HYPOMELANOSIS IN CHICKENS

by

Heather Gaile Marco

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Signed by candidate

September, 1994.

To my Manimals. Especially Chez and the Big White.

Mors janua vitae.

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ABSTRACT

Hypomelanosis, a severe reduction in pigmentation, is a widespread phenomenon which affects many different vertebrate species, including humans and chickens. The cause(s) of various forms of hypomelanosis is(are) not known. The aim of this study was to determine the cause of hypomelanosis in a breed of white chickens (White Plymouth Rock x Pile Game). It was hoped that this hypomelanotic breed may provide insight into the etiopathogenesis of certain human hypomelanotic disorders, such as vitiligo and albinism.

To determine whether melanocytes are present in the hypomelanotic skin, two melanocyte-specific assays were carried out, *in situ* DOPA histochemistry and a sensitive radiometric assay for tyrosinase. The results show that active tyrosinase was present in 8, 9 and 10 day skins. However, unlike normal black skin, the level of tyrosinase did not increase with age, suggesting that the melanocytes either die or that they do not continue to synthesise tyrosinase. Ultrastructurally, these melanocytes appeared to be morphologically normal and they did not show signs of premature degeneration. Unlike black chick melanocytes, however, they contained very few premelanosomes and fully melanised melanosomes were never observed, suggesting that hypomelanosis results from the arrested development (melanisation) of melanosomes *in vivo*.

Two different experiments were carried out to determine whether this blockage in melanogenesis is intrinsic in the melanocyte or whether it is caused by extrinsic environmental factors. The outcome of these studies were conflicting: 1) In culture, white chick neural crest cells produced pigment, suggesting that the melanocyte is not defective. However, ultrastructural examination of these cultured melanocytes showed that they contained a large proportion of partially melanised melanosomes. 2) Black chick neural crest cells migrated into white skin explants and contributed towards pigment in the developing feathers, suggesting that the white chick tissue environment is also not defective.
The results hint that hypomelanosis in the white chicks is caused by the interaction of at least two genetic defects: an intrinsic

mutation of the melanocyte, as well as an extrinsic mutation in the melanocyte environment that, in combination, exert an inhibitory influence on melanin synthesis. This study shows that, *in situ*, white chick melanocytes share some features with ty-pos albino melanocytes and may be representative of this pigmentary disorder. White Plymouth Rock x Pile Game chicks may also be useful as a model for the multi-faceted disorder, vitiligo.

AIM AND OUTLINE

Hypomelanosis, the reduction of pigment, is a phenomenon that affects many species of vertebrates such as chickens, swine, horses, mice, dogs, cats as well as humans (see Nordlund, 1992 and Ortonne and Bose, 1993). Although this does not have a severe effect on most animals, hypomelanosis in man is accompanied by adverse physiological and psychological effects (Kromberg, 1987 and Tripathi *et al*, 1992). Because hypomelanotic disorders occur in such wide range of vertebrates, it suggests that some biological processes common to pigment cells in these species, could be involved in the functional impairment or destruction of melanocytes.

Disorders that result in skin depigmentation can be extremely revealing of the normal processes affected in the pigmentary system. Also, rapid progress can be made in the understanding and possible treatment of human pigmentary disorders when experimental animal models are available for study. The aim of this study then, was to elucidate the etiology of melanocyte dysfunction in developing White Plymouth Rock x Pile Game chicks and to gain insight into some aspects of the developing pigment cell. This domestic fowl breed was selected for investigation as a potential experimental model for human hypomelanotic disorders. The following techniques were applied in this investigation: light and electron microscopy, histochemistry, autoradiography, radiometric assays, primary cell cultures and organ cultures on chorio-allantoic membranes.

CHAPTER 1

1.1 INTRODUCTION TO PIGMENT CELL BIOLOGY

Melanin is an optically dense pigment polymer that protects epithelial cells from the damaging effects of ultraviolet light and also confers colour to the skin, eyes and hair/feathers of vertebrates. In birds, plumage colouration plays an important role in protective camouflage patterning and in sexual dimorphism. The focus of this study is on the severe reduction and even complete absence of pigment that is commonly associated with pigmentary defects, such as albinism and vitiligo. Before one can characterise the aberrant functioning of the pigmentary system, it is necessary to have an understanding of the basics of melanin synthesis.

Melanin synthesis is under complex genetic control. Important events in the melanogenic process in the skin include the following: (a) the migration of melanoblasts from the neural crest tissue to the skin; (b) their differentiation into epidermal melanocytes; (c) the formation and assembly of structural proteins in stage I and II melanosomes; (d) the synthesis of tyrosinase and its transfer to stage I melanosomes; (e) the maturation (melanisation) of melanosomes to form stage III and IV melanosomes; (f) the movement of stage IV melanosomes from the perikaryon to the dendrites of the melanocyte, and (g) the transfer and incorporation of melanosomes from the melanocytes into surrounding keratinocytes. To appreciate the complexity of the controls necessary for normal melanogenesis, a brief synopsis of some of the key events will be discussed in this chapter.

1.1.2 THE ORIGIN AND DIFFERENTIATION OF MELANOCYTES

Melanocytes derive from the neural crest, a transient structure in vertebrate embryos that is associated with the dorsal folds of the developing neural tube. As the neural tube closes in an anterior-posterior direction, the neural crest cells migrate away from the

tube along well-defined pathways in the embryo. In chicks, this occurs between 50 and 55 hours *in ovo* (Weston, 1963). The neural crest cells migrate to specific sites in the embryo where they give rise to diverse phenotypes, such as melanocytes, craniofacial cartilage, Schwann cells and neuroendocrine cells of the adrenal medulla (Bronner-Fraser and Cohen, 1980). In chick embryos, truncal neural crest cells follow three distinct routes (see Erickson, 1988): 1) a dorsolateral pathway between the somites and the ectoderm that is followed by presumptive melanocytes; 2) a dorsoventral pathway through the rostral half of the somite that is followed by cells of the sensory and sympathetic ganglia and cells of the adrenal medulla; 3) a ventral pathway between the sclerotome and the neural tube that is followed by cells of the sympathetic ganglia.

Following their dorsolateral migratory pathway, chick premelanocytes (melanoblasts) arrive in the truncal dermis by day 3 *in ovo*, begin moving into the epidermis from day 4 onward and synthesise pigment from as early as 5 days in certain breeds (Hulley *et al*, 1991). These melanocytes localise in the feather follicles where they are responsible for the colouration of the feathers.

1.1.3 THE EPIDERMAL MELANIN UNIT

Melanin pigment is responsible for black, brown, grey and related shades in birds. In the epidermis, melanins are synthesised by dendritic melanocytes. Melanin granules (melanosomes) move from the melanocyte perikaryon towards the tip of the dendrites from where they are transferred to keratinocytes of the feather follicles (Nakagawa *et al*, 1984). The amount of transferred melanin determines the degree of pigmentation of the feathers. The epidermal melanocyte and its associated keratinocytes have therefore been referred to as the "epidermal melanin unit" (Jimbow *et al*, 1976). Within such a unit, the average ratio of melanocytes to keratinocytes is 1:36 in humans (Cerdan *et al*, 1992). In this way, melanosomes are easily distributed throughout the skin where they contribute towards pigmentation.

In contrast to epidermal or hair melanocytes, feather melanocytes become incorporated into the keratinising epithelium and are shed with the feathers. At the onset of feather regeneration, new melanocytes appear, presumably from a dermal pool of melanoblasts (Foulks, cited in Boissy *et al*, 1986). These feather melanocytes are therefore only active during the initial phases of the feather cycle and eventually degenerate after their melanosomes have been transferred into the keratinocytes.

1.1.4 MELANIN BIOSYNTHESIS AND ITS REGULATION

Two basic types of melanins are produced by mammalian and avian melanocytes. Eumelanins are the black and brown pigments while phaeomelanins constitute the yellow and reddish pigments. Mixed-type melanins, a mixture of eumelanin and phaeomelanin in different proportions, also occur in humans (Hearing and Tsukamoto, 1991). Figure 1 is a schematic representation of the biochemical pathway of melanin formation in mammals. This pathway depicts the formation of melanins from the amino acid, tyrosine, and is thought to be essentially the same in chickens (Bowers, 1988). Tyrosinase (E.C.1.14.18.1), a melanocyte-specific copper-containing enzyme, is of fundamental importance in this pathway. Tyrosinase can catalyse three different reactions in the biosynthetic pathway of eumelanin (Fig. 1) (Korner and Pawelek, 1982): 1) the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA); 2) the oxidation of DOPA to DOPAquinone, and 3) the oxidation of 5,6-dihydroxyindole (DHI) to indole-quinone, a reaction that can also be catalysed by peroxidase (Prota, 1992). The first of these activities, namely tyrosine hydroxylation, is the rate-limiting reaction of the pathway. The remainder of the reaction sequence can proceed spontaneously at physiological pH. Transfection studies have shown that tyrosinase alone is sufficient for melanin synthesis *in vitro*. In these studies, fibroblasts and avian albino melanocytes became highly pigmented after transfection with mammalian tyrosinase cDNA (Whitaker *et al*, 1991). In addition, injection of tyrosinase minigenes into fertilised eggs from an albino mouse

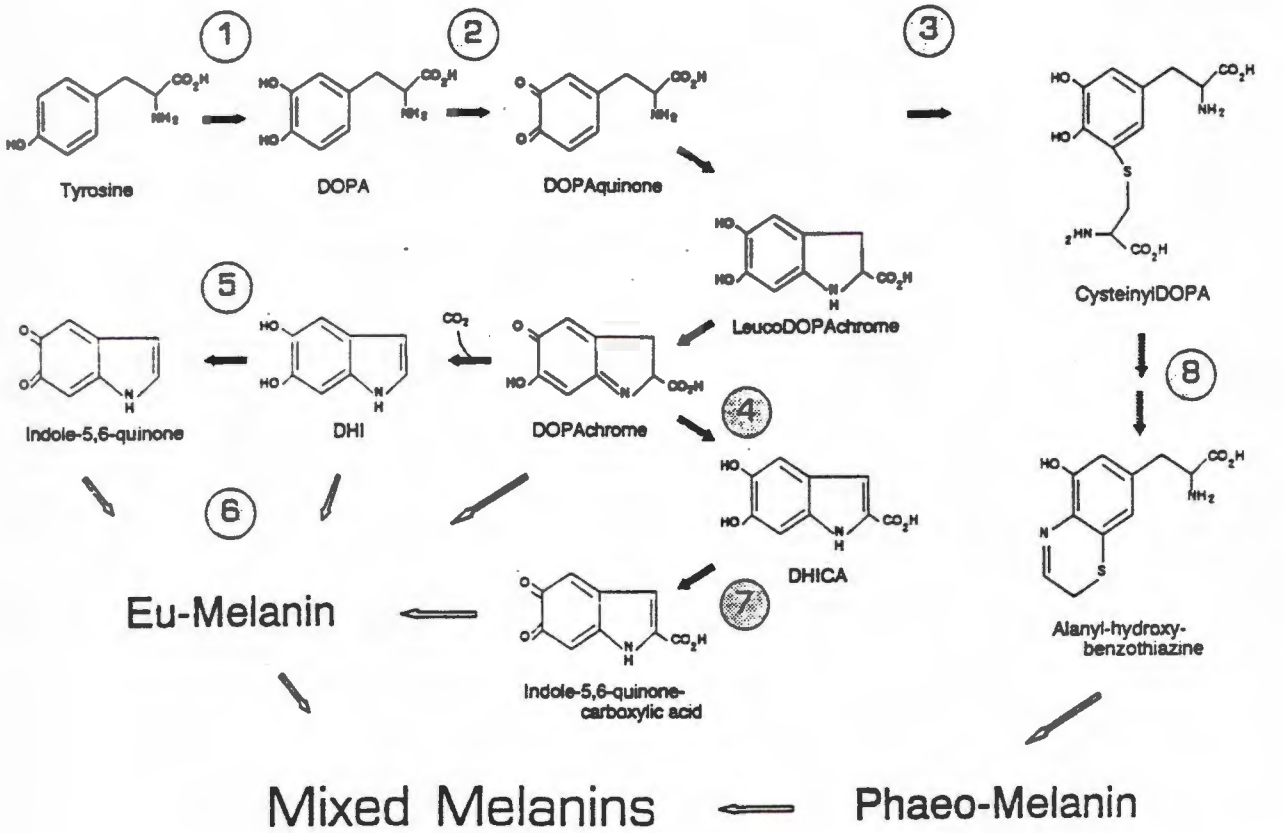

Figure 1. Schematic representation of the mammalian melanogenic pathway (modified from Hearing, 1993). The rate-limiting reaction, tyrosine hydroxylation (1), is catalysed only by tyrosinase, which can also catalyse the DOPA oxidation reaction (2). If glutathione or cysteine is available, DOPAquinone will be diverted to the production of cysteinylDOPAs (3); in their absence, DOPAquinone will cyclise to produce leucoDOPA-chrome, which will then rearrange to form DOPAchrome. In the presence of DOPAchrome tautomerase (TRP-2) or divalent metal cations, DOPAchrome will be diverted to DHICA (4); in the absence of these factors, DOPAchrome will form DHI which is oxidised to indole-5,6-quinone (5), a reaction catalysed by tyrosinase or peroxidase (Prota, 1992). DHICA is similarly oxidised to indole-quinone (7), possibly catalysed by TRP-1. The indoles are incorporated into eumelanin (6), whereas cysteinylDOPA will lead to phaeomelanin production (8). In humans there is a variable mixture of eu- and phaeomelanins, referred to as "mixed melanins".

strain yielded normally pigmented transgenic mice (Tanaka *et al*, 1990).

Apart from the all-essential enzyme, tyrosinase, there are other auxillary enzymes and factors that modify the quality, quantity and type of melanin produced. Briefly, in eumelanin synthesis (see Fig. 1): 1) DOPAchrome is converted to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) in the presence of DOPAchrome tautomerase (E.C.5.3.2.3) rather than to 5,6-dihydroxyindole (DHI) (Fig. 1). This enzyme was previously known by other names (Korner and Pawelek, 1980; Barber *et al*, 1984 and see Hearing and Tsukamoto, 1991) and maps to the tyrosinase-related gene, TRP-2 (tyrosinase-related protein-2) (Jackson *et al*, 1992 and Tsukamoto *et al*, 1991). 2) DHICA is oxidised to a carboxylated indole-quinone under the catalytic activity of TRP-1 (tyrosinase-related protein-1) (Kobayashi *et al*, 1994a). TRP-1 maps to the *brown* (*b*) locus in mice (Jackson, 1988) which was shown to encode a glycoprotein, namely catalase B (E.C.1.11.1.6) (Halaban and Moellmann, 1990). Potential chicken homologues of mammalian TRP-1 and TRP-2 were recently detected and are currently under investigation (Austin and Boissy, 1993 and Austin and Boissy, 1994). 3) The enzymatic conversion of DHICA to melanin by pmel 17, a protein that maps to the murine *silver* locus (Chakraborty *et al*, 1994). This regulatory step remains to be confirmed however, since an immuno-affinity study suggested that the *silver* locus product is a structural melanosomal protein rather than a catalytically functional protein (Kobayashi *et al*, 1994b).

The usual course of eumelanogenesis, as discussed above, can be switched to phaeomelanogenesis by the intracellular levels of sulphydryls such as glutamine and cysteine (Prota, 1993). Under conditions of high sulphydryl content, DOPAquinone is converted to cysteinylDOPA and phaeomelanin is formed (see Fig. 1). In the absence of sulphydryls, DOPAquinone will quickly cyclize to produce leucoDOPAchrome in the eumelanin pathway (see Fig. 1). Other factors, such as the concentration of metal ions (see Prota, 1993 and Martinez et al, 1987) and endogenous inhibitors of tyrosinase and DOPAchrome tautomerase (Imokawa and Mishima, 1986; Kameyama et al, 1993 and Ando et al, 1993), regulate melanin

synthesis by affecting the catalytic abilities of melanogenic enzymes.

Finally, melanin synthesis is also affected by extracellular factors such as melanocyte stimulating hormone (α-MSH) and basic fibroblast growth factor (bFGF), a cytokine produced by keratinocytes. *In vitro*, bFGF appears to be a natural mitogen for human melanocytes (Shih and Herlyn, 1993) but only induces melanogenesis in avian neural crest cells (Stocker *et al*, 1991 and Kalcheim, 1989).

MSH, a peptide produced by the intermediate lobe of the pituitary gland, binds to melanocyte surface receptors to effect a change in melanogenesis. In murine systems, MSH elicits the production of eumelanins rather than phaeomelanins (Burchill *et al*, 1989 and Granholm and Van Amerongen, 1991) and has both a mitogenic and a melanogenic effect on human melanocyte cultures (Abdel-Malek *et al*, 1994 and Hunt *et al*, 1993). Recently, Bowers and Bibosa (1994) produced evidence of increased melanogenesis in cultured feather melanocytes after MSH treatment. This suggests that avian melanocytes possess MSH receptors and ~~that birds~~ also produce MSH-like molecules despite the absence of the intermediate pituitary lobe in birds (Satoh and Ide, 1987).

1.1.5 MELANOSOME ONTOGENY AND ULTRASTRUCTURE

Melanocytes produce specialised organelles called melanosomes in which the biochemical process of melanisation (as described in 1.1.4) occurs. These discreet membrane-bound compartments are essential to protect the cell from the cytotoxicity of the quinoid intermediates of the melanogenic pathway. Four successive stages in the maturation of the eumelanosome have been delineated (Fitzpatrick *et al*, 1969): stage I, the "premelanosome" is a spherical vacuole with absent or ill-defined matrix filaments; stage II, ellipsoid premelanosomes with a well-defined filamentous matrix; stage III, melanosomes with deposition of electron-dense melanin on the matrix filaments, and finally, stage IV, fully

mature melanosomes in which the matrix is completely obscured by dense melanin deposits.

The formation of melanosomes involves the assembly and organisation of structural matrix proteins and the incorporation of tyrosinase and other auxillary enzymes into the melanosomes. Structural elements of melanosomes are synthesised in the rough endoplasmic reticulum (RER) from which premelanosomes originate as smooth out-pockets that are pinched off. The premelanosomes then develop into mature melanosomes by receiving tyrosinase (Hirobe, 1982).

Tyrosinase is synthesised in the RER and post-translational modification (mainly glycosylation) of the enzyme occurs during its transit through the Golgi and the anastamosing tubules of the Golgi-associated endoplasmic reticulum (GERL) (Imokawa and Mishima, 1986). The end portions of the GERL cisternae bud off to form DOPA-positive coated vesicles (Imokawa and Mishima, 1986 and Mishima, 1992). Biochemical analyses of these vesicle fractions indicated that glycosylated tyrosinase as well as eumelanin monomers (5,6-DHI and 5,6-DHI2C) are transferred to premelanosomes (Hatta *et al*, 1988 and Mishima, 1992).

Fusion of the tyrosinase-carrying vesicles with stage II melanosomes is believed to initiate the process of melanogenesis (Maul and Brumbaugh, 1971). The polymerisation of tyrosine to melanin, begins on the matrix lamellae after tyrosinase is released from the coated vesicles into the premelanosome and proceeds until a stage IV melanosome has been produced.

Melanin production also requires the entry of L-tyrosine, the melanin precursor, into the melanosome. Gahl *et al* (1994) demonstrated that tyrosine transport into murine melanosomes is carrier-mediated. The P gene (*pink-eyed dilution* locus) encodes an integral melanosomal membrane transporter protein, thought to be involved in tyrosine transport (Saitoh *et al*, 1994 and Rinchik *et al*, 1993). The transport of tyrosine into the melanosome presents yet another level of regulation in the melanogenic pathway.

1.2 *HUMAN PIGMENTARY DISORDERS AND THEIR CORRESPONDING ANIMAL MODELS*

A number of human pigmentary disorders result in decreased melanin synthesis in the skin, eyes and hair. Vitiligo and oculocutaneous albinism are examples of hypomelanotic pigmentary disorders that reduce the quality of life for its sufferers. Careful analysis of such disorders provides insight into the control and regulation of melanin synthesis and allows the formulation of hypotheses on probable mechanisms of development, as well as providing information on how to correct abnormal function.

The past decade has seen increased interest in murine and avian melanotic systems as models for investigating these pigmentation abnormalities in man (see reviews Bowers, 1988 and Halaban and Moellmann, 1993). The advantages of studying animals with phenotypes resembling human pigmentary disorders are: (a) animals can be bred in large quantities and maintained with minimal cost, (b) animals can be experimentally manipulated to further elucidate the disease process and (c) animals can be used in potential therapeutic studies.

1.2.1 OCULOCUTANEOUS ALBINISM (OCA)

OCA is a congenital, heritable pigmentary disorder in humans that affects the melanocytic system of the integument and eyes. With this disorder melanocytes populate the body in a normal fashion but fail to synthesise adequate amounts of melanin (Witkop *et al*, 1989). Ten types of OCA have been described in humans, but the two major types are: ***tyrosinase positive*** (ty-pos) and ***tyrosinase negative*** (ty-neg) *OCA* (Witkop *et al*, 1989). Both of these forms are transmitted in an autosomal recessive manner.

1.2.1.1 *TYROSINASE POSITIVE OCULOCUTANEOUS ALBINISM (TY-POS OCA)*

This type of albinism affects 1 in 3900 South Africans (Kromberg and Jenkins, 1982). Ty-pos albinos have a pink white skin colour with white-yellow or tan coloured hair. Ty-pos albino hairbulbs have histochemically detectable tyrosinase and contain lightly pigmented stage III melanosomes (Witkop *et al*, 1989). The absence of mature melanosomes has led to the suggestion of a block in the eumelanin biochemical pathway (Witkop *et al*, 1989). The current hypothesis is that ty-pos OCA results from a defect in the transportation of tyrosine into the melanosome (Rinchik *et al*, 1993 and Saitoh *et al*, 1994). Deletions in the *P* gene, which encodes a tyrosine carrier, were recently identified in ty-pos albinos (Rinchik *et al*, 1993 and Kedda *et al*, 1994). In addition, mutations have been identified in the *P* gene of hypopigmented Prader-Willi and Angelman syndromic patients (Brilliant et al, 1993). In an earlier study of hair melanocyte ultrastructure, Witkop and his group (1973) observed autophagocytosis of melanosomes in a sub-group of ty-pos albinos. However, in the light of recent evidence, this self-destruction is thought to be a secondary effect resulting from the obstruction in melanogenesis.

1.2.1.1a AVIAN MODEL FOR TY-POS OCA

A sex-linked recessive imperfect-albino (s^{al}), arising from a mutation at the silver/gold plumage colour locus in fowl, has been proposed as a model for human ty-pos OCA. Oetting *et al* (1985) have shown that sex-linked albinos possess tyrosinase activity and therefore, as in ty-pos albinos, hypomelanosis is not a consequence of a mutation of the tyrosinase gene. Sex-linked albino chick melanocytes have abundant abnormal premelanosomes that display tyrosinase activity (Boissy *et al*, 1987). This contrasts with human ty-pos albinos where melanocytes have an abundance of tyrosinase-active, morphologically normal premelanosomes (Witkop *et al*, 1989). Autophagocytosis of abnormal premelanosomes and parts of the Golgi apparatus in s^{al} melanocytes has also been reported, but whether this degradation of melanin

is the cause of hypomelanosis in these ty-pos albino chicks, is undetermined (Boissy *et al*, 1987).

1.2.1.1b MURINE MODEL FOR TY-POS OCA

Mutations of the murine *pink-eyed dilution* (*p*) locus cause a reduction of eumelanin in skin, eye and coat pigmentation (Silvers, 1979). The cloned murine *p* gene has been identified as the homologue of the human *P* gene (Urabe *et al*, 1993 and Rinchik *et al*, 1993), and encodes a tyrosine transporter in melanosomes (Saitoh *et al*, 1994). The pink-eyed dilute mouse is therefore an ideal model for studying defects of the tyrosine transporter protein and how it may influence pigmentation in ty-pos individuals.

1.2.1.2 TYROSINASE NEGATIVE OCULOCUTANEOUS ALBINISM (TY-NEG OCA)

This is the second most frequent type of OCA in African populations. Human ty-neg albinos have a constant phenotype characterised by the complete absence of visible pigment - all ty-neg albinos have snow-white hair, pink-white skin and grey-blue irides. Hairbulbs from ty-neg patients have no tyrosinase activity and are marked by the presence of unpigmented premelanosomes (stages I and II) (Witkop *et al*, 1989). Hence hypomelanosis results from an enzymatic defect in these albinos. This has been confirmed by several researchers who discovered that mutations in the tyrosinase gene involve the copper-binding sites of tyrosinase and therefore alter the active site of the enzyme (Handoko *et al*, 1992, Tripathi *et al*, 1992 and Oetting and King, 1992).

1.2.1.2a AVIAN MODEL FOR TY-NEG OCA

An autosomal recessive albino allele, c^a, at the C locus in the fowl confers the albino phenotype on avians and has been proposed

as a model for ty-neg OCA in man. Ultrastructural and cytochemical studies of feather melanocytes from these albino chicks suggested that they are tyrosinase negative (Oetting *et al*, 1985 and Boissy *et al*, 1987). Feather melanocytes contained morphologically normal and abnormal premelanosomes that were completely devoid of pigment. Further confirmation that tyrosinase is the key to hypomelanosis in albino chicks was provided by Akiyama *et al* (1992) who demonstrated that cultured c^a chick melanocytes could produce pigment when infected with mouse and quail promoters with mouse tyrosinase cDNA constructs. Hence, autosomal recessive albino chicks are representative of the human ty-neg OCA disorder.

1.2.1.2b MURINE MODEL FOR TY-NEG OCA

In inbred albino mice, tyrosinase activity is not detectable and the immunoprecipitated protein is present at extremely low levels (Halaban *et al*, 1988). A point mutation in the tyrosinase gene is responsible for the complete failure of pigment formation in Balb/*c* albino mice (see Halaban and Moellmann, 1992). The Balb/*c* albino mouse is homozygous for the albino mutation on the tyrosinase gene and is also homozygous for a mutation at the *brown* locus which results in little or no catalase *B* activity (Halaban and Moellmann, 1990 and see Halaban and Moellmann, 1992 for review). These point mutations in conserved domains in the tyrosinase and catalase *B* genes are therefore responsible for reducing enzyme activity below functional levels.

