# *THE CELLULAR BASIS OF THE SOUTHERN AFRICAN FORMS OF RUFOUS & TYROSINASE-POSITIVE OCULOCUTANEOUS ALBINISM*

**FAMIDA RAWOOT**

**Submitted to the Medical Faculty of the University of Cape Town, in fulfillment of the requirements of the degree of M.Sc.(Med) in Cell Biology.**

**Crawford,**
**August 1993.**

I, Famida Rawoot, hereby declare that the work on which this thesis is based is my original work, except where otherwise acknowledged, and that neither the whole work nor any part of it has been, is being, or will be submitted for another degree to this or any other university. I empower the University of Cape Town to reproduce either the whole or part of the contents in any manner whatsoever.

Signed by candidate

Signature Removed

August 1993

To Mama, with love from Famida.

# ACKNOWLEDGEMENTS

A project of this nature is never accomplished single-handedly, and I wish to place on record my deepest gratitude to all those who contributed in one way or another to its success. In particular, the following persons deserve special mention:

Dr Sue H. Kidson, for her invaluable guidance, unfailing enthusiasm and constant encouragement.

Drs J. Kromberg , M. Ramsay and members of staff at the S.A.I.M.R., Johannesburg, for assistance with obtaining the skin biopsies.

Dr A. Harnekar for her warm hospitality during my stay in Johannesburg.

Dr K. Jimbow, Canada, for his generous gifts of antibodies.

All the subjects, albino and normal, who graciously donated skin and hair biopsies.

Bruce Dando and Peter Richards for technical assistance.

Henry Fortuin for assistance with the photography.

Prof.Rawdon, Prof.Omlin, Phillipa, Craig, Christine, Sharon, Heather, Toni and all other colleagues in our laboratory for fruitful discussions, assistance and encouragement.

Members of the Dept.of Pathology, UCT, for help with the immunocytochemistry.

Firzana for assistance with the typing.

My parents and family for everything.

Rafiq for proofreading the manuscript and for being such a wonderful and understanding husband.

# TABLE OF CONTENTS

**PART 2:**



**CHAPTER TWO:**

# LIST OF TABLES

# LIST OF FIGURES

# ABSTRACT

Oculocutaneous albinism is a congenital heritable disorder characterised by hypopigmentation of the eyes, hair and skin, together with photophobia, nystagmus and decreased visual acuity. Ten different forms of oculocutaneous albinism have been described. Of these, the tyrosinase-positive and rufous forms are particularly prevalent in Southern Africa. The tyrosinase-positive form manifests as two distinctly different phenotypes with some individuals developing pigmented freckles (ephelides) on their sun-exposed regions and others never developing these freckles.

To date, studies on the pathophysiology of tyrosinase-positive albinism have been restricted to examination of hairbulbs rather than skin biopsies. The present study utilises light and electron microscopical investigations of both hairbulb and skin melanocytes from the tyrosinase-positive and rufous forms of oculocutaneous albinism to elucidate the cellular aetiology of these disorders. In addition, melanocyte numbers were quantitated in the tyrosinase-positive skin to establish whether the observed hypopigmentation results from a decrease in the size of the melanocyte population. The melanocyte numbers in regions with ephelides were similarly quantitated in order to see whether these freckles were a consequence of increased melanocyte numbers.

The results show, for the first time, that the hypopigmentation observed in tyrosinase-positive albinos is not a consequence of melanocyte paucity and that the regions of ephelides do not contain more melanocytes. Ultrastructural studies show that regions of ephelides and non-ephelides are distinctly different. In regions of ephelides, numerous fully melanised stage IV eumelanosomes, in addition to unmelanised stage I melanosomes, were seen in the melanocyte cytoplasm. Both stage IV and unmelanised stage I melanosomes are transferred to keratinocytes. In ephelis-free regions the melanocyte cytoplasm was filled with numerous unmelanised stage I melanosomes with no evidence of stage IV melanosomes. This suggests that the defect underlying tyrosinase-positive albinism relates to the melanisation process rather than the process of melanosome assembly. In regions of ephelides, the melanocytes are able to produce numerous stage IV melanosomes, and, because these ephelides occur only on sun-exposed regions, it is postulated that U.V. exposure induces a "back mutation" resulting in the restoration of the process of melanosome melanisation in these regions of skin.

Numerous aberrant melanocyte organelles were also observed in tyrosinase-positive skin. These included dilated RER, distorted mitochondria and bloated Golgi. These ultrastructural observations support the recent report of a candidate gene for tyrosinase-positive albinism, which, it

is speculated , encodes an integral melanosomal membrane tyrosine transport protein. A defect in tyrosine transport into melanosomes would explain the observed incomplete melanisation of melanosomes in tyrosinase- positive albino skin.

In rufous albinism, several aberrant melanosomal shapes were also seen, including "racquet"-, "crescent"- and "comma"-shaped melanosomes, all of which were fairly densely melanised. These melanosomes were also about 30% smaller than normal Negroid melanosomes. Upon transfer to keratinocytes, the melanosomes formed membrane-bound "rosette-like" clusters. These findings seem to suggest that the defect in rufous albinism relates to the melanosomal assembly process rather than the melanisation process.

# C H A P T E R  O N E

The purpose of part one of this chapter is to provide a brief background to oculocutaneous albinism (OCA), particularly the tyrosinase positive (ty.pos) and rufous forms. Part 2 of this chapter provides a general outline of pigment cell biology so as to familiarise the reader with the processes that normally occur during melanin formation.

## ***PART 1:***

### ***1.1.1. INTRODUCTION:***

Oculocutaneous albinism (OCA), a condition found throughout the animal kingdom and described as early as 1 A.D., consists of a heterogeneous group of mostly autosomal recessive disorders of pigmentation which are characterised by the reduction or absence of melanin in the skin, hair follicles and eyes, in addition to photophobia, nystagmus and decreased visual acuity (King, & Summers., 1988). Within this broad category of OCA, there are ten subdivisions according to clinical, genetic, and ultrastructural features (Table 1.1). From this table, it can be seen that some skin, hair and eye pigment can be found in most types of albinism except the tyrosinase negative (ty.neg) form. This form of OCA and the tyrosinase positive form (ty.pos) is by far the most common, with the ty.neg form being especially common amongst Europeans while the ty.pos form is more prevalent amongst Africans.

| TYPE | EPONYM | INHERITANCE* | COLOR AND CHANGE WITH AGE | | | TYROSINASE ACTIVITY† |
|---|---|---|---|---|---|---|
| | | | *Hair* | *Iris* | *Skin Pigment* | |
| **Oculocutaneous Albinism with No Associated Defects** | | | | | | |
| IA | Tyrosinase-negative | AR | White | Blue | None | Absent |
| IB | Yellow | AR | White→yellow→blond | Blue→hazel→brown | Pigmented nevi, lentigines, minimal tanning | Low to absent |
| II | Tyrosinase-positive | AR | White→yellow→blond | Blue→hazel→brown | Pigmented nevi, lentigines | Normal |
| III | Minimal pigment | AR | White→yellow tint | Blue | None | Absent |
| IV | Brown | AR | Light brown | Blue→brown | Light generalized pigment | Normal |
| V | Red | AR | Red→auburn | Hazel green→brown | Pigmented lentigines, minimal tanning | ? |
| **Oculocutaneous Albinism with Associated Defects** | | | | | | |
| VI | Hermansky-Pudlak syndrome | AR | White→yellow→blond→brown | Blue→hazel→brown | Pigmented lentigines | Low to absent |
| **Ocular Albinism** | | | | | | |
| I | Nettleship-Falls | XR | Normal | Blue→hazel→brown | Normal (light) | Normal |
| II | Autosomal recessive | AR | Normal | Blue→hazel→brown | Normal (light) | Normal |

*AR = autosomal recessive; AD = autosomal dominant; XR = X-linked recessive.
†Tyrosinase activity determined by tritiated tyrosine assay.[34]

**TABLE 1.1:** **Features of the Ten Different Forms of OCA. Adapted from King & Summers (1988)**

### *1.1.2. ALBINISM IN SOUTHERN AFRICA:*

Very little systematic research had been carried out on OCA in Southern Africa prior to 1971. Since it was so prevalent amongst the black population of Africa and very little was known about it, research on this condition was initiated in 1971 at the MRC Human Ecogenetics Research Unit, Department of Human Genetics, South African Institute for Medical Research (S.A.I.M.R.) and the University of the Witwatersrand. The present study was conducted in collaboration with this group.

### *1.1.3. PREVALENCE:*

Of the ten types of OCA described, three types are particularly common in the South African black population, these being the ty.pos, brown and rufous forms (Kromberg , 1987). Of these three forms, ty.pos albinism occurs most commonly. The prevalence varies among populations and may be as high as 1 in 1300 in some Botswana villages, 1 in 1900 in Swaziland, 1 in 2000 in the Southern Sotho ethnic group and 1 in 4500 in the Zulu population of South Africa. This rate is extremely high when compared to the prevalence of albinism in some European populations, for example, Italy (1 in 29000); Holland (1 in 20000) and Ireland (1 in 10000) (Kromberg, 1987).

### *1.1.4. DESCRIPTION OF THE TWO FORMS OF OCA THAT OCCUR MOST FREQUENTLY IN SOUTHERN AFRICA:*

Ty.pos and rufous albinism occur most frequently in Southern Africa and both forms were investigated in this research project.

*1.1.4.1.Tyrosinase Positive Albinism:* is the most common form of autosomal recessively inherited OCA in Southern Africa with prevalence being as high as 1 in 3000 amongst the black population of Soweto (Kromberg , 1987). These individuals have been found to have normal levels of the enzyme tyrosinase and are able to produce some pigment. The hair colour is yellow and gets progressively darker with age while the skin colour is cream and some individuals develop pigmented freckles (ephelides) on the sun exposed regions. Although these pigmented lesions do not conform strictly to the definition of an ephelis which has been described as being a small (2 - 3 mm) pigmented spot that gets darker on exposure to U.V. light (Kwan, 1990), the term is used throughout this thesis in the absence of a more accurate term. These ephelides are not present at birth and become visible during the first few years of life and grow in size with age. The pigmentation in the regions of these ephelides is the same as that of normal Negroid skin. The eye colour of these individuals is light brown and they have severely impaired visual acuity.

*1.1.4.2.RUFOUS ALBINISM:* is a rare form of albinism reported to occur only in Africa and New Guinea (Kromberg et al.,1990).

The prevalence of rufous albinism was found to be about 1 in 8580 in 34 326 black South African children. It is suspected that the prevalence is in fact much higher than was found in this small sample. The hair colour of these rufous albinos ranges from light brown to ginger. The skin is described as being red-brown. No sun damage was observed in any of the rufous albinos studied, indicating a lack of skin cancer susceptibility in these individuals, nor were any ephelides found in the sun exposed regions of these individuals. None of the rufous subjects showed marked photophobia or iris translucency.

### *1.1.5. SOCIAL ASPECTS OF ALBINISM:*

Attitudes towards albinos have been varied and the condition has been surrounded by old wives' tales and mythology. Because the birth of an albino baby was thought to be a grave punishment to the parents, these babies were frequently killed by the parents to prevent them from being ostracised. Albinos were also thought to be unlucky and were feared. Attitudes have, however, undergone much change in the last century and ignorance, superstition and infanticide have been replaced by a measure of understanding, acceptance and sometimes overprotection (Kromberg et al., 1987).

Against this background of mythology and varying community beliefs, the birth of an albino baby is often a very traumatic experience for the mother. Emotional confusion and the fact that the infant is so different in skin colour from the mother

often interferes with normal maternal-infant bonding and mothers have been known to refuse contact with the infants for up to nine months after birth. This initial rejection by the mother in addition to continued social rejection and physical handicaps often result in albinos achieving delayed milestones.

### *1.1.6. PHYSICAL HANDICAPS:*

A study on the physical problems experienced by South African albinos was conducted by Kromberg, (1987). A summary of her findings are presented here.

#### *1.1.6.1. VISUAL PROBLEMS:*

Clinically, albinos have several problems associated with their depigmented eyes. These include nystagmus (fast rhythmical movements of the eyes), photophobia (intolerance of or sensitivity to light) and strabismus (squint). These problems are especially pronounced in ty.pos albinos. Because near vision is fairly good, many albinos could cope in ordinary schools, but generally their vision is not good enough to drive a motor car.

#### *1.1.6.2. SKIN PROBLEMS:*

The lack of skin pigment makes most albinos susceptible to skin cancer. In a study conducted by Kromberg & CASTLE., (1989), it was found that the incidence of skin cancer increased with age, rising from 6% for individuals below the age of 10, to 14% in those aged 10-19 years, to 21% in those aged 20-29 years, to 62% in the age group 30-49 years and rising to 100% for those

over the age of 50 years. It was found that the skin of the face and neck was most prone to skin cancer because of frequent sun-exposure. Prophylactic counselling include the regular use of sun-barrier skin creams, avoiding sun exposure, especially between 11h00 and 13h00 hours, thus reducing the ultraviolet exposure by 50%, the use of large-brimmed hats, long sleeves and long trousers and cotton rather than nylon clothing. This high susceptibility to skin cancers often restricts them in finding employment since they are required to remain indoors for a large part of the day.

*1.1.6.3. LIFE-SPAN:*

In South Africa, due to limited access to medical care and much sunshine, especially in rural areas, the life-span is slightly shortened. This was found to be more so in males because of their outdoor employment in rural areas. The main cause of premature death in albinos was malignant skin cancer.

*1.1.6.4. FERTILITY:*

Fertility of albinos was found to be greatly reduced. Whether this was the result of physiological defects or simply due to the fact that these albinos are not able to find suitable partners is unclear.

***1.1.7. MODE OF INHERITANCE:***

Albinism is inherited in an autosomal recessive manner, meaning that both parents have to be carriers of the defective gene for the child to be an albino. Since the gene for ty.pos

albinism had not been isolated, there was no carrier test for affected individuals. This situation might change in the near future with the discovery of a candidate gene. If a couple has an affected child, they are warned that there is a 25% chance that they may have another affected child. There is also no pre-natal screening test available for this condition, and the parents have to decide if they want to have any more children that may be affected with this condition. The carrier rate of the ty.pos albinism is about 1 in 120 individuals amongst black South Africans, which is very high. Also, the female carriers of the gene have, by skin reflectance tests, been shown to have a lighter skin complexion and are often regarded as being more desirable by males. This, in addition to the high carrier rate, could explain why the albino prevalence is so high in the black population.

To date, most of the studies on albinism in South Africa have been restricted to social investigations and genetic and prophylactic counselling of albino families. Attempts to isolate the defective ty.pos gene has also been one of the primary aims of the researchers at the Department of Human Genetics, S.A.I.M.R.. Hardly any studies have been done to investigate the cellular defects that occur in ty.pos and rufous albinism. For this reason, this study was initiated and set out to investigate the pigmentation system of affected individuals and to compare this to that of normal Negroid individuals. The first part of the study involved quantitation of melanocyte numbers to assess if albinos have normal numbers

of melanocytes. For this part of the study only ty.pos albinos were investigated since only a few rufous albino subjects volunteered to take part in this study, and for the quantitation data to be statistically significant, at least 5 subjects had to be included. Electron microscopical studies were conducted on 5 ty.pos and 3 rufous subjects. Chapters two and three report on these melanocyte quantitation and ultrastructural studies, respectively. Part 2 of this chapter deals with the processes involved in normal melanogenesis.

***PART 2:***

***1.2.1. INTRODUCTION:***

This part of chapter one provides a background to the process of normal melanogenesis and all the factors that play a regulatory role in this process.

***1.2.2. THE ARCHITECTURE OF HUMAN SKIN:***

The skin has two major components, the dermis and the epidermis. The dermis is basically a thick connective tissue layer comprising collagen, elastic and reticular fibres which are traversed by a rich network of blood and lymphatic vessels. The epidermis consists essentially of a stratified squamous, keratinized epithelium which is devoid of any blood and nerve supply. The keratinocytes (previously known as Malpighian cells) make up 95% of the cell population of the epidermis. The other 5% comprise the Langerhans cells which exhibit an immune or macrophage activity and the melanocytes

which are the pigment (melanin) producing cells of the epidermis.

Melanocytes are dendritic cells that produce melanin in specialised organelles known as melanosomes. These pigmented melanosomes are transferred to keratinocytes via the dendritic processes of the melanocytes. Skin pigmentation relies on the transport of these pigmented melanosomes by the keratinocytes and the melanocytes are merely the site of melanosome synthesis and pigmentation.

*1.2.2.1. THE MELANOCYTES:*

Mammalian melanocytes were shown to arise from the neural crest and not from epidermal cells as had been thought previously (Rawles, 1948). The neural crest is a population of cells which forms during closure of the neural tube and , as the tube closes, these crest cells leave the tube epithelium and migrate in a rostrocaudal wave to various sites in the embryo (Weston, 1971). The migrating neural crest cells follow distinctive pathways towards their destinations and then differentiate into several different phenotypes. These include most of the peripheral, autonomic and central neurons, Schwann cells, connective and skeletal tissue of the face, adrenal medullary chromaffin cells, C-cells of the thyroid, the melanocytes of the skin and irides and numerous other structures of the embryo (Le Douarin, 1982).

Prospective melanocytes, known as melanoblasts, arise from the neural crest by eight weeks of human embryonic life and migrate along either side of the spinal cord to the skin. These cells populate the dermis in increasing numbers by ten to twelve weeks of embryonic life. From about twelve to fourteen weeks, they can be detected in the epidermis (Sagebiel and Odland, 1972). Melanin synthesis usually occurs during the fourth of fifth foetal month. After the sixth month of foetal development, the melanocyte numbers are stabilised, and these cells then occupy their position at the epidermal-dermal junction of the skin. Jimbow et al. (1976) demonstrated that melanocytes have the capacity to divide mitotically. The fact that melanocytes divided in unexposed skin indicates that a turnover of the melanocyte population, albeit low, is necessary and may relate to the need to clear genetic damage induced by intrinsic and extrinsic chemical and physical elements (Jimbow et al., 1976). The mitotic activity of melanocytes is enhanced by ultra-violet (U.V.) light exposure. This aspect of melanogenesis is very important to this study and is discussed in depth later in the chapter.

*1.2.2.2. THE MELANOSOMES:*

Melanin is formed within cytoplasmic organelles known as melanosomes. The reaction that occurs within these melanosomes results in the conversion of tyrosine to melanin. A number of enzymes, including tyrosinase, play a critical role in this reaction.

There are two different types of melanosomes, each producing a different type of melanin. The eumelanosomes produce eumelanin which is the brown-black pigment found in black skin and hair. The pheomelanosomes produce the yellow-red pheomelanin which is found in red hair and Caucasian skin. Eumelanosomes are ellipsoidal in shape and, in humans, they are approximately 0.24-0.5 $\mu$m in length and 0.08-0.19 $\mu$m in width (Everett et al., 1979). Ultrastructural studies reveal that eumelanosomes typically have a very organised internal matrix with thick filaments (30-50 $\text{Å}^0$ units) running the length of the melanosome. These filaments appear to have cross-links between them and they aggregate into several lattices of cross-linked fibres resembling lamellae. In cross-section, the lamellae are seen to be rolled concentrically into a "swiss-roll"-like cylinder. Melanin is deposited on the lamellae and as more melanin is deposited, the internal structure is completely obscured and the melanosome becomes completely electron dense. Pheomelanosomes are spherical or slightly oval in shape and have a diameter of about 0.25 - 1 $\mu$m (Jimbow et al., 1983). Pheomelanosomes do not possess an organised lamellar matrix, but instead, have an amorphous, multivesicular internal structure upon which melanin deposition appears spotty and irregular.

The precise origin of melanosomes was in dispute for many years. One of the earliest viewpoints was that melanosomes were "modified mitochondria" which were derived from promitochondria that acquire melanin in the course of a

"specialised ontogeny" (du Buy et al., 1963; Woods et al., 1963). This theory was disproved by Seiji and Iwashita , (1965) who provided conclusive evidence that melanosomes and mitochondria are distinctive cellular organelles. Furthermore, these investigators demonstrated that there was tyrosinase activity in both the smooth and rough endoplasmic reticulum (ER) and free ribosomes. They , therefore, proposed that tyrosinase is synthesised on the rough ER and transferred via the smooth ER to the Golgi. They further proposed that either by fusion or enlargement of the Golgi-derived vesicles laden with tyrosinase, the earliest form of melanosome (stage I) is formed. According to their theory, the melanosomal matrix proteins and tyrosinase become associated with each other during the initial assembly of the melanosome. A modification of this theory was proposed by Novikoff et al., (1968) who suggested that tyrosinase is transferred through the rough ER to the smooth ER and aggregates in the expanded tip of the Golgi cisternae or in the smooth ER connected with the Golgi, which then enlarges and is pinched off to form the early melanosome. According to this theory, the tyrosinase is initially incorporated into the inner lamellae of the melanosome but remains inactive until melanisation proceeds. Birbeck , (1963) proposed yet another theory which suggested that the melanosomal matrix actually consists of an array of tyrosinase molecules, which implies that tyrosinase behaves simultaneously as a structural protein and active enzyme. A more accepted theory was proposed by Turner et al. (1975) which was based on their studies of *Xanthic* goldfish.

They proposed that melanosomes are formed from large vesicles known as multivesicular bodies (MVB's) which originate from blebbing RER. Golgi-derived vesicles, containing tyrosinase, fuse with these large MVB's and invert to release the tyrosinase. These tyrosinase vesicles reform within the MVB's after releasing the tyrosinase. Melanin synthesis then proceeds.

Several more recent studies have indicated that the melanosomal structural proteins and tyrosinase arise from separate compartments within the melanocyte and that tyrosinase is incorporated only after the assembly of these structural proteins into melanosomes. Stanka STAHLMAN (1981), analysed the pigment epithelium of chick eye and showed that groups of four to ten melanosomes formed from a region of the ER complex called the "premelanosome- forming" centre. An ER origin of the melanosomes was supported by various other studies where newly formed melanosomes were found in continuity with the ER (Maul, 1969; Ide, 1972; Sakurai et al., 1975). Furthermore, cytochemical techniques, using the dopa reaction, which involves incubation of tissue sections in a dopa solution to detect tyrosinase activity when tyrosinase converts dopa to melanin, have shown that melanosome assembly and the incorporation of tyrosinase are separate events. This was further strengthened by the finding that fully formed melanosomes, with completed inner matrix, posses no tyrosinase activity (Maul, 1969; Maul and Braumbagh, 1971; Ide,1972). Ide (1972) reported that tyrosinase activity apparently progresses

from the Golgi sac to vesicles that become attached to the Golgi lamellae and then become numerous in the cytoplasm. Similarly, Stanka (1974) found dopa positive, Golgi-derived coated vesicles close to the melanosomes in human melanocytes. These vesicles have often been observed to fuse with melanosomes and instances where tyrosinase-containing membrane tubules appeared to be connected to premelanosomes have been interpreted as the possible fusion of a coated vesicle with the premelanosome.

All these studies seem to suggest that tyrosinase which is synthesised on the rough ER, is transported to the Golgi where it is packaged and buds off in coated vesicles. This tyrosinase is not in its active form since post-translational modifications of tyrosinase is only complete once the tyrosinase reaches the melanosome. These tyrosinase carrying vesicles then fuse with premelanosomes that have blebbed off the ER. The currently accepted theory is as follows: melanosomes are formed from blebbed portions of smooth ER that harbour the melanosome's structural components. Golgi-derived vesicles containing tyrosinase fuse with these newly formed melanosomes. The fusion of these two membrane compartments initiates the first stage of melanosomal development by combining the key melanogenic enzyme, tyrosinase, with a suitable framework containing the right chemical microenvironment for tyrosinase activity and melanin synthesis (Fig .1.2.1.) (Jimbow et al., 1992).

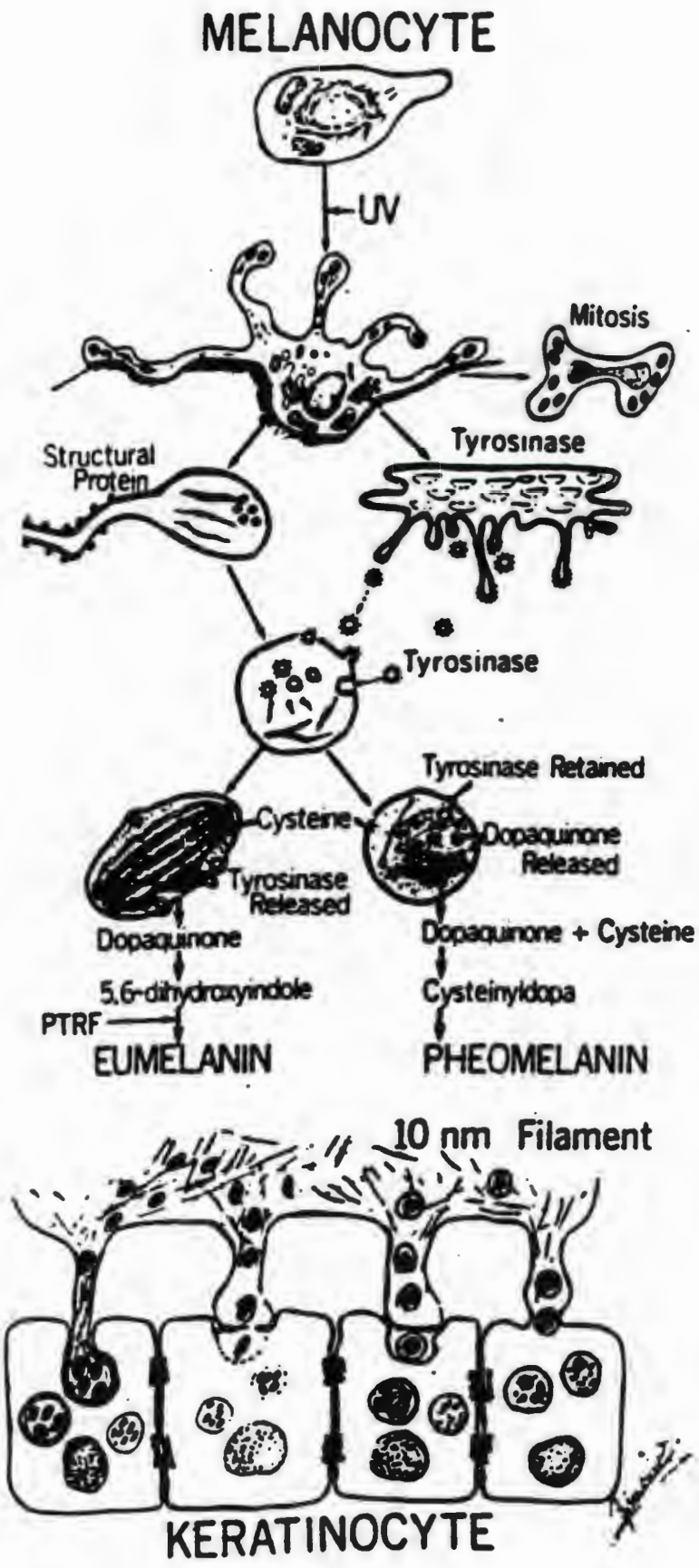


FIG. 1.2.1.: Schematic Representation of Melanogenesis in the Skin. Adapted from Jimbow et al. (1992).

Ultrastructural studies demonstrate that eumelanosomes contain numerous spherical bodies (± 400 Å units) which appear as electron-lucent "holes" (Jimbow and Fitzpatrick, 1974). A comprehensive study of eumelanosomes in chick skin by these investigators proved that these holes are not artefact but are real melanosomal subunits termed vesiculo-globular bodies (VGB's) (Jimbow and Fitzpatrick, 1974). An interesting finding was that VGB numbers vary and depend on the degree of melanisation. The earliest melanosomes of all tissues contain the same amount of VGB's but the highly melanised melanosomes from black skin, hair and feathers eventually contain more VGB's than the melanosomes of white skin and feathers. This suggests that VGB's may be the key units in the organisation and degree of melanisation of the eumelanosomes (Jimbow et al., 1979). It therefore appears that VGB's play an important role in melanosome maturation and it was strongly suggested that these VGB's are the tyrosinase carrying vesicles which are released from the Golgi, but this theory remains uncertain.

Melanosomes were classified into four stages according to their degree of melanisation. The following classification of melanosomal stages was provided by Toda et al.,(1972b): a premelanosome is a newly blebbed melanosome with no internal structure and is dopa-negative; a stage I melanosome was described as a dopa-positive vesicle without recognisable internal structure; a stage II melanosome being a dopa-positive particle with distinctive internal filamentous structure; a stage III melanosome contains melanisation but the

filamentous internal structure is still visible and a stage IV melanosome is a fully melanised granule with no discernable internal structure.

Once the melanosomes have been fully melanised (stage IV) they are transferred to surrounding keratinocytes. Each melanocyte is able to extend its dendritic arms to approximately thirty six keratinocytes. This unit of a melanocyte and its associated keratinocytes is known as the **"epidermal-melanin unit"** (Fig.1.2.2.) (Szabo et al., 1969). The functioning and level of activity of this unit is dependent upon the movement of melanosomes within the melanocyte cytoplasm and dendrites as well as the transfer of these melanosomes by the melanocyte dendrites to the keratinocytes. The exact mechanism involved in these processes is still poorly understood. Seiji et al., (1976) investigated the melanocyte-keratinocyte interactions that occur during melanosome transfer. These cells were observed with the time-lapse cinematography-light microscope, scanning and transmission electron microscopes. From these studies, the transfer mechanism of melanosomes from melanocytes to keratinocytes *in vitro* and *in vivo* was suggested to occur as follows: the tip of the melanocyte penetrates the keratinocytes and remains enfolded with the cell membrane of the keratinocyte. Then the dendrite seems to be squeezed and cut-off by the keratinocyte to form a pouch containing many melanosomes. These melanosomes are then surrounded by two membranes, the inner membrane is derived from the melanocyte and the outer one from the keratinocyte. The

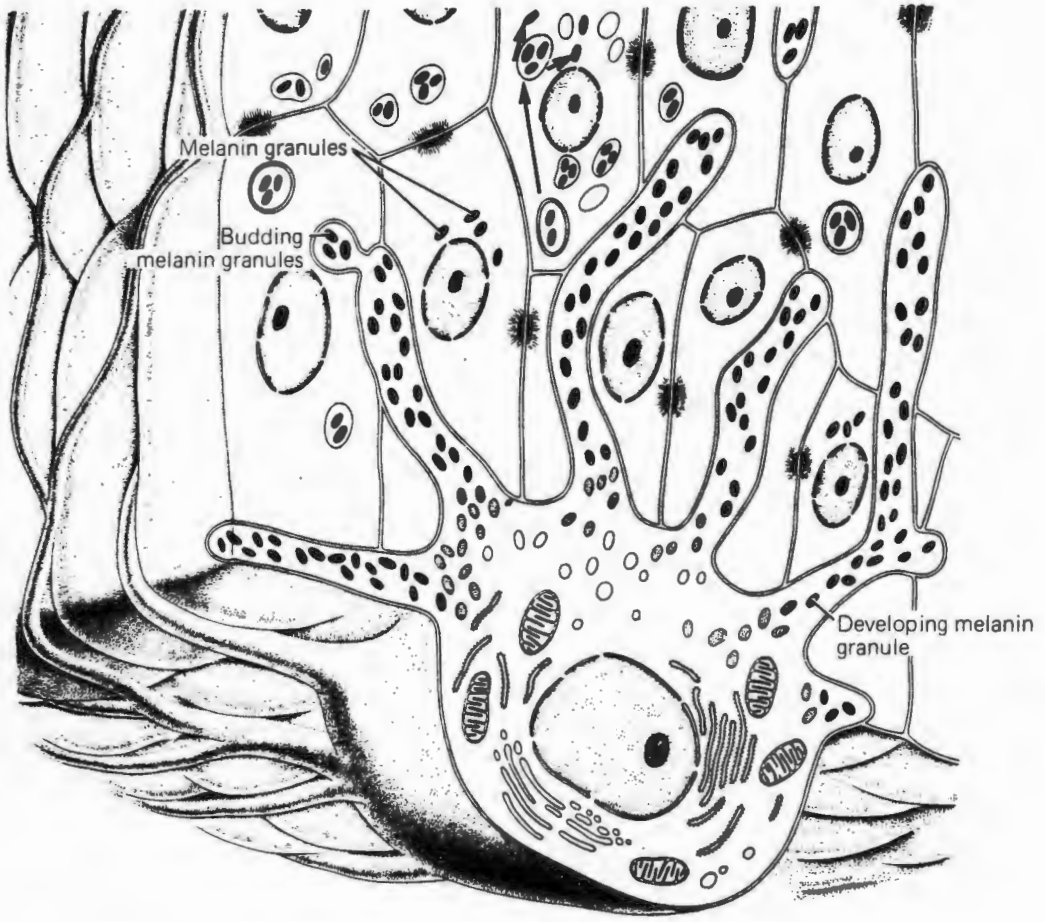


**FIG. 1.2.2.:** **"The Epidermal Melanin Unit." A Melanocyte containing numerous melanosomes transfers stage IV melanosomes to surrounding keratinocytes through its dendritic arms. Adapted from Szabo et al. (1969).**

resulting pouch gradually moves towards the nucleus of the keratinocyte, during which digestion by lysosomes is thought to occur. This degradation process involves disintegration of the inner-melanocyte membrane and melanocyte constituents. The pouch seems to release aggregated melanosomes into the keratinocyte cytoplasm and, depending on the size of the melanosomes, they occur either singly (large melanosomes) or in clusters (smaller melanosomes). Once dispersed in the keratinocyte cytoplasm, the melanosomes are subject to further degradation by lysosomes.

### *1.2.3. THE BIOCHEMISTRY OF MELANIN SYNTHESIS:*

As mentioned previously, two types of melanins are formed within melanocytes, the brown-black eumelanin and the yellow-red pheomelanin. The synthesis of both these forms of melanins occurs in the melanosomes where the amino acid tyrosine is hydroxylated to form L-3,4-dihydroxyphenylalanine (dopa) and the further oxidation of dopa to form dopaquinone. Dopa also acts as a co-factor for these reactions. Both these reactions are catalysed by tyrosinase (monophenol monooxygenase, EC.1.14.18.1) which is a multi-functional copper-containing enzyme (Hearing and Jimenez, 1987). This dual enzymatic action of tyrosinase is most critical since, once dopaquinone has been formed, the other steps in melanin synthesis can occur autocatalytically at physiological pH. Dopaquinone then either enters the eumelanin or pheomelanin synthetic pathways. It is thought that the primary determinant of whether pheomelanin rather than eumelanin is formed, is the presence of sulfhydryls

(glutamine and cysteine). Glutamine and cysteine will bind to dopaquinone as rapidly as it is formed (Prota, 1980). The finding that in the formation of pheomelanin, dopaquinone combines with cysteine residues containing the reactive -SH group is a very fast reaction, whereas the conversion of dopaquinone to leucodopachrome in the eumelanogenic pathway, is not so fast. Prota, (1980) supports the theory that glutamine and cysteine rapidly combine with dopaquinone to form (5-S-, 2-S-, 2,5-di-S and 6-S) cysteinyldopa which are then converted into the corresponding benzothiazines by oxidative cyclisation of the cysteinyl residues. These benzothiazine intermediates then polymerise to form pheomelanin. In the eumelanin pathway the leucodopachrome, that forms dopaquinone, is converted to dopachrome and then to 5,6-dihydroxyindole (DHI). Tyrosinase once again plays a role in the conversion of this DHI to indole-5,6-quinone which polymerises to form eumelanin. These steps of melanin biosynthesis are depicted schematically in Figure 1.2.3.

