CELL INTERACTIONS IN SKIN

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by

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"The spiritual estate of man is so enormous and so inexhaustible in its diversity, only he who stands on the shoulders of great predecessors can claim originality".

Leon Trotsky

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CONTENTS

ABBREVIATIONS

Abbreviations used in this thesis are those recommended in the Biochemical Journal "Instructions to Authors" (1972), with the following exceptions :

bis-MSB	-	p-bis (o-methyl styryl) benzene
BSS	-	balanced salts solution
cpm	-	counts per minute
DOPA	-	L-(β) 3,4-dihydroxyphenylalanine
dpm	-	disintegrations per minute
EDTA	-	ethylene diamine tetraacetic acid
PBS	-	phosphate buffered saline
PPO	-	2,5-diphenyl oxazole
SDS	-	sodium dodecyl sulphate
TCA	-	trichloracetic acid

SUMMARY

1. The literature concerning the growth and differentiation of skin cells is reviewed. The interaction between the dermis and epidermis of postembryonic skin is compared with other forms of mesodermal-epithelial interaction, and the possible involvement of gap junction-mediated transfer of molecules between the two tissue layers is discussed.

2. Cultures of epidermal keratinocytes and dermal fibroblasts were established from newborn mouse skin and adult guinea pig ear skin. The ability of these cells to form gap junctions with one another *in vitro* was examined by autoradiographic analysis of ^{3}H-uridine nucleotide transfer between prelabelled donor cells and unlabelled recipient cells.

3. A novel statistical approach was used to analyse the transfer of radiolabelled molecules between cells in culture. Comparison of the autoradiographic grain counts of recipient cells in contact with prelabelled donor cells and recipient cells not in contact with donor cells was used to generate a probability value which was used as a measure of cell-cell communication.

4. This method was used to demonstrate gap junction formation between epidermal keratinocytes and dermal fibroblasts in culture.

5. Junction formation between skin cells is in contradiction to published proposals of epithelial-fibroblastic specificity To determine whether this is an unusual situation, two other mouse primary cell types, epidermal melanocytes and renal epithelial cells, and cells of several established cell lines, were examined for gap junction formation in vitro. It was shown that epidermal keratinocytes, dermal fibroblasts, renal epithelial cells and epidermal melanocytes communicate non-specifically with cells of the fibroblast line C13 and the epithelial cell line BRL. However, cells of the canine kidney epithelial line MDCK form gap junctions with one another but do not communicate with cells of the junction-competent C13 and BRL lines. From these results it was proposed that specificity in cell communication may be a more general property of established cell lines.

6. The epidermal structural protein prekeratin was used as a biochemical marker for epidermal cell differentiation in culture. Prekeratin from newborn mouse skin was purified by citrate buffer extraction, and shown to consist of polypeptide chains of 61,000MW and 69,000MW in a 2:1 stoichiometry.

7. A further method for the study of keratinocyte differentiation in vitro was developed, based on the relative uptake of the amino acids histidine and leucine. Using these techniques it was demonstrated that epidermal keratinocytes undergo only limited differentiation in dispersed cell culture.

8. The dermal-epidermal interaction was also studied using a transfilter organ culture system. Direct contact between dermis and epidermis was shown to result in stimulation of epidermal protein synthesis. This stimulation does not occur when the two tissues are separated by porous filters with a mean particle retention size of 0.8μm. However, no de novo prekeratin synthesis was detected by labelling of skin organ cultures with ^{35}S-methionine and ^{3}H-leucine.

9. The implications of these observations for the normal growth and differentiation of skin in vivo are discussed.

Chapter 1 - INTRODUCTION

"I pray for inspiration, to tell how it all began"

Virgil

The Aeneid

1.1 The Biology of Skin

Adult mammalian skin is now recognised as a morphologically and biochemically complex organ, consisting of several distinct tissue layers containing cells of widely-varying embryological histories (Hsia, 1971). The functions of skin are similarly diverse, including thermoregulation, sensory perception and secretion, but the general role of skin is as the interface between the aqueous cellular environment of the body and the non-aqueous external surroundings. Thus, normal skin functions as a resistant barrier to physical and chemical damage, and also forms a mechanism of sensory transduction between the organism and its immediate exterior. The resistance of skin is due to the continuous production of an outer epidermal layer of dead cells filled with insoluble keratin filaments; the biological function of the epidermal cell may only be realised by its death.

The mammalian epidermis is a keratinising epithelium, and, as the outermost layer of the organism, may be considered the evolutionary successor of the plasma membrane or cell wall of unicellular organisms. The highly-organised epidermis of vertebrates has evolved from simpler external structures of lower multicellular species. The cell wall of plants consists mainly of the polysaccharide cellulose, which is locally hardened by incorporation of lignin. Similarly, the cuticle of insects is largely composed of the N-acetyl glucosamine polymer chitin (Wigglesworth, 1964). The extreme hardness

of the insect epidermis is due to the presence of sclerotin, which consists of protein chains cross-linked by benzene rings. The disadvantage of the insect exoskeleton is that its rigidity does not permit growth of the immature organism; this is overcome by a series of moults, successive sheddings of the cuticle followed by deposition of a new layer, until the adult stage is reached and growth ceases. In fish, the epidermis is a squamous epithelium composed of several distinct cell types, but no morphological keratinisation appears to occur (Flaxman, 1972). As an adaptation to the terrestial environment, the amphibian epidermis has a uniform cornified layer containing α-type keratin similar to that of mammalian hair. Unlike the mammalian epidermis, cornified cells are not shed continuously, but in a periodic, hormonal-controlled sloughing of the amphibian epidermis which appears analogous to the insect moult. The surface epidermis of birds is similar to that of mammals, but keratinised cells are characterised by the presence of large lipid inclusions.

Normal mammalian skin may be regarded as consisting of four main layers (Hsia, 1971). In order, from exterior to interior of the body, these are the epidermis, dermis, subdermal adipose tissue, and the panniculus carnosus. The epidermis is a stratified squamous epithelium, and is separated from the dermis by an extracellular junctional structure often referred to as the basement membrane or basal lamina. The dermis is a well-vascularised tissue of mesodermal origin, which merges into a layer of subcutaneous fat derived from mesodermal cells of the deep embryonic dermis. In most

animals a region of smooth muscle, the panniculus carnosus, underlies the fat layer, but this structure is vestigial in man. The dermis, epidermis, and dermal-epidermal junction will be considered in more detail below.

1.1.1 The Dermis

The dermis of adult skin is derived from embryonic mesoderm. The free cell population of the dermis includes dermal fibroblasts, which secrete the collagenous matrix or ground substance, and various cells of the immune system (Breathnach, 1978). A phagocytic function is probably performed by the histiocytes, or "dermal macrophages". Mast cells and lymphocytes have also been unambiguously identified in mammalian dermis. The cutaneous appendages of skin comprise another cell population in the dermis. Hair follicles, sebaceous glands and sweat glands are all derived from embryonic ectoderm, but extend downward into the dermis, and the epithelial cells of these appendages are capable of regenerating the epidermis *de novo* in the event of its removal. Also located in the dermis are the arrector pili smooth muscles (associated with the hair follicles), sensory nerve endings, which may extend across the dermal-epidermal junction, and an extensive vascular network responsible for the dissipation of excess body heat.

The cells of the dermis are embedded in a secreted macromolecular matrix, which contains collagen, elastic fibres, and glycosaminoglycans. The elastic fibres are oriented at right angles to the skin surface in the superficial dermis, where

they may be associated with the dermal-epidermal junction, and parallel to the skin surface in the deeper layers. The collagen appears to be relatively unpolymerised, highly hydrated, and may have the physical characteristics of a gel (Jarrett, 1973a). Glycosaminoglycans found in the dermis are mainly dermatan sulphate, chondroitin sulphate, keratan sulphate, and hyaluronic acid, and these may form a proteoglycan complex with dermal tropocollagen (Jarrett, 1973b).

1.1.2 The Dermal-Epidermal Junction

The dermal-epidermal boundary region is seen to be extensively convoluted by protrusions known as dermal papillae or as (epidermal) rete pegs when examined in thin section by light microscopy. In addition, irregularities in the basal epidermal cell plasma membrane constitute a further pattern of invagination visualised by electron microscopy.

The junction between dermis and epidermis consists of four discernable components : (i) the plasma membrane of basal epidermal cells; (ii) the lamina lucida; (iii) the PAS-staining basal lamina; and (iv) the sublaminar dermal filaments (Briggaman and Wheeler, 1975).

The plasma membrane of the cells in the basal epidermal layer constitutes the first component of the dermal-epidermal junction, and is characterised by the presence of hemidesmosomes on basal keratinocytes. A 20-40nm thick "attachment plaque" on the cytoplasmic side of the hemidesmosome appears to be associated with tonofilament bundles, epidermal intermediate filaments containing keratin polypeptides (see section 1.3.1).

The nature of the interaction between desmosomes and tonofilaments is unclear.

Beneath the epidermal cell layer lies an amorphous, electron-lucent zone, the lamina lucida. This region is impermeable to tracers of the intercellular space such as lanthanum hydroxide and ruthenium red (Wolff, 1968). Fine fibres of unknown composition termed anchoring filaments cross the lamina lucida in the vicinity of hemidesmosomes, and are thought to be involved in dermal-epidermal adhesion.

The basal lamina component of the dermal-epidermal junction consists of a fibrillar reticulum embedded in a neutral glycoprotein matrix which stains with periodic acid-Schiff (PAS) reagent. However, it has been suggested that the ultrastructural appearance of the basal lamina is an artefact caused by macromolecular precipitation during fixation (Jarrett, 1973c).

On the dermal side of the basal lamina are found various filamentous structures, including single collagen fibres and dermis-specific anchoring fibrils which contain collagen of irregular periodicity.

The dermal-epidermal junction is thought to function in adherence of the two tissues and as mechanical support for the epidermis (Murray et al, 1979). This structure may also be involved in molecular and cellular transfer between dermis and epidermis. Nutrient and gas exchange is presumed to occur across the junction, as the epidermis is avascular. Passage of macromolecules from dermis to epidermis has been demonstrated

experimentally. Injection into the dermis of horse-radish peroxidase (about 40,000 MW.) was followed by rapid appearance in the lower epidermis, where it freely penetrated the intercellular space (Squier, 1973). The limit to peroxidase permeation in the granular layer correlated with the appearance of membrane-coating granules at cell margins (see section 1.3.2). Particles of Thorotrast (colloidal thorium dioxide) of 5-12.5nm diameter introduced into the dermis were largely retained at the dermal-epidermal junction, but significant penetration into the epidermis was observed (Wolff and Hönigsmann, 1971). Initially, the Thorotrast particles occupied the intercellular space, again excluded at the granular layer, but phagocytosis of the particles by basal keratinocytes resulted in their subsequent appearance in cornified cells. It was surmised, therefore, that the dermal-epidermal junction is at least partially permeable, even to large molecules and small particles. These substances enter the extracellular space of the epidermis except the upper granular layer and stratum corneum, where the lipid matrix derived from membrane-coating granules acts as a diffusion barrier.

Occasional discontinuities in the basal lamina occur, and the dermal-epidermal junction is also interrupted where nerve endings cross (Briggaman and Wheeler, 1975). The presence of dermal lymphocytes in the epidermis, where they are suspected to perform an immunosurveillance role, is observed in adult skin (Breathnach, 1978).

Figure 1. The Epidermis

Electron micrograph of a thin section through human epidermis. The dermis is visible as a lighter area at the bottom of the field, and several layers of cornified cells in the stratum corneum at top left. The small cell with lighter cytoplasm and darker nucleus in the epidermal basal layer is a melanocyte. Note the presence of abundant tonofilaments and numerous granular structures, and the loss of nuclei in the upper epidermal layers. (x 3750)

(Reproduced by permission of Dr. C.J. Skerrow, Department of Dermatology, University of Glasgow).

1.1.3 The Epidermis

Whereas the dermal fibrous network provides the flexibility and resilience of skin (Morejohn and Pratley, 1979), the epidermis is responsible for its physical and chemical resistance. This is achieved by the production of a layer of keratin-filled dead cells, which represents the major differentiative function of the epidermis.

The principal epidermal cell type is the keratinocyte. This epithelial cell is unique to the epidermis, but closely-related cells termed keratocytes occur in the cornea and conjunctiva, and, in common with the gland and follicle cells of the dermis mentioned above, are capable of differentiating into mature keratinocytes upon appropriate stimulus (Sun and Green, 1977).

Other cell types occurring in the epidermis include melanocytes, Langerhans cells and Merkel cells. Melanocytes are derived from primitive neural crest, and migrate to the epidermis during embryogenesis. The function of this cell is the manufacture of the pigment melanin, which provides protection against solar radiation. Melanin granules are transferred to epidermal keratinocytes by the process of pigment donation. *In vitro* analysis of pigment donation has led to the view that this occurs by a cytophagocytotic mechanism, in which blobs of cytoplasm derived from melanocyte dendrites are actively engulfed by neighbouring keratinocytes (Okazaki *et al*, 1976).

Langerhans cells are often included with melanocytes in the epidermal dendritic cell population. However, because of

their resemblance to the sarcoma histiocytosis X, they are possibly of mesodermal origin (Nezelof et al, 1973). The distinguishing features of the epidermal Langerhans cell are its histochemical staining with reagents specific for 5'-nucleotidase (ATPase) and the presence of the characteristic Langerhans cell granules (Wolff, 1972). Potten has suggested that these cells may be involved in the regulation of basal keratinocyte growth and differentiation (Allen and Potten, 1974), but an alternative opinion holds that epidermal Langerhans cells function in the immune response (Silberberg, 1976; Stingl, 1977).

The Merkel cell has a lobulated nucleus and 80-100nm diameter cytoplasmic granules (Winklemann and Breathnach, 1973). Like the melanocyte, this cell is thought to originate in embryonic neural crest and migrate to the epidermis during development. Merkel cells are often associated with Schwann cells and epidermal nerve terminals, and therefore may be tactile receptors.

The epidermis is stratified horizontally, and four major cell layers are generally recognised: the basal layer, the spinous layer, the granular layer, and the outermost stratum corneum (Breathnach, 1975). The ultrastructural differences responsible for this stratification are due to the process of keratinocyte differentiation known as keratinisation. Keratinocytes undergoing this maturational sequence arise by division in the basal layer, successively become spinous and granular cells, and are eventually shed from the surface of the skin as terminally-differentiated stratum corneum cells,

or corneocytes.

1.2 Epidermal Proliferation

Epidermal proliferation occurs continuously in order to replace the cornified cells shed from the surface of the skin. Cell division is localized mainly in the basal layer, but significant mitoses may occur in the suprabasal layers (Penneys *et al*, 1970). A hundred-fold difference in thymidine incorporation has been observed between suspensions of basal and differentiated keratinocytes (Vaughan and Berstein, 1971), and the amounts of nuclear DNA and RNA decrease in the upper epidermal layers (Suzuki *et al*, 1977). Thus the mammalian epidermis may be considered to consist of three stages : a proliferative stage, consisting of the basal (and possibly spinous) layer; a synthetic stage, corresponding to the spinous and granular layers; and a transition stage, in which upper granular cells are converted to stratum corneum cells.

1.2.1 The Epidermal Proliferative Unit

Studies on epidermal proliferation have revealed the existence of a basic, vertically-organized unit of keratinocyte proliferation, the epidermal proliferative unit (EPU). The thin epidermis of furred mammals such as mouse and rat has a regular cellular architecture, in which the flattened cells of the upper epidermal layers are stacked above a group of cuboidal basal keratinocytes (Christophers, 1974; Lawrence and Christophers, 1976). Subsequently the EPU was shown to comprise 10-11 basal cells, surmounted by

one spinous and two granular cells, and 4-6 hexagonal flattened cornified cells (Potten, 1976). Potten and co-workers have identified a Langerhans cell in the centre of the EPU basal group, and speculate that this may be involved in controlling the order of differentiation of the associated keratinocytes (Allen and Potten, 1974). In any case, it appears that one keratinocyte leaves the basal layer from the periphery of the EPU and commences further differentiation. As the keratinocyte flattens into a dehydrated squame and joins the hexagonal cornified cell column in the stratum corneum it interdigitates with squames of adjacent columns, and this may provide a mechanism for ordered desquamation between units (Potten and Allen, 1975). It should be noted that epidermal cell columns do not occur in fast-growing and hairless regions such as the palm and sole, and the central position of the Langerhans cell is apparently restricted to mouse dorsal epidermis (Mackenzie, 1975).

The epidermal proliferative unit could provide an internal epidermal mechanism for regulating epidermal thickness by coordinating basal cell proliferation and corneocyte desquamation, independent of dermis, epidermal nerves, or minor cell types (except possibly the Langerhans cell). However, the existence of conditions in which epidermal proliferation is greatly altered, such as wound healing and epidermal transplantation, suggests that other mechanisms exist regulating epidermal cell growth <u>in vivo.</u>

1.2.2 Epidermal Growth Factor

The rate of epidermal proliferation *in vivo* and in culture is increased by epidermal growth factor (EGF). This substance was isolated by Cohen from mouse submaxillary glands, and shown to cause precocious eyelid opening and tooth eruption in newborn animals (Cohen, 1962). Epidermal growth factor is a polypeptide of molecular weight 6,000 which has the unusual properties of being heat-stable (100°C for 30 min) and lacking lysine and phenylalanine (Cohen, 1962; Savage *et al*, 1972). The effects of EGF on newborn rodents appear to result from a general stimulation of epidermal proliferation. The growth of chick embryo epidermis in organ culture has been shown to be increased by EGF using whole skin, epidermis plus killed dermis, and isolated epidermis (Cohen, 1965). Similarly, the culture lifetime and subculture efficiency of human keratinocytes is enhanced by the addition of EGF (Rheinwald and Green, 1977). Epidermal growth factor is therefore a potent keratinocyte mitogen, but the biological role of this protein is unclear (Carpentier, 1978).

1.2.3 Epidermal Chalone

It has been suggested that epidermal proliferation is regulated by a negative feedback mechanism involving a mitotic inhibitor called epidermal chalone (Bullough, 1962; Lozzio, 1975). Extracts of epidermis with antimitotic activity have been partially purified, and the chalone is thought to be a protein or glycoprotein of molecular weight 30-40,000, and an isoelectric point between 5 and 6 (Duell *et al*, 1975). However, two or more inhibitory substances may be present in

the chalone preparation. Adrenalin stimulates the mitotic inhibition of the preparation, and could be a cofactor of chalone action (Bullough, 1969).

The following model for the chalone-dependent regulation of epidermal proliferation has been proposed (Bullough, 1975). Basal keratinocytes at a certain point in the G1 phase of the mitotic cycle (dichophase) must "choose" whether to undergo mitotic division or to become post-mitotic, differentiate, and eventually die. The choice is governed by the intracellular chalone concentration. At each division in the basal layer, a progeny cell is expelled into the spinous layer, which is a region of higher chalone effectiveness. This cell therefore differentiates, whereas the other progeny cell remains in the basal layer and will divide again. The large increase in epidermal proliferation which follows wounding is explained in this theory by loss of chalone across damaged cell membranes, followed by a consequent wave of mitosis. This model is compatible with the general features of epidermal proliferation and also explains the partial keratinisation which accompanies wound healing. However, it does contain certain inconsistencies, such as the improbability of severely-damaged cells undergoing further division, and in the absence of evidence for the chemical purity and mode of action of epidermal chalone, the chalone theory remains speculative.

An alternative view of the chalone theory is that these substances are extracellular and act via a cyclic AMP second messenger (Iversen, 1969). The evidence for cyclic nucleotide

regulation of keratinocyte growth and differentiation has been derived largely from *in vitro* systems (see section 1.4.2).

Normal homeostatic epidermal growth involves a balance of keratinocyte proliferation and desquamation, which should not require a chalone mechanism. Wound healing, however, may involve either a positive feedback (wound hormone) or negative feedback (chalone) control of keratinocyte proliferation. Regulation of epidermal growth and differentiation by the underlying dermis also occurs (see section 1.3.3).

1.3 Keratinisation

1.3.1 Keratin

Keratin may be defined as the insoluble fibrous protein of the outer epidermal layers, which is characterised by an α-type X-ray diffraction pattern (Baden and Maderson, 1970). The first successful extraction of epidermal protein was probably achieved in 1902 by Ramsden, who observed that : "A dead frog placed in saturated urea solution becomes translucent and falls to pieces in a few hours .... the skin brushes away with the slightest touch" (Ramsden, 1902). Preliminary biochemical analysis awaited the work of Rudall, who extracted bovine epidermis with 6M urea (Rudall, 1952). One fraction isolated retained the α-diffraction pattern, had a low sulphur content, and was termed epidermin.

Matoltsy devised an extraction procedure using citric acid-sodium citrate (CASC) buffer which specifically solubilised the fibrous protein of cow snout epidermis (Maltoltsy, 1965). As CASC buffer only solubilised material from the living cell

layers of the epidermis, the extracted protein was called prekeratin, and thought to represent monomeric units from which the stratum corneum α-keratin was constructed. Bovine prekeratin was subsequently found to have a native molecular weight of 375,000, comprising four subunits of 60,000 MW and two subunits of 72,000 MW (Skerrow, 1974), and is capable of forming filaments *in vitro* (Matoltsy, 1965). Other workers have claimed that "prekeratin" consists of larger numbers of polypeptides, and this may indicate that the native epidermal protein is microheterogeneous (Steinert, 1975; Baden and Lee, 1978).

Fibrous protein extracted from the stratum corneum using urea and mercaptoethanol has an apparently similar polypeptide composition to prekeratin (Baden *et al*, 1976), although no molecular weights were quoted in this study. As the stratum corneum protein is extractable in the absence of disulphide reducing agents (Steinert, 1975), it appears that the association of prekeratin units into α-keratin filaments involves non-covalent interactions.

1.3.2 The Keratinisation Process

The basal layer keratinocyte is a cuboidal epithelial cell, forming desmosomes with its neighbours and hemidesmosomes with the underlying basal lamina. Associated with the desmosomes (in fibre-desmosome complexes) and also free in the cytoplasm are bundles of 10nm diameter tonofilaments. The tonofilament protein is thought to consist of prekeratin polypeptides which form larger structures in the higher epidermal layers. Mitosis of basal layer keratinocytes forces

progency cells upward into the spinous layer.

Spinous layer cells are typified by the presence of villous projections on the cell surface, and by cytoplasmic vesicles containing lamellar structures about 2nm thick. These "membrane-coating granules" are destined to fuse with the keratinocyte plasma membrane (Hayward and Hackemann, 1973).

The granular layer cell contains keratohyalin granules (KHG), the chemical nature and function of which remain unknown. Autoradiographic studies have shown that protein synthesis proceeds in the granular layer cell, and that the amino acids cysteine, histidine and arginine are preferentially incorporated into keratohyalin granules (Fukuyama and Epstein 1966; 1967; 1975). Attempts have been made to isolate KHG and characterise their protein composition. Matoltsy and Matolt (1970), using a citric acid-Brij extraction procedure, found that newborn rat KHG protein contained large amounts of cysteine but very little histidine. Sibrack et al (1974) isolated KHG from newborn rat epidermis with phosphate buffer, but showed that these were histidine-rich and contained no cysteine. The histidine-rich protein of rat KHG has been isolated from the granular layer of the epidermis by extraction with both 1M potassium phosphate buffer and with 4M urea, and shown to have molecular weight 54,000, containing 7% histidine residues (Murozuha et al, 1979). A slightly smaller protein of the cornified layer, the stratum corneum basic protein, has similar antigenic reactivity and amino acid composition to histidine-rich protein, and is thought to be derived from it by a precursor-product relationship (Dale and Ling, 1979). A

lower molecular weight value of 27,000 for mouse histidine-rich protein was reported by Balmain et al (1977), who used the unusual staining properties of the protein with dansyl chloride to localise it in KHG. Further, the histidine-rich protein appeared in extracts of embryonic epidermis concommitant with the first ultrastructural detection of KHG. Messenger RNA thought to code for histidine-rich protein has been shown to occur only in the granular layer, leading to the proposal that control of epidermal protein synthesis is exerted at the level of transcription (Bernstein et al, 1975). The cysteine-rich fraction of KHG isolated by Matoltsy and Matoltsy (1970) may represent a disulphide-bonded protein forming the amorphous component of cornified cells (Matoltsy, 1976).

In the granular layer the membrane-coating granules fuse with the keratinocyte plasma membrane. Their contents, which appear to include polar lipids (Lavker, 1976), are discharged into the intercellular space and a dense layer is formed on the inner surface of the membrane (Hayward and Hackemann, 1973). The cell envelope formed by this process is largely responsible for the resistance of the stratum corneum cell to protein denaturants. Recently, this chemical resistance was explained by the discovery that the corneocyte envelope contains cross-linked protein (Rice and Green, 1977). Approximately 18% of the lysine of cornified cell envelope protein participates in ϵ-(γ-glutamyl) lysine peptide bonds. The formation of this unusual bond is presumably catalysed by the soluble enzyme transglutaminase, which becomes detectable

in the embryonic epidermis concommitant with the onset of terminal differentiation (Buxman et al, 1979).Transglutaminase activity in the epidermis increases in the granular layer as general protein synthesis diminishes (Rice and Green, 1978).

The granular layer represents the end of the synthetic phase of the epidermis, which now enters a transitional phase in which the granular cell is converted to a dead cornified cell. Cells intermediate between the granular and cornified layers are occasionally seen, and are known as transition or T-layer cells.

T-layer cells are considerably flattened, and exhibit organelle degeneration caused by fusion with lysosomes. Also at this stage, keratohyalin granules merge with tonofilaments resulting in an amorphous complex which is the sole intracellular component of corneocytes. Because of the high sulphur content of cornified cells (Matoltsy, 1976), it is thought that a cysteine-rich keratohyalin protein stabilises the keratin filaments. Keratin itself is apparently not disulphide-bonded, as it may be extracted in the absence of reducing agents (Steinert, 1975), and has a very low cysteine content (Matoltsy, 1965). The T-layer cell becomes dehydrated, with consequent loss of much of the cell mass. The resulting corneocyte is a flat hexagonal plate connected to its neighbours by belt-like desmosomes called squamosomes (Allen and Potten, 1974). The physical resistance of the stratum corneum is therefore due to α-keratin filaments stabilised by an amorphous disulphide-bonded protein, the cross-linked protein envelope, and the impermeable intercellular matrix of polar lipid.

1.3.3 The Regulation of Keratinisation

The postulated roles of epidermal proliferative unit formation, cyclic nucleotide levels and epidermal chalone in the normal growth and differentiation of the epidermis have been discussed (section 1.2). Regulation of epidermal keratinisation by the dermis is also thought to occur (Wessells, 1967; Flaxman, 1972). The dermis clearly performs a permissive role in epidermal differentiation, by supplying nutrients to the epidermis *in vivo*. In organ culture the epidermis degenerates except in the presence of dermis (Wessells, 1962; Briggaman and Wheeler, 1968). However, the dermis also has a deterministic effect on epidermal differentiation, by a form of mesodermal-epithelial interaction which determines the type and extent of epidermal keratinisation.

The classical experiments of Billingham and Silvers demonstrated that the dermis is capable of a site-specific induction of epidermal differentiation (Billingham and Silvers, 1967; 1968). Epidermis transplanted to a dermal site elsewhere on the body assumed the morphology of the epidermis normally occurring at that site. For example, guinea pig ear epidermis, which is lightly keratinised, when grafted to sole of the foot dermis became thick and heavily keratinised like normal plantar epidermis. The possibility that the new epidermis arose from transected hair follicles in the dermal bed was eliminated by grafting pigmented epidermis to albino dermis. Ear or sole epidermis transplanted to a dermal site on the trunk were reproducibly transformed to simulate normal

trunk epithelium. However, tongue and esophageal epithelial grafts retain their morphology except when recombined with trunk dermis. In the latter case, the possibility that the newly-arising trunk epidermis developed from residual trunk epithelial cells could not be discounted.

In a similar series of experiments, Sengel and co-workers showed a dominant role of dermis in heterotypic recombinants of chick embryo skin (Dhouailly et al, 1978). Recombinants including dorsal feather-forming dermis produced feathers, irrespective of the origin of the epidermis, and the keratin synthesised was characteristic feather keratin. Recombinants including tarsometatarsal scale-forming dermis always produced scales, containing scale-type keratin. Interestingly, the dermal dominance was interspecific. Mouse plantar dermis recombined with chick dorsal, feather-forming epidermis produced six footpads arranged in a typical mouse pattern. In this case, the keratin synthesised was of the chick scale type (Dhouailly et al, 1978). Thus, the dermis determines the formation of specific cutaneous appendages according to its regional origin, and also induces the corresponding type of epidermal protein synthesis.

Mammalian and avian dermis may be capable of activating specific sets of epidermal genes both in embryonic and adult life. The mechanism of this interaction is unknown, but has usually ben envisaged as involving a diffusible inducer molecule or molecules (morphogen). The inductive interaction may be distinct from nutritive, support, or other functions also performed by the dermis in normal skin.

1.4 Growth and Differentiation of Epidermal Cells in Culture

The cultivation of mammalian epidermal cells *in vitro* has been possible for many years (for review of earlier systems, see Karasek, 1975). Early culture systems consisted of full- or split-thickness skin (that is, epidermis plus all or part of the dermis) floating on a layer of culture medium or attached to a plasma clot (Medawar, 1948). Under these conditions, outgrowth of epidermal keratinocytes occurred over the cut surface of the dermis, a phenomenon known as epiboly. A modification of this technique is explant culture, in which skin attached to a solid surface permits outgrowth over the substratum of multilayered epidermal cell sheets (Stenn, 1978; Halprin *et al*, 1979).

1.4.1 Dispersed Cell Culture

The growth of epidermal cells in pure dispersed cell culture was first achieved by Cruikshank (Cruikshank *et al*, 1960). Many variations of the culture technique have since been developed, but most methods rely on separation of dermis and epidermis followed by disaggregation of the epidermal fragment (Fusenig and Worst, 1974; Prunieras *et al*, 1976a; Liu and Karasek, 1979). It is thought that basal epidermal keratinocytes attach to the culture surface and resume proliferation, whereas differentiated cells from higher epidermal layers are incapable of attachment or division. In support of this, a hundred-fold difference in ^{3}H-thymidine incorporation between basal and differentiated rat keratinocyte suspensions has been reported (Vaughan and Bernstein, 1971). Thymidine incoporation by adult guinea pig keratinocytes has been

observed over two weeks in culture (peaking at day 9 under the conditions used) [Regnier et al, 1973], and with adult mouse keratinocytes Fusenig and Worst recorded a thymidine labelling index of 17% and a mitotic count of 0.6% after 7 days in culture (Fusenig and Worst, 1974). Guinea pig epidermal cell cultures have been maintained over many passages (Regnier et al, 1973).

The question of whether keratinocyte differentiation proceeds in pure dispersed cell culture has not been unambiguously settled. Mouse keratinocytes in primary culture were reported to form a multilayered, keratinising epithelium exhibiting desmosomes and thickened membrane production (Fusenig, 1971). Constable et al (1974) claimed that guinea pig keratinocyte suspensions reform three-dimensional aggregates which show stratification from a lower living cell layer to a fully keratinised layer. Regnier and co-workers have cultured guinea pig epidermal cells for up to eleven passages, demonstrating their epithelial origin by leucine aminopeptidase staining. It was found that primary keratinocytes reconstitute tonofilaments and desmosomes, but that subsequent subculture resulted in the loss of these ultrastructural differentiation markers and epithelial morphology (Regnier et al, 1973). From these results it appears that primary cultures of epidermal keratinocytes are capable of forming multilayered colonies which exhibit a stratification pattern similar to that of normal skin, but on subculture the differentiated phenotype of these cells is not maintained. This is not surprising, as cultured

keratinocytes lack the normal inductive dermal influences of whole skin. Also, the minor epidermal cell types, which may perform an important role in keratinocyte growth organisation (Allen and Potten, 1974), may be lost from epidermal cell cultures. Melanocytes do not appear to divide in culture (Prunieras et al, 1976b), and the fate of the Langerhans cell is uncertain (Regnier et al, 1973).

1.4.2 The Effect of Cyclic Nucleotides

The suggestion has been made that cyclic nucleotides may be involved in the differentiation of keratinocytes in culture. Addition of cyclic AMP has previously been shown to increase neuroblastoma differentiation (Prasad and Hsie, 1971), pigment production by melanoma cells (Johnson and Pastan, 1972), and differentiation of early chick embryo cells (Deshpande and Siddiqui, 1976). Chopra (1977), using metaphase arrest and thymidine incorporation techniques, found that dibutyryl cyclic AMP and theophylline decreased keratinocyte DNA synthesis. Guinea pig epidermal cells pretreated with dibutyryl cyclic AMP in the concentration range 10^{-4} to 10^{-2}M showed reduction in thymidine incorporation by 50-95%, and a similar inhibition was caused by adenyl cyclase stimulators and phosphodiesterase inhibitors (Delescluse et al, 1974). A study of the effect of cyclic AMP on keratinocyte differentiation revealed increased staining with the dye Rhodamine B, which specifically reacts with cornified epidermal cells (Liisberg, 1968), and increased uptake of the amino acids histidine, arginine and cysteine, which are preferentially incorporated into epidermal granular cells (Fukuyama and

Epstein, 1966), by cultures pretreated with cyclic AMP (Delescluse et al, 1977). These effects of cyclic AMP may be relevant to the reciprocal relationship between keratinocyte proliferation and differentiation proposed in the chalone theory (Bullough, 1975), if epidermal chalone works via a cyclic nucleotide-mediated mechanism.

More recently, however, it has been reported that cyclic AMP increases the growth and thymidine incorporation of epidermal cell cultures (Green, 1978; Marcelo, 1979). A stimulatory effect was also induced by the analogue 8-bromo cyclic AMP and by cholera toxin, which raises intracellular cyclic AMP levels, but cyclic GMP had no effect on keratinocyte proliferation. Stimulation of keratinocyte differentiation by cyclic AMP was demonstrated by histochemical methods (Marcelo, 1979).

These results are in clear contradiction. The varied effects recorded by different workers argues against a simple role for cyclic AMP in the proliferation of keratinocytes. However, all these authors are in agreement that cyclic AMP promotes differentiation of epidermal cells. By analogy with the expression of differentiated phenotypes by cyclic nucleotides in other systems, this may indicate mediation of the keratinocyte response to extracellular effectors.

1.4.3 Growth on Collagen Gels

The long-standing hypothesis that epithelial differentiation is induced by mesodermal matrix macromolecules (Grobstein, 1964) has a functional correlate in the technique of growing epidermal keratinocytes on collagenous substrata.

The attachment and proliferation of epidermal cells from rabbit, mouse and human skin have been found to be enhanced by growth on collagen gels (Karasek and Charlton, 1971). In the same study, keratinocyte growth and differentiation were increased by the presence of viable skin fibroblasts or fibroblast-conditioned medium, and this stimulation was subsequently claimed to be localised in a stable low molecular weight fraction of the medium (Melbye and Karasek, 1973).

A role for collagen in epidermal attachment and growth is also indicated by the preferential adherence of keratinocytes to Type IV (basement membrane) collagen (Murray et al, 1979), and the requirement for epidermal collagen synthesis in epidermal migration (Stenn et al, 1979). Guinea pig keratinocytes grown on collagen gels produce an incomplete PAS-staining basal lamina at the cell-collagen interface (Mann and Constable, 1977). Dead pig dermis has also been reported to stimulate human keratinocyte differentiation, and this leads to lysis of the dermis beneath the epidermal cell colonies (Freeman et al, 1976).

1.4.4 Feeder Layer Culture

The studies described above were principally descriptive rather than quantitative, and can not be considered unambiguous proof of epidermal keratinisation in dispersed cell culture. Conclusive demonstration of keratinocyte differentiation in vitro has only been possible using the feeder layer method of Rheinwald and Green (1975a). In this technique human foreskin cells inoculated onto a layer of

lethally-irradiated 3T3 fibroblasts form a keratinising epithelium which can be serially subcultured. Keratinocyte differentiation was demonstrated by electron microscopy and Rhodamine B staining (Rheinwald and Green, 1975a). The feeder layer epidermal culture behaves like a wet-surfaced epithelium, shedding nucleated cells, but suspension of the desquamated cells in methyl cellulose permits nuclear degeneration and terminal differentiation (Green, 1977). The presence of keratin filaments in feeder layer-cultured keratinocytes has been shown by immunofluorescent staining with a specific antiserum raised to human plantar callus protein (Sun and Green, 1978a; 1978b), and purified bovine prekeratin (Franke et al, 1978). Using this culture system, it has been shown that cornea and conjunctiva epithelial cells (keratocytes) are also capable of keratinisation in feeder layer culture (Sun and Green, 1977). A recent modification of the technique employs growth of feeder layer keratinocyte cultures on Millipore filters rather than on plastic substrata (Kondo et al, 1979).

Epidermal cell cultures are thought to consist principally of basal layer keratinocytes. These cells constitute a stem cell population in normal skin, but exhibit ultrastructural markers of keratinisation such as tonofilaments, and therefore must be regarded as partially-differentiated cells. Maintenance of differentiated cells in pure culture is not normally possible, with the exception of teratocarcinomas (Rheinwald and Green, 1975b; Illmensee and Stevens, 1979), and it is consistent with the published data on keratinocyte

differentiation *in vitro* to conclude that pure epidermal cell cultures are capable of limited differentiation only in primary culture. Growth on collagenous substrata appears to facilitate keratinocyte proliferation, but insufficient quantitation of epidermal cell growth and differentiation in this system is available to determine whether this effect relates to the specific mesodermal induction proposed by Grobstein (1964). That the induction of epidermal keratinisation *in vitro* is non-tissue specific and non-species specific is suggested by the growth of human epidermal cells on feeder layers of mouse embryo fibroblasts (Rheinwald and Green, 1975a).

1.5 Mesodermal-Epithelial Interactions

Regulatory interactions between mesodermal and epithelial tissue layers occur at many stages of embryonic development and also in adult life. Mesodermal-epithelial interactions include primary induction, the neuralisation of primitive ectoderm by associated mesoderm, and embryonic induction, the interaction of mesoderm and epithelium to produce differentiated organs in either tissue (Saxén, 1976; 1978).

The early experiments of Spemann showed that presumptive neural plate from early amphibian gastrula transplanted to a new site on the embryo develops according to its new surroundings (Spemann, 1928). At late gastrula stage, however, the grafted tissue develops as an ectopic neural plate. The neurectoderm is therefore determined during gastrulation by the underlying mesoderm.

The study of embryonic induction has led to proposals of possible mechanisms for inductive mesodermal-epithelial interactions : (i) mediation of inductive stimuli by diffusible inducer molecules or by extracellular matrix molecules; (ii) direct physical contact between reacting cells, possibly involving formation of specialised junctional structures. Investigation of mesodermal-epithelial interaction in a variety of tissues has indicated that different mechanisms may apply in different systems.

The development of the mammalian tooth requires a series of interactions between the dental mesoderm and the enamel epithelium. The mesodermal cells differentiate into odontoblasts and secrete predentine, which is mainly collagen, and this induces the epithelial cells to differentiate into ameloblasts and synthesise the organic matrix of enamel. Transfilter culture of mouse tooth mesoderm and epithelium resulted in normal development only across filter which permitted cell penetration (Thesleff et al, 1977). Odontoblast differentiation was dependent upon resynthesis of a basal lamina by the enamel epithelium, and this only occurred when mesodermal cell processes crossed the filter to contact the basal lamina (Thesleff et al, 1978). As differentiation only occurred in explants in which a basal lamina was formed, and direct membrane contacts between mesodermal and epithelial cells did not appear to be required, it was proposed that components of the basal lamina were responsible for the induction of odontoblast differentiation. In this type of study it should be noted that causal relationships are difficult to establish, the experimental conditions which

permit cell-cell contact may also allow expression of the differentiated phenotype independently.

Extracellular matrix macromolecules have also been implicated in the development of chick cartilage and pancreatic epithelium. The somites of the chick embryo differentiate into cartilage by association with notochord or spinal cord. An extract of embryo cartilage which is mainly proteochondroitin sulphate has been shown to increase in vitro somite chondrogenesis (Kosher et al, 1973). This is of interest because notochord and spinal cord normally synthesise proteoglycans. In fact, treatment of these inducer tissues with trypsin or hyaluronidase resulted in the loss of their ability to stimulate chondrogenesis (Kosher and Lash, 1975). The development of chick pancreatic epithelium is induced by a wide range of inducer tissues or even by embryo extract. A fraction of competent mesoderm, which appears to be proteinaceous, stimulates the thymidine incorporation and in vitro development of pancreatic epithelium (Ronzio and Rutter, 1973). In these systems, induction of differentiation appears to be relatively non-specific, and may bear only limited resemblance to mesodermal-epithelial interaction in general.