1.2.2 VITILIGO

Vitiligo is a pigmentary disorder in which melanocytes are selectively eliminated from various regions in normally pigmented individuals. This results in either partial or total amelanosis. The precise mechanism of this elimination of melanocytes is still unknown. Three hypotheses have been postulated to explain the etiology of vitiligo (see Ortonne and Bose, 1993). The first hypothesis, also known as the autoimmune hypothesis, states that

the disappearance of melanocytes is induced by an antigen-antibody reaction. The following findings lend support to this theory: antimelanocytic autoantibody production has been noted in serum of vitiligo patients and vitiligo is frequently associated with autoimmune diseases such as thyroiditis and Type I diabetes mellitus (Grimes, 1993 and Goudie *et al*, 1993). However, antimelanocyte antibodies are not found in all vitiligo patients, suggesting that this hypothesis is not representative of all vitiligo sufferers.

The second theory proposes that vitiligo is a disease involving the nervous system. This neural theory is based on several facts (see Nordlund, 1992). Firstly, melanocytes have a common embryological origin as nerve cells. Secondly, catechols are structurally similar to the melanin precursor, DOPA. Thirdly, nerves transmit catechols which might affect melanocyte function, and finally, vitiligo can be precipitated by severe emotional trauma or stressful events. In addition to these facts, aberrations in B endorphin secretions and elevated urinary levels of catecholamine metabolites were detected in patients with vitiligo (Passi *et al*, 1993; Schallreuter *et al*, 1993 and Moronne *et al*, 1992). Despite all of the above, the role of the nervous system in the pathogenesis of vitiligo is still undefined.

The third theory, also known as the self-destruction or autotoxic theory, suggests that melanocytes are destroyed by a build-up of cytotoxic intermediates of melanin synthesis, leading to cell death (Slominsky *et al*, 1989). This theory is based on the concept that melanin precursors, particularly the indoles and quinones, can be cytotoxic for melanocytes (Urabe *et al*, 1992). Although the addition of these chemicals to melanocyte cultures may cause their destruction, many of these compounds exist in different forms *in vivo* and may therefore not undergo the same reactions as *in vitro* (see Nordlund, 1992). A second version of this theory is that there is an intrinsic defect of the structure and function of the RER in vitiligo melanocytes (Boissy *et al*, 1991a), that leads to aberrant synthesis and trafficking of proteins and eventually result in autophagocytosis. Although ultrastructural defects have been observed in the RER of cultured

melanocytes derived from vitiligo patients, this defect did not appear to be cytotoxic *in vitro* (Boissy *et al*, 1991a). Another version of this theory, proposed by Slominsky *et al* (1989), is that melanocyte destruction results from a cascade of reactions initiated by a deregulation of melanogenesis caused by activation of the melatonin receptor. In rodents, melatonin has been shown to inhibit melanin synthesis (see Ortonne and Bose, 1993). However, little is known about its action on human melanocytes *in vivo* and it remains to be established if normal human melanocytes express melatonin receptors.

From the available data and the different hypotheses, it appears that the loss of melanocytes in vitiligo may be the result of several different pathogenic mechanisms operating alone or in concert. The animal models presented below each represent only one aspect of the complex set of events that might occur in vitiligo.

1.2.2.1 AVIAN MODELS FOR VITILIGO

1.2.2.1a DELAYED AUTOIMMUNE (SMYTH) LINE

The most extensively analysed animal model for vitiligo is the delayed autoimmune or Smyth (DAM-Smyth) line of chickens (see Boissy and Lamoreux, 1988). This line originated in the 1970's and affected chicks display the classic features of vitiligo, namely delayed onset of amelanosis and the production of circulating melanocyte autoantibodies (Boissy *et al*, 1986). In addition to these autoantibodies, immune cells (T-helper cells and cytotoxic T cells) involved in melanocyte destruction, were immunocytochemically located in the skin and feather pulp of Smyth chickens prior to and during depigmentation (Erf *et al*, 1992 and Shresta *et al*, 1992). However, the basic defect in Smyth chicks appears to be inherent in the melanocyte and not in the immune system. This was supported by the report of premature cell death of these melanocytes *in vitro*, whereas melanocytes from control birds continued to thrive (Boissy *et al*, 1986). Since Smyth chick melanocytes developed autophagosomes *in vivo* as well as *in vitro*,

it is evident that the autoimmune response observed *in vivo*, is merely a secondary response to an innate melanocyte defect (see Boissy and Lamoreux, 1988). In spite of this, the Smyth chick is still regarded as a very reliable animal model for vitiligo (see Ortonne and Bose, 1993).

1.2.2.1b WHITE LEGHORN

The White Leghorn (WL) fowl has been proposed as a non-immune, cytotoxic model for vitiligo. White Leghorns have a mutation at the I locus, a dominant white gene that results in decreased amounts of eumelanin (Brumbaugh, 1971). A comprehensive electron microscopy and histochemical study of WL embryonic skin and regenerating feathers revealed that WL melanocytes have morphologically normal and abnormal premelanosomes in which tyrosinase activity was demonstrated (Jimbow *et al*, 1974). These premelanosomes never became fully melanised nor were they transferred to the surrounding keratinocytes. Instead, they were degraded in autophagosomes. This suggested that hypomelanosis resulted from premature melanocyte degeneration initiated by an inherent error in the synthesis and maturation of melanosomes which led to autophagocytosis of melanosomes.

A more recent theory is that the WL melanocytes undergo premature cell death because they are genetically pre-conditioned to be more sensitive to toxic factors such as melanin precursor products. This hypothesis is based on *in vitro* experiments which showed that WL feather melanocytes could survive for 3 months in tissue culture if the culture medium was changed frequently or when antioxidants were added to the media (Harmon *et al*, 1989; Lujan *et al*, 1992 and Bowers *et al*, 1991). When the culture media was not changed for 10 days, 95% of the WL melanocytes had died, compared to 50% of the wild type fowl melanocytes (Harmon *et al*, 1989). These WL melanocytes also showed a greater sensitivity to WL conditioned media than did wild type melanocyte cultures (Bowers *et al*, 1991). This suggested that WL melanocytes are more sensitive to a toxic substance released into the culture medium by the melanocytes. This substance may be a by-product of

melanogenesis, since the addition of antioxidants increased the viability of these cultures (Bowers *et al*, 1991). Studies are currently underway to measure the levels of antioxidants and reactive oxygen radicals in WL chicks in order to confirm the role of antioxidant levels in premature melanocyte death (Bowers *et al*, 1994).

1.2.2.1c BARRED PLYMOUTH ROCK

In the Barred Plymouth Rock (BPR) chicken, white bands appear on otherwise black feathers under the influence of a dominant, sex-linked barring gene (*B*) (Crawford, 1990). These black bands contain numerous melanocytes that are actively transferring melanosomes to keratinocytes, while the white bands have no viable melanocytes. Histological and ultrastructural observations, as well as tissue culture and grafting experiments have shown that the BPR chick melanocytes die in the white bands because the *B* gene causes these pigment cells to be more sensitive to some cytotoxic agent (Bowers, 1989). Thus, the alternating black and white barring pattern in the feathers was ultrastructurally shown to result from premature autophagic degeneration of melanocytes at the proximal edge of a black band. Yet, wild type Jungle fowl embryonic melanocytes did not degenerate prematurely when grafted to BPR embryonic feather follicles, but pigmented the entire feather (Bowers *et al*, 1992). Like WL chick melanocytes, cultured BPR chick melanocytes could survive *in vitro*, but showed a degree of sensitivity to WL conditioned media (Bowers *et al*, 1991).

Bowers (1988) attempted to explain the selective survival of melanocytes in the barred feathers in the following way: high levels of melanin toxins accumulate in the feather due to active melanin synthesis in the black band. These factors may inhibit new melanocytes from entering the growing feather and thus, a white band is formed. After a certain growth period the toxic substance may be "diluted out" in the white band and new melanocytes can enter the growing feather to form a new black band. BPR chickens are therefore also considered as a model for the non-immune, cytotoxic form of vitiligo.

1.2.2.2 MURINE MODEL FOR VITILIGO

The vitiligo mouse, C57BL/6J Ler-*vit/vit*, is homozygous for an autosomal recessive gene and was bred from C57BL/6J (B6) mice with a spontaneous depigmentation mutation. Mice of this genotype are born with white spotting on the back and abdomen and the pigmented areas progressively depigment (Lerner *et al*, 1986). The primary defect in the vitiligo mouse seems to be intramelanocytic and does not involve a systemic immune reaction. This was demonstrated by reciprocal skin transplants between normal B6 and *vit/vit* mice (Lerner *et al*, 1986). The coat colour of these grafts was observed for up to 25 weeks posttransplantation. During that period, *vit/vit* skin that was grafted onto B6 mice turned white, while B6 skin that was grafted onto *vit/vit* mice remained black. This indicated that in the *vit/vit* mouse, melanocytes are not targetted for elimination by a defunct immune system, but that the wheels of destruction are set in motion by a defect inherent in the *vit/vit* melanocyte itself. From the grafting results it is unlikely therefore, that vitiligo in the *vit/vit* mouse is an autoimmune disease. This was further substantiated when cultured *vit/vit* melanocytes displayed structural aberrations and died prematurely, as *in vivo*. (Boissy *et al*, 1991b).

1.3 THE SIGNIFICANCE OF INVESTIGATING GENETICALLY UNDEFINED (VARIABLE) ANIMAL BREEDS

From the above literature review, the value of animal experimental models in elucidating the etiologies of human pigmentary disorders becomes apparent. However, not all the proposed models are, or can be, fully representative of the corresponding human conditions. It is possible that some features observed in the hypomelanotic animal models may have resulted from selection pressure during the development of the experimental animal line (see Boissy and Lamoreux, 1988). In addition, studies involving avian models used regenerating feathers as study material. As a result, important developmental aspects of the pigmentary system may have been overlooked. There is, therefore, space for more

comprehensive studies that may lead to more representative experimental models.

Inbred animal strains are useful in identifying and analysing the underlying cause of a syndrome because there is only one genetic difference between the mutant and the control animal. However, genetically undefined or variable stocks are also valuable since they can provide information on the effect that additional genetic and environmental factors may have on the development of the disorder (Boissy and Lamoreux, 1988).

In the present study a domestic fowl breed, White Plymouth Rock x Pile Game (WPR x PG), was selected for investigation as a model for human hypomelanotic disorders. Pigmented Black Australorp x New Hampshire Red (BA x NHR) chicks were used in this study to compile standard data from a pigmentary normal breed.

1.4 GENETICS OF PLUMAGE COLOUR IN FOWLS OF PRESENT STUDY

Newly hatched White Plymouth Rock x Pile Game chicks used in this study have pale yellow down which later develops into pure white feathers. Pile Game chicks carry an autosomal dominant mutation, dominant white (I). The *I* gene is a so-called "inhibitor of black pigment" and is characteristic of White Leghorns (Hutt, 1949 and Crawford, 1990). *I* suppresses eumelanin production in the plumage whilst phaeomelanin production is unaffected (Brumbaugh, 1971 and Brumbaugh and Lee, 1975).

The other half of the white cross breed, White Plymouth Rock chicks, carry an autosomal recessive mutation at the *c* locus of the fowl known as "recessive white". Tyrosinase activity could not be detected in *c* melanocytes (Oetting *et al*, 1985 and Boissy *et al*, 1987). It is still not certain whether the *c* alleles represent mutations of the structural tyrosinase gene as in human and murine albinos, or whether they exert their effects on the enzyme in a regulatory fashion (Crawford, 1990). White Plymouth Rock chicks are also reported to carry the dominant white mutation of the White Leghorn and the Pile Game chicks (Hutt, 1949 and

Crawford, 1990). Hence, in addition to other pigmentary mutations, the White Plymouth Rock x Pile Game cross-breed is homozygous for the *I* gene and may therefore, show quite a unique series of defects.

To compare the normal series of events in melanogenesis with events in the hypomelanotic mutants, normally pigmented chicks were also studied. Black Australorp x New Hampshire Red chicks were selected for this purpose. These chicks are completely black at the time of hatching, except for a small white throat patch. Later in development, red feathers develop among the black feathers. Black Australorp chicks are solid black birds that carry the *extended black* gene (*E*) (Hutt, 1949 and Crawford, 1990) while New Hampshire Reds carry the dominant wheaten mutation (e^{wh}) that restricts eumelanin to the tail plumage while red is the predominant colour of the plumage (see Crawford, 1990).

CHAPTER 2

MATERIALS AND METHODS

2.1. ANIMALS

Fertile fowl eggs were commercially obtained (Golden Grove Poultry Farm, Malmesbury, and Tokai Breeding Farm), incubated at 37°C (50-60% humidity) and staged either according to Hamburger and Hamilton (1951) for neural tube experiments, or incubated for a stated period of days for other studies.

Black Australorp x New Hampshire Red (BA x NHR) chick embryos and/or White Plymouth Rock x Pile Game (WPR x PG) embryos were used in this study. As described in the Introduction, BA x NHR chicks are pigmented, whereas WPR x PG chicks are unpigmented.

In all the experiments performed and reported here, samples were taken from both BA x NHR and WPR x PG chick embryos, unless otherwise indicated. **Hereafter, the breeds will be referred to as "black" and "white", respectively.**

2.2. HISTOLOGICAL PROCEDURES

Embryonic dorsal chick skin were fixed either in Bouins fixative for routine histology, 4% paraformaldehyde for DOPA histochemistry or in 10% buffered formalin for autoradiography. The fixed tissue samples were then dehydrated through a series of ascending ethanols, cleared in xylol, taken through two changes of wax at 60°C and finally embedded in fresh paraffin wax. Serial sections of 5μm were cut on a Reichert-Jung microtome and collected on glass slides. Slides **containing sections of DOPA reacted skin were** dewaxed in xylol, rehydrated through a series of descending ethanol and lightly stained with eosin for viewing. For autoradiography, alternate slides with sections were stained with haematoxylin and eosin. These slides were viewed under brightfield microscopy to assess tissue morphology of the remaining unstained sections. In this way, tissue sections with

the best epithelial detail and morphology were chosen for autoradiographical processing. After this procedure, the sections were counterstained with haematoxylin. All stained sections were examined with a Nikon Microphot FX microscope.

2.3 DOPA REACTION

The DOPA reaction enhances tyrosinase-positive melanocytes *in situ* and the technique used here is according to the method of Brumbaugh (1971).

Briefly, dorsal skin was dissected from 8- to 13-day old WPR x PG embryos, fixed in 4% paraformaldehyde (in 0.2M phosphate buffer, pH 7.2) for 2 hr and followed by 3 rinses of 5 min each in phosphate buffered saline (PBS, see Appendix), pH 6.9. The skin tissue pieces were then incubated for 3 hr at 38°C in PBS, pH 6.9, containing 5mM L-DOPA (Sigma) and 0.12% D-glucose and then routinely processed for paraffin wax embedding. Control samples consisted of skin pieces incubated in PBS lacking the DOPA substrate. Two embryos per developmental stage were used for the DOPA reaction which was repeated four times for each embryonic stage.

2.4 RADIOMETRIC ASSAY FOR TYROSINASE ACTIVITY

2.4.1 ENZYME PREPARATION

Dorsal skin samples and the intestines were dissected from 9 and 13 day old embryos and stored in sterile chick saline (see Appendix) on ice during the period of dissection. The saline was discarded and the tissue sample tubes were spun briefly (5s) in an Eppendorf centrifuge to estimate the tissue volume of each sample. The tissue samples were then manually homogenised on ice in an equal volume of 0.1M phosphate buffer, pH 7.4 containing 1% Triton X-100. The resulting homogenates were centrifuged at 10 000 x g for 5 min at 4°C. The supernatants were removed and used as a crude source of enzyme. Enzyme samples were always prepared fresh and not stored as frozen aliquots because frozen samples showed reduced enzyme activity. Before using the homogenate preparations

in the assay, they were always adjusted to equal protein concentrations by means of the Biuret dye-binding assay (Bradshaw, 1966).

2.4.2 [^{14}C]TYROSINE ASSAY

Modifications of the technique published by Hearing and Ekel (1976) was used.
For each enzyme preparation, the following mixture was prepared for each assay (final volume 25μl: 10μl of enzyme preparation, 5μl of [^{14}C]tyrosine (25μCi/ml, specific activity 513 mCi/mmol; Amersham), 5μl of L-DOPA (0.05mM in 0.1M phosphate buffer; Sigma) and 5μl of an antibiotic solution consisting of chloramphenicol (1mg/ml), cyclohexamide (1mg/ml), bovine serum albumin (0.1mg/ml) and penicillin (1000 iu/ml).

Samples were incubated overnight in the reaction mix at 37^{o}C. After incubation, 20μl of each sample were spotted onto Whatman (GFC) glass fibre filter discs. The filters were washed twice in 10% trichloroacetic acid for 15 min (to remove unincorporated [^{14}C]tyrosine) and then dehydrated in 90% ethanol and 70% ethanol for 5 min each. The filters were then transferred to scintillation vials and dried for 30 min at 50^{o}C before adding 500μl of Soluene tissue solubiliser (Packard) and incubating the vials at 45^{o}C for 60 min to release acid-insoluble reaction products from the filter discs. Next, 8ml of toluene scintillation fluid (Packard) was added to the vials and the samples were counted in a Packard Tricarb liquid scintillation counter set for 10 min counts on the ^{14}C channel.

Each reaction was carried out in triplicate. The internal abdominal organ samples were used to determine baseline levels of [^{14}C]tyrosine incorporation in tissues known to be tyrosinase-negative. Method controls (blanks) were also carried out. These blanks consisted of reaction mixes in which the enzyme preparation was omitted and replaced by buffer to determine the levels of non-enzymatic incorporation of [^{14}C]tyrosine during the assay. Counts obtained from these blank samples were subtracted from the readings obtained from skin and gut samples as 'background activity'.

For each age category and breed, skin and gut material was dissected from 10 embryos (that is, 40 embryos were dissected per assay).

2.5 ELECTRON MICROSCOPY

2.5.1 CHICK SKIN SPECIMENS

Eggs were incubated for 9-10 days, embryos were removed from these eggs and transferred to a modified Karnovsky's fixative (1.5% paraformaldehyde and 0.5% glutaraldehyde in 0.1M phosphate buffer, pH 7.6). Dorsal skin samples were dissected from these embryos and left to fix at 4°C for 3 hr. After 3 buffer washes (10 min each) the specimens were post-fixed with 1% osmium tetroxide (OsO_4) for 60 min at room temperature, dehydrated through graded ethanol, cleared in propylene-oxide and embedded in Epon-Araldite resin. Ultrathin sections (60 nm) were cut on an UltraCutE microtome with glass knives. Sections were collected on copper grids and stained with uranyl acetate (5 min) and lead citrate (5 min).

2.5.2 ELECTRON MICROSCOPY OF NEURAL CREST CELL CULTURES

Neural crest-derived cells, maintained *in vitro* for 2-5 weeks, were washed twice with 0.1M phosphate buffer, pH 7.6 and fixed *in situ* with modified Karnovsky's fixative for 60 min at room temperature followed by 60 min at 4°C. After two buffer washes (10 min each), the cells were post-fixed with 1% osmium tetroxide for 30 min at room temperature, dehydrated through graded ethanol and cleared in propylene-oxide. The addition of propylene-oxide caused the fixed cells to lift cleanly off the plastic culture dish as an intact sheet. These "sheets" of cells were embedded in Epon-Araldite resin.

2.6 NEURAL CREST CULTURES

Fertile eggs were incubated for 48-50 hr to yield stage 13-14 embryos. Embryos were removed from the eggs and washed in sterile calcium- and magnesium-free buffer (Dulbecco's PBS, pH 7.4, see Appendix) and the trunk was isolated from the rest of the body.

Truncal segments (from the fifth somite to the last somite) were treated with 0.5 mg/ml collagenase (Type IV; Sigma Chemical CO.) for 13-15 min at room temperature. The segments were then transferred into PBS and dissecting pins were used to dissect the neural tube free from the enveloping ectoderm, endoderm and adjacent somites. The cleaned neural tubes were explanted onto 35mm Nunc plastic culture dishes in 2ml of Ham's-F12 medium (Highveld Biological) with 20% foetal calf serum (FCS; Highveld Biological) and 10ng/ml bFGF (Promega). A variety of other supplements were also tested on these cultures and will be discussed in the results. Cell cultures were incubated at 37-38^{o}C (64% relative humidity and 5.8% CO_2), fed fresh medium twice a week and were examined with a Leitz phase contrast inverted microscope. Throughout the culture period, the neural tubes remained in the dish. After 2-5 weeks in *vitro*, some melanocyte cultures were fixed and processed for electron microscopy studies.

2.7 AUTORADIOGRAPHY

2.7.1 NEURAL CREST CELL LABELLING WITH ^{3}H-THYMIDINE

Neural tubes were obtained from stage 13-14 BA x NHR chick embryos as described above. The isolated neural tubes were transferred onto 35mm Nunc plastic culture dishes in 2ml of medium to provide neural crest cells from a pigmented breed. The neural crest cells were cultured in Dulbecco's modified Eagles medium (DMEM; Highveld Biological) supplemented with 10% FCS, 1mg/ml antibiotics (penicillin and streptomycin) and 5μCi/ml of (methyl-^{3}H)thymidine (79 Ci/mmol; Amersham International). The dishes were incubated for 20-24 hr at 37-38^{o}C (64% relative humidity and 5.8% CO_2). The neural tubes were removed from the dish with a tungsten needle and labelled medium was then removed from the culture dish to leave tritium-labelled crest cell populations. These neural crest cells were washed thoroughly with unlabelled medium and chased with fresh, unlabelled medium for 24 hr to remove residual, unincorporated tritiated thymidine from the culture dish. Thus, only the cultured neural crest cells were radioactively labelled.

Unpigmented, recipient skin was then introduced onto the neural crest cells and co-cultured for one day as described below.

2.7.2 PREPARATION OF RECIPIENT TARGET ORGAN

WPR x PG eggs were incubated for 5.5-6 days. Dorsal skin was dissected from these embryos with dissecting pins, cut into squares of $2mm^2$ and placed dermis-side down onto tritium-labelled neural crest cell cultures and incubated together for 24 hr in DMEM + 10% FCS. The recipient skin explants could therefore settle onto the crest cell cultures and provide a potential target organ for migrating cultured neural crest cells. The skin explants were then removed and grafted onto the chorio-allantoic membrane (CAM) of host embryos, see below.

2.7.3 HOST PREPARATION

Fertile fowl eggs were incubated for 72 hr. The eggs were then washed in 70% ethanol. A small window was cut in the shell over the embryo and the vitelline membrane was removed to allow access to the embryo. Two drops of chick saline with antibiotics (1mg/ml penicillin and streptomycin) were pipetted into the egg to prevent infection and dehydration. The window was then sealed with cellophane tape (Scotch's magic tape) and the egg was returned to the incubator for a further six days.

2.7.4 CAM GRAFTING

Skin explants were grafted after 24 hr of co-incubation with cultured neural crest cells. The cultured explants were grafted directly onto the chorio-allantoic membrane (CAM) of 9-day chick embryonic hosts. Well-vascularised regions of the CAM were selected for grafting. To prepare these regions for accepting the skin explants, bloodvessels of the CAM were first ruptured with a dissecting pin. After the excess blood was sucked off, the explants were gently manoeuvered over the ruptured area. The

window of the egg was then resealed and the host returned to the incubator for a further 6 days of development to allow a sufficient growth period for the grafts. The grafts were removed after this period of development, fixed overnight in 10% buffered formalin and hand processed for histological examination and autoradiography.

2.7.5 AUTORADIOGRAPHIC PROCEDURES

Wax embedded grafts were serially sectioned (5μm thick sections). Sections were collected on glass slides, dewaxed in xylol for 20 min followed by 4 min in absolute ethanol and then left to air-dry for 60 min. The dipping protocol and subsequent autoradiographic processing were carried out as recommended by Amersham International. Briefly, liquid photographic emulsion (LM-1) was melted under safelight darkroom conditions in a 43^{o}C water bath and maintained at this temperature throughout the dipping procedure. Slides containing dewaxed, air-dried sections were dipped into the thin photographic emulsion and allowed to gel on a cooled metal tray (5^{o}C) for 20 min and then left to dry in a vertical position for 2-3 hr. The coated slides were then sealed in a light-tight, plastic slide box, covered with a black bag and exposed for 10-14 days at 4^{o}C. After exposure, the slides were developed in Ilford Phenisol developer under safelight conditions for 5 min, then placed in a stop solution (0.5% solution of acetic acid in distilled water) and finally cleared for 8 min in fixer (30% solution of sodium thiosulphate in distilled water). Slides were gently washed in running tap water for 15 min and then transferred to a distilled water bath. After this step, safe-light conditions are not necessary. The autoradiographed sections were counterstained in haematoxylin, dehydrated, coverslipped and viewed with a light microscope to locate tritium-labelled cells.

2.7.6 CONTROLS

As a control for background radioactivity, some unpigmented skin explants were incubated on the plastic culture dish without

contacting the labelled neural crest cells. After the co-culture period, these experimental controls were treated in the same way as the other explants. Because the control explants were never in direct contact with radioactive cells, they should not show any signs of radioactivity other than the expected background levels of the photographic emulsion used for autoradiography.

2.8 STATISTICAL TEST

The Student's t-test was applied to data obtained from the radiometric assay (see 2.4.2) to determine whether or not the difference in tyrosinase activity is significantly different between black and white chick embryos. For this purpose, the significance level was selected as 0.05 (that is, 5% probability).

CHAPTER 3

RESULTS

3.1 LOCALISATION OF MELANOCYTES IN WHITE CHICK SKIN

Light microscopy techniques were used to determine whether (a) melanocytes were present in white chick skin and, if so (b) whether their location contributed to the hypomelanotic phenotype. For this purpose, dorsal skin from 8- to 10-day chick embryos was examined. The control pigmented chick breed used in this study, is known to have visibly pigmented melanocytes at these stages of development (Hulley *et al*, 1991).

