

MELANOTIC DIFFERENTIATION OF
THE AVIAN NEURAL CREST
VOLUME 1

Campbell

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I declare that this thesis is my own composition, and that the work reported herein is mine. Full acknowledgements of the contribution made by others is indicated below.

STEVEN CAMPBELL

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ABSTRACT OF THESIS (Regulation 6.9)

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Title of Thesis MELANOTIC DIFFERENTIATION OF THE AVIAN NEURAL CREST

This thesis is concerned with melanocyte differentiation of the neural crest cells in the embryonic Brown Leghorn fowl (Gallus gallus). Melanocyte differentiation in vivo was compared with that occurring in three experimental systems in vitro. Premigratory neural crest cells were isolated by the neural tube explantation technique of Cohen and Konisberg (1975). Differentiation of these isolated cells was compared with that which occurred in the postmigratory crest cells of the periorbital mesenchyme and corneal stroma.

Examination of melanocyte shape in vivo demonstrated that cell shape is extrinsically determined by features of the local micro-environment contrary to what has been reported in the mouse tissue. Observations of melanocyte shape change in vitro indicated that the environmental control of shape occurs by modulation of locomotory activity.

Melanogenesis within isolated cells was found to be different from that occurring in mesenchymal explants, or connective tissues and ocular epithelia in vivo. The isolated melanoblasts produced an ultrastructurally abnormal brown melanosome with an altered elemental composition, whereas those cells which differentiated within ocular tissue or mesenchymal explants produced black eumelanosomes.

The group of cells which colonise the corneal stroma are not totally committed to their fate as fibroblasts at the time of migration from the orbital mesenchyme. Some of the migratory cells retain the ability to become melanocytes, although this is rapidly lost after stromal colonisation.

Finally, melanocyte and fibroblast differentiation are presented as alternatives within the pigmented mesenchyme and attention is drawn to the fact that the abnormally pigmented crest cells in vitro resemble human malignant melanoma cells.

LIST OF ABBREVIATIONS

bf	=	bright field illumination
c.A.M.P.	=	cyclic adenosine monophosphate
C.E.E.	=	chick embryo extract
dic	=	differential interference contrast
E.C.M.	=	extracellular matrix
F	=	the F statistic
F.C.S.	=	fetal calf serum
H.S.	=	horse serum
i.u.	=	international unit
KeV	=	Kiloelectron volts
μm	=	micrometer
n	=	the sample number
p	=	the probability of a non-significant outcome
ph	=	phase illumination
P.R.E.	=	Pigmented Retinal Epithelium
R.B.C.	=	Red Blood Cell
σ	=	the standard deviation of the sample
<u>si</u>	=	skew illumination
x	=	the sample mean

GLOSSARY OF TERMS

Stage = the stage of embryonic development as described by Hamburger and Hamilton (1951)

Melanoblast = a cell whose presumptive fate is melanocytic

Melanocyte = a cell containing pigment i.e. immature or well formed melanosomes

Premelanosome, early melanosomal stage = melanosome prior to the onset of melanin deposition

Premigratory (Crest Cell) = a crest cell which is located at the dorsal aspect of the neural tube

Postmigratory (Crest Cell) = a crest cell which has migrated away from the neural tube and has become located within a developing 'ectomesenchyme' or a host tissue.

CHAPTER 1

AN INTRODUCTION TO NEURAL CREST DEVELOPMENT AND
MELANOCYTE DIFFERENTIATION WITHIN THE AVIAN EMBRYO

1.1 Scope of thesis

This thesis is concerned with the regulation of melanocyte differentiation in the embryonic fowl (Gallus gallus). Differentiation has been followed from the early stages of neural crest development to the appearance of highly pigmented cells in the late embryo. The principal aim of the work presented here has been to examine the way in which extrinsic environmental factors may influence the course of melanocyte differentiation, and subsequently modulate the phenotype and behaviour of the differentiated cell.

1.2 Origins and Fate of the Neural Crest

The lineage of the presumptive avian neural crest cells has been traced back to the anterior epiblast between the regions which are destined to become epidermis and neural plate (Rosenquist 1981). Histological observation and labelling experiments with vital dyes or tritiated thymidine (Horstadius 1950; Weston and Butler 1963; Weston 1966) have shown that the crest cells then become internalised by migration away from the 'angle' formed by the neural tube and superficial ectoderm. Within the cephalic region the migrating cells move laterally beneath the head ectoderm and ventrally into the cell-free spaces of the head where they will form much of the cranial ganglia and much of the cephalic mesenchyme (Le Lievre 1978). Within the trunk the same crest cells migrate laterally between the somites and the ectoderm, whilst others migrate dorso-ventrally through the somitic mesenchyme and over the surface of the neural tube (Weston and Butler 1963).

In order to follow the migration and fate of the neural crest after this initial dispersion, a more durable labelling procedure was introduced (Le Douarin 1969). This procedure involves transplanting neural tube (and neural crest) between the Japanese Quail (Coturnix Coturnix japonica) and the embryonic fowl from which a segment of neural tube has been removed. Quail cells are easily identified in histological sections, by the presence of a conspicuous heterochromatic nucleolus, and are thus distinguished from the fowl cells with which they intermingle during the course of embryogenesis.

A comprehensive range of neural tube transplantation experiments at all axial levels of the embryo has permitted the progress of crest cells to be followed during their migration into all locations within the embryo over the entire period of organogenesis and differentiation (for review see Le Douarin 1980 1, 2; Noden 1978; Bronner-Frazier 1980). From such experiments it has been possible to construct a definitive fate map of the crest derivatives, and establish which tissues they form either entirely or in part.

The following table is abbreviated from that provided by Noden (1978);

<u>Cell Type</u>	<u>Tissue</u>
Melanocytes	feathers, dermis, internal mesenteries and ocular tissues.
Fibroblasts	cranial connective tissues and corneal stroma.
Endothelium	cornea (but <u>not</u> blood vessels).
Neurones	
Glial cells	ganglia
Stellate cells	
Sheath cells	
Schwann sheath cells	peripheral nerve

<u>Cell Type</u>	<u>Tissue</u>
Chondroblasts	cartilages throughout the cranial region
Osteoblasts	bones throughout the cranial region
Adipocytes (cranial)	subcutaneous adipose
Smooth muscle cells cranial	vasculature and dermis
Striated muscle cells	ciliary body
Secretory cells:	
Type I	carotid body
Calcitonin secreting	ultimobranchial body

The neural tube transplantation experiments also provide direct proof of melanocyte origin, because they may be carried out using a pigmented donor and unpigmented recipient. Tubes had been transplanted between pigmented and unpigmented chicks (Weston and Butler 1963), or between quail and chick (Le Douarin and Teillet 1970; Teillet 1971) and in both cases donor-type pigmentation was observed in the feather germs of white fowl.

1.3 Pluripotency of The Neural Crest

The fate map of neural crest derivatives demonstrates the crest cell population is highly pluripotent. However, it would be wrong to imagine that this cell group is uniquely equipped to produce the range of derivatives listed above, or that by virtue of its position in the epiblast it had retained the totipotency of the oocyte. Although the crest has the ability to form corneal endothelium and the smooth muscle of the cranial vasculature it does not differentiate to form vascular endothelium. One must therefore conclude that either crest does not have the potential to form endothelium or it is not induced to differentiate in this fashion by environments in which blood vessels form. Crest cells are not

unique in their ability to form certain derivatives at particular locations within the embryo. Some of the cranial bones, for example, are of mixed origin consisting of mesodermic mesenchyme and cranial crest (Le Lievre, 1978). Derivative -types formed by the crest are also found at locations from which the crest cells are excluded. This behaviour is particularly characteristic of neural plate cells which differentiate to form the neurones and supporting cells of the retina and central nervous system as well as the melanocytes of the pigmented retinal epithelium (PRE). Pluripotency of the crest population therefore seems to be a feature of its early appearance as a distinct and recognisable cell group, and its subsequent dispersal into a wide variety of environments.

The evidence presented so far has demonstrated that the crest population is pluripotent. However, in order to understand the control of differentiation it is essential to know whether the population consists of numerous cell types of different potentialities or a single pluripotent cell type. Evidence supporting the existence of the latter has come from cloning experiments where the crest population has been isolated prior to the onset of differentiation. Undifferentiated crest cells were first isolated and cloned by Cohen and Konnigsberg (1975) who exploited their natural tendency to migrate away from the neural tube. Segments of neural tube were dissected free of surrounding tissue and explanted for 24 hours. After that period explants were mechanically removed leaving behind a population of stellate cells which were then suspended and replated at clonal density. Individual cells were then physically isolated to prevent interclonal contamination. Three types of colony were identified using these procedures; pigmented, non-pigmented (consisting of flattened

polymorphous cells), and mixed colonies containing both types of cell. In subsequent experiments (Sieber-Blum and Cohen 1980) clones were grown on substrata coated with poly-D-lysine, or poly-D-lysine modified by somitic fibroblasts. Under these conditions about 20% of the unpigmented and mixed cultures contained adrenergic cells. The isolated crest cells have therefore been demonstrated to be clonally bipotent in vitro, giving rise to melanocytes and cells with neural properties. This, of course, is not formal proof of pluripotency, as the authors in fact suggest, or even that all neural crest cells are identical. These experiments do, however, provide a strong indication that multipotent cells exist within the crest population.

It is particularly relevant to the work presented here that the melanoblast has been shown to be at least bipotent for then we may consider what external influences act on the cell and thus induce it to become pigmented or take on some other set of characteristics.

During the course of organogenesis and tissue formation, the neural crest population appears to undergo a progressive restriction of potency. Direct experimental evidence for fate restriction in the nervous system has been obtained by culturing dissociated chick ganglia and peripheral nerves at various stages of development (Nichols and Weston 1977). If these structures are dissociated before 5 days of development, melanocytes will differentiate. After 5 days, the culture remains unpigmented; thus the small stellate cells from which they differentiate must become committed to an alternative fate at this time. These stellate cells are thought to be precursors of the supportive cells found in both nerve and ganglia. When the association between neurones and non-neuronal cells is maintained, no melanin formation occurs (Nichols, Kaplan and Weston 1977). The

pluripotency of the supportive cells therefore appears to become restricted on day 5 of development by some kind of cellular interaction with another crest derivative. Clearly the time at which the neural crest cell loses its pluripotency depends upon the position that it occupies within the embryo and the nature of the inductive interactions to which it is subject.

1.4 Control of Neural Crest Differentiation

Ever since a definitive fate map of the neural crest has been established by the quail-chick marking technique, and the probable pluripotency of the cells demonstrated by cloning, the central focus of research in this field has become the control of differentiation. In general terms, the aim of this work has been to discover which features of the embryonic environment cause the crest cells to adopt a particular set of differentiated characteristics.

At present, some control mechanisms are understood in terms of specific types of embryonic interaction; but in no case has the molecular basis of the inductive mechanisms and of the resulting changes in gene expression been elucidated. The following description is therefore a general consideration of the types of interaction that occur between the crest cells and the embryonic environment which they encounter. It takes as its starting point Weston's (1970) suggestion that the migratory pathway and the terminal site of differentiation may both influence the types of derivative which are found at particular locations in the embryo.

1.4.1 The Migratory Pathway

There is now clear evidence that location of the crest cells along the neural axis influences the migratory pathway and the subsequent fate. Proof of this control has come from experiments where tritiated fowl or quail crest has been transplanted from one

axial level in the host to another in the donor (for review see Bronner-Frazer and Cohen 1980) and the migration followed histologically. Cells transplanted from the adrenomedullary level (somites 18-24) to the vagal level (somites 1-7) migrated in a fashion appropriate to their new location, thus forming the enteric parasympathetic ganglia (Le Douarin and Teillet 1974). Within the cranial region mesencephalic cells which form the maxillary processes and periocular and mandibular mesenchyme can be replaced by metencephalic cells which normally form the cranial sensory ganglia and the first and second visceral arches (Noden 1978). Pluripotency of the crest therefore seems to be accompanied by equipotentiality along the neural axis. The position along this axis influences cell fate because it defines the migratory pathway which the cells will enter and thus the tissue to which they will contribute. An important exception to this general rule is the integumental melanoblast which originates at all axial levels of the neural tube (Teillet and Le Douarin 1970; Noden 1978). Tube transplantation between pigmented and non-pigmented birds, has shown that the limits of the pigmented skin band is not defined by the length of the donor tube segment. Melanoblasts migrate laterally between the ectoderm and somites and spread along the cranio-caudal axis, so that melanoblasts from different axial levels mingle with cells from more posterior and more anterior regions (Teillet and Le Douarin 1970).

The factors controlling these migrations have not yet been elucidated. Two sorts of interactive controls have been suggested. The first of these, cell-cell interaction (Weston and Butler 1963), has not been supported by any experimental evidence. Contact inhibition of locomotion which had been proposed as a feature of homotypic crest cell interaction has not, in fact, been observed to promote migration away from neural tubes when cultured on collagen gels (Davis and Trinkhaus 1981).

The other interaction, which must be a prerequisite for migration, is that which occurs between the cell and the extracellular matrix. Within the cranial region crest cells migrate into the cell free spaces which are abundant in hyaluronic acid (Pratt 1975). Both head ectoderm (Pratt et al. 1976) and cranial crest (Greenberg and Pratt 1971) are capable of synthesising hyaluronic acid in vitro, which suggests that the 'host' tissue and crest cell may both construct the migratory environment. The role of the neural crest in modifying the extracellular matrix was further substantiated by Newgreen and Theiry (1980), who have demonstrated the appearance of fibronectin in the cranial mesenchyme after the neural crest invasion and shown that this is accompanied by the crest cells ability to synthesise fibronectin in vitro.

The migratory pathway should not be thought of as a static structure akin to railway tracks. The route chosen by the cells exists only for short periods of time (Bronner-Frazer and Cohen 1981) and may even be influenced by the synthetic capacity and secreting ability of the cells themselves.

Current evidence suggests that the relative importance of terminal site and migratory pathway depends upon the location and nature of the derivative, and upon the timing of differentiation.

The neuroblast of the sympathetic ganglia is an example of a derivative which may be subject to inductive influences along its migratory pathway. Migration of these cells leads them ventrally between the somites and neural tube and past the notochord. Organotypic experiments recombining neural tube, somites, and notochord with the neural crest (Cohen 1972; Norr 1973) have shown that these structures stimulate neuroblast differentiation whilst

suppressing melanogenesis. The presence of fibronectin along this pathway and over the surface of these tissues (Newgreen and Theiry 1980) is probably significant in neural differentiation for Sieber-Elum et al. (1981) have demonstrated that this glycoprotein, when bound to collagen substrata, will promote adrenergic differentiation of quail cells in vitro. At the time of writing no other purified component of the extracellular matrix has been shown to induce or potentiate the differentiation of crest derivatives.

1.4.2 The Terminal Site of Differentiation

In spite of the evidence that migratory pathway plays a part in the induction of some derivatives, there is also evidence which shows that the terminal site of differentiation determines cell fate completely in other cases. By combining premigratory cranial crest with head ectoderm and pigmented retinal epithelium (Bee and Thorgood 1980; Hall 1981) it has been possible to show that skeletogenic and chondrogenic potential of the crest is expressed in the absence of an in situ migration. At least two crest derivatives (the osteoblast and chondroblast) therefore appear to differentiate independently of crest or migratory influence and rely more upon tissue interactions at the terminal site.

After completing migration to a variety of tissues, the crest cells have, by definition, reached the terminal site where they become frankly differentiated. Weston (1970) proposed that within the terminal site crest cells may be induced to differentiate by interaction with the 'host' tissue. This type of control would seem particularly appropriate to those

derivatives, such as the melanocyte, which differentiate during the later period of organogenesis and long after migration has ended.

Quail-chick neural tube transplantation experiments have shown that the epidermal melanoblast reaches its terminal site about four days prior to the onset of melanogenesis (Teillet and Le Douarin 1970 and Teillet 1971). During the period of intraepidermal 'incubation' the melanoblasts continue to divide, and then increase their cytoplasmic and nuclear volume prior to the onset of melanogenesis on day 9. The fact that the neural crest cells within the epidermis are exclusively melanoblastic makes it clear that some form of terminal site control must operate in this case. As yet we do not know whether this control allows selective colonisation by already determined cells or whether the epidermis itself determines the fate of these cells.

Regardless of the action exerted by the epithelia, it seems likely that the dermis also has an influence upon melanocyte differentiation. Derby (1982) has shown that deoxycholate extracted extracellular matrices from dermal explants or monolayers will overcome the inhibitory effect of heart conditioned medium on melanogenesis. This stimulatory action was found to be tissue specific; being absent from pharynx and umbilical artery. The in vivo situation is likely to be more complex for the quail-chick transplantation experiments (Teillet, 1971) have shown that, at first, only those cells located in the developing feather germ epithelium differentiate, whilst the melanoblast of the intergerm region remain unpigmented. Development of the epithelial barb vane

ridges of the feather germ (Waterson, 1942) therefore seems intimately connected with the melanocyte differentiation in the epidermis. However, the feather germ develops as a result of epithelial-dermal interactions (Kischer 1968), so we must also consider the action of the dermis upon the epithelium, as well as any direct epithelial effect upon the melanoblast, when considering the induction of differentiation.

One of the most developmentally complex situations which arise during crest differentiation is the formation of the highly pigmented ocular tissues (fig. 1/1, 1/2 and 1/3). The first stage of tissue formation within the orbital region is the development of an apparently undifferentiated 'ectomesenchyme' (Le Dourain 1980; Horstadius 1950). This mesenchyme is formed by the mesencephalic crest cells which migrate around the optic cup and penetrate the space between the pigmented retinal epithelium (PRE) and the head ectoderm (day 2). Subsequent development converts this mesenchyme into the choroid sclera, ciliary body, and iridial stroma (Le Lievre, 1978). As a result of secondary migrations from the optic cup, mesenchymal cells colonise the corneal stroma where they form the endothelium (day 4) and stromal fibroblast populations (day 6) (Le Lievre 1978, Noden 1978a). During the course of tissue development six of the derivatives listed in section 1 are formed; these are the chondrocytes (sclera), osteocytes (scleral ossicles), endothelium (cornea), fibroblasts (all uveal tissues), melanocytes (all uveal tissue with the exception of the cornea), smooth muscle cells (vasculature) and striated muscle cells (ciliary body).

A partial explanation of the control mechanism involved in the differentiation of the first two of these cell types is now available, although differentiation of the remainder, including the melanocyte, remains obscure.

On the 5th day of incubation the chondrocytes of the sclera start to form a sheet of cartilage which encompasses the retina from its posterior pole to the posterior edge of the scleral ossicles. The proximity of the retina is of crucial importance, for combinatorial experiments have demonstrated that the pigmented retinal epithelium will induce chondrogenic differentiation of premigratory crest (Bee and Thorogood 1981). Osteogenic differentiation within the ossicles (or membrane bones) of the anterior eye is induced by a ring of papillae which are transient structures within the presumptive conjunctival epithelium. (Coulombre et al. 1962).

Osteogenic and chondrogenic differentiation of crest cells is not restricted to the periorbital tissue, but is found in other parts of the cranial ectomesenchyme (Horstadius, 1950). The mandibular and maxillary mesenchymes for example are induced to differentiate osteogenically by their respective epithelia (Hall, 1981). However the induction is not tissue specific so that these mesenchymes will respond to epithelia from the 'non-osteogenic' sites such as the limb bud, back and abdomen. On the other hand, non-osteogenic mesenchymes will not respond to the mandibular epithelium.