A role for extracellular matrix substances in embryogenesis is also indicated by study of mouse submandibular gland development (Banerjee et al, 1977; Bernfield and Banerjee, 1978). In this tissue, the epithelium develops from a primordial bud of cells into the highly-branched adult salivary gland under the influence of the associated mesoderm. Treatment of the

isolated epithelium with collagenase removes the basal lamina, and on recombination with mesoderm no branching morphogenesis occurs. If the collagenase-treated epithelium is incubated for two hours in organ culture a new basal lamina is synthesised, and on recombination with mesoderm the gland develops normally. A model has been proposed in which site-specific degradation of the basal lamina by an enzymatic activity localised in the mesoderm stimulates local epithelial proliferation and results in the lobulated morphology of the adult gland (Bernfield, 1977). These studies offer more conclusive evidence for an inductive role by matrix components, as the experimental conditions more closely reproduce the in vivo situation.

Chick corneal differentiation appears to depend upon contact between corneal epithelium and the extracellular matrix of the lens capsule. Meier and Hay (1975) showed that lens capsule killed by autoclaving or freeze-thawing was as effective as living tissue in promoting corneal differentiation, and quantitated corneal collagen synthesis in transfilter organ culture with killed lens capsule by the incorporation of ^{3}H-proline into protein. Epithelial collagen synthesis increased directly with the size of the filter pores, and decreased as the number of interspersed filters was increased. Induction readily occurred across Nucleopore filters but not across Millipore filters, and this correlated with the observation that corneal cell processes penetrated the Nucleopore filters and could be visualised by electron microscopy, whereas Millipore filters allowed no cell penetration. These results on the transfilter induction of corneal epithelium development have been interpreted in terms

of a mechanism involving interaction of the corneal cell surface and the extracellular matrix of the lens capsule, but are also consistent with direct cell-cell contact between the tissues.

In several systems studied, embryonic induction may involve different mechanisms. Primary induction of ectoderm neuralisation by archenteron roof mesoderm in the newt Triturus did not occur in transfilter culture, although Nucleopore filters were used which allowed penetration by cell processes (Toivonen and Wartiovaara, 1976). Neuralisation in vivo is associated with the presence of close membrane contacts between cells of mesoderm and ectoderm (Grunz and Staubach, 1979). In contrast, induction of lens development by chick optic vesicle occurred not only in transfilter organ culture, but also across a dialysis membrane which only permitted the passage of molecules less than 12,000MW (Karkinen-Jääskeläinen, 1978).

Direct cell contact between the heterotypic tissues has been implicated in the development of mouse metanephrogenic kidney. Early studies by Grobstein (1957; 1968) showed that induction of mesodermal tubule formation by the associated epithelium could occur across Millipore filters. Subsequent workers used Nucleopore filters, which are thinner than Millipores and have holes of defined size. It was found that transfilter induction of kidney tubule formation by spinal cord only occurred when cytoplasmic processes from both cell types crossed the filter, and these processes formed regions of close apposition between the two tissues (Saxén et al, 1976; Wartiovaara et al, 1974).

Analogous heterotypic contact regions have been found *in vivo*. Discontinuities in the basal lamina between epithelium and mesoderm of rat duodenal mucosa appear during the period of embryonic duodenal differentiation (Mathan *et al*, 1972). Direct mesodermal-epithelial contact was also observed during development of the rat submandibular gland (Cutler and Chaudhry, 1973), morphogenesis of the mouse incisor tooth (Slavkin and Bringas, 1976), differentiation of uropygial ectoderm in the duck preen gland (Bride and Gomot, 1978), and lung development in the embryonic mouse (Bluemink *et al*, 1976).

The mesodermal-epithelial interaction in mouse lung has been studied in more detail *in vitro*. Grainger and Wessells (1974) performed an elegant experiment aimed at the detection of RNA transfer from mesoderm to epithelium during embryonic lung development. A modification was employed of the ^{3}H-uridine labelling technique used by Kelley (1968) to demonstrate the transfer of nucleotides or RNA between chordamesoderm and presumptive neurectoderm of *Xenopus* gastrulae. Mesodermal cells were labelled with ^{13}C- and ^{15}N-substituted ribonucleosides, and with trace amounts of tritiated ribonucleosides. After transfilter culture with responding lung epithelium, the extracted epithelial RNA was analysed by equilibrium centrifugation, and the radioactivity shown to coincide with RNA of normal density. The sensitivity of this technique was such that transfer of 0.01% of labelled mesodermal RNA to the epithelium could have been detected. It was therefore concluded that no transfer of intact RNA molecules had occurred from mesoderm to epithelium during induction. Similar

techniques, at a lower level of sensitivity (0.5%), demonstrated that DNA was also not transferred. This indicates that informational macromolecules are probably not transferred between tissues undergoing embryonic induction, but does not exclude diffusible low molecular weight morphogens.

Mesodermal-epithelial interaction in tissues where direct heterotypic contact is necessary may involve cell-cell transfer of small inducer molecules. One possible mechanism for this would be the formation of permeable gap junctions between cells of the two tissue layers (Wolpert, 1978).

1.6 Intercellular Communication

Communication between cells in higher organisms involves long-range interactions, characterised by extracellular mediator molecules such as hormones and neurotransmitters. Many animal cells also participate in short-range cellular interaction, which involves direct communication between adjucant cells via a specialised membrane structure, the gap junction (Furshpan and Potter, 1959; Revel and Karnovsky, 1967; for recent review see Loewenstein, 1979).

1.6.1 The Structure of the Gap Junction

By thin-section electron microscopy the gap junction is a region of close cellular contact, where the apposed plasma membranes are separated by a 2-4nm "gap" (Revel and Karnovsky, 1967). The total width of the junction is approximately 18-19nm. The gap region is penetrated by lanthanum hydroxide staining, resulting in a pentalaminar structure. Uranyl acetate stains the intercellular gap and also between the

membrane bilayer leaflets, giving a septilaminar appearance. Oblique thin sections reveal the gap junction en face as a hexagonal array of particles with centre-centre spacing of 9nm. Freeze-fracture cleaves the membrane between the bilayer leaflets and results in a hexagonal lattice of protrusions on the A membrane face and a corresponding pattern of indentations on the B face (McNutt and Weinstein, 1970). The junctional particles, connexons, are approximately 5nm in diameter with an electron-dense central core of about 2nm diameter.

Junctional membrane may be purified by its resistance to solubilisation by the detergent n-laurolyl sarcosine (Sarkosyl) [Goodenough and Stoeckenius, 1972]. This procedure has been used to investigate the protein composition of the gap junction. Goodenough (1974; 1976) analysed the junctional protein by SDS-polyacrylamide gel electrophoresis and concluded that the major component consisted of two polypeptides of approximately 9000mw, which were normally disulphide-bonded. It has since been claimed that the 9000MW bands were artefacts caused by the proteolytic enzymes used in the isolation procedure, and that the native junctional proteins have molecular weights 25,000 and 34,000 (Duguid and Revel, 1975). Evans and Gurd (1972) found over 20 polypeptide bands in extracts of liver gap junctions, most protein occurring in the 40,000-80,000MW range. Subsequent purification of hepatic junctions resulted in a major polypeptide component of 38,000MW (Culvenor and Evans, 1977). A recent isolation procedure using no exogenous protease treatment yielded a junctional preparation consisting of 47,000MW and 27,000MW proteins (Hertzberg and Gilula, 1979). The considerable inconsistency of these

studies may be due to the sensitivity of junctional protein to degradation during isolation.

A structure of the gap junction has been proposed, based on correlated electron microscopy, X-ray diffraction, and chemical studies (Caspar et al, 1977; Makowski et al, 1977). X-ray diffraction of pelleted isolated junctions indicated a structure with considerable long-range order, but short-range disorder. Optical diffraction analysis and computer filtration of negatively-stained images suggested that the junctional particles have six-fold symmetry and a densely-staining central core, which may represent the channel pore. The volume of the connexon is approximately 180-200nm^3, and as the junction is 52-57% protein by weight, this corresponds to 140,000-170,000MW of protein. If the connexon is a hexamer, the individual subunit proteins would thus have molecular weight 23,000-28,000. The proposed model for gap junction structure is a hexagonal lattice of connexon particles with centre-centre spacing 8-9nm. Each connexon is apposed to another on the opposite membrane so as to form a hollow tube of outside diameter 5.2nm and inside diameter 2nm joining the cytoplasm of the two cells.

1.6.2 Occurrence

The gap junction is a ubiquitous structure in multicellular animals, being found in phyla as low as Porifera. Invertebrates have a gap junction with distinct structure, having a looser array of particles which freeze-fracture onto the B membrane face (Flower, 1977). The gap junction does not occur in plants, but they have analogous cytoplasmic continuities,

plasmadesmata. As pointed out by Griepp and Revel (1977), the presence of protoplasmic bridges as low in the evolutionary scale as Volvox colonies may indicate a fundamental biological role for this form of intercellular communication.

In higher animals, gap junctions are absent from mature skeletal muscle and circulating blood cells. Skeletal muscle fibres are multinucleate, and electrical coupling between fibres would prevent incremental activation, but gap junctions do form between prefusion myoblasts (Kalderon et al, 1977). Deficiency of junction formation by circulatory leukocytes is indicated by the lack of nucleotide transfer by PHA-stimulated human lymphocytes (Cox et al, 1976), and the lack of uridine nucleotide and ^{51}Cr transfer between murine T-lymphocytes and target mastocytoma cells (Sanderson et al, 1977). A recent report of the occurrence of gap junctions between rabbit lymphocytes probably arose from misidentification of the junctional structure, as these were of the B-type, found only in invertebrates (Kapsenberg and Leene, 1979). However, gap junction formation and ionic coupling have both been observed between macrophages in vitro (Levy et al, 1976; Porvaznik and MacVittie, 1979), although these observations have not been correlated.

An absence of gap junctions in certain cancer cells has led to the proposal that some forms of malignant transformation may be caused by a deficiency of response to normal growth-regulatory signals (see section 1.6.4).

Gap junction formation is relatively non-species specific. Mammalian and avian cells in culture form structurally and

functionally normal junctions with one another, but not with invertebrate cells, which have a morphologically distinct gap junction (Epstein and Gilula, 1977). Junction formation was demonstrated between cells of the insect sub-order Homoptera, but not between cells of Homoptera and either Lepidoptera or Diptera. Although non-species specific, junction formation may be tissue specific. Lack of junction formation between epithelial and fibroblastic cells has led to the suggestion that tissue specificity may ensure the metabolic individuality of different tissue layers in vivo (Pitts and Bürk, 1976; Fentiman et al, 1976).

1.6.3 Junctional Permeability

The presence of gap junctions has been correlated with the ability of adjacent cells to exchange ions and small molecules (Gilula et al, 1972). Cells connected by gap junctions are electrically coupled. Injection of a current pulse via microelectrodes into one cell is followed by a corresponding voltage change in adjacent cells (Furshpan and Potter, 1959; Loewenstein and Kanno, 1964; Sheridan et al, 1978). Ion transfer has also been demonstrated by the acquisition of beating synchrony by myocardial cells (Griepp and Bernfield, 1978), and the "rescue" of cells treated with the Na^+/K^+-ATPase inhibitor ouabain by adjacent resistant cells (Ledbetter and Lubin, 1979). Microinjection techniques have shown the cell-cell transfer of fluorescent dyes, including fluorescein (376 MW) [Loewenstein and Kanno, 1964], Procion Yellow (630 MW) [Johnson and Sheridan, 1971], and Chicago Sky Blue (993 MW) [Potter et al, 1966].

Exchange of other small molecules has been demonstrated by a variety of techniques. Cocultured mouse myocardial cells and rat ovarian granulosa cells cross-respond to hormonal stimulation, presumably due to transfer of the intracellular mediator, cyclic AMP (Lawrence et al, 1978). Cultured animal cells have been shown to correct mutant phenotypes in coculture by exchange of nucleotides (Subak-Sharpe et al, 1969; Cox et al, 1972). An extreme example of this is the survival of cocultures of $HGPRT^-$ and TK^- mutant cells in medium containing hypoxanthine, aminopterin and thymidine which does not support the growth of either cell type alone (Pitts, 1971). Coculture of prelabelled donor cells with unlabelled recipient cells has been used to show the intercellular transfer of nucleotides, sugar phosphates and amino acids, but no DNA, RNA or protein (Pitts and Simms, 1977; Pitts and Finbow, 1977).

The size limit of junctional communication has been investigated using a range of fluorescent peptides. It was found that molecules of less than 1200MW were transferred between Chironomus salivary gland cells, but molecules larger than 1900MW were retarded (Simpson et al, 1977). Assuming that the gap junction pore is an aqueous channel, factors such as shape, charge and solvation may also determine whether a molecule is exchanged between cells.

1.6.4 Properties of Gap Junctions

Estimates of the rate of formation of gap junctions after cells come into contact vary from a few minutes up to an hour (Loewenstein, 1967; Johnson et al, 1974; Griepp and Bernfield, 1978). Junction formation appears to be a passive

process, occurring in the absence of ATP production and protein synthesis (Epstein et al, 1977), and in anucleate cells (Cox et al, 1976). Morphologically, the formation of junctional regions apparently involves the accretion of connexon particles. Particle-free "formation plaques" have been suggested first to occur in areas of forming junctions, and 9-11nm diameter intramembranous particles subsequently appear (Johnson et al, 1974). These are assumed to be native connexons, which then aggregate to form the complete junction. As the unassociated connexons are presumably not porous, an interaction must take place between apposed junctional particles to open the channel pore. This may involve a conformational change in the gap junction protein, or possibly proteolysis of a pre-existing subunit (Revel et al, 1978). The demonstration that the ionic coupling of junction-forming cells increases in a quantal fashion supports the hypothesis that junction formation occurs by gradual recruitment of connexon units (Loewenstein et al, 1978).

Removal of gap junctions occurs by internalisation of the intact junction, including a portion of the neighbouring cell membrane. These appear in the cell cytoplasm as junctional membrane vesicles, or annular gap junctions (Larsen, 1977). The detection of acid phosphatase activity within gap junctional vesicles may indicate that the fate of the annular gap junction is fusion with lysosomes (Larsen and Hai-Nan, 1978).

The structure and permeability of the gap junction have been reported to be altered by the intracellular concentration of ionised calcium. Loewenstein and co-workers found that

microinjection of calcium solutions decreased ionic coupling and dye transfer between Chironomus cells (Dèlèze and Loewenstein, 1976). Quantitation of the cytoplasmic calcium concentration using the calcium-binding fluorophore aqueorin showed that the free calcium concentration in the junctional locale increased from about 10^{-7}M to 5 x 10^{-5}M (Rose and Loewenstein, 1976). Electrical uncoupling was also caused by calcium ionophores and prolonged exposure of the cells to calcium-free medium. Mammalian cells, however, were not significantly uncoupled by calcium ionophores (Gilula and Epstein, 1976). Calcium apparently decreases junctional transfer in a graded manner. Injection of pairs of fluorescent peptides of different molecular weights at a calcium concentration of approximately 10^{-5}M resulted in preferential retardation of the larger molecule (Rose et al, 1977).

Morphological alterations in the structure of the gap junction have been correlated with the calcium-mediated inhibition of ionic coupling. Crayfish septate axon gap junctions exhibit a more regular particle array with decreased centre-centre spacing in the presence of calcium (Peracchia and Dulhunty, 1976; Peracchia, 1977), and similar results have been observed using calf lens junctions (Peracchia, 1978). It is suggested that the increase in lattice ordering associated with calcium binding reflects a conformational change which closes the junctional channels.

Calcium-dependent modulation of junctional permeability is thought to be important in sealing the gap junction in the event of death (and subsequent calcium influx) of adjacent cells.

Indeed, cardiac cells injured by cutting the heart tissue exhibit an ordered junctional structure with decreased particle spacing, similar to that of calcium-treated preparations (Baldwin, 1979). It has been shown, however, that decrease in intracellular pH caused by carbon dioxide treatment of Xenopus embryos causes reversible electrical uncoupling (Turin and Warner, 1977). As injection of calcium causes a drop in pH, the uncoupling mechanism may not involve calcium directly.

1.6.5 Biological Functions of the Gap Junction

The gap junction is a specialised cytoplasmic continuity found between adjacent cells in most solid tissues. The ubiquity of this structure argues for evolutionary conservation of the gap junction, implying an important biological role in multicellular organisms. What this role is, as yet is by no means clear. However, it is reasonable to believe that all cells in a tissue, linked by gap junctions, will share a common pool of ions and small molecules, and retain their individuality only by their macromolecular composition. The junctional network may be important in transporting nutrients from the blood into the cells of an organ. Similarly, response to hormonal stimuli by "second messengers" such as cyclic AMP and calcium may be co-ordinated by having all cells metabolically coupled (Loewenstein, 1973; Lawrence, 1978).

The possibility that growth regulatory molecules are transferred through gap junctions (Loewenstein, 1979) has given rise to the idea that some transformed cells may have

lost the ability to form junctions and therefore be unable to respond to normal growth-inhibitory signals (Weinstein et al, 1976). Malignantly-transformed cells with decreased junction formation have indeed been found (Azarnia and Loewenstein, 1976), but other cancer cells communicate normally (Johnson and Sheridan, 1971; Nicolas et al, 1978). Azarnia and Loewenstein studied the growth and communication properties of hybrids of human fibroblasts and the malignant, communication-defective mouse L cell. The hybrids were junction-forming and non-tumorigenic, but as they spontaneously lost human chromosomes they simultaneously reverted to communication incompetence and tumorigenicity (Azarnia and Loewenstein, 1977). However, as the hybrids tended to lose human chromosomes in groups, no unambiguous correlation is possible between junction formation and malignant transformation. In hybrids of mouse mammary adenocarcinoma cells and normal rat fibroblasts, abundance of gap junctions correlated with density-dependent inhibition of growth (Stamatoglou, 1978).

It has also been proposed that gap junction formation is important in embryonic development (Wolpert, 1978). Neurulation of the amphibian Rana is accompanied by the replacement of tight junctions (zonula occludentes) with gap junctions (Decker and Friend, 1974). In Xenopus embryos, gap junctions occur between retinal cells and pigment epithelial cells throughout the period of retinal specification, after which they disappear (Dixon and Cronly-Dillon, 1974). An analogous disappearance of gap junctions was observed following cell differentiation in the neural retina of the chick (Fujisawa et al, 1976).

Variation in junction formation has been found during liver regeneration following partial hepatectomy in the rat. 28h post-operation gap junctions disappear and only begin to reappear after about 40h , initially in association with tight junctions, reaching normal levels and distribution at 48h . (Yee and Revel, 1978; Yancey et al, 1979).

The relevance of these observations on the presence and absence of gap junctions during embryogenesis and tissue regeneration have not been determined. However, if the loss of gap junctions during cell differentiation and organ specialisation is a general phenomenon, it may be that groups of cells require to be insulated from the general cell mass in order to respond to specific, local, differentiative stimuli, and conversely, the presence of the junctional network may be necessary for the propagation of general developmental effectors. As the molecular mechanisms governing embryonic development are largely unknown, it is not possible to ascertain whether gap junctions are involved in the transmission of developmental signals.

The function of junctional communication in cell growth control and tissue development remains speculative. What can be stated with certainty is that cells joined by gap junctions form a metabolic continuum, and therefore regulatory molecules small enough to traverse the channel pores will equilibrate among the entire cell population.

1.7 Aims of Project

The differentiation of the epidermis in adult mammalian skin is regulated by the associated dermis in a site-

specific manner. This process is analogous to other forms of mesodermal-epithelial interaction, which often involve direct contact between the reacting tissues. As discussed above, it is possible that the dermal-epidermal interaction requires direct heterotypic contact between cells of the dermis and epidermis, perhaps involving formation of permeable intercellular junctions. The possibility has been investigated in this project by determination of the cell communication properties of cells from the dermis and epidermis of newborn mouse skin. This has been related to the proposed tissue specificity of cell communication by study of other mouse primary cell types.

Chapter 2 - MATERIALS AND METHODS

"What's the use of their having names",
the Gnat said, "If they won't answer to them?"
"No use to <u>them</u>", said Alice, "but it's useful
to the people that name them, I suppose".

Lewis Carroll
Through the Looking Glass

2.1 MATERIALS

2.1.1 Chemicals

Trypsin (bovine pancreas, EC 3.4.21.4), alcohol dehydrogenase (yeast, EC 1.1.1.1), uridine, D-valine and dimethyl sulphoxide were obtained from Sigma Chemical Co., Kingston-Upon-Thames. Glutamate dehydrogenase (beef liver, EC 1.4.1.3), phosphorylase a (rabbit muscle, EC 2.4.1.1), ovalbumin and bovine serum albumin were obtained from Miles Laboratories, Slough.

Collagenase (Clostridium perfringens Type IV, EC 3.4.24.3), hyaluronidase (ovine testes, EC 3.2.1.35), L-(β)3,4-dihydroxy-phenylalanine, Rhodamine B, Trypan Blue, toluidine blue, mitomycin C, N,N'-methylene bis acrylamide, and p-bis (o-methyl styryl) benzene (bis-MSB) were obtained from British Drug House, London.

Triton X-100, acrylamide, and 2,5 diphenyl oxazole were obtained from Koch-Light Laboratories, Colnbrook, Bucks.

Coomassie Brilliant Blue was obtained frim Micro-Bio Laboratories Ltd., London.

Amfix was obtained from May and Baker, Dagenham.

Depex mounting fluid and Giemsa stain were obtained from Searle, London.

Millipore filters were obtained from Millipore U.K. Ltd., London.

All other reagents used were of BDH "Analar" grade.

2.1.2 Radiochemicals

Radiochemicals were obtained from the Radiochemical Centre, Amersham, Bucks. These were [5-^{3}H]uridine (specific activity 30 Ci. m mole^{-1}), [methyl-^{3}H] thymidine (18Ci.m mole^{-1}), L-[4,5-^{3}H] leucine (52 Ci. m mole^{-1}), L-[^{35}S] methionine (> 600 Ci. m mole^{-1}), and L-[U-^{14}C] histidine (300 mCi.m mole^{-1}).

2.1.3 Cultured Cell Lines

The following established cell lines were used :

(a) BHK21/C13. Syrian hamster kidney fibroblast (Macpherson and Stoker, 1962).

(b) BRL. Buffalo rat liver epithelial cell (Coon, 1968).

(c) L929/A9. Mouse embryo cell lacking hypoxanthine: guanine phosphoribosyl transferase (Littlefield, 1966).

(d) MDCK. Madin-Darby canine kidney epithelial cell (Rindler et al, 1979).

2.1.4 Scintillation Fluid

The Triton-toluene scintillation fluid used had the composition 70% v/v toluene, 30% v/v Triton X-100, 0.5% w/v PPO and 0.05% w/v bis-MSB.

2.1.5 Phosphate-Buffered Saline (PBS)

NaCl	10g
KCl	0.25g
Na_2HPO_4	1.44g
KH_2PO_4	0.25g
Distilled water to	1l
(pH 7.2)	

2.1.6 Balanced Salts Solution (BSS)

NaCl	6.8g
KCl	0.4g
$MgSO_4.7H_2O$	0.2g
$NaH_2PO_4.2H_2O$	0.14g
$CaCl_2.6H_2O$	0.39g
1% phenol red	1.5ml
Distilled water to (pH 7.2).	1l

2.1.7 Formal Saline

100ml 40% w/v formaldehyde plus 900ml 0.9% w/v NaCl.

2.1.8 D19b Developer

$Na_2SO_3.7H_2O$	144g
Na_2CO_3	48g
KBr	4g
Hydroquinone	8.8g
Metol	2.2g
Distilled water to	1l

2.2 METHODS

2.2.1 Maintenance of Cell Lines

Cell lines were maintained at 37°C in Roux bottles in an atmosphere of 5%CO_2, 95% air using Glasgow modification of Eagle's medium supplemented with 10% foetal calf serum (EFC10), containing 100 units.ml^{-1} penicillin and 100µg.ml^{-1} streptomycin (for details of medium formulation see the Flow Laboratories catalogue, 1978). Cells were subcultured 1:10 every 3-4 days by briefly washing twice with a 1:4 mixture of trypsin (0.25% in 20mM citrate buffer pH 7.8) and EDTA (0.02% in PBS), and inoculation into sterile Roux bottles containing 50ml fresh EFC10. Cells for experiments were grown in 50mm plastic petri dishes (Nunc, Gibco Bio-Cult, Paisley, Scotland) at 37°C in 4ml EFC10 using a humidified incubator containing an atmosphere of 5% CO_2, 95% air.

Suspensions of cells were counted with an "Improved Neubauer" hemocytometer. Viable cell counting was performed by mixing the suspension 1:1 with a solution of 0.1% Trypan Blue in PBS, and counting the cells which excluded the dye.

2.2.2 Culture of Epidermal keratinocytes and Dermal Fibroblasts from Newborn Mouse Skin

Establishment of cell cultures from newborn mouse skin was performed essentially by the method of Regnier *et al* (1973). The skin of 6 1-3 day old mice was sterilised by washing with sterile PBS and 70% ethanol, and then rubbed with antifungal cream ("Nystan", E.R. Squibb, London, or "Ecostatin", F.A.I.R. Laboratories, Merseyside). Mice were killed by decapitation,

and the entire dorsal skin dissected off. The skin was cut into two strips, which were then stuck epidermal side down onto pieces of sterile adhesive tape (Lasso tape, Smith and Nephew, Ltd.) and incubated in 20ml sterile universal bottles (6 skin strips per bottle) containing 10ml 0.2% trypsin in PBS at 37°C for 1h. The dermis and epidermis were separated using forceps, and basal epidermal keratinocytes liberated by agitation of both tissues separately in 10ml EFC10 per 6 mice. The keratinocyte suspensions were filtered through two layers of sterile gauze (Smith and Nephew, Ltd.) and pooled. The cells were pelleted by centrifugation at 500 x g for 10 min and resuspended in 10ml EFC10.

Dermal fibroblasts were prepared by incubation of the dermal tissue from 6 mice in 5ml 0.1% collagenase, 0.1% hyaluronidase in GKN solution (0.1% glucose in 0.8% NaCl, 0.04% KCl; Hinz and Syverton, 1959) in a sterile 20ml universal bottle at 37°C for 1h in a shaking water bath. The resulting cell suspension was filtered, centrifuged, and resuspended in 10ml EFC10 as above.

Viable cell concentrations were estimated by Trypan Blue exclusion, and normally fell in the range 2-10 x 10^6 viable cells per ml (approximately 50% of cells excluded the dye). Total viable cell yield was 2-5 x 10^7 epidermal keratinocytes and 5-10 x 10^7 dermal fibroblasts from the skin of 6 mice. Epidermal keratinocyte and dermal fibroblast cultures were established in 50mm plastic petri dishes containing 4ml EFC10 at 2.5 x 10^5 viable cells per ml. Cultures were incubated at 37°C in an atmosphere of 5%CO_2, 95% air.

2.2.3 Culture of Epidermal Keratinocytes and Dermal Fibroblasts from Adult Guinea Pig Ear Skin

Establishment of cell cultures from the ear skin of adult albino guinea pigs was performed by the method of Regnier *et al* (1973). The hair was removed from the dorsal ear skin using depilatory wax, the ears were washed with 70% ethanol and then with sterile PBS, and then rubbed with antifungal cream (see section 2.2.2). The following day the animals were sacrificed by cervical dislocation, and the dorsal ear skin removed by dissection. Cultures of epidermal keratinocytes and dermal fibroblasts were prepared as for newborn mouse skin (section 2.2.2). Percentages of viable cells were similar to those obtained using newborn mouse skin. Total viable cell yield was 5-10 x 10^6 epidermal keratinocytes and 1-2 x 10^7 dermal fibroblasts from the ear skin of one guinea pig.

2.2.4 Culture of Epidermal Melanocytes from Newborn Mouse Skin

Melanocytes occur as a minor fraction of the epidermal cell population, and may be purified from it by treatment with sodium citrate and serum deprivation (Prunieras *et al*, 1976; Moreno *et al*, 1978). Epidermal cells were prepared from newborn mice of the pigmented strains C3H or C57 (see section 2.2.2). 5 x 10^6 cells were pelleted by centrifugation at 500 x g for 10 min, and the pellet resuspended in 2ml 0.8% sodium citrate in 0.9% sodium chloride. After standing at room temperature for 2 min, the suspension was diluted to

5×10^5 cells per ml with EFC10 and plated out in 50mm plastic petri dishes containing 8 sterile 13mm diameter glass coverslips at 4ml per dish. 6h later the medium was removed and replaced with 4ml serum-free EFC10. 18h thereafter this was replaced with 4ml EFC10 supplemented with 10% foetal calf serum. The resulting cultures consisted of > 95% cells with dendritic morphology which could be identified as melanocytes by dihydroxyphenylalanine (DOPA) staining (see section 2.2.8).

2.2.5 Culture of Baby Mouse Kidney Epithelial Cells

Renal epithelial cells from baby mouse were prepared by the method of Gilbert and Migeon (1975). 9-15 day old mice were killed by cervical dislocation, and the kidneys removed aseptically. 10-12 kidneys were finely minced using scissors, and stirred in 10ml 0.2% trypsin in PBS at 37°C for 15 min. The resulting suspension was pipetted vigorously, dissociating the kidney tissue into individual tubules. The suspension was pelleted by centrifugation at 500 x g for 10 min and resuspended in 10ml D-valine culture medium. This consisted of EFC10 in which L-valine (normal concentration 46 $mg.ml^{-1}$) had been replaced by D-valine (92 $mg.ml^{-1}$), supplemented with 10% foetal calf serum which had been dialysed against 3 changes of 100 volumes of 0.9% NaCl at 4°C over 24h. Contaminating renal fibroblasts, which lack the racemase to interconvert the two isomers (Gilbert and Migeon, 1975), fail to grow in this selective medium. Cultures were established at approximately one kidney per 50mm plastic petri dish in 4ml EFC10, and grown at 37°C in an atmosphere of 5% CO_2, 95% air.

2.2.6 Growth of Newborn Mouse Skin in Organ Culture

Dorsal skin from 1-3 day old mice attached to strips of adhesive tape (Lasso tape) was incubated in 0.2% trypsin in PBS at 37°C for 1h (6 skin pieces per 10ml trypsin solution), as for the preparation of skin cell cultures (see section 2.2.2). The skin was then placed in a petri dish containing sterile PBS and the dermis pulled off using forceps. The epidermis was removed intact from the Lasso tape strip by gentle scraping with a scalpel blade, and both tissues were then floated onto sterile Millipore filters (25mm diameter, 25μm thickness, 0.8μm mean particle retention size). Millipore filters were prepared by brief (1-2min) immersion in 10-14% sodium hypochlorite, then washed 3 times with distilled water and autoclaved. 5 types of organ culture were established : (i) epidermis, (ii) dermis, (iii) cisfilter dermal-epidermal recombinant, (iv) transfilter dermal-epidermal recombinant, (v) whole untrypsinised skin. In the case of the cisfilter and transfilter recombinants, where epidermis and dermis were mounted on the same or opposite sides of one Millipore filter, the dermis was floated on first and allowed to attach for 2-3 min before the filter was reimmersed and the epidermis mounted. Filters were immersed in 4ml EFC10 in 50mm plastic petri dishes, and cultured at 37°C in an atmosphere of 5% CO_2, 95% air.

2.2.7 Establishment of Keratinocyte Cultures on Feeder Layers

Cultures of epidermal keratinocytes on cell line feeder layers were established by a modification of the technique of Rheinwald and Green (1975a). BHK21/C13 fibroblasts,

BRL epithelial cells and L929/A9 cells were set up at 5×10^6 per 90mm petri dish in 10ml EFC10 and incubated for 24h at 37°C. The cells were then incubated in 10ml EFC10 containing 10^{-6}M mitomycin C for 18h, and washed 3 times with sterile BSS. The cells were trypsinised and mixed with freshly-prepared newborn mouse skin keratinocytes in the proportion 1:5 cell line cells : keratinocytes to give a total cell number of 1.2×10^6 per 50mm dish. Feeder layer cultures were grown in EFC10 containing $0.4\mu g.ml^{-1}$ hydrocortisone at 37°C in an atmosphere of 5% CO_2, 95% air.

2.2.8 DOPA Staining

Melanocytes possess the enzyme system to convert L-β(3,4) dihydroxyphenylalanine (DOPA) to the pigment melanin, and may therefore be readily stained in culture by incubation in DOPA solution (Riley, 1970). Cultures of melanocytes on glass coverslips in 50mm petri dishes were fixed by immersion in formal saline at 4°C for 1h. The cells were then washed with distilled water, and incubated in 4ml 0.1% DOPA in 0.1M phosphate buffer pH 7.0 at 37°C for 2h. Fresh DOPA solution was then added, and the melanocytes incubated for a further 2h period. The coverslips were then washed 3 times with distilled water, air-dried, and mounted on glass microscope slides using Depex. The slides were examined using a Leitz Orthomat microscope fitted with plan optics. Control cultures of epidermal keratinocytes exhibited no DOPA-positive reaction.

2.2.9 Analysis of Cell-Cell Communication

The formation of gap junctions between cultured cells in contact was detected by the intercellular transfer of tritiated uridine nucleotides. Donor cells at approximately 5×10^5 per 50mm dish were labelled by incubation in 4ml EFC10 containing $1\mu Ci.ml^{-1}$ ^{3}H-uridine for 3h. Unincorporated uridine was then removed by washing 3 times with sterile BSS. The donor cells were then suspended by trypsin/EDTA treatment (see section 2.2.1), mixed with unlabelled recipient cells in the ratio 1:4 donors:recipients, and a total of 2×10^5 cells plated out in 35mm plastic petri dishes with 3 sterile coverslips, in 2ml EFC10 containing 10^{-3}M nonradioactive uridine. After coculture for 3h the medium was removed and the cells fixed by addition of 2ml formal saline and left at 4^oC for 1h. Acid-soluble nucleotides were removed by washing the coverslips twice in ice-cold 5% TCA, twice in distilled water, and once in methylated spirits. After drying, the coverslips were mounted on glass microscope slides with Depex.

For autoradiography, slides were dipped in a 1:3 dilution of Ilford L4 nuclear research emulsion in distilled water, using a Kodak No. 1 safelight with a 25W bulb, and dried under a stream of cold air. The autoradiographs were stored at room temperature in the dark for 48h, then developed for 5 min with D19b at 20^oC, and fixed for 5 min with a 1:5 dilution of Amfix. After washing for 2 min with tap water, the slides were st ained using a 1:20 dilution of Giemsa (7.5% w/v in 50% glycerol, 50% methanol) for 1.5 min. Further coverslips were subsequently mounted on top with Depex.

Autoradiographs intended for photography rather than grain counting were labelled with 10μCi.ml^{-1} ^{3}H-uridine for 3h, and developed after 3 weeks exposure. Photographs were taken with a Leitz Orthomat photomicroscope using Ilford Pan F film at an exposure rating of 200 ASA. Films were developed in Ilford Microphen developer at 20°C for 5.5 min, and fixed using a 1:3 dilution of Amfix at 20°C for 5 min. The films were rinsed overnight with tap water.

2.2.10 Quantitation of Nucleotide Transfer

Developed autoradiographs show heavily-labelled donor cells, in which the distribution of autoradiographic grains is mainly cytoplasmic, due to the "chase" of nuclear RNA into longer-lived cytoplasmic forms during the coculture period. If cell-cell communication has occurred, recipient cells in contact with donors are lightly labelled over the cytoplasm with heavier labelling over the nucleus, particularly over the nucleoli. Recipient cells not in contact with donors have only the autoradiographic background level of grains.

Uridine nucleotide transfer was quantitated by statistical comparison of the mean grain count over recipient cells in contact with donors (contacting recipients) and the mean grain count over recipient cells not in contact with donors (non-contacting recipients). The autoradiographic grains over 50 contacting recipients and 50 non-contacting recipients were counted, and the mean and standard deviation of both populations calculated. The Student t-test was then applied in order to determine whether the two populations were significantly

different. A P value of < 0.05 (representing a 1 in 20 probability that the two populations are not significantly different) was defined as positive nucleotide transfer. Assessment of junction formation by plotting histograms of grain counts requires much larger numbers of cells to be counted (Burk et al, 1968). The statistical method described can result in significant P values using 10 or fewer cells in each population. 50 cells of each type were counted as this resulted in highly significant differences ($P < 0.001$) even using cells with low transfer efficiency (see Chapters 3 and 4).

$$\text{Mean } \bar{x} = \frac{\Sigma x_i}{n - 1}$$

$$\text{Standard deviation S.D.} = \frac{\Sigma(\bar{x} - x_i)^2}{n - 1}$$

$$\text{Significance t} = \frac{|\bar{x}_1 - \bar{x}_2|}{\sqrt{\frac{S_1^2}{n_1 - 1} + \frac{S_2^2}{n_2 - 1}}}$$

where x_i represents each sample value
n is the total number of samples
Degrees of freedom (d.f.) = $(n_1 + n_2) - 2$
P values derived from statistical tables.

2.2.11 "Pulse-Chase" Experiments

Donor cells labelled as above by incubation for 3h in 4ml EFC10 containing 1μCi.ml^{-1} ^{3}H-uridine were "chased" by incubation for a further 24h in 4ml nonradioactive medium. These cells were then washed 3 times with sterile BSS and cocultured with unlabelled recipient cells as before. Replicate donor cultures were used to determine the distribution of ^{3}H-uridine-labelled material in cellular acid-soluble and acid-insoluble pools and in the culture medium at 0,3 and 24h after the end of the labelling period. Acid-soluble (nucleotide) material was removed by extraction of the cell monolayer with 1ml ice-cold 5% TCA. After a further wash with 1ml ice-cold TCA, the cells were washed with 1ml distilled water, and the acid-insoluble (nucleic acid) material was extracted by incubation of the cells with 1ml 0.1M NaOH for 1h at room temperature. Extracts were acidified by addition of 0.2ml 1M HCl. The acid-soluble and acid-insoluble extracts and a 1ml aliquot of the medium were then mixed with 10ml Triton-toluene scintillation fluid and counted for 4 min in an Intertechnique SL30 liquid scintillation spectrometer.

2.2.12 Polyacrylamide Disc Gel Electrophoresis

SDS-polyacrylamide disc gel electrophoresis was performed according to the method of Weber and Osborn (1969), with the modification of Laemmli (1970). Gel tubes had inside diameter 6mm and length 100mm. The gels consisted of 2ml 7.5% acrylamide, 0.25% methylene bis-acrylamide in 0.375m Tris. HCl pH 8.8. A stacking gel of 0.2ml 3.0% acrylamide, 0.08% methylene bis-

acrylamide in 0.125M Tris. HCl pH 7.0 was also used. Protein samples were dissolved at approximately 1mg.ml^{-1} in 1% SDS, 1% 2-mercaptoethanol at 100°C for 5 min, and 100µl aliquots of protein solution added to 100µl glycerol and 10µl 0.05% bromophenol blue in water. After mixing, 100µl amounts were applied to the tops of gels, and electrophoresed at 4mA per tube for 3h. Gels were stained in 20ml 0.025% Coomassie Brilliant Blue in 10% acetic acid, 25% isopropanol for 16h, then in 20ml 0.0025% Coomassie Brilliant Blue in 10% acetic acid, 25% isopropanol for 8h, and destained in 3 changes of 20ml 7.5% acetic acid, 5% methanol for a total of 48h. Molecular weight marker proteins used were trypsin (23,000 molecular weight), alcohol dehydrogenase (37,000), ovalbumin (43,000), glutamate dehydrogenase (53,000), bovine serum albumin (68,000) and phosphorylase a (93,000).

2.2.13 Polyacrylamide Slab Gel Electrophoresis

SDS-polyacrylamide slab gel electrophoresis was performed by the method of Amos (1976). Polyacrylamide slabs were 80mm x 80mm x 1mm, comprising a running gel of 7.5% acrylamide, 0.25% methylene bis-acrylamide in 0.375M Tris. HCl pH 8.8 and a stacking gel of 3.0% acrylamide, 0.08% methylene bis-acrylamide in 0.125M Tris.HCl pH 7.0. 10µl samples (see section 6.3) were applied per slot and electrophoresed at 15mA per slab for 1.5h. Slab gels were stained and destained in the same way as disc gels (see section 2.2.12).

2.2.14 Fluorography

Fluorography (scintillation autoradiography) of ^{35}S-methionine labelled polyacrylamide gels was performed by the method of Bonner and Laskey (1974). Stained gels were soaked in 20 volumes of dimethyl sulphoxide for 30 min (twice), and then soaked in 20 volumes of 20% (w/w) PPO in dimethyl sulphoxide for 3h. The gels were then immersed in 20 volumes of distilled water for 1h, and freeze-dried. Kodak "X-Omat" X-ray film was placed in contact with the dried gels, and stored at -70°C in the dark for either 1 or 10 days. Fluorographs were developed for 5 min at 20°C in Kodak DX80 developer, fixed for 15 min at 20°C in Kodak FX40 X-ray fixer, and washed by immersion in distilled water for 24h.