3.1.1 EMBRYONIC SKIN MORPHOLOGY

Examination of wax-embedded skin sections showed that the epidermis consisted of an outer layer of flattened peridermal cells and an underlying layer of columnar cells (Fig.2). The epidermis is separated from the mesenchymal dermis by a basement membrane (not always visible without a special stain). Numerous intercellular spaces were evident between the epidermal cells but not between peridermal cells. Changes in skin morphology were seen during feather development. At 8 days, the epidermis consisted of 2-3 layers of cells which bulged slightly at the apical surface (Fig. 2a and 2d). These bulging epidermal placodes formed as a result of local condensations of mesenchyme. At 9 days, the epidermal placodes protruded outwards to form feather buds. The 9 day epidermis was three cell layers thick and dermal condensations were evident only below feather buds (Fig. 2b and 2e). In 10 day embryonic skin, the feather buds were elongate with a central core of mesenchyme. The epidermis between two adjacent feather buds was three cell layers thick, while the bud epidermis was more than three layers (Fig. 2c and 2f). When examined for the presence of melanin, black chick skin contained many aggregates of dark, cytoplasmic granules (Fig. 2a to 2c). These distinctive melanin granules occurred in the cell body and dendrites of numerous

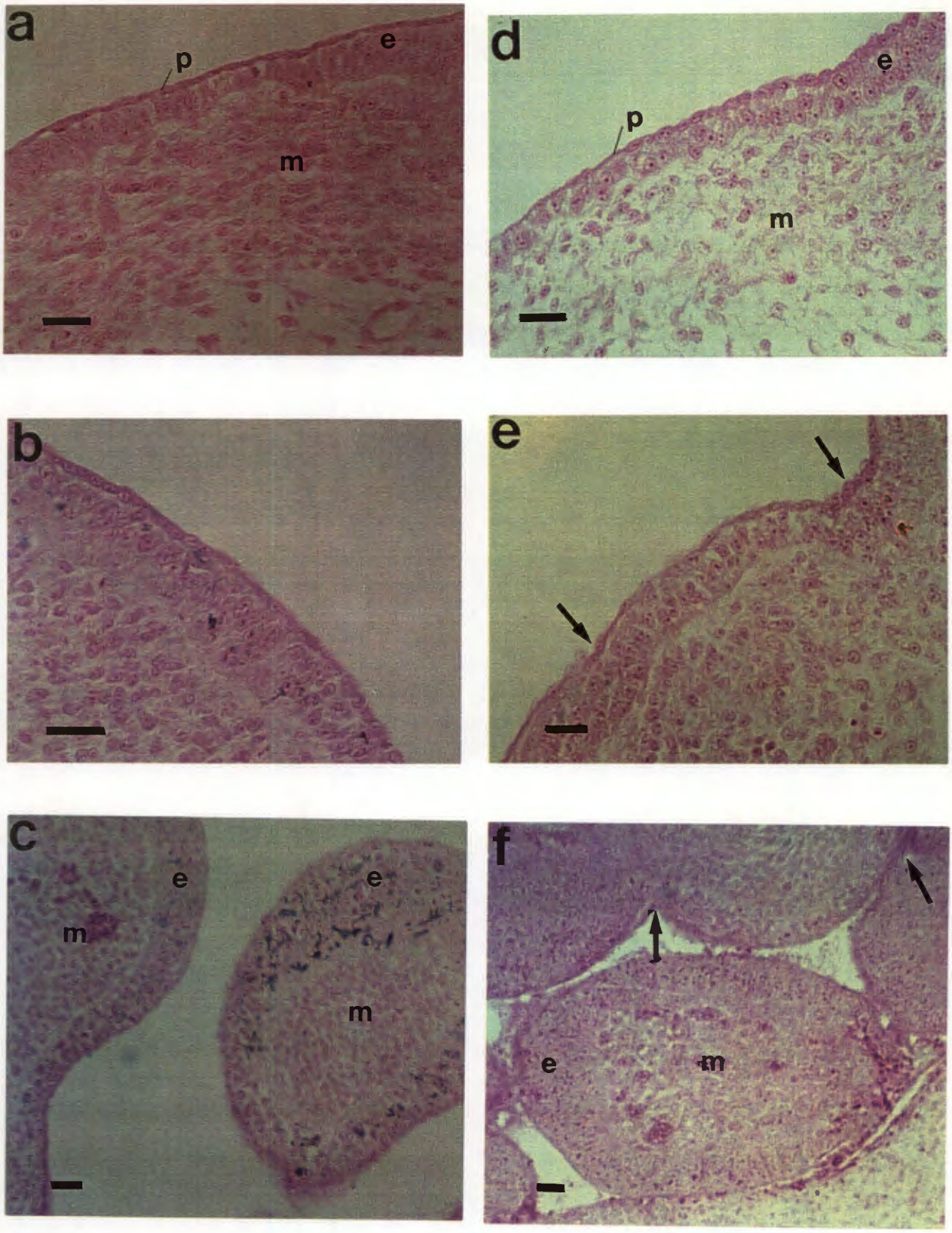

Figure 2. Light micrographs showing the morphology of black (a-c) and white (d-f) embryonic chick skin at 8 (a,d), 9 (b,e) and 10 (c,f) days of development. Notice the squamous peridermal cells and underlying columnar cells in all the sections; pigment in black skin only, and feather buds in cross-section (c and f). p = periderm, e = epidermis, m = mesenchyme. Arrows delineate outer margin of feather bud. Bar=20μm.

melanocytes (Fig. 3a). The presence of pigment therefore distinguished melanocytes from other epidermal cells. In 8, 9 and 10 day black chick skin, melanocytes were typically located in the basal layer of the epidermis. Some melanocytes occurred just below the periderm but, in all the sections examined, none were observed in the periderm itself or in the mesenchyme. Figure 2a-2c illustrates that in black chicks, the amount of pigment increases as development proceeds.

White chick skin on the other hand, did not contain any dark cytoplasmic granules in any of the ages examined (Fig. 2d to 2f). Melanocytes could therefore not be distinguished from other epidermal cells simply on the basis of melanin granules (Fig. 3b). The mesenchyme too, was devoid of melanin pigment granules. Hence, if melanocytes were present in white chick skin they were definitely not synthesising melanin.

3.1.2 DOPA OXIDASE ACTIVITY IN WHITE CHICK SKIN

Although melanin synthesising cells were not detected in white chick skin with routine histology, inactive melanocytes may be present. To determine whether inactive but tyrosinase-containing melanocytes were present in white chick skin, tyrosinase-specific histochemistry (the DOPA reaction) was used at the light microscope level.

Dorsal skins from 8-13 day old white chick embryos were paraformaldehyde-fixed, incubated with DOPA and then prepared for histological examination. The experiment was repeated four times. In each experiment, two embryos per developmental stage were used. Skin samples from 8-10 day old white chick embryos consistently showed a clearly positive reaction after incubation with DOPA. In these tissues, sites of DOPA oxidase (tyrosinase) activity was made visible by brown, DOPA-melanin deposits (Fig. 4a to 4c). DOPA-reactive cells were mainly localised on the basement membrane of the epidermis. However, a few DOPA-positive cells also occurred in the superficial layer of the epidermis, the periderm. The granular DOPA-positive deposits had a perinuclear arrangement

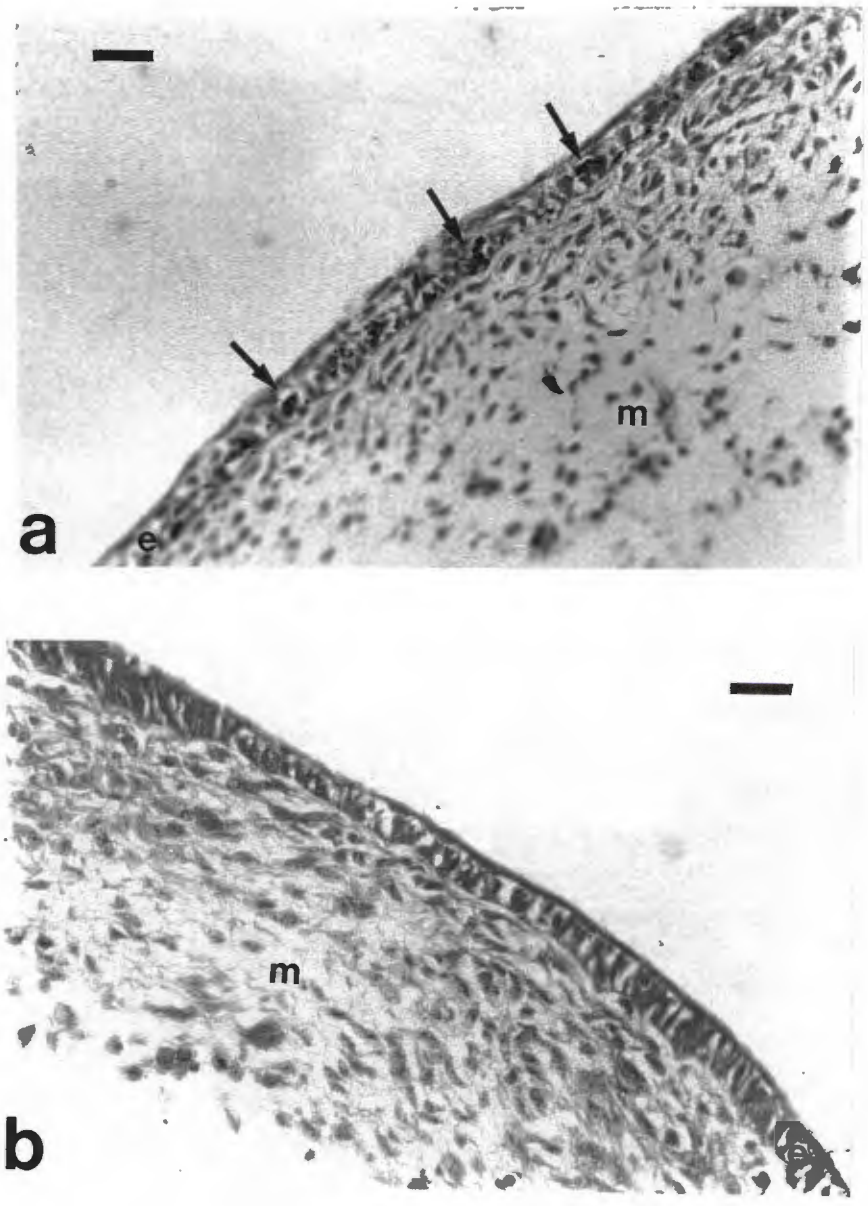

Figure 3. Light micrographs of dorsal embryonic skin from black (a) and white (b) chicks showing absence of pigment in white skin. e = epidermis, m = mesenchyme, arrow = individual melanocyte. Bar=20μm.

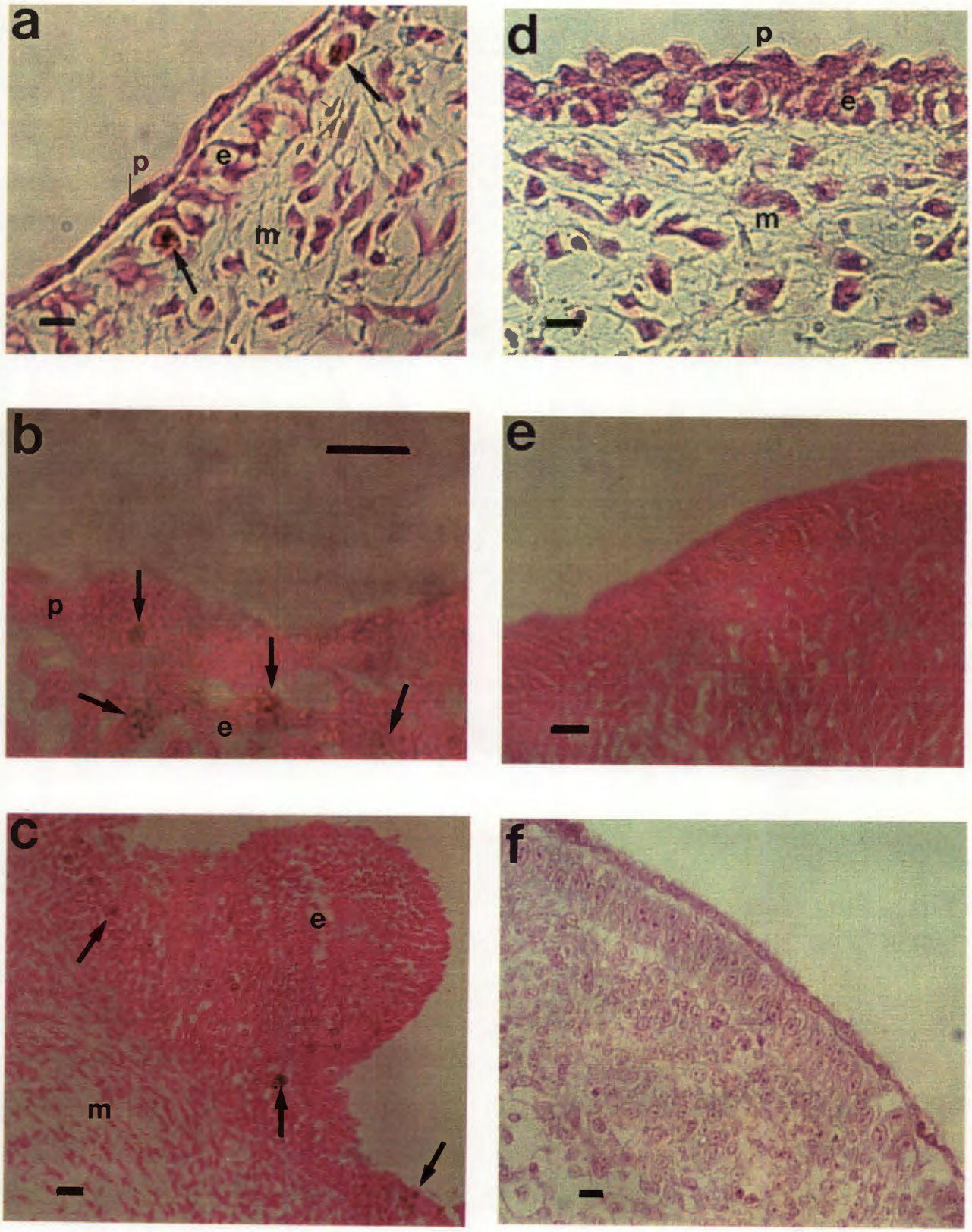

Figure 4. Light micrographs of 8 (a,d), 9 (b,e) and 10 day (c,f) embryonic white chick skin incubated with DOPA (a-c) or without DOPA (d-f).
p = periderm, e = epidermis, m = mesenchyme, arrow = DOPA-positive cell. Bar=10μm.

and were frequently associated with dendritic epidermal cells (arrows in Fig. 4b and 4c).

Examinations of 8-10 day white skin tissue sections suggested that the frequency of DOPA-positive cells increased with age (see Figure 5 a-c). Although not quantified, examination of sections in representative microscopic fields showed that 8 day white skin sections contained the least number of DOPA-positive cells. These cells also had the weakest DOPA-positive reaction compared with cells in 9 and 10 day white skin sections.

Control skin samples that were incubated in buffer alone did not contain any such cytoplasmic granules (Fig. 4d to 4f) indicating that the positive reaction described above was a consequence of the DOPA incubation alone. These results demonstrate that tyrosinase-positive melanocytes are present in the epidermis of 8-10 day old white embryonic chick skin.

DOPA treated skin from 11- to 13-day old white embryos was completely devoid of DOPA-melanin deposits (Fig. 6a to 6c). This indicated that tyrosinase activity was no longer detectable with this particular DOPA-oxidase histochemical technique. The morphology of skin older than 10 days is shown in Figure 6. Feather buds are now elongated, tapering cylinders and the epidermis has thickened to form barb ridges in the feather buds.

To ascertain why tyrosinase activity was not detected in 11-13 day white chick skin, a sensitive radiometric assay for tyrosinase was performed using both black and white skin samples.

3.2 TYROSINASE ACTIVITY IN EMBRYONIC CHICK SKIN

The tyrosinase activity of tissue homogenates from 9 and 13 day black and white chick embryos was determined using the [^{14}C]tyrosine assay of Hearing and Ekel (1976). This assay uses the incorporation of radiolabelled tyrosine into melanin as an indicator of tyrosinase activity. Tissue homogenate samples of equal protein concentrations were freshly prepared from chick skin

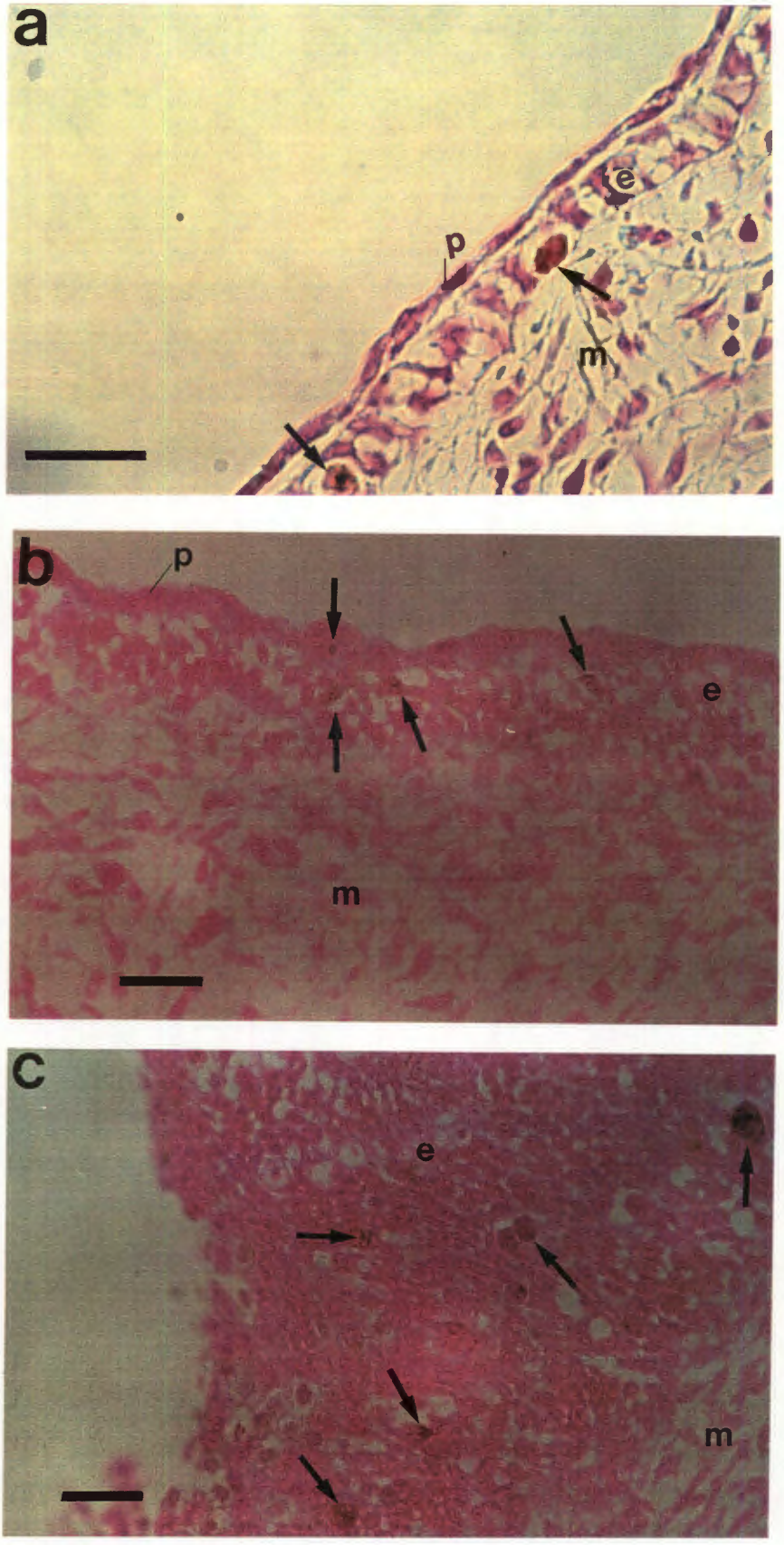

Figure 5. Light micrographs of 8 (a), 9 (b) and 10 day (c) DOPA-reacted embryonic white chick skin showing the numbers of DOPA-positive cells and the intensity of the DOPA reaction.
p = periderm, e = epidermis, m = mesenchyme, arrows = DOPA-positive cells. Bar=20μm.

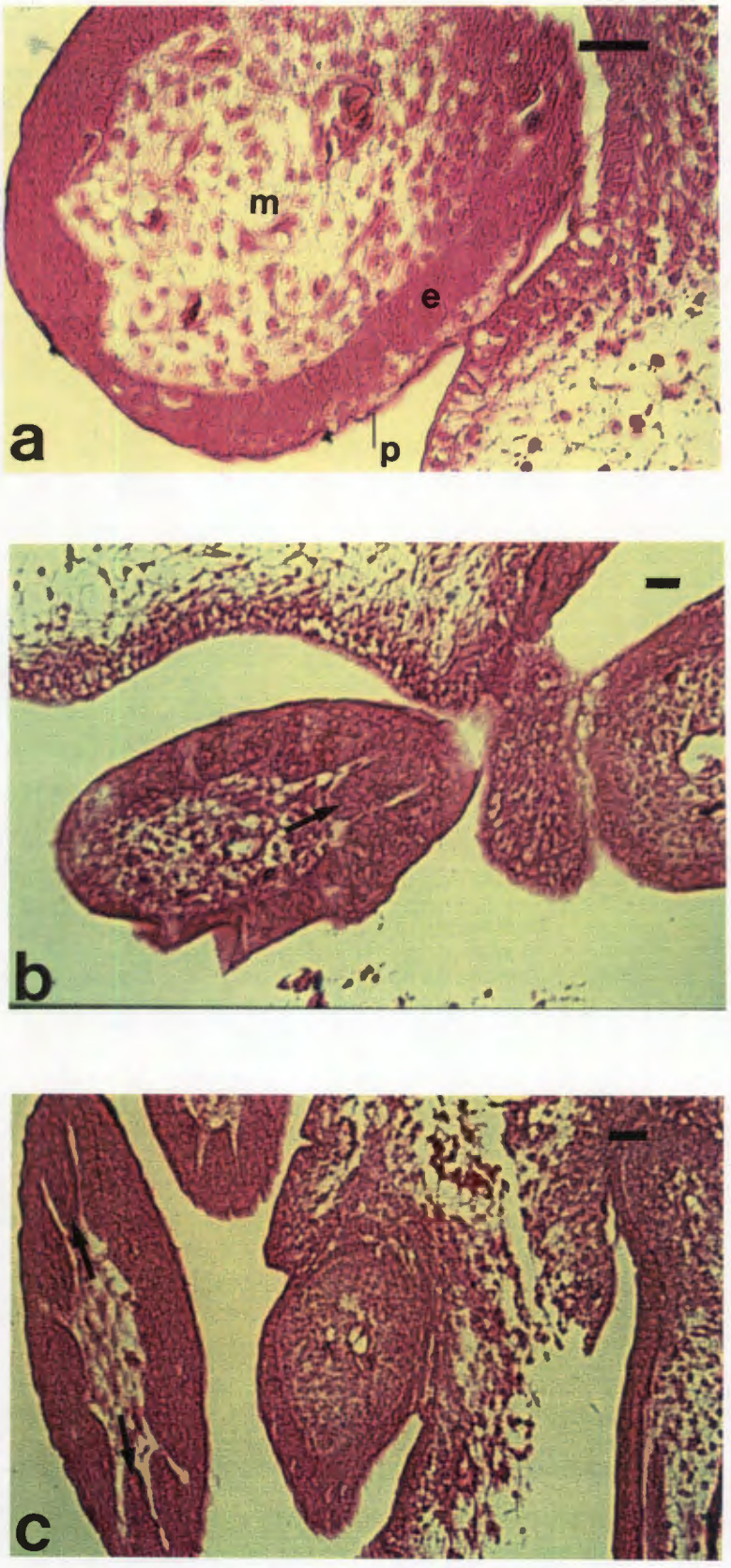

Figure 6. Light micrographs of sections of 11 (a), 12 (b) and 13 day (c) DOPA-reacted dorsal skin from white chick embryos showing skin morphology and the absence of DOPA-positive cells.
p = periderm, e = epidermis, m = mesenchyme, arrows = barb ridges.
Bar=20μm.

and internal abdominal organs. Organ homogenates served as a melanocyte-free control to determine baseline levels of [^{14}C]tyrosine incorporation. Method controls (blanks) were carried out (see Table 1a for readings).
Each sample was assayed in triplicate and the assay was repeated on three separate occasions. The resulting counts per minute per 10 microgram (cpm/10μg) of homogenate were converted to a percentage of the maximum count (cpm/10μg of 13 day black chick skin homogenates) to facilitate comparison between assays. These percentages were used to construct the bar-graph in Figure 7 (see Table 1a for original counts).

It is clear from the graph in Figure 7 that tyrosinase activity increased dramatically in black skin between 9 and 13 days. At 9 days, black skin tyrosinase activity averaged 27.91% of activity in 13 d black skin. This means that tyrosinase activity increased 3.59 times between 9 and 13 days of development. Black skin tyrosinase activity was always clearly detectable above control organ levels (8-9.9% of maximum) (see Table 1b; Fig. 7).

In contrast to the histochemical results (see 3.1.2 above), tyrosinase activity was detected in both nine and 13 day white chick skin samples. Tyrosinase activity in white chick skin was also detectable above organ control levels but by a much smaller margin than was the case with black skin (see Table 1b; Fig. 7). At 9 days, white skin tyrosinase activity averaged 13.92% of maximum (13 day black skin) compared with organ activity levels at 9.92%. At 13 days, tyrosinase activity averaged only 11.4% of maximum and organ activity was 8.17% (Fig. 7).

