TOXICITY STUDIES IN A
DIFFERENTIATING EPIDERMAL
KERATINOCYTE CULTURE

Thesis submitted for the degree of
Doctor of Philosophy (Toxicology)

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1986

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ABSTRACT
ABSTRACT

TOXICITY STUDIES IN A DIFFERENTIATING EPIDERMAL KERATINOCYTE CULTURE

A keratinocyte culture derived from rat sublingual epithelium has been developed and characterised both morphologically and enzymically. Morphologically the culture closely resembles that found in vivo. The culture has been studied comprehensively using light and electron microscopy (scanning and transmission).

The culture procedure has been optimised such that minimal amounts of time and materials are required.

The culture, although heteroploid, shows a high degree of homogeneity with minimal fibroblastic contamination.

Total adhered protein, total DNA content, $^3\text{H}$-thymidine incorporation, ornithine decarboxylase activity, acid phosphatase activity, prolinase and malate dehydrogenase activity have been studied at various phases in the cultures growth cycle.

An initial study using 3,3',4,4' tetrachlorobiphenyl indicated that acid phosphatase, prolinase and total protein may be the best parameters for studying toxic insult in these cells, together with morphological examination by electron microscopy and light microscopy using acridine orange as a stain.
Subsequently, comparisons of the 3,3',4,4' and 2,2',4,4' tetrachlorobiphenyl isomers, benzo(a)pyrene and 20-methylcholanthrene were made. Acid phosphatase and prolinase appeared to be useful markers of the toxic effects of these compounds showing alterations consistent with some \textit{in vivo} findings. These changes were observed at concentrations of the compounds that did not produce significant cytotoxicity. Morphological examination showed changes with some similarities to some carcinomas \textit{in vivo}.

The effects of these compounds on keratin production within the cells has also been undertaken. A comparison has also been made of the relative toxicities of several compounds in the keratinocyte culture and in the human embryonic lung fibroblast line (BCL-D1); the keratinocyte system appeared to be more sensitive to irritant compounds.

Studies of the metabolic capabilities of the culture using benzo(a)pyrene as substrate have also been undertaken. Although the capacity of these cells to metabolise foreign compounds was low, they show hydroxylation, glucuronidation and sulphation activity.

In longer term tests (14-21 day), the system shows enhanced sensitivity, the point of commitment of the culture (ie., differentiation and stratification) being a critical time. The methods used showed good reproducibility and were easily performed. The system may, therefore, provide a useful
sensitive screening test for detection of compounds affecting differentiation (e.g. irritants and carcinogens).
ABBREVIATIONS
ABBREVIATIONS

BP - Benzo(a)pyrene
MC - 20-methylcholanthrene
TCB3 - 3,3',4,4' tetrachlorobiphenyl
TCB2 - 2,2',4,4' tetrachlorobiphenyl
PCB - Polychlorinated biphenyl
SDS - Sodium dodecylsulphate
EDTA - ethylene diamine tetraacetic acid
DMSO - dimethyl sulphoxide
TCA - trichloroacetic acid
PCA - perchloric acid
EC - ethoxycoumarin
ER - ethoxyresorufin
RA - Retinoic Acid
TCDD - 2,3,7,8 tetrachlorodibenzo-p-dioxin
TPA - 12-0-tetradecanoylphorbol-13-acetate
BA - benz(a)anthracene
PMA - Phorbol 12-Myristate 13-Acetate

ECOD - ethoxycoumarin-0-deethylase
EROD - ethoxyresorufin-0-deethylase
ODC - ornithine decarboxylase
MDH - malate dehydrogenase
AHH - Aromatic hydrocarbon hydroxylase
EH - epoxide hydratase
PAGE - polyacrylamide gel electrophoresis
CPD - critical point drying
HPLC - high pressure liquid chromatography
ED - electron diffraction
TEM - transmission electron microscopy
SEM - scanning electron microscopy

SPF - Specific pathogen free
RTE - rat tongue epithelia
PBS - phosphate buffered saline
BSA - bovine serum albumin
FCS - foetal calf serum
MEM - minimum essential medium

H&E - Haematoxylin & Eosin
INTRODUCTION
3 GENERAL INTRODUCTION

The skin together with the lungs and the gut, offers a major route for the absorption of xenobiotics. The skin is a common site of accidental exposure to chemicals such as organic and inorganic solvents, oils and many other similarly related toxicants. For this reason there has been increasing interest in the role of the skin as a protective barrier, particularly since the skin accounts for approximately 16% of total body weight. The skin is also a common target for carcinogenesis. Rodent skin provides a convenient experimental model for the investigation of the multiple stages involved in chemical carcinogenesis.

Development of models of target organs by use of cell culture systems has been important for studies of toxicity. A major advantage of these is the reduction of extraneous factors. In the case of the skin cell culture enables the study of the properties of the dermis and the epidermis.

3.1 Epidermal Histology (Fig 1 and Plate 1)

The skin is composed of an epithelial layer of ectodermal origin (epidermis) and a layer of connective tissue of mesodermal origin (dermis). The junction of the dermis and epidermis is irregular and projections of the dermis (papillae) interdigitate with invaginations of the epidermis called epidermal ridges. The epidermis consists essentially of stratified squamous keratinized epithelium containing
DIAGRAM OF SECTION THROUGH EPIDERMIS
PLATE 1

A - SECTION THROUGH RAT TONGUE SUBLINGUAL EPIDERMIS.  
PAPANICOLAOU STAIN (x100)

B - INSET OF A (x 400)
melanocytes, Langerhans cells and Merkel cells. The keratinocytes (keratinizing epidermal cells) account for the greater proportion of the epidermis.

The thickness of the epidermis varies in different parts of the body, thicker in those areas where there is more abrasion. It may also thicken in response to chemical attack leading to hyperplasia or neoplasia.

From the dermis outwards, the epidermis consists of five layers:-

i) the stratum germinativum consists of basophilic columnar cells, resting on the dermal-epidermal junction. Desmosomes bind these cells in their lateral and upper surfaces. Hemidesmosomes, found in the basal plasmalemma, help bind these cells to the basal lamina. Mitotic activity is high in these cells and they are responsible for the replacement of epidermal cells.

ii) the stratum spinosum consists of polygonal or slightly flattened cells with a central nucleus and a cytoplasm with processes filled with bundles of filaments. These bundles converge into many small extensions, terminating with desmosomes located at the ends. The cells of this layer are firmly bound together by this system termed tonofibrils. It is believed that the filaments protect against abrasion, especially at the sites of desmsomes. The epidermis of areas subject to continuous friction and pressure has a thicker stratum spinosum with more abundant tonofibrils.
iii) the stratum granulosum consists of 3-5 layers of flattened polygonal cells containing centrally located nuclei and cytoplasm filled with coarse basophilic granules called keratohyaline granules containing a histidine-rich protein. The granules are not membrane bound and become part of the interfilamentous matrix in cells of the stratum corneum. Another characteristic structure found with electron microscopy is the membrane-coating granule. These granules formed in association with the Golgi apparatus move to the upper part of the cell near its plasma membrane. They fuse with the membrane and discharge their contents into the intercellular spaces of the granular layer providing a barrier to the penetration of foreign materials.

iv) the stratum lucidum is translucent and composed of a thin layer of extremely flattened eosinophilic cells consisting primarily of densely packed filaments embedded in an electron-dense matrix, desmosomes still being evident.

v) the stratum corneum contains anucleate keratinized cells whose cytoplasm is filled with birefringent filamentous scleroprotein, keratin. This protein consists of elongated protein chains rich in disulphide bonds, present as bundles of 7-8nm packed filaments embedded in a dense amorphous matrix.
After keratinization, the cells consist of only fibrillar and amorphous proteins and thickened plasma membranes. Lysosomal hydrolytic enzymes play a role in the disappearance of cytoplasmic organelles.

### 3.2 Factors affecting epidermal growth and differentiation

All cultured keratinocyte systems possess some characteristics of epithelial differentiation such as desmosomes and tonofilaments, although the degree of similarity to epithelial differentiation in vivo varies. Observations on epidermal growth factors may, therefore, be made from in vitro studies by use of cell and organ culture. Due to the complexity of studying differentiation in vivo, the following observations are, in the majority of cases, from in vitro investigations.

#### 3.2.1 Retinoids

Retinoid deficiency has been found to result in the development of squamous metaplasia in the epithelium of the eye, nasal mucosa, respiratory tract, salivary glands and pancreatic ducts (Wolbach and Howe, 1925; Hicks, 1968; Beitsch, 1970; Hayes et al, 1970; Wong and Buck, 1971 and Harris et al, 1972). Also skin cells that normally produce keratin become hyperkeratotic (Wolbach and Howe, 1925; Mori, 1922; Moore, 1967; Wasserman and Carradino, 1971). In these tissues, effects of Vitamin A deficiency were observed primarily in the germinative layer. Similar effects were observed in vitro where removal of Vitamin A from the growth
medium produced squamous metaplasia in tracheal organ cultures (Marchok et al, 1975).

In the case of retinoid excess, it was observed that organ cultures of chick skin epidermis underwent a mucus metaplasia (Fell and Mellanby, 1952). It has been concluded that retinoid excess suppresses keratinization whilst promoting replacement of squamous cells by actively secreting mucus-producing cells. In studies in mouse epidermal cultures using the ethyl ester of retinol, desmosome and tonofilament formation were inhibited and a proliferation of Golgi complexes and endoplasmic reticulum was evident (Yuspa and Harris, 1974). DNA synthesis also appeared to be inhibited.

Yuspa et al (1981) showed RA to be an inhibitor of Ca$^{2+}$ induced epidermal differentiation, although marked differences in sensitivity to RA with keratinocytes derived from different human tissues (conjunctival, esophageal, vaginal and epidermal) was evident (Green and Watt, 1982).

RA proved to be a potent inducer of transglutaminase, an enzyme important in the formation of isodipeptide bonds during protein cross-linking for production of a cornified membrane (Yuspa et al, 1982). This process appeared to be dependent upon intracellular concentrations of Ca$^{2+}$ (Green and Watt, 1982).
Studies with skin explant cultures produced similar effects (Tammi et al., 1985) 5µM RA inhibited terminal differentiation and produced cell death in the upper epidermal layers.

3.2.2 Calcium

It has been shown that Ca\(^{2+}\) ions have an important role in altering the pattern of proliferation and differentiation in cultured mouse epidermal cells (Hennings et al., 1980) and in human keratinocyte cultures (Tsao et al., 1982). Cells grown in low Ca\(^{2+}\) medium (0.05-0.1mM) retain a flattened morphology and continue to grow as a monolayer, but do not assume the stratified differentiated appearance of cells grown in high Ca\(^{2+}\) medium (> 1mM). Addition of Ca\(^{2+}\) to cells grown in low Ca\(^{2+}\) medium triggers the differentiation process leading to stratification and eventually to cells sloughing from the surface.

Whereas retinoic acid appears to inhibit the production of keratins (Buckley, 1981), it appears that Ca\(^{2+}\) ions are necessary for the activation of cross-linking of membrane constituents by transglutaminase (this may occur by cross-linking of ε-(γ-glutamyl)lysine which has been detected in many proteins (Folk and Finlayson, 1977; Matacic and Loewy, 1979; Rice and Green, 1979). However, keratin synthesis does not appear to be affected by Ca\(^{2+}\) (Hennings et al., 1980).
RNA and protein synthesis were, however, reduced in the presence of low Ca\textsuperscript{2+} levels. Similar findings were reported by Rockwell and Sibatani (1982) and Breitkreut et al. (1984) where the number of cells committed to differentiation (60-70%) in Ca\textsuperscript{2+} concentrations of 1.8mM, decreased to 20-30% in Ca\textsuperscript{2+} concentrations of 0.015mM, keratin profiles remaining unaltered in low Ca\textsuperscript{2+} concentrations. Altered morphology and filament architecture were, however, observable. Watt (1984) proposed that the addition of calcium was necessary for formation of desmosomes and other cell contacts, and was therefore not initially responsible for differentiation, the presence of involucrin being a feature of commitment.

3.2.3 Chalones
From work undertaken by Bullough and Laurence (1960), the existence of an epidermal mitotic inhibitor was in evidence. Further evidence of the existence of such inhibitors came from studies by Fugii and Mizuno (1969). Present evidence indicates the existence of two types of epidermal chalone, G\textsubscript{1} and G\textsubscript{2}. The G\textsubscript{1} type being restricted to the epidermis (Elgjo et al., 1972) and the G\textsubscript{2} type diffusing into the dermis.

It has been postulated that the G\textsubscript{1} chalone regulates the flow of cells into S phases of the cell cycle (DNA replication) and G\textsubscript{2} the transition from the G\textsubscript{2} phase into mitosis.
The G₂ chalone appears to be a glycoprotein with a high content of proline and hydroxyproline and it may be destroyed by the action of trypsin. The G₁ chalone is most likely a mucopolysaccharide-containing glycoprotein.

During stripping of keratinocytes there are profound alterations of desmosomal structures (Potten, 1975) and a temporary loss of responsiveness to epidermal G₁ chalone (Beitsch, 1975). It is possible therefore that the effects of the epidermal G₁ chalone are the inhibition of cell migration with the depression of cell proliferation.

It is therefore evident that the epidermal chalones may have great significance in the growth of epidermis and that they may play an important role in the hyperplastic and neoplastic conditions of skin.

3.3 Growth, differentiation, proliferation and stratification

Together with the factors previously mentioned there are many less thoroughly investigated influences upon epidermal growth. However, the effect of dermis upon epidermis (mesenchymal - ectodermal interactions) as a controlling factor has long been well established (Maderson, 1975). This is most likely due to a combination of retinoids, calcium, chalones and probably magnesium and prostaglandins. Cyclic nucleotides probably play an important role.
The presence of gap junctions has been demonstrated in adult amphibian epidermis (Lowenstein and Penn, 1967) and in human epidermal basal cells (Vanhenkelom et al., 1972; Flaxman and Cavoto, 1973) to play a role in electrical communication systems by allowing the passage of small charged molecules. These may well be important in the morphogenetic movements (i.e., stratification) that take place during the histogenesis of surface epidermis.

Cell-cell interactions, including recognition and adhesion, may also be of great importance in the development of epidermis allowing for organisation of components in order to enable proliferation and stratification to continue. Rapid formation of desmosomal junctions has been seen by Hino (1982) during culture of human epidermis.

3.4 Skin Pathology

The histopathology of contact dermatitis has been thoroughly investigated. Epidermal spongiosis, vesiculation and a vascular response in the dermis characterises the allergic form. In the irritant reaction epidermal necrosis, vesiculation and a separation of epidermis from dermis are seen. A prominent cellular infiltrate can be seen in both types of reactions. The vascular components will obviously play no part in the cell culture system although transformation of cells to a more phagocytic nature may occur.
The initial ultrastructural changes found in contact dermatitis are mainly located in the stratum basale and stratum spinosum. The keratinocytes display a spectrum of changes ranging from mild reactions with cytoplasmic vacuoles and dilated endoplasmic reticulum to a fully developed cell injury with affected mitochondria, nuclei and signs of cytolysis. Tonofilaments are described to be either aggregated or dispersed. An intercellular oedema is a prominent feature but is also often reported to occur in the irritant form.

Loss of cellular contacts between keratinocytes is observed (ie., disappearance of desmosome attachment). This phenomenon is most frequently reported to occur through breakage of cellular processes (microvilli) close to an intact desmosome which is retracted or even engulfed by the keratinocyte. Intracellular desmosomes have been described in allergic contact dermatitis (Kobayasi and Asboe-Hansen, 1974). Dissolution of the intercellular part of desmosomes (or cleavage through the middle) with a subsequent disappearance of the desmosomal residues (Cowan and Mann, 1971) or the disappearance of complete desmosomes (micro-achantolysis) (Braun-Falco and Wolff, 1971) have also been described. In connection with irritative reactions cells with the features of apoptosis are reported after exposure to croton oil (Takigawa et al, 1978). Application of lipid solvents (Lupulescu et al, 1973) and alkali or acid (Nagao
et al., 1972) result in prominent ultrastructural changes in stratum granulosum and stratum corneum.

3.5 Keratins

The keratins are a group of intracellular fibrous proteins associated in filamentous structures called tonofilaments. Epidermal keratins have been shown to consist of a number of different proteins (Baden et al., 1973; Skerrow, 1974; Lee et al., 1975; Huang et al., 1975; Steinert and Idler, 1975; Inone et al., 1976), composed of 2-7 polypeptide chains in the molecular weight range 40,000-75,000. There is also a species dependency in the number of polypeptide chains.

Immunological evidence indicates that all cell layers in the epidermis contain keratins (Gray et al., 1977; Sun and Green, 1977). Similarly cell cultured keratinocytes, including the multiplying basal layer contain keratins. Differing keratins may be found in the different layers of the epidermis (Dale and Stern, 1975; Baden and Lee, 1978; Skerrow and Hunter, 1978; Fuchs and Green, 1980) although the proportion of keratin remains constant during maturation (Taichman and Prokop, 1982). Keratins of the living cells of the epidermis differ from keratins of the stratum corneum in possessing six compared with three disulphide bonds between the chains (Steinert and Idler, 1975; Baden et al., 1976; Sun and Green, 1978). Disulphide bond formation confers stability to keratins during the final stages of differentiation (Green, 1977).
<table>
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<tr>
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<th>B(a)P OH^n</th>
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<tr>
<td>HUMAN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NEONATAL</td>
<td>~2 pmol hr^-1 mg^-1 protein BA inducible (Levin and Conney, 1972)</td>
<td></td>
</tr>
<tr>
<td>ADULT</td>
<td>~0.4 pmol hr^-1 mg^-1 protein BA inducible (Bickers et al., 1984)</td>
<td>Phase I ~5 pmol µg^-1 DNA hr^-1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phase II ~2 pmol µg^-1 DNA hr^-1 (Finnen and Shuster, 1985)</td>
</tr>
<tr>
<td>RAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NEONATAL</td>
<td>Whole skin: 40 pmol hr^-1 mg^-1 protein Epidermis: 35 pmol hr^-1 mg^-1 protein Dermis: 20 pmol hr^-1 mg^-1 protein BP and Aroclor 1254 inducible (Mukhtar and Bickers, 1981)</td>
<td>~2 pmol hr^-1 mg^-1 protein (Mukhtar and Bickers, 1981)</td>
</tr>
<tr>
<td>ADULT</td>
<td>~1 nmol hr^-1 mg^-1 protein MC inducible</td>
<td>NOT DETECTABLE (Vizethum et al., 1980)</td>
</tr>
<tr>
<td>MOUSE</td>
<td>ADULT</td>
<td></td>
</tr>
<tr>
<td>~2 nmol hr^-1 mg^-1 protein TCDD inducible not MC (Pohl et al., 1976)</td>
<td>~1.5 nmol hr^-1 mg^-1 protein (Pohl et al., 1976)</td>
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In addition to the synthesis of new keratins in the maturing cells, there is a small reduction in size of some of the keratins during the last stages of terminal differentiation (Fuchs and Green, 1980).

3.6 Drug Metabolising Enzymes

The biological activity of any given compound is directly dependent upon its metabolism within the organism. The action of one or more metabolising agents may serve to increase toxicity or detoxify.

The complement of drug metabolising enzymes within the skin is of major importance when considering the role of the organ as a barrier to toxins. The metabolism resulting from the presence of these enzymes, is generally divided into two categories. The first is that of oxidations, reductions and hydroxylations (Phase I). The second are those of conjugations and synthesis (Phase II). The Phase I reactions generally produce a product that may undergo Phase II.