RESULTS

"Now the parts of animals are differentiated by means of pneuma; but this is not the pneuma of the mother, nor that of the creature itself, as some of the physiologers allege."

Aristotle
The Generation of Animals

Chapter 3 - COMMUNICATION BETWEEN SKIN CELLS

3.1 Introduction

Junction-mediated intercellular communication may be involved in the interaction between dermis and epidermis in adult skin. As a first stage in examining this possibility, cultures of epidermal keratinocytes and dermal fibroblasts were established from newborn mouse skin and adult guinea pig skin, and their ability to communicate with each other and with cells of standard lines was examined.

3.2 Uridine Nucleotide Transfer Between Skin Cells

Gap junction formation by skin cells was analysed by assaying their ability to exchange radiolabelled uridine nucleotides. In this method, donor cells are prelabelled with ^{3}H-uridine. Washing these labelled cells removes ^{3}H-uridine, but not labelled uridine nucleotides or RNA. Donor cells cocultured with unlabelled recipient cells, either of the same or different type, may form gap junctions with the recipients and therefore transfer tritiated nucleotides. These are incorporated into recipient cell RNA, and may be detected by autoradiography after TCA-washing to remove acid-soluble nucleotides.

Intercellular transfer of radiolabelled molecules has previously been analysed qualitatively by presenting nomograms (Bürk *et al*, 1968), or by defining positive transfer in terms of an arbitrarily-selected autoradiographic background level (Gaunt and Subak-Sharpe, 1979). In this project, cell-cell exchange of uridine nucleotides has been quantitated by a statistical method. Recipient cells in contact with donor cells are labelled if permeable junctions are formed, whereas recipient cells not in contact with donors have only the background level of autoradiographic grains. This is caused by the autoradiographic emulsion background, and could also be due to uptake by isolated recipient cells of ^{3}H-uridine released by donor cells during coculture. The latter possibility has been lessened by coculture in medium containing a thousand-fold excess of non-radioactive uridine. The grain

counts in the two recipient cell populations have been compared by significance testing, and a statistically-significant difference defined as positive nucleotide transfer.

Determination of the nucleotide transfer properties of epidermal keratinocytes and dermal fibroblasts will relate to the hypothesis of tissue specificity in cell communication. It has previously been reported that certain junction-forming fibroblasts fail to communicate or communicate only poorly with junction-forming epithelial cells (Pitts and Burk, 1976; Fentiman et al, 1976). If this is a general phenomenon, such communication specificity may be responsible for the maintenance of the metabolic individuality of different tissue layers in vivo. It was therefore of interest to determine whether communication specificity was exhibited by primary cultures of fibroblastic and epithelial cells from skin. Epidermal keratinocytes and dermal fibroblasts were therefore tested in uridine nucleotide transfer experiments against one another and against two cell lines which exhibit communication specificity BHK21/C13 fibroblasts and BRL epithelial cells. The non-junction forming mouse embryo line L929/A9 was also used in control experiments, as lack of communication with A9 cells shows that observed nucleotide transfer is in fact junction-mediated.

Skin cell cultures were prepared by the method of Regnier et al, (1973). Full-thickness skin was split into dermis and epidermis by trypsinisation. Basal keratinocytes adhering to the dermis were liberated by agitation of the dermal tissue in culture medium. A minor modification of this

method gave an improved yield of epidermal cells, by similar agitation of the epidermal fragment in culture medium. Digestion of the dermis with collagenase and hyaluronidase releases dermal fibroblasts. Primary cultures of these cells were used in assays of cell-cell communication.

Cultures of epidermal keratinocytes and dermal fibroblasts were prepared from newborn mouse skin and adult guinea pig ear skin. The epidermal cell cultures consisted mainly of keratinocytes, identified by their epithelial morphology and their characteristic staining with the dye Rhodamine B (see section 5.6). Dermal cell cultures consisted principally of fibroblastic cells. Initially, a small minority of epithelial-type cells occurred in the dermal cultures, presumably being residual adhering keratinocytes or epithelial gland and follicle cells, but these appeared to be outgrown by the dermal fibroblasts on subculture.

The data shown in Table I, Figure 2 and Figure 3 show that epidermal keratinocytes and dermal fibroblasts from newborn mouse skin exhibit both homologous and heterologous cell communication. This is demonstrated by the large differences in mean grain count per cell between contacting recipient cells and non-contacting recipient cells (Table I). The significance of this difference is shown by the low values of P, which represents the probability of the null hypothesis (that the two populations are not significantly different) being correct. The sample values expressed as means and standard deviations in Table I are plotted on frequency distribution histograms in class intervals of 5

in Figure 2. The difference between the two populations is here shown by the small degree of overlap between the contacting recipient and non-contacting recipient distributions. Epidermal keratinocytes and dermal fibroblasts from adult guinea pig ear skin also exhibit homologous and heterologous communication (Table II).

The transfer of uridine nucleotides between epidermal keratinocytes and dermal fibroblasts from mouse and guinea pig skin indicates that, unlike the non-communicating fibroblast-epithelial cell cocultures previously reported (Pitts and Burk, 1976; Fentiman et al, 1976), no tissue specificity is observed in this system.

Table I. Uridine Nucleotide Transfer Between Epidermal Keratinocytes and Dermal Fibroblasts from Newborn Mouse Skin.

Donor cell cultures were labelled with ^{3}H-uridine ($1\mu Ci.ml^{-1}$) for 3h, then washed 3 times with sterile BSS. The cells were suspended in EFC10 after treatment with trypsin : EDTA, and mixed with suspensions of unlabelled recipient cells in a donor : recipient ratio of 1:4. Cell mixtures were plated out at 2×10^{5} per dish in 35mm plastic petri dishes containing 3 sterile glass coverslips in 4.0ml of EFC10 medium containing 10^{-3}m nonradioactive uridine. After 3h coculture the medium was removed and the cells were fixed with 2ml formal saline at 4^{o}C for 1h. Acid-soluble material was removed by washing the coverslips for 5 min in ice-cold 5% TCA twice, then for 5 min in distilled water twice, and then dipped in methylated spirit. The dried coverslips were mounted on glass microscope slides using DePeX (Searle) and processed for autoradiography. After 48h autoradiographs were developed, fixed and stained with Giemsa. Further coverslips were mounted on top with DePeX.

Autoradiographic grains were counted over 50 recipient cells in contact with donor cells and 50 isolated recipient cells. The means and standard deviations of the grain counts for the two populations were used to calculate t-values using the Student t-test, and the values of P derived from statistical tables.

EK - Epidermal keratinocytes
DF - Dermal fibroblasts
C.R. - Recipient cells in contact with donor cells
N.C.R. - Recipient cells not in contact with donor cells
d.f. - Degrees of freedom.

Donor	Recipient	C.R.	N.C.R.	t	d.f.	P
		Grains/cell (Mean ± S.D.)	Grains/cell (Mean ± S.D.)			
EK	EK	21.8 ± 9.90	4.92 ± 2.22	11.6	98	< 0.001
EK	DF	18.6 ± 5.62	3.76 ± 1.90	17.5	98	< 0.001
DF	DF	20.6 ± 6.01	3.70 ± 1.96	18.7	98	< 0.001
DF	EK	27.4 ± 9.48	4.50 ± 2.21	16.5	98	< 0.001

Figure 2 Uridine Nucleotide Transfer Between Newborn Mouse Skin Cells - Frequency Distribution Histograms of Recipient Cell Grain Counts

The data expressed in Table 1 as mean $\pm$ S.D. are here plotted as histograms of number of cells against number of autoradiographic grains per cell in class intervals of 5, for both recipient cells in contact with donor cells (contacting recipients ; C.R.) and recipients cells not in contact with donor cells (noncontacting recipients ; N.C.R.). Arrows represent mean values.

(a) Donors : Epidermal keratinocytes ; Recipients : Epidermal keratinocytes.

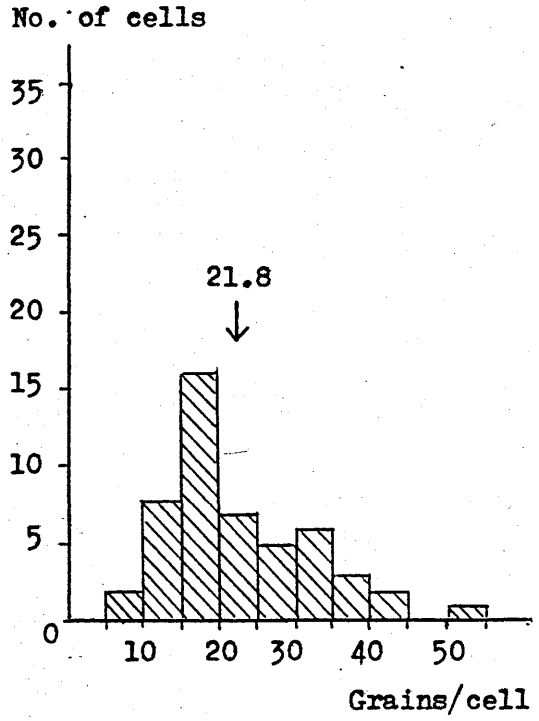
(b) Donors : Epidermal keratinocytes ; Recipients : Dermal fibroblasts.

(c) Donors : Dermal fibroblasts ; Recipients : Dermal fibroblasts.

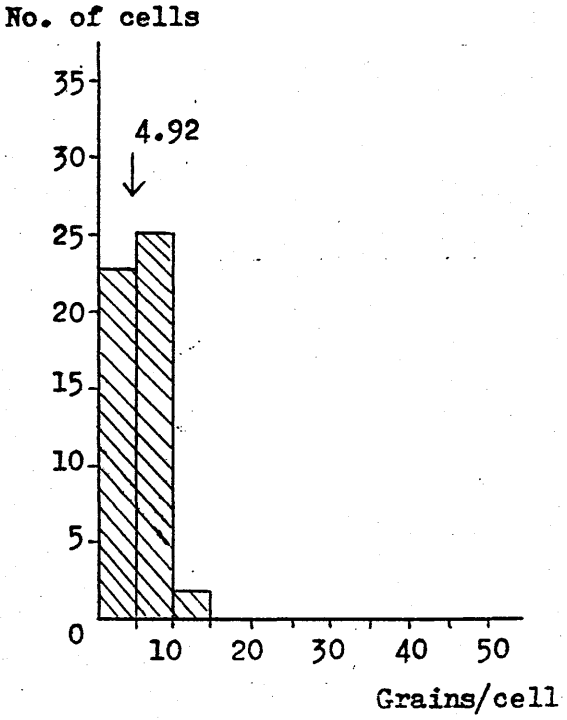
(d) Donors : Dermal fibroblasts ; Recipients : Epidermal keratinocytes.

(a). EK-EK

Contacting Recipients

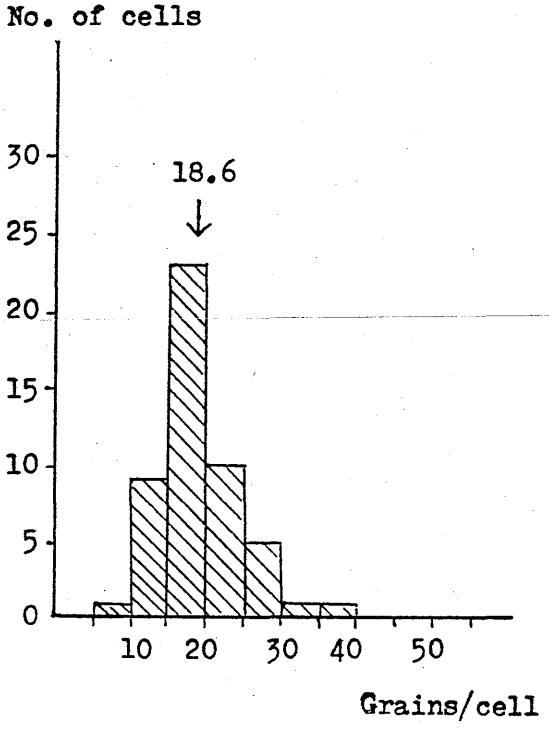


Non-Contacting Recipients

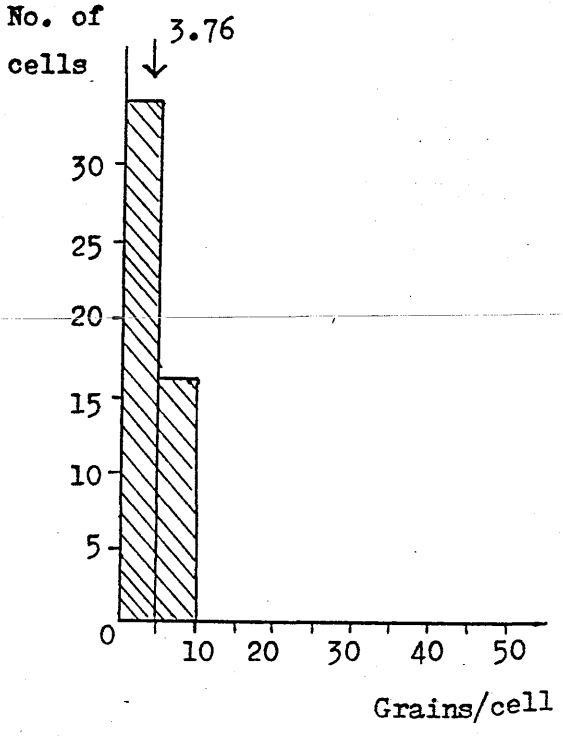


(b). EK-DF

Contacting Recipients

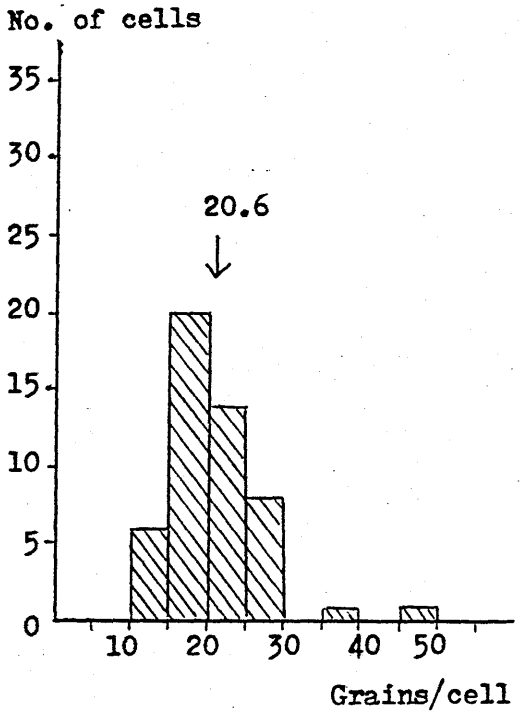


Non-Contacting Recipients

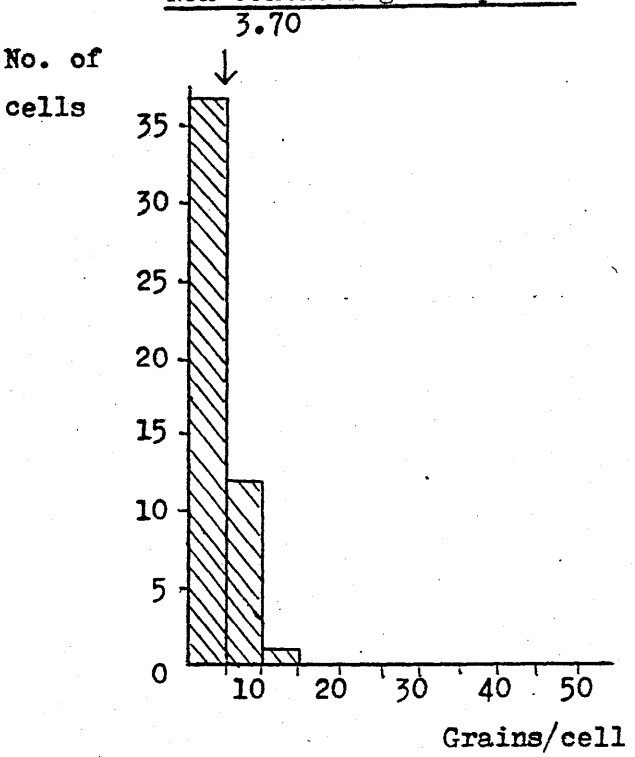


(c). DF-DF

Contacting Recipients

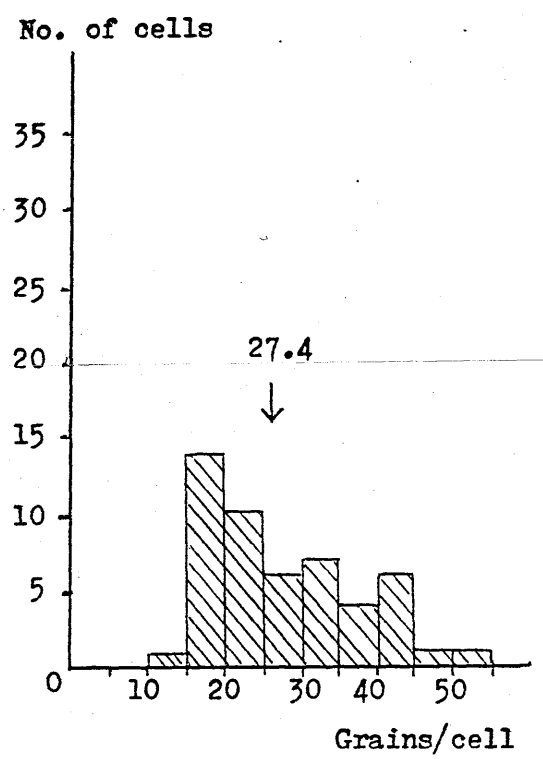


Non-Contacting Recipients



(d). DF-EK

Contacting Recipients



Non-Contacting Recipients

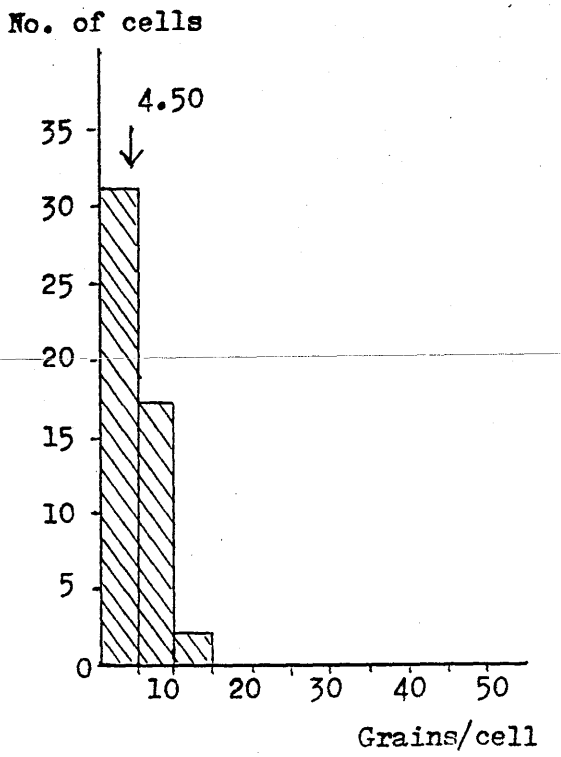


Figure 3 Uridine Nucleotide Transfer Between Dermal Fibroblasts and Epidermal Keratinocytes from Newborn Mouse Skin

Experimental details were as described in the legend to Table I, except that the donor cell cultures were labelled with ^{3}H-uridine at a concentration of 10μCi.ml^{-1} for 3h, and the autoradiographs were exposed for 3 weeks before developing.

(a). Donors : Dermal fibroblasts

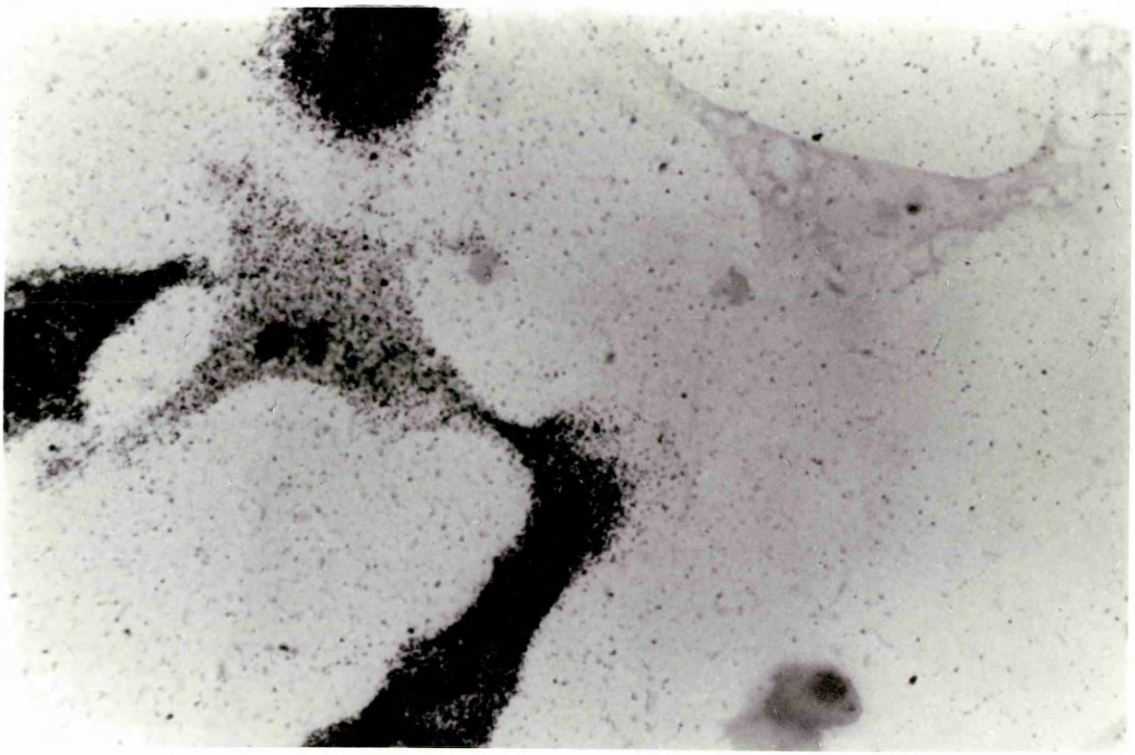
Recipients : Epidermal keratinocytes

(b). Donors : Dermal fibroblasts

Recipients : Dermal fibroblasts

(x1460)

(a).



(x1160)

(b).

Table II. Uridine Nucleotide Transfer Between Epidermal Keratinocytes and Dermal Fibroblasts from Guinea Pig Ear Skin

For experimental details, see the legend to Table I.

Donor	Recipient	C.R. Grains/cell (Mean ± S.D.)	N.C.R. Grains/cell (Mean ± S.D.)	t	d.f.	P
EK	EK	30.3 ± 10.0	3.40 ± 1.70	18.6	98	< 0.001
EK	DF	42.7 ± 12.3	3.28 ± 2.25	21.7	98	< 0.001
DF	DF	35.1 ± 9.22	2.60 ± 1.83	24.2	98	< 0.001
DF	EK	36.3 ± 10.7	2.24 ± 1.49	21.3	98	< 0.001

3.3 Lack of RNA Transfer Between "Pulse-Chased" Skin Cells

To confirm that the ^{3}H-uridine-labelled material transferred between epidermal keratinocytes and dermal fibroblasts was in the form of uridine nucleotides and not RNA, communication experiments were performed using "pulse-chased" donor cells. Donor cultures were labelled with ^{3}H-uridine for 3h, then incubated in non-radioactive medium for 24h and washed before coculture with recipients. The incorporation of ^{3}H-uridine into donor cell RNA was measured by determination of the radioactivity in cellular acid-soluble and acid-insoluble pools. If the ^{3}H-uridine transferred represents nucleotides, the fall in the donor cell acid-soluble radioactivity fraction should correlate with a decrease in transfer of radioactivity to contacting recipient cells.

^{3}H-uridine labelled epidermal keratinocytes and dermal fibroblasts incubated for 24h in non-radioactive medium show increased incorporation of ^{3}H-uridine into RNA (Table III). For epidermal keratinocytes, the acid-soluble (nucleotide) pool decreases from 37% of total cell counts immediately after the labelling period to 5% of total cell counts after 24h. This is accompanied by an increase in the acid-insoluble (nucleic acid) pool (from 61% to 78%) and by loss of ^{3}H-uridine into the culture medium (1.4% to 17%). Dermal fibroblasts appear to synthesise RNA faster than keratinocytes. The fibroblast acid-soluble pool falls from 61% to 7.5% over 24h, with concommitant increase in the acid-insoluble pool (38% to 77%) and the radioactivity

released into the medium (0.9% to 15.5%). Because of the relatively large percentage of ^{3}H-uridine released into the culture medium, pulse-chased donor cells were washed three times with sterile BSS prior to coculture with recipients.

Pulse-chased skin cells used as donors in uridine nucleotide transfer experiments show a decrease in transfer efficiency (Table IV). For example, the acid-soluble pool in epidermal keratinocytes at 24h falls to 12% of the zero time value (33075 to 3942 cpm). Pulse-chased keratinocyte donors in the EK-EK experiment (Table IV) show a decrease in recipient cell grain count to 6% of the unchased level (Table I) [recipient cell grain count minus background : from 16.9 grains per cell at 0h to 1.02 at 24h]. The factor of two difference between the fall in acid-soluble pool and recipient cell grain count is due to the fact that distribution of radioactivity was calculated on a culture basis whereas the nucleotide transfer was calculated on a cell basis, and the donor cells will have undergone mitotic division during the 24h chase period.

The smaller level of nucleotide transfer observed using pulse-chased donor cells is statistically significant (Table IV). This shows that the statistical method used is capable of detecting much smaller differences than those in the experiments to which it is normally applied. As significant differences in recipient cell grain counts are not observed when non-junction forming A9 cells are used (for example see below, Table V), this confirms the suitability of the statistical method.

Table III. Distribution of Radioactivity Between Medium and Cellular Acid-Soluble and Acid-Insoluble Pools After Labelling of Newborn Mouse Skin Cells with ^{3}H-Uridine

Cultures of epidermal keratinocytes and dermal fibroblasts from newborn mouse skin were established at 2 x 10^{5} cells per 35mm dish in 2ml EFC10 and grown at 37°C for 5 days. The cells were labelled with ^{3}H-uridine (1µCi.ml^{-1}) for 3h, then washed 3 times with sterile BSS and incubated in EFC10 containing non-radioactive uridine (10^{-3}M). At 0,3 and 24h thereafter, the radioactivity in the medium and in the acid-soluble and acid-insoluble cellular fractions was measured for 4 replicate cultures of each cell type.

The numbers are total cpm in each fraction (mean of 4 determinations). Numbers in parenthesis are percentage of total cpm at each time point.

(a) Epidermal Keratinocytes

Fraction :	Total cpm/fraction		
	0h	3h	24h
Medium	1205(1.4)	7795(8.3)	13000(16.9
Cellular acid-soluble	33075(37.4)	15775(16.7)	3942(5.1)
Cellular acid-insoluble	54150(61.2)	70150(75.0)	59650(78.0

(b) Dermal Fibroblasts

Fraction :	Total cpm/fraction		
	0h	3h	24h
Medium	890(0.9)	5650(5.7)	11045(15.5
Cellular acid-soluble	58075(61.0)	39500(39.7)	5305(7.5)
Cellular acid-insoluble	53950(38.1)	54300(54.6)	54725(77.0

Table IV Uridine Nucleotide Transfer Between "Pulse-Chased" Newborn Mouse Skin Cells

Donor cell cultures of newborn mouse epidermal keratinocytes and dermal fibroblasts were established as described in the legend to Table III, and labelled with ^{3}H-uridine ($1\mu Ci.ml^{-1}$) for 3h. After washing 3 times with BSS, the cultures were incubated in medium containing nonradioactive uridine ($10^{-3}M$) for 24h. The pulse-chased donor cells were then suspended in EFC10 after trypsin : EDTA treatment, and mixed with suspensions of unlabelled recipient cells in a donor recipient ratio of 1:4. Cell mixtures were plated out at 2×10^{5} per 35mm dish and cultured for 3 h. Fixation, extraction and autoradiography were performed as described in the legend to Table I.

Donor	Recipient	C.R. Grains/cell (Mean ± S.D.)	N.C.R. Grains/cell (Mean ± S.D.)	t	d.f.	P
EK	EK	2.31 ± 1.54	1.29 ± 1.44	3.32	98	< 0.01
EK	DF	1.92 ± 1.35	1.10 ± 1.05	3.29	98	< 0.01
DF	DF	2.25 ± 1.36	1.08 ± 0.88	4.95	98	< 0.001
DF	EK	2.48 ± 1.40	1.15 ± 1.08	5.16	98	< 0.001

3.4 Uridine Nucleotide Transfer Between Epidermal Keratinocytes and Cells of Established Cell Lines

Primary epidermal keratinocytes from newborn mouse skin were tested for gap junction formation by measuring uridine nucleotide transfer with the cell lines C13, BRL and A9. The results are shown in Table V. These data are consistent with the previously-reported communication properties of the three cell lines used. A9 cells do not communicate with any cell type tested, either as donors or recipients. C13 and BRL communicate in homologous coculture, but communicate with low efficiency in heterologous coculture. Thus, whereas the C13-C13 and BRL-BRL experiments show recipient cell grain counts averaging at 30.5 and 18.8 grains per cell, respectively, the C13-BRL and BRL-C13 experiments resulted in recipient counts of only 6.26 and 5.74 grains per cell. The distribution of C13-BRL recipient cell grain counts has been reported to be bimodal, with approximately 95% of recipient cells having only background levels of grains, and the remainder exhibiting high levels of grains similar to that of homologous C13-C13 and BRL-BRL cocultures (Pitts and Burk, 1976). This explains the high standard deviation values for the C13-BRL and BRL-C13 experiments in Table V. The Student t-test is strictly not applicable, as it is based on normal distributions of sample values, but when calculated results in a significant value of P (only at the 0.01 level for the BRL-C13 experiment) indicating that the small fraction of C13-BRL contacts which do result in junction formation is sufficient to be detected by the statistical method used. A high standard deviation

may therefore be diagnostic of a distribution that is not unimodal.

Epidermal keratinocytes communicate with C13 and BRL cells, but not with A9. The amount of nucleotide transfer to C13 cells appears to be greater than that to BRL cells, possibly implying more efficient communication. This does not appear to be due to a bimodal distribution of recipient cell grain counts, as the standard deviation values fall in the normal range. However, variations in cell size and rate of uridine incorporation into nucleic acid will also result in differences in recipient cell grain count.

Epidermal keratinocytes from guinea pig ear skin also communicate with both C13 and BRL cells (Table VI). The heterologous communication between keratinocytes and C13 fibroblasts indicates that epidermal keratinocytes show no tissue specificity of communication.

Table V. Uridine Nucleotide Transfer Between Newborn Mouse Epidermal Keratinocytes and Cells of Established Cell Lines C13, BRL and A9.

The cell lines used were BHK21/C13 (baby hamster kidney fibroblast), BRL (Buffalo rat liver epithelial cell), and L929/A9 (mouse embryo cell). For experimental details, see the legend to Table I.

N.S. - Not significant

Donor	Recipient	C.R. Grains/cell (Mean ± S.D.)	N.C.R. Grains/cell (Mean ± S.D.)	t	d.f.	P
EK	EK	18.8 ± 5.54	3.55 ± 1.80	11.4	98	< 0.001
C13	EK	32.0 ± 8.55	4.28 ± 1.94	22.2	98	< 0.001
BRL	EK	11.1 ± 6.74	3.98 ± 2.32	6.99	98	< 0.001
A9	EK	6.10 ± 2.75	6.60 ± 3.15	0.84	98	N.S.
EK	C13	15.3 ± 6.09	2.96 ± 2.04	13.4	98	< 0.001
C13	C13	30.5 ± 8.33	3.02 ± 1.82	22.6	98	< 0.001
BRL	C13	5.74 ± 3.54	3.78 ± 2.34	3.23	98	< 0.01
A9	C13	5.08 ± 2.86	4.64 ± 2.38	0.83	98	N.S.
EK	BRL	10.9 ± 4.69	3.34 ± 1.80	10.5	98	< 0.001
C13	BRL	6.26 ± 4.12	3.48 ± 1.70	4.36	98	< 0.001
BRL	BRL	18.8 ± 5.15	2.74 ± 1.59	20.9	98	< 0.001
A9	BRL	4.04 ± 1.93	4.14 ± 1.90	0.26	98	N.S.
EK	A9	1.88 ± 1.57	1.68 ± 1.36	0.67	98	N.S.
C13	A9	1.88 ± 1.23	1.62 ± 1.44	0.96	98	N.S.
BRL	A9	1.74 ± 1.18	2.00 ± 1.30	1.04	98	N.S.
A9	A9	2.06 ± 1.16	1.78 ± 1.22	1.16	98	N.S.

Table VI. Uridine Nucleotide Transfer Between Guinea Pig Epidermal Keratinocytes and Cells of Established Cell Lines C13 and BRL.

For experimental details, see the legend to Table I.

Donor	Recipient	C.R. Grains/cell (Mean ± S.D.)	N.C.R. Grains/cell (Mean ± S.D.)	t	d.f.	P
EK	EK	45.9 ± 13.0	5.76 ± 3.06	21.0	98	< 0.001
C13	EK	25.7 ± 8.58	4.04 ± 2.41	16.4	73	< 0.001
BRL	EK	29.9 ± 8.51	3.40 ± 1.50	21.1	73	< 0.001
EK	C13	37.3 ± 12.2	7.08 ± 3.67	15.9	73	< 0.001
EK	BRL	14.8 ± 6.27	4.68 ± 2.66	9.66	73	< 0.001

3.5 Uridine Nucleotide Transfer Between Dermal Fibroblasts and Cells of Established Cell Lines

Primary dermal fibroblasts from newborn mouse skin were tested in uridine nucleotide transfer experiments against cell lines C13, BRL and A9. The results are shown in Table VII. Dermal fibroblasts communicate with C13 fibroblasts and BRL epithelial cells, but not with A9 cells. Dermal fibroblasts from guinea pig ear skin show similar cell communication properties (Table VIII). These results are qualitatively similar to those obtained with epidermal keratinocytes (see section 3.4). Again, an apparently lower level of communication was observed between dermal fibroblasts (DF) and BRL than between DF and C13. This level of nucleotide transfer is statistically significant ($P < 0.001$). Dermal fibroblasts, therefore, exhibit no communication specificity with the cell types tested.

Table VII. Uridine Nucleotide Transfer Between Newborn Mouse Dermal Fibroblasts and Cells of Established Cell Lines C13, BRL and A9.

For experimental details, see the legend to Table I.

Donor	Recipient	C.R. Grains/cell (Mean ± S.D.)	N.C.R. Grains/cell (Mean ± S.D.)	t	d.f.	P
DF	DF	36.3 ± 10.5	3.64 ± 1.99	21.4	98	< 0.001
C13	DF	41.5 ± 16.6	3.64 ± 2.19	15.8	98	< 0.001
BRL	DF	12.9 ± 9.99	5.08 ± 2.73	5.28	98	< 0.001
A9	DF	5.16 ± 2.49	4.76 ± 2.63	0.77	98	N.S.
DF	C13	40.7 ± 15.7	3.25 ± 1.70	16.8	68	< 0.001
DF	BRL	8.86 ± 6.42	3.68 ± 2.20	5.34	98	< 0.001
DF	A9	1.74 ± 1.15	1.50 ± 1.42	0.92	98	N.S.

Table VIII. Uridine Nucleotide Transfer Between Guinea Pig Dermal Fibroblasts and Cells of Established Cell Lines C13, BRL and A9.

For experimental details, see the legend to Table I.

Donor	Recipient	C.R. Grains/cell (Mean ± S.D.)	N.C.R. Grains/cell (Mean ± S.D.)	t	d.f.	P
DF	DF	31.9 ± 9.90	3.08 ± 1.98	14.0	48	< 0.001
C13	DF	21.6 ± 8.72	4.10 ± 3.94	8.96	48	< 0.001
BRL	DF	17.9 ± 8.50	3.00 ± 2.63	8.39	48	< 0.001
A9	DF	3.32 ± 3.19	3.20 ± 1.83	0.16	48	N.S.
DF	C13	44.5 ± 13.5	2.64 ± 1.95	15.0	48	< 0.001
DF	BRL	20.0 ± 5.54	3.24 ± 2.27	13.7	48	< 0.001
DF	A9	1.84 ± 1.28	1.80 ± 1.02	0.12	48	N.S.

Chapter 4 - SPECIFICITY IN CELL COMMUNICATION

4.1 Introduction

As shown in the previous chapter, epidermal keratinocytes and dermal fibroblasts from newborn mouse skin and adult guinea pig skin form gap junctions with one another and with cells of both epithelial and fibroblastic cell lines. The lack of epithelial-fibroblastic specificity in skin contrasts with the deficiency of junction formation reported between the C13 and BRL cell lines (Pitts and Bürk, 1976) and between epithelial duct cells and fibroblasts from human mammary tissue (Fentiman *et al*, 1976). It is not known whether this communication specificity is of biological significance, due to the difficulty of measuring cell communication between different cell types *in vivo*. As an approximation to the *in vivo* situation, cell communication between different cells can be analysed in primary culture. In order to determine whether the absence of communication specificity was a general property of primary cells, an extensive range of cell types would have to be isolated *in vitro* and their communication properties determined. Because of the difficulty in developing techniques for the isolation and culture of different cell types, and because the main aim of this project was to examine cell interactions in skin, only two further primary cell types were examined. Renal epithelial cells, which can be readily cultured free of contaminating fibroblasts (Gilbert and Migeon, 1975), and epidermal melanocytes were tested for gap junction formation in culture. Melanocytes are of an embryological origin distinct from that of keratinocytes and dermal fibroblasts, migrate through the dermis to reach the

epidermis, and are known to interact with keratinocytes by the process of pigment donation. It was therefore of particular interest to determine whether melanocytes are capable of communication with other skin cell types.

Observations of epithelial-fibroblastic specificity have been made using cultured cell lines (Pitts and Burk, 1976; Gaunt and Subak-Sharpe, 1979), and it is possible that communication specificity may not occur *in vivo*. So, in addition to the study of primary kidney epithelial cells, two kidney epithelial cell lines were also investigated, as this may illuminate differences between the communication properties of normal cells *in vivo* and cells of established cell lines derived from the same tissue.

4.2 Uridine Nucleotide Transfer Between Renal Epithelial Cells and Cells of Established Cell Lines

Baby mouse kidney cells were prepared by trypsinisation of the kidney into individual tubules, and then allowing subsequent outgrowth of cells from these explants. Renal epithelial (RE) cells were prepared free of contaminating fibroblasts by growth in medium containing D-valine instead of the L isomer (Gilbert and Migeon, 1975). Attempts to obtain renal fibroblasts in pure culture were made using kidney cells grown in medium containing L-valine, as fibroblasts generally grow faster than epithelial cells and may be preferentially removed from the substratum on sub-culture. This was not achieved as these cultures could not be maintained for a long enough period of time to eliminate renal epithelial cells, due to fibroblast death within 2-3 passages. Renal epithelial cells were tested for formation of gap junctions with the cell lines C13 and BRL by assaying for the intercellular transfer of uridine nucleotides. Renal epithelial cells communicate homologously, and also with both the C13 and BRL cell lines (Table IX). The extent of nucleotide transfer in these experiments, as judged from contact recipient cell grain counts, is similar to that between dermal fibroblasts and epidermal keratinocytes (see Chapter 3). The standard deviations of the recipient cell populations are also similar to those obtained in experiments involving skin cells, where the distribution of grain counts was shown to be unimodal (Figure 2). Communication between renal epithelial cells and cells of the C13 and BRL cell lines does not therefore appear to

involve two populations of contacting-recipient cells. This was confirmed by examination of the distribution of grain count data. Positive junction formation between renal epithelial cells and C13 fibroblasts is shown in Figure 4. Thus, primary epithelial cells from baby mouse kidney also appear to communicate with cell lines with no epithelial-fibroblastic specificity.

Table IX. Uridine Nucleotide Transfer Between Renal Epithelial Cells and Cells of Established Cell Lines C13 and BRL

Cultures of epithelial cells from baby mouse kidney were established as described in section 2.2.5, and grown for 3 days. These cells were then tested for gap junction formation with cells of the C13 and BRL cell lines. For experimental details, see the legend to Table I.