Hall argues that these experiments show that the epithelium does not act "instructively" but induces the expression of osteogenic potential in already "determined" cells. This interpretation of the results seems inappropriate for these experiments do not demonstrate the existence of operationally 'determined' osteoblasts within the mesenchymal population. In fact, the osteoblast could, in principal, retain some degree of pluripotency until the time of differentiation. The evidence thus shows that the inductive agent is not tissue specific, but that the consequence of the tissue interaction is defined by the nature and potential of the mesenchymal cell. These

experiments are of crucial importance, however, in the search for molecular mechanisms of induction, for they indicate that the terminal site need not synthesise 'informational molecules' specific to that tissue or required mainly for the induction of a certain class of crest derivative.

1.4.3 Neural Crest Culture as a System for Studying the control of Melanocyte Differentiation

Neural crest culture seems to have progressed little in the past 40 years. When Twitty began his classic studies of amphibian neural crest melanocyte behaviour in the early 1940s, he used heterogeneous cultures of neural tube and migrant crest cells. Since then, the more refined techniques of crest cell isolation and cloning have been developed (see 1.2). In spite of these improvements within the culture system, the growth medium has remained chemically undefined. Twitty (1966 review) supplemented a simple salt solution with amphibian "body fluid" in order to induce pigmentation in his cultures. More recently, chick embryo extract (Cohen and Konnigsberg 1975) and selected batches of fetal calf serum (Derby and Newgreen 1982) have been used to promote melanotic differentiation in isolated crest cells.

Partial purification of a pigment-inducing factor (PIF) has recently been achieved by gel filtration of chick embryo extract (Derby and Newgreen 1982). The PIF has a molecular weight of more than 400 kilodaltons, but it does not coat the tissue culture substratum and is required continuously in solution. It thus seems rather unlikely that soluble high molecular factors of this nature would be active within the epithelial environment, for example. Further doubts about the similarity of inductive mechanisms in vivo and in vitro must be entertained since Greenberg et al. (1981)

demonstrated that the presence of horse serum, rather than fetal calf serum in the growth medium will result in neural rather than melanoblastic differentiation.

In view of the possible differences between inductive mechanisms in vivo and in vitro, it is pertinent to ask whether the differentiation of the cultured crest cell proceeds in a normal fashion. As yet, this question remains unanswered, because little or no comparison has been made between melanocyte differentiation in vivo and in vitro other than the fact that the cells contain pigment. To appreciate the full complexities of environmental regulation upon melanocyte differentiation (or any other crest derivative), it is essential to compare the character of the cells in vivo and in vitro. As this comparison is central to the study of melanocyte differentiation in vivo, the phenotype of this cell is now described.

1.5 The Melanocyte

The discussion so far has considered the way in which melanocytes arise during the course of neural crest differentiation. However, in order to understand the transition which the crest cell undergoes during the process of differentiation, it is necessary to examine the phenotype of the differentiated cell. The remainder of this chapter is, therefore, concerned with the phenotype and behaviour of the melanocyte and describes how these features of the cell are modulated at the terminal site of differentiation.

The most distinctive characteristic of the melanocyte is its ability to produce pigment and thus provide the superficial tissue of the vertebrate integument with a screen against damaging effects of ultraviolet radiation. The distribution of pigmentation is, as we shall see, also influenced by the cell's shape and behaviour, as well as by its synthetic capability. Melanogenesis, cell shape, and cell behaviour are now considered.

1.5.1 Melanogenesis

Many recent studies of melanocyte differentiation of the avian neural crest have considered the end point of differentiation as the appearance of pigment. It is therefore most surprising that very little attention has been paid to the type of pigment formed. One interesting exception has been a short comment by Greenberg and Pratt (1977), who noted that White Leghorn melanocytes produce a brown pigment in vitro, yet no mention was made of the fact that these cells are derived from animals with white feathers. In fact, White Leghorn are severely hypo-pigmented as a result of a genetic lesion which causes autophagocytosis within black melanocytes (Jimbow et al. 1974, Hutt 1949). Indeed the White Leghorn lesion has been reported to cause cell death within the isolated melanocyte in vitro (Jimbow et al. 1974). It is therefore most surprising that many experimenters have chosen this bird in studies of neural crest differentiation. Clearly it would be better to use the Japanese quail, whose crest differentiation is well studied, or a pigmented variety of fowl.

An early study of 21 breeds of domestic fowl and other birds (Hamilton, 1940) revealed that the embryonic skin of four of these animals contained "red" and "black" melanocytes. Subsequent experiments (Hamilton, 1941), designed to examine the development of both pigment types, demonstrated that the frequency of red melanocytes was increased by estradiol and testosterone, and the frequency of black melanocytes was reduced by both hormones. As a result of these experiments, Hamilton proposed that "red" and "black" precursor melanoblasts existed within the avian skin. No direct evidence of such precursors has been forthcoming in the past forty years, so it is still impossible to say at what stage, if any, the

avian crest cell becomes committed to producing "black" or "red" pigment. These experiments should not be ignored, however, for they help to explain the reason for pronounced sexual dimorphism of pigmentation in adult birds.

Embryonic avian skin has become unpopular for experimental studies because of its variable pigment distribution (Hutt 1949) and morphological complexity (Watterson 1942). A more convenient system for the study of pigment colour modulation has been the Agouti mouse skin. During the neonatal period the Agouti hair colour changes from black (eumelanosomes) to yellow (pheomelanosomes).

Recent ultrastructural observations show that melanocytes are capable of undergoing a pigment transition; thus black and yellow melanosomes may be found in one cell during the transitional period (Sakurai et al. 1975).

Control of this transition is not regulated by the melanocyte itself, but is extrinsically determined by the follicular environment (Silvers and Russell, 1955). In spite of observed transition of pigment type within cultured skin (Cleffmann 1963) the agent producing these changes has not been identified (Galbraith and Patrighiani 1976).

The temporal colour transition observed in agouti hair are relatively simple when compared to the complex colour patterns of the vertebrate integument (Bard 1981). The origins of spatial or temporal pigment transition remain almost totally obscure for, as yet, no colour controlling factors have been identified.

In spite of the problems associated with identifying the spatial or temporal control of pigment type, considerable progress has been made towards describing the difference between the pigments

themselves. Vertebrate melanin pigment is produced by a complex polymerisation of tyrosine and other amino acids, which occurs in a specialised class of membrane-bound organelles known as melanosomes. These organelles vary considerably in shape, ultrastructure and chemistry, depending upon species and genotype concerned. No all-encompassing description of the vertebrate melanosome is possible, but the following account of melanosomal ultrastructure and melanin chemistry will have some applicability to many animals throughout the vertebrate phyla.

1.5.1 i The Ultrastructure of Melanosome Assembly

Melanosomes can be placed into two broad categories, according to colour, shape and ultrastructure. The first of these categories found within the black feather, amphibian retina and human negroid skin, is the elongate eumelanosome. The second category found in brown feathers, xanthic goldfish skin and human red hair, is the round pheomelanosome.

One of the most comprehensive descriptions of these organelle types within the avian embryo was recently produced by Jimbow et al. (1979). In this study, the ultrastructural development of melanosomes was examined in the feathers and retina of Black Minorca and Rhode Island Red fowl and compared with their murine equivalents.

Melanosome production has been split into 4 arbitrary stages, which permit comparison of the 2 developmental pathways to be made (see fig 1/4). At stage I of pheomelanogenesis the pigment producing organelle is a spherical vacuole containing fibrous material. The stage II pheomelanosome accumulates small internal "vesiculo-globular bodies" which are probably membrane vesicles.

These vacuoles contain a partially melanised matrix. By stage IV the melanosomes have become amorphously electron dense and completely melanised. This description probably applies to most birds, mammals and fish, because no significant differences exist between red feathers, yellow mouse hair (Jimbow et al. 1975), and xanthic goldfish skin (Turner et al. 1975). Eumelanosomes at stage I are also round in shape, and contain amorphous material and rare incomplete filaments. At stage II the melanosomes become ellipsoidal, and contain vesiculo-globular bodies, and regularly striated filaments appear oriented along the long axis of the organelle. In stage III melanin is laid down on the surface of the filamentous arrays. By stage IV the melanin matrix is essentially complete, revealing only a few electron lucent areas which correspond to the 'vesiculo-globular' bodies. This developmental pattern applies for most black melanosomes of the vertebrate group, although Moyer (1966) has shown that mutant eumelanosomes of the mouse with disrupted filament organisation may be irregular in shape and brown in colour.

Ultrastructural descriptions of the kind given above, present a rather static picture of a dynamic process which involves membrane flow and compartmentalisation, enzyme synthesis and packaging, and filament formation. The initial stages of melanosome formation are not properly understood, though several plausible hypotheses have been proposed to explain the origin of the stage I melanosomal membrane. Some workers have observed continuity of the melanosomal membrane and endoplasmic reticulum (Maul 1971; Hishima et al., 1979), in mouse. These observations are cited as evidence of a dilation and pinching-off process, which segregates regions of the endoplasmic reticulum which then become the melanosomal membrane.

One other process in melanosomal production, which has been partially described, is the transport of tyrosinase from its site of synthesis to the stage II and III melanosome. Electron histochemistry of human melanoma cells (Hunter et al. 1978) and other melanocytes, has shown that tyrosinase first appears in the golgi associated endoplasmic reticulum (or GERL). From there, it buds off into coated vesicles (Maul and Brumbraugh, 1971) which can be isolated in the coated vesicle fraction of melanoma cells (Mishima, 1979). Fortuitous transverse sections of the early melanosomal membrane suggest that the coated vesicle fuses with developing organelles, 'spill' their contents into the melanosomal lumen, and then invert without the coat, thus forming the multivesicular bodies. Profiles suggestive of vesicle fusion and inversion have been observed frequently in osmotically stressed xanthic gold fish (Turner et al. 1975). However, these events have yet to be observed with equal frequency in normally differentiating avian melanocytes.

1.5.1. ii Melanin Chemistry

Melanins were initially classified into the black acid soluble pigment found in eumelanosomes, and the brown, red or yellow alkali soluble pigment found in pheomelanosomes. These pigments which have been extracted in large quantities from feathers (Minali et al. 1970), bovine hair, and retina, may act as a generalised model of vertebrate melanin.

Despite successful extraction of each pigment type and "an immense amount of work, the general problem of melanin chemistry has not been solved" (Prota and Thompson, 1976). Elucidation of melanin structure has proved such an intractable problem due to the complex and heterogeneous nature of the melanin polymers. These high

molecular weight molecules are insoluble and non-crystallisable and so cannot easily be investigated by spectroscopic analysis or X-ray crystallography. Substantial progress has been made in identifying the precursors of the melanin polymers and determining how they are enzymically modified, as a first step in polymerisation.

The major precursors are the amino acids, tyrosine, cysteine (and glutathione) (Prota, 1980). L-tyrosine which is the major precursor of eumelanin, is oxidised to dihydroxyphenylalanine (L-Dopa), and subsequently to dopa quinone by the copper containing tyrosinase enzyme. Further reactions proceed without enzymic catalysis, although recent work reveals that melanoma cell extracts are capable of regulating 2 of the later reaction steps (Pawlek et al. 1980). As melanin synthesis proceeds, the bouyant density of the melanosomes increases as the tyrosinase activity declines, till no activity remains within the fully melanised organelle (Seijl et al. 1979).

After tyrosinase has generated dopa-quinone it then undergoes spontaneous cyclisation and decarboxylation reactions as part of the multi-step Rapper-Masson scheme shown in fig 1/5. The mechanism of oxidative polymerisation is not known, but it could involve indole-5, 6, quinone, its semiquinones or possibly all precursor quinols and quinones (Prota and Thompson, 1976).

Pheomelanins are structurally more complex and heterogeneous than the eumelanins for they are produced by the co-polymerisation of dopa-quinone with cysteine or reduced glutathione (Agrup et al. 1977) (see fig 1/6). Cysteine reacts rapidly with dopaquinone to give 4 cystinyl-dopa isomers, which in turn, form the highly unstable 1-4, benzothiazine metabolites. These form the low molecular weight

trichochrome pigments (see fig 1/7), present in red feathers and human red hair, or polymerise to form pheomelanin.

This arbitrary classification of pigment chemistry into pheomelanin and eumelanin types has recently been viewed as rather simplistic. According to the more generalised 'intermeshing hypothesis', which has replaced the old classification (Prota, 1983) the melanins are considered to be a series of heteropolymer families with a range of cysteine contents. 'Intermeshing' of the eumelanin and pheomelanin pathways would therefore generate complex groups of polymers which are intermediate in structure between the "pure" eumelanin and pheomelanin types. The degree of 'intermeshing' can be determined by a simple parameterisation of the ESR spectrum (Prota 1982). Using this technique, melanins from a wide variety of sources can be listed on a non-discrete scale. As yet, however, no evidence has been presented which indicates that individuals or different genotypes of birds or mammals produce a continuous range of pigment.

As well as being an organic polymer, matrix eumelanin also contains a proteinaceous filament array. An extraction procedure has recently been developed (Zimmerman, 1982) which allows solubilisation of the melanosomal membrane and the melanin matrix, whilst leaving the filament array morphologically intact. These filaments contain four major low molecular weight polypeptides, with an estimated mass of 12.7 to 14.5 kilodaltons. Pheomelanins which have not been extracted in this manner are presumably deficient in these proteins. How these polypeptides associate with each other or the melanin matrix, still remains to be determined (Whitaker, 1979).

Another important aspect of melanin chemistry which has been neglected during the course of comparative studies of pigment type is the metallic composition of melanin.

Metallic binding to melanin occurs by association to free carboxyl groups, phenolic hydroxyl groups (Bruenger et al. 1967) and free radicals (Francisz et al. 1980) present within the matrix. Energy dispersive X-ray microanalysis (Panessa and Zadunaisky, 1981) and laser microprobe mass analysis (Kauffman, 1980) have demonstrated the presence of high levels of calcium and lower levels of barium, iron, zinc and copper in pigment from various sources. As yet, the full significance of metal binding is not appreciated, although in the case of copper it may be active in the catalytic oxidation of tyrosine and L-dopa either as part of the tyrosinase enzyme or as the free species (Oster and Oster, 1979).

Although this account reveals something of the complexity of melanogenesis it gives little information about the inductive mechanism, and the associated changes in gene expression which define the process of differentiation.

1.5.2 Melanocyte Morphology

The preceding discussion has emphasised that pigment type varies between different environments of the avian embryo, however this may not be the only melanocyte characteristic which is modulated. Recent experiments with cloned quail cells (Cohen and Konnigsberg, 1975) demonstrated that the culture environment is capable of producing dramatic changes of cell shape. Melanocyte cultures were grown on collagen gels and developed into highly ordered epithelial monolayers. After additional growth, dendritic cells appeared on the apical surface of the underlying epithelium. This suggests that a cellular substratum (in this case, a melanocyte epithelium) may provide a significantly different environment from a 'flat' layer of extracellular matrix (the collagen gel).

These experiments run contrary to the opinion of Market and Silvers (1950) who suggested that melanocyte morphology is autonomously regulated and independent of tissue environment. These workers examined 6 tissues in 50 genotypes of house mouse and found no variation in cell shape, with the exception of 2 mutants (dilute and leaden), which had a pronounced flattening of the perinuclear region. Although the melanocyte appeared very similar in each tissue, Market and Silvers failed to demonstrate that these locations constituted significantly different environments.

Clearly a more detailed study of the in situ melanocyte is warranted in order to determine if local variation in morphology can be induced in significantly different embryonic environments.

1.5.3 Melanocyte Behaviour

Two aspects of melanocyte behaviour are worthy of attention, for they appear to influence the overall pigmentation of embryonic and adult tissues. The most significant aspect of melanocyte behaviour is the cell's ability to transfer its melanosomes to neighbouring epithelial cells. By transferring granules in this way, the integumental pigment screen becomes more evenly distributed and therefore of greater protective value against the ionising effect of ultraviolet radiation.

The integumental melanocytes of the avian feather germ start to accumulate melanosomes at the tips of their dendrites on day 10 of incubation (Waterson 1942). These tips become enlarged and pinch off (Hamilton 1940; Waterson 1942) and thus melanosomes become deposited in the spaces between the developing sheath cells. Pinching-off then ceases temporarily, whilst the epidermis thickens to form the barb vane ridge. At a later stage of development, the

barbule cells begin to accumulate melanosomes and the pinching appears to cease, even although transfer of melanosomes between the two cell types continues. As keratinisation occurs within the more peripheral cells, the melanocyte processes are retracted towards the pulp, where they continue to transfer melanosomes to the more centrally located cells. The pattern of pigment deposition in this location therefore appears to be controlled by melanocyte interaction with the differentiating keratinocytes. It remains unclear whether melanosome deposition is caused by keratinocyte-melanocyte interaction, per se, as some workers have suggested (Breathach et al. 1982) or whether it is an intrinsic property of the melanocyte, which is merely modulated by the epithelial environment.

Melanocyte aggregation is a second intriguing aspect of cell behaviour in vitro which, as yet, does not have any proven significance for avian pigmentation. Twitty's pioneering work upon amphibian melanocyte behaviour (for review see Twitty, 1966) revealed that the neural crest "disperse", i.e. migrate away from the neural tube, and then reaggregate around the onset of melanogenesis. Reaggregation occurred only in culture conditions which promoted melanogenesis and in cells from more pigmented animals which possessed prominent flank bands. Twitty therefore proposed that aggregation in vitro was directly linked to the formation of highly pigmented tissue in vivo. Aggregation has since been observed in mutant White Leghorn melanocytes which are derived from severely hypopigmented animals (Jimbow et al. 1974; Howell 1976). Twitty's explanation of the aggregation phenomenon would therefore seem of limited value. In the absence of any mechanistic explanation of the aggregation phenomena, it is tempting to suggest that this behaviour

has no direct in vivo equivalent, but is a consequence of the adhesive and locomotory properties of the in vitro cell.

1.6 The Unresolved Questions Concerning Avian Melanocyte Differentiation

The preceding discussion has mapped out much of what is known concerning the differentiation of the avian neural crest melanocyte. A brief list of the unresolved aspects of this process is now provided in order to familiarise the reader with the context of the experimental work reported in the next four chapters.

1 Isolation, culture and cloning of the neural crest cell have become established techniques in recent years. However, we remain almost totally ignorant about the control of melanocyte differentiation in vivo. We are unable to say whether melanocyte differentiation is influenced by tissue interaction in early embryogenesis or during the course of migration to the terminal site. Once the crest cells have reached the terminal site their differentiation may be locally induced, but as yet, no inductive capacity has been defined or specific factor identified within the epidermis or the presumptive pigmented mesenchymes.

2 Melanosome colour, ultrastructure and composition may vary within different regions of the avian integument, but the control over pigment remains a complete mystery. We also do not know what type of pigment is produced by genetically wild type melanocytes in the absence of a tissue environment.

3 Avian melanocyte shape has been shown to vary dramatically in vitro, but within mouse tissue, no significant variation of melanocyte morphology has been detected. We therefore cannot say whether avian melanocyte shape varies in vivo or whether it is autonomously regulated.

4 Melanocytes have the ability to transfer melanosomes to epithelial cells, but we do not know whether this behaviour is the result of a specific keratinocyte melanocyte interaction.

5 Melanocyte differentiation *in vitro* results in cell aggregation. Until now the mechanism of aggregation and its significance within the avian embryo has not been understood.