### ***1.2.4 FACTORS THAT REGULATE MELANOGENESIS:***

Tyrosinase holds a central position in the biosynthesis of melanin because of its important role in the initial two rate-limiting steps of the pathway (Fig.1.2.3). For a long time, it was believed that the subsequent steps proceed spontaneously to form melanin. Consequently, studies which were aimed at understanding the regulation of melanogenesis were mostly restricted to mechanisms that possibly stimulate or inhibit

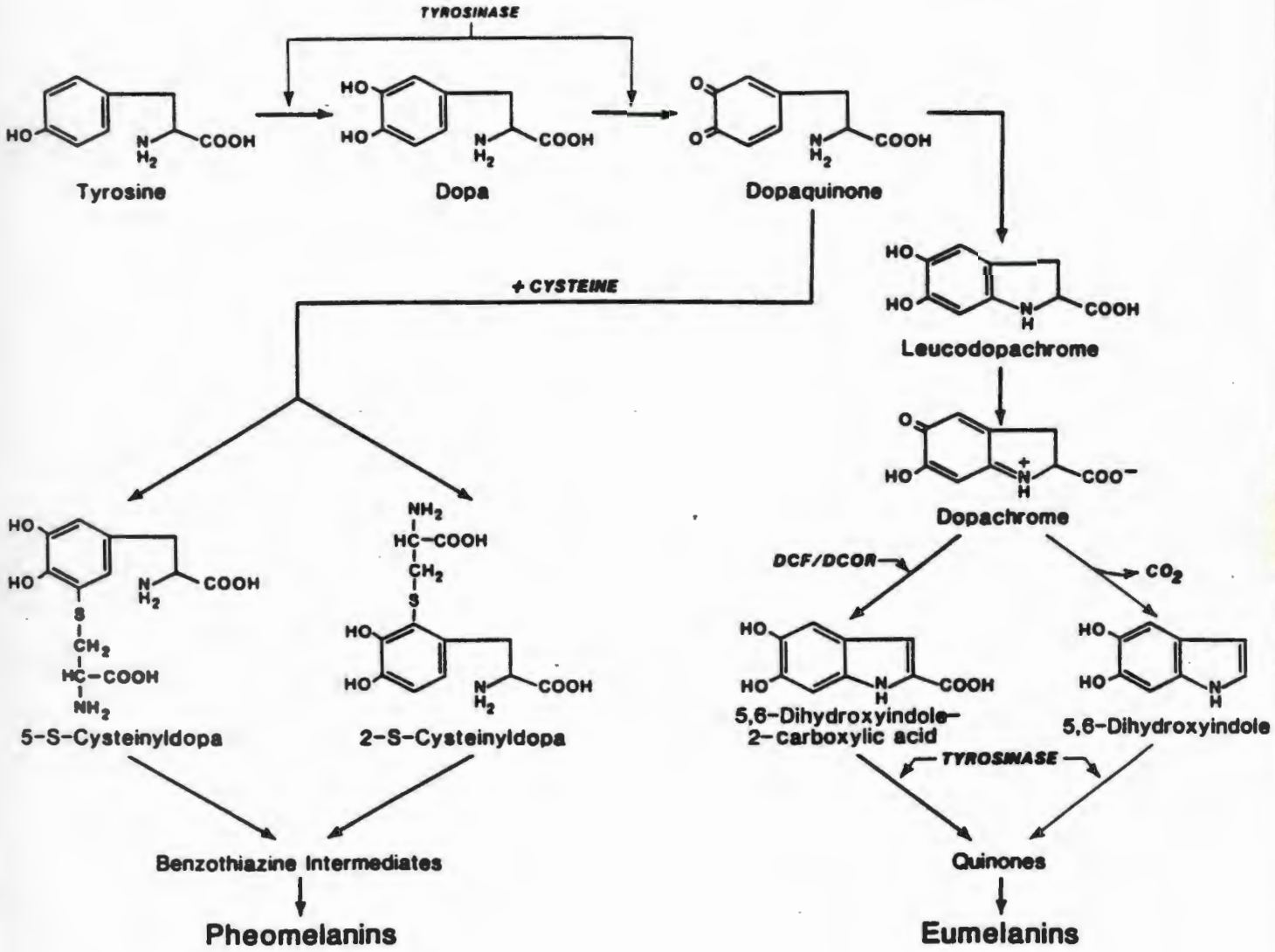


FIG. 1.2.3.: The melanin bio-synthetic pathway.
DCF-DCOR = dopachrome conversion factor-dopachrome oxidoreductase.
Adapted from Witkop et al., (1989).

tyrosinase, which was thought to be the sole controlling factor. Since then, a number of other factors have been recognised that can affect the chemical activity of melanocytes. These factors include a number of intracellular regulators of tyrosinase synthesis and processing; the action of auxiliary enzymes such as dopachrome tautomerase and peroxidase and the presence of certain metal ions, especially copper(II) and iron(II). These factors mostly come into play after dopaquinone has been formed. Some of these factors will be discussed below.

Although it was initially thought that eumelanin consisted of a homogeneous polymer of indole-quinone, recent studies indicate that melanins are heterogeneous polymers with sub-units consisting of all the cyclised derivatives of dopaquinone which may include dopaquinone (Hearing and Tsukamoto, 1991). Also, in addition to eumelanin and pheomelanin, a mixed-type melanin, which is a mixture of eumelanin and pheomelanin in different proportions, was also discovered in humans (Hearing and Tsukamoto, 1991). As mentioned previously, it is thought that the primary determinant of whether pheomelanin rather than eumelanin is synthesised depends on the presence of the sulfhydryls, cysteine and glutamine. The enzymes that influence the metabolism of these sulfhydryls, therefore, also play an indirect role in what type of melanin is being formed.

Furthermore, the melanosome is an intracellular organelle which is membrane bound and the intramelanosomal substrates and

regulatory factors that are required for melanogenesis must be present in their correct quantities for melanogenesis to proceed normally. The melanosomal membrane plays an important role in the regulation of substrate concentration within the melanosomes. For many years it was thought that the uptake of tyrosine, the amino acid required for initiation of melanogenesis, was not regulated by the melanosomal membrane (Jara et al., 1990). However, recently a candidate gene has been found for ty.pos albinism and it is thought that the gene codes for a melanosomal-membrane protein which is responsible for the active channeling of tyrosine into the melanosome (Nicholls et al., 1992). This protein has 11 membrane spanning domains. It is obvious that the absence or low levels of tyrosine within the melanosome will certainly result in absent or reduced pigment production and if this candidate gene is in fact the defective gene causing ty.pos OCA, it would explain the hypopigmentation observed in these individuals.

Other enzymes that operate in the eumelanogenic pathway (other than tyrosinase) include the enzyme that results in the formation of 5,6-dihydroxyindole-2-carboxylic acid (DHICA) instead of DHI which spontaneously forms from dopachrome in the absence of this enzyme. This enzyme was originally called *dopachrome conversion factor.* Later this name was changed to *dopachrome oxidoreductase* , then *dopachrome isomerase* and now it is finally known as *dopachrome tautomerase (E.C. 5.3.2.3)* (Hearing and Jimenez, 1991). This catalytic activity towards dopachrome can also be elicited by metal ions such as $Co^{++}$,

$Cu^{++}$ and $Ni^{++}$ and result in the formation of DHICA rather than DHI. The structural and functional differences between the melanin formed from DHI and DHICA is not exactly known, but must be significant since DHI-derived melanin is black and flocculent whereas DHICA-derived melanin is yellowish-brown and finely dispersed. It has also been shown recently that peroxidase is able to utilise DHI as a substrate to form melanin and its role in melanogenesis is currently under study (Hearing and Jimenez, 1991).

Other factors that influence pigmentation are the melanogenic inhibitors. Any protein or peptide within the melanosome that has an exposed tyrosine or phenylalanine residue can act as a competitive inhibitor of tyrosinase. A variety of exogenous inhibitors of tyrosinase also interfere with the action of tyrosinase. These, in addition to some endogenous inhibitors have been partially characterised and currently much investigation is being carried out to determine the nature of and the role these inhibitors play in disturbing pigmentation. It is thought that these inhibitors play a crucial role in pigmentary disorders where normal levels of tyrosinase are present but pigmentation is disturbed by some other factors.

It is well documented that melanocyte-stimulation hormone (MSH) plays a vital role in increasing the melanogenic potential of murine melanocytes (Pawelek, 1976). This process is mediated by MSH binding to receptors on the melanocyte cell membrane. The hormone-receptor complex is internalised which results in

the activation of protein kinase A with the immediate increase in cAMP. Other enhancers of melanogenesis, such as isobutylmethylxanthine (IBMX) and cholera toxin, have similar effects to MSH and also result in increased cAMP levels. In human melanocytes there are also cell surface receptors for MSH and binding of this hormone to these receptors also increases the cell's cAMP levels, with no marked increase in pigmentation. However, it has recently been shown that human melanocytes can be stimulated to produce pigment through the activation of protein kinase A by means of the diacylglycerol messenger rather than through cAMP (Gordon and Gilcrest, 1989). This finding suggests that the melanogenic stimulation of humans and mice may be significantly different.

### *1.2.5. THE EFFECT OF ULTRA-VIOLET (U.V.) RADIATION ON THE PIGMENTARY SYSTEM:*

When human skin is exposed to U.V., there is a marked increase in the skin melanin content and the skin appears visibly darker. The exact mechanism of how this skin darkening occurs in response to U.V. was dubious and various studies have been conducted to investigate this process.

Early studies on melanocyte quantitation revealed that there was a distinct regional variation of melanocyte numbers in different parts of the body (Szabo, 1954; Staricco & Pinkus, 1956; Rosdahl and Rorsman, 1983). It was found that the face and neck (frequently sun-exposed) has twice as many melanocytes than the upper-arm, thigh and buttock (mostly unexposed). In

view of these findings, Quevedo et al., (1965) tested whether U.V. had any influence on these increased melanocyte numbers in frequently exposed regions. They studied the effect of U.V. irradiation on the buttock of 5 Caucasian males. Each individual was shielded so that only the left buttock was exposed. Twenty-four hours after each exposure, skin biopsies were taken from both the exposed and shielded regions of the buttock. These specimens were mounted and dopa-treated by the method of Starricco and Pinkus (1956). Melanocytes were counted and it was found that there was a significant increase of approximately 350 melanocytes/$mm^2$ in the irradiated as compared to the shielded skin. Furthermore, the irradiated melanocytes were clearly more dopa-positive than melanocytes in the shielded skin. This led Quevedo et al., (1965) to conclude that the increased numbers of dopa-reactive melanocytes after repeated exposure to U.V. may have resulted from either the division of pre-existing melanocytes, the activation of previously dopa-negative melanocytes or the migration of precursor melanocytes from the dermis into the epidermis. They were sceptical that mitotic division or migration of precursor melanocytes from the dermis into the epidermis could result in the melanocyte increase since no mitotic figures were seen nor were any dermal melanocytes observed. They attributed the increase in melanocyte numbers to U.V. stimulation of inactive, dopa-negative (amelanotic) melanocytes to become active.

To confirm whether inactive melanocytes are normally present in the human epidermis, Mishima and Widlan (1967) used a

combination of pre-melanin and dopa pre-melanin techniques to test if some melanocytes that do not react with dopa, will react with premelanin. This study seemed to strongly indicate that U.V. was stimulating a population of dopa-negative melanocytes to become dopa-positive by causing melanin production in these cells. This, rather than mitotic division of melanocytes, was thought to be the reason for the observed increase in melanocyte numbers following U.V. stimulation.

Furthermore, melanocytes were thought to be highly differentiated cells of neural crest origin which were incapable of mitotic activity. This concept was negated when Jimbow et al., (1976) demonstrated that non-neoplastic melanocytes are capable of mitotic activity *in vivo*. This led Rosdahl and Szabo (1978) to re-evaluate the role of mitoses in the increase of the melanocyte population following repeated U.V. irradiation. Cumulative labelling with tritiated thymidine ($^{3}$HTdr) in combination with autoradiography was used to visualise dividing cells. Repeated U.V. irradiation induced a marked increase in the epidermal melanocyte population in the autoradiograms. The results of these experiments by Rosdahl and Szabo (1978) showed that a 4-6 fold increase in the epidermal melanocyte population was associated with $^{3}$HTdr labelling of 65-80% of the melanocytes. This high percentage of labelled cells suggested that the U.V. induced increase in melanocyte numbers was primarily due to mitoses of melanocytes.

More recently, Steirner et al., (1989) demonstrated for the first time in humans, that U.V. induces an increase of melanocyte numbers, not only in exposed skin, but also in the areas that were shielded during the exposure. Twenty-one Caucasians (13 women and eight men) received U.V. irradiation on their buttock. A portion of the buttock was shielded from U.V. irradiation by covering it with plastic tested to be non-transmitting to U.V.B. light. Before the first radiation dose was administered, a punch biopsy was taken from the buttock and the melanocytes were counted (control value). Three weeks after exposure, biopsies were taken from both the shielded and radiated buttock regions. After NaBr and dopa incubation, the melanocytes were counted (method of Rosdahl and Rorsman, 1983). Steirner et al., (1989) found that U.V. irradiation increases melanocyte populations in both exposed and unexposed areas of human skin, but a greater increase was observed in the directly irradiated skin. They also observed that the melanocytes had morphologically changed and became larger and more dendritic, especially in the directly irradiated skin.

That both irradiated and shielded skin responded to U.V. irradiation, was interesting and different from previous reports on the effect of U.V. on melanocytes. It should be noted that all previous investigators took skin biopsies from both shielded and irradiated regions almost immediately after irradiation whereas Steirner et al., (1989) waited for three weeks post-irradiation before taking the biopsies. From these findings, Steirner et al., (1989) postulated that the increase

in melanocyte numbers in the shielded skin, three weeks after irradiation, was initiated by a circulating "factor" which causes melanocytes to divide in response to U.V. irradiation. This "factor" acts on distant, unirradiated sites causing them to increase melanocyte numbers in readiness for further U.V. exposure. In similar studies on C57BL mice (Rosdhal, 1979), it was found that during U.V. irradiation, the mitotic activity of melanocytes and keratinocytes co-vary which suggests that they may be influenced by a common mitosis stimulating factor. Because Steirner et al. (1989) found that there appeared to an independent correlation between the population increase in the exposed and shielded skin, it was thought that this mitosis stimulating factor is derived from proliferating melanocytes in the exposed skin and acts via a paracrine process on melanocytes in shielded areas, causing these cells to divide in preparation for future U.V. exposure. Currently, the exact nature of this mitosis stimulating factor is not clear, but is being investigated.

In addition to serving as a mitogen, U.V. is known to be a very potent mutagen. U.V has been implicated as being a causative factor in melanoma ontogeny, but the exact mechanisms of how U.V. causes the malignant transformation of melanocytes to melanoma cells is not yet fully understood. It is probable that U.V. causes the activation of oncogenes by inducing mutations to the genetic material of the melanocytes. Bredburg et al., (1986) demonstrated that U.V. is a mutagen by exposing melanocytes, from patients suffering from the skin disorder

xeroderma pigmentosum, to U.V. irradiation. Xeroderma pigmentosum cells are extremely sensitive to U.V. and are unable to repair damaged DNA induced by U.V. These investigators propagated a shuttle vector into xeroderma pigmentosum melanocytes and measured the mutational spectrum of U.V. on this shuttle vector. It was found that the U.V. induced multiple mutations which resulted in the malignant transformation of these melanocytes.

Furthermore, it was shown that exposure to U.V. causes increased levels of circulating MSH in humans (Pawelek, 1979). Recently, Pawelek et al., (1992) demonstrated that MSH receptor activity on the surface of cultured Cloudman mouse melanoma cells as well as increased cellular responsiveness to MSH increases upon U.V. exposure. This provides evidence that U.V. and MSH operate synergistically to increase pigmentation. This interaction between U.V. and MSH could explain how exposure to U.V. causes both an increase in the number of active melanocytes, since MSH has been shown to regulate both pigmentation and proliferation of cultured mouse melanoma cells (Pawelek, 1979).

### *1.2.6. GENETIC CONTROL OF PIGMENTATION:*

Many pigmentary disorders in humans are inherited. It is known that over 50 genes are involved in mouse pigmentation (Silvers, 1979). It is not known exactly how many genes are involved in human pigmentation, but it is thought to be numerous since the human homologue for most of the mouse pigmentation genes has

been found. The various effects of gene mutations can be seen in the switches of coat colour of mice. Wild-type mice are termed agouti and bands of eu- and pheomelanin alternate in their hair. Some mutations in mice hair colour elicit the production of pheomelanin only, such as lethal yellow ($A^{Y}/\_$) and recessive yellow (e/e). Non-agouti (a/a) animals are black and produce only eumelanin and appear uniformly black. Other mutations include the brown (b/b) and albino (c/c) where the quantity and quality of melanin produced is further modified. Mutations at the albino locus will be discussed below because of its relevance to this study. Also, other pigmentation genes such as the brown, slaty, silver and the pink-eye dilution, which is thought to be the mouse equivalent to the human ty.pos gene, will be discussed in some detail.

*1.2.6.1. THE ALBINO LOCUS:*

The tyrosinase gene is the key to many forms of albinism. For this reason, there has been a tremendous effort to clone the tyrosinase gene from different species. Early clones were all from non-mammalian species and served as useful tools for structural and functional studies. Several candidate clones were obtained from human and mouse (Shibahara et al., 1986; Kwon et al., 1987; Yamamoto et al., 1987) and it was found that these clones shared significant sequence homology to the non-mammalian clones.

The albino locus, located on chromosome 7 in mice and chromosome 14 in humans, has historically been proposed to be

the structural locus for tyrosinase because of the dramatic lack of pigment production if there is a mutation at this locus. The albino gene in mouse is composed of five exons and four introns and is about 70kb. The processed mRNA is 2.4kb in length and is spliced to delete the introns. This splicing is not always accurate and leads to the production of altered proteins which are not catalytically competent but may act as competitive inhibitors within the melanocytes (Shibahara et al.,1988). It was shown that both the full length and correctly spliced mRNA is required for active tyrosinase (Muller & Ruppert., 1988). That the albino locus protein is expressed in melanocytes has been confirmed by the generation of antibodies to the amino and carboxy termini of the protein. Subsequently, minigenes containing the tyrosinase coding sequence and a competent regulatory sequence were used to produce transgenic mice (Yamamoto et al., 1989; Beerman & Ruppert, 1990). Although these mice were not fully pigmented, there was a dramatic increase in the amount of pigmentation which was specifically restricted to the melanocytes. Following these studies, it is now clear that the protein encoded by the albino gene is tyrosinase. Whether it is the sole protein that can perform as tyrosinase and exactly how it interacts with other melanogenic proteins, remains unclear.

In the mouse, 8 mutations at the albino locus have been discovered. These include the Himalayan (ch) and Chinchilla (cch) mutations which have been studied extensively. In the Himalayan mouse, there is an alteration in glycosylation which

results in a temperature-sensitive phenotype. Tyrosinase is converted into a thermolabile form with greater production of melanin in the colder parts of the body. In the chinchilla mouse, there is an increased sensitivity to proteolytic inactivation and thus a decrease in enzyme function (Halaban et al., 1988). A dramatic discovery was the fact that in mice, albinism results from a single-point mutation whereby a conserved cysteine is substituted by a serine in the first cysteine-rich domain of mouse tyrosinase (Jackson and Bennett, 1990). This implies that a single change in the tyrosinase sequence can result in complete loss of catalytic activity. Similarly, single point-mutations of human tyrosinase have resulted in **tyrosinase negative** (ty.neg) OCA. Recently, it was found that a single base insertion in the human tyrosinase gene resulted in ty.neg OCA (Chintameneni et al., 1991). A single base, thymine, was inserted in exon 5 of the coding region of the tyrosinase gene. This resulted in a shift in the reading frame of 19 amino acids in the 3' end and introduced a premature termination signal which truncated the protein by 21 amino acids at the carboxy terminal. This resulted in the production of inactive tyrosinase which was 3kDa smaller than normal and could not be detected by antibodies that were directed to the carboxy terminus of tyrosinase. Such point mutations now number in dozens. Moreover, these mutations are not confined to a single region or domain of the gene and result from a variety of mechanisms such as point mutations and insertions which may occur in either the structural or the

promoter region of the gene (Tomita et al., 1989; Giebel et al., 1990; Spritz et al., 1990).

*1.2.6.2. OTHER PIGMENTATION RELATED GENES:*

The murine brown locus, when first cloned, was thought to be the structural gene for tyrosinase. It was mapped to chromosome 4 of mouse (Shibahara et al., 1986; Jackson, 1988). The structure and organisation of the gene is remarkably similar to that of the tyrosinase gene and the protein translated from this gene is thought to have all the characteristic features of tyrosinase ((Hearing and Jimenez, 1989). The homologous gene has been found in humans and there is about 90% sequence homology between the murine and human genes (Vijayasaradhi et al., 1991). The specific function of the protein encoded by the brown locus is not clear. A mutation at this locus results in the production of brown rather than black melanin, but the chemical and enzymatic processes that underlie this switch are unclear. This indicates that the function of the brown locus is to promote the formation of black rather than brown melanin. It has been proposed that the brown locus may encode for either dopachrome tautomerase (Jackson, 1988) or DHI conversion factor (Kwon et al, 1988) or a melanosomal specific catalase (Halaban et al., 1990) or another tyrosinase (Jimenez et al., 1989). No human mutations have yet been identified at the brown locus. In mice, there are numerous mutations at the brown locus which result in the production of a brown rather than black animal. The critical molecular lesion results from a point mutation

whereby cysteine is replaced by tyrosine in the first cysteine rich domain of the protein (Zdarsky et al., 1990). This mutation occurs only 3 amino acid residues away from the mutation site of albino mice. This is significant since both these mutations occur in the cysteine rich domain and cysteine has recently been implicated to play a role in iron-binding which may be critical for tyrosinase activation (Halaban et al., 1990).

The slaty locus (TRP-2) gene was identified by Jackson (1988) and was thought to be identical to the brown locus gene. However, it is now known that it is distinct from the brown locus gene but shares homology with tyrosinase. It has been mapped to chromosome 14 of mice. The slaty locus encoded protein has highly conserved features of the albino and brown loci encoded proteins which include a trans-membrane region, highly conserved putative copper binding sites, two conserved cysteine rich domains, potential glycosylation sites, and a signal peptide. This protein is somewhat larger than the albino and brown loci- encoded proteins. It has recently been shown that the slaty locus encodes for dopachrome tautomerase (Hearing and Tsukamato, 1991).

The silver locus, which was originally termed Pmel 17-1, was another clone with significant homology to tyrosinase (Kwon et al., 1987). The expression of its mRNA, which was exclusive to melanocytes, could be induced with MSH or IBMX. This increase in the silver locus mRNA resulted in a marked increase in

pigmentation (Kwon et al., 1987). The protein encoded for by the silver locus is homologous to that encoded for by the albino locus. This protein has a molecular weight similar to that of tyrosinase, putative glycosylation sites and a transmembrane domain. This gene has been mapped to chromosome 10 in mice and chromosome 12 in humans (Kwon et al., 1987). The exact function of the protein encoded by the silver locus is unknown, but it is a thought that it functions as an enzyme in the melanogenic pathway (Hearing and Tsukamoto, 1991).

The mouse pink-eye dilution locus is located on chromosome 7 and is responsible for the mediation of eye and coat colour (Silvers, 1979). Mutations at this locus results in a decrease in pigmentation of the eyes and coat. Mutations at this locus specifically affect eumelanogenesis and the structure of eumelanosomes, but has little or no effect on pheomelanosomes. Many of the mutations at the pink-eye dilution alleles, including spontaneous mutations, are U.V. radiation induced. It was recently shown that the human homologue of the pink-eye dilution corresponds to the human D15S12 locus which is located on the q11-q13 segments of chromosome 15 (Nicholls et al., 1992). Patients suffering from the Angelman and Prader-Willie syndromes, typically have deletions of these segments. Both these syndromes are often associated with hypopigmentation of the skin, hair and eyes. Ramsay et al., (1992) conducted genetic linkage studies with candidate loci, candidate chromosomal regions and random loci. They managed to link the ty.pos OCA locus to two arbitrary loci, D15S10 and D15S13

(human homologue of mouse pink-eye dilution) which is in the same region where mutations occur in the Prader-Willie and Angelman syndrome. To confirm this, it was recently shown that a patient suffering from ty.pos OCA had mutations in both copies of the human pink-eye dilution equivalent gene, D15S12 (Rinchik et al., 1993). Furthermore, it was shown that D15S13 encodes an integral melanosomal membrane transporter protein and it is thought that this protein is responsible for transporting tyrosine into the melanosome (Nicholls et al., 1992).

### *1.2.7. CONCLUSION:*

It is evident that the process of melanogenesis is a complex cascade of events which is controlled by multiple genes. Over the last decade there has been a "quantum jump" in the knowledge of how pigmentation is regulated with the discovery of numerous new regulatory genes and their encoded proteins. The next few years will be used to identify more genes that are involved in the regulation of pigmentation as well as addressing the numerous questions that remain unanswered such as: does alternative splicing play a role in the regulation of tyrosinase activity? What are the intracellular processing and delivery pathways involved in the delivery of these gene products to the melanosome? What is the identity and role of melanogenic inhibitors in disturbing pigmentation? The answers to these questions, in concert with the current knowledge of the pigmentary system, will help to unravel the underlying mechanisms in pigmentary disorders such as albinism, vitiligo and piebaldism.

# CHAPTER TWO

## *2.1 INTRODUCTION:*

When this study was initiated, the cause of ty.pos albinism was not known and the gene/s responsible for this defect had not been found. Although a candidate gene has been found in the last few months and has been linked to the ty.pos locus (Rinchik et al., 1993), the aetiology of this pigmentary disorder remains unclear. Because the defect is known to lie within the pigmentary system, a thorough investigation into the melanocyte system of the skin was made in this study. In this chapter, the light microscopical features of epidermis and melanocytes of ty.pos. individuals is compared to that of normal black individuals. In addition, melanocyte quantitations were carried out to establish whether ty.pos. OCA occurs as a result of a melanocyte paucity.

Much of the published investigations into melanocyte density and distribution in the epidermis of normally pigmented individuals was prompted by interest in the mechanism underlying pathological and physiological changes of human pigmentation. In particular, it was noted that melanocytes have a very distinct regional distribution, occurring most commonly on the face, neck, feet and external genitalia (Szabo, 1954). Quantitation of melanocyte numbers in these and other regions of the body was carried out in order to determine whether higher melanocyte numbers in these regions was the cause of the greater incidence of melanomas observed in these

regions. In order to quantitate melanocyte numbers, it was necessary to establish a means of reliably distinguishing melanocytes from other epidermal cells and to accurately quantitate these. The details of a few of the most important pioneering experiments are outlined here.

***2.1.1. QUANTITATION OF MELANOCYTES IN THE NORMAL EPIDERMIS:***

The first person to endeavour to establish the number of melanocytes in the normal human epidermis was Szabo in 1954. His experiments were based upon the work of Billingham & Medawar (1948) who used the skin splitting technique in conjunction with the dopa reaction to establish melanocyte density in guinea pigs. This method involved splitting the dermis and epidermis of the skin by incubating the skin in a trypsin solution. This resulted in the separation of the epidermis from the dermis in the form of "epidermal sheets" with the melanocytes being exposed at the base of the epidermal sheet. In order to distinguish the melanocytes from the surrounding epidermal cells, the epidermal sheets were incubated in a solution of 3,4 dihydroxy-phenylalanine (dopa). The dopa serves as a substrate for tyrosinase, the melanocyte specific enzyme, responsible for the conversion of dopa to melanin. After a few hours, the melanocytes can be distinguished from other epidermal cells by the presence of large amounts of melanin in their cytoplasms and dendrites. This classic method, known as the "dopa-reaction" is currently still used to detect melanocytes and tyrosinase activity. Using these techniques, Billingham and Medawar showed that the

number of melanocytes in the region of the guinea pig ear was twice as much as the number in the trunk region. Importantly, they also found that there was no difference in the number of melanocytes in darker and paler regions of the guinea pig ear. ***Thus, they found no correlation between melanocyte numbers and observed skin colour.***

These experiments on guinea pig ear prompted Szabo (1954) to do similar tests using fresh human skin from different body regions obtained during plastic surgical procedures with the specific aim of determining melanocyte densities in the different parts of the body . He assumed that there were no racial differences with reference to melanocyte distribution and analysed his subjects without discriminating between different races, ages or sex. He examined a total of 440 samples from individuals of the Negroid and Caucasoid races.

His experiments were conducted as follows: dermis was split from epidermis by trypsin treatment. The whole epidermal sheet was fixed in formal saline and incubated in 3,4 dihydroxy-phenylalanine (dopa) until the melanocytes turned dark brown. The epidermal sheets were then fixed again in formal saline and whole-mounted in Canada balsam. The dopa positive melanocytes were counted in the piece of skin, the area of which was taken to be equivalent to the area of the biopsy. The counts revealed that there was an average of 1504 melanocytes per $mm^2$ in the human epidermis, with numbers in the regions of the head and neck being as high as 2800 melanocytes

per $mm^2$ while 940 cells were counted in the region of the thigh (Table 2.1.).

Although these initial attempts by Szabo (1954) to establish a method for quantitating melanocyte numbers paved the way for future studies, his experimental techniques were flawed in a number of ways. Firstly, the process of fixation can cause shrinkage or swelling of tissues. The skin samples were fixed twice before melanocyte counts were taken and the counts expressed per unit area of the tissue, without correcting for any alterations in tissue area which might have occurred during tissue processing. Another shortcoming of this method was the fact that the melanocyte counts were taken without the aid of square optic fields of known area. Furthermore, it was subsequently found that the method of tissue fixation used by Szabo interferes with the dopa reaction (Starrico and Pinkus, 1956). This may have affected the number of positive staining melanocytes since the tissues were fixed before dopa incubation, which may also have contributed to inaccurate counts. Nevertheless, Szabo's experiments revealed that ***neither the shape, size nor the melanocyte population density differs in the various race groups. Also, marked regional variation in the distribution of melanocytes was found in different regions of the body.***

Starrico and Pinkus (1956) carried out similar quantitation procedures to determine the number of melanocytes per square area in the human epidermis. This was a more comprehensive study using improved techniques and the results were reported

in relation to age, sex, race and body regions. Sixty three skin samples, from various body regions, were obtained from normal blacks and Caucasoids during surgical procedures. A sodium bromide solution, instead of trypsin, was used for splitting skin, a method selected after testing various other salt solutions, because it resulted in clean splitting of skin in reasonable time, the excellent preservation of cell morphology and did not interfere with the dopa reaction of the tissue. They also found that if they dopa treated the tissue before fixation, they had better melanocyte staining. Their method for counting melanocytes was also markedly improved by using a square field with sides of 250 $\mu$m. Dopa positive cells were counted in 8 to 20 fields and converted to number of melanocytes per square millimeter.

They found no differences in the number of melanocytes when comparing the various race groups. ***This confirmed previous findings that there was no correlation between melanocyte numbers and the difference in colour of individuals of different race.*** They found an average of 1548 melanocytes per square millimeter of human epidermis, with the highest numbers being in the region of the upper arm (2500 melanocytes/$mm^2$) and also reported the lowest numbers to be in the region of the thigh (1031 melanocytes/$mm^2$).(Table 2.1.). These results again confirmed the findings of Szabo (1954) that there appear to be distinct differences in melanocyte numbers in the different regions of the body. They also noted that there were greater numbers of melanocytes in the regions of the epidermal ridges

than in the valleys and attributed this to a simple law of optics : by observing the dermo-epidermal junction from above, more melanocytes will be seen in the ridges simply because more cells are projected in these regions.

Another interesting observation made by Starrico & Pinkus (1956) was that there were variations in the size of melanocytes in different regions of the same individual as well as in individuals of different races. Melanocytes of Negroids were found to be much larger and more dendritic than those of Caucasoids. This led to the postulate that there is a causal relationship between the degree of pigmentation and melanocyte size and dendricity. They supported this by referring to the findings of Zimmermann and Cornbleet (1948) who reported that melanoblasts increase in size as fetal age increases. Since pigmentation also increases with fetal age, they correlated increasing pigmentation with increasing melanocyte size.

More recently, Rosdahl and Rorsman (1983) established the total size of tissue mass of the active melanocyte system, regarding this system as a single functional organ system of the body. Their main incentive for carrying out this investigation was to establish a concrete comparison with other organ systems and give an indication of the size of the potential pool for malignant melanoma.

A total of 20 skin biopsies were taken from male donors who had not been exposed to sun-tanning for 5 months prior to donating the biopsies. The skins were split and dopa incubated exactly as had been done previously by Szabo (1954). An ocular plate, of known diameter, containing 25 randomly distributed spots arranged in a circle was used to quantitate the number of melanocytes in a representative area. The skin samples were magnified 40x. Using this plate, the number of spots superimposed on melanocyte cell bodies was counted over ten randomly selected fields in each preparation. It was found that there had been an overall swelling of about 0.2mm in all the skin preparations which is a bit difficult to interpret since swelling is normally expressed as a percentage. Taking swelling into account, Rosdahl and Rorsman estimated the average population density of melanocytes to be between 1000 and 1500 cells per square millimeter (Table 2.1.). They concluded that the total population of active melanocytes in the epidermis of an average size male was in the order of $1.8x10^9$ - $2.7x10^9$ cells which can be compacted into an estimated tissue mass of 1.1-1.6 cubic centimeters with a weight of about one gram.

In summary, the above studies clearly indicate that there are no significant differences in melanocyte numbers when comparing individuals of different races. More specifically, it showed that light skin colouration cannot be attributed to fewer melanocyte numbers in the skin. A difference in the regional frequency of melanocytes was observed and these frequency

***Table 2.1.: MELANOCYTE COUNTS ACCUMULATED FROM VARIOUS STUDIES:***

| BODY REGION | AVERAGE NUMBER OF MELANOCYTES/$mm^2$ | | |
|---|---|---|---|
| | Szabo 1954 | Starrico & Pinkus 1956 | Rosdahl & Rorsman 1983 |
| Thigh | 940 | 1031 | 1700 +/- 139 |
| Upper arm | 1200 | 2500 | 1100 +/- 215 |
| Forearm | 1099 | 1223 | 1130 +/- 160 |
| Head & Neck | 2800 | 1340 | 2900 +/- 249 |
| Genitalia | - | 1668 | 1550 +/- 166 |

*Results from experiments done by various investigators to quantitate melanocyte numbers in different parts of the body. The counts are expressed as number of melanocytes per $mm^2$. Only the average counts are presented.*

distributions were consistent for all races. This difference in regional melanocyte distribution may account for the colour variation in the different regions of an individual.

### *2.1.2. FACTORS INFLUENCING OBSERVED COLOUR DIFFERENCES IN THE DIFFERENT RACE GROUPS:*

An interesting and somewhat unexpected finding arising from the studies on melanocyte quantitation described above, was the absence of a correlation between melanocyte numbers and differences in skin colour amongst the different races. This prompted Szabo (1972) to pursue his investigation into the basis for the difference in observed colour in individuals of different races. His aim was to determine whether there exists any characteristic histological differences in the epidermal melanin units of the various race groups. Samples of skin were obtained from male Caucasoids, Mongoloids, American Indians and Negroids. Each individual donated two biopsies, one from the forearm, a sun exposed region, and one from the buttock, a region usually concealed from sunlight (it has been shown that sun exposure has a profound effect on pigmentation of the skin). Each of the biopsies were divided into smaller pieces. For light microscopical investigation, the sections were fixed and embedded in paraffin wax for histological examination. Some of these sections were also treated with dopa before being embedded in paraffin wax. Some of the pieces of skin were split using a sodium-bromide solution, (method of Staricco & Pinkus, 1957), and the epidermal sheets were whole mounted. Skin samples from all the individuals were fixed in

glutaraldehyde, postfixed in osmium and embedded in Epon for electron microscopy.

The results of the above experiments can be summarised as follows: light microscopical studies revealed that there was considerable variation in the dopa reaction of the skin from individuals of different races. It was found that the dopa reaction was stronger in individuals of darker skin as well as in regions of lighter skinned individuals that had been exposed to sunlight, ie., the forearm as opposed to buttock. Also, skin from darker Caucasoids had very strong dopa reactions. In general, Caucasoid skin showed a variable, but generally weak dopa reaction as compared to that of Negroid skin. Skin biopsies from red-headed Caucasoids also had a very weak dopa-reaction. This finding indicated that the melanocytes of darker skin were more actively producing melanin than that of paler skin. Furthermore, the melanocytes of pale and red-headed Caucasoids were found to be small and had very few dendrites.

Szabo's electron microscopic observations demonstrated differences in the melanogenic activity of melanocytes of different races. His findings corroborated findings of previous researchers that Caucasoid skin, especially if it had not been exposed to sunlight, had almost no stage IV melanosomes. However, numerous stage II and III melanosomes were present in the melanocyte cytoplasm of these Caucasoids. On the other hand, the melanocytes of Negroid and Mongoloid

skin were filled with stage IV melanosomes even in regions not exposed to sunlight while very few stage II and III melanosomes were present. Szabo found that exposure to ultraviolet light serves to increase the relative proportions of stage II and III melanosomes and also increases the overall number of melanosomes in Caucasoid skin. It also results in an increase in the number of stage IV melanosomes in Negroid skin. It was found by Szabo that the most striking ultrastructural difference in individuals of different race existed in the distribution of melanosomes within the keratinocytes. The details of these ultrastructural findings are discussed in greater detail in the next chapter.

### *2.1.3. CELLULAR BASIS OF FRECKLING:*

Freckling is a dominant genetic trait characterised by the presence of specific groups of melanocytes that have the capacity to form melanin more rapidly after exposure to UV than melanocytes from normal skin (Robins, 1991). A freckle can be described as a sharply localised area of relative hyperpigmentation which, as it develops, extends outwards from a centre to involve the neighbouring epidermis (Breathnach, 1957). Freckles (ephelides) are found exclusively on the sun-exposed regions of normal skin of especially fair, red-headed individuals. This phenomenon is of interest to us because ephelides are frequently found on the sun exposed areas of some ty.pos

individuals. The details of freckles that occur in normal Caucasoid individuals are reviewed here to enable a comparison with the freckles observed in ty.pos individuals.

In 1957, Breathnach determined whether there was a numerical difference between the number of melanocytes in the region of a freckle and an adjacent region of normal skin. Five red-headed Caucasian subjects between the ages of 20 and 35 donated skin biopsies from regions of freckles and non-freckles. These biopsies were split into dermis and epidermis and dopa-treated by the method used by Szabo (1954) described previously. It was found that in the regions of the freckle, the overall mean was 951 melanocytes/$mm^2$, while in regions of non-freckles, the overall mean was 1768 melanocytes/$mm^2$. This finding suggests that there are almost 50% fewer melanocytes in the region of the freckle ie., that there is a melanocyte decrease in the region of the freckle. As mentioned previously, early melanocyte quantitation methods used by Szabo and others lacked accuracy and it is therefore of concern that there appears to be no subsequent experiments to confirm these data on melanocyte numbers in regions of freckles.

In addition to these numerical differences, Breathnach (1957) also described a marked difference in the histological appearance of melanocytes in the region of freckles. Light microscopical investigations revealed that these melanocytes were more dopa-positive, suggesting more active tyrosinase in these cells. Furthermore, he provides evidence (which he

refers to as being convincing) that the melanocytes in the region of the freckle were visibly larger and their dendritic processes were more numerous and longer than those found in adjacent paler skin (see Fig. 2.1). However, from his photograph of skin (a whole mount dopa preparation), it is difficult to distinguish individual melanocytes and suggests that his melanocyte counts may be somewhat subjective. Because the melanocytes in the region of the freckle are known to be larger and more dendritic, there may be fewer melanocytes per unit area of skin. This would explain Breathnach's findings that there are fewer melanocytes in the region of the freckle . However, it is somewhat surprising that there are no other studies that report on the number of melanocytes in the region of freckles and points to the need for further investigation in this regard. There are many features of freckling for which no explanations have been found. For example, it is not known what exact local conditions determine the location and size of a freckle in the epidermis. Furthermore, no explanation has been offered as to why some melanocytes are more sensitive to UV than others occurring in the same region of epidermis. The problem could either be a quantitative one, where it would be expected that there would be more melanocytes in the region of the freckle, or it could be a qualitative one where the level of gene expression in these melanocytes would be higher.