3.6.1 Skin Metabolism (Table 1)

Studies of how metabolism might be involved in skin carcinogenesis by chemicals, constitutes the majority of data on xenobiotic metabolising systems in this organ. Skin has lower activity than many organs studied, particularly liver (Mukhtar and Bickers, 1981; Bickers et al, 1981) and as it is extremely difficult to homogenise, the techniques used
are likely to damage many of the enzyme systems. The response of skin xenobiotic-metabolising enzymes to inducers, inhibitors, activators, etc., is far less well studied than with a number of other tissues.

Enzyme assays on fractions rich in basal cells from mouse skin indicated that this is a very poor site for the metabolism of xenobiotics (Pohl et al, 1980). However, rat and mouse skin strips supported de-ethylation, sulphation and glucuronidation reactions (Moloney et al, 1982) and de-ethylation appeared to be inducible by pretreatment with either 5,6 benzoflavone or MC.

Following topical application of BP or the PCB mixture, Arochlor 1254, a greater increase in activity of AHH in skin was observed over that in the liver (Mukhtar, 1981). Activity in skin represented approximately 20% of total body activity when induced with these compounds but control values of AHH, 7ECOD, NADPH-cytochrome C reductase, EH and glutathione S-transferase only contained 2, 0.5, 24, 6 and 1.5% of corresponding hepatic activity. Greater induction of AHH, 7ECOD and EH was reported by Bickers (1982) following topical application of Arochlor 1254 with Cyt P-450 levels exceeding that of liver.
Fig 2

INITIATION AND PROMOTION.
THE TWO STAGE MODEL OF CARCINOGENESIS.

TAKEN FROM BOUTWELL (1974)
3.7 Chemical Carcinogenesis

The model generally used for the description of the mechanism of carcinogenesis is that of a two-stage system. This system is shown by treatment of skin, for example, with a subcarcinogenic dose of an initiator (e.g., benzo(a)pyrene) followed by repeated applications of a promotor (e.g., croton oil).

Figure 2 shows the basis behind the two-stage system, although, each stage comprises many different events, which may or may not result in a neoplastic reaction. Initiation requires only a single application of the carcinogen, and may not be apparent in a tissue from morphological examination. Promotion usually involves a latent period, during which no obvious transformation may occur.

Initiation may remain unexpressed for prolonged periods, but reversal of initiation and promotion results in no tumour formation. Certain agents may only act to propagate the growth of a tumour after application of a promotor, an indication of the complexity of the promotion step.

The mouse skin model of two-stage carcinogenesis has been the model of choice for studying tumour promotion for a number of reasons. In addition to the availability of a series of promotores with a range of tumour promoting activity in the skin, other advantages of the model are that the tumours have a relatively short latent period, that
tumours are visible so that the animals need not be sacrificed to score tumours, and that the number of tumours that develop on the skin reflects the relative potency of the compound to be evaluated.

Research on the mechanisms of two-stage carcinogenesis in mouse skin has been concentrated in the epidermis as the target tissue of the promotor, as well as of the initiator. There have been few studies on the effects of promotors on the dermis and how these compounds might influence tumour formation by affecting the physiology or structure of the epidermis. It is possible, however, that the dermal layer is a target of promotor action in mouse skin and plays a role in the promotion of skin tumours through some type of cell-cell interaction. Tumour formation in the skin may involve both direct and indirect effects of the promotor on initiated cells.

Histological changes that follow promotor treatment appear to be the same whether or not the skin has been initiated. Within a few hours after application of an effective dose of a promotor, such local tissue reactions as the oedema and erythema characteristic of inflammation and irritation are evident, and by 24 hours, leukocytes have infiltrated the dermis (Frei and Stephens, 1968). A sustained stimulation of mitotic activity in the basal cell layer of the epidermis starts within a day or so of treatment and continues for several days, resulting in an increase in the number of
nucleated cell layers from the normal 1-2, to 3-4 or more (Raick, 1973a). The stimulation of proliferation induced by promotor treatment is followed 24-72 hours later by a phase of increased keratinisation of the upper layers of the epidermis (Raick, 1973a; Balmain, 1976). These responses to the promotor, including the marked hyperplasia, all gradually subside, and within two weeks after treatment, the epidermis has regained its normal appearance (Raick, 1973b).

Repeated promotor treatment at regular intervals prevents the reversion of the skin to its normal state. Hyperplasia similar to that induced by promoting agents can be induced in epidermis by weak promoters and inflammatory agents. However, the increase in nucleated cell layers in the epidermis after treatment with a promotor precedes the increase in mitotic index, whereas treatment with a hyperplasiogenic agent results in the reverse situation (Raick, 1973a; Raick, 1974).

Stimulation of macromolecular synthesis by tumour promotors is one of the fundamental parameters. This includes DNA, RNA, protein, plasminogen activator and ODC synthesis. An excellent correlation exists between the tumour promoting ability of phorbol esters and their ability to induce ODC activity in mouse skin (O'Brien et al 1975). While the data indicated ODC induction and the accumulation of putresine by TPA, it was also reported that the application of retinoic acid to the skin of mice inhibited both the induction of ODC
activity and tumour formation (Verma and Boutwell, 1980; Verma and Boutwell, 1979). However, Slaga et al. (1980) showed RA to be ineffective in inhibiting Stage I promotion but effective in inhibiting Stage II promotion by mezerein. Gensler (1984) indicated that the antitumour activity of the retinoids may be carcinogen or co-carcinogen specific.

Most of the cell culture systems employed today are either from cell lines having predominantly fibroblastic morphology or they are fibroblastic. Since approximately 80% of human cancers are of epithelial origin, the fibroblastic nature of such cells represents a serious limitation. Major advances have been made in developing in vitro methods for the detection of carcinogens, including both fibroblastic and epithelial cell populations (Milo and DiPaolo, 1978; Milo et al., 1981) and in the use of many different end points (Indo, 1977; Montesano, 1977; Mass, 1984). However, no definitive test procedure yet exists for the detection of carcinogens in vitro.

Morphological and biochemical responses of mouse skin to phorbol esters are characterised by induction of inflammation and hyperplasia (Boutwell, 1964; Slaga et al., 1976), induction of dark basal cells (Slaga et al., 1980; Raick, 1973a; Raick, 1974), increase in phospholipid synthesis and phosphorylation (Rainer et al., 1973; Rainer et al., 1977), increase in ODC activity followed by an increase in polyamine production (O’Brien, 1975; Lesiewicz
and Goldsmith, 1980), decrease in histidase activity (Troll et al., 1978), and an increase in c-AMP phosphodiesterase activity (Mufson et al., 1979; Perchellet and Boutwell, 1981).

The general effects of tumour-promoting phorbol esters on mouse epidermal cell cultures are increased RNA synthesis (Weinstein, 1980), early delayed DNA synthetic activity followed by an increase in the later stage (Weinstein, 1980; Yuspa et al., 1976; Diamond et al., 1978), increased plasminogen activator production (Weinstein, 1980; Wigler and Weinstein, 1976; Wigler et al., 1978), increased cellular proliferation and phospholipid metabolism (Weinstein, 1980) and a rapid rise of c-GMP and c-AMP (Perchellet and Boutwell, 1980; Trosko et al., 1975; Murad et al., 1971).

A correlation between mouse skin and mouse epidermal cell cultures in the action of phorbol esters to stimulate ODC was also observed by Yuspa et al. (1976). However, variability amongst cell lines in response to TPA were later observed (Yuspa et al., 1980). Thus, it is evident that choice of cell line is an important factor when considering assays of cell transformation particularly when there may be a high basal rate. This has been further proved by studies of TPA on cultured human keratinocytes where no induction of ODC or polyamine synthesis was observed (Fischer et al., 1984). Induction of AHH by BA indicated that ODC was not a prerequisite for this activity and it may therefore be of
less significance than at first proposed (Raunio, 1982). Similarly in other systems RNA and protein synthesis remained relatively normal despite polyamine depletion (Fillingame, 1975; Poso, 1976).

Studies of keratin expression both in vivo and in vitro, as a marker of carcinogenesis have proved useful also. Keratins of papillomas in mice indicated changes in both charge and molecular weight, similarly carcinomas showed changes in the groups of high molecular weight keratins (Nelson and Slaga, 1982; Winter et al, 1980). A study of keratin expression in normal and squamous cell carcinoma also showed alterations of keratin patterns, however, when normal cells were cultured some similarities to the carcinoma keratin profiles were observed (Grace et al, 1985).

3.8 In Vitro Toxicity

Cell culture systems may be divided into three general categories: explant cultures, primary cell cultures and cell lines.

Primary cell cultures may also be obtained by culturing, for more than 24 hours, dispersed cells from tissues or organs. A major advantage of primary cell cultures is that much of the metabolic activity of the original tissue may remain in the culture. However, substantial physiological differences have been observed among primary cell cultures derived from
a given tissue. A major factor influencing primary systems is the method of isolation, physical and chemical means being more disruptive than enzymic means.

Established cell lines derive from primary cultures or finite-lived diploid cell lines by transformation, either spontaneous or induced. The majority of cell lines are heteroploid. Criteria identifying established cell lines include altered morphology, lack of contact inhibition, increased saturation density, loss of anchorage dependence, the capability to undergo an unlimited number of cell divisions and the ability to grow in soft agar medium. There are many cell types that display in vitro biological activity that is characteristic of their original tissues and/or organs (Sato and Yasumura, 1966).

Growth and reproduction in cultures is generally quantitated by measurements of cell number, total protein or DNA content. However, more specific tests may be used, such as tests for the production of a specific biochemical end product, tests for cell surface effects, test for motility and tests for cell differentiation.

Various parameters having been chosen for toxicity assessment, the problem of validation of the in vitro results must be overcome. Many in vivo toxicity investigations were not performed to identify specific mechanisms and target structures in the cell and
Experimental conditions have often not been well described. In the case of a cell culture there are fewer variables and more accurate data may be obtained from any given study. Parameters such as exposure, concentration and duration may be defined precisely.

There are many factors that must be taken into consideration when using the *in vitro* toxicity model such as the metabolic activity of the cells, cell-cycle events and availability of the toxin in question.

Cell cycle events are of less importance in non-synchronous cultures, however, in the case of a differentiating culture a degree of synchrony may be produced when the initial differentiation process begins during the subculture's life. Availability of the toxin is dependent on the general chemical parameters such as lipophilicity, dissociation constants, etc. The influence of metabolic activity will be dealt with later.

Cultures that have not been derived from cloning and non-synchronous cultures will have a greater distribution of toxic response across the culture system. Morphological data must be regarded with caution since this may represent a minor proportion of the cell population whereas such parameters as protein and DNA content represent a more statistical view of the overall situation.
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<td>ACIDS (e.g HF, HCl, HBr)</td>
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<td>ALKALIS (e.g CARBONATES OF NH₄, Ca, K, Na)</td>
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<td>ORGANIC</td>
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Metabolism and Activation Systems

Metabolism of a given xenobiotic is often a prerequisite for its toxic action or detoxification. In the in_vivo situation, the liver is the major site of drug metabolism. Many other tissues have the capability for metabolism of xenobiotics but often this is restricted to minority cell types and therefore the overall rate is lower than liver. In general, freshly isolated cells and primary cultures are more suitable for metabolism studies since they tend to retain much of their original activity but many drug metabolising enzyme activities decrease with time. Alternatively, a metabolising system such as the S9 fraction of liver homogenate may be added to cell lines. Cell cultures with little or no drug metabolising ability are of questionable value for toxicity studies, a problem pertinent to cutaneous toxicity testing. Skin in_vivo has a far lower capacity for xenobiotic metabolism than most of the other major organs, in_vitro this is further reduced.

Dermatotoxicity (Table 2)

Cutaneous toxicity can be divided into problems arising from skin sensitisation (allergic dermatitis) and from primary irritation (irritant dermatitis). The first of these has its basis in the immune system and therefore cannot be studied in a keratinocyte system so will not be dealt with any further here. Primary irritation is the initial toxic response that may provoke an inflammation response and may, therefore, be studied in_vitro where systemic effects are
lacking. Cutaneous toxicants that promote the 'primary irritant' effect may therefore be studied by monitoring changes such as barrier function, cellular differentiation, cellular proliferation and viability. Barrier function may only be carried out in vitro by use of whole skin, the other functions may be studied in cell culture.

Markers of alterations in differentiation of epidermis would seem to be the most useful for screening of irritants and carcinogens, since differentiation appears to be the growth characteristic altered in cases of hyperplasia, transformation and other proliferative changes. Points of interest include phospholipid content; cell membranes of undifferentiated basal cells have normal phospholipid content, while the fully differentiated stratum corneum cells contain very little phospholipid (Gray and Yardley, 1975). A histidine-rich protein is synthesised in the granular layer of the epidermis and its presence appears to be closely linked with the process of differentiation (Sugawara and Bernstein, 1971; Sibrack et al, 1974; Kreig et al, 1974; Balmain, 1976). Keratin, the major protein constituent of stratum corneum, and its precursor pre-keratin exhibit characteristic patterns on SDS-PAGE (Steinert, 1975; Baden et al, 1976; Brysk et al, 1977; Lee et al, 1979).

Increases in $^3$H thymidine incorporation into DNA of skin are observed following cutaneous application of skin promotors (Hennings and Boutwell, 1970; Raick et al, 1972),
hyperplastic agents (Peters and White, 1978) and skin irritants (Middleton and Pratt, 1978). Similarly the enzymes involved in the synthesis of polyamines (ODC and S-adenosyl methionine decarboxylase) increase in tissues undergoing rapid cell turnover in response to various stimuli (Janne et al, 1978). ODC activity in skin increases in response to tumour promotors (O'Brien, 1976) and cell damage following hair plucking (Probst and Krebs, 1975). The effects of cutaneous toxins on ODC activity have been investigated and compared with those provoked by the hyperplastic agent hexadecane (Oliver et al, 1981). Whilst cutaneous toxins examined can stimulate ODC activity, the stimulation is less than that produced by either a promotor or hyperplastic agent, unlike $^3$H thymidine incorporation, where all three treatments appear to produce a similar stimulation (Raick et al, 1972; Peters and White, 1978; Middleton and Pratt, 1978).

3.11 The Epidermal Keratinocyte

There now exist numerous methods of culturing mammalian keratinocytes both from laboratory animals and man (Fusenig and Worst, 1974; Bertolero et al, 1984; Marcelo et al, 1978; Li et al, 1983; Jepsen, 1974; Kondo et al, 1979; Gildchrest et al, 1982; Rheinwald and Green, 1979; Karasek, 1966; Green et al, 1977). However, in the majority of cases, methods have been complicated because keratinocyte culture required added hormones, special substrates, feeder layers (often epidermal fibroblasts) and many other factors to aid
proliferation of the cultured keratinocyte. Interest
developed in the keratinocyte since it was discovered that
the ability to stratify and terminally differentiate was
retained in vitro, even in the absence of instructive
information from the connective tissue stroma (Flaxman,
1967). This ability offered the chance of investigating
epidermal growth and differentiation (Cruickshank, 1960;
Briggaman, 1967; Yuspa et al., 1970; Fusenig, 1974).

Since these earlier investigations, many studies of chemical
toxicity have taken place. These have been predominantly
those of carcinogenicity because the effects of chemicals on
a specific cell function (differentiation) may be
determined. These studies are of more value than the less
specialised type of toxicity investigated with
undifferentiated epithelial or fibroblast-like cell
cultures.

Carcinogens and promoters most studied in epidermal cell
cultures include 7,12-dimethyl-BA, BP, TPA and MC (Li et al.,
1983; Mass et al., 1984; Indo, 1977; O'Brien and Diamond,
1978; Yuspa et al., 1976; Yuspa et al., 1980; Kuroki et al.,
1980; Kuroki et al., 1982; Indo and Miyagi, 1979; Yuspa
et al., 1976; Indo and Miyagi, 1985). A great proportion of
studies are based on the ability of mutated (transformed)
cells to grow in soft agar or following subcutaneous
innoculation into rats or mice.
Examination of the effects of irritants and more general cutaneous toxins have tended to be on epidermal skin slices (Oliver and Pemberton, 1985; Kao et al, 1983; Middleton, 1980) and thus studies of the keratinocyte and its behaviour with irritants have been more thoroughly investigated in these systems which more closely resemble the in vivo situation, than would be found in long-term cell culture.

An irritant stimulus often leads to an initial increase in DNA-synthesis (Middleton and Pratt, 1978) followed by epidermal hyperplasia (Argyris, 1968; Barnes et al, 1968; Cowan and Mann, 1971). If the stimulus is strong, an initial depression of DNA-synthesis (Groth, 1967) and necrosis of keratinocytes occurs. Epidermal hyperplasia is accompanied by an increase of ribosomes (Argyris, 1978; Mueller and Argyris, 1975) and an increased protein and glycoprotein synthesis (Birkin et al, 1979).

3.12 Polychlorinated Biphenyls (PCB's)
The PCB's elicit numerous toxic effects including acnegenic and dermal lesions, a wasting syndrome, immunotoxicity, reproductive toxicity, genotoxic and epigenetic effects, hepatomegaly and related liver damage, and the induction of hepatic and extrahepatic drug metabolising enzymes. The polybrominated biphenyls (PBB's), polychlorinated dibenzo dioxins (PCDD's) and the polychlorinated dibenzofurans (PCDF's) elicit a similar array of toxic and biological properties. The most toxic compound of these halogenated...
aromatics is 2,3,7,8 tetrachlorodibenzo-p-dioxin. The most toxic halogenated aromatics are approximate isostereomers of TCDD (i.e., planar, symmetrical molecules). Chloracne and dermal lesions are regarded as the most important and characteristic signs of PCB toxicity in humans and their effects have also been noted in animal studies. The rabbit ear is particularly sensitive to PCBs, and related halogenated aromatics, typically showing hyperplasia and hyperkeratosis of the epidermal and follicular epithelium (Vos and Beems, 1971; Vos and Notenboom-Ram, 1972). Although rats do not develop acne or skin lesions after exposure to PCB's, hairless mice are highly sensitive (Puhvel et al, 1982). 3,3',4,4' tetra-chlorobiphenyl is highly acneogenic. Inspection of the literature points to TCB3, 3,3',4,4',5 PCB, 3,3',4,4',5,5' HCB as being the most toxic halogenated biphenyls, these compounds closely resembling the structure of TCDD. These three PCB congeners induce AHH in rodents and rat hepatoma H-4-11-E cells in culture and a comparison of their potencies with the cultured hepatoma cells indicated the 3,3',4,4',5 PCB to be 1/13 less active than TCDD (Sawyer and Safe, 1982). These compounds possess several common structural features: those of chloro substituents at both para positions, chloro substitution in at least one meta position of both phenyl rings and no ortho substituents.
Metabolism of the polychlorinated biphenyls generally appears to occur via an electrophilic precursor an arene oxide (Gardner, 1973; Hsu, 1975; Norback, 1976). The end products of this metabolic conversion have been identified as trans-dihydrodiols, phenols and their glucuronides. Isolation of a methyl sulphone derivative (Mio, 1976) also suggests formation of an arene oxide intermediate and further reaction with methionyl residues in proteins with subsequent cleavage (Yoshimura, 1976).