1.7 A brief introduction to the experimental system

The Brown Leghorn fowl was chosen as the experimental animal for this project because of its proven ability to produce melanocytes of different colour in skin culture (Hamilton 1940). Unfortunately, the avian embryonic skin is a highly complex tissue, which makes it unsuitable for defined studies of melanocyte differentiation *in vitro*. A simpler model system was therefore employed in the hope that the factors controlling melanocyte differentiation could be identified. Premigratory neural crest cells were isolated, using the neural tube explantation technique of (Cohen and Konigsberg 1975). Differentiation of these premigratory cells was then compared with differentiation of postmigratory cells at two sites in the developing ocular tissues. The first of these, the anterior periorbital mesenchyme, is a multipotential neural crest derived structure (Le Lievre, 1978), which gives rise to the highly pigmented tissues of the uvea. The second site, the corneal stroma, was chosen because it remains totally unpigmented, in spite of the fact that it is a connective tissue whose cells are derived from the periorbital mesenchyme. Differentiation in vitro was also compared to that occurring in vivo in order to identify the ways in which melanocyte phenotype and behaviour is normally regulated.

CHAPTER 2

MATERIALS AND METHODS2.1 Gallus gallus Embryos

Fertile eggs were supplied from a closed flock of randomly mated J-line Brown Leghorn fowl, kept at the Poultry Research Centre, Roslin. The integumental pigmentation of the adult and embryonic bird corresponded closely to the Brown Leghorn/Red Jungle Fowl 'wild' type described by Hutt (1949). In one particular experiment, reported in chapter 3, Red Minorca embryos were used instead of Brown Leghorn (P.R.C, Roslin).

Eggs were incubated at 38°C and at a low relative humidity (35%), which tended to cause a high mortality rate after day 18 of incubation. Embryos older than that age were therefore rarely examined.

2.2 Microdissection2.2.1 Instruments

Tungsten needles were made by electrolytically sharpening 0.5mm tungsten wire in molten sodium nitrite. A novel form of microscalpel was used as an alternative to tungsten needles where a very fine cutting action was desired. Microscalpels were made by grinding the tips of disposable 19 and 21 gauge hypodermic needles with a hand held rotary polishing wheel (Polisher R22, Identoflex, Switzerland). With practice, a range of flat pointed blades were produced, varying from 20 to 100µm in thickness, depending on the use intended. On occasions the blades were bent, in order to produce a device which allowed an undercutting action, useful in the removal of epithelium.

Small knives were made in order to cut tough tissues such as the corneal stroma. These were made by fracturing brittle steel razor blades (Gillette Blue, courtesy of Gillette Ltd., London), with fine nosed pliers.

Small pieces of tissue were transferred between dishes with fire polished glass capillaries, which were connected to an adjustable micropipette via a small bore silicone tube.

2.2.2 Dissociation Mixture

A combined trypsin-pancreatin dissociation mixture was used to ease tissue separation during dissection. When used at room temperature over short time intervals, the mixture was found to be faster, more effective, and less harsh than either component used alone.

The mixture was prepared by a slight modification of the method used by Bee and Thorogood (1980), 250mg of pancreatin (Sigma, X3) and 150mg of trypsin (Difco, 1:250) were added to 10mls of sterile distilled water and mixed for 5 minutes on a vortexer. 10mls of Tyrodes solution (without magnesium or calcium) was then added, and the solution remixed. A crude filtrate was made by passing the mixture through a prefilter (Sartorius, No. 13400), mounted in a sweeny filter housing. The solution was then sterilised by passing it through a 0.2mm pore filter (Gelman, 'acrodisc'). Two millilitre aliquots were stored at -20°C for up to 2 months.

2.2.3 Isolation of the Trunk Neural Tube from 1:2 to 30 Somite Embryos

Embryos and adjoining blastodiscs were placed in a dish containing phosphate buffered saline (no calcium or magnesium), and the developmental stage determined by counting the number of somites

up to the last well defined furrow. A yolk-free segment was cut from the appropriate region and placed in a 0.2mls drop of dissociation mixture within a 30mm petri dish (Falcon). After 1.5 minutes incubation, the endoderm was cut along the midventral line, to allow access to the notochord and somites. Two or three minutes later the notochord began to buckle, and was then pulled away from the neural tube, with a tungsten needle or microscalpel. In 20 to 30 somite embryos, the neural tube was then freed from the somites by stretching the ectoderm laterally with 2 pairs of watchmakers forceps. . This stretching action caused the somites to detach en masse from the neural tube. They were then removed by cutting the ectoderm at its point of attachment to the neural tube.

In the case of younger embryos, the somites were cut free by inserting a very fine microscalpel at the sides of the neural tube and cutting in an anterior-posterior direction. After 5 to 6 minutes the dish was flooded with 2mls of fetal calf serum (Gibco), and the remaining fragments of endoderm were trimmed away. Care was taken to prevent excessively long digestion of the neural tube, as this resulted in the outgrowth of neuronal processes from the explant within the first day of culture. Some degree of digestion was required, however, to facilitate neural tube-substrata adhesion at the time of explantation.

2.2.4 Isolation of Tube-free Neural Crest in the Cephalic region of 4 to 12 Somite Embryos

Embryos were staged as before and pinned to the base of neoprene lined petri dishes.

In the case of 4 to 7 somite embryos, the cephalic neural folds were cut from the dorsal aspect of the unfused neural tube. At later stages the roof of the mesencephalon was excised, yielding a piece of tissue analagous to that used by Noden (1978) in quail chick transplantation experiments.

2.2.5 Isolation of Periorbital Mesenchyme and Corneal Stroma from Early Embryos

Embryos were staged according to the criteria described by Hamilton and Hamburger (1957). These workers point out that coordination of development varies among embryos, so that it becomes important to assess the developmental status of the organ which is of particular interest. Two additional criteria were therefore used, to improve assessment of eye development between stages 28 and 29 (Bard and Ross, 1982). The presumptive ciliary body region first darkens in late stage 28, whilst the first ciliary folds form about 8 hours later, at the beginning of stage 29.

The anterior part of the eye was isolated along with the attached lens and vitreous (fig. 2/1). Lens and vitreous were then pulled away, along with some attached retinal fragments (fig. 2/2). Any remaining pieces of retina and pigmented retinal epithelium (PRE) were removed with fine forceps (fig. 2/3). The mesenchyme was inspected carefully at high magnification (x62) to ensure that no fragments of PRE were present. (Absence of PRE was subsequently confirmed by comparing 2 specimens in which the epithelium had been removed, with 2 in which fragments remained. Scanning electron microscopy demonstrated an absence of highly villous epithelial cells in the stripped specimens. After a period of culture, no epithelial outgrowth was observed, and no tight junctions were found between melanocytes using transmission electron microscopy). After removal of the retina, the remaining tissue was transferred to a 0.2ml drop of dissociation mixture for 6 to 8 minutes, and the dish was then flooded with 2 to 3 mls of fetal calf serum. This treatment caused detachment and blistering of the corneal and conjunctival epithelium which was then removed with a bent microscalpel.

Squares of anterior periorbital mesenchyme (not illustrated) close to the cornea were cut with a razor blade knife. Mesenchyme was dissected from embryos between Hamburger and Hamilton stages 26 and 32.

Squares of cornea (fig. 2/4) were cut in a similar fashion. Extreme care was taken to avoid inclusion of the adjoining mesenchyme, as this would have resulted in 'contamination' of the corneal square, with melanoblasts from the presumptive limbal region. The corneal tissue fragment therefore consisted of stroma, containing the fibroblasts, and some endothelial cells from its posterior surface. Corneal stromas were dissected in this way from embryos between Hamburger and Hamilton stages 27 and 36.

2.2.6 Isolation of 18 day Corneal Stroma

Two-millimetre corneal squares were cut directly from untreated anterior eye segments from which the lens, vitreous, retina and pigmented retinal epithelium had been removed. The corneal fragment was incubated in dissociation mixture for 10 minutes, and the epithelium and endothelium were then removed with fine forceps. A second more prolonged digestion in either dissociation mixture (60 minutes at 37°C) or in collagenase (in Tyrodes solution, 180iu/ml, Sigma, Type IA), was carried out, in order to facilitate cell migration from the explanted stroma.

2.2.7 Heart Dissection

Hearts were removed from 5 and 6 day embryos, and the pericardium then torn away. The tissue was then crudely dissected by slicing with two scalpel blades. No enzymic digestion was needed to facilitate adhesion or outgrowth.

2.3 Culture Methods

Explants were transferred from the dissecting dish to the culture vessel containing a thin film of medium, and allowed to

adhere for a period of 1 to 4 hours at 37°C, before adding a further 1.5 to 2mls. The culture vessels used were 30mm non-vented petri dishes (Nunclon), sealed with sterilised silicone grease. Sealing in this manner prevented evaporation from the dish, and so circumvented the usual problem of fungal contamination associated with long term incubation of medium in vented dishes in conditions of high humidity.

On occasions, tissues were explanted onto clean glass coverslips, dried rat tail collagen films, or on to collagen gels. (The latter were kindly prepared by Dr D Davidson after the method of Elsdale and Bard (1974)).

Tissues were cultured in Ham's F10 growth medium, supplemented with 1mM glutamine, 10mM sodium bicarbonate, 20mM N-2-hydroxyethylpiperazine-N¹-2-ethansulfonic acid buffer, 100units/ml penicillin, 100mg/ml streptomycin and titrated to pH 7.1 to 7.2. Fetal calf serum (Gibco) was added to a final concentration of 5, 10 or 15% as required. Chick embryo extract, when added, was used at a final concentration of 2 or 5% as required.

All tissue with the exception of the early cornea adhered firmly to the substratum and produced proliferative outgrowths within the first few days of culture. When required explants were removed leaving the cells of the outgrowth. Early corneas consisted of a dense collagenous stroma which was not conducive to cell migration from the explant. Further experimentation showed that brief collagenase (Sigma, type IA) treatment made the stroma too fragile for explantation, whilst a more extensive digestion needed to produce a cell suspension made the cells non-viable.

2.4 Embryo Extract Preparation and Testing

Whole-embryo extract was prepared from 9 day chicken embryo using a similar approach to Sieber-Blum and Cohen (1980). No filter sterilisation was required however as preparation took place in a laminar flow hood. A 50% extract was made using equal weights of embryonic carcass and pre-chilled saline (without magnesium or calcium). The embryos were first homogenised for 1 minute and the resulting 'embryonic soup' then spun at 2000xg for 20 minutes and a crude supernatant fraction collected. This fraction was respun at 27000xg for 20 minutes in a refrigerated Spinco centrifuge, and then allowed to stop, without braking, in order to preserve the integrity of the upper lipid layer. The bulk of the underlying supernatant was pipetted off, yielding a golden coloured transparent liquid. The sterility of the preparation was tested by adding 2 drop samples to bijoux bottles containing bacterial broth, and then incubating for 7 days at 30 to 37°C. After the test period, the stock solution was thawed and dispensed into sterile bijoux bottles and stored for up to 10 weeks at -30°C.

The biological activity of the extract was tested by culturing 7 and 10 day dorsal root ganglia on collagen (rat tendon) coated coverslips. Addition of two per cent extract to the culture medium caused a marked increase in outgrowth similar to that found by Konnigsberg and Cohen (1975).

2.5 Whole Mount Examination of Tissue

All tissues were freshly fixed in 2.5% gluteraldehyde, 0.05M cacodylate buffer, pH 7.2, to minimise distortion of cell shape during dissection.

Fine dissection was then carried out in a 50% ethanol-water solution with fine forceps and embryological spring scissors (courtesy of J Weiss Ltd., London). Thin tissue fragments containing melanocytes were dehydrated through an ascending series of ethanol-water solutions, cleared in xylene and mounted in DPX resin. Examination and photography of the tissues was facilitated by the 'optical sectioning' capability of Nomarski Optics included on a Zeiss Universal microscope.

2.6 Photo-microscopy and Time-Lapse Micro-cinematography

Cultures were examined and photographed on a Wild M40 inverted phase microscope. Owing to the intensity of contrast achieved with phase illumination on this microscope, low levels of pigmentation were difficult to observe and photograph. This problem was overcome by creating an interference contrast-type effect with oblique illumination. Specimen contrast was altered by regulating the condenser aperture and placing a mask above the condenser, so that only a segment of the illuminating cone was used (observed in the back focal plane of the objective lens).

The same microscope was used for time-lapse photography when placed on a vibration-free table in a 37°C hot room.

Initially films were made using cells cultured on glass coverslips placed in Cooper Dishes (Falcon) within a humidified chamber. Latterly however, a much simpler procedure was adopted. The sealed 35mm petri dishes used in routine culture were placed on the open microscope stage. If small water droplets formed on the lid as a result of tight sealing, they were simply removed with a pipette from the area of interest, and filming was then begun. Kodak 16mm Pan F monochromatic film was used and subsequently developed and contact printed by Brent Laboratories Ltd, London.

Positive prints were analysed with a Spectro Mark III analytical projector or a manually operated film editor. Cell shape changes were studied by tracing the outline of cells from 60 or 135-fold projections.

Still photography was carried out with a Nikon F3 camera and panchromatic 35mm film (Ilford, Pan F), which was developed in Kodak D76 developer at 1:1 dilution.

2.7 Histochemical Methods

2.7.1 Tyrosinase (Dopa Oxidase)

Cultures were fixed for 4 hours in 5% gluteraldehyde in 0.1M Sodium Cacodylate buffer of pH 7.3, and then washed 6 times in cacodylate buffered 10% sucrose. Specimens were incubated in 1.0mg/ml L-Dopa (Sigma), in cacodylate buffer for 4 hours at 37°C. Tyrosinase activity, resulted in the formation of a dark-coloured, electron-dense dopa melanin at the sites where the enzyme was present (Hunter et al. 1978). Control cultures were incubated in the same way with D-Dopa (Sigma), in order to distinguish autoxidation of dopa from that catalysed by the enzyme. Cultures were then rewashed in 10% buffered sucrose, and if required, post fixed in osmium tetroxide for transmission electron microscopy.

2.7.2 Melanin Detection Methods on Cultured Cells

Three methods were used in order to establish that melanin, rather than a neuronal lipofuscin (Pearse 1960), was being produced by the neural crest cells in culture. They rely on the fact that melanins do not autofluoresce, are bleached with strong oxidising agents, and do not retain Nile Blue Sulphate staining after bleaching; whereas lipofuscins behave in the opposite fashion.

2.7.2 i Lack of Autofluorescence

Cells were examined live or after fixation in 4%

→ formaldehyde/cacodylate buffer, using a Lietz Ortholux II microscope

with epifluorescent illumination. Excitation was achieved with a Leitz KP 500 or a BG 12 filter, in conjunction with an inbuilt KP 470, and suppression with the dichroic mirror in position 3, giving Leitz TK 510/K 515 barrier filtration. This configuration was used in order to encompass a broad spectrum of emitted light. (Glutaraldehyde fixed cytoplasm produced an intense green autofluorescence when viewed in this way).

2.7.2 ii Bleaching Methods

These methods were modified from the standard techniques which proved destructive to fixed hydrated cells (Pearse 1960). Cultures were therefore air-dried from water or ethanol; the latter giving better explant morphology.

Two methods were used:

- (a) Fixed dried cultures were flooded with 10% H_2O_2 at room temperature while being monitored on an inverted microscope.
- (b) Fixed dried cultures were flooded with 0.25% aqueous $KMnO_4$ for 25 minutes and then washed with distilled water. The dish was placed on the stage of a dissecting microscope and a 0.5% aqueous oxalic acid solution added. When the cells became completely colourless (approximately 1 minute for a monolayer), the reaction was stopped by washing several times in distilled water.

2.7.2 iii Nile Blue Sulphate Staining and Bleaching

The technique used was slightly modified from Lille (1921). Cultures were stained with 0.5% Nile Blue Sulphate in distilled water. The stain was differentiated by incubation in ethanol for 15 minutes. Using this modification, pigmented cells stained dark green, whilst the blue background was removed from the fibroblasts.

The specimen was then bleached with 10% H₂O₂ for 24 hours. Melanin, unlike lipofuscin, does not retain stain after bleaching.

2.8 Glycosaminoglycan Cell Coat Demonstration

Cell coats were visualised by allowing a suspension of marker particles (sheep red blood cells) to settle onto the tissue culture substratum at high density (10⁷ RBCs/cm²). When extensive coats were present, the marker particles were excluded from a region around the cell periphery (McBride and Bard 1979).

In order to demonstrate the hyaluronidase sensitivity of these coats, the marker particles were removed by washing the culture very gently with growth medium. The cells were then incubated in 50iu/ml testicular hyaluronidase (Sigma) in PBS for 10 minutes at 37°C. Another suspension of marker particles was added and allowed to settle again. Sensitivity was defined as the complete absence of a coat after hyaluronidase incubation.

2.9 Histology and Transmission Electron Microscopy

Cultures were fixed in 5% gluteraldehyde in 0.1M cacodylate buffer of pH 7.3, for 4 hours. Tissues were fixed in a similar fashion for 24 hours. Cultures were then post-fixed within their original dishes with 1% osmium tetroxide in cacodylate buffer, and then dehydrated through a graded series of ethanol/water solutions, and embedded in araldite. The dish was removed by immersing the culture in liquid nitrogen and then prising off the cracked plastic with fined nosed pliers and a scalpel. The basal surface was cleaned with xylene to remove traces of plastic and the culture was then re-embedded in araldite. Tissues were processed in a similar fashion, although propylene oxide was used as an intermediate solvent between ethanol and araldite. When serial sections of tissue were required, wax was used as an alternative to araldite.

Serial sections of whole, wax-embedded tissues were cut to 5µm thick. Better quality sections of araldite-embedded tissue and

cultured cells were cut to $3\mu\text{m}$ in thickness, using a Leitz microtome fitted with 28 and 38mm glass knives. Araldite sections were stained with toluidine blue and examined under oil, whilst wax serial sections were stained with heamatoxylin and eosin and mounted in DPX (Gurr).

Semi-thin $1\mu\text{m}$ sections and serial ultra-thin sections (silver interference colour) were cut on an LKB ultramicrotome and then stained with 1% uranyl acetate and Reynolds lead citrate. Ultra-thin sections were viewed on a Philips 300 transmission electron microscope, at an accelerating voltage of 60 or 80 Kv, depending on the magnification used. Specimens were held in a rotating grid holder which was cooled with an anti-contamination device fitted to the goniometer stage.

2.10 Electron Probe Microanalysis

Specimens were processed as for TEM, although the osmium post-fixation and heavy metal staining were omitted, in order to prevent 'contamination' of the elemental spectrum. Thicker 'blue' sections (100nm) were also used, in order to increase X-ray emission from the target zone. These sections were mounted on pure nickel grids, so that copper (present in tyrosinase for example) could be detected.

Specimens were viewed with a Cambridge S180 SEM, used in scanning transmission mode. Elemental analysis of the X-ray emission spectrum was carried out with an energy dispersive electronprobe microanalyser (Link Systems Ltd). Numerical analysis of the spectrum was carried out with the Quantum FLS software package (Link Systems Ltd), which allowed semi-quantitative determination of abundance of each element, so permitting comparison of each element between melanosomes, but not of different elements with respect to one another.