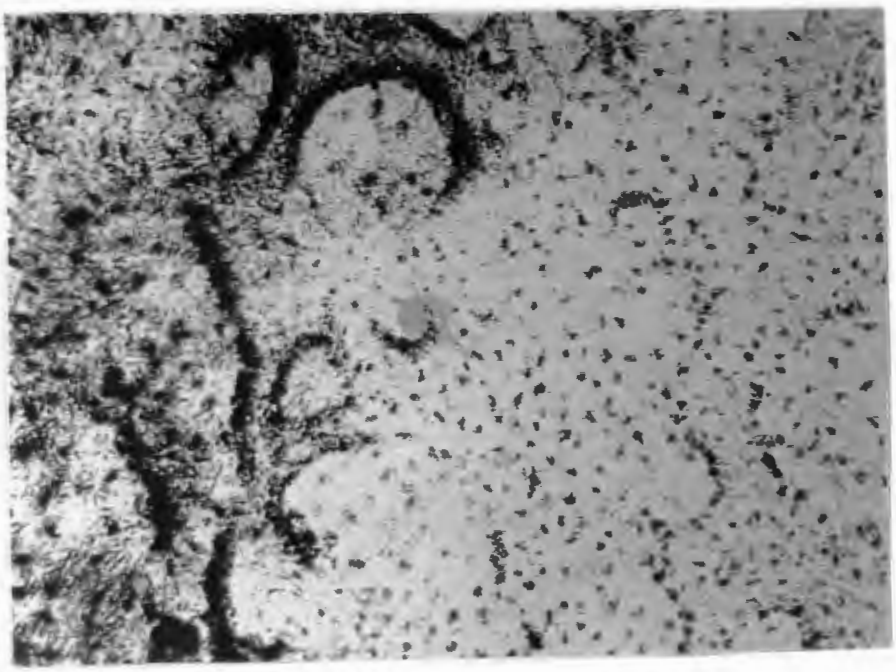

FIG. 2.1.: Whole mount dopa preparation of epidermis treated with "Dopa" and viewed from dermal side to show junction between a freckle(left) and paler epidermis (right). Adapted from Breathnach (1957).

### *2.1.4. EPHELIDES IN TYROSINASE POSITIVE ALBINOS*

As described earlier, ty.pos OCA is characterised by hypopigmentation of the skin and eyes and many individuals suffering from this condition also have ephelides on their sun-exposed regions. The size of these ephelides is not uniform. Density of ephelides varies greatly, with some individuals having only one or two ephelides while others have approximately ninety-five percent of their sun exposed skin freckled (Fig.2.2)

Because of these differences within the ty.pos. phenotype, it becomes important to establish whether there are any significant differences in the number of melanocytes per unit area of skin in ty.pos. individuals in the regions of ephelides and in regions with no ephelides. What also needs to be established is whether there is a significant difference in the number of melanocytes per unit area in ty.pos as compared to normally pigmented individuals. This may help to shed some light on whether the biological basis of albino ephelides is the same as that of normal fair-skinned individuals.

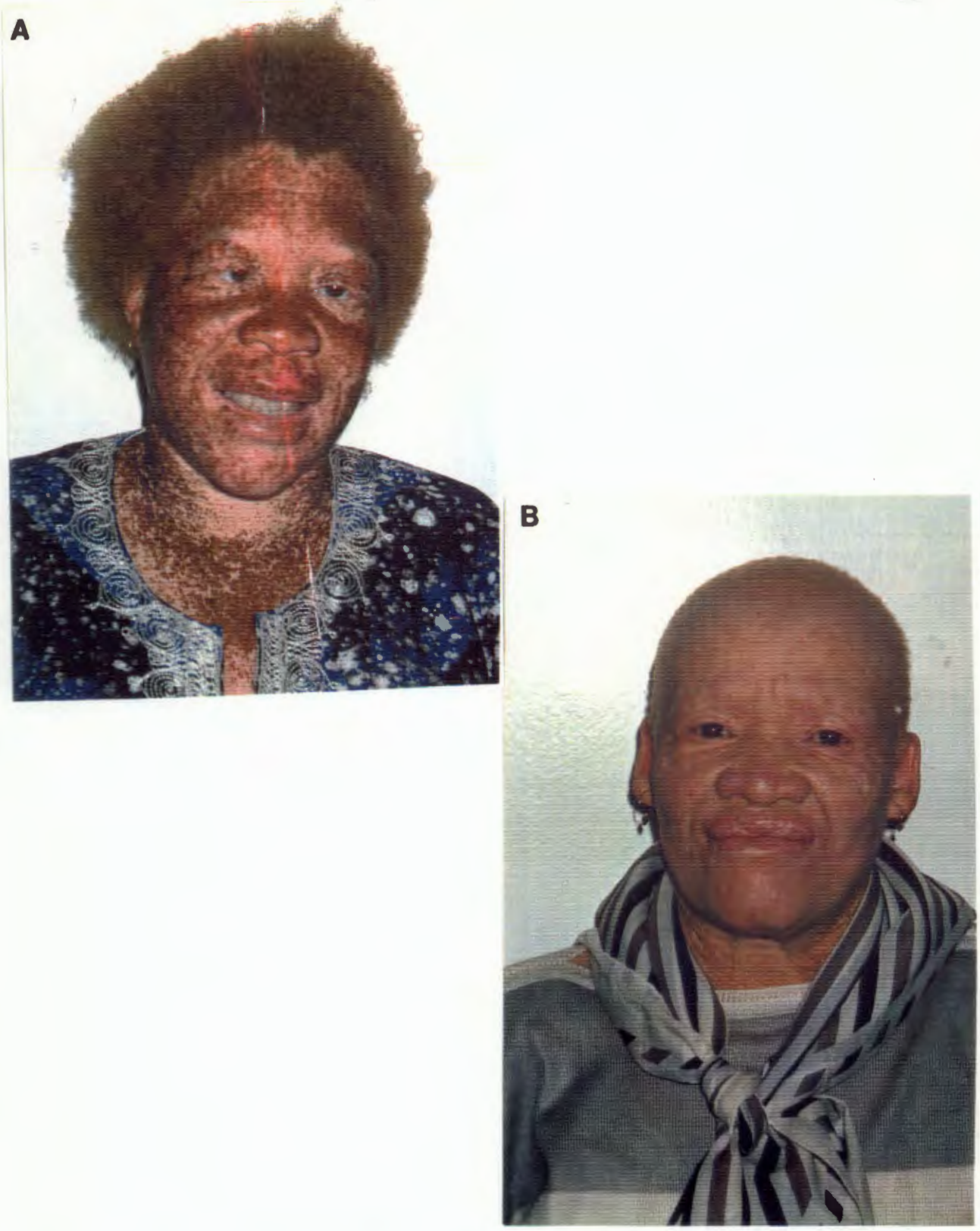


FIG. 2.2.: Tyrosinase Positive Albinos. A. An individual with an extreme case of ephelides. Notice that the ephelides occur only on the sun-exposed regions and the hair colour of this individual is dark brown compared to B. an individual with no ephelides who has yellow straw-coloured hair.

## *2.2. AIM:*

From the literature reviewed in this chapter, it is evident that there is no correlation between melanocyte numbers and degree of pigmentation. However, there is no information on the melanocyte density in skin of ty.pos. albino individuals. To date, most of the descriptive studies on the melanocytes of albinos have been largely restricted to the ultrastructural study of hairbulb melanocytes. This limitation can most probably be attributed to the fact that hairbulbs are more easily accessible than skin. However, since hairbulbs are very small and difficult to manipulate, quantitation of melanocytes in hairbulbs is not possible. Also, the melanocytes are restricted to a ring around the core of the cortex of the hairbulb. The specific aim of the work presented in this chapter was to establish whether there is a correlation between melanocyte numbers and:

1. the hypopigmentation of ty.pos. individuals,
2. the hyperpigmentation in areas of ephelides in these individuals.

This study was also aimed at elucidating any differences in the morphology of the melanocytes, pigment content and any other differences in the epidermis of ty.pos. skin.

## *2.3. MATERIALS AND METHODS:*

### *2.3.1. SOURCE OF SKIN BIOPSIES:*

Skin biopsies from ty-pos albino individuals and a few normal Negroid individuals were obtained from donors who are part of an on-going project at the Department of Human Genetics, South African Institute for Medical Research & University of the Witwatersrand, Johannesburg. Normal (control) biopsies were also donated by Negroid medical students at University of Cape Town Medical School. Donors were informed of the biopsy procedure and signed consent was obtained. All biopsies were done by qualified medical practitioners. Fourteen albino and thirteen normal Negroid control biopsies were obtained (see Tables 2.2 & 2.3 for details).

### *2.3.2. BIOPSY SITE:*

All biopsies, from both albinos without ephelides and control individuals, were taken from the inner upper arm, a region that is not frequently exposed to sunlight. In cases where the albino individuals had ephelides on their sun exposed regions, a biopsy from the forearm was taken to include only the region of ephelis because it was assumed that the adjacent paler regions were the same as that of albinos with no ephelides. All biopsies were taken superficially, ie., not including much dermis in order to minimise scarring. The average size of the biopsies was approximately one centimeter square.

***TABLE 2.2.: DETAILS OF ALBINO SUBJECTS***

| BLOCK NO. | AGE | SEX | EPHELIS STATUS | DEGREE OF EPHELIDES** |
|---|---|---|---|---|
| A1 | 38 | M | NONE | - |
| A2 | 21 | M | NONE | - |
| A3 | 26 | F | YES | few, large |
| A4 | 31 | F | YES | many, small |
| A5 | 31 | F | YES | few, small |
| A6 | 24 | F | YES | many, large |
| A7 | 48 | F | NONE | - |
| A8 | 52 | M | NONE | - |
| A9 | 32 | F | YES | 95%, small |
| A10 | 28 | M | NONE | - |
| A11 | 19 | M | NONE | - |
| A12 | 28 | F | NONE | - |
| A13 | 30 | M | NONE | - |
| A14 | 29 | F | YES | 80%, large |

*Details of albino subjects who donated biopsies. Samples were numbered from A1 to A14. Six males and eight females between the ages of 21 and 52 donated samples.*

**The degree of ephelides of each individual was assessed by visual inspection of the sun-exposed regions. Fig.2.2. shows an albino individual without ephelides and subject A9 who was assessed as having 95% ephelides on the sun-exposed regions.

***TABLE 2.3.: DETAILS OF NORMAL NEGROID SUBJECTS:***

| BLOCK NO. | AGE | SEX |
|---|---|---|
| EC1 | 33 | F |
| EC2 | 32 | M |
| EC3 | 31 | M |
| EC4 | 30 | F |
| EC5 | 36 | M |
| C6 | 19 | M |
| C7 | 19 | M |
| C8 | 20 | F |
| C9 | 18 | F |
| C10 | 19 | M |
| C11 | 18 | F |
| C12 | 18 | M |
| C13 | 17 | F |

*Details of normal Negroid individuals who donated skin biopsies. The sample consisted of 6 females and 7 males between the ages of 17 and 36 years. EC1-EC5 indicate biopsies which were fixed for electron microscopy only, while C6-C13 were fixed for light microscopy only.*

### *2.3.3. PROCESSING OF TISSUES:*

After the piece of biopsy sample had been excised and labelled, it was rinsed in phosphate buffered saline (pH. 7.6). The biopsies were cut into one large piece and a few smaller pieces. The large piece was fixed in 10% buffered formalin while the smaller pieces were fixed in 2.5% glutaraldehyde for electron microscopy (details in chp. 3). Only blocks C6 to C13 of the normal skin samples were fixed for light microscopy and immunocytochemistry. Because the skin samples were very small, processing was done manually. The samples were dehydrated and then incubated in two changes of wax at $60^0$C for 15 minutes each. The sections were then orientated and embedded in fresh wax.

### *2.3.4. LIGHT MICROSCOPY:*

Each block of tissue was sectioned on a Reichert-Jung ultratome. 5 $\mu$m sections were cut and mounted on clean glass slides which were then incubated at $37^0$C for a few hours to facilitate adhesion of the sections to the slide. The sections were dewaxed in two changes of xylol, hydrated in decreasing strengths of alcohol for approximately 2 minutes in each solution and then briefly washed in tap water. Representative sections from each sample was stained with eosin for approximately 5 minutes, cleared, and mounted with DPX mountant. All slides were viewed with a Nikon light microscope.

### *2.3.5. IMMUNOCYTOCHEMISTRY:*

In this study, immunocytochemical techniques were employed to localise and distinguish melanocytes from surrounding keratinocytes at the light microscopical level. Immunocytochemistry (ICC) involves the use of labelled antibodies as reagents for the localisation of specific tissue constituents (antigens) *in situ*. Numerous antibodies have been raised against various different epitopes in human melanocytes. These include antibodies raised against S-100 and HMSA-5 that were used in this study.

#### *2.3.5.1. S-100 ANTIBODY:*

The S-100 protein was initially isolated from brain tissue, but has since been shown to be present in a wide variety of tissue types, including the adenohypophysis, parathyroid gland, pancreas, ovary, testis, blood vessels, heart, kidney, cartilage, adipose tissue and the Langerhans cells and melanocytes of the human epidermis (reviewed by Atoji et al., 1991). It is usually ubiquitous within cells, being found in the cytoplasm as well as being membrane-bound.

The S-100 antibody was initially chosen to stain melanocytes because it is commercially available. However, in addition to melanocytes, it also stains the dendritic Langerhans cells of the epidermis which makes it difficult to distinguish between these two cell types. Thus, it was realised that this antibody was not appropriate for use in melanocyte quantitation, but was nevertheless retained and used to develop the ICC technique for

melanocyte staining as well as for studies on melanocyte morphology.

Rabbit anti-cow S100, the purified immunoglobulin fraction of rabbit antiserum, was purchased from Dakopatts. This company recommends that this antibody be used on tissue that had been fixed in 10% buffered formalin at a concentration of 1:200 (dilution details given later) and that sections be trypsin treated before application of the antibody to enhance the staining.

***2.3.5.2. HMSA-5:***

Dr. Kowichi Jimbow (University of Edmonton, Alberta, Canada) and his collaborators have developed numerous antibodies specific for human melanocytes (Maeda et al., 1990). Currently, there are six murine monoclonal antibodies in this series raised against HMSA-1; HMSA-2; HMSA-3; HMSA-4: HMSA-5 and HMSA-6, (HMSA being the abbreviation for Human Melanosome Specific Antigen). On request, we were sent a generous sample of HMSA-5** from Dr. Jimbow. Recently, Jimbow et al.,(1992) showed by immunoelectron microscopy that this antibody, which was developed against human melanosomes isolated from TPA stimulated melanocytes, was in fact specific for VGB's, the putative tyrosinase carriers described in detail in chapter one. Western blots reveal that HMSA-5 recognises a 69 KDa glycoprotein which is abundant in the cytoplasm of melanocytes. Importantly, it does not cross-react with melanosomes that have been transferred to keratinocytes, nor to

** For the rest of this thesis the HMSA-5 antibody will be referred to as HMSA-5.

melanosomes that have been phagocytosed by melanophages. This makes this antibody specific for melanocytes and ideal for the purpose of identifying and quantitating melanocytes. In the present study, this antibody was used undiluted on 10% buffered formalin fixed tissues as recommended by Dr. Jimbow. Specific staining of only melanocytes was obtained. Furthermore, this antibody was found to cross-react with melanocytes of both normally pigmented Negroid and ty.pos. albino individuals which made possible quantitation and comparison of melanocyte densities between these groups.

### *2.3.6. IMMUNOCYTOCHEMICAL STAINING PROCEDURE:*

Initially, numerous methods of fixation, embedding, antibody dilutions, antibody labelling and detection of ICC products were tested. These included fixation with 4% paraformaldehyde, embedding in epoxy resin, varying the antibody dilutions from 1:200 to 1:500 and the use of fluorescence labelling such as fluorescein isothiocynate (FITC). After much trial and error, a standard protocol was established which worked adequately for both S100 and HMSA-5. This protocol was used for all ICC experiments and can be summarised in sequence as follows :

1. All the biopsies were fixed in 10% buffered formalin and embedded in paraffin wax.

2. 5 $\mu$m sections were cut on a Reichert Jung microtome, mounted onto clean glass slides and left in a $37^0$C oven. Once the sections had adhered to the slide, they were dewaxed with

two changes of xylol for approximately five minutes each. The slides were then dehydrated in alcohol for approximately two minutes each and washed in tap water for five minutes and left in PBS (pH. 7.6).

3. To block endogenous peroxidases, 20 µl of a solution of 1% hydrogen peroxide was pipetted on the tissue sections and left on for ten minutes.
Control experiments using $H_2O_2$ was done and it was found that this step was necessary.

4. All the sections were treated with a solution of 0.1 % trypsin in distilled water. This was done because it is known that the enzymatic action of trypsin breaks protein cross-linkages established during fixation, thereby exposing the antigenic epitope (personal communication, U.C.T. Pathology Department). This was found to be especially necessary in the case of the S-100 epitopes.

5. In the instances that anti S-100 was used as the primary antibody, sections were treated with 20 µl of 10% normal swine serum for ten minutes to reduce non-specific staining.
Although non-specific staining was not a problem, this precautionary step was included.

6. Sections were treated with 20 µl of anti S-100 (1:200). The antibody was diluted with a diluent solution containing 100 ml tris saline, 0.25 g sodium azide, 5 mg bovine serum albumin, 5mg thyroglobulin, 40 mg EDTA and 1 ml normal swine serum. 20 µl HMSA-5 (undiluted) was used on other sections. These sections were sealed in a damp chamber and left overnight in a $4^0$C fridge.

7. The following day, the sections were washed in PBS for approximately five minutes and treated with a secondary antibody for 30 minutes at room temperature. The bridging secondary antibody used was biotinylated donkey-anti-rabbit (1:200) for S100 and biotinylated goat-anti-mouse (1:200) for HMSA-5, both purchased from VectorStain. The slides were then washed in PBS for another five minutes.

8. The Streptavidin-Biotin complex (ABC) method was used for the detection of bound antibodies in the tissue. The complex was purchased from Zymed and was used at the recommended dilution of 1:200, diluted for 30 minutes at room temperature, before applying to sections for 30 minutes at room temperature.

9. The sections were washed in PBS for approximately five minutes. 1 mg of diaminobenzidine (DAB)* was dissolved in 2ml Tris/HCL (pH. 7.6) and 20 µl of a 1% solution of hydrogen peroxide was added to this solution. DAB was applied to the sections for exactly five minutes. * SEE APPENDIX 1

10. The DAB reaction was terminated by washing the slides in running tap water. Thereafter, the sections were counterstained with haematoxylin for approximately five seconds. This serves to stain the nuclei blue. The sections were "blued" in running tap water, increasing the contrasting blue stain of the nuclei from the surrounding tissue. The tissue was then dehydrated by passing the slides through increasing strengths of alcohol and eventually cleared in

xylol. The sections were coverslipped with DPX mountant and were now ready for quantitation.

### *2.3.7. ICC NEGATIVE CONTROLS:*

In any ICC system, a positive appearing result may well be genuine, but there are always risks of non-specific reactions which must be eliminated before the results can be accepted. In all the ICC experiments done in this study, controls were included. The first control involved using diluent instead of the primary antisera. The second control was a positive control whereby the melanocytes of the epidermis stained positively and the negative control was the absence of stain in the dermis. Controls were included in all the experimental runs and serial sections were used for positive and negative staining. In the positive sections, melanocytes stained intensely brick-red making them clearly distinguishable from surrounding keratinocytes.

### *2.3.8. MELANOCYTE QUANTITATION:*

Melanocyte quantitation was done using a KONTRON Digital Analysing System. Essentially, this system comprises a Nikon light microscope, a magnetic digitizer measuring tablet, a computer and a television monitor. This image analyser is designed for the acquisition of data such as object counts and geometric characteristics of objects. The computer programme, M.O.P. Video Plan, is able to calculate various parameters, including length, area, perimeter and volume.

For the purposes of this study, it was necessary to express melanocyte numbers per unit area of skin. Each HMSA-5 positive melanocyte was thus scored as a positive point on the digitizer tablet and the length of basement membrane enclosing the scored points was traced. Only melanocytes with visible nuclei were counted. The counts were expressed as positive cells per length (in millimeters) of basement membrane. The cumulative data was stored until the entire length of basement membrane of a particular tissue section had been covered.

The square area of the tissue section was calculated by multiplying the length of the basement membrane with the thickness of the section which was 5µm (0.005mm). The number of melanocytes per unit area of skin was then calculated. Each section was quantitated twice and the average of these two counts was used. Three sections from each block was quantitated. Only the means of all the counts are presented in the Results section. Statistical calculations, using the F-test and T-test were done to establish if there are any significant differences between the mean melanocyte numbers in ty.pos. albinos and normal black individuals and furthermore, between regions of ephelides and non-ephelides in ty.pos. skin.

## *2.4. RESULTS:*

### *2.4.1. LIGHT MICROSCOPICAL OBSERVATIONS ON THE CELLULAR AND TISSUE MORPHOLOGY OF THE EPIDERMIS:*

#### *2.4.1.1. EOSIN STAINING*

Eosin staining of the skin samples did not render the melanocytes distinguishable from other epidermal cells. However, eosin stained sections did reveal that there was no apparent morphological differences in the appearance of the epidermis of individuals with ty.pos. OCA (Fig 2.3b & 2.3c) as compared to normal Negroid skin (Fig. 2.3a). A very important marked difference revealed by eosin staining was that in the epidermis of Negroid individuals, melanin granules were clearly discernible in the basal keratinocyte layers. These granules were generally very dense in the stratum basale and got progressively more sparse in the upper layers. In the case of the ty.pos. individuals with ephelides, melanin granules were visible in only some regions of the stratum basale (in the region of the ephelis), but there was an obvious decrease in the density of the granules as compared to normal Negroid skin. Here too, the melanin granules were more progressively sparse in the more superficial layers. There were no visible melanin granules in any of the layers of the epidermis of ty.pos. albino individuals with no ephelides.

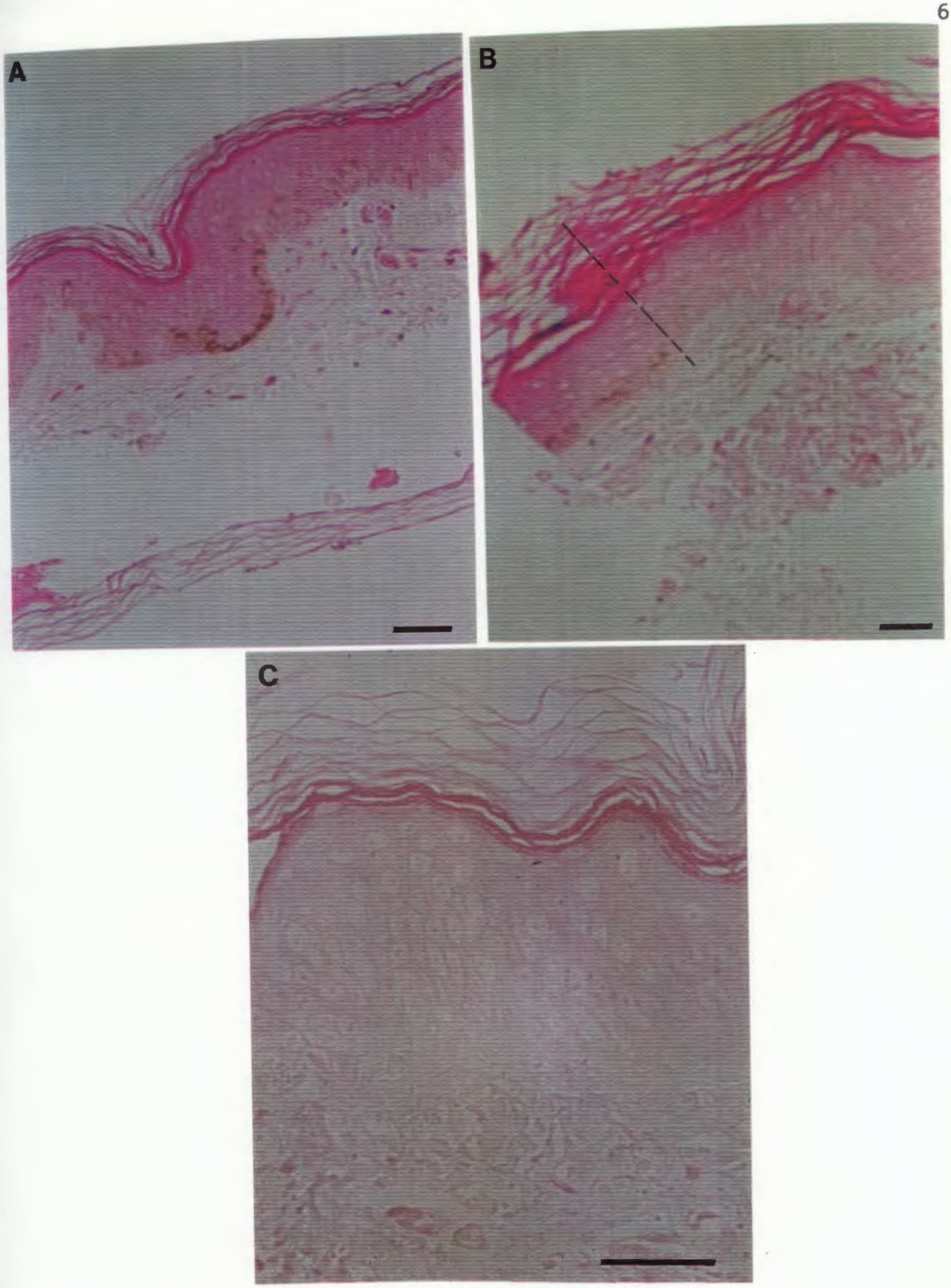


FIG. 2.3.: Eosin stained sections of A.: Normal Negroid skin B.: ty. pos with ephelides and C.: ty.pos without ephelides. In A, melanin granules can be seen in all the basal cells; in B, the dotted line shows the margin of an ephelis which contains numerous melanin granules with surrounding skin where no melanin granules are seen and in C, no melanin granules can be seen in any of the cells. (Bar = 100um)

***2.4.1.2. S-100 STAINING:***

S-100 was stained in both melanocytes and Langerhans cells of the epidermis of normal as well as ty.pos. albino individuals. Although the staining was clear with minimal background, it was difficult to distinguish melanocytes from Langerhans cells since both are dendritic, and although the melanocytes are only located on the basal layer of the epidermis, Langerhans cells can be located in any layer, including the basal layer (Fig 2.4).

The antibody was clearly localised in the cytoplasm of the cell as well as in the membrane, hence staining the dendrites of the melanocytes and Langerhans cells. There appeared to be no difference in the staining intensity and localisation of the antibody within the melanocytes of the three groups of individuals studied. From these S-100 staining experiments, it was evident that in the ty.pos. individual there were numerous fully dendritic melanocytes at the basement membrane of the epidermis, a feature which was not visible on the eosin stained sections. This finding confirmed that these individuals do possess melanocytes.

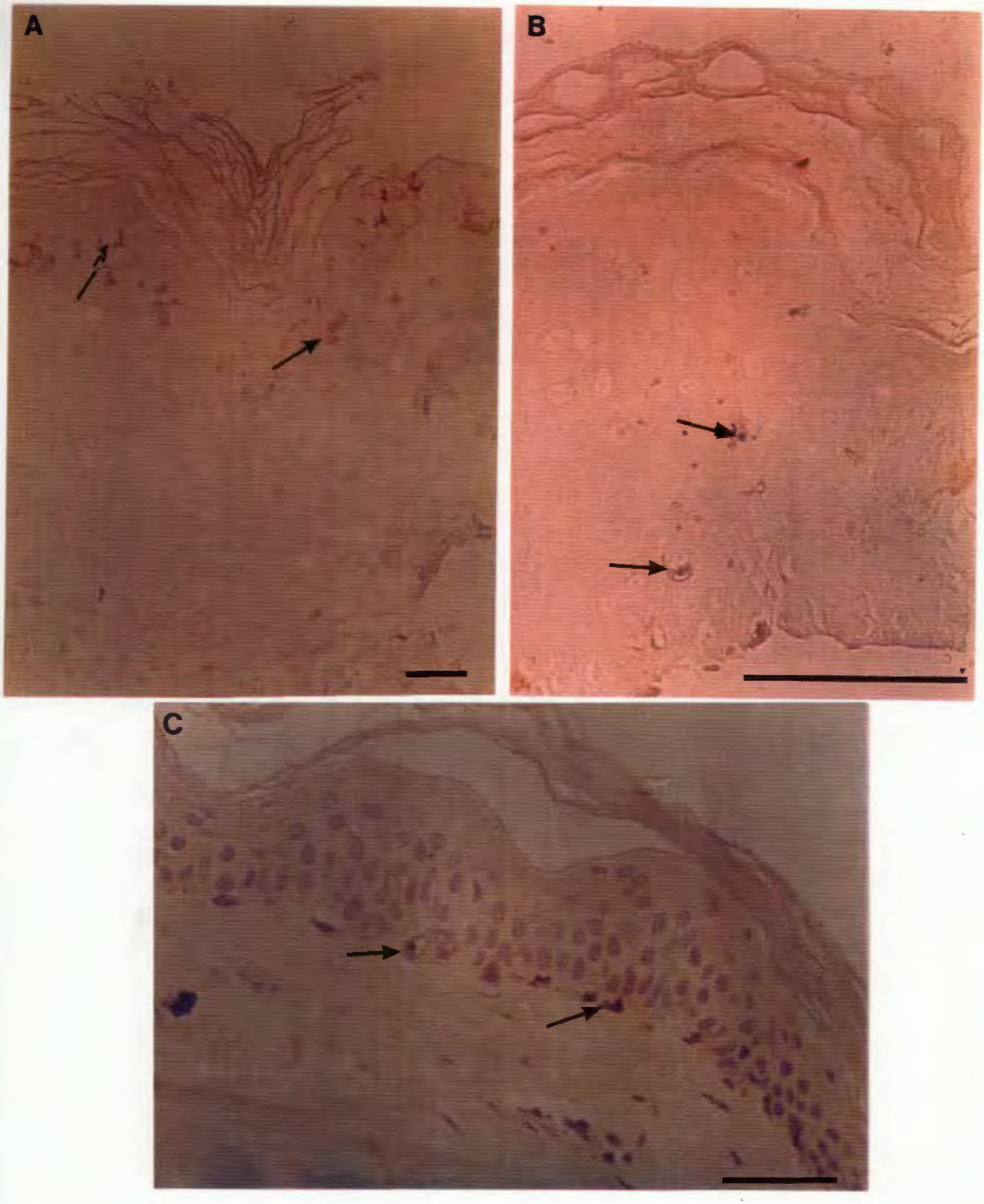


FIG. 2.4.: S-100 stained sections of A.: normal Negroid skin; B.: ty.pos skin with ephelides and C: ty.pos skin with no ephelides. Note that the positive cells are not restricted to the basal layer since both melanocytes and Langerhans cells are stained by S-100. (Bar = 100um).

***2.4.1.3. HMSA-5 STAINING:***

HMSA-5, which was highly specific for melanocytes (Fig.2.5.) stained them in both normal and ty.pos. skin (Fig 2.6a; b & c). HMSA-5 is directed against the VGB's of the melanosomes and is, therefore, expected to localise in the cytoplasm of the melanocytes, but not their cell membranes.

There was clearly a marked difference in HMSA-5 staining intensity in Negroid individuals (Fig.2.6a) as opposed to ty.pos albino individuals without ephelides (Fig.2.6b) and in the region of an ephelis (Fig.2.6c). In melanocytes from normal skin, there was intense staining of the entire cytoplasm as indicated by the dark brown appearance of the entire cytoplasm, and in instances where the dendrites of the melanocytes were visible, these too, were intensely stained. On the other hand, in the ty.pos albino melanocytes, selective staining in only some regions of the cytoplasm and perinuclear area occurred.


FIG. 2.5.: HMSA-5 staining of A.: normal Negroid skin and B: Control section in which HMSA-5 was omitted. Note that HMSA-5 stains only the basal melanocytes (arrows) and staining specificity is confirmed by absence of stain in control section. (Bar = 100um).

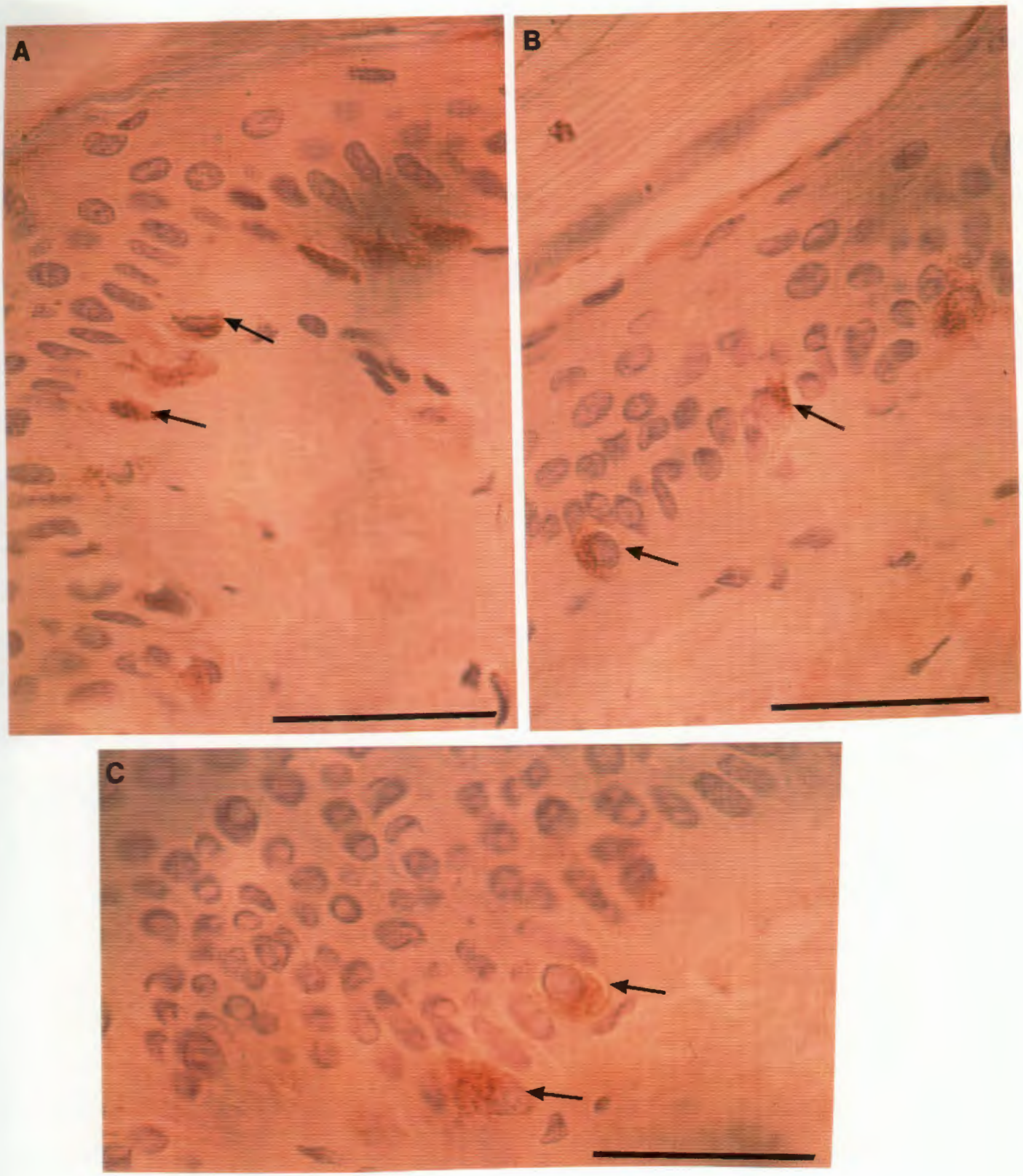


FIG. 2.6.: HMSA-5 staining of A.: normal Negroid skin; B. ty.pos skin with ephelides and C.: ty.pos skin without ephelides. In A., the melanocytes stain uniformly dark brown, while in B and C, only some regions of the cytoplasm stain paler brown. (Bar = 100um).

Interestingly, there appeared to be slightly darker staining of the cytoplasm of the melanocytes from ty.pos individuals with ephelides (Fig. 2.6c) as compared individuals without ephelides (Fig. 2.6b). In both instances, the HMSA-5 localised densely in some regions of the cytoplasm while the rest of the cytoplasm had less stain (Fig.2.6b & c.).

### *2.4.2. MELANOCYTE QUANTITATION DATA:*

Melanocyte counts were accumulated from skin sections stained with HMSA-5 using the KONTRON image analyser as described in the "Materials and Methods" section of this chapter. The results are summarised in Tables 2.4, 2.5 and 2.6.

