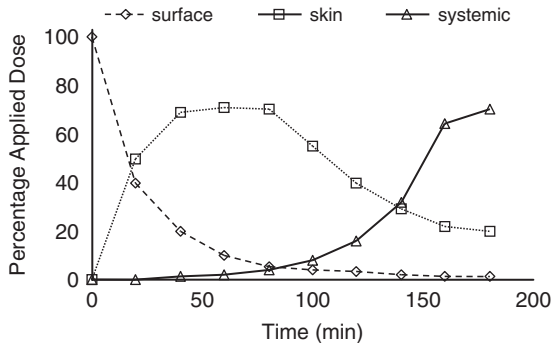


Figure 7.3 Distribution of a non-volatile, ^{14}C radio-labelled test compound recovered from the skin surface of the exposure site ('surface'), within the dermal tissue underlying the exposure site ('skin') and recovered from the systemic circulation ('systemic')

compartment. The term 'minimally invasive' is a frequently used pseudonym for an invasive technique – a needle, probe or device inserted anywhere via a body membrane (no matter how apparently innocuous) is invasive! It should be noted that different techniques monitor different aspects of skin absorption. For example, the rate at which a penetrant 'disappears' from the skin surface may not necessarily correlate with its subsequent appearance in the systemic circulation and vice versa (Figure 7.3). Thus, the actual technique(s) employed should be appropriate to the reason for conducting the experiment.

Some common examples of alternative methods for the measurement of percutaneous absorption *in vivo* include elimination, microdialysis, tape-stripping, spectroscopic, fluorescent and radiometric disappearance techniques, punch biopsies and physiological responses.

7.5.1 Elimination

One alternative method is to measure the rate of urinary elimination of a substance following topical application. Depending upon the kinetics of the penetrant, the urinary excretion of the test compound or its metabolites is measured over several days. An advantage of this technique is that it may be performed on human volunteers. The main limitation is that the elimination method is more a measure of bioavailability than of absorption but it can provide useful information, such as differential permeability according to anatomical location (Maibach *et al.* 1971): Figure 7.4. As some test compounds may not be excreted in the urine solely as the parent compound, analysis should also include the presence of metabolites. It is important that the pharmacokinetics and metabolic characteristics of the penetrant are well defined in order to accurately determine the amount of test compound eliminated by urinary excretion.

7.5.2 Microdialysis

Microdialysis is arguably the most direct technique for the measurement of percutaneous absorption *in vivo* and can be performed either on laboratory animals or human volunteers (Groth 1998; Stahl *et al.* 2002). A small diameter (typically 200 μm) selectively-permeable

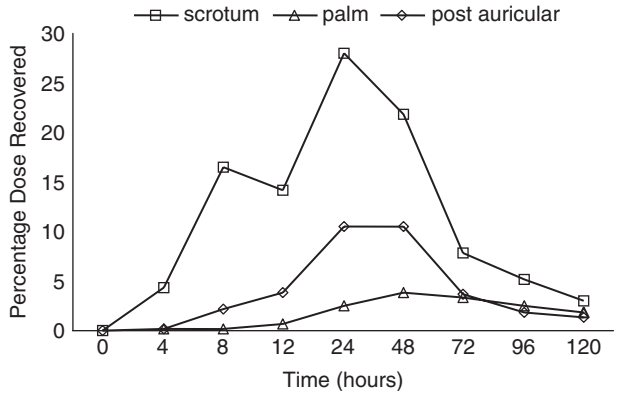


Figure 7.4 Urinary elimination (expressed as a function of time post exposure) of a ^{14}C radio-labelled pesticide administered to skin of the scrotum, palm of hand or back of ear (Reproduced from Maibach H.I. *et al*, (1971) "Regional variation in percutaneous penetration in man", *Archive of Environmental Health*, **23**(3), pp 208–211, by permission of Heldref Publications)

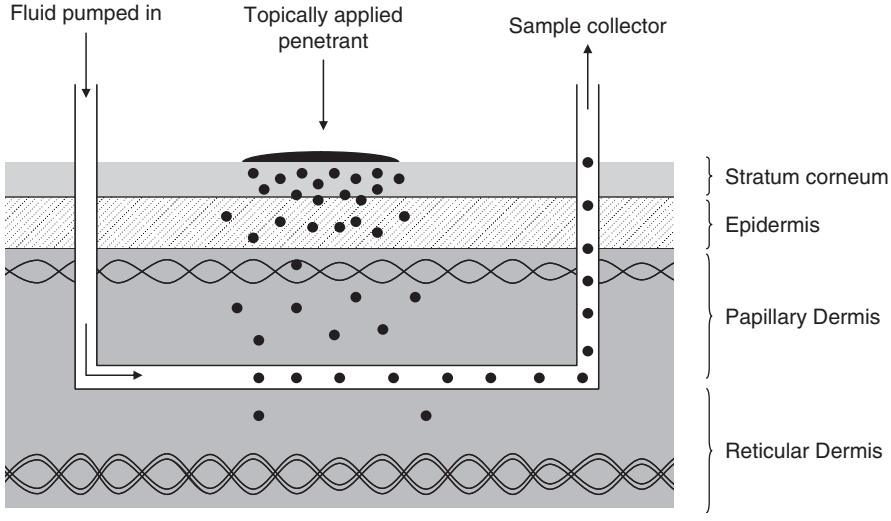


Figure 7.5 Basic concept of microdialysis. An appropriate fluid is pumped through the dialysis tubing (which passes directly beneath the exposure site); the test compound is applied to the skin surface and diffuses through the stratum corneum and epidermis to the dermal tissue where a proportion enters the dialysis tubing; the effluent (dialysate) containing the penetrant is subsequently collected for analysis

probe is inserted into the dermis immediately below the epidermis, parallel to the skin surface (Figure 7.5). In human volunteers this site is commonly on the ventral forearm, for comfort and ease of manipulation. For animal subjects, microdialysis is most commonly performed on a section of clipped skin on the dorsum. The microdialysis probe is perfused with a physiological buffer flowing at a low flow rate and functions as an artificial blood vessel

within the tissue. For microdialysis studies (as with the majority of *in vivo* dermal absorption techniques) the application site for the test compound is limited to a specific area, typically with an o-ring glued in position using cyanoacrylate glue. Any molecules and absorbed compounds that are able to diffuse across the membrane of the microdialysis probe are collected in the dialysate, which can then be analysed by methods such as HPLC or LC–MS. As the microdialysis tubes are continuously perfused, it is possible to determine absorption profiles by collecting fractions of dialysate at regular intervals. A benefit of this technique is the ability to cross-reference the results with those from *in vitro* studies.

The concentration of test compound collected in the dialysate will be representative of the concentration in the extra-cellular fluid. However, in most cases the compound in the extracellular fluid and the perfusate in the microdialysis probe will not completely reach equilibrium. Therefore, the recovery of the compound into the microdialysis probe must be determined to compensate for the dialysate giving an underestimate of percutaneous penetration.

Microdialysis is often described as 'minimally invasive', as the probe is located only just below the level of the epidermis. However, the procedure is actually invasive as the insertion of the probe may cause pain, inflammation and damage to the surrounding tissue. Inflammation resulting from probe insertion may affect skin absorption kinetics of the test compound by increasing blood flow to the site of trauma (vasodilation). The position and depth of the probe is also difficult to ascertain during insertion, and thus probe depth may not be constant at all application sites (although this can be assessed by ultrasound, if available). A further disadvantage of microdialysis is that the tubing material and the flow rate need to be optimised for each individual penetrant, particularly if the test compound is lipophilic. Lipophilic compounds are not readily taken up into the aqueous perfusate and may adhere to the probe material. This problem of low recovery for lipophilic compounds may be addressed by the addition of a physiological lipid emulsion to the perfusate, to more closely simulate the environment within a blood vessel (thereby increasing the affinity of the perfusate for partitioning of the test compound).

7.5.3 Tape-stripping

A commonly used technique for measuring percutaneous absorption is stratum corneum tape-stripping (Rougier *et al.* 1983). An advantage of this technique is that it can be performed on both human volunteers and animals. This method involves the determination of the amount of penetrated compound in the layers of the stratum corneum after a short duration (typically 30 minutes), allowing a calculation of the rate of percutaneous absorption (Rougier *et al.* 1986). The skin is swabbed to remove any residual surface material and, if conducted before the majority of the absorbed dose enters the systemic circulation, the amount of compound recovered is considered to be predictive of the amount that will subsequently be absorbed. Tape-stripping is performed by applying a section of adhesive tape to the skin surface with gentle pressure. On removal, the tape pulls off a layer of superficial corneocytes. Successive layers of the stratum corneum are removed by sequential tape strips, with the total amount of the compound present in each layer being analysed either individually (Figure 7.6) or pooled together as the complete stratum corneum.

The main limitation of the stratum corneum stripping method is that there is a large degree of variability in the number of corneocytes removed with each tape strip; the number of cells removed may not be proportional to the number of tape strips used and the amount of

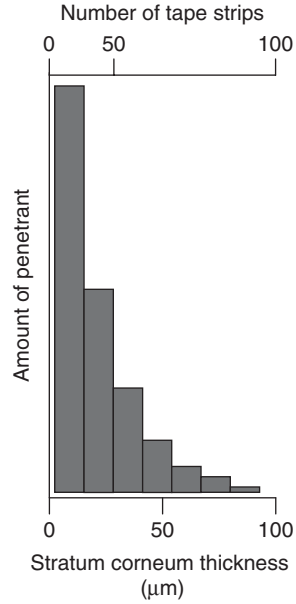


Figure 7.6 Recovery of a topically applied material from tape strips of exposed area (Note that the number of tape strips is not linear in relation to the depth achieved)

cells removed can be influenced by factors such as the force of strip removal, skin hydration, anatomical site and inter-individual variations. As it is not possible to completely exclude any systemic absorption, tape-stripping of animal subjects should be carried out in conjunction with a 'mass-balance' technique, as recommended in OECD 427. The technique allows determination of the amount of compound to have penetrated into the stratum corneum. However, it is not possible to make any distinction between whether the compound is 'bound' or 'free'. Therefore, not all of the penetrated compound may be available for absorption into the systemic circulation, although for risk assessment purposes all recovered material is considered to be bioavailable unless proven otherwise (see Section 8.12).

7.5.4 Spectroscopic, fluorescent and radiometric techniques

A range of techniques can be employed for measuring the disappearance of a compound from the skin surface or diffusion into the outermost layers of the skin. The main principle of these techniques is to monitor the amount of applied compound remaining at the skin surface over time, with a reduction in the amount of compound being considered to be due to dermal absorption (Dutkiewicz and Tyras 1967). Modern techniques can measure the diffusion of penetrants into the upper skin layers using confocal microscopy, UV/Visible spectroscopy or infrared (Raman) spectroscopy. One such application of fluorescence spectroscopy has been to measure the *in vivo* dermal absorption of a fluorescent label encapsulated in lipophilic vesicles (liposomes and micelles) by confocal laser scanning microscopy to determine the optimal vesicle formulation for enhancing penetration (van Kuijk-Meuwissen *et al.* 1998). Historically, the disappearance of emissions from a topically applied, radio-labelled compound at the skin surface, has been used to assess uptake into the skin (Wahlberg 1965) (Figure 7.7).

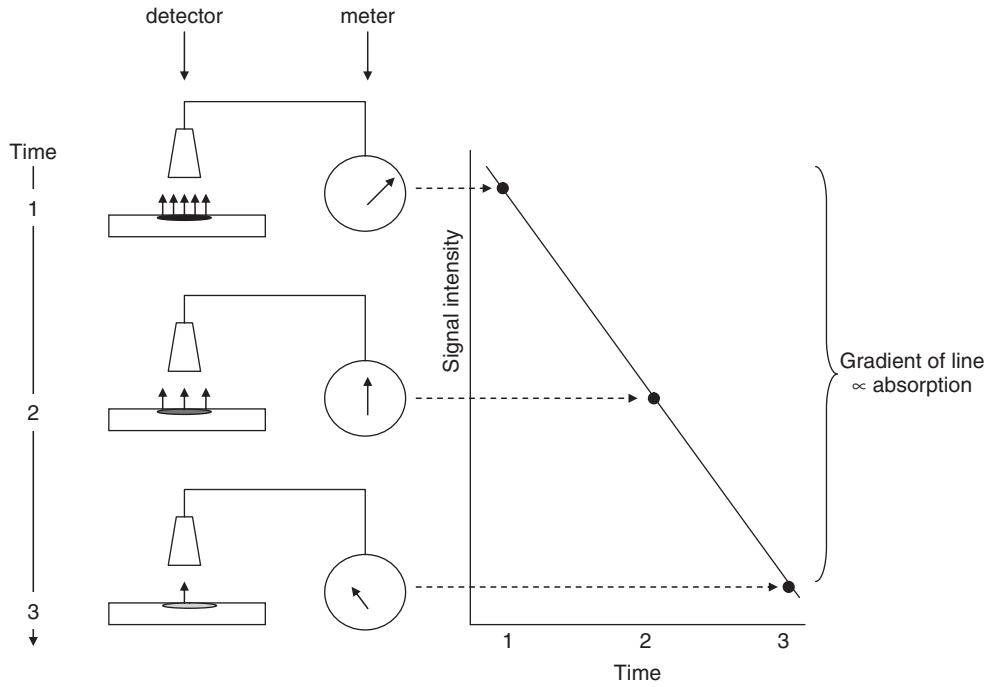


Figure 7.7 Principal of surface disappearance measurement. The signal (fluorescence, radioactivity etc) arising from exposed skin is measured using an appropriate detector; the signal intensity is then plotted as a function of time and the gradient of the line provides information on the rate of disappearance of the substance (which is assumed to be equivalent to absorption)

The main advantages of such methods are that they are non-invasive, can be performed in 'real time', provide quantitative data and may be performed on either humans or animals. The main disadvantage of these disappearance techniques, however, is that they require the assumption that all material which has disappeared from the skin surface will be due to dermal absorption (although volatilisation of test compound from the skin surface must also be considered). An additional disadvantage of the disappearance technique is that the penetrant compound needs to have specific properties to enable detection, such as a fluorescent tracer or radio-label. Another limitation of this technique is that the depth at which the compound can be detected within the skin may be limited to as little as 2 μm, particularly for certain infrared methods, such as fourier transformed infrared (FTIR) spectroscopy. The depth limitation of infrared spectroscopy can be overcome, however, by using the technique in conjunction with tape-stripping of the stratum corneum to determine a profile of absorption with each subsequent tape strip (Higo *et al.* 1993; Stinchcomb *et al.* 1999).

7.5.5 Punch biopsies and tissue sectioning

A punch biopsy of the skin can be used to remove a small section of exposed skin following topical application (Axelrod and Hamilton 1947; Surber *et al.* 1993). The resulting biopsy can then be sectioned at known thicknesses, with the amount of penetrant present in each section

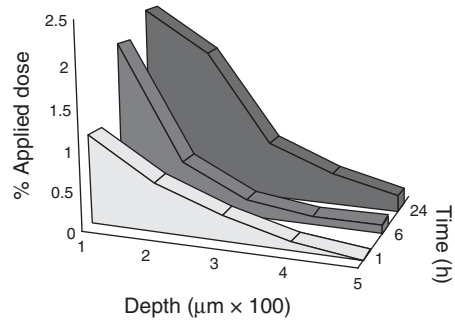


Figure 7.8 Depth profiling of a ^{14}C radio-labelled penetrant (expressed as percentage of applied dose) recovered from 100 μm sections of paraffin-embedded tissue (Skin samples obtained at 1, 6 and 24 hours post exposure)

used to assess the absorption and distribution of the compound through the skin (Figure 7.8). This technique can be used with a variety of analytical methods to determine the level of an absorbed compound in each section and, therefore, the resulting distribution of topically applied compounds through the skin layers. The principle disadvantage, however, of using punch biopsies for the measurement of dermal absorption *in vivo* is that the technique is invasive and can be painful. In this respect, it may be difficult to get either the relevant ethical approval or the consent from volunteers to perform the study. A further limitation for the use of punch biopsies is that it is not possible to perform sequential time studies using the same section of skin. Therefore, further punch biopsies would be required from additional sites in order to determine changes in the distribution with respect to the length of exposure.

7.5.6 Physiological responses

The physiological responses that result from the absorption of a penetrant may be used to assess its dermal absorption *in vivo*. Such responses include changes in blood flow, visual changes to the skin, alteration of the pain response, systemic effects or direct measurement of the test substance in blood or plasma. Measurement of a physiological response can also be used to quantify the effectiveness of protective creams (Figure 7.9). These responses are also referred to as pharmacodynamic responses. It should be noted that the quantification of a pharmacodynamic response as a surrogate for skin absorption carries the inherent possibility that the measurement is being affected by factors not relating to the rate of skin absorption. For example, a vasodilation response may vary between sites on an individual (due to regional variations in receptor densities within the vasculature), may be suppressed by the co-administration of anti-inflammatory drugs such as paracetamol (acetaminophen), or may not exhibit a linear dose–response relationship. Thus, before using a pharmacodynamic response, it is first necessary to validate that the technique suits the application.

Visible changes in blood flow can be seen either as blanching of the skin, commonly seen with the dermal application of steroids (Barry 1976), or erythema resulting from the application of rubefacients such as methylnicotinate (Tur *et al.* 1983). The alteration in blood flow (vasodilation or vasoconstriction) following the topical application of such a compound is commonly measured quantitatively by laser Doppler velocimetry (LDV) (Ryatt *et al.* 1986).

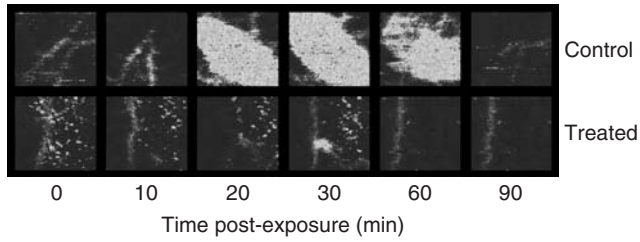


Figure 7.9 Vasodilation (measured by laser Doppler imaging) caused by topical application of a rubefacient (methylnicotinate) to normal skin (control) and following application of a barrier cream (treated). The appearance of a region of bright colours (20–60 minutes; control skin) indicates areas of higher blood perfusion in response to the rubefacient. A full-colour version of this figure appears in the colour plate section of this book

Alteration of the pain response of skin by the application of a topical compound with local anaesthetic properties can be used to determine dermal absorption *in vivo*. However, such a technique is reliant upon the volunteer to express any changes in the pain response and is therefore a subjective measurement.

Summary

- *In vivo* skin absorption studies are necessary to assess the percutaneous or dermal toxicity of chemical substances.
- The use of animals or human volunteers is generally subject to ethical considerations and country-specific legislation.
- The OECD Guideline 427 is the international standard to which *in vivo* dermal absorption studies should be performed.
- Other methods for determining skin absorption *in vivo* are available, but they are not all appropriate for regulatory submissions.

References

Axelrod, D.J. and Hamilton, J.G. (1947). Radio-autographic studies of the distribution of Lewisite and mustard gas in skin and eye tissues. *American Journal of Pathology*, **23**: 389–411.

Barry, B.W. (1976). Bioavailability of topical steroids. *Dermatologica*. **152**(Suppl 1): 47–65.

Dick, I.P., Blain, P.G. and Williams, F.M. (1997). The percutaneous absorption and skin distribution of lindane in man: II. *In vitro* studies. *Human and Experimental Toxicology*, **16**: 652–657.

Dutkiewicz, T. and Tyras, H. (1967). A study of the skin absorption of ethylbenzene in man. *British Medical Journal*, **24**: 330–332.

Groth, L. (1998). Cutaneous microdialysis. A new technique for the assessment of skin penetration. *Curr Probl Dermatol*, **26**: 90–98.

Higo, N., Naik, A. Bommannan, D.B. *et al.* (1993). Validation of reflectance infrared spectroscopy as a quantitative method to measure percutaneous absorption *in vivo*. *Pharmaceutical Research* **10**(10): 1500–1506.

House of Commons Stationary Office. (1986). Animals (Scientific Procedures) Act.

Howes, D., Guy, R. Hadgraft, J. *et al.* (1996). Methods for assessing percutaneous absorption – The report and recommendations of ECVAM workshop 13. *ATLA* **24**: 81–106.

Maibach, H.I., Feldman, R.J., Milby, T.H. and Serat, W.F. (1971). Regional variation in percutaneous penetration in man. Pesticides. *Arch Environ Health* **23**(3): 208–211.

OECD. (2004). OECD guideline for the testing of chemicals. 427. Skin absorption: *in vivo* method. OECD, Paris.

Rougier, A., Dupuis, D. Lotte, C. *et al.* (1983). *In vivo* correlation between stratum corneum reservoir function and percutaneous absorption. *Journal of Investigative Dermatology*, **81**(3): 275–278.

Rougier, A., Dupuis, D. Lotte, C. *et al.* (1986). Regional variation in percutaneous absorption in man: measurement by the stripping method. *Archives of Dermatological Research*, **278**(6): 465–469.

Ryatt, K.S., Stevenson, J.M., Maibach, H.I. and Guy, R.H. (1986). Pharmacodynamic measurement of percutaneous penetration enhancement *in vivo*. *J Pharm Sci*, **75**(4): 374–377.

Scott, R.C., Batten, P.L., Clowes, H.M. *et al.* (1992). Further validation of an *in vitro* method to reduce the need for *in vivo* studies for measuring the absorption of chemicals through rat skin. *Toxicological Sciences*, **19**(4): 484–492.

Stahl, M., Bouw, R. Jackson, A. and Pay, V. (2002). Human microdialysis. *Curr Pharm Biotechnol*, **3**(2): 165–78.

Stinchcomb, A.L., Pirot, F. Touraille, G.D. *et al.* (1999). Chemical uptake into human stratum corneum *in vivo* from volatile and non-volatile solvents. *Pharm Res*, **16**(8): 1288–1293.

Surber, C., Wilhelm, K.P., Bermann, D. and Maibach, H.I. (1993). *In vivo* skin penetration of acitretin in volunteers using three sampling techniques. *Pharm Res*, **10**(9): 1291–1294.

Tur, E., Guy, R.H., Tur, M. and Maibach, H.I. (1983). Non-invasive assessment of local nicotinate pharmacodynamics by photoplethysmography. *Journal of Investigative Dermatology*, **80**(6): 499–503.

van de Sandt, J.J.M., Meuling, W.J.M. Elliott, G.R. *et al.* (2000). Comparative *in vitro–in vivo* percutaneous absorption of the pesticide propoxur. *Toxicological Sciences*, **58**: 15–22.

van Kuijk-Meuwissen, M.E., Mougin, L. Junginger, H.E. and Bouwstra, J.A. (1998). Application of vesicles to rat skin *in vivo*: a confocal laser scanning microscopy study. *J Control Release*, **56**(1–3): 189–196.

Wahlberg, J.E. (1965). Disappearance measurements, a method for studying percutaneous absorption of isotope-labelled compounds emitting gamma-rays. *Acta Derm Venereol*, **45**(6): 397–414.

8 *In vitro* percutaneous absorption measurements

Ruth U. Pendlington

Safety & Environmental Assurance Centre, Unilever Colworth Science Park, Sharnbrook, Bedford, Bedfordshire MK44 1LQ, UK

Primary Learning Objectives

- *Why skin absorption is measured in vitro.*
- *Advantages and disadvantages of in vitro measurement systems.*
- *Salient features of the international guideline for the conduct and interpretation of in vitro skin absorption studies: OECD 428.*
- *Interpretation of data from skin absorption experiments.*
- *Auxiliary methods and techniques.*

8.1 Introduction and scope

Traditionally, percutaneous absorption has been assessed by *in vivo* methods (Chapter 7). This chapter describes and discusses the two most common methods for conducting *in vitro* measurements of skin absorption and provides an overview of standard practices and how these conform with regulatory guidelines.

8.2 Regulatory guidelines

There are a variety of guidelines for the conduct of *in vitro* skin absorption measurements (Table 8.1). The general practices outlined in OECD 428 cover a diverse range of applications but conduct of work in support of specific functions (such as risk assessment of cosmetics or agricultural products) may require conformity with more prescriptive guidelines. The following description of *in vitro* skin diffusion measurements in this chapter is based on OECD 428.

Principles and Practice of Skin Toxicology Edited by Robert P. Chilcott and Shirley Price
© 2008 John Wiley & Sons, Ltd

Table 8.1 Summary of relevant regulatory guidance documentation for conduct of *in vitro* skin absorption studies

Title and reference	Organisation	Description
OECD Guidance document for the conduct of skin absorption studies – Number 28.	Organisation for Economic Co-operation and Development (OECD).	Discusses technical aspects of OECD 428
OECD Guideline for the testing of chemicals: 428 – Skin absorption: *in vitro* method.		Generic protocols for a range of applications.
Opinion Concerning Basic Criteria for the *in vitro* Assessment of Dermal absorption of Cosmetic Ingredients. SCCP/0970/06 (2006)	Scientific Committee on Cosmetic Products (SCCP).	Describes the criteria required for *in vitro* dermal absorption studies of cosmetic ingredients belonging to Annexes III, IV, VI or VII to Directive 76/768/EEC.
Directorate E1. Plant Health. Sanco/222/2000 Guidance Document on Dermal Absorption.	European Commission Health & Consumer Protection Directorate General.	Provides guidance to notifiers and Member States for dermal absorption values to be used in risk assessment of plant protection products reviewed for inclusion in Annex I of Directive 91/414/EEC.
Cosmetic Ingredients: Guidelines for Percutaneous Absorption/Penetration.	European Cosmetic, Toiletry & Perfumery Association (COLIPA).	Describes a general procedure for measuring the penetration of test substances thorough excised mammalian skin.

8.3 Why assess percutaneous absorption *in vitro*?

Measuring percutaneous absorption *in vitro* supports the 'three Rs' of Russell and Burch: reduction, refinement and replacement of animals in scientific research.

The previous chapter on *in vivo* skin absorption highlighted the continuing need for animals and human volunteers in research. However, *in vitro* techniques offer a valid alternative for many important aspects of dermal exposure, can provide an economical and practical alternative for screening large numbers of topical formulations and supports the principles of the 'three Rs' of refinement, reduction and replacement of animal experiments (Russell and Burch 1959). For example, when using *ex vivo* animal skin, less animals are used than in a comparable *in vivo* study and the animals undergo no additional stress or regulated procedures. For some species (such as the pig), *ex vivo* skin can be obtained as a by-product of the food industry and one animal can be used for many experiments (as cold storage of tissue does not disrupt the barrier properties of the skin to a significant degree). Furthermore,

the use of *ex vivo* human skin (obtained from cadavers or cosmetic surgery operations) means that data can be derived using no animals at all.

The need to replace animals with *in vitro* alternatives is a factor which particularly affects companies involved with home, personal care and cosmetics products as a result of the 7th Amendment to the European Cosmetics Directive (Chapter 18), which will prohibit the sale of cosmetics in the European Union containing any ingredients tested on animals. A further motivation is that *in vitro* skin absorption experiments require fewer resources to perform than comparable *in vivo* studies in terms of time, money and staffing.

8.4 Basic principle of *in vitro* percutaneous absorption measurements

In vitro techniques measure the rate at which a penetrant diffuses through an isolated piece of *ex vivo* skin tissue mounted in a specialised cell. Two types of cell are available 'static' and 'flow-through'.

In vitro measurements of skin absorption are based on apparatus comprising a rate limiting membrane (usually skin) sandwiched between two chambers (Figure 8.1). In general, the chamber on the dermal (lower) membrane surface is termed the 'receptor' or 'acceptor' chamber and that on the outer (upper) membrane surface, the 'donor' chamber. The whole ensemble constitutes a diffusion cell. The lower chamber is normally filled with an appropriate solution (receptor fluid/acceptor phase). The penetrant is placed onto the skin surface and its rate of accumulation in the receptor chamber is used to calculate skin absorption kinetics. Methods have been subject to intense development over the last 30 years, culminating in a number of test guidelines (Diembeck *et al.* 1999; Table 8.1).

8.5 Choice of diffusion cell

The principal classification of diffusion cells can be made on the basis of whether the acceptor fluid is confined to the receptor chamber (static; Figure 8.1) or passes through the receptor chamber for subsequent collection (flow-through; Figure 8.1). In the modern literature, static and flow-through diffusion cells are generally associated as being 'Franz-type' (Figure 8.2) or 'Bronaugh-type' (Figure 8.3), respectively, in recognition of their principal proponents (Franz 1975; Bronaugh 1995, 1995a). However, it should be appreciated that diffusion cells of both designs had been around since at least the 1940s (Box 8.1), perhaps even earlier.

The choice of cell often depends on what is available to the researcher. However, if both types are available then the type of experiment usually dictates choice. Flow-through cells are useful for acquiring regular samples of receptor fluid over many hours, whereas static cells (unless linked to an auto-sampler) require manual sampling, which can limit the frequency or duration over which samples can be obtained. Static cells are useful in that they allow accumulation of material within the receptor chamber, which is useful for measuring the absorption of slow-penetrating substances (which may be diluted too much for detection in a flow-through system). Comparisons of the two systems have shown them to provide equivalent results (Clowes *et al.* 1994), although the constant replenishment of fresh receptor fluid makes flow-through cells more amenable to metabolic studies, where the biochemical viability of the skin needs to be maintained.

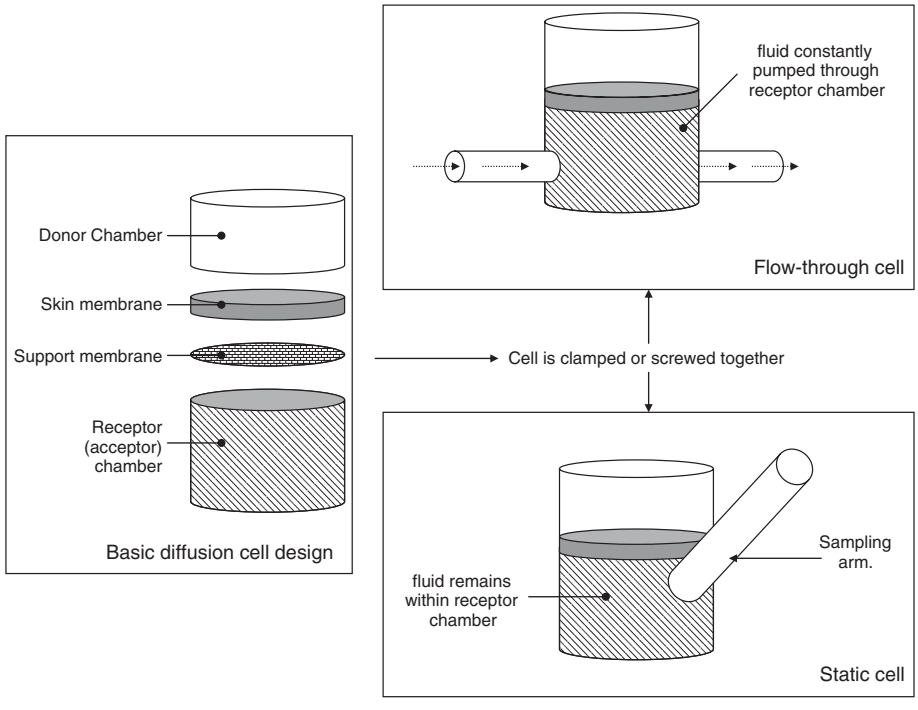


Figure 8.1 Basic design of *in vitro* skin diffusion cell system. A skin membrane is sandwiched between an upper (donor) and lower (receptor) chamber. For fragile tissue preparations (such as epidermal membranes), a support membrane (made of gauze or similar porous material) may be placed underneath the skin. In flow-through designs, the receptor fluid is continually pumped through the receptor chamber and sampled with a fraction collector whereas static cells are manually sampled with a pipette or syringe via the sampling arm

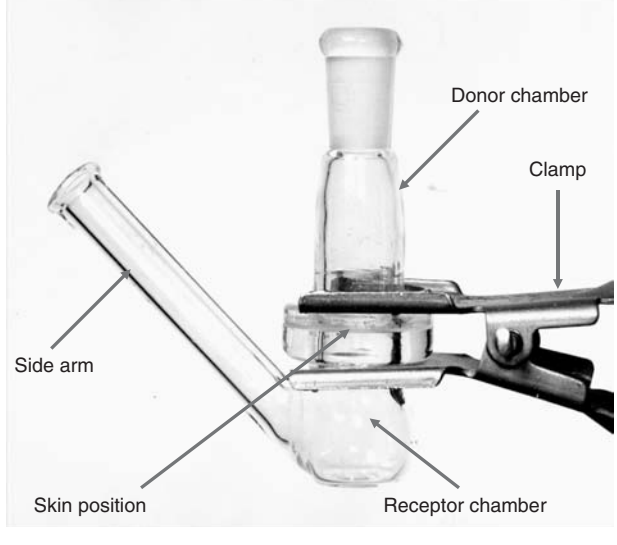
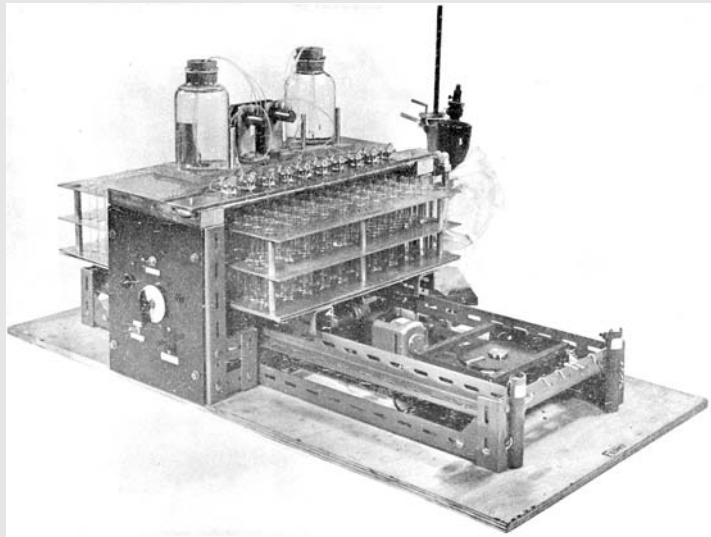


Figure 8.2 Example of a non-jacketed, horizontal, static diffusion cell

Box 8.1

The significance of measuring skin absorption rates was fully realised during and immediately after World War II, due to the potential threat of attacks with chemical warfare agents such as mustard and nerve gases. As these substances are extremely toxic, *in vitro* methods were designed to investigate their human skin absorption so that an accurate hazard assessment and effective medical counter measures could be developed. This led to the construction and use of static and flow-through diffusion cells. An example of the latter is pictured below.



Photograph of flow-through diffusion cell apparatus *circa* 1969. The receptor fluid reservoir bottles are visible on the top of the unit immediately behind the glass flow-through cells. Receptor fluid would have dripped from the cells into a row of glass vials, mounted on a fraction collector comprising a rack that would have been shifted at regular intervals by an electric motor. This design is not dissimilar to that still in use today. Picture reproduced from Allenby *et al.*, 1969.

8.5.1 Static diffusion cells

Static diffusion cells can be sub-divided on the basis of the skin orientation: The membrane can be placed horizontally or vertically. The majority of skin absorption studies are conducted using horizontal cells, with the skin surface open to the air. The use of vertical (or 'side-by-side') cells is more common when evaluating drug delivery systems, such as sonophoresis, iontophoresis or electroporation etc, and requires immersion of both surfaces of the skin preparation, which may result in excessive hydration and possibly skin damage.

An important aspect to any skin absorption experiment is to create a known and constant skin temperature in each diffusion cell. For this reason, static cells can be obtained in 'jacketed' and 'non-jacketed' (Figure 8.2) varieties. The former contains an outer chamber (jacket) that envelops both the donor and receptor chambers. Heated water is circulated through the jacket to control the diffusion cell temperature. Non-jacketed varieties may

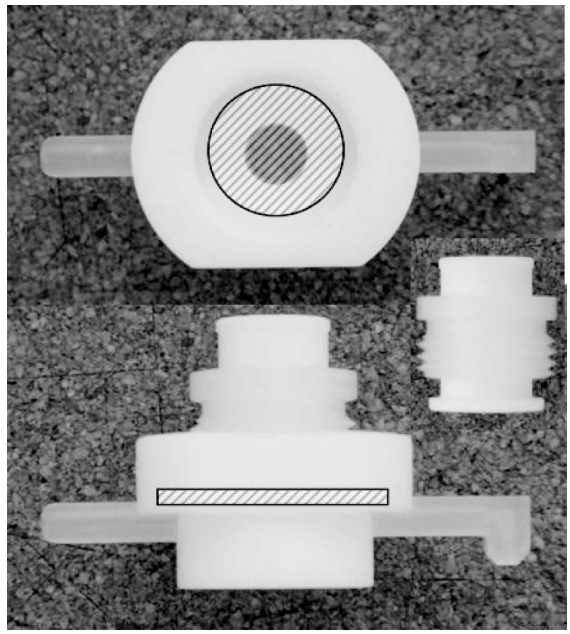


Figure 8.3 Example of a flow-through diffusion cell (the hashed area indicates position of the skin sample)

be immersed in a water bath or be placed into dry heated blocks. Irrespective of the method used, care should be exercised to ensure that the actual skin surface temperature is correct. This is especially important when diffusion cells are used in fume cupboards, as the constant movement of air passing over the apparatus may significantly lower the skin surface temperature. A second, less well documented effect is loss of penetrant through lateral diffusion. This is particularly important when immersing (non-jacketed) static diffusion cells in a water bath, as leakage of penetrant may occur from the skin into the surrounding water.

It is also important to ensure the receptor chamber fluid is adequately stirred. This is frequently achieved by placing a magnetic bar that is rotated via an external magnetic field in the receptor chamber. Inadequate stirring of static diffusion cells can limit the rate of partitioning of chemicals from the skin into the receptor chamber, thus leading to an underestimate of skin absorption.

8.5.2 Flow-through diffusion cells

In flow-through systems, movement of receptor fluid is usually driven by a peristaltic pump (Figure 8.4), enabling an accurate and consistent flow rate through each diffusion cell. A cheap alternative is to elevate the receptor fluid reservoir to a height above the diffusion cells to allow flow by gravity. However, flow rates induced by siphoning are more variable and an internal calibration (see later) should be employed. Siphon-fed (or 'drip') flow-through diffusion cells are now uncommon.

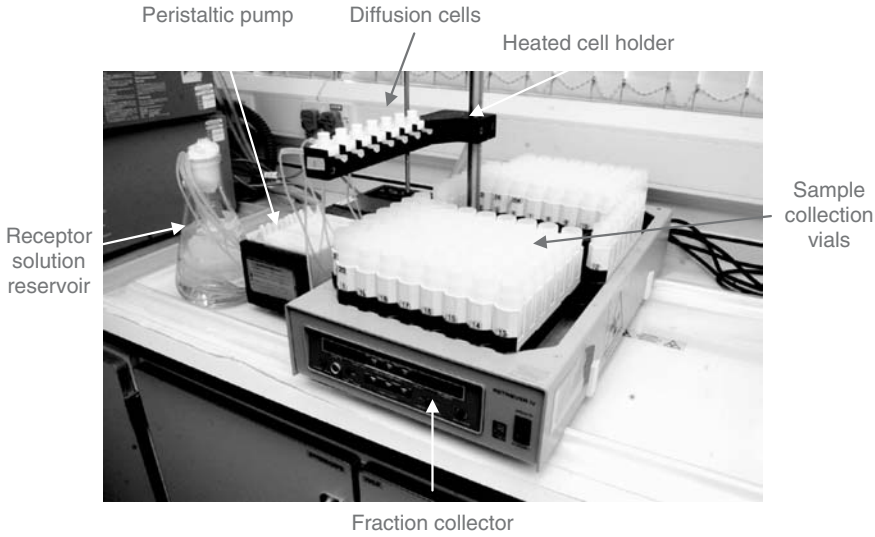


Figure 8.4 Example of flow-through diffusion cell apparatus

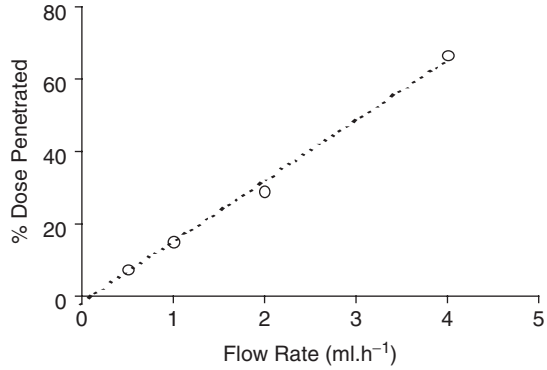


Figure 8.5 Skin absorption of a model penetrant (testosterone) at 24 hours as a function of receptor fluid flow rate (Data from Crutcher and Maibach 1969)

The rate at which fluid flows through a receptor chamber can significantly influence skin permeability (Figure 8.5). In most studies, flow rates generally range from 1–6 ml h^{-1}, with 3 ml h^{-1} perhaps being most commonly used. Ideally, the flow rate used should be sufficiently fast so that it does not affect skin absorption rates and should be regularly calibrated. A simple calibration technique is to weigh the sample vials before and after collection of receptor fluid for a fixed period. The difference in weight, divided by the specific gravity (density) of the fluid and the collection period gives the flow rate (assuming zero loss due from evaporation).

As with static cells, it is important to maintain each flow-through cell at a set temperature and ensure good mixing of the receptor chamber fluid by means of a stirrer bar. In some

designs, the receptor fluid is heated before entering the receptor chamber to ensure that isothermal conditions are maintained. More commonly, the cells are placed on a heated 'arm' that sits over a fraction collector (Figure 8.4).

A potential problem with flow-through cells is the formation of air bubbles within the receptor chamber. Steps can be taken to limit bubble formation, such as adequately de-gassing the receptor fluid or by the addition of broad-spectrum anti-microbial agents. Even with such measures, air bubbles may still form over long periods. Thus, many flow-through cell designs feature a Perspex or glass base, so that the receptor chamber may be visually inspected.

8.6 Skin membrane considerations

Where possible, skin of the species of interest is used; the skin thickness has an impact on the penetration rate of a test material.

Choice of membrane depends on four factors: relevant model, ethics, price and availability. For risk assessment, formulation development and other related applications, human is the species of interest and thus it might be expected that human skin would always be the number one choice if available. On the other hand, assessment of agricultural products (e.g. pesticides for use on animals) or veterinarian preparations (e.g. mange shampoos) means that animal skin (ideally the species of interest) is more relevant.

The skin tissue need not necessarily be viable (i.e. freshly excised) as percutaneous penetration occurs by passive diffusion and the barrier function resides in the dead cells of the stratum corneum. However, if metabolism of the test material during the absorption process is of interest, then the skin must be as fresh as possible and its viability maintained during the experiment (Collier 1989; Bronaugh 1995a).

The thickness of the skin preparation should also be considered. Very lipophilic molecules may be able to penetrate into the stratum corneum (a lipid rich environment) but the epidermis and, especially, the water-rich dermis of full thickness pig or human skin will be a considerable barrier to such molecules and could lead to an erroneously low value for skin absorption. The reason for this is that, in the *in vivo* situation, the point of entry into the systemic circulation is the capillary bed that lies directly beneath the epidermis (Chapter 1; Figure 1.5); in an *in vitro* skin penetration experiment utilising full thickness pig/human skin, the receptor solution flows beneath the dermis of the skin sample (and so the penetrant has to travel an artificially longer distance to penetrate the skin). Thus, epidermal membranes (prepared via heat separation or enzyme digest) are considered to be better models for lipophilic molecules than full thickness human or pig skin. Unfortunately, the harsh conditions required to separate the epidermis/dermis are likely to cause inactivation of any enzymes in the epidermis, so these preparations cannot be used for metabolism studies. Epidermal membranes are also difficult to tape-strip, so measurement of the amount of test material associated with the stratum corneum may not be possible.

A compromise between full-thickness skin and epidermal membranes is using dermatomed skin. In these preparations, much of the dermis is removed by using a surgical instrument to split the skin to about 200–500 μm in thickness. Thus, the receptor fluid lies in closer proximity to the superficial blood capillaries than full thickness skin preparations.

8.7 Integrity measurements

The OECD guidelines recommend that a skin integrity check is carried out; this can be carried out before, during or after the experiment.

8.7.1 Tritiated water permeability

In this method, tritiated water (^{3}H-water) is applied to the skin surface for a short time (typically two hours) and the flux of the ^{3}H-water through the membrane into the receptor solution is measured. If the permeability coefficient (K_p) is less than a certain value (dependent on the type of skin preparation, for example 2.5×10^{-3} cm h^{-1} for dermatomed human skin) then the skin is deemed to be structurally viable. The main advantage of this method is that there is a direct correlation between the K_p of ^{3}H-water and skin integrity. The disadvantages of the method are that it can lead to over hydration of the skin, which can affect the permeability of subsequently applied test materials, and a wash-out period is necessary to ensure there is no interference with the subsequent detection of a radio-labelled test material.

8.7.2 Transepidermal water loss (TEWL)

This method requires the use of a piece of equipment called an evaporimeter (Chapter 12). This is held against the skin until the reading stabilises. Its main advantages are that it is non-invasive and quick. However, readings can be notoriously variable and have been shown in certain cases to have little or no correlation with the actual integrity of the skin membrane (Chilcott *et al.* 2002), although elevated rates of TEWL are considered by many to be representative of skin damage (Levin and Maibach 2005; Netzlaff 2006; Fluhr *et al.* 2006).

8.7.3 Transepidermal electrical resistance (TER)

A volume of saline solution is applied to the skin surface and electrodes from a voltohmmeter are placed into the donor and receptor chambers. The resistance across the skin preparation can then be measured. The method is relatively quick and does correlate with skin damage (Chilcott *et al.* 1995; Lawrence 1997; Davies *et al.* 2004). However, over-hydration of the skin may occur due to the application of the saline solution and subsequent washing of the skin (to remove residual saline).

8.7.4 Concurrent assessment of skin integrity

In this method, a radio-labelled reference compound that poorly permeates skin is added to the test formulation and the absorption of both the test material and a standard penetrant are measured by dual label scintillation counting. Sucrose is a useful standard penetrant, as it is relatively cheap to obtain radio-labelled either with tritium or carbon-14. A scintillation counter that is capable of dual label counting is preferred, although not essential, as long as the liquid scintillation counter can differentiate between tritium and carbon-14 emissions. If any

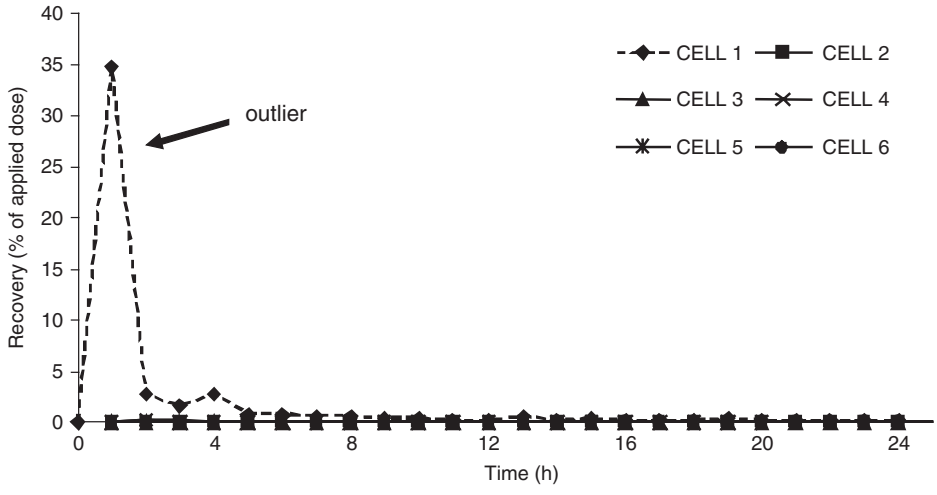


Figure 8.6 Example of post-experimental data outlier. It is clear that Cell 1 contains a damaged piece of skin as the flux through this piece of the test material is many times greater than the flux through the skin in the other cells. The data from this cell would therefore be disregarded

cell exhibits an abnormally high sucrose flux, it can be considered that the barrier function of the skin in that cell is compromised and the data from that cell should be excluded from the overall analysis. This method is particularly useful for studying the integrity of the skin over the length of the experiment, as it will detect any deterioration of the skin with time, which may be an effect of the test material or poor experimental conditions. It should be borne in mind that the use of a standard permeant could affect the penetration of the test material if the two somehow interact.

8.7.5 Post-experimental assessment of data

The above methods all have the advantage that skin samples can be removed from the experiment prior to dosing, thus avoiding waste of test preparation and unnecessary processing of samples at the end of the experiment. However, the disadvantage with these methods is that to compensate for potential loss of cells before dosing, extra cells have to be set up, resulting in wastage of potentially precious skin samples instead.

Post-experimental (retrospective) assessment of skin integrity requires evaluation of the test material flux data at the end of the experiment to identify any obvious outliers, especially within the first hour. For example, in Figure 8.6, it is clear that Cell 1 contains a damaged piece of skin, as the flux through this piece of skin of the test material is many times greater than the flux through the skin in the other cells. The data from this cell would therefore be disregarded.

8.8 Choice of receptor fluid and sampling considerations

If possible, a physiologically relevant receptor fluid should be used; it is critical that the test material is soluble in the receptor fluid.

The test material must be soluble in the receptor fluid. The OECD guidelines state that the test material should be soluble up to ten times the likely maximum concentration achievable in the receptor fluid during the experiment to ensure that sink conditions are maintained (Chapter 5). Solubility of test material in the receptor fluid should be established before the experiment to ensure it is not rate-limiting.

It is important that the receptor fluid does not compromise the skin's integrity. For many applications, physiological receptor solutions (e.g. phosphate buffered saline with or without excipients such as 5% bovine serum albumin, 5% new-born calf serum and anti-microbials) are commonly used. For very lipophilic test materials, a non-physiological receptor fluid may be more appropriate, such as 50% aqueous ethanol; this receptor solution is frequently used by the pesticide industry whose molecules of interest are often highly lipophilic.

Automated processes (e.g. using flow-through cells with a fraction collector) allow sampling of the receptor solution at regular intervals. This allows good flux data to be generated. In the absence of automated systems, flux data have to be generated by manual sampling of the receptor fluid. Given the need for most investigators to sleep (!), this generally means that regular samples are obtained over the first 8–12 hours, with a final time point taken the following day.

8.9 Test material considerations

8.9.1 To radio-label or not?

Use of a radio-label allows a dependable mass balance to be determined, thus assuring the validity of the data.

The first consideration is whether to use a radio-labelled test material or not. The advantages of using a radio-labelled material are that it is easy to monitor the distribution of material in the experimental system, enables simple quantification (by liquid scintillation counting) of mass balance measurements (i.e. all material can be accounted for) and can be used in conjunction with micro-autoradiography to further determine the distribution of the test material within the skin (Section 8.12).

There are a number of disadvantages associated with radio-labelled materials. Radio-chemicals are expensive to synthesise and are only available in small amounts. The knock-on effect of this is that only small amounts of test preparation can be prepared, which may be technically challenging e.g. in the case of a complicated skin cream. The position of the label needs careful consideration; if it is in a part of a molecule that is removed by metabolism within the skin, the remaining portion of the molecule will then become undetectable. Coupled with this is the problem that this method does not allow distinction between parent molecule and metabolite, which might be critical missing information. Furthermore, radio-chemicals can only be used under license in designated areas with necessary safety processes in place.

The two most commonly used isotopes are tritium (^{3}H) and carbon-14 (^{14}C). Both of these are soft β-emitters, making them two of the safest isotopes to work with. Carbon-14 is preferred to tritium, because the latter cannot be detected by a Geiger counter (making monitoring of the work area more arduous) and tritium can exchange with protons on other molecules leading to potentially erroneous results (Essa *et al.* 2002).

8.9.2 Vehicle and penetrant considerations

> The interaction of a test material with the vehicle it is formulated in can have a profound effect on its ability to undergo skin absorption.

The second consideration is which vehicle to use. In general, and certainly for the purposes of risk assessment, a relevant formulation is the vehicle of choice (for example a skin cream formulation for a skin cream ingredient). The reason for this is that the vehicle can have a substantial impact on how much test material penetrates into the skin (Chapter 6). Alternatively, when conducting formulation development studies, a number of different vehicles may need to be evaluated to obtain the desired penetration kinetics.

The test material needs to be in the correct phase of a formulation (for example in the aqueous phase of a water-in-oil emulsion), which can mean having to build a test formulation incorporating the radio-label from scratch. This can be problematic if a factory process has to be scaled down from kilograms of formulation to one or two grams of formulation. If possible, the test material also needs to be at a relevant concentration in the vehicle that models human exposure. This can cause problems if an ingredient is at very low levels in the finished product. However, in these cases, even assuming 100% penetration will often result in a favourable risk assessment to assure safety. The amount of vehicle applied should also be an amount that mimics the exposure scenario.

A final consideration is the physicochemical properties of the test material, as these will have a bearing on which vehicle (in the case of efficacy experiments), membrane, receptor fluid and wash solution to use in the experiment.

8.10 Application of test preparation to the skin

> For risk assessment purposes, it is imperative to use an 'in-use' dosing scenario.

The reason for performing the *in vitro* skin absorption experiment will dictate the protocol for applying the test preparation to the skin. For efficacy or risk assessment purposes, an in-use application time is generally used (e.g. 24 hours for a skin cream formulation, five minutes for a shampoo formulation, eight hours for a pesticide). However, a worst-case scenario could also be reproduced, where the test preparation is applied for longer than would be the normal case.

The amount of test material applied will either be finite (generally the case for in-use applications) or infinite (used if permeability coefficient determination is being carried out; see Chapter 6). A further consideration is whether to occlude the test site or leave it open to the air (Chapter 5). In general, occlusion increases absorption of test material, but this is not always the case (Zhai and Maibach 2001). Occlusion may be used to determine a worse-case scenario, or may be used to mimic in-use, for example an under-arm deodorant application.

It is often assumed that when a penetrant is applied to the donor chamber of a diffusion cell, all the surface area available is covered by the penetrant. When applied in a relatively large volume (such as with infinite dose procedures), this assumption should be true. However, when applied under finite dose conditions, skin surface spreading and/or lateral diffusion may not occur and thus the area over which diffusion is assumed to occur may be incorrect

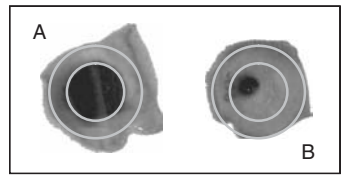


Figure 8.7 Effect of skin surface spreading under finite dose conditions. Two pieces of skin removed from diffusion cells after exposure to the same volume of different ^{14}C radio-labelled compounds ('A' and 'B'), with an autoradiograph overlay indicating area of spread (darkened areas). Concentric circles indicate position of inner and outer borders of donor chamber

(too small), leading to an underestimate of skin absorption rates. The area occupied by the penetrant can be simply measured by a variety of techniques. The more common methods include the addition of a fluorescent or high-contrast dye to the penetrant, although this assumes that the additive spreads homogeneously with the penetrant and does not influence its diffusion into the skin. If the penetrant contains a radio-isotope such as ^{14}C, ^{35}S, ^{32}P or ^{3}H, the area can be accurately quantified using autoradiography (Figure 8.7).

Although experiments are usually run for 24 hours, exposure to the test preparation is often for a shorter period. The scenario that the experiment is attempting to model will dictate whether any intermediary procedures are needed. For example, if a shampoo ingredient is being assessed, then after a five minute application period, the shampoo would be rinsed off the skin surface with water, then the skin swabbed dry with e.g. a cotton swab. Receptor solution samples would continue to be collected for 24 hours, at which point terminal procedures would ensue. Samples for micro-autoradiography may also be taken at intermediary time points to assess the distribution of test material through the skin over time.

At the final time point (often 24 hours post exposure), the skin surface is rinsed irrespective of whether there was an intermediary time point or not. A relevant solvent is used where possible, such as a dilute soap solution for skin creams or deodorants, or an organic solvent for lipophilic substances such as pesticides. The skin is swabbed dry then removed from the cell and divided into the inner, dosed area of skin and the outer, clamped area of skin.

The skin surface can then be tape-stripped to give an indication of the amount of test material associated with the stratum corneum. It is also possible to separate the epidermis from the dermis to get a better picture of distribution of test material within the skin. If radio-labelled test materials are being used, the skin and tape strip samples can be solubilised in a commercial tissue solubilising reagent (e.g. Soluene-350™) and scintillation cocktail added directly to the samples. If HPLC or mass spectrometry are used as an alternative to liquid scintillation counting, then homogenization of the samples, followed by solvent extraction, has to be performed.

The donor and receptor compartments of the diffusion cell should also be rinsed in a suitable solvent to monitor any test material remaining on them. A summary of the method is provided in Figure 8.8.

Once the test material has been measured in every sample, a full mass balance should be performed. A well conducted experiment will result in over 90% of the test material being recovered. To allow for difficult dosing scenarios (e.g. very thick creams where the dose applied may vary between cells) or cases where a test material may adhere to the donor compartment for instance, OECD guidelines permit a range of recovery (85–115%) to be acceptable for regulatory purposes.

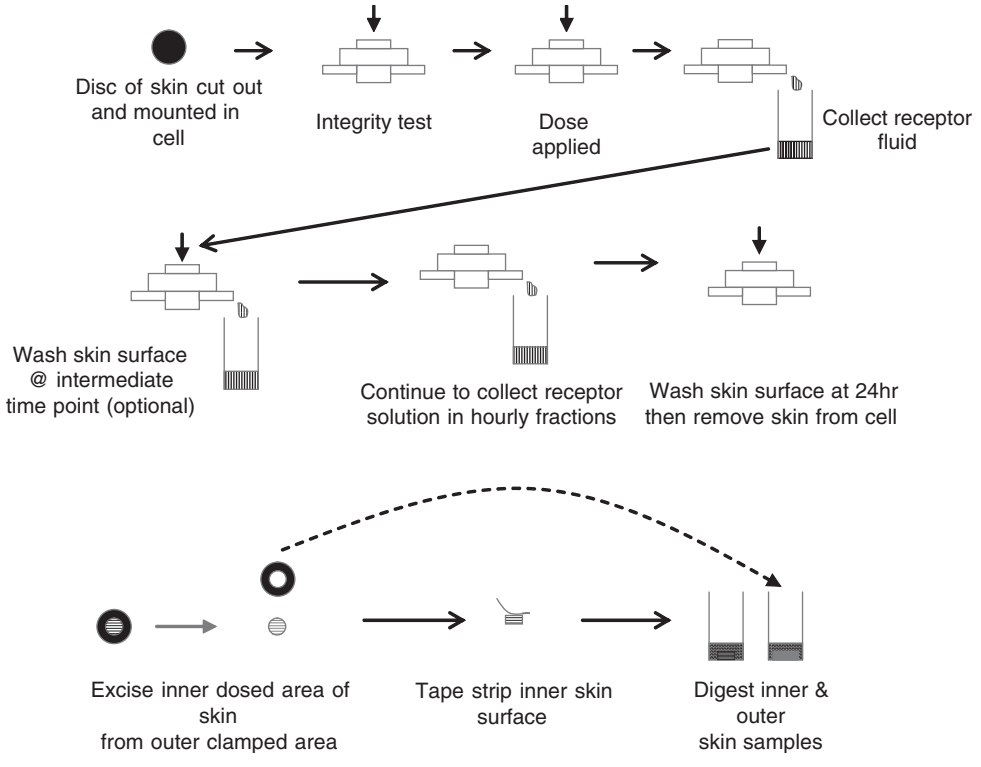


Figure 8.8 Summary of the main steps associated with a standard *in vitro* skin absorption study. An *ex vivo* skin sample is placed into a diffusion cell and a membrane integrity test performed. The test compound is then applied in a suitable manner and samples of receptor chamber fluid are removed and analysed at regular intervals. (It may be appropriate to wash off the test compound at some point during the study if this reflects its intended use.) At the end of the experiment, any residual test compound is washed from the skin surface, the skin is removed and the inner (exposed) area of the skin is excised and subject to tape-stripping. Both the inner and outer (unexposed) skin samples are then digested in an appropriate solvent to determine the dose of test compound remaining in the skin tissue

8.11 Examples of results from *in vitro* skin absorption studies

8.11.1 Infinite dose

In an infinite dose study, the flux will approach or reach steady state during the experimental period (Figure 8.9; see also Chapter 6, Figure 6.2). In this particular example (using a skin cream excipient), the species difference in permeability means that steady state was not attained by the end of the experiment for pig skin. The distribution of the same ingredient at the end of the experiment (Figure 8.10) and distribution within the stratum corneum (Figure 8.11) indicated that the material would have continued to penetrate the skin from the reservoir that had built up in the stratum corneum, if the experiment had been allowed to continue.

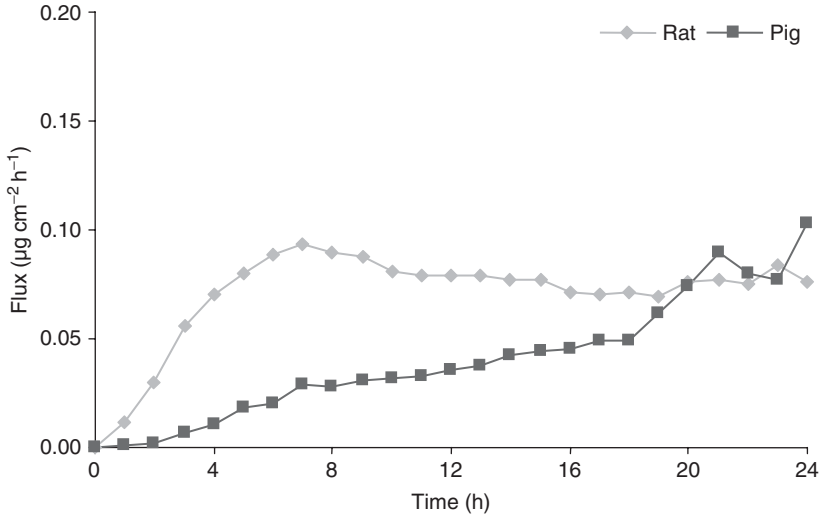


Figure 8.9 Mean flux of a ^{14}C radio-labelled skin cream ingredient through whole rat skin and dermatomed pig skin from PEG300 (24 h exposure)

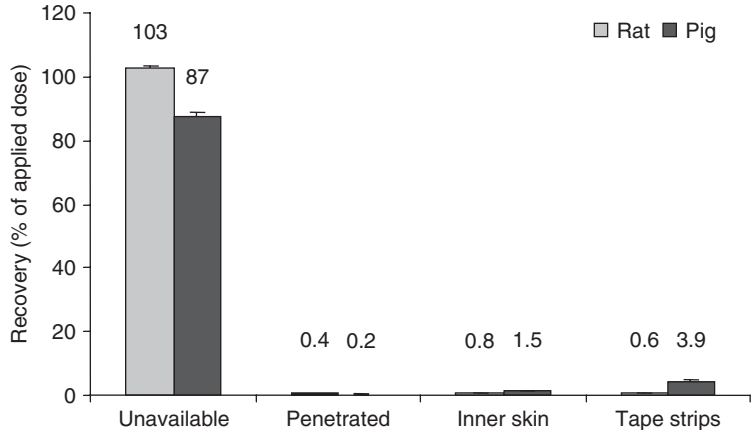


Figure 8.10 Distribution of a ^{14}C radio-labelled skin cream ingredient within whole rat skin and dermatomed pig skin after application in PEG300 (24 h exposure)

8.11.2 Finite dose

P-phenylenediamine (PPD) is a component of many permanent hair dyes. In this example, it was applied as a mixture in a hair dye formulation, mixed immediately before application with a developer. The developer contained hydrogen peroxide, which oxidized the hair dye components during the application time, causing polymerization reactions to occur between PPD and other components in the hair dye formulation. The result of this is the creation of large, coloured molecules, which in use would form within the hair fibre and become

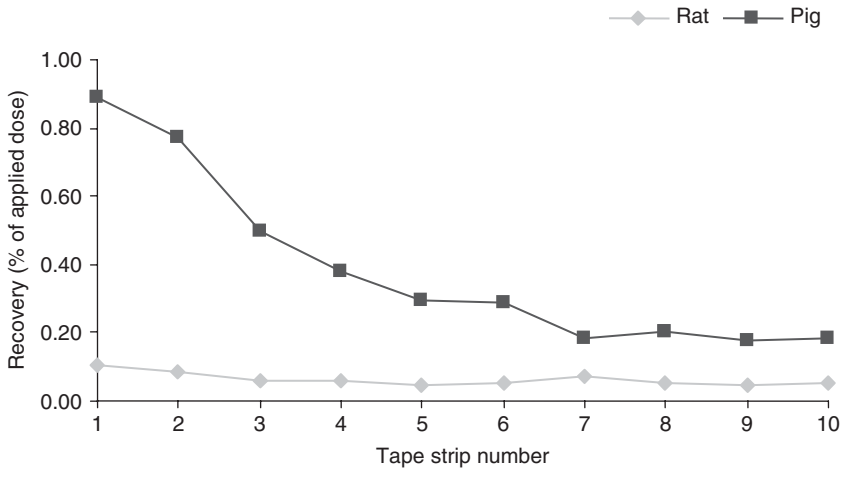


Figure 8.11 Mean recovery of ^{14}C radio-labelled skin cream excipients in tape strips of whole rat skin and dermatomed pig skin after application in PEG300 (24 h exposure)

entrapped as the polymerization reactions continued, leading to a permanent dying of the hair. The dye/developer mixture was applied for 30 minutes, then rinsed thoroughly from the skin. The flux curve in this instance indicated a finite dose (Figure 8.12; see also Chapter 6, Figure 6.3), where the amount of substance on the skin surface becomes the rate limiting factor within a few hours. Over 80% of the dose was washed from the skin surface at the 30 minute intermediary time point (Figure 8.13). The amount penetrated into the receptor solution and remaining within the skin was very low, but a substantial fraction of the applied dose (20%)

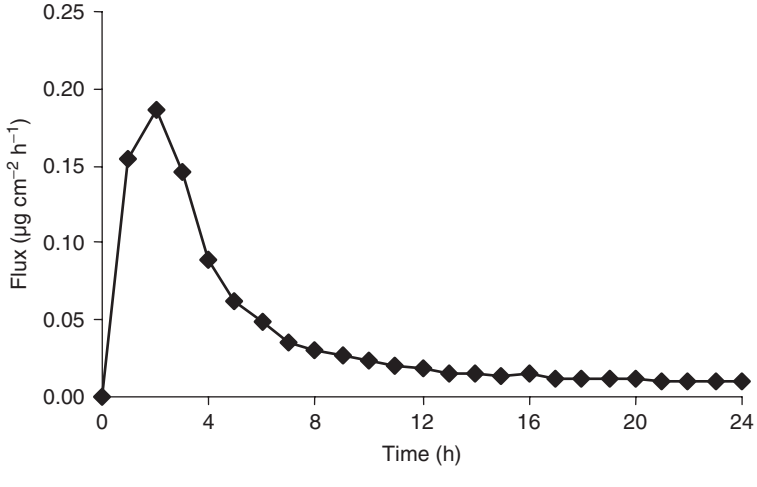


Figure 8.12 Mean flux of [^{14}C] *p*-phenylenediamine through dermatomed pig skin from a commercial hair dye formulation plus developer (30 min exposure)

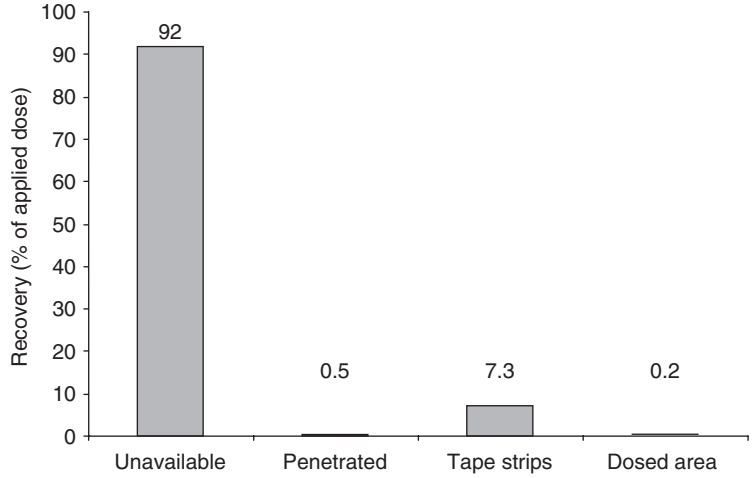


Figure 8.13 Distribution of [^{14}C] *p*-phenylenediamine through dermatomed pig skin from a commercial hair dye formulation plus developer (30 min exposure)

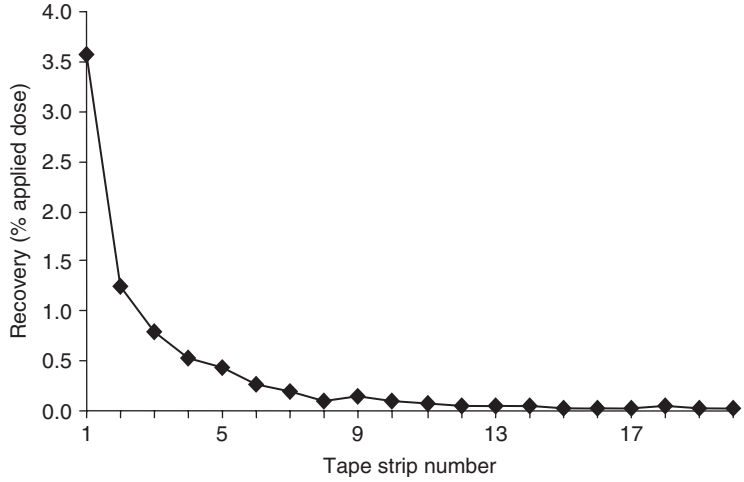


Figure 8.14 Mean recovery of [^{14}C] *p*-phenylenediamine in tape strips of dermatomed pig skin treated with a commercial hair dye formulation plus developer (30 min exposure)

was associated with the stratum corneum. The tape strip recovery profile (Figure 8.14) is very different to the above example (Figure 8.11), with most of the radio-labelled material contained within the first few strips. By considering the flux profile, distribution within the skin and within the tape strips, it could be deduced that the material associated with the stratum corneum would not have continued to penetrate the skin if the experiment had been allowed to continue. Even though there was a substantial amount of PPD in the tape strips, most of it was immobile, being bound to the superficial skin layers.

8.12 What is considered to be absorbed?

Interpreting what proportion of the applied dose has been absorbed has to be considered on a case-by-case basis, taking all aspects of the data into account.

The two examples outlined above are representative of data which stimulate an ongoing debate as to whether material associated with the stratum corneum (tape strips) is or is not available for absorption in the *in vivo* situation. It is generally accepted that material associated with the skin rinses, swabs, outer clamped area of skin and rinsed from the diffusion cell itself are not available for absorption. Material that reaches the receptor solution, plus any remaining within the dosed area of skin can be considered to have been absorbed. However, it is debatable whether material associated with tape strips remains available for absorption, and so examination of the flux profile and distribution within the skin can help to decide what is likely to be bioavailable and what is not. This means that a prediction of percutaneous penetration has to be considered on a case-by-case basis, taking all aspects of the data into account.

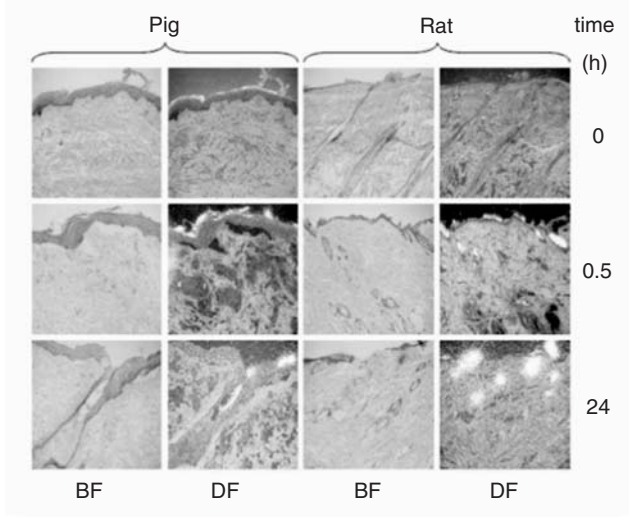


Figure 8.15 Representative micro-autoradiographs of pig and rat skin treated with PPD in a hair dye formulation. Skin sections observed under bright field (BF) and dark field (DF) illumination before exposure (0) and 30 minutes and 24 hours post exposure. Immediately prior to dosing (0 minutes), very few silver grains are visible on the bright field illumination due to natural background radiation. These sections serve as controls against which treated samples are compared. (Note that the stratum corneum possesses some inherent auto-fluorescence.) Skin excised 30 minutes post exposure illustrates deposition of silver grains on the surface of the skin and within the epidermis, with low levels in the dermis. After 24 hours, the distribution of grains can be seen to be associated with the skin surface and hair follicles, with material localised in the follicle opening. A full-colour version of this figure appears in the colour plate section of this book

8.13 Micro-autoradiography

Micro-autoradiography can demonstrate if a test material is localising within particular areas of the skin.

Micro-autoradiography is a technique that allows the distribution of a radio-labelled material within histological sections of the skin to be visualised. The method involves the dosed area of skin being snap frozen in liquid nitrogen and stored at −80°C until it is processed. Skin sections (∼8–10 μm) are produced using a cryostat in a dark room. The sections are then placed on microscope slides coated with a photographic emulsion and left in the dark to expose the emulsion for up to a week or more (Appleton 1964, 1986; Baker 1989). The emulsion is then developed using conventional photographic methods, after which the sections are stained with haematoxylin/eosin, as they would be for standard histology. The slides can be viewed under normal bright field illumination to show the histology of the skin and under dark field illumination to visualise the silver grains formed during a radioactive event; the grains show up as white dots (Figure 8.15).

Summary

- Skin absorption can be measured using *in vitro* methods.
- *Ex vivo* skin is mounted in diffusion cells that contain an upper donor chamber and a lower receptor chamber.
- The receptor chamber is filled with a receptor fluid and the penetration into the receptor fluid of a test material applied to the epidermal face of the skin via the donor chamber is monitored over time.
- At the terminal time point, test material within the receptor fluid, remaining on the skin surface, within each skin compartment and associated with the diffusion cell is measured and a mass balance performed.
- Test material reaching the receptor solution and remaining associated with the skin after rinsing is considered to be absorbed.
- Careful consideration of the flux of material into the receptor fluid and in the tape strip profile will help ascertain whether material remaining in the stratum corneum at the terminal time point is available for absorption into the deeper layers of the skin or not.
- Micro-autoradiography can help visualise whether test material is localising in any specific skin compartments such as hair follicles.

References

Allenby, A., Fletcher, J., Schock, C. and Tees, T.F.S. (1969). The rates of penetration of some V agents through human skin. Porton Technical Report 998. Public Record Office reference WO189/496.

Appleton, T.C. (1964). Autoradiography of soluble labelled compounds. *J R Microsc. Soc*, **83**: 277–81.

Appleton, T.C. (1986). Resolving power, sensitivity and latent image fading of soluble-compound autoradiographs. *J. Histochem. Cytochem*, **14**: 414–420.

Baker, J.R.J. (1989). *Autoradiography: A comprehensive overview*. *Royal Microscopical Society Microscopy Handbooks* Vol. 18, Oxford Science Publications, Oxford, pp. 30–32.

Bronaugh, R.L. (1995) Methods for *in vitro* percutaneous absorption. *Toxicol Methods*, **5**(4): 265–273.

Bronaugh, R.L. (1995a) Methods for *in vitro* skin metabolism studies. *Toxicol Methods*, **5**(4): 275–281.

Chilcott, R.P., Jenner, J., Taylor, C. and Rice, P. (1995). A rapid technique to identify structurally non-viable epidermal membranes during percutaneous penetration studies. *Human and Experimental Toxicology*, **15**: 161.

Chilcott, R.P., Dalton, C.H., Emmanuel, A.J., *et al.* (2002). Transepidermal water loss does not correlate with skin barrier function *in vitro*. *J Invest Dermatol*, **118**: 871–875.

Clowes, H.M., Scott, R.C., Heylings, J.R. (1994) Skin Absorption – Flow-Through Or Static Diffusion Cells *Toxicology in vitro*, **8**(4): 827–830.

COLIPA Cosmetic Ingredients: Guidelines for Percutaneous Absorption/Penetration.

Collier, S.W., Sheikh, N.M., Sakar, *et al.* (1989). Maintenance of skin viability during *in vitro* percutaneous absorption/metabolism studies. *Tox Appl Pharm*, **99**: 522–533.

Crutcher, W., and Maibach, H.I. (1969). The effect of perfusion rate on *in vitro* percutaneous penetration. *J Invest Dermatol*, **53**(4): 264–9.

Davies, D.J., Ward, R.J. and Heylings, J.R. (2004). Multi-species assessment of electrical resistance as a skin integrity marker for *in vitro* percutaneous absorption studies. *Toxicol. in vitro*, **18**(3): 351–358.

Diembeck, W., Beck, H., Benech-Kieffer, F., *et al.* (1999). Test guidelines for *in vitro* assessment of dermal absorption and percutaneous penetration of cosmetic ingredients. *Food Chem Tox*, **37**: 191–205.

Essa, E.A., Bonner, M.C. and Barry, B.W. (2002). Iontophoretic estradiol skin delivery and tritium exchange in ultradeformable liposomes. *Int J Pharm*, **240**(1–2): 55–66.

European Commission Health & Consumer Protection Directorate General – Directorate E1. Plant Health Sanco/222/2000 Guidance Document on Dermal Absorption.

Fluhr, J.W., Feingold and Elias, P.M. (2006). Transepidermal water loss reflects permeability barrier status: validation in human and rodent *in vivo* and *ex vivo* models. *Expt Dermatol*, **15**(7): 483–492.

Franz, T.J. (1975). Percutaneous absorption. On the relevance of *in vitro* data. *J Invest Dermatol*, **64**: 190–195.

Lawrence, J.N. (1997). Electrical resistance and tritiated water permeability as indicators of barrier integrity of *in vitro* human skin. *Toxicol. in vitro*, **11**: 241–249.

Levin, J. and Maibach, H. (2005). The correlation between transepidermal water loss and percutaneous absorption: An overview. *J Controlled Release*, **103**(2): 291–299.

Netzlaff, F., Kostka, K.H., Lehr, C.M. and Schaefer, U.F. (2006). TEWL measurements as a routine method for evaluating the integrity of epidermis sheets in static Franz type diffusion cells *in vitro*. Limitations shown by transport data testing. *Eur J Pharm Biopharm*, **63**(1): 44–50.

