AN ULTRASTRUCTURAL AND LIGHT MICROSCOPIC STUDY OF MELANOCYTE DIFFERENTIATION IN CHICK EMBRYOS

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"The answer is in the journey and not the arrival."

From "Business for the New and the Free"

by Sidney Peimer.

Vir Ma, van Koeka.

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ABSTRACT

The embryonic source and chemical nature of those factor/s directing the *in vivo* differentiation of melanocytes from crest cells are as yet unknown. To begin to address this issue, it is important to establish exactly when and where these signal/s first exert their effects. Therefore, in the present study, overtly differentiated melanocytes containing melanin were quantitated in developing Black Australorp X New Hampshire Red chick embryos. In contrast to previous studies, it was found that embryos synthesize melanin from as early as Day 5 of development, and that at this stage, the melanocytes are predominantly dermally located. Between 5 and 8 days, the numbers of both dermal and epidermal melanocytes increase, after which the dermal melanocyte population declines rapidly while the number of epidermal melanocytes continues to increase. These findings suggest that premelanocytes do not have to be epidermally located to initiate terminal differentiation and implicate the dermis as a possible source of melanocyte inducing factor/s.

The next step was to examine stages of development prior to the onset of pigment production. For this reason, tyrosinase was purified for use as antigen in the production of a polyclonal antibody. The antibody was tested for specificity by western blotting, immunocytochemistry and immunoinhibition procedures. Lack of specificity was demonstrated, rendering it unsuitable as an antibody marker for early melanocytes.

Fowl melanocytes are thought to differentiate into either eumelanosome- or pheomelanosome synthesizing cells. To test the validity of this concept, embryonic skin of the red/black cross breed were screened for possible mixed type melanocytes by electron microscopy. The melanocytes contained melanosomes with a matrix of irregularly arranged filaments amongst typical eumelanogenic melanosomes. This suggests that these chick melanocytes may synthesize both eumelanosomes and pheomelanosomes in single cells. In a further study on pure breeding New Hampshire Reds, it was found that the melanocytes contained a mixture of typical and less typical pheomelanosomes. Outer membrane indentations in the latter melanosome type suggest that tyrosinase may enter these pheomelanosomes by a mechanism related to that proposed for the melanosomes of goldfish.

AIM AND OUTLINE

The development of the vertebrate body is a complex phenomenon during which cells become programmed to differentiate into particular phenotypes. This cell heterogeneity emerges through a precisely regulated cascade of events, which directs cells with identical genomes to express different sets of genes, and so to acquire different functions. A central issue in developmental biology is a search for the mechanisms by which this programming occurs. In this search, a particular embryonic structure called the neural crest has been a very useful model system and tool. The cells belonging to the crest migrate from a localized region in the embryo to various destinations, and then differentiate into several different cell types. Since the migration of these cells can be followed *in vivo* by several marking techniques, and since the cells of the crest are able to migrate from excised neural plates or early neural tubes in culture, their behaviour can be carefully studied under various conditions.

One of the pivotal questions relating to the development of the neural crest is whether the microenvironment through which they migrate plays a decisive role in their fate. As a point of departure, one might begin by asking simple, answerable questions on individual aspects of crest development. For example, knowledge of where and when specific differentiation steps occur can be used to search for the factors that are responsible for such steps.

The focus of this thesis is on one particular neural crest derivative - the melanocytes of the skin. All studies were carried out on Black Australorp X New Hampshire Red embryos. The first aim was to determine the exact time and place during migration when prospective melanocytes first synthesize melanin (chapter 2). This was shown to occur on Day 5. The next question was to determine when melanocytes first synthesize tyrosinase, the enzyme necessary for melanin production. The unsuccessful attempt to produce a polyclonal antibody to tyrosinase leaves this question unanswered (chapter 3). In the last study (chapter 4), melanocyte differentiation was investigated at the ultrastructural level. This study 1/ re-examines the generally accepted viewpoint that fowl melanocytes differentiate into either eu- or pheomelanosome producing cells, but never into mixed type melanocytes as in agouti mice; and 2/ describes the morphology of New Hampshire Red pheomelanosomes. The involvement of vesiculo-globular bodies in the development of the melanosomes from both genotypes is noted and compared with observations in previous reports.

CHAPTER 1.
GENERAL INTRODUCTION

The purpose of the present chapter is to provide a brief background to the formation and development of the neural crest, and to melanocytes and melanogenesis in general. Each of the succeeding chapters have their own, appropriate introduction.

1.1. Formation of the neural anlage and develoment of the neural crest

The neural tube begins to form as a longitudinal infolding in a thickened area of ectoderm along the dorsal midline of the embryo. As the infolding deepens, the folds meet dorsally and begin to fuse into a tube that separates from the overlying ectoderm. The closure of the tube proceeds in a craniocaudal wave, so that the most caudal end of the tube forms last. In the head region, the tube becomes established as the brain vesicles. During neural tube formation, a simultaneous wave of segmentation forms the somites next to the neural tube.

The neural crest is a group of cells that originally lies at the edges of the neural folds. As the folds fuse, the crest cells leave the tube epithelium and move into the area between the dorsum of the forming neural tube and the overlying ectoderm. From here they initiate an extensive rostrocaudal wave of migration to various sites in the embryo. In avian embryos, crest cells begin to migrate from midbrain levels at around 30 hours of development (Newgreen and Gooday, 1985), and the process of migration ceases caudally some 40 hours later (Serbedzija et al., 1989). In general, crest cells commence migration at a level of about three somites rostral to the most recently formed somite (Bronner-Fraser, 1986).

The migrating crest cells follow distinctive pathways towards their destinations and then differentiate into several different phenotypes. These include most of the peripheral autonomic and sensory neurons, Schwann cells, pigment cells of the skin and irides, connective and skeletal tissues of the face, adrenal medullary chromaffin cells, calcitonin producing cells of the thyroid, the parasympathetic postganglionic neurons and support cells of the cardiac ganglia, the aorticopulmonary and truncal septa of the heart, and the musculo-connective tissue walls of the large arteries arising from the heart (for a review see Le Douarin, 1982).

In the trunk region of chick embryos, they travel along three main routes (see Erickson, 1988):

1/ a dorsolateral pathway between the ectoderm and the somites. Cells following this pathway eventually give rise to the melanocytes of the epidermis.

2/ a dorsoventral pathway between the inner side of the somites and the neural tube, from where they invade the rostral half of the somite. Some of these cells form the the sensory ganglia in the dorsomedial sector of the sclerotome, while others migrate down towards the dorsal aorta and form the paravertebral sympathetic ganglia.

3/ an intersomitic pathway between adjacent somites, possibly heading towards the dorsal aorta to contribute to the paravertebral sympathetic ganglia.

The migratory patterns in the head are less well defined due to complex morphogenetic movements (see for example Le Douarin, 1982) and will not be considered here.

1.2. The melanotic system of the skin

Melanocytes are dendritic cells that are specialized to synthesise the pigment melanin within unique organelles called melanosomes. The function of the epidermal melanocytes is to transfer melanised melanosomes to surrounding keratinocytes and to hair or feather follicles.

Pigmentation of the epidermis takes place in the "epidermal melanin unit" (Fitzpatrick et al., 1967), which consists of a melanocyte and a group of basal keratinocytes with which it associates via long dendrites (figure 1.1). In humans, each melanocyte associates with 30 to 40 keratinocytes (Lucky and Norlund, 1985). Melanising melanosomes move from the melanocyte cell bodies towards the tips of the dendrites, from where they are transferred to the keratinocytes (Nakagawa et al.,1984). The keratinocytes, through their divisions, further distribute the melanosomes through the epidermis.

The transferred pigment provides the most common basis for skin, hair and feather colouration. In birds, melanin is responsible for black, grey, brown, or related tints. Apart from coloration, the donated melanosomes play an important protective role. Since melanin has the ability to absorb the harmful ultraviolet rays of the sun, the melanosomes form a type of filter that protects the keratinocytes of the epidermis.

1.3. The chemical pathway of melanogenesis

The polychromy of mammalian hair and chicken feathers is attributable to two basic types of pigment. The eumelanins account for the black and brown colours and the pheomelanins for the yellow to reddish brown colours. Pheomelanin forms by a deviation from the normal course of eumelanogenesis and is chemically distinct from eumelanin.

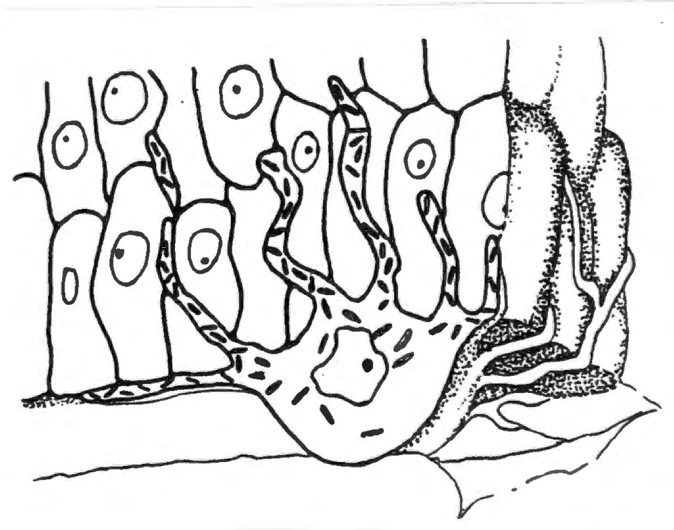

Figure 1.1. Schematic representation of an "epidermal melanin unit." A melanocyte is seen associating with a group of keratinocytes via its long dendrites. Adapted from Fitzpatrick et al. (1967).

Figure 1.2. shows the biochemical pathways of eumelanin and pheomelanin synthesis in mammals, which is thought to be essentially the same in chickens (see Bowers, 1988). Classically, the intermediate reactions in mammalian melanogenesis are known as the Raper-Mason pathway, which depicts the formation of eumelanin from the amino acid tyrosine (figure 1.2A). The first two steps in this pathway are the hydroxylation of tyrosine to 3,4 dihydroxyphenylalanine (dopa) and then the oxidation of dopa to L-3,4 dioxyphenylalanine (dopaquinone). Both of these reactions are catalysed by the enzyme tyrosinase (see chapter 3). Dopa acts as a positive allosteric effector for tyrosine hydroxylation and is the hydrogen donor for the reaction (Hearing and Ekel, 1976). In the rest of the pathway, dopaquinone is converted to eumelanin by a complex series of autocatalytic reactions. Originally it was thought that only 5,6 dihydroxyindole (5,6-DHI) polymerises into eumelanin, but it is now believed that all the intermediates can partake in polymerisation, as indicated in the figure.

The "normal" course of eumelanogenesis can switch to pheomelanogenesis when dopaquinone interacts with cysteine or other related sulfhydryl compounds to produce cysteinyldopas (figure 1.2B). The major product formed by this reaction is 5-S-cysteinyldopa (5-S-cys). In the presence of a catalytic amount of dopa, 5-S-cys is oxidised to 5-S-cysteinyldopaquinone, which converts without enzymatic assistance via a quinoneimine intermediate to a phenolic isomer, or undergoes decarboxylation to form an additional benzothiazine intermediate.

Figure 1.2C depicts an overall view of the two alternative pigmentary pathways. Both pathways begin with the conversion of tyrosine to dopa to dopaquinone. In eumelanocytes, dopaquinone is converted primarily into eumelanin. In pheomelanocytes, some switch mechanism leads to the formation of pheomelanin. Evidence suggests that this switch is determined by the biochemical environment in the pigment cell, which is under genetic control. Also indicated in figure 1.2C is the formation of mixed type melanin, which arises by a copolymerisation process involving both eu- and pheomelanin precursors. Such an intermeshing of the two pathways appears to be a general feature, since purified eumelanic

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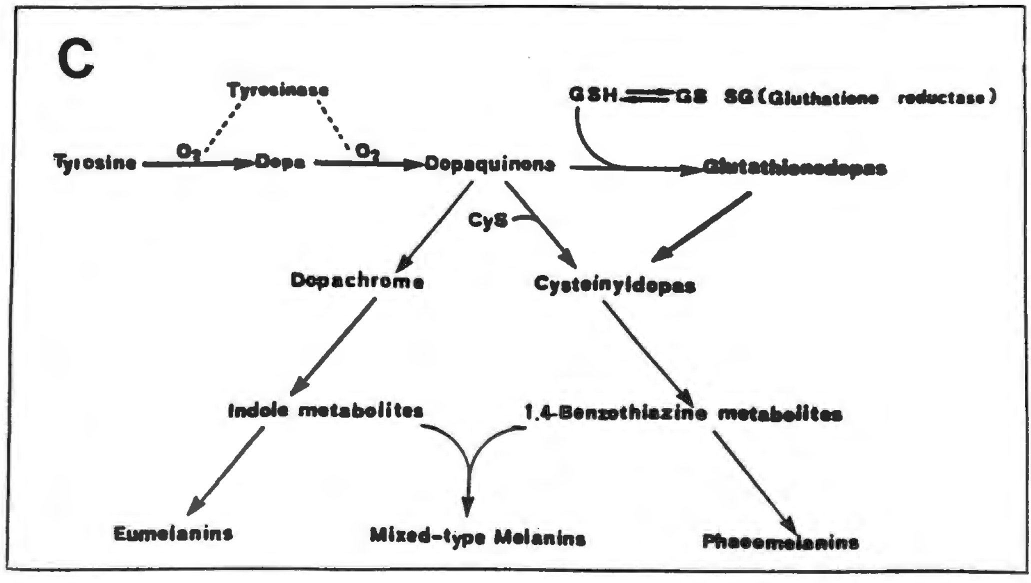
Figure 1.2. Biochemical pathways of eumelanin and pheomelanin synthesis. A: Synthesis of eumelanin from the amino acid tyrosine. B: Switching from the "normal" course of eumelanogenesis to pheomelanogenesis when dopaquinone interacts with sulfhydryl compounds. C: Overall view of the two alternative pathways. Mixed type melanin arises by a copolymerisation process involving both eu- and pheomelanin precursors. Adapted from Prota (1980).

Figure 1.2. Biochemical pathways of eumelanin and pheomelanin synthesis.



pigments almost always contain sulfur (up to 6%) (Ito et al.,1979;Novellino et al., 1980; Ito and Jimbow, 1983). Thus, pigments can be biochemically classified according to their eumelanin/pheomelanin ratio and are considered eumelanic when the amount of pheomelanin is very low, or pheomelanic when the amount of eumelanin is very low (Ito and Jimbow, 1983).

1.4. Regulation of melanin synthesis

An important control point in the regulation of melanogenesis is conceivably at the level of tyrosinase activity. Martinez et al. (1987) assigns a possible regulatory role to the concentration of copper (a constituent of the tyrosinase active site) in the cytosol of melanocytes. Their study showed that the dopa oxidase activity of the cytosol tyrosinases from mouse melanomas increases upon incubation with Cu(II), whereas the activity of melanosomal tyrosinase is unaffected. From this they suggested that non-melanosomal tyrosinase isozymes (see chapter 3) are in the apoenzyme form and that a reconstitution to the functional enzyme could take place in the melanosome. Devi et al. (1987), on the other hand, suggest that, depending on the intramelanosomal pH, tyrosinase may exist in a protonated form (strong cresolase activity) or nonprotonated form (weak cresolase activity), where alterations in the proportions of the two forms may regulate melanin synthesis.

The effect of MSH (melanocyte stimulating hormone) on tyrosinase activity in mammalian pigment cells has been extensively studied. Murine melanocytes respond to MSH by increasing their tyrosinase activity, followed by increased pigment synthesis (see Hearing et al. 1987). Results from Korner and Pawalek (1977) and Pawalek et al. (1980) have suggested that MSH increases tyrosinase activity by inactivating a tyrosinase inhibitor through a cAMP-mediated process. On the other hand, Halaban et al. (1984) concluded from their experiments that the increased tyrosinase activity is a result of an increase in newly synthesized tyrosinase. Recently, however, the possibility came to light that MSH may act by increasing the catalytic activity of a pool of inactive tyrosinase in some melanoma cells (see Fuller et al., 1988).

Other levels of biochemical control over melanin synthesis include the possibility of a feedback regulation through subtle changes in the concentrations of precursors and products of the pathway (Korner and Pawalek, 1982), and the existence of regulatory factors (purified from mouse melanomas) that either inhibit or accelerate various steps of the pathway (Korner and Pawalek, 1980; Pawalek et al., 1980). Briefly,

1/ Dopachrome conversion factor (DCF) promotes the conversion of dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (5,6-DHI2C), which is followed by a spontaneous decarboxylation reaction to 5,6-dihydroxyindole (5,6-DHI)

2/ 5,6-DHI conversion factor (ICF) promotes the conversion of 5,6-DHI to melanin

3/ 5,6-DHI blocking factor (IBF) restricts melanogenesis at 5,6-DHI.

A study by Hearing et al. (1982) showed that IBF activity in murine hair bulbs and melanoma cells is associated with the cytosol tyrosinases whereas melanosomal tyrosinase is associated with ICF activity. They proposed that the precursor tyrosinases are inhibited by complexing to IBF and become active within the melanosome by replacing its IBF with ICF. Appropriately, Hatta et al. (1988) later found substantial amounts of 5,6-DHI and 5,6-DHI2C within the coated vesicle fraction of pig melanoma tissue. Thus, conditions in the coated vesicles appear to allow melanogenesis to proceed up to the level of 5,6-DHI, despite the fact that the intermediates in the melanogenic pathway are cytotoxic (Lerner and Pawalek, 1978).

Finally, Prota (1980) is of the opinion that reduced glutathione, which is responsible for the formation of glutathionedopa from dopaquinone (see figure 1.2C), may play a regulatory role by converting part of the dopaquinone formed in melanocytes into glutathionedopa. The latter molecule cannot give rise to pheomelanins without enzymatic hydrolysis and, in the absence of this latter step, dopaquinone would be sidetracked from producing either pheomelanin or eumelanin.

CHAPTER 2.
TEMPEROSPATIAL MAPPING OF CHICK MELANOCYTES IN EARLY DEVELOPMENT

2.1. INTRODUCTION

The diversification of the dispersing neural crest cell population into different lineages poses the important developmental question of how these cells become restricted to particular phenotypes. Observations from many studies on the *in vitro* behaviour of crest cells support the notion that the environment plays a key role in neural crest differentiation. One way to begin a search for environmental factors that may influence the commitment of a particular crest derivative is to determine when and where such factors might exert their effects. A point of entry into this problem is to determine the time and place when the cells begin to express phenotype-specific genes *in vivo*. In the melanocyte lineage, melanin pigment is a visible marker of differentiation and can be identified with the light microscope. Thus, for the melanocyte lineage, the time and place of this differentiation step can be pinpointed by histological examination of embryos at various stages of development. Following this approach, however, it is important to bear in mind that although the appearance of melanin would reveal the onset of visible melanocyte differentiation, it would not have disclosed exactly when the crest cells became *committed* to the melanocyte lineage (see below).

2.1.1. Possible levels of commitment in differentiating melanocytes

In the literature, the term "melanoblast" usually refers to all crest cells migrating along the dorsolateral crest pathway. This term therefore identifies the probable destiny of these cells by virtue of the migratory route they are following, but does not specify their level of commitment to become melanocytes. Tentatively, the process whereby crest cells become restricted to the melanocyte lineage might therefore be divided into two phases. In the first phase, they may actually make the developmental decision to become melanocytes. It seems appropriate to define this as the differentiation of crest cells into *melanoblasts*. This phase possibly involves the selective activation of genes responsible for the synthesis of the pigment producing organelles (melanosomes), tyrosinase, and other factors involved in melanogenesis (see chapter 1). At this stage, melanogenesis might still be blocked at the transcriptional, or some post-translational level. As defined here, the concept "melanoblast" specifies cells which have become comitted to the melanocyte lineage, but, in response to further cues, are still yet to initiate pigment synthesis. Melanoblasts are possibly more

committed versions of crest cells with "*melanogenic potential*", such as the non-neuronal cells of cultured embryonic chick ganglia that adventitiously differentiate into melanocytes under appropriate culture conditions (Ciment et al., 1986).

In the second phase of melanocyte differentiation, melanoblasts transform into *melanocytes* as pigment synthesis begins (overt differentiation). From available data on fowls it appears that this differentiation step occurs only once the cells are settled in their epidermal destination. Melanocytes have been reported to appear in the chick epidermis at $7^1/_2$ days in Barred Plymouth Rocks (Watterson, 1942; Hopkins-Fox, 1949), at 9 days in Japanese quail epidermis (Le Douarin, 1982; Richardson et al., 1989) and Black Australorp epidermis (Dorris, 1939), and at 13 days in the Rhode Island Red epidermis (Dorris, 1939). Although dermally located pigmented melanocytes have been seen in 9 day Black Australorps (Dorris, 1939) and in $10^1/_4$ day Barred Plymouth Rocks (Watterson, 1942), they have been regarded as ectopic (Dorris, 1939; Sears and Ciment, 1988). Red breeds are reported as never exibiting pigment outside the epidermis (Dorris, 1939; Weiss and Andres, 1952).

That overt differentiation seems to occur in the epidermis does not imply that commitment to the melanocyte lineage (differentiation into melanoblasts) also occurs there - this step may take its course *en route* to the epidermis, in the dermis. With regard to overt differentiation, it might be supposed that the epidermis is the source of the factor/s for this event. Alternatively however, these factor/s may arise elsewhere, the epidermis being the appropriate tissue for reception of and/or response of the cells to these signals.

2.1.2. The dorsolateral pathway

The developmental restrictions that are imposed upon prospective melanocytes occur at some stage between the formation of the crest and the arrival of the cells at the epidermis. The embryonic tissues that supply or contain the appropriate cues for commitment to the melanocyte phenotype are therefore situated along the migratory route taken (the dorsolateral pathway), or within the target tissue. Evidence from several studies suggests that prospective melanocytes follow a dermal route of migration, from where they enter the epidermis. Teillet and Le Douarin (1970) used the quail-chick chimaeric system to study the migration of crest cells. In their system, quail neural tubes are grafted into chick hosts, and quail cells migrating from the crest are recognized by their distinctive nucleoli. It was reported that the quail cells invaded the region between the ectoderm and the somites, migrated through the dermal mesenchyme, and crossed into the epidermis from day 5 onwards. A study by Bronner-Fraser and Fraser (1988b) also support a dermal route of melanoblast migration. Cells of the intact crest were microinjected with lysinated rhodamine dextran, with the subsequent observation of labelled cells beneath the epidermis in $4\text{-}4^1/_2$ day chick embryos. Another endogenous cell labelling technique was used by Serbedzija et al. (1989).

A fluorescent carbocyanine dye (DiI), which irreversably incorporates into the plasma membranes of cells it contacts, was injected into the neural tube lumen at a stage when neural crest cells still have endfeet extended to the neural tube lumen. Once again, labelled cells were observed *beneath* the early chick epidermis. Interestingly, they further found that crest cells populate their derivatives in a ventral-to-dorsal order, with the latest emigrating cells exclusively following the dorsolateral pathway (see also Holmdahl, 1928).

In contrast with the now generally accepted theory that crest cells in the dorsolateral pathway follow a dermal route, observations by Weston (1963) suggested that they may follow an *epidermal* route of migration. In the latter study, the movement of crest cells was traced by transplanting radioactively labelled chick embryo neural tubes into unlabelled hosts. He reported that labelled cells left the crest between 50 and 55 hours of incubation, immediately entered the epidermis above the neural tube and then spread around the body within it. The disparity between these findings and those above has been suggested to result from artefacts of the grafting technique, with crest cells prematurely entering breeches in the ectoderm made during surgery (Erickson, 1988). From the above therefore, it seems that prospective pigment cells enter the dermal mesenchyme as the last emigrating group of crest cells, and then spread around the body within it. It is not known how far laterally the pigment cell precursors move before they enter the epidermis. According to Le Douarin (1982), the most active migration in chick skin occurs before day 6 of incubation, with a subsequent massive seeding of the epidermis by melanoblasts at the end of the 5th day and during day 6 of development.

2.2. AIM OF THE PRESENT STUDY

The developmental timetable of overt melanocyte differentiation in Black Australorp X New Hampshire Red (BA/NHR) fowl embryos has been examined. The aim was:

- to determine the developmental stage at which the melanocytes initiate pigment synthesis in this particular breed of fowl
- to quantitate the increase in melanisation subsequent to the age at which the first pigmented melanocytes appeared
- to determine whether overt differentiation occurs only in the epidermis or whether it can can also occur *en route* to the epidermis, in the dermal mesenchyme.

To this end, the temperospatial distribution of pigmented melanocytes in the skin of 4 to 10 day embryos was recorded and quantitated. The findings in this study contrast in more than one respect with previous reports and shed new light on melanoblast differentiation in fowl.

2.3. METHODS

2.3.1. Melanocyte counts

Black Australorp x New Hampshire Red eggs were incubated at 37°C (50-60% humidity). Embryos were removed and staged according to Hamburger and Hamilton (1951). Embryos aged 4, 5, $5^1/_2$, 6, 8, 9 and 10 days were fixed in Bouin's fluid for 3 hours, dehydrated in alcohols and embedded vertically in wax blocks to obtain serial cross sections (7 μm) through the neck region (just anterior to the front limb buds) and the body region (just posterior to the front limb buds). Sections of all stages were lightly stained with eosin. Haematoxylin staining was omitted since this obscured the melanin in faintly melanised melanocytes. A batch of sections from 8 day embryos were stained by the Periodic acid-Schiff (PAS) reaction to enhance the basement membrane (BM). Sections were viewed with a Nikon Microphot FX microscope and the developing epidermis and underlying mesenchyme (which I shall refer to as dermis) was scrutinised for the presence of melanin.

Neck and body regions were counted separately. Melanocytes were counted in every second section, starting directly lateral to the notochord and ending in the same region on the opposite side of the embryo (figure 2.1). Counts were based on the premise that any melanin visible, albeit only a few granules, represented one melanocyte. This principle was easily applicable in 5, $5^1/_2$ and 6 day skins (4 day skins contained no visible pigment) where differentiating melanocytes were few and scattered and contained small localised clusters of melanin granules, easily allocated to single cells. Examination of consecutive counted sections from these stages revealed that the same cells had not been counted twice. However, the accuracy of melanocyte counts in the 8 day epidermis was reduced in two ways. A dramatic increase in melanocyte number coupled with an intensification of the degree of melanisation (granules filling the cells up to the dendrite tips), made it impossible to always separate neighbouring cells accurately. The 9 and 10 day epidermis were so densely melanised that counts were omitted. In addition, cells in the 8-10 day skins occasionally extended across the intervening uncounted sections and were therefore counted twice. Thus, while the 5, $5^1/_2$ and 6 day counts depict a precise temperospatial pattern of melanocyte differentiation, the 8-10 day counts provide a slightly less accurate but nevertheless useful indication of melanocyte density and distribution.

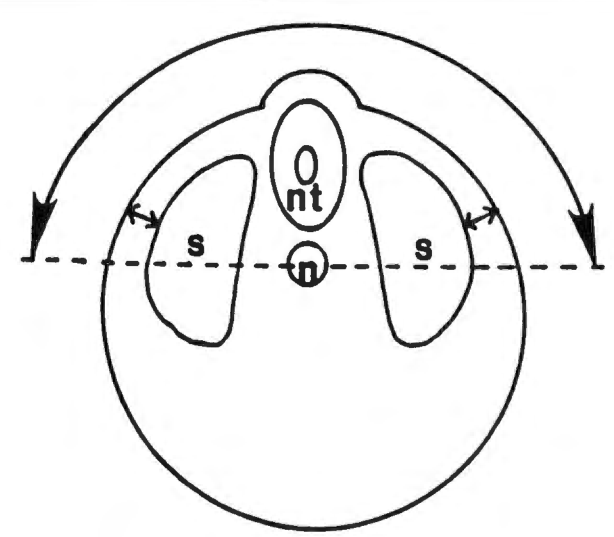


Figure 2.1. Schematic cross section through a chick embryo. Melanocyte counting in the epidermis and dermis (double-headed arrows indicate extent of dermal layer) was started directly lateral to the notochord and ended in the same region on the opposite side of the embryo (curved arrow). nt: neural tube; n: notochord; s: somite.

The counts from all sections of each embryo were averaged to obtain the mean counts per section for that embryo. The counts for all embryos of the same age were then averaged to give the mean regional values for embryos of that age. The results are therefore presented as melanocytes per 7 µm section, where n = number of embryos. To compensate for the age-related expansion of the analysed area, representative sections from the neck and body regions of all embryos were measured to determine the average cross-sectional area counted for each age-group. These values were used to normalise the counts for all ages to the 5 day neck or body equivalent. In addition, the distance of each dermal melanocyte from the epidermis was estimated visually.

2.4. RESULTS

2.4.1. Melanisation of embryonic chick skin

Melanocytes containing visible pigment were counted in sections of embryonic chick skin to determine the time and place of the first appearance of pigmented melanocytes and to quantitate the subsequent increase in melanisation. The first melanocytes were observed in sections from 5 day embryos. These cells contained a few small melanin granules that were faintly melanised (figure 2.2a). Between 5 and 6 days, the melanin granules per melanocyte and the intensity of melanisation increased noticeably. The granules were clustered together in small, localised areas in the cells (figure 2.2b). Melanocytes in the 8, 9 and 10 day skins were densely packed with dark melanin granules that often clearly delineated the dendritic processes of these cells (see figure 2.2c and 2.2d).

