HUMAN MELANOCYTES IN TISSUE CULTURE*

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Tissue culture has furthered the knowledge of cytology in many fields. It is a method by which cells can be studied alive and proliferating outside the body for reasonable periods of time. This discipline seems to offer a promising approach to the elucidation of debated questions concerning melanocytes. However, a review of the literature does not reveal any reports dealing especially with the behavior of normal human melanocytes in tissue culture, although normal pigment cells of lower animals and mammalian and human malignant melanomas (1–9) have been cultured.

The present study reports observations made on human melanocytes from normal skin and benign pigmented nevi grown *in vitro* by the roller tube technic.

MATERIALS AND METHODS

The roller tube method of cultivation of human skin has been reported in an earlier paper (10). Tissues studied included both white and colored preputial skin of infants and benign pigmented nevi of intradermal and compound type. The nevi were removed by excision. Usually, a portion of normal skin was included in the specimen. The specimen was bisected into two parts; one was used for culture and the other for histological examinations. Before explantation, the subcutaneous tissue was trimmed away from the specimen and discarded. The specimen was divided into three portions: the first part consisted of normal skin surrounding the growth; the second part contained the epidermis above the nevus which, of necessity, included a thin layer of dermis immediately beneath it; and the last part consisted only of the dermal portion of the nevus. These three portions were explanted separately.

The culture medium consisted of a plasma-embryonic-extract clot and a fluid nutrient of balanced salt solution, embryonic extract, and human ascitic fluid. Pieces of tissue approximately 2 mm. square were embedded in the clot on a 12 x 50 mm. #1 coverslip which was then placed in a test tube with 2 ml. of nutrient fluid. The tubes were incubated at 37° C. in a roller drum which rotated at a speed of 12 revolutions per hour.

Various methods were employed in the study of these cultures. These included the following:

I. Examination of living cells by the utilization of phase contrast microscopy.

II. Fixed specimens stained with May-Gruenwald and Giemsa reagents according to the procedure described by Jacobson and Webb (11).

III. Dopa reaction for tyrosinase. The procedure followed essentially that described by Laidlaw and Blackberg (12) with the exception that no preliminary formalin fixation was employed.

IV. Silver reaction for demonstration of argentaffine granules. Masson's (13) ammonium silver nitrate technic was employed. A slight modification was required for its use in tissue culture preparations.

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Presented at the Seventeenth Annual Meeting of The Society for Investigative Dermatology, Inc., Chicago, Illinois, June 9, 1956.

A melanocyte, for the purpose of this study, was defined as a cell which either gave a positive dopa reaction or which could be shown to contain argentaffine granules where no granulation had been apparent in the living cell. The presence of uniform-sized brown pigment granules in a cell was considered presumptive evidence only, inasmuch as normal epithelial cells may also contain such granules. With experience gained by using these criteria, melanocytes were recognized also in the unstained living specimens and in preparations stained with the May-Gruenwald-Giemsa technic.

