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The nature of miniature melanocytes in murine epidermis

John Thomas Earnhardt

The Nature of Miniature Melanocytes in Murine Epidermis

by

John Thomas Earnhardt

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Committee Chairman

Examining Committee

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ABSTRACT

In the epidermis of PET/Wmr mice the population of melanocytes reaches a peak and begins to decline during the first postnatal week, and has disappeared within four weeks. During this period a small number of weakly DOPA reactive miniature melanocytes are seen dispersed among the generally large, highly DOPA reactive melanocytes typical of the animal. These miniature melanocytes appear early, are among the last to disappear, and their population remains relatively constant against the drastically changing population of typical melanocytes. In attempts to determine the nature of the miniature melanocytes, heterologous grafting was employed whereby the melanocytes were subjected to morphogenetic impacts. The evidence suggests that these miniature melanocytes are relatively resistant to transformation into typical melanocytes, and may constitute a distinct cell type.

ACKNOWLEDGEMENTS

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I would like to thank my wife, Marian and daughter, Heather for their understanding throughout.

INTRODUCTION

It is well known that the melanocytes of the skin and hair of mammals are derived from melanoblasts which migrate from the neural crest early in embryonic development (Rawles,1947). That mammalian epidermal melanocytes vary somewhat as to shape, size, and color is well established (Markert and Silvers, 1956). Although the mode by which melanocyte morphology is expressed is genotypically dependent, the tissue environment does modify cellular expression. Markert and Silvers (1959) examined melanocyte morphology as related to tissue environment and have shown that cells with a dilute (dd) genotype exhibit a non-dilute morphology if grown in the anterior chamber of the eye. Rovee (1965) has shown that cell size is related to cell density in the melanocyte system of the mouse in that the greater the density, the smaller the size of the melanocytes.

In the melanocyte pattern of an area of freckled epidermis covering a stretched scar, Breathnach (1958) noted the presence of small, weakly DOPA reactive

melanocytes in the pale area of the scar. Also in man, the white macules of tuberous sclerosis have melanocytes which are small and weakly DOPA reactive as compared to normal melanocytes (Fitzpatrick et al, 1968, Figures 5 and 6).

The chorioallantoic membrane (CAM) of chick embryos possesses extensive vascularity which can support the cultivation of isolated tissues (New, 1966). The grafting process involves direct vascular connections between the graft and the chorioallantoic circulation (Coulombre, 1967). The early development of the procedure for CAM grafting (Willier, 1924) has been modified somewhat since its conception. Although the tissue explant is isolated from its original melieu, it does come under hormonal and other influences present in the blood of the host (Rugh, 1962).

One factor which eminates in the blood has been described by Bullough and Lawrence (1962) with the term chalone. In an earlier investigation by Bullough and Lawrence (1960), the question of the presence of growth controlling, tissue specific inhibitors was reviewed. It is now generally accepted that such inhibitors of mitotic activity are present in various

tissue and control cell proliferation by negative feedback inhibition. The action of chalones persists *in vitro* and *in vivo* and although tissue specificity exists, species specificity does not (Maugh, 1972). Reams (1963) has described a "morphogenetic factor" present in humoral fluid of chick embryos that can evoke branching of donor pigment cells within the coelomic lining of the host. In that same investigation the morphogenetic factor has been shown to be non-species-specific with regard to the mouse and the chick.

In the epidermis of the PET/Wmr mice, the melanocyte population reaches a peak and begins to decline during the first postnatal week of life. During this period, a small number of miniature melanocytes (figure 8) have been noted dispersed among the generally large melanocytes (figure 7) typical of the animal. The purpose of this investigation was to attempt to determine the nature of the miniature melanocytes by subjecting them to the impact of the circulating titres of chalone and morphogenetic factor present in the chick embryo via the CAM.

The pigment cell terminology used throughout this communication conforms to that of Fitzpatrick *et al*,

(1966): Melanoblast- a cell which is the precursor of the melanocyte. Melanocyte- any cell that produces melanin; a cell which synthesizes a specialized melanin-containing organelle known as the melanosome. A melanoblast becomes a melanocyte with the formation of melanosomes.

MATERIALS AND METHODS

Mice of the PET/Wmr strain maintained at the University of Richmond were used throughout this investigation. In order to determine the normal pigment cell complement of the epidermis of the mice, microscopic examination of whole mount preparations was carried out. Mice were selected for use when at least three separate litter births occured within 6 hours. At least one animal from each of the three litters was killed by decapitation at the following ages: 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20 25 days and adult. Litters were allowed to age in order that the time period between each day would more closely approximate 24 hours. A specimen of skin between 6 and 10 mm per side was removed from the mid dorsum of each mouse. The hair of the mice in age groups 6 days and older was removed by electric clippers and/or a commercially available depilatory chemical.

Each skin section was removed along with the underlying hypodermis and scraped to remove the subcutaneous tissue. To prevent the skin from rolling up, it was placed dermal side down on a square of

Whatman #1 filter paper slightly larger than the skin specimen. The sodium bromide method for epidermal stripping was employed: the skin was placed in 2 M NaBr for approximately 45 minutes at 37 C after which the epidermis was separated from the dermis with the aid of a dissecting microscope (20 X) and Dumont #5 forceps. The epidermis was then placed in 5% formaldehyde solution (ph 7.2 with $NaHCO_3$) for 30 minutes with a successive washing in distilled water (5 minutes). The skin section was then placed into a 0.1% buffered solution of 1, 3-4 dihydroxyphenylalinine (DOPA). In order to prepare this solution, phosphate buffer was prepared by combining 20 parts of 0.1 M KH_2PO_4 and 80 parts 0.1 M Na_2HPO_4. This stock buffer solution (ph 7.4) was used in the preparation of DOPA solutions throughout, all of which were compounded just prior to use.

Tissues were incubated with the DOPA solution for one hour at which time the DOPA solution was replaced with freshly prepared DOPA solution and incubated for an additional three hours. All incubations were carried out at 37 C.

Subsequent to the DOPA staining, the tissue was placed in 5% formalin and allowed to stand overnight.