***TABLE 2.4: AVERAGE MELANOCYTE COUNTS FOR NORMAL NEGROID SKIN:***

| BLOCK NUMBER | AVERAGE NO. OF MELANOCYTES/$mm^2$ |
|---|---|
| C6 | 1788 |
| C7 | 1232 |
| C8 | 1882 |
| C9 | 1138 |
| C10 | 1129 |
| C11 | 1204 |
| C12 | 1204 |
| **AVERAGE** | **1351** |
| **STD. DEV.** | **318.13** |

*Average melanocyte numbers for 7 normal Negroid subjects per $mm^2$ of epidermis in the region of the inner-forearm. Each sample was counted three times and the averages are presented in this table.*

***TABLE 2.5.: MELANOCYTE COUNTS FOR TY.POS ALBINO INDIVIDUALS IN THE REGION OF EPHELIDES:***

| BLOCK NUMBER | NO. OF MELANOCYTES/$mm^2$ |
|---|---|
| A3 | 672 |
| A4 | 944 |
| A5 | 888 |
| A6 | 1520 |
| A9 | 1059.5 |
| A14 | 1928.5 |
| **AVERAGE** | **1168.7** |
| **STD. DEV.** | **466.61** |

*Melanocyte counts for the epidermis of six albino subjects in the region of an ephelis. Each sample was counted three times and the averages are presented.*

**TABLE 2.6.: MELANOCYTE COUNTS FOR TY.POS ALBINO INDIVIDUALS WITHOUT EPHELIDES:**

| BLOCK NO. | NO. OF MELANOCYTES/$mm^2$ |
|---|---|
| A1 | 1031 |
| A2 | 1377 |
| A7 | 1178 |
| A8 | 927.5 |
| A10 | 1711.5 |
| A11 | 1417.5 |
| A12 | 1506 |
| A13 | 2332.5 |
| **AVERAGE** | **1435.125** |
| **STD.DEV.** | **443.60** |

*Melanocyte counts for ty.pos albino individuals without ephelides from the region of the inner upper arm. The averages of three counts are presented.*

The average number of melanocytes calculated for the region of the inner-upper arm of normal Negroid individuals was ***1351*** melanocytes per $mm^2$ of epidermis. For ty.pos albinos without differentiating between the presence or absence of ephelides, the average number of melanocytes in the region of the inner upper arm was ***1301*** melanocytes per $mm^2$ of epidermis. When distinguishing between individuals with ephelides and without, it was found that an average of ***1435*** melanocytes per $mm^2$ was counted in individuals without ephelides while ***1168*** melanocytes were counted in the region of the ephelis.

In order to establish whether there were any difference existed between the mean melanocyte density of these three sample populations, the F- and T- Tests at 5% significance level were used. This revealed that there was no significant difference between the melanocyte density in normal Negroid skin and ty.pos albino skin for the upper forearm region. Furthermore, using these same statistical tests no significant difference was found between melanocyte densities of ty.pos albino skin with and without ephelides.

At this stage it became apparent that more in depth investigations were required to elucidate the aetiology of ty.pos albinism. Consequently, it was decided to proceed to an ultrastructural investigation of both ty.pos and rufous albinism. The latter was included because the biopsies

became available at this stage, and because it was thought that these would present an interesting comparison with ty.pos biopsies. Furthermore, it became apparent that, in the interest of maintaining the continuity of the progressive unfolding of the aetiology as revealed by this study, it would be more prudent to discuss the findings of this and the next chapter simultaneously. Thus, a complete discussion of the results of chapters 2 & 3 follows in chapter 4.

# C H A P T E R  T H R E E

## *3.1. INTRODUCTION:*

In the previous chapter, light microscopical and immunocytochemical techniques were used to investigate the histology of and to quantitate melanocyte numbers in normal Negroid and ty.pos skin. It was shown that the hypopigmentation observed in ty.pos individuals was not a result of melanocyte paucity. This chapter, in turn, focuses on ultrastructural investigations into the skin and hairbulbs of both ty.pos. and rufous albinos. Although hairbulbs were not useful for melanocyte quantitation purposes, they were used here to simultaneously corroborate ultrastructural findings in skin and to facilitate comparison with the findings of other workers. The results of these electron microscopical investigations of ty.pos skin were, as in the previous chapter, once again compared to those of normal Negroid skin.

The findings of various studies on the ultrastructure of Caucasoid and Negroid skin is reviewed here since it was necessary to establish whether the pale ty.pos albino skin is in fact morphologically identical to Caucasoid skin and also if regions of ephelides, which have the same colouration as normal Negroid skin, are morphologically identical to black skin.

***3.1.1. THE ULTRASTRUCTURAL BASIS OF DIFFERENCES IN COLOURS OF DIFFERENT RACE GROUPS:***

In 1972, Szabo et al., studied the ultrastructural features of skin from individuals of different races. Skin samples were received from three representatives of the Caucasoid group, three Mongoloids of non-American origin, six American Indians and seven Negroids (one African, six Americans). All subjects were males between the ages twenty and thirty. Each individual donated two biopsies, one from the fore-arm which is normally a sun-exposed area and one from the buttock, an unexposed area. These biopsies were further divided for light microscopical studies, whole mount dopa stains and electron microscopy.

In this study, Szabo et al. (1972), classified melanosomal stages according to the classification provided by Toda et al., (1972 b). A stage I melanosome was described as a dopa positive vesicle without recognisable internal structure, a stage II melanosome as a dopa-positive particle with distinctive internal structure, a stage III melanosome showed some degree of melanization and a stage IV melanosome was a fully melanized granule with no discernible internal structure. Szabo et al's study revealed that there were marked differences in the melanogenic activity of melanocytes from men of difference races. It was found that in unexposed Caucasoid skin, the melanocyte cytoplasm contained very few fully melanised (stage IV) melanosomes, although there were numerous fully melanised stage IV melanosomes in the dendrites of these melanocytes. This was found to be true for all Caucasoids, irrespective of

the degree of colour visible to the naked eye; that is, even in the darker skinned Caucasoids, the cytoplasmic stage IV melanosomes were few. On the other hand, a number of stage II and III melanosomes were present in the melanocyte cytoplasm of these individuals. In contrast to this, it was found that the unexposed skin of the Mongoloids and Negroid individuals contained an abundance of stage IV melanosomes and relatively fewer stage II and III melanosomes.

When comparing the ultrastructure of melanocytes from the unexposed regions to that of the exposed regions in Caucasoid skin, it was found that there was an increase in the proportion of stage II, stage III and stage IV melanosomes in the exposed regions. Furthermore, when comparing the sun exposed skin of Mongoloid and Negroid individuals with unexposed skin, there was an even more marked increase in the number of stage IV melanosomes with Negroid skin clearly having the most number of stage IV melanosomes. In both the Mongoloid and Negroid individuals, hardly any stage I, II or III melanosomes were observed. These findings confirmed the previous findings of Szabo (1967), that exposure to ultra-violet light increased the relative proportion of stage II, III and IV melanosomes in Caucasoids, Mongoloids and Negroids. This implies that exposure to U.V. light serves to increase the melanogenic activity of the melanocytes.

In addition to the above mentioned findings, Szabo et al. (1972) also found that there was a marked difference in the

mode of transfer of melanosomes to keratinocytes in individuals from different race groups (Fig 3.1). In all the Caucasoid skin specimens observed, the melanosomes in the keratinocyte cytoplasm were located in complexes surrounded by a membrane. The melanosomes were not very closely packed in these complexes and a granular substance was present between individual melanosomes. It was found
that some stage II and III melanosomes were also transferred to keratinocytes. In the Mongoloid individuals, the melanosomes also occurred in complexes, but the complexes were smaller than those observed in the Caucasoid keratinocytes, with no limiting membrane around these complexes. These melanosomes were also very closely packed with very little granular substance between them. In contrast to this, in Negroid individuals, the melanosomes were mostly individually dispersed with a few melanosomes occurring in doublets or triplets, but clearly not in complexes.
On closer observation of the melanosome sizes in the various types of skin, Szabo et al (1972) reported that the size of
the melanosomes in the Negroid individuals were much larger than the melanosomes of Caucasoid and Mongoloid individuals. However, the precise melanosomal size differences were not calculated.

In a later study Toda et al. (1972a) reported on more precise melanosomal sizes of Caucasoid, Mongoloid and Negroid individuals in exposed and unexposed skin. When the unexposed gluteal skin of a Caucasoid was ultrastructurally examined, it

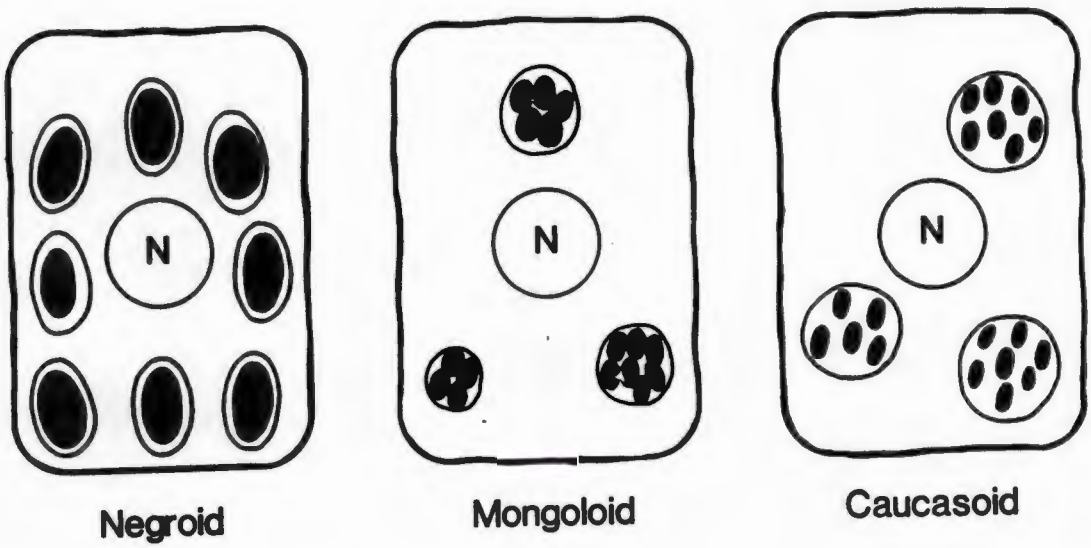


**FIG. *3.1:*** **Racial Differences in arrangement of melanosomes within keratinocytes in unexposed skin. In Negroid keratinocytes, the melanosomes are large and singly dispersed whereas in Caucasoids and Mongoloids, they are smaller and clustered into membrane-bound melanosomal complexes. In Mongoloids, the melanosomes are very closely packed with very little ground substance between them. Adapted from Robins, (1991); diagram based on Szabo et al. (1969).**

showed a predominance of aggregated melanosomes. These investigators then exposed this previously unexposed, gluteal skin to U.V. irradiation and took biopsies from the irradiated sites at 3, 24, 72 and 96 hours, and also at one week, three months and six months. It was found that the melanosomes in the UV irradiated skin regions were no longer aggregated, but were singly dispersed. Additionally, the melanosomes in the irradiated areas increased significantly in size to an average size of 1.1 x 0.6 $\mu$m from 0.6 x 0.3 $\mu$m, and were also more numerous. It was found that even at three and six months after exposure to U.V., the skin still contained larger, singly dispersed melanosomes. This experiment clearly demonstrated that Caucasoid skin , after exposure to U.V. light, synthesizes an increased number of melanosomes of increased size, which no longer formed clusters but occurred singly dispersed.

In addition to the above study, Toda et al. (1972a) also examined the skin from two Mongoloid individuals who were outdoor workers. Skin biopsies were taken from the forearm, a frequently exposed region and from the lower abdomen and buttock, which are usually unexposed regions. On ultrastructural observations of these biopsies, it was found that in the skin from the forearm there was a predominance of singly dispersed, large melanosomes having dimensions of 1.2 x 0.6 $\mu$m. In contrast, the melanosomes in the unexposed regions of the lower abdomen and buttocks occurred aggregated and were smaller (0.7 x 0.3 $\mu$m).

In a companion paper, Toda et al. (1972b) presented results from their study of melanosomes of the unexposed buttock region of fourteen American Negroids (Toda et al. 1972b). These subjects represented a spectrum of visible skin colouration. Of the fourteen subjects, eleven had skin colouration ranging from moderate to heavily pigmented. These eleven individuals showed, without exception, large, fully pigmented stage IV melanosomes that were singly dispersed in the keratinocytes. The other three subjects who had markedly paler skin colouration had both aggregated and non-aggregated melanosomes. These findings were in accordance with those of Szabo et al. (1972) that individuals with paler skin, whether pale Negroid, Mongoloid or Caucasoid is characterised by aggregated melanosomes and dark skinned Negroids and sun-exposed Mongoloids and Caucasoids are characterised by singly dispersed melanosomes.

An Observation made by both Szabo et al. (1972) and Toda et al.(1972b) was that there appeared to be many more melanosomes (about 340 per basal keratinocyte) in the more heavily pigmented skin than the approximately 120 melanosomes per basal keratinocyte observed in the paler skin of Negroid individuals. This is, again, indicative of enhanced melanogenic activity in the melanocytes of darker individuals.

In summary, it was found that the observed colour differences in the different race groups are recognised, at the ultrastructural level, most obviously in the keratinocytes

where melanosomal size and distribution pattern differs characteristically between races. The degree of melanosomal melanization within the melanocyte cytoplasm was also different in the various races with mostly stage II and III melanosomes occurring in the Caucasoids and mostly fully melanised stage IV melanosomes occurring in the Negroids. Lastly, the number of melanosomes in the keratinocytes were found to be much more in darker individuals than in paler individuals, even of the same race, indicating that the melanocytes in the darker pigmented region are more active.

Once it was known that fundamental ultrastructural differences underlie the observed colour differences in individuals of different race, impetus was given to the study of the ultrastructural basis of hypopigmentary diseases such as albinism, vitiligo, phenylketonuria and piebaldism. Since this study focuses on OCA only, the summary below reviews some of the ultrastructural studies reported on various forms of OCA.

### *3.1.2. ULTRASTRUCTURAL FEATURES OF VARIOUS FORMS OF OCA:*

In individuals affected with ***tyrosinase negative (ty.neg)*** OCA, melanocytes have been shown to be present in the skin, hair and eyes, but the melanocytes of the skin were found to contain numerous premelanosomes with no evidence of pigment accumulation on the melanosomal matrix (Witkop et al., 1970; Jung and Anton Lamprecht, 1971; Witkop et al., 1973). Furthermore, it was found that hairbulbs from these patients did not have any pigment discernable by light or electron

microscopy. However, electron micrographs of ty.neg albino hair bulbs did show that they contained numerous melanocytes filled with premelanosomes and stage I intermediate vesicles in which the unmelanized melanosomal matrix was clearly visible. Following incubation in L-tyrosine or L-dopa, there was no increase of the dopa product (dopa melanin) in the stage I melanosomes, nor was there any evidence of dopa product in the Golgi apparatus, endoplasmic reticulum or vesicular bodies (Witkop et al 1970; Witkop 1971; Jung and Anton Lamprecht , 1971). These findings strongly suggested that tyrosinase was absent or inactive in the ty.neg albinos. Furthermore, premelanosomes were packed in the melanocyte dendrites of these ty.neg. individuals and were actually seen to be transferred to the keratinocytes (Witkop et al. 1973). These findings suggest that the lack of tyrosinase in this form of albinism results in retardation of melanosomal development beyond stage I. The finding that premelanosomes are synthesised but remain relatively unmelanised and are transferred in this unmelanised form to the surrounding keratinocytes indicates that the melanocyte to keratinocyte transfer mechanism is functional in these individuals.

In ***yellow mutant*** OCA, the melanocytes present in the hair, skin and eyes contained numerous melanosomes which were more round than ellipsoidal, being more of the pheomelanosomal type. Unlike the melanosomes of the ty. neg albinos, these melanosomes were found develop to up to stage III and an even distribution of pigmentation was seen on the melanosomal

matrix. There were no fully melanized stage IV melanosomes observed in these individuals. Why a developmental arrest occurs at stage III of these melanosomes remains to be established (Hu et al.; 1980).

In a review by Witkop et al. (1989), the ultrastructure of skin and hairbulbs from individuals with ***Brown OCA*** was described. The melanocyte structure was found to be normal in the hair bulbs and skin. The hairbulb melanocytes were examined ultrastructurally and it was found that the melanosomes were classic ellipsoidal eumelanosomes. Fully melanised stage IV melanosomes were seen in this form of OCA, but these were few, with most of the melanosomes having reduced amounts of melanin, almost as if they were arrested at stage I or II of development. Incubation of hairbulbs in dopa showed no effect on melanization of the melanosomes but a slight dopa reaction was seen in the Golgi. In the review on brown OCA, Witkop et al (1989) provide information on skin melanosomes in addition to hair melanosomes. The skin melanosomes had a different architecture to those of normal black skin in that the melanosomes from these individuals were usually round and had incomplete melanization. Also, many of the melanosomes were small and had a granular internal pattern as opposed to the classic banding pattern seen in eumelanosomes of normal black skin. Most of the melanosomes were at stage II and III of development and a few stage IV melanosomes were present but these were not large, single, fully melanised stage IV melanosomes as seen in normal black skin. These findings

suggest that the hair bulb melanocytes of brown OCA individuals are normal and produce normal eumelanosomes. On the other hand, the skin melanocytes produce structurally different melanosomes which seem to be arrested at stage III of development. The stage IV melanosomes which are present seem to be somewhat degraded since they were small and were not fully melanised.

Studies on ***ty.pos*** OCA have been carried out predominantly on the Zuni and Brandywine triracial isolates residing in North America and New Mexico. As mentioned in chapter 1 (Table 1.1), at birth and during the first few months of life, Zuni albinos have white hair and translucent grey-blue eyes (Witkop et al., 1974). At about six months of age, the hair gradually changes colour and by the age of two, has a yellow tinge. The hair continues to darken with age and is a straw colour in adults. Skin colour was observed to be red-white in children while adults had a cream coloured skin. Various forms of visual impairment was also present in these individuals. No mention is made of the presence of ephelides in these types of albinos. This casts doubt on whether these individuals are in fact of the same genotype as the Southern African ty.pos albino under study in this project. It should be reiterated that very little information is available on the ultrastructural features of the Southern African ty.pos albinos since mostly genetic studies have been carried out on these individuals. This study is aimed at providing some information on the ultrastructural features of these albinos and compare these findings to that of Witkop on the Zuni albinos, presented below.

Witkop et al., (1972) described the ultrastructural features of melanocytes of these Zuni ty.pos. albinos. According to their findings, hairbulbs from these individuals frequently had a few pigment granules discernible with the light microscope. Ultrastructural investigation of these hairbulbs revealed the presence of numerous premelanosomes and partially pigmented stage II and III melanosomes in the melanocyte cytoplasm. Fully melanised stage IV melanosomes were rarely seen. A feature noted by Witkop et al. was that the melanocytes of these individuals frequently contained one or more polyphagosome complexes in which aggregates of melanosomes at various stages of development and strands of endoplasmic reticulum, were observed which were apparently undergoing destruction in the cytoplasm of the melanocytes. Unfortunately, Witkop et al. provide no photographic evidence for this finding. Partially pigmented stage III melanosomes were frequently observed in the dendrites of the ty.pos melanocytes, and melanosomes at various stages of development were passed onto the cortical and medullary hair cells where they were found to occupy their normal perinuclear position. In addition to these basic descriptive studies, Witkop et al., (1972) also prefixed freshly epilated hairbulbs from these ty.pos individuals in glutaraldehyde, incubated these hairbulbs in L-tyrosine and then stained ultrathin sections of these hairbulbs with 1% osmic acid and 1% glutaraldehyde. These sections demonstrated that nearly all the melanosomes had been converted to fully melanised stage IV melanosomes. This

incubation procedure also resulted in the staining of the terminal Golgi cisternae which was indicative of the presence of uncomplexed tyrosinase in this structure, a feature well established in the Golgi of normal melanocytes (described in chapter 1).

It is important to note that the above studies on ty.pos albino melanocytes were carried out exclusively on the hairbulbs . The ultrastructural features of the skin melanocytes are not described in the literature although Witkop's review on albinism (1989) mentions that skin melanocytes were studied but no reference is provided for this study. Attempts made to communicate with Witkop have been unsuccessful (Kidson, pers comm) and so the precise origin of this study remains unclear. For this reason and also the suggestion that the population of ty.pos. albinos studied by Witkop et al. may be different from the African phenotype described by Kromberg et al.(1990), it was decided to study the ultrastructural features of hairbulb and especially skin melanocytes from a Southern African population.

Very little ultrastructural information is available on ***rufous*** OCA. Indeed, as previously mentioned, there is a discrepancy between the descriptions provided by Witkop et al.(1989) and Kromberg et al.(1990) on the hair and skin colour of rufous individuals. According to Witkop, these albinos have a hair colour ranging from mahogany red to sandy red, while Kromberg et al. describe the hair colour of these individuals as being

light brown or ginger. Furthermore, Witkop et al. report that nearly all the melanosomes in the hairbulb of a rufous albino are pheomelanosmes whereas preliminary observations on hairbulbs from Southern African rufous albinos revealed a mixture of both eu- and pheomelanosomes at various stages of development (Kromberg et al. 1990). Apart from these findings, no other information was available on the ultrastructure of hair and skin from these individuals. Because these albinos are reported to occur only in Africa and New Guinea, this study also reports on the ultrastructural features of rufous skin and hair melanocytes. Some of the findings presented in this chapter have also been submitted for publication (in press).

### *3.1.3. ULTRASTRUCTURAL BASIS OF FRECKLING:*

As mentioned previously, in the ty.pos individuals studied by Witkop et al. (1989) no information is provided on whether or not these individuals also have the ephelides observed in many African ty.pos. individuals. However, according to the classification of ty.pos individuals in Witkop et al.'s review of albinism (1989), these individuals often have ephelides, but the literature reviewed to date does not elaborate on the features of the ephelides in these individuals. In the present study, the ultrastructural features of ephelides in the African ty.pos. individuals was examined. A brief review of the histological and ultrastructural features of normal freckles (ephelides) is given here to provide a basis for a comparison with the ty.pos ephelides.

Breathnach (1959) reported on the ultrastructural differences between the region of the freckle and an adjacent region of non-freckle in red-headed Caucasians. He observed that the cytoplasm of melanocytes from region of the freckles showed evidence of pronounced melanogenic activity with an abundance of stage IV melanosomes. Furthermore, although these individuals were red-headed Caucasians, the melanosomes in these regions were ellipsoidal eumelanosomes as compared to the round pheomelanosomes observed in the adjacent non-freckled skin. Numerous premelanosomes were also observed in the region of the freckle and it was clear that the melanocytes in the region of the freckle produced many more melanosomes than the adjacent pale regions. Breathnach therefore suggested that there existed two inherently different types of melanocytes in freckled individuals, one of these populations occurring in the region of the freckle and possessing the ability to enhance melanogenesis in response to U.V. stimulation. They also appeared to be synthesising eumelanosomes rather than pheomelanosomes. The other population occurring in the adjacent paler regions, are characteristic of Caucasian melanocytes having a lower melanogenic rate than Negroid melanocytes and synthesising predominantly pheomelanosomes.

In a more recent study, Kwan (1990), studied freckles in pale-skinned, red-haired Caucasians. He, too, found that in the region of the freckle, the melanocytes contained many melanosomes, most of which had the characteristic ellipsoidal shape and dimensions of eumelanosomes, while the surrounding

paler areas characteristically contained predominantly pheomelanosomes. On the other hand, he observed that the melanocytes in the region of non-freckles contained fewer pigmented melanosomes and premelanosomes and that their cytoplasms have a generally empty appearance.

From these findings, it can be concluded that the region of the freckle contains melanocytes that are more active in response to U.V. stimulation than those of the surrounding paler regions. These melanocytes also have a tendency to produce eumelanosomes while those of the surrounding paler skin produce pheomelanosomes more characteristic of Caucasoids.

## *3.2. AIM:*

The present study is aimed at establishing whether ultrastructural defects within ty.pos melanocytes are discernable. A comparison of ultrastructural features of the skin and hairbulbs of ty.pos. OCA (with and without ephelides), rufous OCA and normally pigmented Negroids is presented.

## *3.3. MATERIALS AND METHODS:*

### *3.3.1. SOURCE OF HAIR BULBS AND SKIN BIOPSIES:*

Small pieces of skin were cut from the same skin biopsies, received from ty.pos. albino and normal black individuals, that were used in the melanocyte quantitation experiments described in chapter two (region of the inner upper arm-unexposed skin).

In addition to these, rufous albino skin and hairbulb biopsies were also obtained from volunteers who are part of the on-going research programme on OCA at the S.A.I.M.R. and the Department of Human Genetics, University of the Witwatersrand.

### *3.3.2. PROCESSING OF TISSUES:*

All the tissues were processed in exactly the same way. After the biopsies had been taken , they were washed in Phosphate Buffered Saline (pH 7.6). The biopsies were then primarily fixed in Modified Karnovsky's [2.5% glutaraldehyde and 2% paraformaldehyde in a 0.1M phosphate buffer at a pH of 7.4, (Karnovsky, 1965)] for 3 hours at $4^0$C. After primary fixation, the biopsies were washed in buffer before being immersed in secondary fixative (1% Osmium tetroxide in PBS, for 1 hour at $4^0$C), dehydrated in an ascending alcohol series, and embedded in Spurr's resin (Spurr, 1969) which was left to polymerise for 18 hours at $60^0$C. (Details of this procedure is outlined Appendix 3).

### *3.3.3. SECTIONING AND STAINING OF TISSUE:*

The tissue blocks were trimmed using a LKB Utratome III microtome (Reichert & Jung). 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. These sections were viewed with a Nikon light microscope to ensure that the blocks were embedded correctly and that a favourable angle had been achieved. Using a fresh glass knife, silver to

gold sections (70 - 90 nm) were cut and ribbons of sections were picked up onto copper grids. The sections were then stained with 8% saturated uranyl acetate and Reynolds lead citrate for a period of 10 minutes each (Reynolds, 1963). The sections were then dried on filter paper and viewed on a Hitachi H-600 transmission electron microscope at an accelerating voltage of 50 kV.

### *3.3.4. METHODOLOGICAL PROBLEMS:*

At this stage it should be noted that various problems were encountered during the preparation of skin samples, all of which contributed to the poor quality of some of the micrographs. The main problem experienced was the (understandable) lack of willingness of patients to donate skin biopsies.** Secondly, the biopsies were often taken on field-trips in rural areas and were processed for EM a few days later. This often introduced swelling or shrinkage of tissues which were stored in PBS. Another factor was also the extreme difficulty experienced with producing ultrathin sections of human skin. The skin thickness sometimes resulted in poor resin penetration. A few micrographs presented in this chapter have score marks but were included because of the important features visible and because it was so difficult to find melanocytes in the small pieces of skin used for EM. A "textbook" micrograph of skin is included (Fig 3.2) which can be used as a comparison for the micrographs presented in this chapter.

** Only a few of the rufous hairbulbs that were used in this study were collected on field-trips. All the other tissues were fixed immediately after excision.


FIG. 3.2.: Epidermis of normal Negroid showing a pendulous melanocyte (M) extending its dendritic arms between keratinocytes (K). Note the amount of shrinkage of epidermal cells and formation of cytoplasmic bridges between cells. Adapted from Szabo et al.(1972).

## *3.4. RESULTS:*

### *3.4.1. SKIN OF NORMAL NEGROIDS:*

#### *3.4.1.1. The Epidermis:*

The skin of normal Negroids was viewed and the following features were noted. The keratinocytes had large, oval nuclei which filled most of the cell WITH a very much reduced cytoplasm (Fig.3.3.). At low magnifications (2000x to 5000x) it was apparent that the epidermis of these individuals contained many fully melanised stage IV eumelanosomes (Fig.3.3.A). These eumelanosomes occupied a typical perinuclear position in the keratinocytes and were also mostly concentrated on the apical side of these cells (Fig. 3.3.B.). It is also evident that the melanosome density is greatest in the basal keratinocytes and becomes progressively sparser in the more superficial layers. Also, when comparing the skin in Fig.3.3.a. to that in Fig.3.3.B., it was clear that there were more melanosomes in the epidermis seen in Fig.3.3.A. From clinical records of these individuals, it was found that the individual whose skin is seen in Fig.3.3.A. was visibly more darker than the individual in Fig.3.3.B.

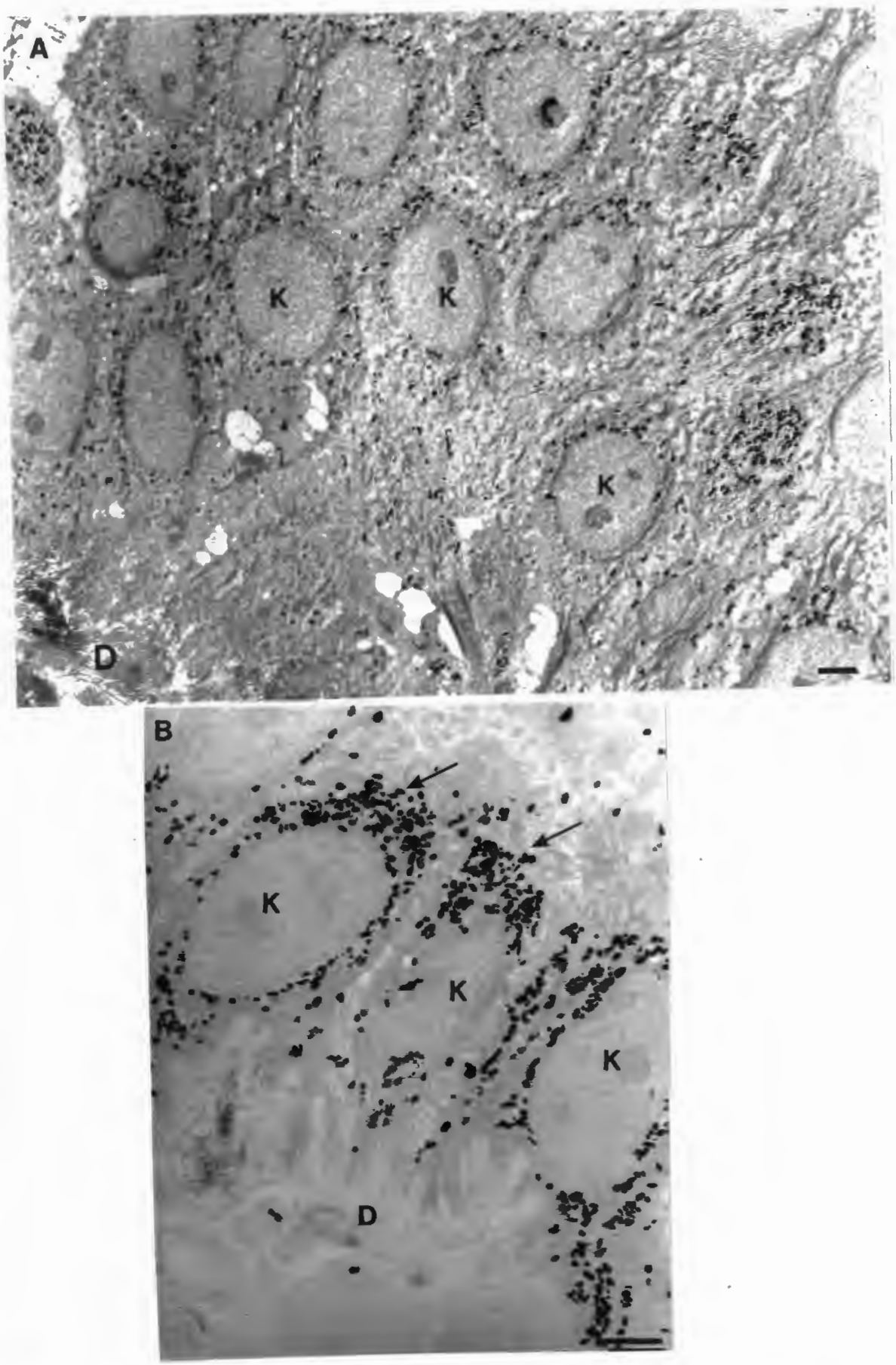


FIG. 3.3: Low power view of epidermis of normal Negroids. Note the abundant presence of stage IV melanosomes in the keratinocytes (K). The melanosomes occupy a typical perinuclear position in the keratinocyte cytoplasm with highest density on the apical side. (b.-arrows). D=dermis Bar=1um

*3.4.1.2. The Melanocytes:*

Initially, it was very difficult to identify melanocytes in skin sections even at very low magnification. This was largely due to the fact that melanocytes make up a very small percentage of the total cell population in the epidermis. The small biopsies used for EM often contained no melanocytes and numerous blocks had to be viewed before any melanocytes were seen. Also, light microscopic observations (chapter 2), revealed that melanocytes occurred as two or three cells in close proximity and for long lengths of the adjacent basement membrane no melanocytes would be seen. This too, could possibly have contributed to the difficulty in locating melanocytes. Interestingly, when melanocytes were located, these were frequently found to be situated more in the superficial dermis than in between the basal keratinocytes. These pendulous melanocytes extend their dendritic arms into the keratinocytes (Fig. 3.4.). (see section 3.3.4. of "Materials and Methods" for comments).

The melanocyte cell bodies were larger than the keratinocytes, and their nuclei were also distinctly different. The melanocyte nuclei were indented while the those of keratinocytes were oval. The melanocyte cytoplasm contained predominantly fully melanised stage IV

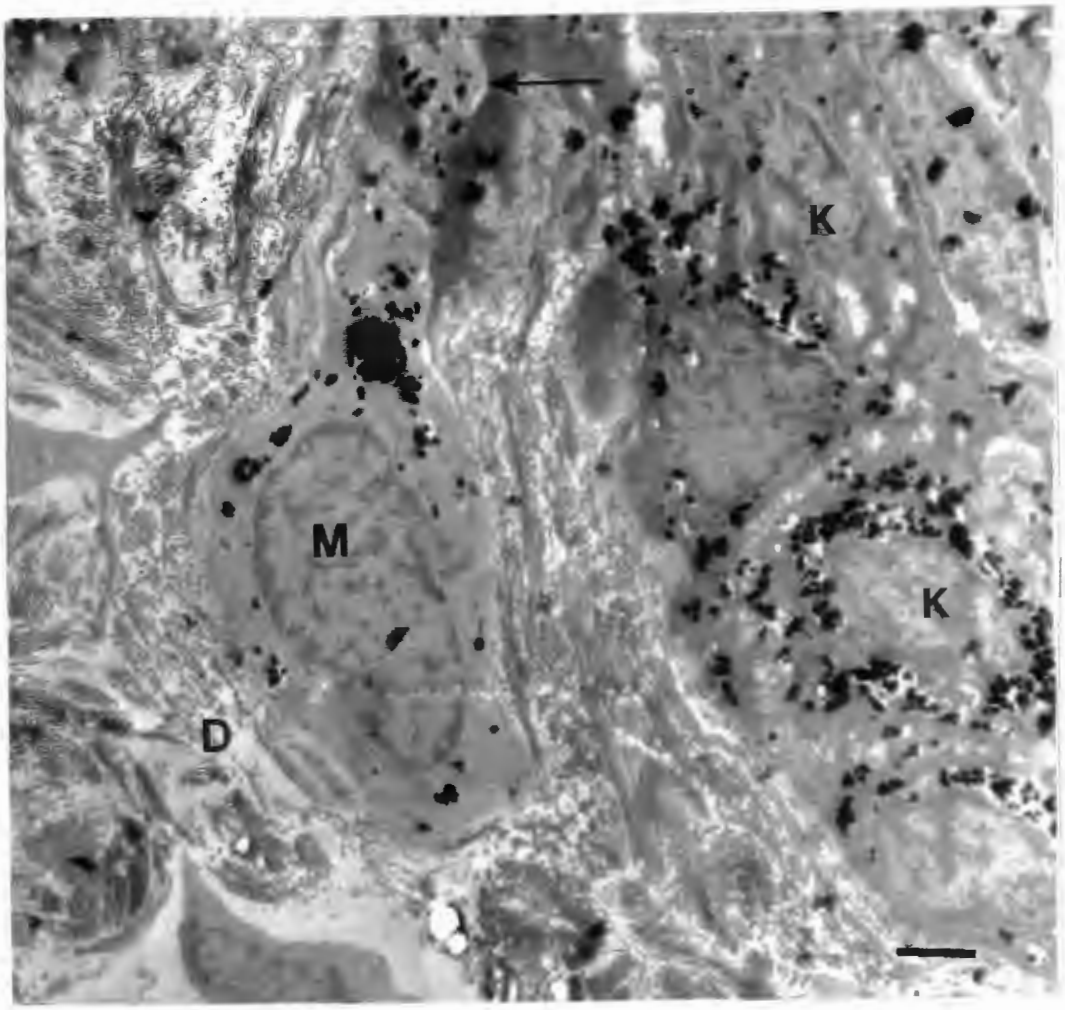


FIG. 3.4.: Pendulous melanocyte (M) of normal Negroid skin extending dendrite in between keratinocytes (K) The melanosome cytoplasm and keratinocytes contain only stage IV melanosomes. Note the typically indented nucleus of the melanocyte. D=dermis. Bar=1um

eumelanosomes, and very rarely, a few intermediate stages were seen. In the dendrites of the melanocytes, only stage IV melanosomes were seen (Fig. 3.5.). On closer observation of the melanocyte cytoplasm, newly formed premelanosomes that appear to have just blebbed off the smooth ER were observed (Fig. 3.5).

All the intermediate stage melanosomes lacked the visible filamentous internal matrix characteristic of eumelanosomes. No pheomelanosome-like structures were observed in these melanocytes of Negroid individuals. Electron lucent spherical bodies were seen on the periphery of most of the mature melanosomes. These bodies most probably represent the putative tyrosinase carrying vesiculo-globular bodies (VGB's).