OECD (Organisation for Economic Co-operation and Development). Guidance Document for the Conduct of Skin Absorption Studies – Number 28 (2004).

OECD Guideline for the Testing of Chemicals 428. Skin Absorption: *In vitro* Method (2004).

Russell, W.M.S. and Burch, R.L. (1959). *The Principles of Humane Experimental Technique* (ISBN 0 900767 78 2), Methuen, London.

SCCP. (2006). Opinion Concerning Basic Criteria for the *in vitro* Assessment of Dermal absorption of Cosmetic Ingredients, SCCP/0970/06.

Zhai, H. and Maibach, H.I. (2001). Effects of skin occlusion on percutaneous absorption: An overview. *Skin Pharm Appl Skin Physiol*, **14**: 1–10.

PART III: Toxicological Assessment

9 Skin immunology and sensitisation

David A. Basketter

St John's Institute of Dermatology, St Thomas' Hospital, London SE1 7EH, UK

Primary Learning Objectives

- *Brief overview of the mechanisms of skin sensitisation.*
- *Historical and contemporary methodology for the identification of skin sensitising agents.*

9.1 Introduction

Skin sensitisation is an immune-mediated response caused by dermal exposure to a sensitising agent (allergen). As a rough rule of thumb, sensitisers tend to be small, reactive molecules.

In this chapter, the focus is on allergic reactions in the skin caused by exposure to chemicals. Mention is also made of other, less common types of skin response such as urticarial reactions. By far the most commonly experienced skin reaction (arising as a consequence of chemical exposure) is a type of delayed ('Type IV') hypersensitivity, usually termed skin sensitisation. An overview of the biological mechanisms underpinning skin immunology is be touched upon (more comprehensive reviews are available elsewhere); the properties of skin sensitisers and their predictive identification and assessment is then detailed. Finally, no review of this topic would now be complete without some consideration of how progress is being made towards the replacement of animal tests. But first, let's start at the beginning . . .

9.2 Definitions

A skin sensitiser/contact allergen is a chemical substance which possesses the intrinsic ability to cause skin sensitisation/contact allergy. Where there has been sufficient exposure to a skin sensitiser, an individual will become sensitised and thus develop a contact allergy. Whilst not necessarily associated with clinical symptoms, this condition can be detected by subjecting the individual to a 48 hour diagnostic patch test with the sensitising chemical. Finally, for an individual who has contact allergy, further skin exposure to a sufficient dose of the sensitising chemical will lead to an eczematous reaction called allergic contact

Principles and Practice of Skin Toxicology Edited by Robert P. Chilcott and Shirley Price
© 2008 John Wiley & Sons, Ltd

dermatitis (ACD). Additionally, a sensitised individual may also experience ACD in response to structurally similar chemicals, a phenomenon called cross-reactivity.

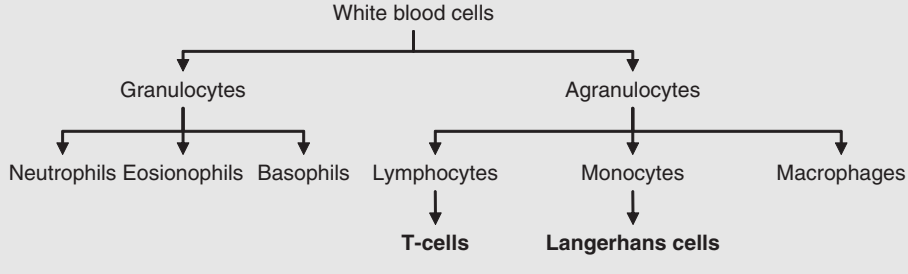
Skin sensitisation is an intrinsic property of a chemical substance and is referred to as a hazard; the likelihood that this hazard will be expressed in humans is referred to as risk and is a function of hazard potency and exposure (Equation (9.1)). The likelihood that a particular individual will become sensitised is also a function of individual susceptibility.

$$\text{Risk} = \text{Hazard} \times \text{Exposure} \tag{9.1}$$

9.3 Skin sensitisation

Skin sensitisation arises from an immune response raised against skin protein which has been modified by covalent attachment of a low molecular weight reactive chemical. There are some exceptions to this, but for practising toxicologists, the working rule is that small, reactive chemicals have the potential to induce sensitisation. The process occurs in two basic phases, induction and elicitation. The detailed immunology of the process has been reviewed relatively recently (Rustemeyer *et al.*, 2006; Friedmann, 2006); what follows here are the salient points necessary for a toxicologist's appreciation of the main type of adverse skin reaction associated with repeated exposure to chemicals[1]. During the induction of skin sensitisation, the immune system develops a heightened propensity to react to a specific chemical penetrating the skin. This may take from weeks to years of skin exposure to develop. During this time, the immune system is developing an expanded population of T lymphocytes (T-cells; Box 9.1) capable of recognising and responding to that chemical. During the elicitation phase, exposure to the chemical evokes the classic inflammatory skin reaction associated with allergic contact dermatitis (ACD), the clinical term for skin sensitisation in humans. This is examined in a little more detail below, and for each of the main stages of this response, recent key references are given to permit more detailed study if required.

Box 9.1 Classification of white blood cells



T-cells (or T lymphocytes) are white blood cells (leukocytes) involved in cell-mediated immunity. The 'T' stands for 'thymus'; all lymphocytes originate in bone marrow tissue, but T-cells migrate and mature in the thymus gland (a small mass of tissue which is situated behind the sternum). There are a number of different sub-types of T-cells, including memory cells, natural killer cells, cytotoxic cells, and helper cells, all of which have particular functions.

[1]For completeness, a summary of the main classification of hypersensitivity reactions is provided in Table 9.1.

Table 9.1 Classification of hypersensitivity reactions with some examples of dermal manifestations

Type	Time to onset	Mechanism(s)	Example of Cutaneous manifestation
I	<30 minutes	IgE antibodies (on surface of mast cells) bind to antigen. Mast cells undergo degranulation (release) of mediators such as histamine. Known as IgE-mediated hypersensitivity reaction.	Wheal-and-flare (urticarial) reaction.
II	4–8 hours	Antibodies bind to cells and trigger an immune response. Known as antibody dependent cell mediated cytotoxicity (ADCC) hypersensitivity.	Pemphigus (blistering sores).
III	2–8 hours	Antigen-antibody complexes precipitate (e.g. in capillaries) and cause mast cell degranulation in capillaries. Also termed the 'Arthus' reaction.	Lupus erythematosus (butterfly rash).
IV	1–3 days	Activation of T-cells. Known as delayed hypersensitivity reaction (see main text).	Allergic contact dermatitis.

Epidermal bioavailability: to behave as a skin sensitiser, a chemical must not just contact the skin, but it must make its way into the viable epidermis. Thus chemicals which are relatively small (<500 Daltons) and with non-extreme log P values are more prone to be sensitising (Chapter 5). The prediction of epidermal bioavailability remains a complex matter however, not least since the specific 'target' sites remain unclear (Divkovic *et al.*, 2005; Basketter *et al.*, 2007a).

Reactive chemistry: once in the epidermis, the chemical (hapten) must react covalently with skin protein (hapten–protein conjugation or 'haptenation') to form a complete antigen which is eventually recognised by the immune system. However, it seems very unlikely that all hapten derivatised proteins are driving the sensitisation response; it is probable that some also contribute to the generation of non-specific ('danger') signals (McFadden and Basketter, 2000). Although there are very limited data on the realities of the *in vivo* reactive chemistry, there undoubtedly exists a good basis of understanding in this area founded on an appreciation of reactive organic chemistry (Lepoittevin, 2006; Roberts *et al.*, 2007a).

Keratinocyte responses: the precise role of this cell is not yet fully understood, but it certainly does produce a number of key cytokines in response to haptenation by chemicals, thereby signalling danger, and may be of particular importance in determining the precise characteristics of the immune response (Matzinger, 2007). A major function of released cytokines is to prompt and to refine the subsequent reactions of Langerhans cells (Griffiths *et al.*, 2005).

Langerhans cell responses: these cells respond to protein haptenation and signals from keratinocytes by migrating from the epidermis to the local lymph node where the hapten it is carrying in the context of MHC molecules is presented to T lymphocytes (Figure 9.1). In

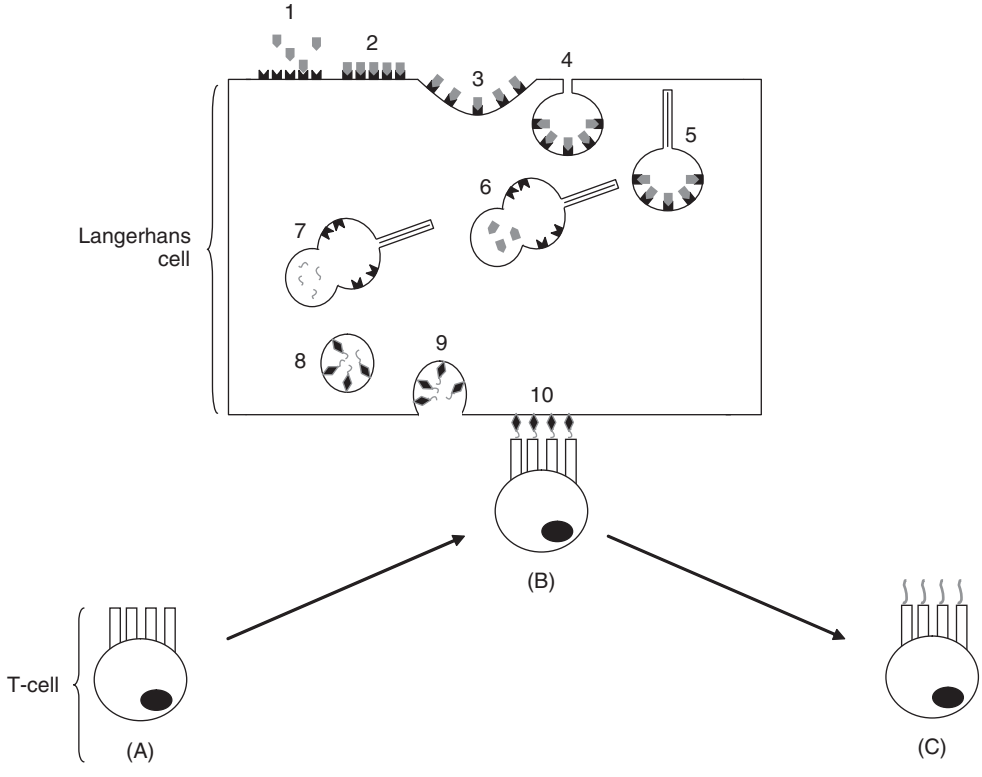


Figure 9.1 Schematic representation of the interplay between Langerhans cells and T-lymphocytes (T-cells). Langerhans cells are antigen presenting cells (APCs) and are attracted to a specific location within the epidermis by the release of cytokines from keratinocytes responding to an insult (e.g. bacterial infection or irritation). The antigens (1) bind to cell surface receptors on the Langerhans cell (2), which subsequently undergo endocytosis (3 & 4) where they form Birbeck granules which resemble tennis racquets when viewed with an electron microscope (5). The granules form lysosomes (6), which cause the antigens to break into small, linear fragments (7) which subsequently bind to a protein (major histocompatibility complex II; MHC II) that is expressed on the internal surface of the vesicle. The vesicles translocate and fuse to the outer membrane of the Langerhans cell. During this time, the Langerhans cell migrates back to the local lymph node where they interact with T-cells (A). The T-cells bind the surface-bound antigen on the Langerhans cell (B) via a protein called T-cell receptor(TCR). [It should be noted that this process is mediated by the presence of adhesion and co-stimulation proteins (complimentary copies of which are present on both the Langerhans and T-cell surfaces).] There are several potential outcomes, one of which is the proliferation and systemic release of activated T-cells (C) which confers sensitivity against the particular antigen

essence, it is the key to the induction of skin sensitisation, where its timely migration and maturation to a dendritic antigen presenting cell ensure efficient antigen presentation to T cells in the paracortical area of the draining lymph nodes (Rustemeyer *et al.*, 2006).

T cell responses: T cells possessing receptors which recognise the hapten presented by the Langerhans cell are triggered into cell division; from this arises an expanded set of daughter memory and effector T cells which recirculate around the body producing systemic sensitisation (Rustemeyer *et al.*, 2006).

The elicitation phase: skin inflammation occurs in a previously sensitised individual in response to dermal exposure to the inducing chemical (or to a chemical of closely similar structure). The skin reaction is typically delayed by several hours to days after the initial contact and is characterised by redness, swelling, itching, papules, vesiculation and blistering and, at the microscopic level, by cellular infiltration, spongiosis and acanthosis. However, the clinical features of allergic contact dermatitis reactions in skin can be very varied, depending not only on the individual responder but also on the allergen and the pattern of exposure and whether the reaction is acute or chronic (Frosch *et al.*, 2006).

9.4 Identification of skin sensitisers

Traditionally, skin sensitisation has been performed using guinea pigs and two particular *in vivo* techniques ('maximisation' and 'Beuhler' tests) have been extensively used. More recently, the local lymph node assay (LLNA) has been developed. This represents a considerable refinement over existing guinea pig protocols, including the potential to quantify the potency of sensitisers.

For the whole of the second half of the 20th century, the guinea pig has been the species of choice for toxicological evaluations of skin sensitising activity of chemicals. As a consequence, many guinea pig tests have been described, of which the guinea pig maximisation test (GPMT; Magnusson and Kligman, 1970) and the occluded patch test (Buehler, 1965) have been the most widely used and most thoroughly characterised. However, a considerable number of variations have been developed by scientists in Europe, the USA and Japan (Andersen and Maibach, 1985). Often, the aim has been to try to increase the sensitivity of the procedure (e.g. Maurer *et al.*, 1980).

Although guinea pig test methods vary with respect to details of the protocol, the principle is essentially identical (in most cases). Groups of animals are exposed by topical or intradermal exposure, or by a mixture of topical and intradermal exposure, to the test material. In some tests, adjuvant is also administered to enhance (maximise) immune responses provoked by the test material. Control guinea pigs receive the relevant vehicle alone, and where appropriate adjuvant treatments. Then, after a rest period of one to two weeks, the test and control groups are exposed topically to the chemical (at the maximum concentration judged not to cause irritant effects) and the elicitation of cutaneous hypersensitivity reactions is determined as a function of challenge induced erythema and/or oedema 24/48 hours after exposure. The sensitisation potential of the test chemical is judged on the basis of the frequency of specific reactions induced by challenge of treated animals. Detailed considerations of guinea pig test methods, including their conduct and interpretation, are available elsewhere (Andersen and Maibach, 1985; Basketter *et al.*, 1998; Steiling *et al.*, 2001). The well characterised guinea pig test methods (GPMT/Buehler) have proven to be a useful toxicological tool, and if conducted properly and interpreted correctly provide an accurate indication of likely sensitisation hazard (Basketter and Kimber, 2007).

Notwithstanding their proven utility, it has to be recognised that guinea pig tests, like all other toxicology tests, do have certain limitations. Quite commonly this can appear in the form of relatively borderline results. An example is shown in Table 9.2. In this case, substance X has displayed a limited level of response at the primary challenge, with up to three guinea pigs responding, but there is a small indication also of slight irritancy in a single control animal. How many guinea pigs would be judged to be sensitised on the basis

Table 9.2 Example of borderline data in guinea pig sensitisation testing: Substance X

Guinea pig no.	Primary challenge		Repeat challenge	
	24 h	48 h	24 h	48 h
1 (T)	0	0	0	1
2 (T)	0	0	0	0
3 (T)	0	1	0	0
4 (T)	1	1	0	1
5 (T)	0	0	0	0
6 (T)	0	0	0	0
7 (T)	0	0	0	0
8 (T)	0	0	0	0
9 (T)	1	2	1	1
10 (T)	0	0	1	1
11 (C)	0	0	0	0
12 (C)	0	0	0	0
13 (C)	0	0	0	0
14 (C)	0	0	0	0
15 (C)	1	0	0	0

T = test; C = control.
Grading scale: 0 = no reaction, 1 = weak, 2 = moderate and 3 = strong.

of this first challenge result? If the control reaction is true, then the responses at 24 hours should be disregarded; but even if it is simply an idiosyncratic reaction, three guinea pigs are positive at the 48 hour time point. Normally, for delayed type hypersensitivity, greater weight is placed on this later time; irritancy tends to appear at the earlier time point and then fade. The repeat challenge is consistent with a weak sensitisation response, in this case, by showing the reproducibility of the response in two of the animals (Kligman and Basketter, 1995; Frankild *et al.*, 1996). This is particularly important, since the threshold for classification of a chemical as a skin sensitiser[2] is a 30% positive rate in the GPMT (15% in the Buehler test).

Examination of Table 9.3 and the corresponding data for substance Y shows how difficult classification decisions can be. In this case, guinea pigs 3, 4 and 9 show weak responses at the primary challenge. The balance, however, is a little more to the 24 hour scoring time point. The reactions seem to be confirmed at repeat challenge in animals 3 and 9, with new weak reactions in a fourth guinea pig at the 24 hour time point, but all the 48 hour reactions in this repeat challenge are negated by the irritation response in a single control. So, although animal 9 may be sensitised, there is inadequate evidence to suggest that a minimum of 30% of the animals are sensitised, and hence substance Y would not be formally classified as a skin sensitiser.

Difficulties of the interpretation of challenge induced erythema are but one complication of guinea pig assays. A second problem is the difficulty of ensuring good skin contact with the sensitiser via proper removal of the fur. This is in addition to the problems associated with a choice of a suitable vehicle system and the selection of suitable

[2]This threshold level applies within the European Union (Dangerous Substances Directive) and the forthcoming 'globally harmonised scheme' (commonly known as GHS).

Table 9.3 Example of borderline data in guinea pig sensitisation testing: Substance Y

Guinea pig no.	Primary challenge		Repeat challenge	
	24 h	48 h	24 h	48 h
1 (T)	0	0	1	0
2 (T)	0	0	0	0
3 (T)	1	0	0	1
4 (T)	1	1	0	0
5 (T)	0	0	0	0
6 (T)	0	0	0	0
7 (T)	0	0	0	0
8 (T)	0	0	0	0
9 (T)	1	2	1	1
10 (T)	0	0	1	1
11 (C)	0	0	0	0
12 (C)	0	0	0	0
13 (C)	0	0	0	1
14 (C)	0	0	0	0
15 (C)	0	0	0	0

T = test; C = control.
Grading scale: 0 = no reaction, 1 = weak, 2 = moderate and 3 = strong.

concentrations. These challenges are discussed in some detail elsewhere (e.g. Magnusson and Kligman, 1970; Kligman and Basketter, 1995), but the practical reality is that vehicle selection and the identification of appropriate irritant induction concentrations and the maximum non-irritant concentration for the elicitation phase are very much open to individual choice. The OECD Test Guideline 406 offers very little advice (OECD, 1992). Because of all the technical variables associated with guinea pig skin sensitisation tests, in 1992 the OECD introduced the requirement for experimenters to use a weak-to-moderate sensitising control substance every six months as a procedural quality check. Example concentrations/vehicles and results have been published for the recommended positive control substances (Basketter *et al.*, 1993). The most commonly used of these is the weak fragrance allergen hexyl cinnamic aldehyde. The experience with this in a single laboratory is shown in Figure 9.2. Note that all the tests gave a >30% positive response.

In addition to the inherent variability of the guinea pig assay, perhaps the greatest limitation of *in vivo* assays is the fact that they do not readily lend themselves to an assessment of relative potency. Some attempts have been made to modify standard guinea pig methods for the purposes of deriving dose–response relationships (Andersen *et al.*, 1995), but these have met with only limited success. The difficulties are that it is not practicable in guinea pig assays to examine multiple induction concentrations of the test chemical: even if this were to be done, an endpoint that comprises a subjective assessment of the frequency of responses (rather than the vigour of responses) is not well suited to defining the inherent potency of a sensitising chemical. These considerations were reflected recently in the recommendations of both industry based and regulatory expert groups (Kimber *et al.*, 2001; Basketter *et al.*, 2005a). Before addressing this aspect in more detail, however, mention must first be made of a

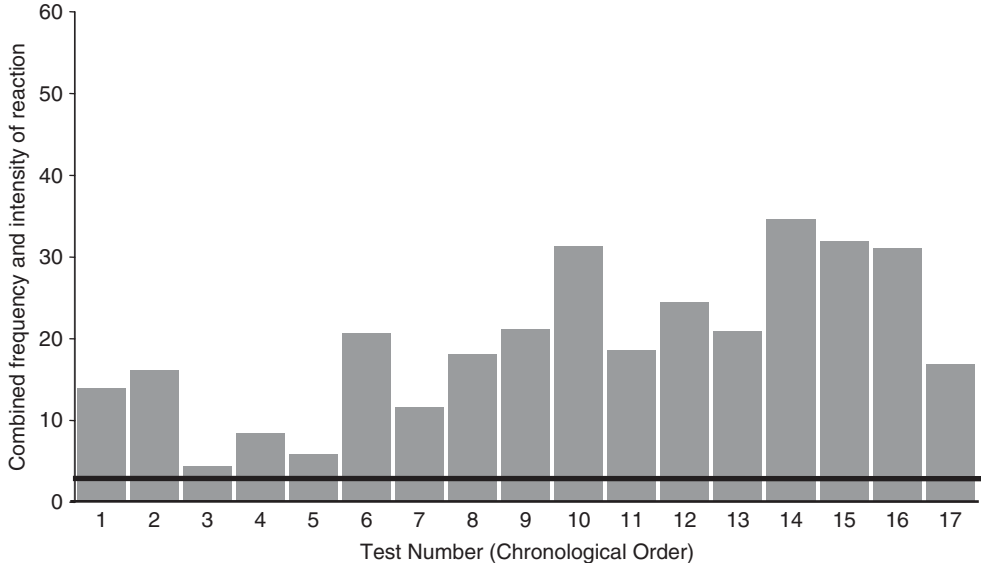


Figure 9.2 Positive control results following exposure of groups of ten guinea pigs to the weak fragrance allergen, hexyl cinnamic aldehyde. Tests conducted between 1985 and 1989 (presented in chronological order), of which all gave a >30% positive response. The y axis shows a combined frequency and intensity of reaction score in which the individual positive animal erythema scores are summated (10 guinea pigs, two scoring times, erythema scale 0–3, equates to a maximum possible score of 60; a score of three on this scale is equivalent to the 30% cut-off limit for identifying phototoxic materials)

non-guinea pig approach to the predictive identification of skin sensitisation hazard, the local lymph node assay (LLNA; outlined in Figure 9.3).

In the last 15 years considerable progress has been made in characterising the immunobiological processes that result in the induction of skin sensitisation and the elicitation of allergic contact dermatitis. In parallel with this more sophisticated appreciation of the relevant cellular and molecular mechanisms, there have emerged opportunities to explore new approaches to skin sensitisation testing. The local lymph node assay (LLNA) (Kimber and Basketter, 1992; Kimber *et al.*, 1994, 2002), is predicated upon an alternative strategy in which activity is judged as a function of responses induced in mice during the induction, rather than elicitation, phase of contact sensitisation. In this method, skin sensitisers are identified as a function of their ability to provoke proliferative responses in draining lymph nodes following repeated topical exposure. In practice, skin sensitising chemicals are defined as those which, at one or more test concentrations, induce a three-fold or greater increase in lymph node cell (lymphocyte) proliferation compared with concurrent vehicle treated controls. The LLNA has been the subject of extensive evaluations and the view currently is that the method provides a reliable and robust approach to the identification of sensitising chemicals and as such represents a stand-alone alternative to guinea pig assays (Basketter *et al.*, 1996; NIH, 1999; Gerberick *et al.*, 2000). Following the formal validation of the LLNA (NIH, 1999; Balls and Helsten, 2000; Dean *et al.*, 2001), it was adopted as OECD Test Guideline 429 (OECD, 2002), which is a good starting point for finding details of the full protocol for test conduct. In brief, the

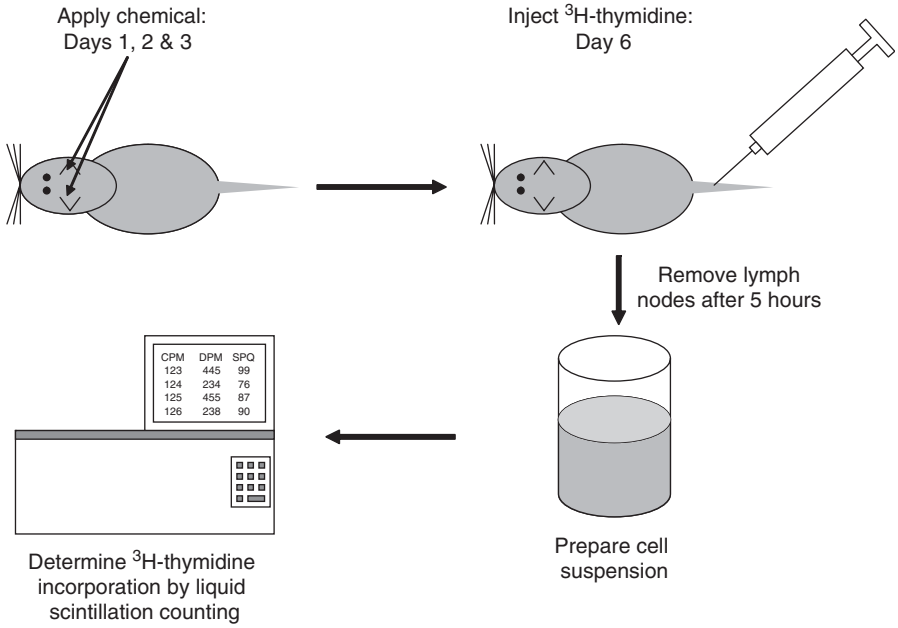


Figure 9.3 Outline of local lymph node assay (LLNA). Groups of four mice are treated topically behind each ear (daily for three days) with test material or vehicle (control). After five days, mice are injected with tritiated (^{3}H) thymidine. Mice are sacrificed after five hours and the draining lymph nodes excised, from which a cell suspension is prepared from which samples are subject to liquid scintillation counting to quantify incorporation of ^{3}H-thymidine

protocol used is typically as follows: groups of four CBA/Ca female mice (7–12 weeks of age) are treated topically on the dorsum of both ears with 25 μl of test material, or with an equal volume of the vehicle (4:1, Acetone:Olive Oil, v/v) alone. Treatment is performed once daily for three consecutive days. Five days following the initiation of exposure, all mice are injected via the tail vein with 250 μl of phosphate buffered saline (PBS) containing 20 μCi (740 kBq) of tritiated thymidine. Mice are sacrificed five hours later and the draining lymph nodes excised and pooled for each experimental group. The lymph node cell suspension is washed twice in an excess of PBS and then precipitated with 5% trichloroacetic acid (TCA) at 4°C for 18 hours. Pellets are resuspended in TCA and the incorporation into lymphocytes of tritiated thymidine measured by liquid scintillation counting.

In guinea pig and murine skin sensitisation assays there exists the possibility of false positive and false negative results, such being the case with any predictive toxicology test; this is also the case for the LLNA. Strategies for dealing with these problems have been published over the years (Kligman and Basketter, 1995; Andersen *et al.*, 1996; Basketter *et al.*, 1998, 2006). Metal allergens, notably nickel, have often been problematic in such predictive tests, but fortunately not many new metals are likely to be invented (!), so the problem is, in practice, relatively insignificant (Basketter *et al.*, 1999a). For organic chemicals the problem is real and involves distinguishing true for false positive results. For the LLNA, the robustness of the assay and the quantitative and objective nature of the endpoint place these matters in sharp relief (Basketter *et al.*, 2006, 2007a, 2007c; Basketter and Kimber, 2007).

For both the guinea pig and murine skin sensitisation assays, substantial databases of test results have been place in the public domain. For the guinea pig maximisation test, data on several hundred substances can be found in two publications (Wahlberg and Boman, 1985; Cronin and Basketter, 1994). For the Buehler test, a smaller data set of results on 40 chemicals has been published (Basketter and Gerberick, 1996). In the case of the LLNA, results on approaching 300 chemicals have been released as part of the validation and in relation to the potency assessment of skin sensitisers (Gerberick *et al.*, 2000, 2004a, 2005, 2007). It is of value to note that recent analysis of the structures of these chemicals serves to demonstrate that they do in fact span all the relevant chemical parameter space associated with skin sensitisation (Roberts *et al.*, 2006, 2007b). This is particularly important in the sense that there is often confusion in the interpretation of an expression such as 'chemical classes'. What matters is not the human use (pesticide, fragrance, surfactant etc), but the organic chemistry determining the reaction mechanism leading to covalent binding with skin protein.

9.5 Risk assessment

There is currently interest in the possibility that, in addition to providing a means for identifying hazard, the LLNA in particular may also be suitable for measurement of relative potency as a first step in the risk assessment process (Kimber and Basketter, 1997). The use of the LLNA for this purpose appears appropriate because the available evidence indicates that the vigour of induced proliferative responses by draining lymph node cells correlates closely with the extent to which skin sensitisation will develop (Kimber and Dearman, 1991). In practice, estimation of relative potency using the LLNA is based upon derivation by linear interpolation from dose response curves of an EC3 value (Figure 9.4), which is defined as the

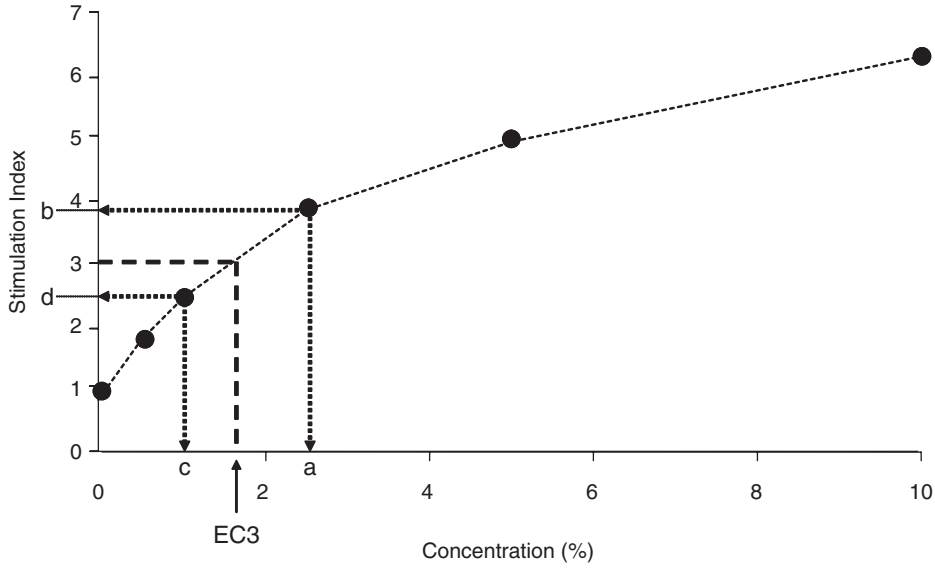


Figure 9.4 Calculation of EC3 value from interpolation of dose–response curve or from the equation $EC3 = C + [(3 - d)/(b - d)](a - c)$

Effective Concentration of chemical required to stimulate a 3-fold increase in lymph node cell proliferative activity compared with concurrent vehicle treated controls (Basketter *et al.*, 1999b). Experience to date indicates that the EC3 value provides a realistic, and apparently accurate and robust measure of relative potency suitable for integration into the risk assessment process (Basketter *et al.*, 2007d).

Two important points must be made here: firstly, potency refers to the intrinsic property of a sensitising chemical, which is thus entirely independent from the frequency with which allergic contact dermatitis occurs in the general or a clinical population (since this depends heavily on exposure as well as potency); secondly, there is a paucity of data indicating the intrinsic potency of chemical skin sensitisers in humans, since this requires possibly unethical experimental studies. Thus, the work that appears in the literature cannot offer the degree of accuracy for human–mouse correlations that could be asked for and professional judgement is needed to compensate for the relatively poor quality of the limited human data. Hence, it has been important that much research in this area has involved independent partners closely associated with the LLNA, including dermatologists, regulators and independent scientists (Basketter *et al.*, 2000, 2005a; Kimber *et al.*, 2001; Gerberick *et al.*, 2001a; Griem, 2003; Schneider and Akkan, 2004).

The potency comparisons referred to above differentiate human skin sensitisers into one of five categories (non, weak, moderate, strong, extreme). To use the LLNA EC3 value to derive the same classification requires the use of cut-off limits. Although this type of analysis can be helpful, more interesting work has been conducted by a number of groups that attempted to compare the LLNA EC3 value with experimental thresholds in humans, typically using a no adverse effect level in a human repeated insult patch test (HRIPT). Neither of these thresholds is of course absolute; they depend very much on the exposure conditions of the protocols. However, since each protocol is standardised (particularly the LLNA), they represent a reasonable basis for a comparison. Two groups published such comparisons in 2003. In one study, over 50 substances were assessed and a satisfactory relationship between the LLNA and HRIPT thresholds shown (Griem, 2003). In a second study, a slightly different approach was chosen, but again a good relationship was demonstrated (Schneider and Akkan, 2004). Lastly, in a more recent analysis, a very critical approach was taken to selection of human data to try to ensure that only good quality HRIPT threshold information was used (Basketter *et al.*, 2005b). This restricted the analysis to just 25 substances, but again a good relationship between EC3 values and HRIPT thresholds was shown. A graphical analysis of all the threshold data from these and other publications is reproduced in Figure 9.5: human predictive test threshold values (expressed in $\mu g\,cm^{-2}$) are plotted against LLNA EC3 values and expressed in the same units.

By using such methods of analysis, information on a new chemical (which may potentially be a skin sensitiser) can be compared with that available for other skin sensitisers. If the latter have previously been safely employed in defined exposure situations, then it may be possible to assume that they could be replaced by the newer chemical if it is of similar or indeed lower potency. For example, the weak sensitising potency of cocoamidopropyl betaine (CAPB) is well understood in terms of data from predictive models. In addition, the very limited extent to which it causes clinical allergy through use in rinse off products such as shampoos at levels up to approximately 10% is also quite well understood. Thus, were a novel material to be proposed for use in shampoos, CAPB could be employed as one potential benchmark for comparison. Similarly, the much stronger sensitising potency of (chloro)methylisothiazolinone is also well understood in predictive models and in man; dose response studies in mice, guinea pigs and

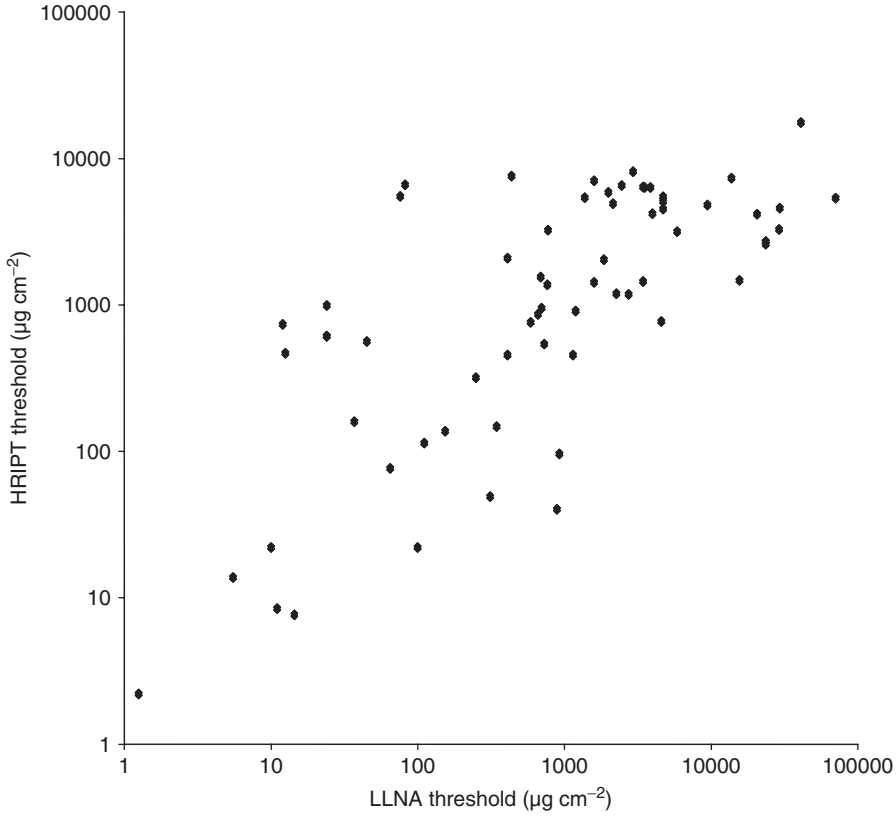


Figure 9.5 Human versus murine predictive test thresholds for skin sensitisation (both expressed as $\mu g\ cm^{-2}$ of test compound applied to the skin)

man exist (Chan *et al.*, 1983; Weaver *et al.*, 1985). Furthermore, there are data on acceptable and unacceptable use concentrations and product types (de Groot, 1990). All of these represent a valuable source of benchmark data for use in risk assessment.

Recently, a more quantitative approach to skin sensitisation risk assessment has been promulgated. In essence, this is founded on the traditional toxicology approach of identifying a no effect level (NOEL) in a predictive model and then appropriate reduction of this NOEL to derive human exposure limits below which the adverse effect (in this case, the induction of skin sensitisation) is unlikely to occur. The approach indicates safe exposure levels for individual sensitising chemicals under well defined exposure conditions; exposure is expressed in dose per unit area and is calculated per diem (each day). Comprehensive details of this new approach have been set out in a short series of publications (Gerberick *et al.*, 2001b; Felter *et al.*, 2002, 2003; Basketter and Kimber, 2006; Api *et al.*, 2008). Given the difficulties concerning the conduct of predictive human testing, this quantitative approach relies heavily on the direct prediction of NOELs from LLNA EC3 values. A number of publications now support the validity of this relationship (Basketter *et al.*, 2000, 2003, 2007b; Gerberick *et al.*, 2001; Griem *et al.*, 2003; Schneider and Akkan, 2004). Quantitative risk assessment for skin sensitising chemicals has been deployed to demonstrate the inappropriately high level of exposure to a

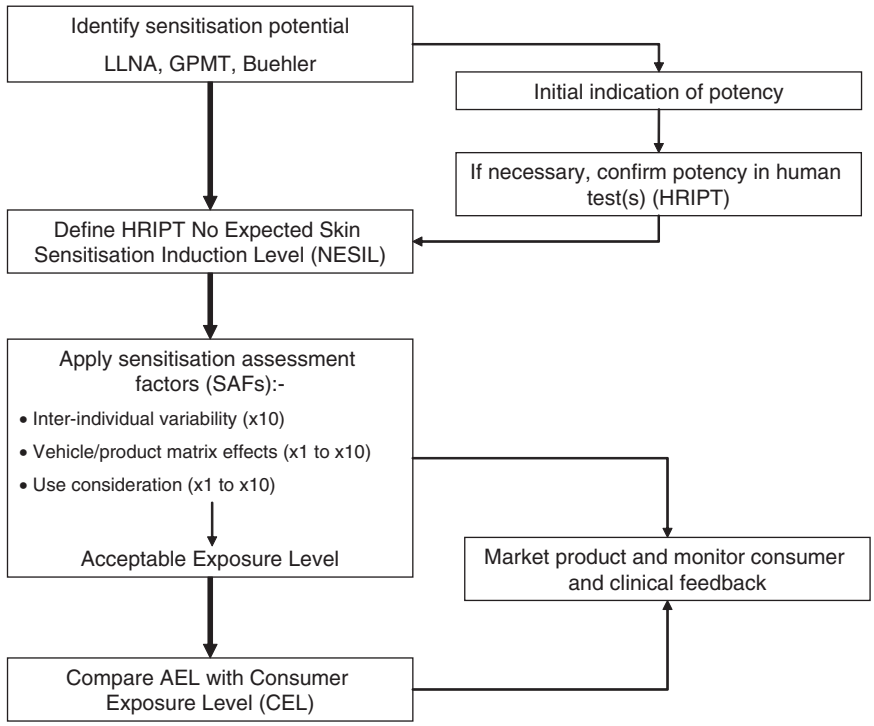


Figure 9.6 Generalised scheme for the conduct of quantitative risk assessment for skin sensitisation

preservative, methyldibromo glutaronitrile, providing an independent demonstration of the utility of the approach (Zachariae *et al.*, 2003). A generic overview of this new quantitative risk assessment strategy is outlined in Figure 9.6.

Use of such a quantitative approach in defining human exposure limits for sensitising chemicals relies heavily on both the accuracy and robustness of the measurement of potency in predictive models such as the LLNA: such appears to be the case (Basketter *et al.*, 2003, 2007b; Basketter and Cadby, 2004). As a further consequence, it is likely that data of this type will form the core sets of material against which *in vitro* alternatives ultimately will be validated (Gerberick *et al.*, 2004a, 2005).

The above methods are all *in vivo* tests. There has also been enthusiasm for the development of *in vitro* approaches to skin sensitisation testing, but although some progress has been made in the context of hazard identification (Kimber *et al.*, 2001), including the identification of chemical structural alerts, the derivation of (quantitative) structure–activity relationships and methods based upon the *in vitro* assessment of cellular responses are not (yet) suitable for consideration of relative potency.

9.6 Other types of allergic skin reaction

Apart from skin sensitisation/ACD, the other form of skin allergy is immunologic contact urticaria, mediated by IgE antibodies. This type of urticaria is a small subset of a large family of

urticarial reactions in skin, of which the contact urticarias form a considerable part (Lahti and Basketter, 2006). In essence, the response is seen as a rapid erythema and oedema, often with itch, which arises within minutes of contact and then fades over an hour or so. Toxicologically, they are usually of minor importance and, although it is possible to classify an immunologic urticant as R43 under the provisions of the EU Dangerous Substances Directive, to this author's knowledge this has never been done. Given the absence of a predictive test method, the prospect seems unlikely in the near future.

9.7 Future prospects

Unlike other areas of toxicology, robust *in vitro* (alternative) assays for sensitisation have not yet been developed.

The main interest for the future is in the development of predictive tests that avoid the use of animals, whilst at the same time provide the same level of human health protection (or better) than the existing animal assays. Within the next few years, toxicologists are likely to have existing approaches to the identification of skin sensitisers supplemented by novel strategies such as hapten peptide binding (Gerberick *et al.*, 2004b, 2007; Divkovic *et al.*, 2005). The details of exactly which approaches are adopted will depend on various considerations, such as the need for accurate identification, the need to limit/avoid animal testing and the need to have data which can be subsumed into an appropriate risk assessment. Under the pressure of forthcoming deadlines in the European Union, by 2013, a substantial reduction should be seen in the use of animals in the predictive identification of skin sensitising chemicals. Nevertheless, the total replacement of *in vivo* tests presents a substantial challenge. For reduction (and refinement) in animal testing, the regulatory acceptance of the reduced LLNA, which uses just four mice, will be critical; however, this will only provide a 'yes/no' hazard identification for the presence of sensitising properties (Kimber *et al.*, 2006). The elimination of animal testing will require the ability to integrate data from a variety of sources (chemical structure, peptide binding, disturbance of keratinocytes, activation of Langerhans cells etc) to provide a perspective on relative potency of test compounds in comparison with benchmark allergens (Basketter and Maxwell, 2007). The challenge of how to combine and use data from *in vitro* endpoints for skin sensitisation has only been addressed so far in theory (Jowsey *et al.*, 2006). Only if toxicologists can develop such methods and have them accepted by other experts and regulatory authorities will progress be made both in the complete replacement of animal tests whilst maintaining, or enhancing, human safety.

Summary

- The main allergic response of the skin is mediated via a (delayed) type IV hypersensitivity reaction.
  - During the induction phase, Langerhans cells present antigens to T-cells which become activated.
  - On re-exposure to the allergen, elicitation of hypersensitivity is mediated by the activated T-cells.
- A less common response is a type II (immediate) hypersensitivity response characterised by the wheal-and-flare (urticarial) reaction.

- Traditionally, the guinea pig has been the species of choice for *in vivo* identification of sensitising agents (allergens).
  - The two most common assays are the maximisation and Beuhler tests.
- The mouse local lymph node assay is a more recent development and has a number of advantages over the traditional assays.
- The development of alternative (*in vitro*) tests has been complicated by the difficulty in reproducing the complexity of the *in vivo* immune response.

References

Andersen, K.E. and Maibach, H.I. (eds). (1985). *Contact allergy predictive tests in guinea pigs: Current Problems in Dermatology*, Karger, New York.

Andersen, K.E., Volund, A. and Frankild, S. (1996). The guinea pig maximization test with a multiple dose design. *Acta Derm Venereol.*, **75**(6): 463–9.

Api, A.M., Basketter, D.A., Cadby, P.A. *et al.* (2008). Dermal sensitisation quantitative risk assessment (QRA) for fragrance ingredients. *Reg. Toxicol. Pharmacol.*, submitted.

Balls, M. and Hellsten, E. Statement on the validity of the local lymph node assay for skin sensitisation testing. ECVAM Joint Research Centre, European Commission, Ispra. *Alternatives to Laboratory Animals*, (2000): **28**: 366–367.

Basketter, D.A. and Cadby, P. (2004). Reproducible prediction of contact allergenic potency using the local lymph node assay. *Contact Dermatitis*, **50**: 15–17.

Basketter, D.A. and Gerberick, G.F. (1996). Interlaboratory evaluation of the Buehler test. *Contact Dermatitis*, **35**: 146–151.

Basketter, D.A. and Kimber, I. (2006). Predictive test for irritants and allergens and their use in quantitative risk assessment, in *Contact Dermatitis*, 4th Edn (Eds Frosch, P.J., Menné, T. and Lepoittevin, J.-P.), Springer Verlag, Heidelberg, pp. 179–188.

Basketter, D.A. and Kimber, I. (2007). Information derived from sensitisation test methods: test sensitivity, false positives and false negatives. *Contact Dermatitis*, **56**: 1–4.

Basketter, D.A. and Maxwell, G. (2007). Identification and characterization of allergens: *in vitro* alternatives. *Expert Reviews in Dermatology*, **2**: 471–480.

Basketter, D.A., Selbie, E., Scholes, E.W. *et al.* (1993). Results with OECD recommended positive control sensitisers in the maximisation, Buehler and local lymph node assays. *Food Chem Toxic*, **31**: 63–67.

Basketter, D.A., Gerberick, G.F., Kimber, I. and Loveless, S.E. (1996). The local lymph node assay – A viable alternative to currently accepted skin sensitisation tests. *Food and Chemical Toxicol*, **34**: 985–997.

Basketter, D.A., Gerberick, G.F. and Kimber, I. (1998). Strategies for identifying false positive responses in predictive sensitisation tests. *Food and Chemical Toxicology*, **36**: 327–333.

Basketter, D.A., Lea, L., Cooper, K. *et al.* (1999a). The identification of metal allergens in the local lymph node assay. *Am J Cont Derm*, **10**: 207–212.

Basketter, D.A., Lea, L., Cooper, K. *et al.* (1999b). A comparison of statistical approaches to derivation of EC3 values from local lymph node assay dose responses. *J Appl Toxicol*, **19**: 261–266.

Basketter, D.A., Blaikie, L., Dearman, R.J. *et al.* (2000). Use of the local lymph node assay for the estimation of relative contact allergenic potency. *Contact Dermatitis*, **42**: 344–348.

Basketter, D.A., Gerberick, G.F. and Kimber, I. (2001). Skin sensitisation, vehicle effects and the local lymph node assay. *Food Chem. Toxicol.*, **39**: 621–627.

Basketter, D.A., Wilson, K., Gilmour, N.J. *et al.* (2003). Utility of historical vehicle control data in the interpretation of the local lymph node assay. *Contact Dermatitis*, **49**: 37–41.

Basketter, D.A., Andersen, K.E., Lidén, C. *et al.* (2005a). Evaluation of the skin sensitising potency of chemicals using existing methods and considerations of relevance for elicitation. *Contact Dermatitis*, **52**: 39–43.

Basketter, D.A., Clapp, C., Jefferies, D. *et al.* (2005b). Predictive identification of human skin sensitisation thresholds. *Contact Dermatitis*, **53**: 260–267.

Basketter, D.A., McFadden, J., Evans, P. *et al.* (2006). Identification and classification of skin sensitisers: identifying false positives and false negatives. *Contact Dermatitis*, **55**: 268–273.

Basketter, D.A., Casati, S., Cronin, M.T.D. *et al.* (2007a). Skin sensitisation and epidermal disposition. *ATLA*, **35**: 137–154.

Basketter, D.A., Gerberick, G.F. and Kimber, I. (2007b). The local lymph node assay EC3 value: status of validation. *Contact Dermatitis*, **57**: 70–75.

Basketter, D.A., Gerberick, G.F. and Kimber, I. (2007c). The local lymph node assay: current position in the regulatory classification of skin sensitising chemicals. *Journal of Cutaneous and Ocular Toxicology*, **26**: 293–301.

Basketter, D.A., Clapp, C.J., Safford, B.J. *et al.* (2007d). Preservatives and skin sensitisation quantitative risk assessment: risk benefit considerations. *Dermatitis*, in press.

Buehler, E.V. (1965). Delayed contact hypersensitivity in the guinea pig. *Arch Dermatol* **91**: 171–177.

Chan, P.D., Baldwin, R.C., Parson, R.D. *et al.* (1983). Kathon biocide: Manifestation of delayed contact dermatitis in guinea pigs is dependent on the concentration for induction and challenge. *Journal of Investigative Dermatology*, **81**: 409–411.

Cronin, M.TD. and Basketter, D.A. (1994). Multivariate QSAR analysis of a skin sensitisation database. *SAR and QSAR in Environmental Research*, **2**: 159–179.

Dean, J.H., Twerdok, L.E., Tice, R.R. *et al.* (2001). ICCVAM evaluation of the murine local lymph node assay. II. Conclusions and recommendations of an independent scientific peer review panel. *Regulatory Toxicology and Pharmacology*, **34**: 258–273.

de Groot, A.C. (1990). Methylisothiazolinone/methylchloroisothiazolinone (Kathon CG) allergy: an updated review. *Am J Cont Derm.*, **1**: 151–156.

Divkovic, M., Pease, C.M., Gerberick, G.F. and Basketter, D.A. (2005). Hapten-protein binding: From theory to practical application in the *in vitro* prediction of skin sensitisation. *Contact Dermatitis*, **53**: 189–200.

Felter, S.P., Robinson, M.K., Basketter, D.A. and Gerberick, G.F., (2002). A review of the scientific basis for default uncertainty factors for use in quantitative risk assessment of the induction of allergic contact dermatitis. *Contact Dermatitis*, **47**: 257–266.

Felter, S.P., Ryan, C.A., Basketter, D.A. and Gerberick, G.F. (2003). Application of the risk assessment paradigm to the induction of allergic contact dermatitis. *Regulatory Toxicol Pharmacol*, **37**: 1–10.

Frankild, S., Basketter, D.A. and Andersen, K.E. (1996). The value and limitations of rechallenge in the guinea pig maximisation test. *Contact Dermatitis*, **35**: 135–140.

Friedmann, P.J. (2006). Contact sensitisation and allergic contact dermatitis: immunobiological mechanisms. *Toxicol Lett*, **15**: 49–54.

Frosch, P.J., Menné, T. and Lepoittevin, J.-P. (2006). *Contact Dermatitis*, 4th Edn, Springer Verlag, Heidelberg.

Gerberick, G.F., Ryan, C.A., Kimber, I. *et al.* (2000). Local lymph node assay validation assessment for regulatory purposes. *Am J Cont Derm.* **11**: 3–18.

Gerberick, G.F., Robinson, M.K., Ryan, C.A. *et al.* (2001a). Contact allergenic potency: Correlation of human and local lymph node assay data. *Am J Cont Derm.*, **12**: 156–161.

Gerberick, G.F., Robinson, M.K., Felter, S. *et al.* (2001b). Understanding fragrance allergy using an exposure-based risk assessment approach. *Contact Dermatitis*, **45**: 333–340.

Gerberick, G.F., Ryan, C.A., Kern, P.S. *et al.* (2004a). A chemical dataset for the evaluation of alternative approaches to skin sensitisation testing. *Contact Dermatitis*, **50**: 274–288.

Gerberick, G.F., Vassallo, J.D., Bailey, R.E. *et al.* (2004b). Development of a peptide reactivity assay for screening contact allergens. *Toxicol Sci.*, **81**(2): 332–43.

Gerberick, G.F., Ryan, C.A., Kern, P.S. *et al.* (2005). Compilation of historical local lymph node assay data for the evaluation of skin sensitisation alternatives. *Dermatitis*, **16**: 157–202.

Gerberick, G.F., Vassallo, J.D., Foertsch, L.M. *et al.* (2007). Quantification of Chemical Peptide Reactivity for Screening Contact Allergens: A Classification Tree Model Approach. *Toxicol Sci.*, **97**: 417–427.

Griem, P., Goebel, C. and Scheffler, H. (2003). Proposal for a risk assessment methodology for skin sensitisation based on sensitisation potency data. *Regulatory Toxicology and Pharmacology*, **38**: 269–290.

Griffiths, C.E., R.J., Dearman, M., Cumberbatch and I., Kimber. (2005). Cytokines and Langerhans cell mobilisation in mouse and man. *Cytokine.* **32**: 67–70.

Jowsey, I., Basketter, D.A., Westmoreland, C., Kimber, I., (2006). A future approach to measuring relative skin sensitising potency. *J. Appl. Toxicol.*, **26**: 341–350.

Kimber, I. and Basketter, D.A. (1992). The murine local lymph node assay; collaborative studies and new directions: A commentary. *Food Chem Toxicol.*, **30**: 165–169.

Kimber, I. and Basketter, D.A. (1997). Contact sensitisation: A new approach to risk assessment. *Human and Ecological Risk Assessment*, **3**: 385–395.

Kimber, I. and Dearman, R.J. (1991). Investigation of lymph node cell proliferation as a possible immunological correlate of contact sensitising potential. *Food and Chemical Toxicology*, **29**: 125–129.

Kimber, I., Dearman, R.J., Scholes, E.W. and Basketter, D.A. (1994). The local lymph node assay: developments and applications. *Toxicol.*, **93**: 13–31.

Kimber, I., Basketter, D.A., Berthold, K. *et al.* (2001). Skin sensitisation testing in potency and risk assessment. *Toxicological Sciences*, **59**: 198–208.

Kimber, I., Dearman, R.J., Gerberick, G.F. and Basketter, D.A. (2002). The local lymph node assay: past, present and future. *Contact Dermatitis*, **47**: 315–328.

Kimber, I., Dearman, R.J., Betts *et al.* (2006). The local lymph node assay and skin sensitisation: a cut-down screen to reduce animal requirements. *Contact Dermatitis*, **54**: 181–185.

Kligman, A.M. and Basketter, D.A. (1995). A critical commentary and updating of the guinea pig maximisation test. *Contact Dermatitis*, **32**: 129–134.

Lahti, A. and Basketter, D.A. (2006). Immediate contact reactions, in *Contact Dermatitis*, 4th Edn (Eds, Frosch, P.J., Menné, T. and Lepoittevin, J.-P.), Springer Verlag, Heidelberg, pp. 83–96.

Lepoittevin, J.-P. (2006). Immediate contact reactions, in *Contact Dermatitis*, 4th Edn (Eds, Frosch, P.J., Menné, T. and Lepoittevin, J.-P.), Springer Verlag, Heidelberg, pp. 45–68.

Magnusson, B., Kligman, A.M. (1970). *Allergic contact dermatitis in the guinea pig. Identification of contact allergens*, Charles C Thomas, Springfield, Illinois.

Maurer, T., Weirich, E.G., Hess, R. The optimization test in the guinea pig in relation to other predictive sensitisation methods. *Toxicology*, (1980); **15**(3): 163–171.

Matzinger, P. Friendly and dangerous signals: is the tissue in control? *Nat Immunol.*, (2007): **8**: 11–13.

McFadden, J.P. and Basketter, D.A. (2000). Contact allergy, irritancy and danger. *Contact Dermatitis*, **42**: 123–127.

NIH (1999). The murine local lymph node assay: a test method for assessing the allergic contact dermatitis potential of chemicals/compounds. NIH No. 99–4494.

OECD Guidelines for Testing Chemicals. (1992). Guideline 406: skin sensitisation. Paris.

OECD Guidelines for Testing Chemicals. (2002). Guideline 429. Skin Sensitisation: Local Lymph Node Assay, Paris, 2002.

Roberts, D.W., Aptula, A.O. and Patlewicz, G. (2007a). Electrophilic chemistry related to skin sensitisation. Reaction mechanistic applicability domain classification for a published data set of 106 chemicals tested in the mouse local lymph node assay. *Chem Res Toxicol.*, **20**: 44–60.

Roberts, D.W., Patlewicz, G., Kern, P.S. *et al.* (2007b). Mechanistic applicability domain classification of a local lymph node assay dataset for skin sensitisation. *Chemical Research in Toxicology*, **16**: 1019–1030.

Rustemeyer, T., van Hoogstraten, I.M.W., von Blomberg, B.M. and Scheper, R.J. (2006). Mechanisms in allergic contact dermatitis, in *Contact Dermatitis*, 4th Edn (Eds, Frosch, P.J., Menné, T. and Lepoittevin, J.-P.), Springer Verlag, Heidelberg, pp. 11–44.

Schneider, K. and Akkan, Z. (2004). Quantitative relationship between the local lymph node assay and human skin sensitisation assays. *Regulatory Toxicology and Pharmacology*, **39**: 245–255.

Steiling, W., Basketter, D.A., Berthold, K. *et al.* (2001). Skin sensitisation testing – new perspectives and recommendations. *Food and Chemical Toxicology*, **39**: 293–301.

Wahlberg, J.E. and Boman, A. (1985). Guinea pig maximization test, in *Contact allergy predictive tests in guinea pigs: Current Problems in Dermatology*, (eds Andersen, K.E. and Maibach, H.I.), Karger, New York, pp 65–106.

Weaver, J.E., Carding, C.W. and Maibach, H.I. (1985). Dose response assessments of Kathon biocide. I. Diagnostic use and diagnostic threshold patch testing with sensitised humans. *Contact Dermatitis*, **12**: 141–145.

Zachariae, C., Rastogi, S., Devantier, C. *et al.* (2003). Methyldibromo glutaronitrile: clinical experience and exposure-based risk assessment. *Contact Dermatitis*, **48**: 150–154.

10 *In vitro* phototoxicity assays

Penny Jones

Safety and Environmental Assurance Centre, Colworth Science Park, Sharnbrook, Bedford, Bedfordshire MK44 1LQ, UK

Primary Learning Objectives

- *How a step-wise testing strategy using in vitro assays can be used to identify the phototoxic hazard potential of test materials.*
- *Details of the individual assays that may comprise an in vitro phototoxicity testing strategy.*

10.1 Introduction and scope

Where substances are intended for uses where they may come into contact with the skin, either accidentally or in products deliberately intended for skin application, it is necessary to carry out an assessment of potential phototoxic hazard. Safety tests for phototoxicity have historically used animal models such as guinea pigs (Lambert *et al.*, 1996; Chapter 9). However, following advances in *in vitro* toxicology, this assessment may now be carried out using a tiered testing strategy involving *in vitro* assays, some of which have been validated. This chapter describes some of the *in vitro* assays which can be used as part of such a testing strategy and their validation status.

10.2 *In vitro* strategies for phototoxicity testing

Phototoxicity testing may be carried out using a step-wise strategy with *in vitro* assays to replace *in vivo* tests using guinea pigs.

An example of the type of step-wise *in vitro* testing strategy suitable for phototoxicity testing is outlined in Figure 10.1. Other similar examples and an extensive review of the background to *in vitro* phototoxicity testing can be found in the reports of the first and second European Centre for the Validation of Alternative Methods (ECVAM) Workshops on *in vitro* phototoxicity testing (Spielmann *et al.*, 1994a, 2000). An essential requirement for phototoxicity is the absorption of light by a test material (Figure 10.1A); the initial assay is always measurement of a UV/visible absorption spectrum to identify absorption at relevant wavelengths (>300 nm) (Lovell, 1993). If a material demonstrates significant UV/visible light

Principles and Practice of Skin Toxicology Edited by Robert P. Chilcott and Shirley Price
© 2008 John Wiley & Sons, Ltd

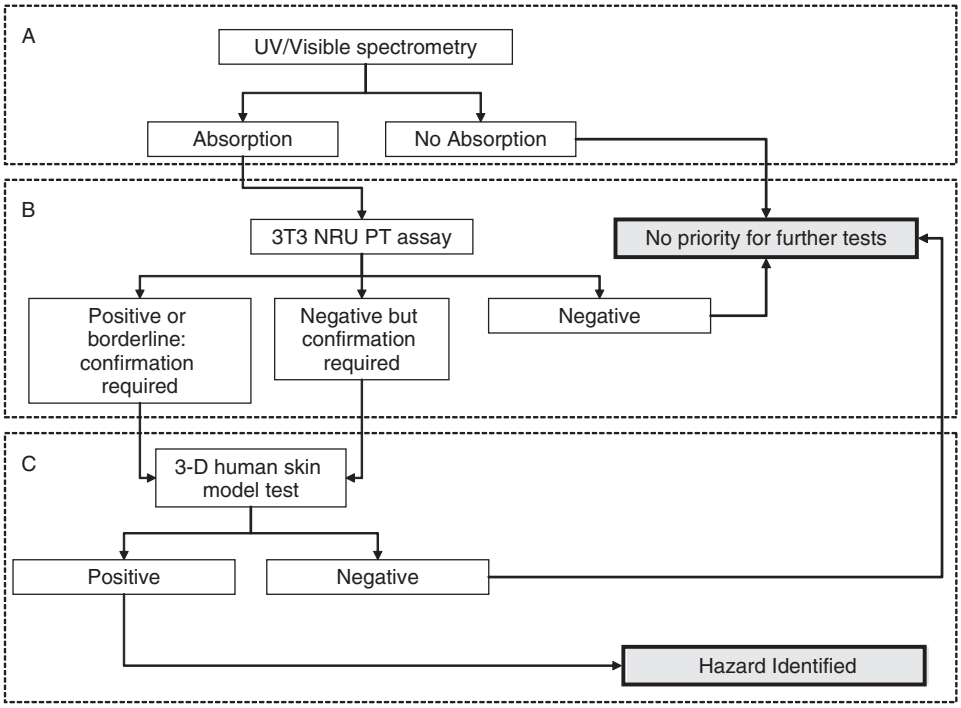


Figure 10.1 Testing strategy for potential phototoxic hazard assessment using *in vitro* assays

absorbance then it may have potential for phototoxicity and should be further tested; materials without significant absorbance are considered not to be of potential phototoxic hazard and do not require testing. For further testing the initial test would be the validated 3T3 cell neutral red uptake phototoxicity test (NRU PT; Figure 10.1B) which has the potential to detect photoirritants and also most photoallergens and photogenotoxins (Spielmann *et al.*, 1994b, 1998a, 1998b). However, if a substance is negative in the NRU PT these data are also useful and represent sufficient evidence that the risk of phototoxic potential is low and therefore no further testing is required (Spielmann *et al.*, 1998a, 1998b). A positive result for a material in the NRU PT provides evidence of possible phototoxic hazard which would feed into the overall risk assessment of the material.

Where a substance gives a borderline result or is positive in the NRU PT more *in vitro* testing may be carried out to further characterise the nature or potency of the hazard. Further confirmatory testing may also be carried even when a material is negative in the NRU PT to add to the weight of evidence demonstrating absence of hazard. If further information on the nature of the hazard is required then a photobinding test for photoallergy may be carried out as detailed below (Lovell and Jones, 2000) and/or by using a phototoxicity test utilising a 3-D human skin model assay (Jones *et al.*, 2001) (Figure 10.1C). It should be noted that 3-D skin models are considered to be more sensitive than human skin and the lack of phototoxicity of a substance in such a model is good evidence that it would not present a phototoxic hazard to human skin *in vivo*. In other words, *in vitro* models provide a conservative approach to assessing phototoxicity. Alternatively, a borderline/positive result may be confirmed as positive

by the 3-D skin model. Dose–response data can then be used to estimate potency through comparison with a phototoxin of known *in vivo* potency (for example, musk ambrette) and analysed in the context of intended human exposure. An example of the hazard assessment process for a test material (ingredient X) using assays given in this testing strategy is described in the following sections.

10.3 The UV/visible absorption spectrum as a pre-screen for phototoxicity

The absorption of light by a test material is an essential requirement for phototoxicity; significant absorption may be defined as the specific absorbance coefficient (A), for a 1% solution with path length 1 cm (A1%1 cm) being >1 or the molar extinction/absorption coefficient being >10.

A UV/visible absorption spectrum is used as a pre-screen to identify test material absorption at relevant wavelengths (usually defined as >300 nm). The wavelengths of interest within sunlight are UVB (280–315 nm), UVA (315–400 nm, absorbed by the majority of phototoxins) and visible light (>400 nm) (Epstein, 1974; Lovell, 1993). If a material has negligible absorption of sunlight wavelengths then photoreaction in sunlight is unlikely, but if sunlight wavelengths are found to be absorbed then further *in vitro* testing is required. Materials are considered to exhibit significant absorption if the specific absorbance coefficient (A), for a 1% solution with path length 1 cm (A1%1 cm) is >1.0. This is similar to OECD guidelines stating that, if the molar extinction/absorption coefficient (ε) of a chemical is less than 10 L mol^{-1} cm^{-1}, then the chemical is unlikely to be photoreactive and need not be tested in the 3T3 NRU PT or any other biological test for adverse photochemical effects (OECD, 1997, 2004). For example, chlorpromazine HCl (often used as a positive control during *in vitro* phototoxicity assays) has an ε of 4200 at 309 nm, whereas sodium lauryl sulphate is non phototoxic and has an $\varepsilon < 1$ (Lovell and Jones, 2000). However, A1%1 cm is more useful where the molecular weight of a material is not known or for the analysis of extracts/mixtures. An example of data for a test material (ingredient 'X') in aqueous solution is given in Table 10.1. Ingredient X absorbs significantly at wavelengths in both near UV (~230 and ~274 nm) and far UV (~300 nm). There is also some absorption at ~340 nm, which was slightly less than the significant level of A (1%, 1 cm) = 1.0 (Jones *et al.*, 2003). The absorbance at 300.4 nm is >1 and sufficient to trigger further testing in the NRU PT.

Table 10.1 UV/visible light absorption by ingredient X using water as solvent

λ_{max} (nm)	A (1%, 1 cm)
230.7 (s)	13.57*
273.8 (p)	12.62*
300.5 (s)	4.18*
340.4 (s)	0.80

λ_{max} = wavelength of maximum absorption; A = absorption coefficient calculated for 1% solution using 1 cm path; p = peak; s = shoulder; * indicates significant absorption.

10.4 *In vitro* assays for phototoxicity using monolayer cultures

The primary test of choice for *in vitro* phototoxicity testing is the validated 3T3 cell neutral red uptake phototoxicity test (the 3T3 NRU PT), which is a comparison of the cytotoxicity of a test material when tested in the presence (+) and absence (−) of exposure to a non-cytotoxic dose of simulated solar light (UVA/visible spectrum).

Development of the 3T3 cell neutral red uptake phototoxicity test (NRU PT) commenced from 1992 and was conceived to enable simple and rapid testing/screening (without the use of animals) of materials suggested to be possible phototoxins by their absorption spectrum. This provides a conservative approach as not all materials that absorb relevant wavelengths are necessarily activated to become phototoxic. The NRU PT is an internationally validated test based on a comparison of the cytotoxicity of a test material when measured in the presence (+) and absence (−) of exposure to a non-cytotoxic dose of simulated solar light[1]. Cytotoxicity is expressed as a concentration-dependent reduction of the uptake of the vital dye Neutral Red (NR) when measured 24 hours after treatment with the test material. Materials identified by this test are likely to be phototoxic following systemic application and distribution to the skin, or after topical application. Applicability of the NRU PT is, however, confined to water soluble materials. Extensive validation of this test has been carried out against human data in three trials. Prevalidation was carried out by eight laboratories in a non-blind trial using 20 chemicals (11 phototoxic and nine non-phototoxic) and developed a prediction model using a photoirritation factor (PIF) to discriminate between positive and negative chemicals (Spielmann *et al.*, 1994b). The PIF is defined as:

$$\mathrm{PIF} = \frac{\mathrm{EC}_{50}\ \mathrm{value} + \mathrm{UV}}{\mathrm{EC}_{50}\ \mathrm{value} - \mathrm{UV}} \tag{10.1}$$

The EC_{50} value is the concentration of test material resulting in a 50% reduction in NRU compared to solvent-treated control cells.

Using a cut-off value of PIF = 5 all of the test chemicals were correctly classified in the 3T3 NRU PT. A formal blind validation trial followed using 30 test chemicals (25 phototoxic and five non-phototoxic) in nine laboratories; this showed that the test was reproducible and that the correlation between *in vitro* and *in vivo* phototoxic potential was very high, with all phototoxic chemicals being correctly identified (Spielmann *et al.*, 1998a). At the request of the then Scientific Committee of Cosmetology and Non-Food Products (SCCNFP), the European expert advisory committee on cosmetics, some of the most commonly used UV-filter chemicals which are not phototoxic *in vivo* and poorly soluble in water, plus known phototoxic chemicals, were tested in a further blind trial with the 3T3 NRU PT (20 chemicals in four laboratories). The test was found to correctly assess the phototoxic potential of modern UV filter chemicals in this third trial (Spielmann *et al.*, 1998b). The NRU PT protocol can also be used with human keratinocytes, as demonstrated in a blind study with the chemicals of the EU/COLIPA validation study and the UV-filter study (Clothier *et al.*, 1999). The successfully validated 3T3 NRU PT is accepted by the European Commission and the EU Member States in Annex V of EU Council Directive 67/548/EEC for the classification and labelling of hazardous

[1]Simulated solar light includes UVA and the visible spectrum, but is commonly referred to as 'UV'.

chemicals (EU, 2000). An OECD guideline for the NRU PT (OECD Test Guideline 432) was accepted and published in 2004 (OECD, 2004). For OECD Test Guideline 432 (TG432) a modified prediction model was developed based on the results of the validation trials; a test substance with a PIF <2 predicts: 'no phototoxicity', a PIF >2 and <5 predicts: 'probable phototoxicity' and a PIF >5 predicts: 'phototoxicity'. TG432 also reflects conclusions of the validation trials that false positive results may be obtained at high test concentrations and recommends a maximum test concentration of $1000\,\mu g\,ml^{-1}$. A list of suggested reference compounds and performance guidelines is given in TG432 to aid in the setting up of the assay by a laboratory.

The following general protocol is used for the NRU PT (for full details see OECD TG432):

- Plate out 10^4 3T3 cells per well in 96-well plates and incubate overnight (37°C; 5% CO_2 in air).
- Expose replicate wells in duplicate plates to test material(s) in Earle's Balanced Salt Solution (EBSS) or similar for one hour at 37°C; 5% CO_2 in air (include blanks and solvent controls).
- Irradiate treated cells for 50 minutes with, for example, a Hönle SOL 500 lamp with Type H1 UVA filter or similar (keeping duplicate plate(s) in dark).
- Rinse all cultures with phosphate buffered saline (PBS) and incubate overnight in fresh medium (37°C; 5% CO_2 in air).
- Remove medium, add $50\,\mu g\,ml^{-1}$ neutral red (NR) to wells and incubate for three hours (37°C; 5% CO_2 in air).
- Rinse cells with PBS, then extract NR from cells with a mixture of acetic acid, ethanol and water (1:50:49) and measure absorbance at 540 nm.
- Calculate results as a percentage of NRU uptake by solvent control treated cells.
- Construct dose–response curves with and without UV and estimate EC_{50} values +/− UV.
- Calculate PIF value (Equation (10.1)).

Example results for Chlorpromazine (positive control for NRU PT) and ingredient X are illustrated in Figure 10.2. The mean results for Chlorpromazine and ingredient X were:

Chlorpromazine : EC_{50} value + UV = $0.43\,\mu g\,ml^{-1}$
EC_{50} value − UV = $16.7\,\mu g\,ml^{-1}$
PIF = 41.5

Ingredient X : EC_{50} value + UV = $5617\,\mu g\,ml^{-1}$
EC_{50} value − UV = $>10\,000\,\mu g\,ml^{-1}$
PIF = >1.8

With a PIF > 5 Chlorpromazine is clearly predicted (as expected) to be phototoxic. There are criteria given in TG432 for chlorpromazine as a positive control which should be met for results to be valid. These are:

EC_{50} value + UV = $0.1 - 2.0\,\mu g\,ml^{-1}$
EC_{50} value − UV = $7.0 - 90.0\,\mu g\,ml^{-1}$
PIF >6

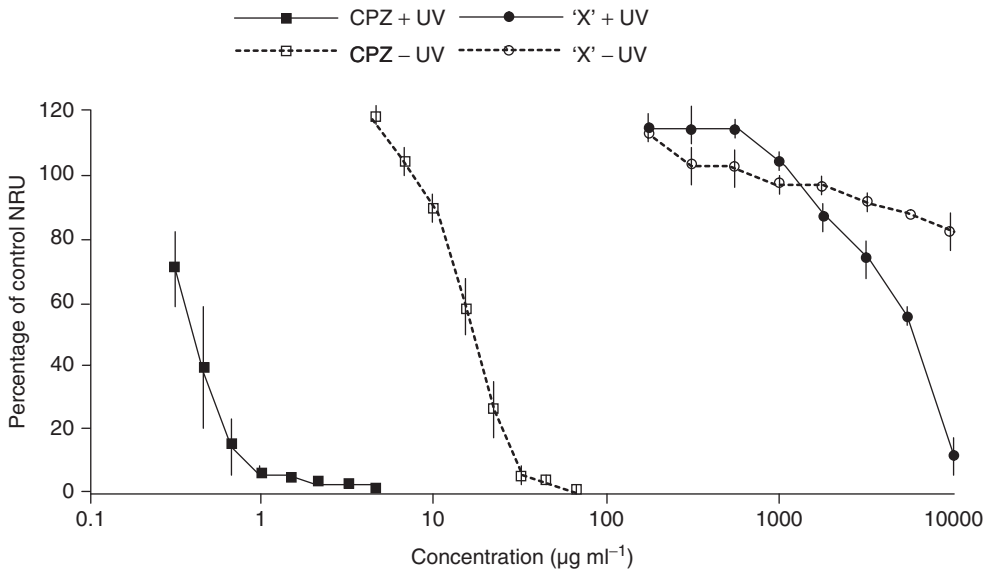


Figure 10.2 Mean dose–response curves in the 3T3 NRU PT for Chlorpromazine (CPZ) and ingredient X, in presence (+UV) or absence (−UV) of UV radiation (Data are expressed as mean ± SEM (n = 3))

Ingredient X is non-cytotoxic at the concentrations tested (up to 10 000 $\mu g\,ml^{-1}$) in the absence of UV. However, in the presence of UV, there is an increase in the cytotoxicity of ingredient X at the highest concentrations tested, giving the mean EC_{50} value indicated. Therefore, the PIF for ingredient X cannot be exactly calculated and is expressed as '>1.8'. These data indicate that further phototoxicity assays should be conducted as the precise EC_{50} value has not been ascertained and the fact that the PIF is >1 indicates that ingredient X may be weakly photoactivated. However, because this occurs at concentrations >1000 $\mu g\,ml^{-1}$ this suggests that this material may not be phototoxic at concentrations likely to be used *in vivo*. To investigate this possibility further an extended battery of phototoxicity assays may be performed.

10.5 *In vitro* assays for photoallergenicity

Photobinding to protein, in conjunction with a test of photo-oxidation of histidine, can be used to help differentiate between photoallergens and phototoxins.

The NRU PT detects both photoirritant and photoallergic test materials. However, there are currently no stand-alone, validated *in vitro* tests for photoallergy. Photoallergy is a delayed-type hypersensitivity reaction with an essential requirement for ultraviolet (UV) radiation (Stephens and Bergstresser, 1985). Photochemical binding of photoallergens to protein is widely accepted as the initial step of the photoallergenic process. Typical photoallergens form free radicals on absorption of UV; these undergo covalent binding to protein and this has been proposed as a test for potential photoallergenicity (Barratt and Brown, 1985; Pendlington

and Barratt, 1990). Photoirritants may also photobind to protein, but in this case other photochemical reactions would be expected to be more significant and photo-oxidation of histidine has also been proposed to identify photo-oxidising potential which may lead to photoirritancy (Lovell, 1993). A general method for photobinding is outlined below (Lovell and Jones, 2000):

- A solution of test material is mixed with 0.05 mM human serum albumin (HSA) and irradiated or kept in the dark (control).
- Irradiation with, for example, an ECETOC lamp; samples are placed in 1 cm cuvettes on a turntable and rotated round a central medium-pressure mercury-metal halide arc lamp (Philips HPM12 400 W) with (Schott WG320 3 mm) UV filters to attenuate UVC and UVB.
- Unbound test chemical is separated from HSA by filtration through a (Sephadex G-25) column.
- The process is monitored by UV spectrometry before and after separation.
- Binding is detected as increased UV absorption of the protein fraction from Sephadex chromatography which is calculated for specific absorption peaks or shoulders.
- A significant increased absorbance is defined as being greater than 5% of the dark control solution absorbance plus 0.01.

For the adjunct histidine oxidation assay the following may be carried out:

- Solutions of histidine (1 mM) and the test material (at a concentration relevant to photobinding) in acetonitrile–water (3:7) are irradiated (for example with an ECETOC lamp).
- Histidine concentration is determined before and after irradiation using a modified Pauly reaction (Johnson *et al.*, 1986).

This photobinding assay using binding to human serum albumin (HSA), in conjunction with a test of photo-oxidation of histidine, has been used to test the 30 chemicals used in the NRU PT validation trial (Lovell and Jones, 2000) against the prediction model given in Table 10.2. Six of seven photoallergens were identified as such by the photobinding assay. Most photoirritants also caused photomodification of protein, but 11 (out of 17) also photo-oxidised histidine efficiently and so were classified as photoirritants. Four photoirritants remained falsely predicted as photoallergens. Two photoirritants were negative for both photomodification of protein and for histidine photo-oxidation, and four chemicals negative

Table 10.2 Prediction model for discrimination between photoallergens and photoirritants

Photobinding	Histidine loss (%)	Prediction
−	<5	Inactive
+	<5	Photoallergen
−	>5 <20	Photoirritant
− or +	>20	Photoirritant

in vivo were negative *in vitro*. The remaining two chemicals could not be classified because of unclear data both *in vivo* and *in vitro*. Conservatively it was concluded that there was good detection of photoallergens although differentiation between photoallergens and phototoxins was not achieved in all cases.