The section was then placed in a position with the hair follicles upward. The follicles were removed by "plucking" with Dumont #5 forceps utilizing magnification (20 X). After washing in distilled water for 15 minutes, the epidermal sheets were dehydrated in successive solutions of 70, 95, and 100% ethanol for a minimum of 15 minutes each. The tissue was then cleared in xylene and a permanent slide prepared with balsam.

The number of melanocytes was determined by microscopically examining five areas on each whole mount preparation of skin using an ocular grid of known dimensions. The areas examined on each control slide were identical although orientation of the slide in the particular plane may have varied. Slides were marked in such a manner as to keep epidermal sheets of the same litter in corresponding groups. Mean values for each age group were determined and a graphic relationship of age versus melanocytes/mm^2 established.

To prepare grafts of mouse skin, newborn PET mice (6 hours: postnatal age) were obtained and sacrificed by decapitation. A small square of skin (5-8 mm/side) was removed from the mid-dorsum and placed in sterile saline containing .06 mg of penicillin (Penicillin G Sodium, Nutritional Biochemicals Company) and .05 mg

of streptomycin (Streptomycin Sulfate, Nutritional Biochemicals Company) per cubic centimeter. The pH of the saline/antibiotic solution was adjusted to 7.2 with $NaHCO_3$. The skin sections were scraped on the dermal side to remove the hypodermis and left in the saline/antibiotic solution while hosts were made ready for receiving the grafts.

In order to determine the effects of environmental change upon the melanocyte population, CAM grafting was employed. Fertile eggs of the White Leghorn fowl were obtained from a local poultry farm. The age of the embryo was determined by establishing the hour of iniation of incubation as the base line for the onset of development.

Fertile eggs were incubated in a Davis Bradley cabinet incubator maintained at 38 C with a consistant level of humidity. Eggs incubated for 12-15 days were used as hosts. Each egg was candled in a darkened room to determine the position of the major blood vessels of the CAM. The area on the shell overlying one of the bifurcations of major blood vessel in the CAM was penciled. Each egg was then placed on a nest of cotton in a Syracuse watch glass and cleaned with cotton soaked in 70% ethanol.

A small triangular hole (1cm/side) was carefully cut in the shell using a 10 cm section of a fine toothed hacksaw blade. The shell was removed, leaving the shell membrane intact. The shell membrane was moistened with saline/antibiotic solution to reduce the tendancy for adherence to the chorioallantois. The shell membrane was removed with extreme care to avoid puncturing the CAM. Previously prepared grafts were removed from the saline/antibiotic, placed onto the flat end of a blunted glass stirrring rod, and carefully lowered onto the CAM with the dermis side down. Orientation of the graft on a well vascularized area between bifurcating blood vessels was accomplished with the aid of forceps. Aseptic technique was used throughout the grafting procedure.

The triquetral opening was sealed with cellophane tape with the edges sealed by applying melted paraffin with a small brush. The eggs were returned to the incubator with the cellophane window directed upward.

The grafts were recovered after 4, 5, 7, and 8 days of incubation. The epidermis was removed by the sodium bromide method and the resulting epidermal sheet subjected to the DOPA procedure explained previously. Whole mount preparations were made and examined microscopically with an ocular grid employed for determining melanocyte populations. Five separate

areas were counted in each epidermal preparation. Mean values were obtained and collated with control values of melanocyte numbers in groups comparable in age to the CAM graft period.

In a preliminary survey, it was found that there was a good correlation between the general size of a melanocyte and the size of its nucleus. Therefore, to qualify as a miniature melanocyte, a pigment cell had to have a nuclear volume no greater than half that of a typical melanocyte.

RESULTS

The control population of melanocytes is presented as a relationship between age of the mice and the number of melanocytes per square millimeter. It can be seen (figure 1) that in the population of macromelanocytes (typical melanocytes) the peak number of cells is reached at day 3 after which there is a gradual decline in the melanocyte population until a relatively constant rate is seen after day 15. From the graph (figure 1) it is evident that the range is much greater for mice in the age groups of 2-3 day. The mean values for each group (day) were obtained from a total of fifteen areas in three different mice.

Figure 1 also relates the relative constancy of the miniature melanocytes from day 0 to day 10 although the group with the largest mean value (day 3) coincides with the peak obtained in macromelanocyte counts. The miniature melanocyte population after day 10 shows mean values that are similar.

A total of 107 CAM grafts were attempted. It is readily apparent from figure 2 that the macromelanocyte population shows a marked increase in melanocyte numbers beginning with an average of 89.7 cells/mm^2 after 4 days of CAM incubation to an average of 442.7 cells/mm^2 after

8 days of CAM incubation and no apparent peak in the number of cells/mm^2. The graph (figure 2) shows the 7 day CAM graft to have the widest range.

The miniature melanocyte population increased slightly before declining with 8.2, 9.3, 10.2, and 7.9 cells calculated per mm^2 for respective CAM incubation periods of 4, 5, 7, and 8 days.

Figure 3 depicts the relationship between the macromelanocyte control population and the macromelanocyte CAM graft population. It is evident that the population of melanocytes in the CAM grafts rises later when an overall comparative picture is presented (see figure 9). Mean values approximate each other at day 5 with the control population averaging 140.8 cells/mm^2 and the CAM graft population averaging 157.8 cells/mm^2. The control range of this group is contained within the range of the CAM graft population; However, the graft indicates divergence at this point. Statistical analysis employing the t-test revealed significance in 3 of 4 in macromelanocyte population when control melanocyte numbers for a given day were compared with CAM graft populations for the corresponding day. Statistically significant figures were recorded for days 4, 7, and 8 when total populations were compared.

A comparison of miniature melanocyte populations can be seen in figure 4. Although the values seem to show only slight differences, significant values were recorded for days 4 and 7. In both of these populations the range of values overlapped when control and experimental groups were compared.

The standard deviation for each population in the miniature group was determined in order to obtain a better understanding of the relationship involved.

DISCUSSION

The present study establishes a normal population of epidermal melanocytes present in the dorsum of the PET/Wmr mice. The macromelanocytes were considered to be the normal type since their size and DOPA reactivity compared favorably with that of previous investigations. Quevedo et al. (1966) in relating the number of epidermal melanocytes per mm^2 in mid-dorsum to the age of dilute black (dd) mice and intense black (DD) mice has shown a graphic relationship with the peak of melanocyte population seen at age 2 days with a steady decline thereafter. The populations presented by Quevedo show that DOPA reactive epidermal melanocytes in the intense black mice averaged $163 \pm 6/mm^2$ at birth and increased to a maximum of $415 \pm 13/mm^2$ at age 2 days. In dilute black mice the number averaged $286 \pm 29/mm^2$ at birth and reached a peak of $555 \pm 48/mm^2$ at day 2.