RE - Renal epithelial cells

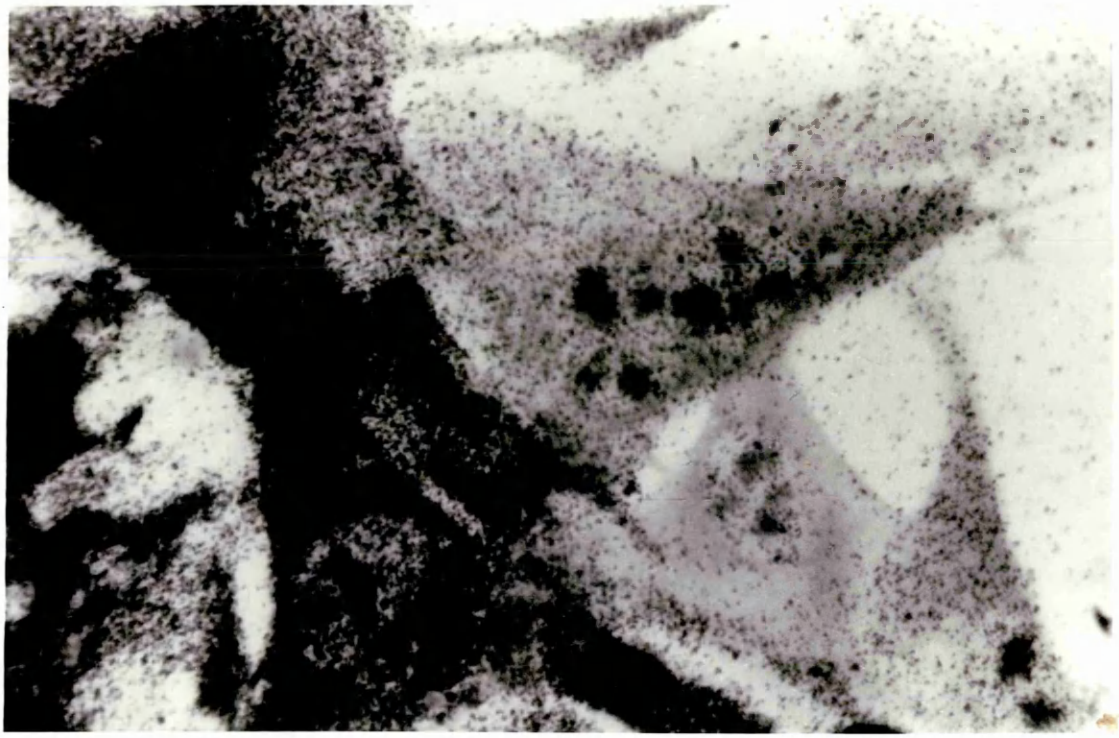
Donor	Recipient	C.R. Grains/cell (Mean ± S.D.)	N.C.R. Grains/cell (Mean ± S.D.)	t	d.f.	P
RE	RE	24.6 ± 5.85	4.42 ± 2.93	21.6	98	< 0.00
C13	RE	18.8 ± 7.09	4.86 ± 2.39	13.0	98	< 0.00
BRL	RE	16.7 ± 7.98	4.20 ± 2.22	10.6	98	< 0.00
RE	C13	24.9 ± 8.56	4.98 ± 2.38	15.7	98	< 0.00
RE	BRL	31.0 ± 8.14	3.24 ± 1.73	23.4	98	< 0.00

Figure 4 Uridine Nucleotide Transfer Between Primary Renal Epithelial Cells and Cells of the C13 Cell Line

Epithelial cells from baby mouse kidney were established as described in section 2.2.5. Experimental details were as described in the legend to Table I, except that donor cell cultures were labelled with $10\mu Ci.ml^{-1}$ ^{3}H-uridine, and autoradiographs were stored for 3 weeks before developing.

Donors : Renal epithelial cells

Recipients : C13 cells



(x1460)

4.3 Uridine Nucleotide Transfer Between Epidermal Melanocytes, Epidermal Keratinocytes, Dermal Fibroblasts, and Cells of Established Cell Lines

Melanocytes comprise the largest minor cell population in the epidermis, and occur as a contaminant of epidermal keratinocyte preparations. Melanocytes may be purified from epidermal cell preparations by suspension of pelleted cells in sodium citrate solution followed by a period of serum deprivation (Prunieras et al, 1976; Moreno et al, 1978; see section 2.2). The dendritic cells produced by this method were identified as melanocytes by DOPA staining (Figure 5). Cultures of melanocytes were tested against epidermal keratinocytes, dermal fibroblasts, and cells of cell lines C13, BRL and A9 for ability to exchange labelled uridine nucleotides. As melanocytes do not subculture successfully (Prunieras et al, 1976), these cells cannot be trypsinised for mixing with other cell types, and were therefore used only as donors in these experiments, unlabelled recipient cells being added to prelabelled melanocyte donor cultures. The results are given in Table X. Melanocytes communicate with both primary skin cell types, and also with C13 fibroblasts and BRL epithelial cells. No communication was detected between melanocytes and A9 cells. The grain counts in recipient cells in contact with melanocyte donors are higher than those recorded in experiments involving epidermal keratinocyte, dermal fibroblast, or renal epithelial cell donors. This may imply more efficient junction formation by melanocytes. However, the higher recipient cell grain

counts may alternatively be due to the fact that in these experiments, unlike previous experiments in which donors and recipients were suspended prior to coculture, the recipient cells were added to attached melanocyte cultures. As the melanocytes were already spread, the chances of recipients making contact with the donor cells may be greater. Recipient cell grain counts *per se* are not a direct measure of the efficiency of junction formation, as factors such as donor cell uptake and phosphorylation of uridine, rate of cell attachment and spreading, rate of nucleotide incorporation by recipient cells, and even cell size, will also be involved in determining the number of autoradiographic grains per recipient cell. The method used here for the quantitation of nucleotide transfer takes account of these differences by only comparing two similar cell populations, recipient cells in contact with donor cells and isolated recipients. This results in a degree of difference between the two populations which should be a reasonable indicator of the efficiency of communication.

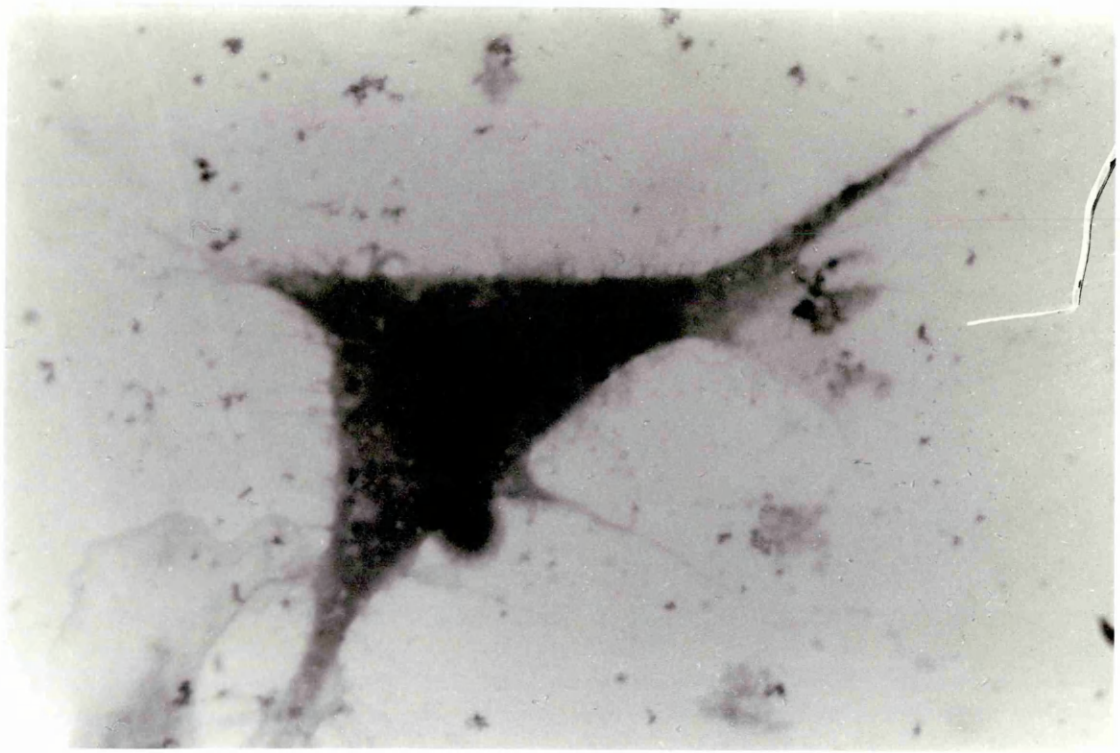
These results show that melanocytes, which are derived from embryonic neurectoderm, communicate with epithelial and fibroblastic cells of both cell line and primary culture origin. In common with epidermal keratinocytes, dermal fibroblasts and renal epithelial cells, melanocytes show no apparent specificity of communication. This suggests that non-specific cell communication is not an unusual property of cells in mammalian tissues.

Figure 5 DOPA Staining of Epidermal Melanocytes

Cultures of epidermal melanocytes from newborn mouse skin were established as described in section 2.2.4, and 2 days later were stained with dihydroxyphenylalanine as described in section 2.2.8.

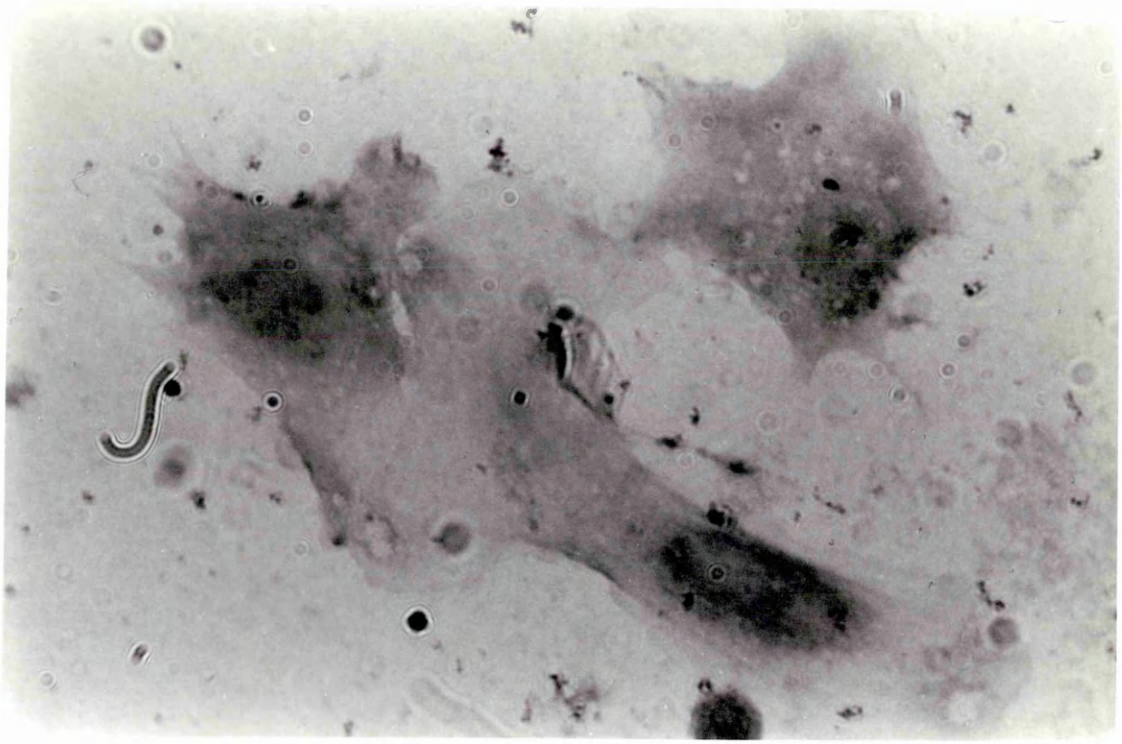
(a). Melanocyte

(b). Keratinocytes



(x1460)

(a).



(x1460)

(b).

Table X. Uridine Nucleotide Transfer Between Epidermal Melanocytes and Epidermal Keratinocytes, Dermal Fibroblasts, and Cells of Established Cell Lines C13, BRL and A9.

Cultures of melanocytes from newborn mouse skin were prepared as described in section 2.2, and incubated on sterile 13mm glass coverslips in 35mm petri dishes for 2 days. The cells were then tested for gap junction formation with epidermal keratinocytes and dermal fibroblasts from newborn mouse skin, and with cells of the cell lines C13, BRL and A9. Melanocyte donor cell cultures were labelled by incubation in EFC10 containing $1\mu Ci.ml^{-1}$ ^{3}H-uridine for 3h, then washed 3 times with BSS. Recipient cell suspensions were then added at 2×10^{5} cells per 35mm petri dish in 2ml EFC10 containing $10^{-3}M$ nonradioactive uridine, and cocultured at $37^{o}C$ for 3h. Fixation, TCA-extraction and autoradiography were performed as described in the legend to Table I.

Mela - Epidermal melanocytes

N.S. - Not significant

Donor	Recipient	C.R. Grains/cell (Mean ± S.D.)	N.C.R. Grains/cell (Mean ± S.D.)	t	d.f.	P
Mela	EK	50.8 ± 14.6	4.44 ± 2.17	21.9	98	< 0.001
Mela	DF	39.6 ± 14.4	4.20 ± 2.76	16.9	98	< 0.001
Mela	C13	46.0 ± 13.7	2.74 ± 1.82	21.9	98	< 0.001
Mela	BRL	39.3 ± 13.9	4.26 ± 2.49	17.4	98	< 0.001
Mela	A9	1.62 ± 1.09	1.44 ± 1.17	0.79	98	N.S.

4.4 Uridine Nucleotide Transfer Between Canine Kidney Epithelial Cell Lines and Cells of Established Cell Lines C13, BRL and A9

The cell communication properties of four primary cell types have been investigated, and no examples of communication specificity were found. It therefore appears possible that the lack of gap junction formation between fibroblasts and epithelial cells in culture may be a more general property of established cell lines. Gap junction formation by two kidney epithelial cell lines was studied, in an attempt to detect patterns of communication different from those of primary kidney cultures. The cultures used were two clones of the Madin-Darby canine kidney epithelial cell line MDCK (Rindler et al, 1979) passaged through 87 and 119 subcultures respectively. These cells were assayed for junction formation with one another and with the cell lines C13, BRL and A9.

The results are given in Table XI and Table XII. MDCK87 cells communicate with each other and with MDCK119 cells, with apparently equal efficiency. Neither MDCK87 or MDCK119 cells communicate with C13 fibroblasts or BRL epithelial cells, or with cells of the non-junction forming A9 cell line. The C13-C13, C13-BRL, BRL-BRL and BRL-C13 experiments all exhibit positive communication, although the interaction between C13 and BRL cells is again much less efficient than the homologous communication (see Table V). The grain counts of recipient cells in contact with donors in heterologous C13-BRL cocultures are characterised by

high standard deviation values, which are due to the non-unimodal distribution of sample values (see section 3.4). Lack of junction formation between MDCK and BRL cells is shown in Figure 6.

MDCK cells therefore exhibit communication specificity, as both clones fail to communicate with the junction-competent C13 and BRL cells, but this specificity is not dependent upon the tissue layer origin of the cells, as it occurs with both epithelial and fibroblastic cell lines. Communication between MDCK cells in characterised by recipient cell grain counts which are slightly lower than those obtained for mouse primary cell types (e.g. Table I). Similarly, in the experiment of Table XI the C13-C13 and BRL-BRL cocultures exhibit higher recipient cell grain counts than homologous MDCK cocultures. This may indicate that MDCK cells have a lower efficiency of junction formation, which could in part explain the lack of interaction between MDCK cells and cells of the C13 and BRL lines. On the basis of the experiments performed the possibility cannot be excluded that the two MDCK clones consist of identical cells.

These results indicate that, unlike primary cell types, which appear to communicate non-specifically, cells of established cell lines in some instances fail to form gap junctions with other junction-competent cells. The possible significance of these observations is discussed below (see section 7.4).

Table XI. Uridine Nucleotide Transfer Between Canine Kidney Epithelial Cell Line MDCK87 and Cell Lines C13, BRL and A9

For experimental details, see the legend to Table I.

MDCK87 - Madin-Darby canine kidney epithelial cells (passage 87)

MDCK119 - Madin-Darby canine kidney epithelial cells (passage 119)

Donor	Recipient	C.R. Grains/cell (Mean ± S.D.)	N.C.R. Grains/cell (Mean ± S.D.)	t	d.f.	P
MDCK87	MDCK87	16.8 ± 7.67	3.24 ± 1.92	12.0	98	< 0.001
MDCK119	MDCK87	15.1 ± 5.54	3.70 ± 1.68	13.7	98	< 0.001
C13	MDCK87	3.54 ± 2.09	3.84 ± 1.92	0.73	98	N.S.
BRL	MDCK87	4.60 ± 1.90	3.94 ± 1.63	0.93	68	N.S.
A9	MDCK87	3.18 ± 2.35	2.90 ± 2.40	0.58	98	N.S.
MDCK87	MDCK119	14.8 ± 6.69	3.36 ± 2.01	11.5	98	< 0.001
MDCK87	C13	4.32 ± 2.61	3.94 ± 2.15	0.79	98	N.S.
MDCK87	BRL	3.04 ± 1.68	2.88 ± 1.49	0.50	98	N.S.
MDCK87	A9	1.84 ± 1.30	2.00 ± 1.55	0.55	98	N.S.
C13	C13	30.6 ± 10.0	2.60 ± 1.85	19.3	98	< 0.001
BRL	C13	6.16 ± 5.02	2.82 ± 1.76	4.40	98	< 0.001
BRL	BRL	19.8 ± 7.64	3.10 ± 1.74	15.1	98	< 0.001
C13	BRL	6.14 ± 4.44	4.16 ± 2.19	2.80	98	< 0.01

Table XII. Uridine Nucleotide Transfer Between Canine Kidney Epithelial Cell Line MDCK119 and Cell Lines C13, BRL and A9

For experimental details, see the legend to Table I.

MDCK119 - Madin-Darby canine kidney epithelial cells (passage 119)

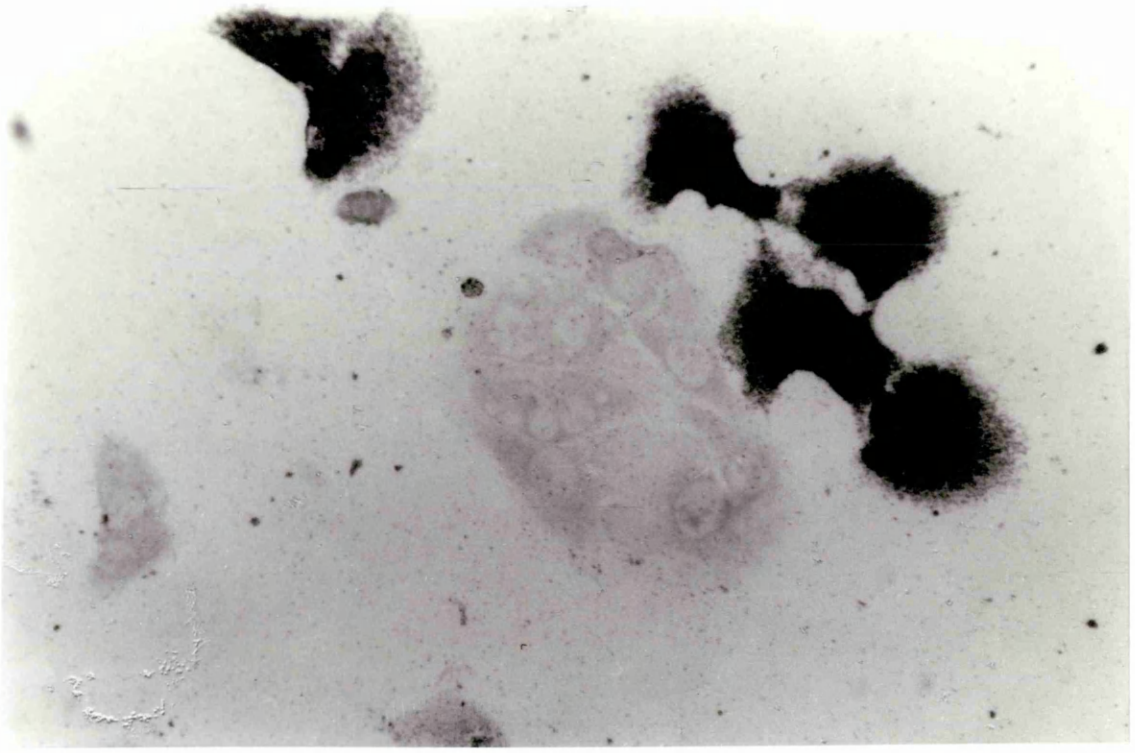
Donor	Recipient	C.R. Grains/cell (Mean ± S.D.)	N.C.R. Grains/cell (Mean ± S.D.)	t	d.f.	P
MDCK119	MDCK119	14.6 ± 4.68	3.04 ± 1.74	16.2	98	< 0.001
C13	MDCK119	3.65 ± 1.98	3.20 ± 1.82	0.73	38	N.S.
BRL	MDCK119	5.10 ± 2.73	4.90 ± 2.05	0.41	98	N.S.
A9	MDCK119	2.42 ± 1.84	2.36 ± 1.76	0.16	98	N.S.
MDCK119	C13	3.52 ± 1.88	3.58 ± 1.99	0.15	98	N.S.
MDCK119	BRL	3.82 ± 1.80	3.40 ± 1.67	1.20	98	N.S.
MDCK119	A9	1.64 ± 1.32	1.58 ± 1.26	0.23	98	N.S.

Figure 6 Uridine Nucleotide Transfer Between MDCK Cells and BRL Cells

Experimental details were as described in the legend to Table I, except that donor cell cultures were labelled with $10\mu Ci.ml^{-1}$ ^{3}H-uridine, and autoradiographs were stored for 3 weeks before developing.

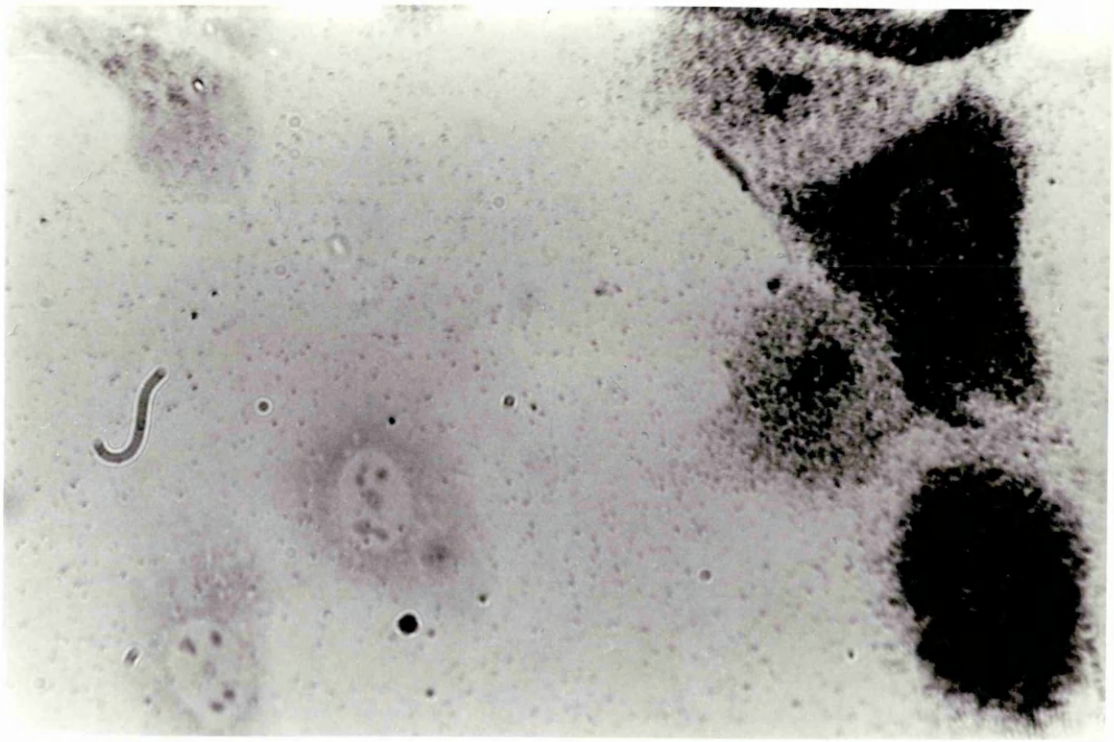
(a). Donors: BRL cells ; Recipients: MDCK130 cells

(b). Donors: MDCK130 cells ; Recipients: MDCK130 cells



(x455)

(a).



(x730)

(b).

4.5 Summary

The results of the uridine nucleotide transfer experiments described in chapters 3 and 4 are summarised in Table XIII. Positive cell communication has been defined as a difference between contacting recipient grain count and non-contacting recipient (background) grain count significant at the P = 0.05 level or lower. Note that there is no rectification; all the cell pairs tested give the same result in both possible combinations of donor and recipient.

These experiments involve only 4 primary cell types and 5 cell lines, and it would be of interest to extend this survey by studying gap junction formation by other primary cell cultures. However, it was decided instead to examine the interaction between dermal and epidermal cells from newborn mouse skin in greater detail, using organ culture and feeder layer culture systems.

Table XIII. Uridine Nucleotide Transfer Properties of Mouse Primary Cell Types and Established Cell Lines

This is a summary diagram of the data in Tables I, V, VII, IX, X, XI and XII. Positive nucleotide transfer is defined as $P \leqslant 0.05$ (i.e., 1 in 20 probability or less that the two populations are not significantly different). Negative nucleotide transfer is defined as $P > 0.05$.

EK - Epidermal keratinocyte

DF - Dermal fibroblast

Mela - Melanocyte

RE - Renal epithelial cell

MDCK87 - Madin-Darby canine kidney epithelial cell (passage 87)

MDCK119 - Madin-Darby canine kidney epithelial cell (passage 119)

C13 - Baby hampster kidney fibroblast line BHK21/C13

BRL - Buffalo rat liver epithelial cell line

A9 - Mouse embryo cell line L929/A9

N.D. - Not determined

		Recipient							
		EK	DF	RE	MDCK87	MDCK119	C13	BRL	A9
Donor	EK	+	+	N.D.	N.D.	N.D.	+	+	-
	DF	+	+	N.D.	N.D.	N.D.	+	+	-
	Mela	+	+	N.D.	N.D.	N.D.	+	+	-
	RE	N.D.	N.D.	+	N.D.	N.D.	+	+	N.D.
	MDCK87	N.D.	N.D.	N.D.	+	+	-	-	-
	MDCK119	N.D.	N.D.	N.D.	+	+	-	-	-
	C13	+	+	+	-	-	+	+	-
	BRL	+	+	+	-	-	+	+	-
	A9	-	-	N.D.	-	-	-	-	-

Chapter 5 - GROWTH AND DIFFERENTIATION OF EPIDERMAL KERATINOCYTES IN DISPERSED CELL CULTURE

5.1 Introduction

Formation of gap junctions has been shown to occur in culture between cells derived from the dermis and epidermis of newborn mouse skin and adult guinea pig skin (see Chapter 3). Junction-mediated intercellular communication therefore represents a possible mechanism of dermal-epidermal interaction *in vivo*, unless this form of communication is prevented by the intervening basal lamina (see below, section 7.3). In particular, dermal regulation of epidermal proliferation and differentiation (see Chapter 1) could involve formation of gap junctions between cells of the two tissue layers.

In order to establish a simple system for the study of epidermal keratinisation, criteria for growth and differentiation of epidermal cells were established and applied, firstly, to epidermal keratinocytes in dispersed cell culture. Previous studies by other workers indicated that dispersed cultures of epidermal cells are capable of at least limited differentiation (Constable *et al*, 1974; Fusenig, 1971; see section 1.4.1). After characterising the properties of keratinocytes in the absence of dermal cells, comparative studies were performed using keratinocytes growing on feeder layers, and subsequently with separated and recombined dermis and epidermis in organ culture.

The principal assay used for the detection of keratinocyte differentiation was based on the incorporation of radiolabelled amino acids into the epidermal structural protein prekeratin. To this end, extraction procedures were developed to isolate protein from newborn mouse skin and the polypeptides correspondin

to prekeratin were identified and quantitated. Epidermal differentiation was also measured by assay for the preferential uptake of histidine into the histidine-rich protein of epidermal cells. Cell proliferation was quantitated by following ^{3}H-thymidine incorporation into DNA.

5.2 SDS-Polyacrylamide Gel Electrophoresis Standard Curve

SDS-polyacrylamide gel electrophoresis of 6 proteins of known molecular weight gave the mobilities plotted in Figure 7. Linear regression analysis of these data using the least squares fit method indicated that the best-fitting straight line was described by the equation :

$$y = -0.403x + 5.01$$

where x = natural logarithm of polypeptide molecular weight

y = R_f (distance migrated by polypeptide relative to marker dye).

The correlation coefficient for this line was -0.996.

Figure 7. SDS-Polyacrylamide Gel Electrophoresis Standard Curve

Trypsin (23,000 molecular weight), alcohol dehydrogenase (37,000MW), ovalbumin (43,000MW), glutamate dehydrogenase (53,000MW), bovine serum albumin (68,000MW) and phosphorylase *a* (93,000MW) were each dissolved at a concentration of 0.1mg.ml^{-1} in 0.5ml 1% SDS, 1% (v/v) 2-mercaptoethanol by heating at 100°C for 5 min. 100µl aliquots of each protein solution were added to 100µl glycerol and 10µl bromophenol blue, mixed, and 100µl amounts of the mixtures electrophoresed for 3h at 4mA per tube on 7.5% polyacrylamide disc gels (see section 2.2.12). Gels were stained with Coomassie Brilliant Blue and destained in 7.5% acetic acid, 5% methanol.

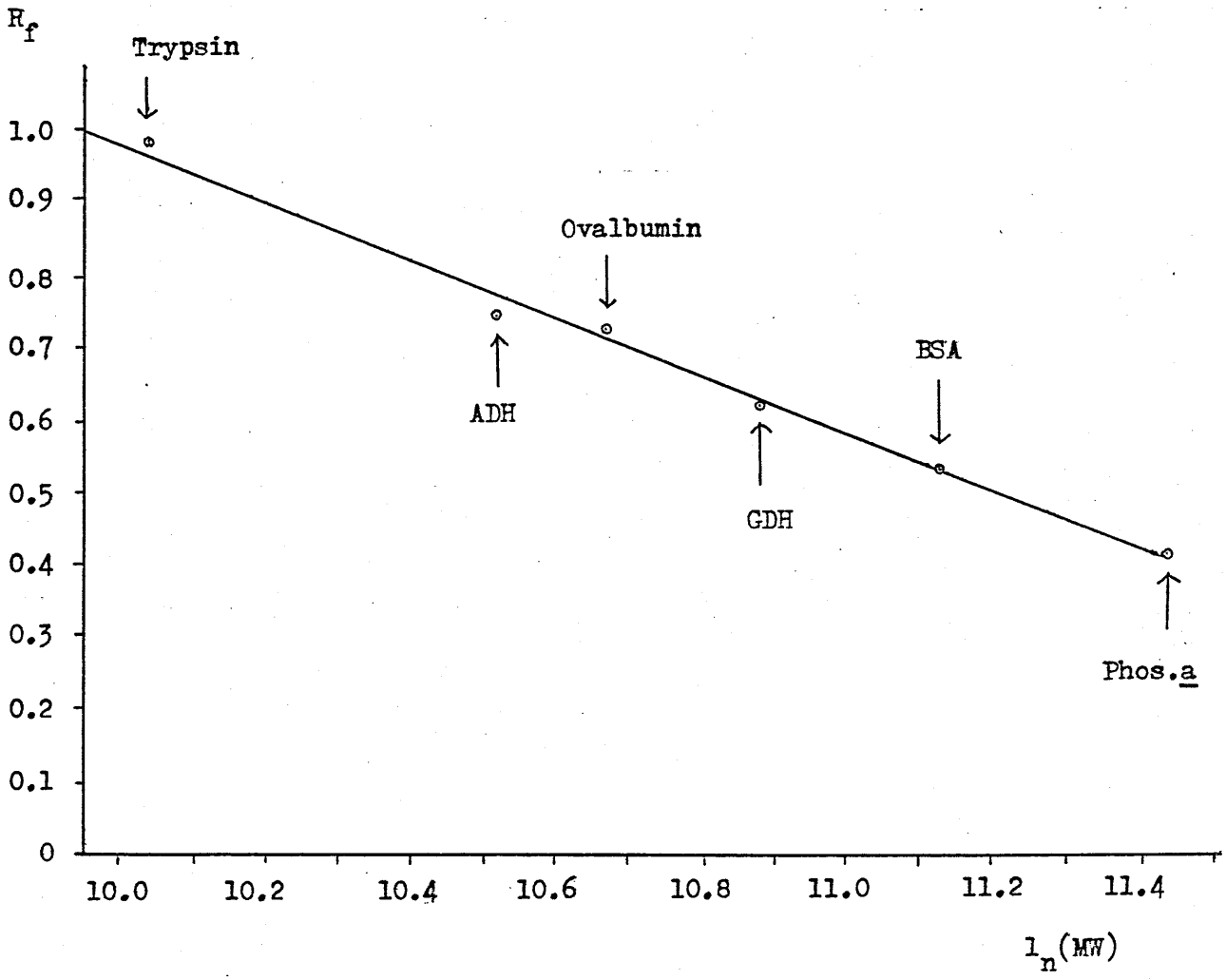
$$R_f = \frac{\text{Distance migrated by polypeptide}}{\text{Distance migrated by bromophenol blue}}$$

ADH - Alcohol dehydrogenase

GDH - Glutamate dehydrogenase

BSA - Bovine serum albumin

Phos. *a* - Phosphorylase *a*


R_f
Trypsin
1.0
0.9
Ovalbumin
0.8
0.7
BSA
ADH
0.6
0.5
GDH
0.4
0.3
Phos.a
0.2
0.1
0
10.0
10.2
10.4
10.6
10.8
11.0
11.2
11.4
l_n(MW)

5.3 Extraction of Epidermis with Citric Acid-Sodium Citrate (CASC) Buffer

Prekeratin has previously been purified from the living cell layers of bovine epidermis by extraction with 0.1M citric acid-sodium citrate pH 2.6 (CASC buffer) followed by serial precipitations from CASC buffer at pH values of 7, 6, 5 and 4.5 (Matoltsy, 1965). When applied to human epidermis, this procedure resulted in precipitates at near-neutral pH which were difficult to redissolve, and the method was modified by performing 3 cycles of precipitation at pH 3.5, at which pH prekeratin was the only protein to precipitate (Skerrow, 1977).

Extraction of newborn mouse epidermis was attempted by the latter method, but no CASC precipitate was obtained. A further modification was therefore adopted. Epidermis was first extracted by homogenisation in 8M urea, 0.1M 2-mercapto-ethanol, 50mM phosphate buffer pH 7.4, and the extract was then dialysed into CASC buffer. Protein could subsequently be precipitated from the CASC extract by raising the pH to 3.5. This procedure yielded approximately 50mg protein from 1g wet weight epidermis.

A Coomassie Blue-stained SDS polyacrylamide gel of the CASC precipitate from mouse epidermis is shown in Figure 8(a), and a densitometer profile of this gel in Figure 8(b). Only two major bands are observed : band I corresponds to molecular weight 69,000 and band II to molecular weight 61,000. The areas of the peaks in Figure 8(b) were calculated with the formula A = $\frac{1}{2}$ base x height, and were band I = 184, band II = 382 (arbitrary units). The ratios of the areas of

band II to band I is 2.08. A true polypeptide stoichiometry of 2:1 would result in a predicted band area ratio of 1.77:1, because of the difference in molecular weights. The molar proportions of the two mouse epidermal polypeptides is therefore likely to be 2:1.

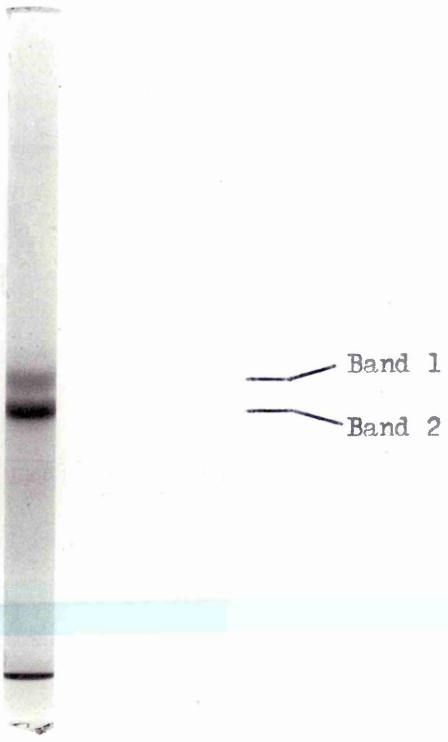
Mouse prekeratin appears to consist of one 69,000MW polypeptide to every two 61,000MW polypeptides. This structure is suggested by the extraction of these polypeptides with the prekeratin-specific CASC procedure. Also, similar three-chain structures have been reported for human and bovine prekeratin. Human prekeratin consists of three polypeptides of molecular weights 70,000, 63,000 and 55,000 (Skerrow, 1977). Bovine prekeratin comprises one 72,000MW chain plus two 60,000MW chains (Matoltsy, 1965), which is very similar to the structure proposed here for mouse prekeratin. Further, extraction of mouse stratum corneum with urea and mercaptoethanol has been claimed to result in two major polypeptides of 68,000MW and 60,000MW (Steinert and Yuspa, 1978; see below, section 7.5). Finally, the 69,000MW and 61,000MW polypeptides are major components of SDS/mercaptoethanol extracts of mouse epidermis, and are absent from similar extracts of other mouse tissues (see below, section 5.4).

Figure 8. Extraction of Prekeratin from Mouse Epidermis By The CASC Procedure

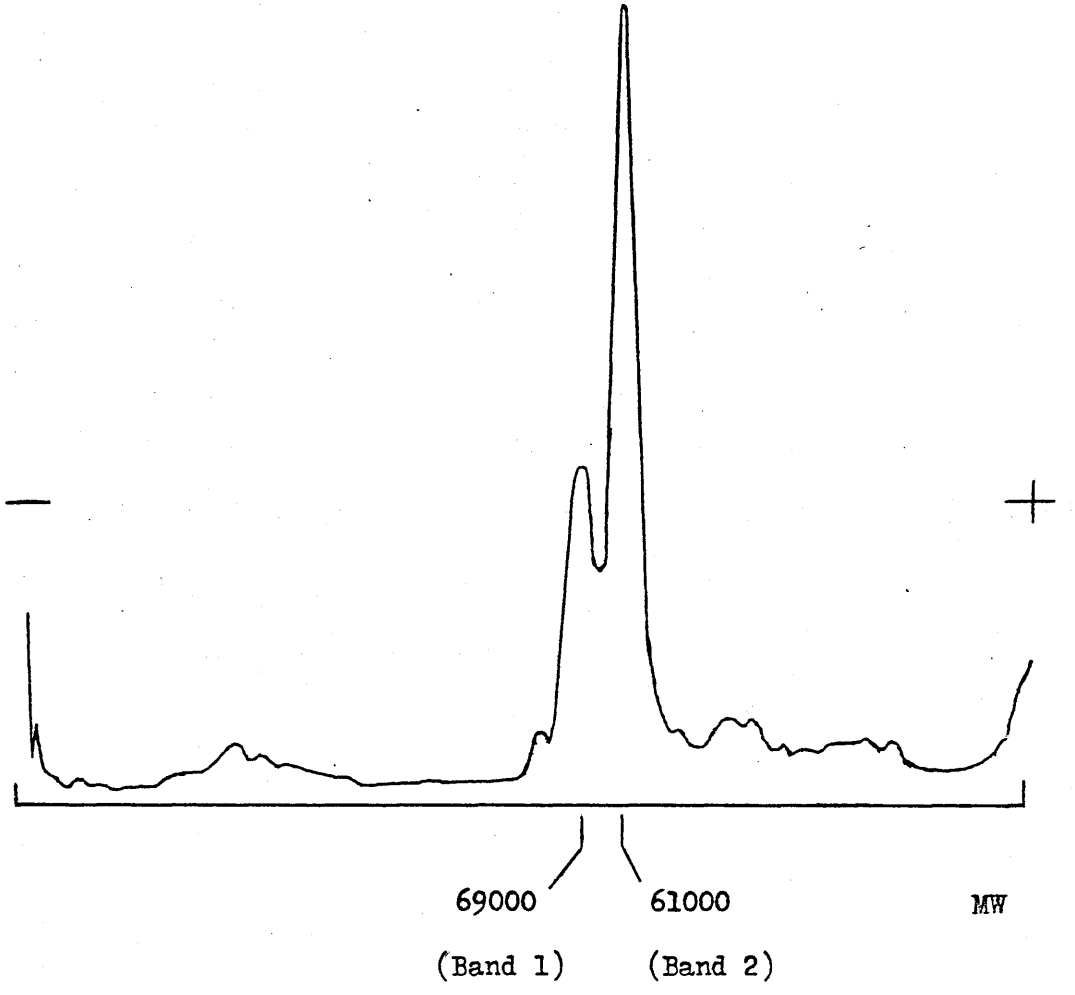
Skin from 1-3 day old mice was separated into dermis and epidermis by soaking in 2M NaBr at 4°C for 6h (Diaz et al, 1977). 1g wet weight of epidermis was incubated at 37°C for 1h in 5ml 8M urea, 0.1M 2-mercaptoethanol, 50mM phosphate buffer pH 7.4. The tissue was then homogenised in a plastic 30ml centrifuge tube in a crushed ice bath using an UltraTurrax TP18/2 homogeniser (setting 150 for 2 min) and sonicated at 1.5A for 5 min using an MSE MT20 ultrasonic probe. The extract was centrifuged at 50,000 x g for 20 min at 4°C, and the supernatant dialysed against 3 changes of 100 volumes of 0.1M citric acid-sodium citrate (CASC) buffer pH 2.6 over a 24h period. After centrifugation of the dialysis residue at 500 x g for 15 min at 4°C to remove CASC-insoluble material, prekeratin was precipitated by dropwise addition of 0.1M NaOH until the pH was raised to 3.5. The precipitate was collected by centrifugation at 500 x g for 15 min at 4°C, redissolved in 5ml CASC buffer at 4°C by sonication for 2 min at 1.5A using an MSE MT20 ultrasonic probe, and reprecipitated at pH 3.5 as before. After 3 cycles of solution and precipitation, the final precipitate was collected and dried under vaccuum. For electrophoresis, prekeratin was dissolved at 0.5mg.ml^{-1} in 1.0ml 1% SDS, 1% (v/v) 2-mercaptoethanol by heating at 100°C for 5 min, and 100µl aliquots electrophoresed as before (see section 2.2.12). Destained gels were scanned at 586nm using a Gilford 250 spectrophotometer fitted with a linear transport accessory.