Analysis of individual melanosomes was made by locating a 100nm electron ($5 \times 10^{-9}A$, 25Kv), in the centre of the organelle, as determined by its position in the afterglow of the scanning transmission image. The microscope configuration was maintained in a standard state for the analysis of each sample.

Melanocyte Migration and Differentiation

Great interest has been shown in the migration and differentiation of some cranial derivatives, such as neurons (Nave 1971), are induced by signals along their migratory pathway. In the case of neurons like the cranial ganglia (Nave and Lund, 1971) and the terminal site of differentiation. In the case of neurons, however, of the relative importance of these signals is still a matter of debate. In the case of neurons, the signals are thought to be important in the early stages of migration, but less important in the later stages.

The present study was carried out using cultured epithelial cells derived from the early embryo and post-migratory cells from a site of differentiation, and then comparing the resulting differentiation with that which occurs in vivo.

Melanocyte distribution under laboratory conditions in the tissue of the eye was studied. The distribution was studied using electron microscopy.

The present study was carried out using cultured epithelial cells derived from the early embryo and post-migratory cells from a site of differentiation, and then comparing the resulting differentiation with that which occurs in vivo.

ENVIRONMENTAL REGULATION OF MELANOCYTE DIFFERENTIATION3.1 Introduction

This chapter is concerned with the way in which the embryonic environment in vivo and the culture milieu in vitro influences melanocyte differentiation.

Organ culture experiments have indicated that differentiation of some crest derivatives, such as neurones (Norr 1973), are induced by structures along their migratory pathway, whilst other derivatives like the cranial osteoblast (Bee and Thorogood 1980) are induced at the terminal site of differentiation. We are still unaware, however, of the relative importance of terminal site and migratory pathway in the induction of melanocytes. Experiments were therefore undertaken to assess the importance of terminal environment in controlling melanoblast differentiation.

The experimental approach adopted involved culturing pre-migratory crest cells from the early embryo and post-migratory cells from a site of differentiation, and then comparing the resulting differentiation with that which occurs in vivo.

Melanocyte distribution, colour and morphology were examined in the tissue of the late embryo using the histological and whole mount techniques described in Chapter 2.

Pre-migratory crest cells were mainly obtained by the neural tube explantation technique of Cohen and Konnigsberg (1975). This technique relies upon the fact that neural crest cells will migrate from the isolated tube onto the culture substratum, whereupon the explant can be removed, leaving behind a population of cells which

are exclusively or largely of crest origin (see chapter 1.2 and chapter 2.2.3.)

Undifferentiated post-migratory crest cells on the other hand, are more difficult to identify and isolate because of their tendency to become intermingled with cells of non-crest origin. Feather melanoblasts, for example, become intermingled with epithelial cells of the barb vane ridges (Waterson 1942), and thus could only be isolated in limited amounts, if at all. Periorbital mesenchyme, however, is composed entirely of cranial crest cells (Le Lievre 1978, Noden 1975), which migrate between the head ectoderm and optic cup during the second day of development. During the next two weeks of incubation, this mesenchyme is transformed into the highly pigmented connective tissues of the uvea. By isolating and explanting this mesenchyme at an early stage of development, one can effectively culture the post-migratory crest cells from a site of prolific melanoblast differentiation (see chapter 2.2.5).

No studies appear to have been published where the differentiation of extra-integumental melanoblast has been studied. The comparison of melanoblast differentiation within presumptive connective tissue, with that occurring in the isolated crest population, therefore seems to be a novel approach to the study of melanoblast differentiation.

Melanocyte shape was re-examined in vitro, and in a variety of tissues, in order to test the claim (Market and Silvers 1955), that morphology of this cell type is autonomously determined and hence independent of environment.

In recognition of the fact that cell shape may change during the course of cell migration or differentiation (Newgreen and Thiery 1980), it was necessary to augment the static description of cell

shape in tissues with a more dynamic time-lapse analysis in the culture situation. By considering both environment and cell behaviour, a more integrated approach to the study of shape control was achieved.

The colour of pigment produced by the premigratory and post-migratory Brown Leghorn crest cell was examined in order to establish whether the mesenchymal environment has a modulating effect on melanogenesis.

The classic work of Twitty (1945) on amphibian melanocyte dispersion and aggregation demonstrated that culture conditions which were favourable for pigment production, resulted in melanocyte aggregation in vitro. This unusual form of social behaviour was reinvestigated, and its dependence on culture conditions examined. The behaviour of the post-migratory cells was compared with that of their early precursor, in order to determine if the population which 'dispersed' (in Twitty's terminology) in vivo, still maintained the tendency to reaggregate.

3.2 Results

3.2.1 The In Vivo Melanocyte

3.2.1 i Melanocyte Distribution and Pigmentation

Reference is often made to the fact that the internal mesenteries and connective tissues of pigmented animals contain neural crest derived melanocytes (e.g. Noden 1978), yet no comprehensive description of their distribution seems to be available. The distribution of melanocytes within the late Brown Leghorn embryo was therefore examined with a view to describing all of the tissue locations in which melanocytes are present.

Dissected 18 day embryonic tissues were examined by serial sectioning, whole mounting, or more directly on the inverted or dis-

secting microscope (xl20). Melanocytes were not observed in the brain, the spinal cord, nor the liver and heart, but were present within the epidermis of feather skin, eyelid, corneal limbus, nictitating membranes, and in the stroma of the dermis, uveal connective tissues (choroid, sclera, ciliary body, iris, corneal limbus, nictitating membrane), and the connective tissues surrounding the skull bones, vertebrae, joints, ribs and intercostal muscle, adipose tissue, skeletal muscle of the leg, foot pad, and the base of the claws.

In all locations, with the exception of some feather germs, the melanocytes were black in colour, and contained melanosomes which appeared elongate in shape (see chapter 4.1). Within the feather germs, pigment was found within the melanocytes and transferred to the epithelial cells of the barb vane ridges (Hamilton 1941). Pigment colour was black and brown and varied non-systematically throughout individual feather germs and between neighbouring germs.

Melanocyte abundance varied among tissues, but was much greater within the choroid than any other connective tissue examined. Dendritic contact was observed when the melanocytes were present at high density. Within the stromas, this contact resulted in the formation of long 'chains' of cells, as previously noted by Hamilton (1940). Contact was particularly obvious within the nictitating membrane where the melanocytes were clustered around the capillaries (fig. 3/1). This clustering was also found in sections of other vascularised stromas such as the corneal limbus, where close association of the blood vessels and melanocytes was observed. Ultrastructural examination of this association revealed a close contact of the melanocyte with extracellular matrix, or basement membrane, surrounding the endothelial cells (see fig. 3/8). The

endothelial basement membrane, therefore, seemed to be a preferred substratum for the melanocyte. In spite of cell clustering, or dendritic contact, in culture, no cell aggregation occurred like that observed by Twitty (1945) in vitro.

3.2.1 ii Qualitative Description of Melanocyte Shape in Different Environments

Epithelia: Within epithelia, the melanocytes had the classic dendritic shape and location. The cell body sat on the basement membrane from where the dendrites spread in an apico-lateral direction. Even here, however, morphology was dependent on the nature of the epithelium in which the melanocyte was located. In non-cornifying epithelia, such as the conjunctiva or that surrounding the nictitating membrane, the melanocytes were very small and contained exceptionally fine dendrites (fig. 3/2). Dorsal epidermis had larger melanocytes with stouter dendrites (fig. 3/3), which tended to run more apically in a thicker epithelium. Although it is difficult to ascertain why cell shape should vary in this way, it seems probable that the difference must be related to a divergence of the epithelia, rather than an intrinsic characteristic of the melanocytes. The most obvious difference observed, was epithelial cell size, as clearly illustrated in figs. 3/2 and 3/3. The epidermis of feathered skin consisted of much larger cells than those surrounding the nictitating membrane or the corneal limbus. Examination of the later epithelium with Nomarski optics revealed a suture-like appearance of opposed cell membranes between the smaller cells. This suggests structural or perhaps functional differences in the intercellular adhesion of each epithelial type.

Connective tissues: Melanocytes of connective tissue stromas were found 'adherent' to capillaries, as indicated earlier, or embedded between the collagen bundles. In both of these locations,

they assumed the classic dendritic shape (fig. 3/4), although those processes attached to capillaries curved as they followed the contours of their walls, running along their length, or around their circumference (fig. 3/5). Where much larger diameter blood vessels acted as a melanocyte substratum some cells were observed to be considerably flatter with less prominent dendrites (fig. 3/6). Melanocytes in the connective tissue surrounding the flatter parts of the skull (i.e. those bits which could be easily whole mounted), were often bipolar, making them more elongate in shape (see quantitative section) than their counterparts in the fold of the nictitating membrane.

Ultrastructural examination of dense collagenous stromas revealed that both fibroblast and melanocytes followed the contours of adjacent collagen bundles or neighbouring cells (figs. 3/7 and 3/8). Cell shape therefore appears to be directly influenced by the gross architecture of the stromal extracellular matrix.

One surprising aspect of stromal melanocyte behaviour was their ability to bud-off melanosomal clumps from the ends of the dendrites (fig. 3/9). This budding-off behaviour was inferred from the presence of bulbous dendrite termini (fig. 3/10), dendrite constrictions (fig. 3/11), and the presence of isolated melanosome clumps. The apparent uptake or transfer of melanosome to epithelial cells has led some workers (e.g. Ereathnach 1982) to suggest that melanosome loss was a function of an epithelial-melanocyte interaction. These observations clearly demonstrate that this behaviour is autonomous to the melanocytes.

Sclera: The avian sclera differs from that of the mammal's because it contains a sheet of cartilage and a ring of flat membrane

bones known as the scleral ossicles. This tissue provided a different sort of environment from that observed during studies of melanocyte shape in the mouse (Market and Silvers 1956).

Close examination of the melanocytes lying on the inner (retinal) or outer surface of the cartilage (fig. 3/12), or more anteriorly within the region of the ossicles, revealed well-spread dendrite-free cells with a more epithelial morphology than that previously recorded (fig. 3/13, 3/14). Examination of transverse and tangential sections demonstrated that these cells tended to be flattened in the plane of the cartilage. This was supported by the fact that the whole mounted cells contained melanosome-free nuclear zones (i.e. the cell was probably little thicker than the nucleus).

3.2.1 iii Quantitative assessment of melanocyte size in three different locations

Three locations were chosen in which all melanocytes had a similar orientation and in which the dendrites tended to run in the same plane.

The temporal fold of the nictitating membrane was a useful location for cell shape measurement because it contained the tiny epithelial melanocytes with laterally spreading dendrites, and sub-adjacent stromal cells oriented parallel to the edge of the membrane. These were compared with the apparently more elongate cells of the connective tissue surrounding the flatter parts of the skull bones.

Cell size was measured with a rotatable ocular micrometer and taken as the maximum distance between two points on the cell periphery (e.g. the distance between the ends of two dendrites). Skull fragments and nictitating membranes from 3 or 4 embryos respectively were examined, and a total of 100 cells measured in each location.

Mean cell size and distribution are presented along with the appropriate statistics in tables below, and displayed in the accompanying histograms (figs. 3/15, 3/16, 3/17).

CELL SIZE IN VARIOUS LOCATIONS

	\bar{x}	σ	n
Skull	67.2	± 1.58	100
Stroma	47.8	± 1.25	100
Epithelium	27.1	± 0.69	100

SIGNIFICANCE OF THE DIFFERENCES IN MEAN AND VARIANCE OF CELL SIZE

	T*	Mean* p	Variance F	p
Skull - stroma	9.08	0.001	1.59	0.05
Stroma - epithelium	14.47	0.001	3.28	0.01
Skull - epithelium	21.50	0.001	5.20	0.01

*The significance of differences in the sample mean were calculated using a T-test with a confidence interval which was modified to account for significant differences in sample variance (Ti52, Instrument Manual, Texas Instruments, Houston, Texas).

These results provide quantitative evidence of variation in melanocyte morphology in different locations within the late chick embryo and thus show that environment modifies cell shape.

3.2.2 Melanocyte Differentiation of the Premigratory Neural Crest In Vitro

3.2.2 i Differentiation of Migrant Crest Cells in Neural Tube Cultures

During the first 24 to 36 hours of explantation, large numbers of (crest) cells migrated from the dorsal aspect of the neural tube onto the culture substratum (fig. 3/18). The tube was then mechanically removed with a microscalpel, leaving behind a population of proliferative stellate cells (fig. 3/19). Melanocytes appeared after 2 to 10 days of culture depending on the composition of the growth medium. They were stellate, rather than dendritic in shape and contained a diffuse light brown pigment (figs. 3/20, 3/21).

This pigment was less dense, and of a different colour, from that observed in whole mounted melanocytes (see chapter 3.2.1) of the connective tissues and the ocular epithelia. Only on rare occasions did the neural crest outgrowth differentiate into highly pure populations of melanocytes (figs. 3/22, 3/23).

After 5 or 6 days of culture, a few well-spread fibroblastic cells were found at the periphery of the outgrowth (figs. 3/24, 3/25). They contained osmophilic lipid vesicles, and possessed prominent ruffling membranes. Greenberg et al. (1980) have also observed these cells and noted that they were not present during the early period of culture, which suggests that they differentiate after a period of growth and proliferation.

Large patches of epithelial cells, like those found by Newgreen (personal communication), sometimes appeared within the outgrowth during the first week of culture (fig. 3/26). Although they may have differentiated in a similar way to the corneal endothelium (Bard et al. 1975), their origin must be the subject of some doubt. Contaminating cells from the neural epithelium or from minute traces of ectoderm could have given rise to epithelial patches. Isolated notochord explants dissociated to form an epithelial island (fig. 3/27). Contaminating notochord fragments could therefore have been another source of the epithelial patches.

Neurones were invariably present if the neural tube was left in place for more than 36 hours. Neuronal processes emerged from the explant, either singly or in bundles, which branched on the substratum (fig. 3/28). These processes were adherent to melanocytes and other non-pigmented cells (fig. 3/29), often zig-zagging their way from one cell to another. In the presence of collagen coated substrata there was a greater tendency for neuronal

processes to move over the free substratum, rather than form adhesions to other cells. When C.E.E. was added as a supplement to the growth media, migrant neurones appeared at the same time as melanocytes. Neurones, unlike melanocytes, disappeared within 7 to 10 days, whether the tube was left in place or subsequently removed.

3.2.2 ii The Influence of Culture Conditions on the Timing of Melanocyte Differentiation

The effect of serum and embryo extract upon melanogenesis was examined because of their effect on crest differentiation (see chapter 1.4.3).

Surprisingly, it was the timing of differentiation rather than pigment type which varied. The timing of differentiation was recorded as the time to the first appearance of pigment within the culture. Serum concentration, rather than embryo extract, defined the time to onset of differentiation over the concentration range used. In high serum concentration, differentiation was precocious in comparison to that found within the embryo, whilst low levels of serum resulted in a later onset, similar to that found in pigmented tissue, such as the periorbital mesenchyme (day 8) or dorsal skin (day 9).

No of Cultures	Serum Concentration	C.E.E. Concentration	Time of Onset
3	0*	0	Cell detachment in 1 day
10 5	5% F.C.S. 5% H.S.	2% 2%	6-10 days
2 10 3	10% F.C.S. 10% F.C.S. 15% F.C.S.	0 5% 5%	2.5-4 days

*denotes transfer into HAM's F10 plus supplements after 1 day of culture.

Although melanocytes were more abundant in the presence of embryo extract (fig. 5/5, and Maxwell 1976), no variation in pigment colour or intensity was detected in the conditions tested above.

Other cultures, containing high concentrations of serum and embryo extract, were supplemented with dibutyryl cAMP, to mimic the action of melanocyte-stimulating hormone (Kitano 1976). After 3 days of culture, dibutyryl cAMP was added to a final concentration of 0.5mM and 1.0mM. Treatment for a further 3 or 4 days caused no change in pigment colour or cell shape. This suggested that the culture medium could not be further optimised by the addition of melanocyte-stimulating hormone-like activity.

3.2.2 iii The Influence of Culture Conditions on Cell Behaviour

Melanocytes formed elevated clusters or aggregates similar to those noted by Twitty (1945). Aggregation was found to be dependent on culture conditions, so that it sometimes occurred prior to the onset of differentiation and sometimes afterward. The culture conditions examined are given below.

10 or 15% F.C.S. plus 5% C.E.E.: Within 36 hours of explantation, small unpigmented clumps of approximately 9 rounded cell diameters formed within the outgrowth (fig. 3/30), and on the surface of the neural tube (fig. 3/31). These clumps started to pigment about one day later (fig. 3/32), at the same time as individual cells within the outgrowth or explant. During the first week of culture most of the melanocytes became located in very large aggregates at the periphery of the culture. Some of these aggregates were loosely attached and became detached after gentle agitation. In two of the cultures containing 15% serum, this peripheral aggregation was so extensive that unusual annular pigmented zones developed (fig. 3/34).

5% F.C.S. or 5% H.S. plus 2% C.E.E.: Outgrowths developing in these conditions did not form unpigmented clumps, and the neural tubes were completely devoid of either clumps or pigmentation. Initially these outgrowths consisted of densely packed overlapping cells, which subsequently aggregated (fig. 3/35).

10% F.C.S.: In the absence of embryo extract few crest cells produced pigment and consequently remained quite remote from each other amongst numerous overlapped unpigmented cells (see fig. 5/5). In these conditions no aggregation of pigmented or unpigmented cells occurred over a 2 week period. This is in accordance with results of Twitty (1945) who suggested that aggregation of the melanocyte only occurred under conditions which favour melanogenesis.

3.2.2 iv Time-Lapse Photography of the Premigratory Melanocytes

Detailed observations of cell shape changes were made in order to determine the location of functional cell substratum attachment. Three events in fig. 3/36 suggest that melanocytes exert tension on the substratum at points of peripheral attachment. Event 1 shows how a cell retracts one of its three dendrites, and how this is accompanied by a change in the orientation of those that remain. The remaining processes change from a skew to a straight orientation, which suggests that tension is exerted along their axes (Harris 1973). This is also accompanied by a change in the position of the nucleus, although the remaining point of free peripheral attachment remains stationary.

A similar change of nuclear position is recorded during event 2 where the nucleus changes from a central to a peripheral location in the cell. Change of nuclear position is then followed by very rapid retraction (event 3) of the cell to its point of free peripheral attachment. These events suggest that the cell is 'tethered'



to the substratum in the dendritic rather than perinuclear regions. These observations are in agreement with the experiments of Harris (1973), which showed peripheral attachment by mechanically deforming cells, and the observations of Curtis (1964) who demonstrated peripheral points of close cell-substratum attachment by interference reflection microscopy.

3.2.2 v The Influence of Dibutyryl cAMP on Red Minorca Neural Crest Cells

Ten Red Minorca neural tubes were explanted and cultured in 5% H.S. and 2% C.E.E. for 24 days. After this period, one prolific outgrowth of unpigmented stellate cells was chosen for study of the relationship between melanocyte differentiation and aggregation. The outgrowth from this culture was suspended with 0.2% trypsin and 0.2% EDTA, in phosphate buffered saline, and then spot-cultured in 100 μ l drops of medium for 4 hours. More medium was added, and the cells allowed to proliferate for 2 days. Four matched pairs were selected and one culture was treated with either 0.5mM or 1.0mM dibutyryl cAMP, whilst the medium was merely changed in the control. After 3 or 4 days, the cells started to pigment and clump (fig. 3/37, 3/38), whereas the controls remained stellate (fig. 3/39, 3/40). Isolated cells started to round up and detach after 5 to 6 days, suggesting a toxic effect of treatment. If the treatment was stopped at this stage, the cells started to proliferate again, and no pigment was observed within 7 to 10 days. These observations demonstrated that aggregation can be induced by an agent which mimics the activity of melanocyte stimulating hormone (Kitano 1976). The continuous presence of such activity is required for the induction of characteristic behaviour in Red Minorca melanocytes in vitro.