2.4.2. Temporal and spatial distribution of melanocytes in developing embryonic chick skin

2.4.2.1. Neck region

In 5 day skin, an average of 0,54 melanocytes/section was counted (figure 2.3a). Of these, 80% were located in the dermis. About half of the dermal melanocytes were found very close to the epidermis while the rest lay scattered up to a depth of more than 120 µm from the epidermis (figure 2.3c). The $5^1/_2$ day skin contained an average of 4,8 melanocytes/section of which 71% were dermal. Dermal melanocytes were not found preferentially located close to the epidermis but were more evenly distributed at all depths. Between $5^1/_2$ and 6 days, the average number of melanocytes had increased to 8,9 melanocytes/section; dermal counts had begun to drop and epidermal counts had risen so that by 6 days, 61% were located epidermally. Melanocytes found very close to the epidermis by far outnumbered those deeper in the dermis. After 6 days melanocyte counts increased dramatically and by 8 days, the average melanocyte counts had escalated to 91,6 melanocytes/section of which 25% were dermal. Of the latter, just under two-thirds were very close to the epidermis.

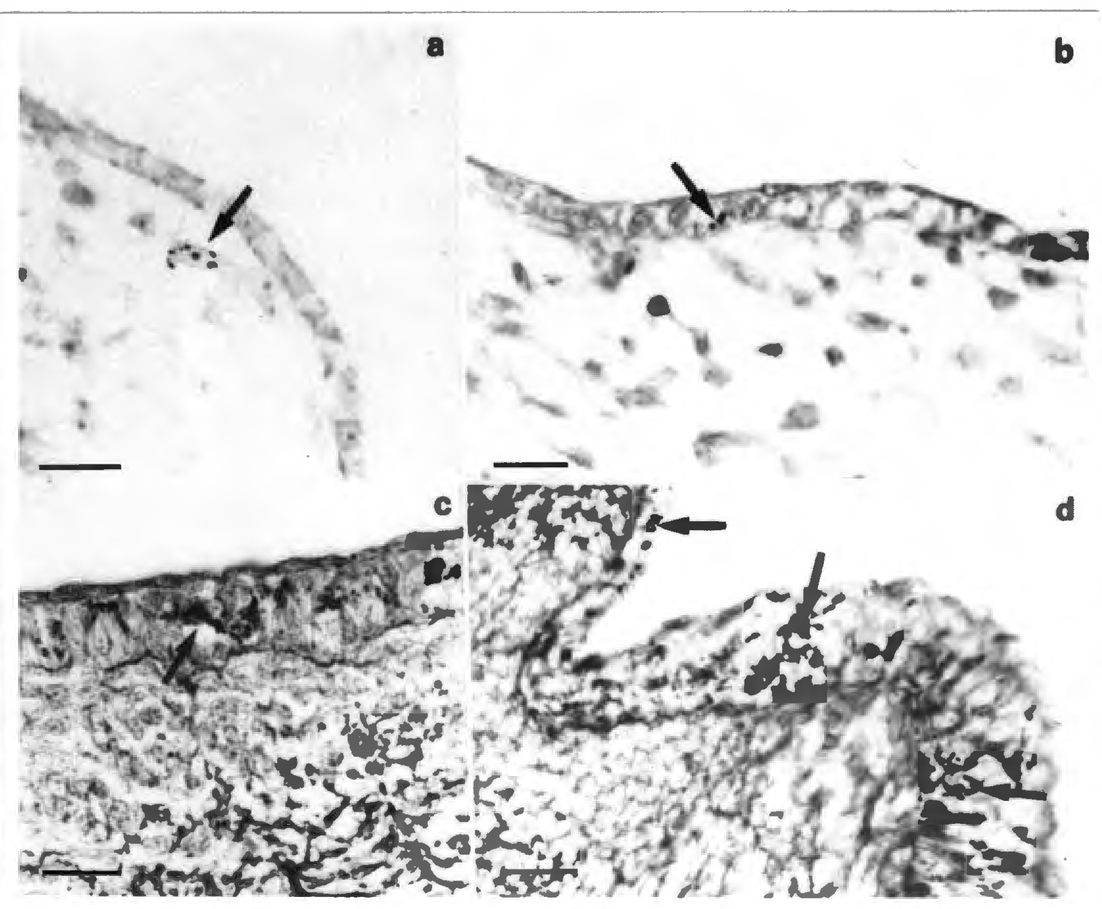


Figure 2.2. Melanisation of embryonic chick skin (arrows). (a) Dermal melanocyte in 5 day skin (eosin). (b) Portion of a melanocyte in 6 day epidermis (eosin). (c) Epidermal melanisation in 8 day skin (PAS). (d) Melanisation of the 10 day epidermis was so pronounced that individual melanocytes could not be distinguished (PAS). Bars = 20 μm.

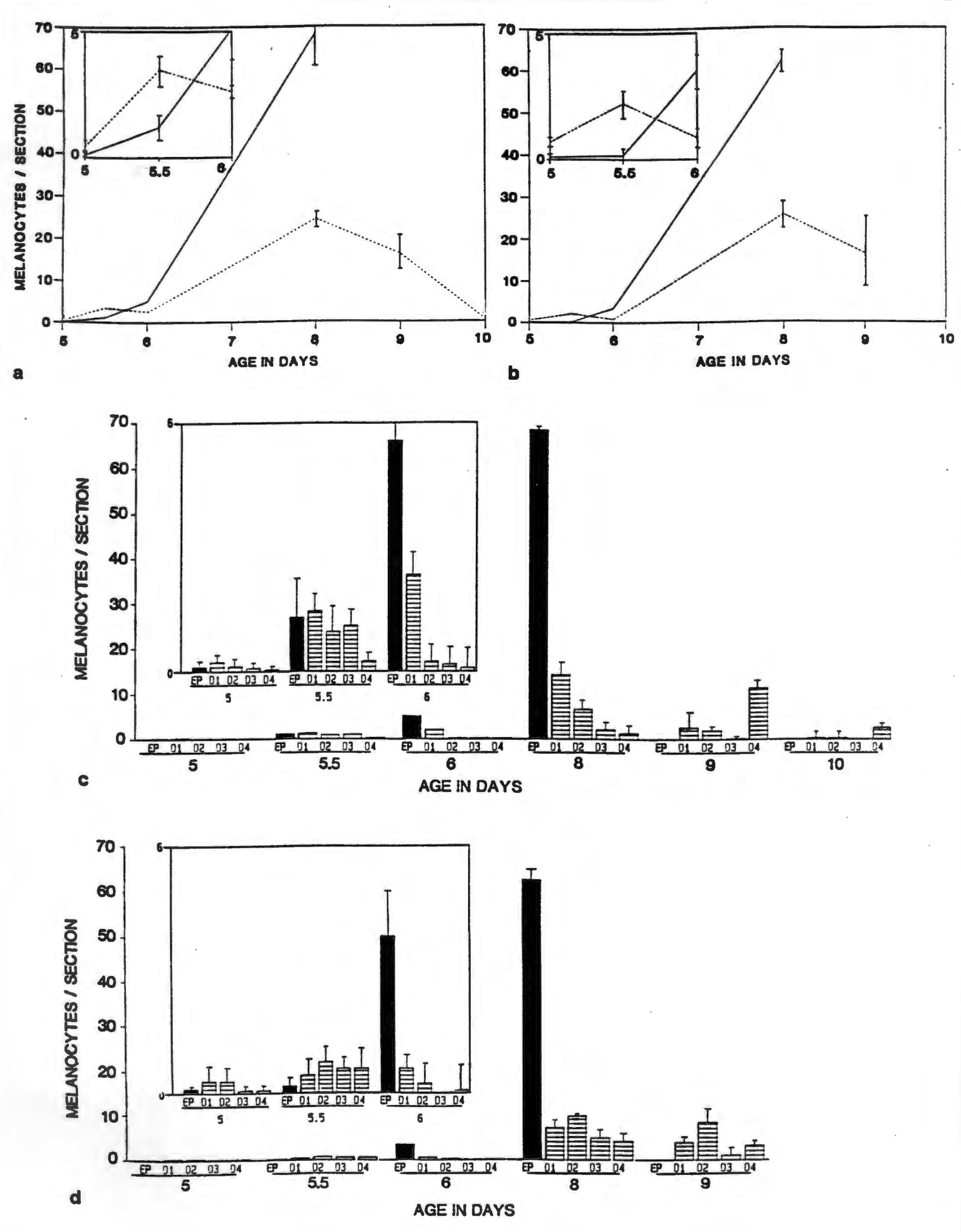
At stages later than 8 days, counts of individual epidermal melanocytes could not be obtained due to their abundance and close apposition (figure 2.2d). The average dermal melanocyte numbers decreased rapidly after 8 days so that only a few scattered melanocytes remained by 10 days (figure 2.3a and 2.3c). In contrast to counts of 5-8 day skin, dermal melanocytes in 9 and 10 day skin were in general not close to the epidermis but tended to lie deeper in the dermis.

2.4.2.2. Body region

The spatial and temporal distribution of melanocytes in the skin of the body region was found to be similar to that of the neck skin (figure 2.3b and 2.3d).

Figure 2.3. Melanocyte counts in embryonic chick skin from the neck (a) and body (b) regions. Solid lines = total epidermal counts; dotted lines = total dermal counts. Insets show enlargements of 5 to 6 day counts. Means were plotted with standard error bars where n = total number of embryos. (a) n values and the total number of sections examined from all embryos together (in brackets) for counts in 4, 5, $5^1/_2$, 6, 8, 9 and 10 day embryos were 5(20); 7(56); 5(37); 6(28); 2(9); 2(6) and 2(6) respectively. (b) n values and the total number of sections examined from all embryos together (in brackets) for counts in 4, 5, $5^1/_2$, 6, 8 and 9 day embryos were 2(12); 4(32); 5(34); 3(23); 2(8) and 2(4) respectively. Dermal melanocyte counts from the neck (c) and body (d) regions for each age of skin were subdivided into groups according to the visually estimated perpendicular distances of dermal melanocytes from the base of the epidermis. Solid bars (EP) = epidermal counts; hatched bars = dermal counts. Distances from the epidermis are indicated as follows: D1 = 0-24 µm; D2 = 36-60 µm; D3 = 72-108 µm; D4 = 108+ µm. Means were plotted with standard error bars where n = total number of embryos.

Fig 2.3. Melanocyte counts in embryonic chick skin.



2.4.3. Melanocytes with abnormal appearance

Many 8 day dermal melanocytes appeared abnormal (pyknotic) and the occurrence of such cells became more frequent in the 9 and 10 day dermis, where at least 60% of the dermal melanocytes appeared pyknotic. At light microscopic level, these cells were distinctly different from other melanocytes in that the cells contained a single large, round clump of melanised material as opposed to the fine, granular melanin of normal melanocytes (figure 2.4a and 2.4b). It is possible that these abnormal cells are either necrotic melanocytes or macrophages with ingested melanocyte pigment.

2.4.4. The 8 day skin contains melanocytes that span the dermal-epidermal interface

In 8 day skin, many melanocytes appeared to span the boundary between the dermis and epidermis (figure 2.4c). The long axis of such cells tended to be at right angles to the epidermis, with the widest part of the cell in the epidermis and an elongated arm lying in the dermis. It seemed that these cells were in the process of crossing the basement membrane (BM).

2.4.5. Anchor filaments in 8 day skin

A batch of 8 day sections was stained by the Periodic acid-Schiff (PAS) reaction to enhance the BM. During the counting procedure, the BM was examined for the presence of visible gaps. The BM appeared to be continuous, except for regions where melanocytes spanned the dermal-epidermal interface. In such regions the BM seemed to end abruptly on either side of the melanocytes. The PAS reaction also revealed numerous PAS-positive, fiber-like structures that merged with the BM and extended perpendicularly deep into the dermis (figure 2.5). Such structures were observed along the entire counting region. They seemed to originate from V-shaped downward protrusions of basal epidermal cells and occured either singly or in pairs (figure 2.5a). At their dermal ends, they often appeared to branch (figure 2.5d) or flare out to run parallel with the epidermis (figure 2.5c).

All the above features correspond to previously observed structures in developing chick skin and by virtue of their suggested anchoring function during feather germ elevation, they have been named "anchor filaments" or "anchor filament bundles" (Wessels 1965; Kischer and Keeter, 1971; Haake and Sawyer, 1982). Interestingly, dermal melanocytes frequently appeared to associate with the anchor filaments (figure 2.5b-2.5e). Several melanocytes were found lying close to or in contact with single filament bundles. On one occasion, a melanocyte appeared to be oriented towards the branched ends of a filament bundle (figure 2.5d).

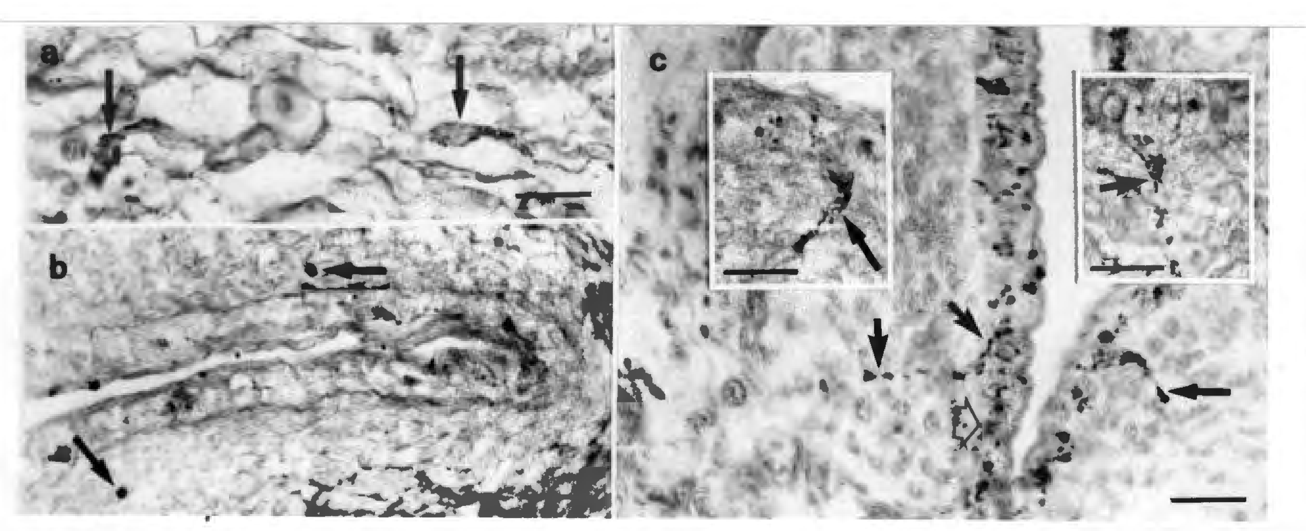


Figure 2.4. Melanocytes in 8 day dermis (arrows). (a) Melanocytes with normal appearance. (b) Abnormal melanocytes in which the melanin is clumped together, giving the cells a pyknotic appearance. (c and insets): Melanocytes spanning the epidermis and dermis in 8 day skin. Open arrow: BM. This photograph was a double exposure, causing the BM to blur. Although not clearly visible, the BM ends abruptly on either side of each melanocyte. Bars = 20 μm; (PAS).

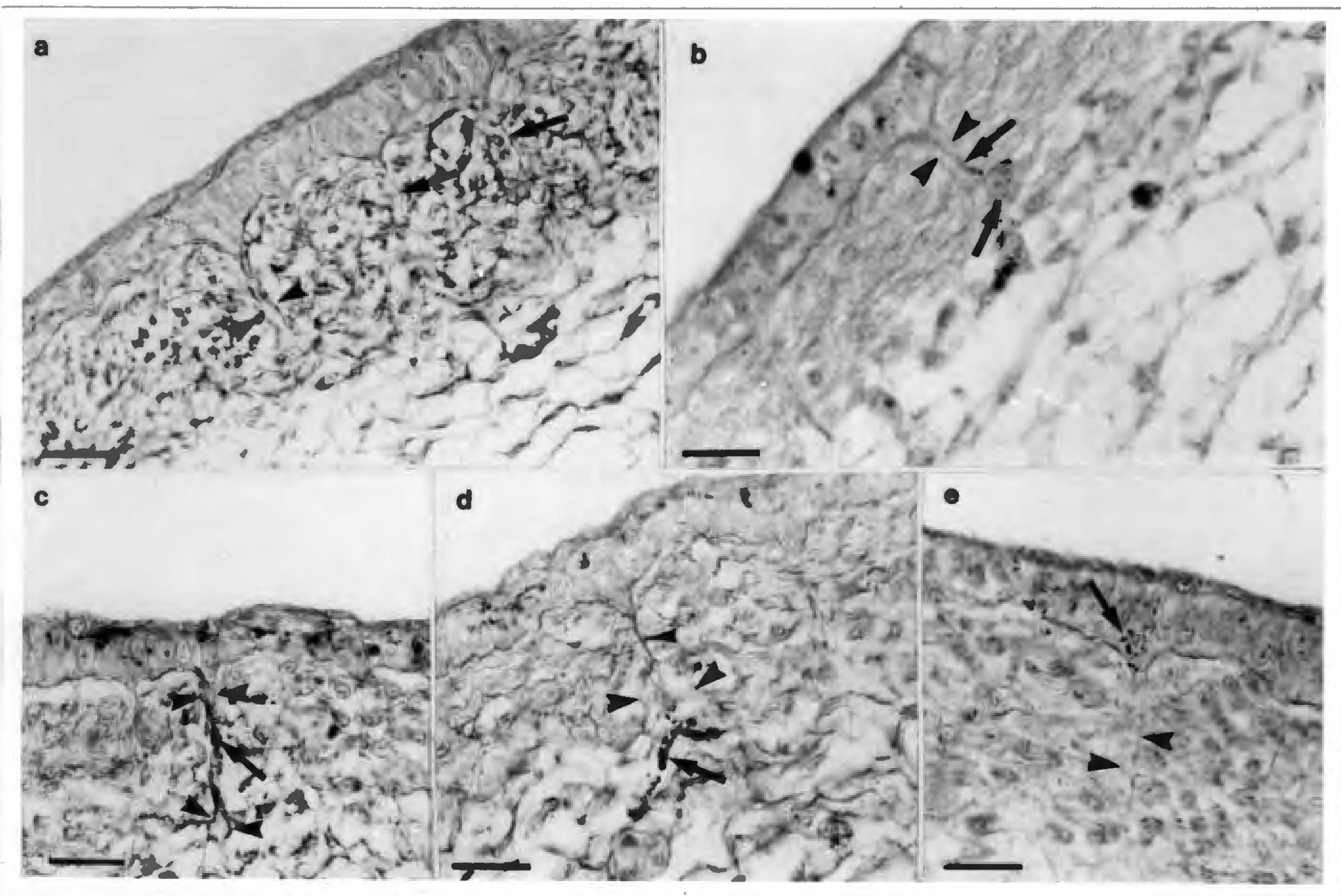


Figure 2.5. Anchor filament bundles in 8 day embryonic chick skin. (a) Paired (arrowheads) and single (arrows) PAS-positive, fiber-like processes (anchor filament bundles) extending perpendicularly from the epidermal BM into the dermis. (b) Two melanocytes beneath a paired filament bundle, one (arrows) with a dendrite extended between the two bundles (arrowheads). (c) Melanocyte (arrow) apparently lying wholly between a pair of filament bundles (arrowheads). Notice that the bundles flare out to parallel the epidermis. (d) Melanocyte (arrow) apparently oriented towards the branched ends of a filament bundle (arrowheads). (e) Melanocyte (arrow) lying in an epidermal outpocketing where a paired filament bundle (arrowheads) merges with the BM. Bars = 20 μm.

The most frequent observation was of melanocytes lying partly or wholly between paired filament bundles (figure 2.5b and 2.5c). Melanocytes were also often found in epidermal outpocketings where single or paired filament bundles merged with the BM (figure 2.5e).

2.5. DISCUSSION

The temperospatial distribution of overtly differentiating melanocytes in the dorsolateral pathway of BA/NHR chick embryos has been recorded by serial observations at regular intervals in 4 to 10 day embryos. By careful examination of the developing skin, it was found that visibly pigmented melanocytes, containing small numbers of faintly melanised granules, first appeared at 5 days of development. From $5^1/_2$ days onwards, the melanin granules per melanocyte increased rapidly in number and intensity of melanisation. The appearance of melanised cells at 5 days is surprisingly early. The earliest reported presence of pigmented melanocytes in other breeds is at $7^1/_2$ days in Barred Plymouth Rocks (Watterson, 1942; Hopkins-Fox, 1949). It is interesting to compare the appearance of melanocytes in the BA/NHR cross breed at 5 days with that reported for Black Australorps at 9 days (Dorris, 1939). These temporal differences between the onset of overt melanocyte differentiation in various genotypes suggest that the initiation of melanogenesis is breed specific (Hulley et al., 1991).

According to previous reports, overt differentiation of melanocytes occurs only once the cells are epidermally located (Dorris, 1939; Watterson, 1942; Hopkins-Fox, 1949; Le Douarin, 1982; Richardson et al., 1989), and occasional pigmented dermal melanocytes (Dorris, 1939; Watterson, 1942) have been regarded as ectopic (Dorris, 1939; Le Douarin, 1982; Sears and Ciment, 1988). In the present study, however, a substantial number of dermally located melanocytes were observed. In fact, pigmentation of melanocytes was found to be predominantly dermal at 5 and $5^1/_2$ days of development. The possibility that these melanocytes had entered the dermis from the epidermis is unlikely, since no pigmented melanocytes were observed in the 4 day skin. It also seems unlikely that the dermal melanocytes had crossed from the epidermis to the dermis as unpigmented melanoblasts that then began melanogenesis in the dermis. In support of this are results from grafting and tritium-labelling experiments of P. Hulley (published in Hulley, Stander and Kidson, 1991), which showed that there is little or no reverse migration of premelanocytes from epidermis to dermis in the embryonic skin of these fowl. Thus, it appears that overt melanocyte differentiation is not dependent on location within the epidermis.

The dermal pigmentation also suggests that the dorsolateral route of crest migration is not within the epidermis, as reported by Weston (1963), but through the dermis, from where the cells enter the epidermis. In addition, the counts provide several indications of a dermal to epidermal movement of pigmented melanocytes. The dermal distribution histograms show that the number of deeper melanocytes decreased between $5^1/_2$ and 6 days, while the

number of melanocytes closest to the epidermis increased, suggesting a movement of deeper melanocytes towards the epidermis. At the same time, the apparent drop in the total dermal count between $5^1/_2$ and 6 days suggests that melanocytes enter the epidermis from the dermis during this time. These findings correlate quite well with earlier observations in quail-chick chimaeric experiments. According to Le Douarin (1982), "the most active migration in the skin takes place before the 6th day of incubation and the advance of the front of migration can be followed in the experimental embryos. Seeding of the epidermis by melanoblasts takes place massively at the end of the 5th and during the 6th day of incubation ... melanocyte differentiation, as evidenced by pigment deposition, takes place from day 9 of development". The difference between the data above and that presented here is that in the BA/NHR embryos, the movement of overtly differentiated melanocytes could be followed, while the latter study described the movement of melanoblasts, as judged by their characteristic quail nucleoli. In the light of the inherent problems in chimaeric or radiolabelling studies, it seems particularly valuable to have discovered a breed of fowl in which visibly differentiated melanocytes are present during the terminal migrational events.

Between 6 and 8 days, there was an enormous increase in the number of dermal melanocytes and in the 8 day skin, several melanocytes were observed lying partly in the dermis and partly in the epidermis. That these melanocytes were moving in a epidermal-dermal direction is unlikely, since the dermal counts declined after 8 days. It would appear that the bulk of dermal to epidermal cross-over of melanocytes between 8 and 10 days occurs shortly after day 8 since no cells spanning the dermal-epidermal interface were found in the older skins. This ties in with the rapid drop in the dermal melanocyte numbers between 8 and 9 days. The drop continued between 9 and 10 days, so that only a few scattered melanocytes remained in the 10 day dermis. A contributing factor to the drop in melanocyte numbers between 8 and 10 days may be that some of the melanocytes die and are ingested by macrophages. Evidence for this is that the 8 day dermis contained a considerable number of apparently pyknotic melanocytes, and these cells became more frequent in the 9 and 10 day skin. Possibly the dermal environment becomes progressively more unsuitable for melanocyte survival after 8 days.

Although the counts show that some melanoblasts initiate melanogenesis first in the dermis and not in the epidermis, they cannot predict whether *all* melanoblasts initiate pigment synthesis in the dermis. Observations by P. Hulley suggest that some melanoblasts indeed cross into the epidermis in an unpigmented state. Pieces of 4 day BA/NHR epidermis were combined with pieces of dermis from an unpigmented strain, and cultured *in ovo* on chorio-allantoic membranes. Subsequent examination of the grafts revealed that pigmented feathers had formed, indicating that there must have been unpigmented melanoblasts in the epidermis by day 4 (Hulley et al., 1991). This latter finding may in part explain why the drop in

the dermal counts between $5^1/_2$ and 6 days cannot account for the simultaneous almost exponential rise in the epidermal counts during this time. Both pigmented and unpigmented melanocytes enter the epidermis, where the latter then presumably initiate pigment synthesis. An epidermal proliferation of either unpigmented or pigmented melanocytes or of both, may then contribute to the rapid increase in the epidermal counts. Le Douarin (1982) states that "epidermal melanoblasts ... divide actively before 9 days". Such a proliferation may relate to the finding that the basal keratinocytes of the mature skin produce basic fibroblast growth factor (bFGF), the natural growth factor and mitogen of melanocytes (Halaban et al., 1988). However, it is not known at what stage of development the keratinocytes begin the production of bFGF.

Crest cells become commited to the melanocyte lineage at some stage between the formation of the crest and their arrival at the epidermis. This commitment has been tentatively divided into two phases - first, the differentiation of crest cells into melanoblasts (committed to the melanocyte lineage), and then the overt differentiation of melanoblasts into melanocytes as pigment synthesis begins. It is not known whether these two events require different cues, but from the present study it appears that all the necessary signals for melanocyte differentiation can be received in the dermis, before cross-over into the epidermis. Whether the dermis provides instructive cues or merely a permissive environment for melanocyte differentiation remains to be determined. It is of course also possible that the actual source of the signals is the epidermis and that the dermal melanoblasts are able to receive them due to their close proximity to the epidermis. In such a case, the dermis would be providing a permissive environment for melanocyte differentiation.

However, the results from an *in vitro* study by Derby (1982) are in support of an *instructive* role for the dermis in avian melanocyte differentiation. Derby examined the differentiation of avian neural crest cells migrating through cell-free, deoxycholate-extracted extracellular matrices produced by various embryonic tissues. He found that crest cells in association with matrices extracted from dermal explants formed melanocytes, while those in association with matrices extracted from epidermal cell cultures failed to do so. Thus, he showed that some deoxycholate-resistant structural component of the dermal, but not epidermal extracellular matrix, is able to induce avian melanocyte differentiation. If the dermis does indeed contain all the instructions for melanocyte differentiation, the question arises whether the cells can only receive these signals in the dermis or whether the signals can also be received by cells having entered the epidermis. The second option would definitely apply if the signals only became available in the dermis at a time when some melanoblasts had already entered the epidermis.

The onset of crest migration occurs in conjunction with the orderly closure of the neural tube from the anterior to the posterior end of the embryo. Thus, the extent of migration varies

at different axial levels - cells in the anterior trunk region will have migrated a considerable distance while those at the most posterior axial levels still reside in the neural tube (Tosney, 1978). The counts in the present study were done separately at two different axial levels of the embryo - the neck region, and the body region. Thus, it might have been expected that the timing of melanocyte differentiation in the body region would lag behind those occuring in the neck region. However, comparison of the timing of neck and body counts show that they exibit identical trends. Perhaps if the second set of counts had been obtained from a more posterior axial level such as the tail region, noticeable temporal differences between the two sets of counts would have emerged. It was noted, however, that while events between all ages were similar in the neck and body, they seemed to occur on a slightly smaller scale between 5 and 6 days in the body. The 8 day epidermal count was also lower for the body than the neck, but the 8 day dermal body counts were almost identical to the head counts.

Since the crest pathways define position only, cues must be available for the presumptive melanocytes to travel in particular directions. Several mechanisms have been proposed to impose directional movement on crest cells (for a review see Erickson, 1988). These include haptotaxis (movement up an adhesion gradient), chemotaxis (diffusable molecules attracting or repelling crest cells), galvanotaxis (directed migration in response to an imposed electrical field) and contact inhibition (directional inhibition due to cell-cell contact). Erickson (1985) grafted cultured crest cells ahead of the wave of crest migration and found that some grafted cells migrated counter to their usual direction, towards the neural tube. This provided evidence against the idea that crest cells follow haptotactic or chemotactic gradients in the crest pathways. In the same study, it was clearly demonstrated that crest cells exibit contact paralysis when touching each other. Thus, contact inhibition was considered the best explanation for crest migration *in vivo* (see also Tosney, 1978 and Newgreen et al., 1982). Erickson (1988) however points out that mechanisms other than contact inhibition are needed to explain directional migration along the dorsolateral pathway. By filming migrating pigment cells in the axolotl embryo, Keller and Speith (1984) found that the cells were always persistent in their directional migration, yet apparently did not contact each other. In the present study also, dermal melanocytes were never seen to contact each other, although this was not confirmed at the ultrastructural level.