(a) Photograph of gel of CASC-extracted mouse prekeratin.

(b) Densitometer scan of gel in (a).



(a).



(b).

5.4 Extraction of Epidermis, Dermis, Liver and Kidney with SDS and Mercaptoethanol

The prekeratin of newborn mouse epidermis has been shown to consist of two polypeptides of 69,000MW and 61,000MW. (see section 5.3). It was attempted to demonstrate the presence of these polypeptides in extracts made using a simpler and more quantitative SDS/mercaptoethanol extraction procedure.

The proteins of newborn mouse epidermis were extracted by heating the tissue at 100°C in 1%SDS, 1%(v/v) 2-mercaptoethanol for 1h. A polyacrylamide gel of the extracted protein is shown in Figure 9(a). The majority of extracted material appears as a series of bands in a region of the gel corresponding to 58,000MW to 73,000MW. This group of proteins included the two prekeratin polypeptides purified by citrate buffer extraction (see section 5.3). These bands, corresponding to 61,000 and 69,000MW, could be identified on gels of SDS/mercaptoethanol extracts by their molecular weights and 2:1 stoichiometry.

SDS/mercaptoethanol extraction of newborn mouse dermis and of baby mouse liver and kidney was performed, and polyacrylamide gels of the extracts are shown in Figure 9(b), (c) and (d). The group of polypeptide bands extracted from epidermis does not occur in the extract of dermis, nor in extracts of the two other mouse tissues. The dermal extract contains a number of high molecular weight polypeptides, in the molecular weight range 120,000-150,000. These may represent procollagen chains (120,000MW) or cross-linked higher order structures of the elastin subunit (70,000MW). The liver and kidney extracts exhibit a large number of polypeptide bands.

A major polypeptide of the kidney extract, also found in extracts of liver, corresponded to 39,000MW, and is most likely the cytoskeletal protein actin (40,000MW). The extracts of dermis, liver and kidney, but not epidermis, contain substantial amounts of incompletely-solubilised material which fails to enter the gel (Figure 9). This may represent extracellular matrix macromolecules. The absence of non-solubilised protein in the epidermal extract may be explained by the fact that even stratum corneum α-keratin is soluble in SDS. It appears that the main insoluble component of the epidermis is the cross-linked protein of the cornified cell envelope (Rice and Green, 1977), and in the extraction procedure used here the cornified cell envelopes may be sedimented by the centrifugation preceding electrophoresis, or washed off the top of the gel during staining.

Figure 9. SDS-Polyacrylamide Gel Electrophoresis of Proteins from Epidermis, Dermis, Liver and Kidney

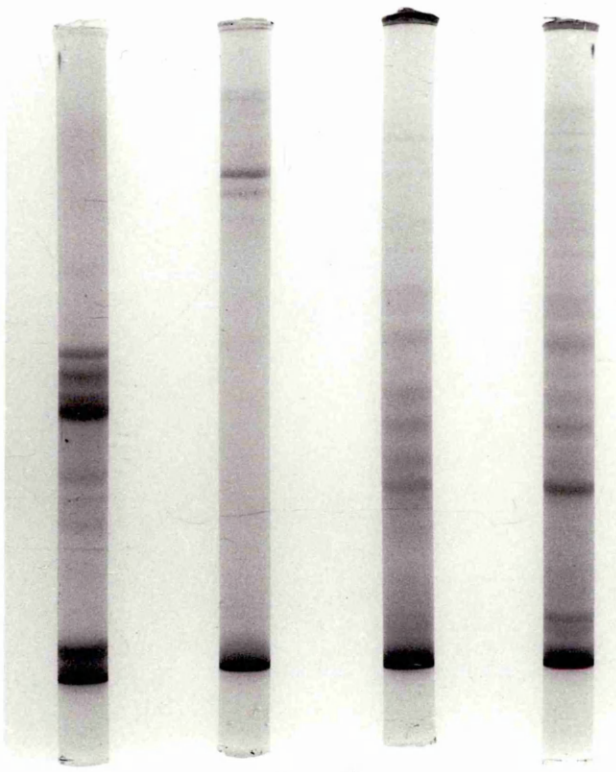
Epidermis and dermis from full-thickness skin of 1-3 day old mice were separated by soaking for 6h in 2M NaBr (Diaz et al, 1977). Liver and kidney were taken from 12 day old mice. These tissues were extracted by heating approximately 100mg wet weight of tissue in 0.5ml 1% SDS, 1% (v/v) 2-mercaptoethanol at 100°C for 1h. The extracts were centrifuged at 500 x g for 15 min at 4°C, and the protein concentration of the supernatants estimated by the method of Lowry et al (1951), using bovine serum albumin as a standard. The protein concentrations of the extracts were adjusted to 3.0mg.ml^{-1} by dilution with 1% SDS, 1% (v/v) 2-mercaptoethanol. Aliquots containing 60µg of protein mixed with glycerol and bromophenol blue were electrophoresed for 3h at 4mA per tube on 7.5% polyacrylamide gels (see section 2.2.12).

(a) Epidermis

(b) Dermis

(c) Liver

(d) Kidney



(a). (b). (c). (d).

5.5 Decrease in Prekeratin in Epidermal Keratinocytes in Culture

From reports in the literature (see section 1.5), the extent of differentiation undergone by epidermal keratinocytes in dispersed cell culture is unclear. In an attempt to clarify this issue in the mouse keratinocyte culture system, the epidermal protein prekeratin was used as a marker for keratinocyt differentiation in culture. CASC extraction (see section 5.3) of dispersed cell cultures of epidermal keratinocytes resulted in no visible precipitation of prekeratin. This is presumably because the small amounts of cellular material obtained from dishes of cultured keratinocytes resulted in too low a concentration of prekeratin in the CASC extract to form a detectable precipitate at pH 3.5. Prekeratin was therefore estimated in SDS/mercaptoethanol extracts after separation of the proteins on SDS-polyacrylamide gels.

Primary cultures of newborn mouse epidermal keratinocytes were established (see section 2.2.2) and medium changed daily. At various times over 8 days in culture, during which time the keratinocytes were subcultured once (at day 7), two 90mm dishes of cells were suspended, pelleted, and extracted with 1%SDS, 1% 2-mercaptoethanol. A pellet of freshly-prepared keratinocytes was also extracted in the same way. Equal amounts of protein from each sample were coelectrophoresed on polyacrylamide gels and the prekeratin bands identified by comparison with gels of CASC-extracted epidermal prekeratin. Total prekeratin was quantitated by densitometric scanning of the gels, and summation of the peak areas of the prekeratin bands. The proportion of prekeratin in total cell protein

is plotted against time in culture in Figure 10.

Over the first day in culture there is a large decrease in the amount of prekeratin relative to total protein. Therefore, there is a large difference between the amount of prekeratin in the original epidermal cell preparation and that in the cells which have attached to the substratum 24h later. This is presumably due to the presence in the original keratinocyte suspension of highly-differentiated cells which do not attach to the culture surface and are lost from the culture at the first medium change. From day 1 onward there is a linear decrease in the proportion of prekeratin in total cell protein, which continues beyond the first subculture.

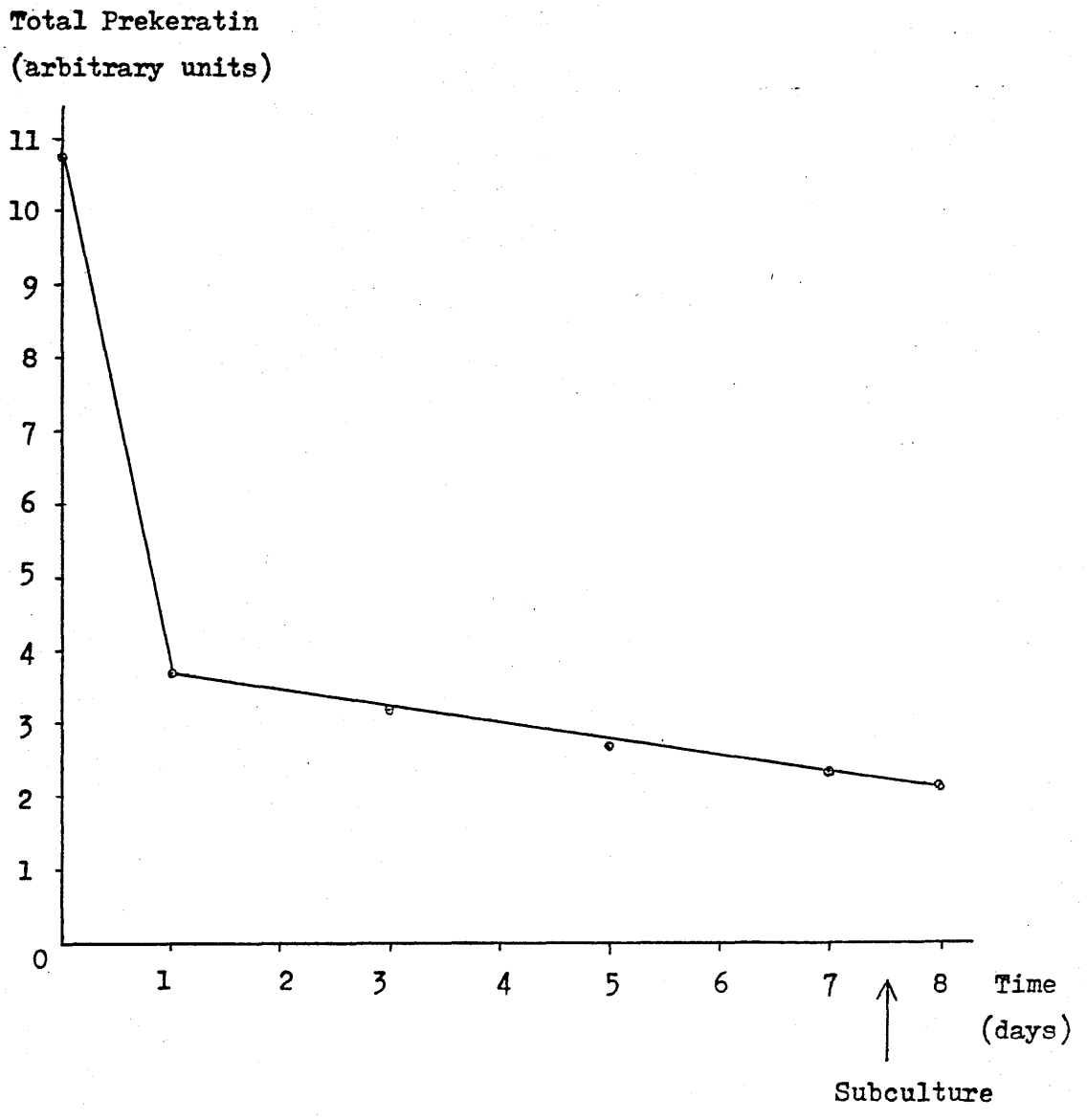
From the rate of thymidine incorporation by newborn mouse keratinocyte cultures (see below, Figure 12), it can be calculated that keratinocytes maintained at 37°C have a mean cell doubling time of approximately 0.9 days. It can be estimated from Figure 10 that the time required for the proportion of prekeratin in cultured keratinocytes to fall by half is approximately 9 days. Since the amount of prekeratin is not being halved at every cell doubling, prekeratin synthesis is probably continuing in cultured keratinocytes. It is also possible that the rate of prekeratin synthesis by these cells is higher than it appears to be but that some of the prekeratin is lost from the culture if differentiated keratinocytes are desquamated into the culture medium. However, the number of dead cells and the amount of cell debris observed in the medium of keratinocyte cultures were very small and did not appear greater than that of other cell types. The normal

level of prekeratin in the epidermis is maintained by a balance of synthesis and desquamation. If keratinocyte cultures are losing differentiated cells into the medium, the prekeratin to replace this loss is not being produced at a sufficient rate to maintain the original level in the remaining attached cells.

These results suggest that de novo prekeratin synthesis occurs in cultures of epidermal keratinocytes, but that these cultures are not capable of maintaining fully the normal level of prekeratin in skin. This may be due to selection of cells in the culture which have increased growth rate and decreased expression of the differentiated phenotype.

Figure 10. Decrease in Prekeratin in Epidermal Keratinocytes with Time in Culture

Freshly-prepared epidermal keratinocytes from newborn mouse skin were established at 4×10^6 viable cells per 90mm petri dish in 10ml EFC10, and incubated at 37°C. At 1, 3, 5, 7 and (post-subculture) 8 days thereafter, 2 dishes of cells were suspended by washing twice with 5ml 0.02% EDTA in PBS at room temperature and scraping with a plastic cell scraper. The cells were pelleted by centrifugation at 500 x g for 10 min at 4°C, and protein extracted by heating the pellet in 0.2ml 1% SDS, 1% (v/v) 2-mercaptoethanol at 100°C for 1h. Similarly extracted was a pellet of freshly-prepared keratinocytes (zero time sample). The protein concentrations of the extracts were measured by the method of Lowry et al (1951) using bovine serum albumin as a standard, and adjusted to $3.5 mg.ml^{-1}$ by dilution with 1% SDS, 1% (v/v) 2-mercaptoethanol. 100µl aliquots of each sample were mixed with 100µl glycerol and 10µl bromophenol blue and 100µl amounts of the mixtures were electrophoresed on 7.5% polyacrylamide gels for 3h at 4mA per tube (see section 2.2.12). Destained gels were scanned at 586nm using a Gilford 250 spectrophotometer fitted with a linear transport accessory, and total prekeratin quantitated as the summed peak areas of the bands corresponding to epidermal CASC-extracted prekeratin.


Total Prekeratin
(arbitrary units)
11
10
9
8
7
6
5
4
3
2
1
0
1
2
3
4
5
6
7
8
Time
(days)
Subculture

5.6 Growth of Epidermal Keratinocytes in Feeder Layer Culture

Epidermal cells from newborn mouse skin appear to undergo only limited differentiation in pure culture (see section 5.5). *In vitro* study of epidermal keratinisation was therefore attempted using a modification of the feeder layer culture system of Rheinwald and Green (1975a). In this method, these workers claim that keratinocytes grown on a layer of killed cell line fibroblasts form a keratinising epithelium which can be serially subcultured. Feeder layer cells are normally fibroblasts, and appear to promote the proliferation of differentiated cell types by secretion of stimulatory factors or by provision of a suitable substratum for growth. The fibroblasts can be killed by X-irradiation or by treatment with mitomycin C (Kubilus *et al*, 1979).

In this study, differentiation of epidermal keratinocytes in feeder layer culture was assayed by synthesis of prekeratin. As the presence of fibroblast proteins in extracts of these cultures made assignment of prekeratin polypeptides more difficult than in pure epidermal cell cultures (see section 5.5), *de novo* protein synthesis was assayed by radiolabelling the cultures with ^{3}H-leucine or ^{35}S-methionine. Proteins were extracted from labelled feeder layer cultures with SDS and mercaptoethanol, separated by polyacrylamide gel electrophoresis, and labelled polypeptides identified by scintillation counting of gel slices.

Feeder layer keratinocyte cultures were established by coculture of newborn mouse skin keratinocytes with C13 cells pretreated with mitomycin C. C13 cells were killed by

incubation in 10^{-6}M mitomycin C for 16h, washed, trypsinised, and then cocultured with freshly-prepared mouse epidermal keratinocytes. A Rhodamine B-stained 5 day feeder layer culture is shown in Figure 11. Rhodamine B is a specific histological reagent for epidermal cells (Liisberg, 1968; Delescluse et al, 1976). The feeder layer cultures exhibit colonies of pink (Rhodamine B-stained) epidermal keratinocytes surrounded by blue (counter-stained) fibroblasts.

The growth rates of mitomycin C-treated C13 cells, epidermal keratinocytes, and both cell types in mixed cultures were determined by labelling with ^{3}H-thymidine. The results are shown in Figure 12. The incorporation of ^{3}H-thymidine at various times in culture by epidermal keratinocytes and killed C13 cells grown separately have been summed for each time-point and plotted in Figure 12(a). Figure 12(b) shows the ^{3}H-thymidine incorporation over the same time-scale of keratinocytes and C13 cells in mixed (feeder layer) culture. The graphs are very similar, being typical sigmoid growth curves. There is an initial "lag" between day 1 and day 2, which is probably due to suboptimal growth of the cells at low density. This is followed by exponential growth until day 5, when the rate of increase in ^{3}H-thymidine incorporation decreases presumably because of density-dependent inhibition of growth. The mean cell doubling time, averaged over the period 1-7 days, of the keratinocytes alone [Figure 12(a)] is approximately 0.9 days. The two graphs are slightly different at the start of the culture period, but by day 3 the two data sets are not significantly different. In view of the close similarity of the two

growth curves, it was concluded that coculture with killed fibroblasts does not significantly affect the growth of mouse epidermal keratinocytes.

These results contrast with the finding of Rheinwald and Green (1975a) that human epidermal cells do not initiate colony formation in the absence of fibroblast feeder layers. This may be due to the relatively small inocula of keratinocytes used by these workers, as non-transformed cells often fail to grow at low culture densities. However, the growth characteristics of pure cultures of epidermal cells found here are very similar to those reported for adult guinea pig epidermal keratinocyte cultures (Regnier et al, 1973).

Figure 11. Rhodamine B-Stained C13 Feeder Layer Keratinocyte Culture

C13 feeder layer keratinocyte cultures were established in 50mm dishes as described in section 2.2.7, and incubated in 4ml EFC10 containing 0.4$\mu g.ml^{-1}$ hydrocortisone at 37°C for 5 days. The medium was then removed, and the cells fixed by immersion in 4ml formal saline at 4°C for 1h. The cells were then washed 3 times with distilled water, and stained with 4ml 1% Rhodamine B in 0.1M acetate buffer pH 3.8 at room temperature for 5 min (Liisberg, 1968). After a further 3 washes with distilled water, the cells were counter-stained with 4ml 1% toluidine blue in distilled water for 5 min, washed 3 times with distilled water and air-dried. Cultures were photographed with a Leitz Orthomat photomicroscope fitted with plan optics.

(x145)

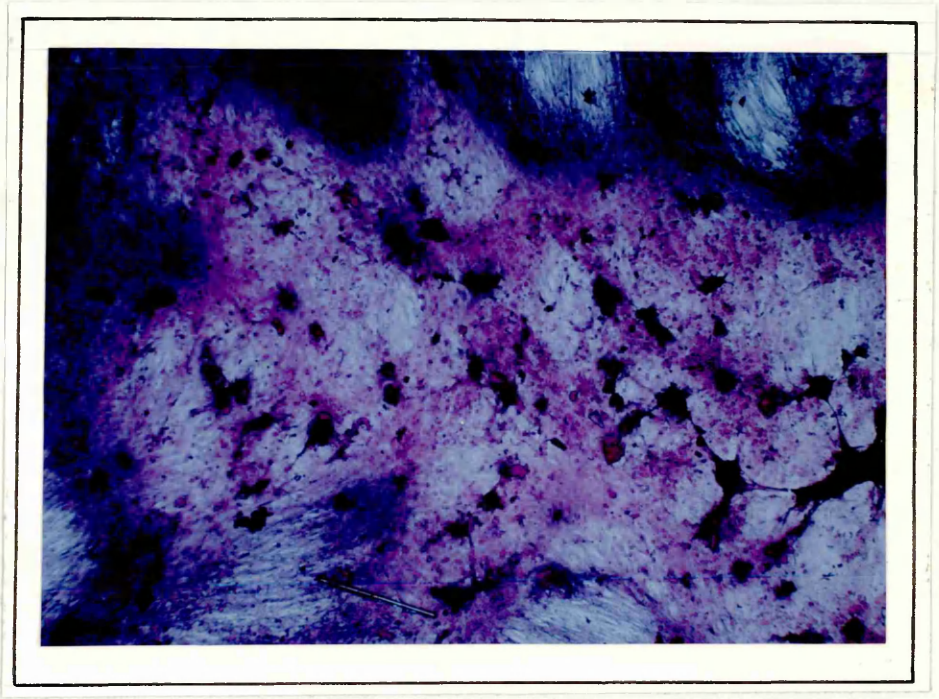
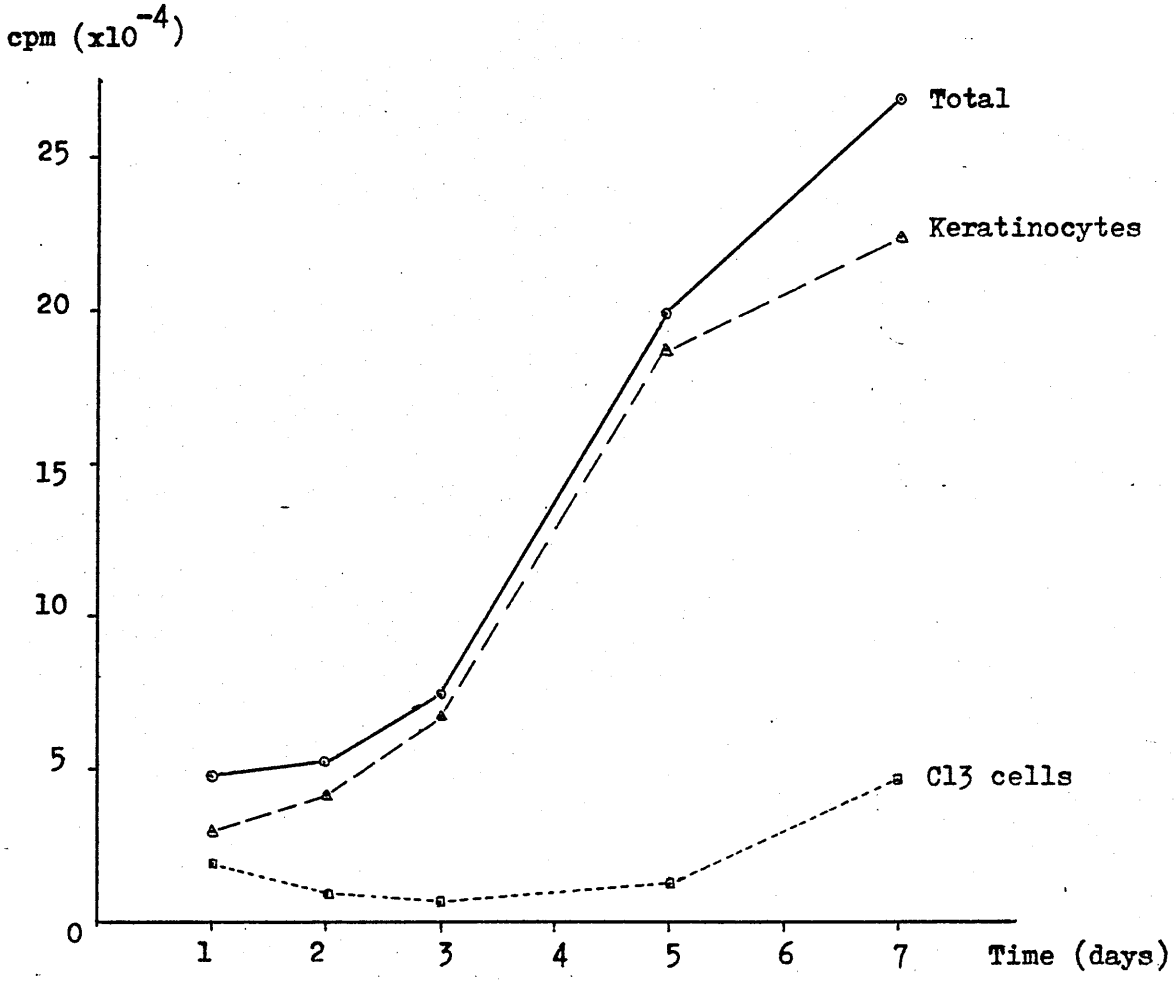


Figure 12. Growth Rates of Epidermal Keratinocytes in Pure Culture and in Feeder Layer Culture

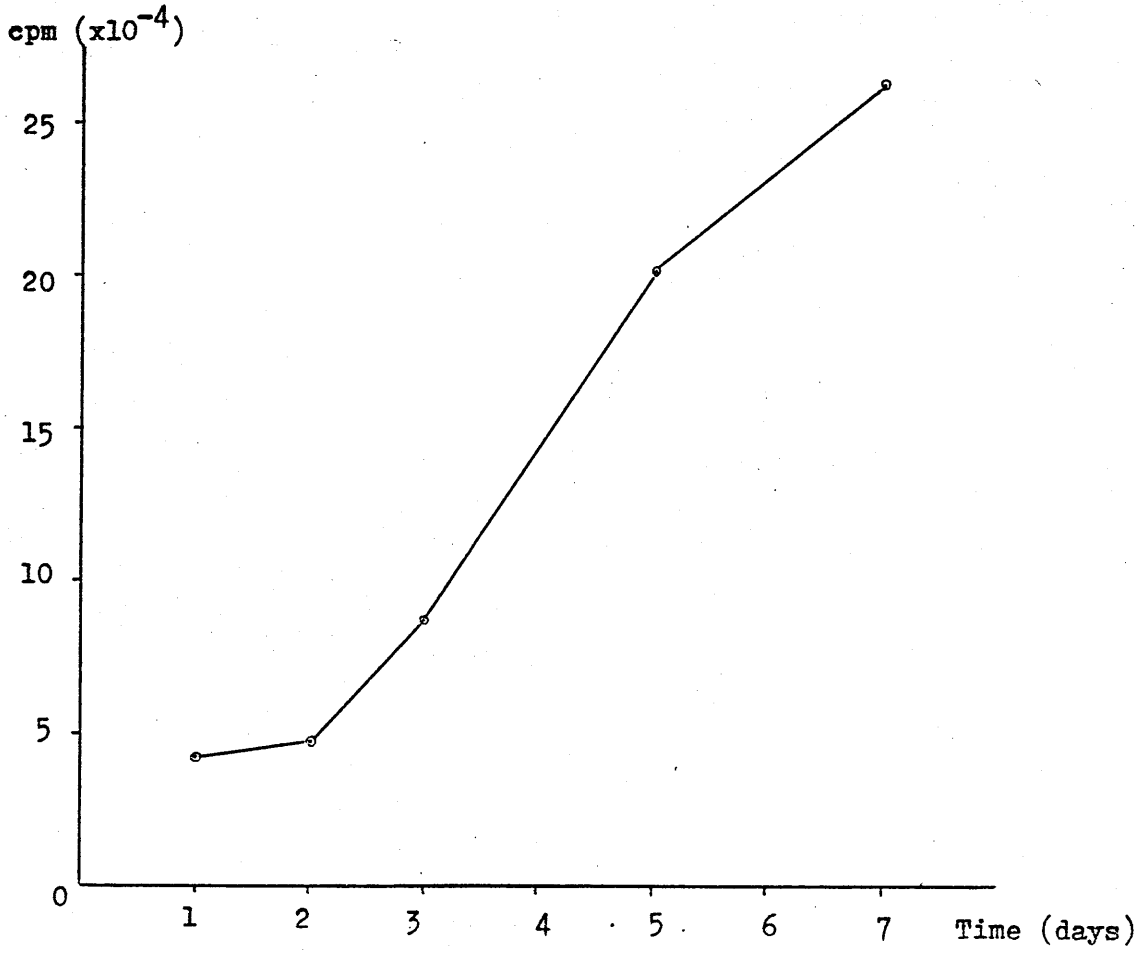
Cultures were established of newborn mouse epidermal keratinocytes (5×10^5 per 50mm dish), mitomycin C-treated C13 fibroblasts (10^5 per 50mm dish), and cocultures of both cell types together (5×10^5 epidermal keratinocytes plus 10^5 C13 cells per 50mm dish). All cultures were grown in EFC10 plus 0 4$\mu g.ml^{-1}$ hydrocortisone, with medium being changed daily. At 1,2,3,5 and 7 days, the rates of growth of the three culture types were estimated by ^{3}H-thymidine incorporation. Duplicate cultures were incubated in EFC10 containing 1$\mu Ci.ml^{-1}$ ^{3}H-thymidine for 1h, then washed twice with 4ml ice-cold 5% TCA, and once with 4ml distilled water. Incorporated thymidine was solubilised by extraction with 1ml 0.1M NaOH, and the extract acidified by addition of 0.2ml 1M HCl. 10ml Triton-toluene scintillation fluid was added, and the samples counted for 4 min in an Intertechnique SL30 liquid scintillation counter.

(a) ^{3}H-thymidine incorporation by epidermal keratinocytes plus ^{3}H-thymidine incorporation by separate C13 cultures.

(b) ^{3}H-thymidine incorporation by epidermal keratinocyte-C13 mixed cultures.



(b). Feeder layer keratinocyte cultures



5.7 Protein Synthesis by Keratinocyte Feeder Layer Cultures

The dermal regulation of epidermal differentiation *in vivo* may involve either deterministic or permissive interaction (Saxén, 1977). In the former possibility, the dermis will induce keratinisation by undifferentiated epidermal cells, and in the latter, the dermis will support the growth of epidermal cells which consequently differentiate. If the fibroblast cells in feeder layer cultures are performing an inductive function similar to that of the dermis of normal skin the mechanism is likely to be a permissive stimulation of epidermal cell differentiation, because of the lack of cell type specificit In the system used in this study, growth on fibroblast feeder layers has no effect on keratinocyte proliferation. However, if growth on feeder layers could be shown to affect prekeratin synthesis, use of different feeder layer cell types may establish whether factors such as tissue layer origin or gap junction formation are involved in the promotion of epidermal keratinisation in culture.

Newborn mouse keratinocytes were cocultured with mitomycin C-treated cells of the C13, BRL and A9 cell lines. C13 cells are fibroblasts which are derived from mesodermal tissue, whereas BRL cells are epithelial. C13 and BRL cells, but not A9 cells, form gap junctions *in vitro* with mouse epidermal keratinocytes (see Chapter 3). Incorporation of the radiolabelled amino acids ^{35}S-methionine and ^{3}H-leucine was used to assay for prekeratin synthesis in feeder layer cultures.

Feeder layer cells were killed by incubation in 10^{-6}M mitomycin C, washed, trypsinised, and cocultured with freshly-

prepared keratinocytes. Epidermal keratinocytes in pure culture were also grown for comparison. After 5 days in culture the cells were labelled with ^{35}S-methionine or ^{3}H-leucine Protein was extracted with 1% SDS, 1% 2-mercaptoethanol and electrophoresed on polyacrylamide gels. The gels were then sliced and counted. Radioactivity profiles of gels containing ^{35}S-methionine-labelled extracts of epidermal keratinocytes and keratinocytes cultured on feeder layers of C13, BRL and A9 cells are shown in Figures 13-16.

Figure 13 shows the ^{35}S-methionine incorporation into epidermal keratinocyte protein. Three major peaks of radioactivity are observed. Only one of these corresponded to a major absorbance peak; this had an R_f value of 0.71 (43,000MW) and is probably actin. Actin occurs in basal epidermal cells (Steinert et al, 1976) and has been shown to increase in cultured keratinocytes (Steinert and Yuspa, 1978). The remaining peaks were at R_f 0.19 (160,000MW) and a "plateau" between R_f 0.37 and 0.48 (76,000-100,000MW). No specific cpm peak corresponded to the prekeratin absorbance maximum.

The C13, BRL and A9 feeder layer keratinocyte cultures all exhibited patterns of protein synthesis similar to that of the pure-cultured epidermal cells (Figures 14, 15, 16). All three showed a major peak of radioactivity at approximately R_f 0.19 (160,000MW) and the actin peak at 43,000MW. The BRL and A9 (but not C13) feeder layer keratinocyte cultures both have a cpm peak and absorbance maximum at R_f 0.46 (80,000MW), which may represent the same labelled protein occurring in the "plateau" region of the keratinocyte culture extract (Figure 13). Again,

no specific peaks of radioactivity are observed in the prekeratin banding region. It appears that coculture with killed cells of these established cell lines does not cause detectable prekeratin synthesis by epidermal keratinocytes.

The absence of a ^{35}S cpm peak corresponding to the prekeratin absorbance band may be due to the low proportion of methionine in prekeratin (8.8 residues per 1000 in human prekeratin, 20.4 residues per 1000 in bovine prekeratin; Skerrow, 1977), although the high specific activity of the amino acid used should offset this disadvantage. Feeder layer keratinocyte cultures were therefore radiolabelled with another amino acid, ^{3}H-leucine. Leucine occurs as 86.8 residues per 1000 in human prekeratin, and 87.9 residues per 1000 in bovine prekeratin (Skerrow, 1977). Radioactivity profiles of gels containing ^{3}H-leucine-labelled extracts from epidermal keratinocyte cultures and keratinocytes cultured on feeder layers of C13, BRL and A9 cells are shown in Figures 17-20.

Figure 17 shows the ^{3}H-leucine incorporation into cultured epidermal keratinocytes. As in the case of ^{35}S-methionine labelling, there was a large cpm peak in the region R_f 0.15-0.20 (150,000-170,000MW), which did not correspond to a major absorbance band. Another peak at R_f 0.44 (84,000MW) may correspond to the 80,000MW protein synthesised by keratinocytes labelled with ^{35}S-methionine (Figures 13, 15, 16), although such a molecular weight difference ought to be distinguished by the gel system used. Actin again was the major absorbance peak at R_f 0.73 (41,000MW), but gave only a small peak of radioactivity. Again, no significant activity was seen corresponding to the prekeratin absorbance peak.

Similar patterns of ^{3}H-leucine incorporation were observed in the C13, BRL and A9 feeder layer keratinocyte cultures (Figures 18, 19, 20). The amount of radioactivity corresponding to the actin absorbance peak was variable, but the high molecular weight band (R_f 0.15-0.20; 150,000-170,000MW) was always the highest radioactivity peak. A definite peak at R_f 0.46 (80,000MW) was observed in all three cases, but minor peaks at R_f 0.55 (64,000MW) in the BRL feeder layer profile at R_f 0.60 (57,000MW) in the A9 feeder layer profile were not reproducibly observed.

These results complement those achieved with ^{35}S-methionine labelling, and confirm that there is no stimulation of protein synthesis in general, and prekeratin synthesis in particular, induced by coculture of newborn mouse epidermal keratinocytes with these mitomycin C-treated cell lines. Prekeratin is observed in extracts of cultured keratinocytes as an absorbance peak on polyacrylamide gels, but is not detectable by incorporation of the radiolabelled amino acids ^{35}S-methionine and ^{3}H-leucine. This indicates that the keratinocytes are not performing detectable <u>de novo</u> prekeratin synthesis in culture. However these cells do incorporate ^{3}H-thymidine (Figure 12) and synthesise other proteins (Figures 13 and 17). This is in agreement with the loss of differentiated phenotype suggested by measure of total prekeratin in keratinocyte cultures (see section 5.5).

Figures 13-16. Characterisation of Protein Synthesis by Feeder Layer Keratinocyte Cultures Labelled with ^{35}S-Methionine

Cultures of C13, BRL and A9 cells were killed by incubation in EFC10 containing 10^{-6}M mitomycin C for 16h, then washed 3 times with sterile BSS (see section 2.2.7). The cultures were then suspended by trypsin/EDTA treatment and mixed with freshly-prepared newborn mouse epidermal keratinocytes in the proportion 2 x 10^5 feeder cells plus 10^6 keratinocytes per 50mm dish. Dishes containing only 10^6 epidermal keratinocytes were also established. Cultures were maintained in 4ml EFC10 plus 0.4µg.ml^{-1} hydrocortisone. After 5 days the cultures were incubated in 4ml methionine depleted medium. This medium consisted of EFC10 containing 1/100th the normal concentration of methionine supplemented with 10% foetal calf serum which had been dialysed against 3 changes of 100 volumes of 0.9% NaCl at 4°C over 24h. The cells were then labelled by incubation in 4ml methionine-depleted medium containing 20µCi.ml^{-1} ^{35}S-methionine for 4h. The cells were suspended by washing twice with 4ml 0.02% EDTA in PBS and scraping with a plastic cell scraper, and pelleted by centrifugation at 500 x g for 10 min at 4°C. The pellets were heated at 100°C for 1h in 200µl 1% SDS, 1% (v/v) 2-mercaptoethanol and the extracts centrifuged at 500 x g for 15 min at 4°C. The protein concentrations of the supernatants were estimated by the method of Lowry et al (1951), using bovine serum albumin as a standard, and adjusted to 5.5mg.ml^{-1} by dilution with 1% SDS, 1% (v/v) 2-mercaptoethanol. Glycerol and bromophenol blue were added, mixed, and 100µl aliquots of the mixtures electrophoresed at 4mA per tube for 3h on 7.5% polyacrylamide gels (see section 2.2.12).

Destained gels were scanned at 586nm using a Gilford 250 spectrophotometer fitted with a linear transport accessory. The gels were then frozen by sprinkling with powdered solid CO_2 and sliced into 1mm sections using a Mickle gel slicer. Gel slices were solubilised in 1ml 0.5M perchloric acid at 60°C for 16h, added to 6ml Triton-toluene scintillation fluid, and counted for 4 min in a Beckman LS8100 liquid scintillation spectrometer.