*3.4.1.3. Melanosomes within the Keratinocytes:*

Keratinocyte cytoplasm was identified by the abundance of tonofilaments ( Fig.3.6.) which are not present in the melanocyte cytoplasm. Melanosomes were singly dispersed in these keratinocytes. These were all eumelanosomes that had either been sectioned transversely and thus had the characteristic ellipsoidal shape of eumelanosomes, or were cross-sectioned and, therefore, appeared more round. No pheomelanosomes were seen. It was noted that the electron lucent VGB's were no longer discernible on the periphery of


FIG. 3.5.: Melanocyte (M) of normal Negroid with dendrite (*) extending into epidermis. In this melanocyte, newly blebbed premelanosomes can be seen (arrows) in addition to stage IV melanosomes. No intermediate stage melanosomes are seen. Nearby, a melanocyte dendrite (d) can be seen in cross-section and contains only stage IV melanosomes.D=dermis, Bar=1um

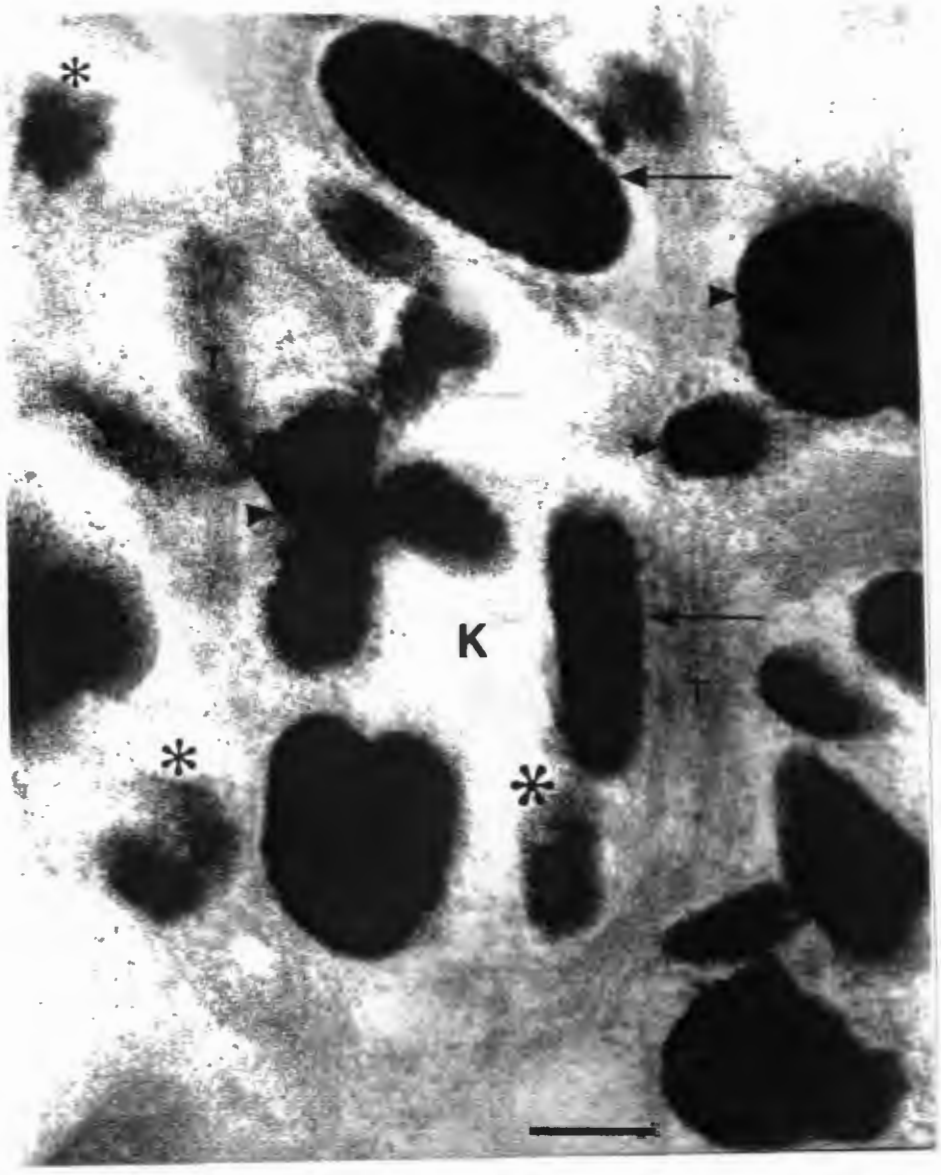


FIG. 3.6.: Keratinocyte cytoplasm (K) of normal Negroid as identified by presence of tonofilaments (T). Numerous ellipsoidal (arrows) and cross-sectioned (arrowheads) eumelanosomes can be seen. No pre- or intermediate stages are present. A few degrading melanosomes (arrowheads) can be seen. Note that the melanosomes occur singly dispersed. Bar=0.5um

the melanosomes. In other words, VGB's were only seen on the periphery of melanosomes in melanocytes and not in those transferred to keratinocytes. It was also noted that some of these melanosomes were diffusely pigmented, evidence that they were subjected to lysosomal degradation in the keratinocytes (degradation of melanosomes in keratinocytes is a normal phenomenon - see Chapter 1 part 2).

### *3.4.2. SKIN OF TY.POS INDIVIDUALS IN REGIONS OF EPHELIDES:*

#### *3.4.2.1. The Epidermis:*

The melanosomal density in the basal keratinocyte of ty.pos individuals with ephelides was similar to that seen in normal black skin (compare Fig. 3.2. & Fig. 3.3. with Fig. 3.7.) The melanosomes typically occupy a peri-nuclear position with highest concentration being in the apical side of the cell (Fig. 3.7.). In the epidermis seen in Fig. 3.7., it appears as if the melanosomal density is much greater in the keratinocytes on the right than those on the left of this micrograph. It is probable that the cells on the left are bordering regions of non-ephelis albino skin
and thus contain fewer melanosomes. Numerous cross-sections of melanocyte arms were seen between keratinocytes, (arrows-Fig.3.7) and these arms mostly contained fully melanised stage IV melanosomes.

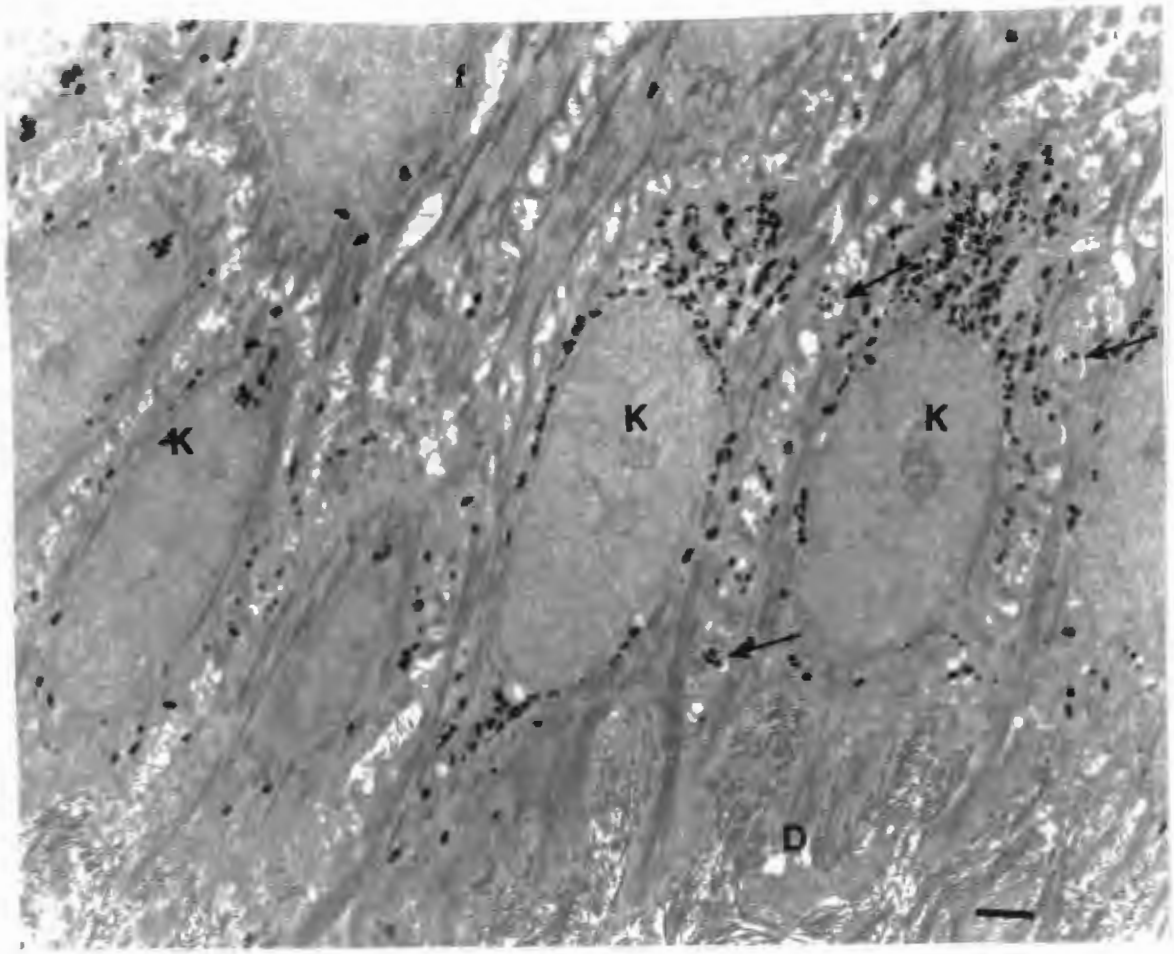


FIG. 3.7.: Epidermis of Ty.Pos albino with ephelides. Stage IV melanosomes occupy a typical perinuclear position in the keratinocytes (K). Numerous melanocyte arms, containing pigmented melanosomes, can be seen (arrows). No intermediate stage melanosomes are seen. Notice that the melanosomal density is greatest on the right and it is possible that the region of the left is bordering onto non-ephelis regions, therefore there are markedly fewer melanosomes per keratinocyte. Bar=1um

*3.4.2.2. The Melanocytes:*

The melanocytes in ty.pos OCA skin were difficult to find for the same reasons as were those of normal Negroid individuals. These melanocytes were mostly situated in the superficial dermis and had dendrites extending into the epidermis (Fig. 3.8.). Their nuclei were large and indented. The cytoplasms were also large and contained numerous melanosomes, most of which were not fully melanised, lacked a clear melanosomal membrane, and showed no evidence of an internal melanosomal matrix. These melanosomes were clearly abnormally developed and was not seen in the melanocytes from skin of normal Negroid individuals. Some fully melanised stage IV melanosomes were also present and these were typically eumelanosomal in shape. On the periphery of a few of the stage IV melanosomes, electron lucent spherical VGB's were seen (Fig.3.8).

In a cross section of a melanocyte dendrite, a few stage IV melanosomes were seen in addition to a premelanosome (Fig. 3.9.). The premelanosome had an amorphous internal structure, suggesting that it was a stage I melanosome (according to classification of Toda et al., 1972). The presence of this stage I melanosome in the melanocyte dendrite would suggest that this stage I melanosome is about to be transferred to a keratinocyte in its unmelanised state.

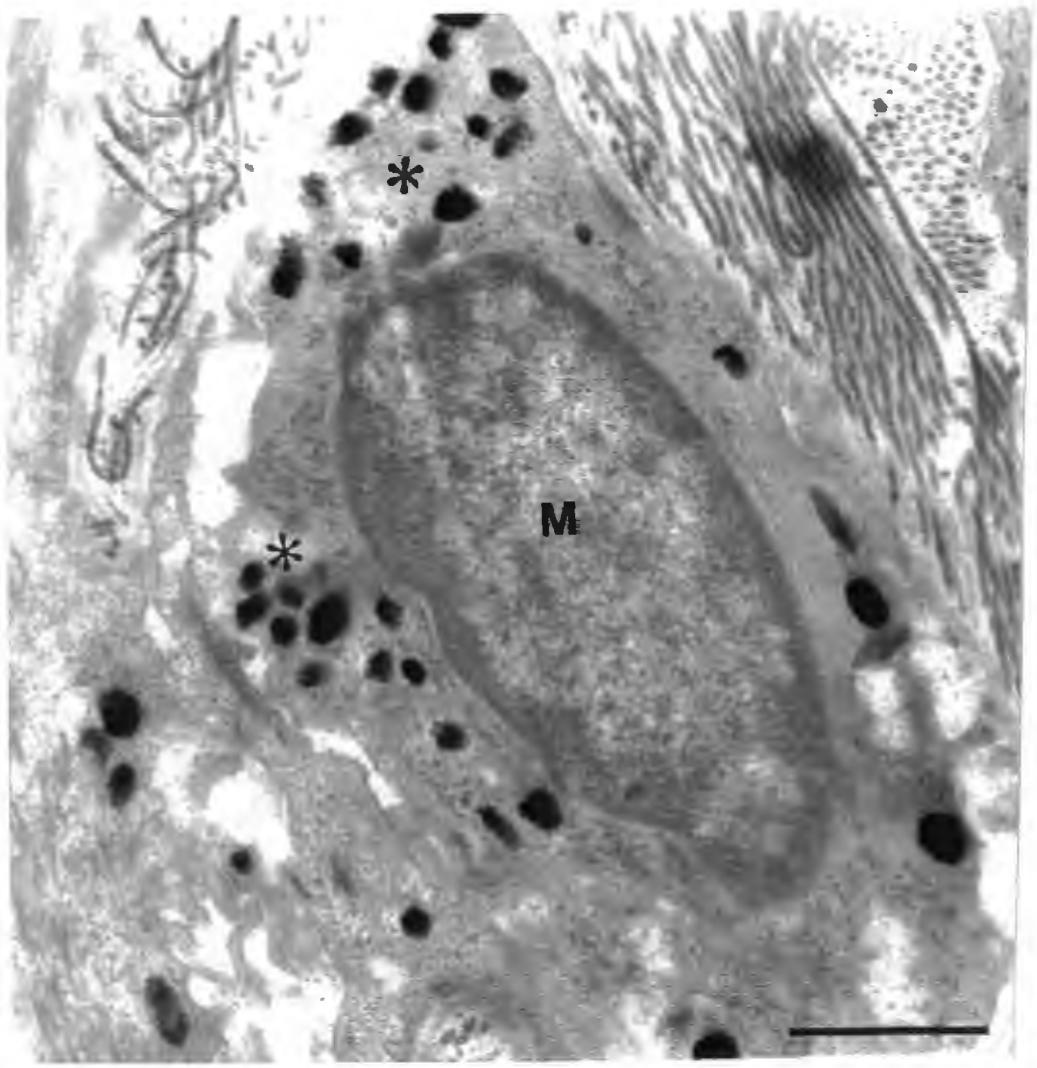


FIG. 3.8.: Melanocyte (M) of Ty.pos albino in the region of an ephelis. Note the typically indented nucleus and large tonofilament free cytoplasm. In addition to some fully melanised melanosomes many partially melanised melanosomes are also seen (*). These could not be staged since they lacked internal filamentous matrixes. Bar=1um

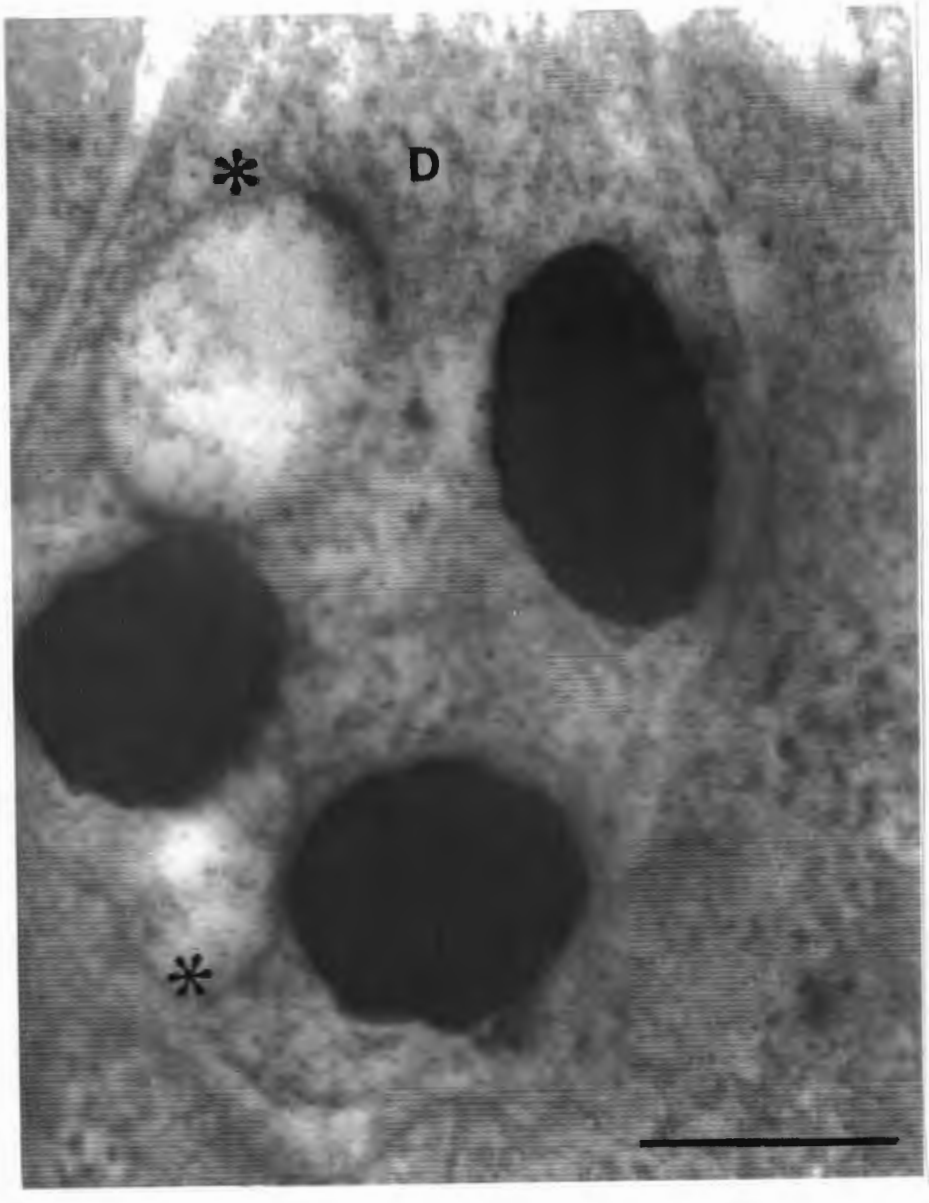


FIG. 3.9.: Cross section of a melanocyte dendrite (D) of a Ty.Pos albino in the region of an ephelis. In addition to three stage IV melanosomes, two empty melanosomes (*) with amorphous internal structures (stage I) can also be seen. Bar=0.1um

*3.4.2.3. The melanosomes within the keratinocytes:*

The keratinocytes of these ty.pos individuals were also recognised by the presence of numerous tonofilaments in the cytoplasm. The melanosomes were singly dispersed in the keratinocytes. In addition to fully melanised stage IV melanosomes, numerous intermediate stage melanosomes were also transferred to the keratinocytes and were dispersed singly amongst fully melanised melanosomes (Fig. 3.10). These premelanosomes contained some amorphous material, suggesting that they were stage I melanosome. Some evidence of melanosomal degradation within the keratinocyte cytoplasm was also visible.

***3.4.3. SKIN OF TY.POS. INDIVIDUALS WITHOUT EPHELIDES:***

*3.4.3.1. The Epidermis:*

At a very low magnification, it was evident that the skin of ty.pos. individuals without ephelides was vastly different from that of normal Negroid skin and ty.pos skin in the regions of ephelides. At low magnification (5000x), there was no evidence of any melanosomes in the keratinocytes (Fig. 3.11. & 3.12). Apart from the lack of melanosomes, the keratinocytes had normal morphology, the nucleus being typically round and occupying most of the cell volume.

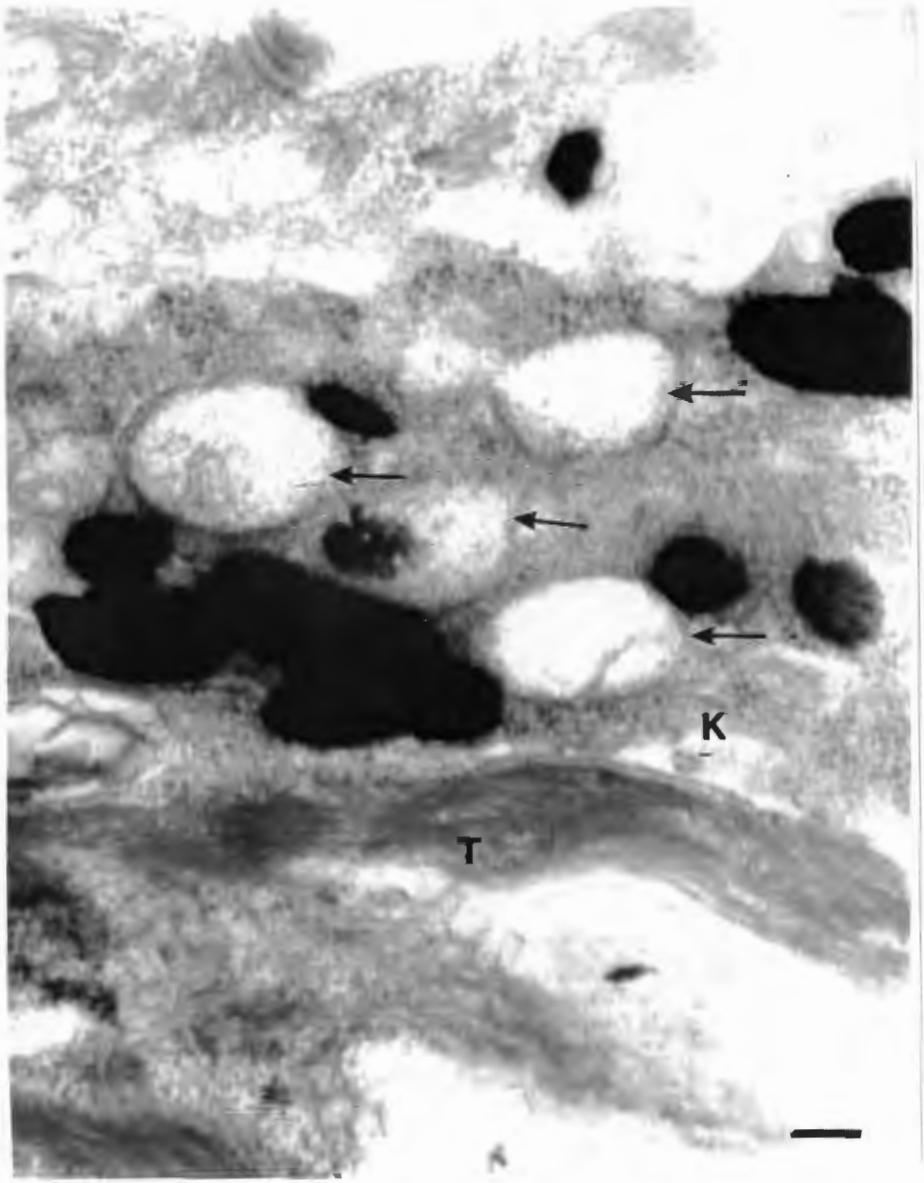


FIG. 3.10.: Keratinocyte cytoplasm (K) as indicated by presence of tonofilaments (T) of Ty.Pos albino in the region of an ephelis. In addition to many stage IV melanosomes, some stage I melanosomes can also be seen (arrows), suggesting that these get transferred from melanocytes in their unmelanised state. Bar=0.1um

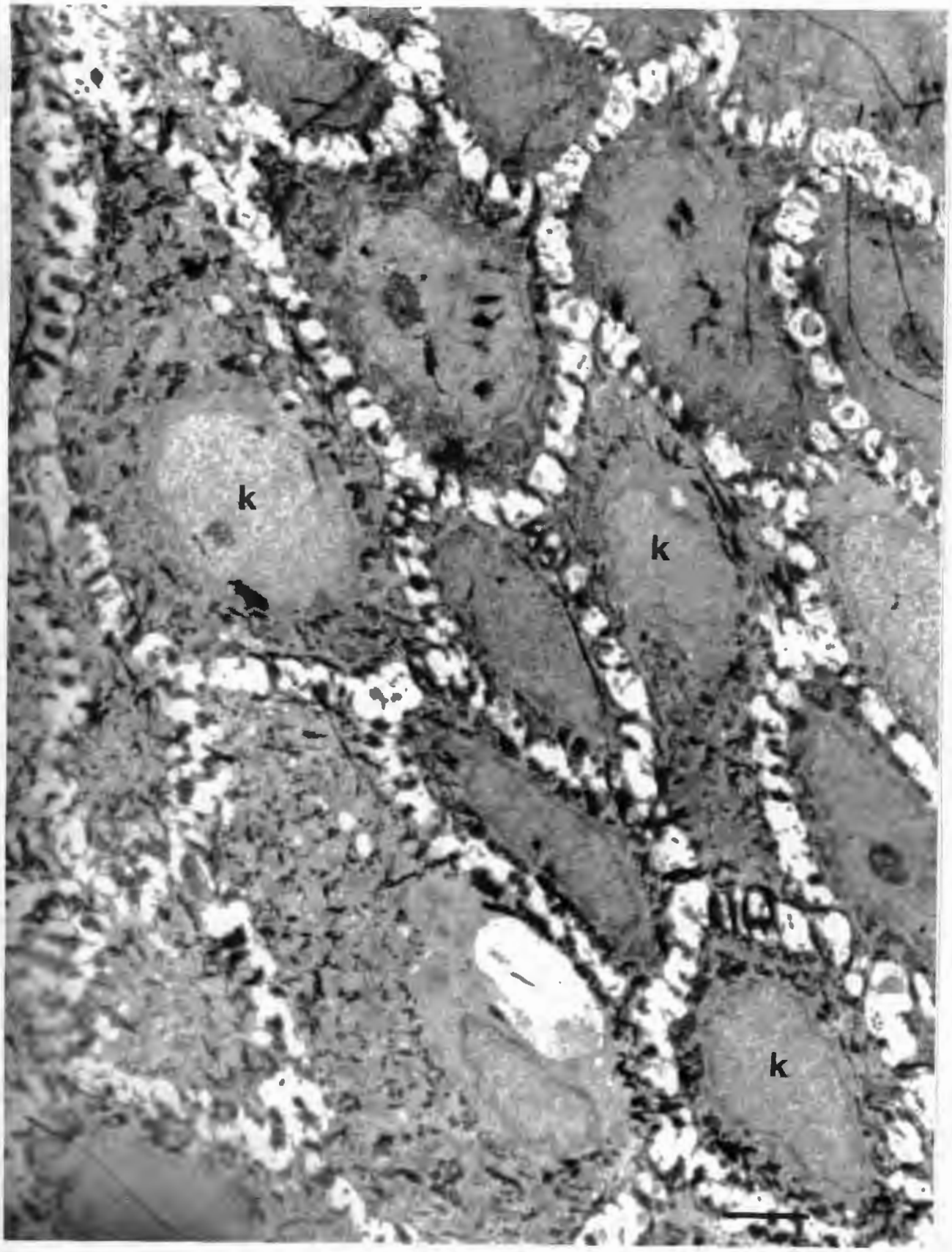


FIG. 3.11.: Epidermis of Ty.Pos albino with no ephelides. Note the absence of pigmented melanosomes in the keratinocytes (K). Also, no intermediate stage melanosomes are seen. Bar=1um

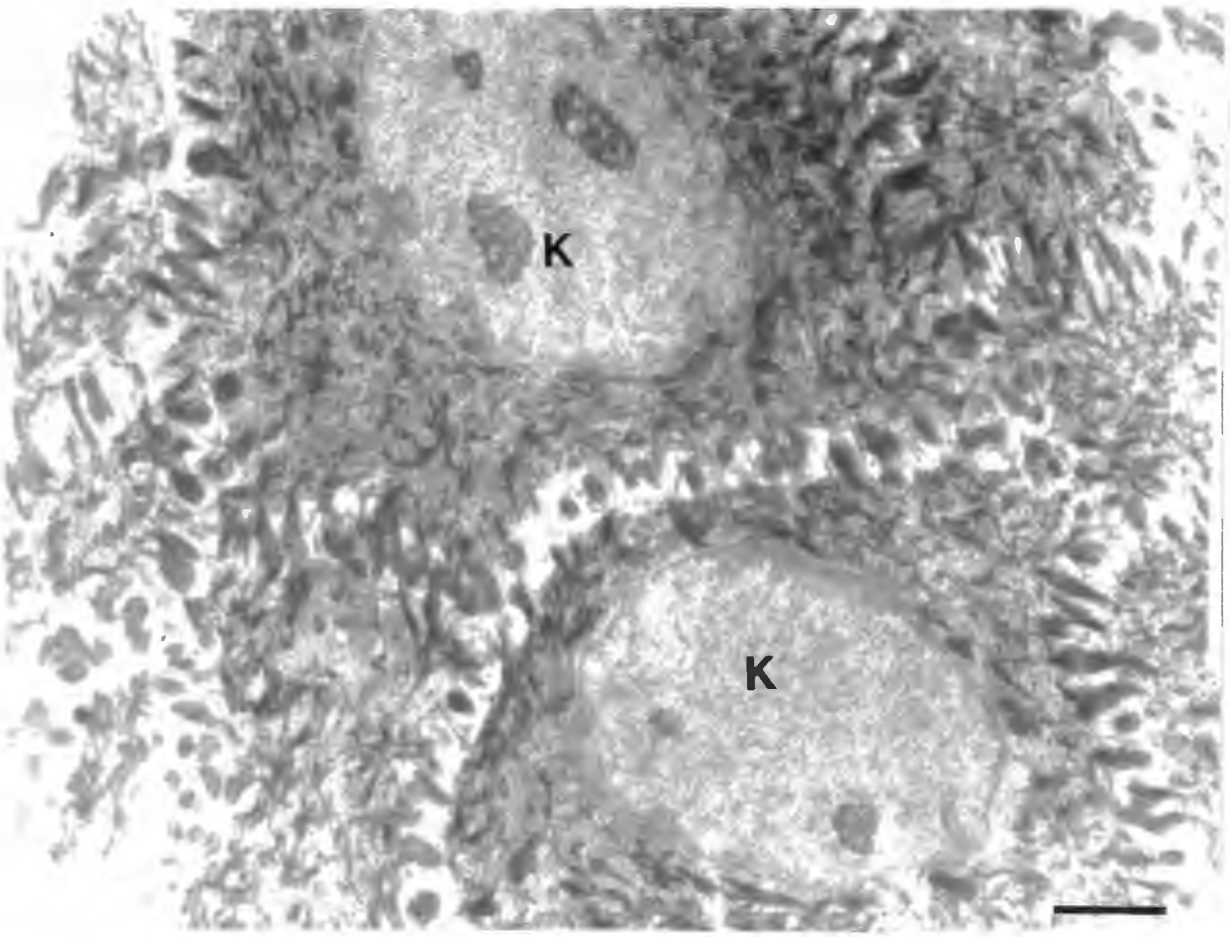


FIG. 3.12.: Two keratinocytes (K) in the epidermis of a Ty.Pos albino illustrating the complete absence of pigmented and intermediate stage melanosomes in these cells. Bar=1um

*3.4.3.2. The Melanocytes:*

It was even more difficult to identify melanocytes in these individuals than in the other skin types studied. This was so because of the lack of melanosomes. From melanocyte locations in the other skin types, the region of the superficial dermis was carefully scanned and , with practice, numerous melanocytes were located in this region. These melanocytes had typically indented nuclei but instead of having melanosomes in the cytoplasm, the cytoplasm contained numerous "empty" vesicles (Fig.3.13.). The exact nature of these empty vesicles are not known although they are most likely to be premelanosomal structures that are not being melanised.

Despite the initial impression that there were no melanosomes in the epidermis, a few electron dense structures, resembling pigmented melanosomes, were seen in the area around the melanocytes (as seen in Fig.3.13). Careful viewing of the epidermis led to the discovery that a few diffusely pigmented melanosomes and some premelanosomes were present in melanocyte arms (Fig.3.13; 3.14; 3.15 & 3.16). These melanosomes had the classic ellipsoidal shape of eumelanosomes but were partially pigmented and clearly aberrant. Most of these melanosomes lacked a clear melanosomal membrane. Some melanosomes were spottily pigmented around the periphery with no pigment in the centre; other melanosomes were pigmented uniformly but seemed to have an unpigmented region around the outside; while in a few melanosomes, pigment deposition was uniformly spotty. In a few instances, the eumelanosomal banding pattern was clearly

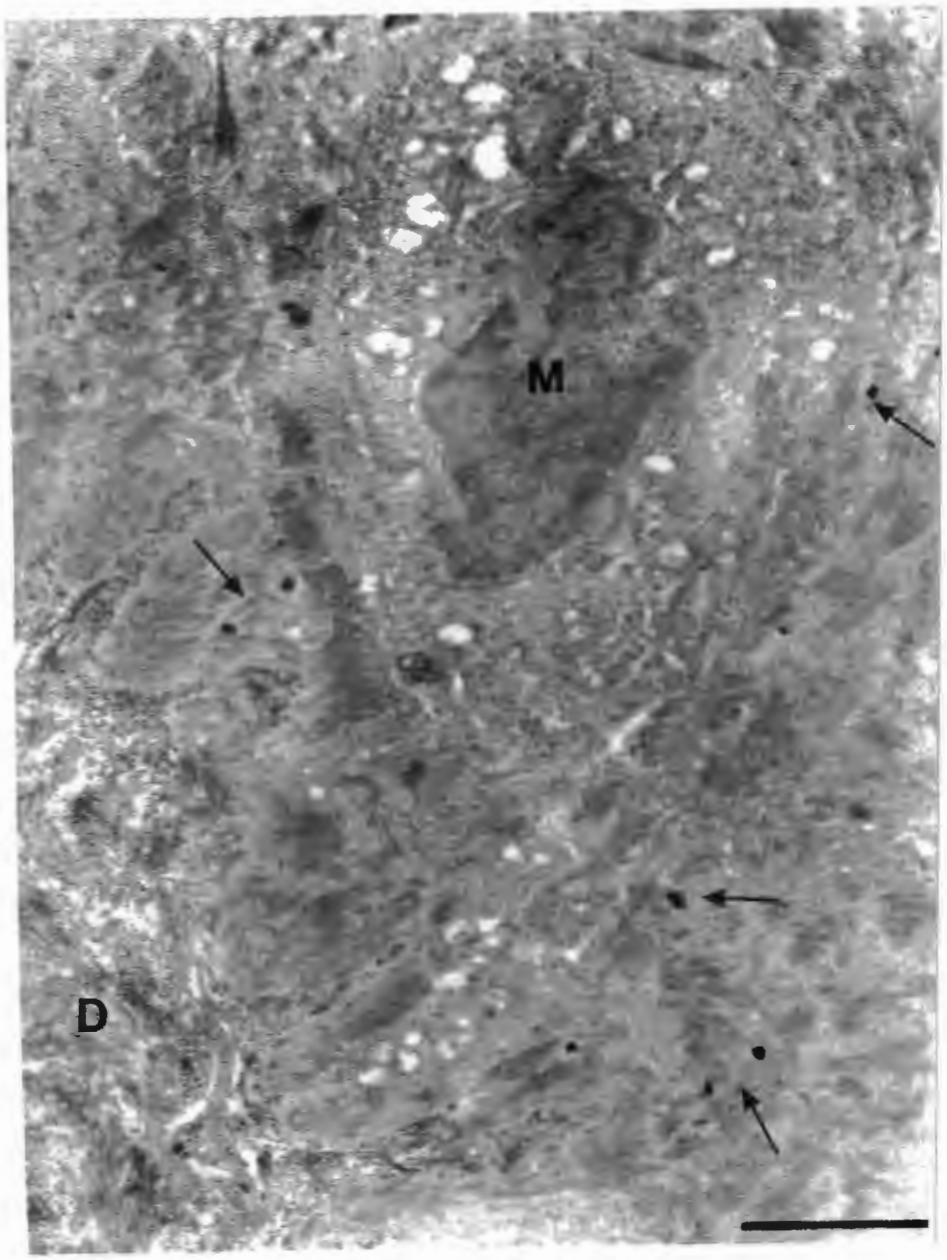


FIG. 3.13.: Melanocyte (M) of Ty.Pos albino with no ephelides. Note the "empty-vesicular" appearance of the melanocyte cytoplasm. Also, a few pigmented melanosomes (arrows) can be seen on the periphery of the melanocyte. Bar=1um

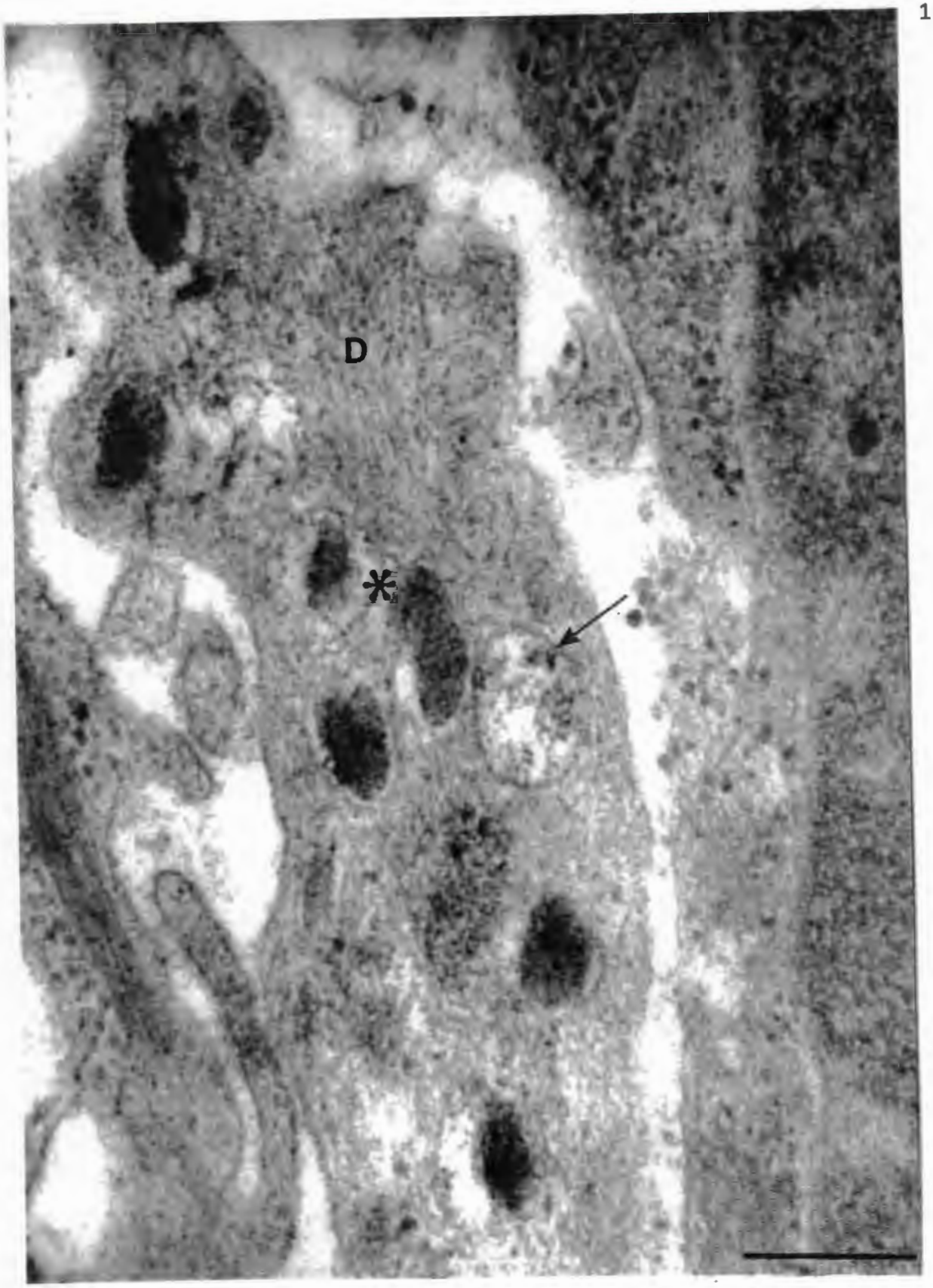


FIG. 3.14.: Melanocyte dendrite (D) of Ty.pos albino with no ephelides containing numerous partially melanised melanosomes (*) and a stage I melanosome (arrow). No internal filamentous structure is seen in these melanosomes. Bar=0.1um