How then, are melanoblasts directed to the epidermis? The mechanics of this process is open to speculation since it has not yet been causally linked to any particular mechanism. It is possible that two different sets of signals are involved. The first set may function to direct the crest cells away from the neural tube, spreading them around the body within the dermal mesenchyme. Another set of cues might then direct the movement of melanoblasts/cytes from the dermis to the epidermis. Results from some previous studies have provided indirect clues about the dermis-to-epidermis component of melanoblast/cyte movements. De Luca et

al. (1988) co-cultured melanocytes with either stratified epidermis or oral epithelium (in the total absence of any underlying connective tissue) and found that the melanocytes invaded both epithelia. This supports the idea that stratified epithelia alone can direct melanocyte invasion. Wachtler (1984) grafted quail neural crest cells to the wing buds of an unpigmented chicken strain and found that differentiated quail melanocytes almost exclusively landed up distal from the graft in the host's epidermis. When the apical ectodermal ridge (AER) region was removed from an operated bud, this directed cell migration ceased, indicating that the excised tissue played a role in this process. The same effect was obtained when gold-leafs (which should not interfere with a supposed electrical field due to its low electric resistance) were implanted beneath the AER, making galvanotaxis an implausable explanation for the directed migration. Since this process could be abolished by interposing an impermeable material between the AER and the graft, it appeared that the AER directed the migration of the melanoblasts by a chemotactic mechanism. However, in all experiments, many dermally located melanocytes of donor origin were found both proximal to and distal from the grafts. This implies that the AER had directed the movements of epidermal donor melanoblasts while apparently having no effect on those in the dermis. Thus, although Wachtler had shown that melanoblasts in the wing bud epidermis may respond to chemotactic signal/s emanating from the AER, we are no closer to understanding the mechanisms that guide melanoblasts/cytes from the dermis into the epidermis.

In order for melanoblasts/cytes to invade the epidermis, they need to cross the epidermal basement membrane (BM). From previous studies it appears that neither crest cells nor melanocytes are able to invade intact BMs. Gehlsen and Hendrix (1987) showed that human amniotic BMs are impenetrable physical barriers to both these cell types *in vitro*. Similarly, when crest cells or melanocytes were confronted with embryonic BMs by grafting them into the neural tube lumen, they were unable to breach the basal surface of the neural tube (Erickson, 1987). It seems from other reports that the passage of crest cells through BMs requires alterations in the composition/condition of the intact BM. Ultrastructural studies on the initiation of crest migration have shown that the basal lamina overlying emigrating crest cells is depleted or absent (Erickson and Weston, 1983; Lofberg et al., 1985). In the mouse, this pattern has been correlated with a deficit of type-IV collagen (Sternberg and Kimber, 1986).

In the present study, as mentioned earlier, several melanocytes were apparently in the process of entering the epidermis from the dermis at 8 days of development. A batch of 8 day skin sections were therefore stained with PAS to enhance the BM. Examination of the 8 day PAS-stained epidermal BM suggested that it was continuous (at least its glycoprotein component), except for regions where melanocytes spanned the dermal-epidermal interface. In such regions, the PAS-stained BM appeared to end abruptly on either side of the

melanocytes. If the apparent gaps in the BM through which these melanocytes were moving had been pre-established, they may have been too small prior to melanocyte entry to be discernable by light microscopy. In this respect, Martins-Green and Erickson (1987) have shown by immunolabelling studies that seemingly continuous BMs (light microscopy) can be misleading, since ultrastructurally, basal laminae from the same regions may reveal gaps.

Another possible means by which melanoblasts/cytes may gain access to the epidermis is by producing BM-degrading enzymes. Erickson (1987) suggested that the crest cells may secrete BM-degrading proteases during the time when they lie beneath the epidermis. In support of this concept is the finding that crest cells begin to produce increasingly higher levels of plasminogen activator (PA) as they differentiate into pigment cells (Erickson, 1988). PA converts inactive plasminogen to plasmin, which is capable of breaking down BM glycoproteins (Saksela and Rifkin, 1988). Finally, it is worthy in the present context to note the recent finding on the effect of epidermal growth factor (EGF) on the embryonic chick epidermal BM. It has previously been shown that EGF receptors are spatially correlated with the basal epidermal cells in embryonic rat skin (Green and Couchman, 1984). Akimoto et al. (1988) found that the BM of chick embryo skin explants cultured in media containing EGF frequently became discontinuous with many gaps. This raises the possibility of *in vivo* EGF-induced disruptions in the epidermal BM, through which melanoblasts/cytes may enter the epidermis.

The original purpose of PAS-staining a batch of 8 day skin sections was to enhance the BM. Interestingly however, the PAS-stain also revealed a frequent apparent association of dermal melanocytes with PAS-positive fibrous processes extending perpendicularly from the BM into the dermis. Such processes have been described in previous studies, but none contain any reference to an association with melanocytes. From his study on the early stages of chick embryo feather development, Wessels (1965) reported that at the time of feather placode formation, long "fiber-like processes" extended deep into the dermis, seemingly originating from downward protrusions of epidermal basal cells. Later, Kallman et al. (1967) described such processes as "anchor filaments". Comparison of the light microscopical appearance of the filaments observed in the present study with descriptions in the literature (Wessels, 1965; Kischer and Keeter, 1971) indicate that the structures are indeed "anchor filaments". Ultrastructural observations of Kallman et al. (1967) offered evidence that the filaments originate from dermal fibroblasts and terminate in the BM. They suggested an analogy of the filaments to a growing tree, where the roots or origins of the filaments are thin and often widely separated in the dermis. As they near their termination in the BM, the filaments converge into bundles to form the main trunk of the tree.

Anchor filaments have consistently been found to be prominent in the early feather germ and absent from the elevated feather germ. This has led to the suggestion that they are

involved in some aspect of feather morphogenesis (Wessels, 1965; Kallman et al., 1967; Kischer and Keeter, 1971; Kischer, 1968; Haake and Sawyer, 1982). Kischer and Keeter (1971) suggested that during feather germ elevation the filaments function to anchor the epidermis to the dermal mesenchyme in the interfollicular skin, allowing differential growth of the feather organ. In 1982, Haake and Sawyer showed by indirect immunoflorescence that anchor filaments are strongly fibronectin positive. Considering the known ability of fibronectin to promote directional migration of fibroblasts (Postlethwaite et al., 1981), they proposed that the filaments may act as substrata for the directed migration of presumptive dermal fibroblasts from the underlying dermamyotome during formation of the dense dermis beneath the epidermal placodes. In support of their suggestion was their frequent observation, as also reported by Kallman et al. (1967), of fibroblasts oriented along the anchor filaments during dermal condensation.

In the present study, dermal melanocytes were often found to be 1/ in contact with single bundles of anchor filaments somewhere along the length of the bundles, or on one occasion, apparently in contact with several "roots" of a filament bundle; 2/ lying partly or wholly between paired filament bundles, or 3/ lying within downward protrusions at the epidermal base, where single or paired filament bundles merged with the BM. These observations raise the possibility that the filaments may play a role in the directional migration of melanocytes toward the epidermis. An interesting question, for example, is why most of the observed associations were of melanocytes or portions of melanocytes lying between paired filament bundles. The distribution of the filaments and dermal melanocytes were however not statistically treated and therefore one cannot predict whether the associations are significant or coincidental. Although such apparent associations were frequently noted, many dermal melanocytes lay long distances away from the nearest filaments. These observations therefore need further investigation. A possible explanation for the associations is that melanocytes have been found to show preference for fibronectin as a migration substrate (Duband and Thiery, 1987). Thus, being in the vicinity of the fibronectin-rich anchor filaments, they may coincidentally use the filaments as a preferred substrate during their migration toward the epidermis.

CHAPTER 3.
PURIFICATION OF CHICK TYROSINASE FOR ANTIBODY PRODUCTION

3.1. INTRODUCTION

An obvious phenotype-specific characteristic in the melanocyte lineage is the synthesis of melanosomes and the deposition of melanin within them. This process cannot occur without tyrosinase, the key enzyme in melanogenesis (see below). The initiation of transcription and translation of tyrosinase gene/s in melanoblasts is therefore an important differentiation step in this lineage. The aim of the present study was to produce a polyclonal anti-chick tyrosinase antibody, to answer the question of where and when tyrosinase is first synthesized in Black Australorp X New Hampshire Red (BA/NHR) embryos.

3.1.1. Tyrosinase - a general background

Tyrosinase is a glycoprotein containing copper at its active site. It is found in a wide range of plant and animal species, and catalyzes the first two steps in the melanogenesis pathway - the hydroxylation of tyrosine to dopa (dihydroxyphenylalanine) and then the oxidation of dopa to dopaquinone (see chapter 1). Evidence has recently been presented that tyrosinase may catalyze a third reaction in the eumelanogenesis pathway, namely, the oxidation of 5,6-dihydroxyindole (5,6-DHI) to indole 5,6 quinone (Korner and Pawalek, 1982). However, this third catalytic activity has not yet been clearly determined (Hearing and Jiménez, 1987). In addition, tyrosinase may also be involved in the conversion process of melanin monomers to the melanin polymer (Hearing et al. 1982).

Extensive research on mammalian tyrosinase has shown that it is synthesized as a unique polypeptide (Ohtaki and Miyazaki, 1973; Hearing et al., 1981) which undergoes an elaborate process of post-translational modification, involving the addition of N-acetyl-glucosamine, α-linked mannose and sialic acid (Ferrini et al., 1987). This post-translational glycosylation presumably primarily determines the specific targeting of the enzyme to the melanosomes (Hearing and Jiménez, 1987). In accordance with this idea is the fact that glycosylation inhibitors have been shown to cause a selective and marked decrease in melanosomal tyrosinase activity and a concurrent depigmentation of melanosomes (Imokawa and Mishima, 1982).

The post-translational processing of mammalian tyrosinase results in multiple isozymic forms (Ferrini et al., 1987). Hearing et al. (1978) purified four isozymic forms of tyrosinase from whole melanoma tissues, and named them T1, T2, T3 and T4* in order of decreasing

* not to be confused with similar abbreviations for the thyroid hormones and their precursors.

electrophoretic mobility in non-denaturing gels. Their report proposed that T3 is the *de novo* form of the enzyme, and that T1 arises from it after glycosylation of T3. T2 is apparently an artefact of the electrophoretic procedure, arising from the deamination of T1. The insoluble form, T4, arises from T1 by associating with melanosomal constituents (see figure 3.1).

Although all details of the post-translational events are still far from clarified, the main sequence of conversions (T3 - T1 - T4) as proposed above has been supported by data from other studies, and this numbering system is widely accepted. However, several reports still refer to melanosomal tyrosinase as "T3". This stems from the nomenclature of earlier studies such as that of Burnett et al. (1967), who isolated three tyrosinases from mouse melanoma and called the two soluble, cytoplasmic forms T1 and T2, and the insoluble, melanosomal tyrosinase, T3.

In vivo, melanin synthesis is restricted to melanosomes (catalysed by the T4 isozyme), whereas all isozymes can initiate melanogenesis *in vitro*, even after trypsin treatment (Miyazaki and Seiji, 1971; Yamamoto and Brumbaugh, 1984) or removal of the carbohydrate residues (see Halaban et al., 1984). Thus, some inhibitory mechanism prevents melanogenesis outside of the melanosomes, despite the presence of active tyrosinase. This mechanism apparently operates at the level of dopachrome formation, since Hatta et al. (1988) have shown that coated vesicles from melanoma tissue contain substantial amounts of 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carbolic acid.

Molecular weight estimations of isolated tyrosinase isozymes vary considerably. While little information is available on avian tyrosinase, cumulative results of many laboratories on mammalian tyrosinases have provided the following approximations, as summarised by Hearing and Jiménez (1987). The *de novo* form has a molecular weight of about 55 kd (T3). After glycosylation, during transferral through the Golgi complex, its weight is approximately 70 kd (T1). The mature enzyme is delivered to the melanosomes, where it aggregates into a high molecular weight form (T4). Table 3.1 summarises some of the reported molecular weight estimations of mammalian and avian tyrosinase.

3.1.2. Problems related to the purification of tyrosinase

Studies on tyrosinase have been facilitated by the relative ease with which its presence can be demonstrated in a given tissue. Since all the steps in the pathway subsequent to the first two (that require tyrosinase) can occur spontaneously, and because melanin is an easily identifiable product of melanogenic activity, tyrosinase activity can be demonstrated visually in test tubes, non-denaturing gels and in subcellular components in tissue sections. This is achieved by adding exogenous dopa to a tyrosinase-containing sample, leading to the autocatalytic formation of dark melanin (the "dopa reaction"). Another, more sensitive assay

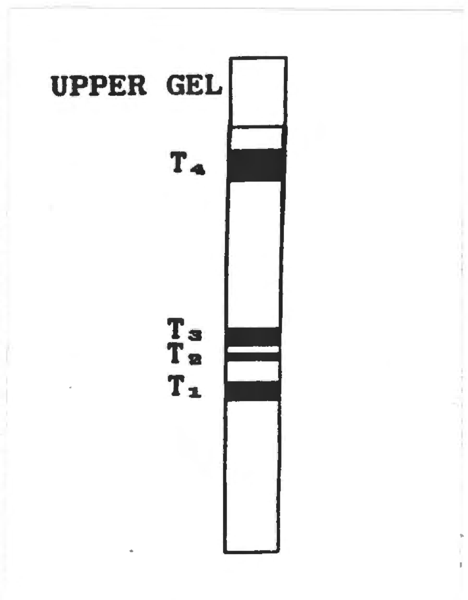


Figure 3.1. Pattern of tyrosinase isozymes after electrophoresis of a crude melanocyte extract in the presence of non-ionic detergents and visualisation by histochemical dopa staining. Adapted from Hearing and Jiménez (1987).

Table 3.1. Some of the reported molecular weight estimations of mammalian and avian tyrosinases.

Reference	source	approximate molecular weight in daltons
Burnett (1971)	Harding-Passey melanoma cells	56 700 66 000
Ohtaki and Miyazaki (1973)	Harding-Passey melanoma cells	56 000 66 000
Hearing et al. (1978)	C57B1 mouse epidermis	70 000 to 85 000 and 54 000 to 70 000
Jimbow et al. (1981)	B16 melanoma cells Harding-Passey melanoma cells	73 000 to 74 000 72 700 to 74 000
Tomita et al. (1983)	Harding-Passey melanoma cells	67 000
Halaban et al. (1984)	Cloudman melanoma cells	68 000 72 000 78 000 80 000
Tomita et al. (1985)	B16 melanoma cells	70 000
Ferrini et al. (1987)	Harding-Passey melanoma cells	70 000
Yamamoto and Brumbaugh (1984)	chick proximal feather sheath tissue	66 000
Boissy et al. (1987)	Avian tyrosinase positive albinos	68 000 82 000

for tyrosinase activity, used in the present study, makes use of ^{14}C tyrosine, and measures radiometrically the formation of melanin from melanin precursors.

In contrast with these advantages, there are several inherent problems to overcome when attempting to purify tyrosinase in order to produce anti-tyrosinase antibodies. To begin with, normal tissues contain very few melanocytes (±1 ml melanocytes/person) and within them, tyrosinase constitutes less than 0,1% of the total protein (Hearing and Jiménez, 1989). For mammalian studies, the scarcity of starting material has mostly been alleviated by using melanoma cell lines. Even so, Jiménez et al. (1988) report that tyrosinase constitutes only approximately 0,04% of the total protein in B16 melanoma cells. In a study of embryonic skin (the present study), this scarcity factor no doubt creates an even greater problem.

A second problem is the electrophoretic behaviour of tyrosinase. Although each of the isozymic forms migrate as a discrete band, the bands have a dispersed appearance (Burnett et al., 1967). This reproducible microheterogeneity results from the existence of multiple sub-isozymic forms, probably varying in degree of post-translational modification (Ferrini and Hearing, 1987).

A further complication is firstly the tendency of tyrosinase to synthesize "sticky" melanin that drags along contaminants (see Lerch, 1988; Hearing and Jiménez, 1989) and secondly, the fact that most of the tyrosinase isozymes have a molecular size common to many other proteins. This latter problem leads to the co-localization of other proteins with similar weight and mobility to tyrosinase during preparative gel electrophoresis. Moreover, since tyrosinase is a conserved protein (Tomita et al., 1985; Boissy et al., 1987) and thus relatively non-immunogenic, it could be expected that minor contaminants in a tyrosinase sample will evoke the production of unwanted antibodies greatly exceeding those against tyrosinase.

3.2. MATERIALS AND METHODS

3.2.1. Partial purification of tyrosinase

3.2.1.1. Preparation of 11 day chick skin homogenate

Batches of BA/NHR eggs were incubated for 11 days at 37°C, 50 - 60% humidity. Embryos were removed from the eggs and placed in dishes containing sterile chick saline (0,12 M NaCl, 5 mM KCl, 1 mM $CaCl_2$, pH 7,4). Skins were dissected in chick saline and collected in tubes which were kept on ice throughout the dissecting period. The skins were then rinsed in 0,1 M sodium phosphate buffer, pH 7,4 (0,1 M PB) to remove excess blood and chick saline.

Two volumes of 0,1 M PB containing 1% Triton X-100 and phenyl-methyl-sulphonyl-fluoride (10^{-6} M) was added to each skin sample. This mixture was violently homogenised in a Polytron homogeniser (2 x 5 seconds) and left on ice for 1 hour to allow membrane solubilization. The mixture was again violently homogenised (2 x 5 seconds) and then centrifuged at 10 000 g for 5 minutes in an Eppendorf micro-centrifuge to pellet cell debris. The supernatant was collected and frozen in aliquots of 50 µl at -20°C until used.

3.2.1.2. Anion exchange chromatography

A. Preparation of the columns

Ion-exchange columns with a bed volume of 8 ml were prepared in 10 ml sterile plastic syringes, using preswollen DEAE-Sephacel anion-exchanging beads (Pharmacia). Glasswool served as a filter at the base of each column. The columns were equilibrated with 20 mM Tris (pH 8) until the OD_{280} was zero and the effluent pH was 8.

B. Chromatography

All columns were run at room temperature under gravity. One milliliter fractions were collected and kept on ice, and the passage of proteins through the columns was followed on a Shimadzu spectrophotometer by monitoring the absorbence at 280 nm. The sample to be applied (as prepared in 3.2.1.1) was mixed with an equal volume of 20 mM Tris (pH 8). One or

two ml of this sample was allowed to run into the column. Unbound substances were then washed through the column with Tris buffer (20 mM, pH 8) (first peak) until the OD_{280} of the effluent was < 0,3. The Tris buffer was then replaced with another buffer, consisting of 0,3 M NaCl + 20 mM Tris (pH 8). This elution buffer removed the bound sample from the column, which consistently emerged as a single peak. Various column fractions from each chromatography run were assayed for tyrosinase activity by the radiometric assay (see below).

High tyrosinase fractions from different experiments were pooled and concentrated against polyethylene glycol (6000) to a final volume of 500 μl, and stored at -20°C until needed.

3.2.2. Radiometric assay for tyrosinase activity

This method makes use of ^{14}C-tyrosine to measure the formation of acid insoluble melanin from melanin precursors and was carried out as a modification of the technique presented by Hearing and Ekel (1976). Each assay (final volume 25 μl) contained:

10 μl sample
5 μl L-(U-^{14}C) tyrosine (25 μCi/ml, specific activity 513 mCi/mmol; The Radiochemical Centre, Amersham)
5 μl of a solution containing:
  1 mg/ml chloramphenicol;
  1 mg/ml cyclohexamide;
  1000 iu/ml penicillin-G;
  0,1 mg/ml bovine serum albumin (Miles-Seravac)
5 μl of 0,05 mM L-dopa in 0,1 M PB

The controls were blanks from which the sample was omitted and replaced with buffer. Assays were run in duplicate, overnight at 37°C. After incubation, 20 μl samples were removed to Whatman GF/C glass fibre disks. The disks were then treated as follows:

- three 10 minute washes in 100 ml of 10% trichlor- acetic acid (TCA) (BDH);
- one 5 minute wash in 95% (v/v) ethanol (Merck);
- one 2 minute wash in acetone.

The filters were dried for 30 minutes at 37°C, then solubilized in 200 μl Soluene (Packard) for 2 hours at 37°C, after which 6 ml of toluene (Packard) was added to each sample. The samples were then counted for radioactivity in a Beckman scintillation counter.

3.2.3. The spectrophotometric dopa assay

This assay is a variation of a technique described by Mitchell (1966), and is based on the oxidation of dopa to dopachrome in the presence of tyrosinase. Samples were mixed 1:10 with a dopa solution (1 mg/ml in 0,1 M PB) and the amount of product at a given time (tyrosinase activity) was given as the OD_{475}, as measured on a Shimadzu spectrophotometer.

3.2.4. Polyacrylamide gel electrophoresis (PAGE)

3.2.4.1. Gel electrophoresis without detergent (non-SDS PAGE)

Preparative gel electrophoresis was carried out according to a modification of the method originally described by Davis (1964). The pooled and concentrated partially purified tyrosinase

sample (see 3.2.1.2B above) was run at 60-80 V on a 7,5% slab polyacrylamide gel in a Tris/glycine buffer devoid of SDS (see Appendix 1).

3.2.4.2. SDS-PAGE

Polyacrylamide gels (10%) containing 0,4% SDS were prepared and samples were run in a Tris/glycine buffer (see Appendix 1) for 3 hours at 120 V and stained for proteins with PAGE blue. Standard molecular weight markers (Pharmacia) were run on all gels. Molecular weights were calculated by plotting the distance migrated against the log of the molecular weight.

3.2.5. Visualization of tyrosinase activity in polyacrylamide gels

After electrophoresis of the partially purified tyrosinase sample on a non-SDS gel, the gel was neutralized in 0,1 M PB for 30 minutes. Two vertical strips, one from each lateral end of the gel, were cut off and incubated in a 1 mg/ml solution of L-dopa in 0,1 M PB at 37°C. The location of active tyrosinase in the gel strips was visualised as darkly stained bands. The strips were lined up with the remainder of the gel and the corresponding horizontal strip containing active tyrosinase was carefully cut out and stored at -20°C until needed.

3.2.6. Preparation of the antigen

The strip of gel containing active tyrosinase was thawed and then crushed into small pieces in a Dounce homogeniser. One milliliter of 0,1 M PB was added to the gel pieces and the mixture poured into the back of a sterile 10 ml syringe which had been sealed at the narrow end. A wide diameter sterile needle was placed on the syringe and, after first expelling most of the buffer, the gel pieces were squeezed into the back of a 5 ml syringe. This process crushed the gel into a much finer pulp. A small diameter needle was placed on the syringe and the content in the syringe forced up to the tip of the needle. The final volume was approximately 2 ml. The syringe, with its needle, was stored at -20°C until needed for immunisation.

3.2.7. Immunization

A white female guinea pig was obtained from the Central Animal Unit, University of Cape Town. The guinea pig was anaesthetised with ether vapour and blood drawn by cardiac puncture to obtain pre-immune serum (see 3.2.8. below). The guinea pig was then injected intraperitoneally with the antigen (one milliliter partially purified tyrosinase in polymerized acrylamide). Two weeks after the first injection, blood was drawn under anaesthesia by cardiac puncture. The serum was separated from the blood and tested positive by western blot for the presence of anti-chicken antibodies (see results). A booster injection (the remaining antigen in the syringe) was now given and, after two weeks, the guinea pig was sacrificed and blood collected by cardiac puncture.

3.2.8. Separation of the serum

The freshly drawn blood was allowed to stand for 2 hours at 4°C, during which time the fibrinogen clotted into a fibrin gel, slowly contracting and expressing the serum. The clot was then separated from the wall of the tube with an applicator stick and the tube stored for two hours at 4°C to permit further clot contraction. The serum was decanted into clean tubes and centifuged at 10 000g for 15 minutes. The supernatant was then removed with a pipette and stored in aliquots at -20°C until used.

3.2.9. Testing of the guinea pig serum for the presence of chicken tyrosinase antibodies

3.2.9.1. Enzyme linked immunoadsorbent assay (ELISA)

The wells of ELISA plates were coated with chick skin homogenate (see 3.2.1.1 above) and ELISA assays carried out according to standard procedures (see Appendix 2), using the immunized guinea pig serum as the primary antibody.

3.2.9.2. Western blot analysis

Homogenates of various 11 day BA/NHR embryo organs (stomach, heart, liver, eye and skin) were prepared as described in 3.2.1.1. above, equalised for protein concentration by the Biuret protein assay (Bradshaw, 1966) and stored at -20°C until needed. Aliquots of various homogenates were subjected to SDS gel electrophoresis, and transblotted in blotting buffer (20 mM Tris; 150 mM glycine; 20% methanol, pH 8,3) to nitrocellulose sheets at 100 mA and 4°C overnight in a Consort blotting chamber. The treatment of nitrocellulose sheets containing transblotted proteins is described in Appendix 3.

3.2.9.3. Immunoinhibition experiments

Details of all immunoinhibition experiments are provided in the results section.

3.2.9.4. Immunocytochemistry

Pieces of skin from BA/NHR embryos were removed and processed for sectioning and indirect immunofluorescence in one of the three following ways:

1. frozen fresh
2. fixed in either methanol or paraformaldehyde (2% in phosphate buffered saline, pH 7,4 (PBS), followed by cryoprotection and gelatin embedding as follows:
    i. $4^1/_2$ hours at 4°C (5% sucrose in PBS)
    ii. overnight at 4°C (15% sucrose in PBS)
    iii. 3 hours at 37°C (7% gelatin in PBS)

The gelatin was allowed to set at 4°C, and the skin pieces cut from the gelatin as small cubes. The cubes were embedded in OCT compound (Tissue-Tek) and frozen in liquid nitrogen. Four micron sections were cut on a Slee cryostat at -20°C and placed on glass slides coated with a solution consisting of 1% gelatin and 2% paraformaldehyde in Tris buffered saline pH 7,4 (TBS), air-dried for 20 minutes at room temperature, and rinsed for 10 minutes in TBS (pH 7,4). Immunocytochemistry was then performed on two sets of sections, the one set having been treated to permeabilise the cells (0,4% Triton-X, 6% sodium chloride and 0,2% sodium azide in 0,4 M PB) for 1 hour, followed by a 5 minute rinse in TBS.

3. quenched in melting isopentane, freeze-dried overnight under vacuum, vapour-fixed with parabenzoquinone vapour at 60°C for 3 hours, infiltrated for 3 hours under vacuum with epon-araldite and embedded in epon-araldite (see Pearse, 1980). One micron sections were cut on an LKB ultramicrotome 3, mounted on polytetrafluoroethylene coated slides (see Rawdon, 1978), deresinated with sodium ethoxide and hydrated through a series of alcohols to water and tris saline.

Indirect fluorescence immunocytochemistry was then carried out on sections prepared as in **1-3** above. The primary antibody (guinea pig antiserum) and secondary antibody (fluorescein-conjugated-rabbit anti-guinea pig IgG(H+L) (Zymed) were diluted in TBS. Frozen sections were TBS mounted, and resin sections were mounted in buffered glycerol. Viewing was done on a Leitz epifluorescence microscope.

Variations in immunocytochemical procedures are summarised in Appendix 4. Despite the variations tried, a melanocyte specific staining could not be obtained (see results). Hudson and Hay (1980, page 15) suggest that in cases of "everything staining everywhere", the "offending" serum should be absorbed with liver membranes. According to a modification of their suggested technique therefore, an aliquot of the guinea pig antiserum was treated as follows:

- C57BL/6J-C/C mouse livers (obtained from the Central Animal Unit, University of Cape Town) were dissected out, briefly homogenised in a Dounce homogeniser, poured into 10 ml of tissue culture medium (Dulbecco's minimal essential medium) and left at 4°C for 30 minutes. The mixture was now centrifuged. After pouring off most of the supernatant, 10 ml of tissue culture medium was again added to the membrane suspension, and the mixture centrifuged. After five such centrifugations, a volume of the packed cell membranes was mixed with an equal volume of antiserum and left on a rotator to mix overnight at 4°C. The cell membranes were now spun off (500 g for 30 minutes at room temperature) and the antiserum retested:

Blocking:	NRS at 10% in Tris saline, pH 7,4 for 2 hours
Rinsing:	2 X 10 minutes in Tris saline (pH 7,4)
Primary:	1:100 in Tris saline (pH 7,4) overnight
Rinsing:	10 minutes in Tris saline (pH 7,4)
Secondary:	1:50 in Tris saline (pH 7,4) for 1 hour
Rinsing:	3 X 10 minutes in Tris saline (pH 7,4)

3.3. RESULTS AND DISCUSSION

It was attemted to raise a polyclonal anti-chick tyrosinase antibody for intended use in a study of melanocyte differentiation from neural crest cells. Tyrosinase was partially purified from chick skin and injected into a guinea pig. The guinea pig serum was then tested for immunoreactivity to chick tyrosinase by the ELISA technique, the western immunoblotting technique, immunoinhibition, and immunocytochemistry.

3.3.1. Partial purification of tyrosinase

3.3.1.1. Anion exchange chromatography

Anion exchange columns were prepared, and tyrosinase partially purified from 11 day chick skin homogenate as described in 3.2.1.2. of methods. Tyrosinase activity of column fractions was measured by the radiometric assay (see methods 3.2.2.) and compared with the activity of the crude homogenate. Fractions with high tyrosinase activity were restricted to the second peak. The results from three such runs are summarised in table 3.2. Figure 3.2 shows an SDS-PAGE analysis of the proteins in peak 1 and peak 2 (high tyrosinase) fractions from a typical chromatography run. It can be seen that in lane B, the major protein is a band at approximately 70 kd.