Figure 13. Epidermal keratinocytes
Figure 14. Epidermal keratinocytes plus mitomycin C-treated C13cell
Figure 15. Epidermal keratinocytes plus mitomycin C-treated BRL cell
Figure 16. Epidermal keratinocytes plus mitomycin C-treated A9 cell

Figure 13. Epidermal Keratinocytes Labelled with ^{35}S-Methionine

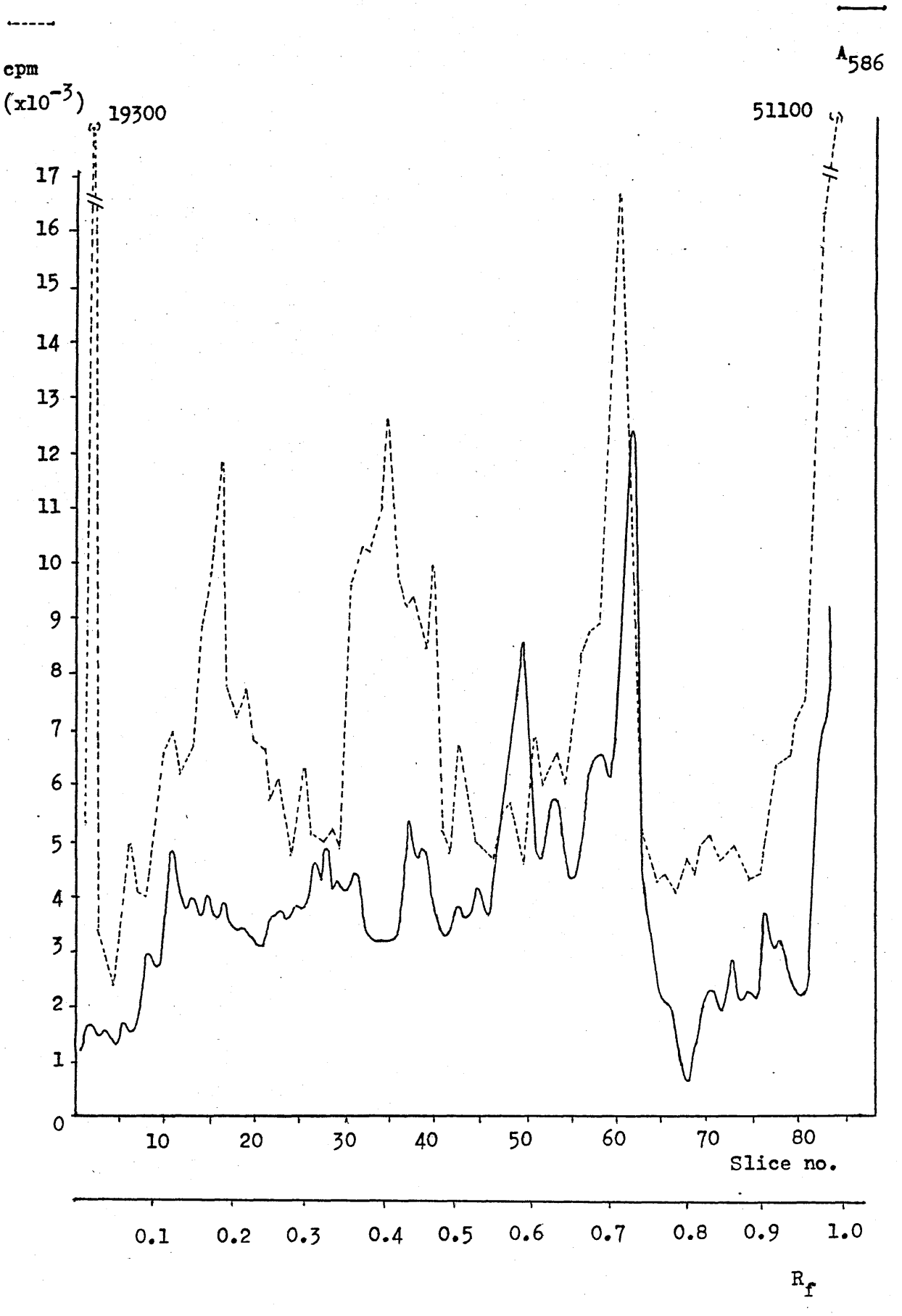


Figure 14. C13 Feeder Layer Keratinocyte Culture Labelled With ^{35}S-Methionine

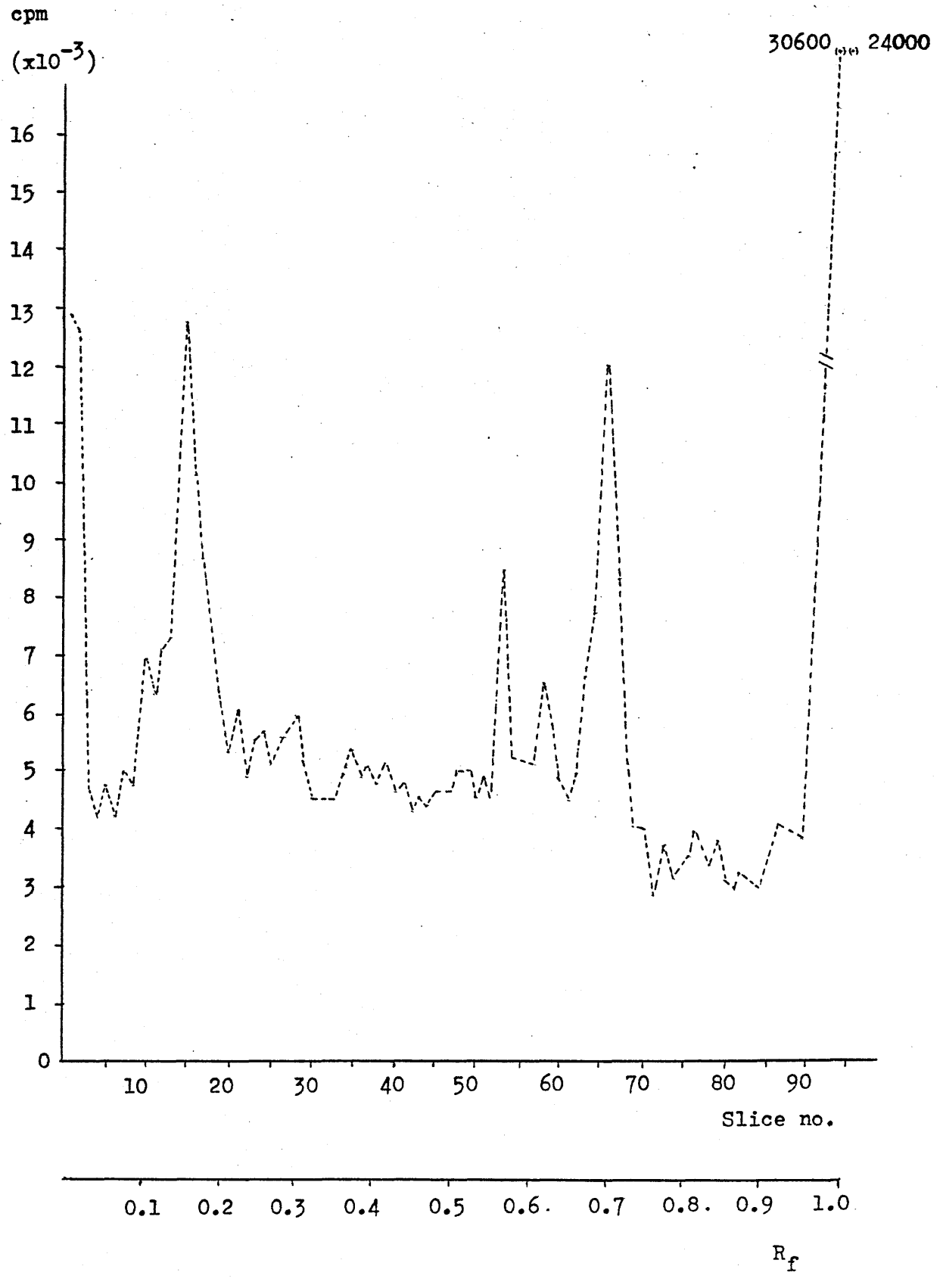


Figure 15. BRL Feeder Layer Keratinocyte Culture Labelled With ^{35}S-Methionine

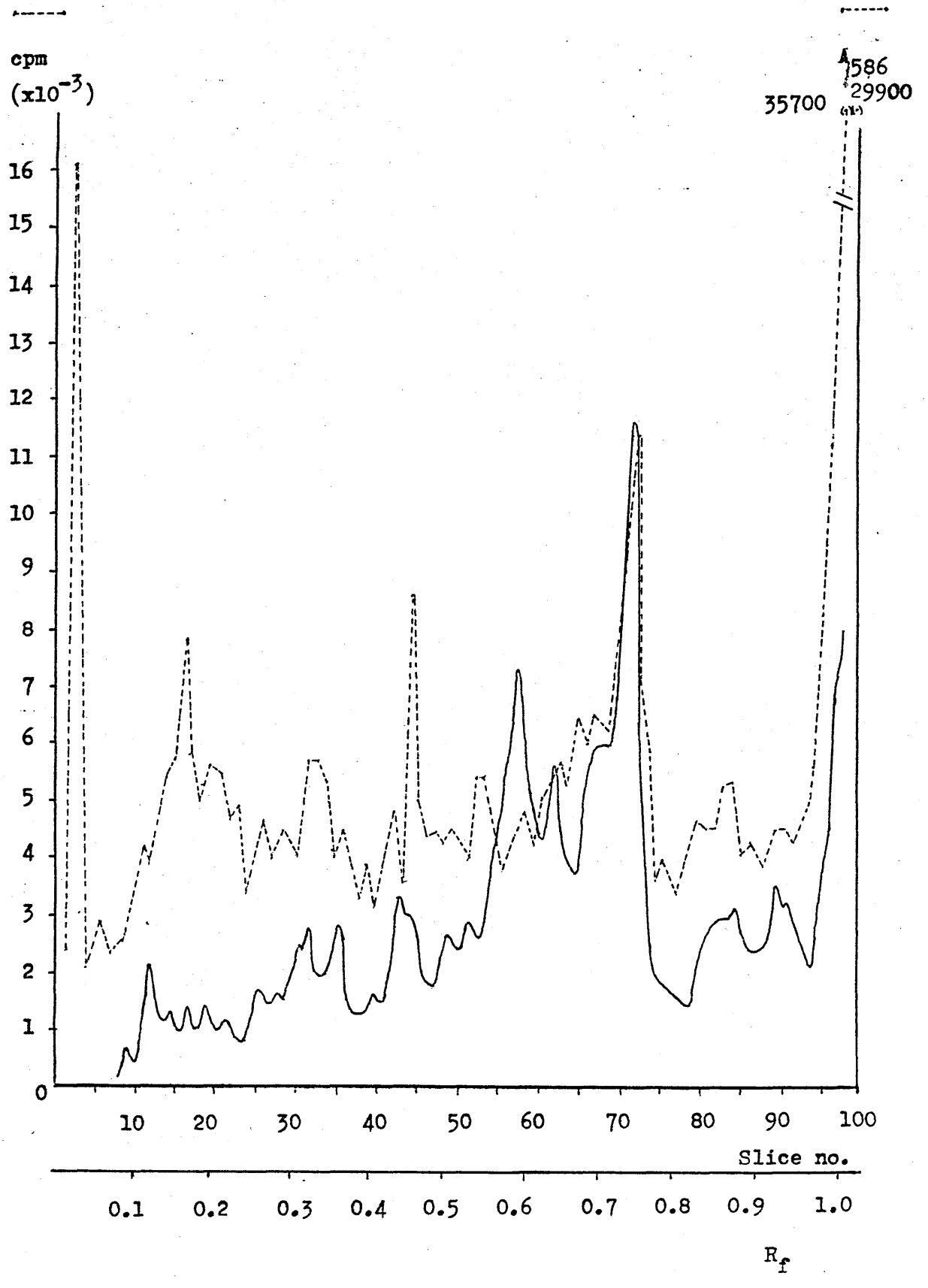
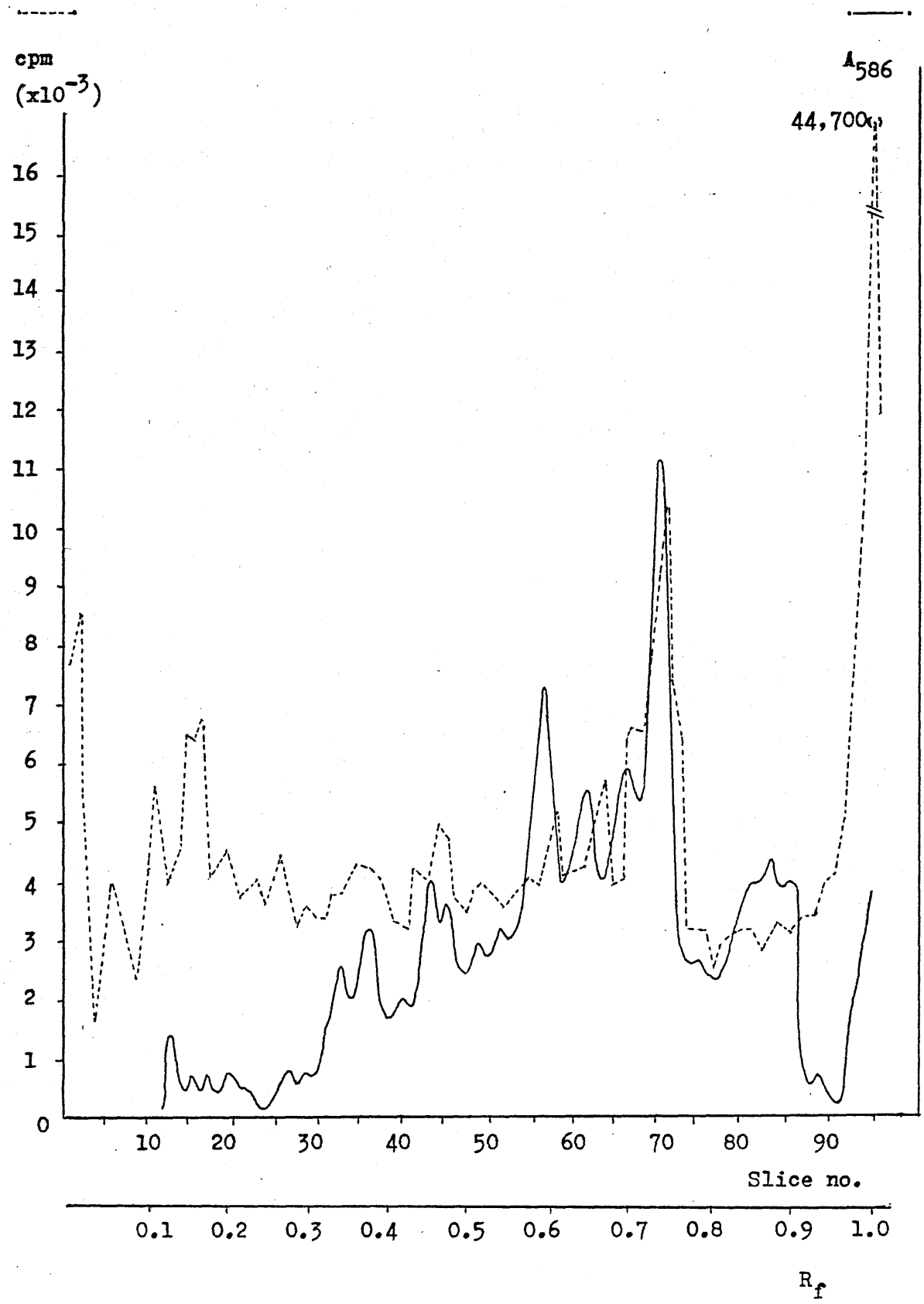


Figure 16. A9 Feeder Layer Keratinocyte Culture Labelled With ^{35}S-Methionine



Figures 17-20. Characterisation of Protein Synthesis by Feeder Layer Keratinocyte Cultures Labelled with ^{3}H-Leucine

Feeder layer cultures of mouse epidermal keratinocytes cocultured with mitomycin C-treated C13, BRL and A9 cells, and pure cultures of keratinocytes, were established as described in the legend to Figures 13-16. After 5 days the cells were incubated in leucine-depleted EFC10 (1/100th normal leucine concentration) for 4h in order to deplete the endogenous leucine pools, and then labelled by incubation in leucine-depleted medium containing 50μCi.ml^{-1} ^{3}H-leucine for 4h. The cultures were suspended, extracted and electrophoresed as described in the legend to Figures 13-16.

Figure 17. Epidermal keratinocytes

Figure 18. Epidermal keratinocytes plus mitomycin C-treated C13 cells

Figure 19. Epidermal keratinocytes plus mitomycin C-treated BRL cells

Figure 20. Epidermal keratinocytes plus mitomycin C-treated A9 cells

Figure 17. Epidermal Keratinocytes Labelled With ^{3}H-Leucine

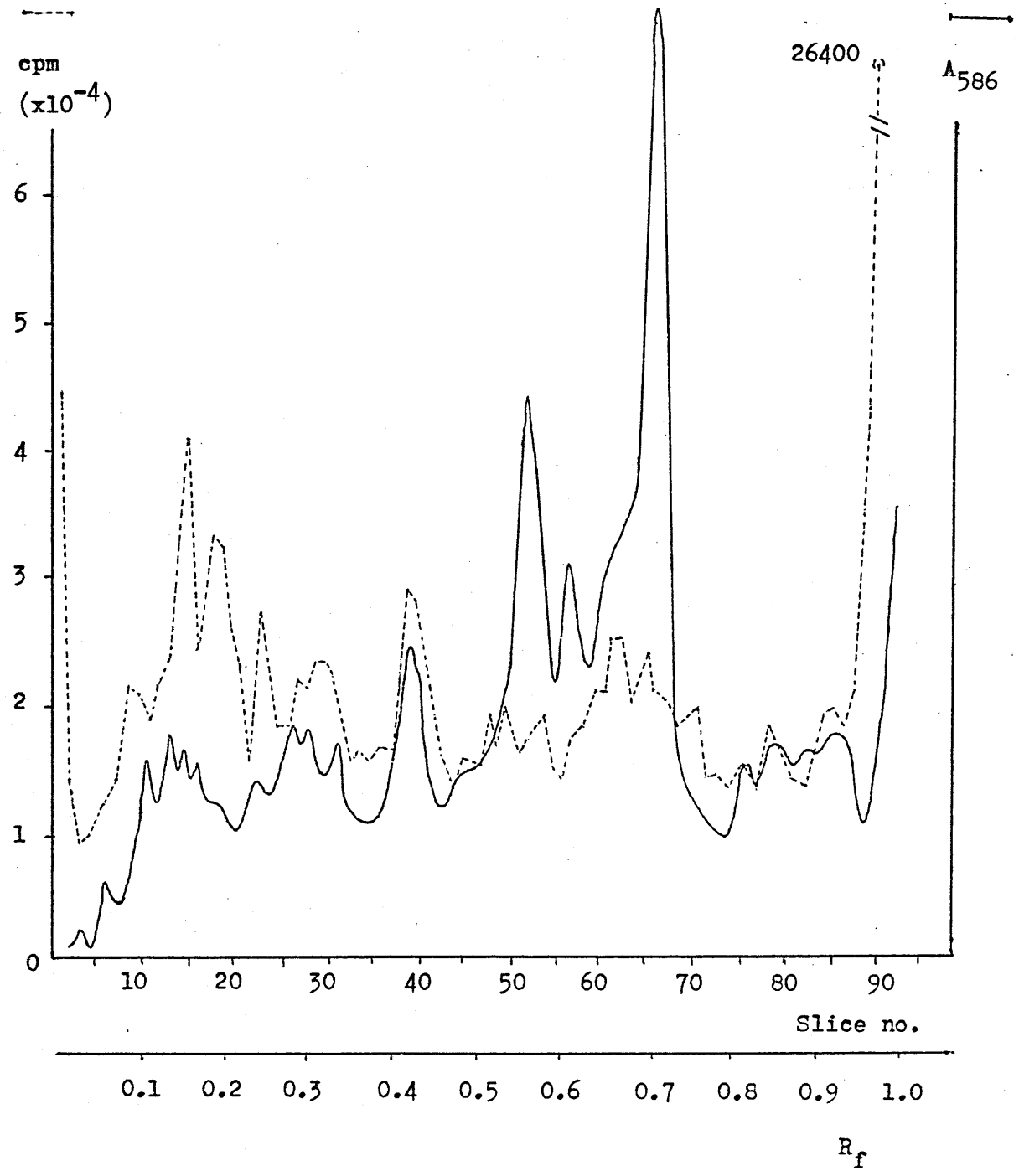


Figure 18. C13 Feeder Layer Keratinocyte Culture Labelled With ^{3}H-Leucine

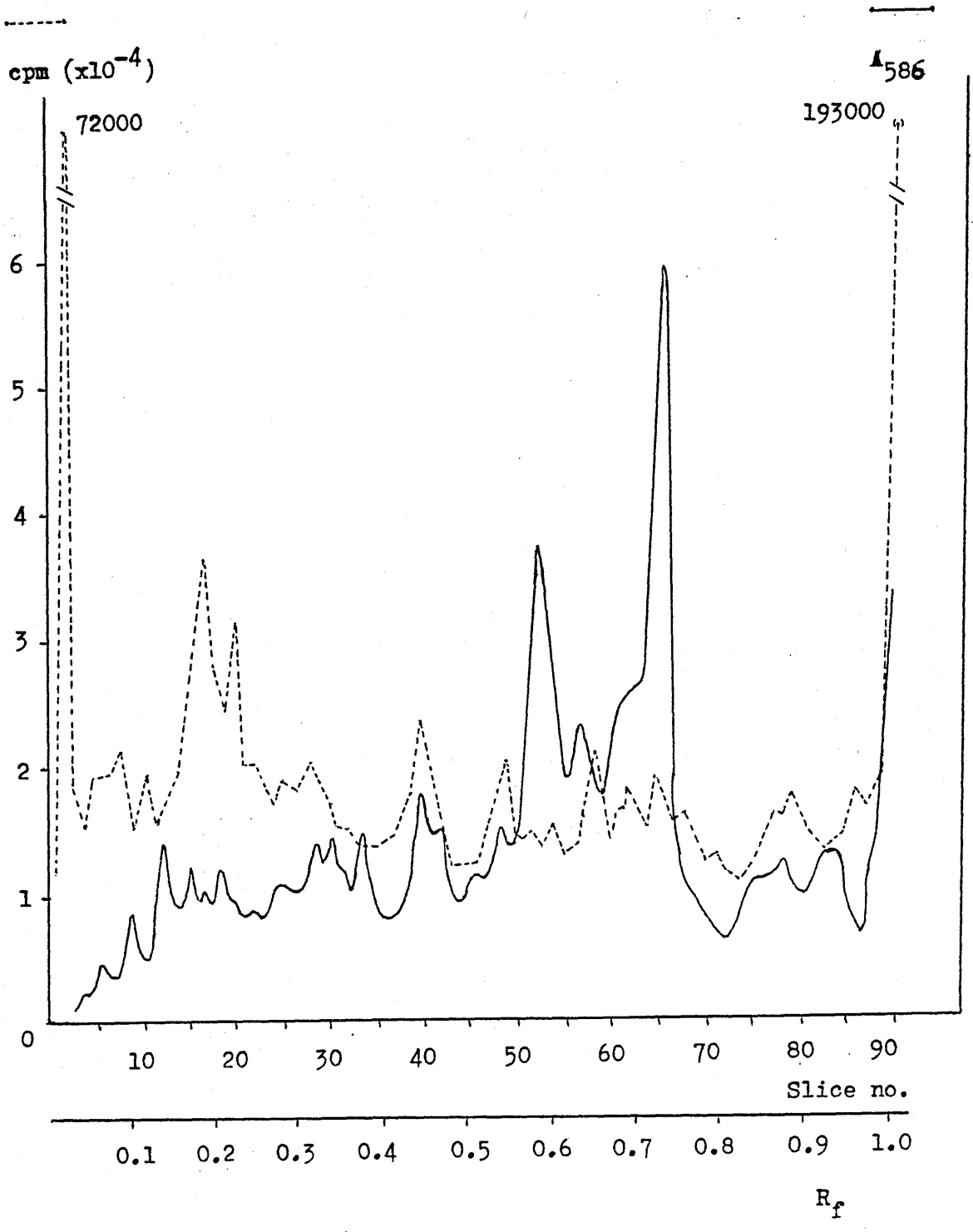


Figure 19. BRL Feeder Layer Keratinocyte Culture Labelled With ^{3}H-Leucine

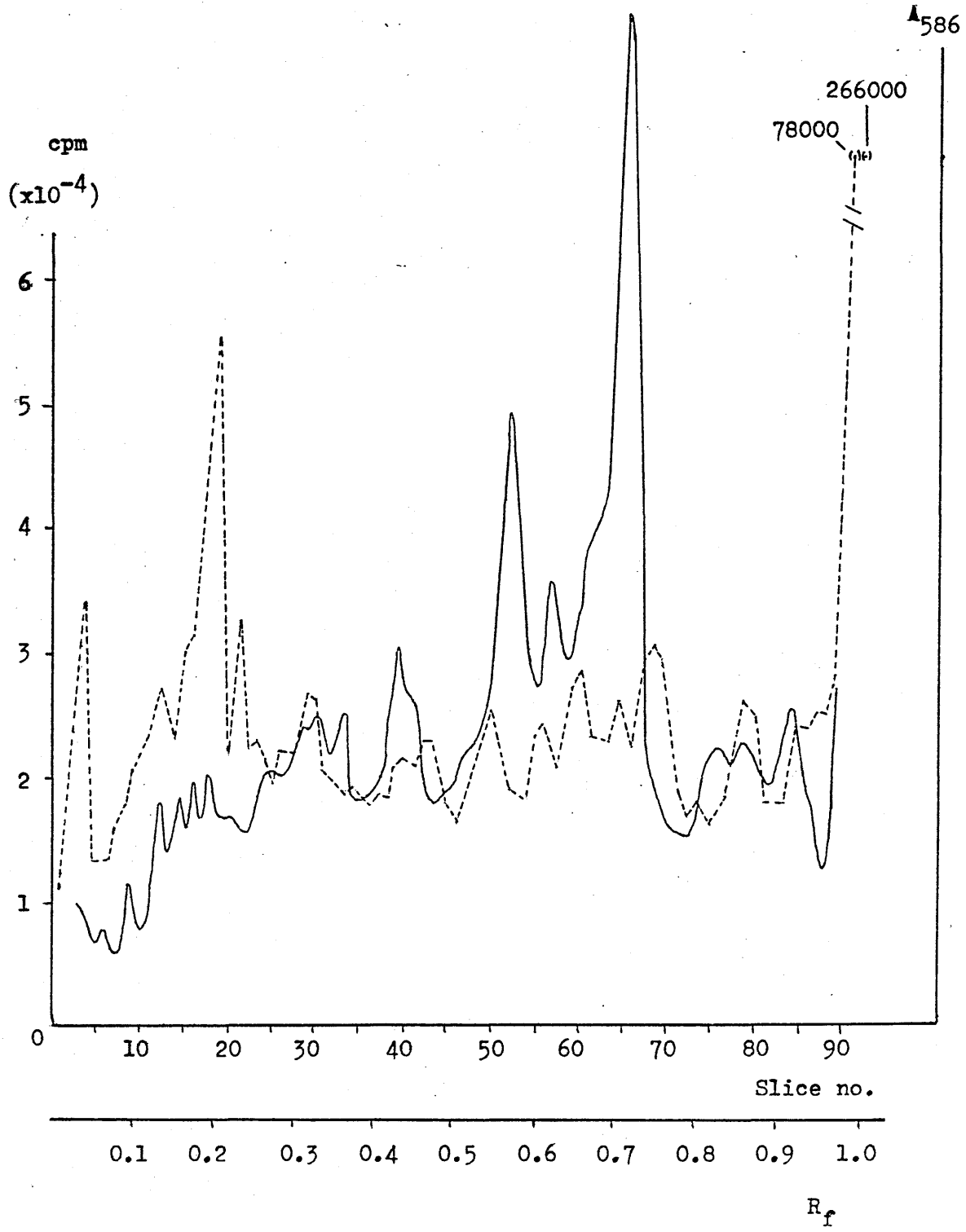
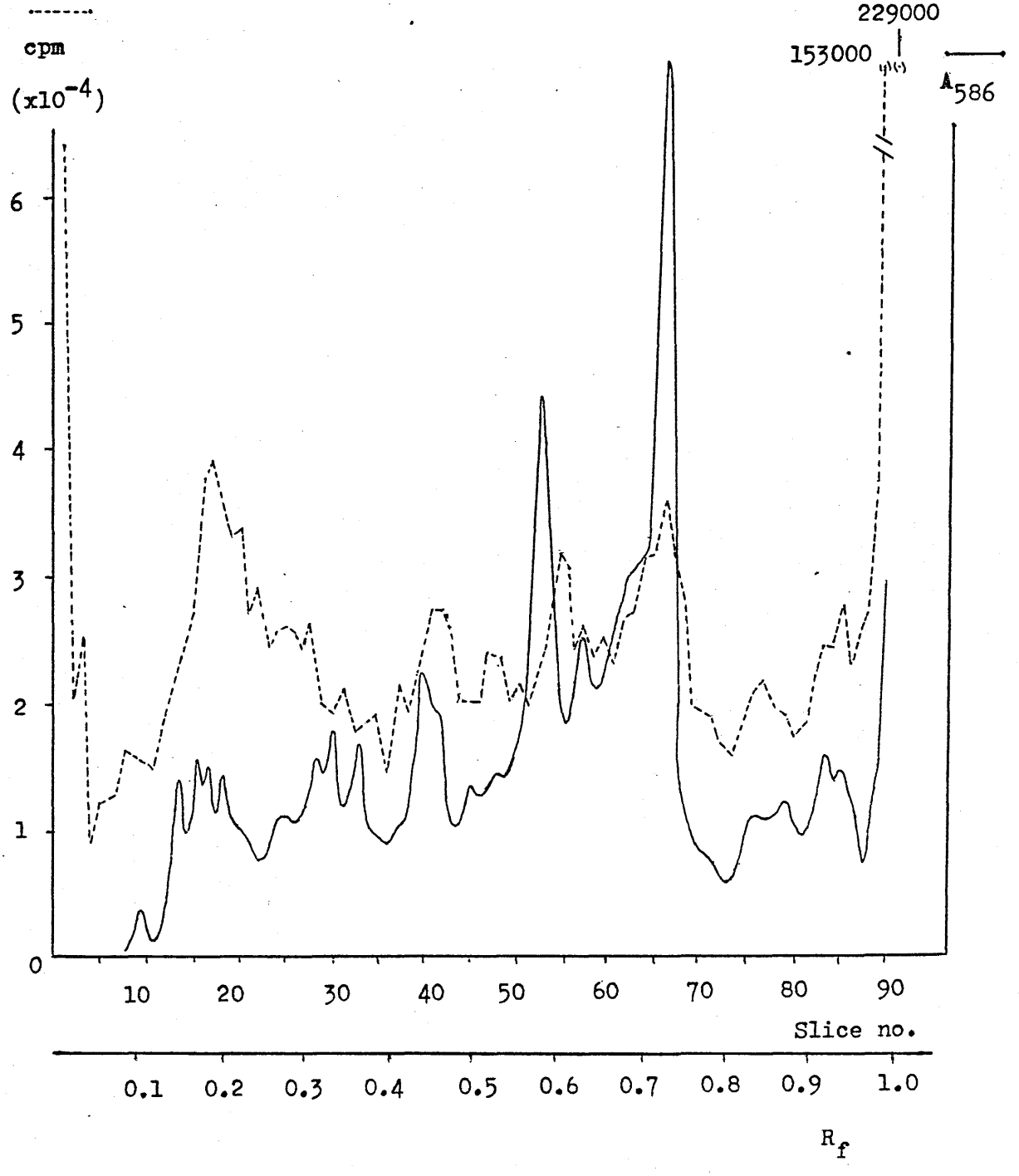


Figure 20. A9 Feeder Layer Keratinocyte Culture Labelled With ^{3}H-Leucine



5.8 Keratinocyte Differentiation Quantitated by the Preferential Incorporation of Histidine.

Certain amino acids, such as histidine, cysteine and arginine, are preferentially incorporated into the granular layer of the epidermis, whereas others, such as leucine and phenylalanine, are incorporated preferentially into the basal and spinous layers (Fukuyama and Epstein, 1966). Histidine is thought to be localised in a histidine-rich protein of keratohyalin granules (Sibrack et al, 1974; Murozuka et al, 1979) which may give rise to a basic protein of the stratum corneum (Dale and Ling, 1979). The preferential uptake of histidine by keratinising epidermal cells has been used as a biochemical marker of keratinocyte differentiation. Delescluse et al (1976) quantitated epidermal keratinisation in vitro by labelling duplicate keratinocyte cultures with ^{3}H-histidine and ^{3}H-leucine, and expressed the incorporation relative to the total DNA content of the culture.

This technique has been modified in this project by using different radiolabels on the two amino acids. This permits expression of results as the ratio of counts derived from the two isotopes with no reference to an outside standard. Cultures of epidermal keratinocytes, mitomycin C-treated C13 cells, and feeder layer cultures of keratinocytes plus C13 cells were labelled by incubation in EFC10 containing ^{3}H-leucine and ^{14}C-histidine. Incorporated radioactivity was evaluated by dual channel scintillation counting. The samples were counted using a programme incorporating automatic quench compensation (AQC). An external standard (H-number) method was used to

calculate counting efficiency, allowing conversion of cpm values to dpm. The energy channel limits were selected automatically on the basis of the calculated efficiency to correct for the decrease in photon energy caused by quenching. The average counting efficiency in the ^{3}H channel was 33% and in the ^{14}C channel 71.5%.

The results are expressed in Table XIV. The mean value of the $^{3}H/^{14}C$ ratio is lower in epidermal keratinocyte cultures and in C13 feeder layer keratinocyte cultures than in mitomycin C-treated C13 cells alone. The difference between the mean feeder layer keratinocyte culture ratio and the mean C13 culture ratio was evaluated statistically by significance testing (see section 2.2.10), and was significant at the level $P < 0.001$ ($t = 6.34$, d.f. = 8). The difference between the mean ratios of keratinocytes alone and keratinocyte feeder layer cultures was not significant ($t = 0.783$, d.f. = 8).

These results indicate that epidermal keratinocytes preferentially incorporate histidine, presumably as part of the keratinisation process. This occurs in the absence of fibroblast feeder layers, and is not significantly altered by the presence of feeder layer cells.

The reproducible preferential incorporation of histidine by epidermal keratinocytes in pure culture is in agreement with the partial differentiation observed in these cultures by quantitation of prekeratin (see section 5.5). However, the comparison with killed C13 cells may not be valid, as the mitomycin C treatment may cause changes in the relative uptake

or incorporation of different amino acids. The lack of stimulation of epidermal keratinisation in culture by cells of the C13, BRL and A9 cell lines is consistent with the results obtained by ^{35}S-methionine and ^{3}H-leucine labelling (see section 5.7).

Table XIV. Incorporation of Histidine and Leucine by Keratinocytes in Pure Culture and in Feeder Layer Culture

Cultures were established in 15mm diameter cell culture tray wells of 3 types : 10^5 newborn mouse epidermal keratinocytes, 2 x 10^4 mitomycin C-treated C13 cells, and mixed cultures of both cell types together (10^5 keratinocytes plus 2 x 10^4 C13 cells). After 5 days, 5 replicate cultures of each type were incubated in EFC10 containing 0.5µCi.ml^{-1} ^{3}H-leucine and 0.5µCi.ml ^{14}C-histidine for 2h. Nonincorporated material was removed by 2 washes with 1ml ice-cold 5% TCA, and one wash with 1ml distilled water, and discarded. Acid-insoluble material was extracted by incubation in 1ml 0.1M NaOH at room temperature for 1h, and the extract acidified by addition of 0.2ml 1M HCl. Samples were mixed with 10ml Triton-toluene scintillation fluid and counted for 4 min in a Beckman LS8100 liquid scintillation counter using a dual channel ^{3}H/^{14}C programme. Counting efficiency was calculated by an external standard (H-number) method, using a programme incorporating automatic quench compensation (AQC). The amount of ^{14}C spillover into the ^{3}H channel was therefore constant, and could be subtracted to give a corrected ^{3}H dpm value.

Culture	^{3}H dpm	^{14}C dpm	$^{3}H/^{14}C$	Mean ± S.D.
Epidermal keratinocyte	4111	638	6.44	
	3972	673	5.90	
	3880	603	6.43	6.23 ± 0.231
	4063	659	6.17	
	4373	678	6.45	
C13	1034	124	8.34	
	1039	136	7.64	
	1124	137	8.20	7.86 ± 0.428
	1105	151	7.32	
	895	115	7.78	
Epidermal keratinocyte	5645	878	6.42	
plus C13	5379	845	6.37	
	5727	934	6.13	6.34 ± 0.143
	5596	859	6.51	
	5533	878	6.30	

Chapter 6 - DERMAL-EPIDERMAL INTERACTION IN ORGAN CULTURE

6.1 Introduction

Measurement of total prekeratin suggests that epidermal keratinocytes undergo limited differentiation in culture (see section 5.5), but no de novo synthesis of prekeratin was detected by labelling with radioactive amino acids (see section 5.7). Growth on feeder layers of cell lines C13, BRL and A9 results in no apparent stimulation of keratinocyte proliferation or differentiation (see sections 5.6 and 5.7). Attempts have therefore been made to examine epidermal keratinisation in vitro using an organ culture system which is more closely related to normal skin. This system has been used in experiments aimed at the detection of in vitro keratinisation in an attempt to determine whether epidermal differentiation, if it does occur in culture, requires direct contact with the dermal tissue layer.

Mesodermal-epithelial interaction (see section 1.5) has been studied in many systems using the transfilter organ culture method developed by Grobstein (1957). In this technique the reacting mesoderm and epithelium are separated by a porous filter (usually Millipore or Nucleopore), the porosity of which can be altered in order to permit or exclude penetration by cell processes. This system was modified to investigate the dermal-epidermal interaction in postembryonic skin. Newborn mouse skin was split into dermis and epidermis by trypsinisation, a method known to produce tissues which are metabolically active (Briggaman and Wheeler, 1968), and the two tissues cultured separately on Millipore filters. These filters are lattices of cellulose nitrate which exclude penetration by cell processes but allow the passage of molecules up to the size of virus particles (Grobstein et al, 1957; Meier and Hay, 1975).

Cultures of dermis and epidermis were also set up either recombined directly and grown on filters (cisfilter recombinants) or grown on opposite sides of filters (transfilter recombinants). Whole untrypsinised skin was cultured for comparison.

Protein synthesis by dermis and epidermis in organ culture was quantitated by labelling the cultures with ^{35}S-methionine and ^{3}H-leucine. Extracted proteins were separated by polyacrylamide gel electrophoresis, and radiolabelled polypeptides identified by scintillation counting of gel slices or by fluorography. It was attempted to detect epidermal keratinisation by de novo synthesis of prekeratin polypeptides. In this way, it was hoped to distinguish between any dermis-dependent differentiation which required direct contact between the tissues, and that which depended only on diffusion of some extracellular dermal stimulus.

6.2 Protein Synthesis by Isolated Dermis in Organ Culture

In living animals, removal of split-thickness skin grafts leaving an exposed dermal site is followed by epidermal regeneration from epithelial cells of hair follicles and exocrine glands (Cruikshank, 1974). Similarly, skin pieces grown in organ culture exhibit epiboly, growth of epidermal cells over the cut surface of the dermis (Medawar, 1948). It was therefore important to establish whether isolated dermis grown in organ culture was capable of total or partial epidermal regeneration. This was performed in the first instance by extracting and characterising dermal protein at intervals during 8 days in organ culture. The proteins were separated by polyacrylamide gel electrophoresis and compared with extracts of whole skin.

Dermal strips produced by trypsinisation of newborn mouse skin were therefore grown on sterile Millipore filters, and at 0,2,4 and 8 days thereafter duplicate cultures were extracted with 1% SDS, 1% 2-mercaptoethanol. Polyacrylamide gels of the extracted protein are shown in Figure 21. Gel 1 is an extract of whole trypsinised mouse skin, gel 2 is an extract of trypsin-separated dermis. Both extracts contain a doublet of high molecular weight polypeptides in the region 120,000-150,000MW, which may represent the dermal matrix proteins collagen and elastin (see section 5.4), and a major low molecular weight band at 40,000MW (actin). The skin extract contains a group of epidermal proteins of 58,000-73,000MW (see section 5.4) which includes two polypeptides of 61,000 and 69,000MW. These two bands correspond to previously-characterised prekeratin and act as internal markers. No bands corresponding to the pre-

keratin polypeptides are observed in the extract of dermis. Gels 3,4 and 5 show dermal protein extracted after 2,4 and 8 days in culture, respectively. In none of these gels are the prekeratin polypeptides seen. Thus, isolated dermis grown in organ culture does not produce amounts of epidermal prekeratin which are detectable in this assay. However, the level of prekeratin synthesis may be too low to give an appreciable absorbance on polyacrylamide gels. De novo prekeratin production may be more readily detected by incorporation of radiolabelled amino acids (see below, sections 6.3 and 6.4).

Figure 21. Lack of Synthesis of Epidermal Protein By Isolated Dermis in Organ Culture

Pieces of dermis were prepared by trypsinisation of new-born mouse skin as described in section 2.2.6, and incubated on Millipore filters in EFC10 at 37°C. At 0,2,4 and 8 days thereafter, cultures were extracted by heating the dermal tissue at 100°C for 1h in 0.5ml 1% SDS, 1% (v/v) 2-mercaptoethanol Similarly extracted was a piece of trypsin-treated whole skin. The extracts were centrifuged at 500 x g for 5 min at 4°C, the protein concentrations of the supernatants estimated by the method of Lowry et al (1951) using bovine serum albumin as a standard, and adjusted to 2mg.ml^{-1} by dilution with 1% SDS, 1% (v/v) 2-mercaptoethanol. After addition of glycerol and bromophenol blue, 100µl aliquots were electrophoresed at 4mA per tube for 3h as described in section 2.2.12.