3.2.2 vi The Absence of Early Embryonic Influences on Melanoblast Differentiation

The experiments reported here were designed to test the hypothesis that melanoblast differentiation of the crest is not influenced by interaction with the tissues of the early embryo.

Crest interaction with the superficial ectoderm was examined by isolating tubes from different axial levels of the same embryo.

Caudal presomitic neural tube segments were isolated from 22 somite embryos, in order to obtain tube where crest had not already formed. Outgrowth formation from caudal segments was compared with that occurring in more cranial tube regions in which the crest had already formed (Weston 1963; presomitic neural tube could not be isolated free of notochord using the methods described previously). Tube segments from three embryos were isolated in this fashion and cultured in 10% F.C.S. and 5% C.E.E. No delay in the outgrowth of the cells was observed, or any difference detected in the amount or type of pigment produced. This suggests that the early ectoderm was not required for crest formation or maturation, and that it did not influence the melanoblastic potential of the premigratory cells.

In order to study the possible inductive effects of the neural tube upon the crest cells, attempts were made to explant 'tube-free' crest. Thirty four explants were taken from very early embryos (17 cephalic neural folds, 8 mesencephalic roof segments, and 7 squares of head ectoderm with underlying migrant crest cells), however only 2 of the roof segments produced outgrowths. Each of these outgrowths contained brown melanocytes which clumped, and were thus indistinguishable from neural tube cultures.

Four whole trunk segments (fig. 3/41) (including neural tube, notochord, somites, ectoderm and endoderm) were explanted in order to determine if the complete embryonic environment of the early embryo influenced melanocyte differentiation. Two segments were partially digested with trypsin-pancreatin in order to promote explant adhesion to the tissue culture plastic (whilst maintaining the gross structure of the tissue).

No enzymic digestion was used on the other 2 segments in order to preserve the integrity of the cultured material. However, this meant that the segments were not adhesive enough to explant onto tissue culture plastic. Trunk segments were therefore explanted onto rat-tail collagen gels (kindly provided by Dr D Davidson). Four out of four segments contained diffuse brown pigmentation (fig. 3/42). Cells which migrated onto the tissue culture plastic or the collagen gel, pigmented and clumped in a similar fashion to that reported earlier. These experiments demonstrated that the tissues of the early embryo could not induce the production of black pigment, similar to that observed in the melanocytes of the connective tissues and ocular epithelia.

3.2.3 Melanocyte Differentiation of the Postmigratory Neural Crest In Vitro

3.2.3 i Differentiation within Periorbital Mesenchyme Explants and their Outgrowths

The mesenchyme of stage 26-32 embryos was isolated from the superficial ectoderm and underlying retinal layers by digestion with a mixture of trypsin and pancreatin (see chapter 2.2.5). This treatment preserved the gross structure of the mesenchyme, whilst making it adhesive enough to explant on tissue culture plastic. Most cultures (94.5%, n=68) consisted of a discrete explant and

outgrowth, although in a minority (5.5%, n=4) of cases the explants dissociated within 5 days and formed diffuse multilayer outgrowths.

All cultures were inspected carefully within 4 hours of explantation; none contained pigment or isolated dissociated melanocytes. After a period of 2 to 3 days, all explants contained black dendritic melanocytes (fig. 3/43, 3/44). Examination of sectioned material showed that the black melanocytes were scattered throughout the entire explant (fig. 3/43, 3/44) but no brown pigment like that observed in neural tube culture (3/46) was found. The outgrowths contained a majority of fibroblastic cells, and a minority of brown melanocytes (fig. 3/47). The colour of the pigment formed in the outgrowth was clearly different from that observed in the explant, but similar or identical to that observed in neural tube cultures. The brown and black melanocytes were also morphologically dissimilar from each other. The brown cells had the stellate appearance characteristic of the premigratory crest, whilst the black cells were more dendritic in shape.

Osmophilic lipid vesicles developed within the fibroblast cytoplasm during the first 5 or 6 days of culture (fig. 3/48), but were never detected in either type of melanocyte.

One day after the development of explant pigmentation, the black melanocytes started to migrate from the explant into the outgrowth. No transition in pigment colour was observed within the outgrowth, so that brown and black melanocytes were still present after 2 weeks of culture (chapter 4.2.3 for contrasting ultrastructural results).

3.2.3 ii The Independence of Differentiation from Culture Conditions

Experiments were conducted, to establish whether culture conditions could influence the colour of pigment produced, or the abundance of melanocyte differentiation.

The five culture regimes used are listed in the table below.

No gross changes in melanocyte abundance or pigment colour were observed in any of the regimes tested. This uniformity of pigmentation was established by the inclusion of a complete series of paired control experiments, in which one explant was taken from both eyes and grown in different media. The independence of pigmentation formation from the stimulatory effect of chick embryo extract was regarded as particularly significant because of the profound influence of this agent in neural tube culture (see section 3.3.1). Minor modulations of abundance may have gone undetected however, because of the difficulty in assessing the number of cells within the explants, or in the outgrowths where they tended to aggregate (see chapter 3.2.3. iii). Tyrosine supplementation of the medium was included in this series, in order to establish that the brown pigmentation was not formed as a consequence of limiting concentrations of this eumelanin precursor.

Condition	No of cultures	Result
5% FCS plus 2% C.E.E.	20	Black dendritic melanocyte appears in 2 days within the explant. Brown stellate melanocytes differentiate within the outgrowth in 3 days
10% FCS plus 2% C.E.E.	11	
10% FCS plus 5% C.E.E.	19	
10% F.C.S.	12	No reduction in melanocyte abundance in the absence of C.E.E.
10% F.C.S. plus 5% C.E.E.	6	Tyrosine 'loading' did not influence the colour of the brown melanocyte

3.2.3 iii Time-Lapse Photography of Post-Migratory Cest Cells In Vitro

The individual and collective behaviour of melanocytes was studied in order to gain a more dynamic perspective upon the in vivo observations of cell shape and distribution.

One day after the onset of pigment formation, melanocytes started to migrate from the explant. On the occasions where large numbers of cells migrated, they rapidly formed clumps similar to that in neural tube cultures. A time-lapse sequence of melanocyte migration (fig. 3/49) shows how aggregation may occur over a short period, in this case 7 to 8 hours. (The detailed migratory behaviour of individual cells could not be followed, due to lack of frame stabilisation of the projection apparatus, and the absence of a fixed reference point within the outgrowth).

Changes in cell morphology were more easily followed by tracing the cell outline at selected intervals. The first set of tracings (fig. 3/50) shows how melanocyte shape is continuously changing by a process of dendrite extension and retraction, so that within a period of 40 to 60 minutes the outline becomes completely reformed. New dendrites may extend de novo from the cell body, or extend as branches from pre-existing structures. Shape changes also occurred over larger areas of the periphery, which lead to lamella-podial rather than dendritic extension. The second sequence (fig. 3/51) was obtained from a 5 day culture in which the explant had disaggregated and produced a dense fibroblastic outgrowth. Dendritic extension and retraction, leading to cell shape change are again clearly illustrated. One additional phenomenon was observed. During the first 5 minutes of the sequence a densely pigmented dendrite tip pinches off, forming a little clump of melanosomes, which subsequently becomes remote from the cell. This phenomenon

was not an artifact of the culture situation, for it was also observed, at different stages, within whole mounted connective tissues (see chapter 3.2.1 ii). Comparison of figs. 3/50 and 3/51 shows that aggregation of melanosomes may occur before or after formation of the dendrite. Melanosome aggregation and dendrite extension therefore appears to have some causal linkage. After 1 week in culture, the melanocytes tended to lose their dendrites and develop a less arborised morphology.

Other sequences presented in fig. 3/52 and 3/53 were obtained by examination of explants, in which morphology of the individual cell could be easily distinguished. Melanocyte morphology varied from dendritic to epithelial and therefore mimicked the situation in vivo. These sequences demonstrate that 'epithelial' cells also undergo changes in cell shapes like those observed in their more dendritic counterparts, either within the explants or outgrowth. Although localised changes resulted in the protrusion of fine processes, activity within the more epithelioid cells was wide-spread over the entire cell periphery. Control of a cell shape in vivo therefore appears to involve the modulation of locomotory behaviour by the local microenvironment.

3.2.3 iv Explantation of Periorbital Mesenchyme with Attached Pigmented Retina

These cultures were produced, in order to examine possible interactions between the retina and melanocytes of the choroidal layer. Within 2 days of explantation, each of the four explants produced an extensive epithelial outgrowth (fig. 3/54) which depigmented at its periphery during the initial 2 day period. Surrounding the depigmented sheet were fibroblast and brown melanocytes, characteristic of the mesenchyme (fig. 3/55). Dendritic black melanocytes (like those found in pure mesenchyme

cultures) were found on the upper surface of the depigmented epithelium (fig. 3/56, 3/57). These cells had prominent dendrites with bulbous endings (fig. 3/58, 3/59), like those observed in vivo or in time-lapse. Unlike their counterparts in purely mesenchymal cultures these cells retained their dendritic morphology for at least one week of culture.

3.3 Summary of Results

1 Melanocytes are almost ubiquitous throughout the Brown Leghorn embryo, being present in connective tissues and the superficial epithelia. Cell size and morphology varied considerably from one tissue to another, although the pigment colour was black in almost all locations. Various stages of dendrite constriction and melanosome shedding were observed within connective tissues.

2 The cultured premigratory crest cells differentiated to produce stellate brown melanocytes. Pigment colour was invariable over a range of culture conditions, although the timing of differentiation and the number of melanocytes could be modulated. Conditions which promoted melanocyte differentiation also caused these cells to form large aggregates. Aggregation occurred prior to or after the onset of differentiation, depending on concentration of serum and chick embryo extract, present in the medium. Red Minorca neural crest cells pigmented and clumped if Dibutyryl-cyclic-AMP was present in the culture medium. Time-lapse photography showed that stable cell-substratum adhesion occurred in the dendritic rather than perinuclear region. The crest cells do not produce black pigment as a result of interaction with early embryonic tissue in vitro.

3 The postmigratory crest cells differentiated normally within orbital mesenchyme explants, producing black pigment and assuming the characteristic range of shapes. Black cells migrated from the

explant to the outgrowth where they aggregated. Outgrowth from explants of mesenchyme consisted of fibroblasts and brown melanocytes similar to those found in premigratory cultures. This brown pigment was uncharacteristic of in vivo connective tissues. Time-lapse photography demonstrated how cell shape within the explant and outgrowth is continually changing and how the process of change is modulated by the environment. Dendritic morphology was stabilised by interaction with the P.R.E. which depigmented in culture. Melanosome shedding occurred by rapid constriction of dendrite tips.

CHAPTER 4

4.1 Introduction

The last chapter demonstrated that isolated Brown Leghorn melanoblasts produce a light brown pigment when cultured outwith tissue explants and a black pigment if they remain within the mesenchyme. This chapter is concerned with the nature of these pigments and how they vary in structure and composition from that found within the pigmented mesenteries and ocular tissues.

The production of this brown pigment was not a special feature of ectopic postmigratory cells, but was also characteristic of the premigratory crest cells which were isolated prior to tissue colonisation. These preliminary observations suggested that presumptive eumelanocytes had become pheomelanocytes when removed from the inductive influence of the developing connective tissues. The aim of the work presented in this chapter was to establish whether the isolated crest cells did, in fact, produce a similar form of melanosome to that found in pheomelanocytes within avian feathers (Jimbow et al. 1979).

Previous studies of pigment colour modulation have involved a comparison of feather melanocytes from different breeds of fowl (Hamilton 1941; Jimbow et al. 1979), or examination of the pigment transition in Agouti follicular melanocytes (Sakurai et al. 1975). In spite of several experimental studies in the later system (Knisely et al. 1975; Galbraith and Patrignani 1976) the control of pigment type remains a mystery (see chapter 1.5.1).

This investigation was novel in approach, for it considered the potentially modulative effect of the connective tissue environ-

ment upon the phenotype of the unpigmented melanoblast. Particular emphasis has been placed on premigratory cultures because of the abundance of melanocytes, which made the identification of different melanosomal stages less time-consuming.

An ultrastructural comparison of the black melanosomes formed in the tissue and those formed in mesenchyme explants was made, in order to establish whether the melanoblasts had differentiated in a similar fashion when removed from the influence of any humoral agents which could regulate differentiation. The brown pigment organelles formed by isolated crest cells were also examined ultrastructurally, with a view to establishing how they differed from their black counterparts, and if they were, in fact, pukka pheomelanosomes. After preliminary investigations revealed a rather unusual substructure in some organelles, the nature of the pigment itself was further investigated. Light and electron histochemistry was used to establish that the pigment was in fact melanin and that tyrosinase activity was detectable at the appropriate sites within the cells. The metallic content of the pigment was then examined, in order to make a more precise chemical comparison of the brown, in vitro, pigment and its black, in vivo, counterpart. Electron probe microanalysis was used as the analytical tool, for it permitted examination of individual melanosomes (Panessa and Zadunaisky 1981), and hence removed the need for bulk chemical analysis which would not have been possible in this situation.

4.2 Results

4.2.1 Melanogenesis within the Ocular Tissues In Vivo

Seven highly pigmented sites were examined so that the normal course of eumelanocyte differentiation within the Brown Leghorn embryo could be described. Stromal melanocytes from 14 and 18 day ciliary body, iris, and corneal limbus were examined because they represented the in vivo equivalent of those melanoblasts which differentiated in cultures of anterior periorbital mesenchyme. Intra-epithelial melanocytes from the corneal limbus and eyelid were also examined because of their equivalence of origin and difference of location.

Stromal melanocytes of the 14 day iris, corneal limbus, and ciliary body, contained melanosomes at all stages of development, ranging from round stage I's to elongate stage IV's (figs. 5/1 and 5/2). The same pattern of melanogenesis was observed in the 18 day eyelid epithelium and the 14 and 18 day epithelium of the corneal limbus (figs. 5/3, 5/4).

Dissection and high power examination (x120) of 60 embryos between days 11 and 16 of development showed that the earliest appearance of pigment within the corneal limbus occurred at H H stage 39 (or 13 days). Incubation of stage 38 anterior eye segments in L-Dopa produced no tyrosinase staining, which indicated that the ability to produce melanin was acquired shortly before the appearance of the pigment. Complete melanisation of some organelles as determined by transmission electron microscopy, would therefore seem to occur within a one day period. Some transferred melanosomes however, were found within epithelial cells on day 14, incompletely melanised. This indicated that transfer was not dependent on melanosomal maturation but occurred rapidly after the onset of melanogenesis.

Examination of a 7 day White Leghorn embryo containing transplanted neural crest cells (by kind permission of Christianne Le Lievre) indicated the presence of neural crest cells within the limbal epithelium at this stage of development. Melanoblasts within the limbal epithelium would therefore seem to undergo a longer 'incubation' phase than they do within epithelium of the dorsal skin (Teillet 1971).

4.2.2 Melanogenesis of the Premigratory Neural Crest In Vitro

4.2.2 i Histochemical Demonstration of Melanin

In view of the unusual brown colour of the pigment in neural crest cultures, 4 tests were made to establish that the pigment was melanin rather than lipofuscin (Nandy et al. 1978).

- 1 Cultures (fig. 4/4) stained with L-Dopa but not D-Dopa (fig. 4/5).
- 2 The pigment bleached with strong oxidising agents (permanganate and hydrogen peroxide).
- 3 The pigment stained green with Nile Blue Sulphate (brown plus blue produces a green colour).
- 4 The cultures did not autofluoresce if examined live or after fixation in formalin.

These results demonstrate that the pigment was not a lipofuscin artefact. Figures 4 and 5 also show that many unpigmented melanoblasts are present in some culture conditions.

4.2.2 ii Ultrastructure of Melanosome Formation

Preliminary examination revealed that the ultrastructural brown pigment organelles did not correspond to that of the pheomelanosomes observed in avian feathers in vivo (Jimbow et al. 1979). Moreover, no variation in the pattern of melanogenesis could be detected using any of the culture media described in the last chapter. This was consistent with the fact that the pigment was brown in colour, regardless of the culture conditions used.

Melanogenesis is now described in two developmental phases which encompass the early and more mature stages of organelle formation.

(a) Immature Melanosomal Stages

In its early stages melanosome formation appeared essentially normal. Numerous small vesicles were found within the melanosomal lumen (fig. 4/6). Fine strands of material interconnecting these vesicles were often evident and may have been involved in the formation of small vesicle clumps (fig. 4/6). Internal continuity of the vesicles with the melanosomal membrane was rarely observed (fig. 4/7). However, the presence of such structures suggests that they may be similar to the proposed "vesicle inversions" which have been detected (probably with much greater frequency) in osmotically shocked Xanthic goldfish (Turner et al. 1975). Numerous vesicles and coated vesicles were observed in the vicinity of premelanosomes (fig. 4/5). In spite of this the proposed external melanosome-vesicle fusion profile was more difficult to detect due to the density of the extra-melanosomal cytoplasm.

The fixation, dehydrating, and embedding procedure preserved an observable mitochondrial matrix (fig. 4/9). This feature suggested that the lack of material within early melanosomes was not caused by leaching from internal membranous compartment, but that it was a genuine feature of the organelle.

(b) The Later Melanosomal Stages

The later stages of melanosome formation were highly variable and essentially incomplete. A mixture of pheomelanosome-like and eumelanosome-like organelles were present, although elongate well-melanised stage IV eumelanosomes were never observed. Some cells displayed a range of granular pheomelanosome-like stages (fig. 4/10, 4/11) in which various degrees of melanin deposition were observed.

The melanin matrix consisted of clusters of electron dense material, similar in size to the internal vesicle. Other cells also contained melanosomes which had internal filamentous structures, characteristic of the eumelanosome (fig. 4/12). Where little or no melanisation had occurred a repeat, or spiral pattern was observed on the filament (fig. 4/13), (Hunter et al. 1978). Subsequent melanin deposition resulted in thickening of the filament and obscured details of its substructure (fig. 4/14). Filament distribution was not characteristic of that demonstrated in the organelles. Normally, they would form a densely packed array oriented along the major axis of the organelle, as shown previously. The filaments present in culture were reduced in number, spaced widely apart, and were often curved or kinked. They became intertwined in a disorganised fashion, rarely showing any close packing or parallel orientation. Melanosomal elongation was severely reduced or absent, so that most melanosomes were either round or slightly oval in section. Roundness of shape is normally characteristic of the pheomelanosome which contains no filaments. Failure to elongate therefore seems related to the absence of an organised filament array.

Both early and later stages of melanosomal formation were observed during the first 2 weeks of culture. These were found within cells which had migrated onto the substratum and within those which formed pigmented clumps upon the neural tube. After longer periods of incubation (5 to 7 weeks), examination with the phase microscope revealed a more punctate distribution. This was correlated with, and increased in, melanisation of the pigment organelles (fig. 4/15), which was associated with a preparative cutting artefact otherwise observed in well-melanised structures. Small holes in the melanin matrix (fig. 4/16), about the size of vesicles, and normally associated with eumelanosomes (Jimbow and Fitzpatrick 1975), were present even although no elongation occurred.