RESULTS

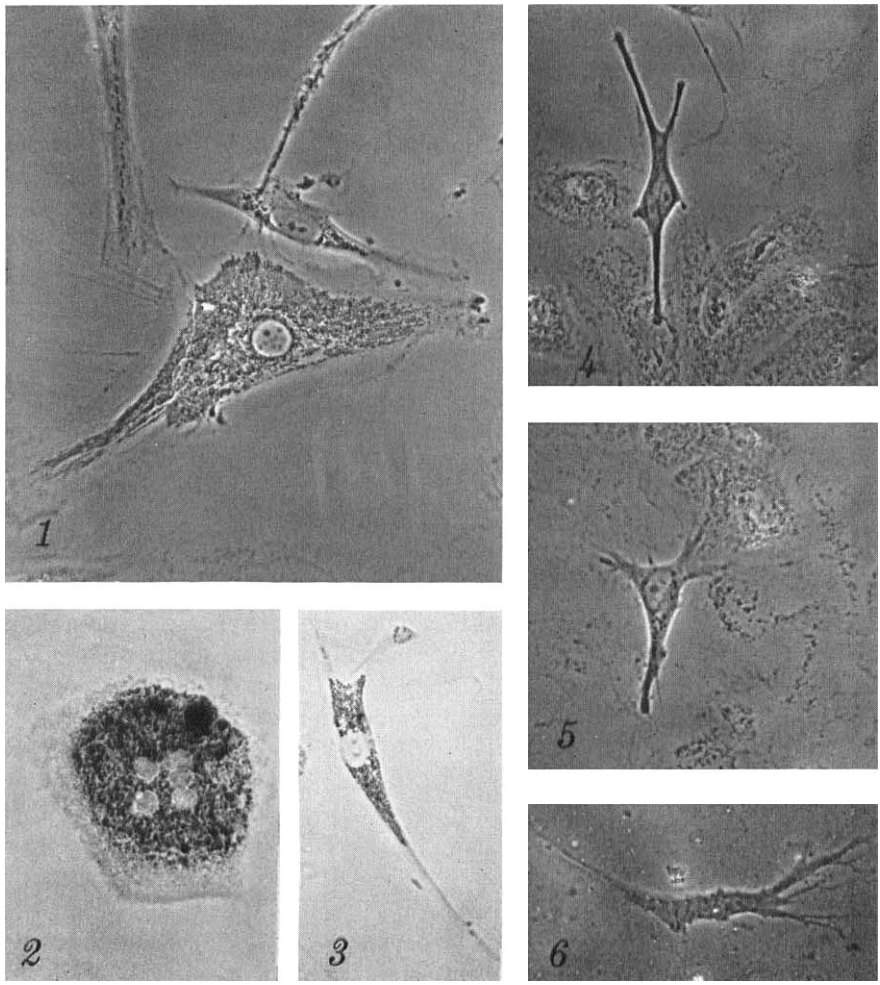
In tissue culture of infantile foreskin, employing the roller tube technic, epithelial outgrowth in sheets usually began to appear in five to six days and continued to increase in size up to three to four weeks. After an incubation of from ten to fourteen days, a sheet of epithelial cells formed evenly around the explant; these sheets of cells usually were about the same width as the diameter of the explant. Mitoses were most numerous in the first two weeks, gradually diminishing in number toward the latter part of the incubation period. The skin portion of the nevus preparations behaved similarly to the normal foreskin. Often the outgrowth was less luxuriant and uniform than that of foreskin. In the later stages of incubation, fair amounts of fibrocytic elements were seen in both normal skin and nevus cultures. The dermal portion of nevi usually had only scanty outgrowth of fibrocytes.

Observations on Cultures of Normal Skin

The melanocytes were more numerous in regions close to the explants. In order to obtain good visualization, the topmost layers of epidermal portions of the explants were removed before examination. This was easily accomplished since, under the conditions of tissue culture, the upper and lower portions of the epidermis tend to separate. The upper strata of cells may be removed without disturbing the outgrowing epithelial sheet or the underlying dermis anchored to the coverglass. This thin layer of dermis tends to contract so that the epidermis above extends out with overhanging edges. The melanocytes were found in great numbers in that portion of the outgrowing epithelial sheet immediately beneath the overhanging edges described above. After the removal of the upper epidermal portion, which consisted mainly of keratinized cells, the pigment cells could be examined in detail. More peripherally, the melanocytes were scattered in between the outgrowing epithelial cells. Here the intimate relationship between the melanocytes and the epithelial cells of the epidermis could be studied.

I. Phase Contrast Microscopic Examination

In living and unstained preparations, even with the use of phase optics, there were difficulties in differentiating fibrocytes, macrophages, and true melanocytes (Figs. 1–6). All may have processes and contain granules. "Lipoprotein" granules are present in many types of cells and are similar in size and appearance to melanin pigment. The distribution of granules within the cells likewise is not a



FIGS. 1–6. Cells other than epithelial cells found in tissue cultures of normal human skin and nevi. Phase contrast microscopic examinations of living unstained preparations. 430×.

FIGS. 1 & 3. Fibrocytes. Note the oval or round nuclei with distinct nucleoli and perinuclear collections of granules. A small amount of granules are also seen in the cytoplasmic processes.

FIG. 2. Macrophages with ingested materials.

FIGS. 4, 5 & 6. Melanocytes in cultures of pigmented nevi. Note the granular cytoplasm, and dendritic processes with secondary branching.

reliable index for their differentiation. One sometimes sees granules in the processes of a fibrocyte. There are also melanocytes which do not contain visible granules.

The macrophages, when filled with ingested materials, were easily recognized. They appeared as large, globular cells containing clumps of granules and vacuoles. Fibrocytes were the main problem; this was especially true when large numbers