In the ventral skin of PET mice, Rovee and Reams (1964) have shown peak population of melanocytes present 3 days after birth. The relative relationship between melanocyte populations for a given day when relating this investigation to others, compares favorably in a way as

to show a general trend in the pattern of the melanocyte population in the first 10 days of postnatal life.

Although small melanocytes have been noted in scattered reports, this investigation brings together their presence in a way which relates them to the control population of melanocytes. Perhaps the lack of their being reported generally has been due to the assumption that small cells become larger when they mature. The control group results indicate a relatively constant population of small melanocytes, suggesting they they are not an intermediate cell type in the sense of the melanoblast.

It has been established that the melanoblasts migrate from the neural crest of the embryo of the mouse (Rawles, 1947). After invasion into an area, the melanoblast is capable of 1) Immediate proliferation, 2) differentiation into a melanocyte, or 3) latentcy with subsequent proliferation or maturation. This varied potentaility of the melanoblast is effected by two main factors: the genotype of the cell and the environment in which the cell resides (Markert and Silvers, 1959).

Quevedo et al. (1966) have shown that many melanocytes which are active shortly after birth continue in an amelanotic state after the early postnatal life. It is possible also that pigment cells may fail to be identified

even by the DOPA reaction since the latent form of the cell is not always expressed by chemical techniques (Mayer, 1965). This may account for the gradual decline in the melanocyte population of the control group. That is, the pigment cells are present but are in an amelanotic form. This suggestion is supported by Quevedo et al. (1966) who have noted the presence of melanocytes showing various degrees of DOPA reactivity.

Since the control population of miniature melanocytes remains constant, it is proposed that there are melanoblasts which give rise only to small melanocytes.

Reams (1956) has shown that in the chick embryo some tissues are able to evoke branching of unbranched melanocytes more effectively than others. That investigation has also shown the coelomic lining as a tissue has an inhibitory effect, holding the branching of a pigment cell in check. Reams (1957) has shown the skin to be an initiator of pigment cell branching and the principle source of a pigment cell branching effector substance. Nichols and Reams (1960) have shown that unbranched melanocytes of PET/MCV mouse embryos changed from the unbranched to branched form when the chick hosts were 15 days old. Reams (1963) has interpreted this response to indicate the time at which a titre of the effector substance sufficient to evoke

branching is present. From data presented in the above mentioned investigation, the present study incorporated fertile eggs incubated 12-15 days in order to extend the full impact of the morphogenetic factor upon the host.

The CAM grafts resulted in pigment cell populations which included miniature as well as macromelanocytes after DOPA incubation. If the titre of chalone and morphogenetic factor was present in the blood of the chick embryo, the effects of the non-species-specific factors should bestow their influence upon the epidermal sheet. Since grafts which came in contact with the CAM were initially close to the newborn age, the regulator factors should influence the entire complement of pigment cells. That is, since miniature melanocytes are present in the first few postnatal days of the control group population the regulator factors should inhibit the mitotic activity of pigment cells in the grafts. Therefore, if the smaller cells were precursors of the larger cells, miniature melanocytes should mature into macromelanocytes without further mitotic activity. This would result in only large cells seen in the CAM graft situation, but this was not the case. The population levels of the macromelanocytes of the CAM grafts show corresponding daily increases directly proportional to the length of CAM incubation.

The population of macromelanocytes in the CAM grafts shows statistically significant differences at days 4, 7, and 8; the population at day 4 being lower while 7 and 8 are higher. Day 7 CAM shows more than a fourfold increase with day 8 CAM showing a ninefold increase over the corresponding daily control group. The data could indicate a stimulatory mitotic effect rather than an inhibitory reaction of the grafting procedure, or merely activation of latent, non-dividing melanoblasts into melanocytes. Although the population of CAM grafted epidermis is much higher, it is within the earlier mentioned range of a previous investigation. In that same investigation, Quevedo et al. (1966) have shown that ultraviolet stimulation of pigment cells evokes the latent population into expression. This would indicate that ultraviolet irradiation would extend the population to its highest attainable peak. For comparable daily population counts, the melanocyte number presented in the communication at hand is well within maximum attainable limits.

Another point can be made about the comparison of the macromelanocyte population. The slope of the line of the CAM graft melanocyte numbers between day 4 and day 8 closely parallels the slope of the line formed by the control population between day 1 and day 3. The controls

and CAM grafts differ here only in that there is continued melanocyte expression throughout the CAM culture period while in the controls there is a later regression.

An even more interesting point can be made concerning the miniature melanocyte population. Since day 4 CAM corresponds to day 1, graphically speaking, the difference is three days. If the graphic representation of the CAM miniature melanocyte population is then moved back three days and superimposed on the control miniature melanocyte population, the values are strikingly similar.

The overall picture presented here may indicate that the activity of chalone and other regulator factors is present in that it represses the population so that it leaves the "starting block" late and with much more impact. The delay is possibly due to the post operative recovery period of the graft.

Breathnach (1957, 1958a and b) has demonstrated that pigment cells of a freckle are larger than normal while those of a scar are smaller than normal. These melanocytes are described as having different lineage and provides evidence that pigment cells in certain areas may comprise distinct cell lines.

It is evident from this investigation that the miniature melanocytes are resistant to transformation into typical melanocytes by the method employed and it is conceivable that they likewise represent a distinct cell line.

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Figure 1. Melanocytes per mm^2 versus age (days) for control populations of macromelanocytes and miniature melanocytes.