An illustration of the data generated by the photobinding assay when testing the phototoxic drug Carprofen (CP) is shown in Figure 10.3 (using methods described in Lovell and Jones, 2000). The initial spectrum (HSA + CP) can be seen to be modified by UV irradiation

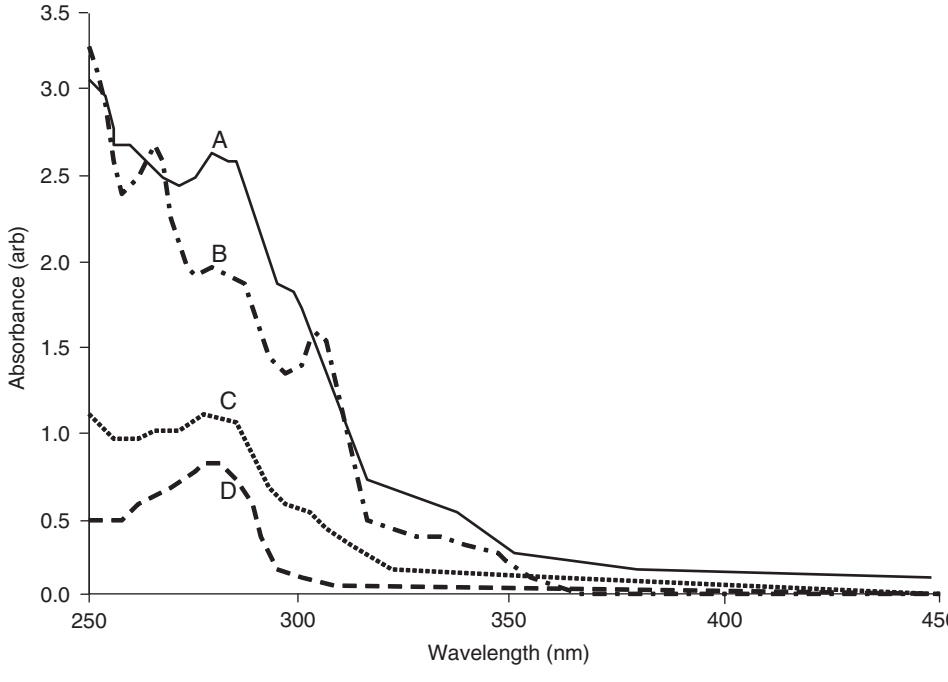


Figure 10.3 Results for Carprofen (CP) for *in vitro* protein binding test for photoallergy: absorbance spectra for CP + HSA (A), CP + HSA + UV (B), CP + HSA + UV post-Sephadex separation (C) and HSA alone (D)

Table 10.3 Results of photobinding assay conducted on Material X to human serum albumin (HAS)

Concentration of ingredient X (%)	−UV		+UV	
	λnm	δA_{min}	λnm	δA
1	278	0.047	278	0.014
	340	0.012	340	0.017*
0.33	278	0.045	278	−0.029
	340	0.011	342	0.017*

λnm = wavelength of peak absorbance due to test material; δA = absorbance at peak wavelength due to test material; δA_{min} = δA minimum = 5% post-Sephadex spectrum – UV + 0.01; *indicates δ A greater than δA_{min}, implying a photobinding reaction.

(HSA + CP + UV). After Sephadex chromatography there is some change in the spectrum, but it still indicates irreversible photobinding to HSA by demonstrating increased absorbance if compared with HSA alone. The dark control, in contrast, separates completely and gives only an HSA spectrum. Results for the same assay carried out on ingredient X are given in Table 10.3. Slight excess absorption is seen at absorption peaks of approximately 340 and 278 nm, which suggests that ingredient X may weakly photobind to protein. These data are consistent with the results from the NRU PT and taken together suggest that there might be some phototoxic hazard associated with ingredient X. In this case, further analysis to investigate the *in vivo* relevance of these results using a 3-D human skin model assay is suggested.

10.6 *In vitro* assays for phototoxicity using human 3-D skin models

A phototoxicity test using a 3-D human skin model can be used to further investigate the relevance of results from the NRU PT to the species of interest; lack of phototoxicity of a test material in a skin model is good evidence that it would not present a phototoxic hazard to human skin *in vivo*.

The NRU PT assay has been demonstrated to detect the phototoxic potential of both strong and weak phototoxins irrespective of their aqueous solubility (Spielmann *et al.*, 1998b). However, in the case of negative or borderline responses there may be some uncertainty, particularly where materials can be tested only at low concentrations because of a lack of aqueous solubility. Cell monolayers are also highly sensitive to toxic effects and so may give false positives results (highest recommended test concentration is 1000 $\mu g\,ml^{-1}$); assays using 3-D reconstructed human skin models can help address these situations when they occur. Owing to their relative (expensive) cost, 3-D models are not generally incorporated into routine testing strategies but do provide a relevant system for assessing the phototoxicity of topically applied test materials, either alone (undiluted or in aqueous or organic solvents) or in commercial formulations, directly to the skin surface and therefore are subject to fewer solubility problems. In addition, higher concentrations (more relevant to *in vivo* usage) can be tested. The 3-D skin models are also considerably less sensitive to UVB than monolayer cells (Cohen *et al.*, 1994; Corsini *et al.*, 1997), allowing the use of a greater proportion of UVB in addition to UVA, thus more accurately modelling sunlight exposure (Jones *et al.*, 2001). Skin^2™ (a now unavailable commercial model) was originally reported to identify phototoxic hazard potential (Edwards *et al.*, 1994; Liebsch *et al.*, 1995; Api, 1997) in a manner similar to the NRU PT assay, with evaluation of phototoxic hazard potential using a MTT viability assay (Mossman, 1983). Similar protocols have now been successfully transferred to currently available 3-D skin models such as EpiDerm™, EpiSkin™ and SkinEthic™. (Cohen *et al.*, 1994; Roguet *et al.*, 1994; Augustin *et al.*, 1997; Liebsch *et al.*, 1997; Bernard *et al.*, 1999; Jones *et al.*, 1999, 2001; Medina *et al.*, 2001). The EpiDerm™ model has also been subject to evaluation in an ECVAM prevalidation study; this gave good predictions in three laboratories using ten chemicals (Liebsch *et al.*, 1999). Three-D skin models also lend themselves to the testing of formulations by direct application to the stratum corneum surface (Medina *et al.*, 2001) and it has been demonstrated that formulations spiked with a known phototoxin can be identified using such a 3-D skin model test (Jones *et al.*, 2001).

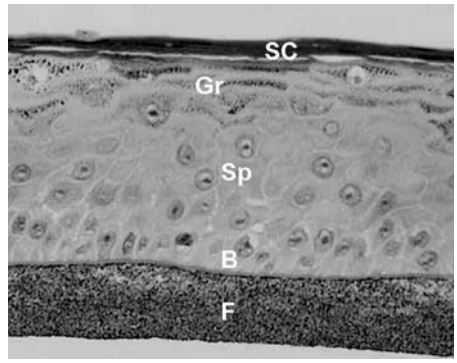


Figure 10.4 Vertical section through EpiDerm™ culture showing support filter (F), basal layer (B), spinosum layer (Sp), granulosum layer (Gr) and stratum corneum (SC)

Three-D human skin models have a similar structure to that of *in vivo* epidermis. For example, EpiDerm™ has a clear basal cell layer resting on a support filter with spinosum cells above, then a clear granulosum layer with stratum corneum at the upper surface (Figure 10.4).

An example method for using 3-D skin models for phototoxicity testing is as follows (for further details see Liebsch *et al.*, 1999 and Jones *et al.*, 2001):

- Place cultures in 6-well plates with 0.9 ml medium for at least one hour incubation (37°C; 5% CO_2 in air).
- Expose replicate cultures in duplicate plates to 20 μl test substance in solvent for ~18 hours at 37°C; 5% CO_2 in air (including solvent controls).
- Irradiate treated cultures for 60 minutes with UVA/B using, for example, a Hönle SOL 500 lamp with Type H2 UVB filter or similar (keeping duplicate plates in dark).
- Rinse cultures with PBS, then incubate overnight in fresh medium (37°C; 5% CO_2 in air).
- Transfer cultures to 24-well plates containing 0.3 ml MTT (1 mg ml^{-1}), then incubate for three hours (37°C; 5% CO_2 in air).
- Rinse cultures with PBS, then extract blue colour by shaking cultures in 2 ml isopropanol (two hours at ~20 rpm).
- Measure absorbance of extracts in triplicate 200 μl samples at 570 nm and calculate results as MTT reduction as a percentage of solvent treated (control) cultures.
- Phototoxic potential is predicted by >30% increase in toxicity in the presence of UV.

Chlorpromazine is again generally used as a positive control in this assay. An example of the type of results obtained with this assay using EpiDerm™ is given in Figure 10.5. Chlorpromazine, 8-methoxypsoralen and unpurified bergamot oil (main contaminant 5-methoxypsoralen; Zaynoun *et al.*, 1977) which are known human phototoxins are clearly phototoxic, whereas bergamot FCF-pure, which is a purified bergamot oil used in fragrance formulations, is non-phototoxic as expected. Figure 10.6 shows results for ingredient X tested using the SkinEthic™ epidermal model. CPZ was phototoxic and reduced MTT conversion

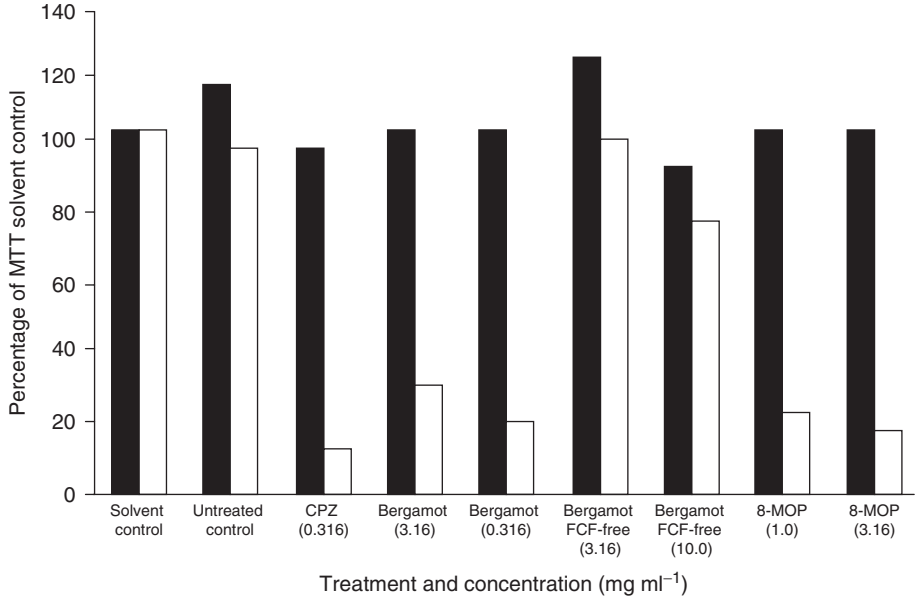


Figure 10.5 Example results for the 3-D human skin model phototoxicity assay; cytotoxicity in the absence (■) and presence (□) of UV light (Data expressed as mean of duplicate cultures)

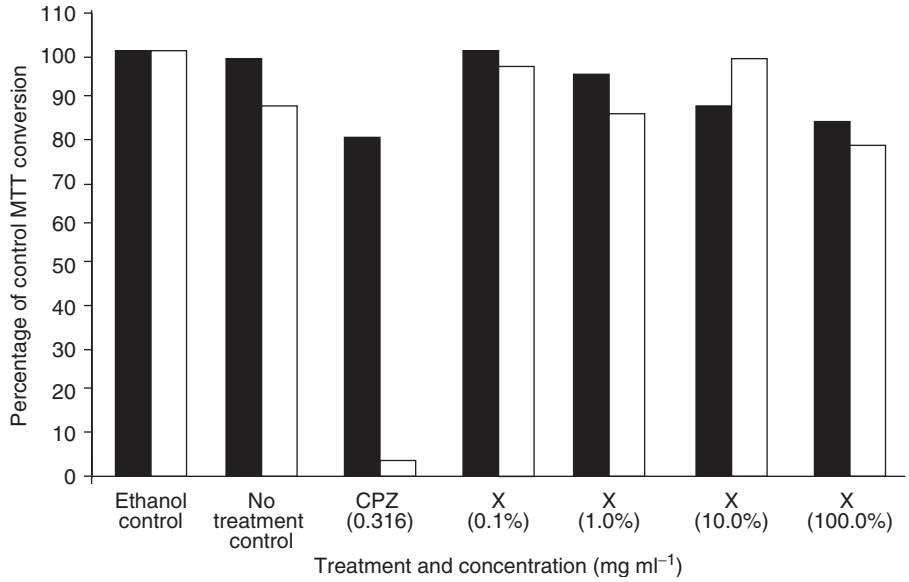


Figure 10.6 Results for the 3-D human skin model phototoxicity assay; cytotoxicity of Chlorpromazine (CPZ) and ingredient X in the absence (■) or presence (□) of UV (Data expressed as mean of duplicate cultures)

Table 10.4 Comparison of results obtained using NRU PT assay and a 3-D skin model

Code	EC_{50} value ($\mu g\,ml^{-1}$)		PIF	Potential phototoxic hazard P/NP	Phototoxicity to 3-D human skin model
1	36.0	39.3	1.1	NP	NP up to $10\,mg\,ml^{-1}$
2	5500	7800	1.4	NP	NP up to $100\,mg\,ml^{-1}$
3	6.36	>97	>17	P	P at $\geq 100\,\mu g\,ml^{-1}$
4	12.95	140	10.8	P	P at $\geq 1000\,\mu g\,ml^{-1}$
5	11.82	534	48	P	P at $\geq 1000\,\mu g\,ml^{-1}$
6	0.62	349	780	P	P at $\geq 1000\,\mu g\,ml^{-1}$
7	0.76	>92	>128	P	P at $\geq 1000\,\mu g\,ml^{-1}$
8	0.69	>100	>206	P	P at $\geq 1000\,\mu g\,ml^{-1}$
9	1612	>2 1477	>13	P	NP up to $100\,mg\,ml^{-1}$
10	1.65	4.35	2.7	P?	P at $\geq 316\,\mu g\,ml^{-1}$

P = Phototoxic; NP = Non-phototoxic; P? = Possibly phototoxic.

to ~5% control, whereas ingredient X was neither phototoxic, nor significantly cytotoxic, at any concentration tested (up to 100%). The difference between the toxicity of ingredient X, with and without UV seen in the NRU PT assay did not, therefore, translate to a similar UV-induced toxicity to the 3-D model, even at 100 times the concentration previously used. This lack of activity in the skin culture model is likely to be related to the presence of an effective stratum corneum barrier function. Toxicity (and phototoxicity) to human skin is affected by penetration rates through the stratum corneum. In general, penetration rates of test materials through *in vitro* skin models are greater than those through human skin (Ponec *et al.*, 1990; Regnier *et al.*, 1992; Michel *et al.*, 1995; Doucet *et al.*, 1998, 1999). Therefore, 3-D skin models would be considered more sensitive to insult than human skin per se and the lack of activity of ingredient X is good evidence that it would not present a phototoxic hazard to human skin *in vivo*. Thus, the overall conclusion from these studies was that, although ingredient X shows a weak potential for photoactivation and photobinding *in vitro*, it was unlikely to present a hazard to human skin.

This example shows how a tiered strategy using *in vitro* assays can be used to provide information on the potential phototoxic hazard of a material which is then available for input, together with other toxicological hazard information, into an overall process for risk assessment. This is of particular help where, as in this case, there are inconclusive results with photoactivity at high concentrations only, the relevance of which to the *in vivo* situation is unknown. Table 10.4 compares the results for other materials tested in both the NRU PT and 3-D skin models and illustrates the different outcomes that can occur. Materials 1 and 2 were confirmed as non-phototoxic up to their maximum solubility. Materials 3–8 were confirmed as phototoxic to human skin models. Material 9 appeared phototoxic at high concentrations in the NRU PT but was non-phototoxic to a human skin model and on the other hand Material 10 was a borderline phototoxin in the NRU PT at low concentrations but was phototoxic in the skin model. These data show that there can be various outcomes to a testing strategy and illustrate the use of a very relevant model in a supplementary assay to help interpret the results of the other *in vitro* phototoxicity assays.

Summary

- Where substances are intended for uses where they come into contact with the skin, either accidentally or in products intended for skin application, it is necessary to carry out an assessment of potential phototoxic hazard.
- This assessment may now be carried out using a tiered testing strategy involving *in vitro* assays instead of the animal tests used historically.
- The initial test is always a UV/visible absorption spectrum to identify absorption by a test material at relevant wavelengths.
- Where testing is considered necessary the primary test is the validated 3T3 cell neutral red uptake phototoxicity test.
- Depending on the outcome of this assay, if further information is required then a photobinding test for photoallergy may be carried out and/or a phototoxicity test using a 3-D human skin model assay.
- The use of human skin models is advised to help interpret the results of other *in vitro* phototoxicity assays.

References

Api, A.M. (1997). *In vitro* assessment of phototoxicity. *In vitro Toxicology*, **10**: 339–350.

Augustin, C., Collombel, C. and Damour, O. (1997). Use of dermal equivalent and skin equivalent models for identifying phototoxic compounds *in vitro*. *Photodermatology, Photoimmunology and Photomedicine*, **13**: 27–36.

Barratt, M.D. and Brown, K.R. (1985). Photochemical binding of photoallergens to human serum albumin: a simple *in vitro* method for screening potential photoallergens. *Toxicology Letters*, **24**: 1–6.

Bernard, F.X., Barrault, C., Deguery, A. *et al.* (1999). Development of a highly sensitive phototoxicity assay using the reconstructed human epidermis SkinEthic, in *Alternatives to Animal Testing II*: Proceedings of the Second International Scientific Conference Organised by the European Cosmetic Industry, Brussels, Belgium (eds Clark, D., Lisansky, S. and Macmillan, R.), CPL Press, Newbury, UK, pp. 167–174.

Clothier, R., Willshaw, A., Cox, H. *et al.* (1999). The use of human keratinocytes in the EU/COLIPA international *in vitro* phototoxicity test validation study and the ECVAM/COLIPA study on UV filter chemicals. *ATLA*, **27**: 247–259.

Cohen, C., Dossou, K.G., Rougier, A. and Roguet, R. (1994). EpiSkin: an *in vitro* model for the evaluation of phototoxicity and sunscreen photoprotective properties. *Toxicology In vitro*, **8**: 669–671.

Corsini, E., Sangha, N. and Feldman, S.R. (1997). Epidermal stratification reduces the effects of UVB (but not UVA) on keratinocyte cytokine production and cytotoxicity. *Photodermatology, Photoimmunology and Photomedicine*, **13**: 147.

Doucet, O., Garcia, N. and Zastrow, L. (1998). Skin culture model: a possible alternative to the use of excised human skin for assessing *in vitro* percutaneous absorption. *Toxicology in vitro*, **12**: 273–283.

Doucet, O., Garcia, O. and Zastrow, L. (1999). Potential of skin culture models for assessing *in vitro* percutaneous absorption, in *Alternatives to Animal Testing II*: Proceedings of the Second International Scientific Conference Organised by the European Cosmetic Industry, Brussels, Belgium (eds Clark, D., Lisansky, S. and Macmillan, R.), CPL Press, Newbury, UK, pp. 246–249.

Edwards, S.M., Donnelly, T.A., Sayre, R.M., Rheins, L.A., Spielmann, H. and Liebsch, M. (1994). Quantitative *in vitro* assessment of phototoxicity using a human skin model: Skin^2. *Photodermatology, Photoimmunology and Photomedicine*, **10**: 111–117.

Epstein, J.H. (1974). Phototoxicity and photoallergy: clinical syndromes, in (Eds.), *Sunlight and Man: Normal and Abnormal Photobiologic Responses* (eds Fitzpatrick, T.B., Pathak, M.A., Harber *et al.*), University of Tokyo Press, pp. 459–477. EU (2000). Commission Directive 2000/33/EC, 25 April 2000, adapting to technical progress for the 27th time Council Directive 67/548/EEC on the approximation of laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances. ANNEX II: B.41. *In vitro* 3T3 NRU Phototoxicity Test. Official Journal of the European Communities L136, 90–107.

Johnson, B.E., Walker, E.M. and Hetherington, A.M. (1986). *In vitro* models for cutaneous phototoxicity, in *Skin models: models to study function and disease of skin* (eds Marks, R. and Plewig, G.), Springer-Verlag, Berlin, pp 264–281.

Jones, P., King, A., Lovell, W. and Earl, L. (1999). Phototoxicity testing using 3-D reconstructed human skin models, in *Alternatives to Animal Testing II*: Proceedings of the Second International Scientific Conference Organised by the European Cosmetic Industry, Brussels, Belgium (eds Clark, D., Lisansky, S. and Macmillan, R.), CPL Press, Newbury, UK, pp. 138–141.

Jones, P.A., Lovell, W.W., King, A.V. and Earl, L.K. (2001). *In vitro* testing for phototoxic potential using the EpiDermTM 3-D reconstructed human skin model. *Toxicology Methods*, **11**: 1–19.

Jones, P.A., King, A.V., Earl, L.K. and Lawrence R.S. (2003). An assessment of the phototoxic hazard of a personal product ingredient using *in vitro* assays. *Toxicology in vitro*, **17**: 471–480.

Lambert, L.A., Wamer, W.G. and Kornhauser, A. (1996). Animal models for phototoxicity testing (reprinted from Dermatotoxicology, 1996). *Toxicology Methods*, **6**: 99–114.

Liebsch, M., Döring, B., Donnelly, T.A. *et al.* (1995). Application of the human dermal model Skin^2 ZK 1350 to phototoxicity and skin corrosivity testing. *Toxicology in vitro*, **9**: 557–562.

Liebsch, M., Barrabas, C., Traue, D. & Spielmann, H. (1997). Entwicklung eines neuen *in vitro* Tests auf dermale Phototoxizität mit einem Modell menschlicher Epidermis, EpiDerm™. *Alternativen zu Tierexperimenten (ALTEX)*, **14**: 165–174.

Liebsch, M., Traue, D., Barrabas, C. *et al.* (1999). Prevalidation of the EpiDerm Phototoxicity Test, in *Alternatives to Animal Testing II*: Proceedings of the Second International Scientific Conference Organised by the European Cosmetic Industry, Brussels, Belgium (eds Clark, D., Lisansky, S. and Macmillan, R.), CPL Press, Newbury, UK, pp. 160–166.

Lovell, W.W. (1993). A scheme for *in vitro* screening of substances for photoallergenic potential. *Toxicology in vitro*, **7**: 95–102.

Lovell, W.W. and Jones, P.A. (2000). An evaluation of mechanistic *in vitro* tests for the discrimination of photoallergic and photoirritant potential. *ATLA*, **28**: 707–724.

Medina, J., Elsaesser, C, Picarles, V. *et al.* (2001). Assessment of the phototoxic potential of compounds and finished topical products using a human reconstructed epidermis. *In vitro and molecular toxicology*, **14**(3): 157–178.

Michel, M., Germain, L., Bélanger, P.M. and Auger, F.A. (1995). Functional evaluation of anchored skin equivalent cultured *in vitro*: percutaneous absorption studies and lipid analysis. *Pharmacological Research*, **12**: 455–458.

Mossman, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, **65**: 55–63.

OECD (1997). Environmental Health and Safety Publications, Series on Testing and Assessment No. 7, *Guidance Document On Direct Phototransformation Of Chemicals In Water*, Environment Directorate, OECD, Paris.

OECD (2004). *Test Guideline 432, In vitro 3T3 NRU phototoxicity test*. OECD, Paris.

Pendlington, R.U. and Barratt, M.D. (1990). Molecular basis of photocontact allergy. *International Journal of Cosmetic Science*, **12**: 91–103.

Ponec, M., Wauben-Penris, P.J.J., Burger, A. *et al.* (1990). Nitroglycerin and sucrose permeability as quality markers for reconstructed human epidermis. *Skin Pharmacology*, **3**: 126–135.

Regnier, M., Caron, D., Reichert, U. and Schaefer, H., (1992). Reconstructed human epidermis: a model to study *in vitro* the barrier function of the skin. *Skin Pharmacology*, **5**: 49–56.

Roguet, R., Cohen, C. and Rougier, A. (1994). A reconstituted human epidermis to assess cutaneous irritation, photoirritation and photoprotection *in vitro*, in *Alternative Methods in Toxicology*, Vol. 10, *In vitro Skin Toxicology – Irritation, Phototoxicity, Sensitization* (eds Rougier, A., Goldberg, A. and Maibach, H), Mary Ann Liebert, New York, USA, pp. 141–149.

Spielmann, H., Lovell, W. W., Hoelzle, E. *et al.* (1994a). *In vitro* phototoxicity testing. The report and recommendations of ECVAM workshop 2. *ATLA*, **22**: 314–348.

Spielmann, H., Balls, M., Brand, M. *et al.* (1994b). EEC COLIPA project on *in vitro* phototoxicity testing – first results obtained with a BALB/C 3T3 cell phototoxicity assay. *Toxicology In vitro*, **8**: 793–796.

Spielmann, H., Balls, M., Dupuis, J. *et al.* (1998a). The international EU/COLIPA *in vitro* phototoxicity validation study – results of phase II (blind trial) – Part 1 – The 3T3 NRU phototoxicity test. *Toxicology In vitro*, **12**: 305–327.

Spielmann, H., Balls, M., Dupuis, J. *et al.* (1998b). A study on UV filter chemicals from Annex-VII of European-Union Directive 76/768/EEC, in the *in vitro* 3T3 NRU phototoxicity test. *ATLA*, **26**: 679–708.

Spielmann, H., Muller, L., Averbeck, D. *et al.* (2000). The Second ECVAM Workshop on Phototoxicity Testing – The report and recommendations of ECVAM Workshop 42. *ATLA*, **28**: 777–814.

Stephens, T.J. and Bergstresser, P.R. (1985). Fundamental concepts in photoimmunology and photoallergy. *Journal of Toxicology: Cutaneous and Ocular Toxicology*, **4**: 193–218.

Zaynoun, S.T., Johnson, B.E. and Frain-Bell, W. (1977). The study of oil of bergamot and its importance as a phototoxic agent. *British Journal of Dermatology*, **96**: 475–482.

11 *In vitro* alternatives for irritation and corrosion assessment

Penny Jones

Safety and Environmental Assurance Centre, Colworth Science Park, Sharnbrook, Bedford, Bedfordshire MK44 1LQ, UK

Primary Learning Objectives

- *Validation status of assays for corrosion and irritation to replace the Draize rabbit skin test currently used for classification purposes.*
- *Details of the individual in vitro assays for corrosion and irritation.*

11.1 Introduction and scope

One of the main focuses of *in vitro* tests in skin toxicology is for the replacement of the *in vivo* Draize rabbit four-hour patch test (Draize *et al*., 1944) as the means of identifying materials which present a skin corrosion/irritation hazard. The development of these assays has recently been given impetus by European Union (EU) legislation, namely the 7th Amendment to the European Cosmetics Directive and the Registration, Evaluation and Authorisation of Chemicals (REACH) legislation, both of which are discussed elsewhere (Chapters 18 and 19). *In vitro* tests for skin corrosion have been validated whereas those for skin irritation have recently undergone validation processes but are not yet adopted into guidelines. This chapter describes these *in vitro* assays and their validation status.

11.2 Acute dermal irritation/corrosion

OECD Test Guideline 404 describes an *in vivo* rabbit test for skin corrosion/irritation and includes a testing strategy to minimise the number of animals required.

Corrosive materials cause irreversible damage of the skin whereas dermal irritation can be defined as the production of reversible skin damage (Table 11.1). Historically, the rabbit Draize test has been used for the classification of test materials which present a skin corrosion/irritation hazard (Draize *et al*., 1944). This test involves the application of undiluted material to the skin

Principles and Practice of Skin Toxicology Edited by Robert P. Chilcott and Shirley Price
© 2008 John Wiley & Sons, Ltd

Table 11.1 Summary of material classifications for the European Union (EU) and Globally Harmonised Systems (GHS)*

Material Classification	EU	GHS
Corrosive	R34/R35: Causes burns/severe burns	Category 1: Corrosive
Irritant	R38: Irritating to the skin	Category 2: Irritant Category 3: Mild irritant*

*GHS Category 3 overlaps with EU 'no classification'.

under semi-occluded conditions for up to four hours followed by assessments of erythema and oedema up to 72 hours in three rabbits. Guidelines for this assay are given in OECD Test Guideline 404 (TG404, OECD, 2002a), which is discussed in Chapter 19 (section 19.7.3). Within TG404, a skin irritation testing strategy is outlined in order to minimise the number of animals used for testing. This allows for classification of some test materials without testing *in vivo*, provided that some of the following information is available:

Existing data. Provided conclusive human or animal data are available, then the test material can be classified with no further testing.

Structure activity relationship (SAR) evaluation. If conclusive, SAR evaluations can predict classification without recourse to further testing being needed, but if prediction is negative further testing is required.

The pH/buffering capacity of test material. If pH ≤ 2 or ≥ 11.5), then the material can be classified as corrosive to skin with no further testing.

Systemic toxicity via the dermal route. If the test material has been determined to be highly toxic via the dermal route then no further testing needed; if not corrosive or irritating when tested up to a dose limit of 2000 mg kg^{-1} or higher using rabbits, then the material can be assumed to be not corrosive or not irritating to skin without further testing. However, if such information is not available or not conclusive then further testing is needed.

Validated and accepted in vitro/ex vivo tests for skin corrosion. If such assays indicate a positive result then corrosivity can be assumed and no further testing is required. However, if the result is negative then further testing is needed (NB: Validated tests are available as described below).

Validated and accepted in vitro/ex vivo tests for skin irritation. If the material tests positive then irritation to skin *in vivo* can be assumed and no further testing is needed. However, if the material tests negative then further testing is needed in an *in vivo* test (NB: Tests are currently undergoing validation, but not yet accepted, as described below).

In vivo test. If an initial test in one animal causes severe damage to skin then the material can be classified as corrosive without further testing. If no severe damage occurs in the first animal then a further confirmatory test in one or two animals is required for final classification.

11.3 Validation/regulatory status of *in vitro* assays for skin corrosion

Three *in vitro* tests for skin corrosion have been validated (the rat skin transcutaneous electrical resistance (TER) test, the human skin model corrosivity test and the Corrositex™ test).

Validation is the process whereby the reliability and relevance of a procedure are established for a particular purpose (Bruner *et al.*, 1996). An essential part of the validation process is the definition of a predictive model that converts each result of a test method into a prediction relevant to the purpose of the test method (in this case corrosive or non-corrosive). These predictions can then be tested during validation trials against known data to give an indication of the accuracy and precision of the outputs of the model. Prior to formal validation there is normally a prevalidation phase, which includes protocol refinement, protocol transfer and protocol performance. The objective of the prevalidation process is to ensure that any method included in a formal validation study adequately fulfils the criteria defined for inclusion in such a study, so that financial and human resources are used most efficiently. The European Centre for the Validation of Alternative Methods (ECVAM) was set up by the European Union in 1992[1] together with the ECVAM Scientific Advisory Committee (ESAC) to oversee the validation process for alternative assays within the European Union. The Interagency Co-ordinating Committee on the Validation of Alternative Methods (ICCVAM), supported by the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), performs a similar (but not identical) function in the United States[2]. The current ECVAM modular approach to validation is described in detail elsewhere (Hartung *et al.*, 2004).

Three *in vitro* methods for assessing skin corrosion have been validated and accepted into international regulations (the use of *in vitro* corrosivity assays is mandatory in the EU). These are: the *ex vivo* rat skin transcutaneous electrical resistance (TER) test; the human skin model corrosivity test; and the Corrositex™ test. The human skin model test uses 3-D reconstructed skin models consisting of primary human keratinocytes grown and differentiated at the air–water interface to form a multi-layered keratinised epithelium similar to human skin. Examples are the Episkin™ or EpiDerm™ models, commercially supplied by SkinEthic Laboratories (France) and MatTek Corporation (USA) respectively. Corrositex™ is a commercially available example of a test using an artificial membrane barrier to mimic effects on skin and is available from *In Vitro* International (USA).

The prevalidation of these assays was reported at the outcome of ECVAM Workshop 6 (Botham *et al.*, 1995). In this blind trial, the rat skin TER, a human 3-D skin model (Skin^2™, a now unavailable commercial model) and Corrositex™ were tested using 25 corrosives and 25 non-corrosives (classified by the *in vivo* rabbit test) by two or three laboratories per test. A formal validation study of these methods was then set up following on from recommendations made by this workshop. In this trial, three laboratories using each of the three assays tested 60 coded chemicals. A second human 3-D skin model (Episkin™) was included in the study (Barratt *et al.*, 1998; Fentem *et al.*, 1998).

The results of the study validated rat skin TER as being predictive of corrosivity in the *in vivo* rabbit test (Table 11.2). The rat skin TER was included by the European Union in Annex V of the Dangerous Substances Directive (EU, 2000) and an OECD guideline has been published (OECD Test Guideline 430; OECD 2002b).

Following the validation study, the ECVAM Scientific Advisory Committee (ESAC) concluded that the Corrositex™ test was a scientifically validated test, but only for those acids, bases and their derivatives which met the technical requirements of the assay (ICCVAM, 1999; ECVAM, 2001) (Table 11.3). Corrositex™ was not adopted into Annex V at the same time as the rat skin TER and human skin model tests, but it has been accepted by the US Department

[1]http://ecvam.jrc.it/index.htm
[2]http://iccvam.niehs.nih.gov

Table 11.2 Predictive capacity of *in vitro* corrosivity models demonstrated during validation (Fentem *et al.*, 1998; Liebsch *et al.*, 2000)

Assay	Sensitivity	Specificity
Rat skin TER	88%	72%
Corrositex™ *	71%	76%
Episkin™	83%	80%
EpiDerm™	88%	86%

Sensitivity = % *in vivo* irritants correctly identified by assay; Specificity = % *in vivo* non-irritants correctly identified by assay. *36% of samples non-qualified (i.e., could not be tested in assay).

Table 11.3 Corrositex™ prediction model

Mean breakthrough time (min)		Corrosivity prediction C/NC	EU risk phrase	UN packing group
Category 1	Category 2			
0–3	0–3	C	R35	I
>3–60	>3–30	C	R34	II
>60–240	>30–60	C	R34	III
>240	>60	NC	No label	N/A

C = Corrosive; NC = Non-corrosive; N/A = Not applicable.

of Transport (US DoT) and a draft test guideline (DTG435 *In Vitro* Barrier Membrane Test), based on the Corrositex™ test method, is under consideration by the OECD.

Unfortunately, during the skin corrosion validation trial, the Skin^2™ skin model ceased commercial production, despite the Episkin™ method being successfully validated. Further validation studies were subsequently carried out using an alternative commercial product; EpiDerm™ (Liebsch *et al.*, 2000). This highlights the importance of validating protocols or approaches that are not reliant on a single, commercially available model. The predictive capacities of these two assays, as found during the studies, are given in Table 11.2. The human skin model method was included by the European Union in Annex V (EU, 2000) and an OECD guideline has been published (OECD Test Guideline 431; OECD 2002c).

11.4 *In vitro* tests for skin corrosion

The rat skin TER corrosivity test uses measurement of electrical resistance as the primary endpoint. Corrosive materials are identified by their ability to produce a loss of normal stratum corneum integrity and barrier function, which is measured as a reduction in the TER below a threshold level (5 kΩ).

The rat skin TER test uses *ex vivo* rat skin as a test system (Oliver *et al.*, 1986). Full details of the recommended method can be found in TG430 (OECD, 2002b). Test materials are applied for up to 24 hours to the epidermal surfaces of triplicate skin discs (Figure 11.1). The skin

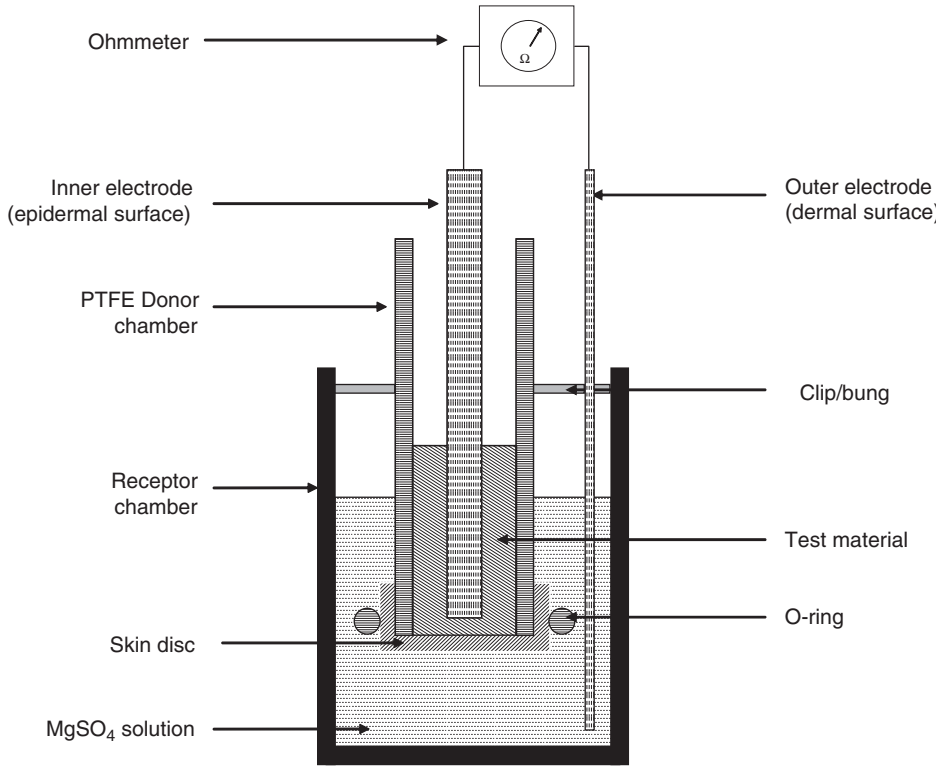


Figure 11.1 Schematic representation of apparatus for measuring transepidermal resistance. The (rat) skin sample is sealed onto the end of an (inner) PTFE tube using an o-ring with the dermal surface facing out. The outer tube is filled with magnesium sulfate solution. The inner (epidermal) surface is first exposed to the test material for 24 hours prior to wash-out and replacement with electrolyte solution (magnesium sulfate). Electrodes are positioned either side of the skin sample and resistance measured using a (Wheatstone bridge) ohmmeter

tissue is obtained (post mortem) from the dorso-lateral skin of 28–30 day old animals and is stripped of excess subcutaneous fat by carefully peeling it away from the skin. The age of the animals is important to ensure that the hair follicles are in the dormant phase before adult hair growth begins (see TG430 for a full description of tissue preparation).

Each skin disc (~20 mm diameter) is fixed over one end of a PTFE tube, with the epidermal surface in contact with the tube and fully submerged in a receptor chamber containing 154 mM of magnesium sulfate heptahydrate ($MgSO_4.6H_2O$) solution (Figure 11.1). Liquid test substances (150 μl) are applied uniformly to the epidermal surface inside the tube. When testing solid materials, a sufficient amount of the solid is applied evenly to the disc to ensure that the whole surface of the epidermis is covered. Hydrochloric acid (10 M) and distilled water are used as positive and negative control substances, respectively. Test substances are applied for 24 hours at 20–23°C. The test substance is removed by washing with a jet of tap water until no further material can be removed. The skin impedance is then measured as TER by using a low-voltage, alternating current Wheatstone bridge. For rat skin TER measured using the apparatus described in TG430, a value of 5 kΩ has been selected as a cut-off to

discriminate between test materials. Generally, materials that are non-corrosive in animals but are irritating or non-irritating do not reduce the TER below this cut-off value. A secondary dye-binding step is incorporated into the test procedure for confirmation testing of positive results in the TER, i.e., values $\leq 5\,k\Omega$. The dye-binding step determines if the increase in ionic permeability is due to physical destruction of the stratum corneum by a corrosive or is merely increased skin permeability due to the test material (this can be caused by some non-corrosive materials). The prediction model for the rat skin TER test is based on the following criteria:

1. The test substance is considered to be **non-corrosive** to skin if:
   a. the mean TER value obtained for the test substance is $\geq 5\,k\Omega$

      OR

   b. the mean TER value is $\leq 5\,k\Omega$ AND the skin disc is showing no obvious damage AND the mean disc dye content is well below the mean disc dye content of the 10 M hydrochloric acid positive control obtained concurrently.
2. The test substance is considered to be **corrosive** to skin if:
   a. the mean TER value is $\leq 5\,k\Omega$ AND the skin disc is obviously damaged

      OR

   b. the mean TER value is less $\leq 5\,k\Omega$ AND the skin disc is showing no obvious damage AND the mean disc dye content is greater than the mean disc dye content of the 10 M hydrochloric acid positive control obtained concurrently.

As stated above, different threshold values may apply if the test conditions are altered or a different apparatus is used. For this reason, TG430 provides set of suitable corrosive and non-corrosive reference chemicals to use when setting up the assay.

The rat skin TER method has shown to be predictive of corrosivity in the Draize rabbit test (TG404), which is conservative with respect to skin corrosivity and skin irritation when compared with the human skin patch test (Basketter *et al.*, 1997). The TER method can also be applied to excised human skin and some differences between human and rat skin have been reported (Whittle and Basketter, 1994).

> The Corrositex™ test assesses membrane barrier damage caused by a corrosive test material after the application to the surface of the artificial membrane barrier which is detected by a colour change to the indicator solution below the barrier.

Corrositex™ is a commercially available example of a membrane barrier test for corrosivity that employs as an endpoint the penetration of test material through a hydrogenated collagen matrix (biobarrier) and supporting filter membrane. This test method is composed of two components: a synthetic macromolecular bio-barrier (proteinaceous gel) and a chemical detection system (CDS). Membrane barrier damage caused by a corrosive test material after the application of the test material to the surface of the artificial membrane barrier is detected by the CDS, in this case a colour change to the indicator solution below the barrier. However, not all classes of test materials can be tested in the assay and there is an initial compatibility test to see if materials can be tested. Many non-corrosive chemicals and chemical mixtures and some corrosive chemicals and chemical mixtures do not qualify for testing (ICCVAM, 1999); test materials not causing a colour change in the CDS cannot be tested with Corrositex™.

The Corrositex™ compatibility test also classifies those materials which can be tested into two categories (1 and 2) depending on their acid/alkaline reserve. The time (in minutes) elapsing between application of the test substance to the membrane barrier and barrier penetration is used to classify the test material in terms of corrosivity and, if applicable, UN Packing Group (as shown in Table 11.3).

> In the 3-D human skin model corrosivity test, corrosive materials are identified by their ability to produce a decrease in cell viability below defined threshold levels at specified exposure periods.

The 3-D human skin model corrosivity assay is based on the hypothesis that corrosive chemicals are able to penetrate the stratum corneum by diffusion or erosion and are cytotoxic to the underlying cell layers. OECD TG431 provides guidance on the general and functional properties required of a skin model in order for it to be suitable for use in the test and on reference chemicals suitable for testing the predictive ability of the model. Following the original validation of the Episkin™ and EpiDerm™ models and publication of the guideline, data for two other human models (SkinEthic™ and CellSystems® EST1000) have been published showing their applicability for corrosivity testing (Kandarova *et al.*, 2006b; Hoffmann *et al.*, 2005). The SkinEthic™ model data have also been reviewed by ESAC and the assay endorsed as suitable for use (ECVAM, 2006).

Human skin model tests use cell viability as the endpoint, measured for example by reduction of MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide or thiazolyl blue) (Mossman, 1983). Uptake and reduction of MTT (producing a colour change from yellow to blue) is carried out by live cells only, and so a decrease in this activity in cells cultured with test materials is an indication of cell death.

Test materials are applied to the surface of the 3-D skin model and corrosive materials are identified by their ability to produce a decrease in cell viability below defined threshold levels at specified exposure periods. General advantages of the use of 3-D skin models in providing a good model of human skin are discussed in Chapter 10 using EpiDerm™ as the example. For comparison, Figure 11.2 shows a vertical section of the Episkin™ epidermal model. In

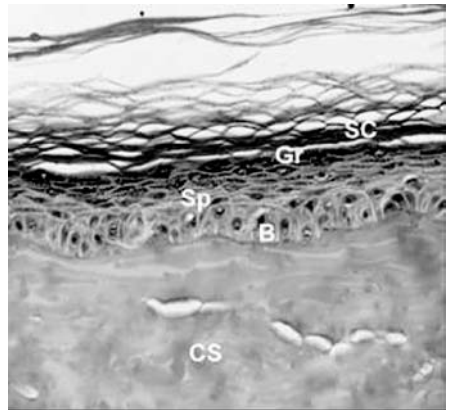


Figure 11.2 Vertical section through Episkin™ culture showing collagen support (CS), basal layer (B), spinosum layer (Sp), granulosum layer (Gr) and stratum corneum (SC)

this case, the basal cell layer grows on a collagen support (rather than a filter used for other models) and differentiates into spinosum and granulosum layers and stratum corneum at the upper surface. The different models vary as to their exact size in terms of surface area and plastic support, which in turn determines the volumes of reagents and test material used for each model (see specific references for details). Test materials are usually tested as supplied or at the specific concentration required to be classified. The general method for the assay is as follows:

- Place cultures in plates with medium to acclimatise (37°C; 5% CO_2 in air).
- Expose surfaces of duplicate cultures to test materials for three minutes (ambient temperature) and 60 minutes (plus four hours for Episkin™ assay) at 37°C; 5% CO_2 in air (include negative and positive controls).
- Rinse cultures thoroughly with PBS.
- Transfer cultures to plates with fresh medium containing MTT (0.5 or 1 mg m^{-1}l), then incubate for three hours (37°C; 5% CO_2 in air).
- Rinse cultures with PBS, then extract blue colour with isopropanol or acidified isopropanol.
- Measure absorbance of extracts in triplicate 200 µl samples at 570 nm and calculate results as percentage of MTT reduction by negative control cultures.
- The results are acceptable if the positive control reduces mean culture viability by the required amount.

The prediction models used with the EpiDerm™ and Episkin™ to predict the corrosivity of test materials from the assay results are given in Tables 11.4 and 11.5 respectively. The EpiDerm™ and Episkin™ models have been shown to discriminate reliably between corrosives and non-corrosives (Liebsch *et al.*, 2000; Fentem *et al.*, 1998). In addition, Episkin™ was also shown in the validation trial to discriminate between European Union classifications R34 (corrosive) and R35 (severe corrosive) or UN Packing Groups Corrosive Classes I, II and III. However, laboratories wishing to use this more complex prediction model should ensure that it is applicable to the types of sample when setting up the assay.

An example of data using the EpiDerm™ model is shown in Figure 11.3. MTT reduction by treated cultures is compared with that for negative control cultures treated with ultrapure water. Potassium hydroxide (KOH; 8 N) is used as a positive control for the EpiDerm™ assay. The criterion for a valid result for this positive control is that it should reduce the mean viability to <30% of control after three minutes; the results shown a reduction to 15% viability, which

Table 11.4 Prediction model for EpiDerm™ corrosivity test

Predicted classification	Criteria for *in vitro* interpretation
Corrosive	If viability <50% after three minutes exposure or viability ≥50% at three minutes and <15% after 60 minutes exposure
Non-corrosive	If viability ≥50% after three minutes exposure and ≥15% after 60 minutes exposure

Table 11.5 Prediction model for Episkin™ corrosivity test

Classification system	Packing group/ risk phrase	Criteria for *in vitro* interpretation
EU	Corrosive class R35	If viability <35% after three minutes exposure
	Corrosive class R34	If viability ≥35% after three minutes exposure and <35% after 60 minutes exposure or If viability ≥35% after 60 minutes exposure and <35% after four hours exposure
	Non-corrosive	If viability ≥35% after four hours exposure
UN	Corrosive class I	If viability <35% after three minutes exposure
	Corrosive class II	If viability ≥35% after three minutes exposure and <35% after 60 minutes exposure
	Corrosive class III	If viability ≥35% after 60 minutes exposure and <35% after four hours exposure
	Non-corrosive	If viability ≥35% after four hours exposure

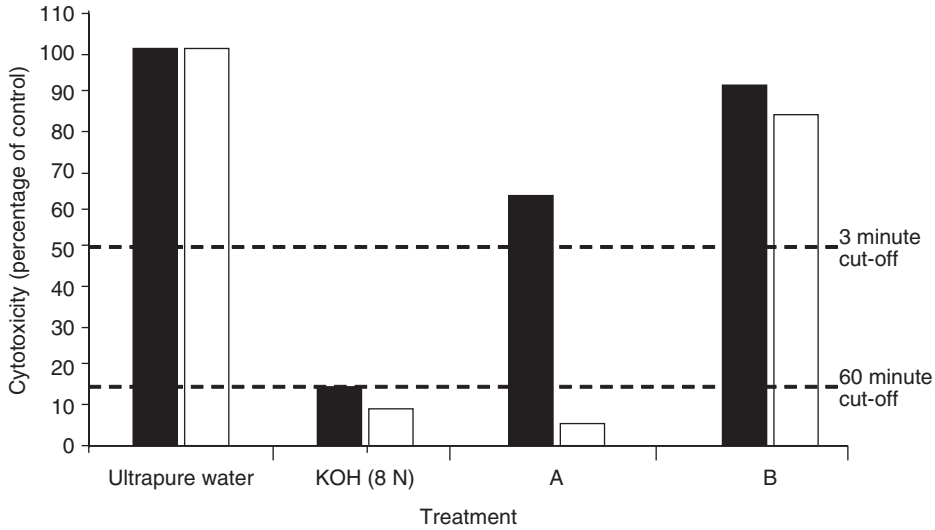


Figure 11.3 Example results for EpiDerm™ corrosivity test expressed as cytotoxicity after three minutes (□) and 60 minutes (■) exposure to test materials A (corrosive) and B (non-corrosive) (Data are expressed as mean of duplicate culture)

would be considered valid. Material A is predicted as being corrosive as although viability was >50% after three minutes, viability after 60 minutes was reduced to 5% (<15%). Material B is much less cytotoxic and after 60 minutes the viability is 88%, which is still >50%. This material would therefore be predicted as being non-corrosive.

An additional control measure for the skin models assays is to check that the test material does not interact directly with MTT (causing a colour change). This can be carried out by

simply incubating a sample of test material (the same volume or weight as used in the assay) directly with the MTT solution; direct reduction of MTT can be clearly seen as the production of a blue/purple solution. If such a test material is not completely removed from the skin by rinsing, then direct reduction of MTT by the test material may mimic that of cellular metabolism and lead to a false estimate of viability (Liebsch *et al.*, 2000). Correction for such test substance interference with the viability measurement can be made by taking 'killed' (previously frozen) cultures with no intrinsic metabolism through the assay, with and with out test material, and subtracting any absorbance obtained from these cultures from the normal control and test material results.

11.5 Validation/regulatory status of *in vitro* assays for skin irritation

A validation study of *in vitro* assays for skin irritation using human skin models has recently been completed and reviewed by ECVAM's Scientific Advisory Committee (ESAC).

An ECVAM-sponsored validation study of *in vitro* methods for acute skin irritation (Fentem and Botham, 2002) has recently been completed and reviewed by ESAC. Prior to this validation, study work was started in 1999 on prevalidation of the then most promising tests (Botham *et al.*, 1998). It is interesting to note that the tests which were considered all involved the use of complex *in vitro* or *ex vivo* models of skin rather than simple monolayer cell cultures. These were two human 3-D skin model cytotoxicity protocols using EpiDerm™ and Episkin™, Prediskin™ (measuring cytotoxicity with *ex vivo* human skin cultures), the non-perfused pig ear model (measuring transepidermal water loss (TEWL) from the skin surface) and the *in vitro* mouse skin integrity function test (SIFT), which uses TEWL and electrical resistance as the endpoints. The conclusion from this prevalidation study was that none of the tests were ready for progression to formal validation (Fentem *et al.*, 2001). Various follow-up activities then took place, which enabled some of the test protocols to meet the criteria for inclusion in a formal validation study (Zuang *et al.*, 2002). The protocols for the two human 3-D skin models, EpiDerm™ and Episkin™, were harmonised to allow a common approach for skin models to be investigated (Portes *et al.*, 2002; Cotovio *et al.*, 2005; Kandarova *et al.*, 2004, 2005). Further details of the human skin model protocols are given below. The SIFT data analysis and prediction model were also modified to enable it to take part in the validation trial (Heylings *et al.*, 2003), but the pig ear model was not included at this stage. The formal validation trial was sponsored by ECVAM.

Phase 1 of the validation study involved testing a set of 20 chemicals (nine irritants and 11 non-irritants) under 'blind' conditions with the three assays by the respective lead laboratories only (Liebsch *et al.*, 2005). However, the predictive ability of the SIFT was not considered sufficient for it to progress to the next phase in which only the 3-D skin models were tested. The performance of the two 3-D skin model tests was considered sufficiently promising for them to progress to Phase 2, experimental testing of 58 coded chemicals (25 irritants and 33 non-irritants) by six laboratories (three per skin model), which took place between October 2004 and April 2005. Since then, independent statistical analysis and management team review of the data and review of the trial outcome by ESAC have taken place (ECVAM, 2007). The review outcome was positive in recommending 3-D skin model assays for assessing skin

irritation (see details for the individual assays below). This successful outcome is likely to lead to eventual adoption into international regulations and guidelines. It is also now likely that other available skin models will undergo performance testing for their suitability for the same approach. Indeed, some data for the SkinEthic™ model have recently been published (Kandarova *et al.*, 2006a).

11.6 *In vitro* tests for skin irritation

3-D human skin model assay protocols for skin irritation under validation involve a short sample application time of 15 minutes followed by removal by rinsing with PBS, then a recovery incubation of 42 hours before cytotoxicity measurement using MTT reduction; IL-1α release may be a useful supplementary endpoint.

Assays using 3-D human skin models have proved to be the most promising for assessment of skin irritation *in vitro*, as shown during the recent ECVAM skin irritation validation trial discussed above. For the trial, a harmonised protocol was used for both the EpiDerm™ and Episkin™ models (Portes *et al.*, 2002; Cotovio *et al.*, 2005; Kandarova *et al.*, 2004, 2005). The general methods were similar to those given above for the 3-D skin model corrosivity assay, but with an altered treatment regime. This approach used a short test material application time of 15 minutes followed by removal by rinsing with PBS, then a recovery incubation of 42 hours before cytotoxicity measurement using MTT reduction as the primary endpoint. The aim of the validation study was the discrimination of those materials that would be classified as irritants (R36) from non-irritants (no classification). However, there will be a retrospective assessment of the data with respect to prediction of the three Globally Harmonised System (GHS) categories (irritant/mild irritant/no classification; OECD, 2001).

Test materials were predicted as irritant if they reduced mean culture viability to <50% of the negative controls. In addition to cytotoxicity (measured by MTT reduction), a second endpoint (release of the cytokine IL-1α measured after the 42 hour post-incubation period), was explored for the Episkin™ model as a secondary predictor of irritants for those chemicals predicted as non-irritant by the MTT assay (Table 11.6). The results of applying these prediction models are given in Table 11.7. Use of the additional endpoint in the Episkin™ assay increased the sensitivity of the assay (correct prediction of *in vivo* irritants). Both the sensitivity and specificity (correct prediction of *in vivo* non-irritants) of the Episkin™ assay were acceptable and as such the Episkin™ assay (MTT assay) has been recommended by ESAC as a replacement for the Draize skin irritation test for the purposes of distinguishing between R38 (skin irritating) and no label (non-skin irritating) test substances (ECVAM, 2007). The IL-1α endpoint has been recommended as an adjunct assay to confirm negatives obtained with the MTT endpoint. The sensitivity (57%) of the EpiDerm™ assay was much lower than the specificity (84%); IL-1α release was initially investigated by the lead laboratory for the EpiDerm™ assay but was not found to be useful in improving these results. These results mean that a high proportion of materials gave a false negative classification using EpiDerm™. Therefore, ESAC recommended that, although an irritant classification was likely to be reliable, a negative classification using the EpiDerm™ assay may require further testing (ECVAM, 2007).

Although formal validation of *in vitro* skin irritation assays has only recently been completed, protocols using 3-D human skin models have been used for a number of years for screening and ranking of ingredients (de Fraissinette *et al.*, 1999; Perkins *et al.*, 1999; Tornier *et al.*, 2006;

Table 11.6 Prediction models used for ECVAM skin irritation validation trial

Classification system	Predicted classification	Criteria for *in vitro* interpretation
EpiDerm™ and Episkin™ 1	Irritant	If viability ≤50% control
	Non-irritant	If viability >50% control
Episkin™ 1	Irritant	If viability ≤50% control or If viability >50% and IL1-α release >60 pg ml^{-1}
	Non-irritant	If viability >50% control and IL1-α ratio release ≤60 pg ml^{-1}

Table 11.7 Predictive capacity of *in vitro* 3-D human skin model skin irritation assays demonstrated during ECVAM skin irritation validation trial (ECVAM, 2007)

Assay	Sensitivity	Specificity
Episkin™ (MTT endpoint)	75%	81%
Episkin™ (MTT + IL-1α endpoints)	91%	79%
EpiDerm™ (MTT endpoint)	57%	84%

Sensitivity = % *in vivo* irritants correctly identified by assay; Specificity = % *in vivo* non-irritants correctly identified by assay.

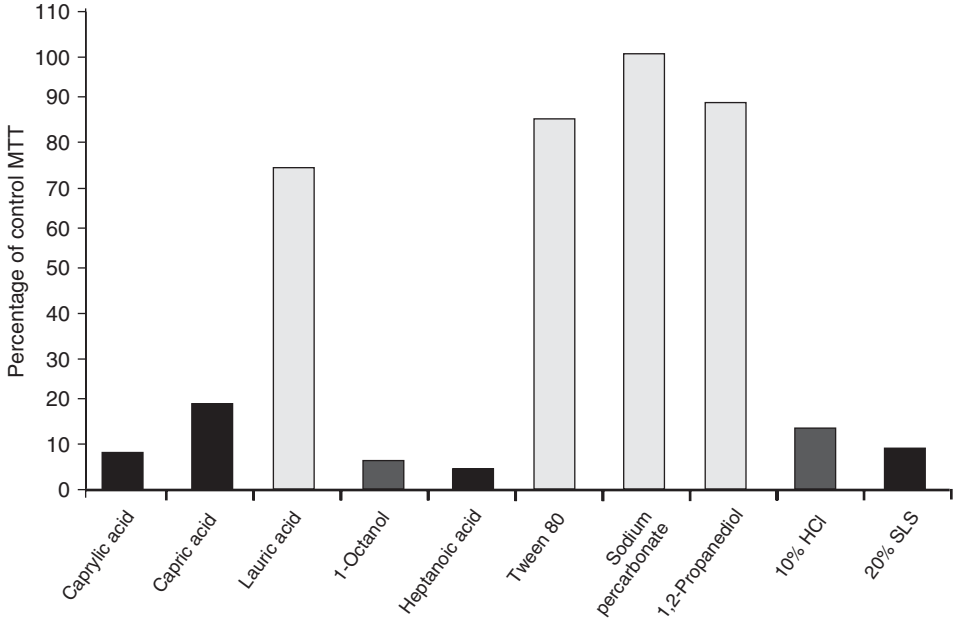


Figure 11.4 Results for irritants (■), slight irritants (■) and non-irritants (□) using an *in vitro* EpiDerm™ human skin model protocol (15 minutes exposure to test material followed by 18 hours recovery incubation) (Data are mean expressed as mean of duplicate cultures)

Table 11.8 Results of screening potential hair product ingredients using an EpiDerm™ human skin model protocol (SDS = Sodium dodecyl sulfate)

Test material	% control MTT reduction	Prediction of skin irritancy
20% SDS	9.3%	Irritant
Code 1	97.9%	Non-irritant
Code 2	91.5%	Non-irritant
Code 3	12.1%	Irritant
Code 4	95.9%	Non-irritant
Code 5	54.1%	Border line irritant?
Code 6	94.6%	Non-irritant
Code 7	95.6%	Non-irritant
Code 8	96.0%	Non-irritant
Code 9	81.7%	Non-irritant

Kejlova *et al.*, 2005) and products (Koschier *et al.*, 1997; Perkins *et al.*, 1999; Faller *et al.*, 2002; Tornier *et al.*, 2006). An example of data obtained using the EpiDerm™ model for some chemicals of known irritation potential (Basketter *et al.*, 1997) in a protocol similar to that in the current validation trial (15 minutes exposure to test material followed by 18 hours recovery incubation) is shown in Figure 11.4. The non-irritants were clearly identified and separated from the irritants by a cut-off value of 50% cytotoxicity (MTT assay). However, the irritants and slight irritants were not differentiated. The assay was further used to investigate the potential irritancy of some potential proprietary (coded) ingredients for hair products, the results for which are shown in Table 11.8. Most of the materials were non-cytotoxic and so would be predicted as non-irritant. However, Material 3 was clearly cytotoxic (similar to the positive control; 20% sodium dodecyl sulfate) and so would be predicted as irritant. Material 5 was a borderline material in the assay. This kind of data can be used to prioritise and rank materials for further development. These 3-D skin models also lend themselves to the testing of formulations (Perkins *et al.*, 1999; Faller *et al.*, 2002; Tornier *et al.*, 2006) to provide information on potentially irritancy prior to human studies.

Summary

- **The current focus of *in vitro* tests in skin toxicology is to replace the *in vivo* Draize rabbit four-hour patch test as the means of identifying materials which present a skin corrosion/irritation hazard.**
- **OECD Test Guideline 404 provides for the use of validated *in vitro* assays in a strategy for skin corrosion/irritation testing.**
- **The development of these assays has also recently been given impetus by EU legislation (the 7th Amendment to the European Cosmetics Directive and the Registration, Evaluation and Authorisation of Chemicals (REACH) legislation).**
- ***In vitro* tests for skin corrosion (rat skin transcutaneous electrical resistance (TER) test, the human skin model corrosivity test and Corrositex™) have been validated and adopted into testing guidelines.**
- ***In vitro* tests for skin irritation using 3-D human skin model protocols are in validation.**
- **3-D human skin model tests can also be used to screen and rank materials to help prioritisation.**

References

Barratt, M.D., Brantom, P.G., Fentem, J.H. *et al.* (1998). The ECVAM international validation study on *in vitro* tests for skin corrosivity. 1. Selection and distribution of the test chemicals. *Toxicology In Vitro*, **12**: 471–482.

Basketter, D.A., Chamberlain, M., Griffiths, H.A. *et al.* (1997). The classification of skin irritants by human patch test. *Food and Chemical Toxicology*, **35**: 845–852.

Botham, P.A., Chamberlain, M., Barratt, M.D. *et al.* (1995). A prevalidation study on *in vitro* skin corrosivity testing. The report and recommendations of ECVAM Workshop 6. *ATLA*, **23**: 219–255.

Botham, P.A., Earl, L.K., Fentem, J.H. *et al.* (1998). Alternative methods for skin irritation testing: the current status. ECVAM Skin Irritation Task Force Report 1. *ATLA*, **26**: 195–211.

Bruner, L.H., Carr, G.J., Chamberlain, M. and Curren R.D. (1996). Validation of Alternative Methods for Toxicity Testing. *Toxicology In Vitro*, **10**: 479–501.

Cotovio, J., Grandidier, M.H., Portes, P. *et al.* (2005). The *in vitro* acute skin irritation of chemicals: Optimisation of the EPISKIN prediction model within the framework of the ECVAM validation process. *ATLA*, **33**: 329–349.

de Fraissinette, A., Picarles, V., Chibout, S. *et al.* (1999). Predictivity of an *in vitro* model for acute and chronic skin irritation (SkinEthic) applied to the testing of topical vehicles. *Cell Biology Toxicology*, **15**: 121–135.

Draize, J.H., Woodard, G. and Calvery, H.O. (1994). Methods for the study of imitation and toxicity of substances applied topically to the skin and mucous membranes. *Journal of Pharmacology and Experimental Therapeutics*, **82**: 377–390.

ECVAM (2001). Statement on the application of the CORROSITEX® assay for skin corrosivity testing. *ATLA*, **29**: 96–97 (also available on http://ecvam.jrc.it/).

ECVAM (2006). Statement on the application of the SkinEthic™ human skin model for skin corrosivity testing. *ATLA*, 17 Nov. (also available on http://ecvam.jrc.it/).

ECVAM (2007). Statement on the validity of *in vitro* tests for skin irritation. *ATLA*, 27 Apr. (also available on http://ecvam.jrc.it/).

EU (2000). Annex I to Commission Directive 2000/33/EC adapting to technical progress for the 27th time Council Directive 67/548/EEC on the approximation of laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances. *Official Journal of the European Union*, **L136**: 91–97.

Faller, C., Bracher, M., Dami, N. and Roguet, R. (2002). Predictive ability of reconstructed human epidermis equivalents for the assessment of skin irritation of cosmetics. *Toxicology In Vitro*, **16**: 557–572.

Fentem, J.H. and Botham, P.A. (2002). ECVAM's activities in validating alternative tests for skin corrosion and irritation. *ATLA*, **30**(Suppl. 2): 61–67.

Fentem, J.H., Archer, G.E.B., Balls, M. *et al.* (1998). The ECVAM international validation study on *in vitro* tests for skin corrosivity. 2. Results and evaluation by the Management Team. *Toxicology In Vitro*, **12**: 483–524.

Fentem, J.H., Briggs, D., Chesné, C. *et al.* (2001). A prevalidation study on *in vitro* tests for acute skin irritation: results and evaluation by the Management Team. *Toxicology in Vitro*, **15**: 57–93.

Hartung T., Bremer S., Casati S. *et al.* (2004). A Modular Approach to the ECVAM Principles on Test Validity. *ATLA,* **32**: 467–472.

Heylings, J.R., Diot, S., Esdaile, D.J. *et al.* (2003). A prevalidation study on the *in vitro* skin irritation function test (SIFT) for prediction of acute skin irritation *in vivo*: results and evaluation of ECVAM Phase III. *Toxicology in Vitro*, **17**: 123–138.

Hoffmann, J., Heisler, E., Karpinski, S. *et al.* (2005). Epidermal-skin-test 1000 (EST-1000) – A new reconstructed epidermis for *in vitro* skin corrosivity testing. *Toxicology in Vitro*, **19**: 925–929.

ICCVAM (1999). *Corrositex®: an in vitro test method for assessing dermal corrosivity potential of chemicals.* NIH Publication No. 99-4495, NIEHS, Research Triangle Park, NC, USA.

Kandarova, H., Liebsch, M., Genschow, E. *et al.* (2004). Optimisation of the EpiDerm test protocol for the upcoming ECVAM validation study on *in vitro* skin irritation tests. *ALTEX*, **21**: 107–114.

Kandarova, H., Liebsch, M., Gerner, I. *et al.* (2005). The EpiDerm test protocol for the upcoming ECVAM validation study on *in vitro* skin irritation tests – An assessment of the performance of the optimised test. *ATLA*, **33**: 351–367.

Kandarova, H., Liebsch, M., Schmidt, E. (2006a). Assessment of the skin irritation potential of chemicals by using the SkinEthic reconstructed human epidermal model and the common skin irritation protocol evaluated in the ECVAM skin irritation validation study. *ATLA,* **34**: 393–406.

Kandarova, H., Liebsch, M., Spielmann, H. *et al.* (2006b). Assessment of the human epidermis model SkinEthic RHE for *in vitro* skin corrosion testing of chemicals according to new OECD TG 431, *Toxicology in Vitro*, **20**: 547–559.

Kejlova, K., Labsky, J., Jirova, D. and Bendova, H. (2005). Hydrophilic polymers – biocompatibility testing *in vitro*. *Toxicology in Vitro*, **19**: 957–962.

Koschier, F.J., Roth, R.N., Wallace, K.A. *et al.* (1997). A comparison of three-dimensional human skin models to evaluate the dermal irritation of selected petroleum products, *Toxicology in vitro*, **10**: 391–405.

Liebsch, M., Traue, D., Barrabas, C. *et al.* (2000). The ECVAM prevalidation study on the use of EpiDerm for skin corrosivity testing. *ATLA,* **28**: 371–401.

Liebsch, M., Botham, P., Fentem, J. *et al.* (2005). The ECVAM validation study of three *in vitro* methods for acute skin irritation – Interim report of the validation management team. *Naunyn-Schmiedebergs Archives of Pharmacology*, **371**: R124-R124 518 Suppl. 1.

Mossman, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, **65**: 55–63.

OECD (2001). *Harmonised Integrated Classification System for Human Health and Environmental Hazards of Chemical Substances and Mixtures*. OECD series on testing and assessment, Organisation for Economic Cooperation and Development, Paris. France.

OECD (2002a). OECD Guideline for Testing of Chemicals. No. 404: Acute Dermal Irritation, Corrosion (revised version, as adopted on 24 April 2002), plus Annex and Supplement. Organisation for Economic Cooperation and Development, Paris, France.

OECD (2002b). OECD Guidelines for the Testing of Chemicals No. 430. *In vitro* Skin Corrosion: Transcutaneous Electrical Resistance Test (TER). Organisation for Economic Cooperation and Development, Paris, France.

OECD (2002c). OECD Guidelines for the Testing of Chemicals No. 431. *In vitro* Skin Corrosion: Human Skin Model Test. Organisation for Economic Cooperation and Development, Paris, France.

Oliver, G.J.A., Pemberton, M.A. and Rhodes, C. (1986). An *in vitro* skin corrosivity test – modifications and validation. *Food and Chemical Toxicology*, **24**: 507–512.

Perkins, M.A., Osborne, R., Rana, F.R. *et al.* (1999). Comparison of *in vitro* and *in vivo* human skin responses to consumer products and ingredients with a range of irritancy potential. *Toxicological Sciences*, **48**: 218–229.

Portes, P., Grandidier, M-H., Cohen, C. and Roguet, R. (2002). Refinement of the EPISKIN™ protocol for the assessment of acute skin irritation of chemicals: follow-up to the ECVAM prevalidation study. *Toxicology In Vitro*, **16**: 765–770.

Tornier, C., Rosdy, M. and Maibach, H.I. (2006). *In vitro* skin irritation testing on reconstituted human epidermis: Reproducibility for 50 chemicals tested with two protocols. *Toxicology in Vitro*, **20**: 401–416.

Whittle, E. and Basketter, D.A. (1994). *In vitro* skin corrosivity test using human skin, *Toxicology in Vitro,* **8**: 861–863.

Zuang, V., Balls, M., Botham, P.A. *et al.* (2002). Follow-up to the ECVAM Prevalidation Study on *In vitro* Tests for Acute Skin Irritation. ECVAM Skin Irritation Task Force Report 2. *ATLA,* **30**: 109–129.

12 Instruments for measuring skin toxicity

Helen Taylor

Enviroderm Services, North Littleton, Evesham, WR11 8QY, UK

Primary Learning Objectives

- *Overview of currently available instrumentation, applications and guidelines for use.*
- *Potential advantages and disadvantages of biophysical skin analysis.*

12.1 Introduction and scope

Traditionally, evaluation of skin toxicity has been subject to visual assessment by experienced individuals. Over the past two decades, technological advances have provided alternative techniques for measuring skin damage that offer a more objective, fully quantitative and (generally) operator-independent analysis of skin damage. Such instruments operate using a variety of biophysical principles.

'Biophysical skin analysis' (or 'skin bioengineering') is the term used to describe the application of scientific instruments for measuring physical skin characteristics. Skin bioengineering is an area which has seen rapid growth over the last two decades, both in terms of the range of available equipment and their increasing applications within dermatotoxicology, dermatology and cosmetology (Beradesca and Distante 1996). Many such techniques are non-invasive and allow the study of skin in real time without damaging or affecting the skin itself (Kligman 1995). The techniques that are becoming available offer innovative ways of conducting skin research that will undoubtedly contribute further to our knowledge of cutaneous physiology. There are many parameters that can now be assessed using biophysical skin analysis. Most techniques require controlled conditions because small changes in ambient conditions can have a significant effect on skin physiology. For example, in a warm environment an individual will have greater blood flow and sweat gland activity leading to an increase in transepidermal water loss (TEWL) and hydration values (Goh 1995). The two main advantages of biophysical instruments are that, when used correctly, they can provide an objective assessment of skin properties and can detect changes which are not visible to the naked eye. The latter benefit is of clinical relevance for detecting subclinical changes in skin function and represents a significant development in the detection and treatment of common skin disorders such as irritant contact dermatitis.

Principles and Practice of Skin Toxicology Edited by Robert P. Chilcott and Shirley Price
© 2008 John Wiley & Sons, Ltd

There are a wide variety of instruments available to measure aspects of skin function. As an introduction to the subject, this chapter is necessarily limited to a relatively small range of techniques. Other, more comprehensive texts should be consulted for further information (Elsner *et al.* 1998; Fluhr *et al.* 2004; Wilhelm *et al.* 2006). The particular techniques that will be considered include skin surface pH, biomechanics (primarily representative of the dermis), sebum measurement, skin surface features, skin thickness, desquamation, hydration (primarily representative of the epidermis), transepidermal water loss (TEWL), skin colour and blood flow.

As each parameter provides information on one specific aspect of skin function, it is more common to find studies where several, complimentary parameters have been measured simultaneously; this provides a more comprehensive analysis of skin condition and, when used in combination with traditional techniques such as histological analysis of skin sections, facilitates a clinical interpretation of the results.

12.2 Skin surface pH

Measurement of skin surface pH is used to assess the acidity of the skin's surface which determines the state of the acid mantle (also referred to as the stratum corneum acid buffer system) and the protective function that this provides (Fluhr *et al.* 2006). Measurement of pH also provides information about the health of the skin's natural microflora. It should be noted that skin surface pH can vary according to the time of day (circadian variation), skin site (anatomical variation; Figure 12.1) and between individuals (inter-individual variation) (Fluhr *et al.* 2006). There are several instruments available for measurement of skin pH (Table 12.1), although any standard, portable pH meter with a planar electrode may suffice.

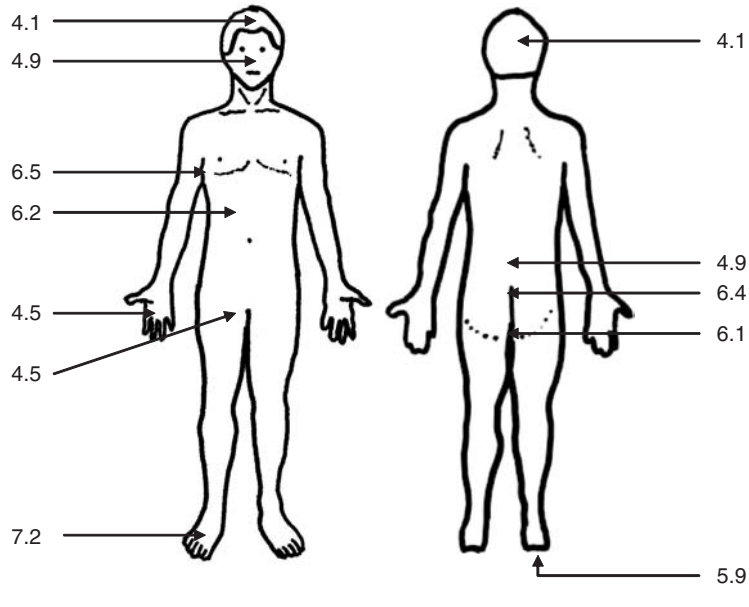


Figure 12.1 Variation of skin surface pH over the human body

Table 12.1 Examples of commercially available skin measurement devices (other instruments are available!)

Skin Characteristic	Instrument type/principle	Examples of Commercial Instruments	
		Model	Manufacturer
Skin surface pH	pH meter	Skin pHmeter	Courage & Khazaka
		827pH Lab	Metrohm
Biomechanical	Suction	Cutometer	Courage & Khazaha
		Dermalab Elasticity Module	Cortex Technology
	Twistometry	Dermal Torque Meter	Dia-Stron
	Levarometry	Not commercially available	n/a
	Indentometry	Torsional Ballistometer	Dia-Stron
	Acoustic wave propogation	Reviscometer	Courage & Khazaka
Sebum	Photometric	Sebumeter	Courage & Khazaka
	Adhesive patch method	Sebutape	CuDerm Corporation
	Photometric	Dermlab Sebum Module	Cortex Technology
	Adhesive patch method	Sebufix	Courage & Khazaka
Thickness	Ultrasound	Dermscan C	Cortex Technology
Transepidermal water loss (TEWL)	Open chamber	Tewameter	Courage & Khazaka
		Evaporimeter	Servo Med
		Dermlab TEWL	Cortex Technology
	Closed chamber; vapour accumulation	Vapometer	Delfin Technologies
	Closed chamber; ventillated	SKD1000	Skinos Co
	Closed chamber; condenser	Aquaflux	Biox Systems
Skin hydration	Capacitance	Corneometer	Courage & Khazaka
		Moisture Sense	Moritex USA Inc
	Conductance	Skicon-200EX	I.B.S. Ltd
		Dermlab Moisture Module	Cortex Technology
	Impedance	Nova Dermal Phase Meter	Nova Technology
Confocal Microscopy	Raman Spectroscopy	Skin Composition Analyzer	River Diagnostics
Skin Colour	Tri-stimulus	Mexameter	Courage & Khazaka
		Erythema Meter	Dia-Stron
		Chromameter	Minolta
	Spectrophotometry	CM-503i	Minolta
Blood flow	Narrow field (point flowmetry)	Laser Doppler Monitor DRT4	Moor Instruments
		Periflux 5000	Perimed
	Wide field (imaging)	MoorLDI	Moor Instruments
		Periscan	Perimed

12.3 Biomechanical properties

Measurements of the biomechanical properties of the skin are used to gain information about the elasticity of the skin. This information can be used to assess the ageing of the skin and for assessment of conditions such as scleroderma, connective tissue disorders, psoriasis and acute oedema. There are a variety of instruments available based on different methods for measuring the mechanical properties of the skin and include suction, twistometry, levarometry, indentometry and the use of acoustic waves (Table 12.1).

12.3.1 Suction

This method applies a partial vacuum to pull the skin into an aperture and then release it. The height to which the skin is pulled into the aperture and the rate at which it comes out of the aperture are dependent on the mechanical properties of the skin. This method can provide information about the elasticity and viscoelastic properties of the skin (Barel *et al.* 2006; Grove *et al.* 2006; O'goshi 2006b).

12.3.2 Twistometry

This method assesses the mechanical properties of the skin through distortion when a disc is stuck to the skin and then twisted (Agache 2006). It provides information about the elasticity and viscosity of the skin.