3.12.1 2,2′4,4′ and 3,3′4,4′ Tetrachlorobiphenyls
The two TCB isomers 2,2′4,4′ and 3,3′4,4′ TCB show marked differences in effects and potency and therefore may be used effectively in studies of the structure-activity relationships of PCB's. Tissue distribution and formation of covalent linkages with cellular macromolecules, covalent binding being higher with the 3,3′4,4′ isomer, differed indicating different pharmacokinetics (Shimada and Siwabe, 1984).

TCB2 is preferentially activated by the mono-oxygenase system containing phenobarbitone-inducible forms of cytochrome P-450 whereas the TCB3 is activated by the MC inducible form of cytochrome P-450 (P-448) (Shimada and Swiwabe, 1983). Both isomers compete with TCDD for the Ah receptor, but with variation in their relative binding efficiencies, 3,3′,4,4′ having the greater binding efficiency (Bandiera et al., 1982).
The Ah Receptor (Induction and Toxicity)  
(Poland and Knutson, 1982)

The Ah receptor is a protein, found in the cytosol, which is thought to be essential for the induction of AHH activity.

The Ah receptor protein is so called because it is produced as a result of the expression of a set of genes (Ah locus) which thus controls the induction of several drug metabolising enzymes by polycyclic aromatic compounds.

The cytosolic receptor for TCDD is viewed as the major regulatory gene product of the Ah complex.

Toxicity of PAH's are, therefore, in part, dependent upon the presence of the Ah locus and receptor protein for metabolic conversion to active compounds.

AIMS

The aims of this work were, primarily, to develop and characterise a keratinocyte culture system; and secondly, to investigate the use of this system as an alternative method to in_vivo toxicity studies on the skin. The way in which this was undertaken was as follows:-

1. Development
Optimisation of culture conditions for producing a differentiating keratinocyte culture.
2. Characterisation
Investigation of the use of certain enzymic parameters to assess the growth patterns of the culture, together with microscopic and ultrastructural examination of the differentiation process.

3. Choice of Parameters of Toxicity
Enzymic parameters were chosen from characterisation studies on the basis of markers of the differentiation process. These parameters were then studied during toxic insult of the culture to assess their suitability.

4. Specificity of the System
a) Compounds of varying potencies and effects were compared within the system;
b) comparisons of the culture with one of a non-specific cytotoxicity system were made.

5. Metabolic Activation
The capacity of the culture for phase I and phase II metabolism were investigated as a comparison to the in vivo situation.
MATERIALS and METHODS
4 MATERIALS AND METHODS

4.1 MATERIALS


3,3',4,4' TCB - Dr Elcombe CTL, ICI, Macclesfield, Cheshire.

2,2',4,4' TCB - Dr Elcombe CTL, ICI, Macclesfield, Cheshire.

MC, BP - Sigma Chemical Co, Fancy Road, Poole, Dorset.

Growth Media - Gibco, Longbridge Way, Uxbridge, Middlesex;
Flow Laboratories, Irvine, Ayrshire, Scotland.

All reagents for Electron Microscopy - TAAB, 40 Grovelands
Road, Reading, Berkshire.

Radiochemicals - Amersham International, Amersham,
Buckinghamshire.

7-hydroxycoumarin, EC - Sigma Chemical Co, Fancy Road,
Poole, Dorset.

7-hydroxyresorufin, ER - Molecular Probes Inc,
24750 Lawrence Road, Junction City,
USA.

Solvents - BDH Chemicals, Freshwater Road, Dagenham, Essex.

Colcemid - Sigma Chemical Co, Fancy Road, Poole, Dorset.

$O_5O_4$ - Sigma Chemical Co, Fancy Road, Poole, Dorset.

Electrophoresis - BDH Chemicals, Freshwater Road, Dagenham,
Essex.

Stains - Paramount Reagents, Liverpool, Merseyside.

All enzymatic and morphological studies were performed on
subcultures 24-27 inclusive unless otherwise stated.

Animals: - Male Wistar/Albino rats,
University of Surrey strain.
4.2 METHODS

4.2.1 Establishment and Maintenance of the Rat Tongue Epithelial Cell Line

4.2.1.1 Primary Cultures

200g male rats (Wistar/albino, University of Surrey strain) were killed by cervical dislocation. Tongues were removed and bisected longitudinally retaining the ventral section. The method used was essentially that of Buckley (1980). Primary cultures of the ventral lingual mucosa of rat were established in 25cm² plastic culture flasks (Falcon, "PRIMARIA") by explanting, with a micromanipulator, 5 pieces of ventral tongue directly onto culture flasks. Each piece of tongue was cut in primary growth media and measured not less than 3 x 2 x 2mm. The tip of the tongue was not used. Tissue pieces were allowed to attach for 120 minutes, then the cultures were incubated with a complete culture medium consisting of MEM with Earle's salt mixture, 20% v/v FCS and 500µg/ml⁻¹ kanamycin. DMSO was added to a concentration of 0.5% (v/v). Cultures were gassed with 5% CO₂ to maintain a pH of 7.4 and incubated at 30°C. Successful growth of the primary cultures required a temperature not exceeding 30°C, selected FCS (ie., those batches best supporting growth of epithelial cells) at a concentration not less than 15% (v/v) dimethyl sulphoxide to prevent vacuolation and cytoplasmic retraction.
4.2.1.2 Subcultures

Primary cultures, 20-30 days old, were rinsed twice (30 seconds each rinse) in sterile medium and incubated with trypsin (0.5% w/v) for 2-3 minutes at 37°C. This initial trypsinisation removed a large proportion of the fibroblasts. Cultures were then incubated with trypsin (0.25%) containing EDTA (1mM) in PBS (Flow, 28-103-05) at 4°C for between 45-120 minutes. The time varied according to the degree of attachment. Epithelial cells were then dislodged from the culture surface by gently shaking. The resultant cell suspension was added to an equal volume of complete tissue culture medium (serum inhibits further trypsin activity) and centrifuged for 4 minutes at 700g. The cell pellet was dissociated by pipetting fresh medium onto its surface and aliquots of cell suspension were added to culture flasks.

Since successful initial subcultivation more often resulted when a "crowding" technique was used, cells derived from 3 or 4 flasks containing 20-30 day old cultures were combined and added to a single flask. Areas of fibroblastic growth were scraped off when the cultures had established. Medium was added, the flasks were gassed with 5% CO₂ and they were then incubated at 30°C. Complete medium containing DMSO (0.5%) was changed every 3 days. Omission of DMSO from the initial subculture medium resulted in vacuolation, cytoplasmic retraction and reduced cell viability (Jepsen, 1974). Subcultures containing several areas (0.5mm²) of
densely packed fibroblasts were discarded. The culture line was subcultured periodically (normally every 30 days) to increase stock. This involved the addition of 1 ml trypsin (0.25%) containing EDTA (1 mM), and incubation at 30°C for 10 to 15 minutes. Cells were dispersed by shaking. Fresh medium was added and cells were harvested by centrifugation (300 g, 1.5 min). New flasks were seeded with 2 x 10^6 cells to which medium was added, and after gassing, the flasks were incubated at 30°C.

4.2.1.3 Cell Freezing and Thawing

In order to maintain a supply of RTE cells and in case of contamination, it was essential to keep a frozen stock in liquid nitrogen.

Cells were trypsinised in the usual manner and 20 ml of medium plus FCS (10% v/v) was added. The cell suspension was decanted into sterile tubes and centrifuged at 700 g for 10 minutes. After removal of the supernatant, the cells were resuspended into freezing medium [complete medium plus DMSO (10%) - pH 7.2, aliquots were added to sterile plastic ampoules and placed in a liquid nitrogen freezer in a cooling adaptor ("Union Carbide"). The cells were cooled slowly for 4 hours (−1°C min⁻¹) and then placed onto storage rods and immersed into liquid nitrogen.
4.2.2 Preparation and Storage of Test Solutions

Stock solutions of test compounds were prepared in DMSO and appropriate amounts added to culture medium prior to the addition to RTE cells as DMSO is toxic and also dissolves "Falcon" plastic. Stock solutions were prepared every 4 weeks and stored at -20°C. Test compounds were added to RTE cultures at each medium change. Controls, which contained an equivalent amount of DMSO were grown in the absence of test compounds.

4.2.3 Histological Stains

4.2.3.1 Oil Red O

Oil red O is a dye that partitions preferentially in tissue fats rather than in the dye solvent. It was used in combination with haemalum (which stains nuclei) resulting in red lipid areas and blue nuclei.

Reagents: Stock solution of oil red O:-(saturated solution in isopropanol); working solution: stock solution (6ml) plus distilled water (4ml) (the precipitate was removed by filtration). The working solution was made immediately prior to use.

Method: (i) Rinse sample in PBS, fix in 10% neutral buffered formalin for 15 minutes; (ii) stain in working solution of oil red O (15-20 minutes); (iii) wash in water; (iv) stain with Mayers Haematoxylin (7 minutes); (v) wash in water; (vi) immerse in 0.1% lithium carbonate; (vii) wash in water and mount with glycerine jelly.
4.2.3.2 Haematoxylin and Eosin
Cultures were fixed in 4% 0.1M phosphate buffered formaldehyde pH 7.4 prior to staining. The following regimen was then followed: (i) dehydrate in ethanol and then rehydrate through graded alcohol to water; (ii) stain in Mayers haematoxylin; (iii) differentiate in tap water; (iv) stain with 1% Eosin 7; (v) dehydrate through 70 and 95% alcohols; (vi) dehydrate in 100% ethanol and mount with glycerine jelly.

Results: nuclei - blue; cytoplasm - pink.

4.2.3.3 Papanicolaou
Cultures were stained by the Papanicolaou method (Papanicolaou, 1942). This stain is used for differentiation between acidophilic and basophilic cells.
Reagents: OG6, EA50 supplied by Paramount Reagents (Liverpool)

Method: (i) Cells were rinsed in PBS and fixed in 95% ethanol (5 min); (ii) rehydrate to water in graded alcohols; (iii) stain in Harris Haematoxylin (3 min); (iv) rinse in tap water; (v) differentiate in 0.5% HCl; (vi) blue in 1.5% NaHCO3; (vii) rinse in tap water; (viii) dehydrate in graded alcohol (70-95%); (ix) stain with OG6 (3 min); (x) wash twice in 95% ethanol; (xi) stain with EA50 (2-4 min); (xii) wash in 95% ethanol then 100% ethanol; (xiii) mount.
Results: nuclei - blue; acidophilic cells - red to orange; basophilic cells - green or blue green.

4.2.3.4 Acridine Orange

Cultures were stained with acridine orange using the method of Von Bertalanffy and Bickis (1956). The stain is an alternative to the Papanicolaou method.

Reagents: Acridine Orange stock - (0.1% in distilled water) - working solution - AO stock (5ml) in 0.07M phosphate buffer pH 6.0 (45ml).

Method: The method is identical to the Papanicolaou method to step (ii). Step (iii) rinse in 1% acetic acid, then distilled water; (iv) stain in acridine orange (5 min); (v) destain in phosphate buffer 0.07M pH 6.0 (1 min); (vi) differentiate in CaCl₂ (0.1M) for 30 seconds, then wash in buffer; (vii) repeat washings in buffer to remove CaCl₂ (viii) mount.

Results: DNA of chromatin of nucleus - green (whitish to yellowish hues); connective tissue fibrils, cornified epithelial structure - greenish; mucus - dull green; leukocytes - bright green; RNA of basophilic structures of cytoplasm, main portion of nucleoli - red and orange fluorescence;
sites of strongly acidic groups - deep carmine;
malignant cells under low power - brilliant flaming red-orange;
proliferating malignant cells under high power - intense fluorescence;
cytoplasm - flaming red-orange, containing reddish granules, patches or fine granules;
nuclei - brilliant orange-red;
degenerating and necrotic malignant cells - characterized by a gradual loss of cytoplasmic RNA and therefore of the flaming red-orange;
cytoplasm - faint orange or brick red, RNA often concentrated at periphery in a rim of dark brick red;
nuclei - brilliant green, green-yellow or pale yellow-grey;
nucleoli - often enlarged and multiple, orange-red.

4.2.4 Preparation of glass substrate for RTE cell culture
Glass coverslips were acid-washed in 1M HCl for 24 hrs, rinsed in distilled water, and then left in distilled water for 18 hrs before a final rinse in distilled water, 70% ethanol, 100% ethanol and acetone respectively. Coverslips were then air dried and polished prior to sterilisation by autoclaving (15 psi - 20 min).
4.2.5 Preparation of RTE Cell Cultures for Electron Microscopy

4.2.5.1 Transmission

An equal volume of glutaraldehyde (4%) in 0.1M cacodylate buffer pH 7.4 was added to the medium in the culture flasks. After fixation for 15 min at 30°C the fixative/medium was carefully removed and the cells were then fixed in glutaraldehyde (4% v/v) Cacodylate HCl buffer 0.1M pH 7.4 for 3 hrs. Cells were then washed in Cacodylate HCl buffer (0.1M) pH 7.4 for not more than 18 hours. Post-fixation was carried out by adding Cacodylate buffered osmium tetraoxide (2%) at room temperature for 2 hours. Flasks were then dehydrated in sequentially increasing concentrations of alcohol and then washed in Epon 812 resin only, for 15 mins. Epon 812 resin was then added and polymerised at 30°C for 48 hours and 60°C for 48 hours. Sections (70-90nm) were cut from the block using an LKB ultratome III. Sections were stained with uranyl acetate and lead citrate and examined with the Jeol 100B electron microscope.

4.2.5.2 Scanning

Cells were grown on glass substrate. The method used was basically that used for samples prepared for transmission electron microscopy. However, post-fixation with osmium tetraoxide was omitted and in place of the wash with Epon 812 resin, the dehydration in 100% ethanol was followed by ethanol/acetone (1:1) and then pure acetone (dried over sodium sulphate).
Samples were then critical point dried in a gas bomb using liquid CO$_2$ by removal of the acetone with CO$_2$(l) and saturating the sample in CO$_2$(l), for 1 hr prior to CPD. Samples were then sputter coated with gold/palladium and examined with the Cambridge Stereoscan 250 electron microscope.

4.2.6 Estimation of Protein and DNA Content and the Rates of Incorporation of L-[U-$^{14}$C]Leucine and [6-$^{3}$H]Thymidine into the Respective Macromolecules of RTE Cell Cultures

4.2.6.1 Preparation of RTE Cultures for determination of protein and DNA

This is essentially the method of Marcelo et al. (1978). Cell cultures were incubated with L-[U-$^{14}$C]leucine and [6-$^{3}$H]thymidine (1μCi/ml) for 1 hour at 30°C. After incubation, cells were washed and removed from the flask by incubation with trypsin (0.25%) containing EDTA (1mM) for 5-10 minutes at 37°C and the cells were then dispersed into medium plus FCS (10%v/v). Cells were washed three times with PBS and the protein precipitated with ice-cold TCA (6%w/v), followed by centrifugation at 1900g for 15 minutes. A sample of supernatant was taken for liquid scintillation counting. The pellet was suspended in PCA (0.5M) and the DNA was hydrolysed twice in a boiling water bath at 90°C for 10 minutes. Samples of the supernatant were counted and DNA content estimated by the method of Burton (1956). The residual precipitate was washed twice with ethanol and dissolved in NaOH (1M) by incubating at 37°C overnight.
Samples were counted and the protein content determined (Lowry et al 1951).

4.2.6.2 Estimation of radioactivity

L-[U-\(^{14}\)C]leucine and [6-\(^{3}\)H]thymidine incorporation into appropriate fractions were measured by counting in Picofluor-30 using a liquid scintillation spectrometer (LKB 1219 Rackbeta). Results were corrected to disintegrations per minute (dpm) using internal standards, and these values were corrected for specific activity (dpm per g protein or DNA). Estimation of L-[U-\(^{14}\)C]leucine and [6-\(^{3}\)H]thymidine incorporation were carried out using the same flask of cells for both measurements. Counts were calculated using a dual label programme:-

Activity of \(^{14}\)C = \(\frac{C_B}{E_{2B}}\)

Activity of \(^{3}\)H = \(\frac{C_A E_{2B} - C_B E_{2A}}{E_{1A} - E_{2B}}\)

where counts in \(^{3}\)H channel = \(C_A\)

counts in \(^{14}\)C channel = \(C_B\)

Efficiency of \(^{3}\)H in \(^{3}\)H channel = \(E_{1A}\)

counts in \(^{14}\)C in \(^{3}\)H channel = \(E_{2A}\)

counts in \(^{14}\)C in \(^{14}\)C channel = \(E_{2B}\)
4.2.7 Pulse labelling the RTE cultures
Cultures were pulse labelled every 24 hrs with \([6-^3\text{H}]\text{thymidine (1}\mu\text{Ci}ml^{-1})\) for 3 hrs at 30°C. The method was as for determination of rate of incorporation of \(^3\text{H}-\text{thymidine, following this incubation. DNA content was determined by the method of Burton (1956).}

4.2.8 DNA assay
The method used was essentially that of Burton (1956) with the following modification:- sample and stock DNA (from calf thymus) were hydrolysed twice in a boiling water bath with PCA (0.5M) at 90°C for 10 minutes and the two volumes of PCA were pooled prior to the addition of diphenylamine reagent.

4.2.9 Protein assay
The residual pellet, after hydrolysis with PCA, was washed with ethanol and then dissolved in sodium hydroxide. Protein content was assayed against BSA standards using the method of Lowry et al (1951).

4.2.10 Electrophoresis
RTE cells or protein extractions were all prepared for SDS-PAGE as follows:-

Cultures were washed three times with PBS then solubilised in SDS (2%w/v) and mercaptoethanol (ME) (1%v/v). A final concentration of 1mg/ml\(^{-1}\) was obtained where possible.
After dissolving in SDS/ME the samples were boiled for 3 minutes at 100°C.

Occasionally in "old" cultures there was a small amount of insoluble material remaining after boiling. This was probably residual cell envelopes of highly differentiated cells. Such envelopes contain (γ-glutamyl-)lysine bonds which are resistant to detergents and reducing agents (Matoltsy and Balsamo 1955; Matoltsy and Matoltsy 1966).

Aliquots of samples were added to SDS-PAGE sample buffer, and appropriate volumes of sample in buffer added to a sample pocket of the upper stacking gel.

Electrophoresis was performed by use of a 3% stacking gel (0.125M Tris-HCl pH 6.8) and a 10% running gel (0.4M Tris-HCL pH 8.8). Bromophenol blue was used as a tracking dye.

A current of 20mA was applied to the gel until the front marked by the Bromophenol blue reached the stacking/running gel interface. At this point the current was increased to 40mA.

The resultant gels were stained with Coomassie blue R-250 (0.5g Coomassie blue R-250, 250ml propan-2-ol, 100ml glacial acetic acid and 650ml distilled water) overnight and destained in a mixture containing 1 vol propan-2-ol, 1 vol glacial acetic acid and 8 vol distilled water.
Gels were stored in a solution containing 3% glycerol (v/v) and 2% sodium azide (w/v).

4.2.11 Keratin preparations from RTE Cells

Most animal cells contain, in addition to microfilaments (5-6 nm diameter) and microtubules (20-25 nm), a third system of cytoskeletal elements, the "intermediate-sized" filaments which include tonofilaments (i.e., keratins), neurofilaments, 10 nm filaments of muscle cells (Franke et al. 1978) and vimentin (Franke et al. 1979).

Samples enriched in keratins of RTE cell cultures were prepared for SDS-PAGE using a method based on that of Franke et al. 1979).