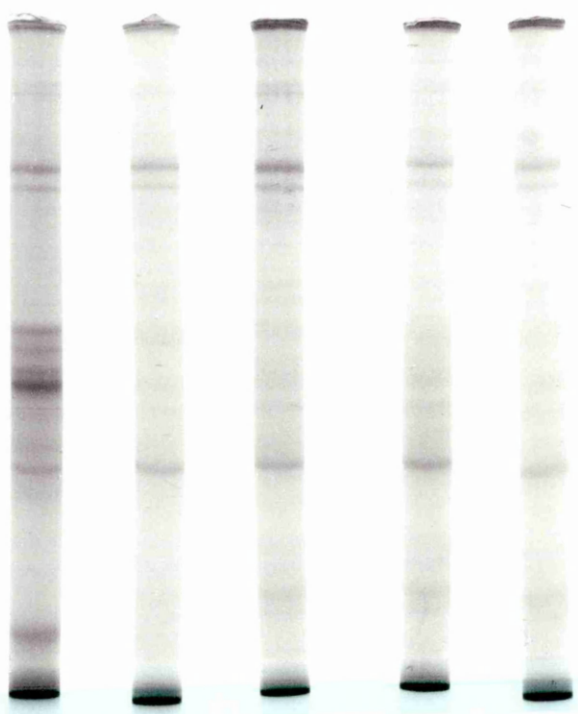
Gel 1. Whole skin

Gel 2. Dermis (day 0)

Gel 3. Dermis (day 2)

Gel 4. Dermis (day 4)

Gel 5. Dermis (day 8)



1. 2. 3. 4. 5.

6.3 Protein Synthesis by Organ Cultures

Newborn mouse skin organ cultures of 5 kinds were established : (i) epidermis, (ii) dermis, (iii) cisfilter recombinant, (iv) transfilter recombinant, (v) whole skin (see section 2.2.6), and incubated at 37°C for 5 days. These cultures were radiolabelled by incubation in EFC10 containing ^{35}S-methionine for 16h, and protein was extracted using 1% SDS, 1% 2-mercaptoethanol. In the case of whole skin cultures, ^{35}S-methionine-labelled epidermis was separated by trypsinisation prior to extraction. As the trypsin treatment may remove some ^{35}S-labelled epidermal protein, the value derived for ^{35}S-methionine incorporation by the epidermis of whole skin may be an underestimate. However, the exclusively intracellular protein prekeratin is unlikely to be affected by external proteolysis.

Duplicate aliquots of each of the extracts were taken for protein estimation and scintillation counting. The amount of radioactivity incorporated per mg protein by each type of culture is given in Table XV. The incorporation of ^{35}S-methionine by dermis (21031 cpm.mg^{-1} protein) was greater than that by any of the epidermal cultures. Epidermis grown alone incorporated 927 cpm.mg^{-1} protein, less than one-twentieth of the radioactivity compared to dermis, and a similar level of incorporation (655 cpm.mg^{-1} protein) was exhibited by epidermis grown transfilter to dermis. Epidermis recombined cisfilter to dermis showed more than twice the ^{35}S-methionine incorporation of epidermis cultured alone (2010 cpm.mg^{-1} protein), and this level was similar to the uptake by the epidermis of whole skin (2116 cpm.mg^{-1} protein).

Aliquots of the extracts containing equal amounts of protein were electrophoresed on a polyacrylamide slab gel, and fluorography performed on the dried gel. Two fluorographs are reproduced in Figure 22. Figure 22(a) shows a fluorograph exposed to the gel for 10 days. The tracks loaded with dermal extract (2 and 9) are very heavily labelled, and no individual bands can be distinguished. Very little labelling is seen in the tracks containing protein from isolated epidermis and epidermis cultured transfilter to dermis (tracks 1 and 10; 4 and 7). The epidermis of whole skin cultures (tracks 5 and 6) exhibits a higher amount of labelling, including at least two distinct bands, which have R_f values 0.73 (approximately 41,000MW) and 0.65 (approximately 50,000MW). The 41,000MW polypeptide is also a major peak in gel slice profiles of ^{35}S-methionine-labelled epidermis, and is likely to be actin (see below, section 6.4). The labelled polypeptide of 50,000MW is not a major component on absorbance profiles of cultured epidermis (see below, section 6.5) and therefore may be methionine-rich, or a protein synthesised in larger amounts by epidermis in culture than <u>in vivo.</u> No bands were observed with R_f values corresponding to the molecular weights of the two prekeratin polypeptides (61,000 and 69,000MW). A similar pattern of labelling is also shown by cisfilter organ cultures (tracks 3 and 8). Therefore, epidermis cultured in direct contact with dermis exhibits uptake of ^{35}S-methionine into proteins similar to those of the epidermis of whole skin, although no synthesis of prekeratin is detected in either culture.

In Figure 22(b), the fluorograph was exposed for only

1 day in order to resolve bands on the dermal tracks (2 and 9). A similar labelling pattern is observed to that of the epidermis of intact skin [tracks 5 and 6, Figure 22(a)], with bands corresponding to molecular weights of 41,000 and 50,000, which coelectrophorese with the epidermal polypeptides. It is not possible to assign exact molecular weights to particular labelled bands, because of shrinkage of the gel during preparatio for fluorography, but the heavily-labelled actin band can be used as an internal molecular weight marker.

In conclusion, culture of epidermis in direct contact with dermis results in a level of protein synthesis comparable with that of the epidermis of intact skin in culture. Epidermis separated from dermis by a permeable filter results in no enhancement of protein synthesis above that of isolated epidermis This contact-dependent stimulation by dermis of epidermal protein synthesis in culture may be analogous to the nutritive and inductive dermal-epidermal interaction <u>in vivo.</u>

Table XV. Incorporation of ^{35}S-Methionine By Mouse Skin Organ Cultures

Organ cultures were established as in section 2.2.6. After incubation at 37°C for 5 days, cultures were incubated in methionine-depleted EFC10 for 8h. This medium consisted of EFC10 containing 1/100th the normal concentration of methionine, and supplemented with 10% foetal calf serum which had been dialysed against 3 changes of 100 volumes of 0.9% NaCl at 4°C over 24h. The cultures were then incubated in methionine-depleted EFC10 containing 20μCi.ml^{-1} ^{35}S-methionine for 16h. The epidermis was removed, washed 3 times in BSS, and extracted by heating at 100°C for 1h in 0.5ml 1% SDS, 1% (v/v) 2-mercaptoethanol. Cultures of isolated dermis were similarly extracted. For cultures of whole skin, the epidermis was separated by incubation of the skin in 0.25% trypsin in PBS at 37°C for 1h. The extracts were centrifuged at 500 x g for 15 min to remove insoluble material. Two 20μl aliquots of the supernatant of each extract were taken : one was used to determine the protein concentration by the method of Lowry *et al* (1951), using bovine serum albumin as a standard; and the second was mixed with 6ml Triton-toluene scintillation fluid and counted for 4 min in an Intertechnique SL30 liquid scintillation spectrometer.

Culture	Protein concn. ($mg.ml^{-1}$)	cpm	$cpm.mg^{-1}$
Epidermis	1.8	1669	927
Dermis	4.2	88331	21031
Cisfilter	1.9	3820	2010
Transfilter	2.3	1506	655
Skin	2.2	4654	2116

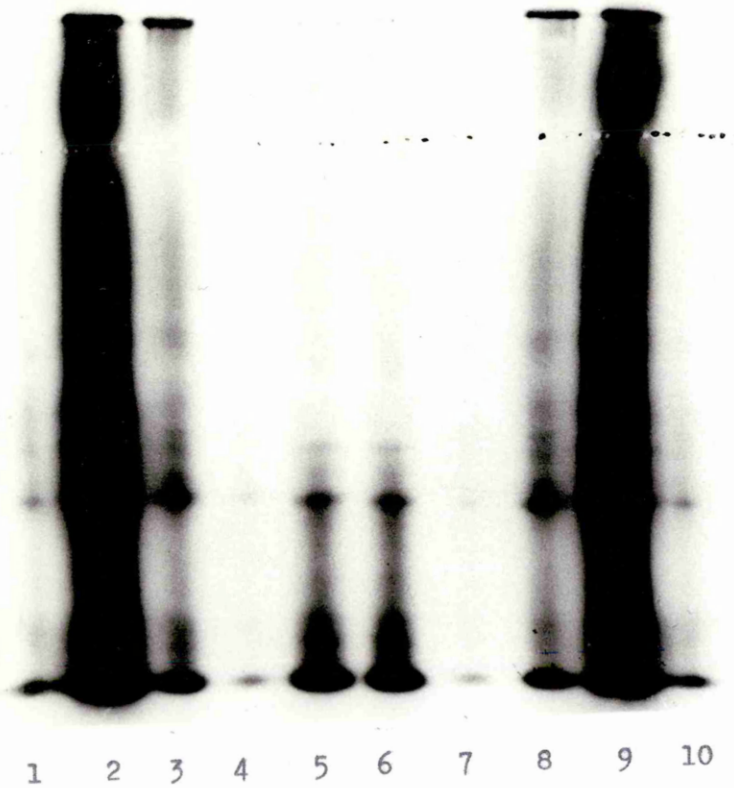
Figure 22. Characterisation of Protein Synthesised By Newborn Mouse Skin Organ Cultures Using ^{35}S Fluorography.

Extracts of newborn mouse skin organ cultures prepared as described in the legend to Table XV were adjusted to a protein concentration of 1.8mg.ml^{-1} by dilution with 1% SDS, 1% (v/v) 2-mercaptoethanol. 100µl aliquots of the adjusted extracts were added to 100µl glycerol and 10µl bromophenol blue, and 10µl amounts of the mixtures were electrophoresed on polyacrylamide slab gels as described in section 2.2.13. Fluorography was performed on the dried gel (see section 2.2.14). The gel was exposed to film for either 1 day or 10 days.

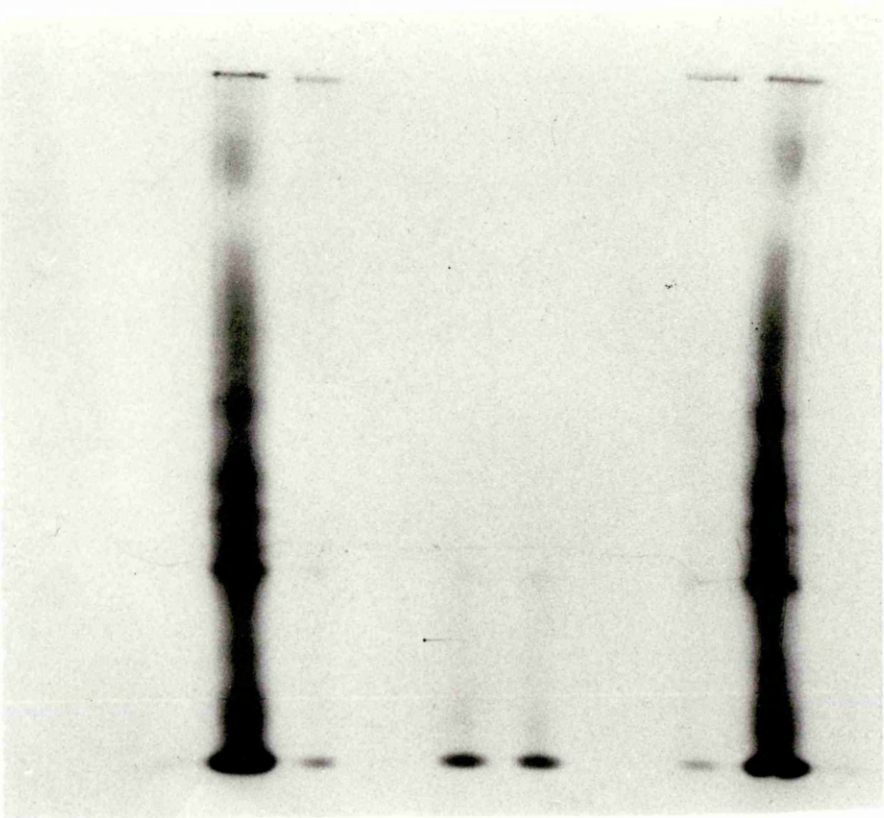
(a) Film exposed to gel for 10 days

(b) Film exposed to gel for 1 day

Tracks 1 and 10	Isolated epidermis
Tracks 2 and 9	Isolated dermis
Tracks 3 and 8	Cisfilter recombinant epidermis
Tracks 4 and 7	Transfilter recombinant epidermis
Tracks 5 and 6	Epidermis of whole skin.



(a).



1 2 3 4 5 6 7 8 9 10

(b).

6.4 Characterisation of Epidermal Proteins Synthesised in Organ Culture - ^{35}S-Methionine Labelling.

In order to characterise further the proteins synthesised by skin organ cultures, cultures labelled with ^{35}S-methionine were extracted with 1% SDS, 1% 2-mercaptoethanol and the extracted proteins separated by gel electrophoresis. The gels were sliced and the incorporated radioactivity quantitated by scintillation counting. Radioactivity profiles of gels of ^{35}S-methionine incorporated into isolated dermis, isolated epidermis, cisfilter epidermis, transfilter epidermis and whole skin epidermis are shown in Figures 23-27.

A large amount of uptake into dermis was observed (Figure 23) confirming the previously-observed higher biosynthetic activity of this tissue (see section 6.3). No major peaks were observed in the prekeratin banding region (61,000-69,000MW; R_f 0.52-0.57) but major peaks were seen at R_f values 0.16 (170,000MW) [presumably collagen, elastin, or other dermal matrix macromolecules - see section 5.4], and at 0.74 (40,000MW), which is probably actin. Isolated epidermis (Figure 24) exhibited very little protein synthesis, although a small peak of radioactivity at R_f 0.53 (67,000MW) may indicate new prekeratin synthesis. Epidermis grown transfilter to dermis was very similar (Figure 26) and no stimulation of epidermal methionine incorporation by possible dermal diffusible substances was observed. Epidermis cultured in direct contact with dermis (Figure 25) again showed a higher general level of ^{35}S-methionine uptake, although less than that observed in the epidermis of whole skin (Figure 27). The latter exhibited a major peak of radioactivity at R_f 0.70 (44,000MW), which was also the major peak of incorporation in cisfilter epidermis. This may be actin, which has been

shown to occur in epidermal cells (Steinert et al, 1976). In both whole skin and cisfilter epidermis, no major peaks occur in the prekeratin region.

This experiment confirms that epidermis cultured in direct contact with dermis exhibits a higher general level of protein synthesis than isolated epidermis. This effect is not observed in epidermis separated from dermis by a permeable filter. However, the amount of uptake of ^{35}S-methionine by these organ cultures is very low, resulting in low counts from polyacrylamide gel slices. It is therefore more difficult to distinguish peaks of radioactivity corresponding to specific proteins. Far higher levels of ^{35}S-methionine incorporation (approximately 1000-fold) are observed in dispersed cell cultures of epidermal keratinocytes (see section 5.7) as the growth of these cells is not constrained by a 3-dimensional tissue organisation, but the amount and nature of the protein synthesised in organ culture may be a more accurate reflection of protein synthesis in vivo.

Figures 23-27. Characterisation of Protein Synthesised by Organ Cultures Labelled with ^{35}S-Methionine

Organ cultures were established as described in section 2.2.6, consisting of isolated dermis, isolated epidermis, cisfilter recombinant, transfilter recombinant, and whole skin. These were incubated at 37°C in EFC10 for 5 days, and then incubated in methionine-depleted EFC10 (see the legend to Table XIV) for 4h. The cultures were radiolabelled by incubation in EFC10 containing 20µCi.ml^{-1} ^{35}S-methionine for 4h, then the epidermis was removed and washed 3 times in BSS. In the case of whole skin, the epidermis was separated by immersion of the skin in 0.25% trypsin in PBS at 37°C for 1h. The epidermal tissue and the isolated dermis were extracted by heating at 100°C for 1h in 0.5ml 1% SDS, 1% (v/v) 2-mercaptoethanol, and the extract centrifuged at 500 x g for 15 min at 4°C to remove insoluble material. The protein concentration of the supernatants was estimated by the method of Lowry et al, (1951), using bovine serum albumin as a standard, and adjusted to 2 mg.ml^{-1} by dilution with 1% SDS, 1% (v/v) 2-mercaptoethanol. Aliquots of the 5 adjusted samples were added to equal volumes of glycerol and 1/20th volume 0.05% bromophenol blue, and 100µl aliquots of the mixtures electrophoresed for 3h at 4mA per tube on 7.5% polyacrylamide gels (see section 2.2.12). After destaining the gels were frozen by sprinkling with powdered solid CO_2 and sliced into 1mm sections using a Mickle gel slicer. The slices were solubilised in 1ml 0.5 M perchloric acid at 60°C for 16h, then added to 6ml

Triton-toluene scintillation fluid and counted for 4 min in a Beckman LS8100 liquid scintillation spectrometer.

Figure 23 Isolated dermis

Figure 24 Isolated epidermis

Figure 25 Cisfilter recombinant epidermis

Figure 26 Transfilter recombinant epidermis

Figure 27 Epidermis of whole skin

The interrupted line represents the scintillation counter background level cpm.

Figure 23. Dermal Organ Culture Labelled With ^{35}S-Methionine

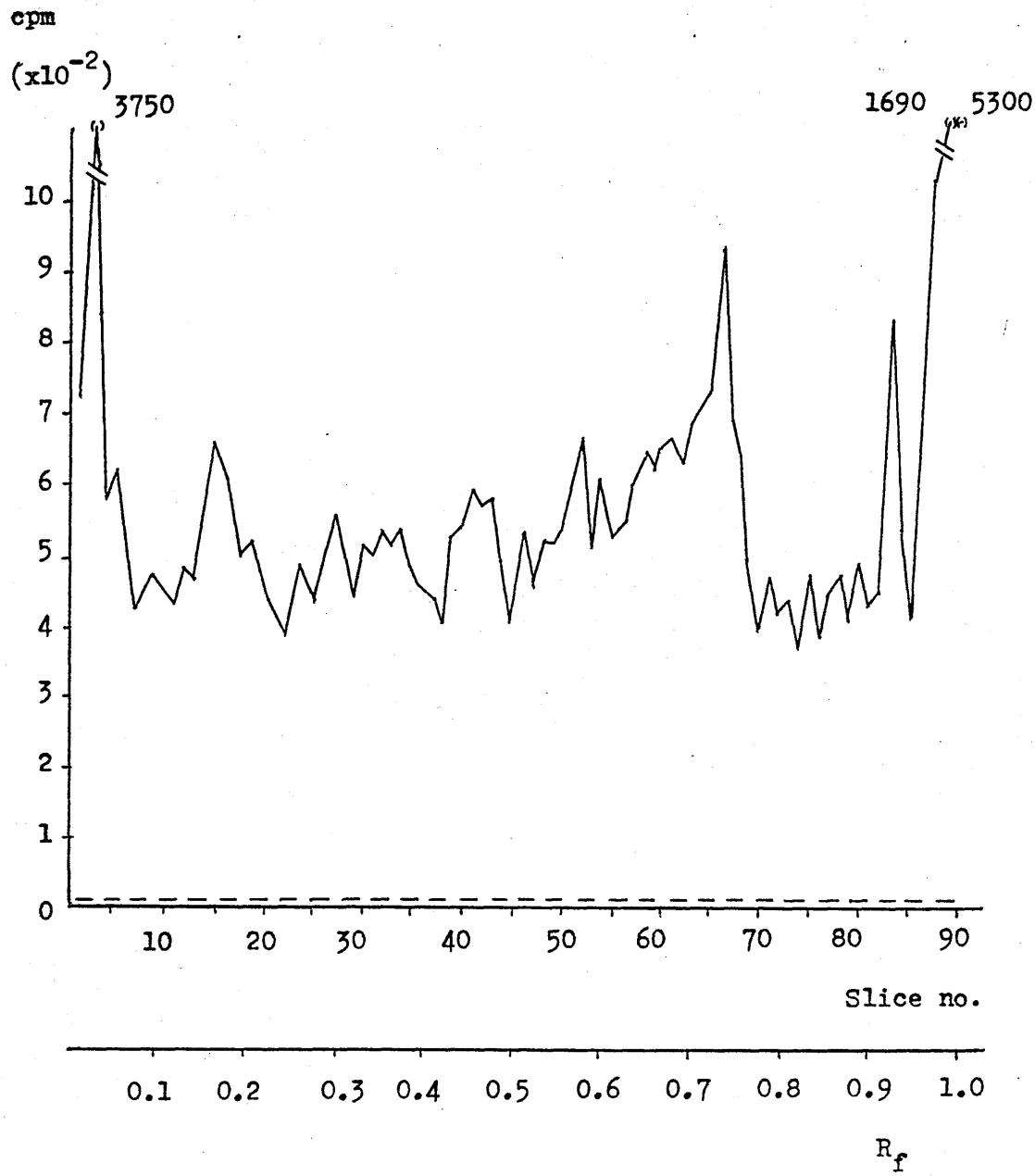


Figure 24. Epidermal Organ Culture Labelled With ^{35}S-Methionine

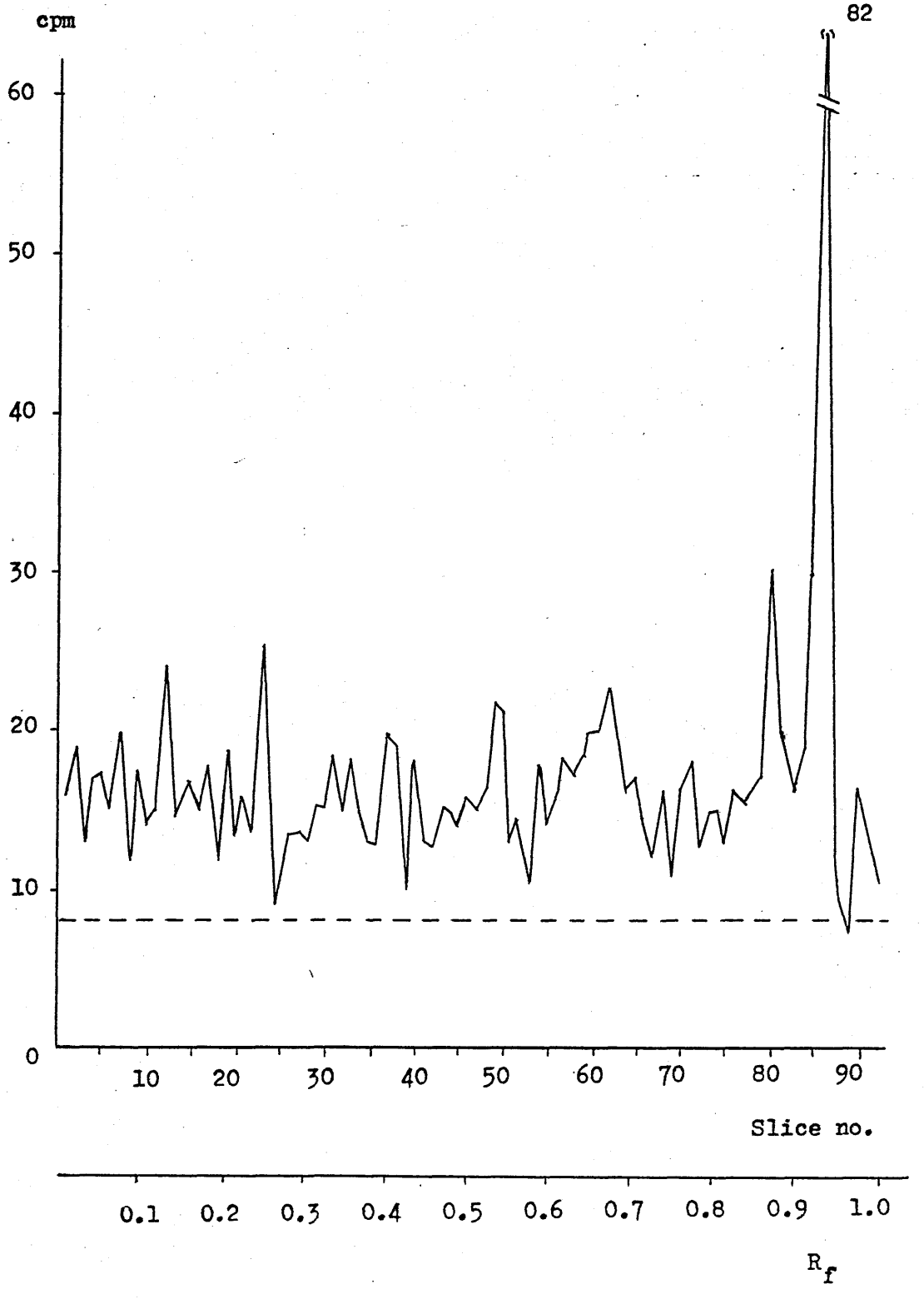


Figure 25. Cisfilter Dermal-Epidermal Recombinant Organ Culture Labelled With ^{35}S-Methionine

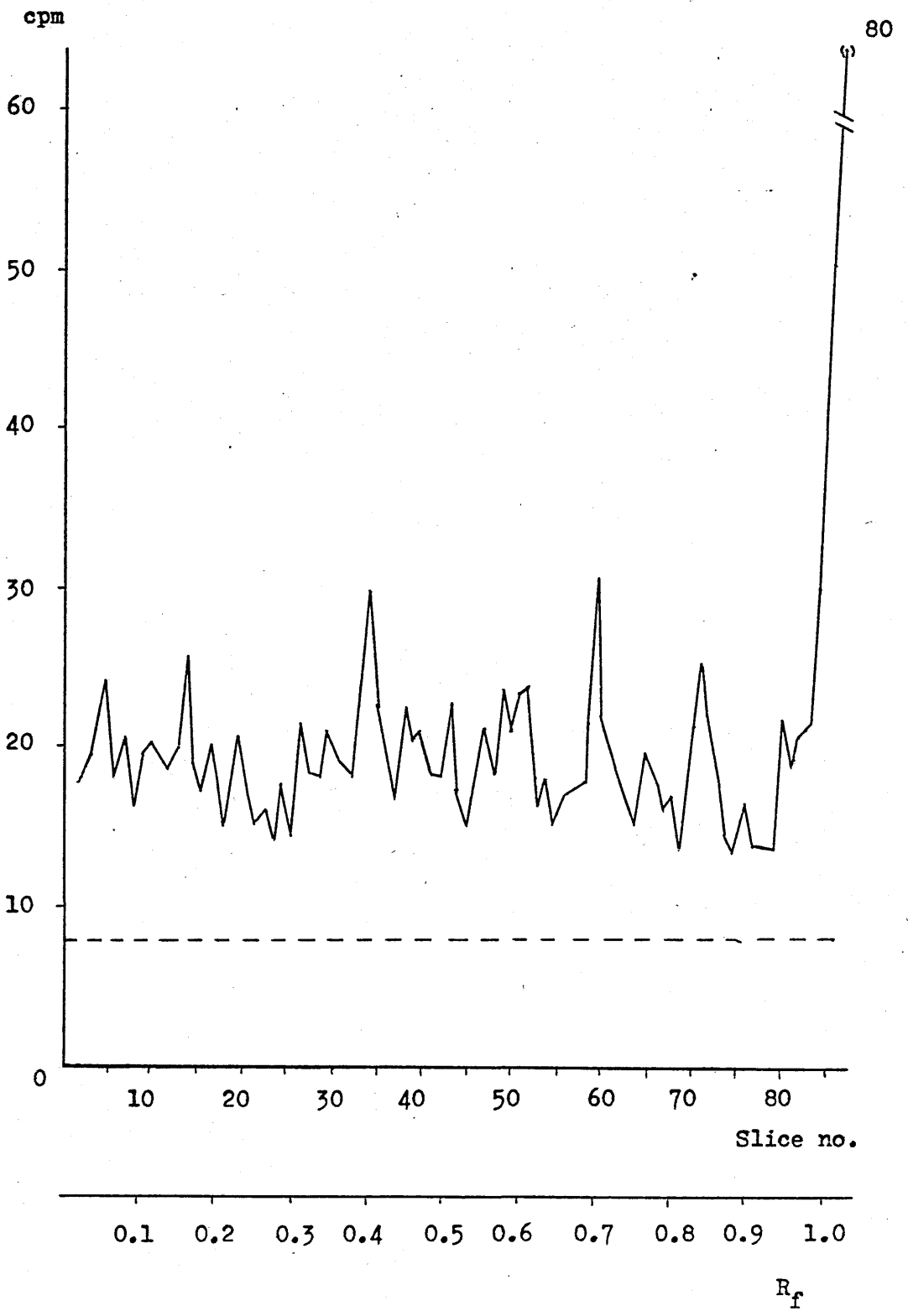


Figure 26. Transfilter Dermal-Epidermal Recombinant Organ Culture Labelled With ^{35}S-Methionine

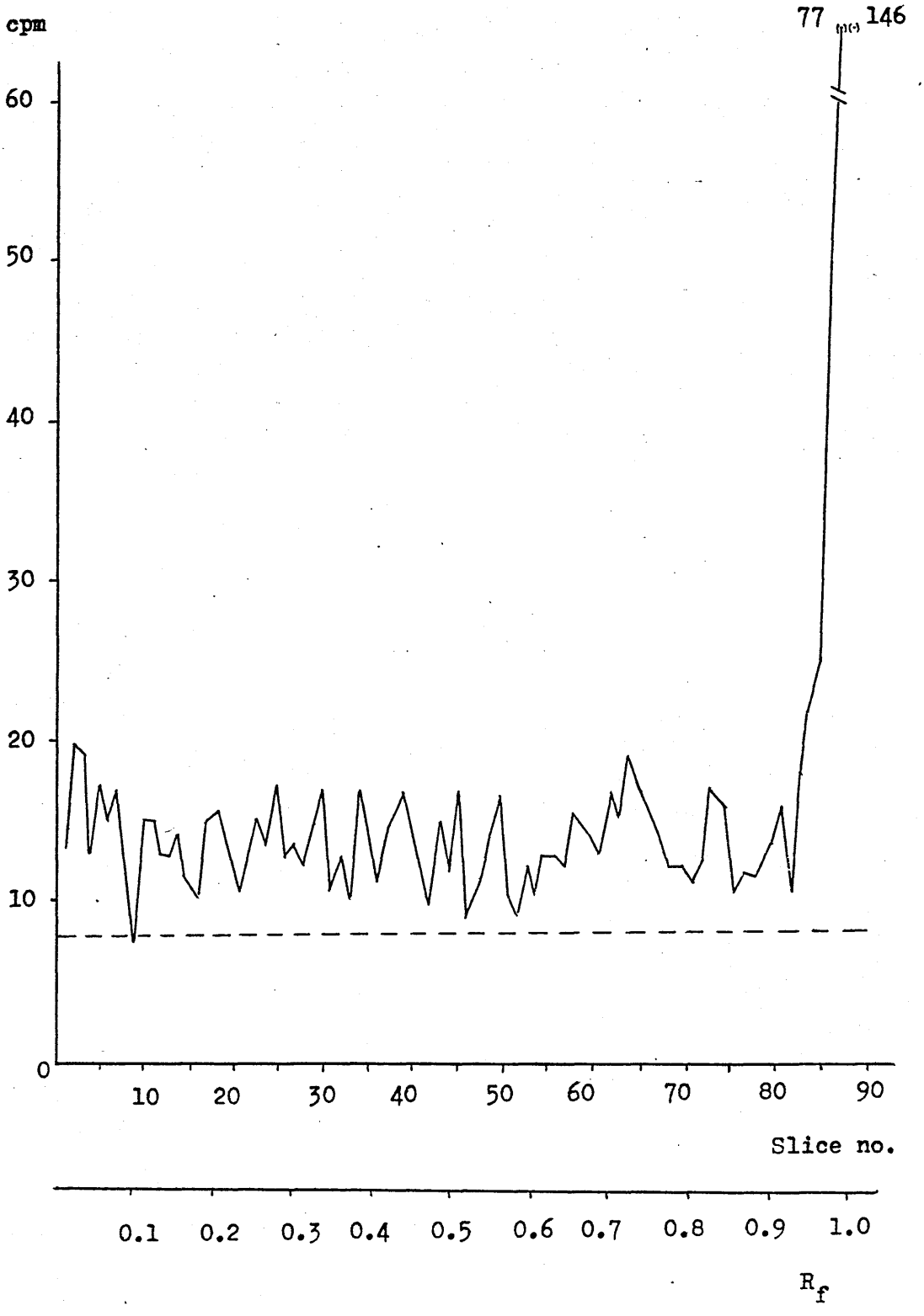
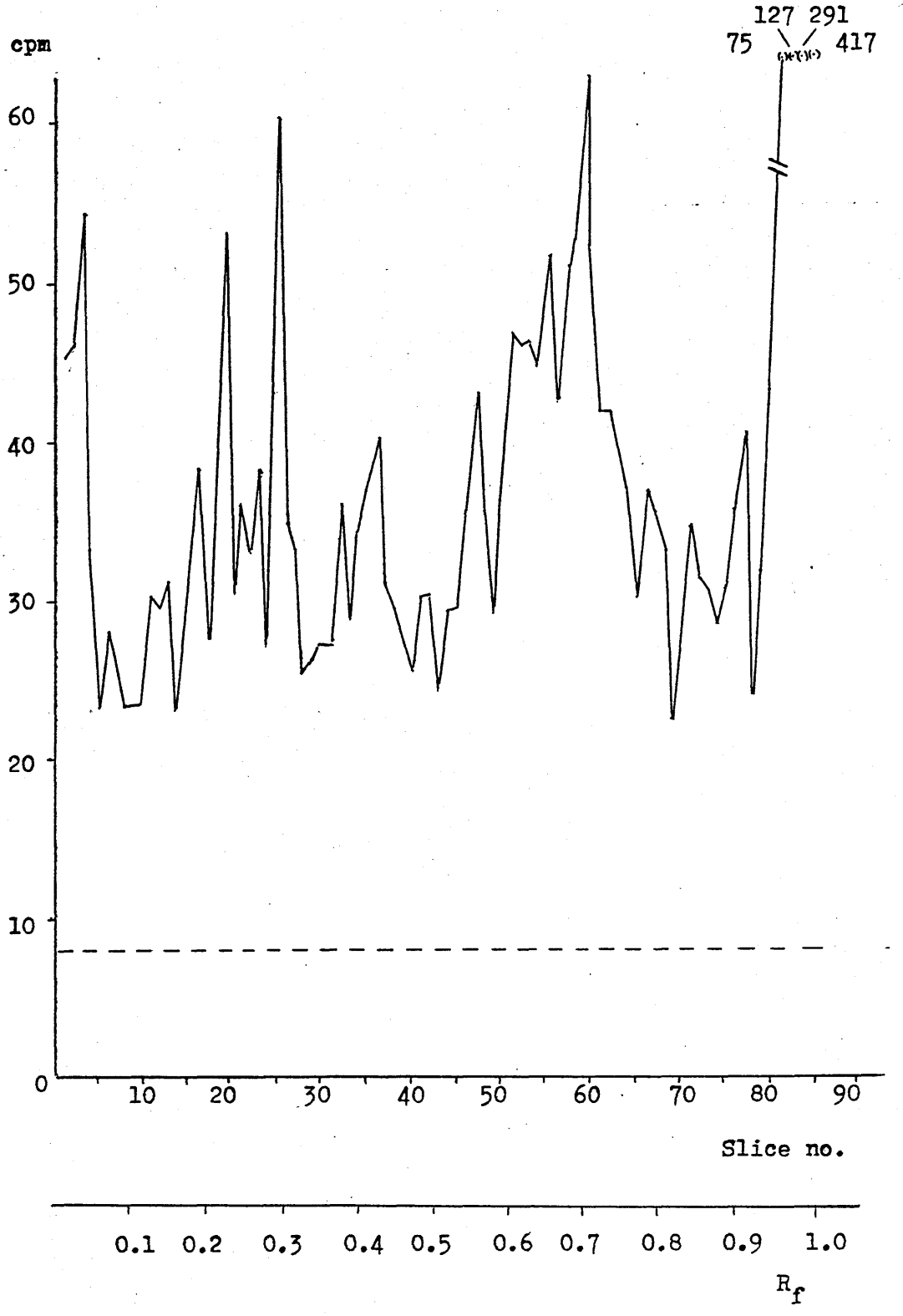


Figure 27. Epidermis of Whole Skin Organ Culture Labelled With ^{35}S-Methionine



6.5 Characterisation of Epidermal Proteins Synthesised in Organ Culture - ^{3}H-Leucine Labelling.

Skin organ cultures consisting of isolated epidermis, isolated dermis, cisfilter epidermis, transfilter epidermis and whole skin were radiolabelled by incubation in EFC10 containing ^{3}H-leucine. Protein was extracted with 1% SDS, 1% 2-mercaptoethanol, and separated by polyacrylamide gel electrophoresis. The gels were sliced and incorporated radioactivity estimated by scintillation counting. The results are shown in Figures 28-32. Dermis exhibited considerable labelling, in a number of well-defined peaks (Figure 28) the principal of which had R_f values 0.33 (110,000MW), 0.43 (86,000MW), 0.56 (62,000MW) and 0.66 (49,000MW) None of these corresponded to the major peaks of ^{35}S-methionine incorporation by dermal organ cultures (Figure 23).

^{3}H-leucine incorporation by isolated epidermis (Figure 29) and by epidermis transfilter to dermis (Figure 31) did not significantly exceed the background level of radioactivity. Cisfilter epidermis exhibited increased incorporation, including a small peak of radioactivity corresponding to the prekeratin absorbance peak (Figure 30). Epidermis of whole skin (Figure 32) showed higher general levels of protein synthesis, with a radioactivity peak corresponding to the prekeratin absorbance maximum, and another major peak at R_f 0.70 (44,000MW), which also may occur in the cisfilter culture, and is probably actin (see section 5.4).

Labelling of newborn mouse skin organ cultures with ^{3}H-leucine confirms the observation made using ^{35}S-methionine

incorporation, that protein synthesis in separated epidermis is stimulated by direct contact with dermis. However, possibly because of the low uptake of radiolabelled amino acids by epidermis in organ culture, it has not been possible to demonstrate induction of epidermal prekeratin synthesis by contact with dermis. The significance of these results is discussed below (see section 7.9).

Figures 28-32 Characterisation of Protein Synthesised in Organ Cultures Labelled with ^{3}H-Leucine

Organ cultures were established as described in section 2.2.6. After 5 days the cultures were incubated in leucine-depleted EFC10 (1/100th normal Leucine concentration) for 2h, then radiolabelled by incubation in leucine-depleted EFC10 containing 50μCi.ml^{-1} ^{3}H-leucine for 5h at 37°C. The epidermal and dermal tissues were extracted, electrophoresed and counted as described in the legend to Figures 23-27.

Figure 28. Isolated dermis

Figure 29. Isolated epidermis

Figure 30. Cisfilter recombinant epidermis

Figure 31. Transfilter recombinant epidermis

Figure 32. Epidermis of whole skin

The interrupted line represents the scintillation counter background cpm.