When analysing these results it is important to bear in mind that tyrosinase activity remains constant (baseline levels) in melanocyte-free organ tissues, regardless of embryonic age and that the results are presented proportional to the maximum enzyme activity levels (13 day black skin). Therefore, to examine the change in tyrosinase activity over time, it is more accurate to obtain the ratio between tyrosinase activity levels of skin and

Tyrosinase Activity in Chick Tissue as Percentage of Maximum

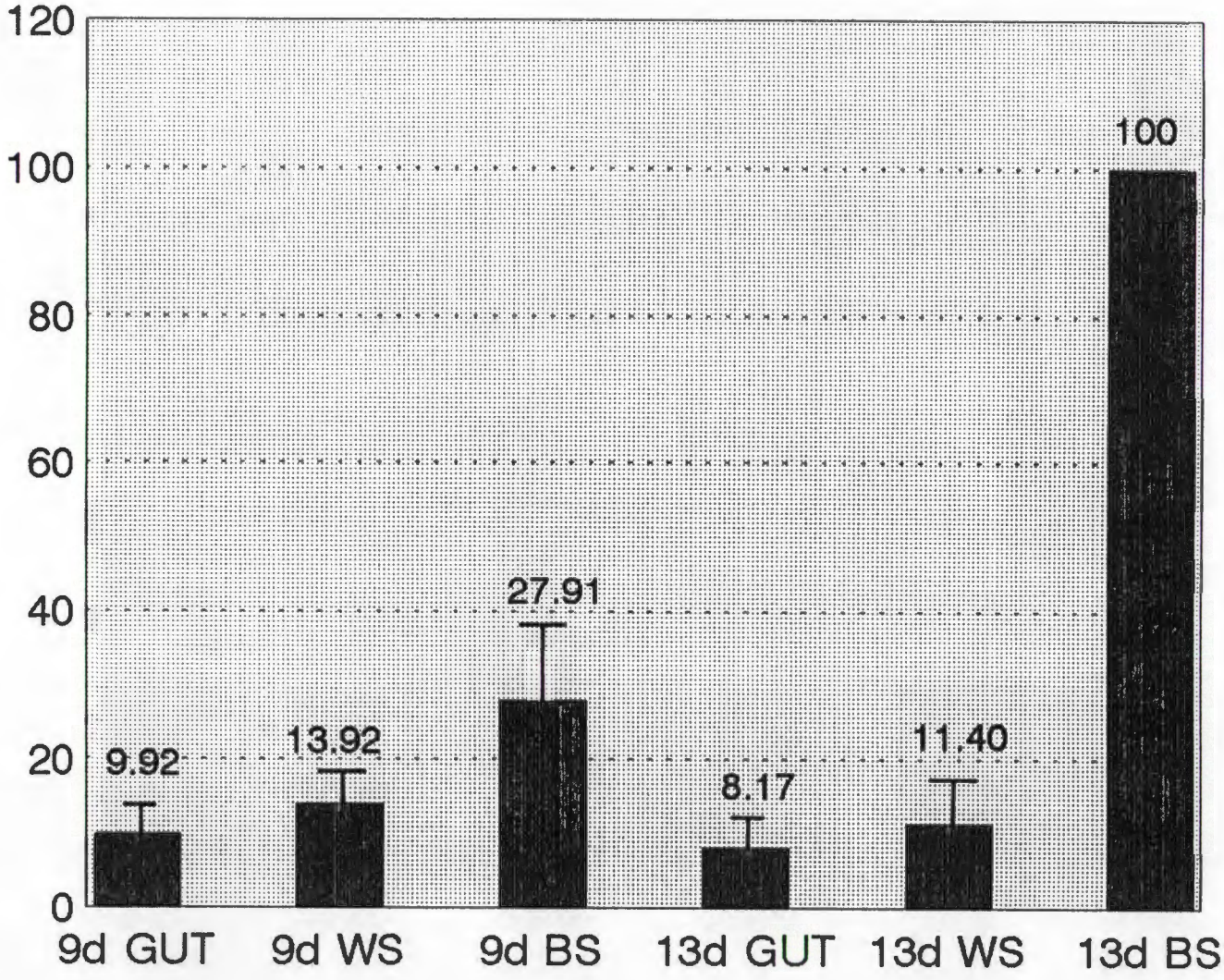

Fig. 7. Radiometric assay of tyrosinase activity in 9 day and 13 day BA X NHR and WPR X PG embryonic chick skin and organ homogenates. Counts (cpm/10μg homogenate) have been converted to percentage of maximum (counts for 13 day B skin). Means given ± SEM, n=3 runs. See Table 1a for original counts.

Table 1a: Tyrosinase activity in cpm/10 μg homogenate

		9 day chick embryo			13 day chick embryo		
Expt No.	BLANK	GUT	W Skin	B Skin	GUT	W Skin	B Skin
1	985.86 ± 124.34	2380 ±283.90	2859.86 ±444.26	7415.27 ±182.60	1381.40 ±301.90	1480.10 ±147.70	19460.80 ±749.40
2	632 ± 5.48	2029.93 ±368.30	3556.23 ±502.70	4835.00 ±718.50	2352.36 ±95.60	3037.60 ±225.00	23231.10 ±275.40
3	1415.03 ± 113.72	1955.60 ±467.30	2609.56 ±306.50	5508.10 ±615.40	1623.36 ±80.00	2998.30 ±310.60	22187.90 ±601.00

Radiometric assay of tyrosinase activity in skin and organ homogenates prepared from 9 day and 13 day BA x NHR and WPR x PG chick embryos. Values for gut and skin samples have been corrected by subtracting the 'blank' counts.
Tyrosinase activity is represented as counts per minute per 10μg of tissue homogenate. Means are given ± SEM, n=3 assay repeats.

Table 1b: Detection of Tyrosinase Activity in Chick Embryos

Tyrosinase activity given as % of maximum						
	9 day chick embryo			13 day chick embryo		
Expt No.	GUT	W Skin	B Skin	GUT	W Skin	B Skin
1	12.22	14.70	38.10	7.09	7.61	100
2	8.74	15.31	20.81	10.12	13.07	100
3	8.81	11.76	24.82	7.31	13.51	100
Ave. ± SEM	9.92 ±1.62	13.92 ±1.54	27.91 ±7.38	8.17 ±1.37	11.40 ±2.68	100

Tyrosinase activity in skin and organ homogenates prepared from 9 day and 13 day BA x NHR and WPR x PG chick embryos. Homogenates were adjusted to equal protein concentrations and the tyrosinase activity was assayed by measuring the incorporation of [^{14}C] tyrosine into insoluble melanin. Tyrosinase activity for each sample has been calculated as a percentage of the maximum count (i.e. 13 day black chick skin) (see Table 1a for original counts). The difference in tyrosinase activity of 9 day white skin and 9 day black skin is not statistically significant, whereas tyrosinase activity is statistically significant in 13 day black and 13 day white chick skin (significance level 0.05).

organs of a particular age group and then to compare this value to the obtained value of another age group. In black chick embryos, this ratio is 2.81 in 9 day samples and 12.2 in 13 day samples. This reflects an increase in black skin tyrosinase activity. In white chick embryos, this ratio is 1.4 in both 9 day and 13 day samples. This implies that tyrosinase activity remained constant in white chick embryos instead of increasing with developmental age.

Another trend that emerged from these quantitative experiments concerned total levels of tyrosinase activity in white chick embryos. Figure 7 clearly shows that white embryonic skin, regardless of developmental stage, had less tyrosinase activity than black embryos. The assay results (Table 1b; Fig. 7) show that 9 day black skin had roughly twice the amount of tyrosinase activity than 9 day white skin. This difference in activity levels further increased to an 8.5 fold difference between 13 day black and white embryos.

3.3 *ULTRASTRUCTURE OF EPIDERMAL MELANOCYTES*

The above results show that there are tyrosinase-active melanocytes in the epidermis of white chick skin. However, these melanocytes never synthesise visible melanin *in vivo*. The ultrastructure of white chick skin was therefore compared with black chick skin to determine if the melanocyte and/or its epidermal environment were structurally abnormal.

Dorsal skin from 9 and 10 day black and white chick embryos were processed for electron microscopy studies. The ultrastructure of nine and 10 day embryonic skin were very similar, consisting of a superficial periderm, a stratified epidermal portion and an underlying mesenchyme (dermis) (Fig. 8a and 8b). The epidermis rests on a basement membrane and is composed of not more than three layers of columnar cells (keratinocytes) with many intercellular spaces. The periderm consists of a layer of closely apposed, squamous cells. The apical membrane of these peridermal

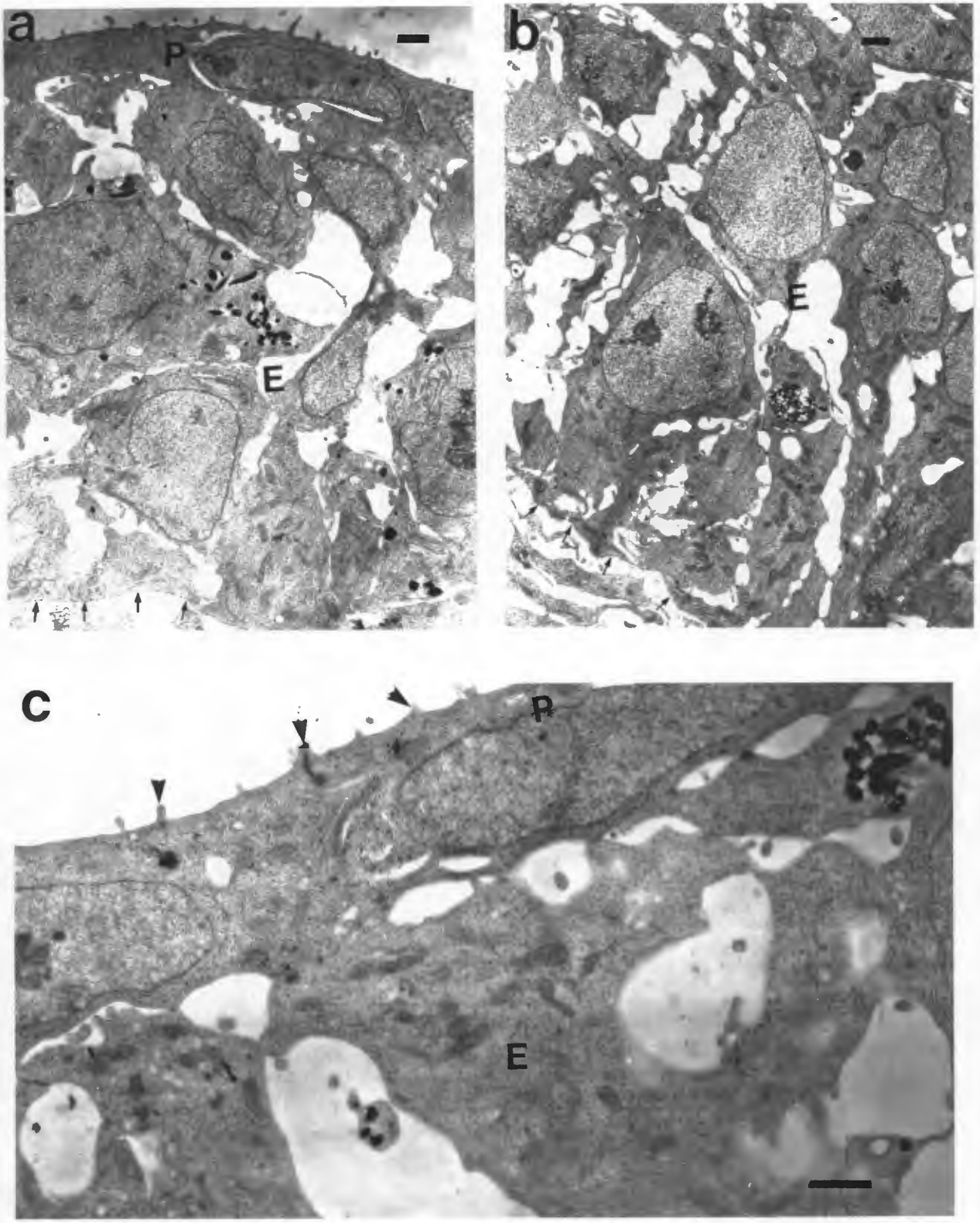

Figure 8. Electron micrographs of 10 day embryonic skin sections from black (a) and white (b) chicks. Notice melanin granules in black skin only. (c) Enlargement of periderm showing microvillous projections (arrowheads).
P = periderm, E = epidermis, arrows = basement membrane. Bar=1μm.

cells frequently display microvillous projections and the cytoplasm is filled with ribosomes and mitochondria (Fig. 8c).

3.3.1 MELANOCYTES IN EPIDERMIS OF BLACK CHICK EMBRYOS

Dorsal skins from 10 day old (n=3) and 9 day old (n=4) black chick embryos were examined ultrastructurally to obtain information about the morphology of normal chick melanocytes and their embryonic environment. At these developmental stages (9 and 10 days), transfer of melanosomes from melanocytes into keratinocytes has not yet begun (Jimbow *et al*, 1974). Therefore all melanosome-containing cells were considered to be melanocytes and the remainder of the epidermal cells were considered to be keratinocytes. Thus, in both semi-thin and ultra-thin sections of skin, melanocytes were easily identified by their dark, cytoplasmic melanin granules (Fig. 8a). Figure 9 illustrates a typical melanocyte with a melanosome-carrying dendrite found in 10 day black chick epidermis. Besides the predominating melanosomes, melanocytes also contained organelles characteristic of protein synthesising cells. An extensive endoplasmic reticulum, numerous ribosomes, a prominent Golgi complex and numerous mitochondria were always present in melanocytes.

In all the skin sections examined, melanocytes were located in the epidermis and were not observed in the dermis. Epidermal melanocytes were often observed close to the basement membrane (Fig. 10a) but melanocytes and their cytoplasmic extensions also occurred in the upper epidermal cell layer, just below the periderm (Fig. 10b). Very infrequently, a melanosome-containing cell was located within the periderm. Figure 11 clearly shows a cluster of melanin granules in the cytoplasm of a typical peridermal cell. This tight cluster resembles packages of transferred melanosomes rather than freely synthesised melanosomes. These peridermal cells were therefore not considered to be melanocytes. This finding contradicts our initial assumption on melanosomal transfer (see above) and will be dealt with in the discussion.

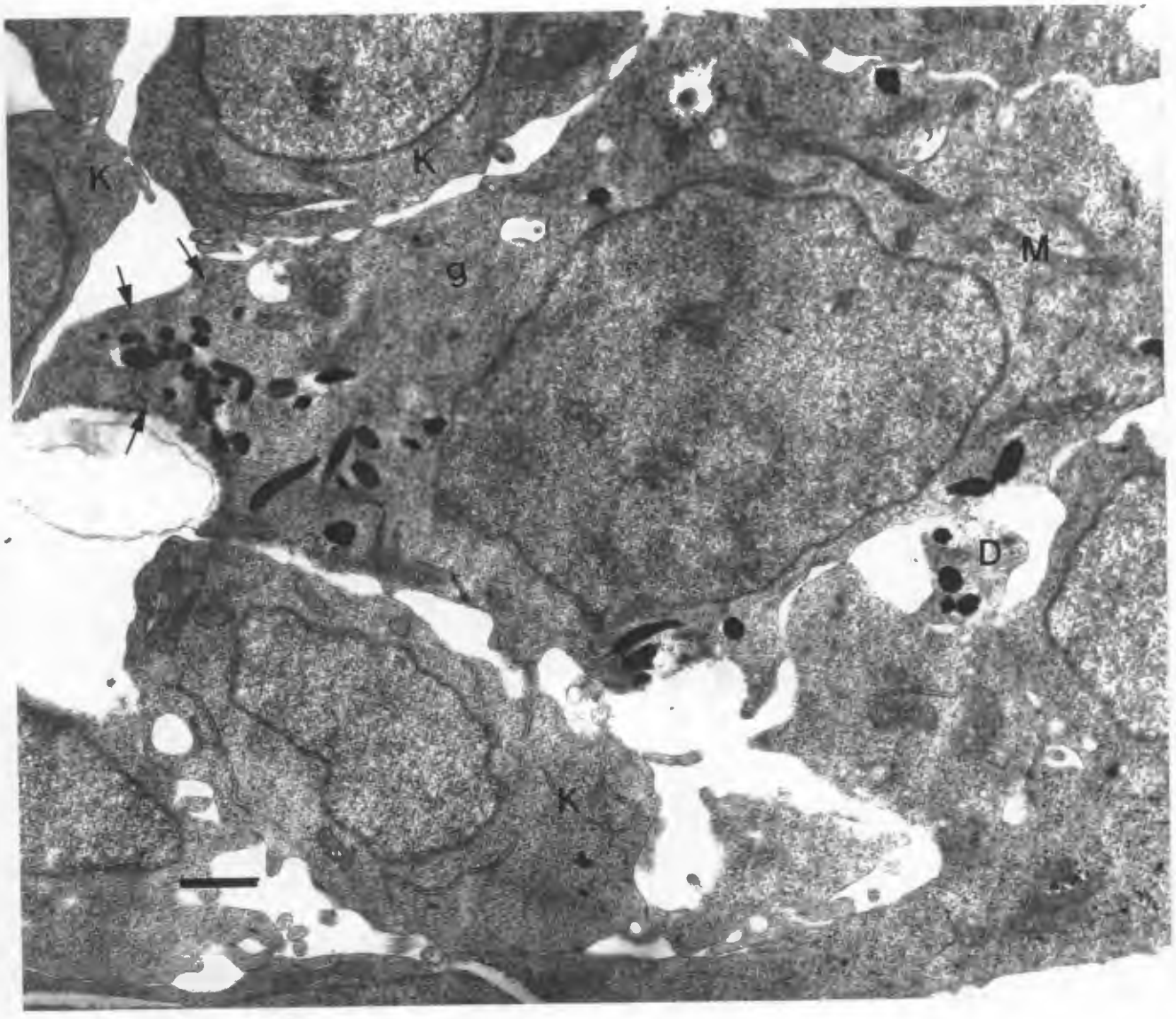

Figure 9. Electron micrograph of a section of 10 day embryonic black chick skin showing an epidermal melanocyte (M) with its dendrite (D) among keratinocytes (K). Melanocytes have distinctive electron-dense melanosomes, well-developed Golgi (g) and endoplasmic reticulum (arrows). Bar=1μm.

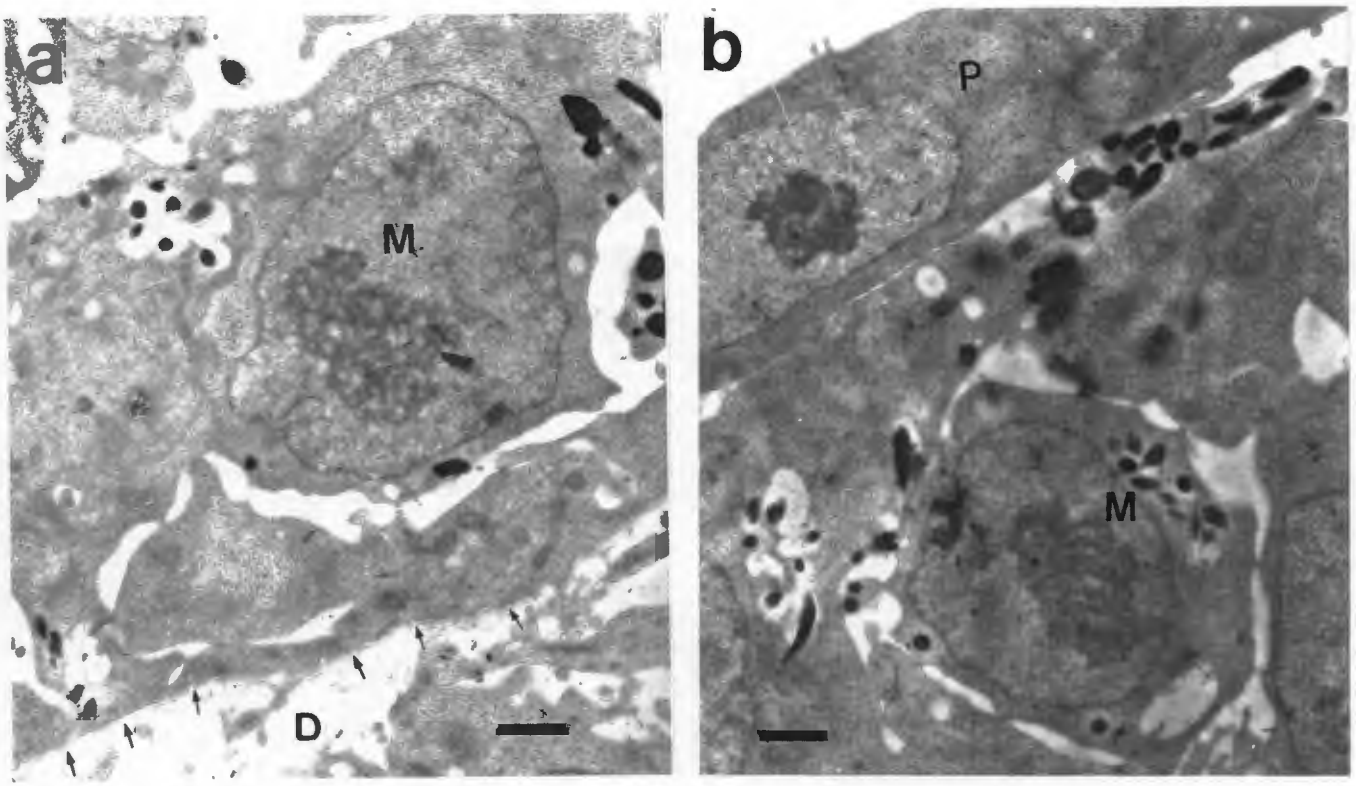

Figure 10. Electron micrographs of 10 day embryonic black chick skin showing the localisation of melanocytes (M) in the epidermis: (a) above the basement membrane (arrowheads), and (b) below the periderm (P). D = dermis (mesenchyme). Bar=1μm.

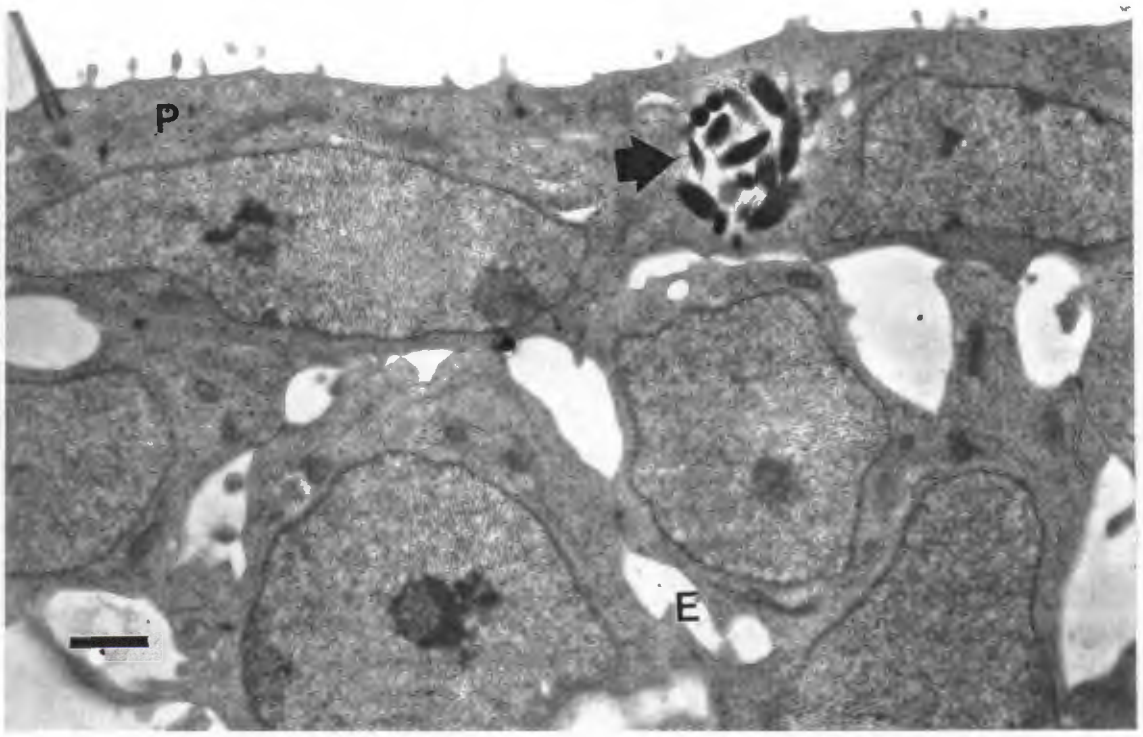

Figure 11. Electron micrograph of 10 day embryonic black chick skin showing a melanosome cluster (arrow) in a peridermal cell (P). E = epidermis. Bar=1μm.

The features of the melanin granules in black skin were characteristic of eumelanin-producing cells. These melanosomes were membrane bound, rod shaped organelles with a regular inner matrix (Fig. 12). This lattice-like matrix, best seen in immature melanosomes (premelanosomes), consisted of fibres arranged parallel to the long axis of the melanosome. These longitudinal strands were not straight but folded into a series of zig-zags and in cross-section, the fibres appeared as concentric lamellae (Fig. 12, inset). In fully pigmented organelles, the lamellar matrix is completely obscured by electron dense melanin deposits (Fig. 12). Sometimes an electron lucent outline could be seen framing such electron dense melanosomes (arrow in inset of Fig. 12). Although spherical melanocytes were also noticed among the rod shaped eumelanosomes (Fig. 12), heavy melanisation obscured all matrix detail of these melanosomes and they could not be positively identified as phaeomelanosomes. No immature phaeomelanosomes were however, observed in 9 or 10 day black melanocytes.

3.3.2 MELANOCYTES IN EPIDERMIS OF WHITE CHICK EMBRYOS

Dorsal skin samples from 9 day (n=4) and 10 day (n=4) white embryos were examined ultrastructurally for structural abnormalities to investigate why the melanocytes do not contribute towards pigment in the feathers.