12.3.3 Levarometery

Levarometry is a technique that gains information about the mechanical properties of the skin through measuring the force required to lift the skin (Dikstein and Fluhr 2006b), although commercial instruments are not yet currently available. This technique assesses the 'slackness' of the skin and can be used to distinguish between young and old skin and can identify the effects of chronic sun exposure.

12.3.4 Indentometry

With indentometry, pressure is applied to the skin with a measuring rod. The pressure required to cause indentation of the skin is related to the mechanical properties of the skin. This technique can be used to assess skin softness, which is related to the water status of the dermis (Dikstein and Fluhr 2006a). It can be also be used to discriminate between young and old skin due to age-related differences in the amount and quality of elastin and collagen fibres of the dermis.

12.3.5 Acoustic wave

The acoustic wave method uses the time taken for the passage of a sound wave through the skin to provide information about the mechanical properties of the skin. The propagation of

acoustic waves through skin tissue is dependent on a number of factors (such as tissue density and water content) that can potentially be affected by a variety of pathological conditions or age-related changes.

12.4 Sebum

Sebum is produced by the sebaceous gland and is the main component of skin surface lipids (Elsner 1995) (Chapter 1). It is thought to have several functions, among which is controlling moisture balance in the stratum corneum (Wood 1985). The production of sebum by sebaceous glands is controlled by androgens and so sebum measurement has been used to investigate skin conditions that may be subject to hormonal influence, such as acne and dry skin.

Sebum can be measured using a gravimetric or a variety of photometric techniques. Acquisition of sebum samples from the skin surface is generally achieved through the use of absorptive tapes which can then be weighed (gravimetric analysis) or subject to a photometric analyses (such as 'grease-spot' photometry or UV–Visible spectrophotometry in the presence or absence of a disclosing dye). Both gravimetric and photometric techniques can provide a measure of the amount of sebum per square centimetre, although the latter can also provide additional information, such as droplet size and distribution (Cunliffe and Taylor 2006; El Gammal *et al.* 2006; O'goshi 2006a).

12.5 Skin surface contours

Skin surface contours can be measured using mechanical profilometry, laser profilometry, replica analysis and high resolution ultrasound. This wide variety of measurements can be used to gain information about skin surface texture, wrinkles, wounds, aging and skin disease, thus enabling a quantitative assessment of the extent of damage and also the effect of a treatment or product.

12.6 Thickness

Skin thickness measurement can be used as an auxiliary parameter to patch tests and in the clinical evaluation of scleroderma, tumours and atrophy. Commercial devices are available for measuring skin thickness (e.g. based on ultrasound) but the traditional method (a measuring calliper applied to a skin fold) is still in common use, although calliper measurements may also include subcutaneous fat.

12.7 Desquamation

Desquamation is the loss of superficial stratum corneum (stratum dysjunctum). It is a natural process, but can be affected by disease. Desquamation can be used to assess normal skin physiology, to identify skin disease and investigate skin disease processes. It is also a useful technique for interpreting the action of drugs. Techniques for desquamation measurement usually involve either stripping layers of the surface for analysis or visual assessment through skin imaging.

12.8 Applications and measurement of transepidermal water loss

The measurement of transepidermal water loss (TEWL) has become ubiquitous in studies requiring an evaluation of skin barrier function. There are a variety of different technical approaches to measuring TEWL rates.

Water passes through the skin to keep the outer layers sufficiently hydrated. This water ultimately evaporates from the surface of the skin and this is termed transepidermal water loss (TEWL), defined as the constitutive evaporation of water from the skin surface in the absence of sweat gland activity. Transepidermal water loss differs from skin surface water loss (SSWL) in that it is not produced 'on demand' from sweat glands as part of the thermoregulatory process. Since the water has to pass through the stratum corneum it is commonly assumed to be representative of basal skin barrier function (Distante and Beradesca 1995b; Lévêque 2002; Pinnagoda and Tupker 1995) and has been used to predict susceptibility of the skin to irritants (Agner 1994; Pinnagoda *et al.* 1989) and in the assessment of chemical injury (Chilcott *et al.* 2000), barrier creams (Chilcott *et al.* 2007) and restorative treatments (Mao-Qiang *et al.* 1996). However, it should be noted that an increase in the rate of TEWL does not necessarily equate to damage of the stratum corneum (Chilcott *et al.* 2002a, 2002b, 2002c), especially where there are concomitant changes in skin hydration or temperature. Conversely, the topical application of occlusive formulations to damaged skin may directly reduce evaporation of water from the skin surface, and so in this instance a reduction in TEWL rates is not necessarily indicative of restoration of skin barrier function.

There are four primary measurement methods for measurement of TEWL, termed 'open chamber', 'vapour accumulation closed chamber', 'ventilated closed chamber' and 'condenser chamber'.

12.8.1 Open chamber

This is one of the most established techniques and is based on Fick's law of diffusion. The measuring chamber comprises a hollow cylinder, with two pairs of sensors measuring humidity and temperature (Figure 12.2). The geometric positioning of the two sensors enables measurement of the water vapour gradient within the chamber, from which a TEWL rate can be calculated (Khazaka 2003).

12.8.2 Vapour accumulation closed chamber

The vapour accumulation closed chamber technique for TEWL measurement consists of a closed cylindrical chamber that contains sensors for relative humidity and temperature. Following application to the skin, there is a linear increase of relative humidity (RH%) within the chamber (Figure 12.3). It is this increase in RH% that is used to calculate the TEWL rates (Nuutinen *et al.* 2003).

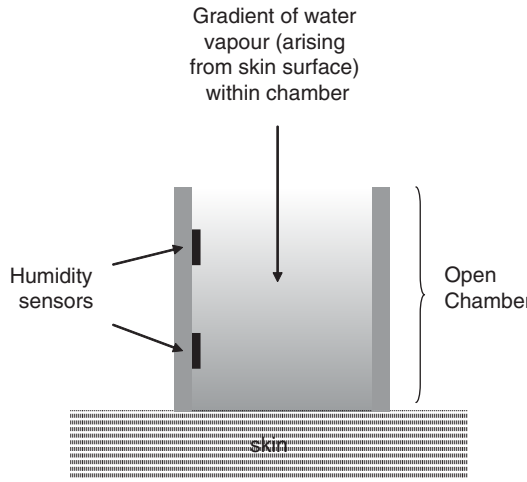


Figure 12.2 Schematic representation of an open chamber probe, indicating position of two humidity sensors (which also measure temperature) which measure the concentration of water vapour at that point arising as a result of evaporation from the skin surface

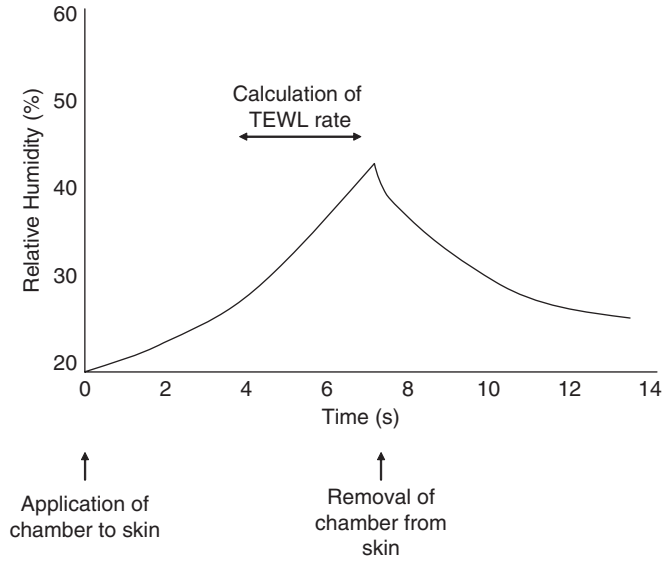


Figure 12.3 Relative humidity (measured within probe chamber) during and after application to the skin

12.8.3 Ventilated closed chamber

The ventilated closed chamber technique measures TEWL using a flow principle (Figure 12.4). It is a closed chamber technique with a flow of ambient air into the device, which is measured for humidity. This air passes through a chamber that is placed on the skin with TEWL being

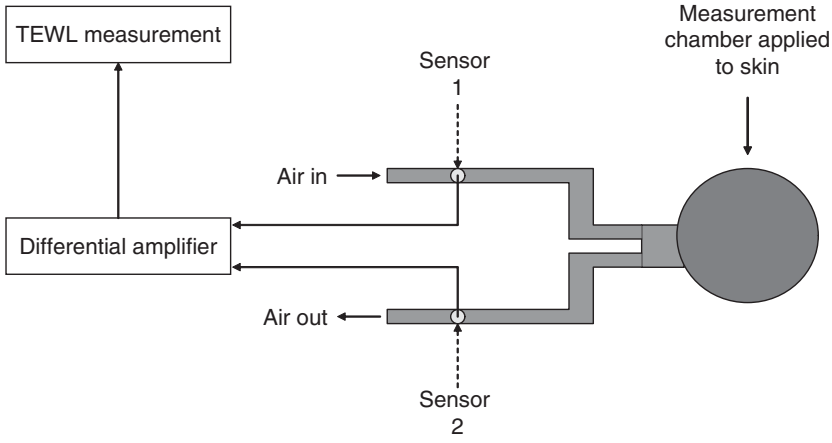


Figure 12.4 Schematic representation of a ventilated, closed chamber measurement principle (Skinos product information). The chamber is applied to the skin. Air is pumped into the chamber and passes a temperature/humidity sensor (sensor 1). The air then passes through the measurement chamber and past a second temperature/humidity sensor (sensor 2). The difference in humidity between the two sensors is measured using a differential amplifier and enables calculation of the corresponding TEWL rate

removed by this flow of air. The air is measured for humidity before being released back into the environment. It is the difference between the humidity before and after contact with the skin that allows the calculation of TEWL rate (Skinos 2002).

12.8.4 Condenser chamber

The condenser chamber technique is the most recent method for measuring TEWL. It is a closed chamber technique with a cold plate that condenses the moisture into ice using a Peltier system. This removal of moisture from the chamber avoids the moisture build up that occurs in unventilated chambers (Figure 12.3). There is one sensor in the chamber (Figure 12.5); the cold plate replaces the second sensor that is normally associated with TEWL measurement devices (Imhof 2001).

12.9 Guidance for TEWL measurements

Many instruments that measure TEWL are sensitive to certain types of experimental artefacts. Therefore, it is essential that TEWL measurements are conducted under standardised conditions using standardised protocols wherever possible.

There are a number of factors to consider when making TEWL measurements. Firstly the measurement of TEWL is dependent on ambient relative humidity, barrier integrity, temperature and stratum corneum thickness. Ambient relative humidity is a key factor: if

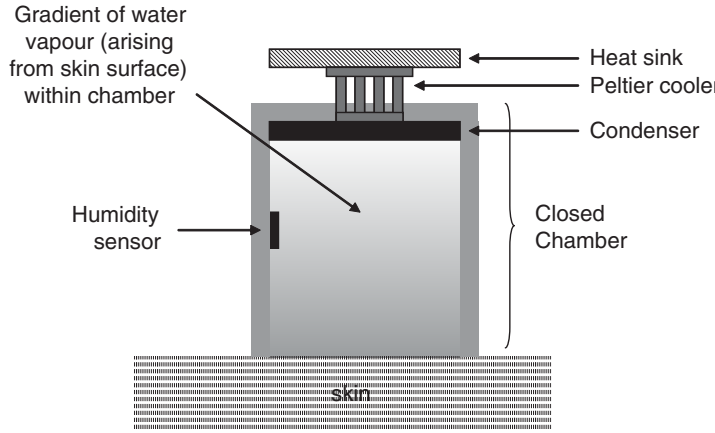


Figure 12.5 Measurement principle of the condenser chamber. A gradient of water vapour is maintained within the measurement chamber by the use of a condenser, powered by a Peltier cooler. Imposition and maintenance of the water gradient by the condenser allows the use of a single sensor to measure humidity and temperature (and thus calculation of TEWL)

the measurement is being made in conditions where the ambient humidity is high, e.g. 90%, then evaporation from the surface of the skin will be much reduced and will lower TEWL rates. The physical integrity of the skin (barrier) will influence measurements, but this may be the factor being investigated. Temperature (body and environmental) will lead to changes in TEWL owing to the effect on the rate of evaporation of water. Stratum corneum thickness is also thought to affect TEWL by altering the distance over which the water must diffuse before evaporation (assuming of course that diffusion is the rate-limiting step, not the rate of evaporation from the skin surface).

Sweat gland activity has a significant effect on TEWL measurements, as instruments cannot distinguish between water vapour arising from TEWL or sweat. For this reason, a number of studies have been performed on volunteers following inhibition of sweat glands by topical application of hyoscine (a muscarinic antagonist). However, instruments that measure TEWL can be used for investigations into sweat gland activity and the effect of products or treatments on their activity.

A précis of current guidelines for measuring TEWL rates is given in Table 12.2.

12.10 Hydration measurement

Hydration is arguably a key measurement for assessment of dermal toxicity (English *et al.* 1999). The hydration status of the stratum corneum is of interest because of the effect it has on barrier function, mechanical properties and drug penetration (Distante and Beradesca 1995a). Both the overall hydration and the hydration gradient within the stratum corneum can be measured. There are several instruments available for measuring hydration, including electrical methods (capacitance, conductance and impedance), optothermal transient emission radiometry (OTTER) and confocal Raman spectroscopy, all of which provide an indirect measure of stratum corneum hydration (Wilhelm 1998).

Table 12.2 General summary of main recommendations for measurement of TEWL rates (Rogiers 2001; Serup 1994; Reproduced with permission, S. Karger AG, Basel)

Volunteer	Use homogenous groups Normal skin areas* 15–30 minutes acclimatisation Dorsal aspect of volar forearm is preferred site with contra-lateral site controls* No concomitant use of topical medicines, cosmetics, etc. Avoid taking measurements in extreme seasonal weather Skin surface should be horizontal during measurements
Operator/instrument	Ideally, same operator throughout study Apply light pressure (to maintain skin contact) between probe and skin Instrument should be set to zero before each measurement session*
Environment	Temperature 20–22°C (±1°C) Relative humidity <60%, ideally 40% Measurement probe should be same temperature as skin test site Reduce or eliminate air turbulence (e.g. enclose measurement in open-top box). TEWL probe should not be touched before or during measurements No direct light sources Conduct single experiment in one session

12.10.1 Electrical methods

Capacitance

Capacitance measurements work by the skin and the probe forming a variable capacitor: changes in the dielectric properties of the skin (resulting from alterations in water content) lead to changes in measured capacitance (Figure 12.6). It is the change in capacitance that enables a calculation of water content.

Conductance

Conductance measurements also use changes in dielectric properties of the skin. A small electric current is passed through the skin and hydration is calculated (using an algorithm) from the measured skin conductance: the greater the conductance the more water is present and so an arbitrary value for the water content of the skin can be calculated.

Impedance

The impedance measurement method is based on the same electrical changes as with conductance and capacitance. However, it is the impedance of the electrical current that is measured instead of the conductance (Distante and Beradesca 1995a). The method works by emitting a span of frequencies from which the impedance is measured at several frequencies

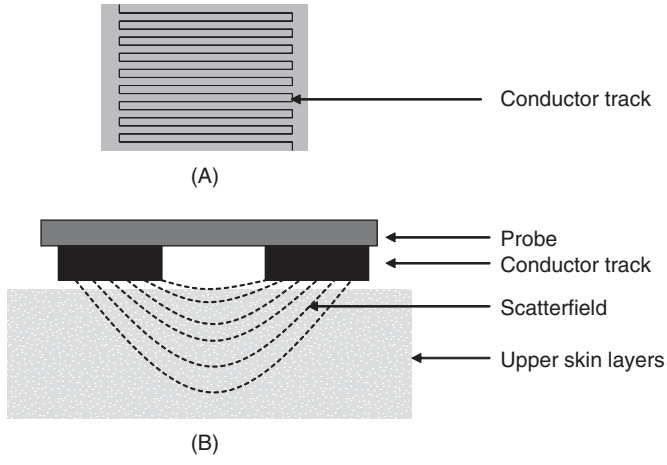


Figure 12.6 Geometry of conductor tracks on a capacitance probe surface (A; front view), with side view (B) of two tracks indicating electric field in relation to upper skin layers (Courage and Khazaka product information)

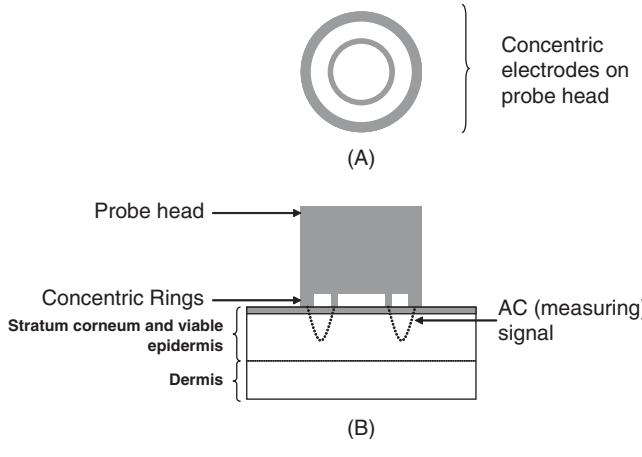


Figure 12.7 Principle of impedance measurement for determination of skin hydration. Two (concentric) electrodes (A; front view) are placed onto the skin surface. Application of an alternating current between the electrodes generates an electric field (B; side view) from which impedance measurements (and hence hydration) are acquired

using concentric electrodes (Figure 12.7) (Wickett 2005). The measurement value relates to the inverse of the overall impedance and increases with increasing skin hydration (Wickett 2005).

12.10.2 Optothermal transient emission radiometry (OTTER)

This is a non-invasive method that is used to acquire information about the stratum corneum, including overall hydration and hydration gradient. The technique uses a laser impulse to

transiently heat the skin. It is only the temperature near the surface of the sample that increases (and by a very small amount). The temperature increase results in an increase in the emission of infrared radiation. The temperature then decays to a steady state with a corresponding decrease in the emitted radiation. The emitted radiation is sensed by an infrared detector (cooled with liquid nitrogen). The detector produces a decay curve signal from which averaging of the signals from many laser pulses produces transient emission decay curves. The decay curves provide information about the properties of the skin sample (Cowen 1999). At present, this technique is limited to laboratory prototypes.

12.10.3 Confocal Raman spectroscopy

Confocal Raman spectroscopy measures the water concentration in the skin. This technique uses a low power laser light that is focused onto the skin by a specially designed microscope objective. Light reflected back through inelastic (Raman) scattering is collected in a spectrometer and provides a skin depth resolution of $<5\,\mu m$. The Raman spectrum produced is representative of the molecular composition of the skin being measured, with signals in the $2500-4000\,cm^{-1}$ spectrum being representative of bond stretching (vibration) in water, proteins and lipids. Water concentration can be determined from the intensity ratio of two spectral intervals within the C–H (carbon–hydrogen bond) and O–H (oxygen–hydrogen bond) stretching regions. By altering the depth of focus, the technique can be used to generate information on the concentration of water in the upper epidermis.

12.11 Guidance for hydration measurements

Hydration measurements require similar standardised procedures as TEWL measurements.

There are a number of factors that can potentially influence measurement of skin hydration and these are broadly similar to those described for TEWL measurements (Table 12.2). Thus, when designing studies for hydration measurements it is important that these factors are controlled as much as possible, with environmental conditions remaining stable for the duration of measurements and for different measurement times. Other factors include human influences, such as stress, which can result in perspiration. Clearly, sweating will influence any hydration measurements and no hydration measurement technique is able to distinguish between the water that is bound in the skin and the water that is passing through (as is the case with perspiration).

When making skin hydration measurements, acclimatisation of the individual to the measurement conditions will be necessary (Bircher *et al.* 1994; Fullerton *et al.* 2002; Serup 1995; Wilhelm 1998; Zuang and Beradesca 1998). A variety of times have been used for acclimatisation, with 15 or 20 minutes being most common (Bircher *et al.* 1994; Fullerton *et al.* 2002; Pinnagoda *et al.* 1990; Zuang and Beradesca 1998).

Regional (anatomical) variation can also be a factor in skin hydration measurements. Therefore, it is important to have a robust experimental design to account for such influences. For example, when using multiple sites on the volar forearm, hydration decreases from the wrist to the elbow; an appropriately balanced study design can reduce the effects of regional variation, for example, by the use of a Latin square to designate treatments to each site.

12.12 Relationship between hydration and dermal toxicity

Measurement of skin hydration has been used to characterise normal and diseased skin. It is used extensively for product efficacy testing as well as product safety evaluation. As a general rule of thumb, abnormal skin exhibits decreased hydration[1]; this is certainly the case with common disorders such as solar actinosis (sun damage) and psoriasis. Acute and chronic skin irritation also result in decreased skin hydration, owing to a combined loss of surface lipids and water-holding substances (so-called 'natural moisturising factors'). Measuring the decrease in hydration can be an effective parameter for investigating irritant contact dermatitis in the workplace: as an individual nears their threshold for irritant contact dermatitis, the inability to bind as much water can be readily measured and so remedial action can be taken before the clinical onset of signs and symptoms.

Hydration of the stratum corneum can be used to characterise the response of normal skin to external influences. This can be important when investigating the effects of a topical product or a working environment. Measurements can also be readily performed on healthy subjects to investigate seasonal influences on skin hydration or regional (anatomical) variations. There have also been studies investigating sex and age relationships with skin hydration (Diridollou *et al.* 2000; Swindt *et al.* 1998) and inter-individual variability of hydration measurements has been well documented (Agner and Serup 1990).

12.13 Colour measurement

Quantification of skin colour is one area where biophysical skin analysis can outperform human evaluations. Contemporary equipment is sensitive, accurate and highly reproducible. Colour measurements are particularly suited to assessing skin irritation.

The two most abundant skin chromophores are haemoglobin (in red blood cells) and melanin; changes in the concentration of either can occur as a result of pathological or physiological responses to external stimuli. Thus, objective skin colour measurement can be a useful adjunct to toxicological studies. For example, quantification of skin colour can be used during the assessment of patch test results (to quantify erythema or blanching) as well as in the clinical assessment of dermatitis, phototype and photosensitivity. Objective skin colour measurements can also be used to quantify the therapeutic effects of skin treatments.

Instrumentation to measure skin colour can be broadly categorised as skin reflectance colorimetry (SRC) or skin reflectance spectroscopy (SRS). Colorimetry generally requires a 'tri-stimulus' device whereas reflectance spectroscopy, as its name implies, requires a spectrophotometer. The basic mode of operation of both devices is similar: the skin is transiently illuminated with light from a well characterised source. Light reflected from within the skin is directed onto a suitable photodetector. The difference between incident and reflected light provides a measure of absorption by skin chromophores such as melanin and haemoglobin. Colorimeters tend to limit their emitted light to three specific elements of the visible spectrum (hence 'tri-stimulus') whereas a spectrophotometer detects and measures reflected light across the whole visible spectrum (400–700 nm). Although generally cheaper

[1]Pathological conditions resulting in an increase in skin hydration can be occasionally observed during the early stages of topical infections in experimental wounds.

than spectrophotometers, tri-stimulus devices may be subject to illuminant metameric failure (i.e. measuring the 'wrong' colour), an effect caused by certain types of indoor lighting (Box 12.1).

Box 12.1 Metamerism

Metamerism

Ever bought a pair of shoes in a shop because you thought the colour was a good match for your suit and then when you've worn them outside find that the colour changed? If so, you've been the subject of illuminant metameric failure!

Put simply, metamerism is an object appearing to take on a different colour when viewed under a different source of light. Fluorescent light tubes are a common cause of metamerism as the light given off is not constant across the visible spectrum.

Skin colour is most commonly expressed in terms of the CIELAB (Commission Internationale de L'eclairage 'Lab') colour scale (Figure 12.8), although other indices of colour such as XYZ, Munsell, RGB (red, green, blue), Hunter-Lab or Swedish National Colour Scale (SNCS) are sometimes used.

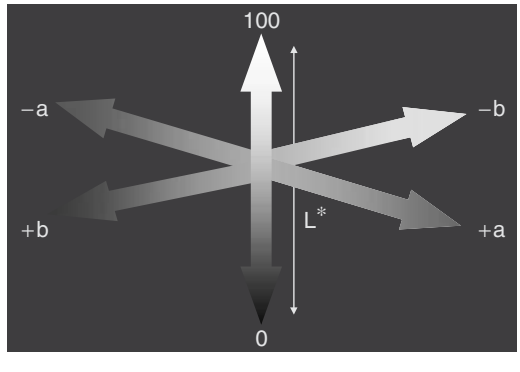


Figure 12.8 CIELAB colour scale. The three basic parameters represent the red–green index (a*), blue–yellow index (b*) and brightness (L*). The a* and L* parameters are most commonly used to quantify erythema and pigmentation, respectively. A full-colour version of this figure appears in the colour plate section of this book

12.13.1 Guidelines for colour measurement

Measurement of erythema (redness) and blanching (the opposite of erythema) are affected by changes in blood flow. Therefore, it is important to control aspects that will result in alterations of vascular perfusion. Similarly, ambient temperature and humidity will impact on colour measurement as will air convection and ambient light (particular instruments subject to metamerism). Stress (or nervousness) on the part of the individual being measured along with exercise and medication can also impact on the measurements through the vasodilation or vasoconstriction.

12.14 Measurement of vascular perfusion

Quantifying changes in vascular perfusion is useful for investigating a range of toxicological end points, including irritation and burn depth assessment.

Blood flow (or more precisely, cutaneous microcirculation) is an important parameter because of the role of the vasculature in thermoregulation and immune defence. In particular, an increase in vascular perfusion can arise as a result of skin irritation caused by the release of inflammatory mediators such as cytokines and histamine. Blood flow measurements can also be used for clinical investigations into disorders such as rosacea and in assessing the depth of chemical, thermal or radiation-induced burns.

At present, the most commonly used instruments for measuring the cutaneous microcirculation are based on laser Doppler velocimetry/flometry (LDV or LDF) or laser Doppler imagery (LDI) (Lomuto *et al.* 1995). These techniques use a laser light source and the Doppler principle (Figure 12.9) to get a relative measure of blood flow (flux). A Doppler shift is observed when

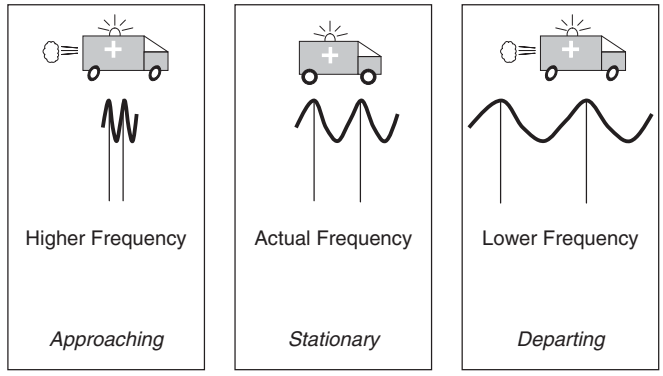


Figure 12.9 When an ambulance rapidly approaches, an observer hears the siren at a higher pitch than after it passes. This is the Doppler effect and is due to an increase or decrease in the perceived frequency of the sound wave resulting from the motion of the vehicle relative to the observer. The greater the vehicle's velocity, the greater the Doppler shift. Laser Doppler instruments are sufficiently sensitive to measure the Doppler shift in blood cells moving through the cutaneous vasculature. The same effect occurs in the Universe; galaxies observed through a telescope appear to be 'red-shifted' due to their movement away from the Earth. This is an example of astrophysics meeting skin biology!

the incident laser beam is reflected by blood cells flowing within the vasculature. The blood flux is proportional to the number of erythrocytes times their velocity (Wahlberg and Lindberg 1995) and is often defined as being the rate of blood flow across a given area (Essex and Byrne 1991; Moor 2005a, 2005b). The laser light source is usually a helium–neon laser producing 632 nm wavelength light (Bircher 2006).

Laser Doppler flowmetry relies on an optical fibre to transmit the laser light to the tissue and allows for continuous assessment of blood flow at a single point. Laser Doppler imagery is a scanning technique in which the laser pulse is rapidly moved across the surface at discrete points of the skin to build up an image of the blood flow in over a wider area. Point measurement devices (LDF) are less expensive than LDI but do not provide information over as wide an area.

A second type of instrument for blood flow measurement is capillary microscopy, which uses a light microscope or videomicroscope to visualise the capillaries (Wahlberg and Lindberg 1995). A drop of oil is placed on the skin area of interest to make the skin more transparent and a microscope image or video recording is made of the skin (Jünger *et al.* 1995). The images that are observed can be assessed for morphology and capillary distribution (Jünger *et al.* 1995). Vascular morphology and capillary distribution can be abnormal in damaged skin and so this technique has been used to evaluate microcirculation in conditions such as scleroderma and psoriasis, microvascular disorders such as Raynauds syndrome and for the assessment of irritation reactions (Humbert *et al.* 2006; Jünger *et al.* 1995).

A similar method to capillary microscopy is fluorescence microscopy; it can be used to investigate morphological and dynamic changes in the microvasculature of the skin and for analysis of trans-capillary exchange (Wahlberg and Lindberg 1995). A fluorescein dye (often sodium fluorescein) is injected intravenously and subsequently appears in the capillaries of the skin (Jünger *et al.* 1995; Wahlberg and Lindberg 1995) where it can be imaged using ordinary videomicroscopy when illuminated by ultraviolet light. Fluorescent imaging has been used to quantify the severity of chemically-induced skin damage (Braue *et al.* 2007).

Photoplethysmography (PPG) is a technique based on light scattering, similar to laser Doppler flowmetry but predating it by about 40 years (Bernardi and Leuzzi 1995). In PPG there is a continuous recording of the light intensity that is scattered from the skin and collected by a detector. The light scattering occurs as a result of absorption by haemoglobin in the red blood cells in the capillaries.

12.15 A final word of caution

Whilst biophysical instruments have many advantages over traditional methods, it is imperative that they are used correctly and subject to proper validation.

The application of new or improved technologies to measure various aspects of skin function have made an enormous contribution to the understanding of cutaneous biology. However, it is essential that users of such instruments understand the basic principles on which they work in order to be able to properly interpret the results. Having a device which reports that some aspect of skin function has changed by a certain number of arbitrary units is essentially useless if the biological relevance of the change is undefined or uncertain. It should also be noted that many instruments have their origins in the cosmetics industry, and so thorough validation

should be conducted to ensure that biophysical instruments are suitable for toxicological applications. In the words of the dermatologist Albert Kligman, 'A fool with a tool is still a fool'.

Summary

- There are many methods for the assessment of skin parameters that can be used to investigate dermal toxicity.
- Such techniques provide a means of objectively evaluating specific properties of the skin and are of immense benefit for investigative research and toxicological assessments.
- Many bioengineering methods are subject to operational guidelines that enable an objective and quantitative assessment of dermal toxicity.
- It is important to validate and fully understand the limitations of individual types of instrument.

References

Agache, P.G. (2006). Twistometry Measurement of Skin Elasticity, in *Handbook of Non-Invasive Methods and the Skin* (eds Serup, J., Jemec, G.B.E. and Grove, G.L.), CRC Press, Boca Raton London New York, pp. 601–611.

Agner, T. (1994). Prediction of Skin Irritation by Noninvasive Bioengineering Methods, in *Hand Eczema* (eds Menné, T. and Maibach, H.I.), CRC Press, Boca Raton London New York, pp. 131–140.

Agner, T. and Serup, J. (1990). Individual and instrumental variation in irritant patch-test reactions – clinical evaluation and quantification by bioengineering methods. *Clinical and Experimental Dermatology*, **15**: 29–33.

Barel, A.O., Courage, W. and Clarys, P. (2006). Suction Chamber Method for Measurement of Skin Mechanics: The New Digital Version of the Cutometer, in *Handbook of Non-Invasive Methods and the Skin* (eds Serup, J., Jemec, G.B.E. and Grove, G.L.), CRC Press, Boca Raton London New York, pp. 583–591.

Beradesca, E. and Distante, F. (1996). Bioengineering: Methods, in *The Irritant Contact Dermatitis Syndrome* (eds Van der Valk, P.G.M. and Maibach, H.I.) CRC Press, Boca Raton London New York, pp. 313–316.

Bernardi, L. and Leuzzi, S. (1995). Laser Doppler Flowmetry and Photoplethysmography: Basic Principles and Hardware, in *Bioengineering of the Skin: Cutaneous Blood Flow and Erythema*, (eds Beradesca, E., Elsner, P. and Maibach, H.I.), CRC Press, Boca Raton, pp. 31–55.

Bircher, A. (2006). Laser Doppler Measurement of Skin Blood Flux: Variation and Validation, in *Handbook of Non-Invasive Methods and the Skin* (eds Serup, J., Jemec, G.B.E. and Grove, G.L.), CRC Press, Boca Raton London New York, pp. 691–696.

Bircher, A., de Boer, E.M., Agner, T., *et al.* (1994). Guidelines for measurement of cutaneous blood flow by laser Doppler flowmetry. A report from the Standardization Group of the European Society of Contact Dermatitis. *Contact Dermatitis*, **30**(2): 65–72.

Braue, E.H., Jr, Graham, J.S., Doxzon, B.F., *et al.* (2007). Noninvasive methods for determining lesion depth from vesicant exposure. *J Burn Care Res*, **28**(2): 275–85.

Chilcott, R.P., Brown, R.F. and Rice, P. (2000). Non-invasive quantification of skin injury resulting from exposure to sulphur mustard and Lewisite vapours. *Burns*, **26**(3): 245–50.

Chilcott, R.P., Dalton, C.H., Emmanuel, A.J., *et al.* (2002a). Transepidermal water loss does not correlate with skin barrier function *in vitro*. *J Invest Dermatol*, **118**(5): 871–5.

Chilcott, R.P., Dalton, C.H., Emmanuel, A.J.P., *et al.* (2002b). Basal TEWL rates and skin permeability, in *The essential stratum corneum* (eds Marks, R., Leveque, J.-L. and Voegeli, R.), Martin Dunitz Ltd, London.

Chilcott, R.P., Patel, A., Ashley, Z., *et al.* (2002c). The effects of chemical damage on TEWL, in *The essential stratum corneum* (eds Marks, R., Leveque, J.-L. and Voegeli, R.), Martin Dunitz Ltd, London.

Chilcott, R.P., Dalton, C.H., Ashley, Z., *et al.* (2007). Evaluation of barrier creams against sulphur mustard: (II) *In vivo* and *in vitro* studies using the domestic white pig. *Cutan Ocul Toxicol*, **26**(3): 235–47.

Courage and Khazaka Product Information: Measurement of Skin, Hair and Nails, Courage & Khazaka Electronic GmbH, Cologne.

Cowen, J.A. (1999). *In Vivo* Opto-thermal Transdermal Diffusion Measurement, Thesis, London South Bank University, London.

Cunliffe, W.J. and Taylor, J.P. (2006). Gravimetric Technique for Measuring Sebum Excretion Rate (SER), in *Handbook of Non-Invasive Methods and the Skin* (eds Serup, J., Jemec, G.B.E. and Grove, G.L.), CRC Press, Boca Raton London New York, pp. 847–852.

Dikstein, S. and Fluhr, J. (2006a). Indentometry, in *Handbook of Non-Invasive Methods and the Skin* (eds Serup, J., Jemec, G.B.E. and Grove, G.L.), CRC Press, Boca Raton London New York, pp. 617–620.

Dikstein, S. and Fluhr, J. (2006b). Levarometry, in *Handbook of Non-Invasive Methods and the Skin* (eds Serup, J., Jemec, G.B.E. and Grove, G.L.), CRC Press, Boca Raton London New York, pp. 613–616.

Diridollou, S., Black, D., Lagarde, J.M. and Gall, Y. (2000). Sex- and site-dependent variations in the thickness and mechanical properties of human skin *in vivo*. *International Journal of Cosmetic Science*, **22**: 421–435.

Distante, F. and Beradesca, E. (1995a). Hydration, in *Bioengineering of the Skin: Methods and Instrumentation* (eds Beradesca, E., Elsner, P., Wilhelm, K.P. and Maibach, H.I.), CRC Press, Boca Raton London New York, pp. 5–12.

Distante, F. and Beradesca, E. (1995b). Transepidermal Water Loss, in *Bioengineering of the Skin: Methods and Instrumentation* (eds Beradesca, E., Elsner, P., Wilhelm, K.P. and Maibach, H.I.), CRC Press, Boca Raton London New York, pp. 1–4.

El Gammal, C., El Gammal, S., Pagnoni, A. and Kligman, A. (2006). Quantification of Sebum Output Using Sebum-absorbent Tapes (Sebutapes), in *Handbook of Non-Invasive Methods and the Skin* (eds Serup, J., Jemec, G.B.E. and Grove, G.L.), CRC Press, Boca Raton London New York, pp. 835–840.

Elsner, P. (1995). Sebum, in *Bioengineering of the Skin: Methods and Instrumentation* (eds Beradesca, E., Elsner, P., Wilhelm, K.P. and Maibach, H.I.), CRC Press, Boca Raton London New York, pp. 81–89.

Elsner, P., Barel, A.O., Berardesca, E., *et al.* (eds). (1998). *Skin bioengineering: techniques and applications in dermatology and cosmetology*. Karger AG, Basel.

English, J.S., Ratcliffe, J. and Williams, H.C. (1999). Irritancy of industrial hand cleansers tested by repeated open application on human skin. *Contact Dermatitis* **40**(2): 84–88.

Essex, T.J. and Byrne, P.O. (1991). A laser Doppler scanner for imaging blood flow in skin. *J Biomed Eng*, **13**(3): 189–94.

Fluhr, J., Elsner, P., Berardesca, E. and Maibach, H.I. (eds). (2004). *Bioengineering of the skin: water and stratum corneum*, CRC Press, Boca Raton London New York.

Fluhr, J., Bankova, L. and Dikstein, S. (2006). Skin Surface pH: Mechanism, Measurement, Importance, in *Handbook of Non-Invasive Methods and the Skin* (eds Serup, J., Jemec, G.B.E. and Grove, G.L.), CRC Press, Boca Raton London New York, pp. 411–420.

Fullerton, A., Stücker, M., Wilhelm, K.P., *et al.* (2002). Guidelines for visualization of cutaneous blood flow by laser Doppler perfusion imaging. *Contact Dermatitis*, **46**(129–140).

Goh, C.L. (1995). Seasonal Variations and Environmental Influences on the Skin, in *Handbook of Non-Invasive Methods and the Skin* (eds Serup, J. and Jemec, G.B.E.), CRC Press, Boca Raton London New York, pp. 27–30.

Grove, G.L., Damia, J., Grove, M.J. and Zerweck, C. (2006). Suction Chamber MEthod for Measurement of Skin Mechanics: The DermaLab, in *Handbook of Non-Invasive Methods and the Skin* (eds Serup, J., Jemec, G.B.E. and Grove, G.L.), CRC Press, Boca Raton London New York, pp. 593–599.

Humbert, P., Sainthillier, J., Mac-Mary, S., A. *et al.* (2006). Capillaroscopy and Videocapillaroscopy Assessment of Skin Microcirculation: Dermatological and Cosmetic Approaches, in *Handbook of Non-Invasive Methods and the Skin* (eds Serup, J., Jemec, G.B.E. and Grove, G.L.), CRC Press, Boca Raton London New York, pp. 679–687.

Imhof, R.E. (2001). Diagram of Aquaflux measurement chamber. Biox Systems Ltd, London.

Jünger, M., Hahn, M., Klyscz, T. and Friedrich Jung, M. (1995). Capillaroscopy and Fluorescence Videomicroscopy, in *Bioengineering of the Skin: Methods and Instrumentation* (eds Beradesca, E., Elsner, P., Wilhelm, K.P. and Maibach, H.I.), CRC Press, Boca Raton London New York, pp. 113–120.

Kligman, A. (1995). Perspectives on Bioengineering of the Skin, in *Handbook of Non-Invasive Methods and the Skin* (eds Serup, J. and Jemec, G.B.E.), CRC Press, Boca Raton London New York, pp. 3–8.

Lévêque, J. (2002). Lipid organization and barrier function, in *The essential stratum corneum* (eds Marks, R., Leveque, J.-L. and Voegeli, R.), Martin Dunitz Ltd, London, pp. 111–117.

Lomuto, M., Pellicano, R. and Giuliani, M. (1995). Equipment available for Bioengineering of the skin. *Clinics in Dermatology*, **13**: 409–415.

Mao-Qiang, M., Feingold, K.R., Thornfeldt, C.R. and Elias, P.M. (1996). Optimization of physiological lipid mixtures for barrier repair. *J Invest Dermatol*, **106**(5): 1096–1101.

Moor, (2005a). Theory of Laser Doppler Imagers and Monitors. Product information, Moor Instruments, Axminster, Devon, *http://www.moor.co.uk/files/Theory/Moor_Laser_doppler_theory_Issue_1.pdf*.

Moor, (2005b). Tissue Blood-flow Mapping with MoorLDI. Product information, Moor Instruments, Axminster, Devon.

Nuutinen, J., Alanen, E., Autio, P., *et al.* (2003). A closed unventilated chamber for the measurement of transepidermal water loss. *Skin Res Technol*, **9**(2): 85–9.

O'goshi, K.I. (2006a). Optical Measurement of Sebum Excretion Using Opalescent Film Imprint: The Sebumeter, in *Handbook of Non-Invasive Methods and the Skin* (eds Serup, J., Jemec, G.B.E. and Grove, G.L.), CRC Press, Boca Raton London New York, pp. 841–846.

O'goshi, K.I. (2006b). Suction Chamber Method for Measurement of Skin Mechanics: The Cutometer, in *Handbook of Non-Invasive Methods and the Skin* (eds Serup, J., Jemec, G.B.E. and Grove, G.L.), CRC Press, Boca Raton London New York, pp. 579–582.

Pinnagoda, J. and Tupker, R.A. (1995). Measurement of Transepidermal Water Loss, in *Handbook of Non-Invasive Methods and the Skin* (eds Serup, J. and Jemec, G.B.E.), CRC Press, Boca Raton London New York, pp. 173–178.

Pinnagoda, J., Tupker, R.A., Coenraads, P.J. and Nater, J.P. (1989). Prediction of susceptibility to an irritant response by transepidermal water loss. *Contact Dermatitis*, **20**: 341–346.

Pinnagoda, J., Tupker, R.A., Agner, T. and Serup, J. (1990). Guidelines for transepidermal water loss (TEWL) measurement. A report from the Standardization Group of the European Society of Contact Dermatitis. *Contact Dermatitis*, **22**(3): 164–78.

Rogiers, V. (2001). EEMCO guidance for the assessment of transepidermal water loss in cosmetic sciences. *Skin Pharmacol Appl Skin Physiol*, **14**(2): 117–28.

Serup, J. (1994). Bioengineering and the skin: from standard error to standard operating procedure. *Acta Derm Venereol* Suppl (Stockh), **185**: 5–8.

Serup, J. (1995). Prescription for a Bioengineering Study: Strategy, Standards, and Definitions, in *Handbook of Non-Invasive Methods and the Skin* (eds Serup, J. and Jemec, G.B.E.), CRC Press, Boca Raton London New York, pp. 17–21.

Skinos Product Information. SKD3000 principle of operation, Skinos Co., Japan.

Swindt, D.A., Wilhelm, K.P., Miller, D.L. and Maibach, H.I. (1998). Cumulative Irritation in Older and younger Skin: a Comparison. *Acta Derm Venereol*, **78**: 279–283.

Wahlberg, J.E. and Lindberg, M. (1995). Assessment of Skin Blood Flow – An Overview, in *Bioengineering of the Skin: Cutaneous Blood Flow and Erythema*, (eds Beradesca, E., Elsner, P. and Maibach, H.I.), CRC Press, Boca Raton, pp. 23–27.

Wickett, R. (2005). Hardware and Measuring Principles: The NOVA Dermal Phase Meter, in *Bioengineering of the Skin: Water and the Stratum Corneum* (eds Fluhr, J.W., Elsner, P., Beradesca, E. and Maibach, H.I.), CRC Press, Boca Raton, pp. 263–274.

Wilhelm, K.P. (1998). Possible Pitfalls in Hydration Measurement, in *Skin Bioengineering: Techniques and Applications in Dermatology and Cosmetology* (eds Elsner, P., Barel, A.O., Beradesca, E., *et al.*), Karger AG, Basel, pp. 223–234.

Wilhelm, K.P., Elsner, P., Berardesca, E. and Maibach, H.I., (eds). (2006). *Bioengineering of the skin: skin imaging and analysis*. CRC Press Inc., Boca Raton.

Wood, E.J. (1985). *The Human Skin*. Edward Arnold, London.

Zuang, V. and Beradesca, E. (1998). Designing and Performing Clinical Studies with Bioengineering Techniques, in *Skin Bioengineering: Techniques and Applications in Dermatology and Cosmetology* (eds Elsner, P., Barel, A.O., Beradesca, E., *et al.*), Karger AG, Basel, pp. 209–216.

PART IV: Clinical Aspects

13 Introduction to dermatology

Manjunatha Kalavala[1] and **Alex Anstey**[2]

[1]*Specialist Registrar in Dermatology, University Hospital of Wales, Heath Park, Cardiff, CF14 4NJ, UK*
[2]*Consultant Dermatologist and Honorary Senior Lecturer, Gwent Healthcare NHS Trust, Royal Gwent Hospital, Cardiff Road, Newport, Gwent NP20 2UB, UK*

Primary Learning Objectives

- *Basic principles of clinical assessment.*
- *Diagnostic procedures.*
- *Common pathological skin conditions.*
- *Standard dermatological therapies.*

13.1 Introduction and scope

Dermatology provides a unique clinical perspective on the practice of skin toxicology and, in this role, is primarily concerned with the pathological consequences of cutaneous or systemic exposures to xenobiotics acquired under a wide variety of circumstances. Empirically, the dermatologist is responsible for identifying cutaneous manifestations of toxicity (diagnosis) and formulating an effective treatment strategy (including recommendations for preventative measures to avoid re-exposure).

Dermatology is the branch of medicine concerned with the diagnosis and treatment of skin disorders. At least 2000 different cutaneous disorders are known to the dermatologist. Skin conditions show diversity in appearance and severity, ranging from cosmetic problems like dry skin and wrinkles to life-threatening conditions like toxic epidermal necrolysis. The practice of dermatology varies throughout the world. In the United Kingdom patients are normally seen by a primary care physician. If the primary care physician is unable to deal with the problem the patient is referred on to a hospital consultant. Around one in seven primary care consultations relate to a dermatological problem. About 12.5 per 1000 of the population are referred to a hospital dermatology department annually, which places dermatology among the highest volume specialties in terms of patient numbers.

The integument is a multifunctional interface between the human body and the environment. A wide variety of environmental hazards like chemicals, plants, animals, parasites, microorganisms, radiation and climatic conditions can adversely affect the skin. These hazards

Principles and Practice of Skin Toxicology Edited by Robert P. Chilcott and Shirley Price
© 2008 John Wiley & Sons, Ltd

may be encountered at work, during home life or through leisure activities. An occupational history may be important in establishing a diagnosis of skin disease. Therefore, it is important to ask patients about the nature of their work, current and past. Similarly, leisure pursuits may be relevant and should be subject to enquiry. The skin may also provide important clues for diagnosing underlying internal disease. Many systemic diseases can affect the skin either directly or as a result of disease complications or side effects of treatment. A good knowledge of internal medicine is therefore useful in recognising these clinical manifestations. Both prescribed medication and over-the-counter medication (including complementary and herbal remedies) can cause rashes. It is therefore important for physicians to enquire about drug history, particularly the recent commencement of new medication and to establish, if possible, a temporal relationship with the onset of the rash. Cessation of a medication often results in prompt resolution of adverse cutaneous effects.

Skin is of major importance in our 'body image'. Cosmetic impairment caused by skin diseases can sometimes be disproportionate to their medical significance. Therefore, it is important for physicians to address these issues during clinical assessment and to plan the management accordingly. In addition, psychological stress can exacerbate, or even be a causative factor of, some skin diseases.

The clinical assessment of patients with skin disease includes a detailed relevant history and clinical examination. Many skin diseases, including warts, acne and psoriasis, are diagnosed clinically without the need for further investigation. In contrast, other dermatological conditions require detailed and time-consuming investigations to reach a diagnosis and rule out systemic manifestations. The extent of clinical assessment and investigations is dictated by the severity of the skin disease, the range of possible diagnoses and the need to individualise the work-up of each patient.

The management of skin disorders starts with an explanation of the diagnosis and management of the disease. Most patients are worried about skin cancer and contagiousness of skin diseases. It is important to address these issues and reassure patients appropriately. Written information leaflets are useful in this regard. Informative patient information leaflets can be downloaded from the British Association of Dermatologists web site[1].

The treatment options for skin disorders include removal of causative agent (where possible), topical preparations, systemic medications, phototherapy, photo-chemotherapy, photodynamic therapy, surgical procedures and laser treatment. All management decisions are planned in consultation with the patient. For complex disorders, close collaboration with other specialists may be required. Examples of disciplines with close ties to dermatology include plastic surgery, oncology, rheumatology, paediatrics, genetics and vascular surgery. In the following sections these aspects of dermatological diagnosis and management are discussed in more detail.

13.2 Clinical assessment of patient with skin disease

The assessment of patients presenting with an overt skin pathology requires a step-wise investigation to identify both the cause and optimal treatment. This involves collating information derived from the patient's history, conducting a clinical examination and identifying causative factors through the judicious use of appropriate tests.

[1] www.bad.org.uk

The clinical assessment of patients with skin disease involves history taking, examination and, sometimes, additional tests. The visibility of skin provides ample information for the diagnosis of many disorders.

13.2.1 The history

A carefully directed history is important for both diagnosis and optimal management. The important components of history include the presenting complaint, history of the presenting complaint and general history. The amount of history that is optimal for each case is variable and clinicians use their judgment. Lumps and bumps, for example, usually have a short history of a few lines. In contrast, a severe rash which waxes and wanes may need a full page of hand written notes to record all of the relevant information.

Itch (pruritis) is the prime dermatological symptom. The severity and description of itch varies greatly between individuals and from one skin complaint to another. Other symptoms include sharp pain, burning or tenderness. During history taking it is important to ascertain the duration, evolution and periodicity of the rash or lesion. Dermatologists record details of treatments used (both prescription and over-the-counter preparations) including herbal and alternative remedies. Some treatments may modify the appearance of skin lesions. For example, the use of topical corticosteroids for cutaneous fungal infections creates a new rash that steadily gets worse and looks different from the untreated condition. Some skin lesions may be completely asymptomatic, but patients may be anxious about the cosmetic appearance or due to misplaced fears about possible skin cancer.

Previous episodes of similar rash, contact allergies and atopy (history of eczema, asthma or hay fever) may all be important in establishing the diagnosis as well as planning management. Previous treatment and response to treatment may also be of benefit as a guide for future therapy. General medical conditions may have cutaneous features. On occasions, an underlying medical condition is first diagnosed while investigating a rash. An example of this is the butterfly rash on the face which characterises systemic lupus erythematosus. Viral or bacterial infections may precede the onset of urticaria (a rash which resembles a stinging nettle rash) or vasculitis (inflammation and leaking of the blood vessels in the skin). The physician must note any recent or current systemic medications, including prescribed, over-the-counter and alternative therapies. This is important for diagnosis, as drug eruptions can mimic a wide variety of skin conditions. A detailed drug history is also important in order to avoid the interaction of newly prescribed treatments with previously prescribed medication. It is important to note medicament and other allergies.

Family history is important if a genodermatosis (familial skin disorder) is suspected. In some conditions, for example psoriasis, a positive family history of the condition may support the diagnosis where genetic factors are known to be important. In acne, family history of severe scarring may influence treatment decisions and result in earlier intervention with potent treatments. For infective conditions like scabies and chickenpox, a history of family contact is important.

An occupational history (both current and previous) and an account of leisure activities (e.g. cooking, gardening etc) may be relevant to the diagnosis of allergic and irritant contact dermatitis. Foreign travel, especially if recent, is a potentially important cause of dermatological disease. Prolonged stay in a sunny climate without adequate photoprotection increases the possibility of subsequent development of skin cancers.

Several disorders have a predilection for specific racial groups; for example, sarcoidosis is more common in Negroes, Afro-Caribbean's and black American people. The morphology of common skin diseases may also be altered by racial pigmentation. Certain cultural practices may offer diagnostic clues; for example the use of hair pomades in African people may lead to hair loss and scalp disorders. Cultural influences also play an important role in acceptance and understanding of disease process and treatments that are offered.

It is most important to understand the patient's main concerns with skin problems. Chronic skin problems such as psoriasis can have a major impact on lifestyle and relationships, apart from significant financial cost to the healthcare system and community due to lost work days. Lifestyle can also adversely affect the skin and the treatment of skin disorders; for example, excessive alcohol intake has an adverse effect on severity of psoriasis and limits therapeutic options.

13.2.2 Examination of the skin

The patient should always be examined in a good light, preferably daylight, and with magnification of lesions if necessary. Ideally, the entire skin should be examined in every patient, particularly if the diagnosis is in doubt. When examining the skin it is helpful to consider the morphology of individual lesions, their overall pattern and spatial relationship to each other, and their distribution over the body. Specific attention to hair, nails and the mucous membranes is also required. Careful description and use of standard dermatological nomenclature is helpful when monitoring these features during follow-up, and in subsequent discussion with colleagues. The commoner descriptive terms applied to cutaneous lesions are listed in Table 13.1.

The shape of each lesion and the pattern in which neighbouring lesions are arranged in relation to each other is often of help to dermatologists when trying to diagnose a new rash. For example, annular lesions (open circles with central skin that has a different morphology) are characteristic of granuloma annulare and tinea corporis. A specific cause of a linear lesion is the Köebner or isomorphic phenomenon. This term is applied when localised, non-specific trauma locally provokes lesions of a dermatosis which is usually spontaneously present elsewhere, and usually in a relatively active or eruptive phase. The arrangement of individual lesions may create a characteristic pattern such as the grouped vesicles of herpes simplex. The overall distribution of lesions in many common dermatoses may be so characteristic that it is of great assistance in clinical diagnosis, e.g. flexural involvement in atopic eczema.

Diascopy

Diascopy is pressing a glass slide or clear stiff piece of plastic onto the skin. This compresses blood out of small blood vessels, to allow evaluation of other colours. In the skin lesions of cutaneous tuberculosis this test alters the appearance producing a nodular lesion that has been likened to 'apple jelly'.

Dermatoscopy

Dermatoscopy or epiluminiscence microscopy is an extension of the use of simple magnification. Dermatoscopes have built-in illumination. Older versions of dermatoscopes are applied

Table 13.1 Common descriptive terms describing cutaneous lesions

Term	Description
Alopecia	Absence of hair from normally hairy area
Atrophy	Loss of tissue from epidermis/dermis/subcutaneous fat
Burrow	A small tunnel in the skin that houses a parasite, e.g. scabies mite
Comedo	A plug of keratin and sebum in a dilated pilosebaceous orifice
Crusts	Dried serum and other exudates
Erythema	Redness of skin
Excoriation	Linear denudation of skin produced by scratching
Lichenification	Thickening of the epidermis due to prolonged rubbing
Macule	A circumscribed alteration in the colour of the skin
Papule	A circumscribed palpable elevation, less than 0.5 cm in diameter
Plaque	An elevated area of skin, 2 cm or more in diameter
Petechia	A punctuate haemorhagic spot 1–2 mm in diameter
Ecchymosis	A macular area of haemaorrhage more than 2 mm in diameter
Pustule	A visible accumulation of free pus
Scale	A flat plate or flake of stratum corneum
Vesicle	Visible accumulation of fluid less than 0.5 cm in diameter
Bullae	Vesicle greater than 0.5 cm in diameter
Wheal	A transient area of dermal oedema. Characteristic lesion of urticaria

to the skin surface with a film of oil on the lesion. The newer versions are more compact and do not require oil application. These devices provide high magnification (up to × 20) and enhance the visibility of cutaneous structures below the outer keratinised layer of the epidermis (stratum corneum). The technique is mainly used in the diagnosis of pigmented lesions. However dermatoscopy is increasingly used in the evaluation of other skin conditions, including scabies and other acquired rashes. The images may be viewed directly, photographed or recorded digitally for subsequent or sequential analysis.

Wood's light examination

Wood's light is a hand held source of ultraviolet A light (at a wavelength of approximately 365 nanometers). Wood's light is used to illuminate the skin of the patient in a dark room; any subsequent fluorescence or change in pigment pattern is observed. Important applications of Wood's light are listed in Table 13.2. It is important not to be distracted by strong fluorescence emitted by white clothes caused by optical brighteners in detergents.

Skin scrapings, KOH preparation of skin scrapings

The laboratory diagnosis of cutaneous fungal infection starts with direct microscopic observation in order to identify the pathogen in skin or nail samples from the affected area. This

Table 13.2 Various applications of Wood's light

Disease state or purpose	Features
Fungal infection	Microspora species and favus – green fluorescence Pityrosporum ovale – yellow fluorescence
Bacterial infections	Corynebacteriium minutissimum – coral pink fluorescence Propionibacterium acnes – coral pink fluorescence
Infestations	Delineation of scabies burrow
Porphyrias	Porphyria cutanea tarda – fluorescence of urine, faeces and occasionally blister fluid Erythropoietic porphyria (congenital porphyria) – fluorescence of teeth Erythropoietic protoporphyria – fluorescence of red blood cells
Pigmentary disorders	Epidermal pigment – accentuated Dermal pigment – less apparent Detection of ash leaf macules in tuberous sclerosis
Drugs and chemicals	Detection of fluorescent contact or photosensitisers on the skin, in the cosmetics and industrial agents, e.g. ball point pen ink, eosin, fuorocumarins Addition of fluorescein to topical medications to investigate sites of application or manipulation, e.g. in dermatitis artefacta Diagnosis of ethylene glycol (antifreeze) poisoning – fluorescence of urine
Photodynamic therapy	To demonstrate conversion of aminolevulinic acid to protoporphyrin IX in the skin tumours

is usually followed by culture, which permits the specific identification of the fungus. A disposable scalpel blade (size 22 or 23) is held vertically to the skin and a repeated scratching motion is used, with the skin held taught in order to obtain the scrapings. If the lesion has a definite edge, the material should be taken from the active margin, otherwise a general scraping is adequate. The scrapings should be collected and transported in folded paper, which keeps the specimen dry, thus preventing contamination. Specially designed commercial transport packs for skin, hair and nail samples are available. In cases of fungal infections of scalp, skin scrapings as well as plucked hair should be sent for examination. In nail infections, the full thickness of nail as well as debris from undersurface of the nail should be obtained.

For routine examination, specimens are usually mounted in 10–30% potassium hydroxide (KOH). Potassium hydroxide dissolves keratin, making visualisation of fungal elements easier. Fungi grow readily on Saboraud's dextrose agar or one of its modifications.

Skin biopsy

Skin biopsy involves taking a sample of skin for examination and is of benefit in a number of situations (Table 13.3). It provides a specimen for light microscopic examination of

Table 13.3 Benefits of skin biopsy

Indication	Benefit
Diagnostic	Confirms clinical diagnosis or aids in establishing the diagnosis where a clinical diagnosis is not apparent
Excision biopsy	Treatment of skin lesions, particularly malignant neoplasms and other lesions removed for cosmetic reasons

Table 13.4 Skin biopsy techniques

Technique	Description or application
Excision biopsy	Removal of single lesion
Incision biopsy	A narrow ellipse including some normal peri-lesional skin
Punch biopsy	Simple and rapid. Accurate sampling essential. Sometimes useful in children.
Curettage	For superficial hyperkeratotic lesions
Shave biopsy	For raised, benign-looking skin lesions

paraffin-embedded tissue. In addition, histochemical special stains, immuno-fluorescence studies and immuno-histochemistry are routinely carried out, when required, in most histopathology laboratories. More specialist studies, not routinely available in all such laboratories, include electron microscopy, tissue culture and molecular biological methods such as *in situ* hybridisation and polymerase chain reaction (PCR). These different investigative techniques require specific specimens and transport conditions and are only offered by specialist laboratories.

The type of biopsy (Table 13.4), the selection of the site of biopsy and the type of lesion to be biopsied all influence the sample, which in turn affects the chances of the histopathologist establishing a diagnosis. Ideally the site to be biopsied should be an early, untreated lesion that is representative of the skin disorder as a whole. Sometimes multiple biopsies may be necessary, especially if there are lesions of different stages of an evolving disease. It may be important to include a sample of normal skin with the biopsy for comparison, for example with localised morphoea where a site-matched sample of normal skin is very helpful to the pathologist. It is also important to make sure that the biopsy is deep enough. If the lesions are widespread and there is a choice of biopsy site it is sensible to avoid areas liable to heal badly, such as skin over bony prominences and skin on the lower limbs. It is also common sense to avoid cosmetically important areas unless absolutely necessary.

Prior to the skin biopsy, written informed consent is normally obtained from the patient. Local anaesthetic, usually 1% or 2% lignocaine with or without adrenaline is injected around the biopsy site. Topical anaesthetics are particularly useful with biopsies performed in children.

Elliptical surgical biopsy

This is one of the most commonly used diagnostic skin biopsy techniques. Equipment required includes scalpel, fine-toothed forceps, needle holder, scissors and eyeless needle with suture. The use of a skin hook greatly facilitates manipulation of the biopsy specimen and avoids undue trauma, thus preventing crush artefact. A reasonable size for an elliptical biopsy is about 30 × 10 mm. The defect is closed by one or two dissolving sub-cutaneous sutures, and a number of fine interrupted nylon sutures to the skin to oppose the wound edges.

Punch biopsy

The biopsy punch is a metal cylinder of variable diameter (3–6 mm) with a sharp cutting edge at one end. This circular cutting blade is mounted on a plastic handle. The punch is pushed in to the skin with a downward twisting movement making a circular cut through the skin. The punch biopsy device is then removed. The tissue specimen is then carefully lifted with a fine skin hook and separated from the underlying tissue. The wound may be closed by one or two sutures or left to heal on its own after securing haemostasis.

Curettage

Curettage is done using a sharp-edged disposable curette. It is a simple and quick technique useful in treating small benign and malignant lesions such as viral warts, actinic keratoses, seborrhoeic keratoses and basal cell carcinomas. The resulting specimen is often fragmented, making it impossible for the pathologist to comment on adequacy of removal.

Shave biopsy

Shave biopsy is a useful technique to remove benign-looking raised skin lesions. It has the advantage of healing to produce a good to excellent cosmetic result in most instances. However, the whole lesion is rarely removed. This biopsy technique is not suitable for the definitive treatment of malignant skin lesions such as basal cell carcinoma, squamous cell carcinoma or malignant melanoma.

Information to be provided with the specimen

Histopathologists rely on the clinicians who send them biopsies to provide the following information to assist with diagnosis: site of biopsy, relevant clinical details, a list of clinical differential diagnoses. Details of previous biopsies, if available, should also be provided.

Immunopathology

The use of immunological methods allows identification of antigens, antibodies and various other cell and tissue components. Hybridoma monoclonal antibody technology has paved the way for the development of numerous antibodies to cell and tissue structures.

Immunofluorescence is a technique for detecting the presence and position of antigens, antibodies and other cell components in tissue sections (Figure 13.1). The principle of the technique is that certain fluorochrome dyes exposed to ultraviolet (UV) light emit fluorescent radiation, the colour of which depends upon the particular fluorochrome. When these dyes are conjugated to proteins that are subsequently added to tissue sections, the position of the

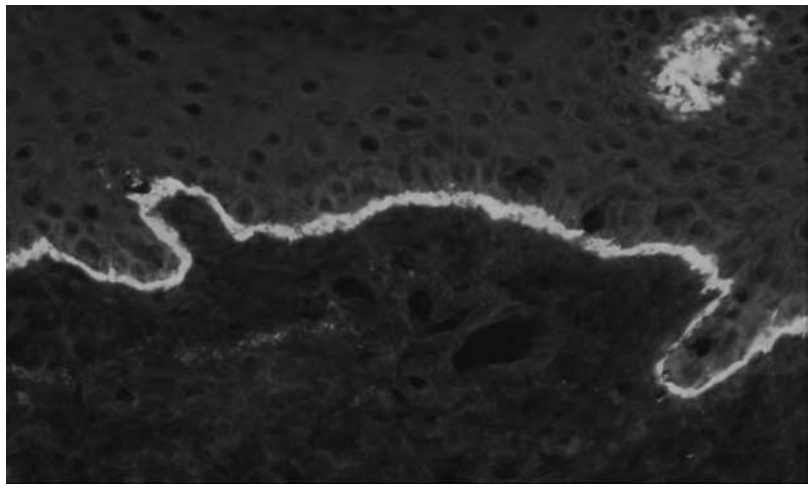


Figure 13.1 Direct immunofluorescence showing linear deposition of Ig G and Complement C3 at the dermo–epidermal junction. A full-colour version of this figure appears in the colour plate section of this book

proteins can be identified microscopically by the fluorescence they emit under illumination with UV light with suitable filters. Two fluorochromes are generally available: Fluoroscein, which emits an apple green fluorescence, and rhodamine RB200, which emits an orange fluorescence. Immunofluorescence has greatly facilitated the diagnosis of auto immune blistering skin disorders such as epidermolysis bullosa. Prolonged or repeated examination reduces the intensity of emission due to photo-bleaching.

Immunoenzyme (immunoperoxidase) methods have the advantage of using a standard microscope and standard white light illumination. The preparations are permanent and do not fade on repeated examination. Positively labelled cells or cellular components are brown coloured on light microscopy against a faint blue counter stain.

Electron microscopy

Transmission and scanning electron microscopy have greatly increased the understanding of the microanatomy of normal skin and many disease processes. However, advances in immunohistochemistry have largely rendered electron microscopy obsolete in diagnostic dermatopathology. Electron microscopy may be of diagnostic help in:

- The classification and sub-divison of bullous disorders.
- Quantitative and qualitative evaluation of melanocytes and melanosomes in disorders of hypo- and hyperpigmentaion.
- Cellular identification in inflammatory and neoplastic disorders, e.g. Birbeck granules in Langerhans' cells.
- Identification of dermal deposits e.g. amyloid.
- Direct visualisation of virus particles.

Patch testing

Patch testing is a well established method of diagnosing contact allergy; a delayed type of hypersensitivity (type IV reaction; Chapter 9). Patients with a history and clinical picture of contact dermatitis are re-exposed to the suspected allergens under controlled conditions to provoke a delayed hypersensitivity reaction (Figure 13.2). Patch test procedure can also be used before recommending alternative medicaments, skin care products, cosmetics or gloves in a particular patient. The patches containing allergens are applied under occlusion on the back and removed after 24–48 hours. The number of allergens applied depends on the clinical picture. The British Contact Dermatitis Society and European Contact Dermatitis Research Group have recommended a series of allergens pertaining to different clinical scenarios. These include a standard series, a textile series, a hair-dressing series, a medicament series, a facial series, a shoe series and others. Patient's own products may also be used in recommended dilutions for patch testing. In addition, allergens may be obtained from the manufacturers of the product in question for patch testing. Two readings are taken: the first after removal of the patches (usually day 2); the second reading 2–5 days later. The common method of recording patch test reactions, recommended by the International Contact Dermatitis Research Group (ICDRG) is shown in Table 13.5. Relevance of a positive reaction should be interpreted in the clinical context and the patient advised accordingly.

Photopatch testing

Photopatch testing is mainly used to diagnose photoallergy to topical agents (see Chapter 14 for a more detailed discussion). The most frequent allergens of relevance are sunscreens. Two identical sets of allergens are applied as parallel series on either side of the back using conventional patch test techniques. Two days later, both are discarded

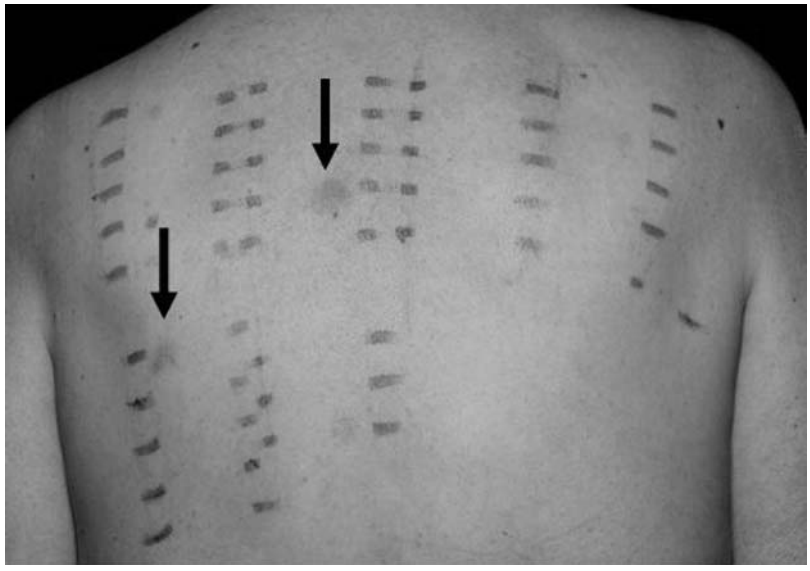


Figure 13.2 Patch testing; reading on Day 4 showing two positive reactions (indicated by arrows). A full-colour version of this figure appears in the colour plate section of this book

Table 13.5 Recording of patch test reactions according to the (ICDRG)

Score	Definition
?+	Doubtful reaction; faint erythema only
+	Weak positive reaction; ertythema, infiltration, possibly papules
++	Strong positive reaction; ertythema, infiltration, papules, vesicles
+++	Extreme positive reaction; intense erythema, infiltration and coalescing vesicles
−	Negative reaction
IR	Irritant reaction
NT	Not tested

and the sites are examined for reactions. One set is then shielded while the other is irradiated with $5\,J\,cm^{-2}$ UVA. A reading shortly after irradiation (up to 20 minutes post illumination) is sometimes performed to detect immediate phototoxic urticarial reactions. In sunny climates, all sites should then be covered with opaque material. Two days later the sites are re-examined and positive reactions noted. A positive photopatch test occurs when the irradiated set shows a positive response while the control (non-irradiated set) does not. If both sets show a positive response of equal severity, this simply represents a standard allergic or irritant response rather that a photoallergic response.

Repeated open application test (ROAT)

This is intended to mimic the actual use situation of a formulated product such as a cosmetic, a shampoo, oil or a topical medicament. The test substances are applied undiluted twice daily for seven days to the outer aspect of the upper arm, the elbow flexure or the skin on the back. A positive response is recorded if an eczematous rash appears within the test period (such a rash usually appears on days 2 to 4).

Atopy patch testing

An epicutaneous patch test with allergens known to elicit IgE-mediated reactions and the evaluation of eczematous skin lesions after 24 to 72 hours is called the atopy patch test. Among the allergens found to be relevant in atopic eczema, aeroallergens and food allergens are the most important. Therapeutic consequences of the diagnosis of allergy are based upon avoidance strategies.

Prick testing

The skin prick test (SPT) is the most convenient and reliable method for detecting clinically significant IgE-mediated allergy (Type I hypersensitivity). Drops of SPT allergen solution are applied to the skin of the back or lower arm, 3–5 cm apart, and pierced with a special prick test lancet. Histamine dihydrochloride ($10\,mg\,ml^{-1}$) is used as a positive control and the base

solution as a negative control. After piercing the skin the drops are wiped off the skin with a soft tissue. After 15–20 minutes the diameter of the wheals, if any, are measured. Reactions greater than 3 mm and at least half the size of that produced by histamine are regarded as positive. A modification of the SPT is the prick-by-prick method used especially for testing fresh foodstuffs. A piece of food is pricked with the lancet and immediately after the skin is pricked with the same lancet. A further variant, the Scratch test, is less commonly used when only non-standardised allergens are available. Scratches approximately 5 mm long are made (avoiding bleeding) and allergen solutions are applied to scratches for 5–10 minutes. The scratch with the allergen may be covered with a Finn chamber.

Chamber test

The chamber test has been used in the diagnosis of immediate contact allergy. The test material is put in to an ordinary patch test chamber, moistened with physiological saline (when needed) and applied to the back or arm for 15–20 minutes. The test is read some minutes after the removal of test chamber. A wheal-and-flare reaction is regarded as positive whereas erythema without oedema is likely to be negative.

13.3 Cutaneous manifestations of disease following exposure to chemicals and pharmaceutical formulations

Although there are literally thousands of chemical substances capable of eliciting a dermatotoxic effect, the skin has a limited 'repertoire' of responses. The most common is a collection of disease states collectively referred to as dermatitis (eczema).

13.3.1 Irritant contact dermatitis

Irritant dermatitis represents the cutaneous response to the physical/toxic effects of a wide range of environmental agents. The skin provides the first and most important line of defense against exogenous noxious agents. The principal barrier is the stratum corneum (Chapter 1). Damage to the stratum corneum results in increased percutaneous absorption. Irritant dermatitis arises when the defense or repair capacity of the skin is exhausted, or when the penetration of chemicals excites an inflammatory response. Strong irritants induce a clinical reaction in almost all individuals. With less potent irritants the response may be physiological rather than apparent, dermatitis only developing in the most susceptible or in situations where there is repeated contact. Immunological memory is not involved and dermatitis occurs without prior sensitisation. Irritants affect everyone, although individual susceptibility with regard to the development of dermatitis varies greatly. Temperature, climate, occlusion and mechanical irritation all have a potentiating effect on the irritant nature of toxic chemicals. Irritants produce a variety of responses in the skin. These range from purely subjective sensations such as stinging, smarting, burning or sensations of dryness and tightness, through delayed stinging or transitional urticarial reactions to more persistent irritant reactions or

Figure 13.3 Severe irritant contact dermatitis (ICD) of lower limbs (Note acute eczematous rash with a sharp cut-off, corresponding to the areas of contact with the irritant). A full-colour version of this figure appears in the colour plate section of this book

irritant contact dermatitis. The same chemical may cause different irritant reactions depending on the concentration.

A chemical burn results when irreversible cell damage and necrosis occurs. There is usually rapid onset of painful erythema, often within minutes, at the site of exposure. This is followed by blistering and development of necrotic ulcers (Figure 13.3). Acute irritant contact dermatitis is often the result of a single overwhelming exposure to an irritant or caustic chemical or a series of brief chemical or physical contacts. Most cases of acute irritant contact dermatitis occur as a result of accidents at work. Cumulative irritant contact dermatitis develops as a result of a series of repeated and damaging insults to the skin. These insults may include both chemical irritants and a variety of harmful physical factors such as friction, microtrauma, low humidity, desiccant effects of powder, soil or water and temperature.

Cumulative irritant dermatitis most commonly affects thin or exposed skin – for example, the dorsa of the hands, fingertips and webs of the fingers or the face and eyelids. Healthcare workers, hair dressers and cleaners are some of the most frequent occupations developing irritant contact dermatitis. Irritant contact dermatitis is essentially a clinical diagnosis. However, a complicating allergic contact dermatitis may need to be excluded by patch testing.

The most important aspect of treatment is avoidance of the cause (see Chapter 16). In an occupational setting, automation of the production process may avoid exposure but can be expensive. A cost-effective compromise is the use of personal protective equipment and/or substitution of the chemical. Once present, dermatitis requires palliation of symptoms with topical steroids and emollients.

13.3.2 Allergic contact dermatitis

Allergic contact dermatitis is due to delayed-type or cell-mediated hypersensitivity. There are two main processes:

1. Sensitisation
2. Elicitation

The induction of sensitivity is the main primary event, which takes place before clinical expression of dermatitis can occur. Contact dermatitis can mimic or be associated with any type of eczematous eruption.

The diagnosis is based on a careful history combined with a sound knowledge of common allergens and irritants in the environment. When a suspected allergen is occupational the history includes a detailed description of the work and working environment, protective equipment used, as well as part time jobs and hobbies. Reference to chemical safety data sheets of the chemicals used at work, visits to the work place and liaison with the occupational medical officer may be necessary (Chapter 15). Background knowledge of different jobs involved in local industries is helpful. When the suspected allergen is not at work, a detailed history of all topical preparations used, including cosmetics, cleansing agents and topical medicaments, is important. Patients often believe new products to be the culprits. However, it is more common for patients to become sensitised to an ingredient in a preparation that has been used for long time. Inspecting all the personal care products used by the patient and patch testing for the individual ingredients may be required to establish the diagnosis. If necessary, ingredients can be obtained by request to the manufacturers. A brief comparison of ICD and ACD is given in Table 13.6.

Table 13.6 Empirical comparison of irritant contact dermatitis (ICD) and allergic contact dermatitis (ACD)

	ICD	ACD
Trigger	Primary irritant; single or cumulative exposure Examples: Caustic liquids (mineral acids and alkalis), solvents, detergents, dyes.	Allergen or sensitiser; repeated exposure only Examples: uroshiol (poison ivy toxin), metals, rubber (anti-oxidants), preservatives (medicinal and cosmetic), plants (primula) and topically applied drugs.
Mediator	Non-immunological	Immune-cell mediated
Time to onset	Immediate	Delayed
Mechanism	Release of inflammatory mediators	Langerhans cells present antigens to T-cells leading to proliferation of lymphocytes
Sign(s)	Erythema, scaling, thickened skin, vesicules and oedematous eruptions.	

See also Chapter 14, Table 14.1 (similarities between photoirritant and photoallergic reactions)

13.3.3 Chloracne

Chloracne is acne caused by topical exposure of skin to halogenated aromatic hydrocarbons. Chloronaphthalenes, chlorobiphenyls and chlorobiphenyl oxides are used as dielectrics in

conductors and as insulators in cables. Chlorophenols are used as insecticides, fungicides, herbicides and wood preservatives. All these halogenated hydrocarbons are contaminated with chlorinated dioxins and the severity of chloracne depends on the degree of contamination and the precise chemical structure of the dioxin. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is the most powerful chloracnegenic agent known and was implicated in the poisoning of the Ukranian president, Viktor Yushchenko[2]. Similarly, the toxicity of polychlorinated biphenyls (PCBs) is largely due to contamination with polychlorinated dibenzofurans (PCDFs). Large epidemics of chloracne have occurred as consequence of industrial or other accidents. Two large epidemics of chloracne are been caused by ingestion of cooking oil contaminated by PCBs. Chloracne predominantly involves the face, even if the chemical has been ingested. The nose tends to be spared, with skin of the malar (cheek) regions, the angles of the jaw and behind the ears often being most severely affected. The typical lesions are small skin coloured cysts, 0.1 mm to 1 cm in diameter, associated with numerous comedones (black heads). Chloracne might persist for few months to a decade depending on the severity of exposure.