3.3.1.2. Further purification by preparative gel electrophoresis

The high tyrosinase fractions from experiments 1-3 (see table 3.2) were pooled and concentrated for further purification by non-SDS PAGE (see methods 3.2.5.). Dopa staining of strips from this gel produced a single thin dopa-melanin band in each strip (lane A, figure 3.3). The thin horizontal portion of the unstained remainder of the gel aligning with these bands was carefully cut out. A piece of this strip was subjected to SDS-PAGE to check

the purity of the antigen. PAGE blue staining revealed a major band at approximately 70 kd, together with some minor bands (lane C, figure 3.3). It was reasonable to assume that tyrosinase had been purified (at least partially) and to proceed with injection of the antigen (see methods 3.2.7), since:

- the major band corresponded to the expected approximate molecular weight of mammalian tyrosinase (Hearing et al., 1978; Tomita et al., 1985; Ferrini et al., 1987) and avian tyrosinase (Boissy et al., 1987), and
- the protein/s present in the injected strip were definitely able to catalyse the dopa reaction (figure 3.3, lane A).

Table 3.2. Partial purification of tyrosinase from Black Australorp X New Hampshire Red 11 day embryonic skin homogenate.

Experiment	volume homogenate loaded	tyrosinase activity (cpm/mg protein)			chromatography x-axis: fraction number y-axis: OD_{280}
		crude homogenate	sample	increase above crude homogenate	
1	2ml	21750 ± 342	64910 ± 630	3x	0,3 M NaCl
2	1ml	45831 ± 497	65984 ± 1809	1,4x	0,3 M NaCl
3	1ml	42283 ± 2037	101500 ± 1713	2,4x	0,3 M NaCl

Figure 3.2. SDS PAGE analysis of proteins in the fraction with the highest OD_{280} of the first peak (lane A) and the second (high salt) peak (lane B) from a typical chromatography run. Molecular weights (kd) are noted on the left.

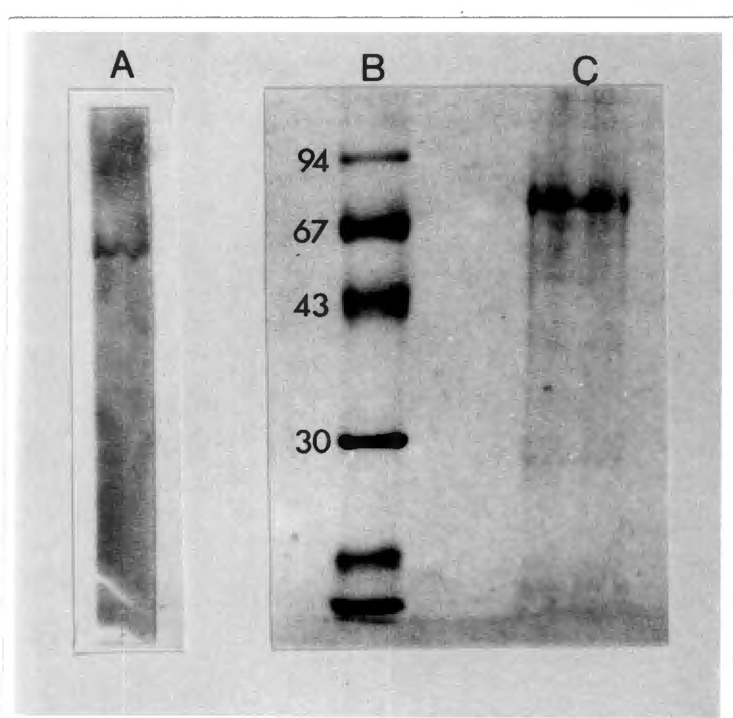


Figure 3.3. Lane A: Dopa reaction of peak 2 (high tyrosinase) column fractions on a non-SDS gel. Lane C: Purity of the injected strip. A piece of the injected strip was subjected to SDS-PAGE. A major band can be seen at approximately 70 kd, with other minor bands above and below it. Lane B: Molecular weights of standard proteins (kd).

3.3.2. Testing of the guinea pig serum for the presence of chicken tyrosinase antibodies

3.3.2.1. The ELISA technique could not be used in the testing of the antiserum

Despite several efforts, this technique consistently failed to provide an appropriate series of readings. There are many possible reasons for this, including a too low yield of the antigen, or an inability of the appropriate antigens to bind to the wells of the plates used.

3.3.2.2. Western blot analysis

Two weeks after the first injection of the antigen, blood was drawn from the guinea pig and the serum was tested by western blot analysis for the presence of antibodies against 11 day chick skin homogenate proteins. The serum tested positive by reacting to transblotted proteins in the 55-70 kd range (figure 3.4 lane C). No reaction was obtained when the immune serum was replaced with pre-immune guinea pig serum or by TBS-milk (not shown). After the booster injection, the reactivity of the serum to the homogenates of various chick tissues was further analysed by a series of western blots, as described below.

A. Age-dependent reactivity of the antiserum to skin homogenate proteins

The reactivity of the antiserum to skin homogenate proteins from BA/NHR embryos of various ages (3 - 8 and 11 days) was tested. Equal proteins were loaded into all lanes. The antiserum showed reactivity to a ±70 kd protein at all ages, the intensity of the reaction increasing with age (figure 3.5A). In addition, a 67 kd and 47 kd immunoreactive protein appeared at Day 6. These results were repeated on four different ocassions with similar findings. Sometimes, the 70 kd and 67 kd bands did not clearly separate (figure 3.5B), probably due to slight differences in the rate of electrophoretic migration in separate experiments. In addition, some blots of 8-11 day skin did not reveal the presence of a 47 kd band at all (not shown). This is possibly due to the fact that in those particular blots, the overall protein concentration of the homogenate was lower than the normal amount loaded (due to variations or errors in preparation), therefore the 47 kd band was below the level of detection.

B. Testing for tissue specific reactivity of the antiserum

In the next series of experiments, it was tested whether the reactivity of the antiserum was specific to proteins present in skin. Homogenates of skin and various BA/NHR organs including eye, stomach, liver and heart, were prepared. If the antibody was tyrosinase-specific, only eye and skin homogenates would be expected to be immunoreactive, (since both tissues contain tyrosinase), while the other homogenates would show no reaction.

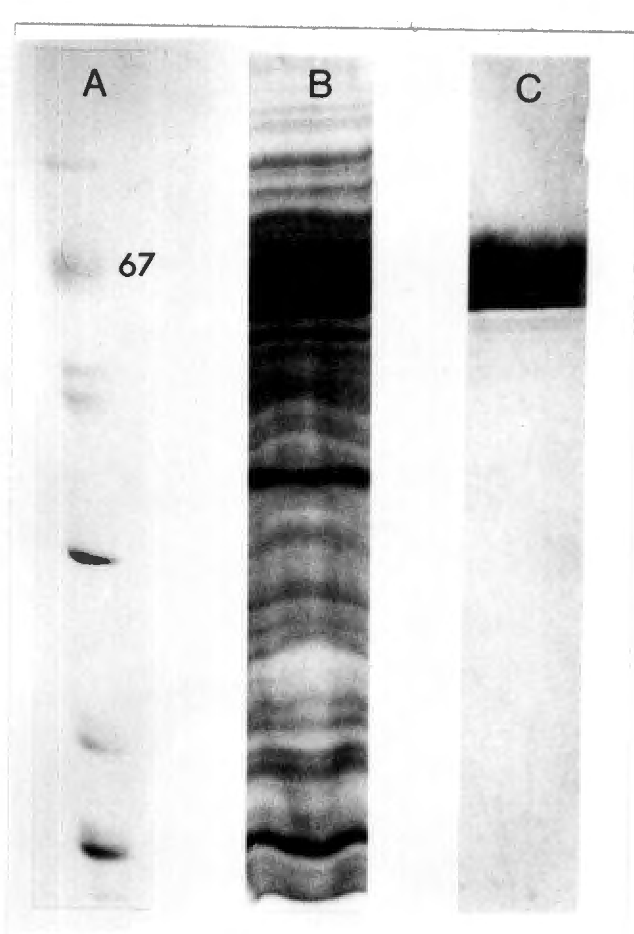


Figure 3.4. Guinea pig antiserum contains antibodies to chick skin homogenate. Lane A: Molecular weights of standard proteins (kd); Lane B: Skin homogenate (PAGE blue). Lane C: reactivity of the antiserum to transblotted skin homogenate. This broad immunoreactive band was subsequently found to separate into two or three individual bands, merged in this gel due to overloading of the sample.

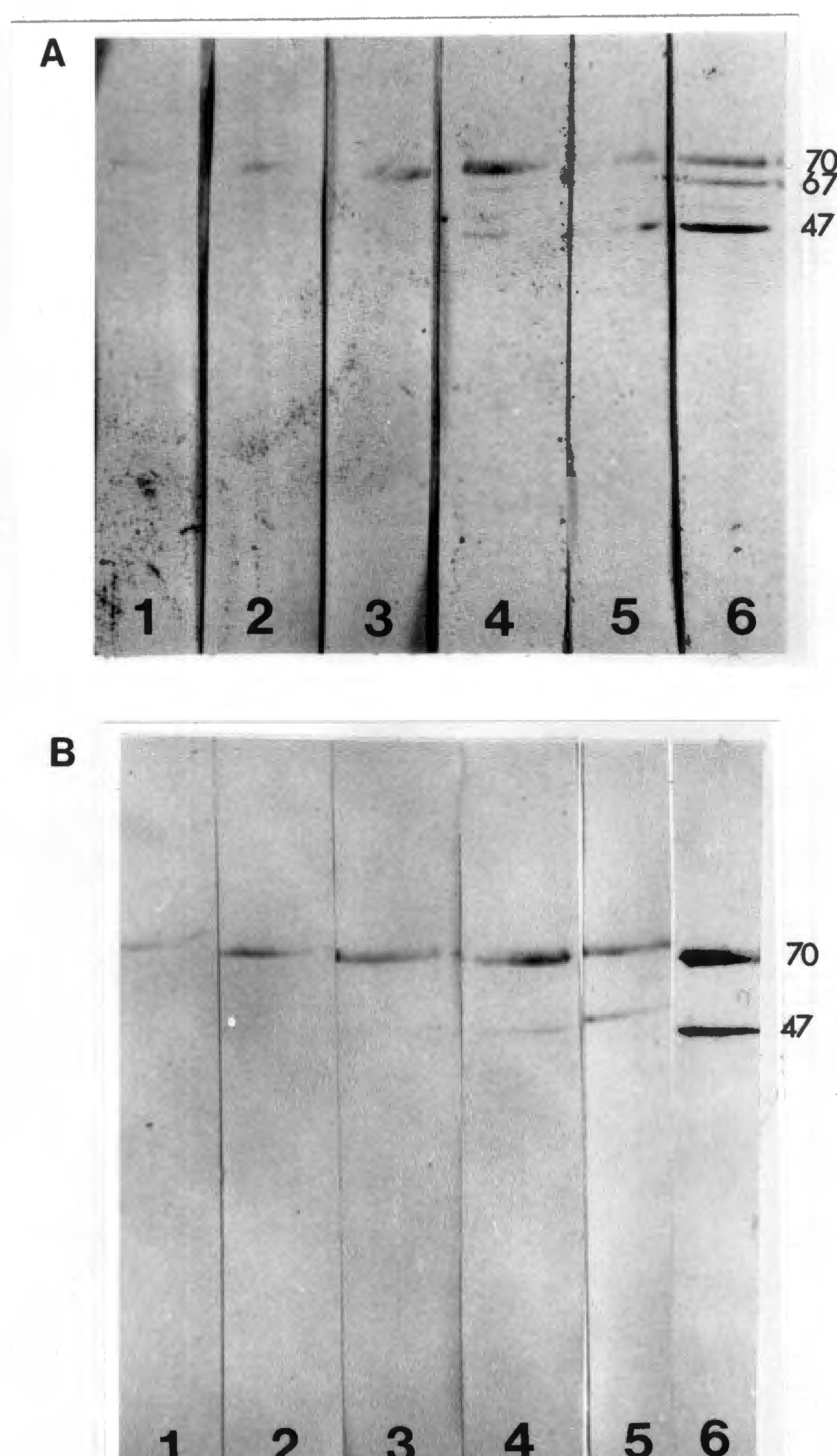


Figure 3.5. Reactivity to skin homogenates of different ages in two separate experiments. A and B: Reactivity to 3 (lane 1), 4 (lane 2), 5 (lane 3), 6 (lane 4), 7 (lane 5) and 8 day skin proteins (lane 6). Molecular weights of immunoreactive bands are noted on the right.

However, suprisingly, this was not the case (see figure 3.6). It was consistently found that the antiserum detected a 47 kd band in all tissues, a 70 kd band in all tissues except liver, and a 67 kd band in all tissues except stomach. These results clearly showed that the antiserum was not skin specific, suggesting that neither the 70, 67 nor the 47 kd immunoreactive proteins was tyrosinase (as was previously assumed). Following this line of thought further however, this implies that the original antigen injected either was not tyrosinase, or alternatively that the tyrosinase was contaminated by another protein or proteins. The former option is eliminated by the fact that the protein/s present in the injected strip was definitely able to catalyse the dopa reaction (figure 3.3). Therefore, one must conclude that the injected tyrosinase was contaminated by other, possibly more immunogenic proteins. In support of this idea is the report that tyrosinase is highly conserved (Hearing and Jiménez, 1989). Thus, the chick enzyme might not have elicited the production of antibodies in the guinea pig. Instead, the guinea pig may have produced antibodies against minor, less conserved contaminating proteins contained in the strip.

Finally, in an attempt to determine whether the contaminating antibody reaction was masking a tyrosinase specific reaction, the following experiments were carried out. Eleven day skin and total organ (excluding skin and eye, but including the entire digestive tract) homogenates were prepared in bulk and tested for immunoreactivity on western blots. As expected, the 47, 67 and 70 kd bands were present in both skin and organ homogenates (lanes 1 and 2, figure 3.7A and 3.7B). Then, with the idea of presorbing (and therefore eliminating) any non-tyrosinase antibodies, the immune serum was mixed with varying amounts of organ homogenate, or as a control, skin homogenate. These mixtures were then tested for immunoreactivity on blots of skin and organ homogenate. It was reasoned that if a tyrosinase specific reaction was present but masked, then the serum presorbed with skin homogenate should show no reactivity to skin or organ proteins, but the serum presorbed with organ homogenate should show reactivity to tyrosinase only. This was not found to be the case. The only result, after many variations tried, was that there was an overall, nonspecific decrease in immunoreactivity to skin and organ proteins in all western blots (figure 3.7A and 3.7B, lanes 3, 4, 5 and 6). This indicated the absence of possible masked tyrosinase-specific immunoreactive bands, and therefore suggested that the serum did not contain tyrosinase antibodies. In this regard however, Tomita et al. (1985) reported that "our experience... has suggested to us that tyrosinase may bind to nitrocellulose in less than an optimal manner... since large amounts of enzyme needed to be applied to the original polyacrylamide gels to demonstrate even the low reactivities in these figures." Since this problem may have also occurred in the experiments described here, a series of immunoinhibition experiments were performed, as described below.

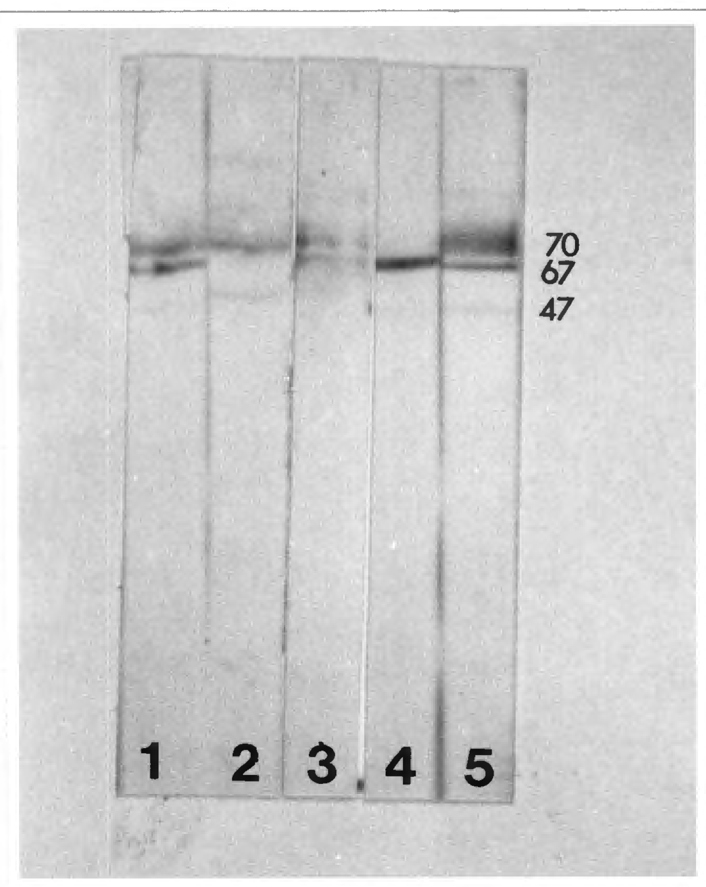
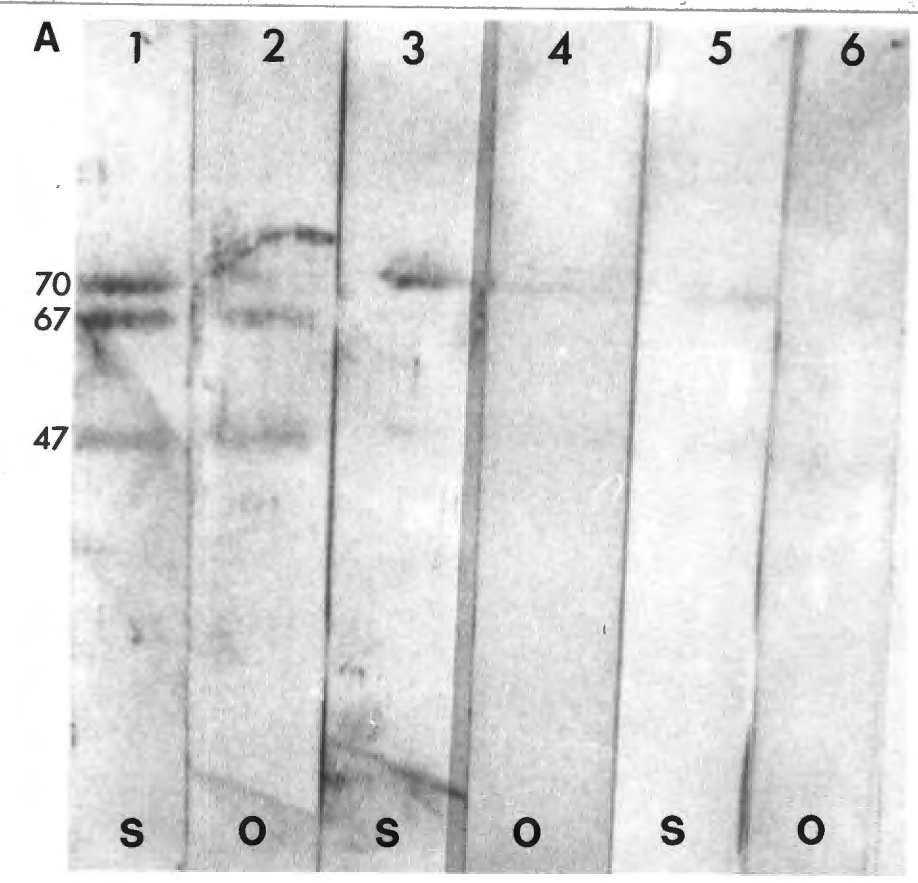


Figure 3.6. Reactivity to homogenates of organs other than skin. Lane 1: eye; Lane 2: stomach; Lane 3: heart; Lane 4: liver; Lane 5: skin. Molecular weights of immunoreactive bands are noted on the right.



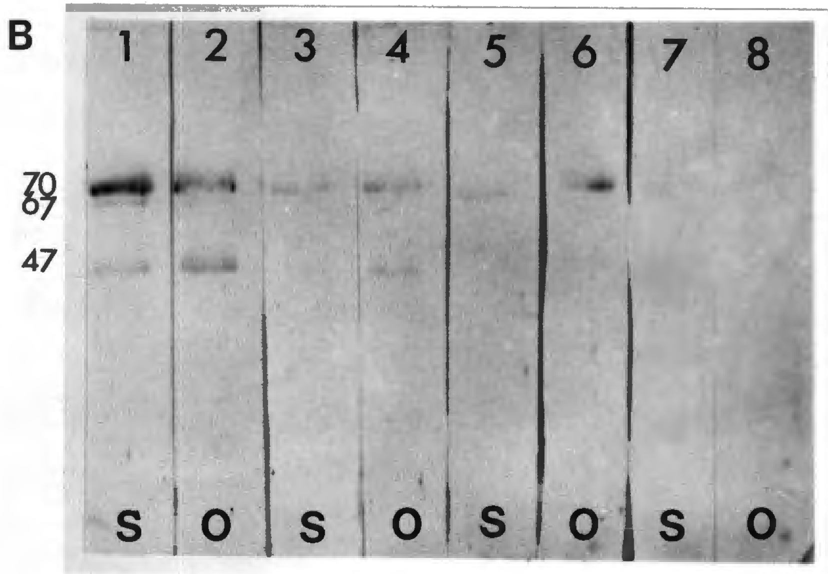


Figure 3.7. A and B: Reactivity of immune serum presorbed with organ or skin homogenate. Either skin or organ homogenate proteins were separated electrophoretically, denoted as S or O on each strip. Lanes 1 and 2 were reacted with untreated serum. Lanes 3 and 4 were reacted with serum that had been presorbed with skin homogenate. Lanes 5 and 6 were reacted with serum that had been presorbed with organ homogenate. In A, lane 7 and 8 were reacted with untreated pre-immune serum. Molecular weights of immunoreactive bands are noted on the left.

3.3.2.3. Immunoinhibition

A series of immunoinhibition experiments were performed in a further search for anti-chicken tyrosinase antibodies in the serum of the immunised guinea pig. In all experiments, 11 day BA/NHR skin homogenate was added to antiserum in various proportions (see below) and the mixtures left for 6 hours at room temperature on a shaker. In control assays, the antiserum was replaced with 0,1M PB (control 1) or with pre-immune serum (control 2). In the pilot experiments, tyrosinase activities of mixtures were measured after various time intervals by the spectrophotometric dopa assay (see methods 3.2.3). In the remainder of the experiments, tyrosinase activity was measured by the more sensitive radiometric assay (see methods 3.2.2). The results of these latter experiments are summarised in figures 3.8 - 3.13.

A. Inability of the antiserum to block tyrosinase activity

In order to determine whether the antiserum can block the catalytic activity of tyrosinase (thereby proving that the antiserum contained tyrosinase antibodies), aliquots of skin homogenate were mixed in various ratios with the antiserum, pre-immune serum or 0,1 M PB and the tyrosinase activity of the mixtures measured.

Four separate experiments were carried out using different serum to homogenate ratios (1:1; 1:2; 6,5:1; 10:1). In no case did the addition of antiserum to the homogenate cause a decrease in tyrosinase activity as compared to controls in which buffer was added to the homogenate. Interestingly however, in the last two experiments (where the ratio of serum to homogenate was high), there was a substantial increase in tyrosinase activity as compared to control samples containing no serum (figure 3.8, IS). Moreover, a similar result was obtained when pre-immune serum was added to the homogenate (figure 3.8, PS). Pre-immune and immune serum on their own showed negligible tyrosinase activity (figure 3.8. ps/is).

It is firstly clear from these experiments that the antiserum is unable to block the catalytic activity of tyrosinase. This implies that the immune serum does not contain anti-tyrosinase antibodies that interfere specifically with the function of the tyrosinase active site, but does not rule out the possibility that it may contain antibodies directed to some other portion of tyrosinase. An example of such an antiserum was provided by Ohtaki and Miyazaki (1972), whose immunoinhibition experiments showed that their rabbit-anti mouse tyrosinase antiserum was unable to block mouse tyrosinase catalytic activity, yet did form tyrosinase-antiserum complexes, since centrifugation could remove the activity from the samples.

Secondly the results showed that, at sufficient concentrations, guinea pig serum is able to enhance tyrosinase activity. No attempt was made to determine what component of the guinea pig serum was responsible for this enhancement. It is significant, however, that the sera of black, red and white guinea pigs have been shown to contain dopa and 5-S-

cysteinyldopa, assumed to be products of melanocyte metabolism (Hansson et al., 1980). The serum of the white guinea pigs contained the least dopa (0,3-0,4 ng/ml) and cysteinyldopa (0,4-0,5 ng/ml). The exact breed of the white guinea pig used in the present study and those used by Hansson et al. above is not known, but if the serum of the guinea pig used here contains similarly small quantities of these amino acids, it would not have had such a pronounced effect on tyrosinase activity. Perhaps the serum also contains other products of melanocyte metabolism capable of enhancing the activity of tyrosinase.

B. The effect of centrifugation

Since it is possible that the immune serum-homogenate mixtures described above did in fact contain tyrosinase antibodies but that these antibodies were not directed at the catalytic site of the enzyme, an attempt was made to remove the putative antigen-antibody complex by centrifugation. These experiments were carried out as described below.

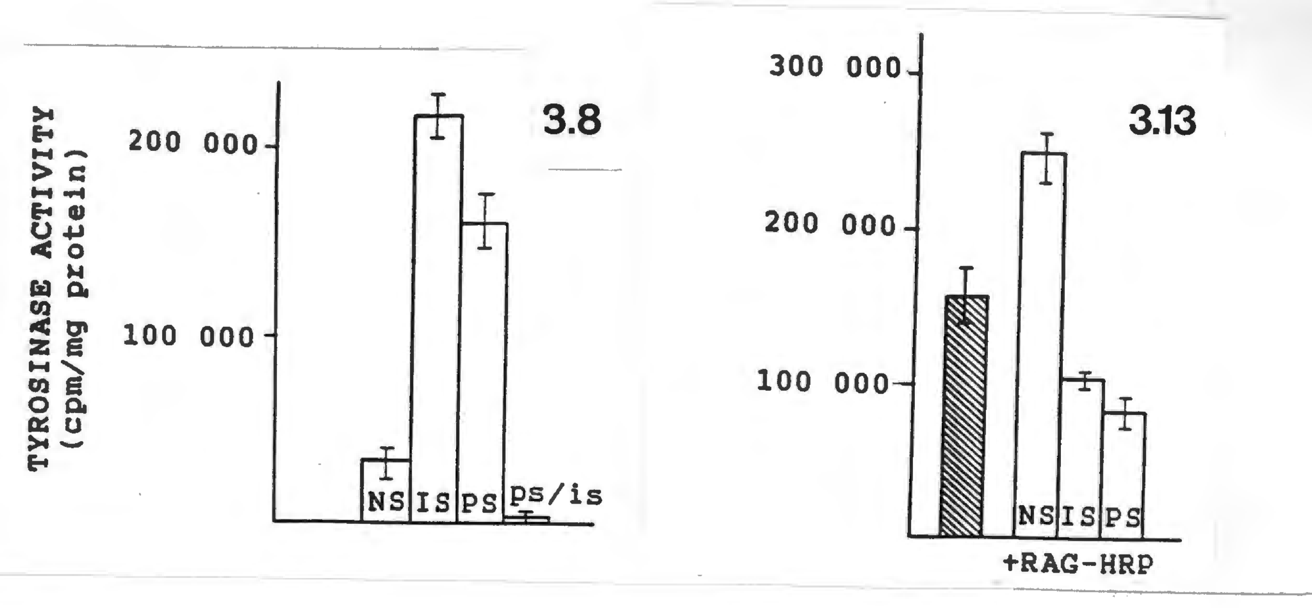
Premixed samples containing 50 µl skin homogenate and 50 µl antiserum were prepared. After 6 hours, the content of each sample was split into two aliquots of 50 µl each. Five microliters of undiluted RAG-HRP (peroxidase conjugated rabbit-anti guinea pig IgG (H+L) (Zymed) was added to each of the one series of samples, and the samples from both series were incubated at 4°C overnight. All samples were then centrifuged at 10 000 g for 10 minutes and the tyrosinase activities of the supernatants determined radiometrically.