Cultures were rinsed twice in TNM-buffer (10 mM Tris-HCl, 140 mM NaCl, 5 mM MgCl$_2$, pH 7.6), and were then incubated for 2-4 minutes in TN-buffer (10 mM Tris-HCl, 140 mM NaCl pH 7.6) containing 1% Triton X-100 (0.5% v/v) and KCl (1.5 M) (pH 7.6). This dispersed cell material was collected by centrifugation at 3,500g for 20 minutes and the pellet washed twice by brief dispersion in 10 mM Tris-HCl (pH 7.6) followed by centrifugation. The samples were then treated in the same manner as protein samples for SDS-PAGE.
4.2.12  Cell Viability

4.2.12.1  Trypan blue dye permeability

The number of cells permeable to this dye equates approximately with the number of dead cells (Pappenheimer 1917: Schreck 1943). The percentage of blue to unstained cells was estimated using a haemocytometer.

4.2.12.2  Malate dehydrogenase activity in medium of RTE cell cultures

Damaged or dead cells "leak" cytosolic enzymes into the medium. Therefore, the amount of MDH activity from a known number of cells, present in medium can be used as an assessment of cell viability (Middleton 1980) when compared with the maximum amount of MDH activity "leaked" from an identical number of homogenised cells (ie., 100% non-viable). MDH (EC 1.1.1.37) activity was determined essentially by the method of Siegel and Bing (1956) using the principle of the conversion of oxaloacetate to malate via the following scheme:

\[ \text{Oxaloacetate} + \text{NADH} \xrightarrow{\text{MDH}} \text{Malate} + \text{NAD}^+ \]

The change in absorbance of NADH $\rightarrow$ NAD$^+$ at 340nm is measured, using the Sigma 340-UV test kit.
4.2.13 Enzyme Assays

4.2.13.1 Ornithine decarboxylase (4.1.1.17)

In all animal tissues that have been examined, a pyridoxal phosphate-dependent enzyme that specifically catalyses the decarboxylation of L-ornithine represents the only mechanism for the formation of putrescine (Williams-Ashman 1969; Williams-Ashman 1972; Pegg 1968). The enzyme appears to be localised almost completely in the cytosol of animal cells. ODC is the first and rate limiting enzyme in polyamine biosynthesis, and in some animal tissues is inhibited in a competitive fashion by putrescine and to a lesser extent by spermidine and spermine. (Pegg 1968; Shrock 1970).

Polyamines were implicated by Cohen (1966) to be important in the regulation of RNA synthesis, and consequently of protein synthesis. Therefore measurement of ODC activity can be used as an indication of the state of the cell at the nuclear level. Other studies of this enzyme induction indicate that it may be of primary importance in tumour promotion (O'Brien 1976; Verma et al, 1979) and is now widely used as a marker for tumour promotion by, for example, TPA (O'Brien et al, 1975).

Estimation of Ornithine Decarboxylase Activity

Cultures were incubated with $^{14}$C ornithine (0.04μCi ml$^{-1}$) for 1.5 hrs at 30°C. Culture flasks were capped with serum stoppers through which a polypropylene centre well was passed containing a filter paper wick.
The incubation was terminated by standing culture flasks on end at 4°C. A solution of *EET (100µl) was then injected through the serum stopper onto the wick and left to take up liberated CO₂ for 1 hr. Injection at this point allowed the pH of the medium to remain stable during incubation. An EET trap avoided the necessity of neutralisation for scintillation counting. Samples were counted in Picofluor-30 on a LKB 1219 Rackbeta liquid scintillation spectrometer. Cells could be trypsinised and measurements of protein made using the method of Lowry et al (1951).

*EET - Jeffay (1961) have described the use of solution of ethanolamine-ethylene glycol monomethyl ether for ¹⁴CO₂ trapping. Ethanolamine is an efficient trapping material and unlike NaOH, does not interfere with the liquid scintillation process. A solvent mixture containing ethanolamine-ethylene glycol monomethyl ether and toluene in a ratio of 1:8:10 by volume was found to be most suitable. Use of the ethanolamine-ethylene glycol monomethyl ether was required to facilitate solubility of ethanolamine carbonate in toluene.

4.2.13.2 Prolinase (3.4.13.8)
During collagen degradation, dipeptides are hydrolysed by prolinase and prolidase. Prolinase acts upon dipeptides of the type prolyl-‘amino acid’ and prolidase upon dipeptides of the type amino-acyl-proline (C). It is therefore a useful marker of collagen-type diseases, collagen having
been indicated as being useful in the determination of psoriatic conditions of the skin (Fleckman et al., 1973).

**Estimation of Prolinase activity**

This is essentially the method of Butterworth and Priestman (1982).

Homogenate of RTE cells was prepared by sonication using a 3mm probe (4mA) for 30 seconds, with cooling (Dawes Ultrasonic Disintegrator 180). 50μl samples of homogenate were added to 50μl 50mM barbitone HCl buffer, pH 7.4 (with 4mmol MnCl₂) and 100μl 30mM prolyl-valine.

Dipeptides used in the initial validation included prolyl-valine, prolyl-alanine and prolyl-phenylalanine, rates of catabolism were approximately equivalent; prolyl-valine, however, was more readily soluble and was therefore chosen for subsequent studies.

After 120 minutes incubation at 37°C, 200μl 7.2g l⁻¹ PCA was added to terminate the reaction. Substrate and extract blanks were run simultaneously. Proline was measured by use of a reagent containing 3ml o-phthalaldehyde (12g l⁻¹ methanol), 3ml 2-mercaptoethanol (0.6%v/v in ethanol) and 95ml 150mM Na₂CO₃-NaHCO₃ buffer pH 9.5, freshly prepared. 10μl samples of incubation mixture were assayed using this reagent. Fluorescence was measured on a Perkin Elmers LS-5 fluorimeter (excitation 365nm, emission 455nm).
4.2.13.3 Acid Phosphatase (3.1.3.2)

Acid phosphatase is one of the few enzymes present in relatively high amounts in the epidermis. The enzyme has been associated with damage to skin by low irritancy compounds (Rutherford 1974).

**Estimation of Acid Phosphatase activity**

This was based on the method of Alvarez (1962).

Cell homogenate (~ 10µl) was prepared by sonication (see prolinase assay). Homogenate was added (sufficient to remain within linearity) to 1ml citrate buffer (0.1M, pH 5.6) and 30µl p-Nitro phenylphosphate (sodium salt 0.6M). The incubation mixture was then incubated for 15 minutes at 37°C and the reaction terminated by addition of 2ml (0.5M) NaOH. Absorption was measured at 405nm on a Perkin Elmer Lambda 5 u.v/Vis spectrophotometer.

4.2.14 Estimation of ATP content of cells

ATP was determined using the decrease in absorbance of NADH occurring during the following reaction sequence:

\[
\text{ATP} + 3\text{-Phosphoglycerate} \xrightarrow{\text{PGK}} \text{ADP} + 1,3\text{ diPG}
\]

\[
\text{NADH} + 1,3\text{ diPG} \xrightarrow{\text{GAPD}} \text{glyceraldehyde-3-P} + \text{NAD}^+ 
\]

Cultures were washed with PBS and 0.5ml 12% TCA added. Cells were removed using a rubber policeman and then centrifuged at 2000rpm for 10 minutes. Activity was measured using the Sigma ATP kit 366.
4.2.15 **Induction of ethoxycoumarin-O-deethylase activity**

Cultures were preincubated with 10 and 25μM MC or 2μM BP for 72 hours prior to estimation of deethylase activity or BP hydroxylation activity analysis by HPLC. Control and solvent controls were also run simultaneously.

4.2.16 **Determination of Ethoxycoumarin-O-deethylase in whole cells**

This method is essentially that of Jacobson *et al* (1974).

Stock solutions of 7-EC were made up in DMSO. EC was added to cultures to give a final concentration of 70μM and incubated for 24 hrs at 30°C. The reaction was terminated by separating the medium from the cells.

Aliquots (1ml) of the medium were incubated for 15 hours at 37°C with 0.5ml 0.2M acetate buffer, pH 4.5, containing β-glucuronidase (2mg.ml⁻¹) to liberate 7-hydroxycoumarin from its glucuronide conjugate. The impure β-glucuronidase preparation contains sufficient sulphatase activity to also hydrolyse the sulphate conjugate of the 7-hydroxycoumarin.

The hydrolysed medium was extracted with hexane (5ml) by shaking for 10 minutes and the hexane layer discarded, as this contained unmetabolised 7-EC. The 7-hydroxycoumarin was extracted into 5ml diethyl ether containing 1.5% isoamyl alcohol (v/v) for 10 minutes. A sample of the ether phase (2ml) was back extracted into 0.2M glycine/NaOH buffer, pH
10.4 (5ml) for 10 minutes. The extracted 7-hydroxycoumarin was measured fluorimetrically in a Perkin Elmer LS-5 spectrofluorimeter, using an excitation wavelength of 370nm and an emission wavelength of 450nm. Blanks of medium from cultures incubated without 7-EC and medium incubated with 7-EC in the absence of cells were taken through the extraction procedure along with standards of 7-hydroxycoumarin added to blank medium to give a final concentration of 500nM.

4.2.17 Determination of 7-Ethoxyresorufin O-Deethylase Activity

Stock solutions of 50μM 7-ER were prepared in DMSO. The compound was added to the cell suspensions and cultures to give a final concentration of 0.5μM. Cultures were incubated with the 7-ER for 24 hrs at 30°C. The reaction was terminated by separating the medium from the cells.

Aliquots (1ml) of the medium were incubated for 15 hrs at 37°C with 0.5ml 0.2M acetate buffer, pH 4.5, containing β-glucuronidase (2mgml⁻¹) to hydrolyse any glucuronide conjugates.

The resorufin was extracted from the hydrolysed medium into ethyl-acetate (3ml) for 30 minutes. A sample of the ethyl acetate phase (2ml) was back extracted into 0.2M glycine/NaOH buffer, pH 10.4 (6ml) for 30 minutes. The extracted resorufin was measured fluorimetrically on a Perkin Elmer LS-5 spectrofluorimeter, using an excitation
wavelength of 530nm and emission wavelength of 585nm. Blanks of medium from cultures incubated without the 7-ER and medium incubated with the 7-ER in the absence of cells were taken through the extraction procedure along with standards of resorufin added to blank medium to give final concentrations of 0.1μM to 1μM.

4.2.18 Determination of Glucuronyl Transferase and Sulphotransferase Activity

7-Hydroxycoumarin was used as a substrate. A stock of 140mM 7-hydroxycoumarin was prepared in DMSO. An aliquot was added to the cell cultures to give a final concentration of 70μM. Cultures were incubated with the 7-hydroxycoumarin for 24 hrs at 30°C. The reaction was terminated by separating the medium from the cells.

After extraction of free 7-hydroxycoumarin as previously described for deethylase activity, aliquots (1ml) of the medium were incubated for 15 hrs at 37°C with 0.5ml 0.2M acetate buffer, pH 4.5, containing β-glucuronidase (2mg.ml⁻¹) to hydrolyse any glucuronide conjugates. The impure β-glucuronidase preparation contained sufficient sulphatase activity to hydrolyse any sulphate conjugates. This produced the total amount of conjugates, after extraction as previously described for 7-ECOD. Aliquots (1ml) were also incubated for 15 hrs at 37°C with 0.5ml 0.1M acetate buffer, pH 4.5, containing 12.9mg.ml⁻¹ aryl sulphatase and 11.5mg.ml⁻¹ saccharolactone. The free 7-
hydroxycoumarin was extracted as previously described, producing the total sulphotransferase activity. The difference between the activity found with glucuronidase and sulphatase was the true value of glucuronyl transferase activity.

4.2.19 Preparation of samples for HPLC

Cultures were treated with 0.5μgml⁻¹ BP and were prepared for HPLC by pooling cell material and medium from 6 flasks. Medium was removed, pooled and subjected to deconjugation (see ECOD). Cells were then trypsinised and pooled, spun down to pellet, resuspended in PBS, homogenised using a Dawes Ultrasonic disintergrator 180 and subjected to deconjugation. Both cell samples and medium samples were then treated with acetone (2 x original volume). This prevented any further reactions and precipitated cellular protein. Acetone fractions were extracted 3 times with an equal volume of ethyl acetate which were then pooled and evaporated to dryness under nitrogen. The residues were then resuspended in methanol (HPLC grade), filtered (0.22μ Gelman Acrodisc CR) and evaporated to dryness under nitrogen. Samples could then be resuspended in methanol and subjected to HPLC. Throughout preparation, all samples were kept in the dark, to prevent any photolysis.
4.2.20 High Pressure Liquid Chromatography

HPLC was performed using a methanol : water gradient with initial composition 65% methanol : 35% water. The gradient was run through a 25cm, 5ODS (type II) 5μ spherisorb, alumina column by two Gilson 302 pumps (Gilson 802 pressure monitor) interfaced with an Apple II PC with a Gilson 702 gradient manager.

The gradient was run with increasing methanol concentrations (0.5% min⁻¹) at 1.5ml min⁻¹, 3000 psi max and at room temperature.

Chromatographed gradient was monitored by a Kratos GM970 fluorimeter with excitation wavelength 254nm and 418nm filter, and a spectromonitor III, type 1204A, spectrophotometer working at 254nm.

Standards of 3 and 9 hydroxy benzopyrene and 7,8 and 9,10 diols of BP were run to establish retention times.

The method used was basically that of Selkirk (1974).

4.2.21 Cytogenetics

Cultures were treated with 0.1μg ml⁻¹ colcemid for 24 hours and then removed by gentle shaking and trypsinisation. Cells were then suspended in water for 30 minutes, spun down at 500g and fixed in methanol : acetic acid (3:1) for 5 minutes. The pellet was further washed in acetic-alcohol
(x2) and spreads prepared on glass slides. Samples were then stained with Giemsa in order to observe metaphase chromosomes.

4.2.22 Toxicity Screening in Multiwell Plates
Twenty four well plates were seeded with $0.4 \times 10^5$ cells ml$^{-1}$ of either BCL-D1 or RTE$_4$, 24 hours prior to treatment of cells with test compound and incubated at $30^\circ C$ 5% CO$_2$.

Cells were then treated with test compound previously added to fresh media. Stock solutions of test compounds were made up freshly in DMSO as solvent. Solvent controls were run simultaneously and measurements of protein content were made after a further 72 hours incubation, using a Dynatech Microelisa minireader MR590 with a 570nm filter. Protein was measured as previously described.

4.2.23 Probe Analysis
Samples prepared for thin film probe analysis were processed by conventional TEM methods. Sections were cut in the thickness range of 100-200nm. They were coated with a 10nm carbon film to render them conducting and examined unstained, using a Philips 200T electron microscope interfaced with a Link systems 860 analyser. For each analysis, a slightly deferred 1 m electron beam was centered on the region of interest and 100 counts obtained. Integrals of iron, zinc Kα, adjacent backgrounds and a region of Bremstrullum (away from major characteristic
peaks) were recorded. The regions analysed were lysosomes and mitochondria.

4.2.24 Electron Diffraction

Diffraction patterns were obtained using a Philips 200T electron microscope with stained and unstained (see Probe Analysis) sections. Areas of both organelles and adjacent background (epon resin) were observed for diffraction bands. Samples were also tilted in several degrees of orientation and analysed.
DEVELOPMENT AND CHARACTERISATION OF A DIFFERENTIATING KERATINOCYTE CULTURE
5 DEVELOPMENT AND CHARACTERISATION OF A DIFFERENTIATING KERATINOBYTE CULTURE

5.1 Introduction
In order to study the effects of irritants and carcinogens in vitro, a keratinocyte culture capable of continuous growth and differentiation in vitro was established.

Cultured keratinocytes possess many characteristics of differentiation in vivo such as desmosomes and tonofilaments (Yuspa et al., 1970; Fusenig and Worst, 1972; Green 1977; Marcelo et al., 1978; Prunieras et al., 1978) allowing correlation of toxic effects on morphology in vitro and in vivo. However, although a keratinocyte culture permits the study of effects specific to this cell type, it is advantageous for toxicity studies if the culture is comparatively simple in nature. Cultures requiring extra growth factors and feeder layers complicate interpretation of toxic effects. Ideally the culture must also possess its own metabolising system and be sensitive to toxic insult of compounds produced, thereby eliminating the need for a two stage system, one of metabolic activation and one of toxic response.

A continuously proliferating and differentiating keratinocyte culture of rat sublingual epithelium was initially developed by Jepsen (1974) and Buckley (1980). This culture system is capable of growth in Eagles MEM with
Earle's salts and 10% FCS with no necessity for added growth factors or feeder layers. Improvements on the method of setting up the cultures have been made here. Detailed examination of the biochemistry and morphology of the culture system was required in order to assess its suitability as a model for the screening of toxic compounds. Therefore, characterisation of the system was undertaken in order to understand the various growth characteristics of these cultured keratinocytes. A moderately simple system for testing toxins could then be developed.

In order to study early effects leading to commitment to differentiation, cultures were more closely screened during their early development stages (1-14 days) during which time commitment was taking place.

RESULTS
5.2 Establishment and maintenance of RTE cells
Primary cultures contained both epithelial and fibroblastic cells and therefore in order to produce a culture made up of predominantly keratinocytes, a series of methods were assessed.

Establishment of the first subculture was greatly enhanced by using preconditioned media. Preconditioned media was prepared by using media from a previously established line and mixing in a 1:1 ratio with fresh medium (0.5% DMSO, 20% FCS).
Differential trypsinisation using 0.25% trypsin removed fibroblasts preferentially to epithelial cells, the resulting culture could then be trypsinised with 0.25% trypsin/1mM EDTA for subculturing. Crowding by pooling several flasks of primary cultures, enhanced the plating efficiency of epithelial type cells, thereby increasing the proportion of epithelial cells to fibroblasts. Incubation at 30°C inhibits growth of fibroblasts (Jepsen 1974) whereas rat tongue epithelial cells will grow continuously at 30°C. The use of medium containing D-valine instead of L-valine as a further measure intended to prevent fibroblast growth (Gilbert and Migeon, 1975) proved to be of little use. It has been suggested that fibroblasts do not possess the D-amino acid oxidase necessary for conversion of D-valine to L-valine and growth should thus be inhibited in the presence of D-valine. However, with 3-4 days of growth in this medium the epithelial cells became enlarged and vacuolated. The use of this medium was therefore discontinued. Similarly Jensen and Jacobson (1984) found D-valine medium to be unsuitable for skin epithelial cells in culture (NB Those epithelial cells do not contain detectable amounts of D-amino acid oxidase). Screening against contamination by viruses and mycoplasma was undertaken using TEM for identification of viral contamination and SEM together with the Hoechst Stain No 33258 using excitation of 360nm and emission of 490-500nm (Kit No. 30-100-00) for identification of mycoplasma.
Confluency of cultures was attained by day 14 of growth with the appearance of areas undergoing stratification. Prior to confluency, focal points of stratification took place observable by phase contrast microscopy and light microscopy using H&E stain, Papanicolaou stain and Acridine Orange. These areas of stratification could be observed as early as day 7 of growth (PLATE 2).

During the initial 14 day growth period, subcultures were made for comparison of plating efficiency. Plating efficiency was greatest during this initial growth period (>95%) and decrease progressively with stratification suggesting only basal layer cells attach. Further to confluency and initial stratification, cells emerging from the basal layer could only be distinguished by their more refractile nature, the picture becoming increasingly more complicated with age of the culture, as basal cells became obscured. Squames could be observed in the media by day 21 of growth. Cells showed typical epithelioid shape once colonies were established and no morphological characteristics constituting fibroblastic growth were observed.