The general morphology of white skin was very similar to that of black skin. The differences lay in the visibility of melanocytes in the epidermis. Dark clusters of melanosomes were not visible in semi-thin nor ultra-thin resin sections of white skin (Fig. 13). Melanocytes were therefore not readily distinguishable from keratinocytes at low microscopic magnifications. To identify melanocytes, white chick skin sections were scrutinised at higher magnifications (more than 15 000 times) to locate melanosomal or premelanosomal structures. Melanosomal structures were observed in both 9 and 10 day white epidermis. These oval membrane-bound organelles had a regular, inner lamellar matrix characteristic of immature eumelanosomes (Fig. 14). All of the observed melanosomes were incompletely melanised and the complete lamellar matrix was

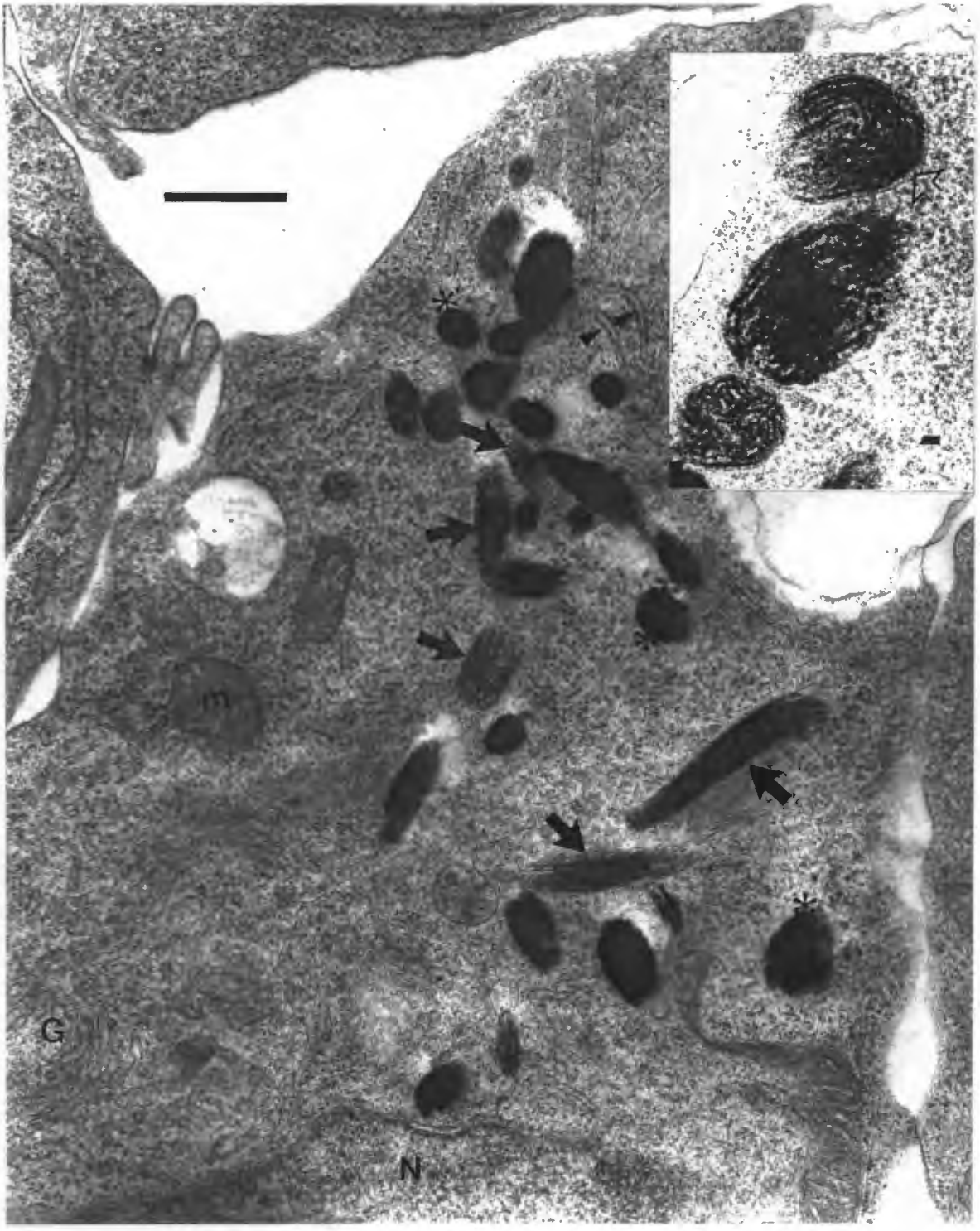

Figure 12. Electron micrograph of 10 day embryonic black chick skin showing typical, ellipsoidal eumelanosomes in a melanocyte. Notice the few spherical melanosomes (asterisk) and the lamellar matrix in premelanosomes (arrows).
G = Golgi, N = nucleus, m = mitochondrion, arrowheads = RER. Inset: cross-section of eumelanosome showing the concentric rolls of lamellae and the electron lucent rim around the melanosome (open arrow). Bar=0.5μm.

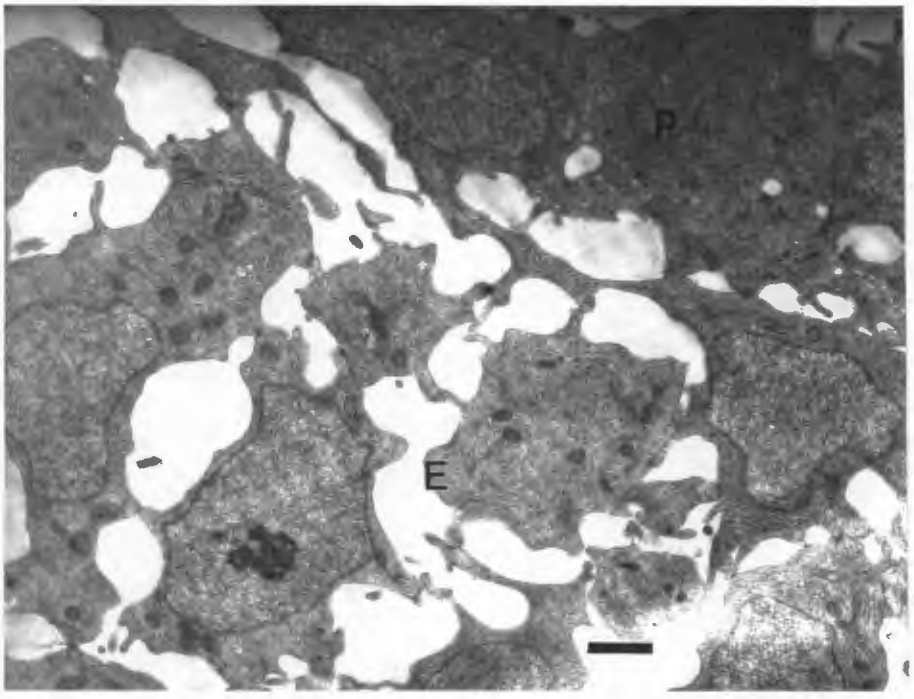

Figure 13. Electron micrograph of 10 day embryonic white chick skin showing general morphology and absence of mature melanosomes. Notice the large extracellular spaces.
P = periderm, E = epidermis. Bar=1μm.

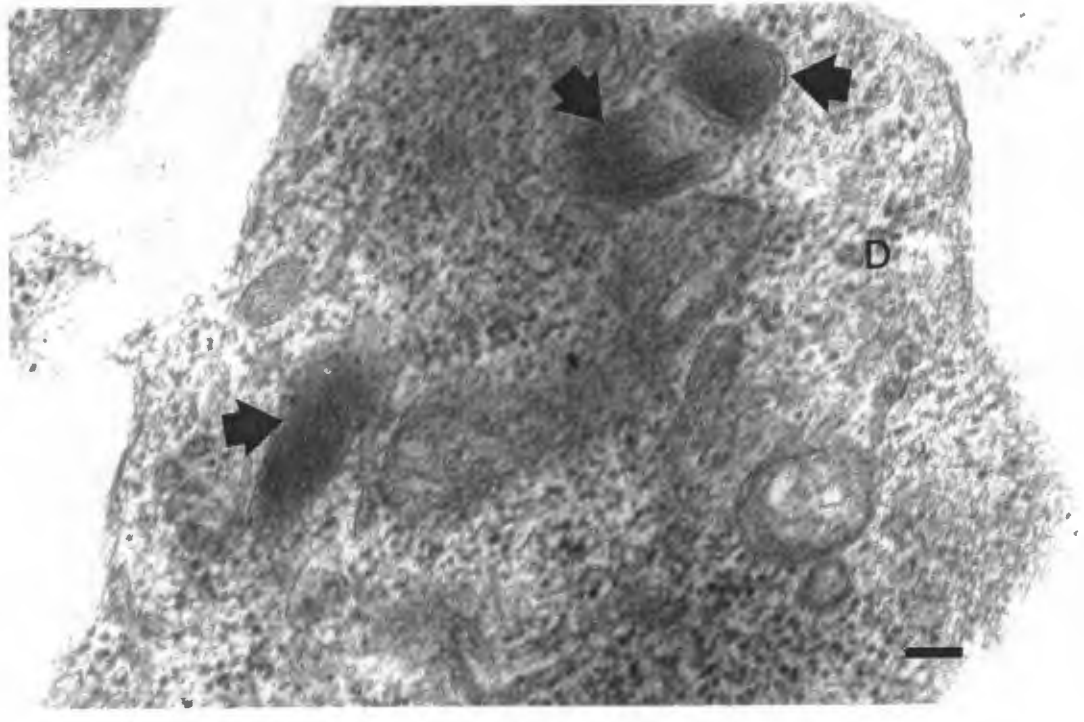

Figure 14. Electron micrograph showing premelanosomal structures (arrows) inside a melanocyte dendritic arm (D) in the epidermis of 10 day embryonic white chick skin. Bar=0.1μm.

visible. Figure 15 shows no evidence of structural abnormalities in white chick premelanosomes - the internal structural pattern was identical to that of black chick premelanosomes.

Both nine and 10 day white skin melanocytes contained only a small number of premelanosomes relative to other organelles. Despite the paucity of specific organelles, white melanocytes contained all the other organelles typical of black melanocytes namely, a prominent Golgi apparatus, endoplasmic reticulum, ribosomes and mitochondria. None of these organelles presented an abnormal morphology - for example, distended endoplasmic reticulum, exploded mitochondria or discontinuous membranes (see Fig. 16).

Melanosomes were never observed in either peridermal or dermal cells. Instead, premelanosomes occurred freely in small clusters in epidermal melanocytes (Fig. 17a) or in melanocyte dendrites (Fig. 17b). However, in a 10 day white chick epidermal section, two normal looking premelanosomes were observed amongst other debris in a large, membrane-bound vacuole (Fig. 18). This vacuole occurred inside a dendritic, cytoplasmic extension. As no other cytoplasmic organelles could be identified from the debris, it is therefore not clear whether this vacuole was a phagosome or not.

3.4 *NEURAL CREST-DERIVED MELANOCYTES IN CULTURE*

The above results indicate that although melanocytes in white chick skin contained active tyrosinase as well as normal premelanosomes, these melanocytes never synthesised pigment *in vivo*. To determine whether hypomelanosis is due to an intrinsic melanocyte defect or the result of a defective environment, white chick neural crest cells were grown in culture to see if they could give rise to viable, pigmented melanocytes. For this purpose, variations of well-established neural crest cell culture methods were tested to determine ideal conditions that would support the growth and survival of chick neural crest cells and their differentiation into pigmented melanocytes.

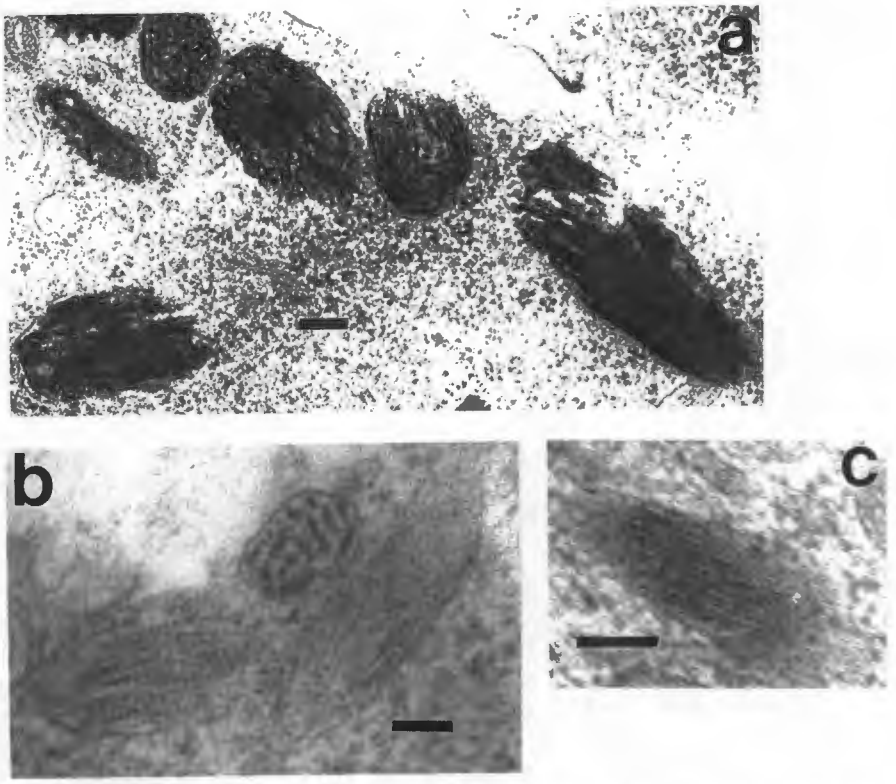

Figure 15. Electron micrographs showing the identical structure of immature eumelanosomes (premelanosomes) from black chick skin (a) and from white chick skin (b,c). Bar=0.1μm.

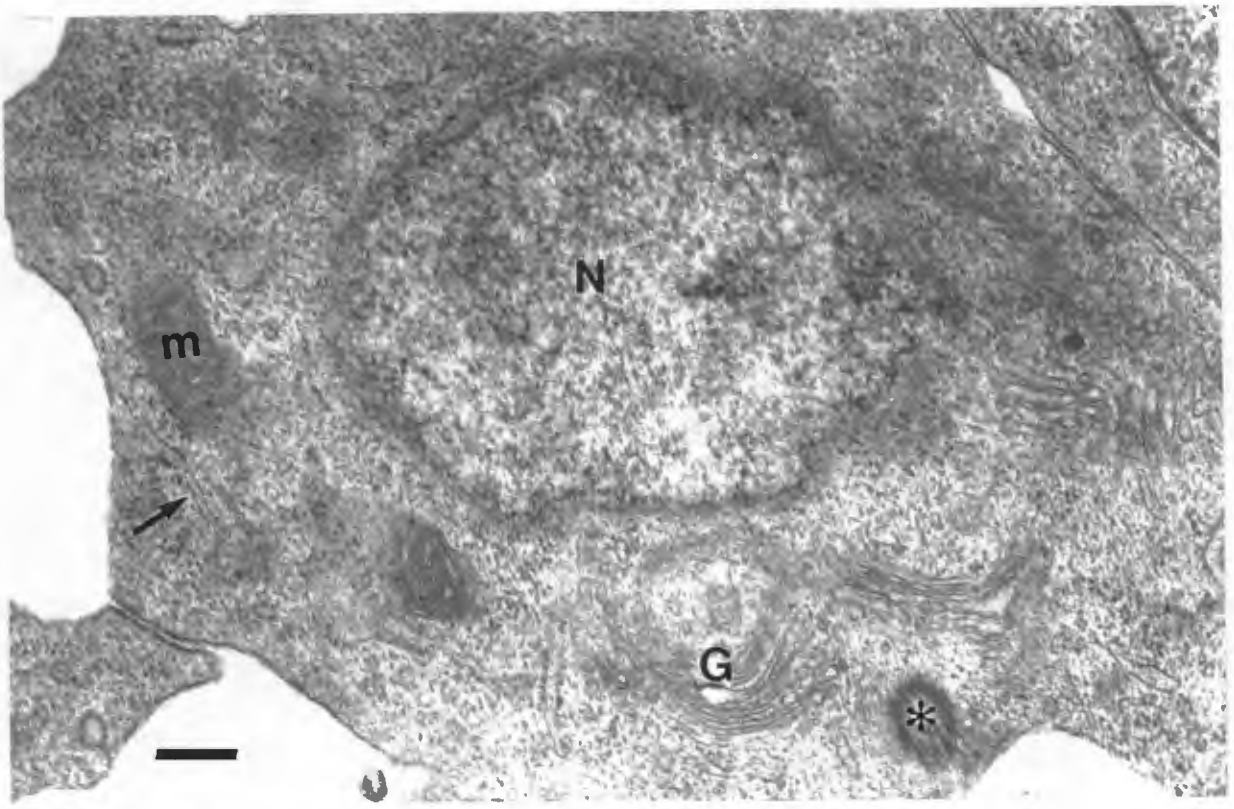

Figure 16. Electron micrograph of a section of 10 day embryonic white chick skin showing an epidermal melanocyte. Notice the absence of melanosomes, also the normal structure of other organelles.
N = nucleus, m = mitochondrion, G = Golgi complex, arrow = ER, asterisk = premelanosome. Bar=0.2μm.

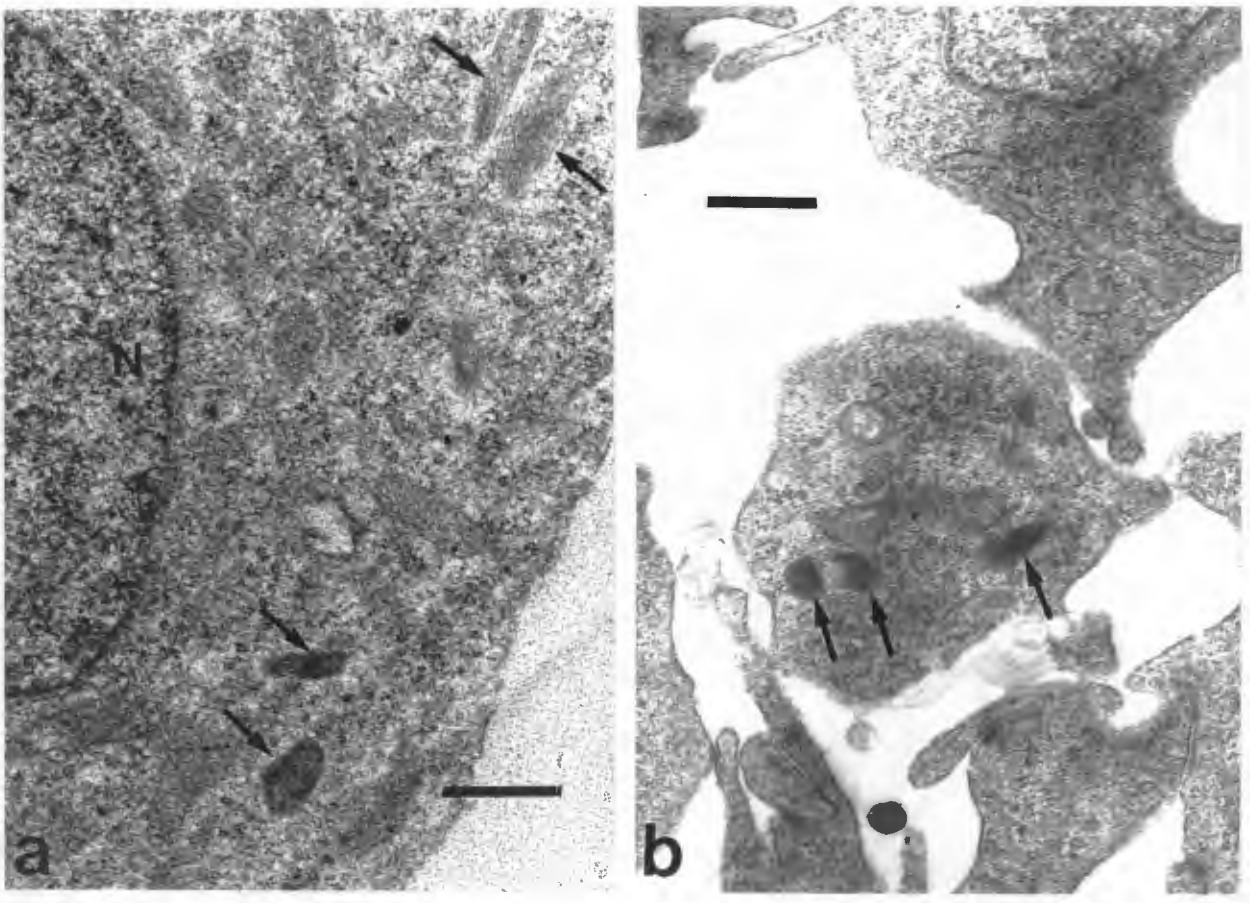

Figure 17. Electron micrographs showing the distribution of premelanosomes in (a) the melanocyte cell body and in (b) the melanocyte dendritic arm.
Arrow = premelanosomes, N = nucleus. Bar=0.5μm.

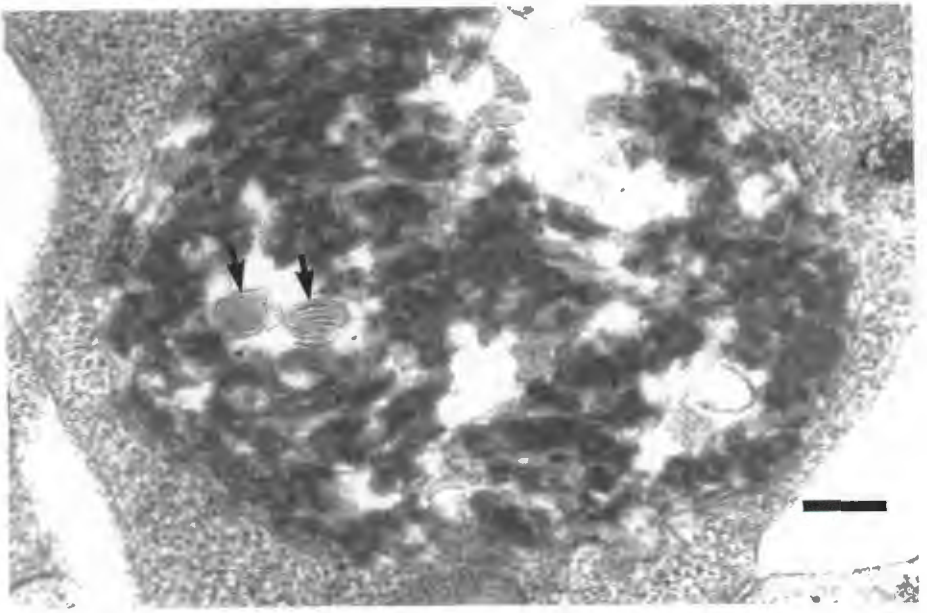

Figure 18. Electron micrograph showing two premelanosomes (arrows) in a large membrane-bound vacuole in a section of 10 day embryonic white chick skin. Bar=0.35μm.

3.4.1 *CULTURE CONDITIONS FOR CHICK MELANOCYTES*

To establish conditions for maintaining long-term chick melanocyte cultures, neural tubes from black chick embryos were explanted into basic medium consisting of Ham's F-12 culture medium supplemented with 20% foetal calf serum (FCS). The following factors were added to the basic medium to examine their effects on melanocyte growth and differentiation: a) 10ng/ml basic fibroblast growth factor (bFGF), b) 100ng/ml α-melanocyte stimulating hormone (α-MSH) and c) 10ng/ml bFGF + 100ng/ml α-MSH. Culture dishes, with at least two neural tube explants, were observed daily with an inverted light microscope. The observations reported here are based on 11 repeat cultures for each culture condition. Each experiment included at least 2, sometimes 3 to 4 neural tubes.

3.4.1.1 *NEURAL CREST CELL MORPHOLOGY AND DISPERSAL*

Under all culture conditions studied, neural crest cells had migrated away from the neural tube and surrounded the explant in a radially symmetric fashion by 24 hr in culture. These small, stellate neural crest cells commonly formed a monolayer on the plastic substrate (Fig. 19a) but sometimes also formed aggregates on or near the neural tube explant (Fig. 19b). After crest cell migration, neural tube explants were left in the culture dishes for the remainder of the *in vitro* studies. This however, resulted in a spread of neural epithelium from the ends of the tube explants onto the dish. In such cases it was difficult to distinguish between neuroepithelial cells and closely surrounding crest cells (Fig. 19b).

Subjective visual assessments of crest cell dispersal was done with the light microscope. Assessments indicated that the area of neural crest cell dispersal from the neural tube, was greater in those cultures fed with bFGF supplemented media than in those with basic medium alone or in cultures fed with α-MSH only.

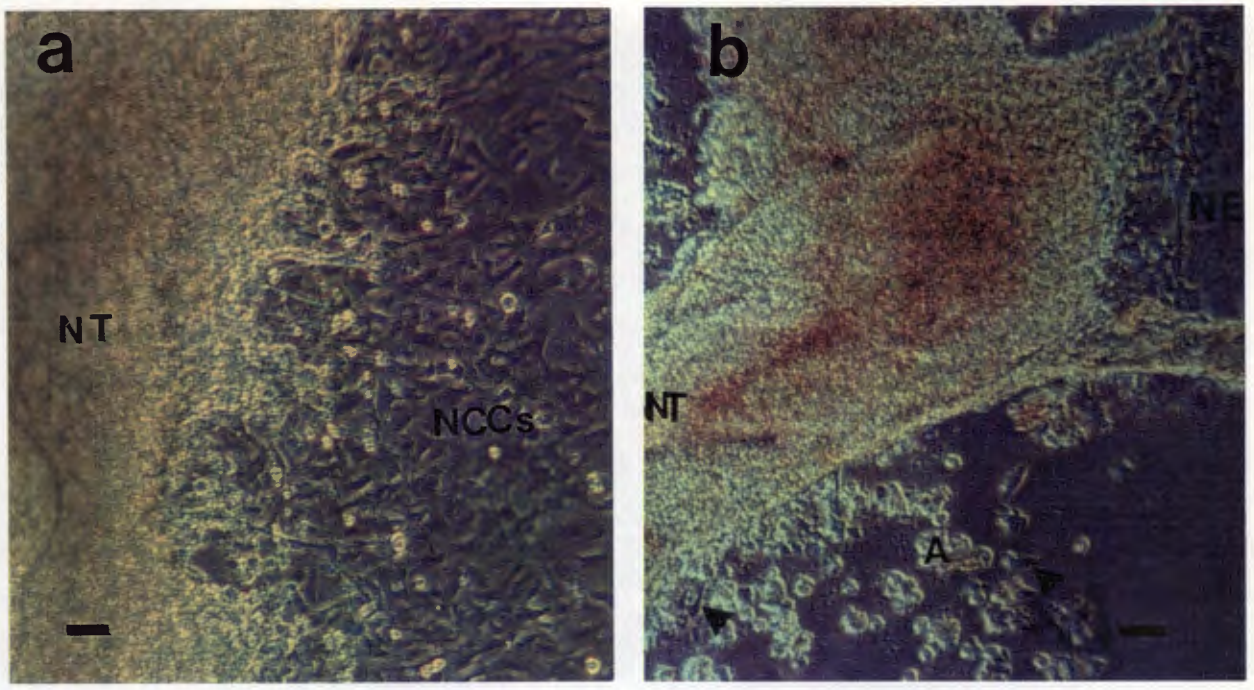

Figure 19. Light micrographs showing the morphology of neural crest cells (NCCs) in culture. (a) Monolayer of stellate NCCs around the neural tube explant (NT). (b) Aggregates of NCCs (A) obscure the more typical monolayer (arrowheads) while the neuroepithelium (NE) has started spreading onto the plastic substrate. Bar=50μm.