The presence of melanosomal filaments, and small holes, as well as the long time course of melanisation suggest the cells exhibit a disrupted form of eumelanogenesis.

(c) Melanosomal Membrane Continuity

Little information is currently available in the literature about the developmental history of the melanosomal membrane. Examination of many thousands of melanosomes revealed rare tubule-like extension of their membrane (fig. 4/17), similar to those occasionally observed by some other workers (Maul 1969; Turner et al. 1975; Sugiyama et al. 1979). These features suggest that the melanosomal membrane is continuous with parts of the smooth endoplasmic reticulum at least during the early stages of its formation. Such observations are consistent with the suggestion that the SER dilates to form the stage I melanosome (Maul 1969).

In addition to those tubular extensions which have been observed in other situations, other abnormal features of the melanosomal membrane were observed. Sometimes the melanosomal membranes were continuous with one another (fig. 4/18), so that the structure formed gave the impression of being either incompletely segregated, or partially fused. More extensive, related structures, took the form of vacuoles with convoluted or smooth profiles (fig. 4/19). On occasions these abutted onto neighbouring melanosomes, giving the impression of melanosome fusion. Some giant vacuoles contained little material, whilst others were filled with a fused mass of early melanosomes (fig. 4/20). Giant melanosomes were also observed (fig. 4/21), and probably constituted a more melanised form of the same structure. As these structures co-existed with individual melanosomes, and did not contain myelin figures, they were presumed to be different from the autophagosomes observed in White Leghorn melanocytes in vivo (Jimbow et al. 1974).

(d) Normality of the Golgi Apparatus and Tyrosinase Distribution

The structure and function of the golgi apparatus was examined with a view to establishing whether the melanosomal abnormality originated at the site of tyrosinase distribution and packaging.

The golgi apparatus within the cultured melanocyte was highly elaborate in structure, consisting of numerous cisternal stacks, each with its associated vesicles budding from the "trans" face (fig. 4/22). The stacks were in close proximity to each other, and enclosed a centriole or cilium or both (fig. 4/23), which radiated microtubules. This highly complex centre of membrane traffic and cytoskeletal organisation was similar in structure to that observed in the melanocytes of the human iridial stroma (Hogan et al. 1971), and must therefore be regarded as morphologically normal.

L-Dopa staining located tyrosinase activity within golgi associated cisternae, vesicles, and melanosomes. Stain was found within cisternae which were separate from the main golgi stack (fig. 4/24), but located nearest to its trans face (using the terminology of Farquhar and Palade (1981)). Similar staining of the golgi has been described by Maul and Brumbaugh (1971), and Mishima et al. (1979), who have described these as anastomosing and cisternal portions of the smooth endoplasmic reticulum, or GERL for short. Some vesicles within the golgi region were so intensely stained that it became impossible to determine if they were coated as other authors have suggested (Mishima 1979). Coated vesicles were clearly involved in other pathways of membrane flow for coated pits were observed on the plasma membrane. Melanosomal staining could only be distinguished from natural melanin when the pattern was significantly different from that observed in the absence of L-Dopa incubation (fig. 4/25).

A minority of vesicles were intensely stained whilst others had a more mottled appearance, containing dense areas which may have corresponded to the melanosomal vesicles. In summary, the golgi apparatus appeared morphologically normal and tyrosinase distribution was characteristic of that reported in melanocyte in vivo (Brumbaugh and Zieg).

4.2.3 Melanogenesis of the Postmigratory Neural Crest In Vitro

Ultrastructural examination of periorbital mesenchyme cultures revealed that the brown pigment formed within the outgrowths consisted of immature granular-type melanosomes similar to that found in neural crest cultures, and that the black pigment formed within the explant consisted of largely mature eumelanosomes.

Most melanosomes within the explant were highly melanised and had reached stage IV of development by the 4th day of culture (fig. 5/26). Those which had not fully melanised contained a densely packed array of filaments with the characteristic repeat pattern. The elongate shape of the organelles was another feature of their development which made them distinct from those found in premigratory crest cultures.

Melanocytes which were found in the vicinity of the explant, and located on the upper surface of the fibroblasts were presumed to be those which had migrated out after differentiation (see chapter 3). Pigmentation within these cells was intermediate between that formed in the outgrowth and that formed in cells of the explant. Dense elongate granules and others containing filament arrays were found alongside poorly melanised granules like those found in neural crest culture (fig. 4/27). Development of eumelanosomes therefore seems to require the continued presence of the melanocyte within the explant even after the onset of pigmentation.

Melanocytes found at the periphery of the cultures corresponded to those which had migrated from the explant prior to differentiation (see chapter 3). These cells, when examined after 18 days of culture, contained round granular melanosomes which were still less dense than those found within the explant after 4 days (fig. 4/28). They were ultrastructurally indistinguishable from those observed in premigration crest culture, containing a mixture of melanosome types.

4.2.4 Electron Probe Microanalysis of the Melanosomes

After demonstrating the melanin-like histochemical properties of the brown in vitro pigment, and showing unusual ultrastructural features of the melanosomes, it became important to determine if the melanin was chemically different from that found in vivo.

The most suitable analytical method available was energy-dispersive electron probe microanalysis, for it permitted examination of single melanosomes using a 100nm beam (see Materials and Methods for a review of biological applications of this technique, see Moreton 1981). Wet processing of the samples limited the analysis to elements which were not detectable within the araldite embedding medium or the neighbouring cytoplasm. The background X-ray spectra of araldite (fig. 4/29), cytoplasm (fig. 4/30) and premelanosomes (fig. 4/31) (within cultured cells), contained sulphur, silicon, phosphorus and chlorine peaks, present in variable quantities. Highly variable contributions from nickel and aluminium, which formed the support grid and microscope fitments respectively, were observed.

The melanosome spectra, in vivo and in vitro, had 5 additional metal peaks (fig. 32-36), which were not detected in the cytoplasm or nucleus. Calcium was consistently present in all melanosomes, whilst the barium, iron, copper and zinc content was more variable. Sodium and potassium were never detected. This

is consistent with studies of synthetic melanins which show binding of di-positive and tri-positive metal ions but very weak binding of monovalent alkali metals (Felix et al. 1978). Qualitative comparison of osmium post-fixed sections of eyelid (18 day), corneal limbus (14 day), choroid (12 day), pigmented retinal epithelium (12 day) and cultured neural crest, revealed calcium was always present, whilst barium was found in greater and highly variable quantities in the cultured material.

Semi-quantitative comparison of a neural crest culture and ocular melanocytes of the choroid and pigmented retinal epithelium was made, in order to determine if there was a significant elevation of barium, iron, copper and zinc in the cultured melanosome. Cultured melanosomes were arbitrarily assigned to an electron dense or an electron lucent group before analysis, in order to ascertain whether there was a correspondence between metal abundance and degree of melanisation. Elemental abundance was expressed as the peak-to-background ratio of the K_{α} emission in order to compensate for variations in section thickness and mass density. The (mass) density of the melanin polymer matrix was assumed to be similar to the plastic embedding resin. When metallic concentration is very high this may not be justified, however, in this case the total area under the melanin peaks was a small proportion of the total background. (Where 2 peaks overlapped, as in the case of nickel K_{α_1} and copper K_{α} emission was automatically 'stripped' by the computer software during the course of analysis).

The mean and standard deviation of the peak to background ratio for the K_{α} emission of calcium, barium, iron, copper and zinc are given in table 5/1. Those means shown to be different at the 95% level (or greater) with the Wilcoxin Runs test are given in table 5/2.

Comparison of the densely melanised and lightly melanised organelles (in vitro) showed a significantly higher level of calcium, copper and zinc in the more melanised group, and a non-significant elevation of iron and copper levels. The latter were highly variable in both groups and so no consistent difference was observed.

Both in vivo groups were expected to be indistinguishable because of their ultrastructural similarity. However, the pigmented retinal epithelium contained significantly more zinc and copper.

When both in vitro groups were compared jointly with both in vivo groups, it was obvious that the cultured melanosomes were much more variable than their tissue counterparts, especially with respect to copper content. Chemical variability would therefore seem to correlate with morphological disorganisation and variation described previously. This disorganisation does not seem to be correlated with a qualitative change in metal composition from the in vivo situation or from that reported in other systems (Kaufman 1980, Paranesa and Zudunaisky 1981, Zeskers 1978). However, the process of melanisation in premigratory cultures would appear to be associated with significantly higher accumulation of barium, iron and zinc.

zinc.

Electron

Electron

Electron

TABLE 5/1

Peak-to-Background ratio for K_α emission

Element	In Vivo		In Vitro	
	Neural Crest (n=9)	PRE (n=7)	Electron dense (n=6)	Electron Lucent (n=7)
Ca	0.756 ± 0.361	0.750 ± .288	0.617 ± 0.221	0.131 ± 0.057
Ba	0.119 ± 0.65	0.096 ± 0.045	0.511 ± 0.314	0.053 ± 0.0270
Fe	0.044 ± 0.31	0.035 ± 0.011	0.141 ± 0.088	0.069 ± 0.034
Cu	0.083 ± 0.032	0.155 ± 0.019	0.339 ± 0.518	0.313 ± 0.326
Zn	0.097 ± 0.016	0.134 ± 0.024	0.439 ± 0.185	0.096 ± 0.070

TABLE 5/2

Significant* Outcomes of Wilcoxin[†] Rank -Sum TestCalcium

Electron Dense >Electron Lucent P>95%
 Neural Crest >Electron Lucent P>99%
 PRE >Electron Lucent P>99%

Iron

Electron Dense >Neural Crest P>99%
 Electron Dense >PRE P>99%
 Electron Lucent >PRE P>99%

Barium

Electron Dense >Neural Crest P>99%
 Electron Dense >PRE P>99%
 Electron Dense >Electron Lucent P>99%
 Neural Crest >Electron Lucent P>95%

Zinc

Electron Dense >Neural Crest P>99%
 Electron Dense >PRE P>99%
 Electron Dense >Electron Lucent P>99%
 PRE >Neural Crest P>95%

Copper

PRE >Neural Crest P>99%

*Non-significant not shown

[†] Lapin (1980)

4.2.5 Summary of Results

1 Ultrastructural examination of eumelanocytes within the embryonic ocular tissues revealed that melanosomes become fully melanised within a short period after the onset of pigmentation and that various melanosomal stages co-existed within the one cell.

2 The brown, in vitro, pigment was shown histochemically to be melanin. However the ultrastructure of the melanosomes differed from that described in avian pheomelanosome (Jimbow et al. 1979). Although some organelles did have the anticipated granular substructure, others appeared to be very poorly developed eumelanosomes. The melanisation process was extremely slow and required several weeks to produce densely pigmented organelles. These abnormalities of structure were accompanied by melanosomal continuity and the presence of giant vacuoles and melanosome. The golgi apparatus was, however, morphologically normal and tyrosinase activity found in golgi associated cisternae and vesicles.

3 The black pigment found in mesenchyme explants resembled that found in eumelanocytes in vivo. Once the eumelanocytes migrated from the explant more granular types of melanosomes appeared.

4 The disorganisation and variability of ultrastructure found in the brown pigment was matched by variation in calcium, barium, iron, zinc and especially copper. The more melanised organelles accumulated greater amounts of barium, iron and zinc.

CHAPTER 5

ENVIRONMENTAL SUPPRESSION OF MELANOGENESIS
WITHIN THE CORNEAL STROMA5.1 Introduction5.1.1 Aims

The work described in previous chapters demonstrated that melanocyte differentiation is locally stimulated within the orbital mesenchyme. This chapter, by contrast, considers why the neighbouring corneal stroma is devoid of melanocytes, even although it is colonised by neural crest cells derived from that mesenchyme (Noden 1978). The main intention of the work reported here was to establish whether the cells which enter the stroma were already committed to fibroblastic differentiation at the time of colonisation or whether they were capable of melanocyte differentiation in vitro. By examining the pluripotency of the early corneal cells in this way, it was possible to study the role of local tissue interactions in the restriction of melanoblastic potency of the crest.

5.1.2 Development of the Corneal Stroma

The primary corneal stroma is laid down by the superficial epithelium during the third and fourth day of development (Dodson and Hay 1973). This acellular stroma consists of about 30 orthogonally arranged layers of collagen fibrils attached to chondroitin sulphate proteoglycan. During the fourth and fifth days of incubation, the posterior surface of the stroma is colonised by neural crest cells (Noden 1978), which migrate from the mesenchyme at the edge of the optic cup. By the end of the fifth day, these cells have flattened and formed an interdigitating endothelial monolayer (Bard, Hay and Heller 1975). The endothelium secretes hyaluronic acid during the following day (Toole and Trelstad 1971; Trelstad et al. 1974), so causing a rapid swelling of the stroma.

After swelling, the stroma is then invaded by neural crest cells from the surrounding mesenchyme during the 5.5 to 6.5 day period. This second wave of cells colonises the entire stroma with the exception of a $2\mu\text{m}$ band of matrix known as Bowman's Membrane, which lies below the corneal epithelium.

Although all of the cells which enter the stroma become fibroblasts at the time of colonisation they show little ultrastructural specialisation (Hay and Revel 1969) which could equip them for their role in collagen and proteoglycan secretion (Conrad and Hart 1976; Bard and Higginson 1977). Between stages 27 to 30 (5.5 to 6.5 days) the cells contain "some endoplasmic reticulum", from stages 30 to 36 (7 to 10 days) the endoplasmic reticulum hypertrophies and free cytoplasmic ribosomes decrease. By stage 36, the "granular endoplasmic reticulum is highly developed in all fibroblasts", and well-developed golgi stacks are observed. These ultrastructural changes suggest that the stromal cells develop an increased synthetic capability after entering the cornea, but at the time of colonisation, they are relatively undifferentiated.

5.1.3 Experimental Approach

The experiments reported below were used to examine the pluripotency of the presumptive fibroblast population upon entering the primary corneal stroma. By explanting epithelium-free stromas at various times after colonisation, attempts were made to follow the commitment to fibroblast differentiation and compare that with the course of melanocyte differentiation within mesenchyme explants (reported in chapters 3 and 4). Heart fragments were also explanted in the same culture medium in order to provide a control population of non-neural crest fibroblasts whose fate could be compared with that in corneal cultures. Differentiation of fibroblasts and melanocytes was assessed by their appearance in the inverted phase microscope, by their ultrastructure, by their ability to

oxidise L-Dopa, and by the presence of glycosaminoglycan-containing coats upon the cell surface. Cell coats, which have been observed in a variety of fibroblasts and tumour cells (McBride and Bard 1979; Toole 1981), were used as a marker for fibroblast differentiation in this system.

5.2.2. RESULTS

The explant developed the typical appearance of a peripheral mesenchymal capsule which was composed of a dense layer of cells with a central area of loose connective tissue. The capsule was composed of a dense layer of cells with a central area of loose connective tissue. The capsule was composed of a dense layer of cells with a central area of loose connective tissue.

The degree of explant pigmentation, typical of that found in mesenchymal culture, was highly significant for two reasons. First, it demonstrated that the explant capsule was "induced" by fragments of Dopa, which were released from the explant.

Finally, the degree of pigmentation was highly significant for two reasons. First, it demonstrated that the explant capsule was "induced" by fragments of Dopa, which were released from the explant.

5.2 Results

5.2.1 Corneal Culture (Hamilton and Hamburger, stages 27-36)

Corneal fragments were explanted as squares which rounded up within the first 24 hours of culture. During this period, outgrowth commenced and occasional mitotic figures were observed. Such proliferative outgrowths developed from 30% of explants (see table 5/1), whilst the remainder had little or no outgrowing cells after 6 days of culture. The low frequency of outgrowth formation probably resulted from the dense collagenous nature of the stroma, for it is known that cells adhere preferentially to collagen fibres, rather than to plastic (Elsdale and Bard 1974).

5.2.1 i Explants

No explant developed the type of pigmentation found in periobital mesenchyme explants within 1 week of culture (i.e. no dendritic black melanocytes developed in the explant or migrated away from it). One exceptional explant, stage 28, however, contained dark brown pigment which could not be localised to particular cells (fig. 5/1). All other explants remained unpigmented for at least 1 week (fig. 5/2).

The absence of explant pigmentation, typical of that found in mesenchymal culture, was highly significant for two reasons. First, it demonstrated that the corneal squares were "uncontaminated" by fragments of limbal mesenchyme, second, it showed the corneal environment would not support melanocyte differentiation in a culture medium which had a stimulatory effect on neural crest cultures (Ham's F10, supplemented with 10% F.C.S. and 5% C.E.E.).

5.2.1 ii Outgrowths

Approximately 28% of cultures which were established from newly colonised corneas (stages 27 and 28), produced a mixed

outgrowth of fibroblasts and melanocytes. The pigmented cultures were indistinguishable from those formed from the mesenchymal explants, in that they contained a minority of melanocytes dispersed amongst a more proliferative group of radially outgrowing fibroblasts. The melanocytes contained a diffuse brown pigment, characteristic of the neural crest or mesenchymal outgrowths. The differentiating fibroblasts tended to be more flattened than their in vivo counterparts (fig. 5/3) (Bard and Hay 1975), and possessed distinct ruffling membranes, whilst the melanocytes tended to be stellate in shape and overlapped each other in dense patches or formed clumps (fig. 5/4, 5/5, 5/6). In one exceptional case, the outgrowth contained an epitheloid patch (fig. 5/7), which was presumably derived from the corneal endothelium. After another 2 days of culture, this sheet rolled up (fig. 5/8) and detached. "Contamination" of the outgrowth by corneal endothelium would therefore seem to have been an insignificant and rare event.

The precision of the staging criteria (see Materials and Methods), was validated by preparing sections of stage 28 cornea (fig. 5/9). These demonstrated that colonisation was at an advanced, but incomplete, stage during the period of corneal melanogenesis. The melanocytes were, therefore, derived from a group of cells, which were still actively migrating in situ.

Explants derived from cornea 1 day after the onset of stromal colonisation (stage 29-30) produced outgrowths which were exclusively fibroblastic in composition (see table 5/1). After stage 28, the cells of the corneal stroma, therefore, seem to diverge from those of the mesenchyme (see chapters 3 and 4), which retain the ability to form brown pigment.

5.2.2 Control Cultures of 6 Day Heart and 18 Day Corneal Fibroblasts

Fibroblasts derived from fragments of 12 six day hearts were grown in separate dishes for 2 weeks. After this time, all cultures had approached confluence, but in no case was there any trace of melanocytes (fig. 5/10). This suggested that the response observed in cornea from an equivalent stage of development was tissue specific.

Six stromal squares from 18 day corneas were explanted and the fibroblast from the outgrowth were subcultured twice into 32 petri dishes (35mm). After 17 days, an approximate total of 4.7×10^6 subconfluent cells were obtained. These were well-spread fibroblastic cells, with prominent ruffling membranes (fig. 5/11), and were like those found in cultures of younger cornea. No melanocytes were found after careful examination of each culture, prior to and after, each replating. The absence of melanocytes suggests that corneal fibroblasts do not transdifferentiate into melanocytes in the culture conditions used.

5.2.3 Characterisation of the Corneal Melanocytes

Melanin was demonstrated histochemically within pigmented cells of the outgrowth, using the criteria described in the last chapter.