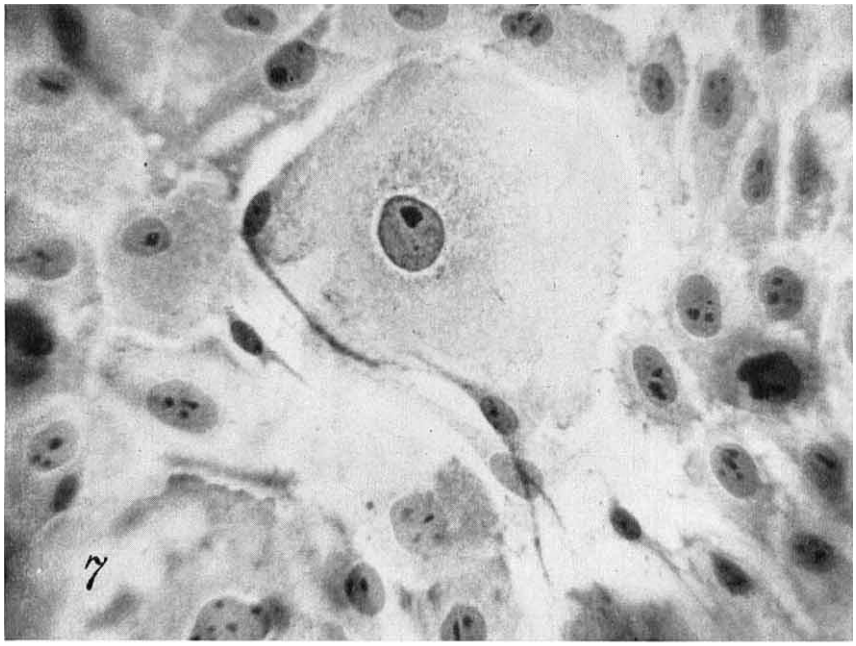
of these cells were present in the culture. The processes of the bipolar melanocytes came off of the cell body more abruptly than those of the fibrocytes. The melanin granules were evenly dispersed in the cytoplasm of the melanocytes, while the granules in the connective tissue and epithelial cells were usually limited to perinuclear zones. The nuclei of melanocytes sometimes were not clearly visible owing to the presence of melanin granules in the same focal plane, while those of epidermal and fibrocytic cells were easily seen with well-defined outline and one or two nucleoli.

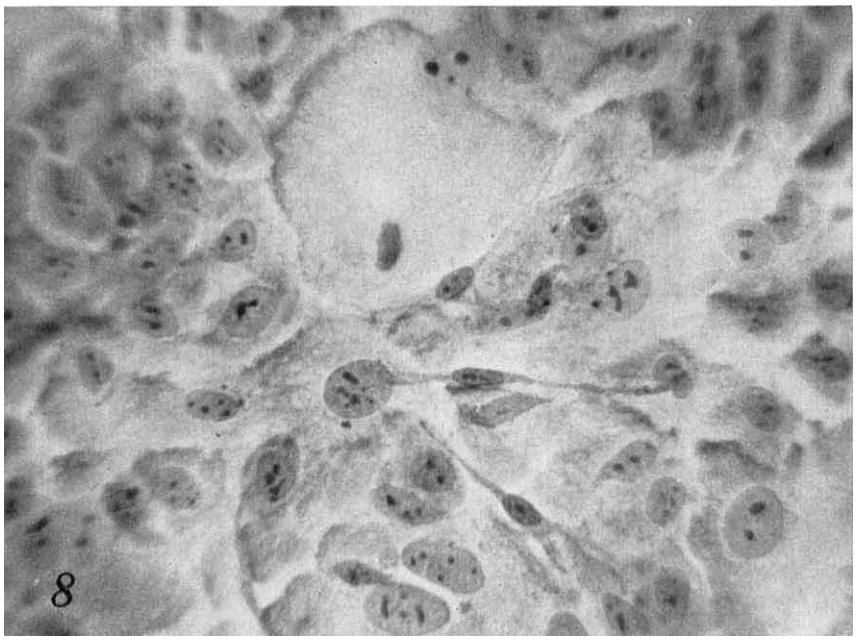
II. Cultures Stained with May-Gruenwald-Giemsa Reagents (Figs. 7–12)

With this stain the thick explant was colored too deeply for visualization of individual cells. Only the cells in the outgrowing sheet could be studied. There was no apparent difference in the number of melanocytes found in cultures of white or Negro skins. The main difference was the amount of melanin granules the cells contained. In cultures of Negro skin, fine granules of greenish-black color were seen evenly distributed in the cytoplasm (Figs. 9–11), while there was little or no pigment seen inside cells derived from white skin. Also, the melanocytes of Negro skin seemed to have more processes and branches; the difference, however, was not great. A few epithelial cells were found with melanin granules clustered around the nucleus, although this was not a constant feature. Clumps of extracellular pigment were often observed in and around the explants of colored skin. Sometimes there was difficulty in differentiating melanocytes from young fibrocytes (Figs. 11–12). With experience, the following features may be distinguished. The nuclei of melanocytes are oval and similar to those of fibrocytes; they differ from those of fibrocytes in being smaller, more uniform in size and shape, and more compact. The cytoplasm of pigment cells, even in the absence of melanin, stains slightly bluer; the nucleus stains more red than that of fibrocytes. There is only a thin shell of cytoplasm around the nucleus, and the cytoplasmic processes come off this small cell body rather abruptly as its filaments. The dendritic processes of melanocytes are very slender and delicate and do not give the tapering fan-like effect usually seen in the fibrocytes. The cytoplasm of melanocytes has a fine, granular appearance, while that of the connective tissue cells is somewhat fibrillar.