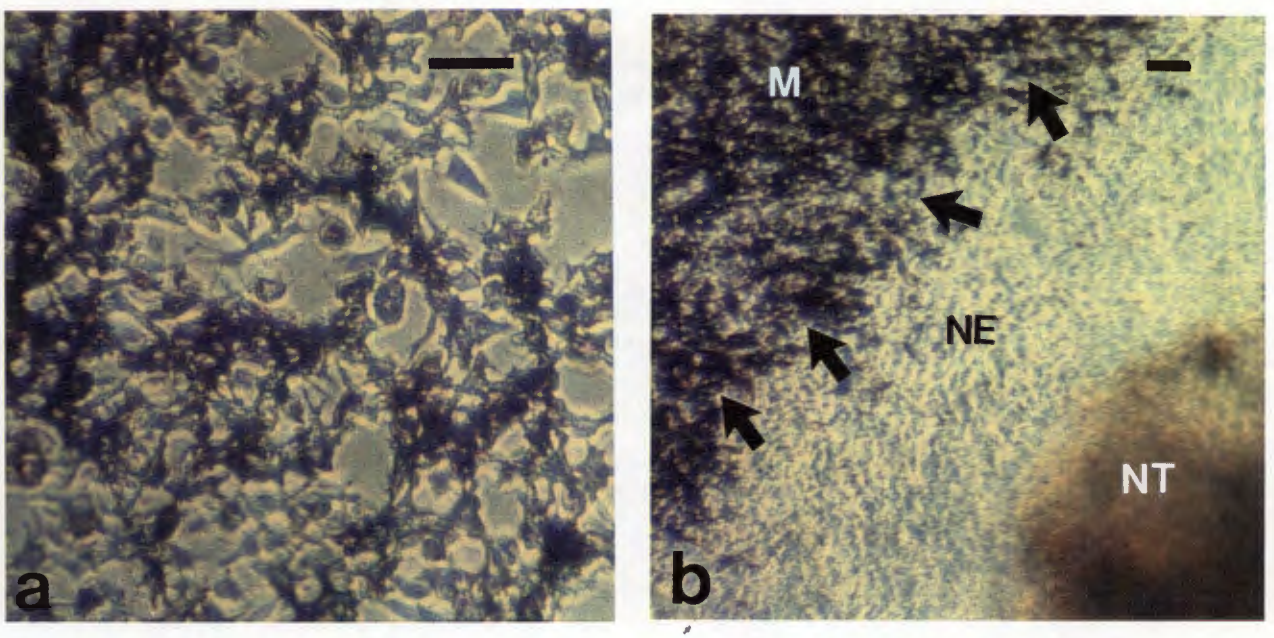

Figure 20. Distribution of cultured black chick NC-derived melanocytes. (a) Phase contrast light micrograph showing pigmented melanocytes growing on neuroepithelial cells (unpigmented cells). (b) Brightfield light micrograph showing dense aggregates of melanocytes at the outer boundary of the neuroepithelial sheet (arrows).
M = areas of melanocytes, NT = neural tube, NE = neuroepithelium. Bar=50μm.

3.4.1.2 MORPHOLOGY AND DISTRIBUTION OF BLACK CHICK MELANOCYTES IN CULTURE

Neural crest cell cultures obtained from black chick embryos were observed daily with an inverted light microscope for the appearance of melanin. With brightfield microscopy, the presence of brown pigment granules clearly distinguished melanocytes from other contaminating cell types visible under phase contrast. Melanocytes were not evenly distributed in culture but occurred in discrete clusters either on the neural tube or associated with the sheet of neural epithelial cells (Fig. 20a). Clusters of melanocytes were most dense at the interface formed between the epithelial sheet and the neural crest cells (Fig. 20b) and seldom extended beyond this interface. The morphology of cultured melanocytes frequently varied with culture conditions but also within the same dish. The most common form of melanocytes in this study was the dendritic type (Fig. 21a), though polygonal and bipolar melanocytes were also observed (Fig. 21b and 21c).

3.4.1.3 EFFECTS OF CULTURE CONDITIONS ON THE ONSET AND EXTENT OF MELANOGENESIS

Black chick neural crest cell cultures were examined daily with a brightfield inverted microscope for traces of visible pigment. The onset of melanogenesis was then determined as the time when melanin granules were first visible microscopically. The proportion of melanised cells relative to unmelanised cells were also visually estimated for each set of culture conditions. As expected, the proportion of melanocytes declined in older cultures. This decline is due to the active proliferation of other unpigmented cell types during the culture period. Melanocyte proportions are therefore represented here as the maximum percentage of melanised cells obtained from a neural tube explant.

Although melanogenesis occurred under all the culture conditions studied, the onset and extent of melanogenesis differed as follows (see Table 2).

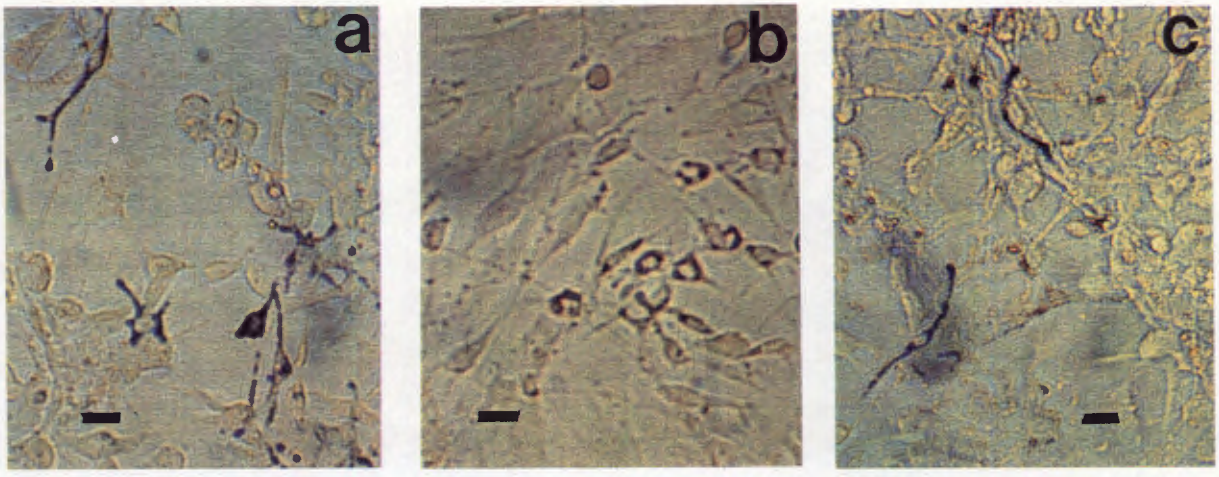

Figure 21. Brightfield light micrographs showing the morphology of black chick NC-derived melanocytes in culture. Dendritic (a), polygonal (b) and bipolar (c), pigmented melanocytes are visible along with unpigmented cells. Bar=20μm.

Table 2: Effect of bFGF and α-MSH on chick neural crest cell cultures

Culture Medium	Onset of melanogenesis (days)	Percentage of melanised cells	Average period of survival (days)
Ham's F12 + 20% FCS	6	10-15	21
Ham's F12 + FCS + α-MSH	5	25	25
Ham's F12 + FCS + bFGF	3-4	62.5	39(+)
Ham's F12 + FCS + bFGF + α-MSH	3-4	80-90	80

Neural crest cells, obtained from BA x NHR chick embryos, were examined daily with an inverted light microscope. The onset of melanogenesis was determined as the time when melanin was first visible. The proportion of melanocytes was visually estimated and presented as a percentage of the total cell population obtained from a neural tube explant.

Basic medium. In neural crest cells fed with basic medium, pigment production was evident six days after explantation. Melanocyte colonies showed a small increase in size and by 10 days in culture, an average of 10-15% of cells had differentiated into pigmented melanocytes. During this period the amount of pigment also increased but was never macroscopically visible. This can be seen in Figure 22a where the areas containing melanocytes are not clearly visible at a low magnification. Melanocyte colony morphology and the degree of pigmentation is shown in Figure 22b. Necrotic cells, evident as round, phase-bright cells with a bubbly appearance, were common after three weeks in culture with basic medium.

α-MSH. When crest cell outgrowths were grown in basic medium supplemented with α-MSH, melanocytes first apeared five days after neural tube explantation - that is, 24 hours earlier than in basic medium alone (see Table 2). Two days after the onset of melanogenesis, the amount of pigment had increased and was macroscopically visible (results not shown). MSH treatment caused a marked increase in the proportion of melanocytes present in black chick neural crest cell cultures. By day 8 in culture, 25% of the cells were pigmented. Treatment with α-MSH did not prolong cell survival beyond 25 days in culture, after which their condition steadily declined.

bFGF. Neural crest cell cultures maintained in bFGF-supplemented medium gave rise to dark clumps of melanocytes 3-4 days after explantation. As early as 5 days in culture, these culture dishes contained areas of melanocytes that were clearly visible with the naked eye. In Figure 22c, areas of pigmented melanocytes are clearly visible in a dish of black chick neural crest cells maintained in bFGF-supplemented medium. Such areas were not visible in cultures grown in basic medium only (Fig. 22a). An average of 62.5% of the total cell population became melanised in bFGF-treated cultures (see Table 2 for a comparison of results obtained with other factors). Figure 22d shows melanocyte colonies in which the cells are so densely intermingled and

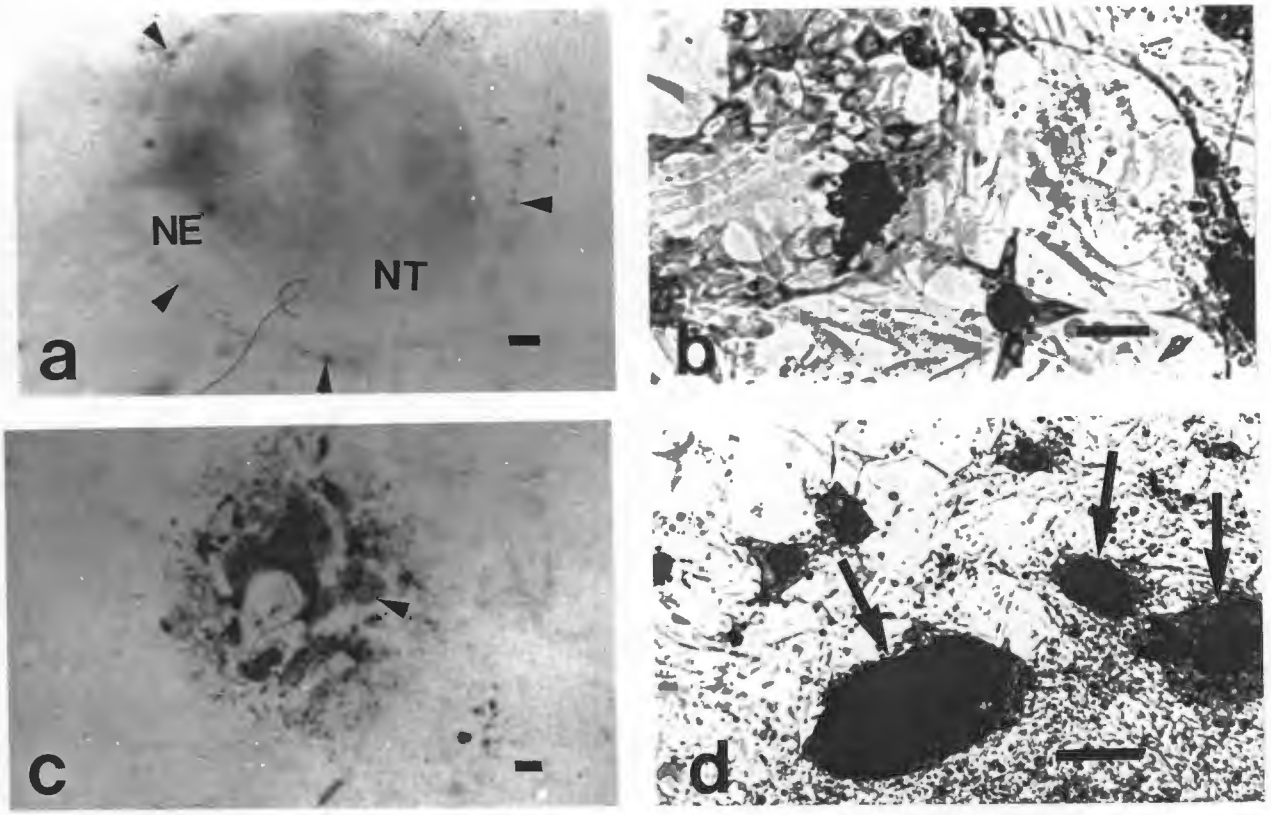

Figure 22. Light micrographs of black chick NC-derived melanocyte cultures grown in medium supplemented with FCS (a,b) and bFGF (c,d). (a) and (c): Low magnification of 4 week old cultures. Groups of melanocytes can be visualised as dark areas (arrowheads) at this magnification. Bar = 500μm.
(b) and (d): Higher magnification of 6 day old melanocyte colonies. Notice the dense clusters of melanocytes (arrows) in (d). Bar = 50μm.

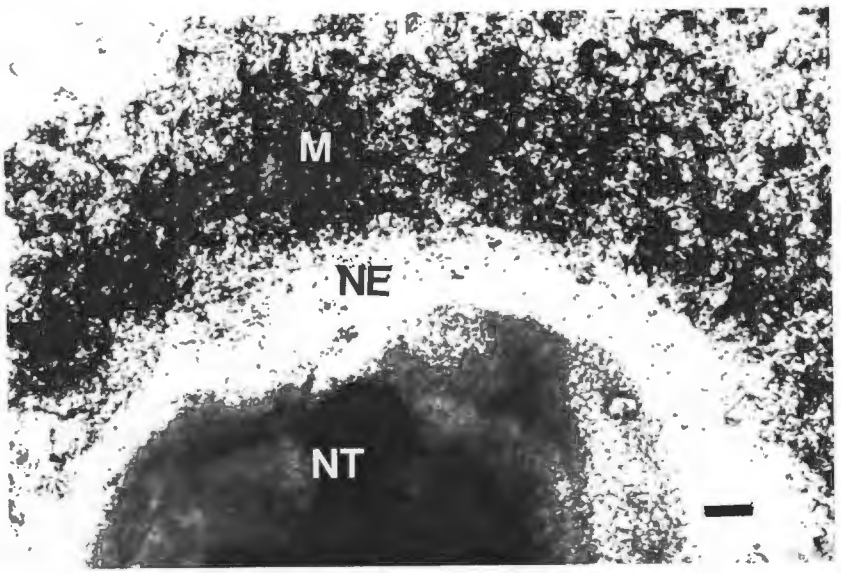

Figure 23. Brightfield light micrograph of black chick NC-derived melanocytes in bFGF + MSH for 6 days showing dense aggregates of pigmented melanocytes.
NT = neural tube, NE = neuroepithelium, M = region of melanocytes. Bar=50μm.

heavily melanised that individual melanocytes cannot be identified. Melanocyte survival *in vitro* was affected by bFGF treatment. Cultured melanocytes were easily maintained for at least 39 days in culture - that is, 2 weeks longer than cultures grown in medium supplemented with α-MSH (see Table 2).

bFGF + α-MSH. Both MSH- and bFGF-supplemented cell cultures showed a marked improvement over basic medium in melanocyte proportions and survival (see above). These factors were used simultaneously in this study to see if this treatment would have an additive effect on black neural crest cell cultures. This set of culture conditions produced the most stunning results as assessed by light microscopic comparisons. Melanocytes were observed in culture 3-4 days after neural tube explantation. These melanocyte colonies rapidly increased in size to yield an average estimate of 80-90% of the cell population. Basic FGF + α-MSH treated cell cultures displayed thick, darkly pigmented macroscopic areas of melanocytes. A similar collar of densely intermingled melanocytes in a 6 day old culture, is shown in Figure 23. The simultaneous use of bFGF and MSH prolonged melanocyte survival up to 80 days in culture.

3.4.2 *FEATURES OF WHITE CHICK MELANOCYTES IN VITRO*

Neural tubes from white chick embryos were explanted in culture to determine if their neural crest cells, when placed in a permissive environment, can differentiate into pigmented melanocytes. Based on the above results, white chick neural crest cells were maintained in Ham's F-12 medium + 20% FCS with either 10ng/ml bFGF or 10ng/ml bFGF + 100μg/ml α-MSH. For direct comparisons, white and black chick neural crest cultures were concurrently established and maintained. Cultures were observed and visually assessed using standard brightfield/phase contrast microscopy. The observations reported here are based on 10 repeat cultures per set of culture conditions.

Under both culture conditions (bFGF and bFGF + α-MSH), white and black chick neural crest cells differentiated into pigmented

melanocytes after 3-4 days in culture. There were no obvious differences in the morphology of cultured black and white melanocytes (see Fig. 24a and 24b). However, there were consistent differences in the proportion of melanocytes and the degree of pigmentation in black and white cultures. Regardless of culture conditions, melanocyte numbers obtained from white chick neural tubes were always less than their black counterparts. In bFGF-supplemented cultures, 62.5% of cells in black chick cultures were melanocytes - only 50% of cells in the white chick cultures became melanised. Similarly with bFGF + α-MSH treated cultures: pigmented cells represented 80% of the cells in black crest cell cultures, while melanocytes formed only 60% of the total white chick cell population. Figure 24c and 24d shows the extent and degree of pigmentation in black and white chick crest cell cultures maintained for a month in bFGF-supplemented medium. Individual melanocytes in black chick cell culture dishes appeared more melanised than white chick melanocytes (see Fig. 24a and 24b). In older cultures, however these differences were not so pronounced and black and white cultured melanocytes appeared visually identical (Fig. 24e and 24f). Furthermore, white chick melanocytes were as viable in culture as black melanocytes. Over a minimum study period of 5.5 weeks *in vitro*, the scale of cell death in white chick melanocyte cultures was equivalent to black chick melanocyte cultures.

3.4.3 *ULTRASTRUCTURE OF NEURAL CREST-DERIVED MELANOCYTE CULTURES*

Melanocyte cultures derived from both black and white chick embryos became melanised and survived for at least five weeks in Ham's-F12 culture medium supplemented with bFGF and MSH + bFGF (see above). Five different sets of these cultured melanocytes were processed for electron microscopy after 1-5 weeks *in vitro*.

The cell cultures were fixed *in situ* (see Methods) and embedded in resin so that the integrity of the intercellular connections remained. Therefore, in sections of melanocyte cultures, the melanocytes were visible as elongate cells with scattered melanosomes and long cytoplasmic extensions that abutted onto

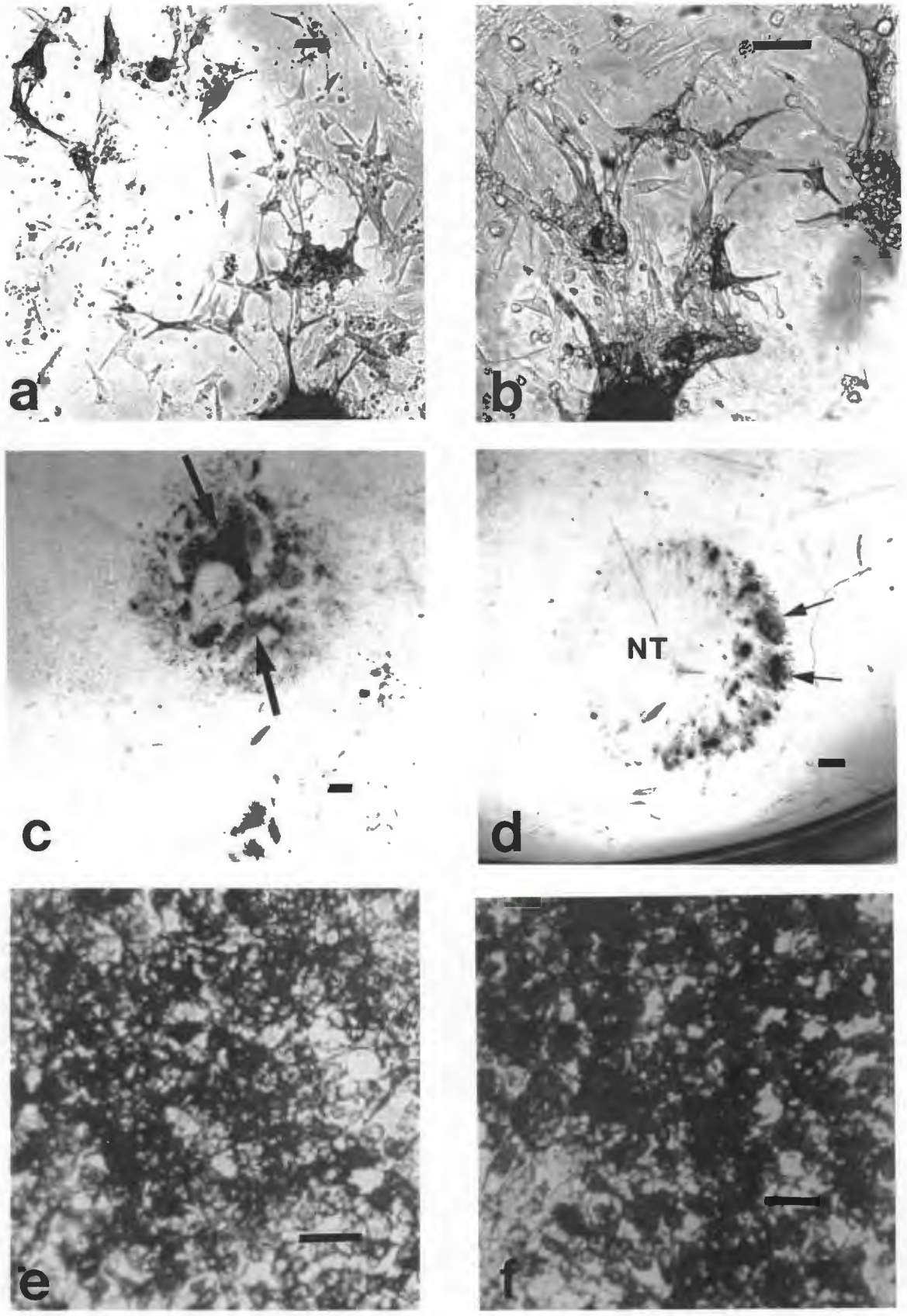

Figure 24. Light micrographs of black chick (a,c,e) and white chick (b,d,f) NC-derived melanocyte cultures treated with bFGF. (a) and (b): Morphologically identical cultures at 6 days *in vitro*, Bar = 50μm. (c) and (d): Low magnification of 4 week old cultures showing only groups of melanocytes (arrows) around the neural tube explant (NT), Bar = 500μm. (e) and (f): visually identical melanocytes at 16 days *in vitro*, Bar = 50μm.

neighbouring cells (Fig. 25). Indications of active melanogenesis in the cultured cells were the extensive Golgi apparatus, RER and the abundance of melanin granules and premelanosomes (Fig. 26). These organelles maintained normal morphologies in black and white melanocyte cultures throughout the *in vitro* periods of study.

Melanosomes of cultured black chick melanocytes were mainly spherical with floccular deposits of melanin that resembled phaeomelanosomes (Fig. 27a). These melanosomes were morphologically different from the oval, evenly pigmented melanosomes of black feather melanocytes *in situ* (see Fig. 12). The premelanosomes of the cultured cells were oval and contained a normal arrangement of matrix filaments (Fig. 27b) identical to that of premelanosomes in melanocytes *in situ*.

Likewise, premelanosomes in cultured white chick melanocytes resembled those found *in situ*. Morphologically normal pre-eumelanosomes occurred throughout the perikarya and dendrites of cultured white melanocytes (Fig. 28a and 28b). Unlike white melanocytes *in situ* however, the cultured white melanocytes contained numerous mature, fully melanised melanosomes. These spherical melanosomes (Fig. 28b) appeared to be morphologically identical to those observed in black chick cultured melanocytes.

Incompletely melanised melanosomes were also more frequently observed in cultured white chick melanocytes than in cultured black chick melanocytes (see Fig. 27b and 28b). This may be an indication of a minor melanocyte autonomous defect that interferes with melanosomal maturation.

3.5 CAN THE WHITE CHICK SKIN SUPPORT MELANOCYTE SURVIVAL?

The culture environment allows the differentiation of white chick neural crest cells into pigmented melanocytes, as shown in 3.4.2, yet these melanocytes do not pigment *in vivo*. This suggests that the white skin environment is not able to support melanocyte differentiation *in vivo*. Therefore, to resolve this issue of whether hypomelanosis in WPR x PG chicks results from an innate

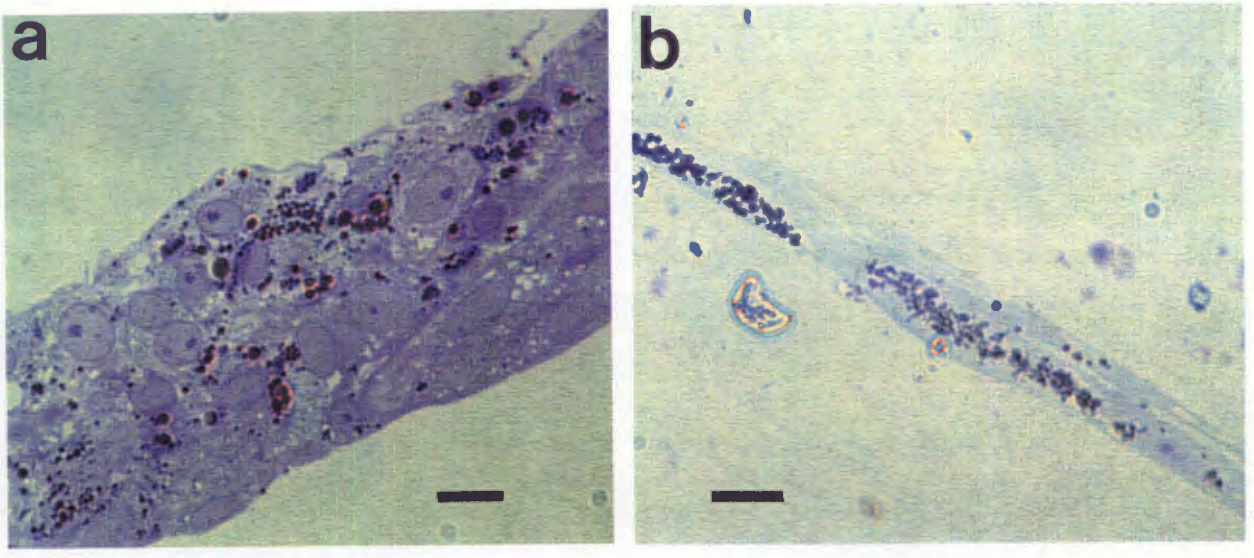

Figure 25. Light micrographs of toluidine blue stained semi-thin resin sections of (a) black chick and (b) white chick cultured melanocytes. Melanin granules appear as dark spots within the cells. Bar = 10μm.