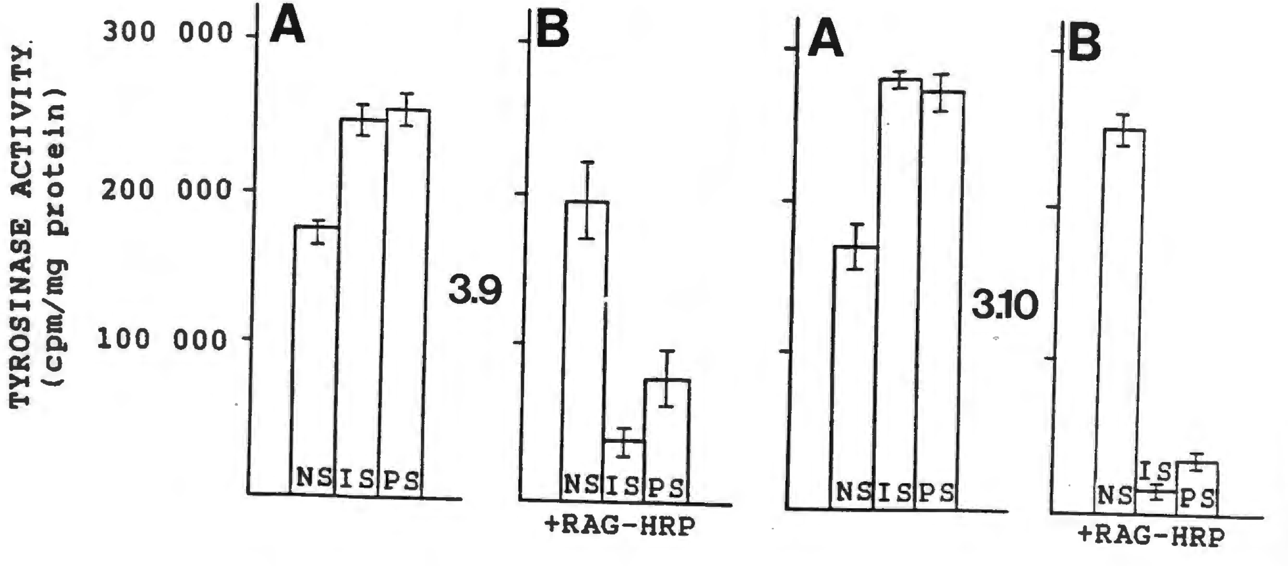
Figure 3.8. The immune serum enhances tyrosinase activity rather than blocks it. IS: Tyrosinase activity after 6 hours of a 6,5:1 immune serum -homogenate mixture. Activities of control samples: immune serum replaced with buffer (NS); immune serum replaced with pre-immune serum (PS); activities of pre-immune serum and immune serum alone (ps/is). Means were plotted with standard error bars where n = activities of duplicate samples.

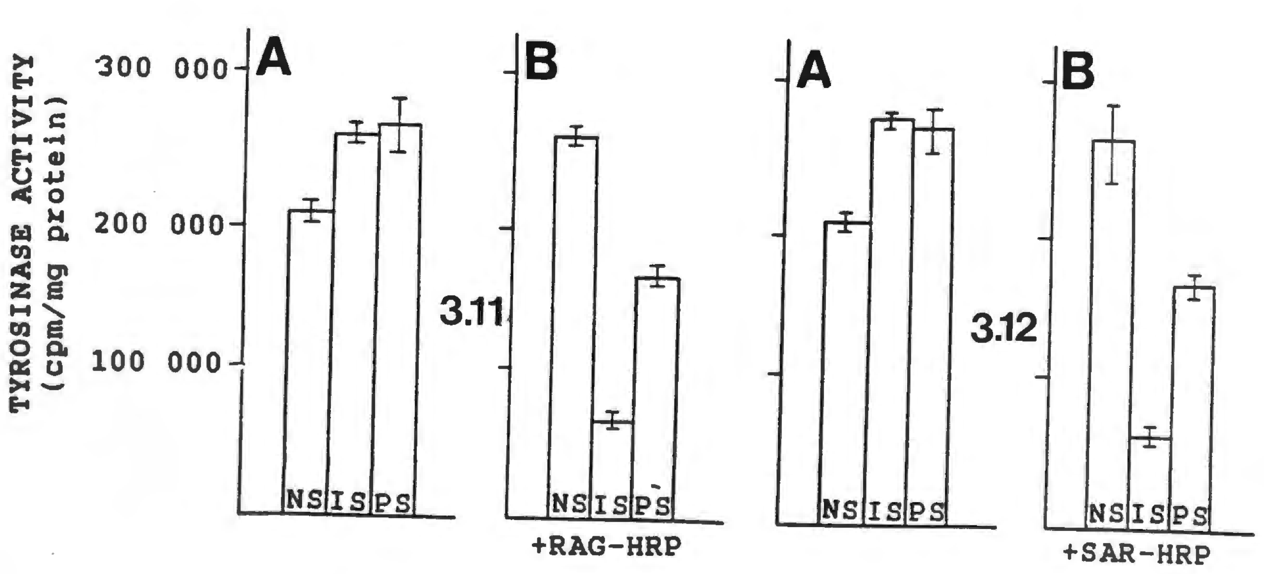
Figure 3.9 - 3.12. Effect of centrifugation. A: Sample series which had not received secondary antibody. B: Sample series which received secondary antibody. IS: tyrosinase activity of 1:1 immune serum-homogenate mixture. Activities of control samples: Immune serum replaced with buffer (NS); immune serum replaced with pre-immune serum (PS). Means were plotted with standard error bars where n = activities of duplicate samples.

Figure 3.13. Effect of double precipitation of the homogenate. Hatched bar: Tyrosinase activity of double precipitated homogenate. Empty bars: Activities of 1:1 immune serum-"cleared" homogenate mixture (IS); and controls in which immune serum was replaced with buffer (NS) or with pre-immune serum (PS). Means were plotted with standard error bars where n = activities of duplicate samples.

Figure 3.8 - 3.13. Immunoinhibition experiments.







This experiment was repeated 4 times, with similar findings (figure 3.9 - 3.12). In figure 3.12, an irrelevant secondary antibody was used (peroxidase conjugated swine anti-rabbit IgG (H + L) (SAR-HRP) (DAKO). Figure 3.9A - 3.12A shows the results for the sample series which had not received secondary antibody. It can be seen that the mixtures containing immune serum (IS) and pre-immune serum (PS) both have an increased tyrosinase activity as compared to control samples in which no serum was added (NS), demonstrating that centrifugation allows the guinea pig serum to enhance tyrosinase activity even at 1:1 serum to homogenate ratios. Centrifugation following the addition to the skin homogenate of antiserum as well as an anti-guinea pig, or an irrelevant secondary antibody (figures 3.9B - 3.11B and figure 3.12B, respectively), resulted in a decreased tyrosinase activity (IS) as compared to control samples containing no serum (NS). A similar pattern of result was obtained for samples in which pre-immune serum was added (PS), but the decrease in activity of samples containing immune serum was in all cases greater than that of samples containing pre-immune serum.

The considerable decreases in activity of the mixtures containing either immune- or pre-immune serum suggests that both the relevant and irrelevant secondary antibodies are able to form complexes with component/s present in guinea pig serum, which is then centrifuged out together with tyrosinase. This curious result is rather difficult to explain. When considering that guinea pig serum enhances tyrosinase activity (figure 3.8, IS and PS) and that centrifugation (without the addition of secondary antibody) further increases activity (figure 3.9A - 3.12A, IS and PS), the following might be suggested. Centrifugation of samples may increase the availability of the factor/s that enhance tyrosinase activity, possibly by removing inhibitory factors, or simply by increasing the relative concentrations of the enhancing factors due to the dilution of other proteins. Centrifugation after the addition of a secondary antibody then dilutes both the tyrosinase molecules as well as the enhancing factors due to the formation of complexes with these factors, which in turn may actually be bound to tyrosinase. However, that the samples containing immune serum consistently showed the lowest activity may imply the additional presence of specific anti-tyrosinase antibodies in the immune serum.

Finally, in order to gain further insight into the effect of guinea pig serum on skin homogenate tyrosinase activity, the effect of a double precipitation of the homogenate with guinea pig serum was examined (figure 3.13). For this purpose, and according to a variation in the method of Boissy et al. (1987), a volume of pre-immune serum was mixed 1:1 with skin homogenate, incubated at 4°C overnight in the presence of RAG-HRP, and then centrifuged. The supernatant was now used as "cleared" homogenate, to which either immune serum, pre-immune serum, or 0,1 M PB was added 1:1, followed by a further addition of RAG-HRP and centrifugation as above. As was found in the previous experiments (figure 3.9-3.12), the activity of the ("cleared") homogenate increased following centrifugation in the presence of

RAG-HRP (figure 3.13, compare hatched bar with NS) and the activities of samples containing immune- or pre-immune serum plus secondary antibody decreased following centrifugation (figure 3.13, compare hatched bar with IS and PS). However, in this case, the activity of the sample containing immune serum was not lower, but slightly higher, than that of the sample to which pre-immune serum was added.

These results contradict the findings in the previous experiments. However, this experiment was not repeated due to lack of time.* Concurrently with the above experiments, a series of indirect immunofluorescence experiments were carried out, as described below.

3.3.2.4. Immunocytochemistry

A series of indirect immunofluorescence experiments were carried out to test the reactivity of the guinea pig antiserum to sections of embryonic chick skin (see methods 3.2.9.4 and Appendix 4). Despite several variations in immunocytochemical procedure, a melanocyte-specific reaction was not obtained.

Briefly, frozen sections consistently showed a strong, general background fluorescence. Within this background staining, a more specific reaction seemed to correspond to the epidermal basement membrane and the dermal extracellular matrix (not shown). In frozen control sections, where the primary was replaced with pre-immune serum, a strong general background fluorescence was also obtained, becoming fainter at higher dilutions of the serum. Resin sections showed a less bright overall staining than frozen sections. A strong perinuclear staining was seen in all cells sectioned through their nuclei, and some cells contained bright, punctate spots of fluorescence (figure 3.14). Sections where the primary antibody was omitted showed no staining, and sections where the primary was replaced with pre-immune serum contained only a very faint background fluorescence. Omission of the primary produced no reaction. Although a less- or more intense fluorescence was produced by some variations such as high dilutions of the immune serum, the general pattern of staining for frozen as well as resin sections remained unchanged.

According to the suggested solution for "everything staining everywhere" by Hudson and Hay (1980, page 15), a volume of the immune serum was absorbed with liver membranes (see methods 3.2.9.4), and retested on resin sections. In this case, a less general pattern of staining was obtained (figure 3.15 and 3.16). Amidst a faint background fluorescence, bright, punctate spots of strong reaction were seen within many of the epidermal and dermal cells. Comparison of sections where the "uncleared" (figure 3.14) and "cleared" (figures 3.15 and 3.16) immune serum was used suggests that the absorption had removed those antibodies/components from the serum which had caused the general- and perinuclear staining, but not those causing the spots of strong reaction seen in some of the cells. Although the spots sometimes closely corresponded to the black melanin pigment visible in

* if this experiment was repeated and results similar to those in 3.13 were obtained, the promise of the findings in figures 3.9 - 3.12 would be negated.

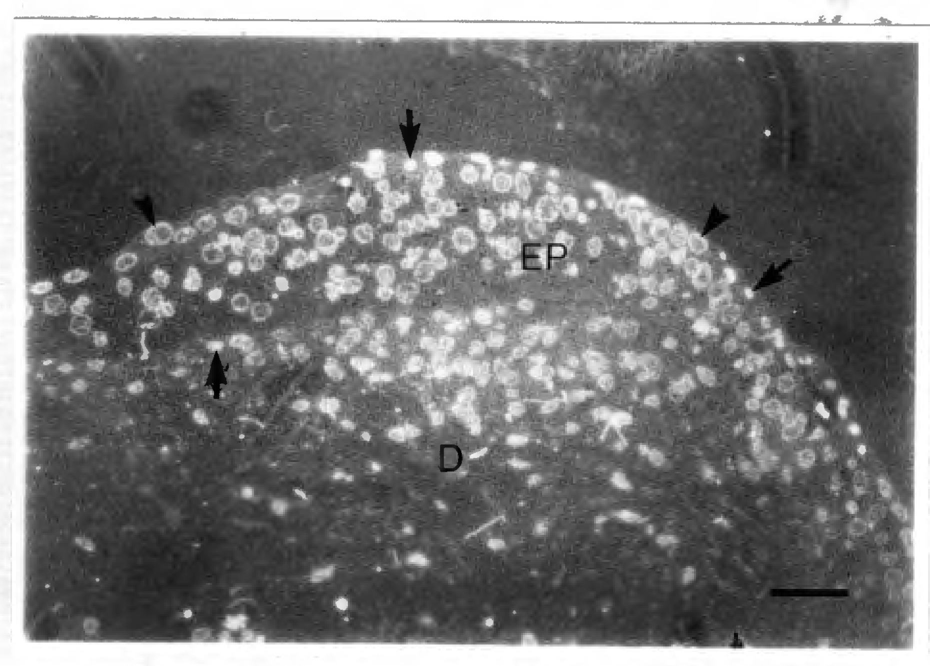


Figure 3.14. Reactivity of the immune serum to 9 day skin (resin). Notice the predominantly perinuclear staining (arrowheads), as well as bright, punctate spots in some of the epidermal and dermal cells (arrows). EP = epidermis. D = dermis. Bar = 20 µm.

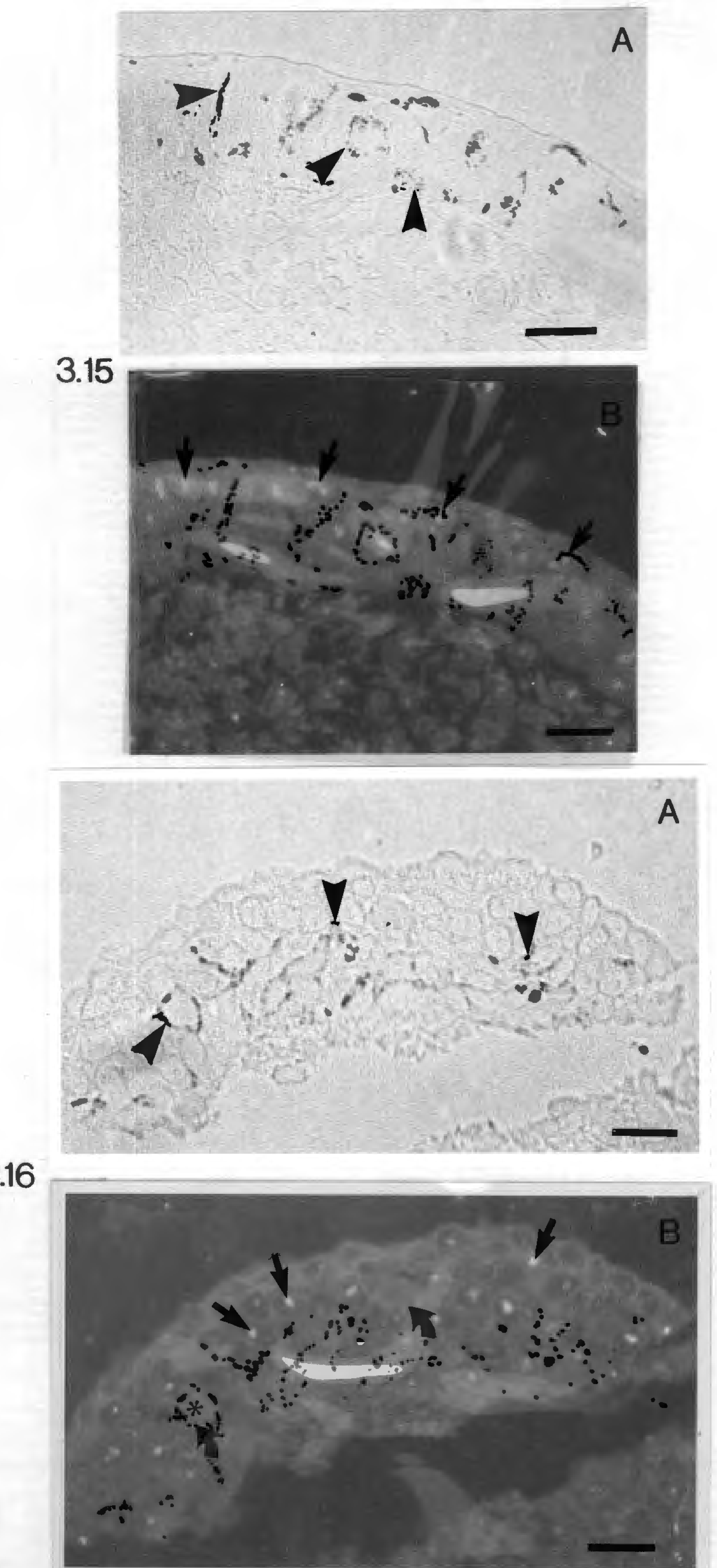


Figure 3.15. and 3.16. Reactivity of membrane-absorbed serum to 9 day skin (resin). A: Position of pigment in the sections (arrowheads). B: Position of pigment (overlay) superimposed on reactivity of membrane-absorbed serum. Notice the bright, punctate spots in some epidermal and some dermal cells (arrows). Curved arrows: epidermal cells showing a specific reaction, one of which is clearly a melanocyte (*). EP = epidermis. D = dermis. Bars = 20 μm.

the epidermis, this was not always the case. The fact that these spots were not always seen in cells surrounded by melanin does not exclude the possibility that the cells were unpigmented melanocytes or that pigment belonging to such cells were not included in the sections viewed. On the other hand however, a large percentage of dermal cells also contained these spots. Although it is possible that some of these dermal cells were unpigmented melanocytes, it seems unlikely that *all* of them were. It was therefore concluded that the spots were probably not melanocyte specific.

A more promising feature of sections stained with the "cleared" immune serum was that the entire cytoplasm of some individual cells in the epidermis also showed a specific reaction. This reaction was not as strong as that of the spots described above, and faded more rapidly during viewing than the spots. Notice that in figure 3.16, one such a specifically stained epidermal cell can clearly be identified as a melanocyte. This result was however only obtained once, and could not be repeated. Thus, it was concluded that the immune serum was not suitable for immunocytochemical localisation of tyrosinase in sections of chick skin.

3.3.3. CONCLUSION

The present study appears to have exemplified the statement by Hearing and Jiménez (1989) that the "art/magic" of making antibodies "depends on a bit of luck". The inescapable conclusion drawn from the results was that the guinea pig antiserum was not tyrosinase specific. Immunoreactive proteins detected by the western blot technique were not specific to skin and eye proteins. In addition, presorption experiments showed that the blots did not contain masked tyrosinase bands. However, since tyrosinase may have been inefficiently or incompletely blotted, further techniques were employed to test for the presence of tyrosinase antibodies in the immune serum. The results from these experiments were somewhat inconclusive. In the immunoinhibition experiments, the pre-immune serum controls behaved in the same way as the immune serum. No immunoinhibition was detected, and in fact it appeared that guinea pig serum itself enhanced tyrosinase activity. The final conclusion was that the antiserum was not suitable for immunocytochemical localisation of tyrosinase in sections of chick skin.

The serendipitous finding that guinea pig serum had an enhancing effect on tyrosinase activity is interesting and warrants further study. In this regard, it may be noted that Jerdan et al. (1985) isolated a factor from calf serum that was shown to stimulate melanin biosynthesis in cultured chick neural crest cells. It was further found that horse, human, rat, and chicken sera lacked this biological activity. This suggests that calf- and guinea pig serum may contain unique tyrosinase-enhancing factor/s.

CHAPTER 4.
MELANOSOME ULTRASTRUCTURE IN RED AND RED/BLACK FOWL

4.1. INTRODUCTION

4.1.1. Melanosomes occur in two basic forms

Pigments of the chicken include two general types, the carotenoids and the melanins. Of the melanins, there are two biochemical forms - the brown to black eumelanins and the yellow to red pheomelanins. These melanins are synthesized and laid down upon the internal structural matrix of specialised membrane bound organelles called melanosomes. Genetic surveys have implicated several loci in the control of mammalian and avian melanosome formation (Silvers, 1979; Takeuchi et al., 1981; Bowers, 1988) and although some mutations lead to variations in melanosome morphology, it has been possible to group these organelles morphologically into two basic forms.

The first form is ellipsoidal in outer shape, and is commonly seen in melanocytes synthesising eumelanin. In humans, they are approximately 0.24 - 0.5 µm in length and 0.08 - 0.19 µm in width (Everett et al., 1979). The structure of these organelles are very similar in mammalian and avian tissues. Eumelanosomes typically have a highly organized matrix. Running the length of the melanosome are 30 - 50 Å thick filaments that have a zigzag appearance. It appears that cross-links form between the filaments so that they aggregate into single or perhaps several lattices of parallel cross-linked fibers, called lamellae. In cross section, the lamellar sheet/s are seen to be rolled concentrically into a cylinder. Melanin is deposited on the lamellae, and gradually obscures their structure until the interior of the melanosome is entirely electron dense.

Much less is known about the second type of melanosome, which is commonly seen in melanocytes synthesizing pheomelanin. These organelles are spherical or slightly oval in outer shape, and in humans have been reported to have a diameter of from 0.25 to 1 µm (Stanka, 1974; Jimbow et al., 1983). Morphological descriptions of mouse (Sakurai et al., 1975; Jimbow et al., 1979) and human (Jimbow et al., 1983) pheomelanosomes are similar to those found in Rhode Island Red fowls (Jimbow et al., 1979). These pheomelanosomes do not possess an organised lamellar matrix, but instead have an amorphous, multivesicular internal structure upon which melanin deposition appears spotty and irreglar. Internal filaments, if present, do not occur in any definite arrangement.

4.1.2. Synthesis of eumelanin and pheomelanin is thought to always occur in separate melanocytes in red/black fowls

In fowls, the major genetic control of red and black melanin distribution is the multiple-allelic E locus (Brumbaugh and Hollander, 1965). The E system of alleles specify a graded series of black and/or red phenotypes. The most dominant allele, E, causes only eumelanin to be produced, while the most recessive allele, e^y , specifies primarily pheomelanin synthesis. The standard/wild type allele, e^+, directs the synthesis of both pigment types, producing fowls in which both red and black melanin exist. Other loci such as "silver" (S) and "dominant white" (I), interact with E locus expression to cause specific phenotypic effects. S bleaches pheomelanin, changing standard red necked, black breasted fowl into white necked, black breasted fowl. I bleaches eumelanin, producing white breasted fowl with red necks (Brumbaugh, 1971).

It is thought that regenerating feathers receive their melanocytes from "reservoirs" of melanoblasts that enter the epidermal collar via the (permanantly established) papilla, and differentiate in the "zone of differentiation" apical to it (Rawles, 1960, page 226). Although grafting experiments have indicated that the E alleles are autonomous in expression (donor specific), it has been shown that the type of melanogenesis in e^+ fowl can be altered by external environmental factors (Brumbaugh, 1967). It was found that e^+ regenerating feathers shifted from eu- to pheomelanin production in response to a reduced feather growth rate induced by fasting, while thyroxine-feeding (increased growth rate) resulted in an increased eumelanin deposition (Brumbaugh, 1967). From this it was proposed that the type of melanin produced in regenerating feathers results from an interaction between the particular E allele of the melanoblasts and the milieu in the zone of differentiation of the growing feather. Rapid movement of melanoblasts through this zone seems to favour eumelanin synthesis, while a slow movement seems to favour pheomelanin synthesis. Alternations in red and black or stippling can be explained as due to diurnal fluctuations of feather growth rate (Brumbaugh, 1967).

The literature suggests that in fowl producing both pigment types, eumelanin-pheomelanin differentiation will occur in seperate melanocytes. Brumbaugh (1968) states that "melanocytes of the normal/standard type fowl can differentiate in either of two directions. They can produce rod-like eumelanin (black) granules, or spherical and ovoid shaped pheomelanin (red or yellow) granules"; and that "once differentiated, these melanocytes will produce only one type of pigment" (Brumbaugh, 1967). Thus, shifts between and eu- and pheomelanogenesis are interpreted as resulting from an (environmentally induced) differentiation of melanoblasts into either eu- or pheomelanocytes (Brumbaugh, 1967).

This concept differs from the reported events occurring during pigmentation shifts in the hair follicles of agouti mice. In mammals, alleles at the agouti (A) locus control whether eu- or pheomelanin is produced by the follicular melanocytes. Non-agouti mice produce only eumelanin. Heterozygotes with the lethal yellow mutation (A^y) produce only pheomelanin. Wild-type agouti (A/A) mouse hair are black or brown, with a subterminal yellow band. This "agouti pattern" is formed by a shift from eu- to pheomelanin production. Electron microscopical studies by Sakurai et al. (1975) on mouse skin revealed that hair bulb melanocytes at the transitional phase between eu- and pheomelanin production contained both rod shaped eumelanosomes such as is seen during the black phase, and spherical pheomelanosomes as is seen during the yellow phase. This indicates that the shift from eumelanosome to pheomelanosome formation occurs within single melanocytes. Mayer and Fishbane (1972) showed by dermal-epidermal recombination grafts of agouti and non-agouti mouse skins that the primary site of the agouti gene action is the dermal component of the follicle. Thus, although the mechanism of the shift is not known, it appears that the agouti alleles are expressed in the dermis, which in turn determines the type of melanin synthesized in the melanocytes of the epithelial component of the hair follicle.

The above ultrastructural observation of eu- and pheomelanosomes within the same melanocytes of agouti mice has been taken as direct proof that wild type mouse melanocytes have a dual potential. This conclusion is based on the predicted morphological differences between eu- and pheomelanosomes, which can only be clearly distinguished at the level of the electron microscope. The generally accepted concept, however (see for example Jimbow and Takeuchi, 1979; Takeuchi et al., 1981), that fowl melanocytes do *not* have the potential to produce both melanosome types simultaneously as do agouti mice, has not been investigated in detail, and no electron microscopical studies of melanosome types during pigmentation shifts have been reported. The environmentally induced pigmentation shifts reported by Brumbaugh (1967) were not investigated at the ultrastructural level, and his assumption that such shifts would not occur in differentiated fowl melanocytes is apparently based on early light microscopical observations described by Rawles (1948) and Hamilton (1952). In general, ultrastructural studies on fowl melanosome types have often involved genotypes with an E or e^y background (Jimbow and Fitzpatrick, 1973; Jimbow et al., 1979) and other studies using e^+ fowl tissues were not concerned with pigmentation shifts.

At this stage it is important to consider observations in the electron microscopical studies of Brumbaugh (1968; 1971). Brumbaugh (1971) presented micrographs of melanocytes from regenerating neck ("pheomelanin producing") feathers of standard fowl, and of fowl with the dominant white or silver mutation. From his figures, it appeared that the melanocytes of all three these genotypes contained both rounded and ellipsoidal melanosomes. The ellipsoidal melanosomes clearly contained lamellar matrices, but due to low magnification, the internal

structure of the rounded melanosomes could not be discerned. It was therefore difficult to judge whether the rounded melanosomes were cross-sectioned ellipsoidal melanosomes or not. In an earlier report however, Brumbaugh compared "pheomelanin formation" in regenerating feathers from standard and e^y/e^y fowl (Brumbaugh, 1968). Representing the pheomelanosomes from standard fowl was a micrograph of an ovoid to ellipsoid melanosome with large globular melanised portions, embedded within parallel "matrix strands". The matrix strands were apparently not cross linked as in typical eumelanosomes (see page 56). The pheomelanosomes of e^y/e^y fowl had a much more disorganised appearance, melanin being deposited upon "short longitudinal strands that may cross each other".

The above observations raise two points. Firstly, e^y/e^y (Rhode Island Red) fowl pheomelanosomes were also described by Jimbow et al. (1979) (see page 56). Their morphology was identical to those of yellow mouse hair (Sakurai et al., 1975; Jimbow et al., 1979), and human red hair (Jimbow et al., 1983). From Jimbow et al. (1979), it would therefore have seemed that the typical morphological differences between mouse pheomelanosomes and eumelanosomes are also applicable to fowl melanosomes. From the above however, it is clear that there is a substantial difference in appearance between the e^y pheomelanosomes observed by Brumbaugh (1968) and those described by Jimbow and his colleagues. Brumbaugh's e^y/e^y pheomelanosomes seem to represent a less organised version of his standard pheomelanosomes, which differ even more from Jimbow's e^y/e^y pheomelanosomes. While Jimbow's "typical" pheomelanosomes are clearly distinguishable from eumelanosomes, those described by Brumbaugh seem to reveal characteristics intermediate between eumelanosomes and pheomelanosomes. Interestingly, Jimbow et al. (1983) reported finding that human hair follicle pheomelanocytes contained, amongst typical pheomelanosomes, "mosaic" melanosomes that also have characteristics intermediate between typical eu- and pheomelanosomes. In this regard, a possibly crucial point to consider is that hair or feathers are often labelled "pheomelanogenic" or "eumelanogenic" simply by their visual colour, and that the melanosomes are then conveniently classified accordingly. Biochemical studies, however, have indicated that there is, in general, an intermeshing of the two melanogenesis pathways. For example, it is typical of all eumelanins so far isolated to contain sulphur in various percentages (see chapter 1). Thus, strictly speaking, pigments can be classified biochemically according to their eumelanin/pheomelanin ratio. This raises the possibility that varieties in "pheomelanosome" morphology may relate to differences in this ratio.

In summary, it seems that as opposed to the situation with eumelanosomes, pheomelanosome morphology is rather variable, even in fowls with the same E-allele. The reason for this is unclear, but unmentioned or unknown genotype differences at loci other than the E locus may very well play a role.

4.2. AIM OF THE PRESENT STUDY

The feathers of BA/NHR (E/e^{Wh}) fowl are black with a reddish lustre, suggesting that both eumelanin and pheomelanin are tranferred simultaneously into the feather barbules. If so, the feathers are either pigmented by mixed-type melanocytes or by separate eu- and pheomelanocytes within the same feather follicles. This cross breed therefore provides an ideal opportunity to explore the possibility that melanocytes synthesize mixed type melanosomes.