Although mitotic figures were found in fair numbers in the outgrowing sheets, no characteristic features were present to indicate their definite origin. It is believed that active proliferation of all three types of cells, i.e., epithelial cells, fibrocytes, and melanocytes, did occur, even though the rate of multiplication may be different. The pigment cells, as described above, are believed to be the relatively younger forms. As will be discussed later, these cells seem to differ somewhat from the cells seen in the original explants. The pigment cells of the latter probably represent a more mature form of melanocyte.

The majority of the melanocytes were bipolar cells with processes extending from the cell body at its greater diameter (Figs. 7–11). Some had more than two processes or showed bifurcation of one or two of the processes. When the cells were bipolar, the dentrites were either short or long. When multiple processes were present, they usually were short and the cell assumed a stellate appearance.

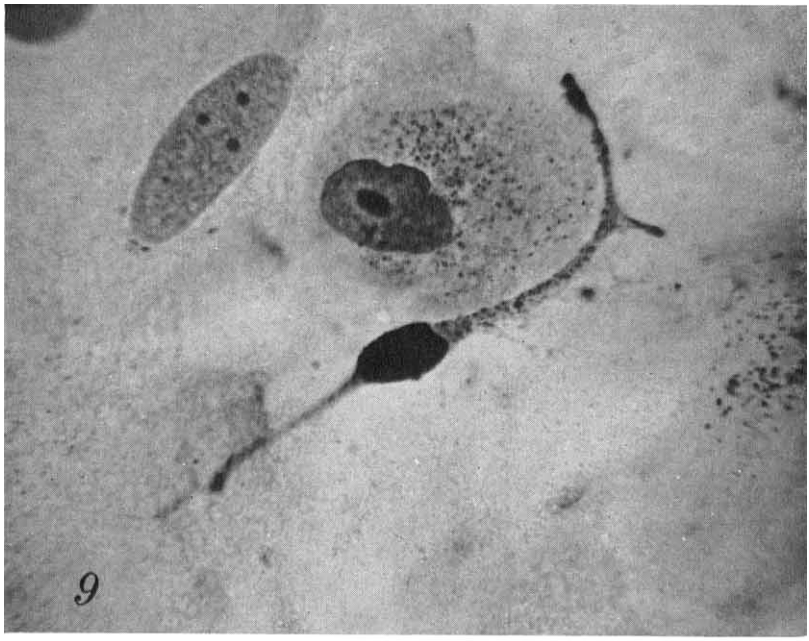


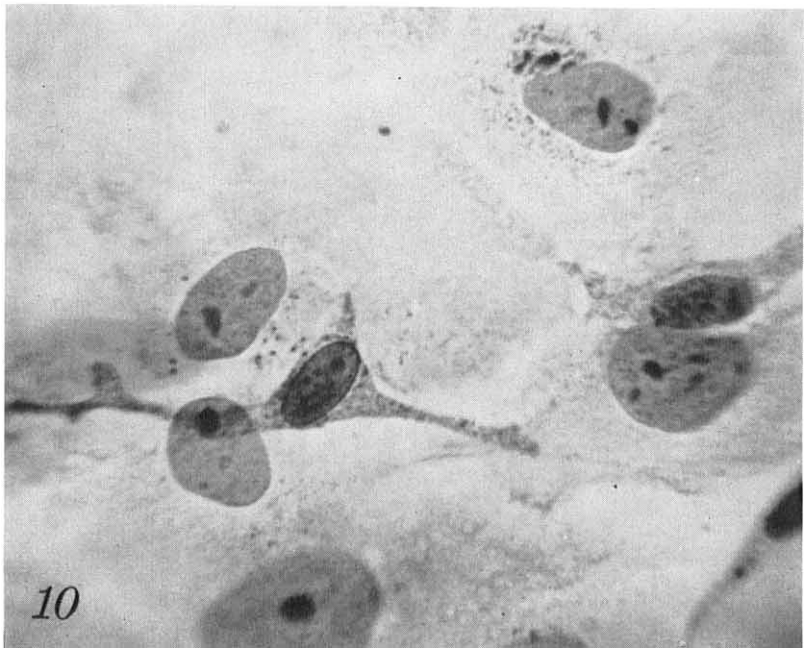


FIGS. 7–8. Melanocytes and epithelial cells of normal Negro foreskin cultures. May-Gruenwald-Giemsa stain. 430×.