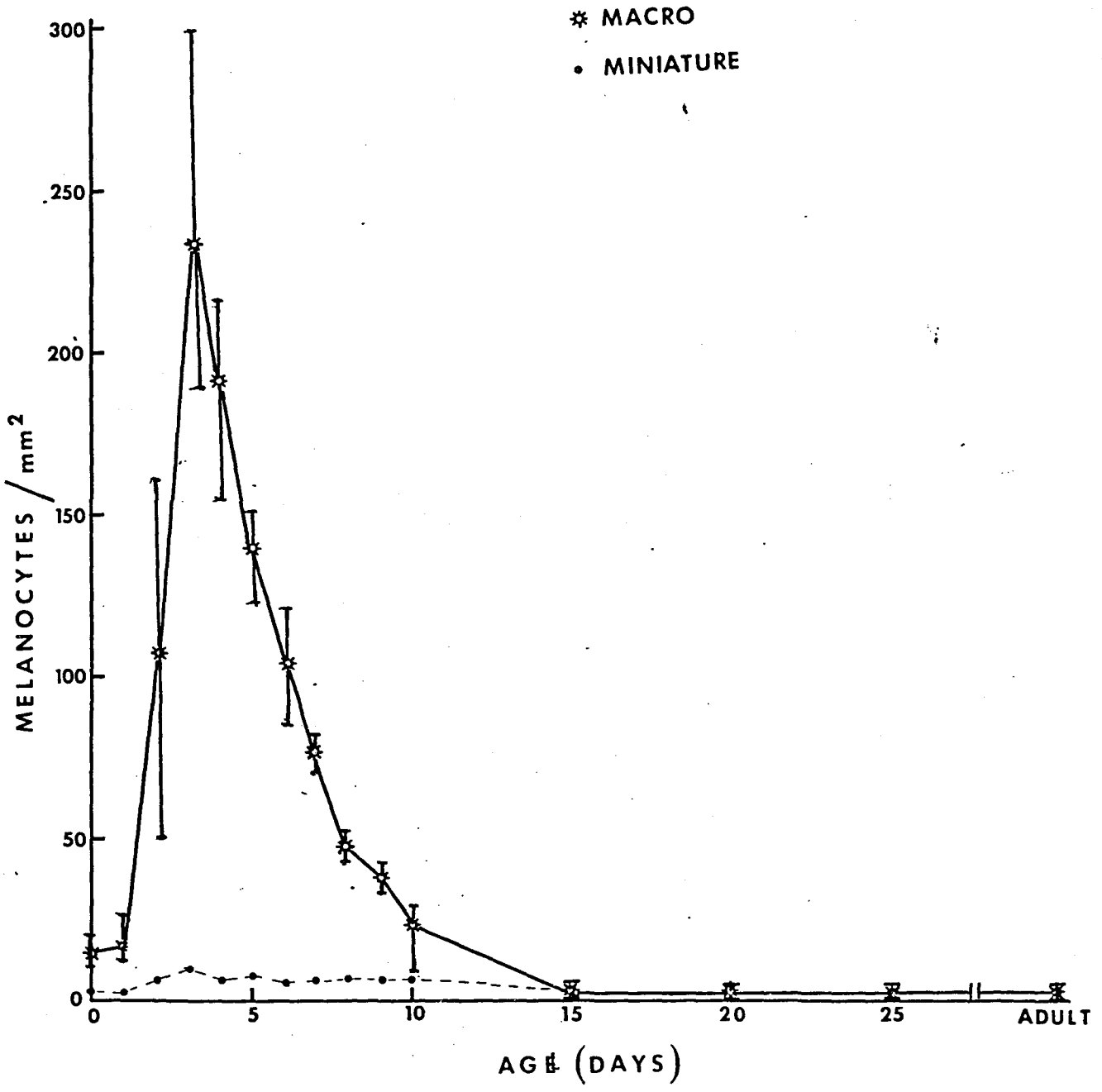

MACRO
MINIATURE
300
250
200
150
100
50
0
MELANOCYTES / mm²
0
5
10
15
20
25
ADULT
AGE (DAYS)

FIGURE 1

Figure 2. Melanocytes per mm^2 versus chorioallantoic graft culture period (days) for populations of macromelanocytes and miniature melanocytes.