When smears of stratified cultures were taken and stained by the Papanicolaou method (PLATE 3), three basic cell populations were observed staining green, orange and red.
H&E OF 14 DAY CULTURE.
B - BASAL CELLS; S - SQUAME; M - AREA OF MULTILAYER.
NOTE OVERLAPPING NUCLEI. (x 400)

ACRIDINE ORANGE STAIN OF 14 DAY CULTURE.
B - BASAL CELLS; S - SQUAMES. (x 200)
PLATE 3

SMEAR OF CONTROL RYE CULTURE: PAPANICOLAOU STAIN. (X 400)

A - BASAL LAYER CELLS

B - INTERMEDIATE LAYER CELLS

C - SQUAMOUS CELLS
5.3 Morphological examination of RTE cell culture

Culture flasks 25cm², were seeded with 0.4 x 10⁵ cells ml⁻¹ (5ml, to maintain correct oxygen tension) at day 0. Growth on glass, using coverslips in Leidig tubes, and plastic substrates were monitored simultaneously using plastic as the control medium. Glass has many advantages over plastic for use in microscopy, since use of organic solvents is often necessary. Light microscopical examination of cultures showed no difference between growth on glass and plastic substrates. Cells showed typical morphology when grown in culture by reduction in size of the cytoplasm with increasing confluency (PLATE 4). Very few multinucleate cells were observed and nucleolar number varied with some cells containing up to 6 nucleoli. The greater proportion of cells, however, contained 2-3 nucleoli. A small proportion of cells contained vacuoles and lipid inclusions as observed using Oil-Red-O stain.

5.3.1 Ultrastructural examination of RTE cell culture

Ultrastructural examination of cultures using SEM was performed with cultures up to 21 days in age. Little tissue shrinkage was observed in subconfluent cultures. Cells appeared regular in size with predominant nuclei, and showed regular surface features, in contrast to those undergoing mitosis or degenerative changes (PLATE 5). Mitotic cells were distinguishable by the appearance of intercellular bridges and microvilli producing irregular surface features (PLATE 6). Degenerative changes were distinguishable by
PLATE 4  PHASE CONTRAST OF RPE CULTURE (PLASTIC SUBSTRATE) (x 100)

A - DAY 6;  B - DAY 11;  C - DAY 16.
PLATE 5  SEM OF RTE CULTURE (GLASS SUBSTRATE)
A - NOTE REGULAR SURFACE FEATURES AND PROMINENT NUCLEI
B - NOTE FORMATION OF DESMOSOMAL JUNCTIONS
PLATE 6  A,B SEM OF RTE CULTURE (GLASS SUBSTRATE) SHOWING INTERCELLULAR BRIDGES AND ROUNDELING UP OF CELLS DURING MITOSIS  B - NOTE SURFACE FEATURES
membrane "blebbing" (PLATE 7). Both mitotic and degenerated cells showed cytoplasmic retraction, although mitosis produced a more rounded appearance.

On very few occasions, cells of a fibroblastic appearance were seen (PLATE 8), although in these cases it was difficult to determine whether they were mitotic cells, since rounding up of cells during mitosis produced cells of similar morphology. Cell borders produced many fibrils (PLATE 9), extending to neighbouring cells when sub-confluent. In more densely packed areas, desmosomal junctions were apparent, through continuity of cell borders (PLATE 9).

With increasing stratification, tissue shrinkage produced fracturing of samples, but squames could be easily identified on surface layers of cells (PLATE 10). Intercellular bridging of sub layers was observed and it was evident that cells in this late phase of development lacked nuclear definition. Cells were greatly enlarged and flattened, although basal cells did not assume the columnar nature that appears in vivo. Squames had more irregular surface features and were partially detached.

Cells underlying the squamous layer could only be distinguished from basal cells by their reduction in cytoplasmic size (PLATE 11).
PLATE 7 (A) SEM OF RTE CELL UNDERGOING DEGENERATIVE CHANGE -
NOTE SURFACE BLEBBING

PLATE 8 (B) SEM OF FIBROBLASTIC TYPE CELL FROM RTE CULTURE
PLATE 9

A - FIBRILS OF RTE CELL BORDER

B - SEM OF DESMOSOMAL JUNCTIONS
PLATE 10  

A - H&E STAIN OF RTE CULTURE SHOWING OVERLYING SQUAMES (S)  
(x 400)  

B - SEM OF EQUIVALENT CULTURE SHOWING SQUAMOUS CELL
PLATE 11

A - SQUAMOUS CELL IN 7 DAY OLD CULTURE (S)

B - SQUAMES IN 21 DAY OLD CULTURE
   NOTE INCREASED CYTOPLASMIC SIZE (S)
Ultrastructural examination of cultures using TEM showed basal cells containing mitochondria, rough endoplasmic reticulum, myelin figures and, dependant upon age, tonofilaments which increased in number with the age of the culture (PLATE 12). Desmosomal junctions were evident amongst cells in contact (PLATE 13). Longitudinal sections of cultures permitted screening of large areas of basal cells but golgi apparatus and smooth endoplasmic reticulum were rarely seen.

Giant cells of the basal layer had a more amorphous nuclear structure but retained many of the characteristics of normal cells within the cytoplasm (PLATE 14).

A distinguishing feature of the basal layer cells was the presence of nuclear invaginations, the predominance of which coincided with the appearance of tonofilaments. Subsequent to the appearance of a tonofilamentous network, there was a subsidence in the number of invaginations until the nuclear structure resumed to that of cells early after subculture. Invaginations appeared to extend through the nucleus and showed an association with nucleoli (PLATE 15). Membrane structure was similar to that of the nuclear envelope with a double membrane, interspersed with nuclear pores and the inner membrane in close association with ribosomal material (PLATE 16). Often the invaginations appeared to form a network within the nucleus.
PLATE 12  TEM OF RTE CELLS
NOTE MITOCHONDRIA (M), ROUGH ENDOPLASMIC RETICULUM (R),
TONOFILAMENTS (T)  (x 18000)
PLATE 13  DESMOSOMAL JUNCTION (D) SHOWING FIBRILS EXTENDING WELL INTO CYTOPLASM (F)  (x 90,000)
PLATE 14 TEM OF GIANT MULTINUCLEATE CELL
NOTE MORE AMORPHOUS NATURE OF NUCLEUS, COMPARED TO NORMAL RTE CELL (N) (x 6000)
PLATE 15  NUCLEAR INVAGINATIONS OF RTE CELLS
NOTE APPARENT ASSOCIATION WITH NUCLEOLI
(A - x 15000;  B - x 12000)
PLATE 16  NUCLEAR INVAGINATION OF RTE CELL SHOWING NUCLEAR FORES (P), ASSOCIATED RIBOSOMAL MATERIAL (R) AND ASSOCIATION WITH NUCLEOLI (N) (x 36000)
Cells of the intermediate layers had increased numbers of tonofilaments and reduced numbers of intracellular organelles. Greater numbers of myelin figures and the appearance of autophagosomes were also indicative of the intermediate layers. The desmosomal junctions were also a common feature (PLATE 17,18).

In the upper layers cells contained an abundance of tonofilaments. Plasma membranes were thickened and cells showed varying degrees of degeneration, with loss of ribosomal material and mitochondria and nuclear pyknosis.

5.4 Growth of RTE cultures

Total adhered protein content of RTE cells increased to a value of approximately 3mg per flask by day 28 of growth. During this growth period, a 'lag' phase was evident during the period from day 14 of growth to day 21 (Fig 3).

Total DNA content increased to a value of approximately 250 μg per flask by day 28, showing no 'lag' phase during growth (Fig 4).

\(^3\)H-Thymidine incorporation increased marginally to a maximum value of approximately 5μmol hr\(^{-1}\) μg\(^{-1}\) DNA by day 21 of growth corresponding to the time of differentiation, followed by a decrease to approximately 3μmol hr\(^{-1}\) μg\(^{-1}\) DNA by day 28. (Fig 5). The rise in DNA content during terminal differentiation of the culture was therefore accompanied by a similar rise in incorporation of thymidine.
PLATE 17 CROSS SECTION THROUGH BILAYER OF RTE CULTURE SHOWING ATTACHMENT TO SUBSTRATE (S) BY HEMIDESMOSOMES (H) AND LOSS OF BASAL CELL ARCHITECTURE IN UPPER LAYER (x 1200)
PLATE 18  CROSS SECTION THROUGH BILAYER SHOWING APPEARANCE OF AUTOPHAGOSOMES (A), INCREASING TONOFILAMENT NUMBER (T) AND INTERCELLULAR DESMOSOMAL JUNCTIONS (D) (x 24000)
PROFILE OF TOTAL ADHERED PROTEIN CONTENT FLASK$^{-1}$ DURING RTE CULTURE

FIGURE 3

$n = 5$ EXPT
MEAN ± SEM
PROFILE OF $^3$H-THYMIDINE INCORPORATION INTO DNA (SPECIFIC ACTIVITY) DURING RTE CULTURE

FIGURE 5

n = 2 EXPT
MEAN ± SEM
5.4.1 Enzyme Profiles of RTE cultures

Specific activity of ODC increased sharply from approximately 5 to 30nmol hr\(^{-1}\) mg\(^{-1}\) protein from day 14 to day 21 of growth. This increase in activity was followed by a fall in activity by day 28 to approximately 12nmol hr\(^{-1}\) mg\(^{-1}\) protein. (Fig 6).

Acid Phosphatase activity also reached a maximum at day 21 at 8µmol hr\(^{-1}\) mg\(^{-1}\) protein. At the three other time points observed specific activity remained within the range 3-5µmol hr\(^{-1}\) mg\(^{-1}\) protein (Fig 7).

Prolinase activity, similarly to the enzymes ODC and acid phosphatase, peaked at day 21 rising to 2.7mmol hr\(^{-1}\) mg\(^{-1}\) protein. The apparent 'base' level was at approximately 1.2mmol hr\(^{-1}\) mg\(^{-1}\) protein (Fig 8).

Leakage of MDH from the cells into the medium expressed as the percentage of the total, decreased during the time of culture. Day 7 produced an enzyme leakage of 17% of total, but this decreased to 3% by day 28. (Fig 9).

5.4.2 Protein profile of cultured cells

SDS-PAGE protein profiles of RTE cultures showed no difference between 7 and 28 day old cultures. The molecular weights of the 5 major proteins were estimated to be 62,000; 60,000; 57,000; 52,000 and 45,000 (PLATE 19). Using the method of Franke et al (1978) these proteins remained
PLATE 19 PROTEIN PROFILE OF RTE CULTURE USING SDS-PAGE.
FIGURE 6

n = 2 EXP
MEAN ± SEM

D A Y  O F  G R O W T H

0 10 20 30 40

mol HR-1 mg-1 protein
PROFILE OF ACID PHOSPHATASE
SPECIFIC ACTIVITY DURING RTE CULTURE

FIGURE 7

n = 3 EXPT
MEAN ± SEM

DAY OF GROWTH
PROFILE OF PROLINASE
SPECIFIC ACTIVITY DURING RTE CULTURE

FIGURE 8

n = 3 EXPT
MEAN ± SEM

DAY OF GROWTH
PROFILE OF MALATE DEHYDROGENASE ACTIVITY DURING RTE CULTURE

FIGURE 9

n = 2 EXPT
MEAN ± SEM

DAYS OF GROWTH

% TOTAL ACTIVITY

0 10 20 30
visible after SDS.PAGE with a marked reduction of minor proteins.

5.5 Cytogenetics
Chromosome spreads of cultures in metaphase indicated a culture heteroploid in nature. The proportion of diploid cells has not been determined.

5.6 Metabolism
Studies of metabolism by RTE cultures failed to detect the ECOD and EROD, in either induced or uninduced preparations.

Cultures showed a much greater capacity for Phase II metabolism as studied using 7-hydroxycoumarin. Rates obtained were 0.47±0.11 and 0.31±0.09nmol hr⁻¹ mg⁻¹ protein for glucuronidation and sulphonation, respectively.

HPLC indicated that cultures had the capability for conversion of BP to the 3-hydroxy, and 9,10 diol derivatives. Some evidence of quinones was obtained although standards were not available. (Appendix I).

Staining for Cyt P-450 and 448, using antibodies, in rat tongue in vivo failed to show any significant levels (Dr C Powell, personal communication). It is therefore unlikely that rat tongue keratinocytes in vitro would show detectable levels by this method.
5.7 DISCUSSION

Establishment of RTE primary cultures was simplified considerably, eliminating the need for SPF animals and plasma clots for attachment of tissue to growth substrates. Omission of the use of plasma clots aided trypsinisation of colonies.

Use of differential trypsinisation, growth at 30°C and seeding at high cell densities produced cultures containing minimal fibroblastic contamination. A combination of these methods appeared the most suitable for establishment of an RTE culture amongst other methods tried, including reduction of free calcium in the growth medium studied by Buckley (1980).

Growth on glass substrates enabled SEM to be undertaken without the introduction of Freon 113 into the procedure as would be necessary for cultures grown on plastic substrates (plastic being susceptible to the solvent effects of Freon). Growth on glass is also more suitable for ED using metal probes, plastic reduces resolution (Dr A Boyd, personal communication).

Long-term cultures of RTE cells showed no gross morphological differences to primary cultures and retained a close resemblance to rat sublingual epithelium in vivo. However, unlike epithelium in vivo, cultures showed only three distinctive layers, although, these layers could grow to more than one cell in thickness. Staining by the
Papanicolaou method also produced evidence of three basic cell populations among which basal layer cells stained green, and increasingly keratinised cells staining with acidophilic properties red to orange, but only when smears were prepared. H&E only distinguished two cell populations as did Acridine Orange and Papanicolaou when used in situ. Evidence of keratinisation was best seen with the Acridine Orange, which provided better definition of major cell components. Thus, for subsequent studies upon effects of toxicants upon differentiation and stratification, this stain was chosen. Keratinisation of cells showed with yellowish hues, basal layer cells had green nuclei (DNA of chromatin) and red cytoplasm (RNA of basophilic structures). Staining of RNA and DNA in this manner may also show malignant changes. Staining by H&E however, demonstrated squamous cells to be anucleate.

Having established cells growing on glass substrates, SEM was used to confirm the light microscopic evidence of stratification. Squames were flattened and also appeared anucleate in many cases. In well-developed cultures, SEM, like light microscopy, was of limited use as a much higher degree of sample destruction due to shrinkage was evident. Although, SEM would probably be of limited use in studying morphological changes during toxicity, it provided the best method of screening against contamination of cultures by mycoplasma.
The occurrence of nuclear invaginations within basal layer cells would appear to be a method of increasing nuclear membrane surface area, and thus the rate at which nuclear material may cross to the cytoplasm. Since it coincided with production of keratin, it would seem likely that this system of nuclear invagination could provide a mechanism by which the rate of keratin production could be increased during the keratinisation and ageing process. The membranes within the nucleus retained the typical structure of the nuclear envelope, with a bilayered membrane and the appearance of RNA granules in close association with the inner membrane. In some cases, evidence of cytoplasmic organelles within the invaginations occurred, confirming that contact with cytoplasmic material had occurred. Such invaginations or pseudo inclusions have also been demonstrated in human synovial intimal cells (Ghadially and Ray, 1969) and leukaemic cells and hepatocytes (Leduc and Wilson, 1959; Bloom, 1967).

RTE cells produce a culture with many similarities to the situation in vivo, showing also many of the typical growth patterns of cultured cells.

From the microscopy studies of RTE cells in culture, it was evident that a proportion of the population of cells was already committed to differentiation before confluency was attained. Thus it would seem that basal cell layer pressure and the relationship of adhesion between post-mitotic and
interphase cells (G_1b phase; Bullough, 1975) may not necessarily be the only factor controlling epidermal structure (Bullough and Deol, 1975). A predetermining, genetic factor may also play an important role in stratification.

The evidence of McLoughlin (1961), Briggaman and Wheeler (1968) and Bullough (1975) that the cells of the epidermis are dependant upon the dermis and its biochemical influences has long been considered not to be correct. Similarly, with respect to the growth of RTE cells without mesenchymal factors, melanocytes, Langerhans cells and Merkel cells of the basal layer, the ability of epidermal cells to undergo stratification through mechanisms at the nuclear level or via interaction between cells is further substantiated.

During the growth period of the RTE cell subcultures, a 'lag' phase in total protein is apparent between days 14 and 21. It would seem likely that this is due to a combination of cell confluency with growth inhibition, the commitment of the culture to stratification and consequent desquamation of anucleate cells into the surrounding media and thus a loss of total adhered protein content. The number of anucleate cells, however, is unlikely to account for this difference alone. Cells of the upper layers do however appear to have a greater nucleus to cytoplasm ratio (PLATE 17,18). This may also be, in part, responsible. Subculturing prior to this period resulted in the closest
relationship between viability and plating efficiency, probably due to the predominance of basal layer cells.

The growth curve given by total DNA content substantiates the fact that the 'lag' phase during growth as shown by total protein may be due to desquamation. If desquamated cells are anucleate, the loss of protein with respect to DNA would be of a much greater proportion, the degeneration processes of the nucleus being of a more 'controlled' nature. The decrease in $^3$H-thymidine incorporation after day 21 of growth is consistent with a state where well differentiated cells cease synthesis of new nuclear material as degradative processes become predominant, particularly if basal cells, only, are capable of cell division (Lever 1970), decreasing specific activity of incorporation.

Since ODC is a measure of the cell at the nuclear level (although an increase in ODC activity may be transcriptional or post-transcriptional (Brandt et al, 1972), it would mean that during the 'lag' phase of growth, the enzyme activity points to increased nuclear activity which might be consistent with a cell undergoing the differentiation process. The drop in ODC activity after day 21 is consistent with the degenerative processes at this stage of growth as with $^3$H-thymidine by dilution of specific activity by squamous cells.
Acid phosphatase provides a fairly early method of monitoring cell viability. During the degradative processes of keratinisation, as cellular autophagy takes place and therefore as lysosomal enzymes are produced by the cell, an increase in acid phosphatase might be expected. On this basis, the results obtained are consistent with degradative processes taking place during the major keratinisation period, as seen by microscopy, reaching a peak at approximately day 21, specific activity then falling due to the increase in squamous cells. Acid phosphatase may thus provide an accurate measure of the maturity and general state of the cells in culture.

Prolinase activity should show a more accurate measure of the state of keratinisation of the culture. Prolinase provides a control mechanism for the keratinisation process. The specific activity may increase with the maturity of the culture as the degree of keratinisation increases thereby compensating for the greatly enhanced production of keratin such that a steady state may be produced in cells before completion of development of squamous cells. At this point specific activity may fall due to this formation of dead, fully keratinised cells.

MDH activity indicated the degree of membrane damage imposed upon the cells during sub-culturing and therefore the trypsinisation process. Although a leakage of 17% of total was apparent at day 7, the plating efficiency of >95% was
maintained. Recovery of the culture was evident by the reduction in MDH released into the media. This enzyme, however, showed no indication of the keratinisation process. This leakage may also suggest a higher level of MDH in basal cells and an inhibition of leakage by other layers. Basal cells may, however, leak more enzyme, thickness of plasma membrane being a determining factor.

The results obtained from chromosome spreads indicate that, although morphological studies show a homogeneous culture, a degree of heterogeneity is present. This may or may not influence the culture in terms of the biochemical response to toxic insult. It would seem unlikely, however, that a genotoxic compound would necessarily show any difference in the ultimate toxic response, since the toxin should bind in the same way, regardless of the chromosome complement.