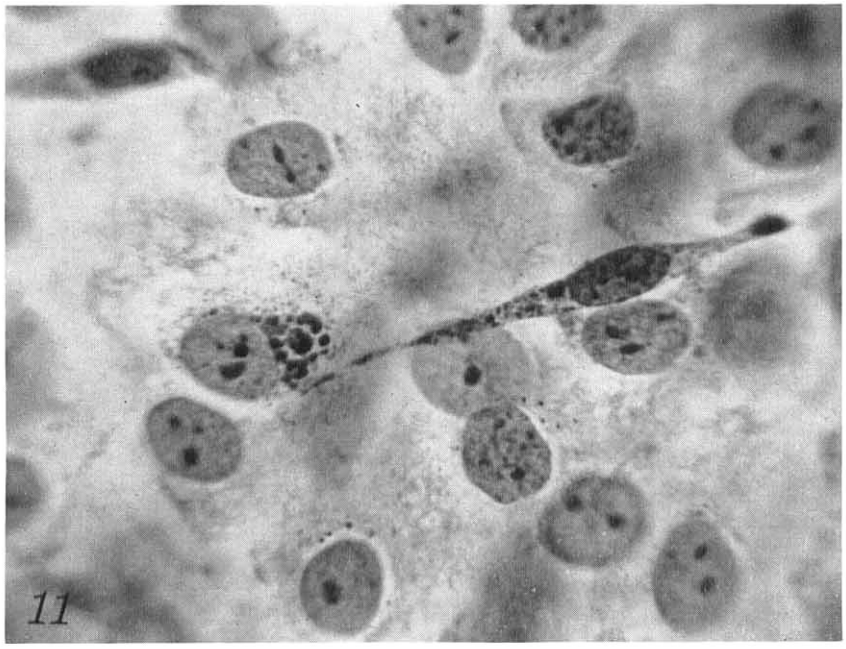
FIG. 7. A melanocyte encircling a large epithelial cell with its two long processes.

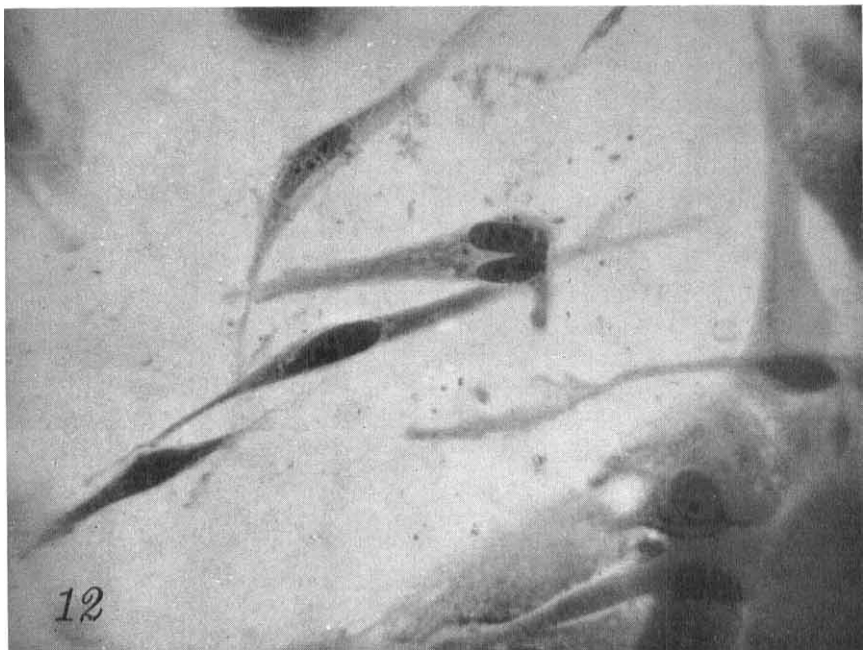
FIG. 8. A bipolar cell with one process closely applied to the periphery of a large epithelial cell and another branch of the same process applied to another epithelial cell close by.





FIGS. 9–10. Melanocytes and epithelial of normal Negro foreskin. May-Gruenwald-Giemsa stain. 970×. High magnifications showing the intimate relationship between the melanocytes and epithelial cells. Note the uniform sized pigment granules (stained greenish black in these preparations) evenly dispersed in the entire cytoplasm of the melanocytes.





FIGS. 11–12. Melanocytes and fibrocytes. May-Gruenwald-Giemsa stain.

FIG. 11. A bipolar melanocytes with one process extending close to the nucleus of an epithelial cell. Note collection of pigment granules in form of nuclear capping adjacent to the process of the pigment-forming cell. 970×.