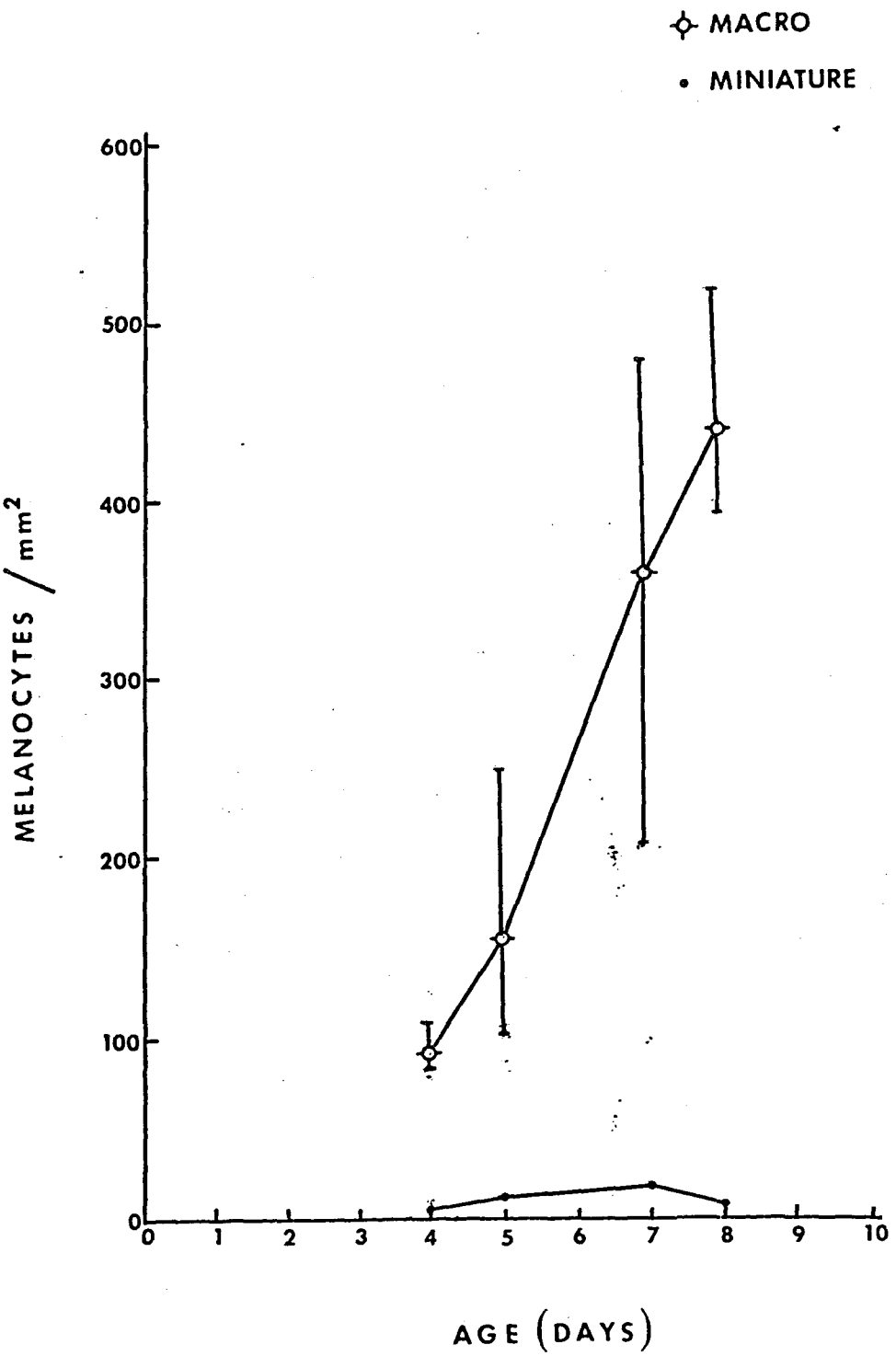

MACRO
MINIATURE
600
500
400
300
200
100
0
MELANOCYTES / mm²
0
1
2
3
4
5
6
7
8
9
10
AGE (DAYS)

FIGURE 2

Figure 3. Melanocytes per mm^2 versus age (days) for populations of macromelanocytes in control and chorioallantoic membrane graft groups.

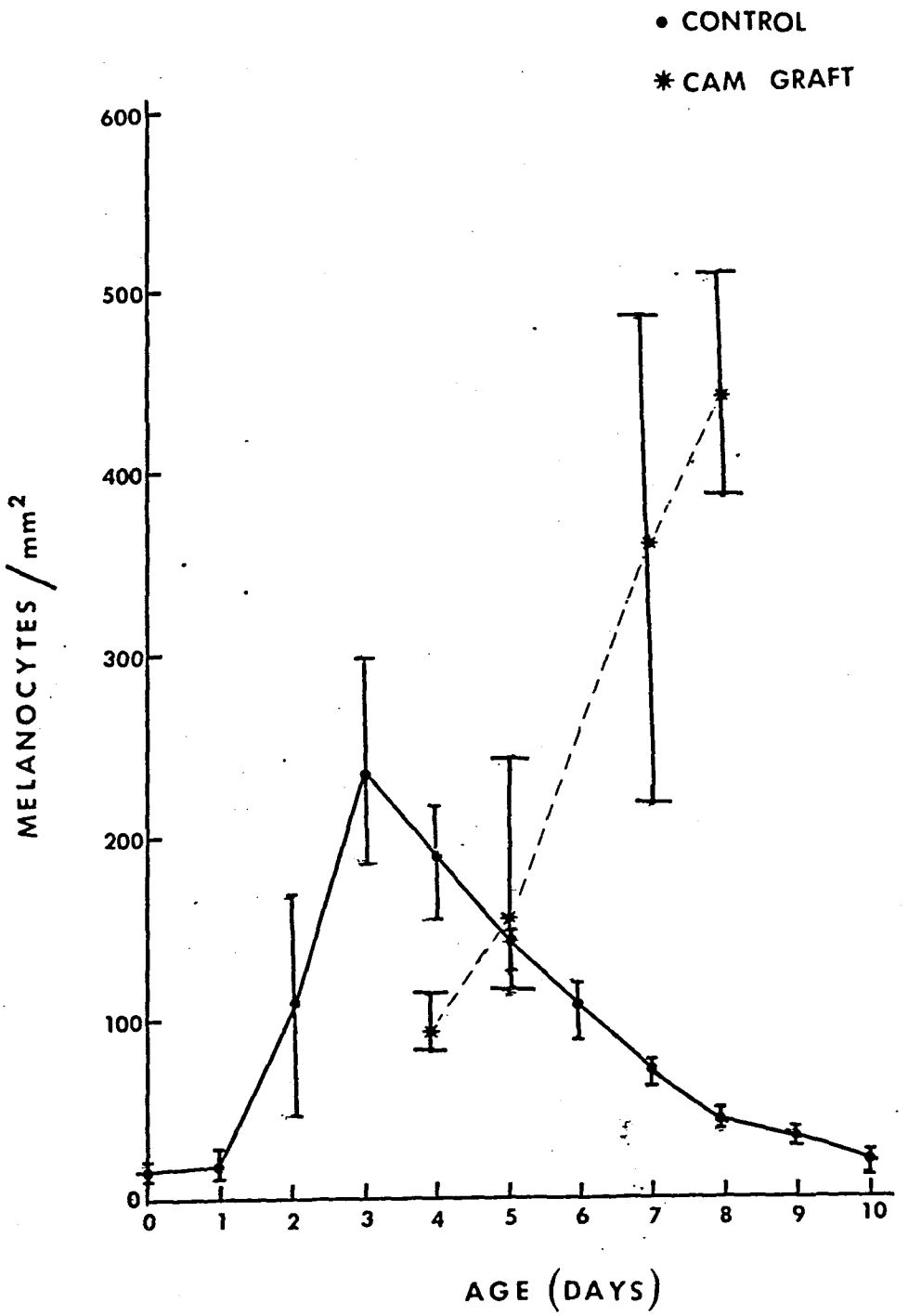

• CONTROL
✱ CAM GRAFT
MELANOCYTES / mm²
600
500
400
300
200
100
0
0
1
2
3
4
5
6
7
8
9
10
AGE (DAYS)

FIGURE 3

Figure 4. Melanocytes per mm^2 versus age (days) for populations of miniature melanocytes in control and chorioallantoic membrane graft groups.