Cytogenetic studies would, however, be more complicated due to the heterogeneity of the culture. Chromosome aberrations would be detectable.

Studies of the metabolic capabilities of the culture indicates the presence of glucuronyl transferase, sulphotransferase and AHH activity. It is of major importance, when considering the use of the culture for subsequent toxicity studies, that the culture retains some the metabolising systems found in vivo. In terms of the compounds studied, the presence of AHH is critical, as
discussed previously, in connection with the Ah receptor protein.
PARAMETERS AND TIMEPOINTS/TOXICITY OF 3,3',4,4' TETRACHLOROBIPHENYL
6.1 INTRODUCTION

As described previously in the Introduction, there are a large number of parameters which might be chosen to investigate toxicity in different culture systems. The section on Materials and Methods has already given the reasons for the choice of parameters to study using the rat tongue epithelial line, and Chapter 5 showed the feasibility of measuring those parameters and the way in which they may be interpreted with regard to the growth cycle of the cell line.

At this point it was decided to investigate the effects of a toxin upon these parameters, in order to determine which were the most sensitive to changes within the cell; changes which may be reversible and therefore not due to non-specific cytotoxicity. Further to this, it was necessary to select various time points for both treatment of cultures and measurement of parameters such that the time taken for identification of toxicity and the practicality of measurements may be optimised.

The compound TCB3 was chosen as a model compound to investigate the use of the system because of some of the more specific effects it produces in the epidermis, namely, hyperplasia and hyperkeratosis (Vos and Beems, 1971). An
assessment of the suitability of the culture model and its sensitivity could then be made, by comparison with existing \textit{in vivo} data.

Another important factor influencing the use of the culture, as mentioned previously, is that of the capacity of the cultured cells to produce similar metabolites to those produced by the epidermis \textit{in vivo}. The possibility of the need for a metabolising system had, therefore, also to be assessed.

Previous work \textit{in vitro} with the PCBs is limited, although studies of the compounds as inducers of cytochrome P-450-dependant monooxygenases and their affinity for the Ah locus have been most closely investigated.

\textit{In vitro} work using the 3,3',4,4' isomer includes the effect on rabbit muscle lactate dehydrogenase showing 50% inhibition at 3\textmu M (Nishizawa and Ohyama, 1980), induction of aryl hydrocarbon (benzo[a]pyrene) hydroxylase activity in the chick embryo \textit{in ovo} (Hamilton \textit{et al}, 1983) and studies of the inhibition of uroporphyrinogen decarboxylase, also in chick embryo but using liver cell culture, significant inhibition (50%) being found at 0.1mM (Swain, 1982). Of these studies, the work by Hamilton \textit{et al} (1983) in the chick embryo \textit{in ovo} possibly has particular importance because induction of AHH activity of up to 30 fold by day 7 at 5\textmu M kg\textsuperscript{-1} was found. The levels of AHH are
critical when considering activation of mutagens and carcinogens. However, studies using the brominated analogues failed to produce mutation in V79 and rat liver cells (Kavanagh et al., 1985) (brominated analogues tend to be more reactive due to the electronegativity of the halogen substituent [i.e., polarisability]). Investigations of the effects of different halogen substituents have been undertaken by Bandiera et al. (1982) who showed that polarisability, stereochemistry and other physico-chemical properties may also be of importance in consideration of potency of the various halobiphenyls. The work of Bandiera et al. (1982) was also carried out in vitro but only induction studies, little is known about subsequent toxic effects. It is therefore necessary to examine the in vitro effects of TCDD. Although TCDD has a much greater potency than TCB3, structural similarities present it as a useful compound for prediction of possible toxic effects of the TCB. Studies of toxicity of TCDD in vitro using HeLa, Balb-3T3, SV101, SV40 transformed 3T3 mouse fibroblast, human skin fibroblasts and human lymphocytes failed to reveal any toxicity when treated with 1pM TCDD (Beatty et al., 1975). Similarly, 0.1pM TCDD failed to produce any toxic effect in another 23 different cell types including 5 liver epithelial lines and 2 human urinary tract epithelial lines (Knutson and Poland, 1980) perhaps surprisingly, since it might be expected that epithelial cells would be more sensitive. A possible reason for this being that the cell types chosen were for the most part derived from a transformed cell type.
Although toxic effects were not indicated in these systems studies of cell proliferation and differentiation, predominantly on cultures of epidermal origin, showed that terminal differentiation was enhanced in TCDD treated cultures, decreasing the basal cell numbers and increasing the degree of keratinisation (Osborne and Greenlee, 1985) although no change in cell protein content was observed, DNA synthesis and epidermal growth factor specific binding (EGF) were also suppressed. This observation was confirmed by the studies of Hudson et al (1985) showing decreased binding of EGF and a concomitant inhibition of EGF-stimulated DNA synthesis. Gierthy et al, (1984) observed a reversibility in the nature of TCDD toxicosis in culture by showing a reversal of the saturation density inhibition of growth observed after treatment with TCDD, also a reduced $^3$H-Tdr incorporation rate relating to a decline in cell proliferation was shown. An important point of this study was that a 14 day exposure time was used to produce a 50% decrease in saturation density of treated cultures.

A closer comparison of TCDD and TCB3 in terms of potency of induction of AHH and Ah receptor binding reveals that TCB3 is 3 fold greater than MC as an inducer (Poland and Glover, 1977) but markedly less potent than TCDD, although all appear to act on the same receptor. TCB2 will also bind at the Ah receptor at higher concentrations but the potency with which it will stimulate AHH is far lower than the 3,3',4,4' isomer. The affinity of the isomers for the
receptor is therefore of more meaning when describing their relative potency (Bandiera et al, 1982). As previously described, the 3,3',4,4' and 2,2',4,4' isomers possess different specificities in terms of the cytochrome P-450 type monoxygenase systems. In vivo studies of the two isomers in the rat showed marked differences in distribution and covalent binding, suggesting very different pharmacokinetic properties (Shimada and Sawabe, 1984). In general, the binding of the 3,3',4,4' isomer appeared greater in the majority of tissues investigated.

TCB3 appears as a more potent inducer of B(a)P hydroxylase, dimethylaminoantipyrine N-demethylase (DMAP-N-demethylase) and cytochrome b5 than the 2,2',4,4' isomer (Parkinson et al, 1980) and might therefore show greater toxicity in a culture system possessing inducible forms of the monoxygenase systems and receptors maintaining their in vivo affinity, in vitro.

A high and low dose of the tetrachlorobiphenyl isomer was used to assess the responsiveness of the parameters chosen to the toxic insult. This therefore provided a basis upon which certain parameters would, later, be chosen.
6.2 RESULTS

6.2.1 Morphological examination of cultures treated with TCB3

Staining of cultures with H&E (PLATE 20) and acridine orange (PLATE 21) did not show marked changes of the cultures treated with 5µM TCB3 from day 1 after subculture for 28 days. The only observable effect was that of a reduction in superficial layers of keratinised cells [and an increased incidence of giant cells within the basal layer].

Ultrastructural examination by SEM showed no changes in surface features (although indicating that cultures were free of mycoplasma contamination).

Examination by use of TEM revealed many observable changes, particularly in the cytoplasm of cells (PLATE 22). These indications of cytotoxicity were only observable in a proportion of cells treated with 5µM TCB3. Cytoplasmic changes in treated cultures consisted of vacuolation, lipid inclusions, nuclear pyknosis, cellular autophagy and crystalline inclusions with a striated structure (PLATE 23). It was concluded that these structures were not artefacts produced during cutting of sections since they did not all run parallel and possessed a fine structure. Myelinated figures were also occasionally observed (PLATE 24,25). No evidence of alterations in tonofilament production were observable, but mitochondrial swelling and disruption were apparent.
PLATE 20

A - H&E OF RTE CULTURE CONTROL AT DAY 14 (x 400)

B - H&E OF RTE CELLS TREATED WITH 5µM TCB3 (x 400)
PLATE 21

A - ACRIDINE ORANGE STAIN OF RTE CELLS AFTER TREATMENT WITH 5µM TCB3
NOTE INCREASE IN GIANT CELLS DAY 14 (x 100)

B - ACRIDINE ORANGE SHOWING TYPICAL GIANT CELL (G)
(x 200)
TEM OF RTE CELL SHOWING AUTOPHAGOSOME (A), LIPID INCLUSIONS (L), NUCLEAR PYKNOSIS, VACUOLES (V) AND 'CRYSTALLINE' INCLUSIONS (C) AFTER TREATMENT WITH 5μM TCB3 (x 30000)
PLATE 23 TEM OF RTE CELL SHOWING STRIATED STRUCTURE OF 'CRYSTALLINE' INCLUSIONS (S) VACUOLE (V), MITOCHONDRIA (SWOLLEN) (M), LIPID (L) (x 51000)
TEM OF RTE CELL TREATED WITH 5μM TCB3 SHOWING AUTOPHAGOSOME (A), 'CRYSTALLINE' INCLUSION (C) AND MYELINATED FIGURE (MF)
(x 25000)
PLATE 25  MYELIANTED FIGURE (MF) SHOWING MEMBRANOUS CONVOLUTIONS WITHIN MAIN BODY (x 36000)
6.2.2 Biochemistry of treated cultures

Total adhered protein (Fig 10) content showed suppression at 14 and 28 day time-points but not 21 days. Using 5μM TCB3 a 50% decrease in protein was observable at both time-points.

ODC activity (Fig 11) showed a general decrease in specific activity during treatment of RTE cultures with TCB3 but the effect was only significant when treating with 5μM. A decrease to approximately 40% of control was observable by day 21.

Prolinase activity (Fig 12) showed marked reductions in cultures treated with 5μM TCB3. At day 21 5μM TCB3 produced a decreased in activity to below 30% of control, a similar decrease was also observable at day 7. 1μM TCB3 however, produced only a 40% decrease at day 7.

Acid phosphatase activity (Fig 13) was increased by day 14 to 140% by treatment of cultures with 1μM TCB3 and decreased to approximately 50% at days 7, 21 and 28 by treatment with 5μM TCB3.

Adenosine triphosphate content (TABLE 3) of cells could not be determined by the method used before day 21 of culture. A luciferin/luciferase method would have to be incorporated at these time points. At day 21 however, ATP showed a 30% increase in cultures treated with 1μM TCB3.
PROTEIN CONTENT PROFILE OF RTE CULTURES TREATED WITH TCB3

RTE CULTURES WERE TREATED WITH TCB3 AND MEASUREMENTS WERE TAKEN EVERY 7 DAYS

FIGURE 10

CONTROL VALUES:

<table>
<thead>
<tr>
<th>DAY</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg protein</td>
<td>0.48</td>
<td>1.43</td>
<td>1.09</td>
<td>2.31</td>
</tr>
</tbody>
</table>

METHODS 4.2.9 MEAN ± SEM * = P < 0.05

- 5μM TCB3
- 1μM TCB3

% SOLVENT CONTROL

DAY
ORNITHINE DECARBOXYLASE PROFILE OF RTE CULTURES TREATED WITH TCB3

RTE CULTURES WERE TREATED WITH TCB3 AND MEASUREMENTS WERE TAKEN EVERY 7 DAYS

FIGURE 11

CONTROL VALUES:

<table>
<thead>
<tr>
<th>DAY</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>nmol hr(^{-1}) mg(^{-1}) protein</td>
<td>6.75</td>
<td>8.30</td>
<td>28.1</td>
<td>17.5</td>
</tr>
</tbody>
</table>

METHODS 4.2.13.1 MEAN ± SEM

\* = P < 0.05

- ■ - 5\(\mu\)M TCB3
- • - 1\(\mu\)M TCB3
PROLINASE PROFILE OF RTE CULTURES TREATED WITH TCB3

RTE CULTURES WERE TREATED WITH TCB3 AND MEASUREMENTS WERE TAKEN EVERY 7 DAYS

FIGURE 12

CONTROL VALUES :
DAY 7 14 21 28
µmol hr⁻¹ mg⁻¹ protein 1366 1259 2642 1364

METHODS 4.2.13.2 MEAN ± SEM * = P < 0.05

■ - 5µM TCB3
○ - 1µM TCB3

% SOLVENT CONTROL

0 100 200 300 400 500 600 700
0 7 14 21 28
DAY
ACID PHOSPHATASE PROFILE OF RTE CULTURES TREATED WITH TCB3

RTE CULTURES WERE TREATED WITH TCB3 AND MEASUREMENTS WERE TAKEN EVERY 7 DAYS

FIGURE 13

CONTROL VALUES:

<table>
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<tr>
<th>DAY</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>μmol hr⁻¹ mg⁻¹ protein</td>
<td>3.34</td>
<td>3.97</td>
<td>9.25</td>
<td>6.02</td>
</tr>
</tbody>
</table>

METHODS 4.2.13.3 MEAN ± SEM * = P < 0.05

- 5μM TCB3
- 1μM TCB3

% SOLVENT CONTROL

DAY
TABLE 3

ATP CONTENT OF RTE CULTURES TREATED WITH TCB3 AND NON-TREATED AT DAY 21

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ATP Content (µmol mg⁻¹ protein ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>0.0165 ± 0.0009</td>
</tr>
<tr>
<td>SOLVENT CONTROL</td>
<td>0.0165 ± 0.0011</td>
</tr>
<tr>
<td>1µM TCB3</td>
<td>0.0227 ± 0.0039</td>
</tr>
<tr>
<td>5µM TCB3</td>
<td>---</td>
</tr>
</tbody>
</table>

Mean of 2 determinations

DAY 7, 14 - undetectable
DAY 28 - not detected
MDH (Fig 14) showed no change from solvent control throughout the time period studied.

$^{3}$H-thymidine incorporation (Fig 15) showed a general decrease in rate during treatment of cultures with 5μM TCB3 to approximately 75% of controls.

Total DNA content (Fig 16) showed a suppression by day 14 of growth to 50% of controls when treated with 5μM TCB3 with an apparent recovery taking place to 80% of control by day 28.

Analysis of the growth cycle of RTE cells (Fig 17) during the first 14 days after subculture revealed two peaks during which incorporation of $^{3}$H-Tdr increased markedly. The maximum rates of incorporation were of similar size (3.5 μmol μg$^{-1}$ DNA hr$^{-1}$) and occurred at the 2nd and 12th day of growth. A smaller peak of 5μmol μg$^{-1}$ DNA hr$^{-1}$ was observed at day 8. DNA content rose steadily over this period.

Treatment of cultures at the 12th day after subculture (Fig 18) resulted in no change in $^{14}$C-leucine incorporation, protein content, $^{3}$H-thymidine incorporation, DNA content or prolinase activity, when assayed at day 21. Acid phosphatase showed an increase of approximately 20% with both high and low concentrations of TCB.
MALATE DEHYDROGENASE PROFILE OF RTE CULTURES TREATED WITH TCB3

RTE CULTURES WERE TREATED WITH TCB3 AND MEASUREMENTS WERE TAKEN EVERY 7 DAYS

FIGURE 14

CONTROL VALUES:

<table>
<thead>
<tr>
<th>DAY</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>% total</td>
<td>15</td>
<td>6</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>

METHODS 4.2.12.2 MEAN ± SEM * = P < 0.05

- 15µM TCB3
- 5µM TCB3
- 1µM TCB3
RTE CULTURES WERE TREATED WITH TCB3 AND MEASUREMENTS WERE TAKEN EVERY 7 DAYS.

**FIGURE 15**

**CONTROL VALUES:**

<table>
<thead>
<tr>
<th>DAY</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>µmol hr⁻¹ µg⁻¹ DNA</td>
<td>4.62</td>
<td>4.73</td>
<td>4.96</td>
<td>2.93</td>
</tr>
</tbody>
</table>

**METHODS 4.2.6.1**

**MEAN ± SEM**

* = P < 0.05

- ■ - 5µM TCB3
- . - 1µM TCB3

---

---
DNA PROFILE OF RTE CULTURES TREATED WITH TCB3

RTE CULTURES WERE TREATED WITH TCB3 AND MEASUREMENTS WERE TAKEN EVERY 7 DAYS

FIGURE 16

CONTROL VALUES:

<table>
<thead>
<tr>
<th>DAY</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg</td>
<td>60</td>
<td>82</td>
<td>124</td>
<td>226</td>
</tr>
</tbody>
</table>

METHODS 4.2.8 MEAN ± SEM * = P < 0.05

- 5µM TCB3
- 1µM TCB3
PROFILE OF $^3$H THYMIDINE INCORPORATION DURING 14 DAY GROWTH PERIOD OF RTE CELL CULTURE

PULSE LABEL EXPT:

METHODS 4.2.7

FIGURE 17

CULTURES WERE GIVEN A 3 HR PULSE LABEL OF $^3$H TdR AT 24 HR PERIODS

MEAN ± SEM 2 DETERMINATIONS
TCB 3, 14 DAYS AFTER SUBCULTURE

**Figure 18**

*Mean ± SD*

A - VALUES OBTAINED AFTER 21 DAYS

B - VALUES OBTAINED AFTER 28 DAYS

- 5μM TCB 3
- 1μM TCB 3
At day 28 for protein content, $^3$H-thymidine incorporation and DNA content, no detectable changes occurred. $^{14}$C-leucine incorporation was however, decreased to 60% of control at 1µM TCB3 and 40% at 5µM. Prolinase activity (Fig 19) was also reduced to 70% of control by 5µM TCB3.

Studies of the keratin profiles of treated cultures (treated at day 1) indicated no change in protein populations from control cultures. (See following chapter.)

6.3 DISCUSSION

Light microscopy of stained cells and phase contrast microscopy of cells during growth indicated that no gross damage had occurred on treatment of cells with TCB3 at the concentrations used. These observations suggest that any changes in biochemical parameters studied would be due to the specific effects of the PCB and not of a more general type of cytotoxicity which may be observed with high concentrations of most compounds. An inhibition or delay of keratinisation was in evidence from light microscopy using both acridine orange and H&E.

The appearance of giant cells indicates an action of TCB3 at the nuclear level. Mutagenesis of the PCBs isomers is not well documented and as yet there have been no definitive experiments to verify whether the PCBs act as promoters or act through epigenetic mechanisms. The
morphological evidence produced here might indicate an alteration in ploidy, although, since the incidence of these enlarged cells is low and the culture has been shown to be heteroploid, this possibility is not proven. The most likely mechanisms are those of alterations in the M or G phase of the cells cycle, but 'refusion' of daughter cells may also be a possibility.

SEM indicated that damage to the cells was probably not at the outer membrane level, since no alterations in surface features were observed. Similarly, no membrane changes were observed using TEM.

TEM indicated gross changes in normal function of keratinocytes. Lipid droplets indicated an altered metabolism. Vacuolation seemed, in some cases, to be a result of changes in the mitochondria. Some areas indicated that swelling of mitochondria had taken place since evidence of the remains of cristae could be seen. Some minor degenerative changes were also observable in mitochondria.

The crystalline inclusions may well represent collagen but as a staining artefact where only the periphery of the fibril is stained or similarly negatively stained collagen. A possibility exists that this may be due to an alteration in collagen production. These filaments certainly show a fine structure, similar to that found in collagen. Interestingly, these structures appear to invaginate the
nucleus in many cases, but the significance of this fact is uncertain. Goldstein et al (1975) found PMA to increase microtubule number in cytochalasin B-treated polymorphonuclear leukocytes, the significance of which appears to be that PMA and possibly other promotors may affect polymerisation of microtubules. Van de Hooff and Tigchelaar-Gutter (1983) attributed similar findings to collagenolysis, this will be discussed further in the following chapter.