FIG. 12. Fibrocytes. The resemblance between melanocytes and some of the fibrocytes is apparent. The fibrocytes are usually larger and their cytoplasm appears somewhat fibrillar. 430×.

Pigment may or may not be present; when present, the granules were very fine and distributed evenly throughout the cytoplasm including the processes. These cells were usually seen closely applied to the surrounding epidermal cells. This might serve as another feature to distinguish them from fibrocytes which were seen scattered in between epithelial cells without establishing intimate relations with them.

III. Dopa Reaction

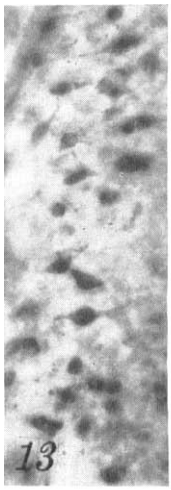
With the use of dopa reagent, the emigrating pigment cells, as well as those on the original skin explant, may be visualized. The melanocytes on the explants were seen in a syncytial arrangement (Figs. 13 & 16) very similar to the picture shown by the split skin technic (14–17). Actually the skin was separated without the additional use of chemical or enzymatic action (18–19), and the split was intraepidermal instead of at the epidermodermal junction. In general, the pigment cells seen on the explants (Figs. 13–16) had larger cell bodies with more and longer branches than those described previously. Outside the explant there were dopa-positive cells very similar to those found in the May-Gruenwald-Giemsa stained preparations, i.e., bipolar and multipolar cells.

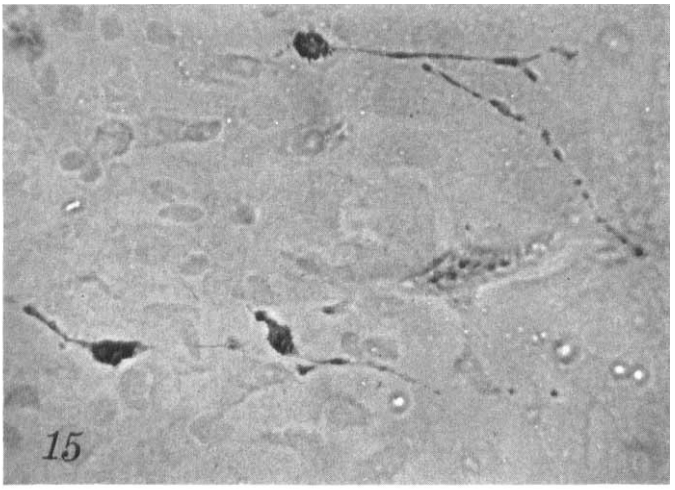
The intensity of the dopa reaction varied with different specimens, but it was always more prominent in pigmented than in white skin. In the latter, the granules were of a very light color, although still distinguishable. The epithelial and fibrocytic portion of the outgrowth were not stained by this technic, thus indicating the specificity of the reaction. It is known that in tissue sections, leukocytes are blackened by dopa because they contain non-specific oxidases (20). Sometimes, in our cultures, round homogeneously black bodies approximately the size of nuclei were evident. The origin of these bodies was not known; these could represent degenerated leukocytes. Also, there appeared cells with darkly stained, pyknotic nuclei and faintly detectable cell boundaries; it is possible that these represented degenerated epidermal or fibrocytic cells. Why such elements take on the black color cannot be explained. This, however, does not represent a true dopa reaction. The melanocytes are always granular instead of solid black. Even in strongly dopa-positive and heavily pigmented cells, the melanin is distinctly visible in form of small particles (Figs. 17–22).

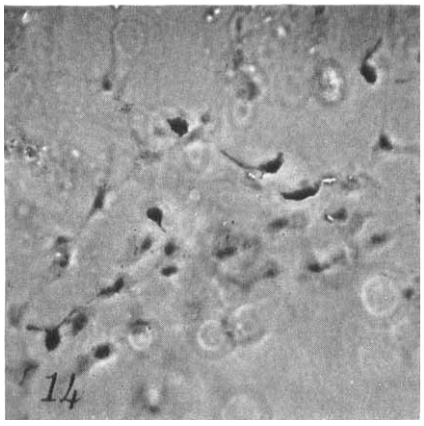
Other than the variation in the degree of dopa-positivity, the melanocytes derived from skins of different colors also differed in size, in length and number of processes, and in degree of branching. Those of white skin generally were smaller, had shorter processes and were less branched. The size and degree of branching of melanocytes found in these cultures seemed to be in direct correlation with the intensity of color of the skin explant used.