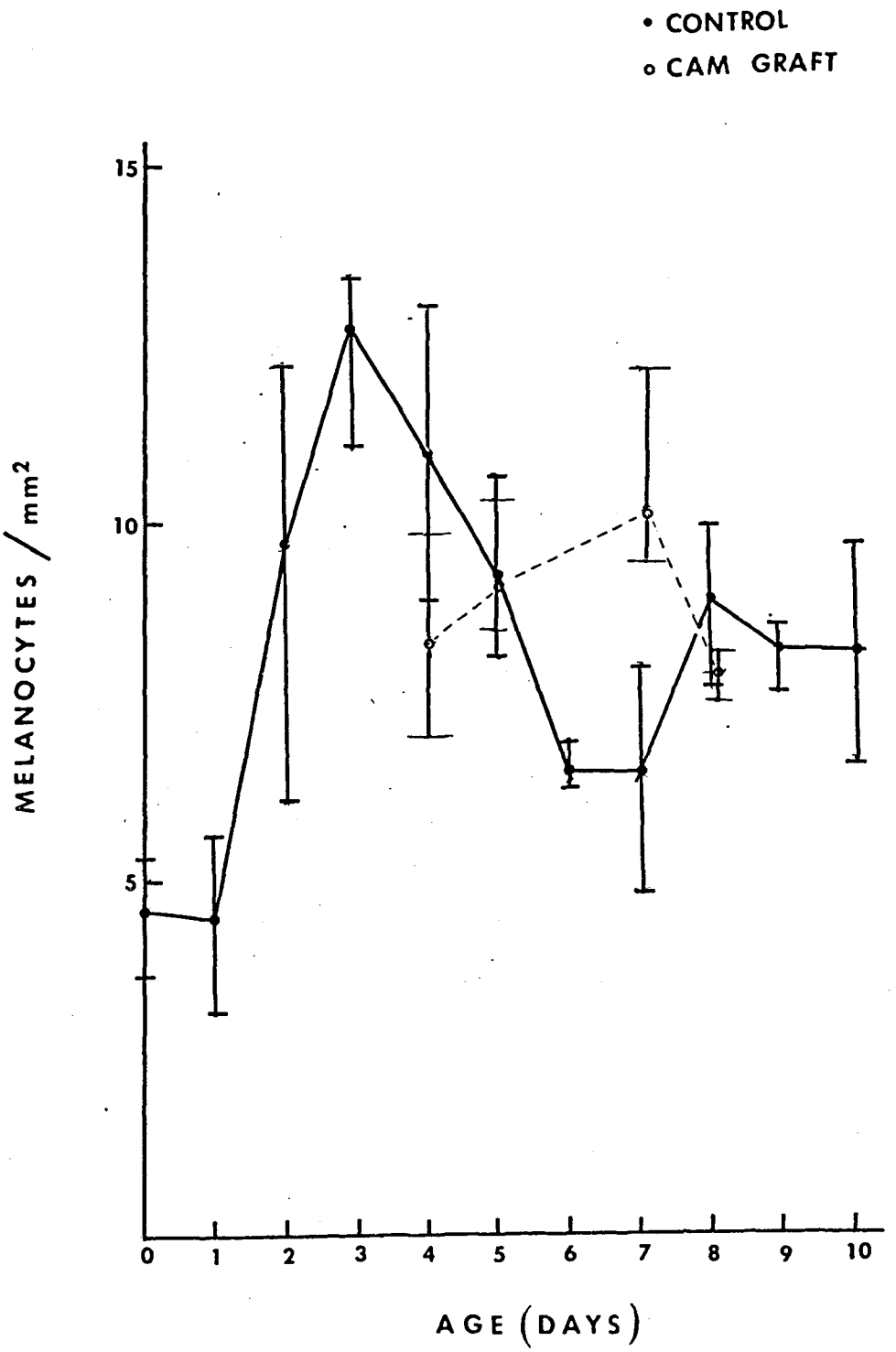
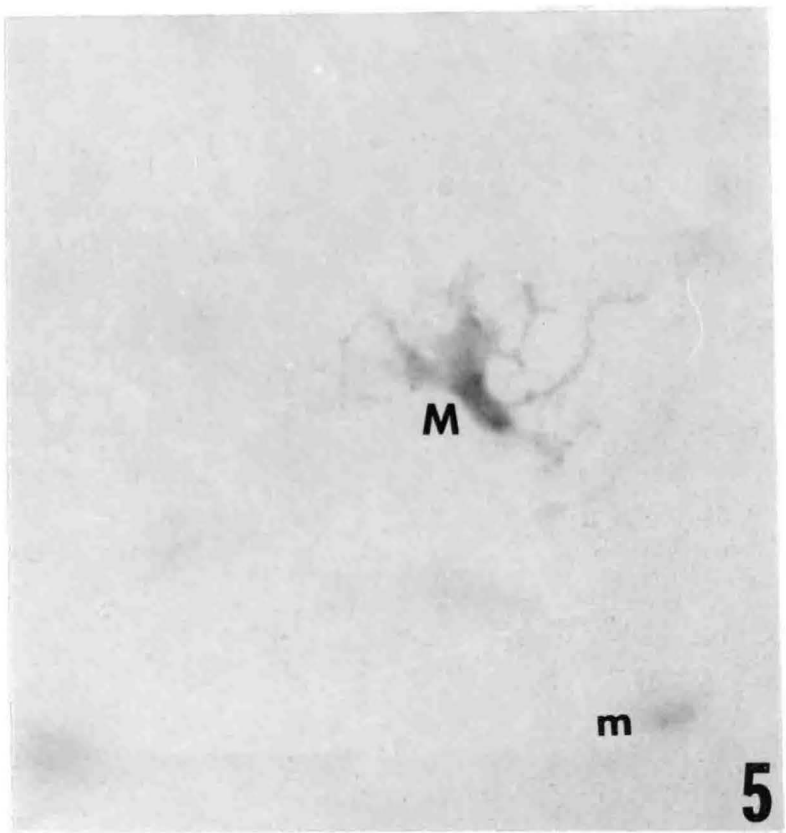

• CONTROL
o CAM GRAFT
MELANOCYTES / mm²
15
10
5
0
1
2
3
4
5
6
7
8
9
10
AGE (DAYS)

FIGURE 4

Figure 5. A typical epidermal melanocyte (M) from a white macule of an individual with tuberous sclerosis. The dark area (m) is a miniature melanocyte not in focus. DOPA reagent. X 1000.