The appearance of autophagosomes certainly indicates an abnormality in a cell where keratin production has not been well established.

Nuclear pyknosis is seen as an indication of necrotic changes, almost certainly, irreversible in this case.

Protein content of cultures indicated, as found in in vivo studies, the slow progressive nature of PCB toxicity. The results presented show the time dependancy for this culture system. Since this culture system can be kept in contact with the toxicant for a much longer time before subculturing than the majority of cell lines, lower concentrations of toxicants may be used for longer periods.

ODC, $^3$H-Tdr incorporation and DNA content indicated that some changes at the nuclear level were occurring but predominantly with the higher concentration. However, as
studied using TEM, irreversible cytotoxic effects were in evidence but only in a small proportion of cells, since no gross change in MDH was observed. Changes in parameters at levels of cytotoxicity are of less importance since it is most probable that changes in the majority of cell parameters will occur during necrotic changes.

MDH indicated that membrane integrity of cultures had been preserved, membrane damage usually reflecting gross cytotoxicity.

Prolinase activity and acid phosphatase activity showed the greatest changes in this study. Although changes in specific activity were observed at high concentrations of PCB, changes were also observed at 1μM TCB3, where no morphological damage was evident. The initial changes in specific activities of these enzymes must be regarded with caution, since small changes in activity lead to a large observed change over controls. It must be recorded that in the first few days after subculture many parameters were only just detectable and consequently errors will be greater.

Suppression of the activities at day 21 is probably of more importance in terms of altered growth patterns. As established, by using light microscopy, stratification occurs at approximately day 14 of growth. The altered activities therefore reflect a change in the stratification process.
As indicated by studies of prolinase and acid phosphatase during normal growth, these enzymes follow trends which reflect the maturity of the keratinocytes. A suppression of these enzyme levels at this point may well, therefore, indicate a delay of the maturing process (i.e., differentiation and stratification). However, these parameters appear to give an earlier indication of this process than do morphological examination and total protein content.

At this point it was necessary to establish if the assay procedure could be reduced in time for more rapid analysis. On the basis of the altered enzyme patterns during the 14-21 day time period it appeared that cells would be most sensitive to toxic insult at this time point. In order to establish a more accurate estimation of the onset of stratification (differentiation) a pulse label analysis was performed. As shown, two major peaks of increased $^3$H-Tdr incorporation were observed, one at day 2 and another at day 12. The peak at day 2 is not remarkable since this is a common feature of cells when first subcultured. The second major peak, however, most probably represents a move towards the differentiation process. The day 12 time point could therefore be used as a marker for treatment of cells to alter their differentiation.

Treatment of cells at this time point resulted in minimal changes in the chosen parameters. Cells appeared to respond
initially by an increased lysosomal activity as shown by acid phosphatase. The possibility that this may also represent a non-lysosomal acid phosphatase activity is not proven. It has been suggested that a non-lysosomal acid phosphatase exists in skin (Braun-Falco and Ruper, 1967; Mishima, 1964) but homogenisation of skin required greater forces than those necessary for most other tissues and therefore non-lysosomal activity may well be due to disruption of lysosomes. The increase in activity observed is most probably due to early damage by treatment with TCB.

Protein content remained unchanged but incorporation of $^{14}$C-Leu indicated that further incubation of cells may result in a reduced protein content. The suppression of prolinase activity also, as previously, indicated an inhibition of the differentiation process.

SDS-PAGE of cultures showed that although an inhibition of the differentiation process may have occurred, it was not at the level of expression of keratin itself, therefore the difference may only be quantitative. Interestingly, cultures of all ages produced the same protein/keratin profiles when examined using SDS-PAGE, this may be due to the process of subculturing where the seeded population will not represent a population of cells of the same age (ie., basal cells at different time points towards commitment).
6.4 Summary and In Vivo Comparison

The study using TCB3 indicated that acid phosphatase and prolinase activity may well be the most sensitive parameters for study of toxicity in the RTE culture before any gross morphological damage is seen or any gross change in total protein.

Due to the cumulative nature of the PCBs, it appears that treatment of cells the day after subculture and the measurement of parameters during the 14-21 day time period may provide the best indication of toxic effects. Cultures appear most sensitive to alterations of the differentiation process by treatment prior to onset of stratification such that expression of the toxic insult may occur at the beginning of differentiation. Alternatively, an accumulation of PCB may be the only requirement.

On comparison with data generated by in vivo studies it would seem the system used reflects the need for prolonged exposure to PCBs to elicit toxic effects. Dermal toxicity studies (Vos and Beems, 1971) showed a more pronounced effect after one week of treatment of mixtures of PCBs with hyperplasia, hyperkeratosis and thickening of the skin with increased desquamation being prominent features. Reduced prolinase activity may be an indication of a hyperkeratotic effect but reduction in protein content conflicts with a hyperplastic reaction, unless due to increased desquamation. Similarly, acid phosphatase appears to show an immaturity of treated cultures.
Comparisons between *in vivo* and *in vitro* situations when considering effective doses/concentrations is difficult. In the case of the epidermal model, possibly the most meaningful representative way of extrapolating *in vivo* data to *in vitro* is by definition of presenting concentrations as moles per unit area, such that 5μM TCB in this system corresponds to 0.001 moles/cm². However, this assumes that all the PCB in solution is in contact with the culture. Other factors which complicate extrapolation include the method of administration of toxicant. *In vitro*, compound and nutrients are transported via the apical membrane to the basal layer, whereas *in vivo*, nutrients reach the basal layer first via the basal membrane. This will almost certainly affect the absorption of toxicant since cells are forced into an abnormal situation. *In vivo*, the compounds will be removed into the systemic circulation and therefore the effective concentration reaching the basal layer will also be different to that found in this *in vitro* system. These factors together with many others, such as possible binding of compound with serum in the media, will all affect the final analysis, so caution must be used in any attempt at comparison.

*In vivo*, Vos et al (1982) reported, after a total TCDD application of 0.09nmole/cm² (4 applications), strong acnegenic activity could be identified in the rabbit ear, but the hairless mouse failed to produce as strong a reaction. In comparison by day 21 of growth a total (high dose) of
7nmol/cm² produced a strong reaction in the RTE system (a reaction observed with the rabbit). In consideration of the relative potencies of TCDD and TCB a reasonable correlation exists for the predicted value for the TCB. A more meaningful extrapolation can only be arrived at by comparing more compounds; this will be made in the following chapter.
COMPARATIVE TOXICITY AND STRUCTURE ACTIVITY RELATIONSHIP OF B(a)P, B-MC, 2,2',4,4' TCB and 3,3',4,4' TCB
7 COMPARATIVE TOXICITY AND STRUCTURE ACTIVITY RELATIONSHIP OF B(a)P, 3-MC, 2,4,2',4' TCB AND 3,4,3',4' TCB

7.1 INTRODUCTION

A general overview of carcinogenesis in the epidermis has been given in the Introduction. In this chapter a comparison of the activities of the two TCB isomers is made to determine the degree of specificity of the culture to structurally related compounds of different potencies. BP and MC were also chosen as 'classic' carcinogens to establish the sensitivity of the culture to these compounds. As previously mentioned in the preceding chapter and introduction, TCB3 also shows similarities to MC in terms of induction of the monooxygenase systems. A comparison of the effects of the PCBs with known carcinogens may then be made also.

Studies using BP and MC (Berwald and Sachs, 1965; Heidelberger and Type, 1967; Chen and Heidelberger, 1968) in a B prostrate line from C3H mice, showed transforming ability of these compounds to be maximal at approximately 1μgml\(^{-1}\) with no evidence of toxicity below 5μgml\(^{-1}\). Increasing the concentration of carcinogen resulted in a diminished transforming ability and thus an indication that transformation and toxicity were unrelated events.
Mondal and Heidelberger (1970) used PMA to study promotion in vitro by scoring colony formation in the mouse C3H10T1/2 cell line. MC and BP proved ineffective at 0.1µgml⁻¹ in producing foci but following PMA treatment, MC treated cells developed foci. If MC concentration was raised to 0.25µgml⁻¹ development of foci also occurred.

In studies of the cytotoxic dose response of rat tracheal epithelium, a system closely resembling that of the rat tongue epithelium, neither BP nor MC inhibited colony forming efficiency at concentrations up to 30µgml⁻¹ (Gray, 1983). Cultures were, however, only treated once, unlike a study using rat lung epithelium where a concentration of 0.67µgml⁻¹ MC failed to show a proliferative response for up to 21 days (Indo, 1977).

Allen-Hoffmann and Rheinwald (1984) showed BP to be cytotoxic in human SCC-137 epidermal keratinocytes at a concentration of 1µgml⁻¹ after only three days of treatment. However, a 3T3 variant, 3T3M1, was used as a feeder layer for this system and this may, therefore, modify the toxicity of the BP, such that initial cytotoxicity of carcinogens may not, necessarily, be totally unrelated to the secondary mutagenic response.

BP treatment of foetal rat keratinising epidermal cells caused shifts in chromosome structure towards the hypodiploid range and parakeratotic changes in the
keratinisation process, although, truly malignantly transformed cells tended towards the hypotetraploid range (Indo, 1979). These alterations were produced over 30 days using $1\mu g ml^{-1}$ BP. It would, therefore, seem that concentrations of BP and MC, sufficient to produce biochemical alterations in cultured cells fall in the low $\mu g ml^{-1}$ range, a level at which minor cytotoxic damage may occur.

A comparison of acnegenic activity of TCDD, BP and MC gave values for a moderate to strong reaction (as quantified by histology) of $0.09 nmoles/cm^2$, $2.4 nmoles/cm^2$ and $0.9 nmoles/cm^2$ respectively by 21 days (Vos, 1982). These results are of importance when considering the binding of these compounds to the cytosolic (Ah) receptor protein, supporting the hypothesis that the forementioned responses of skin are mediated through that receptor, since all three compounds will bind. Interestingly, the difference in magnitude in the acnegenic potency of TCDD and MC correlates well with their potency to induce AHH activity (Poland, 1974).

3,3',4,4' and 2,2',4,4' TCB have been discussed in more detail in the previous chapter and will, consequently, not be discussed further here.
From the results obtained previously, acid phosphatase and prolinase activity were chosen to monitor toxicity in the culture system to further assess their possible use.

7.2 RESULTS

7.2.1 Morphological examination of cultures treated with BP, MC and TCB2

Morphological examination of RTE cultures treated with TCB2 or with MC showed no gross observable changes. MC (0.93μM) did however appear to show an increased mitotic index, as indicated in PLATE 26B, in basal layer cells. BP (1.96μM) treatment produced changes as shown using acridine orange (PLATE 26 A). A degree of colonisation of cells was also evident. Cells did, however, survive and differentiate.

SEM (PLATE 27,28) showed no change in surface features of those cultures treated with TCB2 but treatment with MC and BP produced cells with numerous microvilli. These cells showed no indication that they were undergoing mitosis such that those features might be expected (prominent microvilli are a feature of cells undergoing mitosis).

TEM showed that in both MC and BP treatment of RTE cultures, an inhibition of keratinisation had occurred with increased vacuolation (PLATE 29). Vacuoles contained much membraneous material and were probably, in part, the result of mitochondrial damage (PLATE 30 A). Some cellular autophagy
was also evident. An increased incidence of osmophilic inclusions as apparent within nucleoli, showed no indication of being viral in nature. The possibility that these may have been produced as artefacts of the staining procedure can, however, not be discounted (PLATE 30 B).

In a smaller proportion of cells, inclusions within nucleoli were seen (PLATE 31 A,B), containing heavily myelinated bodies (PLATE 32). Very occasionally a diskeratotic cell was observed, but this is probably of less significance (PLATE 33).

During treatment of cultures with the TCB3 isomer, an inhibition of keratin production was observed with mitochondrial swelling and lipid inclusions (PLATE 34). Some organelles contained osmophilic foci (PLATE 35). A proportion of these foci appeared to be within mitochondria, others appeared to have the appearance of hepatocyte peroxisomes. Vacuolation due to mitochondrial damage was also in evidence.

Nuclei in TCB2 treated cultures lacked signs of invagination and showed a more regular morphology. Cell contacts however appeared disrupted, either through cytoplasmic retraction or broken desmosomal linkages (PLATE 36,37). Some desmosomes, however, appeared intact whilst the surrounding membranes were separated.
PLATE 26  ACRIDINE ORANGE STAIN OF CONTROL (DMSO 1%) CULTURE
(x 400)
PLATE 26

A - ACRIDINE ORANGE STAIN OF RTE CULTURE TREATED WITH 1.96μM BP (x 400)

B - ACRIDINE ORANGE STAIN OF RTE CULTURE TREATED WITH 0.93μM MC
    NOTE INCREASE IN MITOTIC FIGURES (M) (x 400)
PLATE 27 SEM OF CULTURES SHOWING TYPICAL MORPHOLOGY OF CELLS TREATED WITH BP OR MC
PLATE 28  SEM SHOWING MICROVILLUS STRUCTURES OF CULTURES TREATED WITH BP OR MC
PLATE 29

A - TEM OF CONTROL CULTURE (1% DMSO) DAY 14 (x 5000)

B - TEM OF CULTURE TREATED WITH BP (1.96μM) SHOWING LACK OF TONOFILAMENTS AND VACUOLATION (V) (x 5000)
PLATE 30

A - TEM SHOWING VACUOLATION (V) WITH MEMBRANOUS INCLUSIONS SOME OF MITOCHONDRIAL APPEARANCE (M) AND LYSOSOMAL (L) (x 14000)

B - OSMOPHILIC INCLUSIONS IN NUCLEOLI SHOWING NO EVIDENCE OF BEING VIRAL IN NATURE (x 30000)
PLATE 31  A, B - NUCLEOLAR INCLUSIONS SHOWING CYTOSOLIC MATERIAL (C) WITHIN THE NUCLEOLUS (N) OF THE CELL

(A - x 16000;  B - x 16000)
PLATE 32  NUCLEOLAR INCLUSION SHOWING MYELINATION OF CYTOPLASMIC MATERIAL
(from PLATE 31A x 36000)
TEM OF TYPICAL DISKERATOTIC CELL FOUND DURING BP OR MC TREATMENT. THE DISKERATOTIC CELL APPEARS TO BE UNDERGOING PHAGOCYTOSIS BY THE ADJACENT KERATINOCYTE (x 18000)
TEM OF RTE CELLS TREATED WITH TCB2 (5µM) SHOWING LIPID INCLUSIONS (L), MITOCHONDRIAL ENLARGEMENT AND PROLIFERATION (M) AND LYPOSOMAL BODIES (O) WITH OSMOPHILIC INCLUSIONS (x 5000)
TEM SHOWING PEROXISOME LIKE STRUCTURES FOUND DURING TREATMENT WITH TCB2. Zn AND Fe NEGATIVE BY PROBE ANALYSIS (x 300000)
PLATE 36  TEM OF RTE CELLS TREATED WITH TCB2 (5µM) SHOWING
LOSS OF CELL-CELL ADHESION (C)  (x 5000)
TEM OF RTE CELL CONTACT SHOWING DESMOSOMAL DISRUPTION AFTER TREATMENT WITH 5μM TCB2 (D) (x 24000)
PROFILE OF ACID PHOSPHATASE ACTIVITY DURING TREATMENT OF CULTURES WITH MC

CULTURES WERE TREATED THE DAY AFTER SUBCULTURE AND MEASUREMENTS TAKEN EVERY 7 DAYS

FIGURE 19  MEAN OF 3 DETERMINATIONS

METHOD 4.2.13.3

- 0.37 μM
- 0.93 μM
- 1.86 μM
PROFILE OF ACID PHOSPHATASE ACTIVITY DURING TREATMENT OF CULTURES WITH BP

CULTURES WERE TREATED THE DAY AFTER SUBCULTURE AND MEASUREMENTS TAKEN EVERY 7 DAYS

FIGURE 20 MEAN OF 3 DETERMINATIONS

METHOD 4.2.13.3

° - 0.39 µM
- - 0.99 µM
■ - 1.98 µM

% SOLVENT CONTROL

DAY
PROFILE OF ACID PHOSPHATASE ACTIVITY DURING TREATMENT OF CULTURES WITH TCB2

CULTURES WERE TREATED THE DAY AFTER SUBCULTURE AND MEASUREMENTS TAKEN EVERY 7 DAYS

FIGURE 21 MEAN OF 3 DETERMINATIONS

METHOD 4.2.13.3

- - 1 \( \mu M \)
. - 3 \( \mu M \)
■ - 5 \( \mu M \)
PROFILE OF ACID PHOSPHATASE ACTIVITY DURING TREATMENT OF CULTURES WITH TCB3

CULTURES WERE TREATED THE DAY AFTER SUBCULTURE AND MEASUREMENTS TAKEN EVERY 7 DAYS

FIGURE 22 MEAN OF 3 DETERMINATIONS

METHOD 4.2.13.3

- 1 μM
- 3 μM
- 5 μM
Some evidence of extracellular material with a ultrastructural morphology similar to that of glycogen was also observed.

7.2.2 Biochemistry of Treated Cultures

7.2.2.1 Acid Phosphatase (Figs 19,20,21,22)
At day 7 of growth, acid phosphatase activity showed significant effects only with high concentrations of BP and TCB3, 1.98µM and 5µM respectively. Activities with these compounds were reduced by 40% of solvent control. At day 14 of growth only low concentrations of TCB3 produced an increase in activity of approximately 50% and MC at 1.86µM reduced activity by 30%. At day 21 of growth 3,4,3',4' produced a 50% decrease in specific activity at 5µM, MC producing a 30% increase at 1.85µM.

7.2.2.2 Prolinase (Figs 23,24,25,26)
At day 7, TCB3 decreased specific activity with increasing concentration to 30% of control at 5µM. The TCB2 isomer increased activity by approximately 30% at 1µM.

At day 14, BP and MC increased activity by 30% at 1.98 and 1.86µM respectively. Cultures treated with the TCB3 isomer showed a recovery in activity whilst the 2,4,2',4' isomer produced an overall decrease.