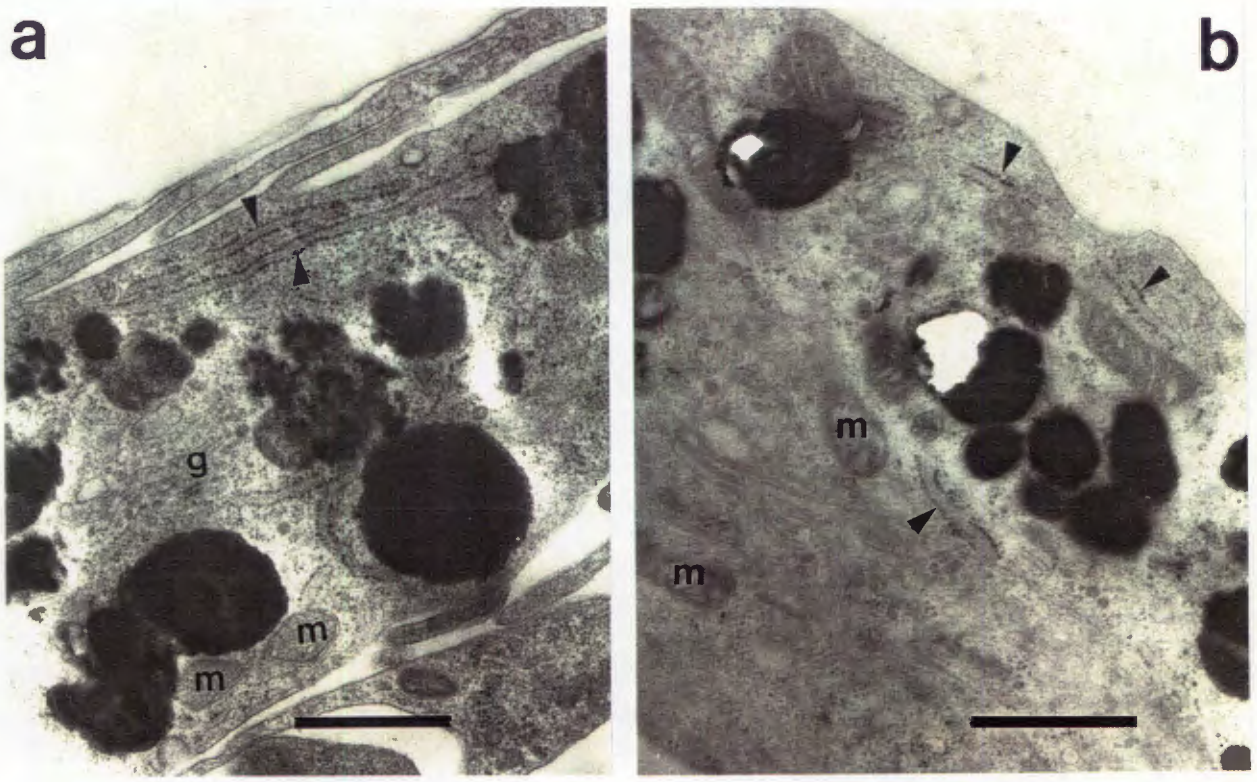

Figure 26. Electron micrographs of (a) black chick and (b) white chick cultured melanocytes treated with bFGF, showing identical (normal) appearance of organelles. Large pigment granules (melanosomes) are dispersed throughout the cytoplasm.
m = mitochondrion, arrowhead = RER. Bar = 0.75μm.

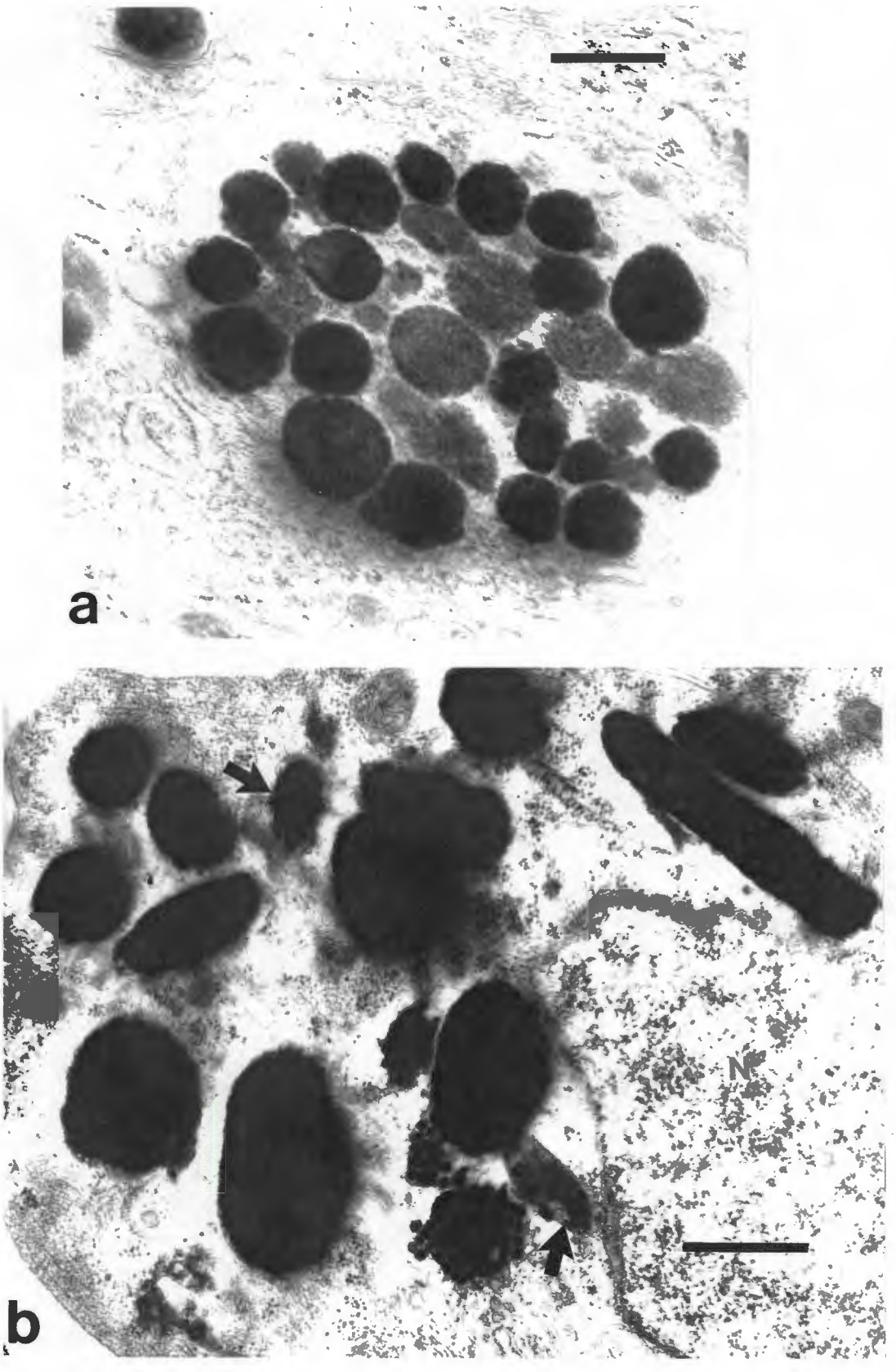

Figure 27. Electron micrographs showing the different morphologies of melanosomes *in vitro*. (a) Spherical, flocculent melanosomes in black chick melanocyte dendrite. (b) Oval melanosomes and premelanosomes in black chick melanocytes.
Arrow = premelanosomes, N = nucleus. Bar=0.5μm.

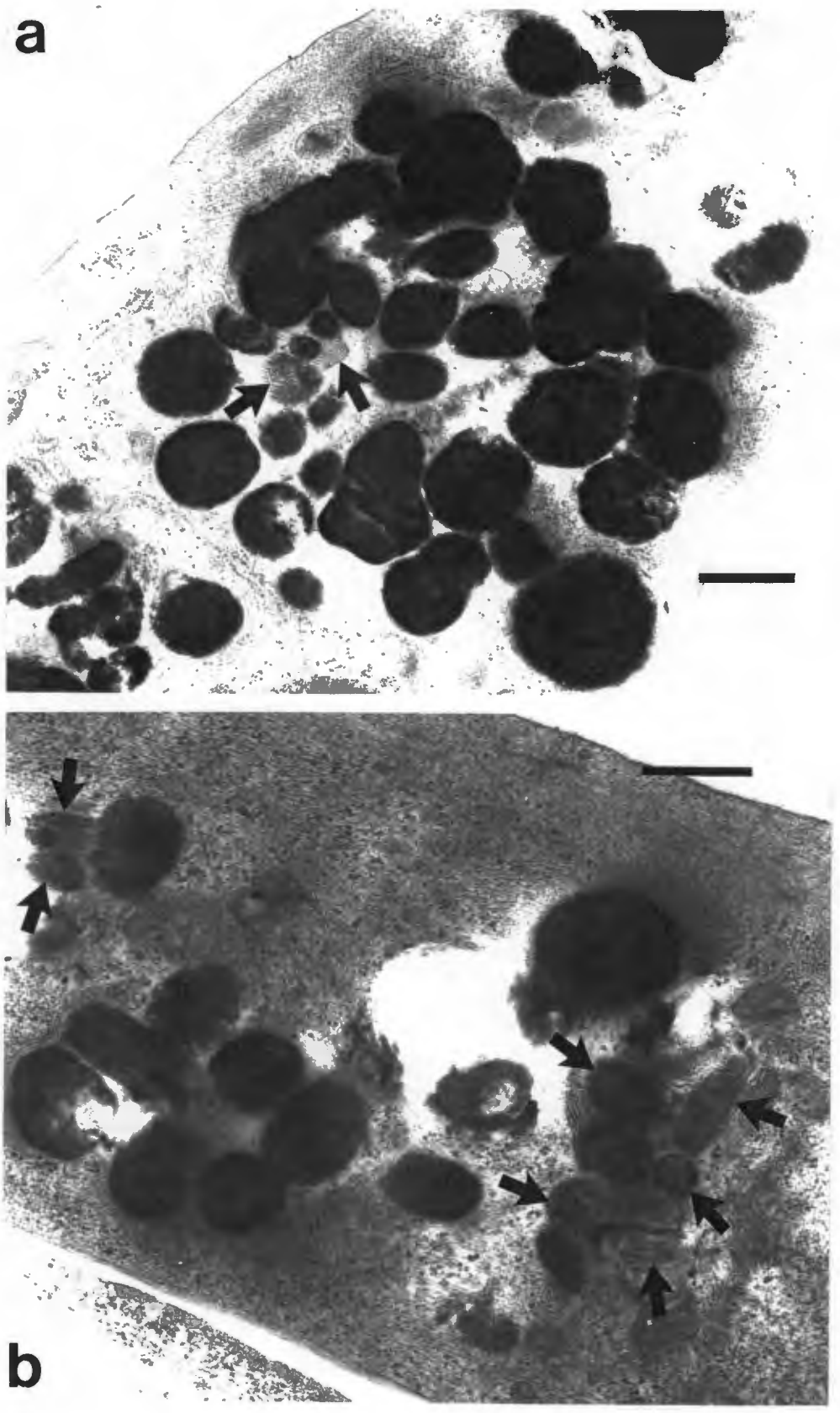

Figure 28. Electron micrographs of cultured white chick melanocytes showing (a) Mature, spherical melanosomes in a melanocyte dendrite. (b) Normal looking melanosomes and premelanosomes in a melanocyte. Notice the high number of premelanosomes. Arrow = premelanosome. Bar=0.5μm.

melanocyte defect or an environmental defect, a novel study was undertaken to determine if black chick neural crest cells can develop into pigmented melanocytes in a white skin environment. Pilot studies to establish these conditions had previously been carried out (Marco, 1991). In these experiments, cultured neural crest cells from black chicks were allowed to populate white embryonic chick skins by placing 5 day old white skin explants on top of the cultured cells for 24 hr. The skin were then removed from the culture dish, grafted onto a CAM membrane and allowed to develop until the feather formation stage. If the white skin environment is normal, it would support the differentiation of the black neural crest cells into functional epidermal melanocytes and give rise to black feathers. On the other hand, if the white skin environment is defective, it would be unable to support the differentiation of the black crest cells into epidermal melanocytes and the developing feathers would remain white. However, the success of this study design depends on: 1) the migration of neural crest cells from the culture dish into the skin explant, as well as, 2) the differentiation of these cells into melanocytes and 3) their final localisation in the epidermis of the skin explant. Therefore, to interpret the occurrence of unpigmented grafts correctly, the cultured neural crest cells from black chick embryos were firstly labelled with tritiated thymidine and the presence of labelled cells in the white skin was later determined by autoradiography. These experiments were repeated four times and the results are presented below.

Neural crest cell labelling with [^{3}H]-thymidine and co-culture with white chick skin. To label neural crest cells, neural tubes from black chick embryos were cultured in medium containing [^{3}H]-thymidine. Cultures were examined 18-24 hr after explantation with an inverted phase contrast microscope. By this time, the neural crest cells had migrated from the neural tubes to form a homogenous monolayer on the plastic culture dish. The crest cell cultures displayed the typical stellate morphology with numerous filopodia extending from the neural crest cell body (Fig. 29). After 24 hr *in vitro*, the neural tubes were removed from the dishes and the remaining neural crest cell populations were rinsed and chased with fresh, unlabelled culture medium. Next, dorsal

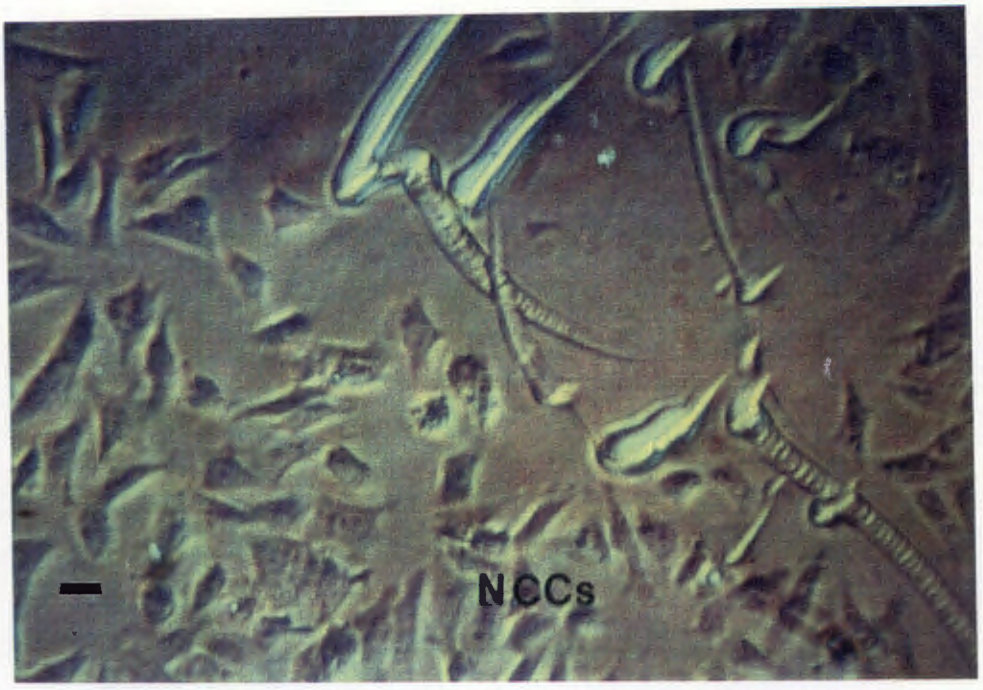

Figure 29. Light micrograph of neural crest cells (NCCs) cultured on plastic substrate for 1 day. Note their typical stellate shape and cytoplasmic extensions. (Scratch marks indicate former position of neural tube). Bar=20μm.

Figure 30. Whole mount photograph of a recovered CAM graft, showing black feathers among white feathers. Bar = 500μm.

skin, dissected from white chick embryos were co-cultured with the labelled black neural crest cell cultures for 24 hr, allowing neural crest cells to leave the culture dish substrate and migrate into the skin. After 24 hr of co-culture the skin was grafted onto host chick embryo CAMs. After removal of the skin explants from the culture dish, the dishes were inspected microscopically. Although a large number of neural crest cells appeared to have migrated into the skin, a substantial number still remained in the dish.

CAM grafting of white skin explants. A total of 31 grafts were allowed to develop on the chorio-allantoic membrane of host embryos until the grafts were approximately 12 days old. Table 3 provides a summary of the grafting results.

Table 3: SUMMARY OF CELL CULTURE AND GRAFTING SUCCESSES

Expt No.	NCC outgrowth from NTs	No. of CAM grafts retrieved	Condition of graft: Feather formation	Condition of graft: Pigmented
1	4/5 = 80%	3/6 = 50%	3	0
2	7/10 = 70%	7/8 = 87.5%	4	1
3	7/14 = 50%	6/6 = 100%	4	0
4	8/10 = 80%	8/11 = 72.7%	2	0

Embryonic skin from WPR x PG chicks were first co-cultured with tritium labelled neural crest cell cultures, derived from BA x NHR chick embryos, then CAM grafted. After 6 days, the grafts were removed and fixed for further examination.

The results indicate that on average 75% of the grafts survived and most of these had developed feathers. Of the original 31 grafts, 24 were retrieved of which 13 had good feather development. These represented 3 control grafts and 10 experimental grafts. Only one of these experimental grafts had clearly visible black feathers (Fig. 30). Similar results (3 pigmented grafts out of a total of 20 grafts) have also been obtained by others (Kidson, pers. comm.). The reasons for the

high number of white grafts obtained in this study was revealed by autoradiographic analyses of the skin grafts, presented below.

Analyses of recovered grafts. Only feathered grafts were further analysed. Histologically, a typical feathered graft consisted of an epidermis of at least 3 layers of columnar cells with superficial flattened peridermal cells and an underlying mesenchyme (Fig. 31a). In cross-section, feathers appear as rounded structures with the epidermal cells surrounding a central core of mesenchyme in which blood vessels are frequently seen (Fig. 31b). In unpigmented skin grafts melanin was not visible in the feathers or in the mesenchyme (Fig. 31a), whereas the pigmented grafts contained dark, densely packed melanin granules in the epidermis of the developing pigmented feathers (Fig. 31b). To detect tritium labelled cells in these skin grafts, 8 unpigmented, 1 pigmented and 1 control graft were selected for autoradiography. Specific radioactivity in the tissue was indicated by coarse black grains that formed clusters over tritium labelled cell nuclei (Fig. 32a). This was easily distinguished from background radioactive signals which occurred throughout the emulsion as fine, randomly scattered granules. With darkfield microscopy, the positive grains appeared as bright clumps, whereas background signals were visible as less intense individual spots (Fig. 32b).

Autoradiography on sections of the sole pigmented graft revealed tritium-labelled cells in the epidermis around the melanin (Fig. 33a). As can be seen from Figure 33, it was difficult to clearly identify positive silver halide grains from the melanin clusters. The examination of unpigmented grafts did not reveal any positive grains within the epithelium of the white feathers. In these sections, positive grains were localised just outside the epidermis, near the base of developing feather follicles (Fig. 34a). Tritium-labelled cells were also detected in the mesenchyme and clustered around cartilagenous structures (Fig. 34b), indicating that the black neural crest cells had migrated into the white skin but failed to migrate into the epidermis and differentiate into melanocytes.

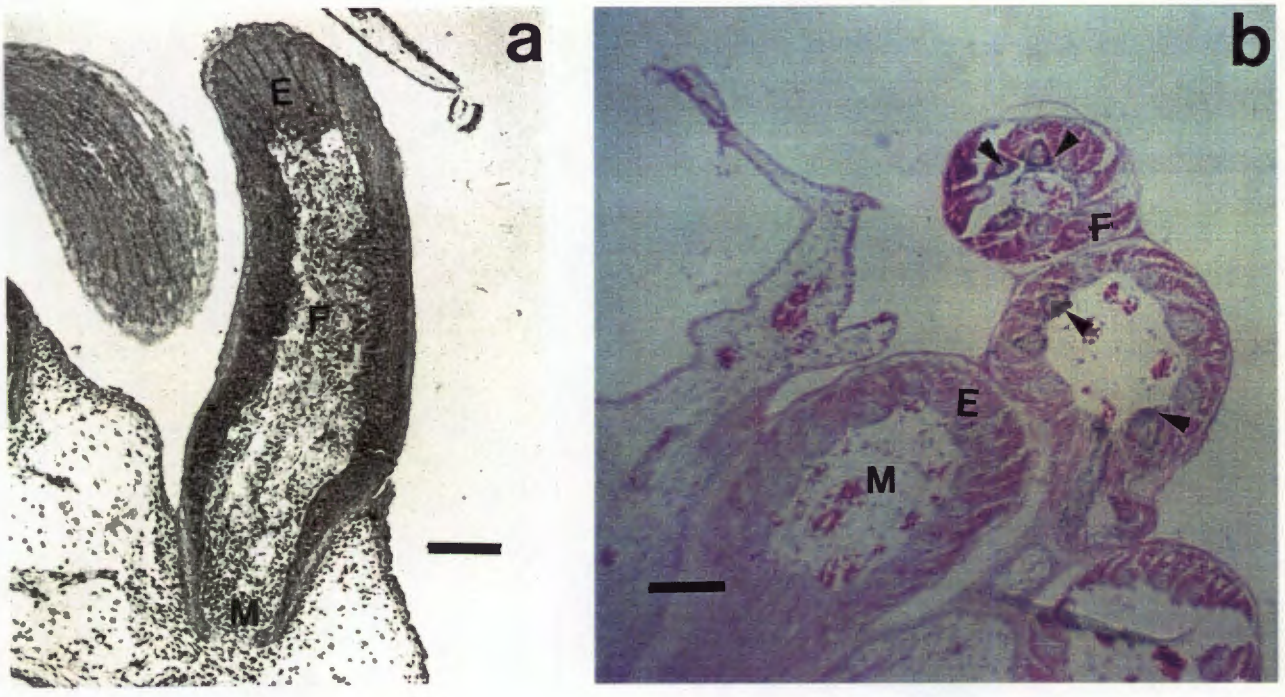

Figure 31. Micrographs of recovered CAM grafts showing a longitudinal section through an unpigmented graft (a) and a cross-section through a pigmented graft (b).
F = feathers, E= epidermis, M = mesenchyme, arrowhead= melanin. Bar=100μm.

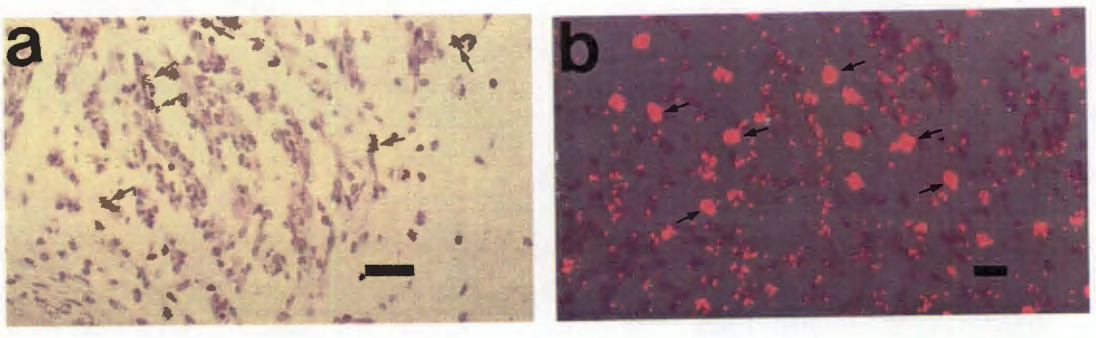

Figure 32. (a) Brightfield and (b) double-exposed light micrographs showing specific autoradiographic grains (arrows) over the nuclei of tritium-labelled cells only. Double-exposure micrographs were first taken under darkfield with a red filter, then under brightfield without the filter. Bar=10μm.

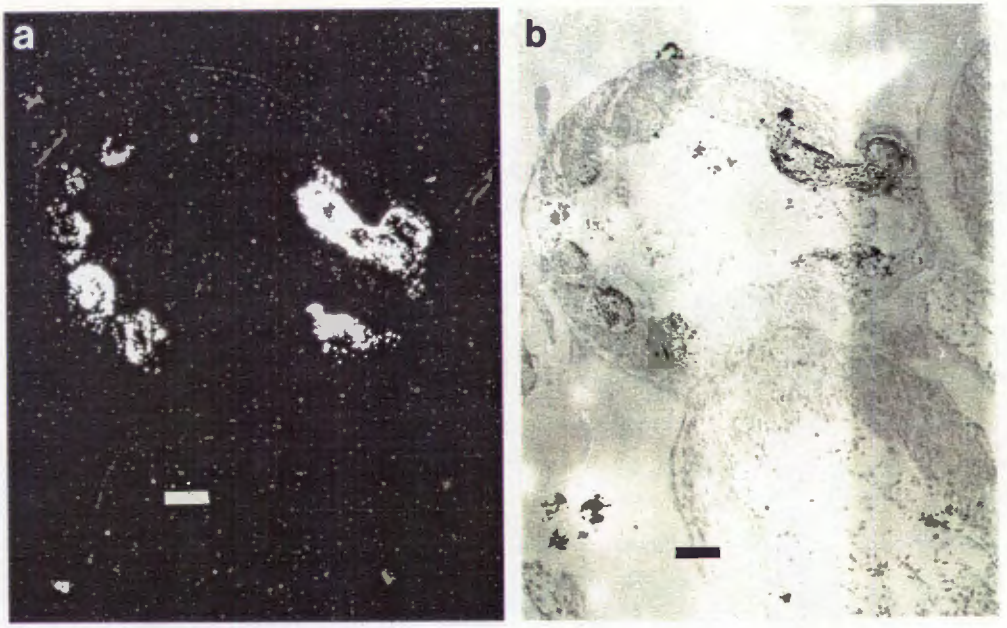

Figure 33. (a) Darkfield and (b) brightfield micrographs of autoradiographed cross-sections through feathers of the pigmented skin graft. Notice the scattered background granules and the bright clusters in (a) that are associated with the distribution of melanin in (b). (The poor quality of micrograph (b) is due to drying marks on the film negative.) Bar=20μm.