13.3.4 Drug rashes

An adverse drug reaction (ADR) is an undesirable clinical manifestation resulting from administration of a particular drug. ADRs may be predictable (type A, e.g. side effects, overdose) or unpredictable (type B, e.g. idiosyncracy, intolerance) manifestations. About 80% of drug reactions are predictable, dose dependent and are a known pharmacological action of the drug. The skin has limited morphological reaction patterns of drug reactions to a wide variety of drugs. Hence it is often impossible to identify an offending drug, or the pathological mechanisms involved on the basis of clinical appearance alone. The assessment of a potential ADR involves taking a careful history, trial of drug elimination, skin tests (including prick and intra-dermal testing), patch testing and *in vitro* tests. Challenge by re-exposure is rarely justified and is seldom performed. However, skin tests and *in vitro* tests are not reliable: frequently the diagnosis is an assessment of probability.

Exanthematous (maculopapular) reactions are the most frequent of all cutaneous drug reactions and can occur after any drug at any time up to three weeks post administration. They may be accompanied by fever, pruritus and eosinophilia. The eruption is generalised and symmetrical. If the administration of the drug is continued, an exfoliative dermatitis may develop. Anaphylaxis and anaphylactoid reactions usually develop within minutes to hours, are often severe and may be fatal. Along with urticaria and angioedema they are accompanied by bronchospasm and vasomotor collapse. The most common causes are contrast media for X-ray examination and antibiotics such as penicillin. ADRs can mimic common dermatoses including psoriasis, lichen planus, eczema, acne vulgaris and lupus erythematosus. Patients with AIDS are more likely to demonstrate multiple cutaneous drug reactions. It is important to be familiar with the reaction patterns caused by commonly used drugs in local clinical practice. To gather reliable data, reporting of ADRs and collating reported ADRs are essential. The World Health Organisation's Adverse Reaction Collaborating Centre in Uppsala provides a very large database, as does the Adverse event reporting system of the United States Food and Drug Administration (FDA) and the United Kingdom's 'Yellow card' reporting system.

[2] *http://student.bmj.com/issues/05/02/education/58.php*

Yellow reporting cards are available at the end of British National Formulary and may be obtained electronically[3].

13.3.5 Acute urticaria

The urticarias are characterised by a short-lived eruption of well-demarcated superficial erythematous or pale swellings known as wheals (synonyms: nettle rash, hives). Wheals are due to plasma leakage in to skin owing to a local increase in permeability of capillaries and venules mediated by release of pro-inflammatory mediators (such as histamine) released from cutaneous mast cells. Angio-oedema affects the deeper dermal, subcutaneous and submucosal tissues. Angio-oedema is usually painful rather than itchy, is usually poorly defined and pale or normal in colour.

Urticaria is the second most common type of adverse cutaneous drug reaction. Urticaria occurs within minutes of drug ingestion. It is most commonly caused by penicillins, sulphonamides and non-steroidal anti-inflammatory drugs (NSAIDs). Drug-induced urticaria is also seen in association with anaphylaxis and serum sickness. Angio-oedema is more rarely seen as an adverse drug reaction. Sometimes an unsuspected agent, for example the yellow dye tartarazine, may be responsible for an urticaria wrongly attributed to a drug. Food and drug additives such as benzoic acid, butylated hydroxyanisole, butylated hydroxytoluene, sulphites, aspartame and tartarazine dyes may be responsible for urticaria. Certain drugs such as opiates and radiocontrast media may stimulate the release of mast cell mediators directly. Cyclo-oxygenase inhibitors, such as aspirin and indomethacin, and ACE inhibitors may cause urticaria and angio-oedema via pharmacological mechanisms.

13.3.6 Vasculitis

Vasculitis is inflammation and necrosis of blood vessels (arteries, veins or both). Vasculitis may be localised to skin or may be systemic, involving the internal organs. The cutaneous manifestations depend on the size of the blood vessel involved. Small vessel vasculitis is characterised by palpable purpura, petechiae, necrosis and urticarial lesions on dependent areas such as the ankles and lower legs. In more severe cases the lesions may be more extensive. Larger vessel vasculitis is characterised by necrosing livedo reticularis and multiple sites of peripheral gangrene.

Vasculitis of various morphological types may be caused by drug ingestion. A variety of drugs including antibiotics, diuretics, NSAIDs and antiretroviral drugs have been implicated in causing cutaneous vasculitis. The clinical pattern is often that of a small vessel cutaneous vasculitis, characterised by multiple small bruises on the lower legs. It is important to be aware that illicit drugs, drug excipients, vaccines and food additives can also cause vasculitis. For example, cocaine is recognised as a cause of systemic vasculitis. Stopping the suspect drug may be all that is required to treat cutaneous vasculitis. Acute generalised exanthematous pustulosis is a pustular reaction reported in association with a number of drugs, usually penicillins and macrolides. Some cases of this reaction pattern may exhibit histological signs of vasculitis.

[3] *http://www.mca.gov.uk/yellowcard*

Figure 13.4 Erythema multiforme. A full-colour version of this figure appears in the colour plate section of this book

13.3.7 Erythema multiforme

Erythema multiforme is characterised by macular, papular, urticarial lesions, as well as the classical iris or 'target lesions', distributed preferentially on the distal extremities (Figure 13.4). Stevens–Johnson syndrome comprises extensive erythema multiforme of the trunk and mucous membranes, accompanied by fever, malaise, arthralgia and myalgia. Erythema multiforme is a well recognised pattern of adverse cutaneous drug reaction. A wide variety of drugs have been implicated, including antibiotics, non-steroidal anti-inflammatory drugs, anticonvulsants and antihypertensives. Drugs are sometimes blamed on inadequate and anecdotal evidence. Confirmation requires re-exposure to the drug, which may carry an unacceptable risk.

Erythema multiforme can also be provoked by topical medications containing chloramphenicol, neomycin, ophthalmic anticholinergic preparations, contact sensitisers like primula obconica and rubber gloves.

13.3.8 Toxic epidermal necrolysis (TEN)

Toxic epidermal necrolysis (TEN) is characterised by extensive sheet-like erosions of skin, with severe involvement of conjunctival, corneal, iridial, buccal and genital mucous membranes. Nikolsky's sign, the ability to extend the area of superficial sloughing by gentle lateral pressure on the surface of the skin at an apparently unaffected site, may be positive. The entire skin surface may be involved, with up to 100% of the epidermis sloughing off. Complications of TEN include wound infections, pigmentary changes, scarring alopecia and hypertrophic scarring.

The mortality for TEN is up to 40%, due to sepsis, renal failure, pulmonary embolism and gastrointestinal bleeding. A large number of drugs have been implicated. The most common triggers are antiepileptic drugs, β-lactam antibiotics, non-steroidal anti-inflammatory drugs, sulphonamides, trimethoprim and pentamidine. Early withdrawal of the causative drug improves the prognosis. The management of TEN is best carried out in an intensive care or a burns unit, with daily supervision of topical and systemic treatments by an experienced dermatologist and an experienced dermatology clinical nurse specialist. Treatment is mainly supportive, with attention to fluid balance, nutrition, skin care and anti-infectious therapy.

13.3.9 Fixed drug eruption (FDE)

A fixed drug eruption characteristically occurs at the same site(s) each time the drug is administered. However, the number of sites may increase with subsequent exposures. The lesions usually develop 30 minutes to eight hours after ingestion of the drug. The lesions are sharply demarcated round or oval itchy plaques of erythema and oedema. Later, the colour changes to dusky violaceous or brown. Vesicular or bullous lesions may sometimes be seen. The hands, feet, genitalia and peri-anal sites are commonly involved. As healing occurs, crusting and scaling are followed by pigmentation, which may be very persistent. The number of drugs capable of producing FDE is large. The important groups of drugs implicated are antibacterials such as sulphonamides and tetracyclines, barbiturates and other tranquilizers and non-steroidal anti-inflammatory drugs. Cross-sensitivity to related drugs may occur, for example between tetracycline-type drugs. There may be a refractory period after the occurrence of a fixed eruption.

13.3.10 Phototoxic drug reaction

Phototoxic drug reactions occur 5–20 hours after the first exposure and resemble exaggerated sunburn. They can be produced in almost all individuals given a high enough dose of the drug and ultraviolet radiation. Erythema, oedema, blistering, weeping, desquamation and residual hyperpigmentation occur on exposed areas. There may be photo-onycholysis (separation of the distal nail from the nail bed due to the phototoxic reaction at this site). A representative list of recognised phototoxic drugs is presented in Table 13.7.

13.3.11 Photoallergy to sunscreens

Photoallergy is a type IV hypersensitivity reaction to an antigen generated by the interaction of sunlight with a topically applied substance (Chapter 9). The most common photoallergens are ultraviolet filters in sunscreens and cosmetics. Benzophenone-3 and benzophenone-10 are the most common UV filters causing photoallergy. Chlorpromazine, promethazine and topical NSAIDs are also reported to cause photoallergy. Some photoallergens are also contact allergens, and some also cause phototoxic reactions. Photoallergy nearly always manifests as eczema. The latent period between exposure and appearance/deterioration of eczema is usually 2–48 hours. Exposed areas tend to be most severely affected though there may be spread to

Table 13.7 Summary of recognised causes of phototoxic drug reactions

Antibacterials	Tetracyclines, sulphonamides, fluoroquinolones
Phenothiazines	Chlorpromazine, promethazine
Diuretics	Furosemide
NSAIDs	Ibuprofen, piroxicam, naproxen, ketoprofen, diclofenac
Anticancer drugs	Dacarbazine, 5-fluorouracil, mitomycin, vinblastine
Fibric acid derivatives	Bezafibrate, fenofibrate
Miscellaneous	Psoralens, amiodarone, flutamide, tricyclic antidepressants and quinone derivatives

unexposed sites resulting in a generalised eruption. Diagnosis is confirmed by careful history and examination followed by patch testing and photo-patch testing.

13.4 Overview of standard treatments

As a fully externalised organ, the skin is unusual in that it can be treated by direct application of a range of standard medicaments, although it is sometimes more appropriate to administer certain therapeutic agents via the systemic circulation.

13.4.1 Topical therapy

Skin offers the advantage of being accessible to treatment with a wide range of physical and topical treatments. Topical therapies are simple, easy to use and achieve high concentrations of active agent at the site of disease with minimal systemic side effects.

Topical medications contain an active pharmaceutical ingredient(s) in a suitable vehicle. Topical medicaments can be categorised according to the characteristics of the vehicle. Various topical preparations are summarised in Table 13.8.

Table 13.8 Examples of topical formulations

Ointments	Semi-solid vehicles composed of lipid. Typically greasy and occlusive
Creams	Semi-solid emulsions. These are less greasy and less occlusive
Pastes	Semi-solid, high proportion of finely powdered material. These are occlusive, protective and hydrating
Lotions	Liquid formulations. On application to skin the liquid evaporates leaving a film of medication on the surface
Gels	Semi-solid, contain high molecular weight polymers. On application to skin the liquid evaporates leaving a film of medication on the surface, cosmetically more acceptable
Powders	Applied directly to skin, reduce friction or excessive moisture
Paints	Liquid preparations, applied with a brush to the skin

The choice of vehicle depends on the anatomical site to be treated and the condition of the skin. It is necessary to specify the concentration of active ingredient, the vehicle, the frequency of application and the quantity to be supplied in the prescription. Patients need to be educated on the quantity to be used, where it should be applied and the frequency of application. If more than one topical medicament is used it is important to advise when each application should be applied relative to the other(s).

Topical medicaments are usually safe to use under guidance. However, it is important to be aware of associated hazards. The most frequent adverse effect is localised irritant or allergic reaction. Irritation can be minimised by using the right quantity in an optimal vehicle. Contact allergy to the active ingredient as well as any component of the vehicle may occasionally develop. Contact allergy might manifest as worsening of the dermatitis or a failure to respond to treatment. Long-term use of potent topical corticosteroid preparations may result in atrophy, striae, telangiectasia, hypopigmentation and acneiform eruption. Systemic side effects from topically applied medications are relatively rare, but may occur with topical corticosteroids and topical antipruritis agents. Systemic absorption is greater in children and in the elderly, particularly if used under occlusion.

Physical modalities of treatment used in dermatology include cryosurgery, curettage, electro-surgery, infrared coagulation, application of caustics, chemical peels, lasers and light therapy. It is beyond the scope of this chapter to discuss these therapies in detail.

13.4.2 Systemic therapies

Topical therapies are usually preferred to systemic therapies in the treatment of skin conditions. However, a number of drugs, such as methotrexate, cyclosporin and acitretin, are only effective when administered systemically. In addition, topical therapy alone may not be sufficient to treat an extensive dermatosis. Whenever systemic therapies are used the prescribing physician

Table 13.9 Examples of systemic medications used in dermatology; uses and potential adverse effects

Medication	Indications	Adverse effect(s)
Corticosteroids	Anaphylaxis, auto-immune connective tissue and blistering diseases, severe drug reactions	Gastrointestinal upset, osteoporosis, Cushing's syndrome, diabetes mellitus
Retinoids	Severe nodulo-cystic acne, disorders of keratinisation, psoriasis	Teratogenicity, hepatotoxicity, hyperlipoprotenemia, skeletal hyperostosis
Methotrexate	Psoriasis and psoriatic arthritis, sarcoidosis, cutaneous lupus erythematosus, systemic sclerosis, dermatomyositis	Bone marrow suppression, hepatotoxicity, pneumonitis
Azathioprine	Dermatomyositis, systemic lupus erythematosus, pemphigus vulgaris, eczema	Bone marrow suppression, hypersensitivity, gastro-intestinal upset, pancreatitis, skin cancer
Ciclosporin	Psoriasis, eczema, pemphigus and pemphigoid, dermatomyositis	Nephrotoxic, hypertension, lymphoma, hirsutes, gum hypertrophy, skin cancer

should be alert to the possibility of adverse effects and drug interactions. The dosage needs to be carefully adjusted in children, the elderly and patients with chronic liver or renal disease. Patients on systemic medication are monitored by clinical assessment and investigations (e.g. periodic blood tests, bone scan). Important systemic medications used in dermatology, their uses and adverse effects are summarised in Table 13.9.

A wide variety of other systemic agents like antihistamines, antibiotics, antifungal and antiviral agents are used in dermatological practice. It is important to be aware of the potential side effects of these medications. Biological agents like infliximab, etanercept, efaluzimab and adalimumab are recent additions to systemic therapies for psoriasis and are all injectable drugs. These medications are expensive and the anti-TNF alpha agents (etanercept, infliximab and adalimumab) carry the risk of reactivation of latent tuberculosis.

Summary

- **Diagnosis of conditions indicative of a dermatotoxic response requires an evaluation of patient history, careful examination and the use of appropriate clinical tests.**
- **Whilst there is a wide variety of skin pathologies that can result from exposure to toxic substances, the most common include dermatitis (irritant or allergic), chloracne, characteristic rashes, urticarial (wheal-and-flare) reactions and frank necrosis.**
- **For contact dermatitis, measures to prevent re-exposure to the causative agent are an essential part of effective management.**

14 Clinical aspects of phototoxicity

Anthony D. Pearse[1] and **Alex Anstey**[2]

[1]*Cutest Systems Ltd, 214 Whitchurch Road, Cardiff, CF14 3ND, UK*
[2]*Consultant Dermatologist and Honorary Senior Lecturer, Gwent Healthcare NHS Trust, Royal Gwent Hospital, Cardiff Road, Newport, Gwent NP20 2UB, UK*

Primary Learning Objectives

- *Outline of photoreactive skin conditions and analogous pathologies (irritant contact dermatitis and allergic contact dermatitis).*
- *Potential causes and clinical manifestations of conditions characterised by photoreactivity of the skin.*
- *Standard clinical tests for identifying photoirritant and photosensitising substances.*

14.1 Introduction and scope

Skin exposure to ultraviolet radiation can elicit an irritant or allergic skin reaction through photoactivation of xenobiotics present in skin tissue. Such adverse effects are referred to as phototoxic (photoirritant) and photoallergic (photosensitive) reactions, respectively.

The subject of this chapter is the development of cutaneous reactions to ultraviolet radiation (UVR). Sources of UVR (such as direct sunlight) have the capacity to energise molecules and, in this photoactive state, these molecules can produce phototoxic (photoirritant) and photosensitive (photoallergic) reactions that are analogous in many ways to irritant contact dermatitis (ICD) and allergic contact dermatitis (ACD), respectively (Table 14.1).

In addition to the growing number of toiletries, cosmetics and topical dermatologicals in the marketplace, there are increasing numbers of new drugs available. Despite modern drug pre-registration screening, a small number slip through the net and are capable of eliciting phototoxic (photosensitive) reactions. The information on photosensitising drugs is collected by various national and international reporting systems for adverse drug reactions. This is usually a voluntary reporting procedure involving the medical profession, pharmacists and pharmaceutical companies. The major bodies being the World Health Organisation Programme for International Drug Monitoring database, the Medicines Healthcare Regulatory Authority (MHRA) Committee on the Safety of Medicines in the United Kingdom and the United States Food and Drug Administration (FDA). It is also likely that a large number of

Principles and Practice of Skin Toxicology Edited by Robert P. Chilcott and Shirley Price
© 2008 John Wiley & Sons, Ltd

Table 14.1 Empirical comparison of photoirritation (phototoxicity) and photoallergy (photosensitisation) [see also Chapter 13, Table 13.6; similarities between irritant contact dermatitis and allergic contact dermatitis]

	Photoirritation	Photoallergy
Trigger	Primary photoirritant; single or cumulative exposure	Photoallergen or photosensitiser; repeated exposure only
Examples	Plant extracts (e.g. furocoumarins such as psoralens), essential oils (e.g. bergamot oil due to presence of bergapton) Coal tar extracts (e.g. antracene, pyrene, fluoranthene) Drugs such as quinoline-based antimalarials, the cardiac antiarrythmic amiodarone, tetracycline antibiotics (e.g. doxycycline and chlortetracycline), thiazide diuretics and some non-steroidal anti-inflammatorys (particularly those containing a propionic acid group).	Drugs such as antibiotics (halogenated salicylanilides), fungicides (fentichlor) and local anaesthetics (benzocaine) Sunscreens (para-aminobenzoic acid and derivates) Essential oils (sandalwood) and plant extracts (coumarins and derivatives).
Mediator	Non-immunological	Immune-cell mediated
Time to onset	Immediate	Delayed
Mechanism	Release of inflammatory mediators	Langerhans cells present antigens to T-cells leading to proliferation of lymphocytes
Sign(s)	Erythema, scaling, thickened skin, vesicules and oedematous eruptions	Generally similar to photoirritation, although a less common reaction (solar urticaria) can result in a wheal-and-flare reaction

mild episodes of drug-induced photosensitivity pass unnoticed by patients or are assumed to have been an episode of mild or moderate sunburn (Ferguson, 2002). There are also several diseases which are initiated or exacerbated by UVR exposure. These include autoimmune diseases, such as lupus erythematosus, and idiopathic photodermatoses, such as polymorphic light reaction, chronic actinic dermatitis and solar urticaria (Epstein, 1999).

Some materials require UVR to activate them before they are capable of eliciting adverse reactions. These photoirritant or photoallergic reactions are probably less common than contact dermatitis reactions. In phototoxic reactions, the chemical or drug may absorb radiation and transfer that energy to membranes of skin cells inducing cell damage. Alternatively, some phototoxicity reactions are mediated by chemicals or compounds that become intercalated into DNA, with the result that the DNA or nucleus become damaged by the energy of absorbed UV radiation. In photoallergic reactions the energy of absorbed radiation leads to a compound capable of reacting as a hapten and inducing an immunological reaction. This can potentially stimulate the immune system and produce an allergic response such

as a Type I immediate or Type IV delayed hypersensitivity reaction. Despite the range of safety tests carried out by pharmaceutical companies and to a lesser extent by cosmetic and toiletry manufacturers, it may be necessary to further test the final product in the hospital or laboratory environment. Tests to assess the phototoxicity potential of a product should not be carried out in isolation, but in conjunction with tests designed to assess the irritant or allergic potential of the product in question. Before a human test is undertaken, every potential risk to the subjects is assessed. This evaluation is based on all known data, including animal and *in vitro* information (Chapters 9 and 11). However, the most valid data are gained from the species for which the product was designed, namely man.

14.2 UV-induced skin reactions

Sunlight is a continuous spectrum of radiation and humans react to its entirety, sometimes with erythema or sunburn in exposed skin. The paler the skin, the greater the erythematous response for any given dose of UVR. Thus, skin type I burns more readily than skin type VI (Table 14.2). UVB is primarily responsible for the erythema seen in human skin following excessive sun exposure. A more detailed review of the biological skin effects of UV exposure is given in Chapter 3. In contrast to UV-induced erythema (which can be considered to represent a 'natural' response to solar irradiation), UV exposure in the presence of an exogenous or endogenous chemical (chromophore) which can absorb UV or visible light may lead to a pathological responses known as a phototoxic (photoirritant) or photosensitive (photoallergic) reactions.

14.3 Phototoxicity (photoirritancy) reactions

Phototoxicity arises following exposure to a photoirritant. Such substances include plant and coal tar extracts and a number of drugs. The onset of photoirritation can be immediate and is mediated via a non-immunological mechanism.

It is possible that topically applied or systemically administered chemicals or drugs that have little or no potential to promote an irritant or allergic reaction in the skin, may do so in the presence of sunlight due to the effects of photoactivation. Although photosensitive reactions may be rare relative to irritant or sensitisation reactions, the development of such reactions can

Table 14.2 Boston classification of skin phototype (Compare with the detailed Fitzpatrick phototype classification; Chapter 3, Table 3.1)

Skin type I	Always burns and never tans
Skin type II	Always burns and tans with difficulty
Skin type III	Often burns and tans moderately
Skin type IV	Burns minimally and tans easily
Skin type V	Rarely burns and tans profusely
Skin type VI	Insensitive, never burns, deeply pigmented

lead to withdrawal of products from the market (e.g., the antirheumatic drug, benoxaprofen) (Allen, 1983).

Phototoxicity refers to skin irritation that is produced through the interaction of chemical substances and radiant energy in the ultraviolet and visible ranges. Phototoxic or photoirritant effects are immediate and non-immunological, and in most instances are mediated by UVA. It is for this reason that when testing for the phototoxic potential of topically applied chemicals, the output from the required radiation source is UVA only. This is best accomplished by using a suitably filtered solar simulator. The clinical identification of phototoxic reactions in humans relies on both morphology and the clinical evidence or suspicion of the presence of possible phototoxic chemicals. Phototoxicity usually causes erythema or even bullae, increased skin temperature and pruritus and is followed by hyperpigmentation that, in some instances, may be long lasting.

A range of phototoxic agents have been reported in the literature, many of which are naturally occurring plant substances, such as the furocoumarins, including the psoralens (Avalos and Maibach, 1999). Psoralens occur in a variety of plants, such as parsley, celery and citrus fruits. A plant-induced phototoxic reaction is known as phytophotodermatitis. Perfumes that contain bergapton, a component of bergamot oil, a well-known photoirritant, can be phototoxic. Other phototoxic agents include coal tar derivatives, pyrene, anthracene and fluoranthene (Kochevar *et al.*, 1982). The cardiac antiarrhythmic agent amiodarone has produced phototoxic effects (Rappersberger *et al.*, 1989), as have quinoline anti-malarial drugs (Ljunggren and Winestrand, 1988). Tetracyclines, including demethylchlortetracycline, doxycycline and chlortetracycline, may also prove phototoxic when taken orally (Bjellerup and Ljunggren, 1985). The thiazide diuretics have also exhibited a phototoxic potential in experiments involving cardiovascular patients with hypertension and heart failure (Diffey and Langtry, 1989). Some non-steroidal anti-inflammatory drugs (NSAIDs) have a phototoxic potential, both when administered orally or topically (Bosca *et al.*, 1994; Stern, 1983). This is particularly true of propionic acid-related NSAIDs, which produce a unique wheal-and-flare response (Diffey *et al.*, 1983; Kaidbey and Mitchell, 1989).

Most of the information gained on the underlying mechanisms of phototoxic reactions has been derived from *in vitro* animal models (Chapter 9). For example, a recent study evaluated 13 compounds by *in vitro* and animal *in vivo* methods including the fibroblast 3T3 test, the photo hen's egg test, a guinea pig test for measuring acute photoreactions and a modified Local Lymph Node Assay, an integrated model for the differentiation of skin reactions (Neumann *et al.*, 2005). Other methods have included *Candida albicans* (Daniels, 1965; Mitchell, 1971), photohaemolysis of red blood cells and isolated normal human fibroblasts (Lock and Friend, 1983). Such *in vitro* models are, however, limited, as they lack the complexity of a fully integrated physiological response, which presently can only be modelled in a living animal. Human skin equivalent models have also been used in an attempt to predict the phototoxic potential of topically applied personal care products (Chapter 11). Whilst these models have attained some success in identifying phototoxicity, false negative results were seen with well-known phototoxic agents such as amiodarone hydrochloride, 6-methycoumarin, bithionol and piroxicam (Edwards *et al.*, 1994). Methods such as these may become more accurate predictors if they rely on more than one marker of cell damage for the prediction of chemical irritancy or, in this case, photoirritancy. However, the most reliable results are still obtained when using the whole animal as a model. If the goal is to predict phototoxicity in humans, then the preferable test method should also use, when possible, human subjects.

14.3.1 Phototoxicity assays

A standard method for clinical assessment of phototoxicity includes the topical application of test compounds under occluded conditions prior to irradiation with UVA followed at set intervals by visual assessment.

The development of procedures for assessing both the phototoxic (and photosensitive) potential of chemicals has, in general, been carried out in laboratory animals. A wide range of animal species has been used, including rabbits, mice, guinea pigs, squirrel monkeys, opossums and swine. All methods require the skin to be irradiated with UVA following application of the test substance. However, the measurable end result is not always similar to that obtained from human experiments. Increases in guinea pig (Stott *et al.*, 1970) or mouse ear thickness (Cole *et al.*, 1984; Gerberick and Ryan, 1989) have been used to quantify phototoxic responses. Dermatitis and the increase in weight of the mouse tail has also been used (Ljunggren and Muller, 1976). However, hairless mice and albino guinea pigs have been used where simple erythema was the toxicological endpoint for assessing a phototoxic response (Forbes *et al.*, 1977); the results obtained from the use of such atypical models may be false negative or false positive when extrapolated to humans.

Kaidbey and Kligman (1978) proposed a method for identifying potential topical phototoxic agents in humans. Human testing can be justified as only small areas of skin are irradiated and clinical experience of phototoxic reactions indicate that when the stimulus is removed the erythema subsides. As with all clinical trials the informed consent of the subject and ethical committee approval must be obtained. The recommended method is based on that of Kaidbey and Kligman and is suitable for assessing the phototoxic potential of topically applied drugs, chemicals, transdermal and skin care products.

In the protocol for phototoxicity testing (Figure 14.1), a test panel consisting of a minimum of 12 healthy white adults with untanned back skin is required. As the dermatitis of a phototoxic agent can be produced in almost every subject, given sufficient exposure, it is not necessary to employ a large test panel. Two sets of test products and appropriate controls (usually vehicle or base alone) are applied under occlusion to the mid-back area (one set on the left and one set on the right). Aluminium Finn™ chambers (12 mm diameter) affixed with Scanpore™ tape are used by many testing laboratories and are suitable for product application. They are occlusive and ensure optimal product contact with the skin. An empty chamber should also be fixed to each side of the back as an additional negative control. Six hours after administration of the materials and chambers, they are removed and one set of applications irradiated immediately with UVA. The chemical-to-skin contact time is relatively short compared with, for example, the 24 hours needed in a vasoconstriction assay. If longer times are used (e.g., 24 hours), light exposure will often produce negative results. The test sites should be irradiated with 20 J cm^{-2} of UVA. The radiation source should ideally be a xenon arc lamp solar simulator with a well characterised spectral output.

Such a system typically consists of a 1000 W ozone-free xenon arc lamp, the output from which is filtered with a Schott WG 345 filter of 2–3 mm thickness. This filter blocks all erythemogenic UVC and UVB wavelengths below 320 nm. In addition, unwanted longer wavelength visible and infrared radiation are removed using a combination of a suitably coated dichromic mirror, water filter and UG11 filter. In the absence of these devices subjects may feel heat and or pain from the irradiations. It is often convenient to deliver the UVA

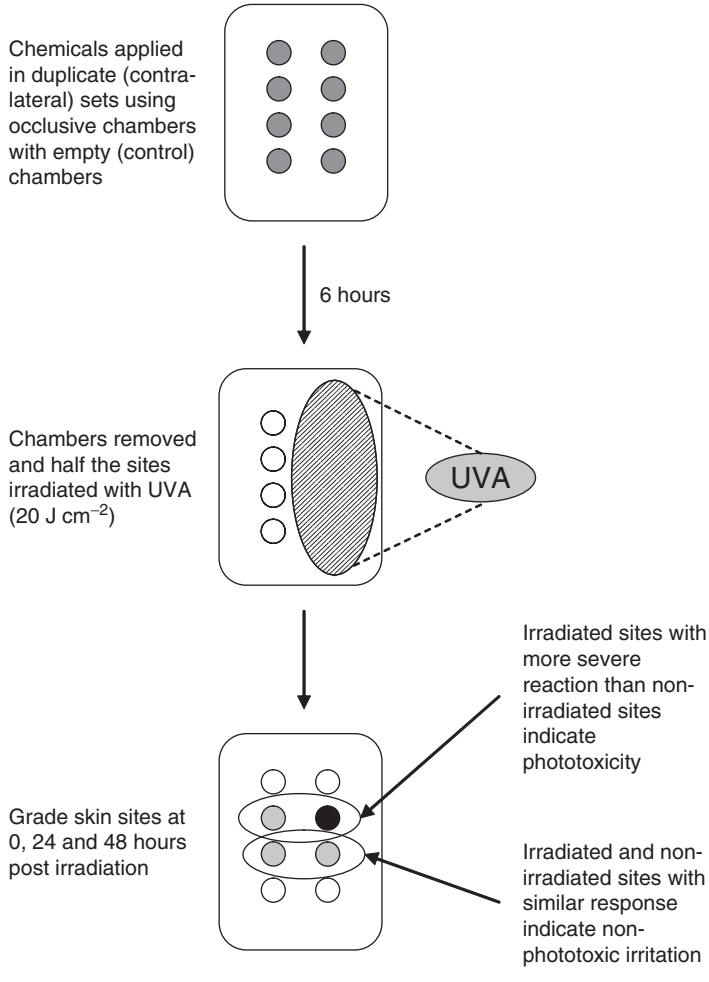


Figure 14.1 Summary of basic procedures for performing standard photoirritation (phototoxicity) test involving human volunteers. After obtaining informed consent, at least twelve volunteers are subject to test compound exposure using occlusive chambers. Test compounds are applied in duplicate. Six hours later, the chambers are removed and one set of treated sites is exposed to UVA (20 J cm^{-2}). The skin response at each site is then scored at 0, 24 and 48 hours post irradiation using the grading system described in Table 14.3

radiation to the skin surface using an 8 mm diameter liquid light guide. A broad-spectrum thermopile should be used to measure the output of energy from the solar simulator (typically expressed as mW cm^{-2}). The thermopile should be calibrated against a known standard from, for example, the UK National Physics Laboratory. The time of irradiation necessary to administer a dose of 20 J cm^{-2} to the skin should be calculated from Equation (14.1):

$$t(s) = \frac{\text{mJ cm}^{-2}}{\text{mW cm}^{-2}} \tag{14.1}$$

where t(s) is the exposure duration (expressed in seconds).

Table 14.3 Phototoxicity grading system

Grade	Cutaneous reaction
0	No reaction
1	Mild erythema, possibly with scaling
2	Moderate or strong erythema
3	Moderate or strong erythema with a papular response
4	As grade 3, but with definite oedema
5	Vesicular or bullous eruption

For example, if a thermopile reading of 200 mW cm^{-2} and a dose of 20 J cm^{-2} is required, then the duration of exposure would be $(20\,000/200) = 100$ s.

Skin assessments should be made immediately following irradiation and at 24 and 48 hours after photoexposure. The grading system in Table 14.3 is suitable for recording any cutaneous reactions. The irradiated sites should be compared in each subject with the non-irradiated sites. If the response in any one subject at the irradiated site is greater than that seen at the non-irradiated site, then that product or chemical is deemed phototoxic. Phototoxicity is relatively easy to detect and therefore prevent. However, it should be noted that phototoxic reactions may sometimes mask or contribute to photoallergic reactions.

14.4 Photosensitive reactions

Photoallergy arises following exposure to a photosensitiser. Such substances include a number of drugs, sunscreens and essential oils. The onset of photosensitisation is delayed and mediated by cells of the immune system.

Photoallergy refers to an allergic dermatitis to an allergen produced through the interaction of a chemical substance in the skin with UVR or visible light. The chemical substance may be orally ingested or topically applied (photocontact allergy). Unlike phototoxic reactions, photoallergic reactions are typically delayed in onset and immunologically mediated, and are less dose-dependent. Photoallergic and particularly photocontact photoallergic reactions are relatively uncommon compared to phototoxic reactions. Clinically, photocontact allergic reactions produce a dermatitis that resembles allergic contact dermatitis, appearing as an acute dermatitis affecting primarily, but not exclusively, light-exposed skin. A characteristic histological feature of photocontact allergy is a dense perivascular round cell infiltrate in the dermis, which helps distinguish this dermatitis from a phototoxic reaction (Epstein, 1985). A second and rare type of photoallergic reaction is solar urticaria (Sams, 1970). This occurs after only brief exposure to light and is characterised by an immediate urticarial wheal-and-flare reaction within minutes of exposure. The reaction usually subsides within 1–2 hours, is associated with degranulation of mast cells at the site of exposure and the release of neutrophil chemotactic factors and histamine into venous blood near the reaction sites. Photoallergic reactions, when they occur, may apparently be triggered by irradiation alone (in the absence of known sensitisers) or may be due to exogenous chemicals and UVR. Photoallergy, whether photocontact allergic dermatitis (delayed hypersensitivity: Type IV

reaction; Table 9.1) or solar urticaria (immediate hypersensitivity: Type I reaction; Table 9.1), is an acquired reactivity dependent on cell-mediated hypersensitivity or antigen–antibody interaction (Epstein, 1983).

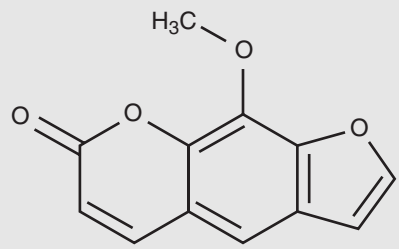
Test procedures designed to identify potential photosensitising chemicals were developed in response to the outbreak of reactions caused by the use of anti-bacterial halogenated salicylanilides in the early 1960s (Wilkinson, 1961). A minority of affected individuals developed a persistent photodermatitis that lasted several years despite the avoidance of contact with photosensitising phenolic compounds (Smith and Epstein, 1977). Therefore, it became clear that there was a requirement for a laboratory test to detect potential photosensitising agents and avoid such situations.

Several photocontact sensitisers have been identified, including coumarins (and derivatives), musk ambrette, fentichlor, bromochlorosalicylanilide, chloro-2-phenylphenol and benzocaine (Kaidbey and Kligman, 1981; Raugi *et al.*, 1979; Epstein, 1972). Certain sunscreens have also been reported to produce photocontact allergic dermatitis, most notably *para*-aminobenzoic acid (PABA) and derivatives, benzophenone-3, mexenone and cinnamates (Parry *et al.*, 1995; Thune, 1984; Szczurko *et al.*, 1994). A recent review on the prevalence of contact allergy and photoallergy to sunscreen agents concluded that, as sunscreen use increases, the problem has become more widespread (Scheuer and Warshaw, 2006). Several essential oils have also produced photoallergic reactions, e.g., sandalwood oil (Starke, 1967). Also, 8-methoxypsoralen, which is used in PUVA phototherapy (psoralen + UVA) (Box 14.1), can also be photoallergic as well as phototoxic (Fulton and Willis, 1968).

Box 14.1 Bath PUVA therapy – getting the dose right

8-Methoxypsoralen (8-MOP) is a compound administered transdermally in a treatment for psoriasis known as PUVA therapy. The treatment involves the patient bathing in a solution of 8-MOP (or applying a cream containing the drug) followed by irradiation with ultraviolet A (hence, PUVA stands for psoralen ultraviolet A). The treatment utilises the phototoxicity of 8-MOP; it substantially lowers the dose of UVA required to give the same therapeutic effect than UVA exposure alone and, in comparison with oral 8-MOP UVA therapy, produces less severe side effects.

However, this is not as straight forward as it seems: skin absorption of too little 8-MOP will prevent accumulation of a therapeutic concentration of 8-MOP at the site of action (epidermis). Conversely, absorption of too much drug will result in delivery to the dermis and systemic circulation (which is not good for a toxic compound!). This problem has largely been resolved by the appropriate use of *in vitro* and *in vivo* studies (e.g. Anigbogu et al., 1996).



The incidence, mechanism, prevention and management of drug-induced cutaneous photosensitivity have been reviewed recently (Moore, 2002). The photochemical and photobiological mechanisms underlying the adverse reactions caused by the more photoactive drugs are mainly free radical in nature, but reactive oxygen species are also involved. Drugs that contain chlorine substituents in their chemical structure, such as hydrochlorthiazide, furosemide and chlorpromazine, exhibit photochemical activity that is traced to the UV-induced dissociation of the chlorine substituent, which leads to free radical reactions with lipids, proteins and DNA. The photochemical mechanisms for the NSAIDs that contain the 2-aryl proprionic acid group involve decarboxylation as the primary step, with subsequent free radical activity. In aerated systems, the reactive excited singlet form of oxygen is produced with high efficiency. This form of oxygen is highly reactive towards lipids and proteins. NSAIDs without the 2-aryl proprionic acid group are also photoactive, but with differing mechanisms leading to a less severe biological outcome. Antibiotics can also be photoactive with tetracyclines, fluoroquinolines and sulfonamides being most photoactive. Prevention of contact photosensitivity from other topically applied materials, such as cosmetic ingredients, local anaesthetics and anti-acne agents, involves adequate protection from the sun with clothing and possibly sunscreens.

14.4.1 Photoallergenicity assays

A standard method for clinical assessment of photosensitisation involves a two-stage (induction and elicitation) procedure. Sensitisation is induced by the repeated topical application of test substances followed by exposed to UVR (UVA and UVB). Elicitation is subsequently promoted by irradiation of sites topically exposed to test compounds followed by irradiation with UVA, with grading of skin responses at set intervals thereafter by visual assessment.

Test procedures to identify potential photoallergic photosensitisers have received less attention than methods designed to detect either ordinary contact sensitisers or chemicals with the potential to cause phototoxicity. Landsteiner and Chase (1942) demonstrated that low molecular weight haptens can produce contact dermatitis in guinea pigs. They also observed that allergic contact dermatitis could be conferred on immunologically naive guinea pigs by passive transfer of mononuclear cells from non-sensitised animals. Furthermore, guinea pigs develop oedema and erythema after contact with topically applied sensitisers and, to some extent, develop a response similar to the clinical response in humans. These observations became the cornerstone of photocontact allergic dermatitis research over many years and led to the guinea pig becoming the most commonly used animal in photoallergy studies (Harber *et al.*, 1967; Harber and DeLeo, 1985). On the other hand, mouse ear swelling is claimed to be a more sensitive model (Maguire and Kaidbey, 1982), but is even further removed from the human response than that of the guinea pig. To induce contact photosensitivity in any animal it has to be repeatedly exposed to the test molecule in the presence of UVR. For this induction phase a broad-spectrum source is necessary, which should include UVB as well as UVA. The period of induction is similar to that for testing contact sensitisers, followed by a rest period and then a challenge on a previously untested site with the test chemical and UVA alone.

The photomaximisation test for the prediction of photosensitisers is conducted on humans and is similar in design to an irritancy and sensitisation test, but with the addition of exaggerated UVR exposure of both the chemical and the skin to which it is applied (Figure 14.2). In an

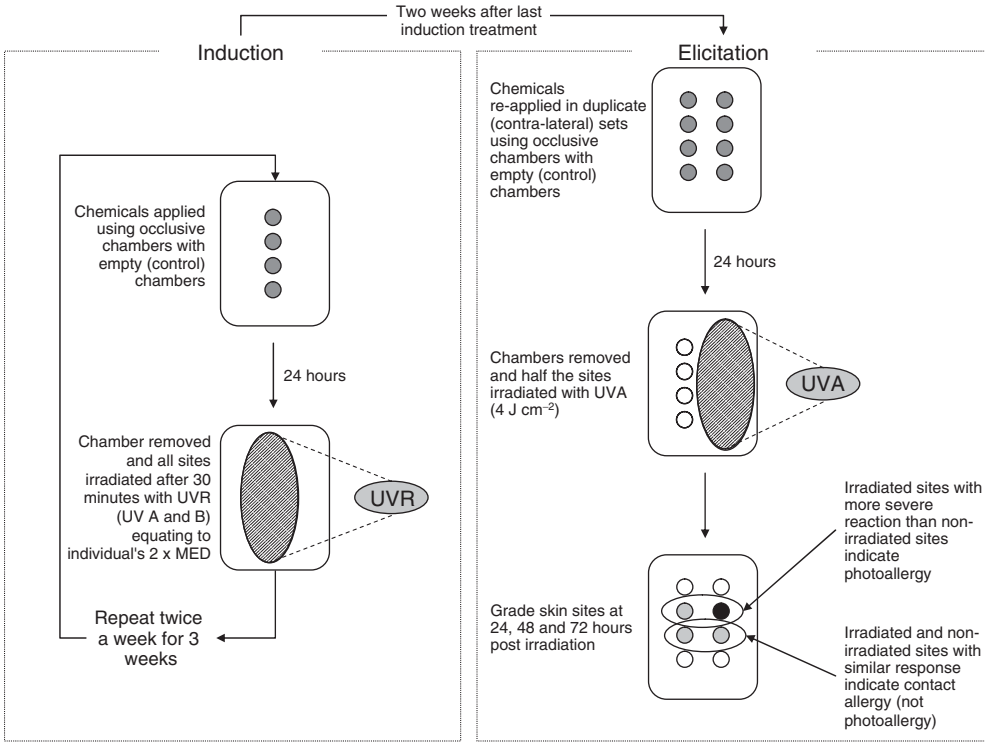


Figure 14.2 Summary of a standard photoallergy (photosensitisation) test involving human volunteers. Two phases are involved: induction and elicitation. After obtaining informed consent, at least twenty six volunteers are subject to test compound exposure using occlusive chambers. After twenty four hours, the chambers are removed and all sites exposed to UVR (UVA and UVB) at a dose corresponding to twice the individual's minimum erythema dose (2 × MED). The process is repeated six times. Two weeks after the last induction phase treatment, subject to test compound exposure using occlusive chambers. Test compounds are applied in duplicate. Twenty four hours later, the chambers are removed and one set of treated sites are exposed to UVA ($4\,J\,cm^{-2}$). The skin response at each site is then scored at 24, 48 and 72 hours post irradiation using the grading system described in Table 14.3

ideal world, this type of test would be carried out on a large number of subjects (>100) to more accurately predict the incidence of photosensitisation reactions in the populations at large. From a practical point of view this is not possible because of the demanding nature of the protocol; therefore, a test panel of 26 is normally recommended. The method described here is based on that of Kaidbey and Kligman (1980) and is suitable for identifying topical photocontact sensitisers.

The photomaximisation test is a six-week study and is divided into an induction phase and an elicitation phase. A solar simulator, as described earlier, is an ideal source of UVR. During the induction phase a 1 mm WG320 filter and a 2 mm UG11 filter are used, allowing both UVA and UVB (290–400 nm) to reach the subject's skin. For the elicitation phase, a 2–3 mm WG345 and a 1 mm UG11 filter are used to allow only UVA (320–400 nm) to reach the skin. The test chemical together with the vehicle control is

applied occlusively to the mid-back of each subject, again using 12 mm aluminium Finn™ chambers on Scanpore™ tape. Twenty-four hours later, the patches are removed and the test sites wiped clean and allowed to air-dry for approximately 30 minutes. Each site is then exposed to twice the subject's minimal erythema dose (MED) of solar-simulated UVR. The sites are then left uncovered and exposed to the air for approximately 48 hours. This procedure of application and irradiation is repeated such that each subject has six applications and irradiations over a three-week period. The sites are evaluated 24 hours after each irradiation and, if the reaction becomes severe such that further application and irradiation is undesirable, then application of the material and subsequent irradiation is carried out at an adjacent site.

Following completion of the induction phase, there is a two week rest period, with no applications or irradiations. Approximately 14 days after completion of the induction phase, two sets of test materials are applied to previously untreated sites on the mid-back, again using 12 mm Finn™ chambers on Scanpor™ tape. Twenty-four hours later the patches are removed, the skin wiped dry with a gauze swab, and one set of applications irradiated with 4 mJ cm^{-2} of UVA. The sites are then evaluated at 24, 48 and 72 hours after the elicitation irradiation. The grading system for all irradiations (both induction and elicitation) is summarised in Table 14.4. If one or more subjects develop a reaction at an irradiated site during the elicitation phase that is greater than the corresponding unirradiated site, then that chemical is considered to be a photosensitiser. In practical terms there are usually many or no reactors in a test panel, making the decision as to whether a product is a photoallergen relatively easy.

Table 14.4 Grading system for photoallergy test: induction and elicitation phase

Score	Description
0	No reaction.
1	Reaction readily visible, but mild unless letter grade appended (see E and F grades). Mild reactions include weak, but definite erythema, and weak superficial skin responses such as glazing, cracking, or peeling.
2	Definite papular response (appended E, F, or S if appropriate).
3	Definite edema (appended E, F, or S if appropriate).
4	Definite edema and papules (appended E, F, or S if appropriate).
5	Vesicular-bullous eruption (appended E, F, or S if appropriate).
E	Strong erythema at patch site.
F	Strong effects on superficial layers of the skin including fissures, a film of dried serous exudates, small petechial erosions, or scabs.
S	Reaction spreading beyond test site.
I	Itching.
B/S	Burning or stinging

Notes: Applications must be either terminated or moved to an adjacent non-irradiated site if a reaction score of two or higher occurs. Descriptive letter designations may be added to the numerical score if experienced at the test site. Any other signs or symptoms (e.g., wheal-and-flare responses) may be described separately.

Summary

- Skin exposure to ultraviolet radiation can cause dermal reactions analogous to ICD and ACD; photoirritation and photosensitisation, respectively.
- Standard methods of clinical assessment are available to identify the causative agents of such photoreactive skin conditions.

References

Allen, B. (1983). Benoxaprofen and the Skin. *Br J Dermatol*, **109**: 361–364.

Anigbogu, A.N., Williams, A.C. and Barry, B.W. (1996). Permeation characteristics of 8-methoxypsoralen through human skin; relevance to clinical treatment. *J Pharm Pharmacol*, **48**(4): 357–66.

Avalos, J. and Maibach, H.I. (eds). (1999). *Dermatologic Botany*, CRC Press, Boca Raton, 51–65.

Bjellerup, M. and Ljunggren, B. (1985). Photohaemolytic potency of tetracyclines. *J Invest Dermatol*, **84**: 262–264.

Bosca, F., Miranda, M.A., Carganico, G., *et al.* (1994). Photochemical and photobiological properties of Ketoprofen associated with the benzophenone chromophone. *Photochem Photobiol*, **60**: 96–101.

Cole, C., Sambuco, C., Forbes, P. and Davies, R. (1984). Response to ultraviolet radiation: ear swelling in hairless mice. *Photodermatology*, **1**: 114–118.

Daniels, F.A. (1965). Simple microbiological method for demonstrating phototoxic compounds. *J Invest Dermatol,* **44**: 259–263.

Diffey, B.L. and Langtry, J. (1989). Phototoxic potential of thiazide diuretics in normal subjects. *Arch Dermatol*, **125**: 1355–1358.

Diffey, B.L., Daymond, J.J. and Fairgreaves, H. (1983). Phototoxic reactions to piroxicam, naproxen, and tiaprofenic acid. *Br J Rhematol*, **22**: 239–242.

Edwards, S.M., Donnelly, T.A., Sayre, R.M. and Rheims, L.A. (1994). Quantitative *in vitro* assessment of phototoxicity using a human skin model, Skin^2. *Photodermatol Photoimmunol Photomed*, **10**: 111.

Epstein, J.H. (1972). Photoallergy: a review. *Arch Dermatol,* **106**: 741.

Epstein, J.H. (1983). Phototoxicity and photoallergy in man. *J Am Acad Dermatol,* **8**: 141.

Epstein, J.H. (1985). Photoallergy and photoimmunology, in *Dermatologic Immunology and Allergy* (ed. Stone, J.), CV Mosby, St Louis.

Epstein, J.H. (1999). Phototoxicity and Photoallergy. *Sem Cutaneous Med Surg,* **18**(4): 274–284.

Ferguson, J. (2002). Photosensitivity due to drugs. *Photoderm Photoimmunol & Photomed,* **18**: 262–269.

Forbes, P.D., Urbach, F. and Davies, R.E. (1977). Phototoxicity testing of fragrance raw materials. *Food Cosmet Toxicol*, **15**: 55–60.

Fulton, J.E. and Willis, I. (1968). Photoallergy to methoxsalen. *Arch Dermatol,* **98**: 445–450.

Gerberick, G. and Ryan, C. (1989). A predictive mouse ear-swelling model for investigating topical phototoxicity. *Food Chem Toxicol,* **27**: 813–819.

Harber, L.C. and DeLeo, V.A. (1985). Guinea pigs and mice as effective models for the prediction of immunologically mediated contact photosensitivity, in *Models in Dermatology: Dermatopharmacology and Toxicology* (eds Maibach, H.I. and Lowe, N.J.), Karger, New york.

Harber, L.C., Targovnik, S.E. and Baer, R.W. (1967). Contact photosensitivity patterns to halogenated salicylanilides in man and guinea pigs. *Arch Dermatol,* **96**: 646.

Kaidbey, K.H. and Kligman, A.M. (1978). Identification of topical photosensitising agents in humans. *J Invest Dermatol*, **70**: 149.

Kaidbey, K.H. and Kligman, A.M. (1980). Photomaximisation test for identifying photoallergic contact sensitisers. *Contact Dermatitis,* **6**: 161.

Kaidbey, K.H. and Kligman, A.M. (1981). Photosensitisation by coumarin derivatives: structure activity relationships. *Arch Dermatol,* **117**: 258.

Kaidbey, K.H. and Mitchell, F. (1989). Photosensitising potential of certain non-steroidal anti-inflammatory agents. *Arch Dermatol,* **125**: 783–786.

Kochevar, I., Armstrong, R.B., Einbinder, J., *et al.* (1982). Coal tar phototoxicity: active compounds and action spectra. *Photochem Photobiol,* **35**: 65–69.

Landsteiner, K. and Chase, M.W. (1942). Experiments on transfer of cutaneous sensitivity to simple compounds. *Proc Soc Exp Biol Med*, **49**: 688.

Ljunggren, B. and Muller, H. (1976). Phototoxic reaction to chlorpromazine as studied with the quantitative mouse tail technique. *Acta Derm Venereol,* **56**: 373.

Ljunggren, B. and Winestrand, L. (1988). Phototoxic properties of quinine and quinidine: two quinoline methanol isomers. *Photodermatology,* **5**: 133–138.

Lock, S.O. and Friend, J.V. (1983). Interaction of ultraviolet light, chemicals and cultured mammalian cells: photobiological reactions of halogenated antiseptics, drugs and dyes. *Int J Cosmet Sci,* **5**: 39–49.

Maguire, H.C. and Kaidbey, K.H. (1982). Experimental photoallergic contact dermatitis: a mouse model. *J Invest Dermatol,* **79**: 147–152.

Mitchell, J.C. (1971). Psoralen type phototoxicity of tetramethylthiur-ammonosulphide for *Candida albicans*: not for man or mouse. *J Invest Dermatol,* **56**: 340.

Moore, D.E. (2002). Drug-Induced Cutaneous Photosensitivity. Incidence, mechanism, prevention and management. *Drug Safety,* **25**(5): 345–372.

Neumann, N.J., Blotz, A., Wasinska-Kempka, G., *et al.* (2005). Evaluation of phototoxic and photoallergic potentials of 13 compounds by different *in vitro* and *in vivo* methods. *J Photochem Photobiol B: Biology,* **79**: 25–34.

Parry, E.J., Bilsland, D. and Morley, W.N. (1995). Photocontact allergy to 4-*tert*-butyl-4'-methoxydibenzoylmethane (Parsol 1789). *Contact Dermatitis,* **32**: 251.

Rappersberger, K., Honigsmann, H., Ortel, B., *et al.* (1989). Photosensitivity and hyperpigmentation in amiodarone treated patients: incidence, time course and recovery. *J Invest Dermatol,* **93**: 201–209.

Raugi, G.J., Storrs, F.J. and Larsen, W.G. (1979). Photoallergic contact dermatitis to mens perfume. *Contact Dermatitis,* **5**: 251.

Sams, W.M. (1970). Solar urticaria: studies of the active serum factor. *J Allergy Clin Immunol,* **45**: 295.

Scheuer, E. and Warshaw, E. (2006). Sunscreen allergy: A review of edipemiology, clinical characteristics and responsible allergens. *Dermatitis*, **17**(1): 3–11.

Smith, S.Z. and Epstein, J.H. (1977). Photocontact dermatitis to halogenated salicylanilides and related compounds; our experience between 1967 and 1975, *Arch Dermatol,* **113**: 1372.

Starke, J.C. (1967). Photoallergy to sandlewood oil. *Arch Dermatol,* **96**: 62.

Stern, R.S. (1983). Phototoxic reactions to piroxicam and other nonsteroidal anti-inflammatory agents. *N Eng J Med,* **309**: 186–187.

Stott, C.W., Stasse, J., Bonomo, R. and Campbell, A.H. (1970). Evaluation of the phototoxic potential of topically applied agents using longwave ultraviolet light. *J Invest Dermatol,* **55**: 335–338.

Szczurko, C., Dompmartin, A., Michel, M., *et al.* (1994). Photocontact allergy to oxybenzone: ten years of experience. *Photodermatol Photomed,* **10**: 144.

Thune, P. (1984). Contact and photocontact allergy to sunscreens. *Photodermatology,* **1**: 5.

Wilkinson, D.S. (1961). Photodermatitis due to tetrachlorosalicylanilide. *Br J Dermatol,* **73**: 213.

15 Occupational skin diseases

Jon Spiro

Capita Health Solutions, Didcot, Oxfordshire OX11 0TA, UK

Primary Learning Objectives

- *Overview of the main pathological conditions associated with occupational exposures.*
- *Clinical methods associated with the identification and remediation of chemical exposure in the workplace to reduce or eliminate occupational skin disease.*

15.1 Introduction and scope

Skin diseases associated with occupational exposure are a major cause of work-related illness. 'Occupational exposure' includes any form of repeated exposure and may arise from domestic, industrial or leisure activities.

While mental ill health (including stress-related conditions) and musculo-skeletal disorders have, in recent years, accounted for the majority of occupationally related ill health and injury, occupational skin disorders continue to be common and affect significant numbers of working people in a wide variety of workplaces. Recent estimates (McDonald *et al.*, 2006) suggest that the average incidence rates for occupational skin disease overall in the United Kingdom may be as high as 623 per million. It has also been estimated that the number of working days lost per year from occupational skin disease is four million at a cost of £200 million (English, 2004). The vast majority of these cases are of occupational contact dermatitis, which mirrors the relative abundance of dermatitis as a whole in the general population. It is, of course, the case that skin disorders that can be acquired occupationally can also be acquired away from the workplace because of exposure to causative factors in the home, in pursuit of hobbies and pastimes or for unknown reasons. Occupational and non-occupational skin disorders thus frequently co-exist.

Long-standing experience and surveillance of workplaces and individuals by occupational physicians and dermatologists have yielded extensive knowledge of workplace factors, among them chemical, physical, biological and psychosocial, which have been found to give rise to or aggravate occupational skin disorders. This chapter describes, in outline, how these conditions arise and how individuals with such conditions should be investigated, with a case of hand dermatitis being used as an example of how to assess causative factors in the workplace.

Principles and Practice of Skin Toxicology Edited by Robert P. Chilcott and Shirley Price
© 2008 John Wiley & Sons, Ltd

15.2 Dermatitis

The most common skin pathology arising from occupational exposure is dermatitis (eczema), of which irritant contact dermatitis (ICD) and allergic contact dermatitis (ACD) are the most common manifestations.

Dermatitis is, in simple terms, inflammation of the skin and is also known as eczema[1]. It is both common and occurs in a variety of forms. Many cases arise from unknown or constitutional factors. Common amongst these is the condition of atopic dermatitis, which is associated with hay fever and asthma. Dermatitis is not, in itself, an infectious or contagious condition but is not uncommonly complicated by infection. Where it occurs mainly or entirely as a result of exposure to external factors that lead to skin inflammation, it is referred to as contact dermatitis (Chapter 13). This, in turn, may arise from direct damage to the skin due to 'attack' by chemical or physical agents, where the result is known as irritant contact dermatitis, or as a result of allergy or hypersensitivity in which the body's immune system plays a part in the genesis of the condition and which is thus known as allergic contact dermatitis (Chapters 9 and 13). Most cases, not surprisingly, arise on skin that is directly exposed to the causative factor(s) which make the hands the most common site for the condition, although other sites can be affected due to secondary spread, extension of the reaction or due to permeation of clothing. Data from a recent survey (McDonald *et al.*, 2006) indicate that approximately 82% of reported cases of occupational skin disease in the United Kingdom are contact dermatitis.

A variety of changes in the properties of the skin have been found to occur in the development of dermatitis. The normal barrier function becomes impaired as a result partly of altered skin permeability, itself associated with changes in lipid (fat) composition and proliferation of cells in the epidermis. As a result of altered barrier function, penetration of environmental agents into the skin is enhanced, increasing the probability of developing allergic dermatitis (Proksch *et al.*, 2006). Changes in the (normally acidic) pH of the skin have been reported to be associated with the development of dermatitis and skin pH can be altered by external agents which can cause the condition (Schmid-Wentner and Korting, 2006). The concentration of chemical agents in contact with the skin and the duration and frequency of contact are also relevant to the risk of developing dermatitis.

15.2.1 Irritant contact dermatitis

This is much the commoner of the two types of contact dermatitis, although both types can occur together. Factors which give rise to irritation of the skin and which can, therefore, cause this form of dermatitis are to be found in many workplaces, homes and elsewhere. The main factors are mostly either chemical or physical and can affect the skin either singly or in combination.

Chemical irritants vary in their potency to cause skin irritation and dermatitis. The most potent are corrosive or comparably severe irritants, which will cause a prompt reaction in almost all exposed people, often causing burns, and which include mineral acids and alkalis,

[1]The origin of the term 'eczema' is from the Greek words 'ek' (out of) and 'zeein' (to boil); the 'out-boiling' of skin is an accurate description of a severe eczematous reaction.

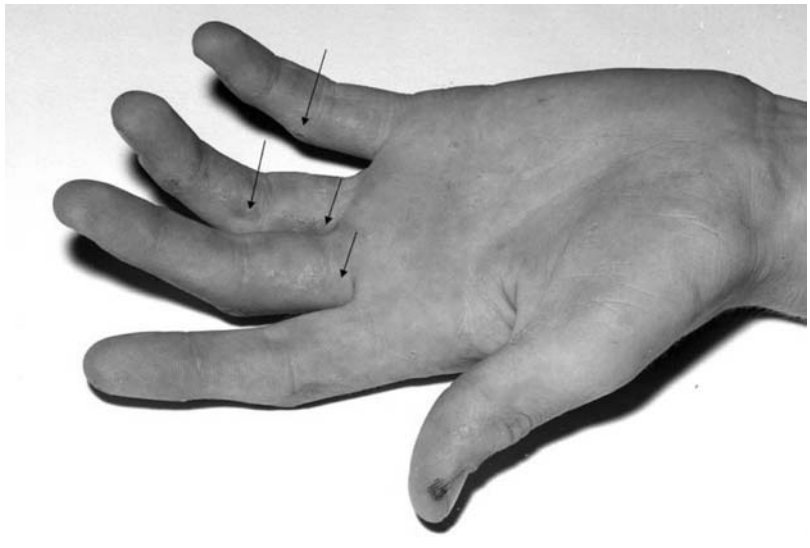


Figure 15.1 Early probable contact dermatitis due to chronic exposure to mild irritant (Arrows indicate regions of red, dry, scaly skin). A full-colour version of this figure appears in the colour plate section of this book

even in dilute form. These are referred to as primary irritants. Less potent irritants include organic solvents, such as acetone, oils, particularly mineral oils, and detergents. Soaps and other agents used to clean the skin can themselves lead to dermatitis. Milder irritants do not invariably cause harm to those who come into contact with them and, if they do, this often arises after prolonged and/or repeated exposure leading to cumulative skin damage (Figures 15.1 and 15.2), which can span many years during which there appears to be no evidence of harm (see Figure 16.2).

Physical agents (or other factors implicated in irritant dermatitis) often act as co-factors with chemical irritants, rather than as the sole causes of the condition. These include heat, which can adversely affect the skin by causing sweating and thus overhydration (if sweat evaporation is impeded by clothing), a condition which can also arise in environments where the relative humidity is high. Occlusion of the skin, as can occur when the skin is covered by a dressing or by tight-fitting gloves, can have a similar effect. Conversely, cold conditions and low relative humidity lead to dehydration of the skin. Immersing the skin in liquids, including water, for long periods can be detrimental, also causing skin overhydration. Additionally, activities that subject the skin to repeated friction cause local damage which can lead or contribute to dermatitis. Among the causes of friction, exposure to dust in significant concentration may, apart from any chemical irritant effect, aggravate dermatitis, as may exposure to fibres, such as man-made mineral fibres.

In many people exposed, even repeatedly, to relatively mild irritants, the skin may simply react by 'hardening' or thickening with little if any evidence of inflammation. Other people will, instead, more readily develop active and prolonged dermatitis, thus suggesting that some individuals are pre-disposed to develop the condition. It is well established that those who have had atopic dermatitis fall into this category.

Figure 15.2 Acute dermatitis of unknown origin. A full-colour version of this figure appears in the colour plate section of this book

15.2.2 Allergic contact dermatitis

In this condition, substances in the environment react with the body's immune system, which then leads to dermatitis (Figure 15.3). Many are not intrinsically harmful to the skin and do not cause irritation. They instead induce sensitivity in the affected individual by inducing reactivity in certain subtypes of T lymphocytes (Chapter 9). Once sensitivity has been induced, further contact with the substance that has caused it – the allergen – leads to an inflammatory response in the skin produced by chemical mediators released by lymphocytes. Like irritants, allergens vary in potency. The inflammatory response manifests as dermatitis, which is generally similar to irritant dermatitis in appearance. Only very small exposures to an allergen that has induced hypersensitivity are necessary to produce this reaction, which begins after 12 hours of the exposure and reaches a peak within 48 to 72 hours. Thus, this is an example of a 'delayed hypersensitivity reaction'. Most agents that induce sensitivity in this situation are of relatively low molecular weight, typically less than 500 (Chapter 5).

A considerable variety of substances have now been found to cause contact allergy, of which nickel is responsible for the largest individual number of cases and affects many more females than males. Others include fragrances (by no means confined to perfumes and after shaves), epoxy resins, compounds used in the manufacture of rubber and chromates. Many are found in a wide variety of places, while others are confined to specific workplaces or work processes. Once induced, contact allergy is considered to persist indefinitely and affects the whole area of the skin and not just the part where exposure has taken place. It is unclear if individual factors contribute to the risk of developing this condition. Many cases of contact allergy arise from

Figure 15.3 Localised areas of allergic contact dermatitis of the face and neck due to epoxy resin exposure. A full-colour version of this figure appears in the colour plate section of this book

non-occupational exposure, as in the case of nickel, commonly found in jewellery, watches, coins, keys and many other household objects.

15.3 Development of occupational dermatitis

The onset of dermatitis in response to a chemical exposure may be sudden or gradual. Identification of the causative agent may be complicated if an individual is exposed both in the home and work environments. The most effective treatment strategy for dermatitis is prevention or avoidance of exposure to the causative agent.

As already indicated, dermatitis can arise as a result of exposures that occur in the workplace, outside it or both. A single exposure to a powerful irritant or corrosive agent can produce prompt and significant changes in the skin – this may be as severe as a burn, although a marked inflammatory response is more usual – which may leave it vulnerable indefinitely to the adverse effects of other agents which may be less harmful, but which may thereby be more likely to induce dermatitis. While it is often the case that this will occur at work, often due to an accident or a failure of control measures (or failure to implement them; Chapters 16 and 17), it may easily result elsewhere, for example handling or kneeling in cement to lay a path at home without adequate protection. If dermatitis arises primarily from activities and exposures that occur at work, whether at an early stage in employment or after a number of years, it can be readily aggravated even by routine activities outside, such as gardening (chemicals in

plants or used in gardening include both irritants and allergens), cooking, 'do-it-yourself' home maintenance or even routine household cleaning. The converse is also true in that any of the aforementioned domestic activities can initiate dermatitis which workplace activities can then aggravate, a possibility that does not always occur to many individuals! Thus the onset of dermatitis can be sudden or gradual and follow a brief period of relevant exposure or occur after many years of it.

Mention has been made of the increased personal risk of the development of contact irritant dermatitis. While this may arise either from exposures and practices at work or elsewhere, the presence in an individual of the already mentioned 'atopic' tendency is of significance. A large number of people in most western populations suffer with any or all of the triad of eczema, hay fever and asthma. The eczema, in affected individuals, generally occurs initially at a very young age and can affect any area of skin, although a very common pattern is to be found in the 'flexures' or skin folds, notably at the front of the elbows and backs of the knees. Those with this medical history are at increased risk of the development of irritant dermatitis and in those who suffer with the latter, a history of atopic conditions, particularly eczema, should always be sought.

While those who have had an episode of irritant dermatitis and recovered can be subject to continuing exposure to mild irritants without necessarily suffering a reactivation of their condition, the situation for contact allergic dermatitis is somewhat different in that, as already stated, even minimal exposure to the allergen will provoke a flare up of the condition. Control of exposure thus has to be much more rigorous and it is often necessary to attempt to eliminate further exposure; this does not always require a change of job, although this can sometimes be necessary.

The influence of treatment for dermatitis should be considered. While elimination of the cause, as far as practically possible, should be the principal aim, the use of treatments, particularly emollients (moisturisers) and anti-inflammatory agents (generally in the form of steroid creams and ointments), will usually have a major beneficial effect on the condition (Chapter 13). This does, however, depend not only on compliance with treatment, but using it at the earliest opportunity and taking all other available steps to protect the skin, from safety controls at work to the use of protective clothing. Even care in how hand washing is carried out will play a part in managing dermatitis (Chapter 16).

15.4 Patterns of occupational dermatitis

It has been mentioned already that the most common sites for contact dermatitis are the hands, as these are most commonly in contact with the possible causes of the condition. This does not, however, preclude the occurrence of the condition at other sites. The face is also normally exposed to the environment and can be affected both by airborne agents, including splashes, and by indirect contact – an individual may handle a substance and then touch his or her face, often repeatedly, a particular risk if exposure control or hygiene measures are deficient. Agents that can penetrate clothing, for example many chemicals in liquid form, or allergens such as nickel, for example in the form of keys or coins in pockets, can cause dermatitis on non-exposed skin.

The site of dermatitis is normally the specific site of contact of the causative agent or agents, for example the particular part of the hand that is used to handle the agent. This may be on the palms of the hands, the backs or the fingers, or a combination depending on how

work is carried out. In addition, other factors may play a part, such as the common retention of moisture in the finger webs from 'wet work' and inadequate techniques for drying the skin afterwards, which may thus produce a pattern of skin abnormality which is strongly suggestive of the cause. Dermatitis does, however, readily spread from the initial point of contact, particularly in allergic cases. It is thus useful to see cases at an early stage, as the signs then may be easier to interpret.

15.5 Incidence of occupational dermatitis

Much has been learned about the incidence and prevalence of occupational dermatitis and its causes in different occupations. A number of the latter are almost notorious for the number of cases they yield.

Data from the EPIDERM and OPRA surveys (McDonald *et al.*, 2006) indicate that the industries giving rise to the highest number of cases of occupational dermatitis for the years 1996 to 2001 include agriculture, forestry, fishing, food manufacture, petrochemical (including rubber and plastics manufacture), metal and automotive product manufacture and health services (especially nursing). Hairdressing has long been held to be responsible for many cases. The occupations most highly represented in these surveys were technical, craft and related, plant and machine operatives and personal and protective services.

The agents most frequently reported in the same surveys as being responsible for occupational dermatitis were rubber chemicals, soaps and cleaners, wet work, resins and acrylics, nickel, petroleum and products and cutting oils and coolants. Many other chemicals or products were cited.

15.6 Effects of dermatitis on work

Dermatitis can vary in severity. In its most extreme form, dermatitis can prevent an individual from working with a particular process or environment.

Many of those with dermatitis in fact report no difficulties, especially if the effects are minor or the condition is considered to be a fact of life in their occupation. Some of this number, however, fear that if they report the condition, or if it is otherwise discovered, they will lose their job and their livelihood. A certain amount of suffering is, therefore, concealed.

Symptoms and difficulties with work due to dermatitis are, however, common. The visible presence of the condition in those who provide services directly to outsiders may cause problems, as many consider the condition to be not only unsightly but infectious – some will not wish to touch the skin of an affected person. In those affected, there will often be discomfort or itching, which may be aggravated by the presence of secondary infection. The latter often causes the skin to exude a straw-coloured fluid or even pus, while bleeding can occur from uninfected dermatitis. The discomfort will cause variable, but sometimes considerable, difficulties with work, for example, with grip or machine operation. This is worsened if the skin cracks (which occur in more severe cases) in several places and to a marked depth. Such cracks (fissures) are often slow to heal and painful.

The occurrence of bleeding or skin exudate also leads to impediments in work processes where infection control is paramount, for example in food or pharmaceutical manufacture.

Those affected are then barred from their jobs for a period. If work, for practical purposes, becomes impossible and temporary redeployment to alternative duties which have little effect on the skin condition is not possible, absence from work results, usually for relatively short periods, such as less than three weeks. It can sometimes, however, be for much longer (Adisesh *et al.*, 2002). Furthermore, the psychological impact should not be overlooked, as sufferers of dermatitis may be unaccustomed to the condition and readily develop a very negative view of the situation, often fostered by their own fears about the future of their work and the opinions, often ill-informed, of others.

15.7 The outlook in occupational dermatitis

The prognosis for individuals affected by dermatitis can vary significantly, from complete recovery without relapse to chronic and persistent pathology in the absence of exposure to the original causative agent.

Once dermatitis has begun to develop, if the onset is gradual, or has actually developed in a frank manner, the natural progression of the condition will often largely depend on the level of subsequent exposure to the causative agent or agents. Reducing the level and frequency of exposure significantly can slow the progression of the condition. It may then recede and only recur if significant exposures subsequently occur. This pattern may often be recognisable to the worker, where the condition will improve away from work. If, however, the condition is well established, an absence from work may not lead to any improvement even if exposure to irritants away from work is minimal. Eventually, even a change of job to one where irritant exposure is insignificant may not bring about an improvement in, let alone a resolution of, dermatitis. Therefore, the prognosis in such situations is poor and some individuals who reach this stage may be unable to work in any occupation.

Overall, around 20% of cases of occupational dermatitis result in time off from work and the outlook may be worse with increasing age, being atopic or having longer exposure to the causative agent (Adisesh *et al.*, 2002). In particular, oil-related dermatitis tends to persist in most cases, whether or not the individual is still working (Pryce *et al.*, 1989). In a review of other relevant studies, the vast majority of individuals improved and clearance of the condition was seen in up to 40% of cases (Cahill *et al.*, 2004). The outlook was improved by early diagnosis and improved knowledge on the part of the individual.

15.8 Identification of occupational dermatitis

While it will be self-evident that a proper diagnosis of dermatitis and corresponding remedial action should be done as soon as possible (and that prevention is even better!), a clear strategy needs to exist to accomplish this. Education of the workforce and management is essential and should include the provision of information and training on possible workplace causes, the means of prevention (discussed in detail below), how to recognise the condition and what to do if it appears. Most workplaces will have a health and safety officer or manager who will be responsible for drawing up the strategy, although everyone is, in effect, responsible for implementing it. Vigilance is also of paramount importance as the early signs of dermatitis can easily be overlooked.

Proper identification of the condition is normally a medical matter. If the workplace has an occupational health nurse or doctor, they will be best placed to advise. The matter will otherwise be in the hands of the individual's general medical practitioner who will also, in any case, take care of any treatment required, advising on and certifying absence from work with referral to a dermatologist if necessary.

The diagnosis of dermatitis is normally quite straightforward, although a number of other common and less common skin conditions can have a similar appearance. The medical and occupational history given by the individual will be important in establishing whether or not the condition has been occupationally acquired and in helping to decide if it is an irritant or allergic condition which, as has already been seen, makes a difference as to how the situation will be managed overall.

No diagnostic test exists for irritant dermatitis, the condition being diagnosed on the history and evidence of exposure to irritants. The signs of the condition will not usually be specific. Allergic contact dermatitis can, however, be investigated as to a specific cause by the use of patch tests (Chapter 13). The process is not without its pitfalls as there can be false positive and false negative reactions, even when approved procedures have been followed rigorously. Furthermore, a reaction may not be from any substance encountered at work. The procedure is, nonetheless, an important part of the process of looking for and identifying allergens that may have been responsible for an episode of dermatitis and, wherever these are encountered, the individual is best advised to reduce exposure to a minimum or, ideally, to avoid further exposure, thus reducing the risk of further episodes, at least of contact allergic dermatitis.

15.9 Other occupational skin disorders

Whilst dermatitis is one of the most common types of occupational skin disease, there are a variety of other pathological conditions which are seen in the workplace. These include urticarial (wheal-and-flare) reactions, acne, cancer, vitiligo and conditions arising from frequent exposure to physical insults.

While these form the minority of skin conditions associated with the workplace, they are nonetheless important and should be recognised and dealt with promptly to prevent further adverse consequences for the individual and to address deficiencies in workplace practices. The better known 'non-dermatitis' skin conditions are discussed briefly below.

15.9.1 Contact urticaria

Urticaria, in its various forms, is commonly known as 'hives'. It is also well known that it can be caused non-occupationally, and in many cases the cause is never determined. It occurs within minutes of exposure and settles usually within hours (Chapter 13). It occurs either as a non-sensitivity reaction (i.e. without provoking the immune system) or as an immediate allergic response, indicating that previous exposure and sensitisation must have occurred. The former type of reaction can be provoked by exposure to plants and chemicals such as cinnamic aldehyde. The latter, which can be associated with more severe reactions, including those provoked by foods, is also caused by a number of chemicals, such as antibiotic preparations.

The condition can be investigated by skin testing using prick tests (Chapter 13). Blood tests can also help identify sensitising agents that cause urticaria.

The significant problem of allergy to natural rubber latex can be considered under this heading. This reaction, most often encountered in users of gloves made of this material, such as healthcare workers, may be associated with other allergic conditions such as rhinitis (hay fever is another example of this) and eyelid swelling. It may lead to severe anaphylactic reactions that can be life threatening.

15.9.2 Acne

This condition is much better known in adolescents and occurs in association with a number of naturally occurring changes in the skin in that age group. However, the worker may experience it in association with chemical exposures, most notably oils and chlorinated hydrocarbons; in the latter case, the condition is referred to as chloracne. The appearance of the condition is not unlike that of the condition that affects adolescents, but the distribution on the skin can be very different – in the case of oil acne, the limbs may be extensively affected (due to heavy oil exposure at those sites).

15.9.3 Skin cancer

As one of the earliest work-related medical disorders, occupational skin cancer was first described in 1775 on the scrotum of chimney sweeps; it was due to chronic exposure to soot (Box 15.1). Since then, other causes and occupations have been recognised and linked with various chemicals such as polycyclic hydrocarbons (sources of which include tar and petroleum derivatives) (Gawkrodger, 2004) and inorganic arsenic. Physical factors (notably ionising and ultraviolet radiation) have also long been found to be causative of skin cancer. While it must be acknowledged that in the case of ultraviolet radiation, this is more likely to be a problem with recreational rather than occupational exposure, prolonged outdoor working in hot climates without adequate protection undoubtedly poses a risk. Nonetheless, the proportion of skin cancers that can be shown to have a clear occupational cause is actually small. The length of time between exposure and the onset of the condition (the 'latent period') is relatively long and is usually measured in years, or even decades; this makes linking specific chemicals and skin cancers very difficult. The situation is further complicated by the fact that in most workplaces there is often simultaneous exposure to a range of materials, and not just one substance.

Specific types of skin abnormality may be observed under the general classification of skin cancers, notably the focal scaly changes known as keratoses (associated with occupational arsenic exposure) and cutaneous melanomas (Perez-Gomez *et al.*, 2004).

15.9.4 Infections

These can complicate other occupational and non-occupational disorders, as mentioned above. Otherwise, there is much potential in the workplace for infection to cause skin problems in its own right. Some cases are caused by micro-organisms, including bacteria and viruses that also infect animals. Such diseases (e.g. anthrax or brucellosis [Harries and Lear, 2004]) affect those who work closely with animals, fish or derivative products (such

Box 15.1 Sir Percival Potts and scrotal cancer of chimney sweeps

Sir Percival Potts (*circa* 1714–1788) was amongst the first to draw an association between occupational exposure to a harmful substance and the onset of a specific pathological response and in doing so laid the foundations of epidemiology.

Whilst working as a surgeon at St Bartholomew's Hospital in London, he noted a high incidence of squamous cell carcinomas in those whose work involved intimate contact with soot and tar arising from the combustion of coal (the condition was referred to as soot wart). This disease was particularly prevalent on the scrotal area of chimney sweeps. Incidentally, the great fire of London (1666) was partly responsible for the young age of the sweeps who were employed in this practice; the rebuilding of London was affected by the architectural fad for long and tortuous chimneys. Consequently, such structures could only be accessed by small, nimble children and it was not uncommon for boys from the age of 5 to be cruelly exploited for this purpose. It is interesting to note that this occupational disease was not as prevalent within other industrialised European countries, a fact attributed to better hygiene arrangements; washing off the soot reduced exposure and hence limited the absorbed dose.

Unfortunately, although Potts had made a landmark discovery, no great remedial action was taken to reduce the incidence of soot wart and it was nearly a century later (1833) before an act of parliament was passed that finally limited the use of children in such work activities (sponsored by Anthony Ashley Cooper, who became the 7th Lord of Shaftesbury in 1851).

as hides). Parasitic diseases of the skin, particularly from insects, may similarly affect such workers. Yeast infections commonly affect the hands of those who undertake wet work and who fail to dry them adequately, the signs being visible commonly in the finger webs and around the nails.