Figure 28. Dermal Organ Culture Labelled With ^{3}H-Leucine

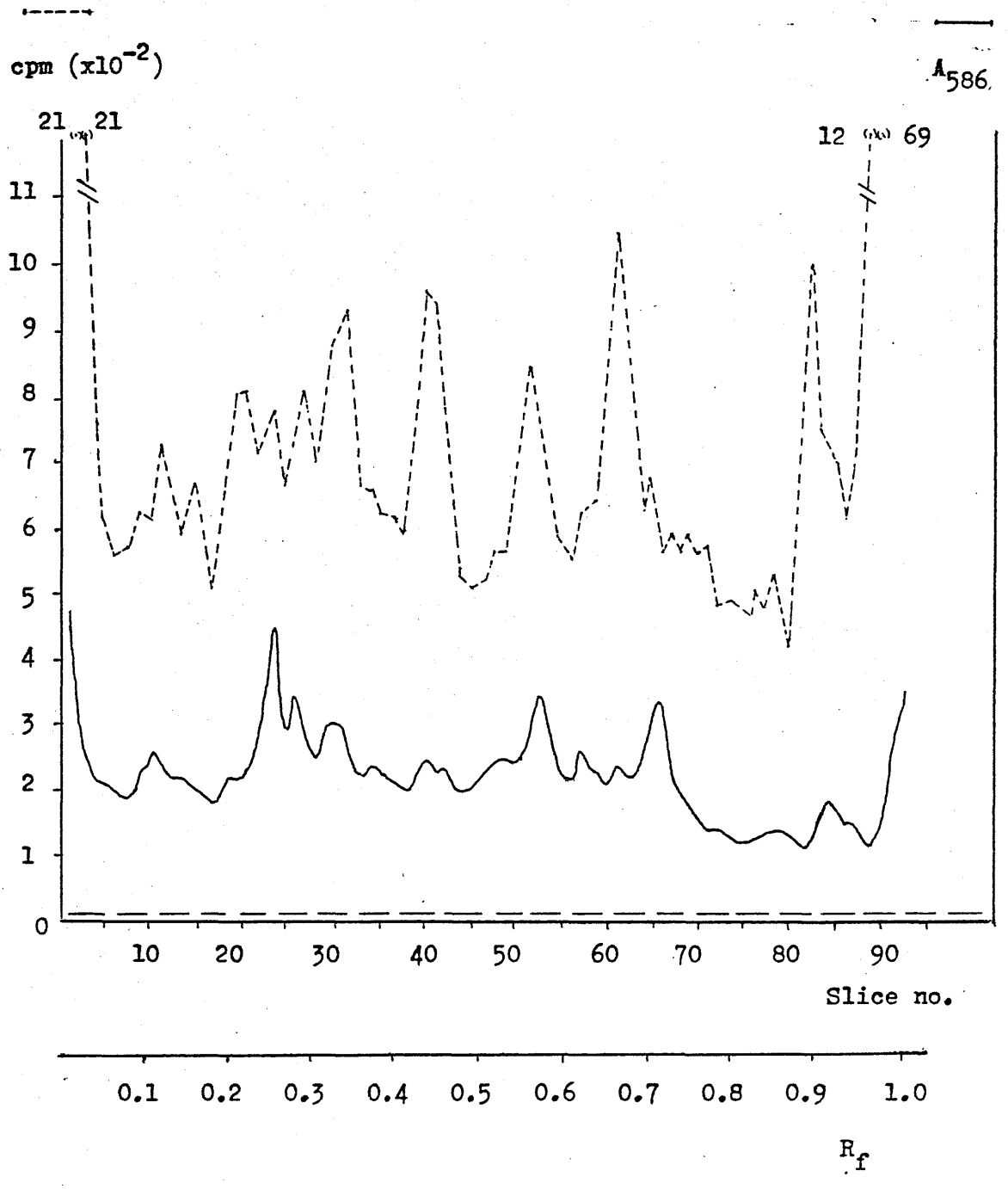


Figure 29. Epidermal Organ Culture Labelled With ^{3}H-Leucine

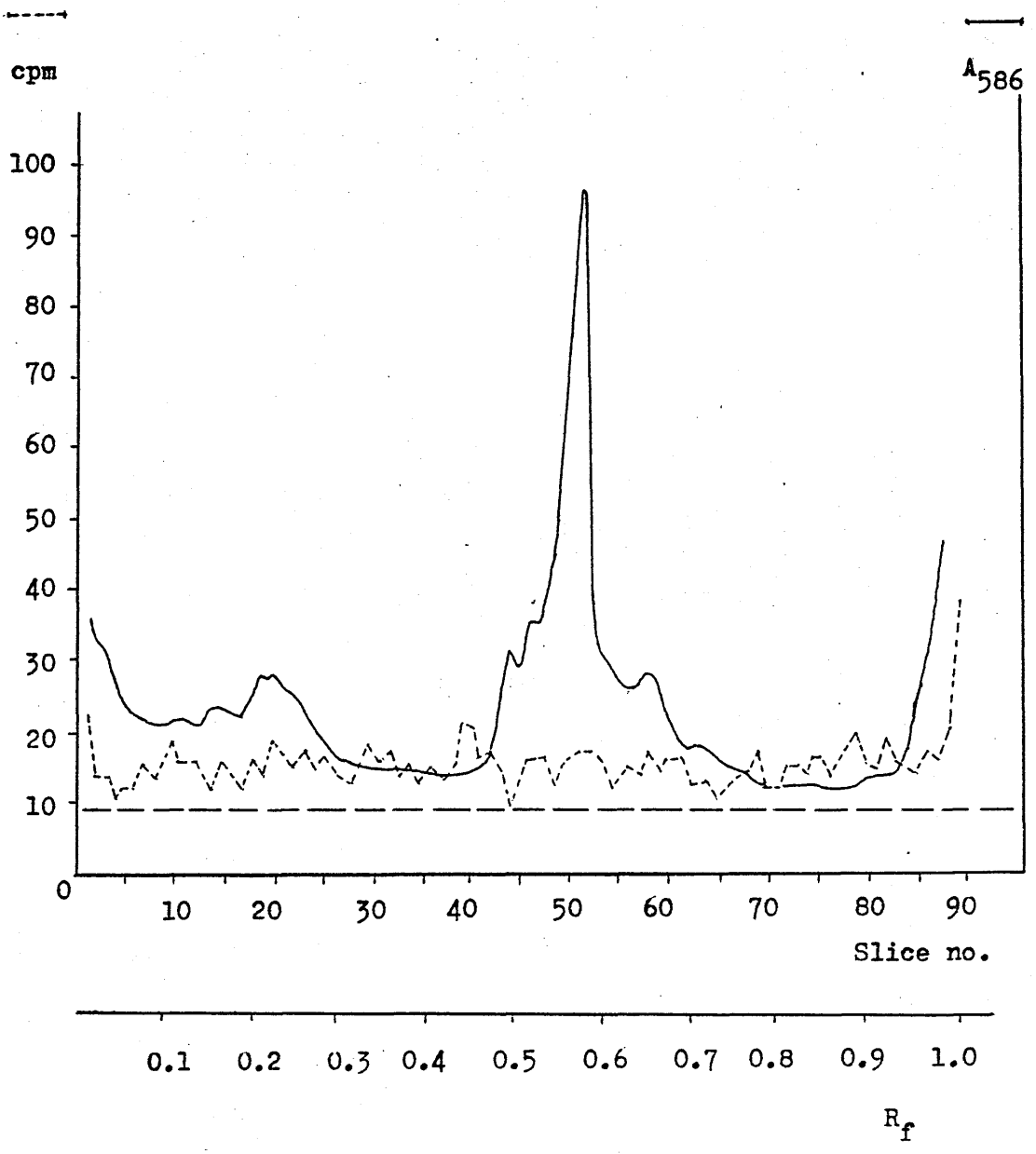


Figure 30. Cisfilter Dermal-Epidermal Recombinant Organ Culture Labelled With ^{3}H-Leucine

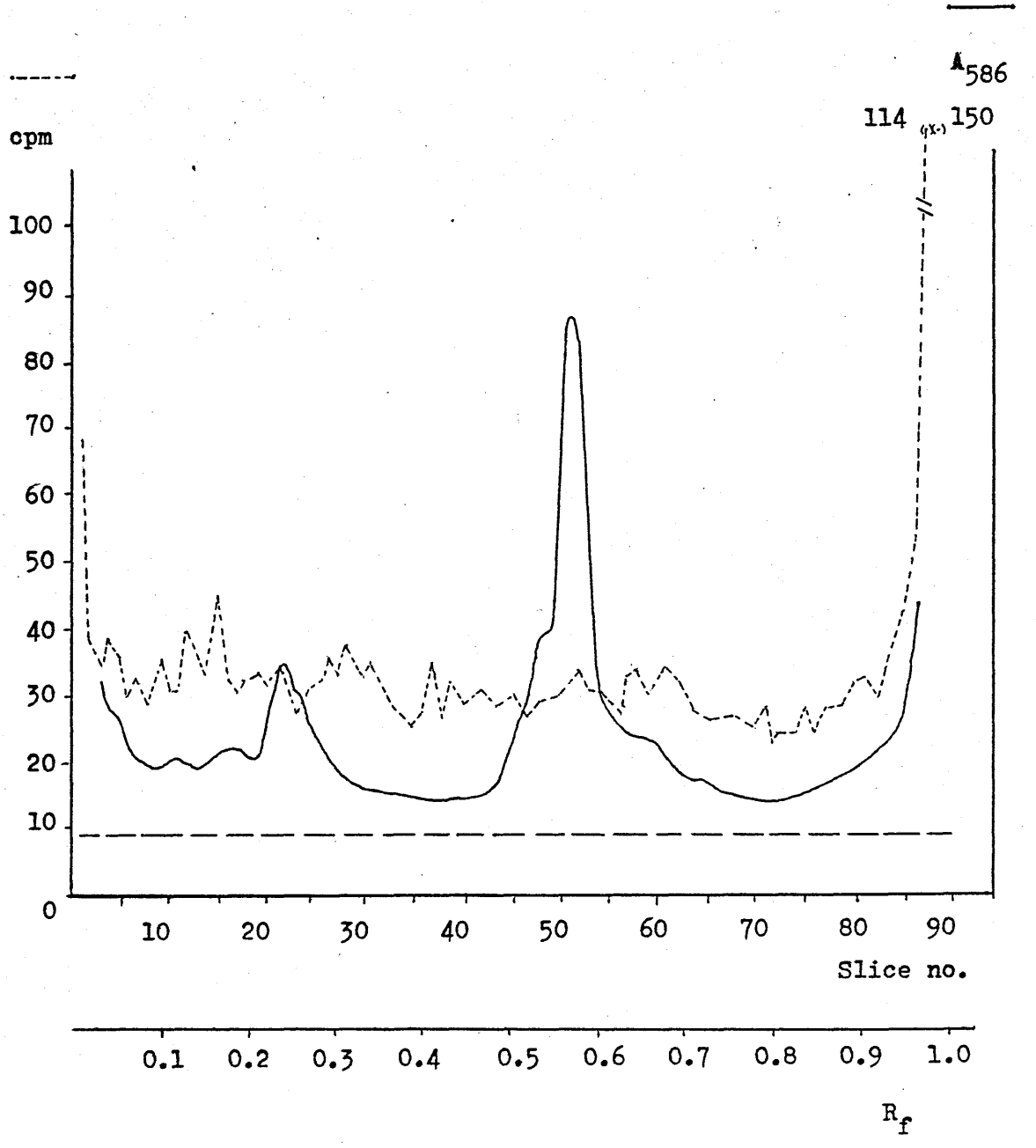


Figure 31. Transfilter Dermal-Epidermal Recombinant Organ Culture Labelled With ^{3}H-Leucine

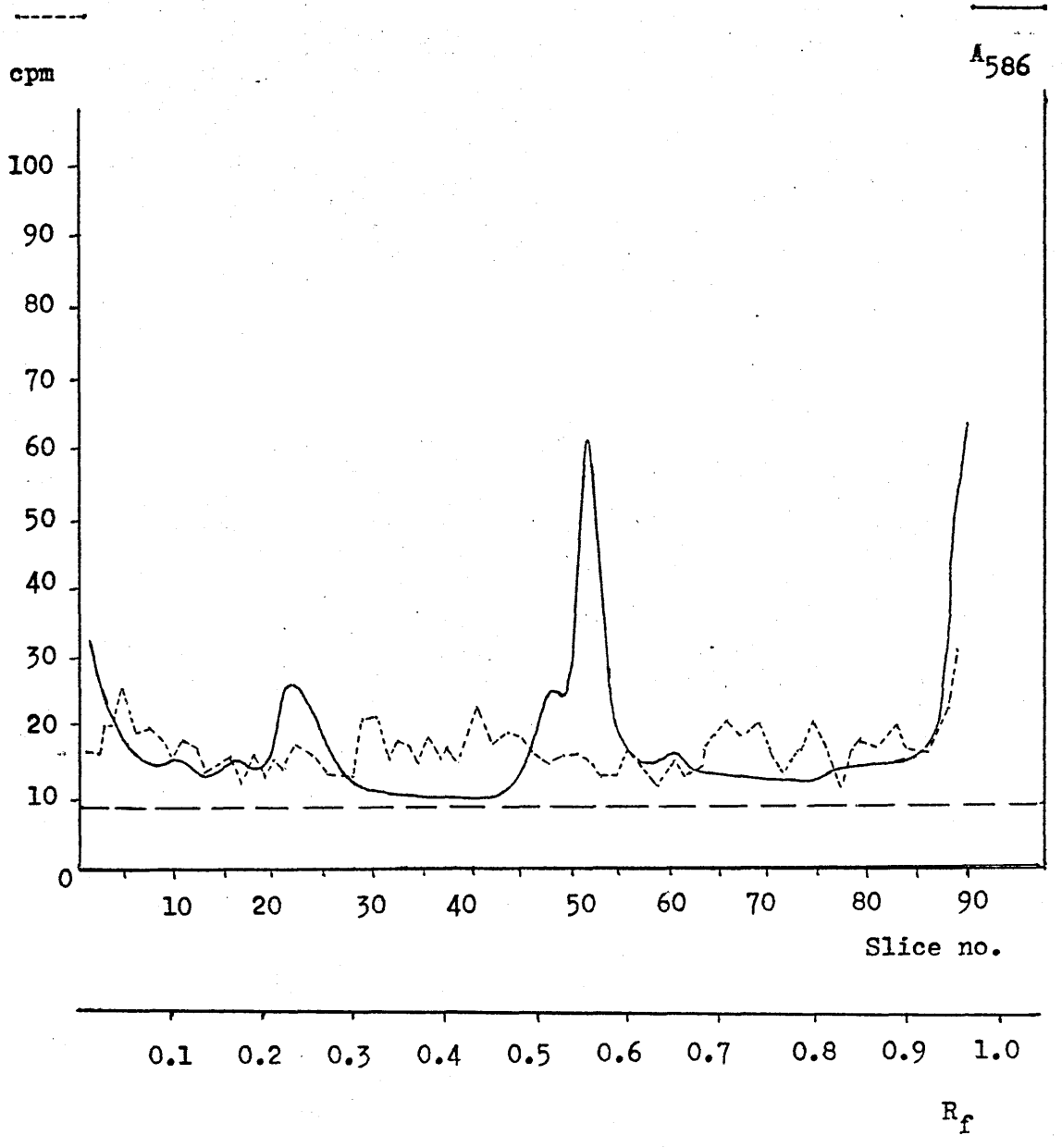
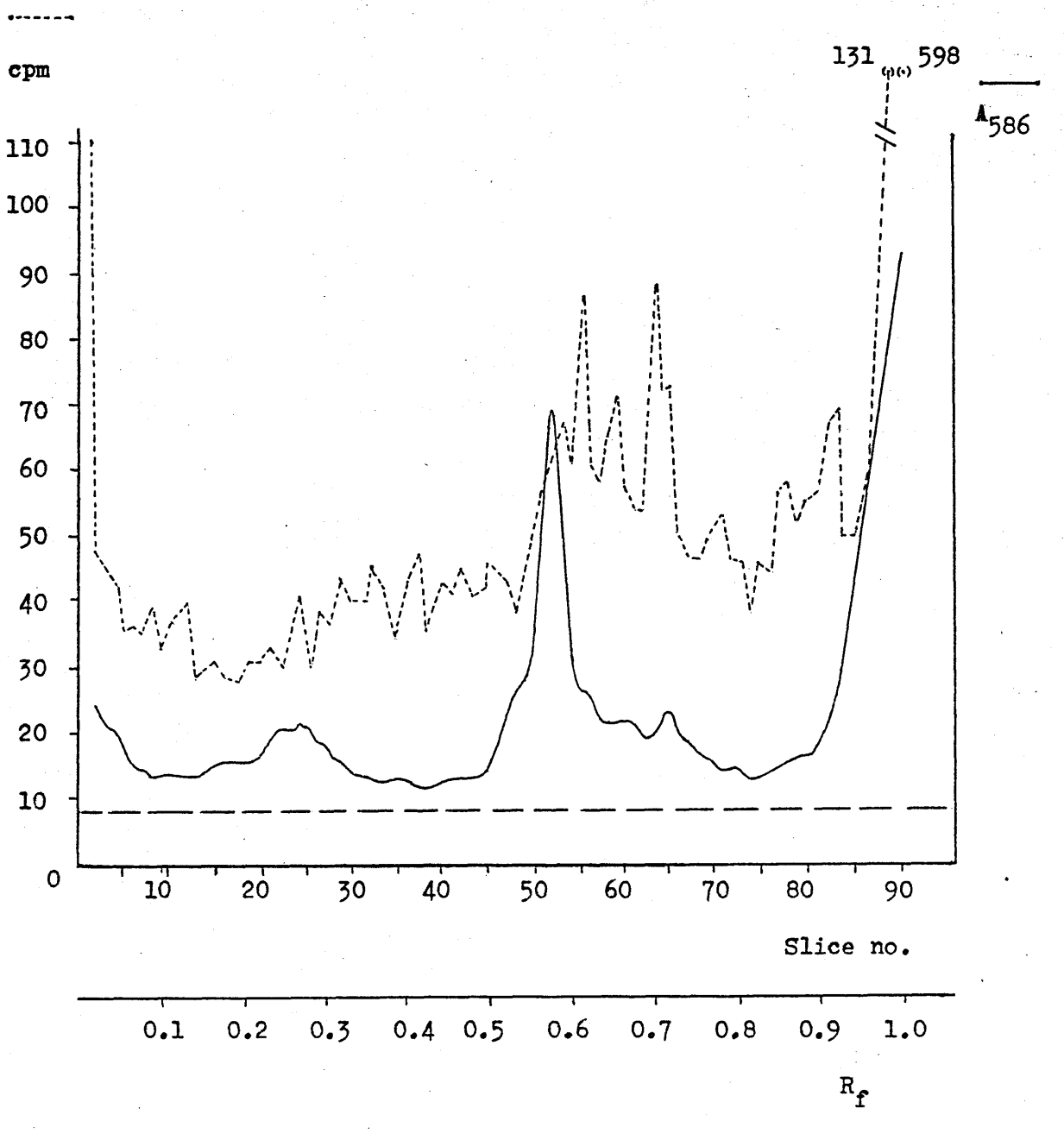


Figure 32. Epidermis of Whole Skin Organ Culture Labelled With ^{3}H-Leucine



6.6 Lack of Transfer of Dermal Tissue to Epidermis in Cisfilter Organ Culture Recombinants

Apparent dermal stimulation of epidermal protein synthesis in organ culture has been observed by labelling with ^{35}S-methionir and ^{3}H-leucine. This phenomenon could be due to the attachment of dermal tissue to the epidermis during dermal-epidermal separation at the end of the labelling period. The extent of transfer of dermal cells to epidermis was measured by radio-labelling the dermis with ^{3}H-thymidine prior to recombination with epidermis both in the cisfilter and transfilter orientations.

Pieces of trypsin-separated dermis were labelled by incubation in EFC10 containing ^{3}H-thymidine for 1h, washed, and placed in cisfilter and transfilter culture with epidermis. At various times thereafter the epidermis was removed and its dry weight estimated. The tissue was solubilised with 1M hyamine hydroxide and counted. Results expressed as dpm per mg dry weight epidermis are given in Table XVI. After 2 days in culture, the radioactivity in epidermis grown in cisfilter and transfilter culture were similar. At 4 days, the values were again similar, although both had decreased from the day 2 values. After culture for 6 days, the cisfilter epidermal dpm had decreased slightly, and the transfilter epidermal dpm had fallen sharply, and was approximately two-thirds of the cisfilter value. This probably indicates degeneration of the epidermis grown in transfilter culture. Therefore, no detectable attachment of dermal cells to epidermis occurs on separation of dermal-epidermal recombinants.

However, this experiment does not exclude the possibility of protein being transferred from dermis to epidermis during coculture. This appears unlikely, as the patterns of labelling of dermis and epidermis are dissimilar (Figures 28 and 32). Also, if dermal protein were transferred in the absence of nucleic acid transfer, the protein presumably would be extracellular matrix protein, which is normally of high molecular weight. In both ^{35}S-methionine and ^{3}H-leucine labelled cisfilter organ cultures (Figures 25 and 30), the stimulation of epidermal protein synthesis occurs over the entire molecular weight range. An alternative possibility, that dermal cells exhibit increased uptake of exogenous amino acids, which are then transferred to epidermal cells via a junctional pathway and incorporated into epidermal protein, cannot be excluded on the basis of the above experiments.

Table XVI Lack of Transfer of Dermal Cells to Epidermis in Cisfilter Organ Culture Recombinants

Newborn mouse skin was separated into dermis and epidermis by trypsinisation (see section 2.2.6). Dermal pieces were labelled by incubation in EFC10 containing $5\mu Ci.ml^{-1}$ ^{3}H-thymidine at $37^{o}C$ for 1h, then washed 3 times in sterile BSS. The dermis was then recombined on Millipore filters with freshly-prepared epidermis in either cisfilter or transfilter orientation (see section 2.2.6), and incubated at $37^{o}C$ in EFC10 containing $10^{-4}M$ nonradioactive thymidine. At 2,4 and 6 days thereafter, the epidermis was removed and the transfer of thymidine-labelled material from dermis estimated. Epidermis was dried at 100 ^{o}C for 6h and the dry weight recorded. The tissue was then solubilised by immersion in 1ml hyamine hydroxide (1M solution in methanol) at $60^{o}C$ for 3h, mixed with 5ml Triton-toluene scintillation fluid, and counted for 4 min in a Beckman LS8100 liquid scintillation counter. Because of colour quenching by the hyamine hydroxide-solubilised epidermis, quench correction analysis was performed by the channels ratio method to convert cpm values to dpm.

Time in culture (Days)	Culture type	Epidermal dpm	Dry weight (mg)	cpm.mg^{-1}
2	Cisfilter	48352	16	3022
	Transfilter	44825	14	3201
4	Cisfilter	18285	9	2032
	Cisfilter	22123	10	2212
	Transfilter	14542	8	1818
	Transfilter	37067	18	2059
6	Cisfilter	31415	15	2094
	Cisfilter	12238	7	1748
	Transfilter	16188	12	1348
	Transfilter	16443	15	1096

Chapter 7 - DISCUSSION

"I hae nae doot some foreign philosopher
Has wrocht a system oot to justify
A' this: but I'm a Scot wha blin'ly follows
Auld Scottish instincts, and I winna try".

Hugh McDiarmid
A Drunk Man Looks At The Thistle

7.1 Communication Between Cells

The gap junction is a ubiquitous structure in the tissues of animals, where it acts as a partial cytoplasmic continuity between adjacent cells. Invertebrate cells possess a morphologically and functionally similar structure, and analogous intercellular connections are found between cells of organisms lower in the evolutionary scale. The evolutionary conservation of the gap junction implies an important biological role for cell-cell communication, but it is not clear what functions are performed by gap junction-mediated communication in multicellular organisms. It has been suggested that junctional channels permit the transmission of developmental signals during enbryogenesis (Wolpert, 1978). There is no direct evidence to support this but it is consistent with the hypothesis that intimate cell-cell contact regions are correlated with the onset of organ development during embryonic induction (Saxén, 1977). The dermal induction of epidermal differentiation in skin, which is an example of a post-embryonic mesodermal-epithelial interaction, is associated with occasional discontinuities in the basal lamina between the two tissues, although no junctional structures have been reported between dermal and epidermal cells (Briggaman and Wheeler, 1975).

In this project it has been attempted to determine the role, if any, of gap junctions in the normal growth and differentiation of skin, and to relate this to the more general question of the biological significance of junctional communication. The first stage of this work was to isolate cells from dermis and epidermis in primary culture, and determine their junction-

forming properties. This was performed by labelling of donor cells with ^{3}H-uridine prior to coculture with unlabelled recipient cells and detecting junctionally-transferred nucleotides by autoradiography. A statistical approach was devised to quantitate the intercellular transfer of radiolabelled molecules. Autoradiographic grains were counted over recipient cells in contact with donors and over recipient cells not in contact with donors, and the two populations compared by significance testing. This method permits quantitation of the degree of transfer between different cells. However, the data obtained by the counting of autoradiographic grains are often rather scattered, and statistical analysis may only be appropriate at a semi-quantitative level. As discussed above (see section 4.3), the recipient cell grain count in these experiments depends not only upon the efficiency of junction formation, but also upon other factors such as cell size and rate of recipient cell nucleotide incorporation. The t-test method used takes account of some of these factors by comparing two similar cell populations recipient cells in contact with donors and recipient cells not in contact with donors. One disadvantage of this method is that bimodal sample distributions alter the derived t values (see section 3.4); however, bimodality can be detected by histogram analysis or by the consequently high standard deviation values.

7.2 Gap Junction Formation Between Cells of Dermis and Epidermis

Study of the transfer of uridine nucleotides between skin cells showed that exchange of nucleotides occurs between dermal

fibroblasts and epidermal keratinocytes in culture (see section 3.2). Therefore, formation of gap junctions between dermis and epidermis of adult skin is a possible mechanism of dermal-epidermal interaction. Direct contacts between dermal fibroblasts and epidermal basal cells in skin are infrequent but do occur (Briggaman and Wheeler, 1975), and may be sufficient to permit transmission of regulatory signal molecules. Theoretical calculations suggest that diffusion of a small (300-500MW) molecule between cells could extend to approximately 1mm (about 50 cell diameters) in 3h, decreasing to 1% of the "source" concentration (Crick, 1970), and gradients of junction-transmitted molecules have been generated over similar distances in 16-24h by cultured cells (Michalke, 1977). Further, the minimum rate of flux of purine nucleotides through cells linked by gap junctions has been estimated at 10^6 molecules per cell pair per second (Pitts and Finbow, 1977).

7.3 Lack of Specificity of Cell Communication in Primary Culture

Nucleotide transfer between cells of dermis and epidermis contrasts with several reports of tissue specificity in gap junction formation; that is, lack of molecular transfer between fibroblastic and epithelial cells, each of which is junction-competent. Primary cultures of human mammary fibroblasts fail to communicate with epithelial cells of the same tissue (Fentiman et al, 1976). Certain malignantly-transformed mammary epithelial cells, however, have been shown to communicate non-specifically with breast fibroblasts (Fentiman and Taylor-Papadimitriou, 1977). C13 fibroblasts and BRL epithelial cells exchange nucleotides much more efficiently in homologous coculture than in heterol-

ogous coculture (Pitts and Burk, 1976). A study of the nucleotide transfer properties of a wide range of cell lines revealed several examples of communication specificity (Gaunt and Subak-Sharpe, 1979). This communication deficiency between cells derived from different tissue layers has been proposed as a means of maintaining the metabolic individuality of different cell layers (Fentiman et al, 1976).

The generality of tissue specificity in cell-cell communication is qualified by several reports of positive junction formation between different cell types. Ionic coupling between various combinations of lens epithelial cells, liver epithelial cells, BHK21 fibroblasts, and two virus-transformed cell lines was demonstrated by electrophysiological techniques (Michalke and Loewenstein, 1971). Primary cultures of mouse myocardial cells and rat ovarian granulosa cells respond coordinately to hormonal stimuli by the intercellular transfer of cyclic AMP (Lawrence et al, 1978). Also, synchronous beating of mouse embryonic myocardial cells was shown to be transmitted through heart fibroblasts (Goshima and Tonomura, 1969). Synchrony could also be mediated by cells of a human amniotic cell line or the monkey kidney epithelial line BSC-1.

In the work reported here, it has been shown that epidermal keratinocytes communicate with dermal fibroblasts from newborn mouse skin and adult guinea pig skin. Also, both primary cell types communiate non-specifically with cell lines derived from mesodermal and epithelial tissue (see sections 3.4 and 3.5). In order to determine whether this lack of specificity was a general property of primary cell cultures, two further mouse

cell types were investigated : renal epithelial cells and epidermal melanocytes. Renal epithelial cells form gap junctions with fibroblast and epithelial cell lines (see section 4.2) ; melanocytes communicate with both epidermal keratinocytes and dermal fibroblasts, and also with fibroblast and epithelial cell lines (see section 4.3). Melanocytes are derived from neural crest, from which they migrate to the epidermis during embryogenesis. These cells are therefore of neurectodermal origin, and the demonstration of junctional transfer with epidermal keratinocytes and dermal fibroblasts provides further support for the hypothesis that cell-cell communication occurs non-specifically between normal cells. Interaction between melanocytes and keratinocytes is also seen in the phenomenon of pigment donation; however, this appears to be an unrelated process involving phagocytosis of melanocyte cytoplasm by the keratinocyte (Okazaki et al, 1976).

These results suggest that tissue specificity of communicatio is an exceptional rather than a general occurrence. This is in agreement with the apparent universality of the gap junction structure. The biochemistry of the junction has been examined in depth in only a few tissues, but many detailed morphological studies suggest that the gap junctions of different vertebrate cells are invariant.

7.4 Specificity of Communication in Cell Lines

As the majority of observations on tissue specificity have been made with cultured cell lines, rather than in vivo or with primary cell cultures, it is possible that lack of communication

between junction-competent cells is a property of transformed cell lines, perhaps as a consequence of the transformed state. This was investigated using two clones of the Madin-Darby canine kidney epithelial cell line MDCK (see section 4.4). The two lines exhibit efficient heterologous communication with one another, but fail to transfer nucleotides to either the fibroblas line C13 or the epithelial line BRL. These observations support the hypothesis that communication specificity is non-tissue dependent, and is a more general property of cell lines than normal cells *in situ*. Cultured cell lines are normally derived from spontaneously-arising cells having transformed growth properties, and the transformed state may be associated with altered junctional communication properties. Lack of gap junction formation leading to a deficient response to growth-regulatory signals has been proposed as a cause of malignant transformation in certain cells (Azarnia and Loewenstein, 1977; Loewenstein, 1979). However, the acquisition of communication specificity by cells of transformed growth properties may be a coincidental phenotype associated with the clones selected by growth in culture. In agreement with this hypothesis, embryonal carcinoma cell lines which interact to varying extents in culture show no communication with differentiated derived cell types (Nicolas *et al*, 1978). This does not explain the lack of communication between primary cultures of fibroblasts and epithelial cells from human mammary gland (Fentiman *et al*, 1976); however, this now appears to be an exceptional case.

The results obtained in this study of cell-cell communication in primary cell culture and cell lines are

consistent with the conclusion that normal cells *in vivo* are capable of forming gap junctions with no tissue (or other) specificity. If metabolic segregation of different tissue layers does in fact occur, it is therefore likely to be caused by the physical separation of tissues by basal laminae and other extracellular structures. The molecular mechanism by which communication specificity is mediated is not clear; however, it may be significant that poorly-communicating C13-BRL mixtures show greatly-enhanced transfer upon prolonged coculture (Pitts and Bürk, 1976). It is possible that communication specificity may be caused by defective alignment of the two cell surfaces rather than genetic inability to form junctions. Thus, an essential first step in the formation of gap junctions between a cell pair may be the close apposition of the plasma membranes, which then permits interaction of junctional particles and aggregation into a complete macular junction (Johnson *et al*, 1974). Such a two-step model is compatible with theories of gap junction formation involving *de novo* protein synthesis (Griepp and Bernfield, 1978), proteolysis of a pre-existing subunit (Reve *et al*, 1978), or allosteric interaction between apposed junctiona subunits.

The communication specificity of cells with transformed growth properties would therefore be attributed to differences in the cell surface architecture, which would slow or prevent correct orientation of adjacent cell membranes. Such difference could include the expression of cell-surface tumour antigens (Nicolas *et al*, 1978), or the loss of the transformation-sensitive glycoprotein, fibronectin.

7.5 The Structure of Mouse Epidermal Prekeratin

Differentiation of newborn mouse epidermal keratinocytes in cell culture has been analysed in this study mainly by the synthesis of the epidermal protein prekeratin. The term prekeratin has been used as denoting a soluble protein of the lower epidermal layers which aggregates through non-covalent interactions to give the insoluble α-keratin filaments of the stratum corneum.

Prekeratin was purified from newborn mouse epidermis by extraction with urea and 2-mercaptoethanol followed by dialysis into CASC buffer. Protein precipitated from the CASC extract was characterised electrophoretically, and shown to consist of two polypeptides of molecular weights 69,000 and 61,000, in the proportion 1:2 (see section 5.3). This suggests a minimum prekeratin unit of two 61,000 chains plus one 69,000 chain, of total molecular weight 191,000. A similar structure has been obtained for bovine prekeratin, which contains one 72,000MW polypeptide plus two 60,000MW polypeptides (Matoltsy, 1965). A three-chain prekeratin unit has also been reported in human epidermis, comprising polypeptide chains of 70,000, 63,000 and 55,000MW (Skerrow, 1977).

A recent study of mouse epidermal protein by Steinert and coworkers, using urea/mercaptoethanol extraction of stratum corneum, confirmed the above conclusion, showing that epidermal α-keratin consisted of two polypeptides of 68,000 and 60,000MW, termed K1 and K2 respectively, and that these two chains occurred in equal quantities (Steinert and Yuspa, 1978). These molecular weights are not significantly different from those found in this

project. It was subsequently shown that the K1 and K2 polypeptides had the same N- and C-terminal residues, similar α-helix content and amino acid composition, and cross-reacted immunologically (Steinert et al, 1979). Reformation of α-keratin filaments by mixing different amounts of purified keratin polypeptides resulted in filaments with K1/K2 chain composition of either 1:2 or 2:1. The equal stoichiometry of mature stratum corneum α-keratin was attributed to a mixture of the two forms. However, epidermal keratinocytes maintained in culture exhibit decreased synthesis of the K1 protein, which resulted in a K1/K2 chain stoichiometry of between 1:2 and 1:3 (Steinert and Yuspa, 1978). It therefore appears likely that the prekeratin of living epidermal layers (if not stratum corneum) is composed of two polypeptides of 60,000-61,000MW (K2) and one polypeptide of 68,000-69,000MW (K1). By analogy with bovine prekeratin (Matoltsy, 1965), the native prekeratin structure may be a dimer of the minimum unit, with total molecula weight 376,000-382,000.

7.6 Differentiation of Keratinocytes in Culture

The presence of the two prekeratin polypeptides of mouse epidermis was used to quantitate differentiation of epidermal keratinocytes in vitro. The total amount of prekeratin was found to decrease with time in culture (see section 5.5). This decrease could not be explained by a total cessation of prekeratin synthesis, as in that case the amount of prekeratin would be expected to be halved every keratinocyte generation, which is 0.9 days at 37°C (see section 5.6). The observed effect could be

due to a gradual general decrease in the synthesis of prekeratin with time in culture, caused by the selection of rapidly-growing non-differentiated cells from the culture population. This would be in agreement with the disappearance of tonofilaments from secondary and subsequent passages of guinea pig keratinocyes (Regnier et al, 1973). However, a permanent keratinocyte cell line has recently been described in which intermediate filament arrays can be visualised using a prekeratin antiserum (Franke et al, 1979).

An alternative explanation for the decrease in total prekeratin in cultured keratinocytes would be preferential loss of one of the polypeptides. Such an imbalance in prekeratin chain synthesis by mouse-epidermal keratinocytes was observed by Steinert and Yuspa (1978), and a decrease in the production of the largest prekeratin polypeptide in feeder layer cultures of human keratinocytes was reported by Sun and Green (1977) and by Kubilus et al (1979). It may be relevant that decreased production of the heaviest human prekeratin chain is associated with the hyperproliferative epidermal disease, psoriasis (Skerrow and Hunter, 1978). In any case, it can be concluded that normal levels of prekeratin polypeptide synthesis do not proceed in epidermal keratinocytes in dispersed cell culture.

7.7 Feeder Layer Keratinocyte Culture

Interaction between mesodermal cells and skin epithelial cells was further studied using a feeder layer culture system. The effect on keratinocyte growth of coculture with killed cell line fibroblasts was first determined (see section 5.6). Rheinwald and Green (1975a) claimed that no growth of human

keratinocytes occurred in the absence of mesodermal cells; other workers have postulated a reciprocal relationship between keratinocyte proliferation and differentiation (Bullough, 1975; Delescluse et al, 1976). In the experiments reported here, no effect on keratinocyte proliferation by killed fibroblasts was observed. The effect on keratinocyte differentiation of feeder layer culture was analysed using three feeder layer cell types (see section 5.7). C13, BRL and A9 cells all failed to produce any detectable alteration in keratinocyte protein synthesis.

Epidermal cell keratinisation in vitro was examined using another biochemical marker of keratinocyte differentiation, the preferential incorporation of histidine (Delescluse et al, 1976; see section 5.8). The molecular basis of histidine incorporation by the epidermis appears to be the synthesis of a histidine-rich protein (HRP) in the keratohyalin granules of the granular layer (Sibrack et al, 1974; Murozuka et al, 1979). The HRP appears to give rise to a stratum corneum basic protein whose function is unknown (Dale and Ling, 1979). Keratinocytes in dispersed cell culture were shown in the work reported here to exhibit a higher relative incorporation of histidine than mitomycin C-treated C13 cells. Coculture of keratinocytes with feeder layer C13 cells resulted in no increase in histidine incorporation. This, again, suggest that some degree of keratinocyte differentiation occurs in dispersed cell culture.

The lack of stimulation of keratinocyte proliferation by coculture with killed cell line fibroblasts is in contradiction to the findings of Rheinwald and Green (1975a). This may be because the promotion of epidermal cell proliferation and

keratinisation is induced by only a few cell types, such as mouse 3T3 cells and human diploid fibroblasts. 3T3 cells differ from the C13 fibroblast line mainly used in this study in that they are grown by a subculture regime which retains their marked density-dependent inhibition of growth, and in that the cell morphology suggests that they may have been derived from endothelial rather than mesodermal tissue. Another reason for the poor differentiation by newborn mouse keratinocytes in feeder layer culture may be that these cells lack the strong substratum adherence shown by human (and also guinea pig) epidermal cells, and therefore may not so readily initiate formation of keratinising colonies by pushing back the fibroblast monolayer.

7.8 Dermal Stimulation of Epidermal Protein Synthesis

The interaction of dermis and epidermis has been studied using a transfilter organ culture system. In principle, any effects of the dermis on the growth and differentiation of epidermis may be distinguished as long-range or short-range interactions by interspersion of a porous filter between the reacting tissues. Such filters can be selected to permit the free diffusion, even of large molecules, between the tissues, but exclude direct physical contact. Organ cultures were established by enzymatic separation of dermis and epidermis from newborn mouse skin, and recombination either in direct contact or separated by Millipore filters. The effect on epidermal protein synthesis was quantitated by labelling the cultures with ^{3}H-leucine and ^{35}S-methionine (see sections 6.4 and 6.5). It was

found that isolated epidermis exhibited very low levels of protein synthesis. This was increased on recombination with dermis, and was similar to the level of protein synthesis by the epidermis of whole skin. However, epidermis grown transfilter to dermis showed no enhancement of protein synthesis above that of isolated epidermis. Previous studies in other systems have yielded contradictory results. Wessells (1962) reported that chick epidermis in organ culture degenerated unless maintained in contact with dermis or muscle. Epidermal maintenance by dermis was possible when the tissues were separated by Millipore filters. Briggaman and Wheeler (1968) found that human epidermis grafted to chick chorioallantoic membrane rapidly degenerated. Epidermis recombined with dermis developed normally, but transfilter dermal-epidermal recombinants resulted in no effect on epidermis by dermis. The contradiction in these two observations may be explained by the different culture systems used, or by differences between mammalian and avian skin, as the results obtained in this project using mouse epidermis are in general agreement with those of Briggaman and Wheeler for human epidermis.

On characterisation of the labelled epidermal protein by polyacrylamide gel electrophoresis, no specific induction of prekeratin synthesis was detected in cisfilter recombinants. Major peaks occurred of high molecular weight protein, and of actin. Thus, it appears that dermal support of epidermis in organ culture involves a general stimulation of epidermal protein synthesis.

7.9 The Dermal-Epidermal Interaction

An interaction occurs between dermis and epidermis in organ culture resulting in stimulation of epidermal protein synthesis. This interaction is dependent upon direct physical contact between the tissues, as interpolation of porous filters permeable to molecules up to the size of virus particles fails to result in stimulation. The mechanism of the dermal stimulation of epidermal biosynthesis remains unclear. By analogy with mesodermal-epithelial interaction in other systems, the mechanism seems likely to involve either direct communication between the reacting cells, perhaps by formation of intercellular junctions, or a regulatory effect mediated by extracellular matrix macromolecules (Grobstein, 1968). In support of the former mechanism, gap junction formation and cell communication have been demonstrated between dermal and epidermal cells *in vitro* ; the latter mechanism may be indicated by the stimulation of keratinocyte differentiation by freeze-thawed dermis (Briggaman and Wheeler, 1968) and by growth on collagen gels (Karasek and Charlton, 1971).

The means by which epidermis is stimulated by dermis is equally uncertain. It is of some heuristic importance to distinguish between permissive and deterministic interactions (Saxén, 1977), but in practical terms these probably cannot be separated. The grafting experiments of Billingham and Silvers (1967 ; 1968), which showed that the type of epidermal differentiation was altered by the underlying dermis, appear to implicate a specific deterministic induction of epidermal keratinisation. However, the differentiation of epidermis is too specialised to distinguish different types of keratinisation from different extents, and in fact certain epidermal tissues

retain their morphology after grafting to a new dermal site (Billingham and Silvers, 1968).

The dermis of skin interacts with epidermis in two general ways : the regulatory interaction referred to above, and transmission of nutrients from the vascular system. The organ culture system developed in this project has been used to show that the dermis exerts a general stimulatory effect on epidermal protein synthesis. From this it may be proposed that the dermal influence is principally a permissive one. The regulation of keratinisation may therefore occur by endogenous epidermal mechanisms, such as the ordering of epidermal cell columns and the putative chalone system. The dermis permits the expression of normal keratinisation by providing nutritive functions and perhaps acting as a mechanical support. Thus, epidermal cell keratinisation can proceed, albeit abnormally in dispersed cell culture, and epidermal differentiation may be promoted by killed mesodermal cells.

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