- The first aim of the present study was to determine whether melanocytes with mixed type melanosomes occur in the developing embryonic epidermis of BA/NHR fowl.
- An essential component of research on the relationship between genotype (E locus alleles) and pheomelanosome morphology is to accumulate information on pheomelanosome ultrastructure from fowls with known differences at the E locus. The present study contributes to this field by describing the ultrastructure of New Hampshire Red (NHR) (e^{Wh}/e^{Wh}) melanosomes.
- Vesiculo-globular bodies (VGBs) are reportedly subunits of maturing mammalian and avian melanosomes, and certain evidence indicates that they contain tyrosinase (see below). The fate of the VGBs during the development of the two melanosome types apparently differs, since in mature pheomelanosomes, the VGB interiors become melanised, while they remain unmelanised in mature eumelanosomes. Observations in the present study on BA/NHR melanosomes suggest that this generally accepted view may not hold for all genotypes.

4.3. GENERAL BACKGROUND

4.3.1. Terminology

In the literature, the term "melanosome" usually refers to the fully pigmented organelle only, while the term "premelanosome" is used to describe all the earlier stages in the genesis of melanosomes. However, "melanosome" is sometimes used to refer collectively to the developing, as well as the apparently completely melanised organelles. This term seems more appropriate in the sense that one cannot be sure that melanosomes appearing uniformly electron dense have entirely completed melanisation. Thus, for the sake of simplicity in the present chapter, premelanosomes and melanosomes as defined above will both be designated "melanosomes", some of which are unpigmented and others that have pigmented to various degrees of maturity.

4.3.2. Melanosome ontogeny

Information concerning the origin of melanosomes and of when and how tyrosinase becomes associated with them has largely involved ultrastuctural observations of pigmented bird and mammal tissues. One of the earliest viewpoints was based on the similarities between melanosomes and mitochondria. It was proposed that melanosomes were "modified mitochondria" derived from "promitochondria that aquire melanin in the course of a specialized ontogeny" (du Buy et al., 1963; Woods et al., 1963). However, a study by Seiji et al. (1963) on mouse melanoma cells provided conclusive evidence that melanosomes and mitochondria are distinctive subcellular organelles. Their alternative suggestion was that tyrosinase is synthesized on ribosomes and transferred through the endoplasmic reticulum (ER) to the Golgi area, where it is packaged into membrane bound units "within which tyrosinase is condensed and loaded onto or into the network". This theory implies that the melanosomal matrix and tyrosinase become associated with each other during the assembly of the melanosome. Novikoff et al. (1968) suggested a modification of the above theory in which the site of union of tyrosinase and matrix proteins is the GERL and not the Golgi. GERL is defined as Golgi-associated, tubular, anastomosing and cisternal portions of smooth ER (Maul, 1969). From observations on human hair bulb melanocytes, Birbeck (1963) proposed yet another theory, in which it was suggested that the melanosomal matrix actually consists of an array of tyrosinase molecules, implying that tyrosinase may behave simultaneously as a structural protein and active enzyme.

Common to all the above conflicting opinions on melanosome ontogeny is the idea that the structural proteins and tyrosinase co-exist in the melanosome from the onset of melanosome assembly. However, several more recent studies have shown that these two principle components of the melanosome arise from separate compartments of the

endomembrane system. Ultrastructural reinvestigations implicate the ER and not the Golgi or GERL as the direct precursors of melanosomes. By analysing serial sections of chick eye pigment epithelium, Stanka et al. (1981) showed that continuous cisternal ER complexes ("premelanosome forming centers") transform simultaneously into groups of four to ten melanosomes that pinch off from the cisterna. Images obtained in other studies also support an ER origin of the melanosome: Sakurai et al. (1975) found early pheomelanosomes in mouse hair follicles in continuity with ER; Ide (1972) observed continuities between ER and early eumelanosomes in chick embryo eye pigment; and Maul (1969) reported that cultured human melanoma cell melanosomes frequently possessed a cisterna-like appendage of tubular smooth ER. Cytochemical studies employing the dopa reaction, by which the location of active tyrosinase can be visualised as electron dense "dopa-melanin" deposits, have shown that melanosome assembly and the incorporation of tyrosinase are separate events. This conclusion was strengthened by the finding that some early melanosomes with completed inner matrices posses no tyrosinase activity (Ide, 1972; Maul, 1969; Maul and Brumbaugh, 1971; Sugiyama et al., 1979).

Cytochemical studies indicate that the dopa-positive product first appears in the vicinity of the Golgi apparatus. According to some reports, this site is the Golgi cisternae (Ide, 1972; Stanka et al., 1981), while other reports claim that it first appears in the GERL (Maul and Brumbaugh, 1971; Sakurai et al., 1975; Sugiyama et al., 1979). However, by tilting thick sections of dopa-reacted melanoma tissue with a goniometer, Mishima et al. (1979) concluded that dopa positive tubules in the Golgi area are actually GERL that lies in close apposition to, but separated at an angle from the Golgi apparatus. A compromising conclusion was drawn by Bowers and Chun (1981) from their ultrastructural study of regenerating chick feathers. They describe tyrosinase as originating in the Golgi apparatus, followed by transportation through the GERL.

A consistent observation in dopa reacted tissues is the budding of dopa-positive vesicles from Golgi- or GERL cisternae. Ide (1972) reported that tyrosinase activity apparently progresses from the Golgi sac to vesicles that are first attached to the Golgi lamellae and then become numerous in the cytoplasm. Similarly, Stanka (1974) found dopa positive, Golgi derived (coated) vesicles close to melanosomes in human pheomelanocytes. Studies on regenerating feather melanocytes (Maul and Brumbaugh, 1971) and melanoma cells (Mishima et al., 1979) have shown that such cytoplasmic coated vesicles contain the highest concentration of dopa reaction product of all subcellular compartments, suggesting that they carry highly concentrated tyrosinase. Furthermore, such vesicles have often been observed to fuse with melanosomes (Maul and Brumbaugh, 1971; Ide, 1972; Sugiyama et al., 1979). Instances where tyrosinase-containing membrane tubules appeared to be connected to premelanosomes (Maul, 1969; Maul and Brumbaugh, 1971; Ide, 1972) have been

interpreted as the possible fusion of a coated vesicle still attached to the precursor membrane system (Maul and Brumbaugh, 1971).

All the above evidence seems to indicate that tyrosinase, newly synthesized on rough ER, is transported to the Golgi and/or GERL, where it budds off in coated or uncoated vesicles. These vesicles then fuse with early melanosomes that have been preformed from ER (figure 4.1).

4.3.3. Vesiculo-globular bodies: descendants of cytoplasmic tyrosinase carrying vesicles?

It has been noted in several reports that eumelanosomes contain numerous spherical, ±400 Å units, appearing either as non-lucent bodies or as electron lucent "holes" (for references consult Jimbow and Fitzpatrick, 1973). A comprehensive study of eumelanosomes in chick skin, feather and eye tissue showed that these bodies are not artefacts of the electron image but real melanosomal subunits, which have been termed "vesiculo-globular bodies" or VGBs (Jimbow and Fitzpatrick, 1973). The VGBs are in fact not empty, but contain a fine granular substance. However, since "the electron density of the surrounding matrix makes the electron lucency of the VGBs more apparent than real", VGBs often appear "empty" (Jimbow and Fitzpatrick, 1973).

Ultrastructural studies on maturing chick and mouse eumelanosomes and pheomelanosomes suggest that VGBs become associated with melanosomes at the very early stages of melanosome development. From their examination of hair, retinal and feather melanosomes, Jimbow et al. (1979) described melanosome development as follows. The youngest eumelanosomes are round, empty vacuoles that accumulate randomly distributed, incomplete lamellae and a few scattered VGBs within an amorphous proteinaceous material. The melanosomes then elongate as the lamellae become organized, and VGB numbers increase. VGBs attach in a linear fashion to the surface of the lamellae and appear to become incorporated into the construction of the inner matrix as melanin deposition on the lamellae commences. In completely matured eumelanosomes, VGBs can be discerned around the entire surface of the melanosomes and all VGB cores remain unmelanised. An interesting further finding was that VGB numbers vary greatly depending on the genetic background and degree of melanisation. For example, although the earliest melanosomes from all tissues start off with about the same number of VGBs, the highly melanised melanosomes from black feathers and black hair eventually contain more VGBs than the less melanised melanosomes of white feathers and white hair, and melanosomes from dark pink eyes eventually contain more VGBs than those of light pink eyes. These results suggest that VGBs may be the key units in the organisation and degree of melanisation of the eumelanosomal inner matrix, their mode of involvement apparently being under genetic control (Jimbow et al., 1979).

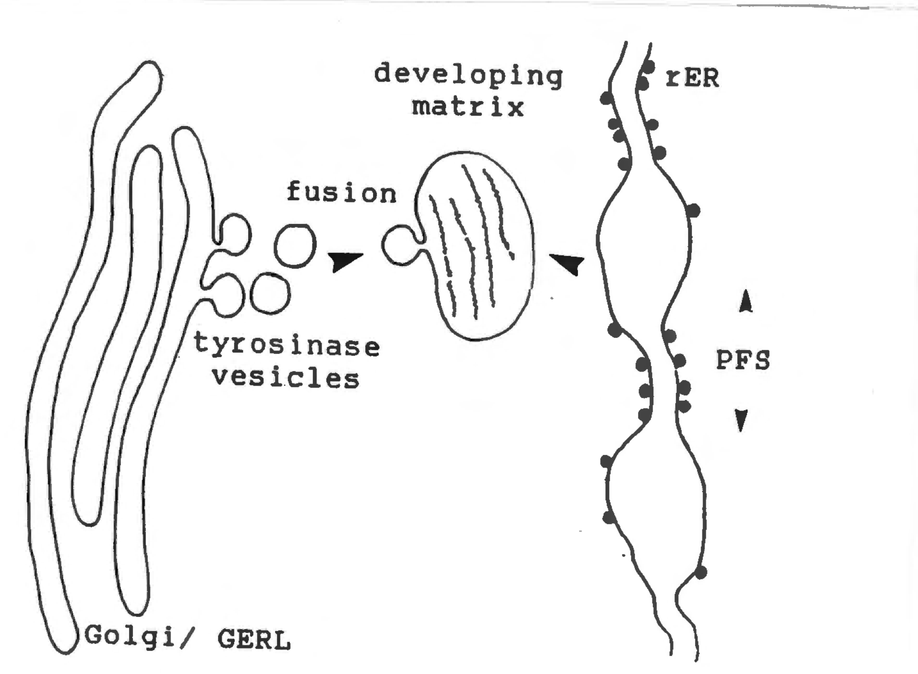


Figure 4.1. Schematic representation of melanosome ontogeny. rER = rough endoplasmic reticulum. PFS = "premelanosome forming center".

As with the development of the eumelanosomes, the earliest pheomelanosomes in red (e^Y/e^Y) feathers and yellow hairs are also spherical, membrane limited vacuoles (Jimbow et al., 1979). The vacuoles become aggregated with an amorphous proteinaceous material, some VGBs, and sometimes a few disorganized and incomplete filaments. As the melanosomes develop, the proteinaceous materials and the number of VGBs increase. The VGBs then appear to fuse with each other and with the surrounding materials, followed by a "spotty" melanisation of the proteinaceous material as well as the VGB cores, until the entire melanosome is amorphous and electron dense.

From the above it appears that VGBs may play an important role in both eumelanosome and pheomelanosome maturation. In fact, evidence suggests that they are tyrosinase containing units. VGBs have been shown to contain dopa reaction product in the pheomelanosomes of yellow mouse hairs (Sakurai et al., 1975) and in the eumelanosomes of black regenerating feathers (Maul and Brumbaugh, 1971). In the latter study it was found that in the early melanosomes, a few of the VGBs contained dopa-reaction product, while in the more mature melanosomes, all the VGBs were dopa positive. To reconcile these observations, it was speculated that VGBs do contain tyrosinase, but that the tyrosinase may be initially inhibited, followed by a gradual release of this inhibition, until all the VGBs are dopa positive.

Jimbow and Fitzpatrick (1973) report that the eumelanosomal VGBs from various tissues do not melanise within themselves at any stage of melanosome melanisation. Thus, if VGBs carry tyrosinase, as indicated above, it is conceivable that tyrosinase must be released from them to melanise the lamellar matrices. Since the VGBs are present at all stages of melanisation, it would appear that for such a release, a breakdown of the VGBs is not necessary. In fact, it has been reported that once incorporated into the inner matrices, the VGBs were resistant to degradation by phenol and thioglycolic acid and prolonged boiling at 130°C (Jimbow and Fitzpatrick (1973). The reason for the "toughness" of the VGBs and the mode of the possible release of tyrosinase from these vesicles has not yet been clarified and requires further study.

Unlike the situation in eumelanosomes, melanin deposition in pheomelanosomes occurs both within and around the VGBs. While VGBs are associated with the inner lamellae of eumelanosomes, they apparently fuse with each other in pheomelanosomes to form an amorphous matrix for melanin deposition (Jimbow et al., 1979; 1983). The "spotty" melanisation pattern of mammalian and avian pheomelanosomes (Jimbow et al., 1979; 1983) is not well understood, but Bagnara et al.(1979) believe that the proposed events in the development of goldfish melanosomes may provide some clues to understanding this process. A study by Turner et al., (1975) showed that the assembly of goldfish melanosomes

begins with vesicles blebbing from rough ER, simultaneous with the budding of dopa positive vesicles from the Golgi complex. The small and large vesicles fuse so that the former become incorporated into the membranes of the large vesicles. Once incorporated, the small vesicles invert and reform within the larger vesicles, which are then called multivesicular bodies. During the inversion, tyrosinase is thought to be transposed from the interior to the exterior periphery of the vesicles. As their theory then suggests, they found that melanin deposition first appears around the periphery of the inverted vesicles, followed by gradual deposition around the intervesicular spaces, giving the melanosomes a "moth-eaten" appearance. Eventually the interiors of the inverted vesicles are also melanised.

In the above scheme therefore, the internal vesicles carry tyrosinase (enzymatic function) and serve, in the absence of an organised fibrillar matrix, as the structural frame for melanin deposition. Since mammalian and avian pheomelanosomes also form from multivesicular bodies, and lack an organized lamellar matrix, the VGBs may very well serve as a frame for melanin deposition as proposed for goldfish melanosomes. However, consensus on this idea has not been reached since reports vary as to whether melanisation begins within or between the VGBs (see Bagnara et al., 1979). In this respect, the finding by Sakurai et al. (1975) of dopa positive VGBs in early mouse pheomelanosomes suggests that tyrosinase is localised *within* the VGBs and therefore that the VGBs from these pheomelanosomes are not inverted tyrosinase containing vesicles.

4.4. METHODS

4.4.1. Skin specimens

Black Australorp X New Hampshire Red and purebred New Hampshire Red eggs were incubated for 10 to 13 days at 37°C (50 - 60%). Embryos were removed from the eggs, rinsed in sterile chick saline (0,12 M NaCl, 5 mM KCl, 1 mM $CaCl_2$, pH 7,4), and then transferred to primary fixative (a modified Karnovsky's fixative - Karnovsky, 1965). Skin samples from each embryo were then dissected from the lateral neck area and diced into ±1 mm^3 cubes. The cubes were transferred to fresh primary fixative and left at 4°C for the remainder of the primary fixation period.

4.4.2. Feather follicle tissue

Red feathers were plucked from the breast of a New Hampshire Red cock. Tissues from the bases of feather follicles were squeezed directly into primary fixative (modified Karnovsky's). After approximately 30 minutes, the tissue pieces were diced into ±1 mm^3 cubes, transferred to fresh primary fixative, and left at 4°C for the remainder of the primary fixation period.

4.4.3. Processing

After primary fixation for 3 hours at 4°C, tissue specimens were washed in buffer before being immersed in secondary fixative (1% Osmium tetroxide in phosphate buffer, for 1 hour at 4°C). They were then dehydrated in an ascending alcohol series to Spurr's resin (Spurr, 1969). The specimens were embedded in fresh Spurr's resin and polymerised for 18 hours at 60 °C, according to the schedule outlined in Appendix 5.

4.4.4. Sectioning

Blocks were trimmed on an LKB Utratome 3 with glass knives. One micron sections were cut and stained for 5 to 10 seconds with 1% toluidine blue in 1% borax after which they were rinsed in distilled water, dried, and mounted in DPX. Sections were inspected for areas sectioned at favourable angles. Block faces were now trimmed to include such areas. Silver to gold sections (70 - 90 nm) were cut with glass knives and ribbons were picked up onto 200 mesh copper grids. Sections were stained with 8% uranyl acetate and Reynolds lead citrate (Reynolds, 1963) before being viewed on a Hitachi H600 transmission electron microscope at an accelerating voltage of 75 kV.*

4.5. RESULTS

4.5.1. Melanosomes in the 10 day embryonic Black Australorp X New Hampshire Red (E/e^{Wh}) epidermis

Melanosome morphology was studied in the 10 day Black Australorp X New Hampshire Red (BA/NHR) (E/e^{Wh}) embryonic epidermis to determine whether the melanocytes produce both eumelanosomes and pheomelanosomes, or alternatively, whether separate eu- and pheomelanocytes exist in this phenotype.

Melanocytes were readily distinguishable in the epidermis as large cells containing several melanosome clusters, surrounded by melanosome bearing dendrites and melanin free keratinocytes (figure 4.2). In all melanocytes, the majority of melanosomes had the basic morphological features of "typical" eumelanosomes; ie they were membrane bound, ellipsoid or rod shaped organelles with organized lamellar matrices (figure 4.3A and B). The matrix lamellae had a zigzag appearance and were arranged parallel to the melanosome long axis (figure 4.3B). Melanosomes sectioned at right angles to their long axes (ie cross sectioned) revealed that what appeared to be individual lamellae in longitudinal sections, in fact represented a single or a few lamellar sheets, rolled concentrically within the melanosomes (inset, figure 4.3B).

* In the case of Black Australorp X New Hampshire Red specimens approximately 5 sections (non-consecutive, with varying intersection gaps) from each of 3 different embryos were examined. Approximately 3 sections (non-consecutive, with varying intersection gaps) from specimens of each of 4 different New Hampshire Red feather follicles were examined.

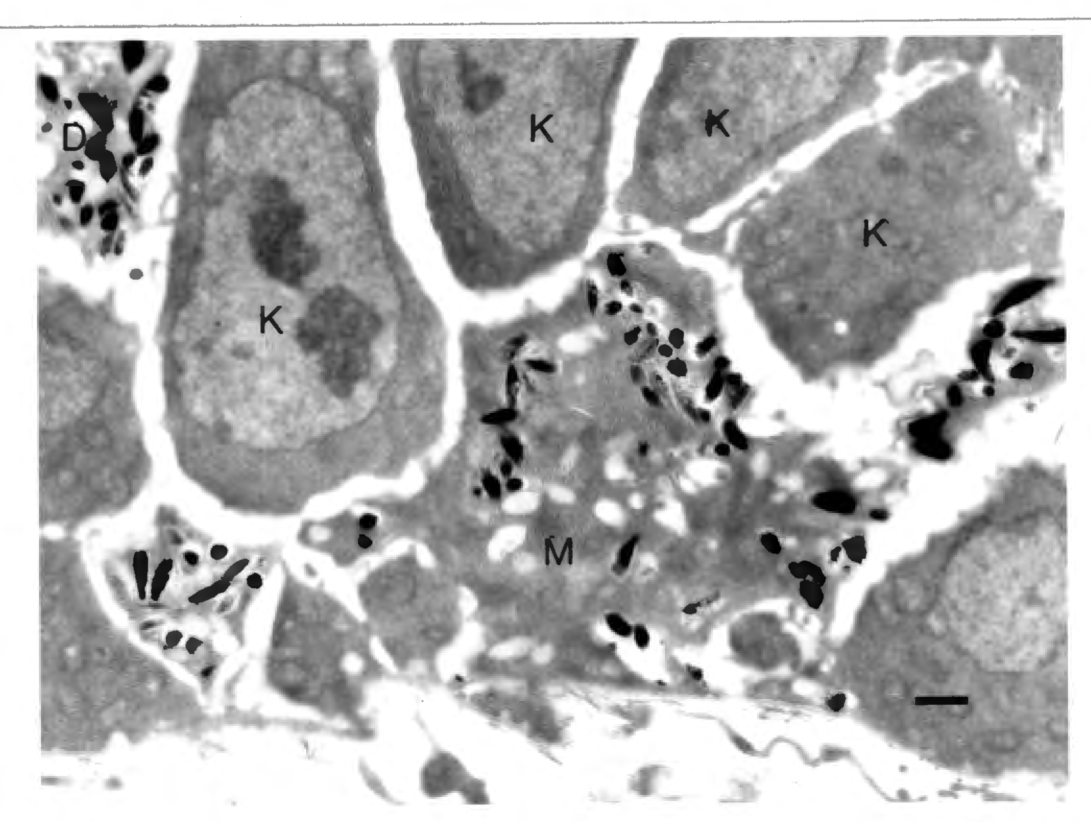
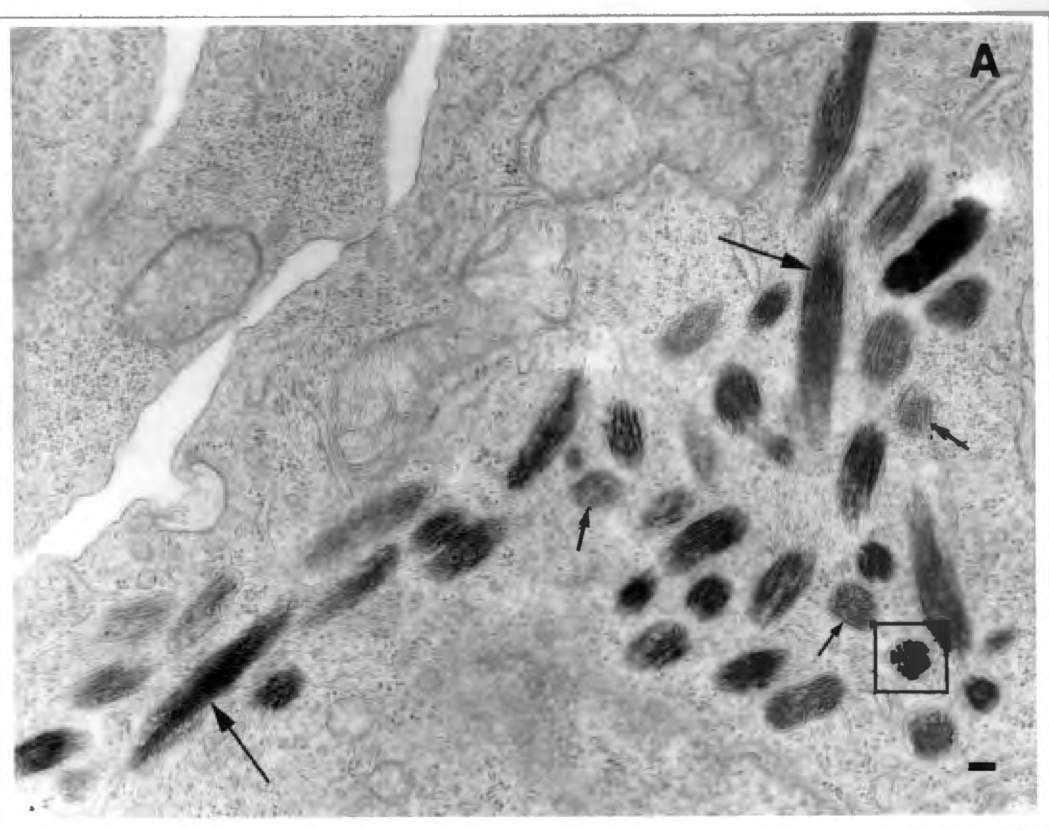


Figure 4.2. Section of 10 day BA/NHR epidermis. Melanocytes (M) were readily distinguisheable from the keratinocytes (K) as large cells with many melanosomes, surrounded by melanosome bearing dendrites (D). Bar = 0,1 μm.

Most of the melanosomes were found to be at advanced stages of melanisation, while the remainder were at very early stages of development. Very few of the melanosomes were at intermediate developmental stages. A portion of a possible "premelanosome forming center" (Stanka et al., 1981) is presented in figure 4.3B. An early melanosome appears to be pinching off from the center, and a coated vesicle (carrying tyrosinase?) is seen fusing with it. Multivesicular bodies were also observed in some melanocytes (figure 4.4 and inset).

In melanosomes at advanced stages of melanisation, all detail of the internal matrix was obscured by melanin, giving them the appearance of uniformly electron dense ellipsoids. Often, small electron lucent regions were visible within the electron dense ellipsoids, the significance of which will be discussed later. A thin electron lucent rim was present between the lamellae and the outer limiting membrane during the entire course of melanin deposition (figure 4.3B), although the outer membrane was not always visible.

Apart from those that were ellipsoidal with lamellae, all melanocytes also contained melanosomes that appeared to be spherical in outer shape (see figure 4.3A, 4.4 and 4.5). These melanosomes were either distributed amongst groups of ellipsoidal melanosomes or formed groups where they predominated over the ellipsoidals. While some of the apparently spherical melanosomes could be identified as obliquely sectioned eumelanosomes, (short arrows, figure 4.3A and asterisks, figure 4.4), the identity of most were uncertain due to advanced melanisation which obscured all internal detail. In some of those that were less melanised however, the ordered lamellar eumelanosomal pattern of pigment deposition was not always evident. Instead, melanisation within such melanosomes appeared to occur upon irregularly arranged, filamentous, but non-lamellar matrices (see insets, figure 4.4).



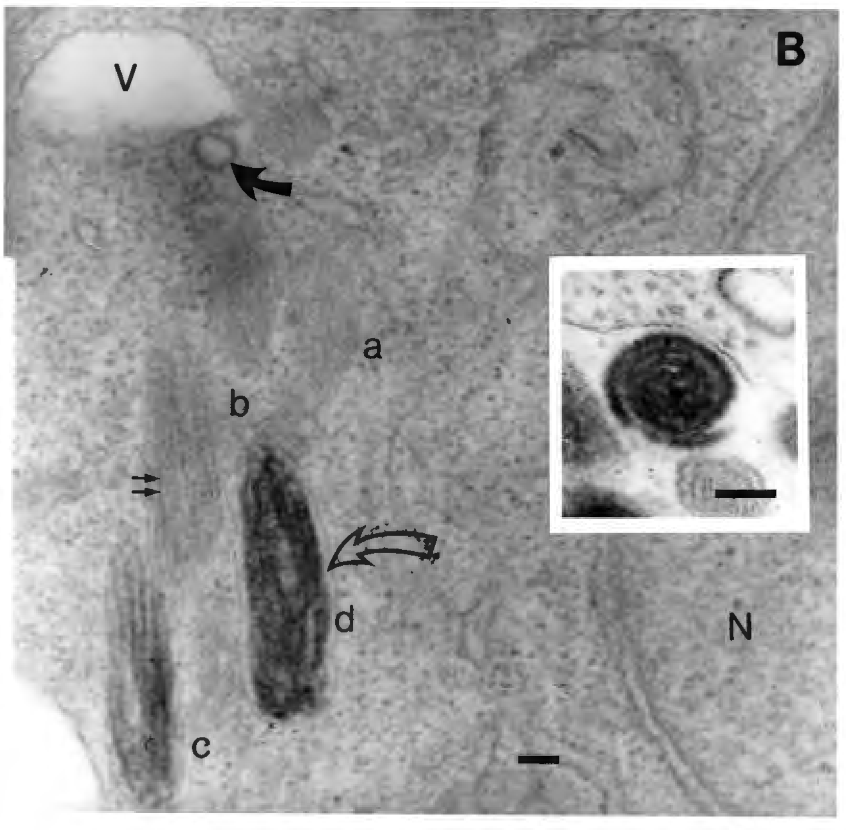


Figure 4.3. Melanocytes in 10 day BA/NHR epidermis. A: Group of melanosomes, some of which are sectioned longitudinally (long arrows), and others obliquely (short arrows). Square: This melanosome is not typically eumelanogenic (see inset, ~~2.8B~~ 4.4C) and appears to possess a non-lamellar matrix of short, irregularly arranged filaments. B: Early eumelanosomes at progressive stages of development (a, b, c, d respectively). Small arrows: zigzag appearance of matrix lamellae. A coated vesicle (curved arrow) is seen, apparently fusing with an empty vacuole (V), possibly representing a forming eumelanosome. Open arrow: electron lucent rim between lamellae and outer melanosome membrane. N: nucleus. Inset: cross-sectioned melanosome showing that the lamellae are rolled concentrically. All bars = 0,1 μm.