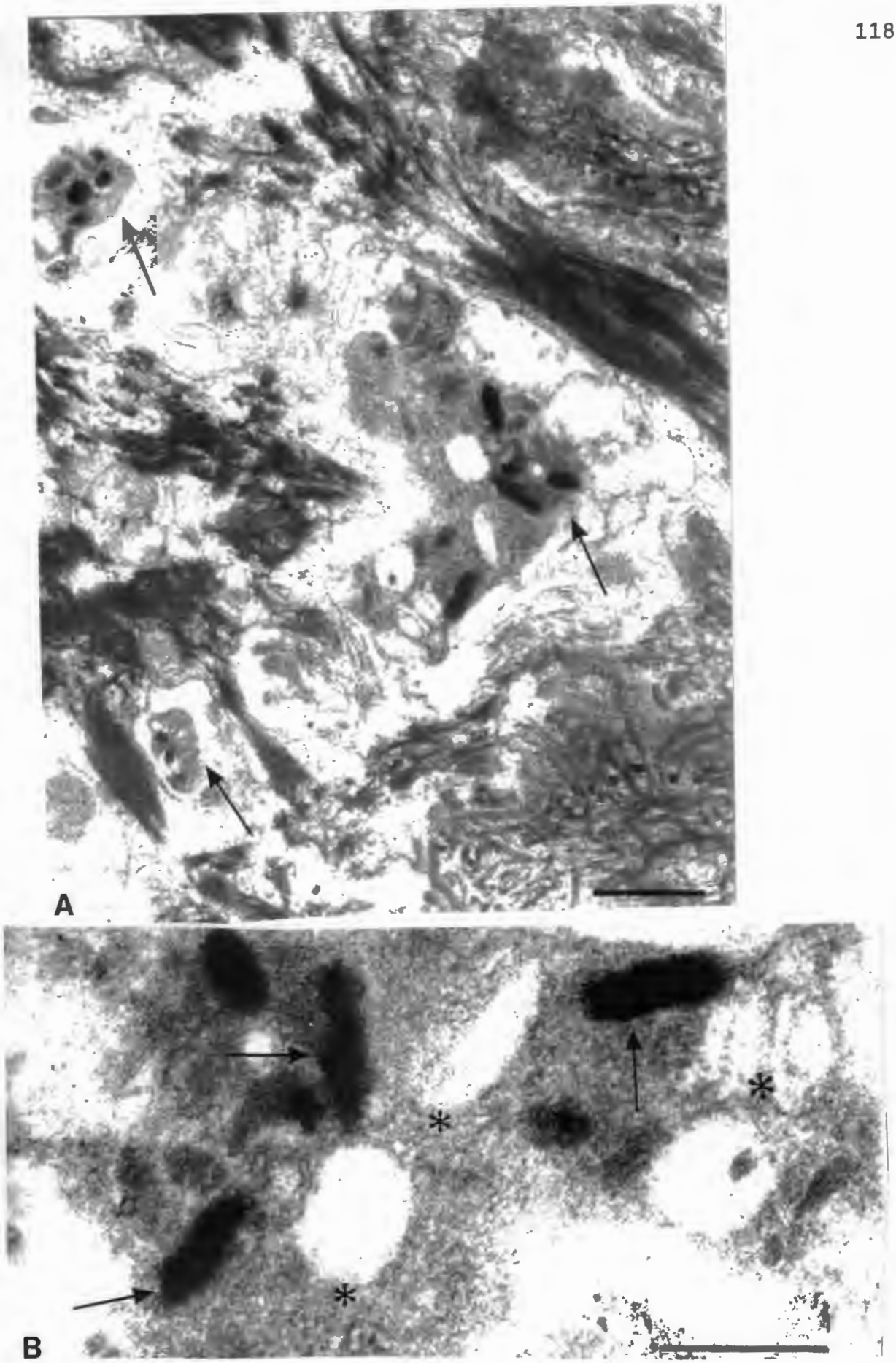


FIG. 3.15.: Cross-sections of melanocyte arms (arrows) of Ty.Pos albino with no ephelides. (B.) shows a high power view of one of these arms which contains both empty melanosomes (*) and pigmented melanosomes (arrows). Bar=0.5um

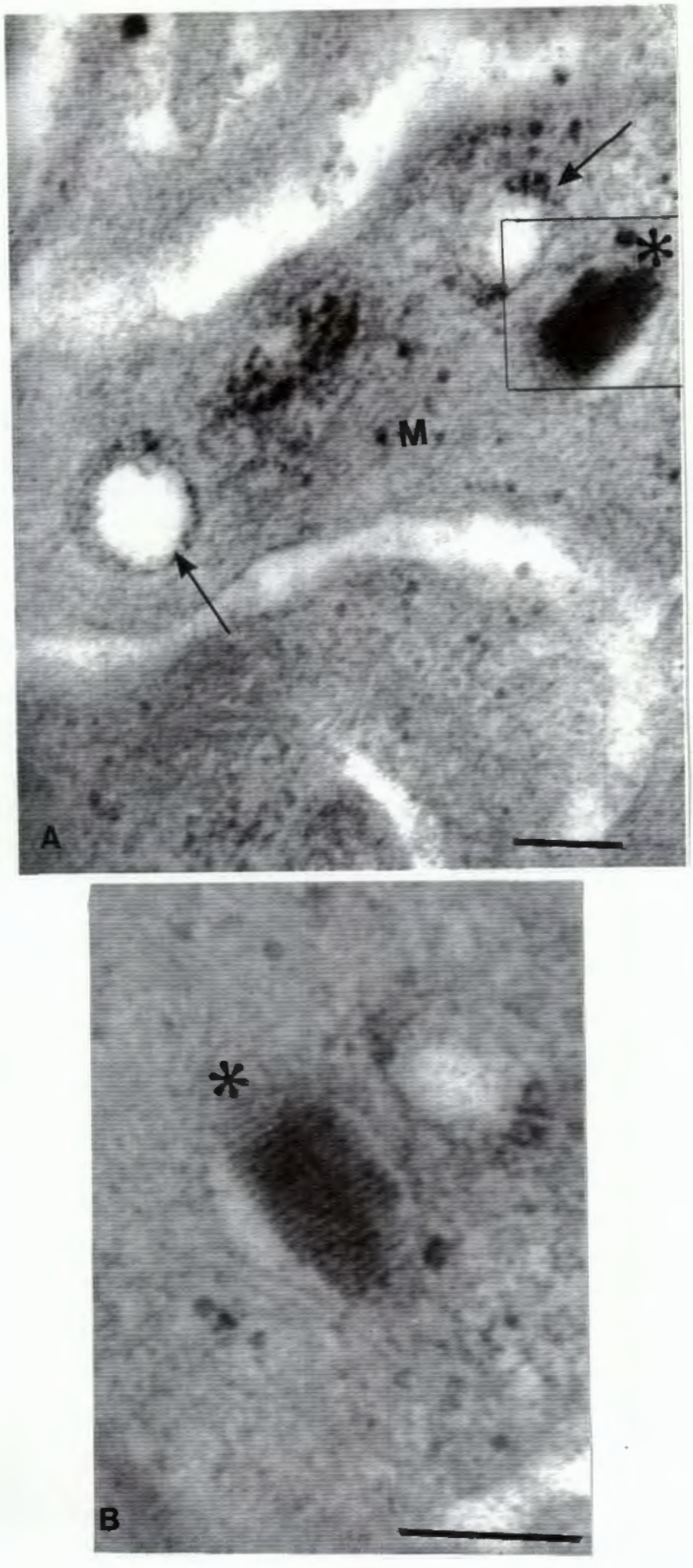


FIG. 3.16.: Melanocyte arm (M) containing a partially pigmented melanosome with clear cross banding internal structure (*) and some melanosomes where spotty melanin deposition has occurred on the periphery (arrows). Bar=0.1um

seen. This indicates that the melanosomal protein assembly and internal matrix structure is probably normal. It must be stressed that the number of melanosomes observed in these ty.pos individuals was very few in comparison to the other skin types studied (approximately 10-15 melanosomes per section compared to an average of about 200 melanosomes per basal keratinocyte in normal black skin and regions of ephelides). Furthermore, these melanosomes were not observed in all the sections viewed, indicating that only certain regions of the epidermis contained pigmented melanosomes.

*3.4.3.3. Aberrant Features observed within these Melanocytes:*

Melanocytes of these ty.pos individuals contained abnormal cellular organelles. Structures recognised as being mitochondria because of their double limiting membrane and internal cristae, seemed to have a decreased or distorted internal membranous system (Fig. 3.17.). When comparing these structures to normal mitochondria (inset - Fig.3.17), it was clear that these mitochondria were vastly abnormal. These abnormal mitochondria were numerous within the melanocytes of these ty.pos individuals, and were never observed in melanocytes from normal black skin nor in melanocytes in regions of ephelides. Furthermore, the number of (abnormal) mitochondria seen in these melanocytes appeared much more than would normally be found in melanocytes. The Golgi-apparatus seen within these melanocytes was also somewhat large and bloated (Fig. 3.17).

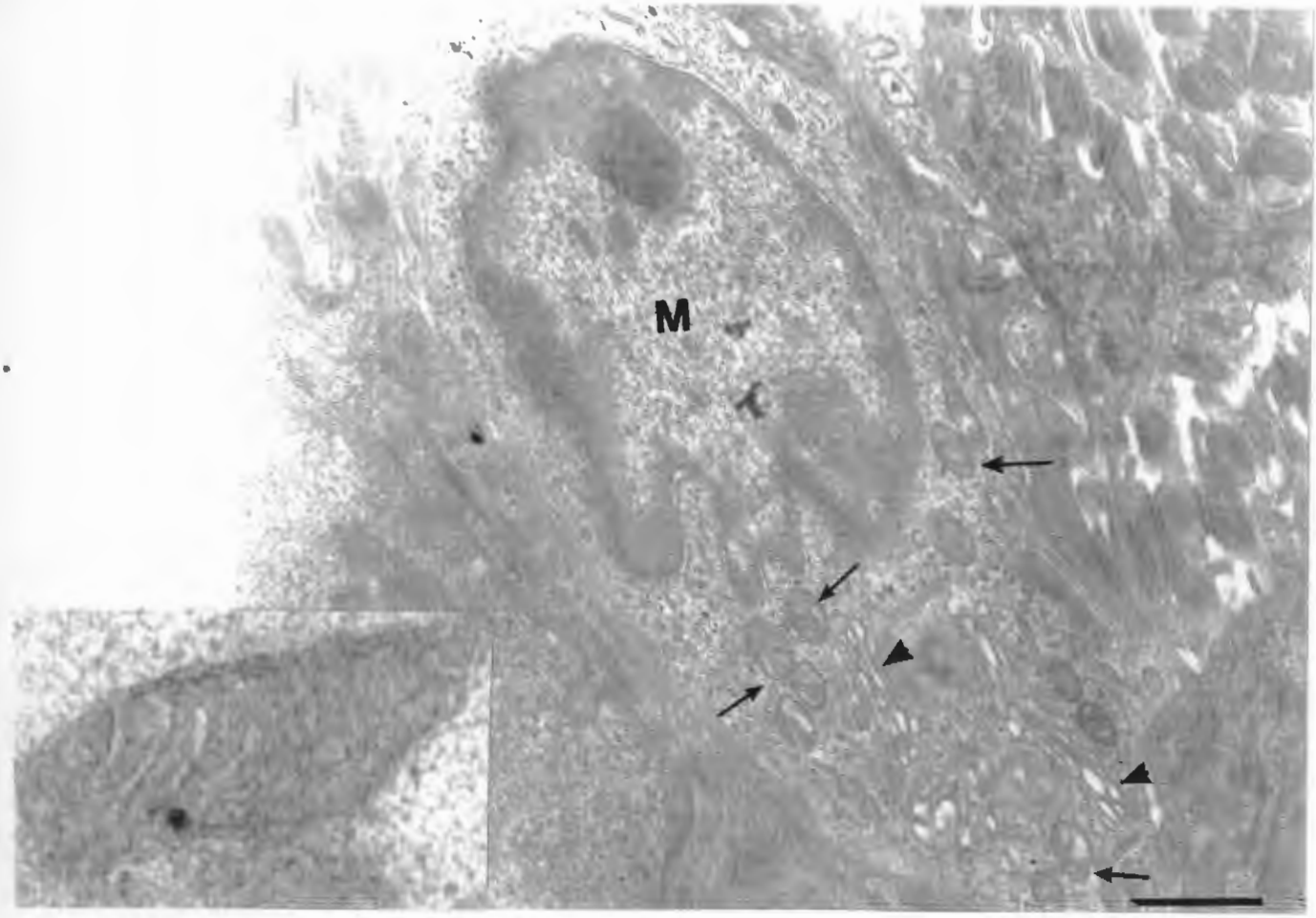


FIG. 3.17.: Melanocyte (M) of a Ty.Pos albino with no ephelides showing aberrant mitochondria (arrows) and bloated Golgi (arrowheads). Inset shows a normal mitochondrion from Negroid skin for comparison. Bar=1um

A higher power view of these distorted mitochondria is presented in figure 3.18. Here it can be clearly seen that some of these mitochondria have discontinuous outer membranes and distorted internal cristae.

In addition to these abnormal mitochondria and bloated Golgi, dilations of the RER was also observed in the melanocytes of these ty.pos. individuals (Fig. 3.19 & 3.20). These dilated RER were not observed in the melanocytes of Negroid individuals nor in melanocytes from regions of ephelides. Similar reports of dilated RER and "blown" mitochondria were made by Boissy et al. (1991) in melanocytes of vitiligo patients. A photograph from Boissy's paper is included in the discussion (Chapter 4) in order to compare these dilated RER and abnormal mitochondria to the aberrant organelles observed in this study.

Figure 3.21. illustrates the margin between a region of an ephelis and surrounding non-ephelide skin. It is obvious that in the region of the ephelis, there are active melanocytes that produce fully pigmented melanosomes as seen in the keratinocytes. In the region of non-ephelis, an amelanotic melanocyte can be seen. This melanocyte has the typical "empty-vesicular" feature of melanocytes seen in ty.pos skin with no ephelides. Also, no pigmented melanosomes can be seen in the cytoplasm of the melanocyte, nor in any of the surrounding keratinocytes.

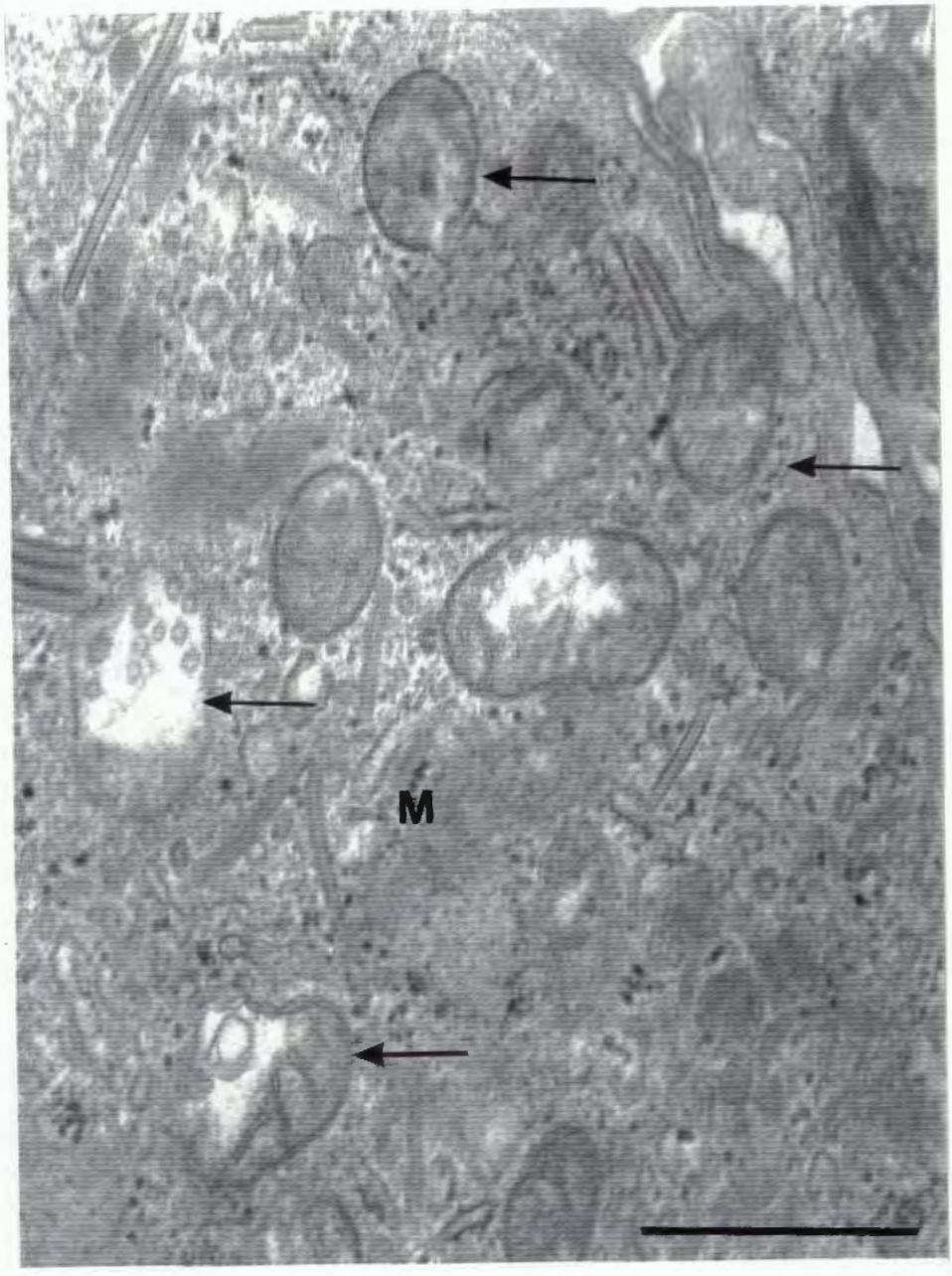


FIG. 3.18.: Higher power view of melanocyte cytoplasm of a Ty.Pos albino with no ephelides showing mitochondria with discontinuous outer membranes and distorted cristae. Bar=0.5um

M = MELANOCYTE

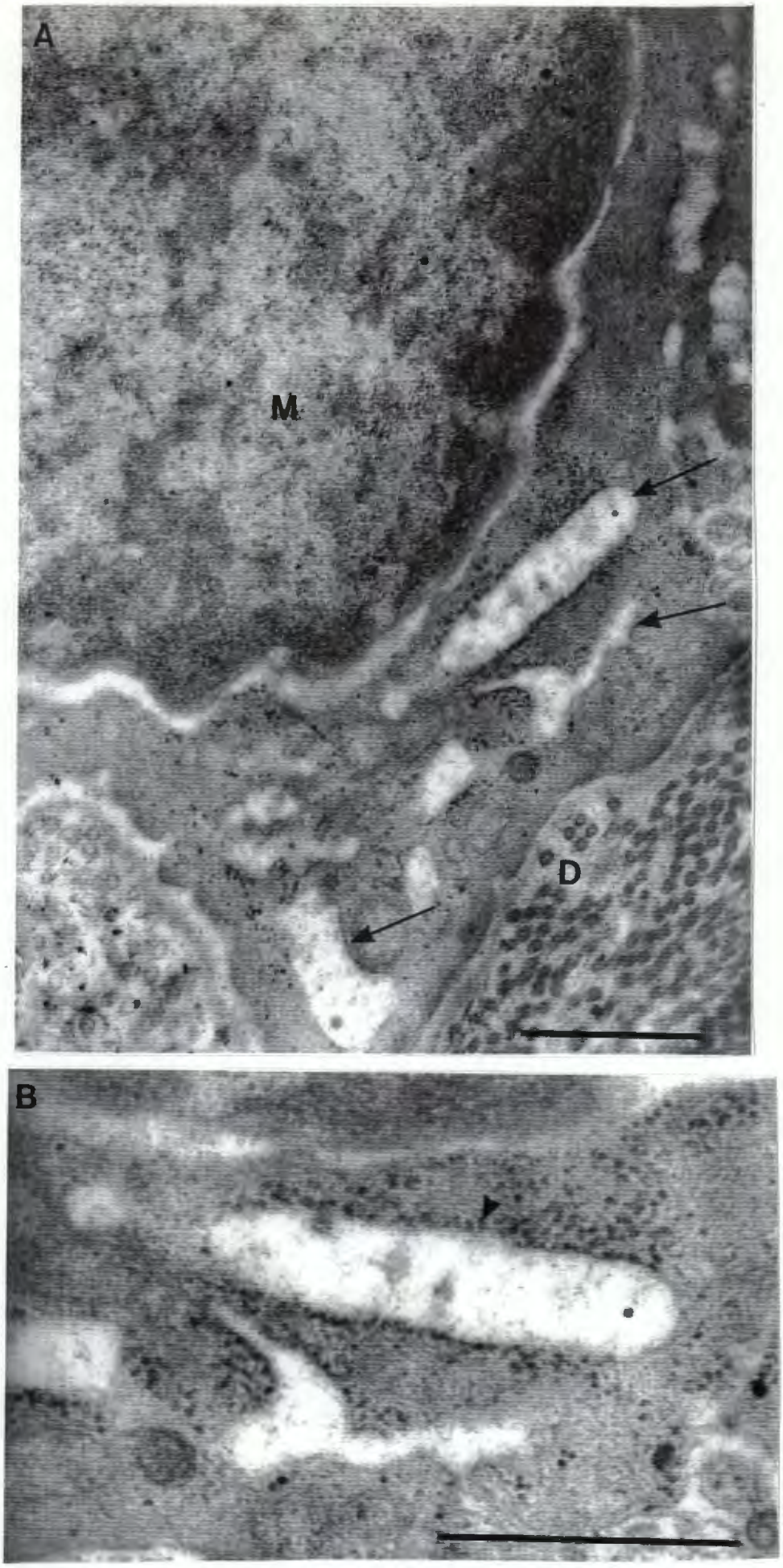


FIG. 3.19.: Melanocyte (M) of a Ty.pos albino with no ephelides showing dilated RER (arrows). (B.) ribosomes can be clearly seen (arrowheads). D=dermis Bar=0.5um

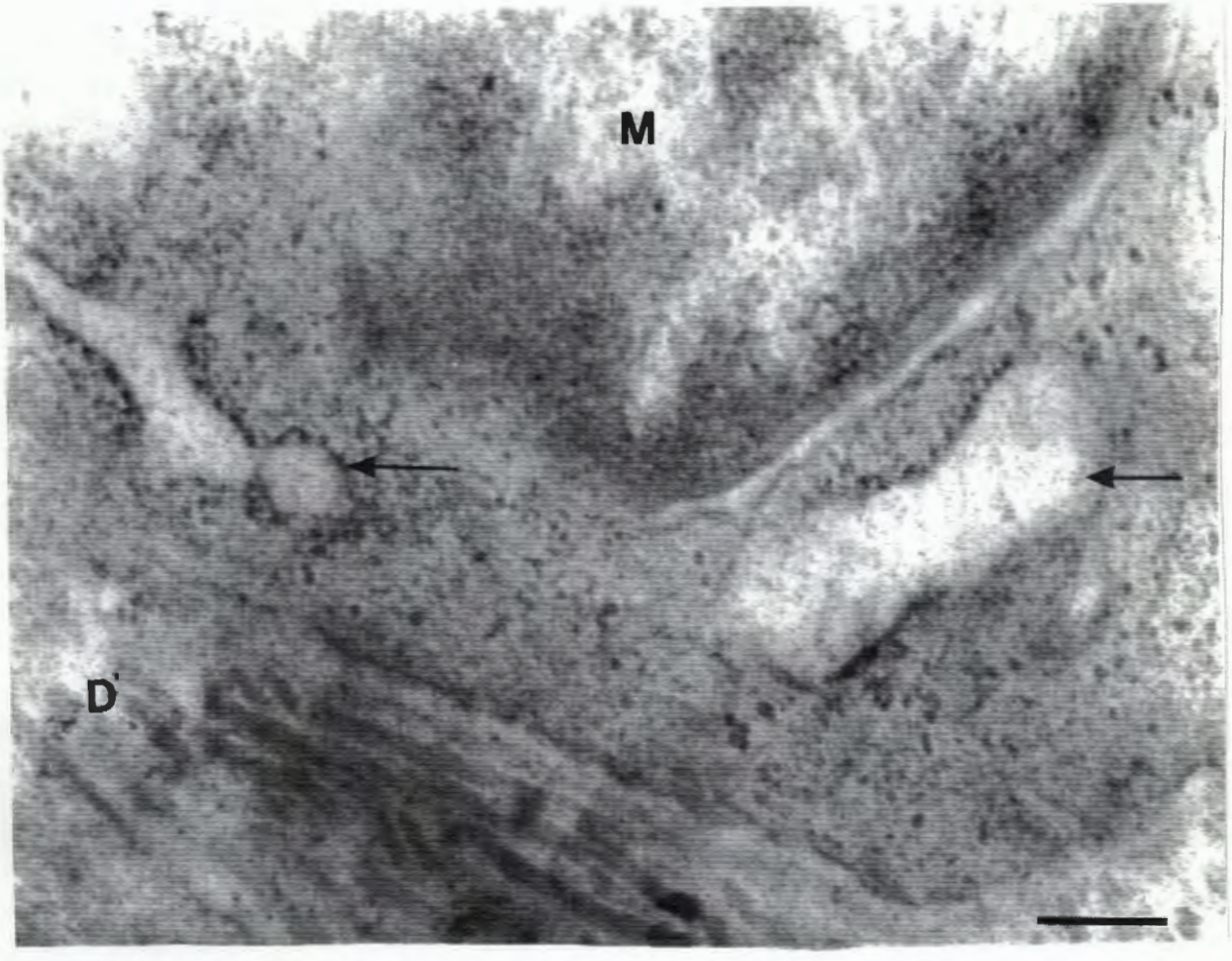


FIG. 3.20.: Melanocyte (M) of ty.pos albino with no ephelides showing another example of dilated RER (arrows). D=dermis Bar=0.1um

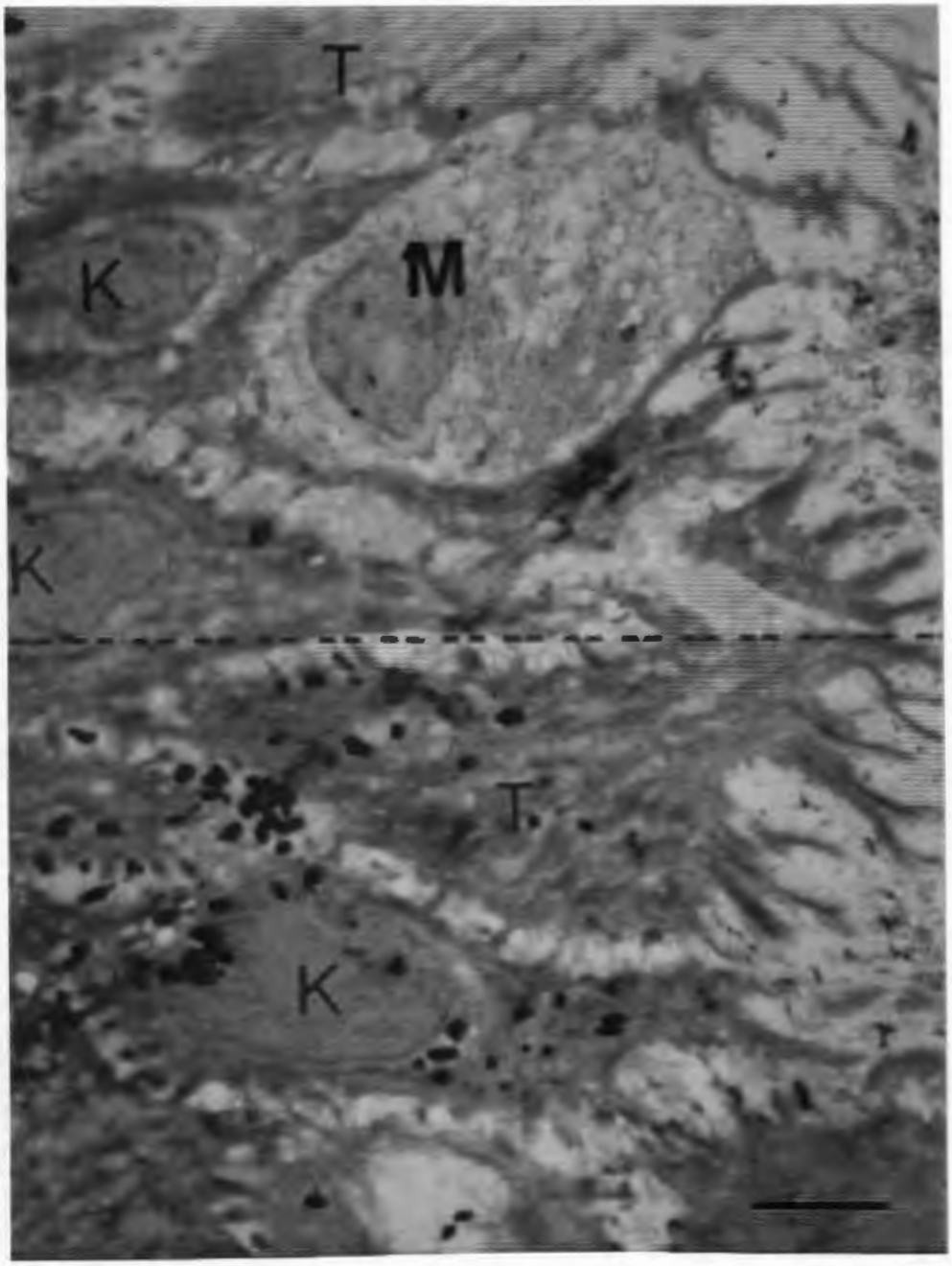


FIG. 3.21.: Margin between region of ephelis and non-ephelis (dotted line) showing an amelanotic melanocyte (M) with typical "empty vesicular" appearanceand no pigmented melanosomes in any of the keratinocytes (K) and many Stage IV melanosomes in the keratinocytes of the region of an ephelis. T=tonofilaments Bar=1um

### *3.4.4. SKIN OF RUFOUS ALBINOS:*

#### *3.4.4.1. The Epidermis:*

The epidermis of rufous individuals contained numerous fully pigmented melanosomes which were situated in a typical perinuclear position in the keratinocytes. The number of melanosomes was greatest in the basal layers and became progressively less in the more superficial layers.

#### *3.4.4.2. The Melanocytes:*

The melanocytes of rufous individuals were situated mostly in the superficial dermis and were easily recognised by the presence of dendritic arms which extended into the epidermis. The nuclei of these cells were also typically indented (Fig. 3.22.a,b.). In addition to numerous fully melanised eumelanosomes, many intermediate stage melanosomes were present in the melanocyte cytoplasm (Fig. 3.22.). Frequently, the melanosomes formed clusters in the melanocyte cytoplasm.

Sometimes, abnormal mitochondria and dilated RER were also seen in these melanocytes. However, these aberrant cellular organelles were not as numerous within these melanocytes as in of those of ty.pos. individuals (no ephelides).In addition to fully melanised stage IV melanosomes, numerous aberrant melanosomal structures were also seen (Fig. 3.23.a,b,c & d) within the melanocyte cytoplasm. These included "crescent" or "racquet" shaped melanosomes with a melanised matrix and a central core lacking in melanin. It appeared that this central core was also completely lacking in matrix, almost as if these

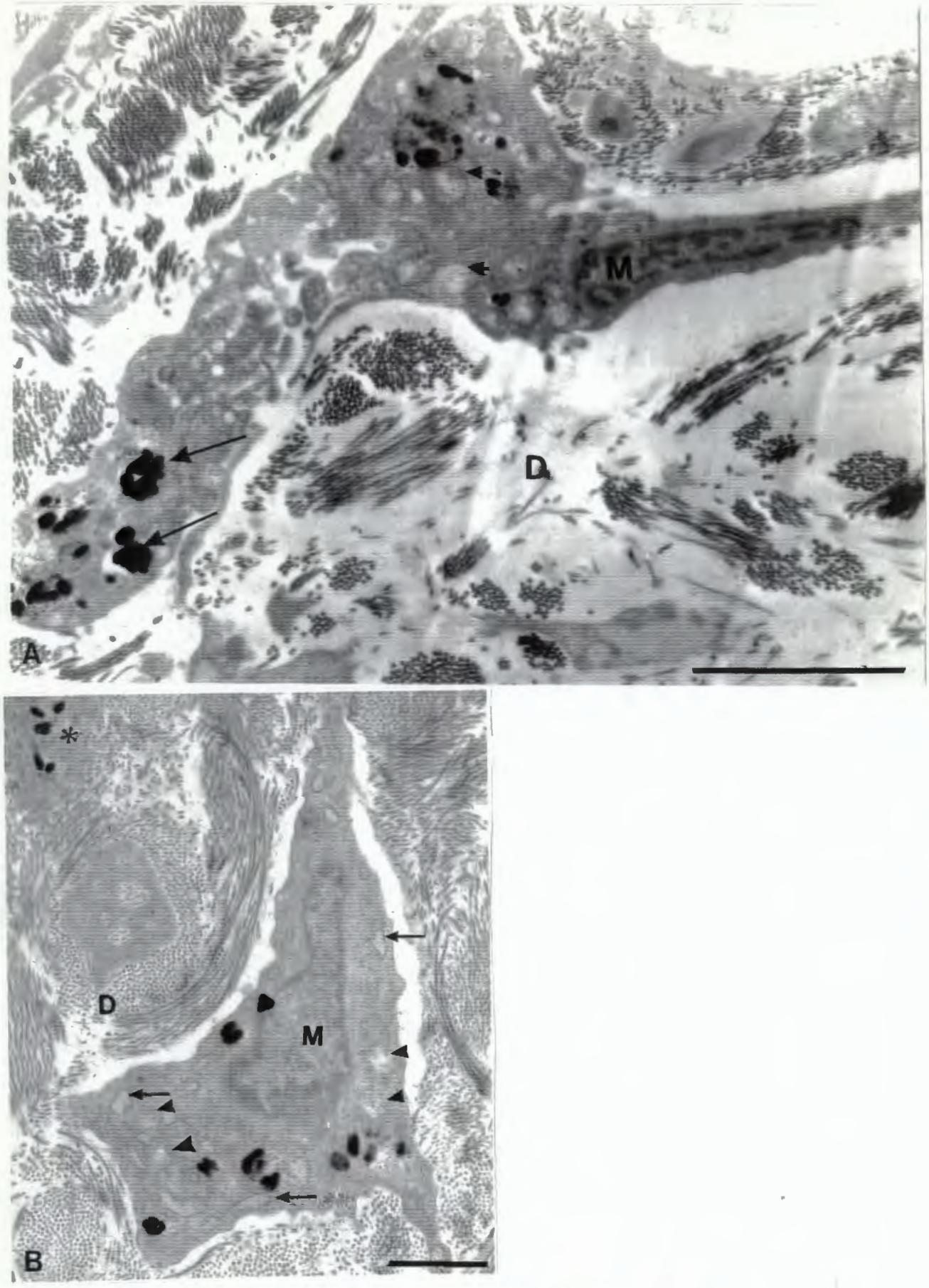


FIG. 3.22.: Melanocytes (M) of Rufous albinos. In (A) melanosomes can be seen forming clusters within the melanocyte cytoplasm (arrows). Some aberrant mitochondria can also be seen (arrowheads). In (B) some melanosome clusters can be seen in the melanocyte cytoplasm as well as dilated RER (arrows) and aberrant mitochondria (arrowheads). A cross section of a melanocyte dendrite can also be seen (*) and contains pigmented melanosomes. Bar=1um
D= DERMIS

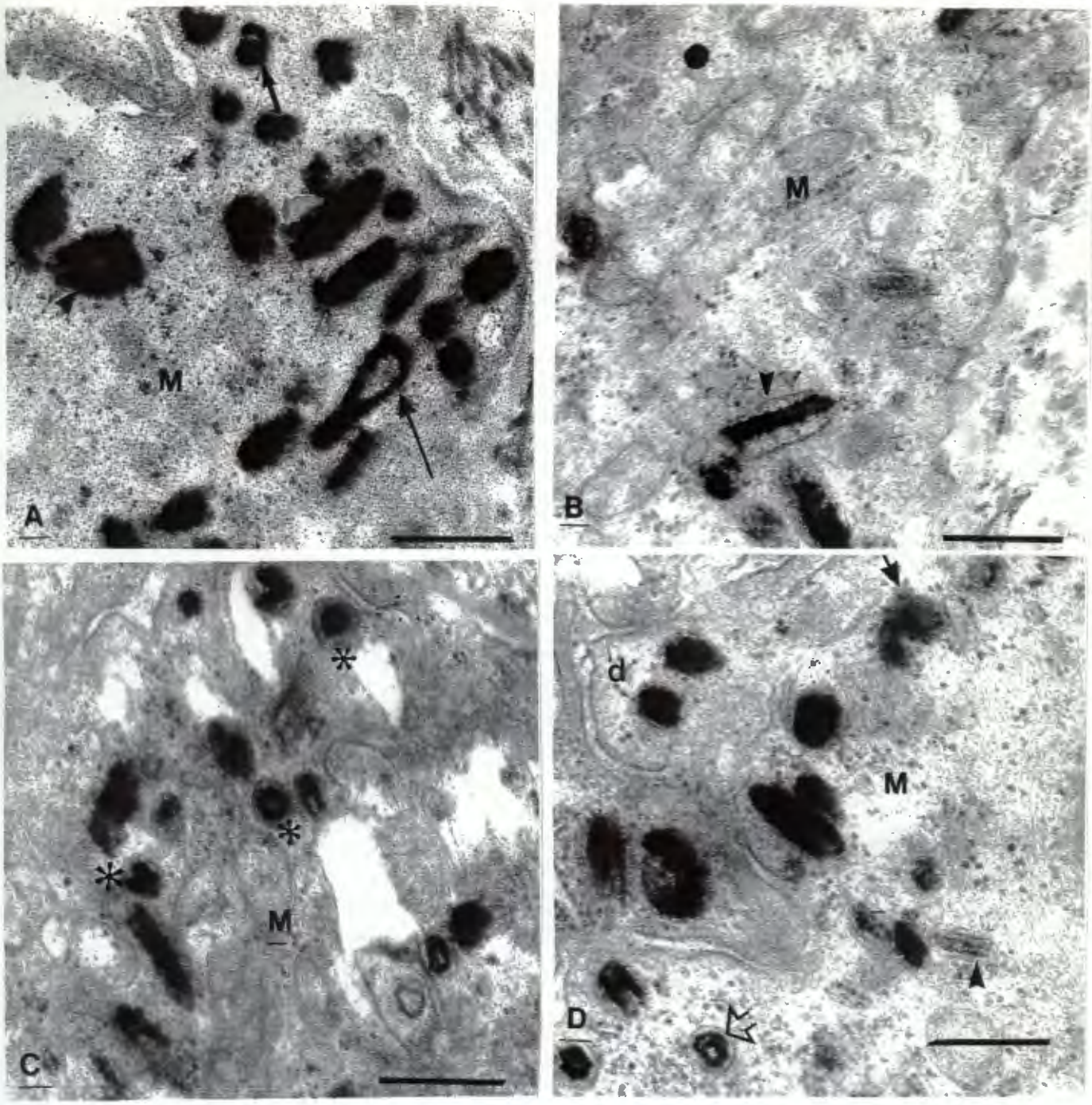


FIG. 3.23.(A-D): Melanocyte cytoplasm (M) of Rufous albinos showing various aberrant forms of melanosomes. In (A) "racquet" and "comma" shaped melanosomes can be seen (arrows); in (B) melanosomes with only a central band of melanin can be seen (arrowhead); in (C) cross-sectioned melanosomes with only a central band of melanin can be seen and in (D) "crescent" shaped (arrows) and clear melanosomal banding (arrowhead) can be seen. Bar=0.5um

melanosomes contained a central "hole". Other forms included eumelanosomes in which pigment deposition occurred merely as a central band with no pigment in the periphery. A clear internal matrix was observed in an early stage eumelanosome (Fig.3.23c). In addition to the normal eumelanosomes and aberrant melanosomes, a few pheomelanosomal-like structures were also seen in the melanocytes of rufous skin. The pheomelanosomes were recognised on the basis of their rounded shape as well as their tendency to deposit pigment in the centre first and then spread outwards.