Figure 6. A miniature epidermal melanocyte (m) from a white macule of an individual with tuberous sclerosis. DOPA reagent. X 1000.



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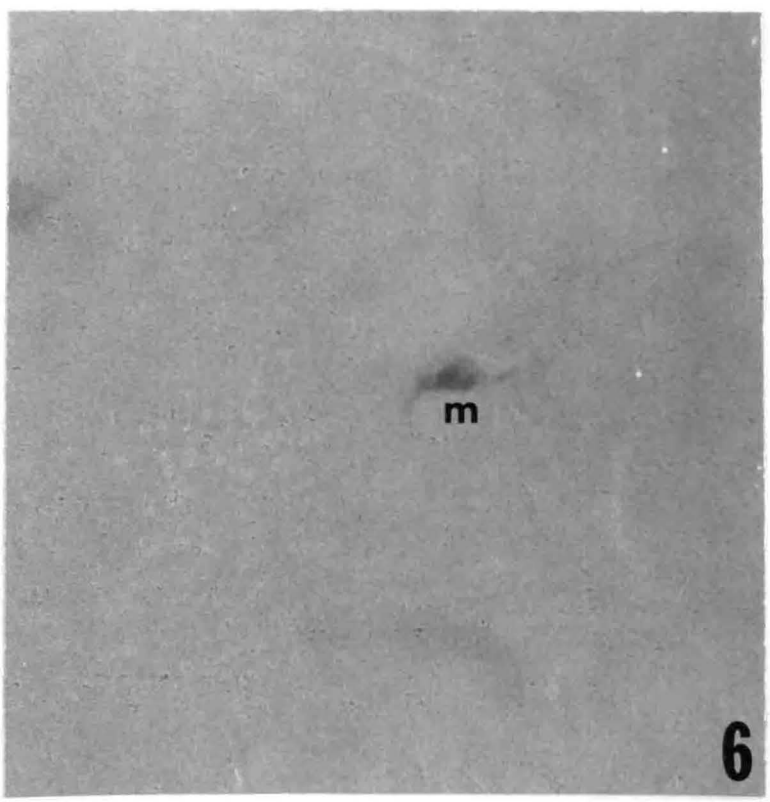

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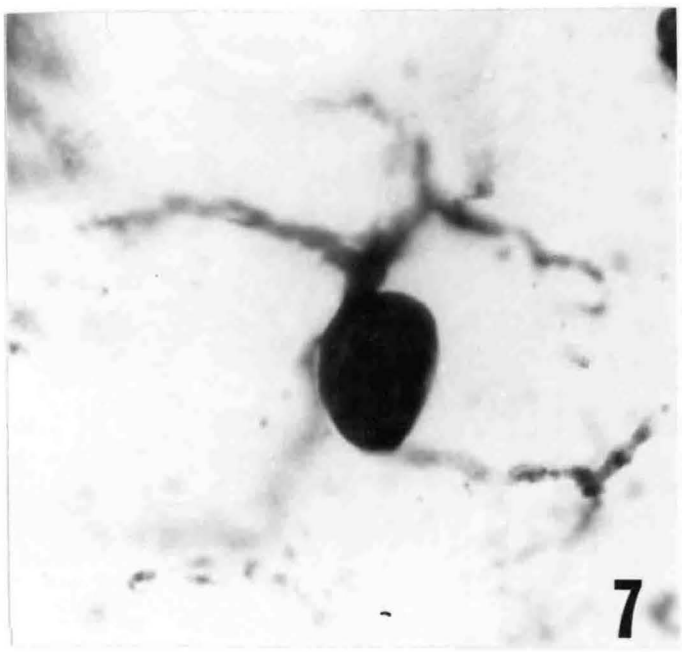
Figure 7. A typical epidermal melanocyte in the dorsal region of the PET/Wmr mouse. DOPA reagent. X 450.

Figure 8. A miniature epidermal melanocyte in the dorsal region of the PET/Wmr mouse. Note DOPA reactivity and size with respect to figure 7. DOPA reagent. X 450.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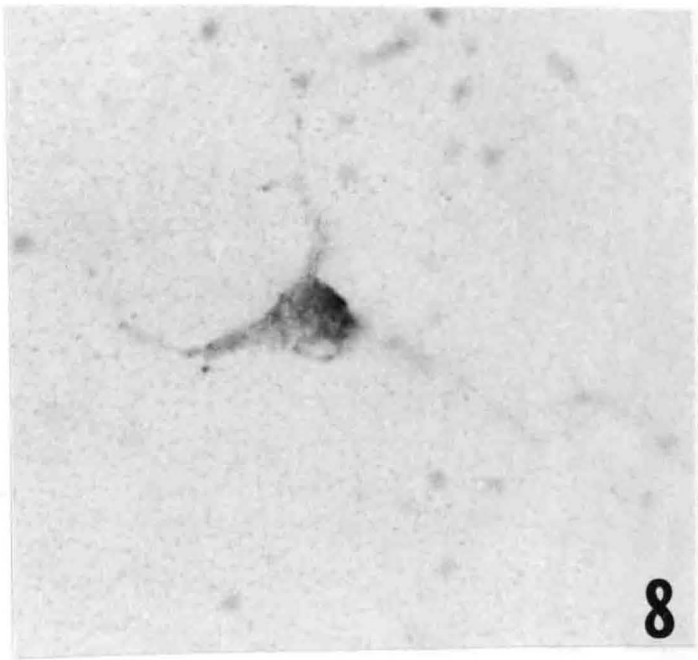
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Figure 9. Epidermal melanocytes of a PET/Wmr dorsal skin section incubated 7 days on the chorioallantoic membrane. DOPA reagent. X 450.