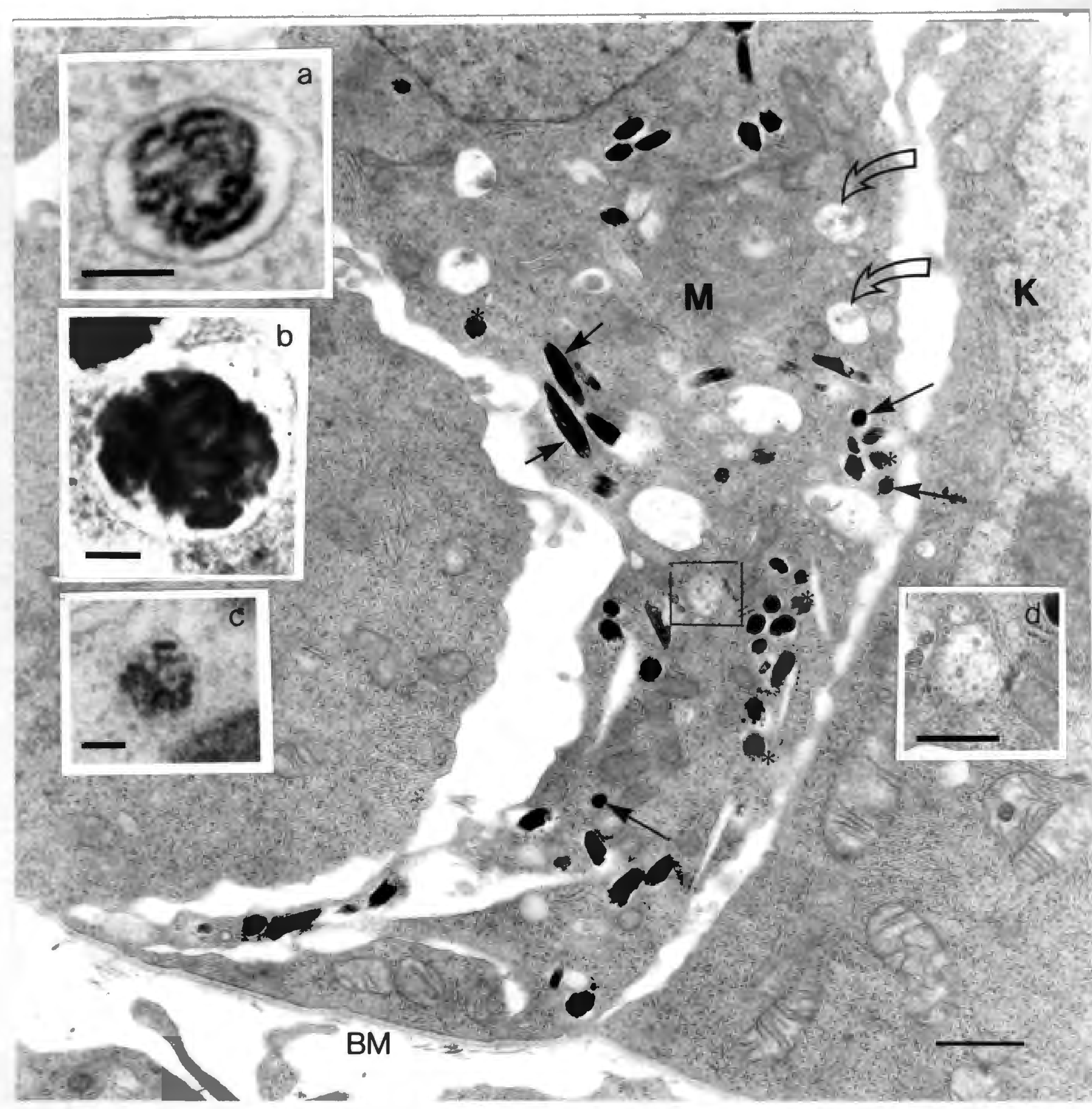


Figure 4.4. Melanocyte (M) in 10 day BA/NHR epidermis. Both ellipsoidal (short arrows) and apparently rounded melanosomes (long arrows) are seen. Some of the rounded melanosomes can be identified as obliquely sectioned eumelanosomes (*). Open arrows and square: multivesicular bodies containing numerous internal vesicles. Notice how large some of these bodies are compared to the mature melanosomes. Bar = 1 µm. Thick arrow and insets a, b, and c: Rounded melanosomes with irregularly arranged filamentous, but non-lamellar matrices (inset c is a high power view of the square in figure 4.3A). Inset d: higher power of squared multivesicular body. BM = basement membrane. K = keratinocyte. Bars insets a, b and c = 0,1 µm. Bar inset d = 0,5 µm.

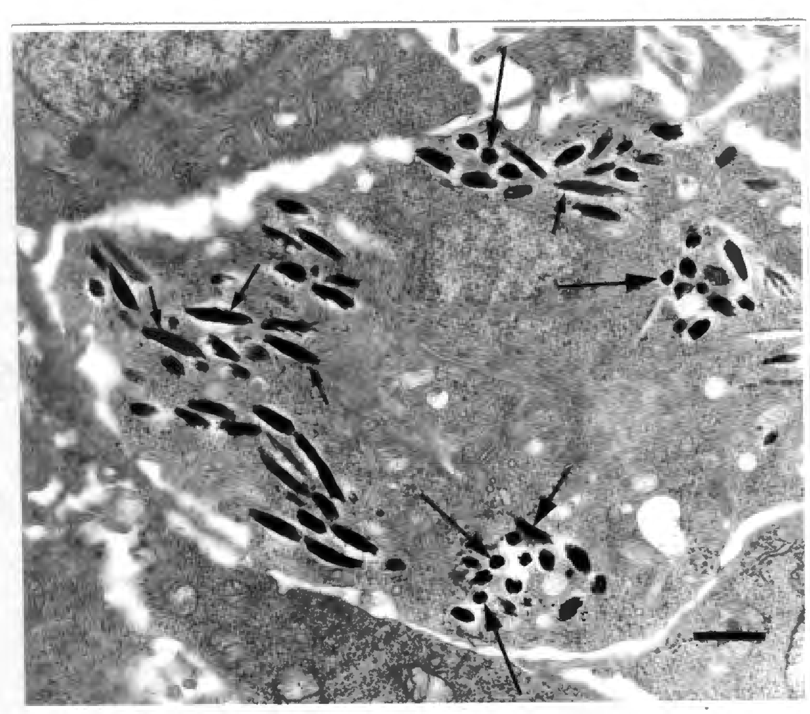


Figure 4.5. Portion of melanocyte in 10 day BA/NHR epidermis. Apparently rounded melanosomes (long arrows) are seen amongst ellipsoidal melanosomes (short arrows). Notice that most of the melanosomes are at advanced stages of melanisation. Bar = 1 μm.

4.5.2. Vesiculo-globular bodies (VGBs) in Black Australorp X New Hampshire Red eumelanosomes

The presence of vesiculo-globular bodies (VGBs) was evident in many of the ellipsoidal-lamellar BA/NH-R eumelanosomes. Observations made on these melanosomal subunits are described below.

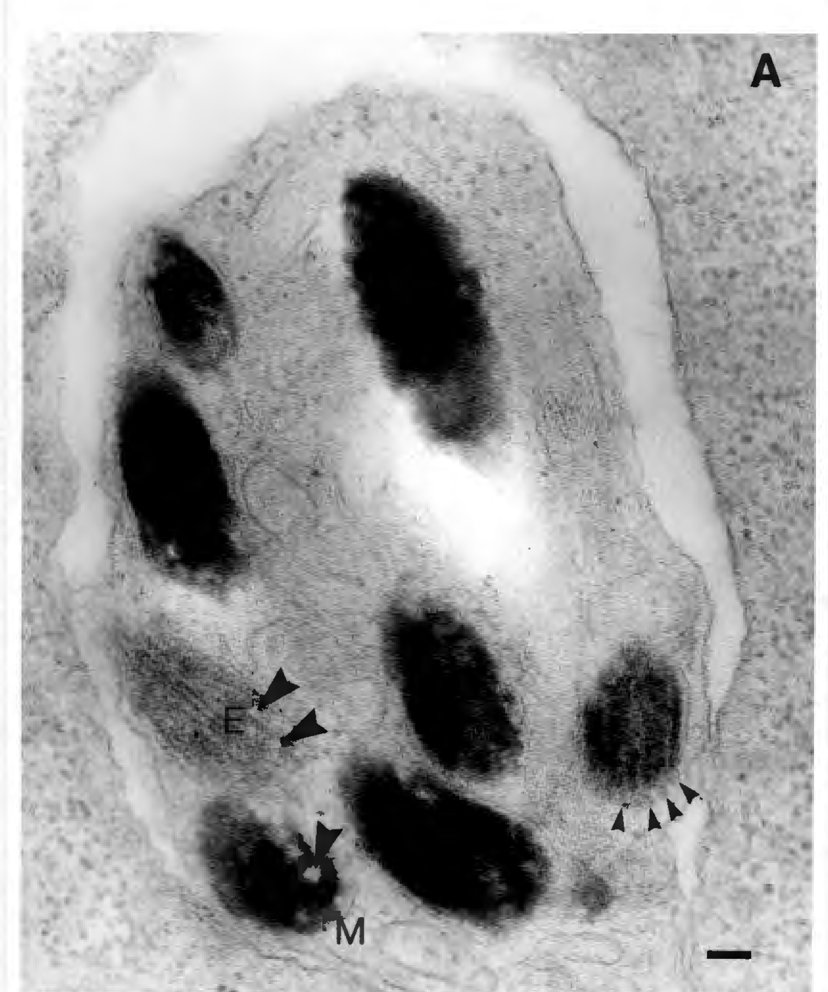
The interiors of early as well as mature melanosomes were often studded with electron-lucent "holes"/VGBs (figure 4.6A). In some cases, such "holes" appeared to indent the outer melanosome surfaces (figure 4.6B). Another frequent observation was that of spherical bodies/VGBs along the melanosome peripheries (figure 4.6B). These bodies contained varying amounts of a fine granular material. Contrary to previous reports that eumelanosomal VGBs do not melanise within themselves (see introduction), VGBs with completely electron dense contents (presumably melanin) were seen in many of the melanosomes examined in the present study (figure 4.6C and figure 4.7). Such VGBs were always associated with densely pigmented melanosomes, and appeared as electron dense globular protrusions beneath the outer melanosome membranes. Due to their electron density, the VGBs often appeared to be continuous with the inner melanosomal mass.

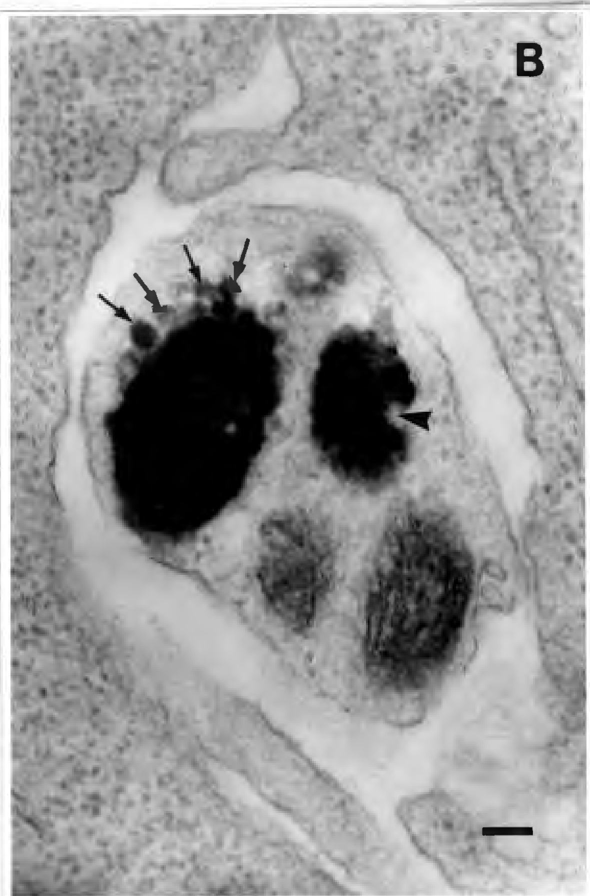
Comparative measurements of outer limiting membrane thicknesses in fowl eumelanosomes at various stages of development (Stanka and Sahlman, 1981) have shown that the limiting membrane is transformed from a thin (ER), to a thick (early melanosome) and again to a thin (mature melanosome) state. Figure 4.8 includes a possible example of a developing eumelanosome at the "thick" membrane stage: a group of spherical bodies/VGBs (diameter ±40 nm) appears to be encased within a thick, rod-shaped membrane. However, the identification of this structure as an early melanosome is dubious, since the ultrastructural texture inside the putative eumelanosome is similar to the texture of the surrounding cytoplasm.

4.5.3. Melanosomes in the 13 day embryonic feather bud epidermis and in mature feather follicles of New Hampshire Red (e^{Wh}/e^{Wh}) fowl

Embryonic and mature feather tissues of New Hampshire Red (NHR) (e^{Wh}/e^{Wh}) fowl were examined to investigate the morphology and development of the melanosomes in these fowl.

Figure 4.9 shows portions of two melanocytes in a 13 day embryonic feather bud. As is evident in these micrographs, most of the melanosomes were found to be at advanced stages of melanisation, while a small number of very early and intermediate melanosomes were also seen. The melanosomes were in general spherical or slightly oval, while a few were more irregular in shape. No ellipsoidal-lamellar melanosomes were observed in this tissue.





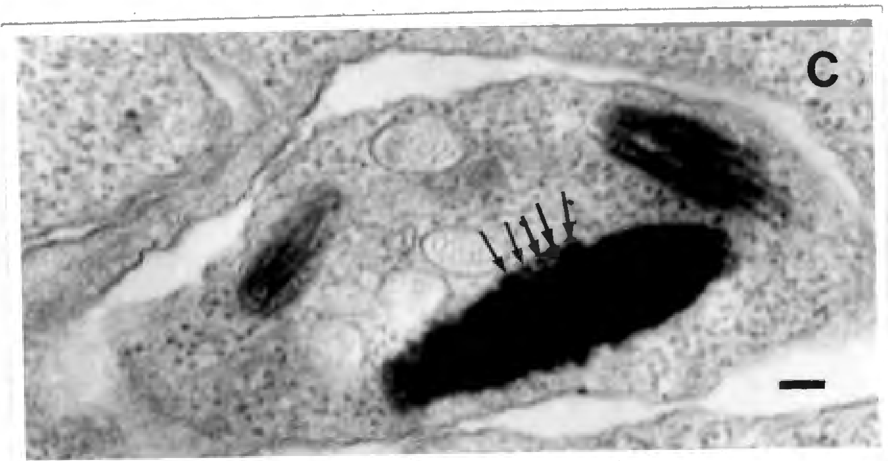


Figure 4.6. VGBs in BA/NHR eumelanosomes. A: VGBs appear as electron lucent "holes" (larger arrowheads) in early (E) and more mature (M) melanosomes, or as spherical bodies with a fine granular content (smaller arrowheads). Notice that the latter VGBs appear to align with the lamellae. B: A VGB "indents" the surface of one melanosome (arrowhead). VGBs along the periphery of an adjacent melanosome appear as spherical bodies containing varying amounts of a fine granular material (arrows). C: Completely electron-dense VGBs protrude from the outer surface of a melanosome (arrows). All bars = 0,1 μm.

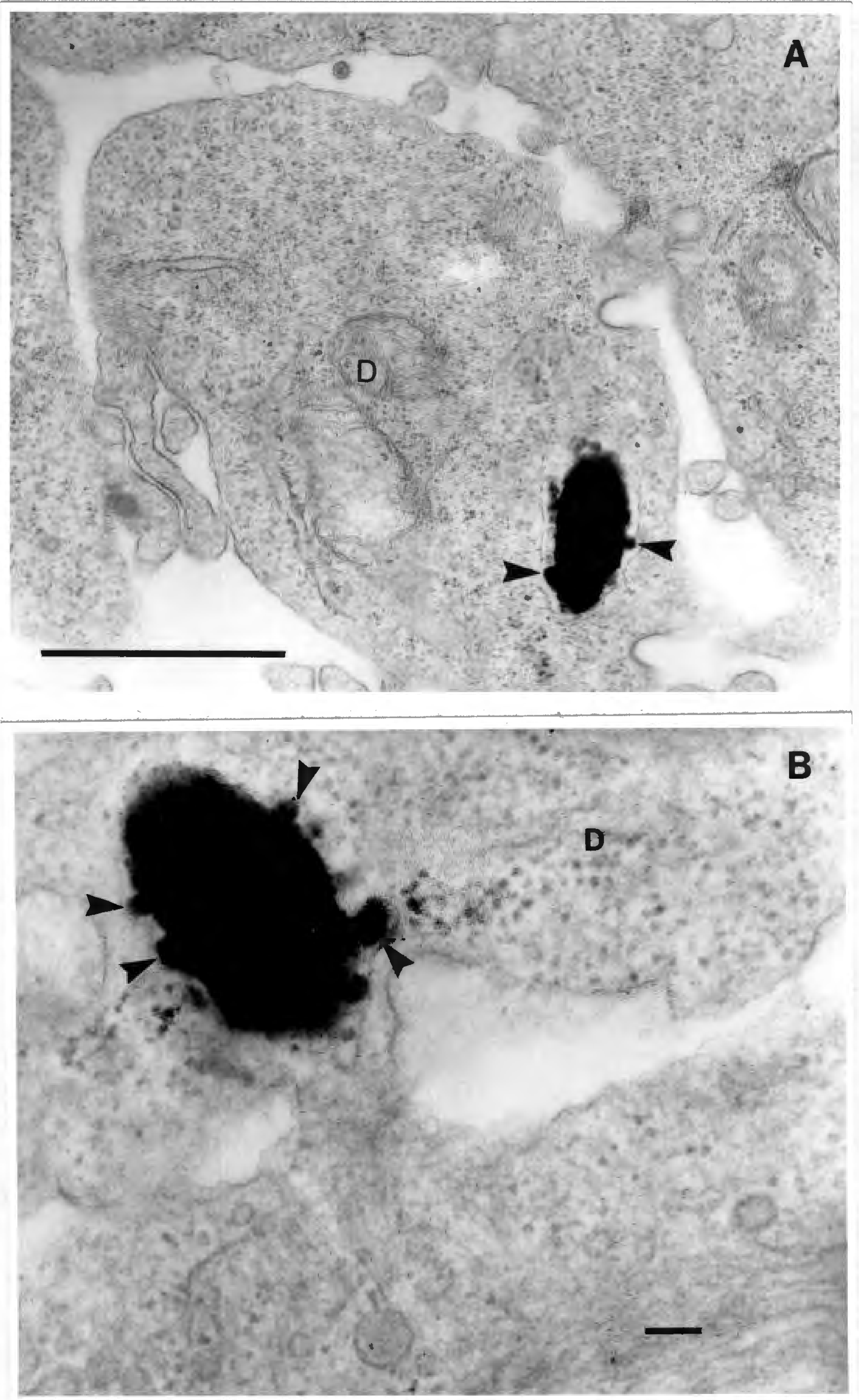


Figure 4.7. A and B: Portions of melanocyte dendrites (D) in 10 day BA/NHR epidermis, within the section each containing a single melanosome. VGBs (arrowheads) appear as electron dense globular protrusions beneath the outer melanosome membranes. Bar in A = 1 µm. Bar in B = 0,1 µm.

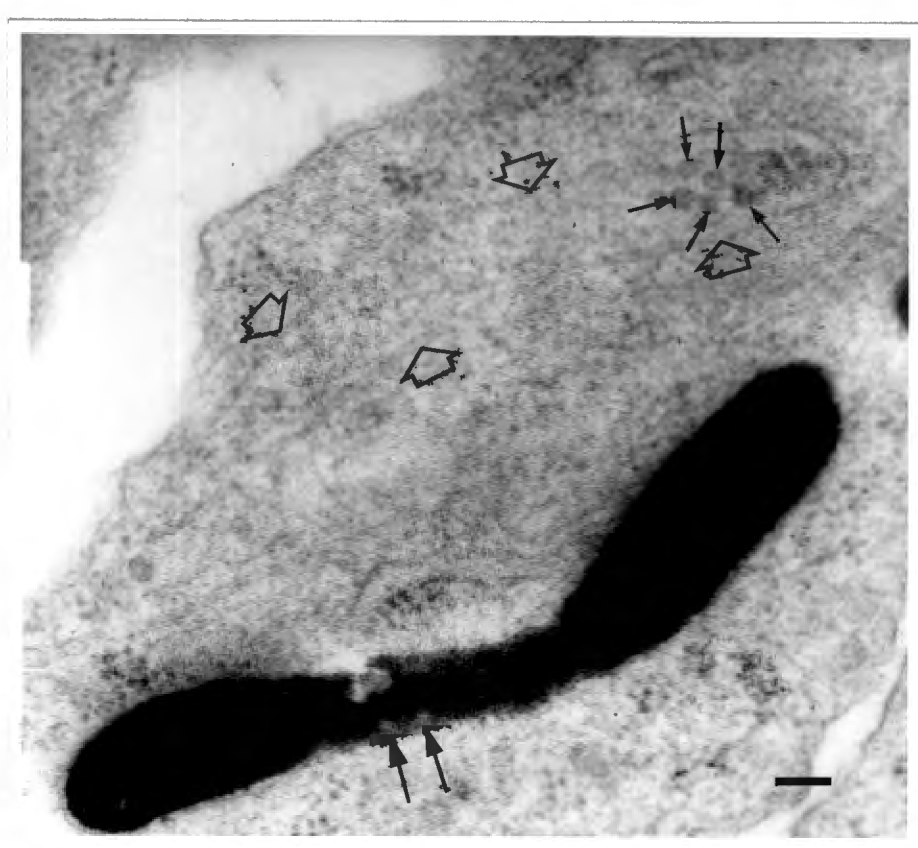
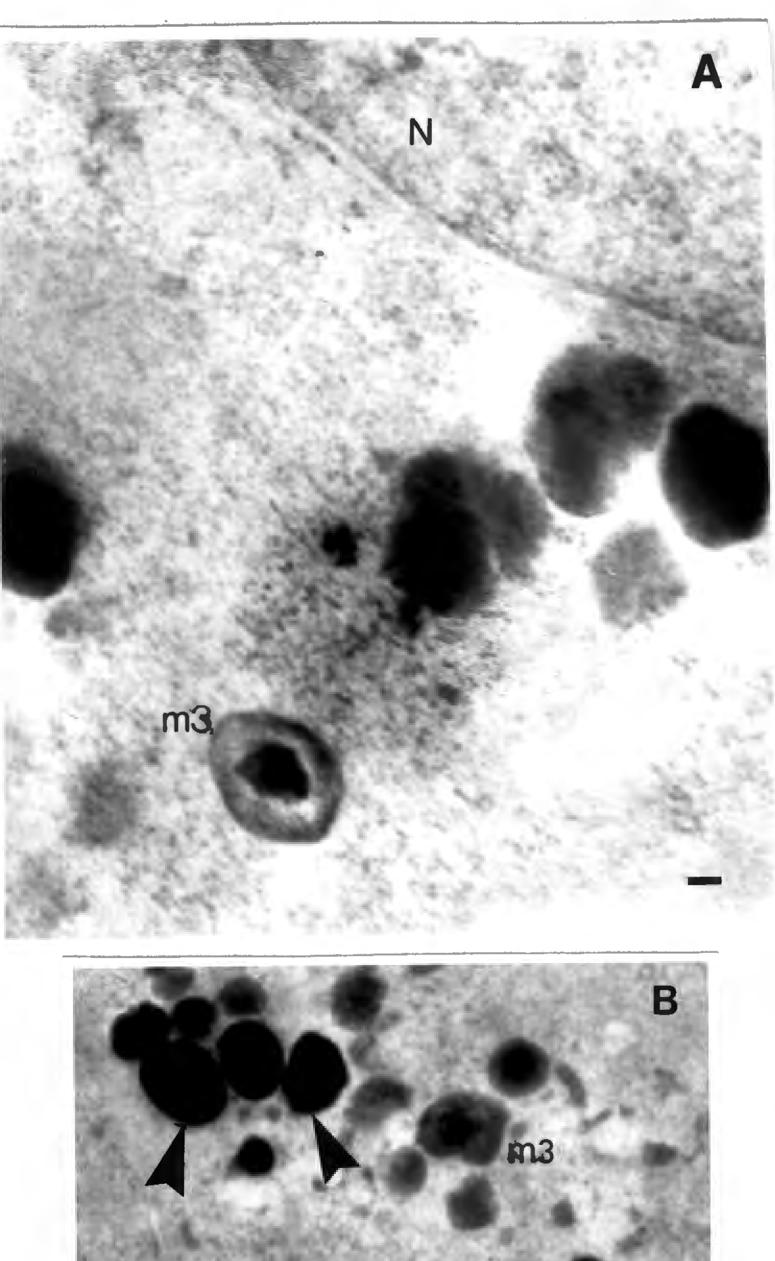


Figure 4.8. Melanocyte with mature, very elongated eumelanosome. Spherical, less electron-dense "holes" (VGBs) are seen within the electron-dense melanin (larger arrows). Open arrows delineate what appears to be a thick, rod-shaped membrane, encasing a group of possible VGBs (diameter ±40nm) (small arrows). Bar = 0,1 μm.



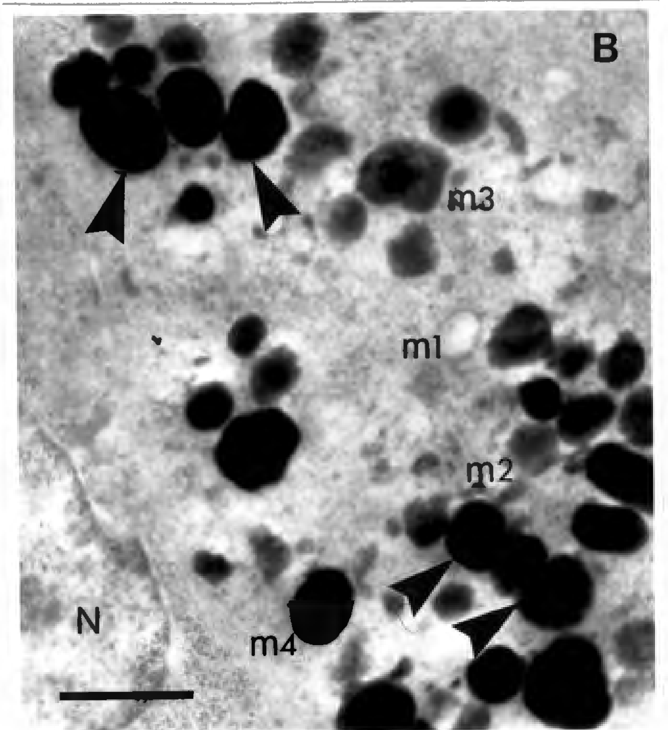


Figure 4.9. A and B: NHR pheomelanosomes in 13 day embryonic feather bud. The melanosomes appear to develop from empty vacuoles (m1), that accumulate a fine granular material (m2), followed by the appearance of an electron dense mass in the center of the melanosome (m3). This mass expands until the melanosomes are entirely electron dense (m4). Arrowheads: melanosomes occuring in groups, as if having formed together as units. N = nucleus of melanocyte. Bars = 1 µm.

The earliest melanosomes were empty, membrane bound vacuoles (figure 4.9). Intermediate stages of development seemed to involve the accumulation of a fine granular material, followed by the appearance of an electron dense mass, usually in the center of the melanosome. This electron dense mass expanded until the melanosomes were entirely electron dense. In some mature melanosomes, a thin outer rim of the fine granular material was present.

The melanosomes often occured in groups of two or more, as if having formed together as units (fig 4.9A). This grouping of melanosomes was also evident in sections of the follicular tissue of mature NHR feathers (figure 4.10). Many such units appeared to be embedded within an amorphous material. Interestingly, the individual melanosomes within the units were ~~were~~ not always at the same stage of development.

As with the melanosomes in the embryonic feather buds, those observed in the mature feather follicles were spherical, slightly oval, or irregular in shape. However, one single ellipsoidal-lamellar melanosome was also found (see later). While the embryonic material contained mostly mature melansomes, the mature feather follicle tissue examined provided ample examples of melanosomes at all stages of development. This allowed for a closer inspection of processes such as pigment deposition and the accumulation of VGBs.

Figure 4.11 shows two groups of developing melanosomes from the latter tissue. An accumulation of globular structures (VGBs) is evident in two of the melanosomes (small arrows, figure 4.11). The VGBs contain varying amounts of an electron dense substance (presumably melanin), and are aggregated tightly together, as if fusing with each other. Melanisation apparently begins as a small mass in the central melanosome core, surrounded by varying amounts of a fine granular material (figure 4.11A and B). The initially small melanised cores then enlarge as melanisation proceeds, until the melanosomes are electron dense spheroids. In many mature melanosomes, a thin outer rim of the fine granular material is present.

The above developmental scheme seemed to apply to most of the melanosomes observed in the embryonic as well as the mature feather tissues examined. In a smaller number of melanosomes, however, melanisation did not proceed through this "central core expansion", but appeared to occur more "randomly" as in the pheomelanosomes described by Jimbow et al. (1979;1983). This latter term is used in the sense that such melanosomes contained varying quantities of melanising globular structures/VGBs that were scattered throughout the melanosome interiors (figure 4.12). Also seen in this figure, as noted above, is an ellipsoidal melanosome with a lamellar structure.