At day 21, TCB3 decreased activity to 30% of solvent control.
PROFILE OF PROLINASE ACTIVITY DURING TREATMENT OF CULTURES WITH MC

CULTURES WERE TREATED THE DAY AFTER SUBCULTURE AND MEASUREMENTS TAKEN EVERY 7 DAYS

FIGURE 23   MEAN OF 3 DETERMINATIONS

METHOD 4.2.13.2

\[
\begin{align*}
\circ & - 0.37 \, \mu M \\
\cdot & - 0.93 \, \mu M \\
\square & - 1.86 \, \mu M
\end{align*}
\]
PROFILE OF PROLINASE ACTIVITY DURING TREATMENT OF
CULTURES WITH BP

CULTURES WERE TREATED THE DAY AFTER SUBCULTURE AND MEASUREMENTS
TAKEN EVERY 7 DAYS

FIGURE 24  MEAN OF 3 DETERMINATIONS

METHOD 4.2.13.2

° - 0.39 μM
. - 0.99 μM
■ - 1.98 μM
PROFILE OF PROLINASE ACTIVITY DURING TREATMENT OF CULTURES WITH TCB2

CULTURES WERE TREATED THE DAY AFTER SUBCULTURE AND MEASUREMENTS TAKEN EVERY 7 DAYS

FIGURE 25 MEAN OF 3 DETERMINATIONS

METHOD 4.2.13.2

- 1 μM
- 3 μM
- 5 μM
PROFILE OF PROLINASE ACTIVITY DURING TREATMENT OF CULTURES WITH TCB3

CULTURES WERE TREATED THE DAY AFTER SUBCULTURE AND MEASUREMENTS TAKEN EVERY 7 DAYS

FIGURE 26 MEAN OF 3 DETERMINATIONS

METHOD 4.2.13.2

\[ \text{\% SOLVENT CONTROL} \]

- \( - 1 \ \mu\text{M} \)
- \( - 3 \ \mu\text{M} \)
- \( - 5 \ \mu\text{M} \)

DAY
PROFILE OF PROTEIN CONTENT DURING TREATMENT OF CULTURES WITH MC

CULTURES WERE TREATED THE DAY AFTER SUBCULTURE AND MEASUREMENTS TAKEN EVERY 7 DAYS

FIGURE 27 MEAN OF 3 DETERMINATIONS

METHOD 4.2.9

- 0.37 μM
- 0.93 μM
- 1.86 μM
PROFILE OF PROTEIN CONTENT DURING TREATMENT OF CULTURES WITH BP

CULTURES WERE TREATED THE DAY AFTER SUBCULTURE AND MEASUREMENTS TAKEN EVERY 7 DAYS

FIGURE 28 MEAN OF 3 DETERMINATIONS

METHOD 4.2.9

- 0.39 μM
- 0.99 μM
- 1.98 μM
PROFILE OF PROTEIN CONTENT DURING TREATMENT OF CULTURES WITH TCB2

CULTURES WERE TREATED THE DAY AFTER SUBCULTURE AND MEASUREMENTS TAKEN EVERY 7 DAYS

FIGURE 29 MEAN OF 3 DETERMINATIONS

METHOD 4.2.9

° - 1 µM
- - 3 µM
■ - 5 µM
PROFILE OF PROTEIN CONTENT DURING TREATMENT OF CULTURES WITH TCB3

CULTURES WERE TREATED THE DAY AFTER SUBCULTURE AND MEASUREMENTS TAKEN EVERY 7 DAYS

FIGURE 30  MEAN OF 3 DETERMINATIONS

METHOD 4.2.9

○ - 1 μM
. - 3 μM
■ - 5 μM
7.2.2.3 Protein (Figs 27, 28, 29, 30)

At day 7, TCB2 produced an increase in total protein of 30% at 1μM.

At day 14, total protein was suppressed in all treatments except BP and MC at 0.39 and 0.37μM respectively where no effect was seen and TCB2 where an increase of 30% at 1μM was still observed.

At day 21, total protein remained suppressed by MC and BP, resumed to control values with TCB3 but remained elevated by 40% of control with 1μM TCB2.

7.2.2.4 Further Observations

$^3$H-thymidine incorporation showed no change with either BP or MC at any of the concentrations tested.

Protein/DNA ratios of cultures treated with BP and MC showed no alteration except at 1.98μM BP when the ratio was altered to 70% of control.

Keratin profiles of treated culture showed no changes from control (PLATE 38).

Comparison of the RTE culture with that of the BCL-D1 cell line indicated that the RTE culture was more sensitive to irritant compounds (Table 4).
PLATE 38  SDS-PAGE OF PROTEIN PROFILES OF TREATED CULTURES

1 - TCB3
2 - MC
3 - BP
4 - SOLVENT CONTROL

ALL AT HIGHEST CONCENTRATION
### TABLE 4

**ID₅₀ VALUES:- (μM)**

<table>
<thead>
<tr>
<th>Compound</th>
<th>RTE₄</th>
<th>BCL-D1</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBT</td>
<td>0.16</td>
<td>4.39</td>
</tr>
<tr>
<td>DBT</td>
<td>2.58</td>
<td>8.82</td>
</tr>
<tr>
<td>DNP</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>TCB2</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>TCB3</td>
<td>&gt;10 (70%)</td>
<td>&gt;10</td>
</tr>
<tr>
<td>CH</td>
<td>0.33</td>
<td>&gt;10</td>
</tr>
<tr>
<td>CDNB</td>
<td>10.06</td>
<td>14.27</td>
</tr>
</tbody>
</table>

**Abbreviations:**
- TBT - TRIBUTYLTIN CHLORIDE
- DBT - DIBUTYLTIN DICHLORIDE
- DNP - DINITROPHENOL
- CH - CYCLOHEXIMIDE
- CDNB - CHLORODINITROBENZENE
7.3 DISCUSSION

Light microscopy of treated cells indicated that with BP, MC and TCB2, a proportion of the cultures population were resistant to the cytotoxic effects of these compounds. The observations of the changes occurring with 1.98μM BP were the only indication that cytotoxicity may have occurred.

The increased mitotic index in MC treated cultures failed to be confirmed by $^{3}\text{H-TdR}$ incorporation rate thus this may not be a significant change. This result, together with results of small proportions of cells showing more marked cytotoxic effects, suggests a heterogeneity of the cultures response to toxic insult. This remains an advantage of the transformed cell line where the population becomes more homogenous due to selection of 'colonies' through subculturing. The further possibility exists, that treatment of cells at specific points in the cell cycle has marked effects on the subsequent toxicity produced, but, since the cultures are observed after several cell cycles and remain in contact with the test compounds throughout this period, the probability of a cell cycle effect is low.

The apparent cytotoxic effect observed using BP may well correlate with that found by Allen-Hoффmann (1984) where recovery of cell colonies leads to a transformed cell type or resistant cell type which will then continue to grow, and in the case of RTE culture, differentiate.
SEM produced morphological changes similar to those found by Williams (1973) on treatment of TR1 cells with nitrosomethyl urea. In other studies, increased surface complexity, although not necessarily a consistent feature, appears as a possible marker of oncogenic transformation (Malick, 1976; Saxholm, 1979; Narayan, 1984). The RTE culture appears to emulate these findings, but the significance of this morphological heterogeneity is, as yet, uncertain. It would seem that altered surface features are not a prerequisite for transformation but may present, in some cases, an early method of detection of such changes. The changes may be an indication of an initiation process only, cells still maintaining the capability of differentiation and proliferation. Subsequent growth in soft agar may provide an answer.

The reduced filament content of cultures treated with BP and MC, and studies by TEM would seem to reflect the general findings in vivo. During tumour promotion in mouse skin with TPA, collagen degradation and synthesis are affected, degradation being more greatly affected at the beginning of treatment, such that collagenolytic activity is greatly increased (Marian and Mazzucco, 1985). Similar findings were observed by Van den Hooff (1962) and Van den Hooff and Tichelaar-Gutter (1983). It would, therefore, appear that morphologically the RTE cultures reflect this increased collagen breakdown through the absence of observable fibres.
Mitochondrial damage observed was probably due to an initial toxic response but the accompanying lysosomal material may be of importance in the initial collagenolytic activity present. Lysosomal activity may play an important role in collagen degradation together with the more specific collagenase activity (Van den Hooff and Tigchelaar-Gutter, 1983).

Osmophilic nucleolar inclusions were probably of little significance, since similar morphological signs could be observed in control cultures. Close investigation of these osmophilic foci revealed an amorphous nature, thus viral contamination was unlikely.

The presence of cytoplasmic material within the nucleus may be of significance with regard to the keratinisation process. The possibility of pseudoinclusions being of importance in the keratinisation process has already been discussed. The heavy myelination or organelles of the cytoplasm within the nucleus may be as a result of these pseudoinclusions becoming true inclusions, by pinching of the nuclear membrane, thus leading to some degenerative changes. If this were true, it might be expected that keratin formation would be reduced due to the decrease in nuclear-cytoplasmic transfer. Alternatively this may be a simple degenerative process observed during toxic insult. The increase of cytoplasmic material within the inclusions is probably due to an increase in cytoplasmic size due to
toxic insult forcing cytoplasmic material into the inclusions.

TCB2 produced morphological damage closely resembling that found in squamous cell carcinoma, Bowen's disease. In a study of Bowen's disease by Olson (1968) widened intracellular spaces, decrease in intact desomosomes with replacement by villous projections, increase in number of cytoplasmic organelles and some diskiratosis, were all observed in these treated cultures. Accumulations of glycogen were also observed by Olson et al (1968) a feature also observed in Ewing's sarcoma (Ghadially, 1980). Confirmatory work on the nature of the glycogen-like material observed in treated cell has not been carried out, therefore any similarities drawn up in respect to glycogen accumulation must be tentative.

The appearance of organelles with osmophilic inclusions is of unknown significance. Some appear to be a result of mitochondrial condensation, others may be lysosomal in nature. Acid phosphatase was, however, unaltered. Electron diffraction and probe analysis of cores was unable to elucidate the true nature of these osmophilic inclusions (APPENDIXII), most probably due to the small amount of sample that can be analysed by the method used. Zn (uricase) and Fe (catalase) were looked for to determine the possibility of peroxisome-like organelles appearing.
In diskératotic cells, features observed with a cribiform appearance are most probably due to a dilation of endoplasmic reticulum (PLATE 39). However, due to the ingress of water, the dilation and vesiculation of the rough endoplasmic reticulum leads to a degranulation and the consequent change in appearance of rough endoplasmic reticulum to smooth endoplasmic reticulum. Similar observations include the rough endoplasmic reticulum in the pancreatic acinar cell during islet cell adenoma (Ghadially, 1980) and in the synovial membrane of the knee joint during lipohēmaarthrosis (Ghadially, 1969).

Acid phosphatase and prolinase activity tended to show the same overall trends with any given compound throughout the 21 day time period studied. For the TCB3 isomer and BP, the general trend was one of a fall in enzyme activities (day 7) followed by an increase (day 14) and a further fall (day 21). Relationships to control values may not be of great importance here but changes in the relationships of values between time points for any given treatment is probably significant.

If it is assumed that prolinase and acid phosphatase activity relate to keratin and collagen degradation, then a decrease in activity should result in an increased content of keratin/collagen, if biosynthethic pathways remain unaltered. Similarly, the reverse is true for increased activity. The results obtained would, therefore, reflect an
PLATE 39  DISKERATOTIC CELL SHOWING CRIBIFORM APPEARANCE OF ENDOPLASMIC RETICULUM (ER)  (x 8000)
increase in keratin/collagen followed by a further decrease and increase (i.e., a three phase response). A similar result was obtained by Van den Hooff (1962) whilst studying carcinogenesis in mouse skin following topical application of MC. Phase 1 was attributed to a low dose toxic stimulation; Phase 2 to either inhibition of fibre production or active enzymic breakdown and Phase 3 to stimulation of collagen production coinciding with malignant degeneration. The rise in prolinase and acid phosphatase activities at day 14 would therefore substantiate the hypothesis of enzymatic breakdown during the second phase.

Another possibility for the changes in activity observed may be that of alterations in biosynthetic pathways of keratin/collagen altering the observed activities by a feedback mechanism, such that the observed effects are secondary to the initial response.

If the levels of these enzymes are compared to the total protein values, an inverse of the trends is observable. When enzyme levels are reduced, protein content is raised, a change that might be expected if protein content relates to collagen production. If collagen biosynthesis is raised a rise in protein content may ensue; however, a change in protein may simply be due to a change in cell number.
7.3.1 SUMMARY AND IN VIVO COMPARISON

A number of possibilities exist for comparison of the results with the in vivo data. In terms of the relative concentrations necessary to produce a toxic reaction, the relationship between the PCB isomers correlates well with that found in vivo, TCB3 being more toxic than the TCB2 isomer, TCB3 having the structure more closely resembling TCDD. Similarly, TCB3 produces a greater response in the RTE system than both MC and BP, both MC and BP being several orders lower in magnitude at producing an acnegenic response than TCDD (Vos et al, 1982).

On a basis of the enzyme profiles obtained following treatment with the PCB isomers, the RTE culture findings indicated a difference of action between the two. TCB3 more closely resembles that of the classic carcinogens BP and MC.

The RTE system agrees with the findings in the literature that concentrations of BP and MC, sufficient to produce alterations in the biochemical parameters, fall in the low \( \mu \text{g ml}^{-1} \) range. The system does not, however, appear to be any more sensitive than other systems used, as found in the literature (ie., fibroblastic cell lines).

The measurement of acid phosphatase and prolinase would seem to provide a suitable method of investigating the relative potencies of carcinogens and acnegen in this culture
system. Acid phosphatase and prolinase provide a measure of collagenolysis, which may therefore be a sensitive method of detection of carcinogens. Electron microscopy is also of value in supporting the enzyme data.


Similarly, enzymic measurement of the collagenolytic behaviour of tissues during carcinogenesis confirms these alterations in vivo (Ma, 1949; Wirl, 1977; Marian, 1985).

Studies in vitro using chick embryo fibroblasts (Delclos and Blumberg, 1979) showed an inhibition of collagen synthesis as measured using the rate of formation of hydroxyproline, although this could not be attributed solely to prolyl hydroxylase inhibition. These effects were observed by treatment of the culture with PMA as used in studies in rabbit synovial fibroblasts (Brinkerhoff and Harris, 1981). However, in these studies, both increased collagenase activity and reduced collagen synthesis were observed.
FINAL DISCUSSION
The present studies have indicated that acid phosphatase activity, prolinase activity, protein content and morphological examination may well provide sensitive methods for the detection of toxic insult by irritants and carcinogens in the RTE cell culture. These parameters may then be related to the state and maturity of the growth of RTE cells and thus be a measure of alterations in the differentiation process.

For compounds where chronic toxicity may present a problem, the long life time of the RTE subculture offers the possibility of studying the effects of such compounds over extended periods (i.e., 28 days). This is seldom possible in the majority of cultures where the period between sub-culturing may be only a few days.

Morphologically the cultures resemble the **in vivo** situation closely and during toxic insult, show effects that may be consistent with changes found **in vivo**. There is, therefore, a possibility of direct comparison between the **in vivo** and **in vitro** situation, although the changes **in vitro** may not give a full representation.

The RTE culture system, also, allows a comparison of the relative toxicity of compounds and shows different reactivity to structurally related compounds. Thus,
toxicities observed may not only produce a comparative scale but also show mechanistic differences in the mode of action of compounds.

The RTE culture has been shown to possess a number of metabolic functions, both phase I and phase II, a requirement necessary for conversion of certain compounds to active metabolites. The system therefore, possesses both a 'metabolising' and 'response' system. Thus, the culture provides potentially a complete system for screening of toxins without any additions to the test system. However the full complement of drug metabolising enzymes present in the culture should be investigated.

The capacity of the test system to metabolise test compounds may be of major importance. Although a low level of metabolism of BP has been shown in mature cultures (passages 24-27) a much greater activity might be expected in earlier passages of RTE. Autrup et al (1985) found that the established keratinocyte RTE line, derived from rat sublingual epithelium appeared to have lost most of its metabolic activity. A similar finding was shown by Heimann and Rice (1983) in late passages of rat epidermal cells. When comparing skin from different sites of the rat, it appears that the tongue possesses least inducible and inactive cytochrome P450 and P448 (Dr CPowell, personal communication). The cytochrome contents of the skin, oesophagus and tongue appear to be in the general order:
Skin > oesophagus > tongue

thus tongue may not be the most useful site for preparation of cultures. However, due to the diversity of cell types in the rat skin, further complications in attempts to culture keratinocytes from this site occur. Sobin (1970) and Wiebel et al (1975) have also shown different skin areas to exhibit different rates of xenobiotic metabolism.

Inducibility of AHH activity is especially pertinent, as discussed earlier (Section 5.7) to the toxicity of many halogenated aryl hydrocarbons such as the polychlorinated dibenzofurans (Mason et al, 1985) and PCBs. Induction of AHH activity in mature RTE cultures was not detectable although it may well occur in earlier passages.

The RTE cultures responded to structurally related compounds (PCBs, alkylltins) with a sensitivity similar in order to that observed in skin \textit{in vivo}. The responses observed also showed a marked similarity to the effects observed \textit{in vivo}, this is an important finding because it allows extrapolation of results from \textit{in vitro} to \textit{in vivo}.

Ultrastructural examination by TEM, acid phosphatase activity, prolinase activity and protein content appeared to be useful markers of toxic response in the system although they may still not provide the best method. Collagenolytic activity, therefore, may provide a method for observing toxicity.
Keratin profiles did not appear to be a useful marker in this system, but since the compounds used did not change the direction of differentiation, an altered keratin complement may not be expected. Pulse labelling with proline, leucine or histidine may detect more subtle changes. Keratin profiles, however, do show variation within populations both in vitro and in vivo (Grace et al., 1985; Wild and Mischke, 1986) a complication when comparing tests and controls.

Non-specific in vitro toxicity tests should be simple, reproducible, reliable and of low cost. Reproducibility and reliability (i.e., minimal false negatives) are also of importance in a specific target organ test such as the RTE system. Good reproducibility has been shown with the compounds tested in the RTE culture but due to the small number used, reliability cannot be assessed fully. The methods used in this system are simple to perform, which may not necessarily be expected for such a specific test system. The time taken to produce results, however, is on a scale of weeks, which is less desirable when screening large numbers of test compounds.

Further work should include studies upon other compounds, including those that directly effect differentiation (e.g., retinoids), structurally related compounds of differing toxicities, specific target cell toxins that would enable the specificity of the cell type to be investigated and skin irritants and carcinogens. The end points used should also be investigated for their specificity with such compounds.
Further studies on the induction of the cytochrome P-450 monooxygenase system and AHH induction should be made at different stages in the culture development together with investigations of the presence of the Ah receptor. If induction of AHH through the receptor is necessary, toxic effects are most likely to relate to the *in vivo* situation if the Ah receptor is present *in vitro*. Such studies should initially be performed in primary cultures to reveal the change in metabolic activity and inducibility with time in culture.

Incorporation studies using proline, leucine, histidine and cysteine may be of value, as discussed previously, but changes in differentiation must be attained first (e.g., with the retinoids). Since the proportions of these amino acids change with the maturity of the culture, an alteration in the direction of differentiation may well be shown by their relative contents in the keratins.

An investigation of the connection between prolinase action and the degradation of keratin/collagen would be of value. The true relevance of the action of prolinase in the RTE culture system is still not understood.

Preliminary comparative studies of the RTE system with the finite cell line BCL-D1, showed some indication that the RTE system was somewhat more sensitive to irritant compounds and may thus indicate a specificity greater than non-specialised
cell types. A blind trial at this point may be of value in determining the effectiveness of this system, using perhaps, just one of the chosen end-points.

In conclusion, the results of enzyme profiles, morphological examination, metabolism and the comparison of the RTE system with the BCL-D1 cell line, indicate that this culture may be of use in investigating target organ toxicity of the skin.

The present studies have elucidated the complex nature of the RTE culture as a model system. Growth of the culture encompasses three basic phases. The first is the growth of the basal layer cells to confluency; the second is the subsequent stratification. Finally, terminal differentiation, producing dying, squamous epithelia occurs.

During this time, many changes in biochemical parameters occur partially reflecting these growth characteristics. Toxicity in the system seems to accentuate these changes such that the observed effects reflect the maturity of the culture. Further work in validation is still required.

These initial observations may now be used in a human keratinocyte model, where extrapolation to human in vivo toxicity results may be less complicated and possibly more meaningful.
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LEGEND  
3 : 9 : 7,8 : 9,10 : : - RESPECTIVE ALCOHOLS OF BP
Q : - QUINONES