15.9.5 Nail disorders

These arise in an occupational background for similar reasons to non-occupational abnormalities – from physical injury, chemical insults and infections. Physical factors at work (including heat, cold, friction, pressure, vibration and direct injury from sharp objects) harm nails just as they can harm the skin. Chemical agents that damage the skin around the nail, such as turpentine, may cause alterations in nail growth. Infections that affect this area and cause local inflammation have a similar effect. Various nail anomalies can result, such as brittle nails and premature separation of the nail from the nail bed. Ridging of the nails, general changes in appearance such as the development of a concave deformity and discolouration may also occur.

15.9.6 Vitiligo

This term refers to depigmentation of the skin, generally patchy, and often without obvious cause. Occupational associations are well established, however, and include chemicals such as hydroquinone. The condition is not only unsightly, but affected areas of skin lose their natural protection from ultraviolet light and so are at risk of severe sunburn. Thus, affected individuals have to be rigorous about protection from all forms of ultraviolet light, especially as vitiligo is irreversible.

15.9.7 Other skin conditions of physical causation

A miscellany of conditions comes under this heading, some amounting to simple injury, others being more complex. Burns may be caused by heat, cold, ultraviolet light, electricity or chemicals – among the latter, hydrofluoric acid is a particularly destructive cause. Heat may also lead to a skin eruption caused by sweat retention and a form of urticaria. Cold may cause urticaria, as well as chilblains, frostbite and a circulatory abnormality known as Raynaud's phenomenon. A variant of the latter may affect those who frequently use vibrating tools. Ultraviolet light exposure can cause a variety of other skin conditions and is more likely to do so in those taking certain medications.

15.10 Investigation of a case of dermatitis at work

Health professionals can take a series of steps to investigate and eliminate occupational skin diseases once a problem has been identified. These include an assessment of the clinical manifestations of the disease, an overview of the workplace and practices, recommendations for remedial action and subsequent review(s).

For the purposes of discussing how this would be carried out for an occurrence of dermatitis in a workplace setting, a hypothetical situation is taken where an individual working in a factory has reported a skin condition affecting the hands, necessitating the following actions:

15.10.1 Clinical assessment of the individual

This would ideally be done by an occupational health practitioner (if one is assigned to the workplace). It would otherwise be done by the individual's general medical practitioner in the latter's surgery or consulting room. An enquiry should be made of the individual covering the points summarised in Table 15.1. An examination of the individual's skin should then follow and be sufficiently thorough as to ensure that a correct clinical diagnosis can be made. Investigations (e.g. patch testing) may be indicated; if so, or there is doubt about the diagnosis or treatment, referral to a dermatologist should be arranged. At the conclusion of this process, there should be a clear outcome with certainty as to whether or not the skin disorder is occupational and, if so, what condition is present. An allergic cause, if there is one, may also have been identified.

Table 15.1 Factors to consider when conducting a clinical assessment of a patient with dermatitis potentially arising from an occupational exposure

Factor	Description
Anatomical location	The site of origin of the current skin condition, when it started and on exactly which part of the skin.
Spread	Whether or not the skin condition is present on other areas of skin and, if so, which?
Family history	Any past or family history of skin disorders in the individual. Is there a history of allergy of any kind?
Individual's history	The general medical history of the individual and any medication used, both on the skin and orally, if this is not already recorded in the medical records for that individual.
Exposure scenarios	Details of the individual's work history, past and present, including details of the current job and workplace. Details of any hobbies, pastimes, domestic activities or even second jobs.
Causality	Of key importance is to establish the possible relationship of the skin condition to work. The individual needs to be asked if the condition varies between working and non-working days and, particularly, if there is any improvement when a holiday of at least one week is taken (unless, of course, this involves other activities which might also be detrimental to the condition of the skin).
Frequency	Also of significance is if there are any others in the same workplace who are also experiencing skin problems or who are known to have done so.

If there is no occupational health service associated with the individual's place of work, this may be as far as the overall management of an occupational skin condition goes. However, if this is the case an opportunity to deal with the causes of the problem will have been missed, as will a chance to remedy problems in the workplace that may potentially have had an adverse health effect on other employees and, by implication, adverse (economic) effects on the employing organisation. The availability of an occupational health service allows this state of affairs to be rectified, particularly as occupational health practitioners can be expected to have not only a thorough understanding of how occupational diseases and conditions arise in various workplaces, but also a respectable working knowledge of the workplaces they serve and how to assist the management of the workplace to take the appropriate steps to deal with skin conditions or, indeed, any other occupational health matter.

15.10.2 Workplace assessment

It is assumed here an occupational health specialist is available to carry this out. (Some dermatologists who have a special interest in occupational skin conditions also involve

themselves in this activity.) It is also assumed that the clinical assessment of any individual who has presented with a skin problem has already been carried out. The management of the workplace, who may well have initiated the referral to the doctor in the first place, may already know this. A workplace visit by the latter may then be expected and, indeed, welcomed. Otherwise, it will be a matter of protocol to inform the workplace management that the occupational health practitioner wishes to visit the workplace as the help and co-operation of management is essential. If the workplace has a health and safety manager or officer, they will be best placed to assist in a workplace assessment as this will be a major part of their day-to-day work. In the absence of such an individual, the manager of the section of the workplace where the individual works (or the general manager of a small workplace) is likely to 'host' a visit.

Before the actual inspection begins, a number of points should be established and discussed as necessary (Table 15.2). The inspection itself will then follow. Ideally the affected individual will be at work, allowing inspection not only of the work activities, hazards and control measures, but also of how the individual actually goes about their work. What appears to be a safe and healthy workplace does not guarantee that all those who work in it practise what the management preaches! If the individual reporting the disorder is unable to attend work, other

Table 15.2 Factors to be considered and discussed with managers, supervisors or other workers prior to inspection of the workplace

Factor	Description
Activities	If not already known to the visiting occupational health practitioner, what are the main work activities? What is made or carried out there?
Hazards	What are the main hazards of the workplace – chemical, physical, biological or psychosocial?
Risks	What risk assessments have been carried out? These are a requirement of workplaces under UK health and safety legislation, although compliance with this tends to be variable. Any documented evidence should be available for review during the visit.
History	What evidence is there of past health problems in the workplace, be they illnesses, conditions or injuries? Such evidence can be gathered from records of sickness absence and accidents and may give useful insights into what may underlie the problem under current consideration.
Concerns	Do the workplace managers have any particular concerns of their own, such as possible risks arising from any new processes, including new chemicals or even about the state of the workforce in general? Litigation may sometimes be a background concern.
Other	Other information, such as the size of the workforce, its age and sex distribution and details of the organization of work will often be enquired about at the preliminary stage.

Table 15.3 Factors to be considered during the conduct of a workplace inspection

Factor	Description
Specific products	Any chemicals used in any part of the work process and how they are handled. Information about these can be expected to be readily available in the workplace.
Environmental/physical factors	Physical factors, such as the temperature and humidity of the workplace. Is wet work carried out and is the skin of workers subject to friction?
Control measures	How is exposure to harmful agents controlled? Are these controls in proper use and effective? (These may range from enclosure of a process to simple measures such as splash guards.) What, if any, ventilation systems are in use and do they appear to be working properly (important where fumes or dusts are present)?
Personal protective equipment (PPE)	What personal protective equipment is available and actually in use? Of major relevance to an individual with a skin condition of the hands are gloves, although other protective measures such as overalls, goggles and visors may be important. Gloves, if indicated, should be made of the correct material for the job, worn and removed correctly and be intact.
Hygiene	What washing facilities are present and how are they sited in relation to the work area? Poorly maintained and/or inconveniently situated wash rooms may actually contribute to a skin problem, whereas those that have the opposite characteristics will probably be playing a part in the prevention of such problems. Other practices relating to worker hygiene should be observed, e.g. if refreshments are consumed in an area separated from work and if personal protective clothing is properly looked after.
General housekeeping	Workplace 'housekeeping' should be noted – if this is not of a high standard, for example if chemical agents are not stored correctly, uncontrolled exposure of the skin to irritants or allergens may occur.
Additional patients	If there are any other employees who may have a skin problem they can, providing that suitable facilities are available and everyone is in agreement, also be questioned, examined and advised.

workers who do the same job should be observed, with attention being paid to certain points (Table 15.3).

At the conclusion of a workplace visit, the occupational health practitioner should have gained a reasonably accurate view as to what, if any, are the deficiencies in workplace practices or the workplace itself. These may exist in spite of the efforts of even a highly knowledgeable and diligent health and safety officer, although some workplaces clearly suffer from the absence of such an individual. In any case, exposures to irritants and allergens may have been observed

and been seen to be inadequately controlled, or the advantages of suitable and sufficient control measures that already exist in the workplace may have been found to be negated by improper practices such as poor personal hygiene.

Following the visit, a report detailing observations and recommendations is likely to be produced. This should lead to actions being proposed which relate firstly to the affected individual and secondly to the workplace.

15.10.3 Actions following clinical and workplace assessment

These will be absolutely indicated for the individual with hand dermatitis (and very possibly if dermatitis is present on other exposed sites) if any factor identified in the workplace appears to be causing or contributing to the skin condition. If no workplace assessment has been carried out, recommendations for the individual may still come forward from any physician who has been involved in the case outside the workplace, although these will be unlikely to be as specific and wide-ranging as will be the case if the workplace has been inspected. Such actions are summarised in Table 15.4. Additional actions to be considered in the workplace will follow logically from the observations and recommendations referred to in Table 15.3. The health and safety officer or manager, working with the workplace management, will need to look closely at what should be done. In some cases, this may mean reviewing compliance

Table 15.4 Summary of changes that can be recommended to mitigate the incidence of occupational dermatitis

Remedial factor	Description
Procedures	Changes in the way the job is done by the individual, particularly the correction of any faulty practices.
PPE	Better use of personal protective equipment, especially gloves.
Hygiene	Improvements in hygiene practices, e.g. hand washing techniques.
Staff	Restriction of the employee from a certain part of the work, especially that which poses the greatest risks to someone with dermatitis. Redeployment of the individual, on a temporary basis, to a different job. Advising that the employee takes absence from work altogether for a short period.
Therapy	In any event, treatment is likely to have been recommended and should be used as recommended. This can extend to the workplace, as everyone with dermatitis should benefit from the use of emollients or moisturising agents, which should be provided not only outside the workplace but in it in the form of 'after work creams'. (These will also be beneficial to those who have previously had or indeed have never had dermatitis, as a preventive measure.)

with the law, thereby avoiding criticism or even prosecution by health and safety inspectors. In general, steps that assist in managing risks more effectively might include:

- Elimination or substitution of an allergen or potent irritant.
- Enclosure or modification of a process, thus cutting down or eliminating exposure to hazards in that area.
- Installing or improving devices that diminish the risk of significant skin contact with chemical agents or physical factors, such as dust or metal 'swarf' (sharp pieces of discarded metal).
- Installing or upgrading ventilation systems and checking maintenance schedules.
- Reviewing the storage and disposal of hazardous agents.
- Looking at workplace cleaning schedules and 'housekeeping'.
- Reviewing the provision and use of personal protective equipment, such as introducing gloves that give better protection.
- Reviewing washing facilities and those provided for refreshment breaks.
- Looking at the training provided to the workforce, which may need to be revised.
- Considering health surveillance – either introducing or improving this essential method of periodic assessment of the health of the workforce. This would, in this case, consist of regular skin inspections of all employees at risk. It would ideally be undertaken by an occupational health practitioner, but a 'responsible person' in the workplace, such as a manager, can be trained to carry this out.
- Taking a general critical look at how health and safety is managed overall.

15.10.4 Follow up

This also is indicated, particularly for the individual with dermatitis but also for the workplace, especially if any remedial actions have been necessary.

For the individual, a review of the skin condition and his or her work will be needed. The former is a medical matter, while the latter is matter of shared concern – an occupational health practitioner would, if available, play a key part, but do so in conjunction with the relevant workplace manager. The health and safety officer or manager and, in some workplaces, the human resources professional will also be involved. The key issues to address will be:

- Has the dermatitis improved? Follow-up medical assessment would be expected to occur approximately weekly in the early stages after diagnosis and the commencement of treatment.
- If there has been no improvement, there will be a need to review the diagnosis, treatment (or compliance with it) and whether or not any actions taken at work have been adequate, such as restrictions placed on the individual's work. Referral to a dermatologist may be considered.

- If the dermatitis has improved, the debate will be about when the individual can return to their normal work, if they have been restricted or kept away from work, and for how long the treatment, if indicated, should continue. It will be essential to consider what steps must be taken to avoid a recurrence, which will also be of relevance to others undertaking the same work.
- Close supervision of the individual will be advisable at work to reinforce any changes made and to ensure that any recurrence of the dermatitis is detected as early as possible. The skin remains vulnerable for a number of months after an episode of dermatitis and may, in long standing cases, be permanently susceptible to the adverse effects of workplace (and other) hazards.
- Does the individual need to be redeployed? Restrictions placed on an employee may not be sustainable indefinitely and a permanent change of work may be necessary. In some cases, an employee who has been absent from work for any significant length may be unable to return – a possibility for those with chronic, severe dermatitis which has not responded favourably, or even at all, to treatment.

The outlook in occupational dermatitis has been discussed above. It can be seen that efforts to bring about a favourable outcome in the short, medium and long term can be considerable, have a reasonable chance of being rewarded.

Summary

- While occupational skin disorders are common, they are largely preventable.
- The duty to attempt to minimise the substantial burden arising from them that affects both individuals and their employers falls to everyone involved.
- While the identification and management of occupational skin disorders requires knowledge and experience, what matters more is a combination of vigilance and adherence to some basic principles that underpin the way any medical condition that occurs at work should be prevented and managed.
- The benefits of applying these basic principles to major occupational health problems such as dermatitis and other occupational skin conditions are clear. So, too, are the risks of failing to do so.

References

Adisesh, A., Meyer, J.D. and Cherry, N.M. (2002). Prognosis and work absence due to occupational contact dermatitis. *Contact Dermatitis*, **46**(5): 273–9.

Cahill, J., Keegel, T. and Nixon, R. (2004). The prognosis in occupational contact dermatitis in 2004. *Contact Dermatitis*, **51**(5–6): 219–26.

English, J.S.C. (2004). Occupational dermatoses: overview. *Occupational Medicine*, **54**: 439–40.

Gawkrodger, D.J. Occupational skin cancers. (2004). *Occupational Medicine*, **54**: 458–63.

Harries, M.J. and Lear, J.T. (2004). Occupational skin infections. *Occupational Medicine*, **54**: 441–49.

McDonald, J.C., Beck, M.H., Chen, Y. and Cherry, N.M. (2006). Incidence by occupation and industry of work-related skin diseases in the United Kingdom. *Occupational Medicine*, **56**: 398–405.

Perez-Gomez, B., Pollan, M., Gustavsson, P. *et al.* (2004). Cutaneous melanoma: hints from occupational risks by anatomic site in Swedish men. *Occup Environ Med*, **61**: 117–26.

Proksch, E., Folster-Holst, R. and Jensen, J.M. (2006). Skin barrier function, epidermal proliferation and differentiation in eczema. *J Dermatol Sci*, **43**(3): 159–69.

Pryce, D.W., Irvine, D., English, J.S. and Rycroft, R.J. (1989). Soluble oil dermatitis: a follow-up study. *Contact Dermatitis*, **21**(1): 28–35.

Schmid-Wentner, M.H. and Korting, H.C. (2006). The pH of the skin surface and its impact on the barrier function. *Skin Pharmacol Physiol*, **19**(6): 296–302.

16 Prevention of occupational skin disease

Chris Packham

Enviroderm Services, North Littleton, Evesham, WR11 8QY, UK

Primary Learning Objectives

- *The roles of supervisors and operators in tasks involving potentially harmful materials.*
- *An understanding of the range of measures that can be introduced to limit the incidence of occupational skin diseases.*
- *Limitations of personal protective equipment (PPE).*

16.1 Prevention of occupational skin disease

Whilst protective creams, cleansers, conditioners and gloves can be used (with varying levels of success) to limit occupational skin diseases, the best form of protection is to prevent exposure.

In the European Union it is estimated that occupational skin disease costs around €6 million annually. A recent study to ascertain the prevalence of occupational skin disease in printers indicated that 41% of respondents reported having had a skin problem at some time, with 26% reporting a concurrent skin problem (HSE, 2000). Very few of these had been formally reported to the work place manager. Also, there may be difficulties in specifically relating skin disorders to an occupation: when does dry, cracked skin officially become occupational dermatitis? Thus, all statistics need to be regarded with some degree of caution. However, the fact remains that the available statistics indicate that occupational skin disease remains a significant cause of occupational ill health.

Of a total number of 62 472 cases (of occupational ill health) reported in Germany in 2002, nearly one third were skin diseases, the vast majority (90%) of these being hand eczema (Weisshaar *et al.*, 2006). It is also necessary to keep in mind that occupational skin disease is just part of the problem: many chemicals can penetrate the skin and cause damage to internal organs. Statistically, there is no way of determining what contribution dermal uptake makes to systemic damage in comparison with the body burden arising from respiratory and oral absorption.

Principles and Practice of Skin Toxicology Edited by Robert P. Chilcott and Shirley Price
© 2008 John Wiley & Sons, Ltd

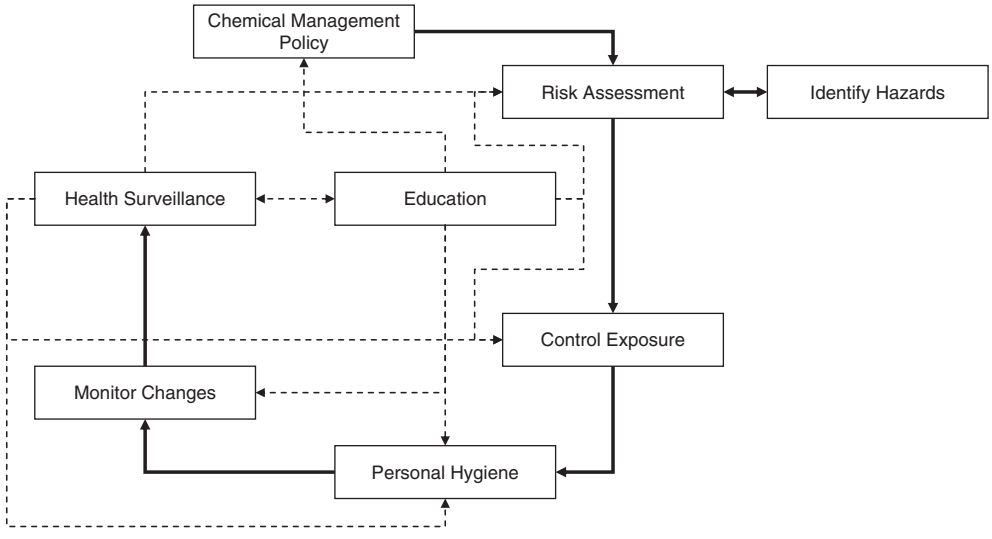


Figure 16.1 Elements of a chemical exposure management system

Overall, the available data suggest that the 'traditional' approach of barrier or protective creams, cleanser, conditioner and gloves has not produced the desired reduction in occupational ill health from skin exposure. This approach can be called 'skin care'[1]. What is needed is a more comprehensive approach, which might be called 'skin management'[2] (Figure 16.1).

The fact is that damage to health from dermal exposure to chemicals can be largely prevented. The simple answer is that if there is no exposure of the skin to the chemical then there will be no risk of damage to health. The rest of this chapter deals with the principles and practice of managing dermal exposure.

16.2 Defining the problem

Occupational skin damage (such as dermatitis) is generally the result of a cumulative exposure to chemicals and there is wide inter-individual variation in the dose required to elicit a dermal response.

Should we be striving to prevent all exposure to all chemicals? In other words, should we be seeking to achieve zero exposure? Not only is this unrealistic, but it is actually undesirable; there are many substances to which skin exposure can be beneficial up to a certain level but where excessive exposure presents a significant risk of damage to health. For example, water is needed to wash other, more harmful, chemicals off the skin. The skin actually needs a certain moisture level to function properly as a barrier. Yet excessive exposure to water is one of the major causes of occupational skin disease. Of course, there are some chemicals to which any

[1]The provision and use of products to be applied to the skin for the purposes of cleaning, protecting and conditioning.

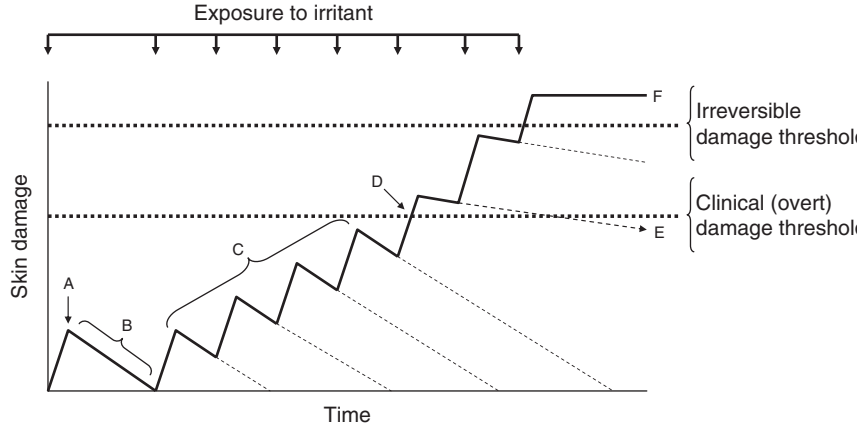
[2]The process of structuring the workplace, equipment and the work done so as to minimise any risk of skin exposure causing damage to health.

exposure of the skin is unacceptable. However, with many of those chemicals used regularly in an occupational setting what is sought is to find a balance between what is acceptable, and possibly beneficial, and what represents an unacceptable risk of damage to health.

So, it can be said that what it is necessary to achieve is to control any exposure to a level where the risk of damage to health is outweighed by the benefits that can be obtained. The problem then is to define what level of exposure fits this requirement. Unfortunately, for the employer there is very little practical guidance on this. There are no limits for dermal exposure, nor are there any simple, validated methods for quantifying this. In many cases the problem may not be due to exposure to a single chemical, but to many different chemicals, often repeatedly and over a long period. This is particularly true of the most common form of occupational skin disease, irritant contact dermatitis.

Irritant contact dermatitis occurs as a result of damage to the skin where contact occurs with one or more irritants (Chapter 13). This damage may not be apparent immediately and it may only be after repeat exposures that cumulative damage is noticed (Figure 16.2).

The current hypothesis (outlined in Figure 16.2) proposes that every time the skin is exposed to an irritant a finite level of damage is done (Figure 16.2; A). The extent of the damage will depend upon both the strength of the irritant and the duration of exposure. In simple terms, the longer and stronger the exposure, the greater the damage. As and when contact with the irritant ceases, the skin will begin to recover (Figure 16.2; B). Given time the damage can be completely restored. However, if there is subsequent contact with the same or a different irritant before this process is completed, then there will be a cumulative effect (Figure 16.2; C). At this point, the damage is 'sub-clinical', that is there will be no visible sign of any damage. The skin will *appear* to be completely normal. However, eventually the damage may accumulate to the point where the skin appears to lose its ability to resist any further. Then, over a relatively



After first exposure, a finite amount of (sub-clinical) skin damage occurs (A). With time, the skin undergoes repair (B). Repeated exposure can cause cumulative damage (C). Again, up to a certain point, the damage may be sub-clinical and reversible. However, if damage reaches a certain threshold (D), then repeat exposure produces clinical dermatitis and damage requires longer to repair (E) or becomes irreversible (F).

Figure 16.2 Effect of cumulative exposure to irritants

short period, perhaps only a few days or a week or so, the skin will start to show symptoms of the damage. There will be a dryness, cracking, flaking and redness. Irritation will start and eventually perhaps there may even be ulceration and blistering. Now it is a full-blown irritant contact dermatitis that is being faced (Figure 16.2; D).

If contact with irritants is eliminated then the skin will start to recover. Within a relatively short period it may return to a normal appearance (Figure 16.2; E). It is important to recognise that it is still extremely vulnerable: Premature exposure to irritants will quickly result in a re-appearance of the dermatitis. It may be necessary to keep the individual away from contact with irritants for several weeks, even after the skin has apparently returned to normal, so that by then controlling contact with irritants the individual can be kept from exceeding the threshold again. If, when the dermatitis appears, action is not taken and the damage continues to accumulate, a point can eventually be reached where the skin seems to lose its ability to repair itself (Figure 16.2; F). Now it is what is sometimes called a chronic irritant contact dermatitis that is being faced. This may take an inordinately long time to heal or, indeed, may turn out to be a permanent condition.

Since each person's skin is unique to them, it follows that each person will have their own threshold. What may cause one person no problem may, in another, result in a quick appearance of dermatitis. The problem arises when break-through of the threshold occurs only after a long period. This may extend to several years. The reaction by managers and workers is to try to identify some recent event or change that has triggered the problem, whereas in reality it is the result of a long term build-up of invisible (sub-clinical) damage.

It is important to realise that irritant contact dermatitis generally results from exposure to a whole range of irritants, both in the workplace and at home. Whilst it is tempting to seek a single substance as a cause, this does not reflect reality.

Almost all chemicals can, under certain conditions, act as an irritant. This has been recognised in Germany, where there is a specific requirement contained in Technische Regel für Gefahrstoffe Nr. 401 (Technical regulation for hazardous substances no. 401) covering the measures to be taken where exposure to water or wet work is for an extended period[3]. Incidentally, this regulation also considers the wearing of occlusive gloves for extended periods to be equivalent to wet work, due to the hyperhydration of the skin that may occur.

16.3 Material safety data sheets

> The provision of a MSDS cannot be assumed to provide relevant or factually correct information on a given material.

Traditionally, most employers will have used the material safety data sheet (MSDS) provided for a chemical by their supplier as their source of information for assessing the risk of damage to health. Unfortunately, as will be shown, this is often inadequate or even misleading. In the first place, several studies have shown that safety data sheets are frequently inaccurate; 42% of a sample of MSDSs were found to be 'non-compliant' (Keegel *et al.*, 2005). In a study in the United States (Kolp *et al.*, 1995) 150 safety data sheets were examined; 53% contained

[3] An English translation of this German document is available on the Federal Institute for Occupational Safety and Health (BauA) website: *http://www.baua.de/nn_41278/en/Topics-from-A-to-Z/Hazardous-Substances/TRGS/TRGS-401.html__nnn=true*

inaccurate information on the use of PPE. Only 37% contained accurate information on the health effects of the substances involved. In a quick examination of safety data sheets posted on websites the author found that over 50% contained inaccurate information. Thus safety data sheets must always be treated with caution. However, there is a further problem in that safety data sheets are written to cover hazards, *as supplied*. This can be very different from the hazards that occur when the product is used. It may be heated, mixed, diluted, oxidised etc with the result that the hazard data in the safety data sheet becomes largely irrelevant!

In the United Kingdom, the Control of Substances Hazardous to Health (COSHH) Regulations contain several definitions of what constitutes a substance hazardous to health which can be paraphrased as 'a substance which because of its chemical or toxicological properties and the way it is used or is present at the workplace creates a risk to health'. Perhaps it is prudent to keep in mind what Paracelsus said so many years ago that: 'All things are poisonous and there is nothing without poison. Only the dose determines whether it is poisonous.' This is certainly true of chemicals in contact with the skin (Box 16.1).

Box 16.1 Extract from Control of Substances Hazardous to Health Regulations; Regulation 7(3): guidance on when to employ PPE

Where it is not reasonably practicable to prevent exposure to a substance hazardous to health, the employer shall comply with his duty of control . . . by applying measures appropriate to the activity and consistent with the risk assessment, including in order of priority:

(a) the design and use of appropriate work processes, systems and engineering controls and the provision and use of suitable work equipment and materials;

(b) the control of exposure at source, including adequate ventilations systems and appropriate organizational measures; and

(c) where adequate control of exposure cannot be achieved by other means, the provision of suitable personal protective equipment in addition to the measures required by sub-paragraphs (a) and (b).

16.4 Chain of responsibility

If a system is to be created that minimises the risk of damage to health from workplace skin exposure, then it is necessary to determine who, within an organisation, is actually responsible on a day-to-day basis for ensuring adequate control of skin exposure. In most countries, and certainly in the United Kingdom, the overriding responsibility under law will rest with whoever is ultimately in charge of the organisation, e.g. the Chief Executive Officer, Chairman of the Board, Managing Director. However, it is extremely unlikely that this person can take day-to-day responsibility, nor that they will have the specialist knowledge and expertise needed. In almost all cases they will have secured the assistance of one or more specialists in health and safety. In larger organisations there may be a sizeable team comprising occupational hygienists, occupational physicians, occupational health nurses, safety specialists etc. However, even a sizeable team can only act to develop systems, advise on specific situations and audit the organisation's performance. It is extremely unlikely that the team can monitor and assess all the minor changes and decisions that are being made by line/area managers, team leaders etc on a day-to-day basis.

In practice it will be these people who will have to make decisions on how actual activities are carried out, or how a defined activity or procedure might be modified to fit a change in conditions. For most organisations it would be impractical to have to involve someone from health and safety to assess every such minor change. What is needed, therefore, is to ensure that these managers are able to ensure that any minor changes do not constitute an unacceptable increase in risk to those who might be affected by the change.

In most organisations, therefore, it is necessary to address questions such as:

- Do they understand their responsibility for health and safety?
- Do they have the ability to identify any exposure occurring in work under their control?
- Do they have the knowledge and tools to conduct a simple risk assessment?
- Do they know what is needed to adequately control exposure?
- Do they have the time and resources to do this?

Those responsible for an organisation's health and safety will need to develop and implement systems and training schemes to ensure that these questions are adequately dealt with.

16.5 Managing dermal exposure

A reduction in the incidence of occupational skin diseases can be attained through the effective implementation of improved workplace environment procedures and exposure control measures. Effective training of managers and workers is central to all these actions.

Fundamental to managing exposure, whether to the skin or by the other routes, is the concept that we should strive to manage the process, not the person. In other words, in line with the basic concept of occupational skin management, we should attempt to make the process intrinsically safe rather than rely upon the individual worker to act in an appropriate manner. There are important reasons for this approach (Table 16.1).

If a process is intrinsically safe, then the behaviour of the worker can have no influence on his or her safety. Furthermore, in most cases the process can be so arranged that should there be a failure the system will 'fail to safety', i.e. the worker's health will not be impaired.

Table 16.1 Elements of process and person controls for mitigating dermal exposure

Controlling the process	Controlling the person
Control lies with management	Control lies with the person
System can be made to 'fail to safe'	System is always 'fail to danger'
Initial costs may be higher, but medium/long term costs are almost always lower	Initial costs may be lower, but long term costs will usually be higher

Table 16.2 Strategy for risk management. (Items 1 to 3 represent controlling the process; item 4 can be either controlling the process or the person, depending upon the nature of the handling equipment. Items 5 to 7 represent controlling the person. It must be stressed that all of these are valid techniques)

Item	Description
1	Design workplace and equipment to eliminate exposure
2	Select chemical(s) for minimum hazard
3	Install engineering/process controls
4	Provide handling equipment
5	Establish safe working procedures
6	Control exposure with personal protective equipment
7	Minimise any effect by limiting exposure and monitoring effect

As soon as reliance is placed on the worker acting in a certain, safe manner, then neither absolute control nor 'fail to safety' can be guaranteed. To ensure that the worker is continuing to operate in a safe manner, management needs to ensure supervision. This will involve management time, which, of course, entails cost. In addition, actions such as merely providing personal protective equipment, for example gloves, would not necessarily result in compliance with COSHH regulations, as is explained elsewhere in this chapter. It is certainly advisable to work to a clearly defined strategy when attempting to manage skin exposure risks effectively (Table 16.2); as a general rule, an attempt should be made to concentrate the first three items. In practice, what generally provides the most satisfactory solution is a combination of some, occasionally all, of the seven items.

16.5.1 Design workplace and equipment to eliminate exposure

Design of the workplace and equipment is one aspect of risk management that is often overlooked. There are many occasions where, had the health and safety team been involved at the planning stage, a building, process or equipment could have been modified with little or no added cost to ensure that it could be used or operated safely. Unfortunately, those who design buildings, processes or equipment frequently have little training or expertise in health and safety. Once the building is erected, the process initiated or the equipment installed, it is often an extremely expensive operation to introduce modifications so that intrinsic safety can be assured.

16.5.2 Select chemicals for minimum hazard

This is often an approach encouraged by health and safety authorities. However, it is not always as simple as many assume. Unfortunately, there are no short term tests that will reveal with certainty what the health effects of long term use might be. Thus, care must be exercised when selecting chemicals so that the a chemical does not introduce a new, or unexpected, hazard.

For example, in certain workplaces solvent-based paints have been replaced by water-based types, with the assumption being that the latter would represent a lesser hazard. What was overlooked was that water-based paints generally contain a biocide, often either one based on isothiazolinones or a formaldehyde releaser. Even after the paint has dried, the biocide can remain active and be released from the paint, with the result that where the paint has been applied in a closed environment significant levels of airborne contamination can accumulate. In one study involving isothiazolinones, these were still being released in significant amounts into the atmosphere ten days after the paint had been applied (Bohn *et al.*, 2000). In a factory manufacturing kitchen furniture, this strategy caused both respiratory and skin reactions in an enclosed area where painted panels had been stored, resulting from the release of formaldehyde. Previously, there had been no such contamination of the environment with solvent-based paints.

In some cases it may be possible to eliminate the hazardous chemical altogether. For example, in a printing works, a mixed, reclaimed solvent was being used to clean printing ink off rollers and other parts of the printing press after each print run. As will be seen from the section on gloves later in this chapter, no glove would protect the hands effectively for more than a few minutes. An alternative cleaning process was introduced. This used carbon dioxide (CO_2) combined with compressed air. The carbon dioxide was converted on site into small, solid pellets (dry ice). These were directed at the parts to be cleaned at high velocity by the compressed air. The physical impact combined with the extremely low temperature caused the dry ink to fall away from the rollers and other parts. It then fell on to a sheet of plastic placed underneath the press. The carbon dioxide pellets evaporated (adequate ventilation had to be ensured) leaving behind just the dry ink, which could then be disposed of simply. Not only did this remove the exposure to the solvent but it also resulted in considerable cost savings, since there was no hazardous solvent to store and dispense and no waste solvent for disposal as hazardous waste.

16.5.3 Install engineering or process controls

Assuming that an existing situation is being faced and that the risk assessment has shown an unacceptable risk of dermal exposure, can anything be done to modify the equipment or process to eliminate or reduce exposure to an acceptable level? In principle there are a number of possible approaches.

Automation

By automating the process the need for the worker to be in the direct presence of the chemicals is removed. At worst, exposure will be limited to the time spent loading or unloading a component. Even this may be automated or at least the design can be such that contact between worker and chemical is minimal.

Enclosure and guarding

By providing a physical barrier between worker and chemical it is possible to ensure that any direct exposure is prevented or substantially limited. Again, consideration must be given to loading and unloading.

A factor that must be considered with both of the above methods is that set-up and maintenance activities can seldom be automated. In such cases systems must be developed that ensure that the set-up or maintenance fitter is not unduly exposed. In many cases this should not present a significant problem, as the amount of time spent setting up or repairing the equipment should be limited. One simple procedure that it is often found has not been considered in maintenance and setting-up work is to introduce a procedure whereby any item of equipment that has to be worked on is cleaned before work starts. This may appear a time consuming activity, but experience shows that in many cases the total time taken to complete the maintenance or setting-up task is actually reduced, since the worker will find carrying out the task on clean equipment much easier and quicker.

Local exhaust ventilation

Airborne exposure must also be considered: contact dermatitis as a result of exposure to spray, aerosol or dust is common and well documented. It must also be kept in mind that, whilst maintaining levels of exposure to below the legal level TLV (threshold limit values), PEL (permissible exposure limits), MEL (maximum exposure limits), WEL (workplace exposure limit), MAK (Maximale Arbeitsplatzkonzentration; maximum airborne concentrations) etc may be adequate for control of respiratory exposure, dermal effects, particularly allergic reactions, can occur at much lower levels.

Automatic cleaning plant

Instead of a worker having to use a solvent to manually degrease a component, with the inevitable risk of dermal contact, automatic component washing could be used. There are many different types available, so it should be possible to find a suitable design. There are even simple, relatively inexpensive devices for the automatic cleaning of paint spray guns, thereby eliminating the most common source of dermal exposure to solvents in paint spray and automotive body shops. Of course, where the degreasing is relatively rare, it may not be possible to economically justify such equipment. In such cases, simpler methods may offer a more practicable solution.

Control dilution and concentration

In many cases, chemicals will be diluted with another (possibly less hazardous) chemical. Thus the potential to cause damage to health from skin exposure is reduced. Since the higher the concentration the greater the potential to cause such damage, it follows that it is important to ensure correct dilution and that the concentration is controlled during the life of the mixture.

A typical example of this is metalworking fluid, a ubiquitous product where metal is being cut, ground or otherwise worked. The metalworking fluid is essential in cooling both the cutting tool and work-piece, in removing the metal that has been removed (swarf), in lubricating the process to reduce wear and in preventing corrosion. Usually the fluid is based either on mineral oil or some form of synthetic compound similar to oil. In addition, there will be a variety of additives, many of which are irritant and sensitising if placed in contact with the skin. The fluid is generally supplied as a concentrate and is mixed with water. Typical concentrations of mixture in water can be between 1% and 15%. To ensure a stable emulsion,

the formulation usually contains a detergent (emulsifying agent), which can potentially defat (delipidise) the skin. Obviously, the stronger the mixture, the greater the effect if in contact with the skin.

Since contact with the skin is impossible to avoid in many machining operations (e.g. centreless grinding), it is important that the concentration is controlled to the minimum needed for the machining operation. Therefore, dilution should be performed in such a way that the correct concentration is ensured. However, when in use, the fluid will remove heat from the process. This can cause the water to evaporate, leading to an increase in the concentration of additives with lower volatility. It is important, therefore, to check the concentration regularly, using an instrument called a refractometer. This is just one illustration of how a fluid management system may be required to ensure minimum effect.

Create a 'safe working distance'

There are many ways in which work can be conducted with the skin being kept sufficiently remote from the chemical to eliminate exposure. For example, workers have traditionally used rags and solvent to clean equipment, with gloves being used for hand protection. The protection afforded by gloves against solvents can be poor (see below). Thus, many workers were being exposed to cleaning solvents and subsequently running a very high risk of developing dermatitis. Contact between the gloved hand and the solvent could have been eliminated by replacing the rag with a paint pad, reducing the gloves to the function of protection against splashes. In workplaces adopting this practice, skin exposure was reduced to an acceptable level and the risk of dermatitis essentially eliminated.

16.5.4 Provide handling equipment

It is not uncommon to see workers placing their hands into chemicals, for example when removing components from a degreasing chemical tank. Whilst they may rely upon gloves to provide protection, the protection may not be adequate, as will be seen elsewhere in this chapter. A simple change would eliminate the need for such exposure.

For example, a wire basket with long handles could be provided. Components could be placed into this and then into the degreasing chemical. If necessary, the basket could be agitated to increase the degreasing effect. With such a system the hands need not be anywhere near the degreasing chemical.

Other handling equipment could be simple tongs to hold a component contaminated with metalworking fluid, jigs for moving contaminated components from one machine to another etc.

16.5.5 Establish safe working procedures

Where reliance is on the worker to carry out the process in a safe manner, this will have been assessed for the risk of dermal exposure. It is then necessary to ensure that the operation is conducted in such a way that any exposure is eliminated or minimised. It is sensible to involve the operator in the development of the system, as this helps to ensure that he or she is able (and willing!) to comply. Once the procedure has been established, the worker will need instruction and training to ensure that (s)he can act appropriately. There will also be a need

for management to check from time to time to ensure that workers are adhering to the agreed procedure.

16.5.6 Control exposure with personal protective equipment

Where control cannot be achieved by any of the above methods, then reliance may have to be placed on the use of personal protective equipment. This may take the form of protective clothing, such as coveralls and gloves, face shields etc.

One point that is often overlooked is that when considering protection against inhalation of a sensitiser, exposure of the facial skin must also be included. Whilst a half-mask might provide adequate protection against inhalation, the facial skin would remain exposed and this could result in sensitisation and an allergic reaction.

Selection and use of personal protective equipment is a complex topic and is dealt with in some detail later in this chapter.

16.5.7 Minimise any effect by limiting exposure and monitoring effect

This is often an acceptable approach when dealing with less hazardous chemicals, such as detergent and cleaning products that might be found in a kitchen. In one hospital kitchen, for example, cutlery and crockery went through an automatic washing process, but work surfaces and the large pans were cleaned manually. Workers took turns at this, each spending a complete day on cleaning. As a result, the extended exposure was causing skin damage, which resulted in several cases of irritant contact dermatitis. By reducing the time each worker spent at this activity to half a day the level of damage was reduced and the dermatitis problem solved. Wherever skin is exposed to a situation where damage to health could occur, then regular skin health surveillance is essential. How and when this should be done is dealt with elsewhere (Chapter 15).

16.6 Selection and use of personal protective equipment

Personal protective equipment (PPE) can be an effective control measure. However, it is imperative that the PPE chosen for a specific task is appropriate and used correctly!

Probably the most common form of personal protective equipment used to protect the skin against chemical hazards is gloves. It is also one form of personal protective equipment where failure to protect is common, simply because those selecting and deciding how gloves may be used do not understand how gloves work and fail.

Firstly, it is important to recognise that gloves should be one of the last methods selected to provide chemical protection. In many countries, such as the United Kingdom, this is a legal requirement (Box 16.1). Thus, merely providing gloves as protection without first attempting to control exposure by other means would not result in regulatory compliance.

In theory, the safety data sheet for the chemical should indicate the type of glove and the length of time for which it can be used. In practice many simply state: 'Use suitable gloves' or some other vague phrase. Even where the glove material has been specified, treat this

with caution (see section 17.6). As will become clear, such recommendations may result in inappropriate glove use.

It is also important to recognise that the use of the incorrect glove, or the correct glove incorrectly, can result in greater damage than would have occurred had no glove been worn: 'Wearing internally contaminated gloves led to higher systemic absorption than was gained from the equivalent skin contamination when not wearing gloves' (Rawson *et al.*, 2005).

A further consideration is that gloves will always 'fail to danger'. In other words, if a glove fails then the wearer will be exposed. The results may be catastrophic, as illustrated by the case of Professor Karen Wetterhahn who died as a result of dermal exposure to dimethylmercury following contamination of a latex glove (Blayney, 2001). Thus, where the outcome of glove failure may result in acute, severe and possibly irreversible damage to health, it is essential to consider gloves only as secondary protection; control of exposure must be achieved by other means. The only exception to this would be an extreme emergency when other methods are not available.

Whilst gloves can perform an important and excellent role in preventing or limiting exposure to chemical hazards, this will only be achieved if those who select and decide how gloves can be used have an understanding of the function and limits of such PPE.

Ensuring that the correct glove is selected and used only within its real protective capacity is not as simple as most assume. In practice there are four main ways in which a glove can fail: misuse, physical failure, degradation and permeation. In practice, it is often a combination of these factors that results in the worker being exposed to a chemical, often without worker or employer being aware until damage occurs either to the skin or to the general health of the glove user.

Misuse

Use of the correct glove is essential. The provision and control of gloves in the workplace (so that only the correct glove is used for a specific task) is something that is essential but is often missing or inadequate in many workplaces.

Most workers have never been trained to don or remove gloves. It is common to find that the procedure they use results in contact between the hand and the contaminated surface of the glove. Dirty hands may be inserted into gloves, ensuring that the soiling on the hand can cause maximum damage. In one study (Babb *et al.*, 1989), it was clear that the incorrect method of glove removal was causing significant contamination of the hands (Figure 16.3).

Thus it is obvious that training in the use of gloves is essential if adequate protection is to be achieved. Glove storage, when used intermittently, is often ignored with the result that the gloves become damaged or contaminated. Gloves that have been in contact with hazardous chemicals must be disposed of as hazardous waste, something that does not always occur.

Physical damage

It is not uncommon to see workers wearing gloves with holes, splits or cracks. It is also not uncommon to see workers wearing gloves from which they have removed the fingers (Figure 16.4) so as to improve dexterity! Normally such damage is obvious. More insidious are the tiny holes that are often impossible to detect visually, but which require other detection techniques. These may be large enough to permit passage of bacteria or chemicals. It is important to note that such defects may be present in new, unused gloves, so where there is a significant hazard glove *in situ* testing should be considered.

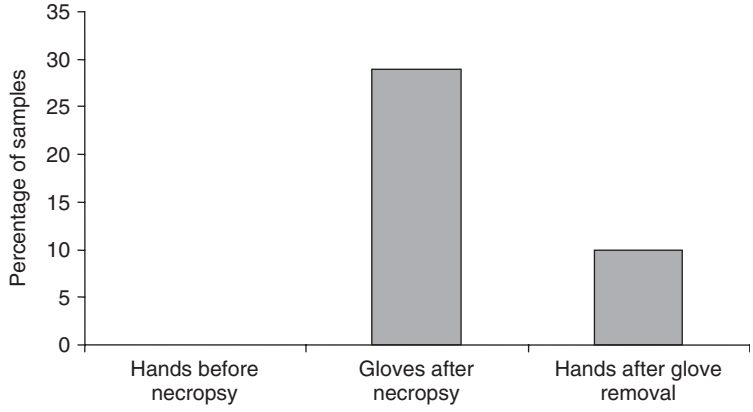


Figure 16.3 Bacterial contamination of the hands (defined as being >10 colony forming units [cfu] per sample) expressed as the percentage of samples obtained from hand swabs of pathologists conducting post mortem examinations (Babb *et al.*, 1989; reproduced with permission from BMJ Publishing Group)

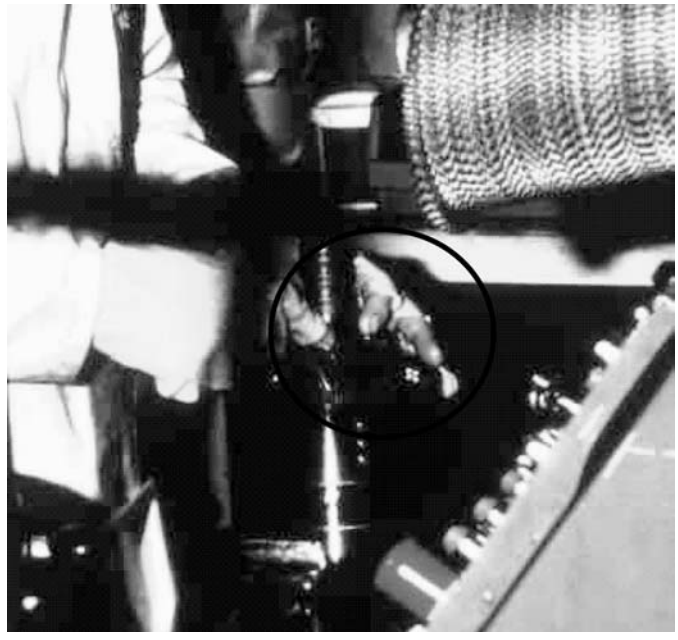


Figure 16.4 Whilst increasing the wearer's tactility, these gloves certainly do not provide adequate protection! A full-colour version of this figure appears in the colour plate section of this book

Degradation

Since there is no material known to man which is suitable for use as a glove and which is resistant to all known chemicals, it follows that many chemicals will attack and destroy a particular glove. For example, all hydrocarbon solvents will degrade natural rubber latex. Usually such degradation is visible in the form of swelling, loss of flexibility, tackiness etc.

Permeation

Less easy to detect is permeation, the diffusion of the chemical through the glove. The chemical then partitions into the glove's interior as a vapour. Permeation, itself, does not result in any change in the glove's appearance or feel and is thus generally undetectable by the wearer until the chemical inside the glove causes some damage. Since there is no glove material that is impermeable to all chemicals, what is needed is to know for a given glove how long the permeation breakthrough of a specific chemical will take. This is generally known as the permeation breakthrough time (BTT) and is generally expressed in minutes, although in the European Union there is now a system of six different categories (Table 16.3). Unfortunately, the standard specifies that gloves shall be tested at room temperature, defined as 23°C ± 1°C. Hands tend to be warmer than this. A thermometer placed against the skin inside a glove may register temperatures around 35°C. Permeation at this temperature may be significantly different than that at 23°C (Table 16.4).

Protection data published by the manufacturer of gloves must be regarded with caution. Unfortunately, there is no simple method for calculating the different permeation breakthrough times at different temperatures. It is important to recognise that permeation must be regarded as an absolute phenomenon. That is, permeation starts from first contact and continues, irrespective of whether the glove is being worn or not. Thus a permeation breakthrough time of, say, two hours does not mean that the glove can be used for fifteen minutes each morning for four days.

Table 16.3 Standard (EU) classification of gloves based on breakthrough times as measured using the EN374-3 test method

Breakthrough time (minutes)	Class (protection index)
>10	1
>30	2
>60	3
>120	4
>240	5
>480	6

Table 16.4 The effect of temperature on glove breakthrough times

Chemical	Breakthrough time (mins)	
	at 23°C	at 35°C
n-Butanol	>480	>240
Diethylamine	60	6
Dipentene (*d*-limonene)	>480	36
Isobutanol	>240	>240
Methyl Ethyl Ketone	>1440	>240

One of the main problems with permeation is the perception of the user. Since the glove will appear to be in 'as new' condition, it is often difficult to persuade that person that the glove is at the end of its useful life and should be discarded.

It is also important to recognise that the manufacturer's published permeation data cannot be used to indicate the actual performance that will be achieved in practice. In addition to the problems associated with the actual test procedure, there are many other factors that will affect how a glove performs when used. If gloves are to be used as protection against chemicals, then it is essential that they are selected and used within their true protective capability. Unfortunately, this is not always easy to establish. There are gloves that will provide extended permeation breakthrough times but that will degrade and thus split within a very short time.

There is a range of factors (Table 16.5) that indicates the complexity of this subject. In the author's view, establishing the true protective life of a glove requires testing under the conditions of actual use. This can be done, using detector pads inside the glove (Figure 16.5).

Table 16.5 Some factors affecting the performance of chemical protective gloves

Duration of Protective Effect	
Decreased	Increased
High temperature	
Flexing and stretching	Intermittent or incomplete contact
Mechanical damage, including abrasion	Volatility
	Low temperature
Poor maintenance	Frequent glove washing
Ageing	Mixture strength
Mixtures	

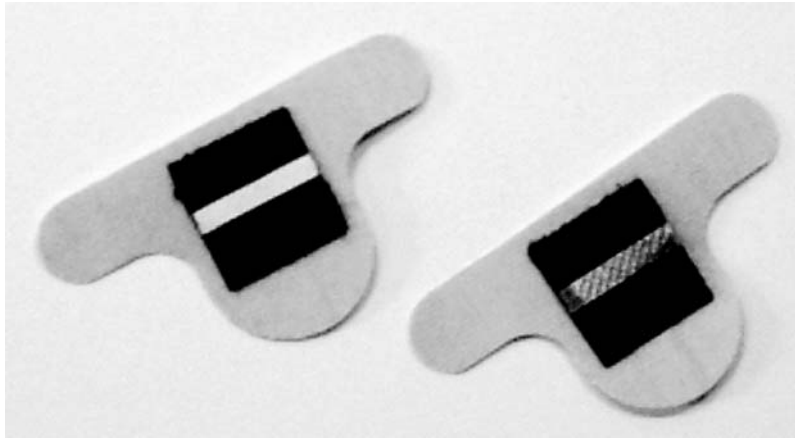


Figure 16.5 Example of under-glove pads used to establish duration of protection

16.7 Protective or 'barrier' creams: do they have a role?

There are still those who believe that protection can be achieved by the use of so-called 'barrier' or 'protective' creams. This is despite all the evidence that indicates that any protection that might be achieved will be minimal and transitory, and that in many cases more absorption into the skin can be shown on the skin treated with the cream than on untreated skin (Chilcott *et al.*, 2002; Frosch and Kurte, 1994; Schlüter-Wigger and Elsner, 1996; Treffel *et al.*, 1994). Furthermore, even were there to be a cream that did actually provide a barrier, its effectiveness would be dependent upon correct application. Several studies have shown this not to be the case. In one study 85% of those applying the cream missed areas of the hands (Wigger-Alberti *et al.*, 1996). An analogy would be the provision of gloves with holes!

16.8 The role of education and training

If any skin management system is to function effectively, it is essential that both management and workforce have a basic understanding of how the skin interacts with the working environment, what is involved in preventing interaction resulting in damage to health, and their respective roles. Thus, education must play a major role in any skin management system.

16.9 Conclusions

It is possible to significantly reduce the probability of skin exposure in a working environment resulting in damage to health. In most cases this requires both an understanding of the skin's interaction with the environment and of the processes and chemicals present in that workplace.

However, creating an effective system will never completely eliminate the potential for a problem to arise. Each person's skin is unique to them, so it is always possible for one person to react to a situation that leaves all the others in the workplace unaffected. This is why skin health surveillance is an essential element in any skin management system (Chapter 15).

Summary

- The most effective way of dealing with occupational skin disease is through prevention.
- Prevention can be effectively achieved through the introduction of good working practices, appropriate training and by the judicious use of physical measures to reduce exposure to hazardous materials.

References

Babb, J.R., Hall, A.J., Marlin, R. and Ayliffe, G.A.J. (1989). Bacteriological sampling of postmortem rooms. *J Clin Pathol*, **42**: 682–8.

Blayney, M.B. (2001). The need for empirically derived permeation data for personal protective equipment: the death of Dr. Karen E. Wetterhahn. *Appl Occup Environ Hyg*, **16**: 233–6.

Bohn, S., Niederer, M., Brehm, K. and Bircher, A.J. (2000). Airborne contact dermatitis from methylchloroisothiazolinone in wall paint: abolition of symptoms by chemical allergen inactivation. *Contact Dermatitis*, **42**: 196–201.

Chilcott, R.P., Jenner, J., Hotchkiss, S.A.M. and Rice, P. (2002). 'Evaluation of barrier creams against sulphur mustard: (I) *In vitro* studies using human skin', *Skin Pharmacology and Applied Physiology*, **15**: 225–235.

Frosch, P.J. and Kurte, A. (1994). Efficacy of skin barrier creams (IV). The repetitive irritation test (RIT) with a set of 4 standard irritants. *Contact Dermatitis*, **31**(3): 161–168.

Keegel, T., Saunders, H. and Nixon, R.L. 2005). *Material safety data sheet accuracy: Reporting of skin irritants and skin sensitizers*, Poster at the Occupational and Environmental Exposures of Skin to Chemicals Conference, Stockholm, June 2005. *http://www.cdc.gov/niosh/topics/skin/oeesc2/AbPost053Keegel.html*.

HSE (Health and Safety Executive). (2000). The prevalence of occupational dermatitis amongst printers in the Midlands. *Contract Research Report 307/2000*. Available at *http://www.hse.gov.uk/research/crr_pdf/2000/crr00307.pdf*.

Kolp, P., Williams, P. and Burtan, R. (1995). Assessment of the Accuracy of Material Safety Data Sheets (MSDSs). *Amer Ind Hyg Ass*, **56**: 178–183.

Rawson, B.V., Cocker, J., Evans, P.G. *et al.*, (2005). Internal Contamination of Gloves: Routes and Consequences, *Annals of Occupational Hygiene*, **49**: 535–541.

Schlüter-Wigger, W. and Elsner, P. (1996). Efficacy of 4 commercially available protective creams in the repetitive irritation test (RIT). *Contact Dermatitis*, **34**(4): 278–283.

Treffel, P., Gabard, B. and Juch, R. (1994). Evaluation of barrier creams: an *in vitro* technique on human skin. *Acta Derm Venereol*, **74**(1): 7–11.

Weisshaar *et al.* (2006). Educational and dermatological aspects of secondary individual prevention in healthcare workers, *Contact Dermatitis*, **54**: 254–260.

Wigger-Alberti, W., Maraffio, B., Wernli, M. and Elsner, P. (1997). Self-application of a protective cream. Pitfalls of occupational skin protection. *Arch Dermatol*, **133**: 861–4.

PART V: Regulatory

17 Occupational skin exposures: legal aspects

Chris Packham

Enviroderm Services, North Littleton, Evesham, WR11 8QY, UK

Primary Learning Objectives

- *The responsibility of supervisors and operators in tasks involving potentially harmful materials.*
- *An understanding of the range of legislation and guidelines which influences occupational skin exposures.*
- *Overview, implications and practical impact of relevant (United Kingdom and European Union) legislation.*

17.1 Introduction and scope

There are several regulations and guidelines that affect the handling and use of chemicals in the workplace. However, many are not statutory, some are conflicting and some are imprecise and so difficult to interpret.

This chapter is not a legal treatise. It is intended to explain how, in practice, the various acts and regulations need to be considered by those attempting to ensure compliance. It is concerned with how the United Kingdom law affects how skin exposure is managed in the working environment. In this connection it must be pointed out that significant changes will occur in the next few years, owing to the implementation of the new European Union Regulation on Registration, Evaluation, Assessment and Restriction of Chemicals (REACH). At the time this chapter was being written, it was not clear how and over what period the relevant parts of REACH will be implemented, nor of its impact on how occupational skin exposure is managed.

Principles and Practice of Skin Toxicology Edited by Robert P. Chilcott and Shirley Price
© 2008 John Wiley & Sons, Ltd

17.2 Brief overview of current United Kingdom legislation

In the United Kingdom, the two main legal requirements are a 'general duty of care' and the Health and Safety at Work Act. The latter includes the Control of Substances Hazardous to Health (COSHH) regulations, which are primarily concerned with the use of chemicals in the workplace.

In the United Kingdom, employers have to comply with two separate legal requirements. One is a general duty of care, the other is the Health and Safety at Work Act (1974). The Act has been expanded and updated by the introduction of various regulations, such as the Management of Health and Safety at Work, Control of Substances Hazardous to Health (COSHH), Personal Protective Equipment Regulations etc. These are not legal statutes in their own right. The Act places duties upon the employer, but also upon the employee. In this context, a self-employed person is considered also to be an employer and thus subject to the provisions of the Act and the subsidiary regulations.

Section 2-1 of the Act provides the fundamental duty placed upon the employer, namely that '*it shall be the duty of the employer to ensure, so far as reasonably practicable, the health, safety and welfare at work of his employees*'. Note the phrase, 'so far as reasonably practicable'. This allows the employer to claim that the measures in place to meet its duties under the Act are sufficient for it to have fulfilled this duty, even if the resulting situation could represent a potential cause of damage to health. It is probable that in the near future this phrase will no longer be allowed under European law.

However, it is not always realised that under United Kingdom legislation, where chemicals are concerned, this phrase no longer applies. Section 7-1 of the Control of Substances Hazardous to Health states that '*every employer shall ensure that exposure of his employees to a substance hazardous to health is either prevented or, where this is not reasonably practicable, adequately controlled*'. Note that the phrase 'reasonably practicable' only relates to prevention and not to 'adequately controlled'. In other words, if the employer cannot prevent exposure it must adequately control it. The employer cannot argue that the exposure has been controlled so far as reasonably practicable. Unless control is 'adequate' the employer will not have complied with its responsibilities under the Act.

This raises two questions:

What is a substance hazardous to health?

Before looking at the definitions of a substance hazardous to health, it is prudent to ensure that the terminology which the various regulations use is clearly understood; the more common terms are summarised in Table 17.1.

The current version of the Approved Code of Practice for COSHH (ACoP) defines a substance hazardous to health in a number of ways (Box 17.1). Paragraph (e) is particularly relevant when it comes to skin and exposure to chemicals. It implies that all chemicals in a workplace must be evaluated by considering the properties of the chemical and the actual or potential exposure that is occurring. This is correct. It is well documented that exposure to water, in the form of wet work, is a major cause of occupational contact dermatitis. It can be plain water but, of course, if the water also contains other chemicals such as detergents, then the problem is likely to be more severe. In Germany this has been formally recognised

Table 17.1 Common terminology and definitions in use under current COSHH Regulations

Substance	'Substance' means a natural or artificial substance whether in solid or liquid form or in the form of a gas or vapour (including micro-organisms)
Preparation	'Preparation' means a mixture or solution of two or more substances
Hazard	'Hazard', in relation to a substance, means the intrinsic property of that substance which has the potential to cause harm to the health of a person, and 'hazardous' shall be construed accordingly
Risk	'Risk', in relation to the exposure of an employee to a substance hazardous to health, means the likelihood that the potential for harm to the health of a person will be attained under the conditions of use and exposure and also the extent of that harm
Inhalable dust	'Inhalable dust' means airborne material which is capable of entering the nose and mouth during breathing, as defined by BS EN 481 1993
Respirable dust	'Respirable dust' means airborne material which is capable of penetrating to the gas exchange region of the lung, as defined by BS EN 481 1993

Box 17.1 Extract from the Approved Code of Practice (ACoP) pertaining to the Control of Substances Hazardous to Health (COSHH) Regulations (amended 2002): definition of hazardous substances

A substance hazardous to health means a natural or artificial substance (including a preparation):

(a) which is listed in Part I of the approved supply list as dangerous for supply within the meaning of the CHIP Regulations and for which an indication of danger specified for the substance is very toxic, toxic, harmful, corrosive of irritant;

(b) for which the Health and Safety Commission has approved a workplace exposure limit;

(c) which is a biological agent;

(d) which is dust of any kind, except dust which is a substance within paragraphs (a) or (b) above, when present at a concentration in air equal to or greater than –

(i) 10 mg/m^3, as a time-weighted average over an 8 hour period, of inhalable dust, or

(ii) 4 mg/m^3, as a time-weighted average over an 8 hour period, of respirable dust;

(e) which, not being a substance falling within sub-paragraphs (a) to (d), because of its chemical or toxicological properties and the way it is used or is present at the workplace creates a risk to health.

(Box 17.2). Incidentally, the latter considers the wearing of occlusive gloves to be synonymous with wet work.

Basically, 'risk phrases' cannot be relied on to indicate which chemicals in a workplace are hazardous to health. This has implications for the time taken to conduct a risk assessment for dermal exposure, since this must consider all chemicals in a workplace and not merely those with risk phrases.

Box 17.2 Extract from Technische Regel für Gefahrstoffe (Technical regulations for hazardous substances) no. 401: definitions of substances hazardous to the skin

Substances and preparations are hazardous to the skin if they can damage the skin following skin contact (e.g. by causing burns and/or irritant effects). The following R-phrases indicate that this property applies to the relevant material: R 34, R 35, R38, R66.
Other substances or preparations that do not satisfy the conditions for the above R-phrases, but may have damaging effects on the skin in the event of prolonged or repeated contact can also be hazardous to the skin. This classification may also apply in the event of mechanical influences (friction, microlesions).
Dermal risks are present if

- *wet work, or*
- *activities involving hazardous substances that are hazardous to the skin, absorbed through the skin or sensitising to the skin are carried out under such circumstances that health risks for the employees cannot be excluded.*

What is meant by adequately controlled?

The ACoP to the COSHH Regulations defines the routes of exposure (Box 17.3) and, when considering absorption through the skin, states that '*In handling any substance which has been assigned an 'Sk' notation, the employer's application of good practice controls, work methods and other precautionary measures should prevent the substance coming into contact with the employee's skin. The plan should draw on any information and advice provided by the supplier on the particular characteristics and properties of the substance and how to deal with spillages etc*'.

Box 17.3 Extract from ACoP to COSHH (5th Edition): definitions of routes of entry

'COSHH requires that employers prevent or adequately control exposure by all routes, not just the inhalation route and deals with substances which can be hazardous to health by:

(a) absorption through the skin or mucous membranes; or
(b) contact with the skin or mucous membranes, e.g. dermatitis, chemical burns, antimicrobials infection; or
(c) ingestion'

ACoP to COSHH (5th edition), paragraph 135S

The 'Sk' notation is found in the Health and Safety Executive (HSE) publication 'EH40', which provides workplace inhalation exposure limits (HSE, 2005); it specifically indicates those substances which can penetrate the skin. Note that EH40 does not list those substances which do not have an exposure limit but which are skin penetrants, so the 'Sk' notation does not constitute a comprehensive list.

So for inhalation/respiratory uptake there are exposure limits for those substances that are most likely to be encountered in the normal workplace. It must be recognised that United Kingdom workplace exposure limits (WELs) apply only to inhalation exposure, and not to

airborne exposure. It is possible with some sensitisers to have airborne skin exposure (for example of the face) which is below the WEL that will elicit an allergic contact dermatitis in a previously sensitised person.

When it comes to dealing with 'adequately controlled' for skin exposure, COSHH and the ACoP are much less precise. This is an area where considerable difficulty is encountered. Unfortunately, the way in which the skin reacts to chemical exposure is extremely complex. Many different factors can influence whether exposure will represent a risk of damage to health. It has not been possible yet to develop a system that allows a no effect level for many of the chemicals normally found in a workplace to be determined. Thus it is not possible to define 'adequately controlled' for any specific chemical. In effect, the employer is left in a situation where there is a regulation with which it must comply, but where the regulation does not state clearly what is needed to ensure compliance. In practice this places increased importance on the employer's skin health surveillance system to detect any damage prior to the appearance of the contact dermatitis (Chapter 15).

This is further complicated by the fact that most data on hazards are limited to individual substances, whereas most workers will be handling mixtures. These may vary over time, and thus present differing characteristics. For example, a solvent penetrating the skin may act as a vehicle and carry other, possibly more toxic, chemicals with it into the body, whereas on its own the toxic chemical would not have been able to do this.

17.3 The employer's perspective

Basically, the employer is in the unenviable position of being presented with a regulation with which it has to comply, but which does not clearly state what the employer has to do to achieve compliance! Thus, at no point is it possible for any employer, or any health and safety practitioner, to state with certainty that the regulations are being complied with. In theory, any occupational ill health arising from skin exposure could be construed as showing non-compliance, but since it is clearly impossible to eliminate skin reactions altogether, this is hardly a practical position.

It's all a question of balance!

One of the complexities of dealing with skin exposure is that with some substances skin exposure can be beneficial up to a certain level and harmful above this level. For example, overexposure of the skin to ultraviolet radiation can cause adverse skin effects. However, a certain level of ultraviolet radiation is needed to initiate the synthesis within the skin of precursors for vitamin D_3. Without it people will start to suffer from Vitamin D deficiency. Ultraviolet radiation is even used in the treatment of certain skin diseases. Yet excessive exposure will not only lead to premature skin ageing but increases the risk of developing skin cancers.

The problem arises in that the threshold level of exposure (above which harm occurs) is difficult to define for a great number of chemicals and can vary, not only from individual to individual, but within the same individual due to a range of factors (Table 17.2). This is why there are no dermal exposure levels.

In practice, what an employer will need to do is to demonstrate that it has taken as much care as it could to identify and manage any skin exposure, to identify any problems at the

Table 17.2 Some factors which influence an individual's susceptibility to adverse health effects following dermal exposure to hazardous substances

Individual	Workplace
Genetic variability	Substances to which exposed
Endogenous skin conditions	Substance control standards
Concomitant non-occupational exposure	Duration and frequency of exposure
Past exposure and acquired sensitivity/resistance	Location of contact on body
	Ambient conditions
Working habits	General workplace hygiene
Personal hygiene standards (at work and at home)	Personal protective equipment
	Knowledge and training
Variations in overall skin condition	Skin surveillance systems
	Hygiene facilities

earliest possible stage and to have responded promptly to any indication of a possible problem arising from skin exposure.

17.4 Hazard identification

Hazard identification and risk assessment are key processes to providing a safe working environment. However, these can only be useful if the data on which they are based are accurately reported and actually reflect the purpose for which a material is being used.

If a level of exposure management that can be construed as adequate for compliance is to be achieved, one of the first concerns must be to ensure that adequate information on the hazards represented by the many different chemicals found in all workplaces is available.

This is where further problems are encountered. Many employers will tend to regard the material safety data sheet as the source of information needed for this purpose. This is not the case! The material safety data sheet is actually produced for compliance by the supplier with the Chemicals (Hazard Information and Packaging for Supply) Regulations 2002 (CHIP). Note the words 'for supply'. This is a very different requirement than 'for use'.

Regulation 5 of CHIP 2002 states:

> *Subject to paragraph (7), the supplier of a dangerous substance or dangerous preparation shall provide the recipient of that dangerous substance or dangerous preparation with a safety data sheet. . .*
>
> and
>
> *Subject to paragraph (7), the supplier of a preparation of the type specified in paragraph (3) shall provide free of charge to a professional user a safety data sheet which. . .*

(Paragraph 7 refers to supply to the general public and paragraph 3 refers to a preparation which is not in itself dangerous but which contains substances that are dangerous above certain concentration levels.)

A dangerous substance is either one which has been included in the approved supply list [Information Approved for the Classification and Labelling of Dangerous Substances and Dangerous Preparations (Seventh Edition)] or one which the supplier has identified as effectively meeting the criteria for such inclusion. If the ACoP for COSHH is then consulted, in paragraph 13 the following statement is found:

> *'Many commonly supplied substances, classified in one or more of the ways described above, are listed in Part I of the Approved Supply List: Information for the classification and labelling of substances and preparations dangerous for supply. However, that document should not be regarded as a complete listing of chemicals covered by COSHH as it deals only with substances subject to CHIP and even then omits many substances and all preparations'.*

In other words, there are many substances and preparations that would fall into one of the categories for a substance hazardous to health for COSHH that will frequently not appear on the safety data sheet.

However, it is important not to lose sight of the fact that the overriding statute is the Health and Safety at Work etc Act 1974. COSHH and CHIP are merely extensions of this Act. Section 6-1 of the Act provides a definition of the duties of the supplier with regard to the information that must be provided for the user (Box 17.4). Of particular relevance is paragraph (c); what it means is that the supplier must firstly know what the product is being supplied to do and then provide sufficient information about both the risks to health that might arise out of that use and how to manage them. In other words, the supplier will need to be concerned about what happens to the chemical after it has been supplied.

Box 17.4 Extract from Health and Safety at Work (HASAW) 1974, Section 6-1: definition of the duties of a supplier with regard to information that must be supplied for the user

It shall be the duty of any person who designs, manufactures, imports or supplies any article for use at work:

(a) to ensure, so far as is reasonably practicable, that the article is so designed and constructed as to be safe and without risks to health when properly used;

(b) to carry out or arrange for the carrying out of such testing and examination as may be necessary for the performance of the duty imposed on him by the preceding paragraph;

(c) to take such steps as are necessary to secure that there will be available in connection with the use of the article at work adequate information about the use for which it is designed and has been tested, and about any conditions necessary to ensure that, when put to that use, it will be safe and without risks to health.

The significance of this can, perhaps, best be illustrated by the following example:

> *In an engineering workshop metalworking fluid was causing a skin problem. The water-mixed metalworking fluid contained formaldehyde as a biocide. A worker had become allergic to the formaldehyde. As a result, every time the worker was in contact with the fluid the allergic reaction occurred, resulting in two to three weeks away from work. The employer contacted the supplier, requesting a fluid that did not contain formaldehyde. One was duly supplied and put into the worker's*

machine. It was expected that there would be no reaction, but the allergic reaction occurred once again. Examination of the safety data sheet revealed no mention of formaldehyde. However, there was a biocide mentioned. This was found to be what is termed a 'formaldehyde releaser', i.e. a chemical that in time breaks down and releases formaldehyde. Thus, although the safety data sheet was correct and legally compliant, it could be argued that the supplier had failed under section 6-1 of the Act to provide the necessary information.

In consideration of these various factors it can be concluded that:

- Suppliers will need to know much more about the potential risks arising out of the use of their products.
- There has to be much closer liaison between supplier and end user.
- Suppliers may also need to liaise with each other, since products from two different suppliers may be mixed by the end user, resulting in a preparation with very different properties to each of the individual products.

Concerning the latter, it is not clear from the regulations who actually bears responsibility for ensuring that the information is sufficient to meet the requirements of the Act.

One factor must be kept in mind when considering risk assessment for skin exposure – it is not sufficient to rely upon risk phrases. One of the most common causes of occupational contact dermatitis is wet work, i.e. exposure to water, probably in combination with soaps, shampoos, cleaning products etc, none of which will themselves have a risk phrase. The following example illustrates why this is important:

In a food factory employing some 200 workers there were 20 cases of dermatitis of varying severity, some sufficient for the affected person to be unable to work. The plant in question merely sliced and packed cooked meat. There were no chemicals with risk phrases in the particular area. Investigation showed the cause of the dermatitis to be an extremely low ambient temperature (3–5°C), very low relative humidity, almost continuous hand contact with water and frequent hand washing. A risk assessment based on risk phrases, such as is suggested in the ACoP for COSHH, or the COSHH essentials or RISKOFDERM toolkits, would have revealed no significant risk, as no risk phrases could be applied.

17.5 Risk assessment

In the United Kingdom the Management of Health and Safety at Work Regulations require every employer to carry out a risk assessment for the hazards that may exist in its workplace (Box 17.5). The terms hazard and risk have been defined earlier (Table 17.1). However, this is an aspect of health and safety where confusion still seems to occur. So what exactly is meant by 'risk assessment'?

The definition of 'risk' is the probability that an event, in this case damage to health from dermal exposure to workplace conditions, will occur. It does not take account of the severity of the risk. This only becomes relevant when the management of the consequences of the exposure is considered.

Box 17.5 Extract from Management of Health and Safety at Work Regulations, Regulation 3(1): guidance on risk assessment

Every employer shall make a suitable and sufficient assessment of:

(a) the risks to the health and safety of his employees to which they are exposed whilst they are at work; and

(b) the risks to the health and safety of persons not in his employment arising out of or in connection with the conduct by him of his undertaking,

for the purposes of identifying the measures he needs to take to comply with the requirements and prohibitions imposed upon him by or under the relevant statutory provisions.

This can best be explained by an example:

> *A lion will represent a significant hazard, particularly if hungry. The consequences of exposure could be life threatening. At the other extreme of the cat family, a kitten represents only a minor hazard, but some people will develop skin or asthmatic reactions if contact occurs. However, the risk of meeting a lion is, at least for most people, minimal, whereas the risk of exposure to kittens is much higher. Given this exposure it is more probable that we will see reactions to kittens than to lions. In other words our risk assessment would show a higher risk from kittens than from lions!*

Considering how this risk is then managed requires that the hazard be taken into account. Thus a much more stringent standard of exposure management for lions than for kittens would be needed. However, for most people, exposure management for lions will be straightforward. (Don't go to Africa, stay outside the cage at the zoo and keep the windows closed when driving through the lion enclosure at the safari park!) The risk and risk management when dealing with a lion tamer or zookeeper may be more difficult, emphasizing again how important the task is in risk assessment and risk management.

With kittens, the risk management standards may not need to be so stringent as with lions. However, it is probable that more people will be exposed and, given the fact that there will almost certainly be many kittens (and cats) in our environment, avoiding exposure may be difficult to achieve. It may be possible to identify those people who are known to react to exposure to kittens, but it will not be possible to identify those people who, at the time, have never shown a reaction but who may subsequently become allergic to kittens and cats. Thus, whilst the standard may not need to be so stringent, managing the risk of exposure to kittens may actually be more problematic and require more time and effort.

So where does the employer start with risk assessment for dermal hazards? Certainly not with the hazard and certainly not with what is contained in the safety data sheet. The hazard of a chemical only becomes relevant once it has started to be used and thus a situation when exposure may occur has been created. Thus what it is necessary to start with is the task.

Simple, sequential approaches to risk assessment commence by defining the task, then identifying (task by task) what chemicals are used and their corresponding hazards (Figure 17.1). Scenarios that may result in dermal exposure are not always easy to identify (Table 17.3). Moreover, there are no simple, validated methods for measuring skin exposure. In fact, this

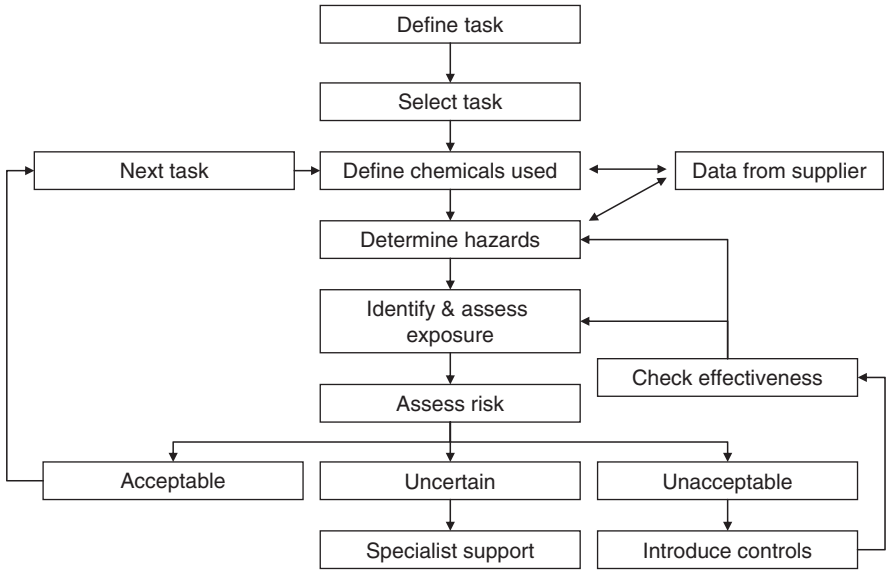


Figure 17.1 Proposed sequence for dermal exposure risk assessment

Table 17.3 Summary of different scenarios that may lead to dermal exposure

Gas	Airborne – direct Airborne – indirect, e.g. absorption into substances, such as clothing, filters in masks Condensation to liquid
Liquid	Direct – through immersion, as spray or as contamination on surfaces Indirect – contamination of clothing, absorption into clothing etc Permeation – through gloves or other personal protective equipment
Solid	Direct – if sufficient free ions available, as dust from mechanical action, as fume or smoke particles, e.g. from soldering or welding Indirect – e.g. as contamination in metalworking fluids

is more complex than many realise. It is necessary to consider many factors, such as whether a chemical can remain on the skin, partition into the skin or be absorbed by the skin. It is also necessary to consider where on the skin exposure will occur, since different areas of skin will react differently to exposure to chemicals, as well as inter-individual variations in skin condition etc.

When determining the hazards it is necessary to consider those that arise out of the use of the chemicals. These can be significantly different from those of a chemical as supplied. A chemical may be mixed, diluted, heated, reacted or otherwise processed. Chemicals may become contaminated during use, e.g. a solvent in a degreasing tank. The result may be that the original hazard is increased or decreased, or new hazards introduced. It is also necessary to establish whether a constituent within a chemical is actually bio-available, as illustrated in the examples below.