A feature observed only in rufous skin melanocytes, was the presence of large empty vacuoles. These were frequently seen in cross sections of melanocyte arms (Fig. 3.24). The exact nature and function of these large vacuoles remains unclear. In addition to containing these large vacuoles, these melanocyte arms also contained the previously described aberrant melanosomes.

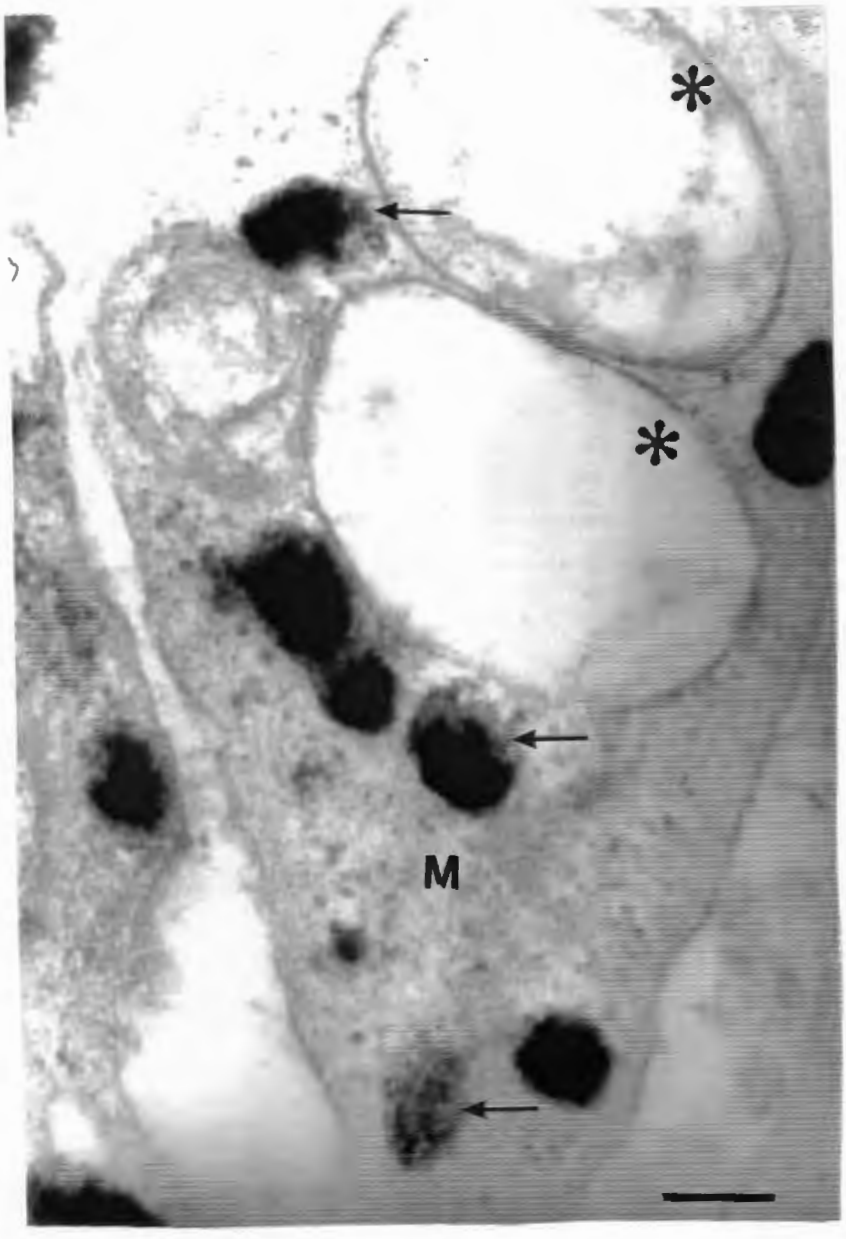


FIG. 3.24.: Cross section of a melanocyte dendrite (M) showing partially pigmented melanosomes (arrows) and large vacuoles containing amorphous material (*) only seen in Rufous skin melanocytes. The nature and function of these vacuoles remain unclear. Bar=0.1um

### *3.4.4.3. Melanosomes within the Keratinocytes:*

A distinguishing feature of rufous skin was the fact that the melanosomes of these individuals were found in "rosette" like clusters in the keratinocytes (Fig.3.25.a). This clustering phenomenon was not observed in any of the other skin types studied. These clusters occupied a typical perinuclear position in the keratinocytes. A higher power view of these clusters revealed that both fully melanised melanosomes and aberrant, partially melanised, melanosomes made up the clusters (Fig. 3.25.b). The melanosomes were very tightly packed in these clusters, leaving room for very little agranular matrix. It was not clear whether or not these melanosome clusters were surrounded by a limiting membrane, but in some cases, a very thin membrane was visible. The size of the melanosomes in these clusters was determined and compared to the size of melanosomes in normal Negroid skin. It was found that the rufous melanosomes had average dimensions of 300nm x 156nm while those of normal black individuals were 463nm x 183nm. These measurements revealed that the melanosomes of rufous individuals were approximately 30% smaller than those of normal Negroid individuals.

### ***3.4.5. COMPARISON OF HAIR BULB MELANOSOMES OF TY.POS; RUFOUS AND NEGROID INDIVIDUALS:***

The melanosomal type and stages were compared in Negroid, ty.pos.(with and without ephelides) and rufous individuals. In the hair bulb of a normal black individual (Fig. 3. 26.a), all

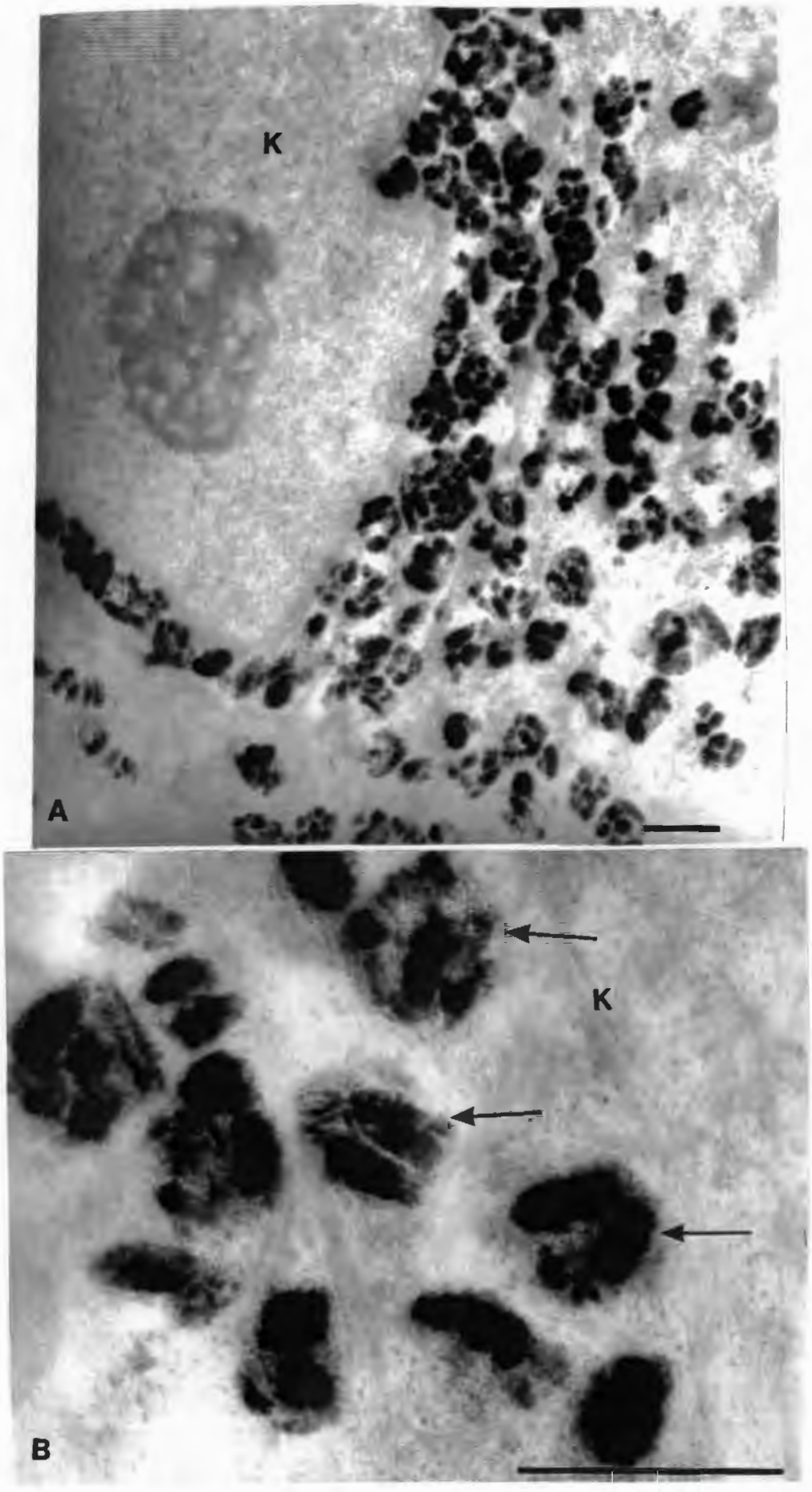


FIG. 3.25.: Keratinocyte (K) cytoplasm of Rufous albino skin showing melanosomal clustering into membrane-bound complexes. In (B) it can be seen that the melanosomes are very closely packed in these complexes with very little granular material between them, resembling the complexes seen in Mongoloids. Bar=0.5um

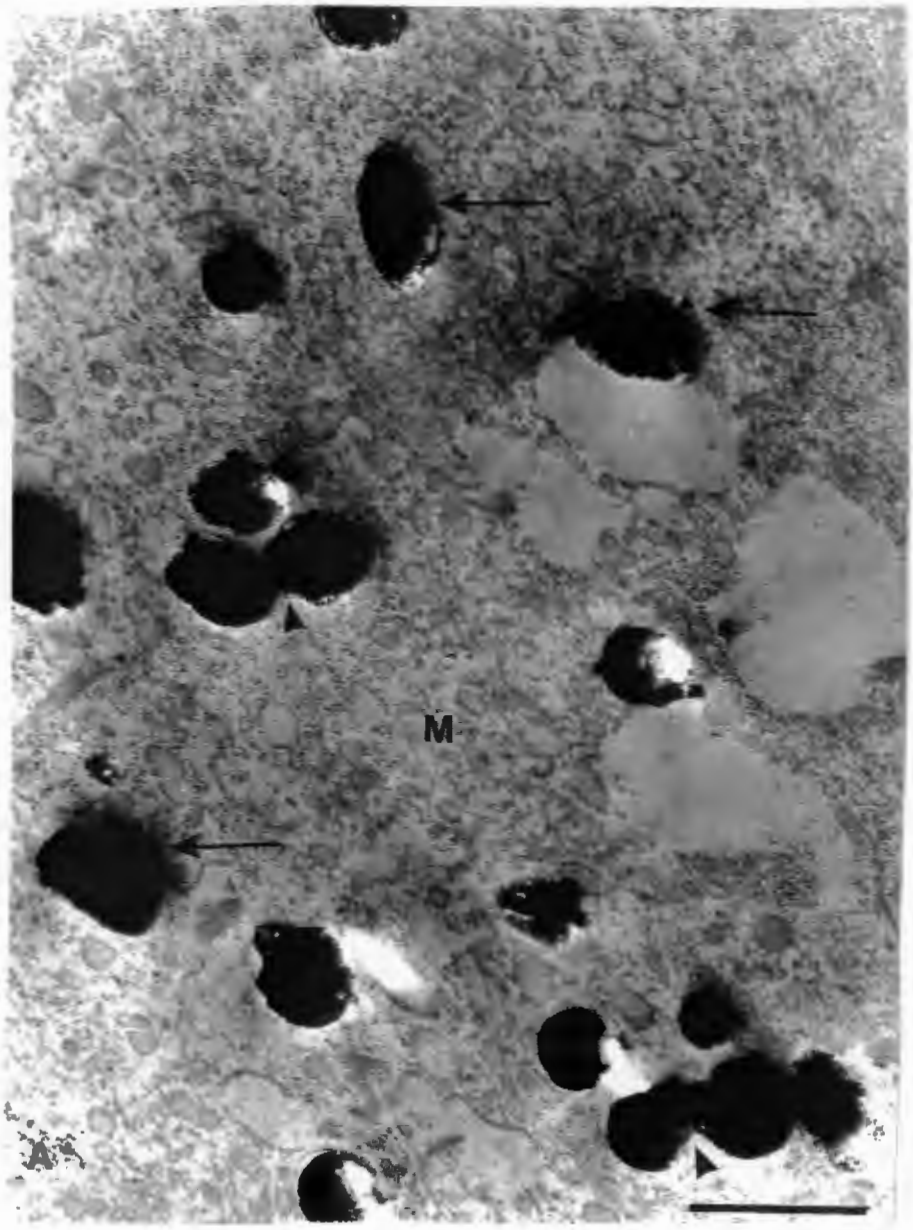


**FIG. 3.26.(A):** Hairbulb Melanocyte (M) of a normal Negroid individual showing the presence of only stage IV melanosomes (arrows). Bar=0.5um

the melanosomes were fully melanised, longitudinal and cross sectioned, eumelanosomes. Neither intermediate stage melanosomes nor pheomelanosomes were observed in hairbulbs of these individuals.

In the hair bulbs of ty.pos. individuals with ephelides (Fig. 3.26.b), numerous fully melanised stage IV melanosomes were present. In addition to these, numerous intermediate stage melanosomes were also seen. These included stage I,II and III melanosomes. The internal filamentous structures of these intermediate stage melanosomes were clearly visible. No pheomelanosomes were seen in these individuals.

In the case of ty.pos. individuals without ephelides (Fig. 3.26.c), there was a predominance of intermediate stage eumelanosomes in the hair bulb melanocytes. These included stages I,II and III melanosomes, all of which showed a very clear internal filamentous structure. In contrast to Negroid and ty.pos. (with ephelides) hair bulbs, it was found that hardly any stage IV melanosomes were present. Here too, no pheomelanosomes were seen.

In the hair bulb melanocytes of rufous individuals (Fig 3.26.d), a few fully melanised stage IV eumelanosomes were seen. In addition to these, the aberrant melanosomal forms observed in the skin melanocytes of these individuals, were also present. These included melanosomes with a central, unmelanised core. Numerous intermediate stage melanosomes were

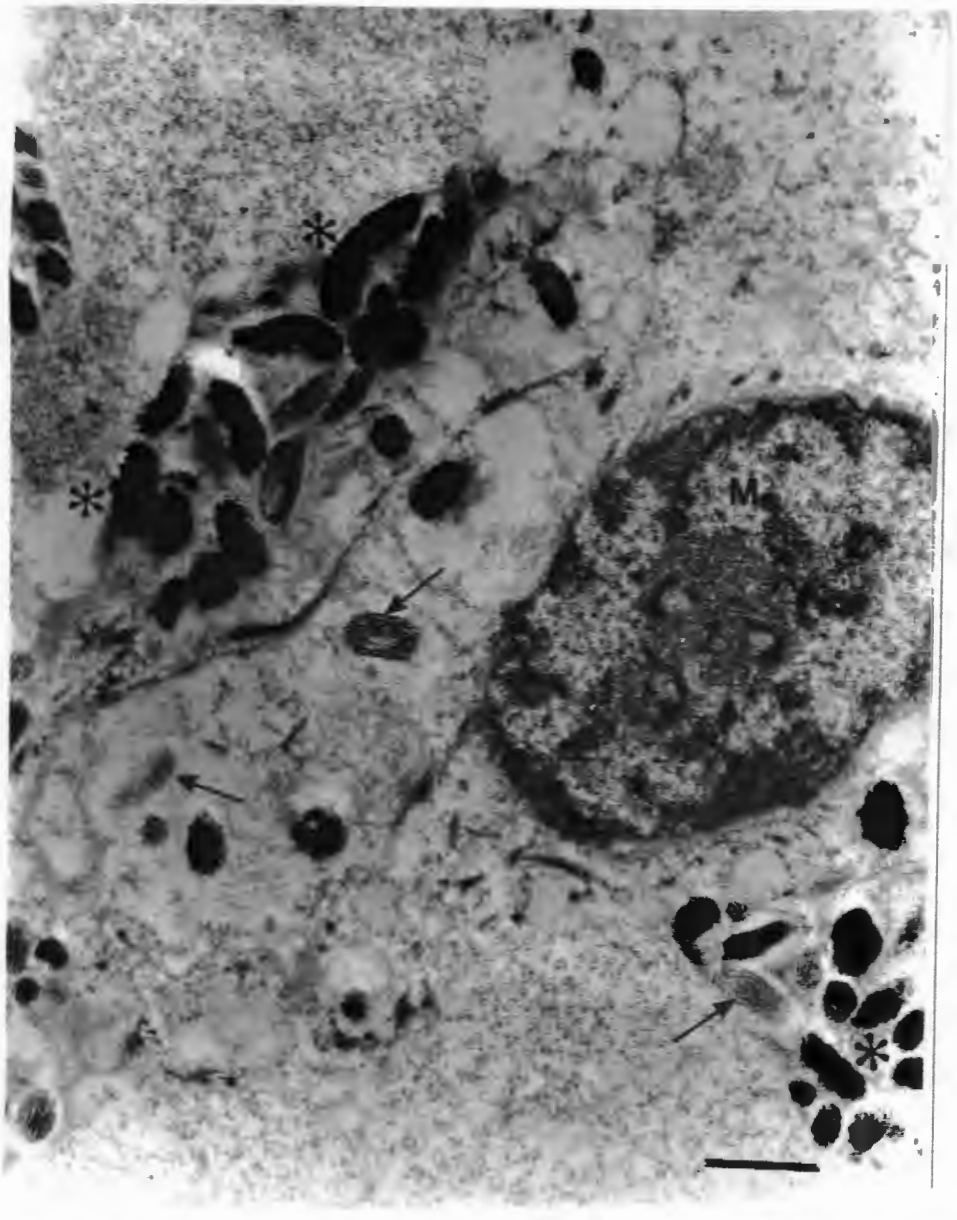


FIG. 3.26.(B): Hairbulb melanocyte (M) of a Ty.Pos albino with ephelides. Numerous stage IV melanosomes are present (*) in addition to unmelanised melanosomes (arrows). This mixture of melanised and unmelanised melanosomes presumably results in the dark-brown hair colour of these individuals. Bar=0.5um

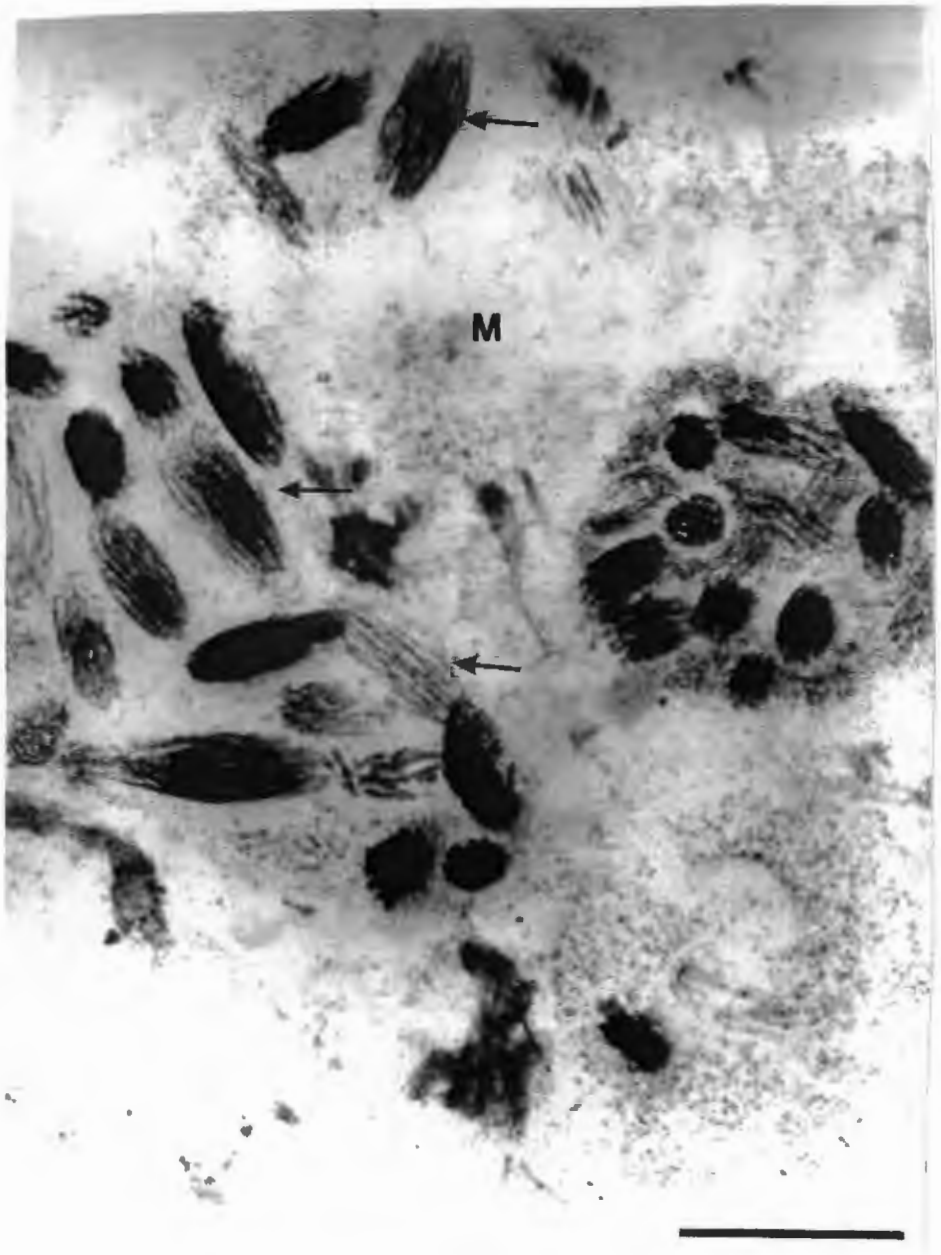


FIG. 3.26.(C): Hairbulb melanocyte (M) of Ty.Pos albino with no ephelides. Here, most of the melanosomes are unmelanised, resulting in the yellow straw coloured hair observed in these individuals. Bar=0.5um

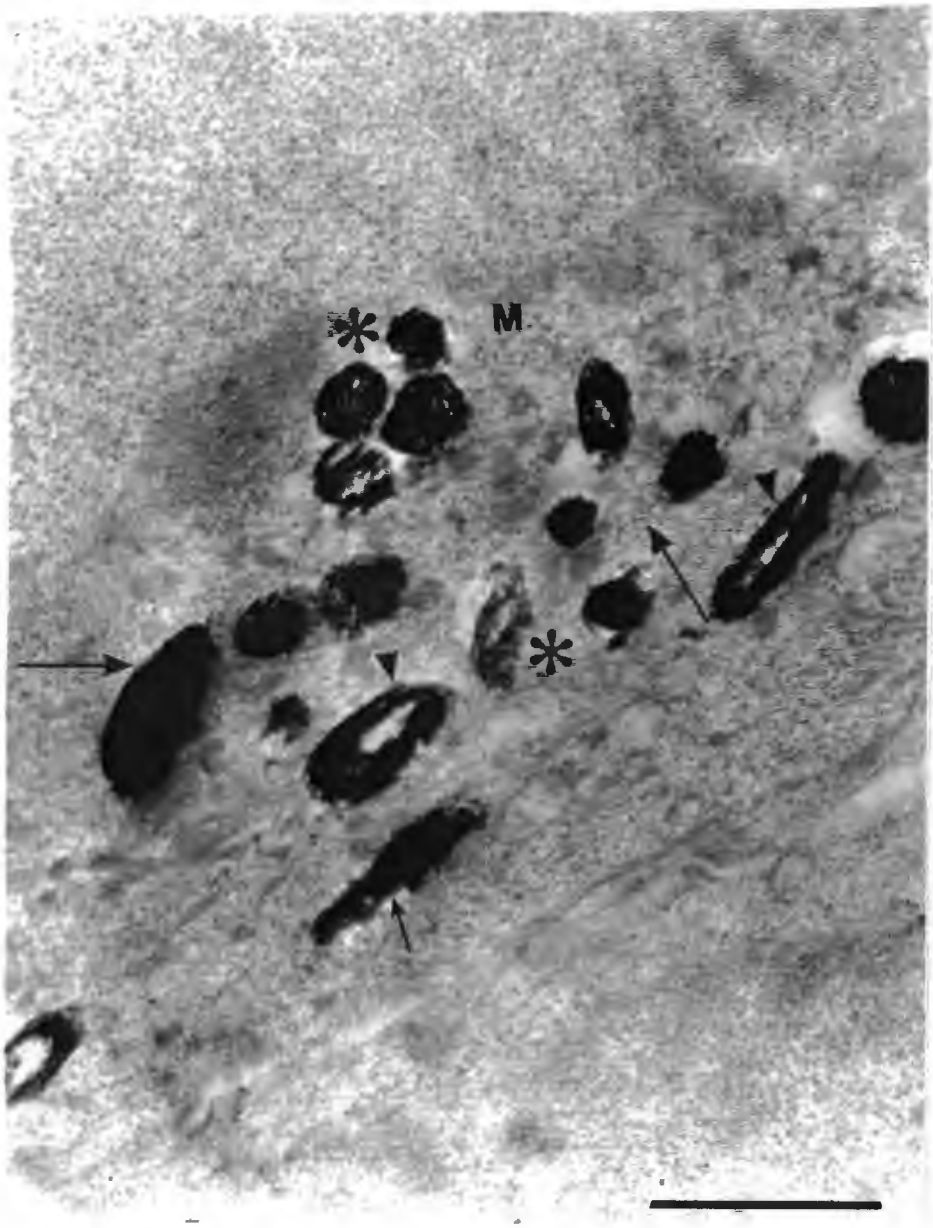


FIG. 3.26.(D): Hairbulb melanocyte of a Rufous albino showing the presence of similar aberrant melanosomes as that seen in the skin melanocytes. These include "racquet" shaped melanosomes and melanosomes with an unmelanised central core (arrowheads). Some partially melanised melanosomes (*) were also seen. Bar=0.5um

also present. These too had a visible filamentous internal structure typical of eumelanosomes. There was no evidence of any pheomelanosomes in the hair bulb melanocytes of these rufous individuals.

# CHAPTER FOUR

***DISCUSSION:***

This project set out to establish the aetiology of the tyrosinase-positive and rufous albinism forms in Southern Africa. Skin and hairbulb samples of individuals inflicted with these conditions were examined histologically and ultrastructurally. In this chapter the results of chapters 2 and 3 will be considered and conclusions drawn about the aetiology of ty.pos and rufous albinism.

## *4.1 Histological Features and Melanocyte Quantitation of Ty.Pos and Normal Negroid Skin:*

To ascertain whether there were any morphological differences between the epidermis of ty.pos and Negroid individuals, eosin stained sections were viewed with a light microscope. No obvious histological differences were seen and the number of epidermal layers, thickness of the stratum corneum and shapes of the epidermal cells appeared similar. The only marked difference was that normal Negroid skin contained numerous melanin granules, particularly in the stratum basale, whereas ty.pos skin without ephelides contained no melanin granules. However, in the region of an ephelis, some melanin granules were observed, suggesting that the melanocytes in these regions are melanotic whereas those in ephelis free regions are amelanotic.

The next step in the investigation of the morphology of the epidermis involved the use of the melanocyte-specific antibodies S-100 and HMSA-5. Although S-100 could not be used for melanocyte quantitation, it proved useful in elucidating some important aspects of melanocyte morphology. Firstly, it confirmed the presence of melanocytes in ty.pos skin, both in regions of ephelides and no ephelides. Furthermore, S100 staining revealed that the melanocytes of all ty.pos individuals were dendritic, suggesting that the melanosomal transfer mechanism was not impaired. However, this conclusion could not be confirmed from these observations alone since melanosomal movement and transfer is dependent on other factors such as cytoplasmic filaments, cytoplasmic sol-gel transformation and intra-melanocytic current flows (Jimbow et al.,1976). For example, the murine **dilute** mutation, which manifests in the production of a lighter coat colour, is caused by abnormal adendritic melanocytes which cause an uneven release of pigmented melanosomes into the hair shaft (Silvers, 1979). It was found that the dilute gene encodes for a myosin heavy chain with a C-terminus which has both type I (non-coiled) and type II (coiled) myosin heavy chains. Myosin binds actin and produces mechanical force through ATP hydrolysis and these forces are associated with organelle movement and pseudopod extensions of the cytoplasm. Mutations of this gene alter the configuration of these myosin heavy chain molecules at the C-terminus and prevent the formation of dendrites.

Furthermore, it is possible that ty.pos OCA might occur even in the presence of normal melanosome transfer mechanisms. For example, in ty.neg albinos, it has been shown that it is possible for unmelanised melanosomes to be transferred to keratinocytes (Witkop et al., 1973).

The antibody HMSA-5 (Jimbow et al., 1992) was useful for both the purposes of quantitation and elucidation of some important morphological aspects of melanocytes in Negroid and ty.pos skin. In the melanocytes of albino and normal Negroid skin, this antibody was detected mainly in the cytoplasm and around the nucleus, an expected result since vesiculo-globular bodies (VGB's), for which it is specific, are found in the peri-nuclear position near the RER and in the cytoplasm (site of VGB localisation in the melanosome). Since VGB's are thought to be tyrosinase containing vesicles (Jimbow et al., 1979), the presence of these HMSA-5 positive vesicles in the melanocytes of ty.pos individuals seems to support the notion that these albinos have normal levels of tyrosinase (Witkop et al., 1989). However, it should be cautioned that HMSA-5 staining merely confirms the presence of VGB's and does not necessarily indicate that they contain tyrosinase. It is possible, for example, that the tyrosinase detected by dopa is restricted to the RER and is not passed into the VGB's. For confirmation one way or another, double-immunolabelling of ty.pos melanocytes with HMSA-5 and a human tyrosinase specific antibody would have to be carried out.

An interesting observation was that the melanocytes of Negroid individuals rendered a stronger immuno-reaction with HMSA-5 than ty.pos melanocytes. The cytoplasm of Negroid melanocytes stained uniformly dark brown while, in that of ty.pos melanocytes, only some regions stained pale brown. It also appeared as if the melanocytes in the region of an ephelis stained somewhat darker than those in non-ephelis regions. The stronger immuno-reaction to normal Negroid melanocytes could imply the presence of more VGB's in these cells. On the other hand, this stronger immuno-reaction might be artefactual since Negroid melanocytes also contain more melanin granules which might enhance the observed dark brown DAB staining. However, Jimbow & Fitzpatrick, (1973), reported that VGB numbers increase with increasing melanisation. Thus, the observed paler staining in ty.pos melanocytes is probably indicative of the presence of fewer VGB's than in Negroid melanocytes. This, in turn, would imply a concomitant decrease in the amount of tyrosinase in ty.pos melanocytes. Indeed, slightly lowered serum tyrosinase levels have been reported for ty.pos albinos (King et al., 1988 - see Table 1.1, p.2). This might offer one explanation for the decreased melanin formation in ty.pos albinos. However, clinical records of the albino subjects in this study show that they have low to normal levels of tyrosinase. Alternatively, the defect resulting in ty.pos albinism might lead to a decreased availability of the substrate for tyrosinase. This decreased demand for tyrosinase might in turn result in fewer VGB's.

Quantitation of melanocytes that had been stained with HMSA-5 revealed that an average of approximately 1200 +/- 100 melanocytes/$mm^2$ (of basement membrane) was found in both normal Negroid and ty.pos albino skin. Statistical tests found no difference between, firstly, the mean melanocyte numbers of ty.pos individuals compared to normal Negroids, and, secondly between ty.pos individuals with and without ephelides. Although differences in these melanocyte counts were found to be statistically non-significant, it must be taken into account that the sample sizes used in this study were very small (due to unwillingness of ty.pos individuals to donate skin biopsies) and this introduced the possibility of a false negative result (type II error). However, if these are not false negative findings, the results would suggest that there is no correlation between the hypopigmentation observed in ty.pos albinos and melanocyte numbers. This finding corroborate previous reports that there is no correlation between melanocyte numbers and skin colour (Starrico & Pinkus, 1956; Szabo, 1969; Toda, 1972.a.). Furthermore melanocyte counts obtained for the inner-upper arm of both normal Negroid and ty.pos skin without ephelides were also in the same range (+/- 1100) as those reported by previous investigators (Szabo, 1954; Rosdahl & Rorsman, 1983). This strengthens the validity of the quantitation technique used in this study and argues against the possibility of a type II error.

It was interesting to note that slightly **fewer** melanocytes were found in the region of ephelides. This finding is in

accordance with that of Breathnach (1957) who found that there were fewer, but larger and more dendritic dopa-positive melanocytes in the regions of Caucasoid freckles.

The quantitation carried out in this study indicated that the hypopigmentation observed in ty.pos individuals occurred not as a result of decreased melanocyte numbers, but rather as a consequence of defective melanin synthesis.

***4.2 Ultrastructural Studies of Melanocytes of Ty.Pos, Rufous & Normal Negroid Epidermis:***

The next step in the investigation was to examine melanocytes at an ultrastructural level. In the epidermis of normal Negroids, many fully melanised (stage IV) melanosomes were seen inside the keratinocytes. This melanosomal density highest in the basal layers and became more sparse in the superficial layers. Furthermore, it was noticed that even within this group of normal Negroids, there were inter-individual variations of melanosomal densities. For example, a lighter skinned woman (Fig. 3.3a.) had visibly fewer melanosomes than a darker skinned man of the same race (Fig. 3.3b.). This observation agrees with those of Szabo et al., (1972) and Toda et al., (1972b) who found that there were many more melanosomes (about 340 per basal keratinocyte) in darker skin than in paler Negroid skin (about 120 melanosomes per basal keratinocyte). Both these and the present study were done using unexposed skin (buttock and inner-upper arm respectively) and, thus, the observed increase in melanosome density cannot be attributed to U.V. stimulation. In the light of this finding it can be

concluded that observed skin colour is a function of the inherent melanogenic activity, which is presumably genetically predetermined, rather than melanocyte numbers.