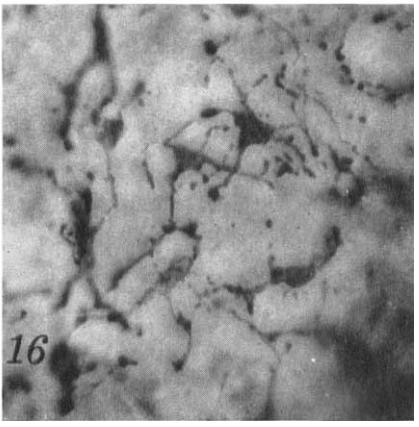
IV. Silver Reaction

By silver impregnation, melanin granules appeared in the form of black deposits (Figs. 23–27). The melanocytes had a similar appearance to those in the dopa reaction. Pigment particles were also found inside epithelial and fibrocytic cells. The pigment in these cells showed the characteristic supranuclear "cap"







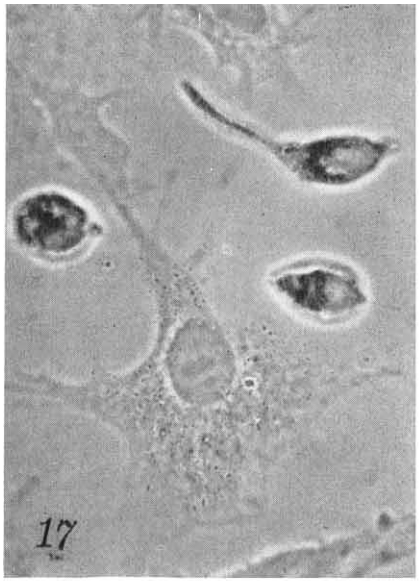


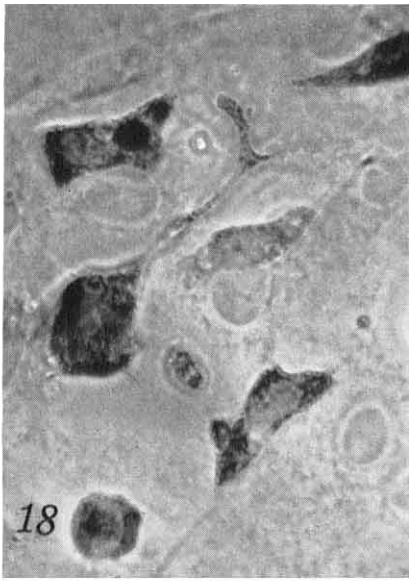
FIGS. 13–16. Dopa reaction. 430×. Phase contrast microscopy. These pictures illustrate the differences of sizes and shapes of melanocytes in cultures of white and pigmented foreskins.

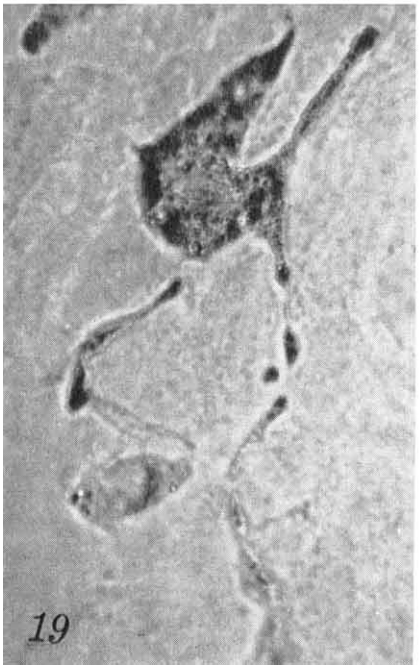
FIGS. 13 & 14. Dopa-positive cells in white foreskin cultures. Fig. 13. Melanocytes in the split portion of original explant. Fig. 14. Melanocytes in the outgrowing sheet of same. The pigment cells in the outgrowing sheet of Negro skin explant are often of approximately same size and shape as seen here, although larger ones are also present. (Fig. 15).

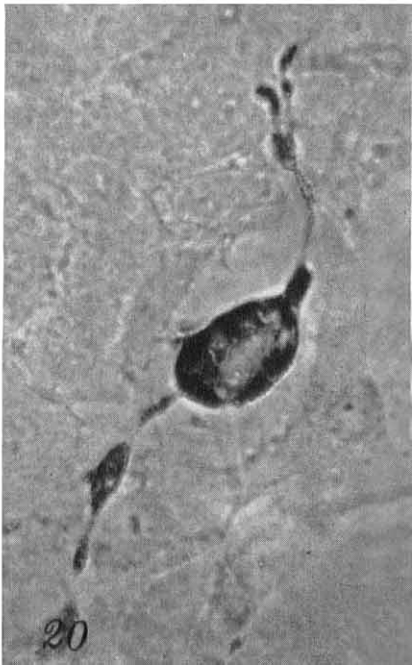
FIG. 15 & 16. Darkly pigmented foreskin. Note the highly branched cells in a syncytial arrangement in the split portion of epidermis (Fig. 16), and the slightly smaller and less branched melanocytes in the outgrowing sheet of epidermis. (Fig. 15).