In a factory manufacturing components for the aerospace industry, there was concern that a case of allergic contact dermatitis was due to contact with tetraglycidyl methylene dianiline (TGMDA) contained in a pre-impregnated carbon fibre mat. This was cut and formed into moulds prior to heating and curing in an autoclave. The workers carrying out this task wore gloves knitted from a synthetic material (Dyneema). Since this process had been used for several years without any reported skin problems, the question that had to be asked was whether the TGMDA was actually the cause of the reported allergic contact dermatitis. Gloves that had been worn for four days were analysed for the presence of TGMDA. Whilst this was present, it was in such a minute quantity that it was not felt that this could be sufficient to represent a sufficient dose per cm^2 *to sensitise or elicit an allergic reaction. In other words, the TGMDA was not sufficiently bio-available. This illustrates the caution which anyone concerned with risk assessment for dermal exposure must employ when identifying chemical hazards. It also illustrates the need to concentrate upon the task in order to determine the hazard. Were a solvent to be used when handling the mat there would be a significant release of TGMDA, and it would be necessary to manage the exposure, possibly by the use of chemical protective gloves.*

In a separate incident at an engineering plant, several workers had simultaneously developed dermatitis of the hands. Patch testing had shown them all to be positive to methacrylates. However, the only methacrylate that could be identified in the workplace was in an area where components were assembled into a pump. Since the affected workers were machinists and not involved in the assembly tasks, was the diagnosis of methacrylate allergy relevant? Investigation revealed that it had been the practice that where castings which these workers had machined were found to be porous, they were sent to another works where they were vacuum impregnated with a methacrylate based sealant. The components were then autoclaved, with the result that the sealant was fully cured. Shortly before the outbreak it had been decided on economic grounds to have all castings impregnated before delivery. The heat generated at the tip of the cutting tool when the castings were subsequently machined was, in some way, releasing methacrylate, with the result that the metalworking fluid being used to cool tools and work-pieces during this machining operation became contaminated, resulting in exposure of these workers to methacrylate. Thus the diagnosis was relevant, but in a different way than had been originally assumed.

As has been described elsewhere in this chapter, the actual chemicals to which exposure occurs may not be the same as those that were purchased, and so the care with which dermal exposure risk assessment must be made should be apparent. However, as it is the only way in which it can be decided what control measures must be implemented, risk assessment is essential, both from a practical aspect as well as to ensure legal compliance. What is essential is a carefully structured approach so that each task is assessed in a consistent way.

17.6 Gloves: a note of caution

When it comes to skin exposure, all guidance needs to be treated with caution. This applies, for example, to the selection and use of gloves as protection against chemical hazards. The Approved Code of Practice for CHIP states with regard to the safety data sheet (paragraph 73, item (b)) that the supplier shall:

'Specify clearly the type of gloves to be worn when handling the substance or preparation, including:

(i) the type of material

(ii) the breakthrough time of the glove material, with regard to the amount and duration of dermal exposure.'

Given the many variables that will apply and the different uses to which one preparation may be put in any one workplace, it is difficult to see how any supplier can actually comply with this requirement! A more detailed consideration of the use of gloves is given in Chapter 16.

Summary

- Although we all have to comply with the law, this is not as simple as many assume.
- Unfortunately (and for good scientific reasons) the law is not specific as to what we have to do with regard to skin exposure.
- The law can also mislead, since compliance with some of the requirements could actually result in placing workers at risk.
- All that the employer, or the health and safety practitioner, can do is to attempt to minimise risks of damage to health in order to be able to claim that they have done their best to comply with their legal duties.

References

HSE (2005). EH40/2005 *Workplace exposure limits*. ISBN 0-7176-2977-5.

18 Safety assessment of cosmetics: an EU perspective

Jo Larner

ForthTox Ltd, PO Box 13550, Linlithgow, West Lothian EH49 7YU, UK

Primary Learning Objectives

- *Understanding of what constitutes a cosmetic product in the European Union (EU).*
- *Overview of EU legislation concerning cosmetic products.*
- *Role of the safety assessor in the EU.*
- *Considerations during a safety assessment of a cosmetic product.*

18.1 Introduction and scope

Cosmetics have been part of human culture for thousands of years. Modern cosmetics have to comply with a variety of national and international laws, of which the EU Cosmetics Directive provides guidance on the safety aspects of cosmetics for both their intended purpose and 'reasonably foreseeable' use.

Cosmetics have long been part of our history, ever since our ancestors first mixed ashes and animal fat and discovered the cleansing properties of soap. Today, cosmetics continue to play an integral part in all cultures. Indeed, the way we look and present ourselves is important to our sense of well-being and social interaction. The cosmetics industry is a major business area for the European Union (EU), with an estimated turnover of ~€65 billion in 2005 (COLIPA, 2006).

When the average member of the public considers the characteristics of a typical cosmetic product, it is common for initial thoughts to be of items such as eyeshadow, mascara or lipstick. Whilst these are indeed cosmetics, the formal European Union definition covers a significantly wider range of products, and most of us come into deliberate and/or accidental contact with a selection of them everyday (Table 18.1). However, many cosmetics help protect us from potentially harmful consequences. For example, hand washing with soap helps reduce the transfer of bacteria and sunscreens help reduce the effects of exposure to ultraviolet radiation.

Principles and Practice of Skin Toxicology Edited by Robert P. Chilcott and Shirley Price
© 2008 John Wiley & Sons, Ltd

Table 18.1 Example of cosmetic product categories which may result in daily skin application/exposure

Morning	Daytime	Evening	Other
Toothpaste Mouthwash Shower gel Shampoo Hair Conditioner Antiperspirant/Deodorant Hairspray/gel Shaving gel Perfume/aftershave Skin toner/Moisturiser (twice a day) Facial make-up (foundation, eyeshadow, mascara, lipstick)	Handwashes (several times) Other peoples' cosmetics: perfumes, lipstick/gloss	Make up remover Bubble bath Moisturiser/body lotion Toothpaste Mouthwash	Sunscreen Aftersun lotion Depilatory cream Spray tan/Self-tanning lotions Nail polish and removers Hair dyes and perming lotions

The widespread use of cosmetics means that the safety of the ingredients in cosmetic products and, therefore, the finished product, must be assured. With the exception of food, there is no other product class to which we so repeatedly and deliberately expose ourselves. Therefore, it is crucial that cosmetic products do not pose a threat to health but are safe, not only for their intended use but also for their reasonably foreseeable uses. This is the cornerstone of the EU Cosmetics Directive (Article 4a). Unlike pharmaceuticals or medical devices though, the industry is largely self-regulated with no pre-marketing approval by a competent authority or notified body required. Instead, the onus for the safety of each product lies with the end supplier to the public, whether or not they actually manufacture the goods. Acquiring the assurance of safety requires the collation and review of a significant amount of information, and culminates in a formal safety assessment by an appropriately experienced and qualified assessor. In this chapter, the requirements for the safety assessment of cosmetic products are examined in more detail.

18.2 Overview and scope of Cosmetics Directive 76/768/EC

The EU Cosmetics Directive is comprised of articles which set out various definitions and requirements (including the manufacturers' obligation to market only safe products). The directive also contains annexes which specify the use (or exclusion) of certain cosmetic ingredients.

In the European Union, cosmetic products are legislated by the Cosmetics Directive 76/768/EEC[1]. In turn, this legislation is transposed into the national laws of the EU member states. It is this directive which defines what is meant by a cosmetic:

> *'A 'cosmetic product' shall mean any substance or preparation intended to be placed in contact with the various external parts of the human body (epidermis, hair system, nails, lips and external genital*

[1] *http://ec.europa.eu/enterprise/cosmetics/index_en.htm*

organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance and/or correcting body odours and/or protecting them or keeping them in good condition.'

Council Directive 76/768/EEC

Any therapeutic claim or inclusion of pharmacologically active substance can classify the product as a drug. It might seem somewhat obvious and clear cut on reading, yet the definition of a cosmetic can vary subtly in other major market regions, for example in the United States:

'The term 'cosmetic' means (1) articles intended to be rubbed, poured, sprinkled, or sprayed on, introduced into, or otherwise applied to the human body or any part thereof for cleansing, beautifying, promoting attractiveness, or altering the appearance, and (2) articles intended for use as a component of any such articles; except that such term shall not include soap.'

Food, Drug & Cosmetics Act, Sec. 201 (i)

However, if such a product affects the body's structure or functions, then the product is considered a drug and must comply with both the drug and cosmetic provisions of the Food, Drug and Cosmetics Act. Sunscreens and anti-dandruff shampoos are both considered cosmetic drugs for example, yet are deemed cosmetics, not medicines, in Europe.

In Japan, cosmetic products are defined as:

'Articles intended to be used by means of rubbing, sprinkling or by similar application to the human body for cleaning, beautifying, promoting attractiveness, and for keeping skin and hair healthy, provided that the action on the body is mild.'

Japanese Pharmaceutical Affairs Law

The definition of 'mild action', is, however, left to the interpretation of the Japanese authorities. To complicate matters further, whilst therapeutic products are considered drugs, Japan has a third category of so-called 'quasi drugs', which are defined as substances with a mild therapeutic action, and in the European Union some quasi drugs would be considered cosmetics, e.g. depilatories and deodorants.

Such differences in definition throughout the world can lead to confusion and the potential for expensive mistakes when considering the global marketing of cosmetic products. The rise of internet and TV shopping bring their own perils. Even within the European Union, there are borderline areas between cosmetics and other classes of products such as biocides/human hygiene products, detergents, pharmaceuticals or even medical devices. When not only ingredient content and manner of use but also presentation of a product (e.g. label claims) can influence classification there can be a need for careful negotiation of regulatory hurdles. It should also be appreciated that despite all member states transposing the same common directive, there is still the potential for national differences, especially with label claims.

The EU Cosmetics Directive is an example of an 'old approach' directive. Rather than indicate the application of harmonised standards and CE marking for assurance that essential requirements are met, as with toys, medical devices or personal protective equipment, it contains 15 Articles which lay down the obligations of cosmetic suppliers, be they manufacturers or importers, and of the EU member states. Suppliers are duty-bound to market only safe products, appropriately labelled, and have the supporting information to

prove this as part of their due diligence. On request by a competent authority, such as Trading Standards in the United Kingdom, this should be made readily available to them.

In return for compliance with the EU Cosmetics Directive, it is expected that member states will permit free movement of cosmetic products within Europe yet will maintain a system of in-market controls permitting rapid notification and withdrawal of products if it is deemed necessary. Under the European-wide rapid alert system for dangerous non-food products (RAPEX[2]), individual member states or suppliers can instruct withdrawal of the product from the market, recall the product from consumers or issue warnings.

The Directive comprises a set of main text (the 'Articles') followed by a series of Annexes which provide negative and positive lists of ingredients that may be banned, or restricted in use (Table 18.2). In particular, only ingredients listed in Annex IV may be used to colour cosmetics and these are further divided into four categories with decreasing exposure levels permitted in their fields of application:

1. Colouring agents allowed in all cosmetic products.
2. Colouring agents allowed in all cosmetic products except those intended to be applied in the vicinity of the eyes, in particular eye make-up and eye make-up remover.

Table 18.2 Structure of the EU Cosmetics Directive

Text	Contents
Articles	The text of the rules and requirements
Annex I	Examples of cosmetics
Annex II	Banned ingredients (*negative* list >1200 chemicals)
Annex III	Restricted ingredients Part 1. typically the *actives* e.g. fluoride Part 2. provisionally allowed but restricted
Annex IV	Permitted colours (*positive* list) Part 1. permitted colours typically by CI number with requirements Part 2. provisionally allowed colours
Annex V	Substances excluded from the Directive
Annex VI	Permitted preservatives (*positive* list) Part 1. Permitted preservatives with limits and requirements Part 2. provisionally allowed but restricted
Annex VII	Permitted UV filters (*positive* list) Part 1. Permitted preservatives with limits and requirements Part 2. provisionally allowed but restricted
Annex VIII	Symbols e.g. hand and book (period after opening)
Annex IX	List of validated alternative methods to animal testing

[2] *http://ec.europa.eu/consumers/dyna/rapex/rapex_archives_en.cfm*

3. Colouring agents allowed exclusively in cosmetic products intended not to come into contact with the mucous membranes.
4. Colouring agents allowed exclusively in cosmetic products intended to come into contact only briefly with the skin.

Note, however, that hair colorants are not covered by this list as they are intended to colour the consumer, not the product. Similarly, only those preservatives and UV filters listed in Annexes VI and VII may be used in products for the primary function of preservation or as sunscreen actives, providing they meet the requirements described. UV absorbers (which may be included in a formulation for the purpose of protecting the product rather than the consumer) are not listed in the Annexes. There are also many cosmetic ingredients which may have colorant, preservative or UV absorbent properties secondary to their primary function in a product. For example, ethanol has an inherent anti-microbial activity at concentrations above 15–20% yet it is not included on the positive preservative list in Annex VI. Such substances may be employed provided they are used for their primary function. Since ethanol is most commonly used as a solvent it thus may be included in cosmetic formulations despite its preservative action provided it has been appropriately denatured. The formulator, of course, is at liberty to exploit such dual characteristics and this may permit a reduction in the use of other preservatives in the product.

It is important to realise that the Annexes are not exhaustive in listing all of the ingredients which may be used or which are restricted or banned from use in cosmetics. There are thousands of other possible ingredients, for example the 11th edition of the Cosmetic, Toiletry and Fragrance Association's (CTFA) Ingredient Dictionary lists over thirteen thousand! The decision as to whether the ingredients used are suitable, for safety or regulatory reasons, is left to the safety assessor taking into account all that (s)he knows about the ingredients, the specification of the final product, its presentation to the consumer and the intended or foreseeable use.

18.3 Overview of the requirements of the EU Cosmetics Directive

The EU Cosmetics Directive dictates both general and a number of particular requirements for cosmetics and lays down the mandatory obligations for manufacturers and importers.

18.3.1 General requirements

Products must be safe under:

- *normal* conditions of use; or
- *reasonably foreseeable* conditions of use, including the product's presentation, labelling, any instructions for use and disposal and any other information or indication provided by the manufacturer, his agent or the person who supplies the product on the first occasion that it is supplied in the European Union.

For example, a foreseeable use of a blusher could be as an eyeshadow, whereas a hand moisturiser could conceivably used as a facial cream.

18.3.2 Particular requirements

- No ingredients listed in Annex II may be used in cosmetic products.
- Restricted ingredients described in Annex III must be within their prescribed limits.
- Any colour ingredients, preservatives and UV filters used must be included in their respective positive lists and meet any requirements noted in their respective Annexes.
- No product should contain any transmissible spongiform encephalopathy (TSE) risk materials (exception: tallow derivatives, providing appropriate manufacture has been certified).
- No finished products which have been tested on animals since September 2004 may be marketed.

In addition, cosmetic suppliers are expected to meet a number of other obligations (Appendix 18.1).

18.4 Scientific advice

The SCCP is responsible for the EU Cosmetics Directive and provides advice to the EU and industry on the safety of a range of non-food products.

In the European Union, the Scientific Committee on Consumer Products (SCCP[3]) currently advises the European Commission as to the safety of consumer products (non-food products). Prior to 2004, this committee was known as the Scientific Committee on Cosmetic Products and Non-Food Products (SCCNFP) which developed from the Scientific Committee on Cosmetology (SCC) in 1997. In the United States, the Cosmetics Ingredient Review (CIR[4]) plays a similar role.

The SCCP responds to questions from the European Commission and industry concerning the safety of cosmetics, toys, textiles, clothing, personal care products, household detergents and a number of consumer services. The ingredients in the various Annexes of the EU Cosmetics Directive fall under the responsibility of the SCCP. In addition, the Committee may act independently to bring issues of concern to the notice of the Commission. Subsequent scientific opinions are published through the European Commission's website and these should be borne in mind when assessing any product for safety. Whilst SCCP recommended restrictions are not in themselves law they frequently preclude subsequent amendments or adaptations to technical progress to the directive.

Further advice on regulatory and safety matters can often be obtained through national or European trade associations, for example, the European Cosmetic, Toiletry and Perfumery Association (COLIPA[5]), which publishes a number of guidance documents.

[3] *http://ec.europa.eu/health/ph_risk/committees/04_sccp/sccp_opinions_en.htm*
[4] *http://www.cir-safety.org*
[5] www.colipa.com

18.5 Influence of other legislation

Other national or international legislation can affect the supply and marketing of cosmetics. Of general relevance are the EU's Dangerous Substances Directive and REACH. In the Unite Kingdom, compliance with the Trading Standards Act is also required.

Whilst cosmetic products are legislated by the Cosmetics Directive 76/768/EC, it should be appreciated that other laws may also apply (Chapter 19). In particular, two areas are of relevance for cosmetics; (1) permitted or restricted ingredients and (2) packaging and labelling.

18.5.1 Permitted or restricted ingredients

Notified chemicals undergo a classification process and chemicals deemed dangerous are subsequently listed in Annex I of the Dangerous Substances Directive (67/548/EC). The European Chemicals Bureau offers an electronic means of searching this Annex[6]. As part of the package of changes brought about by the 7th amendment to the EU Cosmetics Directive in 2004, a specific link with the Dangerous Substances Directive was introduced. Chemicals that are classified as Class 1 or 2 carcinogens, mutagens or toxic to reproduction (CMRs) are not permitted as ingredients in European Union cosmetic products. Class 3 CMRs are only deemed permissible in cosmetic products if used as formally approved by the SCCP. Note that, in addition, Council Directive 76/769/EEC (which controls the restrictions on the marketing and use of certain dangerous substances and preparations) also applies.

18.5.2 Packaging and labelling

The EU Cosmetics Directive dictates what specific labelling is required. In addition, labelling of cosmetic products must also comply with national requirements. In the United Kingdom for example, the Trade Descriptions Act (1968) prevents the use of claims for product characteristics which the product does not have. This Act requires claims substantiation testing to be conducted, for example where a product declares a sun protection factor rating (e.g. SPF20). There is a variety of additional, pertinent legislation that should be considered (Table 18.3).

18.5.3 REACH

Mention should be made perhaps of the forthcoming new European Union chemicals policy, REACH, which stands for the Registration, Evaluation, Authorisation of Chemicals[7]. In force introduced in June 2007, the use of all chemicals (including cosmetic ingredients), above one tonne are subject to registration and subsequent compilation of chemical safety reports and/or assessments. As a consequence of the REACH process, certain chemicals may only be authorised for specific uses. This could potentially affect the cosmetic industry and the subsequent manner in which some ingredients are used. The reports must include information not only on the

[6] *http://ecb.jrc.it*
[7] *http://ecb.jrc.it/reach*

Table 18.3 Examples of additional legislation pertinent to the manufacture, packaging and marketing of cosmetic products

Directives	Regulations	Application/Description
EU Directives 75/106/EC and 76/211/EEC		Labelling/packaging of liquids and solids.
2007/45/EC		Standardisation of sizes of pre-packed goods.
75/324/EEC		Aerosols directive; safety requirements for pressurised containers.
	EC 2037/2000	Ozone-depleting propellants.
87/357/EEC		Food Imitations Directive; prevent products being mistaken for foods and subsequently being ingested.
88/378/EEC		Safety of toys: cosmetic products intended for use in play by children (e.g. face paints, cartoon bubble bath containers).
94/62/EC		Packaging and Packaging Waste Directive: reducing the impact of packaging on the environment. Several EU member states also have additional legislation, e.g. the German 'green dot' scheme.
Directive 2006/121/EC (amending 67/548/EEC)	**EC 1907/2006**	REACH; Registration, Evaluation, Authorisation of Chemicals.

chemical's impact upon human health but also on the environment. Whilst safety assessment under the EU Cosmetics Directive may be considered to satisfy the requirement for evaluation of the former, the environmental impact of cosmetic ingredients will have to be considered closely under REACH.

18.6 Adverse effects from cosmetics

Whilst the adverse effects of cosmetics are predominantly dermal, systemic toxicity (via inhalation or ingestion) can also occur. Thus, each ingredient in a formulation must be subject to a full toxicological assessment.

Significant injuries from cosmetics are, thankfully, rare[8], but products can pose physical and microbiological hazards as well as chemical ones. For example, mascara wands can potentially scratch the surface the cornea if used clumsily; this introduces the risk of infection from both the product and the external environment. Microbiological contamination of products, especially water-containing cosmetics like moisturisers that are intended for repeat use, is virtually unavoidable. Thus, preservatives are usually employed to control microbial growth. To reflect the vulnerability of certain body areas or consumers, the industry has more stringent criteria for preservative performance for eye area or mucosal membrane products and for products intended for children under three years of age.

[8]This is not necessarily the case throughout history (see Box 18.1).

Box 18.1 The (not so informed) use of ancient cosmetics

Cosmetics are known to have been used in African, Asian and European cultures since before the bronze age (~5000 BC), possibly even earlier. Indeed, the ancient Egyptians, Greeks and Romans were renowned for their manufacturing skills and trade in facial cosmetics, amongst which was a substance called kohl used to accentuate eye brows, eyelids and eyelashes. Kohl and many other cosmetics were essentially toxic time bombs, containing various forms of finely powdered carbon (pot black, lamp black), mercury (cinnabar, vermilion), lead (galena), antimony (stibnite), copper (malachite), arsenic and cadmium–to name just a few! Whilst the vogue for cosmetics diminished somewhat following the fall of the Roman empire (5 AD), the renaissance period in Europe (circa 1500 AD) stimulated interest in facial cosmetics (sometimes to mask skin blemishes caused by diseases associated with the use of the very same cosmetics). In particular, unblemished white skin was the vogue in Elizabethan times and the use of whitening powders manufactured from toxic salts of lead, antimony and cadmium was not uncommon.

Since cosmetic products, as defined in the European Union, are applied topically to the skin, hair, nails, oral cavity or genitals, the nature of any adverse effect is mostly an issue of local tolerance, i.e. irritation or allergy. However, respiratory routes of exposure can also be seen with volatile chemicals (perfumes) or fine dusts (talcum powder) that are potentially able to be inhaled into the lungs. Therefore, adverse effects can include systemic as well as local manifestations of toxicity.

Dermal effects can be immediate (e.g. irritation), cumulative (e.g. allergic) or a combination (e.g. phototoxic response which occurs on exposure to sunlight). The clinical aspects of such skin reactions are considered in detail in Chapters 13 and 14. It is important to appreciate that when cosmetics are implicated in an adverse skin response it may be due either to just one causative ingredient or the overall influence of other components in the mixture upon one causative ingredient. Therefore, a safety evaluation must consider both the individual ingredients as well as the final formulation (see later). In the case of allergic reactions, sensitisation can originate from contact with a non-related product which contains the same allergen (or a cross-reacting substance) as the cosmetic product identified as causing the problem.

A notorious example of the potential for injury can be seen with PPD (p-phenylenediamine, an oxidising agent used in hair dyes) when used in combination with a natural dye, henna (extracted from the plant *Lawsonia inermis*). Traditionally, the skin is painted directly with henna and after a few hours the colour develops leaving a design which can lasts several days. However, the recent trend for temporary tattoos has lead to the use of henna adulterated with PPD to accelerate colour development and increase persistency of the tattoo. Unfortunately, PPD is a known contact sensitiser (Chapter 8) and it is known to be the cause of serious allergic reactions resulting from topical exposures. The use of henna adulterated with PPD has been the subject of review by the Council of Europe and is now considered illegal by most EU member states.

If a consumer is aware of their sensitivity to a particular ingredient, then careful reading of the product label permits them to select alternative formulations lacking the ingredient and so avoid such reactions. It was for this reason that the recent 7th Amendment of the EU Cosmetics Directive brought in an additional requirement for 26 fragrance allergens (with a known propensity to trigger allergic responses) to be specifically indicated in the ingredient listing if present above a certain threshold.

The recent marketing predilection for 'all-natural' cosmetic products is a minefield. Natural does not mean safe! Many medicines have been derived from natural sources and can be quite potent. Digitalin from the Common Foxglove (*Digitalis purpurea*) has potent cardiac effects, and atropine from the Deadly Nightshade (*Atropa belladonna*) has anticholinergic activity. Anyone who has had the misfortune to come into contact with Poison Ivy (*Toxicodendron radicans*) will attest that it is far from harmless. The attraction for aromatherapy-based cosmetics (even for baby products) has brought numerous essential oils and exotic plant extracts to the interest of suppliers, yet for a vast number there is very limited information about their constituents and potential side effects and so caution is warranted.

18.7 Toxicity of cosmetic ingredients

Although there are exceptions to which ingredients need to be included on a product label, all ingredients should be evaluated in the product assessment – as far as practically possible.

Whilst it seems that there can be an almost infinite number of different types of cosmetics, they generally tend to make use of a small repertoire of common ingredients, many of which are considered the workhorses of the industry (e.g. surfactants). It is for this reason that the pragmatic approach taken for safety appraisal of the finished product is the evaluation of the toxicology of ingredients rather than of the end product, with the inherent assumption that knowledge of the properties of the ingredients will adequately describe the likely properties of the mixture. This is formally stated in the EU Cosmetics Directive:

> *'To that end the manufacturer shall take into consideration the general toxicological profile of the ingredients, their chemical structure and their level of exposure. It shall take particular account of the specific exposure characteristics of the areas on which the product will be applied or of the population for which it is intended.'*

Cosmetic ingredients are substances or preparations that are intentionally included in a cosmetic product and the safety assessment of a final product is based upon knowledge of their identity, quality, regulatory acceptability and overall suitability in a mixture of ingredients given their toxicological profile and proposed use in a final product. Whilst European Union regulations identify some banned and restricted ingredients and set down positive lists for colorants, UV filters and preservatives as noted earlier, this is not the end of the responsibilities of the manufacturer or supplier when it comes to the ensuring the acceptable use of chemicals as ingredients in their products.

18.7.1 Identity

Characterisation of the physicochemical properties of each ingredient is essential for the safety assessment process since some parameters may be able to predict certain toxicological properties. For example, low molecular weight molecules may demonstrate higher propensity for percutaneous absorption (Chapter 5). Also, physicochemical parameters may indicate physical hazards, such as flammability. When the SCCP reviews the toxicological dossier for

a new chemical for one of the Annexes[9], to clearly characterise the ingredient the following specifications are expected to be described:

1. Chemical identity (CAS No., EINECS/ELINCS No.).
2. Physical form.
3. Molecular weight.
4. Characterisation and purity of the chemical (including methodology).
5. Characterisation of the impurities or accompanying contaminants.
6. Solubility.
7. Octanol/water partition coefficient (Log P).
8. Additional relevant physical and chemical specifications, e.g. appearance, odour, flash point, density.

Cosmetic ingredients can be obtained from various sources. Some may be manufactured (synthetic) whilst others may be derived from petroleum, minerals or organisms such as plants, animals, algae or bacteria. The complexity of some naturally derived materials leads to obvious complications in identification owing to the number of possible components. For example, above what concentration should ingredients be identified and how does processing these materials affect these ingredients? Clearly, it is important to understand the manner of preparation when considering such ingredients. For example, where ingredients are 'natural mixtures', details of the raw material (e.g. part of plant) and preparation process (e.g. collection, solvent extraction, distillation) should be available. There is an irony with the current distaste for 'synthetic' ingredients in that it is more likely that the properties of a synthetic chemical will be better characterised than most naturally occurring ingredients.

All ingredients used in a formulation are required to be detailed in an ingredients list on the product label. Yet there are exceptions as to what are actually considered ingredients and thus excluded from this requirement. These include impurities in the raw materials used, subsidiary technical materials used in the preparation (but not present in the final product) and materials used in strictly necessary quantities as solvents or as carriers for perfume and aromatic compositions.

While some materials, such as impurities, may not require formal identification on the product label, they should still be identified, quantified and taken into account during the safety assessment of the finished product. For example, carbomer, a polymer of acrylic acid, typically retains residual solvent from its production, albeit at low concentrations ($<0.1\%$). It is obviously preferable to have a solvent like ethyl acetate rather than benzene as a residue. It is also important to ensure that all ingredients in a raw material have been identified. Frequently, however, these 'hidden' ingredients are not always clear from raw material documentation and can require specific enquiry of the raw material supplier by the manufacturer or assessor. For instance, preservatives (e.g. methylparaben, propylparaben) are commonly added to aqueous plant extracts to preserve the raw material, and anti-oxidants (e.g. BHT, tocopherol) are added to oils to prevent rancidity. Even though they are included at low concentrations, these additives are deemed cosmetic ingredients and must be included in the ingredient labelling

[9]Ideally, such information should be also available from the chemical supplier for the independent safety assessor to review non-annex materials.

and taken into account by the assessor. Use of preservatives in this way will also contribute to the total preservative content and can potentially cause a legal restriction to be exceeded.

18.7.2 Quality

The safety of cosmetics can be assured by the use of best practices during manufacture.

In addition to the general and particular requirements for cosmetics, there is an obligation to manufacture cosmetic products in accordance with cosmetic Good Manufacturing Practice (GMP). This must also, however, extend to the production of cosmetic raw materials if the safety, quality and efficacy of cosmetic products are to be assured. The European Federation for Cosmetic Ingredients (EFfCI) issued guidance in 2005[10] highlighting the importance of ingredient GMP and the control of the ingredient specification. A GMP compliant raw material supplier should be able to supply relevant documentation to assure the manufacturer that the intended ingredients are fit for purpose in cosmetics but, as intimated in the previous section, this requires two-way communication between both parties and an understanding of the market region requirements in which the final product is to be sold.

With increasing globalisation of both raw material and finished product production combined with delegation of purchasing to non-scientific departments, it is crucial that the necessary standards for the ingredients in a product are maintained. Many of the pigments permitted in cosmetics are available in various grades and some of these are not suitable for cosmetic purposes. For example, such pigments can have the potential to contain unacceptably high levels of heavy metals; perhaps fine for car paint, not for skin! European Union requirements are not identical to those in the United States either, with many being required to meet additional food purity specification, e.g. titanium dioxide (CI 77891) should meet the E171 specification.

Ingredients are assigned specific names (International Nomenclature Cosmetic Ingredient, INCI) for use on product labelling in the European Union. These names are assigned by the American Cosmetic Toiletry and Fragrance Association's International Nomenclature Committee. The INCI names are based on chemical structure and composition information provided by the raw material supplier applying for the name. It is important to note, however, that having an INCI name formally assigned does not infer that an ingredient meets an official standard or purity or that it has been assessed for safety, or imply that it is approved, certified or endorsed by any organisation or authority. It is the responsibility of the cosmetic manufacturer or importer to ensure that the ingredients used are appropriate for the intended finished product.

18.7.3 Toxicological profile

Safety testing of cosmetic ingredients has traditionally been conducted using animal studies and a minimum set of requirements regarding specific toxicological aspects is specified by the SCCP.

In addition to a clear understanding of the physicochemical parameters of the ingredients, the toxicological potential of each ingredient also must be reviewed (and not limited to

[10] *http://www.ipec-europe.org/docs/pdf/news/effcigmp.pdf*

dermal toxicity). As with most other industries, this is still largely achieved by the conduct of toxicological studies in laboratory species for the evaluation of no observed adverse effect levels (NOAEL), although there is increasing use of *in vitro*, *ex vivo* and Quantitative Structure Activity Relationship ((Q)SAR) and other *in silico* techniques. Test materials are administered using the same exposure route expected in man, bearing in mind that not all cosmetic related exposure is solely dermal, there may also be systemic exposure following percutaneous absorption, oral contact and intake, or inhalation. Target organs and potential mechanisms of actions are identified (e.g. induction of sensitisation) and margins of safety are then estimated.

A cosmetic ingredient dossier for submission to the SCCP should contain data for assessment of the following:

1. Acute toxicity.
2. Irritation and corrosivity.
3. Skin sensitisation.
4. Percutaneous absorption.
5. Repeated dose toxicity.
6. Mutagenicity/genotoxicity.
7. Carcinogenicity.
8. Reproductive toxicity.
9. Toxicokinetics.
10. Photo-induced toxicity.
11. Human data.

Points 1 to 6 are considered the minimal base-set requirements for the SCCP but information for points 7 to 9 is expected when there may be significant oral ingestion or dermal penetration leading to systemic exposure. Phototoxicity data is specifically necessary when it is expected that the ingredient will be applied to sun exposed skin, whilst human data is always valuable if available. The methodology for examining these issues is not discussed here but studies follow guidelines in Annex V part B of the Dangerous Substances Directive and should comply with Good Laboratory Practice.

For other ingredients not intended for review by the SCCP but for assessment of safety by the safety assessor there can often be difficulties owing to a lack of information. Based on current European Union legislation for chemicals, the size of the data package is dependent on manufacture or importation tonnage levels of the chemical. For example, for a chemical imported at between 0.1 and 1 tonne only information on acute toxicity, skin and eye irritation, sensitisation and mutagenicity is required, which leaves notable gaps for preparing a competent safety package (as outlined in points 1 to 11, above). The REACH policy further complicates matters, since it will only be a requirement to assess the four points above for those chemicals imported at between 1 and 10 tonnes per year. This lack of information poses difficulties for cosmetic manufacturers and importers, as it may be problematic to demonstrate satisfactory evidence of safety for their respective products as required by the EU Cosmetics Directive and highlights a need for raw material suppliers to provide at least a minimum of data to their customers.

There has been recent discussion of the application of the Threshold of Toxicological Concern (TTC) in the assessment of risk from cosmetic ingredients where no NOAEL has been calculated. The TTC is based upon a concept developed from the food industry that, subject to the absence of structural alerts or evidence to suggest serious toxicity like carcinogenicity, there can be safe level of exposure which will have a low risk of actually causing any appreciable harm. The acceptable exposure level, estimated from a number of chronic oral studies, is $1.5\,\mu g\,kg^{-1}\,day^{-1}$ for life based on an increase in risk of cancer of 1×10^{-6}. The main advantage of the proposal is that it requires no formal testing, thus it avoids the legal and ethical issues of animal use. Also, being based on oral studies, the extrapolation of the acceptable exposure level to dermal exposure for cosmetics is thought to be conservative, since topical administration should result in lower bioavailability. The drawback is that life is rarely that simple. The database for topical effects is limited, the potential for other toxicological endpoints such as neurotoxicity, reproductive toxicity, allergenicity or endocrine disruption requires judicious use of lower acceptable exposure levels, there is no adjustment for potency, and the TTC is not considered scientifically justified for some chemicals e.g. proteins, metals and pharmacologically active substances. That said, the TTC has potential if used in the full knowledge of its weaknesses and may yet play a role in the safety assessment of cosmetic ingredients.

18.7.4 Testing requirements and restrictions for cosmetic ingredients

According to the 7th amendment of the EU Cosmetics Directive, the use of animals in cosmetic toxicity testing is to be phased out and replaced with alternative techniques by 2009. However, this is likely to conflict with other international laws.

The evaluation of the toxicological properties of ingredients has traditionally required the use of animals. Yet the use of animals raises serious ethical and socio-political issues – more so if the generally perceived benefit of the substance is commonly thought to be unnecessary (as many would consider the case for testing cosmetic products). The principle of the 3Rs proposed by Russell and Burch (1959) underpins the ethical use of animals in toxicological studies (Chapter 11). However, what should not be forgotten is a fourth R; responsibility to the general public to ensure that appropriate studies are conducted to adequately predict the risks to humans from exposure to cosmetic chemicals in the finished products.

With the adoption of the 7th amendment to the EU Cosmetics Directive, a timetable for the banning of animal testing of ingredients and the subsequent marketing of products containing such tested ingredients was introduced and this legislation is one of the key drivers for the development of alternative methods (Chapter 11). To date, only a small range of alternative methodologies (Table 18.4) has been formally validated by the European Centre on Validation of Alternative Methods (ECVAM) and included in the list (Annex V) of approved methods in the Dangerous Substances Directive 67/548/EEC.

Animal testing for other toxicological endpoints may continue until March 2009 unless an alternative is validated by ECVAM. From then, ingredients may only be tested on animals for purposes of investigating reproductive toxicity, toxicokinetics and repeat dose studies. The marketing ban for products that are formulated with animal-tested ingredients has the same timeframe. It is anticipated that alternative tests for skin and eye irritation, skin absorption and photogenotoxicity will be validated before 2009, but that leaves a number of endpoints without alternative method approval. Restrictions in the testing of ingredients will prove a

Table 18.4 Alternative models validated by ECVAM (For further details of alternative methods see Chapter 11)

Annex V Reference No.	Type of Test (Toxicological endpoint)	Alternative Test Method
B40	Skin corrosion	Rat skin transcutaneous electrical resistance assay
B40	Skin corrosion	Human skin model assay
B41	Phototoxicity	*In vitro* 3T3 neutral red uptake phototoxicity test

significant hurdle in the development of innovative products with new ingredients and could be exacerbated by the removal of currently approved ingredients (for example, due to the future identification of adverse effects).

It should be noted that the timetable only applies to testing for the requirements of the EU Cosmetics Directive. Whilst the 3Rs will continue to be applied by toxicologists, animal testing on cosmetic ingredients to satisfy other legislation, e.g. REACH, may continue. Quite how that may affect the cosmetic marketing ban, however, remains to be clarified.

18.7.5 Sources of information

Data on the physicochemical and toxicological properties of ingredients can be sourced from a number of areas but at the top of the list is the raw material supplier, which should be able to provide at least a basic set of information if it complies with the European Union chemicals legislation. This can lead to problems, however, with increasing subcontracting of manufacture to non-EU countries as indicated above when data may be scant. Material safety data sheets from the actual supplier used or alternative reputable suppliers also may provide indications of potential side effects that should be considered.

Special mention should be made of fragrance or flavour ingredients at this point. It is not common for the exact composition of such ingredients to be readily available, usually due to confidentiality reasons, but also sometimes because of the inherent complexity of the material. Fragrances can contain a large number of ingredients and the individual labelling of each ingredient in a fragrance would be totally impractical. For this reason they are simply identified as *parfum* or *aroma* on the product's ingredient listing. In the absence of such details, assurance of good quality is expected from the perfume house in the form of a signed and dated compliance statement stating that the fragrance of flavour meets the latest code of practice of the International Fragrance Association (IFRA[11]). Since perfume ingredients are frequently the cause of allergic reactions and may themselves contain ingredients restricted by the EU Cosmetics Directive, the perfume house should additionally recommend a maximum use level for the fragrance in order to limit consumer exposure. For product flavourings (where ingestion is likely), assurance should also be obtained from the supplier that the flavouring components are of food grade and the European Fragrance and Flavour Association (EFFA[12]) have issued guidance.

[11] *http://www.ifraorg.org*
[12] *http://www.effa.be*

Supplementary information can be obtained from scientific literature in the public domain via on-line search engines like ToxNet and PubMed, scientific databases like the Hazardous Substances Database, and books like Patty's Toxicology, Sax's Dangerous Properties of Industrial Materials, and the Royal Society of Chemistry's Dictionary of Substances and Their Effects. The advisory scientific committee opinions of the SCCP and the CIR also contain valuable data as well as judgements on the use of a large number of ingredients, besides those listed in the Annexes.

Other ingredients, developed by the manufacturer, may require the compilation of in-house data to support their use. This can be from testing data of the ingredient itself (as detailed in the previous section) or evidence of safety in use from other products, including those of competitors.

18.8 The safety assessment

Since suppliers have the obligation to only place safe products onto the market, they have an obligation to ensure that all of their cosmetic products are formally evaluated for safety by a suitably qualified and experienced cosmetic safety assessor. Absolute safety is an impossible goal, but the assessor must appraise the risk/benefit of the product with the aim of ensuring that that the risks are reduced to as low as possible given the composition and presentation of the product and its probable use.

18.8.1 Safety assessor

Cosmetic product safety evaluation must be undertaken by a competent person.

The assessor may be an employee of the manufacturing company, the importer or an external consultant, but in whatever circumstance it is essential that the assessor must be able to work independently without undue pressure from the supplier and is not concerned with the commercial aspects associated to the product. The safety assessor's first responsibility is to the safety of the user of the product and not to the supplier. The EU Cosmetics Directive makes it clear that the assessor must have the appropriate credentials but it is also expected that the assessor has relevant experience and should not act outside of their sphere of competence. With respect to qualifications, the cosmetics assessor must hold a diploma as defined in Article 1 of Directive 89/48/EEC in the field of pharmacy, toxicology, dermatology, medicine or a similar discipline. However, to approach a locally registered pharmacist or medical practitioner and request that they assess and approve a new product would not be likely to be considered acceptable, despite their qualifications, because they would not be deemed to have appropriate and sufficient expertise with the cosmetic formulations. European Union member states may also recognise specific scientific qualifications in their national enactment of the Directive. For example, in the United Kingdom, the regulations state that an EU-qualified pharmacist, medical practitioner, chartered chemist or chartered biologist may assess cosmetics although there are rules for acceptance of non-EU medical or pharmacy diplomas. Curiously, toxicologists are actually omitted from the United Kingdom list.

18.8.2 The safety assessment process

The safety assessment should be in accordance with COLIPA guidelines and the cosmetic product must be deemed safe for both intended and reasonably foreseeable uses.

According to Article 2 of the EU Cosmetics Directive:

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

This makes it clear that the scope of the safety assessment is not expected to cover deliberate misuse or abuse. What is considered a foreseeable use can vary significantly between different types of products and is influenced by presentation (both packaging and labelling) as well as the competence of the intended consumer. For example, a product may be labelled with appropriate instructions and warnings for safe use such as 'Do not use to dye the eyelashes or eyebrows. Rinse eyes immediately if product comes into contact with them'. If such instructions are ignored by the consumer, then the manufacturer is not likely to be considered responsible. However, if severe eye damage occurs because the product is formulated to be so concentrated that eye contact would invariably cause injury, then the manufacturer could be considered liable if eye contact is a foreseeable consequence of normal use.

An assessment of the individual chemicals in the product forms the mainstay of the assessment (as indicated in Section 18.7, *Cosmetic Ingredients*). How much information about the ingredients and the final product the safety assessor deems necessary for a competent assessment is left to them. Products manufactured by a new factory and containing some novel ingredients are more likely to raise questions than generic cosmetics with commonly used ingredients sourced from reputable raw material suppliers and produced by a reliable GMP-compliant factory.

Where NOAEL have been determined, then the Margin of Safety (MOS) can be determined using the Systemic Exposure Dosage (SED) ($mg\,kg^{-1}$ bodyweight) for each ingredient in a product (Equation (18.1)).

$$\mathrm{MOS} = \frac{\mathrm{NOAEL}}{\mathrm{SED}} \tag{18.1}$$

The SED attempts to take into account the specific exposure scenario expected for a product, with estimations for parameters like frequency and quantity of application, total area of skin contact, retention of product, degree of percutaneous absorption and consumer body weight amongst others (Table 18.5). The relevant exposure parameters can differ when considering for example, skin irritation or phototoxicity against systemic toxicity. For the former an understanding of the exposure per unit area of skin is more important than exposure per unit body weight and vice versa. There are inherently errors in these calculated figures; not all consumers use the products in the exact same way, nor are all consumers the same. Some ingredients, like preservatives, can also be expected to be used in other cosmetic products requiring the calculation of a global daily exposure value.

Table 18.5 Example of calculation of daily exposure to cosmetics using COLIPA data (taken from SCCPNFP/0321/02)

Product Type	Amount of Substance Applied (g)	Frequency of application (times per day)	Retention Factor	Daily Exposure Calculated (g/day)
Shampoo	8.0	1	0.01	0.08
Make-Up Remover	2.5	2	0.1	0.5
Lipstick	0.01	4	1.0	0.04

18.8.3 Safety assessment – conclusions

The quantity and quality of the information available to the safety assessor can vary significantly from product to product and a successful positive outcome of the safety assessment process is not a forgone conclusion. At the end of the assessment process, the safety assessor can make one of a number of conclusions. These have been summarised by COLIPA in its guidelines for safety assessment:

- The product is safe as such without additional special warnings or precautions.
- The product is safe provided a given type of packaging is used or provided a warning is added or the or the mode of use and usage instructions are defined more precisely or provided a complementary test with favourable results is obtained;
- The product is not safe for the proposed use.
- That available data are not sufficient to determine whether or not the product will be safe and that further studies need to be carried out to obtain the required information.
- Specific safety claims may or may not be used.

Within larger companies, much of the responsibility for the checking some of the safety issues is commonly delegated to other departments (GMP, product labelling), but it is the duty of the manufacturer to see that pertinent instructions from the safety assessor are communicated to and acted upon by these departments, including the conduct of further studies if thought necessary. The assessor should also receive reports of any relevant production issues that may introduce risks, or of any product problems associated with the use of the product by the consumer in order for re-evaluation of safety.

18.9 A final consideration

Following marketing, many companies conduct cosmetovigilance to ensure the ongoing safety of products.

It should be realised that no cosmetic product can ever be considered 100% safe for the entire population. The competence of the integument varies with age, some individuals

may be atopic or have sensitive skin, and others just don't read or follow instructions. Consequently there will, unfortunately, always be somebody who will react to the product and, as with any other product, be it a food, drug, toy or biocide, the risk of an adverse effect from a cosmetic is the product of the item's inherent hazard and any extent of subsequent exposure (i.e. dose). Cosmetic suppliers and their safety assessors are aware of this and expect occasional adverse reports. This is why monitoring (cosmetovigilance) of the frequency and severity of such reports should be collated. If there is a noticeable increase in the number or nature of reaction over that expected or historically seen, then investigation and action, including safety re-evaluation, can then be taken to reduce the risk to other consumers. Suitable actions can range from a simple change in instructions for use, recall of specific problem batches of product, change in packaging, or complete removal of product and subsequent reformulation. Whilst competent authorities can be involved in the withdrawal of unsafe products from the marketplace, most reputable suppliers act swiftly to effect changes or remove a product if its safety is in question to protect both the consumer and their brand/company reputation.

Summary

- Cosmetic products represent a category of materials which are deliberately and often repeatedly applied to the skin yet are not expected to pose a threat to health.
- Although no chemical product is entirely without risk, adverse effects from cosmetics are infrequent and usually restricted to short-lived irritation or allergy.
- Safety assurance for products in the European Union is derived from enactment of the EU Cosmetics Directive (76/768/EC).
- The Directive obliges manufacturers and importers to have stringent control over the quality of their products and to ensure that a formal safety assessment is undertaken for each one prior to marketing.
- The safety assessment is a key part of the compliance process and requires a collation and review of the information about: regulatory restrictions, identity, quality, physicochemical/toxicological properties of the ingredients, characteristics of the finished product and an appreciation of the purpose and presentation of the product to the consumer.

References

Colipa Activity Report 2006, The European Cosmetic, Toiletry and Perfumery Association Avenue Herrmann-Debroux 15A - B-1160 Brussels

Council Directive of 27 July 1976 on the approximation of the laws of the Member States relating to cosmetic products (76/768/EEC), OJ L 262, 27.9.1976, p. 169

International Cosmetic Ingredient Dictionary and Handbook, 11th Edition (2006), Cosmetic, Toiletry and Fragrance Association

Council Directive 89/48/EEC of 21 December 1988 on a general system for the recognition of higher-education diplomas awarded on completion of professional education and training of at least three years' duration, *OJ L 19, 24.1.1989, p. 16–23*

EFfCI GMP Guide for Cosmetic Ingredients, (2005) The European Federation for Cosmetic Ingredients, 2, Avenue de Terveuren, 1040 Brussels, Belgium

Russell, W.M.S. and Burch, R.L. (1959). *The Principles of Humane Experimental Technique*, Methuen, London

SCCNFP/0321/00, Final: Notes of Guidance for Testing of Cosmetic Ingredients for Their Safety Evaluation, 4th revision, (2000), Scientific Committee on Cosmetic Products and Non-Food Products

Notes of Guidance for Testing of Cosmetic Ingredients for Their Safety Evaluation, 6th revision, (2006), Scientific Committee on Consumer Products

Guidelines for the safety assessment of a Cosmetic Product, (1997), The European Cosmetic and Perfumery Association

Appendix 18.1 Additional obligations for cosmetic suppliers

1. Notify the competent authorities in the country of manufacture or the first country of importation within Europe. There is an expectation that the type of product will be identified during the notification process and that poison centres are supplied with the formulation breakdown.
2. Maintain a dossier of information about the cosmetic product (called the Product Information Package)[13] which should contain, as a minimum:
   (a) the qualitative and quantitative composition of the product;
   (b) the physicochemical and microbiological specifications of the raw materials and the finished product and the purity and microbiological control criteria of the cosmetic product;
   (c) show that the method of manufacture complies with good manufacturing practice, with the person responsible for manufacture or first importation into the Community possessing an appropriate level of professional qualification or experience;
   (d) assessment of the safety for human health of the finished product and the name and address of the qualified person or persons responsible for the assessment;
   (e) existing data on undesirable effects on human health resulting from use of the cosmetic product;
   (f) proof of the effect claimed for the cosmetic product, where justified by the nature of the effect or product;
   (g) data on any animal testing performed by the manufacturer, his agents or suppliers, relating to the development or safety evaluation of the product or its ingredients, including any animal testing performed to meet the legislative or regulatory requirements of non-member countries.
3. Label the product with the following information:
   (a) the name or style and the address or registered office of the manufacturer or the person responsible for marketing the cosmetic product;
   (b) the nominal content at the time of packaging, given by weight or by volume;
   (c) the date of minimum durability **or**, for cosmetic products with a minimum durability of more than 30 months, an indication of the period after opening for which the product can be used without any harm to the consumer (open jar symbol).

[13] It is also expected that this dossier would be made readily available to the competent authorities (e.g. within 72 hours) although it is not required to be sent to or reviewed by them prior to marketing. It is important to note that this dossier is a 'live' document that should be reviewed and maintained throughout the life of the product.

(d) particular precautions to be observed in use including mandatory ingredient-triggered warnings or instructions;
(e) the batch number of manufacture or the reference for identifying the goods;
(f) the function of the product, unless it is clear from the presentation of the product;
(g) a list of ingredients, using international cosmetic nomenclature, in descending order of weight above 1%.

There are allowances for products that are too small to contain all of the above labelling and/or those which have additional outer packaging, but it must be clear to the consumer so that they can see the composition of the product and how to use the product appropriately.

Additional information about the cosmetic product besides that placed on the product label may also be requested by members of the public, and suppliers are obliged to fulfil this request although they are not required to release the entire contents of the dossier. This additional information includes details of existing data on undesirable effects on human health resulting from use of the product (e.g. irritation, allergic response), and a quantitative declaration of ingredients which have been classified as dangerous in Annex 1 of the Dangerous Substances Directive (67/548/EEC).

19 Regulatory dermatotoxicology and international guidelines

Adam Woolley

ForthTox Limited, PO Box 13550, Linlithgow, West Lothian EH49 7YU, UK

Primary Learning Objectives

- *Overview of dermal toxicology from a regulatory perspective and how guidelines impact upon product development.*
- *Appreciation of different types of dermatological study in relation to different product categories.*
- *Introduction to basic dermal toxicity study designs.*

19.1 Introduction

In general, dermal toxicology studies fall into two categories, repeated dose and specific endpoint. The former are frequently employed in strategies to develop a chemical product whereas the latter can be used to demonstrate the safety of a final product under 'in use' conditions.

Regulatory dermal toxicology has elements of similarity to other toxicity disciplines but there are also important differences. In essence, the field can be divided in two: repeat dose and specific endpoints. The former have a similar design to other repeat dose studies and differ only in terms of the route of administration whereas the latter examine specific toxicological endpoints such as irritation and corrosion.

While specific endpoints may be examined in repeat dose studies, these studies have a far wider brief and use more animals than studies for specific endpoints. This last sentence highlights another difference between what might be called 'mainstream repeat dose dermal toxicity' and those studies looking at specific endpoints; tests involving specific endpoints have shown more scope for the adoption of *in vitro* or alternative techniques. Repeat dose studies investigate many more endpoints than the specialist studies and they require the presence of inter-relationships between organs, tissues and processes that cannot currently be reproduced using *in vitro* systems. At present, it is unlikely that repeat dose toxicity studies can be emulated *in vitro*; however, it would be hubristic to say that it will never occur.

Principles and Practice of Skin Toxicology Edited by Robert P. Chilcott and Shirley Price
© 2008 John Wiley & Sons, Ltd

In the same way that it is possible to define two broad study types, it is possible to state that there are broadly two purposes to dermal studies:

- Repeat dose programme during chemical development, such as is common with topical pharmaceuticals.
- More focussed shorter studies, which are usually conducted to assess occupational safety with materials such as agrochemicals and industrial chemicals.

Broadly speaking, when considered in the context of pharmaceutical or chemical development, dermal administration is generally a less common route than others, particularly oral or intravenous. However, large numbers of short studies are conducted to cover the needs of chemical notification and occupational safety, for which dermal administration is essential as this is one of the main routes of foreseeable exposure in a workforce.

19.2 Regulatory context

Regulation and guidelines covering dermal toxicology are broadly summarised by the division in purpose of the intended study type and intended use of the chemical.

For human and veterinary pharmaceuticals the relevant guidelines are encapsulated in those promulgated by ICH (The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use). This is complemented by the veterinary side, VICH (The International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products).

As implied above, the tests for such compounds may be best considered to be routine toxicity studies in which the route of administration is dermal. This is predicated on the similarity of design to a regular, for example, oral gavage study in rats. The numbers of animals are the same, the durations of treatment do not differ and the endpoints investigated are also no different. What is different is that the route of administration is more specialised and this is associated with more complex clinical and histopathological observations of the application site. The general similarity of design and purpose extends to acute toxicity studies conducted by dermal administration; these contrast with the more specialist endpoint studies in that the endpoint is crude in comparison (e.g. death or severe toxicity).

The other main area for dermal regulation is in the field of chemical notification and the new European chemicals legislation REACH (Registration, Evaluation, Authorisation (and restriction) of Chemicals). This is a regulatory device for ensuring that producing companies expend effort in the safety assessment of existing chemicals and will also encompass new chemical notifications in the European Union. While it may be necessary to conduct repeat dose dermal toxicity studies in some cases, the main dermal effort in chemical notification is on the so called 'six pack' studies, the three dermal studies of which comprise skin irritation and sensitisation and acute dermal toxicity (the others are eye irritation and acute toxicity by the oral route and, when relevant, by inhalation).

Cosmetics form a slightly different – and more awkward – group. For many cosmetics, the skin is the major route of exposure and it is clear that evaluation of repeat dose toxicity is relevant to the long term use by human consumers. However, the safety evaluation of cosmetic ingredients is increasingly constrained by the forthcoming European ban on the use of animals for testing ingredients or products. This may be a Europe-only initiative but,

given the importance of the European cosmetics market, the effects will be global. This also means the use of the most awkward, least controlled, highly variable and most expensive test species of all – human volunteers. The assessment of cosmetics is dealt with comprehensively in Chapter 18 and will not be explored further here.

19.3 Product groups and the human context

It is important to ensure that the type of dermal toxicity study being performed is appropriate to the particular category of product for which a safety assessment is being sought.

To put the regulation of dermal toxicology into context, it is useful to consider the various product groups and the characteristics and extent of human exposure to each group in terms of route and level. Broadly speaking, product groups can be summarised as follows:

- Pharmaceuticals for human use
- Pharmaceuticals for veterinary use
- Medical devices
- Agrochemicals
- Biocides
- Industrial chemicals
- Cosmetics

In general terms, exposure is either controlled or uncontrolled, intentional or unintentional. Exposure to human pharmaceuticals and medical devices is usually intentional and controlled; in contrast the exposure to cosmetics is intentional but – at the point of consumption – essentially uncontrolled. Unintentional human exposure is seen with agrochemicals, veterinary pharmaceuticals and industrial chemicals.

Exposure to pharmaceuticals is mostly well controlled; the size of each dose and the duration of treatment are both known, although this knowledge tends to break down as patient compliance slips. Also, while this may be broadly true of oral or parenteral formulations, the exposure assessment for dermal products may well be less precise. This is due to the presentation of topical products in tubes, the lack of precision of the amount applied and the variation in the area of skin to be treated. An oral dose of ibuprofen may be readily defined in milligrams, whereas the dose of the same active ingredient applied in a gel cannot. This is because the amount applied will vary according to the severity of the pain and the area to which the gel is applied, not to mention variation in the knowledge of the consumer and the extent to which the instructions have been read. In addition, the topical dose is unlikely to equate to the systemic or internal dose due to differences in absorption from different regions of the skin and the local conditions (erythema, moisture, heat) at the site of application.

Medical devices pose a series of challenges in safety evaluation that are unusual in the context of the other chemical groups listed above. They are intended to be pharmacologically and biologically inactive (that is, they should be safe) and the result is that there should be no effects due to administration; the consequence of this is that no dose response can

be assessed. The final assessment of safety is based on negative data. If there is any effect, this is unlikely to be very welcome. In the context of dermal toxicology, relevant medical devices include examination gloves, wipes, surgical drapes used to protect the skin around operation sites, wound dressings and liquids such as cleansers or antiseptics. In contrast to a classic pharmaceutical, which is usually a single chemical with an intended biological action and, probably, a range of unintended effects, medical devices are complex mixtures that are expected to be biocompatible with the skin and not to have any adverse effect.

While it can be said with reasonable certainty that exposure to medical devices, pharmaceuticals or cosmetics is intentional, exposure to most of the other groups is likely to be unintentional – always making allowance for the fact that unintentional is not the same as unexpected. This group of products is the subject of two groups of people who are exposed to them – production workers and consumers.

Production worker exposure is limited by occupational safety legislation, which sets threshold limit values and similar concentrations for the work place. These seek to define a concentration that can be tolerated in the course of a normal 8-hour working day (LTEL; long-term exposure level) or a maximum concentration that can be tolerated for 15 minutes (STEL; short-term exposure level). There is the need in setting these figures to undertake some toxicological testing and biological monitoring.

Exposure of consumers to veterinary pharmaceuticals and plant protection products (pesticides) is possible through consumption of plants or animals which have been treated with the various products. Consumers have little control over this, other than to consume 'organic' produce which should not have been treated with the products of concern. Otherwise, the concentrations of these products in foods and beverages is controlled by the imposition of intervals between treatment and harvest and the use of Maximum Residue Limits (MRLs) in conjunction with acceptable daily intakes (ADIs, usually expressed in mg person^{-1} day^{-1}).

Industrial chemicals, the subject of the recent European REACH initiative, are of dermatological interest in respect of occupational exposure and, in the case of chemicals used in household products, of dermal exposure of consumers. Intermediates in pharmaceutical synthesis are a subgroup in these chemicals as they are not intended for human exposure but are clearly of interest (unless used in closed systems) for occupational safety issues.

19.4 Dermal toxicology with the different product groups

The intention in this short section is to give an overview of the philosophy and strategy that drives testing in each of the main product groups mentioned above.

One study type is common to a greater or lesser extent for all the product categories and that is absorption studies. For human or veterinary pharmaceuticals that are applied topically, absorption through the skin is a vital aspect of their behaviour. For some products that are intended only to be active at the skin surface (such as topical steroids), extensive absorption into the systemic circulation is undesirable. In contrast, topical formulations designed to deliver drugs across the skin into the systemic circulation (e.g. nicotine and hormone replacement patches), transdermal absorption is clearly very important. For these two product groups, it is likely that the studies would be in animals rather than *in vitro*, although that would not rule out the use of *in vitro* work in the early stages of development (see Chapters 7 and 8 for experimental details). For cosmetic ingredients, absorption studies will increasingly have to be carried out *in vitro* due to the forthcoming ban on the use of animals in the testing of these

chemicals in Europe (Chapter 18). The remaining products fall into intermediate positions, with the absorption of chemicals being of clear importance to production workers in all the relevant industries, including intermediates in synthetic pathways.

19.4.1 Pharmaceutical agents

Studies on pharmaceuticals for human and veterinary use fall into two areas. The first is the need to predict safety for human patients by the conduct of non-clinical studies in support of clinical development plans. The second area of dermato-toxicological focus is the need to perform studies for worker protection. The former category covers the full range of toxicity studies conducted in a normal pharmaceutical development, with the probable exception of *in vivo* genotoxicity and safety pharmacology studies. All other study categories – general toxicology, reproductive toxicology and carcinogenicity studies – can be conducted by dermal administration. Having said that, there is likely to be pressure to avoid dermal administration in some study categories (e.g. reproductive toxicity testing). The strategy adopted depends on whether the development is a new chemical for topical administration that has no previous safety data by other routes or a topical formulation of an existing and well characterised drug substance. In the latter case, it is likely that careful characterisation of dermal absorption *in vivo*, together with an appropriately designed bridging study to examine toxicity at the site of application, will give sufficient dermal data for a submission.

Dermal administration in carcinogenicity studies with pharmaceuticals is not uncommon; fortunately the extension of these to encompass photo-carcinogenicity is rare, as well as being complex and expensive. If a case can be made for supporting dermal administration in the clinic with an oral carcinogenicity programme, many companies would take that option in view of the benefits it would bring in terms of simplicity of dosing, observation and animal housing.[1]

The studies for these two compound groups explore the local and systemic toxicity of repeated administration with products intended for topical use. Dermatological testing of cosmetics is moving towards the exclusive use of *in vitro* methods in toxicity tests and human volunteer studies to assess tolerability and, in some cases, sensitisation.

19.4.2 Medical devices

Dermal testing of medical devices is not constrained by the need to avoid animal testing but is limited in that dermal exposure of patients is rarely going to be long term, thereby avoiding the need for long, repeat dose toxicity studies. Another factor in this is the form and intention of medical devices, namely as complex, often solid, agents without intended activity. The main emphasis on evaluation and testing, therefore, is on local effects in short studies. Note the use of the word evaluation in conjunction with testing; biological evaluation is a necessary step in the assessment of medical devices and for the most part the emphasis is on irritation, sensitisation and cytotoxicity. For the more invasive devices, especially those intended for long term implantation such as heart valves or prosthetic joints, the main areas of toxicity testing have to be considered, although possibly not conducted.

[1]For most dermal studies, individual housing of animals is the preferred option in order to avoid interference by cage mates with the application site, self-ingestion of the dose and possible exacerbation of any local effects.

It is possible that testing may be avoided completely if the device uses known chemicals and is going to be used in a similar manner to existing devices of similar composition. For solid devices the conduct of dermal toxicity studies poses several problems in terms of method of application and dose escalation. It is not easy to apply a solid to an area of skin in any way that will result in meaningful exposure of the animal; in addition, the form of the device is likely to make increasing the dose difficult, the only options being increased duration of exposure or increased area of exposure. Dose escalation for liquid devices such as hand cleansers is easier but as the concentration of the active ingredients increases the formulation tested becomes less relevant to the one to be used clinically.

19.4.3 Other chemicals

For the other groups, dermal exposure is mostly unintended or brief and the objectives of testing are not to support clinical development but to explore worker safety, in the industrial context. For chemicals to which the public may be exposed, such as domestic biocides and domestic plant protection products, the emphasis is likely to be more on dermal absorption and short term toxicity. There is unlikely to be a requirement for repeat dose dermal toxicity studies of more than four weeks. For the most part, therefore, dermal toxicity in these chemicals entails the conduct of studies for acute dermal toxicity, irritation and corrosion, and dermal sensitisation.

19.5 Factors in dermal toxicity

Having selected the appropriate study type, it is essential to ensure that practical elements of the study design meet any regulatory obligations or can provide an adequate indication of product safety representative of 'in use' conditions.

The following is a brief consideration of some of the factors that affect toxicity and the conduct of dermal toxicity studies (many of which influence skin absorption and so are examined in more detail in Chapters 5 and 6).

Formulation. This is a critical factor that affects absorption into or across the skin. Often, industrial materials are used 'as supplied' in irritation or sensitisation studies where the chief focus is on worker protection. For pharmaceuticals, elaborate formulations may be used to mimic the clinical situation. In contrast, cosmetic safety does not require a final product to be evaluated if there is sufficient information on the individual ingredients and their behaviour in the final formulation is predictable.

Area exposed and local concentration of test material. The area in square centimetres and the concentration of the formulation are probably more important than the dose in mg kg^{-1}, in respect of local effects.

Location of exposure. While the location of exposure is important in the human context, the site of application in non-clinical dermal toxicity is always the back, which has been clipped or shaved of hair.

Local conditions at exposure site. Warmth and humidity will tend to increase absorption (and hence local effects) and this is usually achieved by occlusion of the application site. Local vasodilation may also increase absorption by maintaining a diffusion gradient.

Skin condition. The presence of broken skin or inflammation is likely to increase absorption. Abrasion of the treatment site may be used in some study designs.

Metabolic capability of skin. The skin has significant capacity for metabolism and this may enhance the local toxicity or potential for sensitisation through production of reactive haptens.

Local reaction may be limiting. Repeat dose toxicity studies may be stopped due to excessive reaction at the application site. If the intention is to explore systemic toxicity, this is likely to lead to reformulation or use of a different route of administration.

Physicochemical properties of the test substance. The lipophilicity of the chemical is an important driver of absorption, as are molecular weight and pKa. Reactivity with endogenous molecules will also bring about local effects that may limit bioavailability.

19.6 Repeat dose dermal toxicology

As indicated above, these studies are essentially standard toxicity studies – general, reproductive, carcinogenicity – that have designs that are very similar to those of other studies, the only difference being the route of administration and the complexity of site observations. The most frequent repeat dose dermal toxicity studies undertaken are those for general toxicity; frequently, these studies have an emphasis on determining local tolerance to the test substance and, to some extent, the formulation. Typical outline designs for these studies for pharmaceuticals are illustrated in Table 19.1.

For carcinogenicity studies, the design is likely to follow that of similar studies by other routes, namely 50 or 60 animals per sex per group, randomised to three treated groups and two controls groups. Treatment is for two years in rats and mice (or for a shorter period in transgenic mice). For dermal studies with transgenic animals, the Tg.AC Model is considered as a suitable model for dermally administered pharmaceuticals by both the United States Food and Drug Administration (FDA) and the European authorities, although doubts have been expressed about the phenotypic stability of this model. The number of transgenic animals per group and sex has risen over the years from a statistically weak 15 to a more powerful 25.

As reproductive studies are rarely conducted by the dermal route, they are not considered further here; the designs are similar to those using other routes and these are given in a number of standard texts (see Bibliography).

Test systems for repeat dose dermal toxicity studies have been used based on their similarity of skin structure to man; in the past this meant the rabbit was sometimes used, together with the rat. The current non-rodent species of choice is the minipig, based on the close similarity of skin structure to man; the rodent of choice is still the rat, although mice may still have their uses. The minipig is an excellent model for dermal toxicity studies as it is large, relatively easy to handle, is now well understood and – perhaps more importantly – well accepted by regulatory authorities. The mouse has a long history of use in dermal toxicity, especially in carcinogenicity testing; its use in skin cancer promotion studies is well documented. Of the other species available, the guinea pig (the OECD favourite), the dog and the non-human primate are rarely used.

Table 19.1 Summary of basic designs for general toxicology

Species		Acute[†]	Subacute	Subchronic/Chronic
Group size[a]	Rodent	5 m 5 f[b]	10 m + 10 f[c] (Rev 5m + 5f in 3 groups)[d]	10 m + 10 f (Rev 5m + 5f in 3 groups)
	Non-rodent	Not used	3 m + 3 f (Rev 2m + 2f in 2 groups)	4 m + 4 f (Rev 2m + 2f in 2 groups)
Clinical pathology	Rodent	Not done	Wk 4 + end rev.	Wk 4 or 6, 13, rev.
	Non-rodent	Not done	Wk 0, 4 + end rev.	Wk 0, 4 or 6, 13, rev.
Ophthalmoscopy	All	Not done	Wk 0, 4, end rev	Wk 0, 13, rev
Necropsy	All	All groups	All groups	All groups
Histopathology[e]	Rodent	Gross lesions retained but not examined	Control, high dose and affected tissues at low and mid dose groups.	
	Non-rodent	Not done[f]	Affected tissues only in reversibility groups. All animals are examined.	

Abbreviations: M = male; f = female; rev = reversibility studies; Wk = week.
[†]Acute is single dose. [a]Except in acute studies there are usually three treated groups and a control. [b]Groups of 2 m 2 f used for dose selection. This is OECD but new designs use fewer animals. [c]This may be reduced to 5 m + 5 f in some cases. [d]Reversibility studies usually controls, mid and high dose for rodents, controls and high dose for dogs (or mid and high dose only – see text); examinations normally conducted in final week. [e]In addition, organ weights are recorded on all except acute studies. In-life observations on all studies include clinical signs, bodyweight and food consumption. [f]Acceptable data on acute toxicity may be obtained from early dose range finding studies. Adapted with author permission from Woolley, 2003.

Although there may be similarities of test systems and basic study design in dermal toxicity to studies conducted by more routine routes, there are differences:

- The vehicle may have a disproportionate effect on absorption and thus toxicity. For pharmaceuticals, it is very important to test a formulation that is as close as possible to the clinical intention.
- Additional endpoints must be added in to cover local reaction at the application site.
- Administration may involve occlusion of the test site leading to stress.
- Some assessments, such as the intensity of erythema, are subjective but there is a move towards electronic assessment of application sites, which will overcome this shortcoming.
- Occlusion of the application site is recommended for studies up to 28 days but not for longer unless there is a specific reason. The OECD guidelines for a 90-day dermal toxicity study (Guideline 411, 1981) suggest occlusion of the application site for six hours a day. Occlusion of the site enhances absorption and prevents ingestion but is stressful for the animals and an additional practical procedure that adds to the time and cost of these studies. For local toxicity, the concentration of the test substance and the dose in mg cm^{-2} is probably more important than the dose in mg kg^{-1}.

19.7 Classic short-term dermal toxicity studies

These are probably the most frequently conducted dermal toxicity studies and are intended to examine the classic endpoints of irritation, corrosion and sensitisation. The differences between these endpoints may be summarised as follows:

- Irritation is reversible and dose dependent and not mediated by any immune response.
- Corrosion is dose dependent but not readily reversible, depending on the extent of the lesion.
- Sensitisation is dose dependent during the induction phase, but may not have any detectable threshold when sensitisation has been achieved. It is mediated by the immune system in the shape of sensitised lymphocytes and/or antibodies.

19.7.1 Test systems in short-term dermal toxicology

As usual in toxicity testing, the choice of test system is driven by a combination of experience, conservatism, regulatory acceptance and practicalities (including availability and cost – both of purchase and husbandry). Leaving aside the *in vitro* alternatives (Chapter 11), the main animal species used are the rat, rabbit and mouse. The guinea pig, which has a long history of use in sensitisation studies, features in some of the older OECD guidelines but is now rarely used.

The decline in use of the guinea pig has been largely brought about by the inception of the local lymph node assay for sensitisation (Chapter 9). This has replaced the Buhler and Magnusson-Kligman tests, which used large numbers of animals and were mostly subjective in their assessments. The rabbit remains the species of choice for dermal irritation and corrosion studies and the rat is used for acute dermal toxicity studies.

19.7.2 Acute toxicity

The approach to acute toxicity has changed in recent years, with fewer animals being used as the intensive LD_{50} (or median lethal dose test) has been laid aside. The result has been a progressive approach, typically using two or three animals of one sex to establish a dose at which toxicity is seen and then treatment of more animals at that dose to give a final group size of five males and five females. While the number of animals has been reduced and the lethality endpoint abandoned, the basic design of the test has not changed.

Twenty-four hours before treatment, the backs of the test animals are shaved or clipped of fur, taking care not to damage the skin; the OECD guidelines indicate that this should cover at least 10% of the body surface. On the day of treatment, the test substance is administered and the site occluded by wrapping the animal in gauze and protective tape for 24 hours. After 24 hours the sites are unwrapped and inspected and the animals are then observed for a further 14 days before being killed and subject to a necropsy, during which any abnormal tissues may be retained for possible histopathological processing and examination. Bodyweights are recorded at appropriate intervals; food consumption may be recorded but is not essential from the point of view of some regulations or guidelines. Clinical observations are recorded at appropriate intervals, including assessment of the application sites.

While this approach is still important for some chemical groups, there is a movement away from acute toxicity testing in pharmaceutical development. This is because there is increasing recognition of the limited utility of the classic approach to acute toxicity and the increasing acceptance by regulatory authorities that data relevant to acute toxicity can be derived from other sources, especially dose range finding studies.

19.7.3 Irritation and corrosion

This is a contentious area, in terms of the ethics of animal experimentation, although perhaps not as contentious as ocular irritation. The OECD guidelines for acute dermal irritation and corrosion (no. 404, adopted in 2002) put forward a strategy for testing that is intended to avoid severe reactions in animals; these are set out in detail in Chapter 11. The guidelines indicate that known irritants or corrosive substances and those with clear evidence of no corrosivity and which are non-irritant need not be tested *in vivo*. A typical design for such a study is as follows:

- Two rabbits exposed for four hours to the material applied to water-moistened trunk under semi-occlusive patches.
- Typically expose one animal then the second.
- Separate sites as within animal controls.
- Observations.
- Observe sites at predetermined intervals – 1, 24, 48 and 72 hours after patch removal up to 14 days to determine reversibility of any effect.
- Score erythema, eschar and oedema.

Approximately 24 hours before the test, the backs of the animals are clipped or shaved to remove the fur, ensuring that the skin remains intact. The test substance is applied to an area

of about 6 cm^2, covered with a gauze patch, which is then held in place with non-irritating tape. In some circumstances it is more appropriate to apply the substance to the gauze patch rather than the skin. The form of the chemical that is used is important. Liquids (0.5 ml) should be used without dilution and solids (0.5 g) should be moistened with 'the smallest amount of water' or another suitable solvent. At the end of the four-hour exposure period, the patches are removed and any remaining test substance removed with water or an appropriate solvent; it is important that this process does not affect the existing response.

A refinement of this approach may be used to further investigate effect where deemed necessary. In this, three patches are applied sequentially to one animal; the first for three minutes, the second (if there is no reaction to the first) for an hour and, if appropriate, the third patch is applied for four hours. The presence of corrosivity at any of the three stages results in an immediate end to the test. In the absence of reaction, observation is continued for 14 days.

The evaluation of the results of these tests is subjective and, by implication, potentially contentious. As a result, it is important that the person doing the assessment has plenty of experience or long training in order to get around the potentially poor reproducibility of such a system. Although this evaluation is currently subjective, there is likely to be increasing use of instrumentation that can assess skin colour optically and so produce a numerical result based on objective measurement (Chapter 12).

The evaluation of the dermal effects is aimed at assessing the duration, and by implication the reversibility, of any effect as well as its intensity. While the classic indication of irritation is erythematous reddening of the skin, if responses such as hair loss over a limited area, hyperkatosis, hyperplasia or scaling persist to the end of the observation period, the substance should be considered to be an irritant.

A typical scoring system is given in Table 19.2 and is similar to those used for clinical applications as discussed in Chapters 13 and 14. The values derived from such indices may be used to calculate a Primary Irritation Index (PII), for which there are various methods broadly based on the adding together of all the erythema and oedema scores for all the animals at

Table 19.2 Typical scoring index for subjective quantification of skin lesions resulting from corrosion/irritation testing (Similar systems are used for clinical applications; Chapters 13 and 14)

	Grade or severity	Score
Erythema	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
Oedema	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 mm)	3
	Severe oedema (raised >1 mm, extending beyond the area of exposure)	4

Table 19.3 European Union score system for determining the classification of irritants and corrosives

0	Non-irritant
<2	Slightly irritating
2–5	Moderately irritating
>5	Severely irritating/corrosive

all observation points and dividing the total by the number of test sites and scoring times. Some systems combine the scores for erythema and oedema, while the European Union treats them separately. In the European Union the scores from this process are used to produce a standard classification (Table 19.3). However, there is a plethora of classification systems and more detail is given in reference texts such as Deralenko and Hollinger (2002).

19.7.4 Sensitisation

Dermal sensitisation has traditionally been assessed in guinea pigs using the maximisation test of Magnusson and Kligmann and the Buehler test. However, the murine local lymph node assay (LLNA) has superseded the rather long-winded and animal hungry guinea pig protocols. The LLNA has the huge advantage that it is an objective and quantitative method. Both the traditional approach and the LLNA are discussed in depth in Chapter 9. Overall, the LLNA is a quick and relatively inexpensive method of assessing skin sensitisation. Although it uses animals, it uses fewer than the more traditional methods and is quantitative. It is expected that the use of the LLNA and experience will increase in the future.

19.8 Pragmatic considerations

In vivo dermal toxicity testing requires that animals are exposed to test products by topical administration. This raises a number of pragmatic issues including how the exposure site is prepared, how it is dosed and how the animals are housed.

Dermal toxicity studies pose a number of practical challenges which are not seen with more usual routes of administration. Amongst these are the issues of shaving versus clipping, abrasion of the skin, dose in terms of area, site occlusion and housing.

In regulatory tests it is more usual to clip the hair from the application site, although shaving may be employed as an additional measure in some circumstances. For the most part it is important that the integrity of the skin remains intact as dermal damage may affect the validity of the test. Abrasion, where the skin is deliberately damaged in a carefully controlled manner, may be used to enhance absorption across the skin.

In dermal toxicity, the local concentration of the dose may be more important than the dose in terms of mg kg^{-1} bodyweight. This needs to be considered in designing the test to meet the study objectives.

There is some debate about the desirability of occluding the test site and this is normal in shorter tests. For repeat dose studies, it may be used up to 28 days but is not advisable beyond

that. The consequence of not occluding the test sites in repeat dose toxicity studies is that the animals should be housed separately to prevent interference with the application sites.

In some contrast to other areas of toxicological testing, the vehicle may have a substantial influence on what is tested in repeat dose studies. For pharmaceuticals, it is particularly important to test a formulation that is as close as possible – if not actually identical – to that to be used in the clinic and/or in the finally marketed product. This is because excipients can have a profound effect on skin absorption. For tests such as the LLNA, the OECD guidelines provide a list of approved vehicles and reasons for deviation from this guidance must be given.

For other chemical groups, the test material is often used 'as supplied', which can have some apparently bizarre consequences. For example, practically insoluble powders may be further pulverised, moistened and applied to the back of the animal under an occlusive patch. Equally, some substances such paints and other industrial liquids may be used as supplied, meaning that visual observation of the exposure site may by impossible!

Summary

- The type of dermal toxicity study to be performed should be commensurate with the category of test product being assessed, which in turn is dependent on the particular regulatory requirement.
- Dermal safety assessments are broadly similar to other toxicological studies, especially in terms of available test systems and basic study design. However, there are several notable differences, including:
  - The vehicle used in dermal toxicity studies can be a significant factor in affecting the outcome of studies, especially in pharmaceutical development.
  - Site occlusion has the disadvantage of stressing the animals but protects the application site and may enhance adverse effects (leading to a conservative estimate of toxicity).
  - Animal husbandry considerations to ensure integrity of exposure site.
- Whilst experimental endpoints of dermal studies may be more limited in number and type, common gross pathologies (e.g. erythema and oedema) present their own technical challenges, especially when using subjective measurements.
- Objective methods such as biophysical skin analysis are being introduced to address this problem.