The melanocytes were initially difficult to locate but, with practice, they were usually found just below the basal epidermal layer with dendrites extending in between the overlying epidermal keratinocytes (Fig 3.2 & 3.4). They were recognised on the basis of their indented nuclei, larger and tonofilament-free cytoplasm (when compared to that of keratinocytes) and the presence of numerous premelanosomes. The melanosomes in these normal Negroid melanocytes were mostly fully melanised stage IV eumelanosomes (Fig 3.4 & 3.5). Other than these, only a few empty stage I premelanosomes, but no stage II or III melanosomes were seen. Presumably, this was a consequence of the extremely rapid rate at which melanin deposition occurs in normal Negroid skin.

In normal skin, only the stage IV melanosomes were transferred to surrounding keratinocytes (Fig 3.4. & 3.6). Inside the keratinocyte cytoplasm, melanosomes occured singly dispersed (Fig 3.6), exactly as described by Szabo et al., (1972) (Fig. 3.1) and Toda et al., (1972b). They also reported that, in contrast to this, the melanosomes of Caucasoids and Mongoloids occur in membrane-bound complexes. The significance of these findings in normal Negroid skin will become apparent when they are compared to those of ty.pos and rufous albinos. Furthermore, in the hairbulbs of normal Negroids, only stage IV

eumelanosomes were seen (Fig 3.26a), resulting in the observed black hair. This observation accords with that in normal Negroid skin where only stage IV melanosomes were seen.

The ultrastructural features of ty.pos skin in the region of an ephelis was next examined ultrastructurally. The first and most striking feature of ty.pos skin in the region of an ephelis was the numerous fully melanised stage IV melanosomes seen inside the keratinocytes (Fig. 3.7). This observation simultaneously corroborated the light microscopical observation of the presence of melanin granules and confirmed the presence of melanotic melanocytes in these regions. The melanocytes were again recognised by their basal location, indented nuclei and extensive tonofilament-free cytoplasm (Fig. 3.8). They also contained numerous stage IV melanosomes in addition to a few premelanosomes and some partially melanised melanosomes. These somewhat aberrantly melanised melanosomes also displayed no clear internal filamentous matrix and could, therefore, not be staged (Fig 3.8 & 3.9). This co-existence of stage IV and premelanosomes was also seen in cross-sections of melanocyte dendrites (Fig 3.9) as well as in keratinocytes (Fig 3.10). This finding suggests that premelanosomes, despite being unmelanised, are also transferred to surrounding keratinocytes in ty.pos albinos, an observation similar to that of Witkop (1973) in ty.neg albinos. It further suggests that the melanosomal structural protein synthesis and assembly mechanisms, as well as the transfer mechanisms are intact, and that this transfer mechanism does not exclusively export stage

IV melanosomes only. Presumably, the presence of so many unmelanised melanosomes also suggests a retarded rate of melanin synthesis and deposition.

In the hairbulbs of ty.pos individuals with ephelides, stage IV melanosomes in addition to numerous intermediate stage ones were seen (Fig 3.26b). The combination of stage IV and numerous intermediate stage melanosomes presumably explains the dark brown hair colour of these ty.pos individuals with ephelides. In contrast to the observation in skin, these intermediate stage melanosomes displayed a clear internal filamentous structure, permitting their classification into stage II and III. Why, despite identical specimen preparation, the melanosomal matrix of hairbulbs should be clearer than that of skin, remains unclear. Perhaps it is normal for skin melanosomes to display less internal matrix than hair melanosomes and since most studies on melanosome structure have been carried out on hairbulbs, it might have been assumed that skin melanosomes display similar banding patterns to hair melanosomes. This point needs further investigation.

Ty.pos skin in regions without ephelides were also examined ultrastructurally. Here, the most striking feature was the complete absence of stage IV melanosomes (Fig 3.11 & 3.12). The resultant lack of melanised melanosomes made the identification of melanocytes even more difficult in these sections. However, from the quantitation data, it was known that these biopsies contained normal numbers of melanocytes.

Thus, with careful scanning, these were located once again in their basal positions and on the basis of their typical nuclear and cytoplasmic features. Higher magnification of the cytoplasm revealed numerous "empty vesicles", which gave the melanocytes a vacuolated appearance (Fig 3.13). These were speculated to be unmelanised premelanosomes. In addition, a few pigmented melanosomes were also seen. However, these had very hazy outlines, lacked a clear internal filamentous matrix and were incompletely melanised, making accurate staging difficult (Fig 3.14; 3.15a & b; 3.16a & b). In one unusual case, (Fig. 3.16) cross banding of the internal filamentous matrix can clearly be seen. This once again, as in the case of ty.pos skin with ephelides, suggests that the melanosomal assembly mechanisms are probably functional and that the defect seems to lie with the melanisation process. In this regard, the presence of a few, albeit aberrantly, melanised melanosomes would suggest that very low levels of pigment formation does indeed occur and that there is not a complete block in the melanin synthetic pathway.

Figure 3.21 represents an interesting section through a region flanked by an ephelis on one side and non-ephelis skin on the other. In the region of the ephelis, there are numerous stage IV melanosomes in the keratinocytes, whereas, on the other side, no melanosomes are seen. Instead, a large vacuolated amelanotic melanocyte is seen. This apparently confirms the premise that in ty.pos skin with ephelides, the skin

surrounding these ephelides is similar to ty.pos skin without ephelides.

In the hairbulbs of ty.pos albinos without ephelides, there were virtually no stage IV melanosomes, but an abundance of partially melanised intermediate stage ones (Fig 3.26c), corroborating similar findings in skin. Again, the matrix of the intermediate stage melanosomes was clearly discernible. Perhaps the predominance of intermediate stage melanosomes rather than full melanised stage IV ones explains the yellow, straw coloured hair of ty.pos albinos without ephelides.

It was decided to include skin and hair bulbs from rufous albinos for comparison with ty.pos skin and also because this rare form of OCA is very prevalent in Southern Africa. In view of the small size and limited quantity of biopsies available, these were used only for ultrastructural investigations.

As in the case of ty.pos skin in regions of ephelides, the most striking feature once again was the abundance of fully melanised melanosomes. The melanocytes were found in their usual basal epidermal location (Fig 3.22a & 3.22b). Closer scrutiny of the melanosomes revealed that their shape, size and packaging in keratinocytes were different from that seen in normal Negroid and ty.pos skin (Fig 23 a-d). Some of the melanosomes were "racquet" or "crescent" shaped with a central core that lacked both a filamentous matrix and melanin. Most of the melanosomes also lacked a clear outer limiting membrane.

It was further noticed that although most of the melanosomes were aberrant in structure, all were fairly densely melanised. An additional observation was that the melanosomes were much smaller than those of normal Negroid skin. On average, they were found to be 30% smaller.

A final observation was the clustering of melanosomes into "rosette-like" aggregations in the keratinocytes (Fig 3.25 a-b). This arrangement was never seen in either normal Negroid or ty.pos skin. As reported earlier in chapter 3, Toda et al., (1972a), measured melanosomal sizes in different races and found that the melanosomes of Caucasoids and Mongoloids were relatively smaller than those of Negroids, and that these smaller melanosomes tended to occur in clusters whereas larger melanosomes occurred singly dispersed (Fig 3.1). These workers proposed that clustering resulted in a paler skin colour. The closely packed melanosomes of rufous skin also had very little ground substance between them, reminiscent of that observed in Mongoloid skin. On the basis of this, the striking similarity between Mongoloid and rufous skin colour is not surprising.

In the case of the rufous albinos hair bulbs, numerous stage IV melanosomes together with the aberrant forms seen in the skin were found (Fig 3.26d), again corroborating the findings of this study in skin. Also, the finding that all rufous melanosomes, irrespective of whether they were aberrant in structure or not, were always melanised to a greater or lesser extent, lends further support to the postulate arrived at after

investigating rufous skin, that the defect resulting in rufous OCA lies with the melanosomal structural protein assembly and not with melanin formation. No pheomelanosomes were seen, contradicting the findings of Witkop et al., (1989) that rufous hairbulbs contain only pheomelanosomes. Also, Witkop's description of rufous albinos having deep mahogany to sandy - red coloured hair is contrary to that of Kromberg et al., (1990) that African rufous albinos have light to ginger brown coloured hair. Both these apparent discrepancies seem to support the view that the African rufous albino might represent a different form of albinism to that described by Witkop (Kidson et al., 1993, in press).

### *4.3. Aberrant Organelles within Melanocytes:*

In addition to the melanosomal abnormalities, other melanocytic organelles were also seen to be abnormal in ty.pos skin without ephelides. The mitochondria appeared distended with loss of the typical internal cristae and outer membrane continuity (Fig 3.17 & 3.18). In addition, the RER appeared dilated (Fig 3.19 & 3.20), as was the Golgi apparatus (Fig 3.17). Dilated RER were also observed in the skin of Rufous albinos. It was possible that these aberrant organelles were not artefactual since they were not found in the melanocytes of normal Negroid nor in ty.pos regions with ephelides. Similar observations of the dilation of RER and "blown mitochondria" was reported by Boissy et al., (1991a & 1991b) who studied a murine model for vitiligo. Figure 4.1. is a micrograph presented by Boissy et

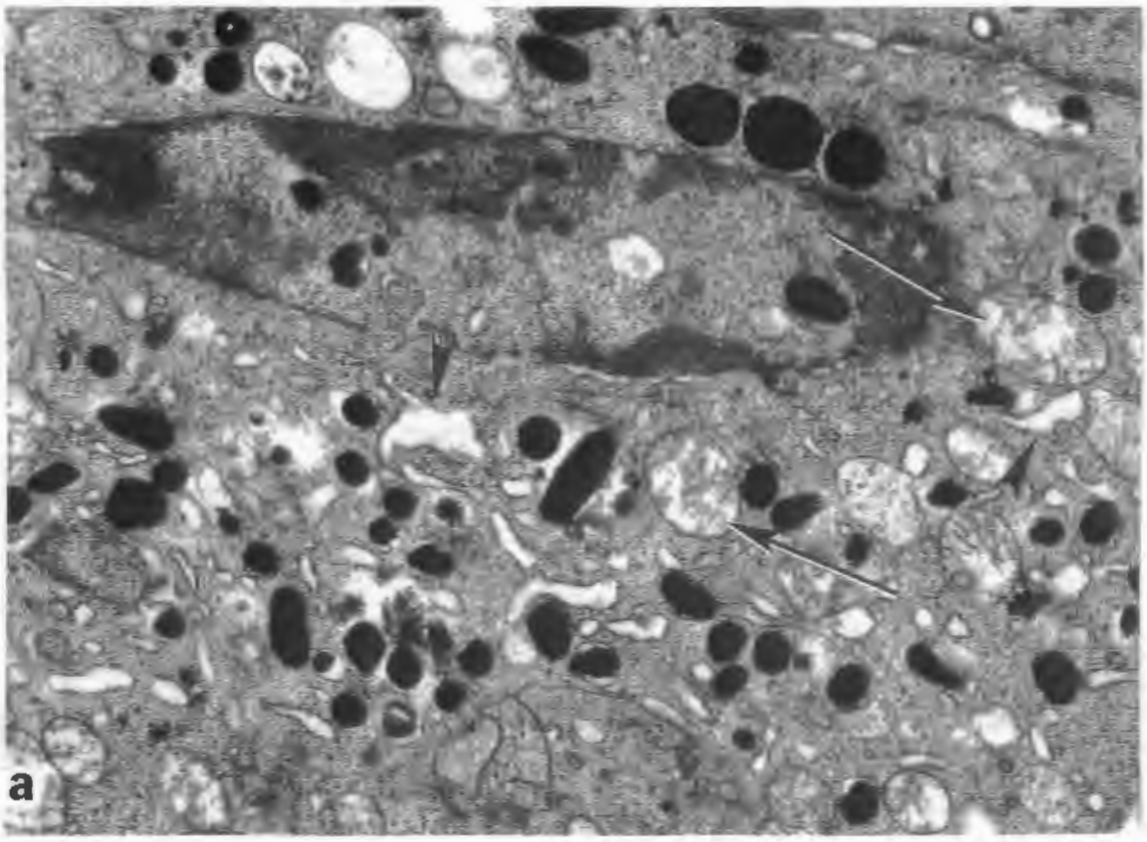


***Fig 4.1. Micrograph of Melanocyte Cytoplasm, showing dilated RER & aberrant mitochondria in Vitiligo mice. From Boissy et al.,(1991a).***

ARROWS → "BLOWN MITOCHONDRIA"
ARROWHEADS → "DILATED RER"

al., (1991a) and is included for comparison. They reported that the dilation of the RER was reversible by cyclohexamide treatment. Since cyclohexamide is a protein synthesis inhibitor, they suggests that the dilation is a consequence of abnormal protein flow through the RER.

In terms of the melanin synthetic pathway, essentially two important protein types pass through the RER. Both melanosomal structural proteins and tyrosinase are synthesised in the RER. In this study, evidence for the apparently normal structure of premelanosomes in ty.pos albinos has already been presented. Consequently, it could be that these dilations of the RER seen in melanocytes from these individuals result from the accumulation of tyrosinase. If this were so, the two most plausible explanations for this accumulation would be that, firstly, an abnormal tyrosinase is being synthesised, or, secondly, that there is a decreased availability of substrate for and utilisation of tyrosinase. Dopa incubation tests of hairbulbs of the ty.pos subjects of this study, which were done during the initial screening procedures, indicate that they have normal levels of functional tyrosinase. This argues against the possibility that the dilatation occurs as a result of an abnormal tyrosinase, and thus renders the theory of a decreased substrate availability more plausible. However, the results of this ultrastructural investigation alone did not prove this conclusively.

It was postulated that the dilated RER seen in rufous melanocytes resulted from abnormal melanosomal protein assembly and hence abnormal melanosomal structure. The fact that these aberrant melanosomes were fairly densely melanised suggests that rufous albinism results from a defect in the melanosomal structural protein synthetic pathway rather than the melanin synthetic process as in the case of ty.pos OCA..

## *4.4. Concluding Remarks*

This project set out to investigate the cellular basis of ty.pos and rufous OCA. Both light and electron microscopical techniques were employed to this end. It was demonstrated that ty.pos albinism does not result from a paucity of melanocytes, and that the epidermis of ty.pos subjects, both with and without ephelides, was not obviously different from that of normal Negroid control subjects, with the exception of the absence of melanin granules in ty.pos skin without ephelides. At ultrastructural level, ty.pos skin in the region of ephelides, appeared very similar to normal Negroid skin in that numerous stage IV melanosomes, characteristic of normal Negroid skin, were also present here. However, unlike normal Negroid skin, it was found that the melanocytes of ty.pos skin with ephelides were able to transfer stage I melanosomes also, implying a slowing in the rate at which premelanosomes are melanised in these cells. In ty.pos skin without ephelides, on the other hand, no stage IV melanosomes were seen. Instead,

numerous unmelanised premelanosomes, some with clear filamentous matrices, were seen, together with some aberrantly melanised melanosomes. All of this was interpreted as implying that the melanosomal structural protein synthesis and assembly was intact, and that the defect resulting in ty.pos albinism seemed rather to be one in the melanosomal melanisation process.

During the course of this study, the discovery of a candidate ty.pos OCA gene was reported (Rinchik et al., 1993). The human *P* gene ( analogous to the mouse pink-eyed dilution (p) locus) was shown to correspond to the D15S12 locus within the chromosome segment 15q11-q13 , and mutation of this gene was shown to result in ty.pos OCA. Of particular relevance to this study was the speculation that this gene encoded an integral melanosomal membrane transport protein responsible for the transport of tyrosine, the precursor of melanin synthesis, into the melanosome. Several ultrastructural features noted in this study would appear to support this notion of an abnormality in the melanin synthetic pathway. Firstly, in ty.pos skin without ephelides, the presence of numerous unmelanised premelanosomes suggested a defect in the melanisation process, which might be explained by a lack or low levels of intra-melanosomal tyrosine. Presumably, the finding that some melanisation did take place might be explained by the fact that in ty.pos OCA there appears to be a mutation, rather than a complete deletion of the *P* gene. This would result in the formation of a protein that is partially functional rather

than a complete absence of this protein. This would explain why low levels of melanin deposition were seen in most ty.pos melanosomes.

It is further speculated that this reduction of substrate availability to tyrosinase might explain several other abnormalities noted here. The dilated RER profiles seen might well be a consequence of the accumulation of tyrosinase in these organelles, in turn a consequence of the decreased demand for and utilisation of this enzyme. In the final analysis, therefore, it would appear that the ultrastructural findings of this study support the notion that ty.pos OCA results from a defect in the transport of tyrosine into the melanosomes.

In ty.pos skin with ephelides, which occurred only in sun exposed regions, the co-existence of stage IV melanosomes and premelanosomes suggested an increased capability for melanisation as compared to ty.pos skin without ephelides. It is speculated that this increased potential for melanogenesis is a consequence of random back mutations of the *P* gene, induced by U.V. stimulation. Support for this speculation was derived from the murine ***p* unstable** gene ($p^{un}$) which is the final allele at the *p* locus, on which the murine homologue, *p*, for the human ty.pos (*P*) gene is found (Silvers, 1979). Mutations at $p^{un}$ produces a phenotype consisting of areas of dilute and intense pigmentation (similar to regions of ephelides and non-ephelides). This bicoloured pattern was found to be due to a very high frequency of spontaneous

somatic reversion to wildtype. The proportion of coat affected with this bicolour followed a bimodial pattern with some individuals having hardly any pigmented spots while others had a predominance of pigmented spots. This again correlates to the ephelide:no ephelide ratio seen in ty.pos albinos. Because the *P* gene encodes a fairly large integral membrane protein (11- membrane spanning domains) and it is known that this gene is mutated rather than deleted, it is probable that U.V. could cause reversions to wildtype in some parts of the gene, allowing it to encode a protein which has retained a functional domain. This U.V.- induced "back-mutation" is most likely a random event which can occur at any developmental stage, being more prevalent in older individuals as a consequence of their prolonged exposure to U.V. stimulation. This would explain why only some individuals develop ephelides and, furthermore, why only some sun-exposed regions develop ephelides.

The other form of OCA studied here was rufous or red albinism. Here, the most striking feature was the abundance of fully melanised melanosomes. However, closer scrutiny revealed abnormalities of shape, size and clustering. Some melanosomes were "racquet" or "crescent" shaped, with no discernable internal matrix or clear outer limiting membrane. Despite these structural aberrations, most of these melanosomes were densely melanised. This was interpreted as suggesting that the defect resulting in rufous OCA was one of the melanosomal structural pathway, rather than of the melanin synthetic pathway.

In conclusion, it is obvious that many more investigations are required to validate many of the findings of this study. An important priority, currently being pursued by Rinchik et al.(1993), is the raising of an antibody to the *P* polypeptide suggested to be defective in ty.pos OCA. Using immunolabelling procedures, this antibody would then be used to localise and confirm the presence of this protein in the melanosomal membrane. This, in turn, would serve to confirm or disprove the proposed aetiological basis of ty.pos OCA and would also test the validity of several of the findings of the present study. In view of the scarcity of skin biopsies alluded to earlier, attempts are currently underway in this laboratory to establish ty.pos melanocyte cultures, which might circumvent this difficulty.

# REFERENCES

Atoji,Y.; Shirogane, D.; Kurano, T.; Suzuki, Y. & Sugimura,M. (1991). S-100 - Immunoreactive giant macrophages in lymphoid tissue of guinea pig. *Acta Anatomica* 140, 17-25.

Beermann,F.; Ruppert, S. (1990). Rescue of the albino phenotype by introduction of a functional tyrosinase gene into mice. *EMBO J.* 9, 2819-2826.

Billingham,R.E.; Medawar P.B. (1948). Pigment spread and cell heredity in guinea pig's skin. *Heredity* 2. 29-47.

Birbeck,M.S.C.; (1963). Electron microscopy of melanocytes: The fine structure hair-bulb premelanosomes. *Ann. New York Acad. Sci.* 100, 540-547.

Boissy, R.E.; Beato, K.E.; Nordlund, J.J. (1991 a). Dilated rough endoplasmic reticulum and premature death in melanocytes cultures from the vitiligo mouse. *Am. J. Pathol.* 138, 1511-1525.

Boissy, R.E.; Liu, Y.Y.; Medrano, E.E.; Nordlund, J.J. (1991 b). Structural aberrations of the rough endoplasmic reticulum and melanosome compartmentalisation in long-term cultures of melanocytes from vitiligo patients. *J. Invest. Derm.* 97, 395-404.

Breathnach, A.S. (1957). Melanocyte distribution in forearm epidermis of freckled human subjects. *J. Invest. Derm.* 29, 253-261.

Breathnach, A.S. (1959). An attempt to induce pigmented spread in freckled human skin. *J. Invest. Derm.* 33, 193.

Chentameneni,B; Halaban,C.D.; Kobayashi,Y; Witkop,C. *Proc. Natl. Acad. Sci. U.S.A.* (88) 12, 5272-5276

du Buy,H.G.; Showacre,J.L.; Hesselbadh,M.L. (1963). Enzymatic and other similarities of melanoma granules and mitochondria. *Ann. New York Acad. Sci.* 100, 569.

Everette,M.A.; Nordquist,R. (1979). Melanosome size and distribution in American Indians. *Pigment Cell* 4, 291-298.

Giebel,L.B.; Strunk,K.M.; King,R.A.; Spritz,R.A. (1990). A frequent tyrosinase gene mutation in classic, tyrosinase negative (type IA) OCA. *Proc. Natl. Acad. Sci. U.S.A.* 87, 3255-3258.

Gordon,P.R.; Gilchrest,B. (1989). Regulation of human melanocyte growth, dendricity, and melanization by keratinocyte derived factors. *J.Invest. Derm.* 92, 565-572.

Halaban,R.; Moelmann,G. (1988). Tyrosinases of Murine melanocytes with mutations at the albino locus. *Proc. Natl. Acad. Sci. U.S.A.* 85, 7241.

Halaban,R.; Moelmann,G. (1990). Murine and Human B Locus pigmentation genes encode a glycoprotein (GP 75) with catalase activity. *Proc. Natl. Acad. Sci. U.S.A.* 87, 4809-4813.

Hearing,V.J.; Jimenez,M. (1989). Analysis of mammalian pigmentation at the molecular level. *Pigment Cell Res.* 2, 75-85.

Hearing,V.J.; Tsukamoto,K. (1991). Enzymatic control of pigmentation in mammals. *FASEB* 5, 2903-2909.

Hu,F.; Hanifin,J.M.; Prescott,G.H.; Tongue,A.C. (1980). Yellow mutant albinism: Cytochemical, ultrastructural and genetic characterisation suggesting multiple albinism. *Am. J. Hum. Genetics* 32, 387.

Ide,C. (1972). Development of melanosomes in the pigment epithelium of the chick embryo. *Z.Zellforch* 131, 171-186.

Jara,J.R.; Martinez-Liarte,J.H.; Salano. (1990). Transport of L-tyrosinase by B16 F10 melanoma cells. *J. Cell Sci.* 97, 479-485.

Jackson,I.J. (1988). DNA encoding tyrosinase-related protein maps to the brown locus in mice. *Proc. Natl. Acad. Sci. U.S.A.* 85, 4392-4396.

Jackson,I.J.; Bennett,D.C. (1990). Identification of the albino mutation of mouse tyrosinase by analysis of an in vitro revertant. *Proc. Natl. Acad. Sci. U.S.A.* 87, 7010-7014

Jimbow,K.; F itzpatrick,T.B. (1974). Characterization of a new melanosomal structural component, the vesiculoglobular body, by conventional transmission, high voltage and scanning electron microscopy. *J. Ultrastruct. Mol. Struct. Res.* 48, 269-283.

Jimbow,K.; Roth,S.I.; F itzpatrick,T.B.; Szabo,G. (1976). Some aspects of melanin biology - 1950-1975. *J. Invest. Derm.* 67, 72-89.

Jimbow,K.; Takiyi Takeuchi. (1979). Ultrastructural comparison of pheo- and eumelanogenesis in animals. *Pigment Cell* 4, 308-317.

Jimbow,K.; Ishida,O. (1983). Combined chemical and electron microscopic studies of pheomelanosomes in human red hair. *J. Invest. Derm.* 81, 506-511.

Jimbow,K.; Oikawa,O.; Sugiyama,S.; Kakeuchi,T. (1992). Comparison of Eu- and Pheomelanogenesis in retinal and follicular melanocytes: Role of VGB in melanosome differentiation. *J. Invest. Derm.* (73) 4, 278.

Jimenez,M.; Hearing,V.J. (1987). Mammalian tyrosinase: The critical regulatory control point in melanocyte pigmentation. *Int. J. Biochem.* 19, 1141-1147.

Jimenez,M.; Maloy,W.L.; Hearing,V.J. (1989). Specific identification of an authentic tyrosinase clone. *Chem.* 264, 3397-3403.

Jung,E.G.; Anton Lamprecht,I. (1971). Investigation of a case of OC Albinism. *Birth Defects* (7) 8, 26-30.

Karnovsky,M.J. (1965). Formaldehyde - Gluteraldehyde fixative of high osmolarity for use in electron microscopy. *J. Cell Biol.* 27, 137.

Kidson,S.H.; Richards,P.D.G.; Rawoot,F; Kromberg,J.G.R. (1992). An ultra-structural study of hairbulb and skin melanosomes in rufous albinos. *Pig. Cell Res.* (1993,in press).

King,R.A.; Summers,C.G. (1988). Albinism. *Dermatologic Clinics* (6) 2, 217-288.

Kromberg,J.G.R. (1987). Albinism in South Africa. Infama.

Kromberg,J.G.R.; Castle,D. (1989). Albinism and skin cancer in Southern Africa. *Clinical Genetics* 36, 43-52.

Kromberg,J.G.R.; Castle,D.; Kidson,S.H. (1990). Red or rufous albinism in South Africa. *Ophthalmic Paediatrics and Genetics.* (11) 3, 229-235.

Kwan,T.H. (1990). Hypermelanoses in: *Pathology of the skin.* Chapter 38, pp 493 Ed. E.R. Farmer & A.F. Hood. Appelton and Lange Publishers.

Kwon,B.S.; Haq,A.k.; Promerantz. (1987). Isolation and sequence of a cDNA locus for human tyrosinase that maps at the mouse c-albino locus. *Proc. Natl. Acad. Sci. U.S.A.* 84, 7473-7477.

Kwon,B.S.; Wakulchik,m.; Haq,A.K. (1988). Sequence analysis of mouse tyrosinase cDNA and the effect of melanotropin on its gene expression. *Biochem. Biophys.* 153, 1301-1309.

Le Douarin,N. (1982). *The neural crest.* Cambridge University Press, Cambridge.

Maul,G.G. (1969). Golgi-melanosome relationship in human melanoma in vitro *J. Ultrastructure. Res.* 26, 163-176.

Maul,G.G.; Brumbaugh,J.A. (1971). The possible functions of coated vesicles in melanogenesis of the regenerating fowl feather. *J. Cell Biol.* 48, 41-48.

Mishima,Y; Widlan,S. (1967). Enzymatically Active and inactive melanocyte populations and ultraviolet irradiation : combined dopa - premelanin reaction and elecron microscopy. *J. Invest. Derm.* 49, 273-281.

Muller,G.; Ruppert,S. (1988). Functional analysis of alternatively spliced tyrosinase gene in mice. *EMBO* 9, 2819-2826.

Nicholls,R.D.; Rinchik,E.M.; Driscoll,D.J. (1992). Genomic Imprinting in mouse and man. *Seminars Dev. Biol.* 3, 139-152.

Novikoff.A.B; Albala,A. & Biempica,L. (1968). Ultrastuctural and cytochemical observations on B-16 and Harding Pascey mouse melanomas. *J. Histochem. Cytochem.* 16, 299-319.

Pawelek,J.M. (1976). Factors regulating growth and pigmentation of melanoma cells. *J. Invest. Derm.* 66, 201-209.

Prota,G. (1980). *Cysteine and Glutathions in Mammalian Pigmentation.* pp 391-398. Plenum. New York.

Rawles,M.E. (1948). Origin of melanophores and their role in development of colour patterns in vertebrates. *Physiol. Rev.* 20, 383.

Reynolds,E. (1963). The use of lead citrate at high pH as an electron - opaque stain in electron microscopy. *J. Cell Biol.* 17, 208-212.

Rinchik,E.M.; Bultman,S.J.; Horsthemke,B.; Lee,S.T.; Strunk,K.M.; Spritz,R.A.; Avidano,K.M.; Jong,M.T.C.; Nicholls,R.D. (1993). A Gene for the Mouse Pink-eyed Dilution Locus and for Human Type II OCA. *Nature* 361, 72-76.

Robins,A.H. (1991). *Biological Perspectives on Human Pigmentation.* Cambridge University Press.

Rosdahl,I.; Szabo,G. (1976). Ultrastructure of the human melanocyte system in the newborn, with special reference to racial differences. *Pigment Cell* 3, 1-12.

Rosdahl,I. (1979). Local and systematic effects on the epidermal melanocyte population in UV irradiated mouse skin. *J. Invest. Derm.* 73, 301-309.

Rosdahl,I.; Rorsman,H. (1983). An estimate of the melanocyte mass in humans. *J. Invest. Derm.* 81, 278-281.

Sagebiel,R.W.; Odland. (1972). *Ultrastructural Identification of Melanocytes in Early Human Embryos.* pp 43-50. Appelton Century Crofts; New York.

Sakurai,T.; Ochiai,H.& Takeuchi,T. (1975). Ultrastructural change of melanosomes associated with Agouti pattern formation in mouse hair. *Devel. Biol.* 47, 466-471.

Seiji,M.; Iwashita,S. (1965). Intracellular localization of tyrosinase and site of melanin formation in melanocyte. *J. Invest. Derm.* 45, 305.

Seiji,M.; Okazaki,K.; Sugiyama,M. (1976). Melanocyte - Keratinocyte interaction in pigment transfer. *Pigment Cell* 3, 308-317.

Silver,W.K. (1979). *The Coat Colour of Mice: A Model for Mammalian Gene Action and Interaction.* Springer Verlag, New York.

Shibahara,S.; Tomita,Y.; Sakakura,T. (1986). Cloning and expression of cDNA encoding mouse tyrosinase. *Nucl. Acids Res.* 14, 2413-2427.

Shibahara,S.; Tomita,Y.; Tagumi,H.; Muller,R.M. (1988). Molecular basis for the Heterogeneity of human tyrosinase. *J. Exp. Med.* 156, 403-414.

Spritz,R.A.; Strunk,K.M.; Giebel,L.B.; King,R.A. (1990). Detection of mutations in the tyrosinase gene in a patient with Type 1A OCA. *N.E.J.M.* (June 14).

Spurr,A.R. (1969). A low viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* 26, 31.

Starrico,R.J.; Pinkus,H. (1956). Quantitative and Qualitative data on the pigment cells of Human epidermis. *J. Invest. Derm.* (June 9).

Stanka,P. (1974). Ultrastructural study of pigment cells of human Red Hair. *Cell Tiss. Res.* 150, 167-178.

Stanka,P.; Sahlmann,B. (1981). Electron microscopic study on the origin of melanosomes: The nature of their matrix and differentiation of their limiting membrane. *Pigment Cell* 11, 291-298.

Steirner,U.; Rosdahl,I.; Augustsson,A. (1989). UVB irradiation induces melanocyte increase in both exposed and shielded human skin. *J. Invest. Derm.* 92, 561-564

Szabo,G. (1954). Number of melanocytes in human epidermis. *B.M.J.* (May 1). 1016-1017

Szabo,G.; Quevedo,W.C.; Virk,S. (1969). Influence of age and UV on the population of Dopa-positive melanocytes in human skin. *J. Invest. Derm.* (52) 3, 287.

Szabo,G.; (1967). Photobiology of melanogensis. *Advances in Biology of Skin* (8) pp 379-391. Permagon Press.Oxford.

Szabo,G.; Gerald,A.B.; Pathak,M.A. (1972). The ultrastructure of racial colour differences in man, in Riley. *Pigmentation : Its Genesis and Biologic Control.* pp 23-41. Appelton Century Croft. New York.

Toda,K.; Pathak,M.A.; Parrish,J.A. (1972 a) Alteration of melanosome distribution in human epidermis after exposure to ultraviolet light. *Nature New Biol.* 236, 143.

Toda,K.; Fritzpatrick,T.B. (1972 b). Ultrastuctural and biochemical studies of the formation of melanosomes in the embryonic chick retinal pigment epithelium, in Riley: *Pigmentation: Its Genesis and Biological Control.* pp 125-141. Appelton Century Crofts.

Tomita, Y.; Takeda, A.; Okinaga,S.; Tagami, H. & Shibahara,S.(1989). Human OCA caused by a single base insertion in the tyrosinase gene . *Biochem. Biophys. Res. Commun.* 164, 990-996.

Turner,W.A.; Taylor,J.D.; Tchen,T.T. (1975). Melanosome formation in the goldfish : The role of multivesicular bodies. *J. Ultrastruct.Res.* 51, 16-31.

Vijayasaradhi,S.; Doskoch,P.M.; Houghton,A. (1991). Biosynthesis and Intra-cellular movement of the Melanosomal Membrane Glycoprotein gp75, the Human (Brown) Locus product. *Experimental Cell Research* 196, 233-240.

Weston,J.A. (1971). Neural crest cell migration and differentiation. *UCLA Forum Med. Sci.* 14, 1

Witkop,C.J.; Nance,W.E.; Rawles,R.F. (1970). Autosomal recessive OCA. *Am. J. Hum. Genetics.* 22, 55.

Witkop,C.J.; White,J.G.; Desnick. (1971).
Classification of Albinism in man.
*Birth Defects: Original Article Series*, (7) 8, 13.

Witkop,C.J.; Niswander,J.P.; Bergsma,D.R. (1972).
Tyrosinase positive Albinism among the Zuni and the Brandywine Triracial isolate.
*Am. J. Phys. Anthropol.* 36, 392.

Witkop,C.J.; Hill,C.W.; Desnick. (1973).
Ophthamologic, Biochemical, Platelet and Ultrastructural defects in the various types of OCA
*J. Invest. Derm.* 60, 443-455.

Witkop,C.J.; White,J.G.; King,R.A. (1974).
*Oculocutaneous Albinism in Nyhan.* pp 177-261.
John Wiley and Sons, Inc. New York.

Witkop,C.J.; Quevedo,W.C.; Fŕitzpatrick,T.P. (1989).
Albinism. *Metabolic Basis of Inherited Disease.*
pp 2905-2947. Mc Graw-Hill, New York.

Woods,M.; Burks,D.; Hunter,J. (1963). The Ontogenic status of melanin granules. *Ann. New York Acad. Sci.* 100, 534-547.

Yamamoto,H.; Takeuchi,S.;Kudo,T.; Makino,K. (1987).
Cloning and sequencing of mouse tyrosinase cDNA.
*Jpn. J. Genetics.* 62, 271-274.

Yamamoto,H.; Takeuchi,S.; Kudo,T. (1989).
Melanin production in cultured albino melanocytes transfected with mouse tyrosinase cDNA.
*Jpn. J. Genetics* 64, 122-135.

Zdarsky,E.; Favour.; Jackson. (1990).
The molecular basis of brown, an old mouse mutation, and of an induced revertant to wild type.
*Genetics* 126, 443-450.

Zimmermann,A.A.; Cornbleet,T.H. (1948).
The development of epidermal pigmentation in the Negro Fetus. *J. Invest. Derm.* 11, 383-392.

# APPENDIX 1

## Solutions used for Immunocytochemistry

**Phosphate Buffered Saline (PBS):**
42.5g NaCl
6.4g $Na_2.HPO_4$
0.78g $NaH_2PO_4.2H_2O$
make up to 5 litres distilled water

**Trypsin:**
0.2g CaCl
0.2g porcine trypsin powder(Sigma)
200ml distilled water
(pH 7.8)

**Diluent for Antisera:**
100 ml Tris saline
0.25g sodium azide
5 mg bovine serum albumin
5 mg thyroglobulin
40 mg EDTA
1 mgl swine serum

**Diaminobenzidine tetrahydrochloride (DAB)**
1 mg DAB
2 ml 0.05 M Tris-HCl (pH 7.6)
20 µl 1% hydrogen peroxide

* DAB IS HIGHLY CARCINOGENIC. GLOVES SHOULD BE WORN WHEN HANDLING DAB AND ALL SYRINGES, PIPETTES AND GLASSWARE USED SHOULD BE STERALISED WITH JIK.

# ***APPENDIX 2***

**Preparation of sections for Light Microscopy:**

| | |
|---|---|
| **Primary Fixative:** | **4-6 Hours** |
| 10% Buffered Formalin | |
| **Dehydration:** | |
| 70% ethanol | **overnight** |
| 95% ethanol | **1 Hour** |
| 100% ethanol | **4 X 30 minutes** |
| 50:50 ethanol:xylol | **30 minutes** |
| xylol | **4 X 15 minutes** |
| melted paraffin wax | **2 changes - 15 min.** |

Embed in fresh wax and cool blocks.

# APPENDIX 3

## Processing of Specimens for Electron Microscopy

| | |
|---|---|
| **Primary Fixation** | **3 Hours at 4°C** |
| Modified Karnovsky's: | |
| 2.5% Gluteraldehyde | |
| 2% Paraformaldehyde | |
| 0.1 M Phosphate Buffer | |
| (pH of solution: 7.4) | |
| **Wash** | **3 X 20 minutes** |
| 0.1 M phosphate buffer | |
| (pH 7.4) | |
| **Secondary 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 | |
| **Embedding** | |
| Spurr's resin | **30 minutes** |
| Spurr's resin | **30 minutes at 60°C** |
| fresh Spurr's resin | **1 Hour at 60°C** |