The melanocytes:

- a) Stained green with Nile blue sulphate, whilst the fibroblasts remained unaffected (2 cultures)
- b) Bleached with hydrogen peroxide (2 cultures)
- c) Did not autofluoresce prior to above treatment (2 cultures)
- d) Stained with L-Dopa (2 cultures).

One outgrowth from a stage 28 corneal explant was examined after 27 days in culture. During this time, the cells had multilayered and produced elongate processes, which were found on the apical surface of the upper cells. The melanosomes, which were incompletely melanised, were mostly granular in substructure (fig. 5/12), and showed little evidence of elongation (fig. 5/13). Large melanosome clusters, similar to those in neural crest cultures, were also observed. Well-developed golgi apparatus, and associated centrioles were another feature shared with differentiating premigratory neural crest cells.

The features described above, demonstrate that the corneal melanocytes were, histochemically and structurally, indistinguishable to those found in neural tube cultures.

5.2.4 Characterisation of the Corneal Fibroblasts

5.2.4 i Lack of Dopa Oxidising Capability

As previously indicated, only melanocytes oxidised L-Dopa. Before examining a sample of unpigmented cultures more closely, it was necessary to establish that they contained no unpigmented melanoblasts. Six cultures were therefore incubated with L-Dopa and 4 controls with D-Dopa. Careful examination of these cultures at high power (x400) with the phase microscope, revealed no tyrosinase activity.

5.2.4 ii Glycosaminoglycan-Containing Cell Coats

Cell coats, of the type found on fibroblastic cells, were observed on the surface of fibroblasts from the cornea and periorbital mesenchyme when examined after 1 week in culture (figs. 5/14, 5/15). Mitotic cells had "halo" regions, which extended well beyond the retraction processes, onto the neighbouring substratum

(fig. 5/16). Cell coats totally disappeared after hyaluronidase digestion, indicating the importance of glycosaminoglycans in maintaining the integrity of this structure. Large fibroblastic cells which had migrated from 10 day dorsal root ganglia, and had then been maintained for 28 days in culture, also possessed very prominent halos (fig. 5/17). Thus, both trunk and cranial crest fibroblasts synthesise enough surface associated glycosaminoglycan to form halos, in spite of these differences in their developmental history. Heart fibroblasts, on the other hand, after 1 week in culture, had no halos. This is consistent with the metabolic labelling experiments (Conrad et al. 1977), which demonstrate proportionately less hyaluronic acid synthesis, by heart and skin fibroblasts, than that occurring in corneal fibroblast. Melanocytes from the orbital mesenchyme or neural crest culture did not possess halos (fig. 5/18). The undifferentiated neural crest cells grown for 36 hours (fig. 5/19) or 1 week in medium, supplemented with 10% F.C.S., were also deficient in these structures.

Fibroblasts therefore appear to acquire halos after differentiation from the neural crest, although melanocytes or the precursor cells are "uncoated".

5.2.4 iii Ultrastructure

Two unpigmented cultures, which had been established for 9 days, were examined with a view to ascertaining whether the in vitro cells develop ultrastructural characteristics of the corneal fibroblasts in situ.

The structure of the in vivo cells were found to correspond to that observed in "White Rock" embryos (Hay and Revel 1969). The 18 day stromal fibroblasts were elongate and bipolar in shape and lay

in a plane parallel to the corneal epithelium. They possessed prominent stacks of rough endoplasmic reticulum (fig. 5/20), like that reported during earlier development.

Cultured cells were quite different in structure. They contained large clusters of osmophilic vesicles (fig. 5/21), which were also observed as clear vesicles in the light microscope, prior to post-fixation. Ruffling membranes, characteristic of motile cells (Bard and Hay 1975), were observed in the light microscope. When viewed in profile, these were complex in structure (fig. 5/22), and contained convoluted microfilament bundles. Microfilament bundles also ran below the subapical membrane (fig. 5/23), traversed the cytoplasm, and impinged upon the basal cytoplasm, toward the cell periphery. The overall shape and cytoskeletal organisation of the cultured cell seemed characteristic of the "fibroblast" in vitro.

TABLE 5/1
PIGMENTATION IN CORNEAL CULTURES

Stage	Total number of cultures	Number with explant pigmentation	Total number with outgrowths	Number of unpigmented outgrowths	Number of pigmented outgrowths	% pigmented
27	25	0	3	2	1	27.6%
28	67	1*	26	19	7	
29-30	15	0	6	6	0	0%
32-36	19	0	3	3	0	

* The pigment appearing in this explant was not equivalent to that observed in mesenchymal cultures (see text and fig. 5/3).

5.2.5 Summary and Discussion of Results

The results presented in this chapter demonstrate that the fate of the presumptive corneal fibroblast can be manipulated in vitro if they are removed from their normal environment during the period of stromal colonisation. Once colonisation of the stroma is complete (around stage 29), the cells become committed to their fate as fibroblasts, and will no longer differentiate into melanocytes in culture. In spite of this initial lack of commitment, it is noteworthy that only a minority of cells differentiated into melanocytes. This behaviour was also characteristic of the periorbital mesenchyme outgrowths, and may therefore have been typical of the postmigratory crest within the ocular region. The low frequency of pigmented outgrowths was, however, significantly different from that recorded in mesenchymal cultures where the vast majority of the outgrowths contained pigmented cells. This difference suggested that significant interactions occur within the stroma during the colonisation, or early culture phase, which reduce still further the number of cells able to pigment in culture.

The evidence presented here for early restriction of fate within the stroma is reinforced by ultrastructural and biochemical indicators of fibroblast differentiation within the newly invaded stroma. By stage 30, there is an increase in the number of collagen fibrils within the stroma, which suggests that the stromal cells "begin to secrete collagen as soon as they cease migration" (Hay and Revel 1969). Keratan sulphate production is another potentially useful marker of fibroblast differentiation, for its synthesis commences after stromal colonisation. Metabolic labelling experiments have demonstrated that keratan sulphate synthesis starts to rise above base on day 7 (Hart 1976), thus placing the onset of biochemical differentiation around stage 30.

In view of the fact that the results presented here are supported by careful scrutiny of the literature, the reader may wonder why corneal melanogenesis was not previously detected in such a well-studied tissue. Three reasons can be offered for this apparent oversight. Firstly, fibroblasts are not usually cultured in the presence of C.E.E. (which is used to stimulate melanocyte or neuronal differentiation). Secondly, White Leghorn chicks, which are often used as a source of tissue, are deficient in their ability to form viable melanocytes (see chapter 2). Thirdly, little attempt would appear to have been made to culture isolated stromal cells from the early cornea.

CHAPTER 6

Discussion6.1 Introduction

The results presented in the last three chapters have been concerned with the importance of environmental regulation of melanocyte differentiation. This process has been examined from the early stages of crest migration to the later stages of embryogenesis when particular melanocyte characteristics are expressed. The major findings regarding the induction of melanocytes, and the modulation of melanogenesis, cell shape and behaviour, have demonstrated the importance of terminal environment in controlling cell fate and a variety of specialised characteristics.

The following discussion considers three major topics:-

- 1 The environmental control of cell fate with particular reference to melanoblastic and fibroblastic differentiation as alternatives within the pigmented mesenchymes.
- 2 The environmental control of melanocyte morphology and behaviour by extrinsic factors.
- 3 The environmental control of melanogenesis in vitro and the significance of abnormalities in that process.

6.2 Control of Cell Fate

During the course of development the neural crest cells lose their pluripotency (Nichols and Weston 1977). Within crest-derived pigmented connective tissue this loss of potency must be associated with melanocyte and fibroblast differentiation. The following discussion tries to establish that during the course of development from the premigratory crest to mesenchyme and subsequently to the corneal

stroma, there is a progressive shift from melanocyte to fibroblast differentiation. Some speculations are advanced in an attempt to explain the control of differentiation in these situations.

6.2 i Early Development

At the onset of migration the crest cells encounter a tissue environment in which neuroblast differentiation is induced (chapter 1, and Norr 1973; Cohen 1972). Migrant neurones were observed in neural tube cultures when the explant was left in place for more than 48 hours. Since the appearance of these cells was also accompanied by the emergence of neuronal processes from the neural tube, the migrant neurones could be "contaminants" from the neural epithelium, as suggested by Le Douarin (1980) in her critique of the neural tube explantation method. However, it was not possible to distinguish the differentiation of tube cells from that of crest cells which may be subject to an inductive effect resulting from prolonged incubation of the tube. Subsequent failure to survive was probably influenced by the substratum. Polylysine- and polyornithine-coated plastics have proved more suitable for the culture of neurones for they promote neural cell adhesion (Hawort 1980) and maintain locomotory behaviour (Wessels et al. 1980).

After 5 or 6 days of culture cells of fibroblastic morphology containing osmophilic lipid droplets appeared at the edge of the crest outgrowth. This was considered to be a genuine, although minority, form of differentiation within the isolated population for cells of this nature were never found in early outgrowths. One other group of workers has obtained fibroblastic cells which stained with antibodies to procollagen type I (Greenberg et al. 1980), thus suggesting that they may undergo some degree of functional differentiation.

When chick embryo extract was included in the culture medium, the melanocyte was the most predominant differentiated cell. Its differentiation was apparently unaffected by the neural tube, although Derby and Newgreen (1982) have reported that precocious removal of the neural tube (<18 hours) prevented melanogenesis. Similar results were obtained in preliminary experiments, although this was associated with very restricted outgrowth and a rapid decline in cell numbers (a phenomenon also detected by Maxwell, 1976). The neural tube may therefore provide some trophic stimulus as well as acting as a source of migrant cells.

When more caudal tube was explanted prior to the formation of crest in vivo, differentiation proceeded normally. This suggested that the presumptive epidermis does not have a role in crest cell maturation, even although it may be involved in the regulation of early migration as the cells emerge from the dorsal aspect of the neural tube (Newgreen and Theiry 1980). The fact that whole trunk segments could not induce the formation of black pigment in the same way as periorbital mesenchyme suggests that mesenchymes of the early trunk are significantly different from the crest-derived ectomesenchyme of the cranial region. Considerable development of feather germ epithelial cells must occur before a suitable environment for melanocyte differentiation develops within the trunk tissues.

6.2 ii Differentiation within the Periorbital Mesenchyme

Crest differentiation within the periocular mesenchyme is influenced by the head ectoderm and pigmented retinal epithelium (see chapter 1). The experiments reported here were carried out after

these "inducer" tissues were removed, although this may have been after they had already exerted some influence upon the mesenchyme. Anterior mesenchyme, which was isolated at 5 to 7 days would normally form membrane bone on the day after induction by the scleral papillae (Coulombe et al. 1962). As yet no effect of the PRE is known within this region, for the cartilage is restricted to that part of the sclera posterior to the ossicles. Normally, the anterior mesenchyme participates in the formation of the ciliary body (Bard and Ross 1982) and iris, which contains melanocytes, fibroblasts, and smooth and striated muscle (Noden 1978).

Upon isolation of the mesenchyme abundant melanocyte differentiation ensued, so that on occasions the entire tissue became a dense black mass within 48 hours. Whether this pigmentation resulted from morphological change of the explanted tissue, increased induction of melanocytes, or unrestricted proliferation of melanoblast or melanocytes, remains to be determined. Variation in culture conditions had no obvious effect upon the appearance of the explants, which suggested that the development of pigmentation was maximal for that environment, and determined by local inductive influences.

Melanocyte differentiation in the outgrowths was severely reduced when compared to that found in neural crest culture, whilst fibroblast differentiation was greatly increased. This change in the proportion of cell types within the outgrowths may have 3 explanations.

The first explanation concerns rates of proliferation and migration of each differentiated cell type. The cell cycle of the crest cell slows down after the onset of melanogenesis (Maxwell 1976) so that the fibroblasts could quickly come to outnumber melanocytes

merely by more rapid proliferation. This will certainly apply at extended times after the onset of melanogenesis (3-7 days), however, it would not significantly influence the observed ratio within the first day.

If the differentiated fibroblasts were capable of more rapid migration than uncommitted or undifferentiated melanoblast then the number of fibroblasts within the outgrowth could become relatively elevated. This also seems unlikely because crest cells (Davis and Trinkhaus 1981) and melanocytes are highly motile cells. The latter congregated in large numbers within one day after the appearance of black melanocytes in the explant.

The most probable reason for the change of ratio, is that it represents a commitment to fibroblastic differentiation within a large proportion of the motile mesenchymal population. A definitive demonstration of the fibroblast commitment will require clonal fate analysis of dissociated mesenchymal cells at this stage of development.

6.2. iii The Corneal Stroma

The experiments reported in chapter 5 suggest that the cornea is colonised by a population of cells which is representative of the anterior mesenchyme, that is, the majority of colonising cells are already committed to their fate as fibroblasts. Within 2 days of entry commitment to fibroblast differentiation becomes complete. The acellular nature of the cornea suggests that it is the epithelium derived extracellular matrix (Dodson and Hay 1973) which causes commitment of the invading cells and brings about ultrastructural differentiation within 3 days of entry (Hay and Revel 1969).

When viewed from a functional standpoint it becomes evident that suppression of melanogenesis within the cornea is an essential evolutionary adaptation. Melanocyte differentiation must be

suppressed so that an optically transparent tissue may form.

The existence of a highly pigmented band at the corneal limbus is less easy to understand. One plausible explanation may be that the epithelial and stromal melanocyte at this location reduce the transmission of internally scattered light between the cornea and sclera. The corneal culture experiment shows how melanocyte differentiation is suppressed within the stroma. However, examination of the pigmented limbal epithelium suggests that there may also need to be some mechanism which suppresses the development of pigmentation within the corneal epithelium. The limbal epithelium contains basally located melanocytes which rest on a highly convoluted membrane (as shown in chapter 4). The basal surface of the corneal epithelium is quite different, for it rests on Bowman's zone (Hay and Revel 1969). Bowman's zone is a narrow band of dense extracellular matrix which remains uncolonised by the crest during stromal invasion. It may be that this zone has a previously unrecognised significance. By acting as an impervious barrier to migration, Bowman's zone may "protect" the corneal epithelium from melanoblast invasion and subsequent melanisation. One way to test this possibility experimentally would be to co-culture isolated corneal epithelium and periorbital mesenchyme.

In spite of the obvious parallel between differentiation in vivo and in vitro, melanocyte and fibroblast differentiation within the outgrowth was somewhat abnormal. Both melanocyte and fibroblast differentiation was abnormal to some extent. Melanocytes produced aberrant pigment organelles, characteristic of the neural crest in vitro. Fibroblasts, on the other hand, acquired lipid vesicles and lacked well-developed RER or golgi apparatus. Although collagen synthesis was not specifically examined, its failure to occur (to the same extent as it does in vivo), was apparent from the lack of

ultrastructural differentiation and the conspicuous absence of extracellular matrix. In spite of these faults, the ocular fibroblasts had clearly undergone some form of differentiation from the premigratory neural crest, for they possessed surface coats and were morphologically dissimilar from their more stellate precursors.

Failure to synthesise and secrete collagen was almost certainly due to the inappropriate nature of the culture medium, which was not designed to maintain fibroblastic function.

Experiments with primary avian tendon cells have shown that collagen synthesis is inhibited by the lack of ascorbic acid, and by the presence of significant amounts of fetal calf serum and embryo extract (Schwartz and Bissell 1977; Shwartz et al. 1976; Schitz and Ward 1980). The requirements for melanocyte and fibroblast differentiation are therefore difficult to reconcile in vitro.

The evidence presented here also shows that the fibroblast of this tissue becomes committed long before the onset of melanocyte differentiation in the neighbouring limbal region (day 13). If the differentiation of corneal fibroblast can be extrapolated to the surrounding ocular connective tissues, then it seems likely that fibroblast differentiation precedes melanocyte differentiation within any particular location.

The evidence now discussed suggests that fibroblast differentiation may be a prerequisite for melanocyte differentiation.

6.2 iv The Nature of the Melanocyte Inducer

The evidence presented here regarding corneal and periorbital mesenchyme suggests that some local factors are involved in the control of melanocyte and fibroblast differentiation. These factors did not seem to exert an effect upon the migrant cells, which suggested they were either immobilised in the tissue, or did not

diffuse out in high enough concentration to become effective.

If corneal fibroblasts can become committed and differentiated as a result of cell-matrix interaction, then it is relevant to know whether the other mesenchymal cell type (the melanocyte) is influenced by similar events. Current evidence supports this possibility. Detergent extracted ECM from dermal cells (but not pharynx or gut) will overcome the inhibition of melanogenesis caused by heart-conditioned medium (Derby 1982). These experiments provide evidence that fibroblast products may influence melanoblast commitment to melanogenesis. The tissue specificity of the dermal ECM is of crucial significance for it implies tissue variation in the fibroblast population, and at the same time, provides an explanation for differences in melanocyte distribution throughout the embryo.

The difference in cell coat production by 7 day corneal and heart fibroblast provides more evidence for tissue variation amongst fibroblasts, and suggests that hyaluronic acid production varies between tissues. This is, in fact, supported by biochemical evidence of fibroblast variation between 14 day fibroblast of the avian skin, heart and cornea (Conrad and Hart 1975; Conrad et al. 1977). Corneal fibroblasts incorporate a greater proportion of ^3H -glucosamine into hyaluronic acid than do heart fibroblasts, which, in turn, incorporate more than skin fibroblasts. Conversely, skin fibroblasts incorporate a greater proportion of $^{35}\text{S}\text{O}_4$ into chondroitin-6-sulphate than corneal cells. The ratio of glycosaminoglycan types within the mesenchymal extracellular matrix may therefore be partially responsible for the observed tissue specificity in the stimulation of melanogenesis.

In view of the difference between induction of melanocytes within the orbital mesenchyme and cornea, one might expect to find important differences between those environments.

One of the unique features of the corneal stroma is its avascularity. This characteristic, more than any other, distinguishes it from the highly vascularised pigmented tissues such as the choroid, which contains an elaborate plexus of blood vessels (Leplat 1912). The evidence presented in chapter 3 demonstrated close association of melanocytes with the subendothelial matrix of these blood vessels. Whether this association develops after or before differentiation has still to be established. However, it is tempting to speculate that vascular endothelial cells, which produce this matrix are involved in melanocyte induction.

More gross differences between corneal and mesenchymal matrix have been detected by indirect immunofluorescence studies. At the time of presumptive fibroblast invasion the corneal stroma contains a mixture of type I and II collagens (von der Mark et al. 1977), although it is deficient in fibronectin except for Descemet's membrane overlying the endothelium (Kurinen et al. 1979). This contrasts with the sclera where type I and III collagen, and fibronectin are present. Fibronectin distribution is probably unimportant as an inducer of melanocytes in connective tissues, for the early neural crest cells have the ability to synthesise fibronectin (Newgreen and Theiry 1980), as do the cells invading the cornea (Kurinen et al. 1979). The absence of collagen type II, or the presence of type III (and possibly IV in the subendothelial matrix of the vascular endothelium), therefore may be important in the induction of melanocyte differentiation.

The role of the PRE at an earlier stage of development (2-5 days) has also been neglected, even although it synthesises matrix which induces cartilage differentiation (Newsome 1976), and is in intimate contact with the highly pigmented choroidal layer. Culture

of cranial crest upon retinal extracellular matrix would help identify any retinal effect upon fibroblast or melanocyte differentiation within the mesenchyme.

Further work is required to establish whether cell-matrix rather than cell-cell interaction is the determining factor in the induction of these derivatives.