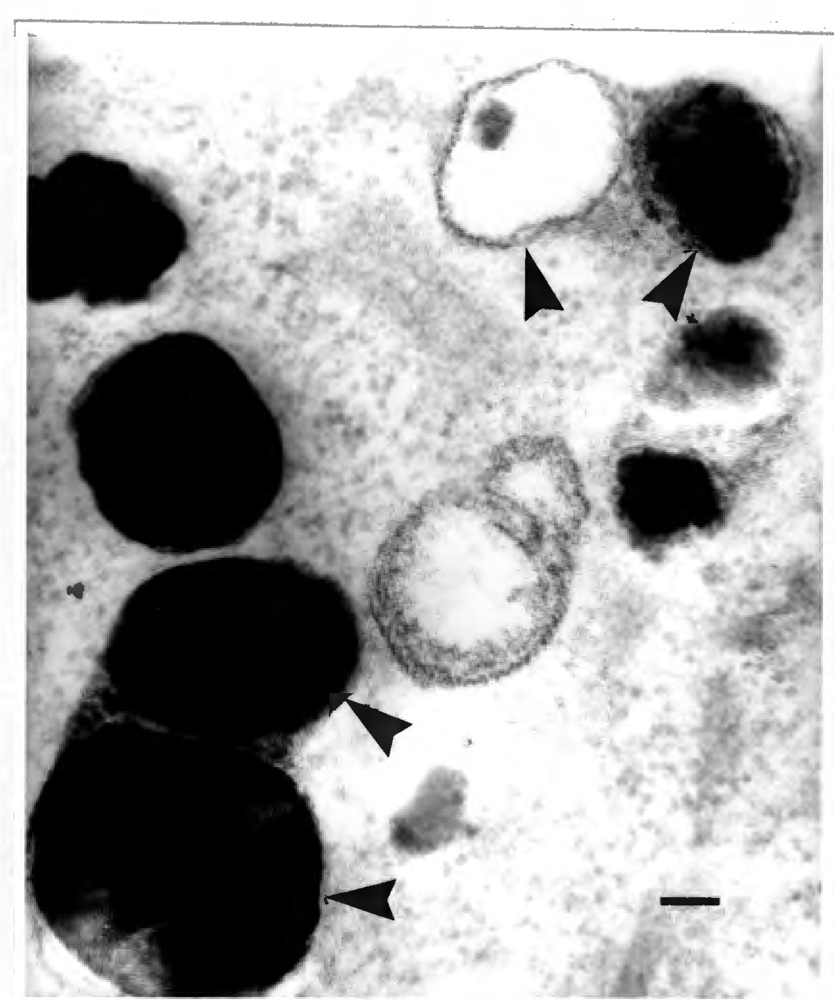


Figure 4.10. Pheomelanosomes in mature NHR feather follicle. Notice the melanosome "doublets" (arrowheads), apparently embedded in an amorphous material. The individual melanosomes within the upper doublet are clearly at different stages of development. Bar = 0,1 µm.

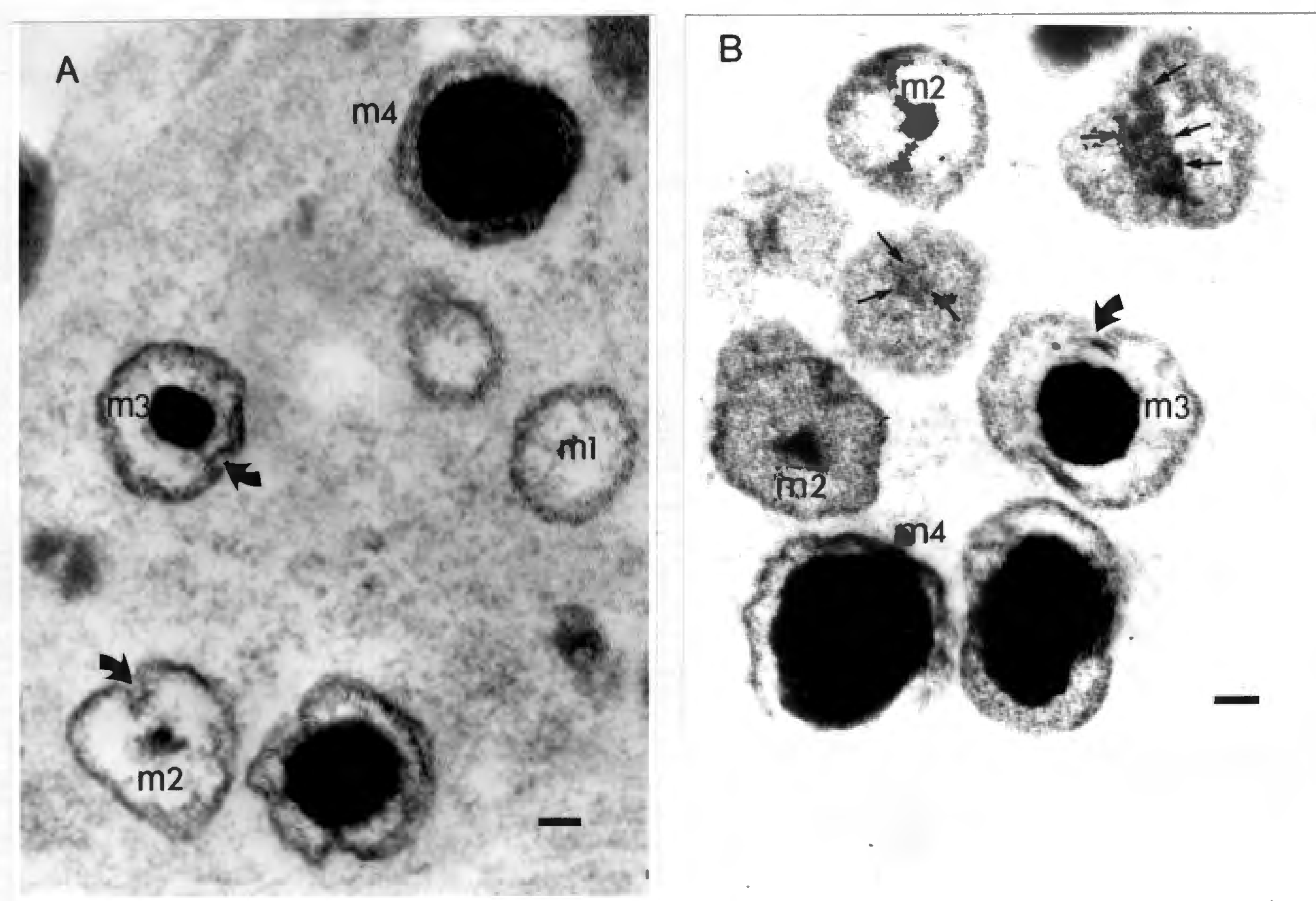


Figure 4.11. A and B: Pheomelanosomes at various stages of development in mature NHR feather follicle tissue. The earliest melanosomes are spherical vacuoles that contain varying amounts of a fine granular material (m1). Melanisation begins as a small mass in the melanosome core (m2), and then expands towards the periphery (m3, m4). Globular bodies/VGBs are seen in some early melanosomes (small arrows). Melanosomes frequently possess outer membrane indentations (curved arrows), resembling inverting tyrosinase vesicles in goldfish melanosomes. Bars = 0,1 μm.

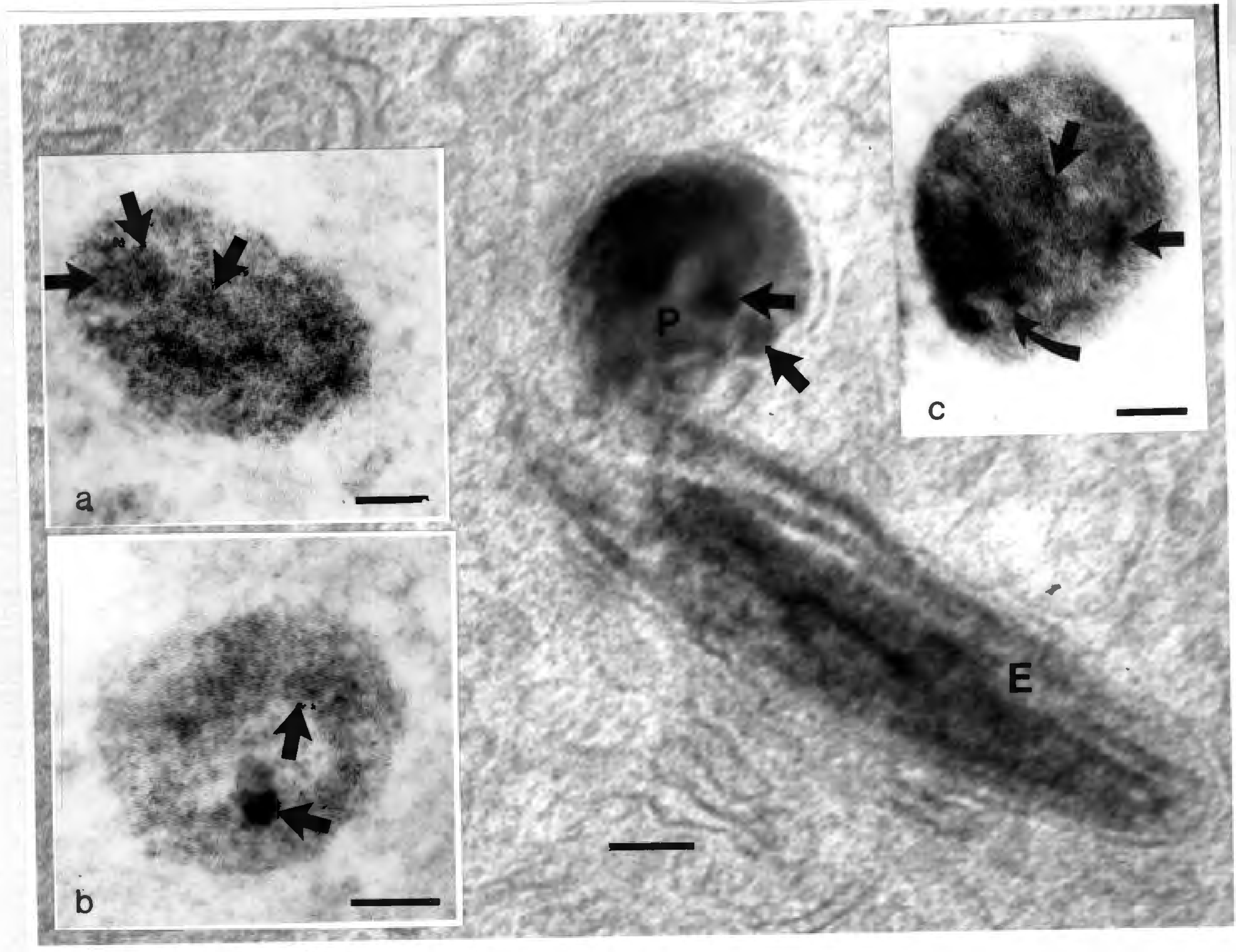


Figure 4.12. Insets and P: Pheomelanosomes with "randomly" arranged VGBs (arrows) in melanocytes of mature NHR feather follicles. As seen in the insets, the outer melanosome membranes and outer VGB borders were often indistinct. The VGBs appear to accumulate an electron dense substance (presumably melanin), and seem to fuse with each other in the more mature melanosomes (curved arrow). An ellipsoidal-lamellar, eumelanosome-like organelle is also present (E). All bars = 0,1 µm.

Finally (figure 4.11A and B), it was noted that the outer membranes of the early "symmetrical" type pheomelanosomes were often indented, resembling the inverting tyrosinase vesicles seen in the membranes of early goldfish melanosomes (see introduction). However, in no case was there evidence for pigment deposition around the *peripheries* of internal vesicles (as proposed for goldfish melanosomes).

4.6. DISCUSSION

4.6.1. Melanosomes in 11 day Black Australorp X New Hampshire Red (E/e^{Wh}) melanocytes are not all typical eumelanosomes

The Black Australorp X New Hampshire Red (BA/NHR) (E/e^{Wh}) cross breed produces feathers that are black, with a reddish lustre. This colouring suggests that the feathers are pigmented primarily by eumelanin, but that some pheomelanin is also present. The first question asked in the present study is whether the melanocytes of these fowl differentiate into separate eumelanosome- and pheomelanosome synthesizing cells, or alternatively, into mixed type melanocytes. This has been investigated in the 11 day embryonic epidermis.

The melanocytes observed in the above tissue were found to contain mostly mature melanosomes, with few examples of melanosomes at intermediate stages of melanisation. The significance of this finding is not clear, but such a paucity of intermediate stages was also reported by Maul and Brumbaugh (1971). In the present study, examples of very early melanosomes included possible "premelanosome forming centers", and multivesicular bodies that were observed amongst groups of melanosomes. These bodies consisted of rounded vacuoles containing numerous internal vesicles/VGBs, and were often much larger than the more mature melanosomes.

In all E/e^{Wh} melanocytes, most of the melanosomes had typical eumelanosomal features. They were ellipsoidal or rod shaped, and contained an organized matrix consisting of longitudinally arranged lamellae with a zigzag appearance. In cross sections, the matrix appeared to be organized into single, or perhaps more than one consentrically rolled lamellar sheets. The lamellae of the matrix became gradually obscured by melanin, until the melanosomes were completely electron dense with no internal detail visible. A thin electron lucent rim appeared to persist between the lamellae and the outer limiting membrane.

Amongst these typically eumelanogenic melanosomes, varying numbers of melanosomes with a spherical outer shape were also seen in all E/e^{Wh} melanocytes. In many cases, such apparently spherical melanosomes could be identified as obliquely or cross sectioned eumelanosomes by their ordered lamellar internal structure. Often however, their internal detail was completely obscured by melanin, giving them the appearance of membrane limited, electron dense spheroids. Such melanosomes were therefore similar to mature

mouse (Sakurai et al., 1975; Jimbow et al., 1979), human (Jimbow et al., 1983) and Rhode Island Red fowl (Jimbow et al., 1979) pheomelanosomes (see introduction). Further possible candidates for developing pheomelanosomes were the multivesicular bodies described above, since they were never found to contain developing filaments, as in early eumelanosomes (Jimbow et al., 1979). However, these bodies may simply not yet have begun the synthesis of filaments.

The abovementioned similarity between the rounded, completely melanised melanosomes and mature pheomelanosomes does not prove that the numerous organelles of this type were in fact pheomelanosomes and not cross-sectioned eumelanosomes. Furthermore, despite thorough searching, no rounded melanosomes with a morphology such as described for the earlier stages of the mature pheomelanosomes above (ie "spotty" melanisation upon and within a structural matrix of fused VGBs), were found. This suggests that the rounded melanosomes in question were probably cross-sectioned eumelanosomes. The occurence of rounded melanosomes in *groups* is probably due to the cross-sectioning of several ellipsoidal melanosomes aligned in the same plane within the cell. The likelihood of such an alignment was not investigated, but groups of longitudinally sectioned ellipsoidal melanosomes were often observed, suggesting that alignment does occur.

Morphologically, the least typically eumelanogenic melanosomes observed were some incompletely melanised ones in which the matrix appeared to consist *not* of *ordered* lamellae, but of irregularly arranged filaments, as seen in the e^+ and e^y pheomelanosomes described by Brumbaugh (1968). Such melanosomes were present only in a small percentage of the melanocytes. Similar to Brumbaugh's melanosomes, these melanosomes revealed characteristics intermediate between "typical" eu- and pheomelanosomes. Whether these organelles were indeed pheomelanic is an open question. For example, they may have represented cross sections of eumelanogenic melanosomes in which the filaments had, for some reason, not become organized into neat, concentrically arranged lamellae. In this sense, and in the absence of more "typical" pheomelanosomes, the evidence seems to weigh in favour of an absence of mixed type melanocytes. Furthermore, since no melanocytes were found to contain *only* rounded, pheomelanosome-like melanosomes, one may safely conclude that separate eu- and pheomelanocytes also do not exist in the 11 day skin of these embryos. It is possible that in the *mature* feather follicles, separate pheomelanocytes or even mixed type melanocytes differentiate to produce the red lustre of the feathers. However, due to an unavailability of fresh material, this was not investigated.

4.6.2. Vesiculo-globular bodies in Black Australorp X New Hampshire Red eumelanosomes

In agreement with previous reports (Jimbow and Fitzpatrick, 1973; Jimbow et al., 1979), vesiculo-globular bodies (VGBs) in the melanosomes of the red/black cross 1/ were present at all stages of melanosome development, and 2/ appeared as electron lucent "holes" against a background of melanin, or 3/ as non-lucent, spherical bodies containing varying amounts of a fine granular material. However, in contrast with the finding in E/E chick skin, feather and eyes that VGBs do not become melanised (Jimbow and Fitzpatrick, 1973; Jimbow et al., 1979), VGBs with an electron dense content (presumably melanin) were frequently observed in the present study. Such VGBs were only seen in mature melanosomes, and appeared as globular protrusions beneath the outer limiting membranes.

The above observation suggests that, similar to the VGBs in mouse and avian (Jimbow et al., 1979) and goldfish pheomelanosomes (Turner et al.,1975), the VGBs in E/e^{Wh} fowl may eventually become melanised. The fact that many mature melanosomes with electron lucent "holes" were also observed can be interpreted in several ways. For example, such VGBs may have simply not yet become melanised, or alternatively, the VGBs in some melanosomes may remain unpigmented, while those in other melanosomes will eventually melanise. Future studies may provide the answer to this question.

4.6.3. Ultrastructure of New Hampshire Red (e^{Wh}/e^{Wh}) melanosomes

New Hampshire Red (NHR) (e^{Wh}/e^{Wh}) embryonic feather buds and mature feather follicle tissues were examined to investigate the morphology and development of the melanosomes in these fowl.

Morphologically, only pheomelanosome-like (non-lamellar and rounded, slightly ovoid, or irregularly shaped) organelles were observed in both the embryonic and mature tissues studied, except that in one melanocyte from mature feather follicle, a single ellipsoidal-lamellar melanosome was found directly next to a pheomelanosome. This finding bears on the issue of whether mixed type fowl melanocytes exist or not, and, although conclusions cannot be drawn from an isolated incident, may prove a fruitful subject for future research on this genotype.

It may be surmised from the similarities between mouse (Sakurai et al., 1975; Jimbow et al., 1979), human (Jimbow et al., 1983) and Rhode Island Red fowl (Jimbow et al., 1979) pheomelanosomes that the melanisation of "typical" pheomelanosomes is "spotty" and occurs upon and within a matrix of fused VGBs. In this sense, some of the developing melanosomes observed in this study bore resemblance to "typical" pheomelanosomes. Such melanosomes contained varying numbers of randomly scattered, melanising VGBs, which

appeared to later fuse with each other. Most of the other melanosomes, however, appeared to follow a more "symmetrical, central core expansion" type of developmental sequence: melanisation began as a small, uniformly electron dense mass in the center of the organelle, and from there expanded outward as pigmentation continued. It seems that in this type of melanosome, the growing melanin cores are formed by a continual process of aggregation, fusion, and melanisation of VGBs (beginning in the melanosome center), as evidenced by the presence of melanosomes containing tightly packed central accumulations of globular bodies with varying electron densities.

It is not clear why the pigmenting VGBs in some melanosomes were scattered, while in most others, they appeared to immediately fuse in the melanosome center. Since the melanin cores were always uniformly electron dense, it seemed that no pigment deposition occured around the *peripheries* of internal vesicles (such as in goldfish melanosomes). However, the outer melanosome membranes were often indented so as to resemble inverting tyrosinase vesicles in early goldfish melanosome membranes (Turner et al., 1975). Speculatively, the indentations in e^{Wh}/e^{Wh} pheomelanosomes may contain tyrosinase which is bound to their *extramelanosomal* side, and reform as vesicles/VGBs with tyrosinase attached *internally*, so that pigmentation begins *within* the VGBs, and not on their peripheries (see figure 4.13).

Finally, an observation worth noting is that the melanosomes in both the embryonic and mature tissues frequently occured in groups of two or more, as if having formed together as units. Within such units, the individual melanosomes were sometimes at very different stages of maturation. This finding may relate to the report by Stanka et al. (1981) that chick eye melanosomes form from continuous cisternal ER complexes ("premelanosome forming centers") that pinch off to transform into groups of four to ten separate eumelanosomes. It is possible that in the pheomelanosomes of the present study, the ER of similar "centers" pinch off but that sometimes the individual melanosomes so formed do not move apart. Thus, the melanosome groups may be the consequence of an incomplete separation of individual pheomelanosomes from "premelanosome forming centers".

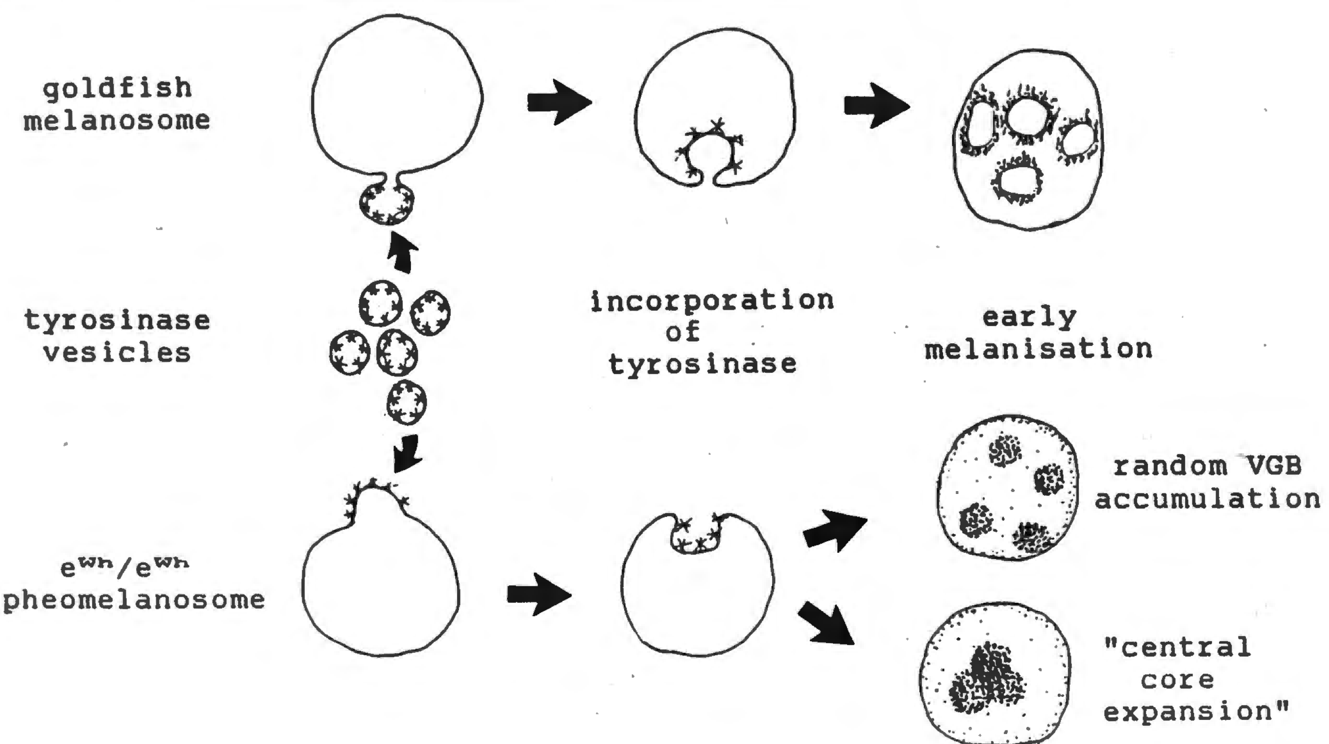


Figure 4.13: Schematic representation of possible positioning of tyrosinase molecules (*) in the membrane indentations of e^{Wh}/e^{Wh} pheomelanosomes, as compared with goldfish melanosomes.

5. FUTURE INVESTIGATIONS

The Black Australorp/New Hampshire Red cross breed has proved to be a valuable asset to the study of melanocyte differentiation. Not only does terminal differentiation occur at least $2^1/_2$ days earlier than is reported for other breeds, but this event was also found to be independent of location in an epidermal environment. The finding that some melanocytes fully differentiate in the dermis by Day 5 implies that tyrosinase synthesis must have begun earlier than or on Day 5. To answer this question requires the successful production and immunocytochemical utilisation of a tyrosinase antibody. Alternatively, chick tyrosinase cDNA probes would be ideal for mapping the temperospatial distribution of early melanocytes by *in situ* hybridization. Such studies are already underway (P. Hulley, personal communication).

In terms of future ultrastructural studies on chick melanosomes, the occurence of atypical eu- and pheomelanosomes and of melanised eumelanosomal VGBs needs statistical evaluation to determine their prevalence and significance. Similarly, a comprehensive study on different breeds of fowl might reveal further instances of mixed type avian melanocytes.

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Appendixes

APPENDIX 1.

POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

Non-SDS gels (7,5%) were prepared as follows:

Lower gel

8 ml distilled H_2O

4 ml 1,5 M Tris-HCl (pH 8,8)

4 ml acrylamide/bisacrylamide (Schwarz/Mann Biotech)

60 µl fresh ammonium persulphate (APS) (Bio-rad)

8 µl N,N,N1,N1,-tetra-methyl-ethylene-diamine

(TEMED) (Merck)

Upper gel

1,6 ml distilled H_2O

0,6 ml 0,5 M Tris-HCl (pH 6,8)

0,25 ml acrylamide/bisacrylamide

8 µl fresh APS (Amersham)

3 µl TEMED (Amersham)

SDS gels (10%) were prepared as above, except that the Tris-HCl buffers contained SDS at a final concentration of 0,4%.

APPENDIX 2.

ELISA ASSAYS

The wells of Falcon or Nunc plates were coated overnight at 4°C with 100 µl skin homogenate (as prepared in methods 3.2.1.1.). After flicking out the homogenate, the plates were blocked with 200 µl of 10% normal rabbit serum and 1% bovine serum albumin in PBS (phosphate buffered saline, pH 7,4) for 1 hour at room temperature. The plates were washed 5 times with 0,1 M TBS (tris buffered saline, pH 7,4), after which 50 µl of serum at various dilutions (1:50; 1:200; 1:500; 1:1000; 1:10 000) in 0,1 M PB (sodium phosphate buffer, pH 7,4) was added to each well. Controls were wells in which the antiserum was replaced with 0,1 M PB or pre-immune serum. After 4 hours at room temperature, the plates were washed 5 times with 0,1 M TBS containing Tween-20 at a final concentration of 0.05%. Fifty microliters of RAG-HRP (peroxidase conjugated rabbit-anti-guinea pig IgG(H+L)(Zymed), diluted 1:200 in PB, was now added to each well and left for 30 minutes at room temperature. The plates were then

washed 10 times with 0,1 M TBS, after which the substrate was added (1.2 mg ABTS (2,2-azino-di-(3)-ethylbenzthiazoline sulphonate) + 4 ml citrate buffer (0,1 M, pH 7,4) + 10 µl of a 30% hydrogen peroxide solution). After 30 minutes, the absorbance was read at 492 nm on a Titertek multiscan.

APPENDIX 3.

TREATMENT OF NITROCELLULOSE SHEETS CONTAINING TRANSBLOTTED CHICK PROTEINS:

- **Blocking:**
  One hour in TBS-milk-Tween (milkpowder and Tween-20 both at 3% in TBS, pH 7,4)

- **Brief rinsing**
  in 30 ml TBS

- **Guinea pig antiserum:**
  1:200 in TBS-milk (3% milkpowder in TBS) for one hour

- **Washing:**
  2 X 15 minutes in TBS-Tween (Tween-20 at 0.05% in TBS)

- **Secondary antibody:**
  RAG-HRP (peroxidase conjugated rabbit-anti-guinea pig IgG(H+L) (Zymed), 1:500 in TBS-tween for one hour

- **Washing:**
  4 X 10 minutes in TBS, pH 7,4

- **Substrate:**
  6 mg 4-chloro-N-napthol dissolved in 2 ml methanol into which 10 ml 0,1 M TBS and 6 µl of a 30% hydrogen peroxide solution was added.

  Controls included substitutions of:
  - guinea pig antiserum by pre-immune serum;
  - guinea pig antiserum by TBS-milk;
  - guinea pig antiserum and RAG-HRP by TBS-milk

APPENDIX 4.

Immunocytochemical procedures on sections of 6 or 9 day Black Australorp X New Hampshire Red chick embryonic skin.

parameter of variation	variation	detail
fixation and processing	frozen sections resin sections	✱ ✱
blocking	composition	variations in content of: NRS (0-20%); BSA (0-1%); milk powder (0-1%) in TBS
	time	0 hours 2 hours 4 hours
	frequency	overnight no blocking blocking before and/or after primary
rinsing after primary and secondary	time	none 1x, 2x or 3x for 5 minutes or 15 minutes in TBS
primary	time	1 hour overnight
	dilution	1:50 1:500 1:32 000
secondary	time	30 minutes 1 hour
	dilution	1:20 1:50

Controls were sections in which the primary antibody was replaced with TBS (pH 7,4) or pre-immune serum. NRS = normal rabbit serum; BSA = bovine serum albumin; ✱ see methods.

APPENDIX 5

PROCESSING OF SPECIMENS FOR ELECTRON MICROSCOPY

Step	Time
Primary fixation	3 hours at 4°C
Modified Karnovsky's: 2,5% glutaraldehyde and 2% paraformaldehyde in 0,1 M phosphate buffer (pH of solution: 7,4)	
Wash	3 X 20 minutes
0,1 M phosphate buffer (pH 7,4)	
Post-fixation	1 hour at 4°C
1% OsO_4 in 0,1 M phosphate buffer (pH 7,4)	
Wash	2 X 5 minutes
0,1 M phosphate buffer (pH 7,4)	
double distilled water	1 minute
Dehydration	
50% ethanol	10 minutes
70% ethanol	10 minutes
80% ethanol	10 minutes
95% ethanol	10 minutes
3 X 100% ethanol	15 minutes each
Infiltration	1 hour
1:1 100% ethanol: Spurr's resin	
Impregnation	
Spurr's resin	30 minutes
Spurr's resin	30 minutes at 60°C
fresh Spurr's resin	1 hour at 60°C