References

Ballantyne, B., Marrs, T. and Syversen, T. (Eds) (1999). *General and Applied Toxicology*, 2nd Edn, MacMillan Reference Ltd, Basingstoke and Oxford.

Deralanko, M.J. and Hollinger, M.A. (Eds) (2002). *Handbook of Toxicology*, 2nd Edn, CRC Press, Boca Raton.

Hayes, A.W. (Ed.) (2001). *Principles and methods of toxicology*, 4th Edn, Taylor and Francis, London.

Klassen, C.D. (Ed.) (2001). *Casarett and Doull's Toxicology: the basic science of poisons*, 6th Edn, McGraw Hill, New York.

Woolley, A. (2003). *A Guide to Practical Toxicology: evaluation, prediction and risk*, Taylor and Francis, London.

Zai and Maibach (2004). *Dermatotoxicology*, 6th Edn, CRC Press, Boca Raton.

OECD Guidelines Relevant To Dermal Toxicology

OECD Guidelines for the Testing of Chemicals, available free at http://www.oecd.org/document:

- Test No. 402: Acute Dermal Toxicity (1987)
- Test No. 404: Acute Dermal Irritation/Corrosion (adopted April 2002)
- Test No. 406: Skin Sensitisation (1992)
- Test No. 410: Repeated Dose Dermal Toxicity: 21/28-day Study (1981)
- Test No. 411: Subchronic Dermal Toxicity: 90-day Study (1981)
- Test No. 427: Skin Absorption: *In Vivo* Method (2004)
- Test No. 428: Skin Absorption: *In Vitro* Method (2004)
- Test No. 429: Skin Sensitisation: Local Lymph Node Assay (2002)
- Test No. 430: *In Vitro* Skin Corrosion: Transcutaneous Electrical Resistance Test (TER) (2004)
- Test No. 431: *In Vitro* Skin Corrosion: Human Skin Model Test (2004)
- Test No. 432: *In Vitro* 3T3 NRU Phototoxicity Test (2004)
- Test No. 435: *In Vitro* Membrane Barrier Test Method for Skin Corrosion (2006)

20 Glossary of main terms and abbreviations

James C. Wakefield

Chemical Hazards and Poisons Division, Centre for Radiation, Chemical and Environmental Hazards, Chilton, Oxfordshire, OX11 0RQ, UK

This section summarises the main terms and abbreviations commonly encountered in dermal toxicology. This is by no means an exhaustive list and many other terms can be found in the index section.

3Rs: The three R's of replacement, refinement and reduction; a set of principles relating to the humane use of animals for research

Absorption: uptake of a substance into an organism or tissue to the site of action or systemic absorption by diffusion down a thermodynamic gradient

ACD: allergic contact dermatitis, an eczematous inflammation reaction of the skin following repeat exposure to a sensitising chemical

Acid mantle: a superficial layer on the skin surface formed from a thin film of sebum, corneocytes debris and residual material from sweat

Actinic: pertaining to skin damage caused by sun (ultraviolet radiation) exposure.

ACoP: Approved Code of Practice

ADH: alcohol dehydrogenase, a group of phase 1 metabolising enzymes responsible for oxidising alcohols into aldehdehydes

ADI: Acceptable Daily Intake

ADR: adverse drug reaction

ALDH: aldehyde dehydrogenase, a group of phase 1 metabolising enzymes responsible for oxidizing aldehydes into carboxylic acids

Allergic contact dermatitis: see ACD.

Apical migration: movement of cells from a lower layer to the surface, eg, during differentiation of the epidermis from the stratum basale to the stratum corneum

Principles and Practice of Skin Toxicology Edited by Robert P. Chilcott and Shirley Price
© 2008 John Wiley & Sons, Ltd

Apoptosis: the process of pre-programmed cell death. The opposite of necrosis.

Appendage: collective term for structures in the dermis which protrude through the epidermis, such as hair follicles, sebaceous glands and sweat ducts

Atopic: An individual's genetic predisposition to hypersensitivity reactions such as eczema and asthma.

Beuhler test: see guinea pig maximisation test.

BHT: butylated hydroxytoluene, an anti-oxidant used to preserve topical formulations.

Blanching: loss of colour (redness) of the skin due to vasoconstriction of the superficial plexus

Cancer: a group of diseases characterised by the uncontrolled growth and spread (metastasis) of abnormal cells.

CHIP: Chemicals (Hazard Information and Packaging for Supply) Regulations

CIELAB: Commission Internationale de L'eclairage LAB. A standardised colour scheme using 3 axis to quantify colour (denoted L*, a* and b*). In general, L* indicates skin brightness and a* is used to quantify erythema. The b* parameter can be used to quantify hypopigmentation.

COLIPA: the European Cosmetics and Toiletry and Perfumery Association. A trade association promoting the interests of European companies associated with the industry.

Corneocyte: the 'dead' cells comprising the stratum corneum, resulting from the terminal differentiation of keratinocytes

Corneodesmosome: a structure which mediates cell to cell adhesion, specifically between corneocytes in the stratum corneum, derived from the epidermal desmosomes. See desmosome.

Corrosive: a substance which, when brought into contact with the skin surface, will cause overt, indiscriminate damage. Opposite of non-corrosive.

COSHH: Control of Substances Hazardous to Health

Cosmetovigilance: process of monitoring and the frequency and severity of adverse effects arising from the use of cosmetics

CTFA: Cosmetic, Toiletry and Fragrance Association. The former name of the Personal Care Products Council.

CYP: cytochrome P450, a key group of phase 1 metabolising enzymes responsible for mixed function oxidation reactions (some of which are expressed in the skin)

Dermatitis: inflammation of the skin, also known as 'eczema'

Dermatoglyphs: the pattern of ridges on the surface of the skin, particularly on the fingertips, commonly known as fingerprints

Dermatomed: a technique for the preparation of skin for *in vitro* percutaneous absorption studies, which removes the majority of the dermis from a skin section to leave the epidermis

and a small portion of dermis, in contrast to full thickness skin. Also known as 'split thickness skin'

Dermis: the layer of skin beneath the epidermis consisting of connective tissue and containing hair follicles, sebaceous glands, sweat glands and blood vessels

Desmosome: a largely externalised cell structure with the role of adhering adjacent cells and preventing them from shearing apart. When present in the stratum corneum, these are specifically known as corneodesmosomes.

Desquamation: the process of removal or loss of the uppermost corneocytes from the stratum corneum to be replenished by underlying corneocytes. Also known as 'sloughing'

Differentiation: a cell changing into those of a different morphology, eg, migration of keratinocytes from the stratum basale progressing and transforming into the cells of the stratum spinosum

Diffusion cell: equipment used for *in-vitro* percutaneous absorption studies consisting of a donor chamber and a receptor chamber

Donor chamber: the chamber of an *in-vitro* diffusion cell situated above the skin section on the outer surface to which a penetrant is applied

EC3: the effective concentration of a chemical which is required to stimulate a 3-fold increase in lymph node cell proliferation, compared to controls in the local lymph node assay

ECVAM: European Centre for the Validation of Alternative Methods

EFfCI: European Federation for Cosmetic Ingredients.

EH40: a HSE publication which prescribes maximum permissible air concentrations in the workplace for short (*STEL*) or long (*LTEL*) term durations of exposure.

Endobiotic: a chemical which is formed or produced within a given organism. Also known as 'endogenous'

Endocytosis: the process by which material is transported into a cell via a membrane-bound vesicle, either as particles (phagocytosis) or liquids (pinocytosis). The opposite of exocytosis.

EPIDERM: A system, funded by the UK *HSE*, for the collection of case reports pertaining to occupational skin diseases.

Epidermis: the outermost layers of the skin, comprising the stratum corneum, stratum granulosum, stratum spinosum and stratum basale

Erythema: increased redness of the skin due to vasodilation of the superficial blood capillaries.

ESAC: ECVAM Scientific Advisory Committee

Exocytosis: the process by which material is transported from within a cell via a membrane-bound vesicle. The opposite of endocytosis.

Ex-vivo: pertaining to an experiment using living cells or tissues taken from an organism and used in an artificial environment, eg, skin removed from an animal and used in an *in-vitro* system

FDA: Food and Drug Administration (USA)

Finite dose: a concentration or amount of a penetrant compound that when applied to the skin surface is significantly depleted by absorption that the concentration gradient and rate of absorption are diminished over time. Opposite of infinite dose

Flow-through: a type of diffusion cell in which the receptor fluid is pumped through the receptor chamber, constantly replenished and can provide regular automated samples over many hours, also known as 'Bronaugh-type'

Flux: the rate at which a chemical diffuses through the skin, commonly scribed as *J*

Fugacity: the tendency of a molecule to escape a certain environment. Related to the *thermodynamic activity* (α).

Full thickness: a type of skin preparation used for *in vitro* percutaneous absorption studies, in which the epidermis and dermis are used intact, in contrast to dermatomed skin

Genodermatosis: a familial (inherited) skin disease.

Glabrous: skin which is devoid of hair follicles, eg, the palm of the hand, sole of the feet and the lips

GLP: Good Laboratory Practice.

GMP: Good Manufacturing Practice.

GPMT: *guinea pig maximisation test*.

Guinea pig maximisation test: a standard test protocol for evaluating the skin-sensitising activity of a substance. A modification of the Beuhler test.

Hapten: a molecule which, when bound to a larger carrier molecule (such as a protein) will elicit an immune response

Hazard: the intrinsic characteristic of a substance to cause harm. For example, cyanide is a hazardous compound, but the risk of an adverse health effect can only arise through exposure. See *risk*.

Hives: see urticaria.

HRIPT: human repeated insult patch test

HSE: Health and Safety Executive. An Agency of the UK Government

Hydrophilic: describing the character of a substance having an affinity for water, also known as 'lipophobic'. The opposite of lipophilic. See also, log P.

Hyperproliferation: an abnormally high replication rate of cells.

Hypersensitivity: see *skin sensitisation*.

Hypodermis: the innermost layer of skin beneath the dermis, containing mostly adipose cells

ICD: irritant contact dermatitis, an eczematous inflammation reaction of the skin following exposure to an irritant chemical

ICH: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use.

INCI: International Nomenclature Cosmetic Ingredient.

Infinite dose: a high concentration or large amount of a penetrant compound that when applied to the skin surface is not significantly depleted over time by absorption thus maintaining a constant concentration gradient. Opposite of finite dose

Insensible water loss: see TEWL.

In-silico: pertaining to data generated using computer modelling

Integument: the skin. From the Latin 'integumentum' meaning covering.

Intercellular: a route of dermal absorption of a penetrant, diffusing through the stratum corneum along lipid- filled spaces between adjacent corneocytes

In-vitro: in glass, pertaining to a study in a laboratory usually involving isolated organ, tissue, cell, or biochemical systems

In-vivo: in the living body, referring to a study performed on a living organism

Irritant contact dermatitis: see ICD.

Jss: *steady state* flux.

Keratohyaline granules: small intracellular vesicles of the stratum spinosum which contain profilaggrin, a protein which controls keratin bundling during terminal differentiation.

Keratinocyte: the major cell type within the epidermis, responsible for around 90% of the epidermal cells

K_{ow}: see Log P.

K_p: permeability coefficient, a coefficient associated with diffusion through a membrane, which is proportional to the partition coefficient (log P) and diffusion coefficient (concentration gradient) and is inversely proportional to the thickness of the membrane

Lag-time (τ): the period which elapses before *steady-state* conditions are achieved under *infinite dose* conditions.

Lamellar: fine alternating layers, such as the arrangement of intercellular lipid bilayers with polar head groups aligned and the hydrophobic tail groups aligned, forming stacked sheets

Langer's lines: the orientation of collagen fibres within the human skin along which it has the least flexibility, also known as 'cleavage lines'

Langerhans cells: a cell type in the epidermis which aids with the immune response

LCt_{50}: indicates an inhaled dose which is lethal in half the exposed population

Lentigines: plural of lentigo; hyperpigmented spots which superficially resemble freckles but do not darken on exposure to UVR.

LD_{50}: Median lethal dose; indicates a dose which is lethal in half the exposed population. See also LCt_{50}.

LDV: laser Doppler velocimetry, a technique for measuring the relative cutaneous blood flow, also know as 'LDF – laser Doppler flowmetry'

Lipophilic: describing a substance with a propensity to dissolve in fatty tissues, also known as 'hydrophobic'. Lipophilic is the opposite of hydrophilic. See also, Log P.

LLNA: local lymph node assay, a mouse model used to predict the skin sensitisation hazard of a compound

Log P: the partition coefficient between octanol and water, used as a measure of the relative lipophilicity or hydrophilicity of a chemical, also known as 'K_{ow}'

LTEL: long term exposure limit. A *WEL* prescribed in *EH40* which gives the maximum permissible exposure concentration for an 8 hour working period.

Maximisation test: see guinea pig maximisation test.

MRL: maximum residual limit

Melanin: a pigment distributed in epidermal tissue and hair which provides protection against sunlight by absorbing ultraviolet radiation.

Melanocyte: a cell type in the epidermis responsible for the production of melanin

Melanogenesis: the biosynthetic processes involved in the production of melanin.

MHRA: Medicines Healthcare Regulatory Authority

Microautoradiography: a technique which allows visualisation of the distribution of a radiolabelled material within histological skin sections

Microdialysis: an *in-vivo* technique used to measure percutaneous absorption by the recovery of the compound in a porous capillary tube inserted into the dermis, immediately beneath the skin exposure site of a test subject

MOS: Margin of Safety

MSDS: material safety data sheet

MTT assay: a colourimetric assay to measure cell viability, the colour change is only produced from active mitochondria in live cells, so a reduction in colour change indicates an increase in the number of dead cells. MTT is an abbreviation for the tetrazole compound 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Necrosis: uncontrolled death of cells (or tissues) which can be a consequence of exposure to a noxious substance. The opposite of apoptosis.

NOAEL: No Observable Adverse Effect Level. See NOEL.

NOEL: no effect level, the level of exposure to a chemical which has no adverse effects upon the health of the test subject, also known as *NOAEL*.

Non-corrosive: see corrosive.

Non-glabrous: skin which possesses hair follicles

NRU PT: neutral red uptake phototoxicity test, an *in-vitro* test with the potential to detect photoirritants

NSAIDs: non-steroidal anti-inflammatory drugs

Occlusion: an air or water-tight covering over the skin surface preventing evaporation of water to the external atmosphere

OECD: Organisation for Economic and Co-operative Development

OPRA: Occupational Physicians Reporting Activity. A system which collates information on a broad range of occupational diseases. *EPIDERM* is one scheme which reports to OPRA.

OTTER: optothermal transient emission radiometry. An experimental technique that can be used to determine residence time of chemicals on the skin surface or examine the water gradient within the stratum corneum.

Palmar: pertaining to the palm of the hand

Partitioning: the transfer of a chemical from one compartment to another, usually from the solvent or vehicle into the epidermis.

Patch test: a test for allergic sensitivity in which a small quantity of a suspected allergen is applied to the skin

Pelage: the hair, fur or wool coat covering an animal

Penetrant: a generic term for a compound or chemical which is absorbed into the skin

Penetration: action of a substance entering and passing through a membrane, eg, stratum corneum (compare with permeation and absorption).

Percutaneous: through the skin following application to the surface

Permeation: action of a substance entering into a membrane, eg, into the stratum corneum

Photoallergy: a form of *ACD* requiring irradiation of the skin with ultraviolet light.

Photoirritation: a form of *ICD* requiring irradiation of the skin with ultraviolet light

PIF: photoirritation factor, a predictive model developed to identify whether chemicals are likely to be positive or negative for phototoxicity

PII: primary irritation index

pKa: acid dissociation constant. Represents the pH at which half the number of acid molecules in a solution are dissociated.

Plantar: pertaining to the sole of the foot

PPE: personal protective equipment, eg, gloves

Proliferation: the generation of cells by division and replication, eg, the formation of cells of the stratum basale prior to differentiation

Psoriasis: a chronic skin condition which essentially involves thickening of the skin as a result of hyperproliferation of underlying keratinocytes.

PUVA therapy: treatment for psoriasis (and other skin conditions) based on administration of psoralen followed by timely exposure to UVA radiation.

QSAR: see SAR.

Quantitative Structure Activity Relationship: see SAR.

REACH: Registration, Evaluation and Authorisation of Chemicals. An EU directive.

Receptor chamber: the chamber of an *in-vitro* diffusion cell situated beneath the skin section which holds the receptor fluid, also known as 'acceptor chamber'

Receptor fluid: the culture medium which sits beneath a skin section in an *in-vitro* percutaneous absorption study, also known as 'acceptor phase'

Rete ridge: a protrusion of the dermis, which interdigitates into the epidermis carrying blood vessels and providing a larger surface area for transfer of nutrients, oxygen and waste products

Risk: the probability of harm arising from exposure to a *hazard*.

RISKOFDERM: A project, funded by the European Commission, to develop a risk assessment and risk management toolkit for occupational dermal exposures

Risk Phrases: standardised system identifying the risk(s) of a substance or material. Examples include R21 (harmful in contact with skin), R24 (toxic in contact with skin), R27 (very toxic in contact with skin), R34 (causes burns), R35 (causes severe burns), R38 (irritating to skin), R43 (may cause sensitisation by skin contact) and R66 (repeated exposure may cause skin dryness or cracking).

ROAT: repeated open application test

ROS: reactive oxygen species.

Rubefacient: a substance which induces erythema when topically applied to the skin.

SAR: structure activity relationship, also known as 'QSAR – quantitative structure activity relationship'

SCC: Scientific Committee on Cosmetology. The 1997 precursor of the *SCCNFP*.

SCCNFP: Scientific Committee on Cosmetic Products and Non-Food Products. The pre-2004 precursor to the *SCCP*.

SCCP: Scientific Committee on Consumer Products, the EU group responsible for the EU Cosmetics Directive.

Scintillation: an emission of light (photon) produced by a material absorbing ionising radiation, used for detection and quantification of a radiolabelled compound, eg, analysis of receptor fluid samples by liquid scintillation counting following percutaneous absorption

Sebaceous gland: a duct attached to hair follicles responsible for the release of sebum onto the skin surface

Sebum: an oily substance containing lipids and waxes, released onto the skin surface from a sebaceous gland

SED: Systemic Exposure Dose

Shunt pathway: a route of dermal absorption of a penetrant, diffusing along appendages through the epidermis, such as hair follicles and sweat glands, also known as the 'appendageal' or 'follicular' route

Sk: a notation found in the *HSE* publication "*EH40*" which indicates that a substance which can readily penetrate skin.

Skin irritation: the result of a non-immunologically mediated response to a noxious substance (irritant). Compare with *skin sensitisation*. If sunlight is required to elicit this type of response, it is known as skin photoirritation (caused by a photoirritant).

Skin sensitisation: the result of an immune response to a substance (allergen or sensitising agent) or modified endogenous skin protein (caused by a reaction with a sensitiser). Compare with *skin irritant*. If sunlight is required to elicit this response, it is known as skin photosensitisation (caused by exposure to a photosensitiser).

SPF: sun protection factor. A measure of the effectiveness of a sunscreen.

SSWL: skin surface water loss. This is water vapour above the skin surface arising from a combination of sweat gland activity and transepidermal water loss (TEWL). Compare with TEWL.

Static: a type of diffusion cell in which the receptor fluid contained in the receptor chamber requires manual sampling, also known as 'Franz-type'

Stratum basale: the lowest layer of the epidermis providing the source of keratinocytes for differentiation into the upper layers

Stratum compactum: The lower region of the stratum corneum in which adjacent corneocytes are held firmly by competent corneodesomosomes.

Stratum corneum: the outermost layer of the epidermis comprised of corneocytes, also referred to as the 'horny layer'

Stratum dysjunctum: the outermost region of the stratum corneum in which adherence between adjacent corneocytes has been weakened by proteolytic cleavage of corneodesmosomes.

Stratum granulosum: the layer of the epidermis above the stratum spinosum, histologically has a characteristic 'granular' appearance

Stratum spinosum: the layer of the epidermis above the stratum basale, histologically has a characteristic 'spiny' appearance

Steady-state: refers to the condition when the amount of substance penetrating the skin per unit time is constant. Quantified by the steady-state flux (*Jss*).

STEL: short term exposure limit. A *WEL* prescribed in *EH40* which gives the maximum permissible exposure concentration for a 15 minute working period.

Structure Activity Relationship: see SAR.

Sunscreen: a substance or formulation containing a substance which, following topical application, can absorb UV radiation and thus provide protection of underlying skin from actinic damage.

Superficial plexus: the network of capillaries and blood vessels in the dermis

Tape-stripping: a technique used to measure the distribution or extent of percutaneous absorption into the stratum corneum. Performed by the repeated application and removal of adhesive tape to remove sequential layers of stratum corneum

TER: transepidermal electrical resistance, an *in-vitro* technique to measure the electrical impedance across a skin section as a marker of the structural integrity of the skin barrier

Terminal differentiation: the process by which cells undergo irreversible morphological and functional changes. Terminal differentiation of keratinocytes leads to the formation of the stratum corneum.

TEWL: transepidermal water loss (also referred to as insensible water loss); water vapour above the skin surface arising from evaporation of water from the skin surface to the air in the absence of sweat gland activity. TEWL is often used as a surrogate measure of the integrity of the skin's barrier function.

Thermodynamic activity (α): a measure of the effective concentration of molecule in a solution. Quantitatively, the thermodynamic activity of a molecule is equal to the product of concentration and activity coefficient.

Tight junction: see desmosome.

Transcellular: a route of dermal absorption of a penetrant, diffusing through the stratum corneum across the corneocytes and intercellular lipids, also known as 'intracellular'

Transepidermal water loss: see TEWL.

Transgenic: An organism which has undergone a genetic modification

TTC: Threshold of Toxicological Concern.

Urticaria: an immediate, immune-mediated skin reaction which can be caused by topical exposure to chemicals. Also known as the "wheal-and-flare" response or "hives".

UVR: ultraviolet radiation, can refer to either UVA or UVB, although is commonly used to indicate visible and UVA wavelengths of the solar spectrum.

Vasoconstriction: the narrowing of arterioles or capillaries, normally accompanied by a decrease in blood flow.

Vasodilation: the widening of arterioles or capillaries, normally accompanied by an increase in blood flow.

Vehicle: the solvent matrix or formulation in which a penetrant compound is applied to the skin surface

VICH: International Conference on Harmonisation of Technical Requirements for Registration of Veterinary Products

Vmax: the maximum velocity of an enzyme when all active sites are saturated with substrate

WEL: workplace exposure limit. See also, STEL and LTEL.

Wheal and flare: see urticaria.

Xenobiotic: a compound with a chemical structure which is foreign to a given organism, also known as 'exogenous'

Index

Note: page numbers in *italics* refer to figures and tables

absorption
  kinetics 94–5, *96*
  profile 94–5
  see also percutaneous absorption
acetyl coenzyme A 41
N-acetyltransferases 41–2
acid mantle 6, 8, 347
acitretin 242
acne
  occupational 274
  see also chloracne
acoustic wave propagation *203,* 204–5
actinic dermatitis 246
  hydration effects 213
acute toxicity studies 342
adherens junctions 18
adverse drug reactions (ADRs) 237–8, 347
  photosensitisation 252, 253
  topical therapy 242
  toxic epidermal necrolysis 240
adverse event reporting system (US FDA) 237
age, skin barrier function 76–7
ageing of skin 56
airborne exposure, occupational 287
alcohol dehydrogenase 36–8, 347
  activity 37, 38
aldehyde dehydrogenase 36–8, 347
  activity 37, 38
allergens
  patch testing 232
  sensitisation 236
  see also contact allergens
allergic contact dermatitis 151–2, 236, 347
  clinical features 155
  occupational 262–3
  photoallergy 251
allergy/allergic skin reactions 163–4
  cosmetics 319
  topical therapy 242
anaphylaxis/anaphylactoid reactions 237
angio-oedema 237, 238
animal studies
  analyses 119
  animal care/management 116, *117*
  ban for cosmetics 324–5, 336–7
  corrosion 342–4
  data analysis/reporting 119
  exposure duration 118
  fluorescent techniques 124
  guinea pig sensitisation 155–7
  irritation 342–4
  local lymph node assay 158–60
  microdialysis 120–2
  photoallergenicity assays 253
  phototoxicity assays 248, 249
  physiological responses 125–6
  punch biopsy 124–5
  radiometric techniques 124
  sampling 118, 119
  sensitisation assays 158–60, 341, 344
  short-term dermal toxicity 341–4
  skin absorption measurement 111, *112–14,* 115–19, 120–6
  skin membrane 136
  spectroscopic techniques 124

Principles and Practice of Skin Toxicology Edited by Robert P. Chilcott and Shirley Price
© 2008 John Wiley & Sons, Ltd

tape-stripping 122–3
terminal procedures 119
test substance 116
application to skin 117–18
tissue sectioning 124–5
see also guinea pig(s); local lymph node assay
Animals (Scientific Procedures) Act (1986) 115
anions, penetration 88
anthralin 61–2
anticancer drugs 61–2
antioxidants 59
apoptosis 61–3, 348
mitochondrial role 61–2
Approved Code of Practice for COSHH (AcoP) 300–3, 305, 347
arsenic, inorganic 274
atopic dermatitis 19, 260
atopy 225, 348
patch testing 233
automated procedures 286
cleaning 287
azathioprine 242

bacterial infections, occupational 274
barrier creams 294
barrier function, age effects 76–7
basal cell carcinoma 57
bases 88
bergapton 248
biobarriers 190
biological agents 243
biomechanical properties of skin *203,* 204–5
biophysical skin analysis 201
biopsy techniques 228–30
bleeding, occupational dermatitis 265–6
blood flow measurement *203,* 215–16
bulk transport pathways 10

cancer *see* skin cancer
cancer cells
DNA damage 62
mitochondria 62
ROS stress 59, 62
capacitance *203,* 210
capillary microscopy 216
carbon dioxide (dry ice) cleaning process 286
carboxyl esterases 34–5
carcinogenicity studies
pharmaceuticals 337
repeat dose dermal toxicity 339
carcinogens, mutagens or toxic to reproduction (CMR) 317
Carprofen 176–7
cations, penetration 88
CellSystems® 191
ceramides 17, 19–21, *22*
acyl chains *22*
formulae *20*
nomenclature 21
stratum corneum extracellular lipid matrix 12, 72
CES family 34–5
chamber test 234
charged molecules 86–8
chemical detection system (CDS) 190–1
chemical hazard limitation 285–6
chemicals
acute toxicity studies 342
carcinogens, mutagens or toxic to reproduction 317
concentration control 287–8
dermal toxicity 338
dilution control 287–8
EU policy 317–18
exposure limitation 289
handling equipment 288
industrial 336, 338
irritants 260–1, 282
monitoring 289
skin exposure reduction 288
use conditions 308–9
see also Registration, Evaluation, Authorisation of Chemicals (REACH)
Chemicals (Hazard Information and Packaging for Supply) Regulations (2002) 304, 348
chimney sweeps, scrotal cancer 274, 275
chloracne 236–7, 274
chlorinated hydrocarbons 236–7, 274
chlorobiphenyls 236
chloronaphthalenes 236
chlorophenols 237
chlorpromazine
human 3-D skin models 178, *179,* 180
neutral red uptake phototoxicity test 173–4
cholesterol
free 21
regional distribution 75
stratum corneum extracellular lipid matrix 12, 72
cholesterol esters 21
CIELAB colour scale 214, 348
cleaning, automatic 287

cocoaamidopropyl betaine (CAPB) 161–2
collagen, photoageing 56
conductance *203,* 210
confocal Raman spectroscopy *203,* 212
contact allergens 38, 151–2
  patch testing 232
  sensitisation 236
contact allergy 225
  occupational 262–3
  topical therapy 242
contact dermatitis *236*
  irritant 234–5, *236*
    occupational 260–1, *262,* 281
  see also allergic contact dermatitis
Control of Substances Hazardous to Health (COSHH), Regulations (UK) 283, 300–3, 305, 348
corneocyte envelope 17–18, 72
  proteins 19
corneocytes *9,* 10, *11,* 12, 17–18, 348
  cell–cell adhesion 23
  stratum corneum structure 71–2
corneodesmosomes 18, 23–4, 348
corrosion 185–6, 348
  animal studies 342–4
  scoring system 343–4
  in vitro assays 186–94
Corrositex™ test 186, 187–8, 190–1
corrosivity prediction 190, 192, *193*
corticosteroids, systemic/topical 242
Cosmetic, Toiletry and Perfumery Association (COLIPA) 316, 348
cosmetics 311–31
  adverse effects 318–20, 329
  allergy 319
  ancient use 319
  animal testing ban 324–5, 336–7
  anti-oxidants 321–2
  aromatherapy-based 320
  colouring agents 314–15, 316
  definitions 313
  environmental impact 318
  exposure levels 324, 327, *328*
  flavours 325
  fragrances 319, 325
  impurities 321
  information dossier 323, 330
  ingredients
    dossier for submission 323
    identity 320–2
    information sources 325–6
    listing 319, 321
    nomenclature 322
    restrictions 324–5
    testing requirements 324–5
    toxicity 320–2
    toxicological profile 322–4
  irritation 319
  labelling 317, *318,* 321, 322, 330
  marketing *318*
  monitoring 329
  'natural' products 320, 321
  packaging 317, *318*
  permitted ingredients 317
  plant extracts 320, 321
  preservatives 315, 316, 318, 321–2
  product categories *312*
  quality 322
  regulation 334–5
  restricted ingredients 317
  safety assessment 326–8
  safety assessor 326
  suppliers 313–14
    obligations 330–1
  UV absorbers 315, 316
  see also European Union Cosmetics Directive 76/768/EC
Cosmetics Ingredient Review (CIR, US) 316
cosmetovigilance 329, 348
curettage *229,* 230
cyclosporin 242
cytochrome P450 isoenzymes *25–6,* 27–9, *30–2,* 33–4, 348
  activity 28–9
    regulation 33–4
  expression 28
    regulation 33–4
  immunochemical detection 29
  localisation 29, 33
  molecular biological techniques 29, *30–2*
  percutaneous absorption effects 33
  selective probe substrates 28–9
  toxicity effects 33

Declaration of Helsinki 111
delayed-type hypersensitivity reactions *see* hypersensitivity reactions, Type IV
depigmentation, occupational vitiligo 276
dermal exposure management, occupational 284–9
dermal toxicity
  exposure 338–9
  factors 338–9

formulations 338
product groups 336–8
repeat dose 339, *340,* 341
short-term studies 341–4
study designs 339, *340,* 341
dermatitis, occupational 260–3
actions after assessment 271–2
clinical assessment 267–8
development 263–4
diagnosis 267
follow up 272–3
identification 266–7
incidence 265
mitigation *271,* 272
investigations 267–73
outlook 266
patch testing 267
patterns 264–5
psychological impact 266
treatment 264
work impact 265–6
workplace assessment 269–71
dermatoglyphs 4, *6,* 348
dermatology 223–43
dermatomed 349
dermatoscopy 226–7
dermis 13, *14,* 349
dermo-epidermal junction 13
desmosomes *9,* 10, 349
desquamation 18, 23–4, 349
measurement 205
detector pads 293
diascopy 226
diffusion
concepts 97
up a gradient *99*
see also Fick's laws of diffusion
diffusion cells 349
classification 131
penetrant application 140–1
in vitro skin absorption measurement 140–1
choice for 131, *132,* 133–6
diffusion coefficient (D) 94, 101, 102–5
hydrogen bonding 102–5
phenols 104–5
diffusivity 102–5
3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide *see* MTT
dioxins 237
DNA damage
cancer development 62
ultraviolet radiation 55, 56–7, 58
drug reactions
erythema multiforme 239
fixed eruptions 240
photosensitisation 245–6, 252, 253
phototoxic 240, *241,* 248
rashes 237–8
toxic epidermal necrolysis 240
see also adverse drug reactions (ADRs)

EC3 values 160–1, 162, 349
efaluzimab 243
electron microscopy 231
elicitation, allergic contact dermatitis 236
elimination studies 120, *121*
elliptical surgical biopsy 230
employers, occupational skin exposure 303–4
EpiDerm™
corrosion assays 187, *188,* 191, 192–3
irritation assays 194, 195, *196,* 197
phototoxicity assays 178, *179*
epidermis 8–13, *14,* 17, 349
apical migration 9–10
cells *9*
differentiation 18–19
function 8–9
layers 9
lipid synthesis 19–20
protein synthesis 18–19
thickness *5, 7*
epiluminescence microscopy 226–7
Episkin™
corrosion assays 187, 188, 191, 192, 193
irritation assays 194, 195, *196*
epoxy resin exposure, allergic contact dermatitis *263*
equipment design, workplace 285
erythema multiforme 239
ester pro-drug delivery 36
esterases 34–6
activity 35–6
esters, hydrolysis 35–6
Etanercept 243
ethics, *in vivo* measurements of skin absorption 110–11, 115
eumelanin 52, 53
synthesis 53, *54*
European Centre on Validation of Alternative Methods (EVCAM) 324, *325,* 349

European Federation for Cosmetic Ingredients (EFfCI) 322, 349
European Union Cosmetics Directive 76/768/EC 312–16
  animal testing ban 324–5, 336–7
  ingredient listing 319
  requirements 315–16
  safety assessment 326–8
  safety assessors 326
  scientific advice 316
  structure 314
European Union Dangerous Substances Directive (67/548/EC) 317, 323
European Union Regulation on Registration, Evaluation, Assessment and Restriction of Chemicals (REACH) 299
examination of skin 226–34
exanthematous reactions 237
  pustulosis 238
excision biopsy *229*
exudate, occupational dermatitis 265–6

Fick's laws of diffusion 79, 97–8
  partitioning 101
filaggrin *9,* 18, 76
finite dose
  conditions 95, *96*
  percutaneous absorption study 143–5
Fitzpatrick skin colour scale 52
fixed drug eruptions 240
FLG (filaggrin) gene 18
flow-through diffusion cells 131, 133, 134–6, 350
fluorescence microscopy, vascular perfusion measurement 216
fluorescent techniques 123–4
fluorochrome dyes 230–1
flux (J) 94, 95, 350, 351
Food and Drug Administration (FDA, US) 237, 350
formaldehyde 305–6
formulations 338
Fourier transformed infrared (FTIR) spectroscopy 124
fragrances 319, 325
free fatty acids 23
  function in stratum corneum 23
  stratum corneum extracellular lipid matrix 12, 72
fugacity 98–9, 350
furocoumarins, phototoxicity 248

gangrene, peripheral 238
Gaucher disease, type 2 19
genodermatosis 225, 350
Germany, legislation 300, 301–2
gloves 289–93
  damaged 290
  degradation 290
  detector pads 293
  disposal 290, 293
  guidance for use 309–10
  hand contamination 290, *291*
  misuse 290
  performance *293*
  permeation 292–3
  selection 289–90
  skin exposure reduction 288
  storage 290
glucocerebrosidase 19
glucosyl ceramides 19–20, 21
glucuronyl transferase 40
glutathione transferase 39–40
Good Laboratory Practice (GLP) 323, 350
Good Manufacturing Practice (GMP) 322, 350
Göttingen minipig 14
guinea pig(s)
  short-term dermal toxicity 341
  skin sensitising chemicals testing 155–7, 160
guinea pig maximisation test 155, 156, 350
  test results 160

hair dyes 319
hair follicles 4, *6,* 11–12
  percutaneous absorption 77–9
hand washing, occupational skin disorders 306
handling equipment 288
hCEs 34–5
Health and Safety at Work etc Act (1974) 300, 305
healthcare workers, rubber latex allergy 274
henna 319
histidine, photo-oxidation 175–6
hives 273–4
human 3-D skin models 170–1, 177–8, *179,* 180
  corrosivity assay 186, 187, 191–4
  irritation assays 194–5, *196,* 197
  methods 178
  neutral red uptake phototoxicity test comparison 180
human repeated insult patch test (HRIPT) 161, *162,* 350

human serum albumin (HSA), photobinding assays 175–7
human skin model corrosivity test 188
human studies
fluorescent techniques 124
microdialysis 120–2
photoallergenicity assays 253–5
phototoxicity assays 249–51
physiological responses 125–6
punch biopsy 125
radiometric techniques 124
skin absorption measurement 111, 120–2
skin membrane 136
spectroscopic techniques 124
tape-stripping 122–3
tissue sectioning 124–5
hydration of skin
dermal toxicity relationship 213
electrical methods *203,* 210–11
measurement *203,* 209–12
guidance 212
stratum corneum 213
hydrogen bonding 88–9, 102–5
position of groups 103–4
hydroquinone 276
6-hydroxysphingosine 20, *21*
hypersensitivity reactions
classification *153*
Type I (immediate) 233, 247, 252
Type IV (delayed) 174, 240, 247, 251
allergic contact dermatitis 262
patch testing 232
photoallergy 174

ichthyosis
lamellar 23
vulgaris 18
X-linked 24
immune response 152
immunoenzyme (immunoperoxidase) methods 231
immunofluorescence techniques 230–1
immunologic contact urticaria 163–4
immunopathology 230–1
impedance *203,* 210–11
in vitro mouse skin integrity function test (SIFT) 194
indentometry *203,* 204
infection
occupational skin disorders 274–5
secondary in occupational dermatitis 265
infinite dose
conditions 95, 351
percutaneous absorption study 142, *143, 144*
infinite sink condition 95
inflammation, skin sensitisation elicitation phase 155
infliximab 243
inhalation exposure 302–3
instruments for skin toxicity measurement 201–17
International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) 334, 349
International Nomenclature Cosmetic Ingredient (INCI) 322, 351
involucrin 19
ionising radiation 274
irritant(s)
chemical 260–1, 282
occupational exposure 281–2
irritant contact dermatitis 234–5, *236,* 351
occupational 260–1, *262,* 280
irritation 185–6, 355
animal studies 342–4
cosmetics 319
hydration effects 213
prediction 195, *196*
scoring system 343–4
topical therapy 242
in vitro assays 194–5, *196,* 197
itch 225

Japan, definition of cosmetics 313

keratinocyte transglutaminase 1 gene 19
keratinocytes 10, 351
differentiation 28–9
photoageing 56
response to skin sensitisers 153

lamellar bodies *9,* 17, 19–20
Langerhans cells 10, 352
response to skin sensitisers 153–4
Langer's lines 4, 6, 351
laser Doppler velocimetry/flowmetry *203,* 215–16, 352
latex allergy 274
LD_{50} 342, 352

legislation
occupational exposure 299–310
United Kingdom legislation 300–3
in vivo measurements of skin absorption 110–11, *112–14,* 115
levarometry *203,* 204
lipids 17
stratum corneum 23
synthesis during epidermal differentiation 19–20
lipophilicity 85–6, 352
livedo reticularis 238
local lymph node assay 158–60, 341, 344, 352
EC3 values 160–1, 162
relative potency assessment 160, 161, 163
Log P 85, 86, *87,* 352
diffusion up a gradient *99*
loricrin 19

machining operations 288
maculopapular reactions 237
malignant melanoma 53, 56–8
occupational 274
Management of Health and Safety at Work Regulations (UK) 306–7
Margin of Safety (MOS) 327, 352
material safety data sheets 282–3, 352
hazard identification 304, 306
personal protective equipment recommendations 289–90
medical conditions with cutaneous features 225
medical devices 335–6
dermal toxicity 337–8
melanin 352
photoprotective role 53
skin colour 52
melanocortin 1 receptor (MC1R) gene 63
melanocytes 10, 53, 352
photoageing 56
melanogenesis 51, 53, *54,* 55, 352
melanosomes 53
transfer 55
metalworking fluids 287–8, 305–6
metamerism 214
methacrylate allergy 309
methotrexate 242
micro-autoradiography *146,* 147, 352
microdialysis 120–2, 352
mitochondria
apoptosis 61–2
cancer cells 62
drug damage 62–3
function modulation as drug treatment 62–3
toxicity 63
mitochondrial DNA (mtDNA)
mutations in human cancers 62
photodamage 60–1
rearrangements 59
sun exposure biomarker 60–1
molecular weight 85, 86
monolayer cultures 172–4
MTT 352
corrosion assays 191, 192–4
irritation assays 195, *196,* 197
phototoxicity assays 177, 178, *179*

N-acetyltransferases 41–2
NAD(P)H quinone reductase 38–9
nails
occupational disorders 275
photo-onycholysis 240
nanoparticles 77, 79–80
natural moisturising factor (NMF) *9,* 18, 213
neutral red uptake phototoxicity test (NRU PT) 170, 353
human 3-D skin model comparison 180
monolayer cultures 172–4
protocol 173
Nikolsky's sign 239
no effect level (NOEL) 162
no observed adverse effect levels (NOAEL) 323, 324, 327, 353
non-melanoma skin cancer 57
mtDNA biomarker 60–1
non-perfused pig ear model 194
non-steroidal anti-inflammatory drugs (NSAIDs) 353
phototoxicity 248, 253
Nuremburg Code 111

occluded patch test 155
occlusion 140, 353
dermal toxicity studies 341
percutaneous absorption 90
topical therapy absorption 242
occupational health practitioners 270–1
occupational health service 267, 268
occupational skin disorders 259–76
acne 274
airborne exposure 287

barrier creams 294
chain of responsibility 283–4
chemical hazard limitation 285–6
contact urticaria 273–4
definition 280–2
dermal exposure management 284–9
dermatitis 260–3, 281
development 263–4
diagnosis 267
identification 266–7
incidence 265
investigations 267–73
outlook 266
patch testing 267
patterns 264–5
psychological impact 266
treatment 264
work impact 265–6
education 294
engineering controls 286–8
exposure elimination 285
infections 274–5
legislation 299–310
material safety data sheets 282–3, 289–90
nails 275
prevention 279–94
process controls 286–8
safe working practices 288–9
skin cancer 274, 275
skin care 280
skin management 280
training 294
vitiligo 276
water exposure 280–1, 282, 306
see also chemicals; gloves; personal protective equipment
occupational skin exposure
adequately controlled 302–3
employers 303–4
hazard identification 304–6
individual susceptibility 303, *304*
legal aspects 299–310
production workers 336
threshold level 303
UK legislation 300–3
octanol–water partition coefficient (Log P) 85, 86, *87,* 352
diffusion up a gradient *99*
oils
acne 274
see also metalworking fluids
optothermal transient emission radiometry (OTTER) *203,* 211–12, 353
Organisation for Economic and Co-operative Development (OECD) 353
Guideline 404 185–6
Guideline 427 115–19
Guideline 428 129, *130,* 157
Guideline 429 158
Guideline 431 188, 191
Guideline 432 173
oxidative stress 58

paints, solvent-/water-based 286
panniculus carnosus *14*
Paracelsus 83, 84
parasitic skin disease, occupational 275
partitioning 101, 353
patch testing 232–4, 353
occupational dermatitis 267
see also human repeated insult patch test (HRIPT)
pelage 353
density 13–14
penetrants 353
in vitro skin absorption measurement 140–1
penetration 94–5, *96,* 353
maximum rate (Jmax) 95, *96*
profile *95, 96*
rate 95
percutaneous absorption
age effects 76–7
alcohol dehydrogenase activity 37
aldehyde dehydrogenase activity 37
animal studies 111, *112–14,* 115–19, 120–2
data 105–6
elimination studies 120, *121*
esterase activity 35–6
exposure 89–91
fluorescent techniques 123–4
hair follicles 77–9
human studies 111, 120–2
intra-/inter-individual variation 75–6
microdialysis 120–2
molecular weight 85
occlusion 90
partitioning 101
passive process 83–4
penetration prediction 146
physicochemical factors 83–91

percutaneous absorption (*continued*)
physiological responses 125–6
punch biopsy 124–5
radiometric techniques 123–4
in silico models 109, 110
skin appendages 77–80, 87
skin treatment 90–1
species differences 75
spectroscopic techniques 123–4
tissue sectioning 124–5
vehicles 89–90
in vitro measurement 129–47
data assessment 138
diffusion cell choice 131, *132,* 133–6
finite dose study 143–5
infinite dose study 142, *143, 144*
mass balance 141
micro-autoradiography *146,* 147
models 109, 110
principle 131
radiolabelling of test compound 139
receptor fluid 138–9
regulatory guidelines 129, *130,* 131
sampling 138–9
skin application 140–2
skin integrity measurement 137–8
skin membrane 136
tape-stripping 141
vehicles 140
in vivo measurements 109–26
alternative methods 119–26
ethics 110–11, 115
legislation 110–11, *112–14,* 115
OECD Guideline 427 method 115–19
volatility 90
percutaneous toxicity 83
permeability coefficient (Kp) 94, 351
permeability of skin 6
regional/species variations 74–5
permeation breakthrough time (BTT) 292
personal protective equipment 288, 289–93, 354
guidance for use 309–10
selection 289–90
pesticides 336
petroleum derivatives 274
pharmaceuticals 335
acute toxicity studies 342
dermal toxicity 337
veterinary 336
see also adverse drug reactions (ADRs); drug reactions
Phase I metabolism 24, 27
detoxification enzymes 27–39
Phase II metabolism 27
enzymes 39–42
phenols
diffusion coefficients 104–5
planes of symmetry *105*
P–phenylenediamine (PPD) 319
finite dose study 143–5
pheomelanin 52, 53
synthesis 53, *54*
photoageing 56
photoallergenicity assays
in vitro 174–7
in vivo 253–5
photoallergens 251–3
discrimination from photoirritants 175–6
neutral red uptake phototoxicity test 170
photoallergy 174, *246,* 251–3, 353
grading *255*
sunscreens 240–1
photobinding assays 175–7
photobiology of skin 51–65
photocontact sensitisers 252
photodamage, mtDNA 60–1
photodermatoses, idiopathic 246
photogenotoxins, neutral red uptake phototoxicity test 170
photoirritants
discrimination from photoallergens 175–6
neutral red uptake phototoxicity test 170
photobinding to protein 175
photoirritation *246,* 353
human tests 249–51
reactions 247–8
photoirritation factor (PIF) 172, 173, 174, 353
photomaximisation test 253–5
photo-onycholysis 240
photopatch testing 232–3
photoplethysmography (PPG) 216
photoprotection 51–3, *54,* 55
melanin role 53
skin cancer 63, 64
photosensitisation, drug reactions 245–6
photosensitive reactions 251–3
phototherapy, safety 61
phototoxic drug reactions 240, *241,* 248
phototoxicity assays, *in vitro* 169–81
human 3-D skin models 177–8, *179,* 180
monolayer culture use 172–4
strategies 169–71

phototoxicity assays, *in vivo* 248, 249–51
animal models 248, 249
phototoxicity reactions 247–8
agents 248
grading system 251
physical agents, irritant dermatitis 261
physiological response measurement 125–6
phytophotodermatitis 248
phytosphingosine 20, *21*
pig ear model, non-perfused 194
plant extracts, cosmetics 320, 321
plant protection products 336
platysma muscle *14*
polychlorinated biphenyls (PCBs) 237
polycyclic hydrocarbons 274
polymorphic light reaction 246
potassium hydroxide (KOH) preparation of skin scrapings 228
Potts, Sir Percival 275
Potts and Guy equation 85, 86
Prediskin™ 194
preservatives, cosmetics 315, 316, 318, 321–2
prick testing 233–4
contact urticaria 273
Primary Irritation Index (PII) 343–4, 354
process controls 286–8
product groups 335–6
dermal toxicity 336–8
factors 338–9
repeat dose 339, *340,* 341
formulation 338
Product Information Package, cosmetics 330
production worker exposure 336
profilaggrin *9,* 18
protective creams 294
protein synthesis, epidermal 18–19
proteins, photobinding assays 175–6
pruritis 225
psoralens 252
phototoxicity 248
psoriasis 354
hydration effects 213
impact 226
mitochondrial apoptosis induction 61–2
psychological impact, occupational dermatitis 266
punch biopsy 124–5, *229,* 230
pustulosis, exanthematous 238
PUVA phototherapy 252, 354

quinones 38–9

radiolabelling of test compound 139
radiometric techniques 123–4
Raman spectroscopy *203,* 212
random molecular motions 98
rapid alert system for dangerous non-food products (RAPEX) 314
rash 225
drug 237–8
rat skin transcutaneous electrical resistance (TER) test 186, 187, 188–91
reactive nitrogen species (RNS) 58
reactive oxygen species (ROS) 58, 354
cancer cell stress 59, 62
receptor solution 138–9
refractometer 288
Registration, Evaluation, Authorisation of Chemicals (REACH) 299, 317–18, 323, 325, 334, 354
exposures 336
regulation 334–5
repeated open application test (ROAT) 233, 354
respiratory uptake 302–3
resveratrol 59
retinoids *242*
risk assessment
chemical exposure management system *280*
chemicals 307–9
COSHH Regulations *283*–4
in vitro phototoxicity assays 170, 180
local lymph node assay 160–1, *162*
occupational skin exposure *272*, 306–9
percutaneous absorption 105–6
RISKOFDERM 354
sensitisation 160–4
sensitisation assays 160–4
skin absorption 109, 123
skin membrane considerations 136
vehicle and penetrant considerations 140
risk phrases 306, 354
rubber latex allergy 274
rule of 500 12, 85

safe working practices 288–9
safety data sheets 282–3, 352
hazard identification 304, 306
personal protective equipment recommendations 289–90
sarcoidosis 226
Scientific Committee on Consumer Products (SCCP, EU) 316, 323, 355

scintillation counting 355
dual label 137–8
scrotal cancer, chimney sweeps 274, 275
sebaceous glands 4, 8, 355
sebum 6, *7,* 355
measurement *203,* 205
sensitisation 151–64, 355
allergic contact dermatitis 236
elicitation phase 155
photocontact 252
risk 152
quantitative 163
sensitisation assays
animal studies 158–60, 341, 344
false positive/negative 159
murine 158–60
see also guinea pig(s); local lymph node assay
sensitisers 151
epidermal bioavailability 153
identification 155–60
keratinocyte response 153
Langerhans cells response 153–4
reactive chemistry 153
T cell response 153–4
shave biopsy *229,* 230
shunt transport pathways 10, 11–12, 79, 355
Sk notation 302
skin
anatomical regional differences 4
anatomy 3–15
bioengineering 201
biopsy 228–30
biotransformations 24, *25–6,* 27–9, *30–2,* 33–42
care in occupational skin disorders 280
characteristics 4, *5*
cleavage lines 4, 6
entry route 71–80
glabrous 4, 350
muscle layer 14
non-glabrous 4
phototypes *247*
physicochemical properties 84–9
species differences 13–14
structure 8
regional/species variation 72, *73,* 74
surface features 3–4, *5,* 6, *7,* 8
thickness measurement *203,* 205
see also percutaneous absorption
skin appendages 348
percutaneous absorption 77–80, 87
skin cancer 56–8, 274, 275, 348
early warning 61
non-melanoma 57
mtDNA biomarker 60–1
sun protection 63, 64
see also malignant melanoma
skin colour 52
CIELAB colour scale 214, 348
measurement *203,* 213–15
guidelines 215
skin disorders 223–43
clinical assessment 224–34
cracks in occupational dermatitis 265
drug history 225
examination of skin 226–34
familial 225
family history 225
history taking 225–6
management 224
occupational 259–76
management 280
occupational history 225
treatment 224, 241–3
skin integrity
measurement 137–8
receptor fluid 139
in vitro mouse function test 194
skin membrane, *in vitro* skin absorption measurement 136
3-D skin model *see* human 3-D skin models
skin prick testing 233–4
contact urticaria 273
skin reflectance colorimetry 213–14
skin reflectance spectroscopy (SRS) 213–14
skin sandwich *in vitro* model 78–9
skin scrapings 227–8
skin surface
contours 205
pH 202, *203*
skin surface water loss (SSWL) 206, 353
skin treatment, percutaneous absorption 90–1
SkinEthic™ 191
solar actinosis 246
hydration effects 213
solar urticaria 246
photoallergy 252
solubility 85, *87*
solute concentration
stratum corneum penetration *101*
thermodynamic activity 100
solvents
occupational skin exposure 303

reclaimed 286
skin exposure reduction 288
spectrophotometry *203,* 213–14
spectroscopic techniques 123–4
sphingolipids, regional distribution 75
sphingosine 20, *21*
squamous cell carcinoma 57
static diffusion cells 131, *132,* 133–4, 355
steady state 93, 95, 356
Stevens–Johnson syndrome 239
stratum corneum 9, 10–11, 355
adhesion 23–4
anatomy 71–2
brick and mortar model 10, 71–2
concentration profile of penetrant *101*
desquamation 18, 23–4, 349
measurement 205
extracellular lipid matrix 12, 72
guinea pig *14*
hydration 213
hydrogen bonding groups 88–9
ion absorption 86–7
lamellae 23
lipid lamellae 12
lipids 20–1, *22,* 23
molecule size penetration 12–13
pH 88
renewal 18
routes for chemical passage 72
structure 17–18
tape-stripping 122–3
thickness 72, 74, 79
kinetic parameters 106
TEWL effects 209
turnover 23–4
stratum corneum chymotryptic enzyme (SCCE) 24
stratum corneum tryptic enzyme (SCTE) 24
stratum granulosum 9, 17, 356
stratum spinosum 9, 356
structure activity relationship (SAR) evaluation 186, 354
sucrose, dual label scintillation counting 137–8
suction of skin *203,* 204
sulphotransferases 41
sun exposure
mtDNA biomarker 60–1
see also solar *entries*
sun protection 63–4
sun protection factor (SPF) 63–4, 355
sunscreens 63–4, 356
photoallergy 240–1
surface area, whole body 105–6
sweat glands 4, 8
TEWL effects 209
sweating, hydration measurement effects 212
Systemic Exposure Dosage (SED) 327, 355
systemic lupus erythematosus (SLE) 225
UVR exposure 246
systemic therapies 242–3
systemic toxicity 186

T cells, skin sensitiser response 153–4
tanning, delayed/immediate 55
tape-stripping 122–3, 141, 356
in vitro skin absorption measurement 145, 146
tar derivatives 274
test compound 125–6
application to skin 140–2
buffering capacity 186
corrosive 190
elimination studies 120, *121*
fluorescent techniques 123–4
microdialysis 120–2
non-corrosive 190
OECD Guideline 427 115–19
pH 186
physiological responses 125–6
punch biopsy 124–5
radiolabelling 139
radiometric techniques 123–4
receptor fluid 138–9
spectroscopic techniques 123–4
tape-stripping 122–3
tissue sectioning 124–5
vehicles 140
tetraglycidyl methylene dianiline (TGMDA) 309
thermodynamic activity 98–9, 356
different vehicles 100
maximum 99
thermodynamic gradient 93
thermodynamics 93–106
thermopile 250–1
Threshold of Toxicological Concern (TTC) 324, 357
tight junctions 18
tissue sectioning 124–5
topical therapy 241–2
toxic epidermal necrolysis 239–40

toxicity, percutaneous 83
transcutaneous electrical resistance test, rat skin 186, 187, 188–91
transdermal drug delivery 90, 336
transepidermal electrical resistance (TER) 137, 356
transepidermal water loss (TEWL) 74, 137, 356
  applications *203,* 206–8
  closed chamber method
    vapour accumulation *203,* 206, *207*
    ventilated *203,* 207–8
  condenser chamber *203,* 208, *209*
  measurement *203,* 206–8, *210*
    guidance 208–9
  non-perfused pig ear model 194
  open chamber method *203,* 206, *207*
  in vitro mouse skin integrity test 194
transferases 39–42
transglutaminases 19
transmissible spongiform encephalopathy (TSE) risk materials 316
triglycerides 23
tri-stimulus *203,* 213
tritiated water permeability 137
twistometry *203,* 204
tyrosinase 53, *54,* 55
tyrosine 53, *54,* 55

ubiquinone 59
UDP-glucuronyl transferase 40
ultraviolet radiation (UVR) *52,* 53, 55–9, 357
  ageing of skin 56
  cutaneous reactions 245–56
  DNA damage 55, 56–7, 58
  environmental exposure 55–9
  exposure 55
  oxidative stress 58
  pathological effects 55–9
  skin cancer 56–8, 274
  skin reactions 247
  sunscreens 63–4
  see also UVA; UVB
United Kingdom legislation 300–3
urinary excretion, test compound 120, *121*
urticaria 357
  acute 238
  contact 273–4
  drug-induced 237, 238
  immunologic contact 163–4
  solar 246, 252
UVA 55
  measurement 171
  oxidative stress 58
  photoageing 56
  photoallergenicity assays 253
  phototoxicity 248
    assays 249, *250*
  skin damage 63
  sunscreens 64
UVB 55
  measurement 171
  oxidative stress 58
  photoallergenicity assays 253
  skin damage 63
  sun exposure 247
  sunscreens 64
UV/visible absorption spectrum
  measurement 169–70
  pre-screen for phototoxicity 171

vascular perfusion measurement *203,* 215–16
vasculitis 238
vehicles 357
  absorption of substance from 99–100
  dermal toxicity studies 341
  percutaneous absorption 89–90, 140
  topical therapy 242
  in vitro skin absorption studies 140
ventilation, local exhaust 287
veterinary pharmaceuticals 336
viral infections, occupational 274
vitamin E, delivery 6
vitamin E acetate 76
vitiligo, occupational 276
volatility, percutaneous absorption 90

water exposure, occupational skin disorders 280–1, 282, 306
weak acids 88
white blood cells, classification 152
whole body surface area 105–6
Wood's light examination 227, *228*
workplace design 285
workplace exposure limits (WELs) 302–3, 357

xenobiotic metabolism 24, *25–6,* 27, 357

yeast infections, occupational 275
Yellow Card system (UK) 237–8

zwitterions 88

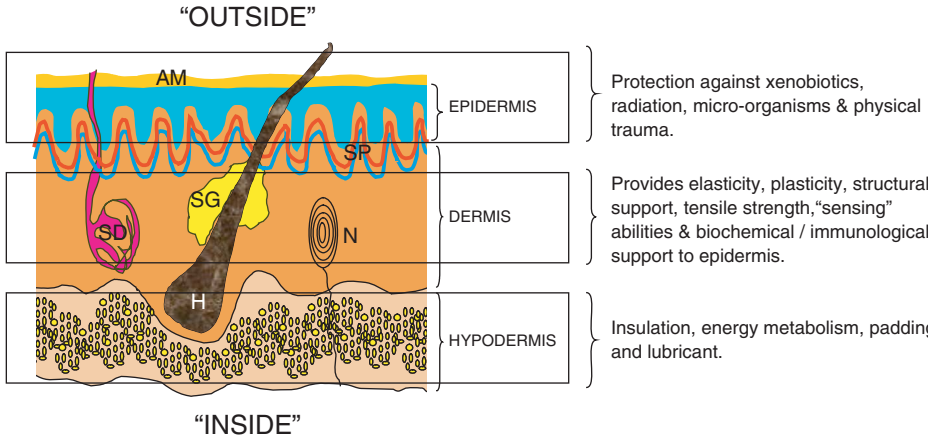


Plate 1 Schematic representation of skin structure and associated functions. Note that the relative thickness of each layer is not to scale (see text). Several adnexal structures are shown (SP = superficial plexus; SG = sebaceous gland; SD = sweat duct; N = Pacinian corpuscle; H = hair). In humans the skin is covered with a thin layer of lipids known as the acid mantle (AM), which comprises sebum, cell debris and sweat residua

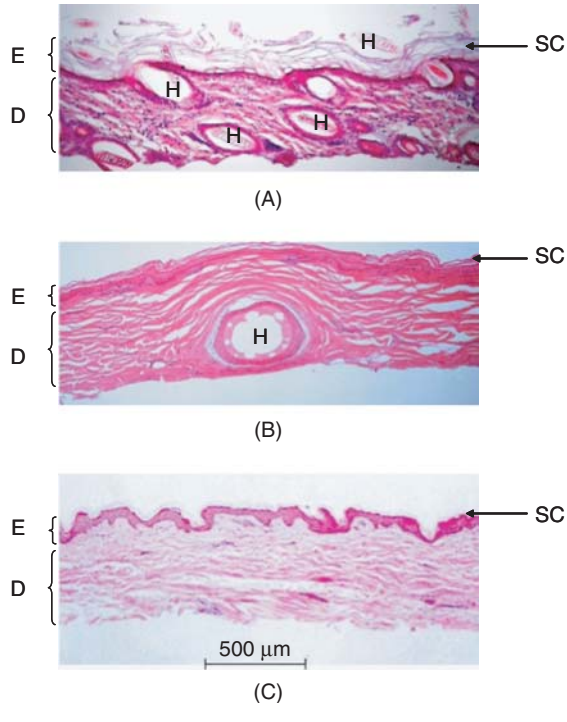


Plate 2 Representative sections of dermatomed guinea pig (A), pig (B) and human (C) skin. Two principal layers are discernible in each section: the epidermis (E) and dermis (D). Note that guinea pig stratum corneum (SC) appears as an incoherent, flaky layer whereas SC of pig and human retains a flatter, more compact appearance. A large number of hairs (H) are present in the guinea pig section

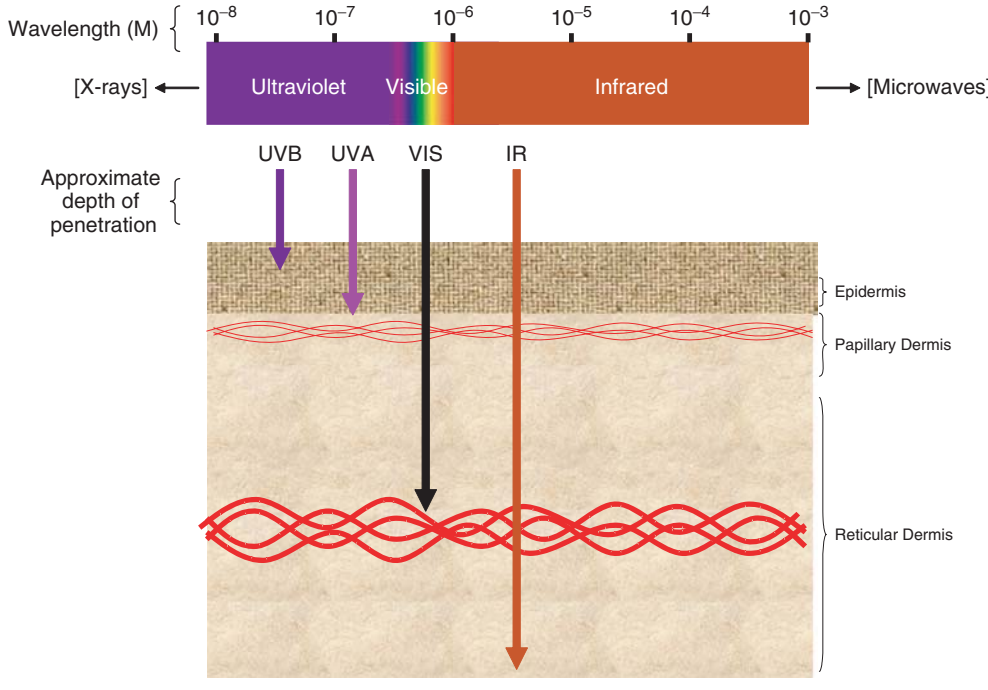


Plate 3 Representation of the solar spectrum and approximation of the relative depth to which UVB, UVA, visible and IR radiation penetrates cutaneous tissue. Note that the representation of skin tissue is not to scale: the epidermis is actually much thinner in comparison with dermal tissue

Paracelsus, also known as Theophrastus Phillippus Aureolus Bombastus von Hohenheim (circa 1493 – 1541), was a largely self-taught polymath who recognised the dose-response relationship which is an underpinning principal of modern toxicology.

A somewhat interesting character, he roamed Europe, north Africa and parts of Asia in his pursuit of alternative medical knowledge. His published works, personal activities and outspoken criticism of contemporaneous medical practices did not particularly endear him to his peers!

Plate 4 Paracelsus

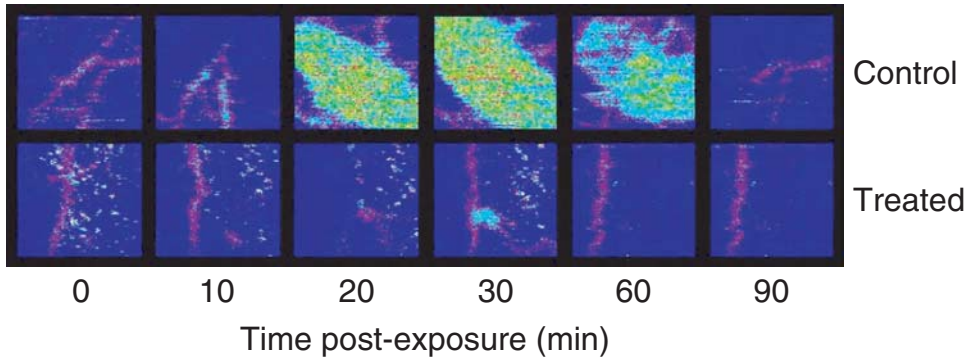


Plate 5 Vasodilation (measured by laser Doppler imaging) caused by topical application of a rubefacient (methylnicotinate) to normal skin (control) and following application of a barrier cream (treated). The appearance of a region of bright colours (20–60 minutes; control skin) indicates areas of higher blood perfusion in response to the rubefacient

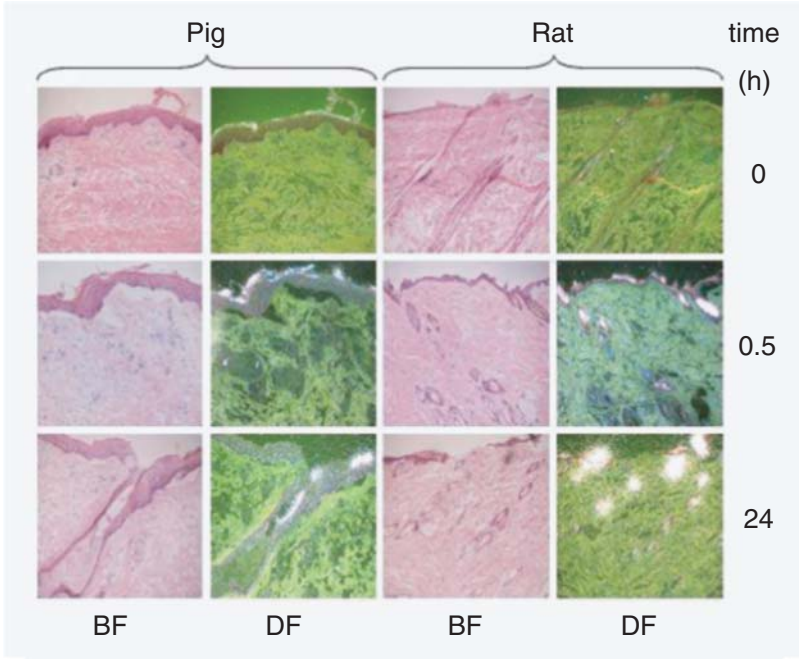


Plate 6 Representative micro-autoradiographs of pig and rat skin treated with PPD in a hair dye formulation. Skin sections observed under bright field (BF) and dark field (DF) illumination before exposure (0) and 30 minutes and 24 hours post exposure. Immediately prior to dosing (0 minutes), very few silver grains are visible on the bright field illumination due to natural background radiation. These sections serve as controls against which treated samples are compared. (Note that the stratum corneum possesses some inherent auto-fluorescence.) Skin excised 30 minutes post exposure illustrates deposition of silver grains on the surface of the skin and within the epidermis, with low levels in the dermis. After 24 hours, the distribution of grains can be seen to be associated with the skin surface and hair follicles, with material localised in the follicle opening

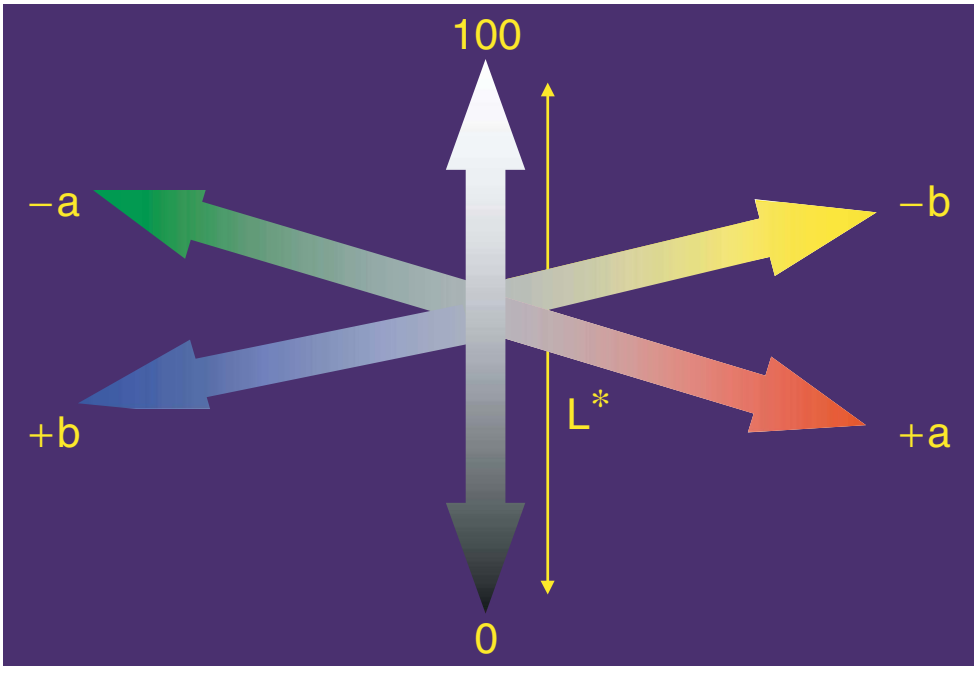


Plate 7 CIELAB colour scale. The three basic parameters represent the red–green index (a*), blue–yellow index (b*) and brightness (L*). The a* and L* parameters are most commonly used to quantify erythema and pigmentation, respectively

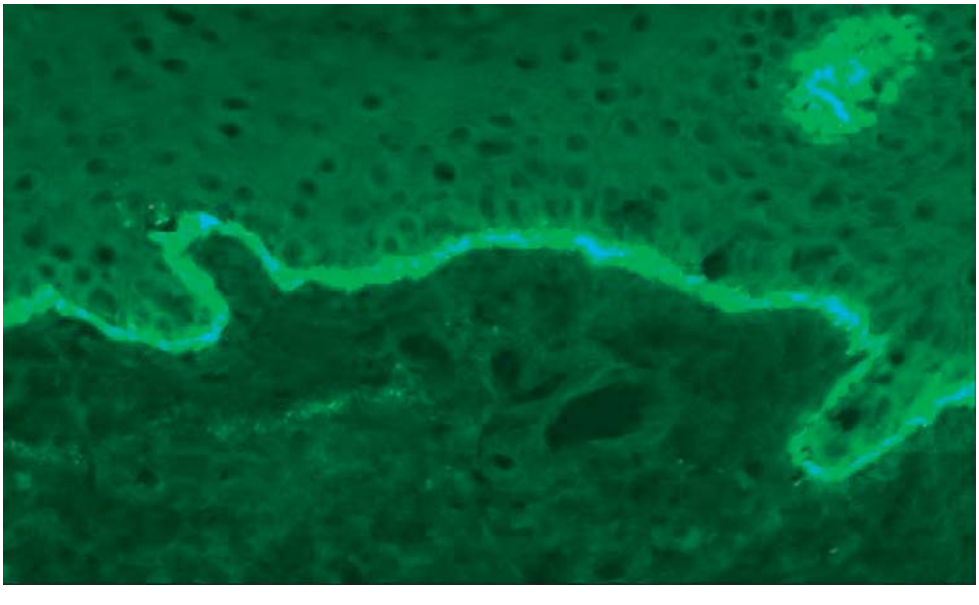


Plate 8 Direct immunofluorescence showing linear deposition of Ig G and Complement C3 at the dermo–epidermal junction

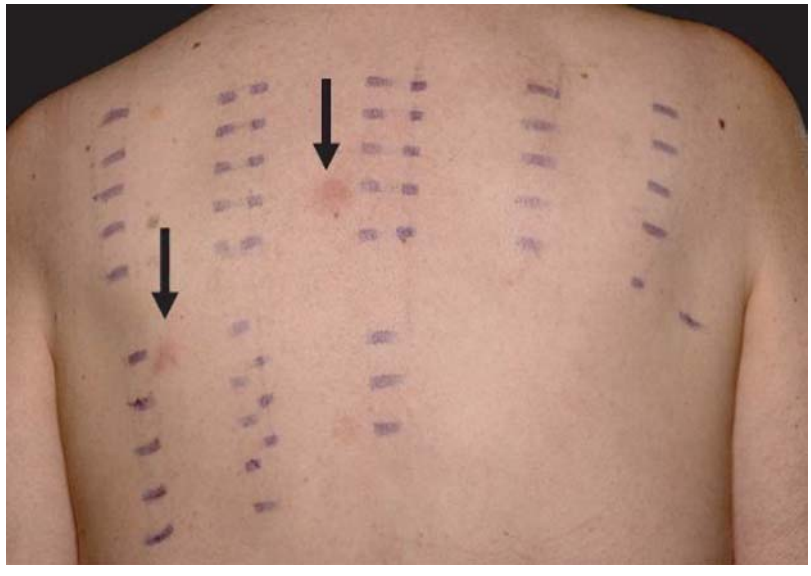


Plate 9 Patch testing; reading on Day 4 showing two positive reactions (indicated by arrows)

Plate 10 Severe irritant contact dermatitis (ICD) of lower limbs (Note acute eczematous rash with a sharp cut-off, corresponding to the areas of contact with the irritant)

Plate 11 Erythema multiforme

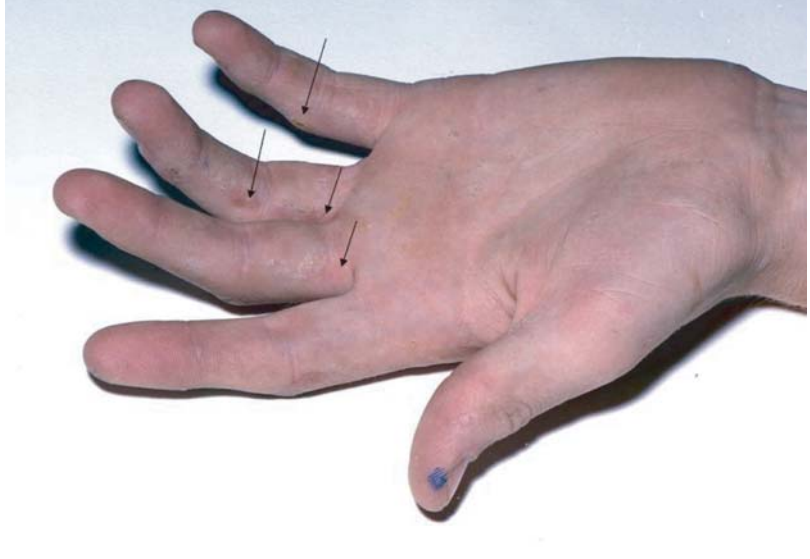


Plate 12 Early probable contact dermatitis due to chronic exposure to mild irritant (Arrows indicate regions of red, dry, scaly skin)

Plate 13 Acute dermatitis of unknown origin

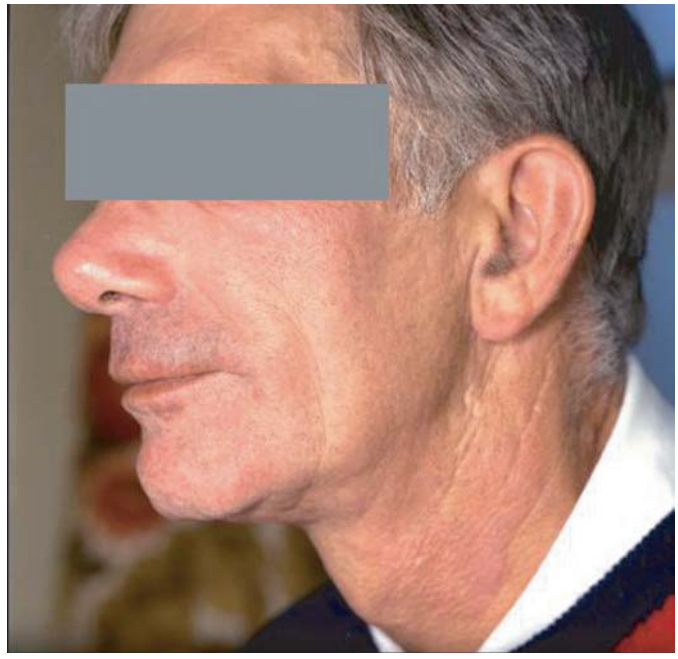


Plate 14 Localised areas of allergic contact dermatitis of the face and neck due to epoxy resin exposure

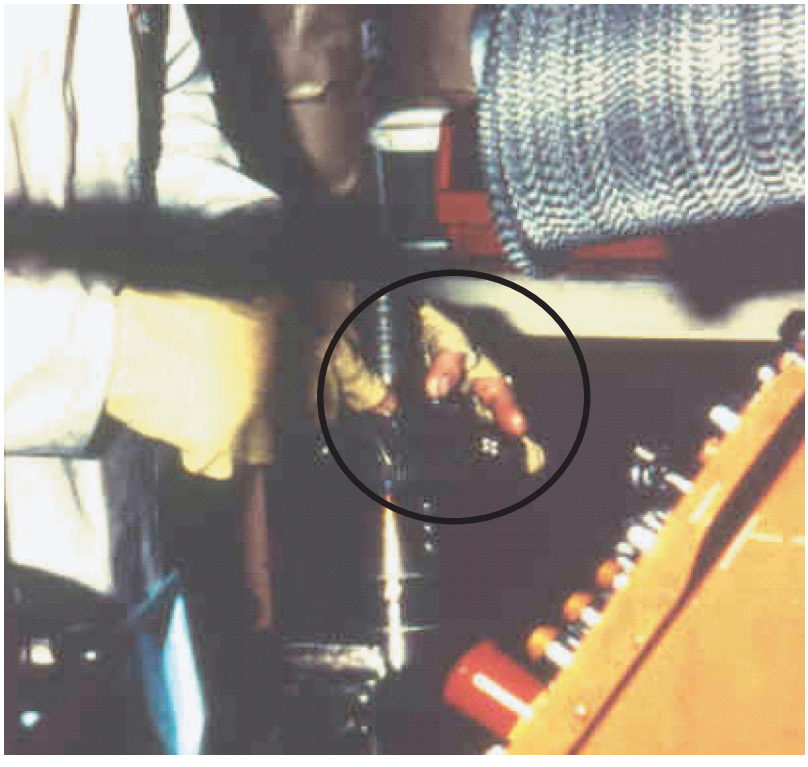


Plate 15 Whilst increasing the wearer's tactility, these gloves certainly do not provide adequate protection!