6.3 The Extrinsic Modulation of Melanocyte Behaviour and Morphology

6.3 i Melanosome Deposition

The observations upon cultured melanocytes and those present within connective tissues show that melanosome deposition occurs in the absence of direct epithelial interaction as had been formerly assumed by some workers (Breathnach 1982). Melanosome shedding was not found to occur in neural tube cultures and therefore appeared to require the formation of proper dendrites, characteristic of cells differentiating within the mesenchyme or epithelia. Time-lapse photography, on occasions, demonstrated that melanosomes accumulated rapidly (20 minutes) in the perinuclear cytoplasm and then acted as a focus for dendrite extension.

Melanocytes are not the only type of cultured cell to lose parts of their extremities. Parts of cultured fibroblast are, on occasions, left behind during the process of locomotion. Whilst the cell is actively locomoting the leading edge of the cell moves forward and exerts tension on a point of cell substratum attachment toward the trailing edge. Normally the trailing edge attachment is retracted as the cell moves. In a small minority of occasions this attachment is not withdrawn and the cytoplasm becomes stretched and eventually breaks leaving a part of itself behind. Time-lapse photography showed that melanosome shedding did not occur by this

mechanism but resulted from a constriction of the dendrite. Pinching off of the dendrite ends happened over a period of 2 to 5 minutes, which makes it equivalent in speed to the pinching of the cleavage furrow during cytokinesis. Both of these processes can be reversibly blocked by the action of cytochalasin B and therefore appear to be brought about by the contractile activity of a microfilament network (Wiksuro and Szabo 1973; Sanger and Sanger 1980). Analogy with cleavage furrow suggests that contraction within a circumferential band of microfilaments may cause dendrites to bleb off at their ends. The existence of such a ring structure has still to be demonstrated by electron microscopy or immunohistological staining of actin.

6.3 ii The "Social" Behaviour of Melanocytes

The most obvious consequence of homologous melanocyte interaction was the production of large aggregates of cells. Time-lapse photography, histological and ultrastructural examination of these aggregates in neural tube cultures and mesenchymal outgrowths demonstrated that aggregates were largely of melanocyte composition. This confirms the work of Loring et al. (1981) who showed that clumps isolated from quail neural tubes could be dissociated into a pure suspension of melanocytes. These workers have also shown a similar dependence of aggregation and culture conditions reported here, although their claim that the ratio of serum to extract is important does not seem to be borne out by the data presented. Derby and Newgreen (1982) suggest that aggregation is caused by pancreatin treatment of the neural tube, because dissociation carried in collagenase produced clump-free cultures. Although pancreatin treatment may promote aggregation it is not exclusively responsible for this behaviour, for trunk segment cultures in which no pancreatin

was used, produced peripheral aggregates of melanocytes. Clumping of Red Minorca melanocytes by the action of dibutyryl-cyclic AMP shows that melanocyte differentiation and aggregation are closely related, as initially suggested by Twitty (1966 review).

Collagen type I substrata did not alter melanocyte behaviour although it did change the behaviour of neurones. When collagen was absent, neuronal processes maintained contact with other cell types. In the presence of a collagen-coated substratum, neurones migrated freely without cell contact.

The driving force of cell contact formation was melanocyte locomotion, which brought cells close enough to adhere. No contact inhibition of locomotion was observed by time-lapse photography. Melanocyte aggregation was not restricted by a paralysis of the locomotory apparatus observed in other cell types (Bellairs et al. 1982).

Twitty's attempt to correlate aggregation in vitro with flank band formation in the amphibian epidermis was probably unjustified, for no counterpart of this is present within avian embryo, and no melanocyte clumps were observed even in highly pigmented tissue. This sort of behaviour would seem, therefore, to be an artefact of melanoblast culture at high density. Loring et al. (1981) blocked neural crest migration from the tube, by explantation on agar, and have demonstrated a large increase in aggregate formation. A local increase in cell number on the neural tube would seem to account for the increased aggregation after blocking migration.

Why do melanocytes aggregate? Starving slime moulds aggregate in waves by releasing cAMP (Gerisch 1982). It is interesting, although perhaps coincidental, that Red Minorca cells clumped after the exogenous administration of dibutyryl-cyclic AMP. The response of aggregating slime moulds is a chemotactic one induced by food deprivation, and as such, it has an obvious significance for

the survival of this species. Aggregation of melanocytes in vivo would, however, have a deleterious effect on the animal because it would tend to reduce the effectiveness of the integumental pigment screen. Sponge cells produce soluble aggregation factors (Humphreys et al. 1977) which promote cell-cell adhesion. Although there is no obvious reason why such agents would be needed to control melanocyte distribution within the avian, there may be cell surface molecules which facilitate interaction between melanocytes and keratinocytes during the course of melanosome transfer. The simplest explanation may be that in the absence of a physiological substratum in vitro, the force of cell-cell adhesion is greater than that of cell-substratum adhesion, so that the equilibrium situation is shifted in favour of aggregation when frequent cellular interactions are possible.

6.3 iii Control of Cell Shape

The observations presented here contradict the hypothesis of Market and Silvers (1950), which stated that cell shape was determined autonomously by the melanocyte. Comparison of different tissue locations and culture situations demonstrated a marked variation in shape which was related to features in the microenvironment. Morphology and size in vivo varied enormously, which may have resulted in differences of cell volume between the tiny melanocytes of corneal epithelia and subadjacent stroma.

The isolated melanoblast did not develop complex dendritic processes like those found in mesenchyme or epithelia. A more polarised dendritic shape developed in the black melanocytes which form with mesenchymal explantation. Maintenance of that shape was enhanced by interaction with the PRE rather than tissue culture plastic, although the reasons for this remain unclear. Long-term maintenance of a particular shape therefore appears to require interaction with components of the E.C.M. or with epithelial cells.

Changes of cell shape within human melanomas have also been attributed to changes in the microenvironment of the transformed cell (Paul 1980). The characteristic dendritic shape of the epithelial melanocyte is only maintained when the tumour cells are at low density and when melanocyte contact with the epithelial cell is maintained. At high density "nests" or aggregates of melanoma cells form (Clark 1969). Nest formation in melanoma is the equivalent of melanocyte aggregation in vitro, in both of these situations dendrites are absent (see Paul 1980 and fig. 3/38).

The flat epithelioid melanocytes of the eye were apposed to flat substrata such as cartilage or bone, while their more dendritic counterparts followed the contours of collagen bundles or blood vessels, or were located between the epithelial cells of the eye or skin. The fact that the physical nature of the substratum is important has been confirmed by culturing chick corneal fibroblasts (Bard and Hay 1975) upon glass and within collagen gels. Fibroblasts migrating in gels maintain the characteristic filopodial morphology of the in vivo cell, whilst those cultured on glass become flattened and develop wide lamellipodia on their leading edges.

The composition of the substratum as well as its physical properties, are important. Melanoma cells, for example, become more flattened when transferred from tissue culture plastic to collagen substrata (Dr Seith Schor, personal communication).

Melanocyte shape is not a static property, for it changes during cell division and as a result of locomotory behaviour of the cell. Epidermal melanocytes of mouse ear retract their dendrites during the course of cell division and consequently develop a rounded profile (Rosendal and Lindstrom 1980). Regrowth of the dendrites occurs during telophase and is completed by the end of cell division.

This behaviour, which is characteristic of many cells in vitro (e.g. Harrison and Allen 1980), emphasises the importance of respreading activity in the determination of cell shape. Subsequent activity of the interphase cell, like that reported here, changes the outline of the cell but tends to preserve a similar type of morphology to that which already exists. The more dendritic cells maintained their morphology by continual extension and retraction of fine processes, whereas the epithelioid cells changed shape over larger areas of their periphery. Morphology is not determined in a fixed unalterable fashion, by the substratum, but it acts in a regulatory fashion by modulating the locomotory behaviour of the cell.

6.4 Melanogenesis In Vitro

The observations upon Brown Leghorn tissue revealed that black eumelanocytes were ubiquitous throughout the connective tissues of the late embryo. This contrasted with the behaviour of isolated in vitro melanoblasts which produces a brown pigment in all conditions examined. The production of brown pigment was not related to whether the cells were premigratory or postmigratory, neither was it influenced by the concentration of tyrosine in the culture medium (the major eumelanin precursor) or the absence of melanocyte stimulating activity (dibutyryl-cyclic AMP).

L-Dopa staining revealed that many non-pigmented tyrosinase positive cells were present within primary crest culture lacking chick embryo extract. Melanoblast differentiation of the crest cells can therefore occur without the appearance of differentiated melanocytes. Differentiation may be operationally defined as a two stage process; the first stage being the development of a committed, but unpigmented cell, and the second stage the expression of the

pigmented phenotype by the production of melanosomes.

Both aspects of differentiation are completed rapidly when the cells are located within the mesenchyme, either in vivo or in vitro. The colour and ultrastructure of the isolated crest cell in vitro demonstrated that the second part of the process remained incomplete over long time intervals. This happens in spite of the fact that at high serum concentration pigment appears at the equivalent of 5 days of development approximately 2 to 3 days before it was observed in choroidal melanocytes (Leplat 1912). At lower serum concentration the timing of pigment appearance is approximately equivalent to that occurring in vivo, so that disruption of melanogenesis can not be easily explained by temporal constraints in vitro.

The experiments showing dibutyryl-cyclic AMP stimulation of Red Minorca melanoblasts and the ultrastructural changes observed when black melanocytes migrate from the crest, confirms that melanocytes need continuous "stimulation" in vitro to permit expression of the pigmented phenotype (Derby and Newgreen 1982). The exact nature of this requirement is genetically determined, for Brown Leghorn and Red Minorca behaved quite differently in similar culture conditions. In mutant cells, stimulation may induce the short-circuiting of normal humoral control mechanisms which produce activation of tyrosinase within the melanosome (Lerner 1980).

Retinal-mesenchymal explantation also revealed that the control of pigment expression was different in the PRE for the epithelial melanocyte totally depigments in conditions which promote expression in the neural crest cell.

6.4 i The Nature of the Brown Pigment

At a superficial level the brown pigment, present in Brown Leghorn crest cultures, may have been similar to that observed in skin cultures from the same breed (Hamilton 1941), or in crest

cultures of White Leghorn melanocytes (Greenberg et al. 1977). However, it seems likely that the main similarity between the White Leghorn and Brown Leghorn in culture was the failure to fully melanise, hence the appearance of less dense pigment. The ultrastructure and elemental spectrum of the melanosome found here also brings into question the normality of the brown pigment found in skin cultures.

In view of the disruption of melanogenesis on isolated crest cells it is relevant to enquire whether their pigment organelles were a default type of eumelanosome or whether they were a mixture of different types of melanosomes. These two explanations have not been distinguished, although the evidence does show that melanosome production in vitro was defective in the genotype examined. Neural crest cells from different breeds of fowl do vary in their ability to produce pigment in vitro (Derby and Newgreen 1982) although no obvious difference in pigment type has been detected (D. Newgreen, Tubigen, personal communication).

Granular melanosomes, normally indicative of pheomelanocytes were found in the same cell as organelles containing reduced numbers of misaligned melanosomal filaments. Neither organelle type developed over the normal time course, which would be, at most, 2 days from the onset of pigment formation. After long periods in culture (35 days) more completely melanised round organelles, similar to pheomelanosomes were observed. The variation in barium, iron, copper and zinc showed that chemical abnormality of the pigment also developed.

Histochemical staining demonstrated that tyrosinase was present within the golgi associated cisternae and transport vesicles, as well as in the melanosome, where it became active. Although this

technique could not be used to quantify synthesis of tyrosinase or measure its activity a reduction in enzyme function was suggested by the unusually long time course of melanin deposition. With a tissue or explanted mesenchyme well melanised organelles were observed within 1 or 2 days after the onset of pigmentation. In outgrowths, melanisation occurred over a period at least ten times longer than that observed in situ.

Normally tyrosinase becomes inactivated as the melanosome becomes melanised and physically dense (Seji et al. 1973 and 1980). Within the cultured cells, inactivation did not occur, except perhaps over long time intervals.

Tyrosinase loading of mesenchymal cultures had no effect on pigment colour which suggested that hypomelanisation resulted from a reduction in tyrosinase activity rather than from a lack of available substrate.

Melanosome aggregation observed in culture was significantly different from that observed in commercial stocks of White Leghorn fowl. White Leghorn melanocytes sequester the developing melanosomes into acid phosphatase positive autophagosomes (Jimbow et al. 1974), which is followed by death both in vivo and in vitro (Maxwell 1976). Melanosome aggregation in Brown Leghorn cultures probably occurred by a different mechanism, for it was infrequent, much less extensive, and did not cause an obvious decline in cell numbers. Continuity of the melanosomal membrane between neighbouring organelles, and the formation of giant melanosomes suggested that melanosomal membrane fusion occurred.

The evidence presented here does not vigorously support any mechanistic explanation of melanogenesis in vitro, however the following speculative explanation may act as a useful working

hypothesis upon which future work could be based.

When melanocytes differentiate in an ectopic environment devoid of the normal inductive cues the pattern of gene expression is disrupted. As a result, melanogenesis is abnormal, so that typical eumelanosomes and pheomelanosomes fail to be produced. The 4 matrix polypeptides (Zimmerman 1980) are not produced in sufficient quantity (Brumbaugh et al. 1979) to form an abundant, elongate, well-ordered filament matrix. Tyrosinase, although present within the organelles is not properly activated (Lerner, 1980) or inactivated (Seji et al. 1973) so that melanin synthesis continues at a low level for an extended period. Associated with these abnormalities, are changes in the melanosomal membrane, which results in melanosomal continuity and eventually giant melanosome formation.

Circumstantial evidence for the abnormalities of gene expression postulated above is now discussed.

6.4 ii The Significance and Possible Causes of the Melanosome abnormality

Although abnormalities of the Brown Leghorn melanocyte are an in vitro artefact, similar disruptions to melanogenesis are observed in mutant pigment types and in human malignant melanoma. These similarities suggest that gene expression within the cultured cells may be disrupted in a similar fashion to that which may occur as a result of inherited or somatic mutation. The pathological situation is, in fact, more complex for both the initial mutation and subsequent environmental changes during the histogenesis of the tumour contribute to the abnormal phenotype (Foa and Aubert 1977).

Mutant genotypes of both fowl (Brumbaugh et al. 1973 and 1979) and mouse (Moyer 1966) exhibit defects of melanogenesis in vivo, which are similar to those seen in the cultured cells.

Analysis of the 'wild type' red jungle fowl and two dominant mutations has been carried out on feathers from various regions of the adult bird (Brumbaugh 1971). The white (I) mutation affects eumelanosomes and the silver (S, sex linked) affects pheomelanosomes. The I mutant which affects crest cells, but not PRE, causes a reduction in the number of melanosomes, and a reduction of melanosomal filaments and results in the formation of irregularly shaped and partially melanised organelles. The isolated Brown Leghorn cell often bore a resemblance to this mutant, although it was not subject to a reduction in the number of melanosomes. Pheomelanocytes with the S mutation were reduced in number and poorly melanised. Defects in the expression of both pigment types could operate in culture, although the patterns observed can be largely explained by a disruption of eumelanogenesis similar to that observed in the I mutation.

Yellow pheomelanin is produced in the mouse by the presence of mutations at the A (Agouti) locus, which acts upon the follicular environment rather than on the melanocyte (Silvers and Russell 1955). "All other (mutant) melanocytes produce eumelanin which may either be black or brown", (Moyer 1966). Mutant eumelanosomes of the mouse, although brown in colour, do not appear as similar to the I mutant of the fowl. Nevertheless, this supports the idea that the brown pigment observed in culture may be an aberrant form of the black eumelanosome.

The other type of disruption to gene expression is that which occurs during the evolution of the transformed phenotype within the malignant melanocytes. Phenotypic transformation of this kind changes the ultrastructure and elemental composition of the melanosome (Zekers et al. 1975). Electron probe microanalysis has demon

strated elevated levels of iron and calcium in intradermal nevus cells. This change of composition was associated with variations in melanosomal ultrastructure which consisted of "spherical pre-melanosomes, spotted variants (and striated fully melanised organelles". Abnormalities of melanogenesis are a frequent feature of malignant melanoma cells and have been classified into 3 types (Hunter et al. 1978); type I contains "relatively normal ellipsoidal melanosomes", type II contains "numerous abortive melanosomes with filaments oriented in a haphazard fashion" and type III contains "granular melanosomes as well as the occasional abortive and lamellar organelles". Electron micrographs of these types are reproduced in figs 6/1, 6/2 and 6/3 (with kind permission of Professor Hunter). The brown pigment present in the cultured cells corresponds more to that found in type II and III cells from the more aggressive relatively undifferentiated tumours (Hunter et al. 1978 and Foa and Aubert 1977). Giant melanosomes have also been described in malignant melanoma and in other pigmentary disorders within the human (Ortonne and Perrot 1968) which provides yet another link between the pathological and ectopic melanocytes.

Melanogenesis within the human tumour cells does not appear to be characteristic of the normal process, and seems unrelated to hair colour (Aubert et al. 1977). Unfortunately, the ultrastructure of melanosomes within normal cells of the melanoma patients have not been reported on a systematic basis, thus it is not possible to say whether the pattern observed in any individual case is a degeneration of eumelanogenesis or pheomelanogenesis.

The precise cause of these abnormalities is not easy to define for the experimenter cannot isolate or even identify the newly mutated cell. Even when the transformed cell remains within the

epithelium its microenvironment may still change significantly (Paul 1980). Thus it is hard to know whether the faults of melanogenesis within primary tumours arise because of genetic changes, clonal selection (Woodruff 1983), or environmental modifications. Melanoma phenotype does however, appear to change during the progression of metastatic disease. Cells excised from different invasive levels of nodular melanoma, for example, show variation in melanosome ultrastructure (Foa and Aubert 1977; Rouge and Aubert 1979). These changes may be brought about by "pathological alteration due to intrinsic (genetic) modification or (be) related to extrinsic factors" (Foa and Aubert 1977).

Two lines of experimental evidence suggest that "extrinsic factors" (within the environment) may modulate the phenotype of melanoma cells. The first line of evidence has come from experiments where cell lines derived from metastatic lymph node melanoma have been injected into nude mice (Aubert et al. 1976). In one case, a cell line, which initially had granular melanosomes produced tumours with fibrillar melanosomes, 40 days after injection. Although cell selection may be involved in the production of tumours in the nude mouse, it seems unlikely that this arose by selection of a karyotypically abnormal cell population. Chromosome studies on bladder carcinoma cells have shown that highly differentiated cells have near-diploid karyotypes whilst the poorly differentiated cells are polyploid (Hasting and Franks 1981).

More direct evidence of environmental regulation of tumour phenotype has come from culture experiments in which fragments of nodular melanosome have been explanted. Cells which migrate from these tumours are initially pigmented. After 8 days in culture the cells change enzymically (Janiaud et al. 1973), depigment, and become

fibroblastic in appearance, even although they continue to synthesise the melanin precursor 5-S-cysteinlydopa (Rouge and Aubert 1979). Although avian cells never totally depigmented the parallel with human tumours is quite striking, for those cells which migrated from the mesenchyme explants after differentiation developed an intermediate type pigmentation in 4 days (see Fig. 4/27).

The melanoma injection experiments suggest that the pattern of gene expression in abnormal cells is not fixed, and that suitable exposure to the appropriate environmental cues may cause the cell to revert to a more normal phenotype. If avian melanocytes can be made to revert in a similar way, an important link between embryonic and tumour cells will have been established.