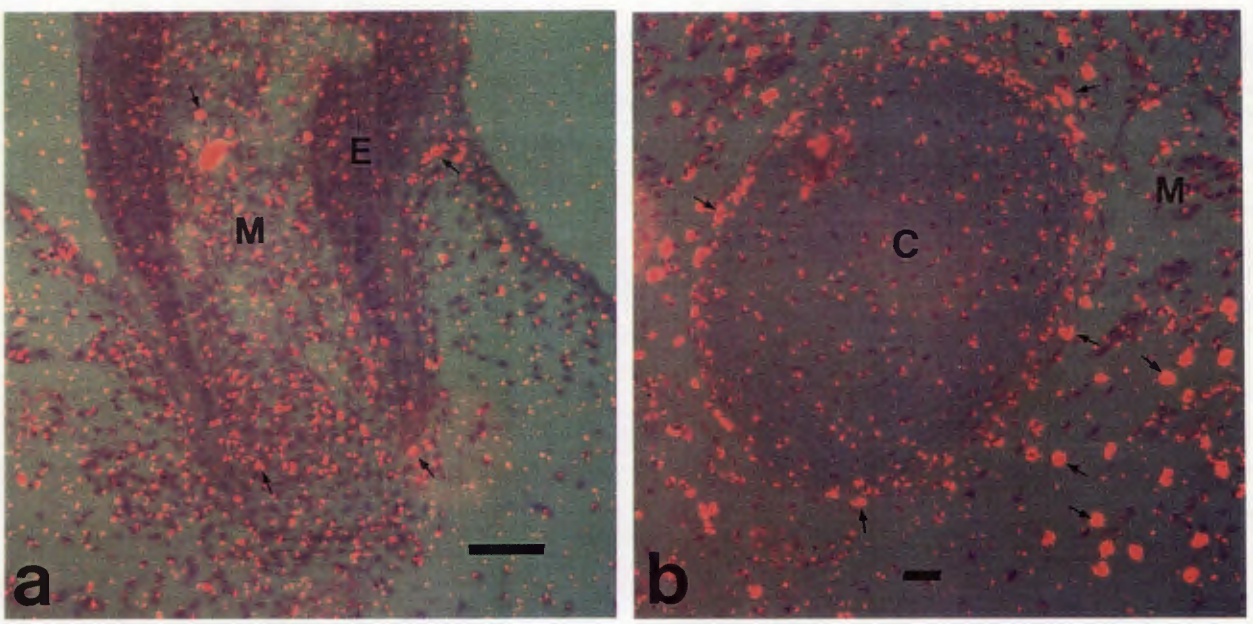

Figure 34. Double-exposed light micrographs showing sections through unpigmented grafts. (a) Longitudinal section through a feather showing positive cells (arrows) outside the epidermis. (b) Positive cells (arrows) clustered in the mesenchyme and around cartilage.
E= epidermis, M= mesenchyme, C= cartilage. Bar=20μm.

During the co-culture period, control skin were placed in culture dishes without contacting the cultured cells. This was to demonstrate that only black neural crest cells were labelled with tritiated thymidine. Microscopic examination of such control graft sections were devoid of specific silver-halide clusters (Fig. 35). This result shows that the white skin grafts contained tritium-labelled cells that originated from the crest cell cultures only.

From these crest cell-transplant studies, it can be concluded that the white chick skin environment is able to support both the migration of neural crest cells and their differentiation into epidermal pigment cells. In other words, the hypomelanotic defect does not seem to be within the white skin environment but is probably in the melanocyte itself. This is however, in conflict with the results obtained from long-term melanocyte cultures (presented in 3.4.2), which showed that the white chick melanocytes are not defective *in vitro*.

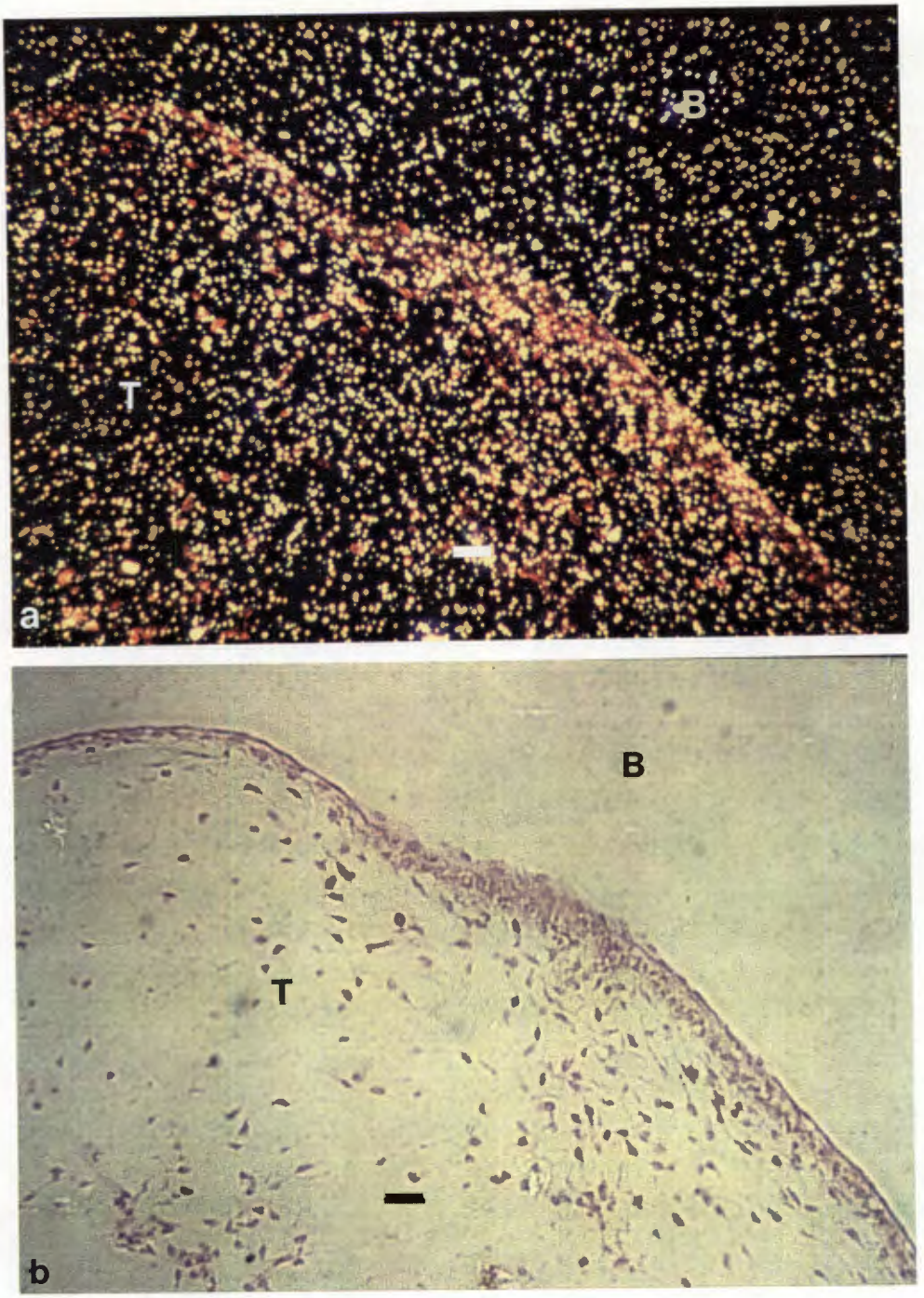

Figure 35. (a) Darkfield and (b) brightfield micrographs of a control skin graft processed for autoradiography. Non-specific radioactive granules can be seen throughout the tissue area (T) and in the background (i.e. off the tissue area) (B). Bar=20μm.

CHAPTER 4

DISCUSSION

From the available literature on human and non-human animal studies, it seems that hypomelanosis may be induced by a multitude of different mechanisms such as: 1) failure of embryonic melanoblasts to reach the epidermis; 2) reduction in melanosome synthesis and melanisation; 3) defective enzymes that are critical for normal melanin synthesis and 4) the selective elimination of functional melanocytes (Hearing and Jiminez, 1989). The aim of this study was to characterise the mechanism(s) that lead to hypomelanosis in a locally available fowl cross-breed, White Plymouth Rock x Pile Game (white) chicks. *In situ* and *in vitro* techniques were employed to establish which critical processes of melanogenesis were affected by the hypomelanotic defect and also to investigate whether this hypomelanotic fowl breed would be a suitable experimental model for human hypomelanotic disorders.

The results from this study presented an interesting contradiction as to the underlying cause of hypomelanosis. On the one hand, the findings allude to an intrinsic melanocyte defect and on the other hand, there is evidence to suggest that the extrinsic environment is defective and not the melanocyte itself. These issues have not been completely resolved in this study and will be discussed below.

4.1 IS HYPOMELANOSIS IN WPR x PG CHICKS DUE TO AN ABSENCE OF EPIDERMAL MELANOCYTES?

Pigment produced by melanocytes can only contribute towards plumage colouration if active melanocytes are localised in the epidermis. Melanocytes in turn, can be detected either by the presence of melanins, or by specific enzyme activities or by immunocytochemistry. White chick skin is unpigmented, therefore melanocytes could not be detected on the basis of melanin granule presence. It was also not possible to locate white chick

melanocytes by immunocytochemistry because avian melanocyte markers have only recently been developed and could not be obtained from the authors, Nataf *et al* (1993) and Kitamura *et al* (1992), nor were they commercially available. Another strategy, based on specific enzyme activity, had to be adopted to detect melanocytes in white chick skins. The tyrosinase-specific histochemical technique, the DOPA reaction, was therefore used to locate the presence of tyrosinase in white chick skin. This reaction revealed the presence of melanocytes in the skin of 8-to 10-day white chick embryos, indicating that hypomelanosis is not due to the failure of neural crest cell migration and localisation in the skin epidermis, nor is it primarily due to an absence of tyrosinase protein. Thus, melanocytes are present, contain active enzyme, but never synthesise melanin *in vivo*.

DOPA-reacted white skins of 11-to 13-day chick embryos were DOPA-negative. This result cannot be interpreted as a disappearance of white skin melanocytes or the down-regulation of tyrosinase activity, because assayable quantities of tyrosinase in 13 day skin w detected with the more sensitive [^{14}C]tyrosine assay. It is possible that the absence of DOPA product in 11-to 13-day white skins is due to the fact that the reactions were carried out on unsplit skin specimens in which the melanocyte-bearing barb ridges are not exposed to the effects of the DOPA solution unless further dissections are carried out (Brumbaugh and Zieg, 1971).

The presence of DOPA-positive peridermal cells in 8-10 day white skin was somewhat surprising. As far as can be established, melanocytes have never been observed in this superficial, protective layer of embryonic skin (Parakkal and Matoltsy, 1968 and Mottet and Jensen, 1968). Although peridermal cells of 14-to 17-day chick embryos contain cytoplasmic granules, these granules do not resemble melanosomes since they are irregular in form, of varying size and consist of interlacing strands that produce a mesh-like structure (see Parakkal and Matoltsy, 1968 and Mottet and Jensen, 1968). To confirm that these positive cells were not artefactual and to establish the nature of these DOPA-positive peridermal cells, black chick embryonic skin was examined ultrastructurally for the occurrence of peridermal melanocytes. A

cluster of electron dense granules, morphologically recognisable as eumelanosomes, was indeed observed in some of the peridermal cells. However, the subcellular structure of these cells was not typical of melanocytes and the cluster of melanosomes resembled packages of transferred melanosomes rather than freely synthesised melanosomes (compare Fig. 9 and Fig. 11). These results strongly suggest that melanosomal transfer had occurred between the melanocytes and the peridermal cells. The functional significance of melanin transfer to a transient embryonic structure such as the periderm, is not apparent.

4.2 *IS HYPOMELANOSIS DUE TO STRUCTURAL ABNORMALITIES OF THE WPR x PG CHICK MELANOCYTES?*

If hypomelanosis is not due to the absence of epidermal melanocytes as shown histochemically, then perhaps it is a consequence of an intrinsic, structural defect as in some forms of vitiligo. To investigate this probability, white chick skin was examined ultrastructurally.

Electron microscopy showed morphologically normal premelanosomes inside white chick skin melanocytes that occurred in smaller numbers than in black skin melanocytes and did not become melanised and mature. The absence of mature melanosomes and the presence of normal premelanosomes have been observed in ty-pos and ty-neg albinos (Witkop et al, 1989) as well as in some vitiligo animal models. In an ultrastructural study, Brumbaugh (1971) and Jimbow *et al* (1974) reported that feather melanocytes of dominant white chickens such as White Leghorns and White Leghorn-derived stocks contain unmelanised melanosomes at significantly low populations. They further observed that these melanosomes were degraded within autophagosomes from day 10 of incubation onward. In this ultrastructural study of 9 and 10 day WPR x PG chick skin, there was no evidence of melanosomal or melanocytic degradation, nor were there any visible structural abnormalities of the ER and Golgi that could account for the absence of pigment. These findings are different to those reported observations of ultrastructural defects of the RER in some vitiligo skin samples

and melanocyte cultures (Boissy *et al*, 1991a). The results of this study then, suggest that the structural locus for premelanosomal assembly is not defective in WPR x PG chicks. However, the low melanosome number and absence of melanised melanosomes may be an indication that the rate of melanosomal synthesis is affected and that melanin synthesis is inhibited within these melanosomes. Ultrastructurally, WPR x PG chick melanocytes closely resemble melanocytes from ty-pos albinos and both are DOPA-positive (Witkop *et al*, 1989). It may be possible that they also share a similar defect that results in a blockage of eumelanin synthesis. Mutations of a melanosomal tyrosine transporter that impede the entry of tyrosine into the melanosome and in this way prevent melanin synthesis, have already been identified in ty-pos individuals (Rinchik *et al*, 1993 and Kedda *et al*, 1994). The possibility of a shortage of melanin precursors in the WPR x PG chick melanosomes was, however not directly explored and remains a speculative factor in this study.

The ultrastructural studies did not reveal evidence of melanocyte degradation by autophagocytosis in 9 and 10 day WPR x PG chicks. This is in contrast with reports on other Dominant white chickens (see Crawford, 1990; Jimbow *et al*, 1974 and Brumbaugh, 1971) and Barred chickens (see Crawford, 1990; Bowers, 1989 and Bowers *et al*, 1992) that show premature degeneration *in vivo*. Both of these chick breeds are models of the cytotoxic forms of vitiligo and, since WPR x PG chick melanocytes are not prematurely destroyed, it is likely that the observed blockage in melanogenesis is not toxic to the melanocytes.

4.3 *IS THERE A BIOCHEMICAL BASIS FOR HYPOMELANOSIS IN WPR x PG CHICKS?*

If the severe reduction in pigment synthesis is not due to structural defects of the melanocyte and their melanosomes, then it may be due to a defect in the biochemical pathway of melanin synthesis. Since tyrosinase is the rate-limiting enzyme in the biosynthesis of melanin, tyrosinase activity is a key determinant of pigment production in skin, as shown by studies of racial

differences in human skin colour (Pomerantz and Ances, 1975 and Talwar *et al*, 1993). The [^{14}C]tyrosine assay was used to quantify and compare the tyrosinase activities in 9 day and 13 day embryonic skins from black and white chicks. The assay results showed that white chicks differed from black chicks in respect of tyrosinase activity. Firstly, white skins showed less tyrosinase activity (approximately 50% less) than black skin and secondly, tyrosinase activity did not increase with embryonic development, but remained constant in white chicks.

This reduced enzyme activity may either be due to melanocyte intrinsic factors or due to melanocyte numbers. The rate of enzyme synthesis and its degradation within the melanocyte can determine the level of tyrosinase activity. This was shown by Halaban *et al* (1983) who compared these factors in cultured melanocytes from black and white individuals. It is possible then, that the lower levels of tyrosinase activity in WPR x PG chick melanocytes is due to a low rate of tyrosinase synthesis and a higher rate of tyrosinase degradation. This was, however, not further investigated in this study. Another explanation for the observed enzyme activity may be that individual white chick melanocytes have normal levels of tyrosinase activity but if there are fewer melanocytes per area of white skin than in black skin, the total activity would be less. Should this be the case, it means that fewer melanoblasts migrated into the white skin or that melanoblast survival is reduced. DOPA-positive melanocytes could not be statistically quantified in this study because 11-to-13-day white chick skin specimen were DOPA-negative. However, melanocyte numbers seemed smaller in 9 and 10 day white skins than in black skins but this does not constitute proof of the cause for reduced tyrosinase activity.

It has been shown that the amount of melanin correlates well with assayed levels of tyrosinase activity (Halaban *et al*, 1983 and Iozumi, 1993). The extent of pigmentation therefore, correlates positively with tyrosinase activity. It follows then that BA x NHR chicks will have a greater melanin content than white chicks. However, the two-fold difference in tyrosinase activity between 9 day black and white chick embryos cannot fully explain the

complete absence of pigment in the white chick melanocytes. It can be concluded from this study that hypomelanosis is not due to the total absence of active enzyme (like the structural tyrosinase gene mutations of human ty-neg OCA individuals), but may be due to the reduced levels of tyrosinase activity in white chick skin.

4.4 *A CELL INTRINSIC VERSUS AN EXTRINSIC MUTATION IN WPR x PG CHICKS*

The *in situ* findings presented above, indicate that melanocytes are present in white skin and though not grossly abnormal, with respect to morphological and enzymatic profiles, these cells are different from black chick melanocytes. A number of possible causes of hypomelanosis have been eliminated during the course of the study but many questions still remain unanswered. To correctly interpret the *in situ* results, one must know whether a melanocyte intrinsic or an extrinsic environmental defect is responsible for hypomelanosis. A number of studies were carried out to determine whether the observed block in melanin synthesis is an intrinsic melanocyte defect or whether it results from systemic or environmental factors. Results from these studies present an interesting paradox: 1) Neural crest-derived melanocytes from white chicks synthesised pigment *in vitro*, indicating that the melanocyte itself is not defective. 2) Black chick neural crest cells migrated into the epidermis of embryonic white chick skin and survived to pigment the developing feathers, implying that the local tissue environment of the white skin is also not defective. These contradictions will now be discussed below.

4.4.1 EVIDENCE FOR AN EXTRINSIC DEFECT IN WPR x PG CHICKS

The technique of cell culture, given the right conditions, provides the ideal way to examine the innate behaviour of cell populations when removed from the modifying influences of their natural environment. To see whether white chick melanocytes are able to synthesise pigment at all, neural crest cells were

maintained *in vitro* in media either supplemented with bFGF or with bFGF and MSH. These culture conditions were selected after pilot studies with black chick neural crest cells confirmed the positive results of these factors on melanogenesis and survival of avian crest cells, as reported by others (Satoh and Ide, 1987; Bowers and Bibosa, 1994; Stocker et al, 1991 and Kalcheim, 1989).

In the cell culture environments, white chick melanocytes not only survived as long as black melanocytes, but also produced pigment in amounts that nearly equalled that in black chick cell cultures. These results clearly showed that white chick melanocytes have the potential to be fully functional melanocytes when in a supportive environment. This suggests that the underlying defect may not be intrinsic in the melanocyte but may act extrinsically on the local tissue environment to inhibit melanogenesis *in vivo*. In contrast with these findings, some animal pigmentary models such as Smyth chicks, White Leghorn chicks and the *vit/vit* mouse did not survive *in vitro* but mimicked their respective *in vivo* situations and died prematurely (Derby and Newgreen, 1982; Maxwell, 1976; Boissy *et al*, 1986; Boissy *et al*, 1991b and Bowers *et al*, 1992). This indicates that in these animal models, a change in the environment could not alter the fate of their intrinsically defective melanocytes.

4.4.2 EVIDENCE FOR A MELANOCYTE INHERENT DEFECT IN WPR x PG CHICKS

If the cause of hypomelanosis in WPR x PG chicks is not intrinsic in the melanocytes as suggested by the *in vitro* results, then it must be caused by an extrinsic, environmental defect. To determine whether the white skin is defective, black chick neural crest cells were allowed to populate embryonic white chick skin which was then CAM grafted and left to develop normally until feather formation had started. By transplanting melanoblasts from one chick breed to another, it is therefore possible to obtain information regarding their behaviour in a genotypically different environment. Thus, if the cutaneous environment is normal within white chicks, it would be able to support the differentiation of

black neural crest cells into functional epidermal melanocytes and give rise to pigmented feathers in otherwise unpigmented skin. In this study, about one out of every ten grafts became pigmented, indicating that the white chick skin environment is able to support melanocyte survival. This, in turn, suggests that the white melanocyte must be intrinsically defective. This, however, contradicts the conclusion, drawn from the behaviour of white chick melanocytes *in vitro*, that the white skin environment cannot support functional melanocytes.

The resulting paradox of a melanocyte inherent defect versus an environmental defect can be explained if these defects are not mutually exclusive. In other words, hypomelanosis in WPR x PG chicks may result from minor defects in the melanocyte environment *and* within the melanocyte itself. On their own, these defects may not be severe enough to affect melanogenesis, but together they may exert an additive effect that blocks melanin synthesis. This would explain why black chick neural crest cells could survive as functional melanocytes in this defective environment and why white chick melanocytes could complete melanogenesis when placed in a permissive environment *in vitro*. Evidence of such a minor melanocyte autonomous defect was supported by ultrastructural investigations of cultured WPR x PG chick melanocytes. These cultured melanocytes were found to contain a larger proportion of unmelanised melanosomes when compared with black chick melanocyte cultures of an identical age. This suggests that the favourable *in vitro* conditions could only partially correct the melanogenic defect of the white chick melanocytes.

The low numbers of pigmented grafts (only one out of 10) obtained in this study, is not unusual for this type of study design, as indicated by results from similar studies (Kidson, pers. comm.). This may be due either to a flaw in the experimental design that prevents the migration of neural crest cells from the culture dish into the skin explant, or it may result from migrating neural crest cells that did not enter the epidermis in time to be incorporated into the feathers as melanocytes. In order to distinguish between these options, cultured black chick neural crest cells were labelled with tritiated thymidine and were later

located in the white chick skin explants by autoradiography. The autoradiography results from the unpigmented grafts showed that labelled cells were indeed present in the grafts, but not in positions that would allow pigmentation of the feathers. It is possible that developmental changes in the tissue environment restricted the migration of neural crest cells into the feather follicle environment (Bronner-Fraser and Cohen, 1980).

4.5 *CONCLUDING COMMENTS*

This study attempted to elucidate the mechanisms leading to hypopigmentation of White Plymouth Rock x Pile Game chickens by analysing the melanotic system of developing chicks. Because of the complexity of melanogenic control, all the different levels of control could not be investigated in this limited study. However, based on experimental data presented here, certain levels can be excluded as potential cause of hypomelanosis: 1) incorrect migration and localisation of melanoblasts outside the skin epidermis, 2) structural and/or functional mutations of tyrosinase, 3) structural aberrations of subcellular organelles such as melanosomes, endoplasmic reticulum and Golgi complex, and 4) obstruction of tyrosine entry into the melanosomes, perhaps due to defective transporters. This study further showed that most of the white melanocytes have the potential to complete the melanisation process *in vitro* although this never occured *in vivo*. It was also shown that white skin can induce and support black chick melanocyte differentiation. It is therefore proposed that hypomelanosis in White Plymouth Rock x Pile Game chicks results from a dual defect: a minor defect in the extracellular environment creates subtly altered conditions that will inhibit melanisation only in genetically susceptible melanocytes. This unique situation may be attributable to the influences from the different genotypes that contribute to this particular fowl cross-breed.

Although the gross phenotype of WPR x PG chicks is similar to that of human hypomelanotic disorders, they only share some of the characteristic features of vitiligo and ty-pos albinism in humans

and other animal models. In particular, the absence of cytologic aberrations of melanocytes and the persistance of white chick melanocytes *in vivo*, contrasts sharply with other reports. However, since none of the other vitiliginous animal models could provide a universal model for vitiligo, WPR x PG chicks may be used to improve the understanding of the etiopathogenesis of vitiligo in genetically variable animals. The WPR x PG chick breed may also be representative of ty-pos albinos, as shown by their shared ultrastructural features. It remains to be seen whether melanocytes from ty-pos individuals can become fully pigmented *in vitro* as did WPR x PG melanocytes. Studies are currently underway to address this issue (Kidson, pers. comm.). Other crucial studies that can be done to determine the level of the defect in white skin, would be to investigate the possibility of programmed cell death (apoptosis) in the initial melanoblast population as well as later in development. The question of cell death was not easy to address without available melanocyte-specific markers. However, research on this issue has recently been made possible with the use of the TUNEL technique that specifically labels apoptotic cells.

5. *REFERENCES*

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APPENDIX

1. *PHOSPHATE BUFFERED SALINE : pH 6,9; 1 litre*

8,00 g NaCl
1,45 g $Na_2HPO_4.12H_2O$
0,20 g KCl
0,20 g KH_2PO_4

Autoclave

2. *CHICK SALINE (Howard Ringers' Solution) : pH 7.45; 1 litre*

7,25 g Sodium Chloride (NaCl)
0,37 g Potassium Chloride (KCl)
0,18 g Calcium Chloride ($CaCl_2.2H_2O$)

Autoclave for 30 minutes.
Before use, add 10 ml pen-strep with sterile pipette.
Store at 4^oC.

3. *DULBECCO'S PHOSPHATE BUFFERED SOLUTION (Calcium and Magnesium free) : pH 7.4; 1 litre*

8,00 g NaCl
0,20 g KCl
1,15 g Na_2HPO_4
0,20 g KH_2PO_4

Add distilled water to make up volume.
pH to 7,4
Autoclave.