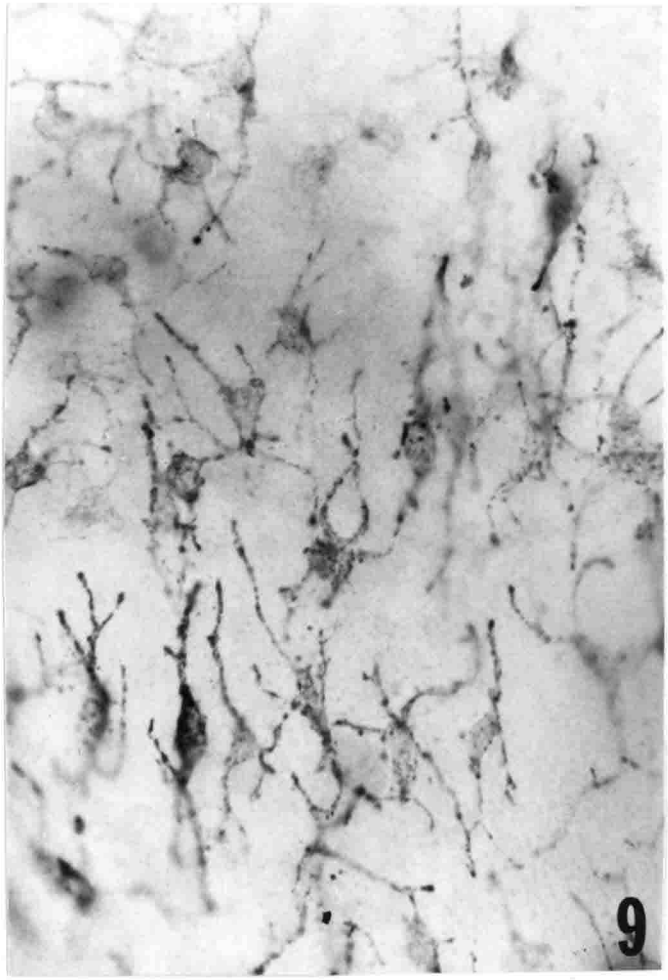
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Figure 10. A 7 day chorioallantoic membrane graft of PET/Wmr dorsal skin showing typical (M) and miniature (m) melanocytes in the epidermis. DOPA reagent. X 450.

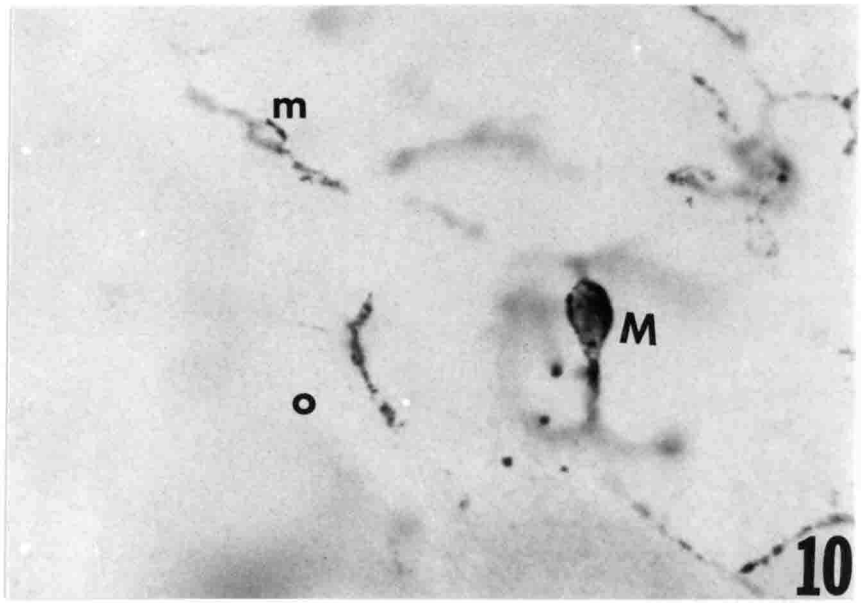
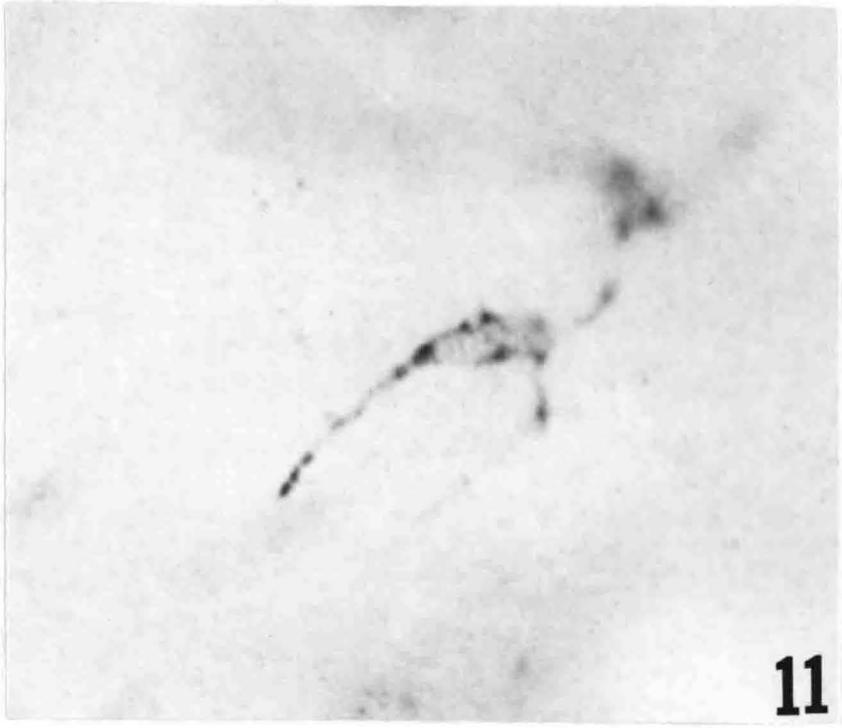

m
M
o
10

Figure 11. A miniature melanocyte in the epidermis of a PET/Wmr dorsal skin specimen incubated 7 days on the chorioallantoic membrane. DOPA reagent. X 450.



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VITA

John Thomas Earnhardt was born in Salisbury, North Carolina June 6, 1949. He received his elementary and secondary education in the Salisbury City School System graduating from Boyden High School in 1967. He entered Lenoir Rhyne College in Hickory, North Carolina and graduated with a B. S. degree in Biology in May 1971. In September of the same year he entered the Graduate School of the University of Richmond. During this time he became a member of Beta Beta Beta Biological Honor Society, was a recipient of a Williams Fellowship, and assisted various laboratories. He was invited and attended the International Pigment Cell Conference at Yale University during his final semester of graduate studies. He received the M. S. degree in Biology in May 1973.