pattern (15, 17). Extracellular small collections of pigment granules were sometimes seen a short distance from some of the dendritic processes. This probably represents a process first described by Ranvier known as clasmatosis (21). Clasmatosis was reported to occur in melanoblasts of the Harding and Passey mouse melanoma by Grand (22). He considered this process to be a mode of physiological excretion and one of the ways in which the melanoblasts of this particular tumor eliminate melanin.





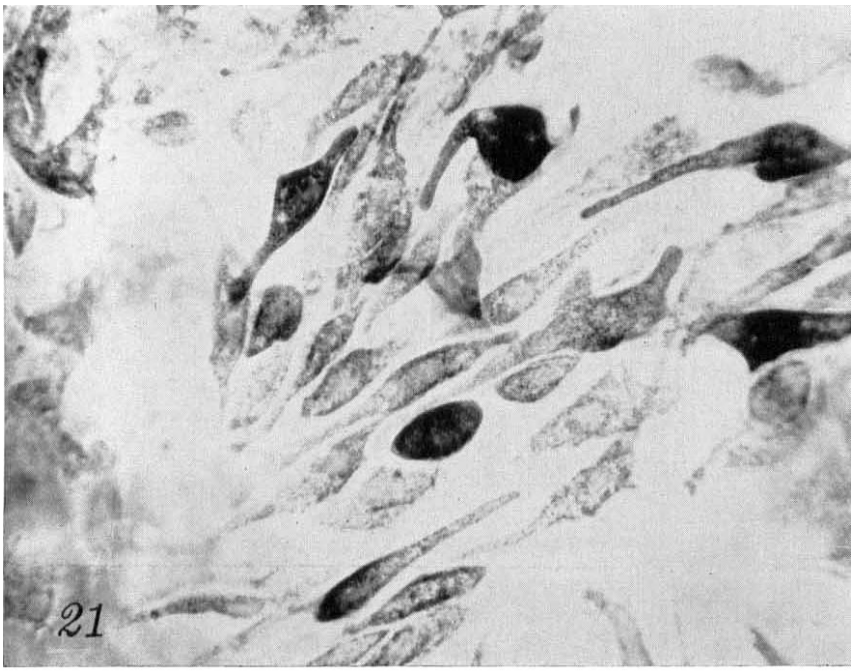


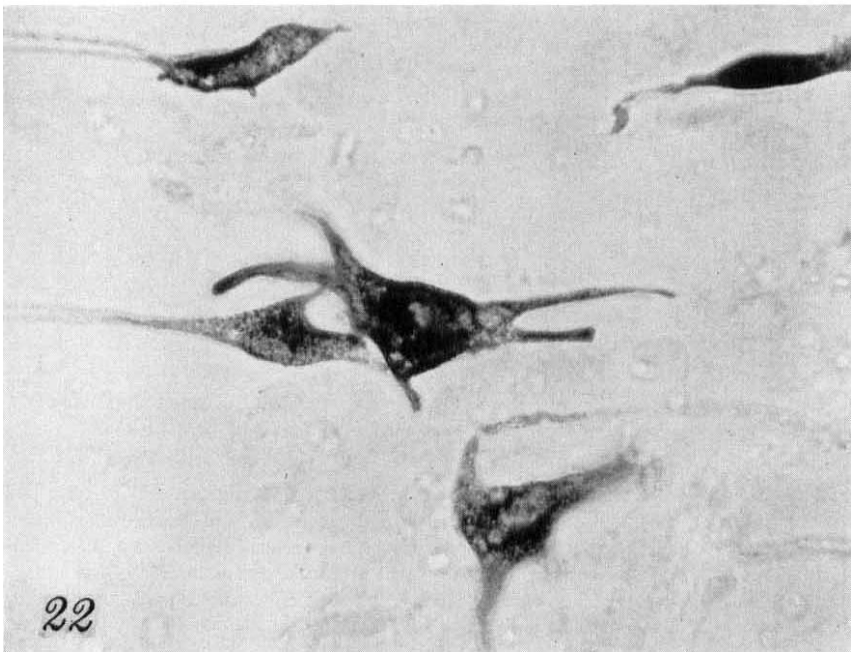


FIGS. 17–20. Dopa reaction. 970×. Phase contrast microscopy. Comparison of sizes and shapes of melanocytes in normal pigmented skin and those in pigmented nevi.

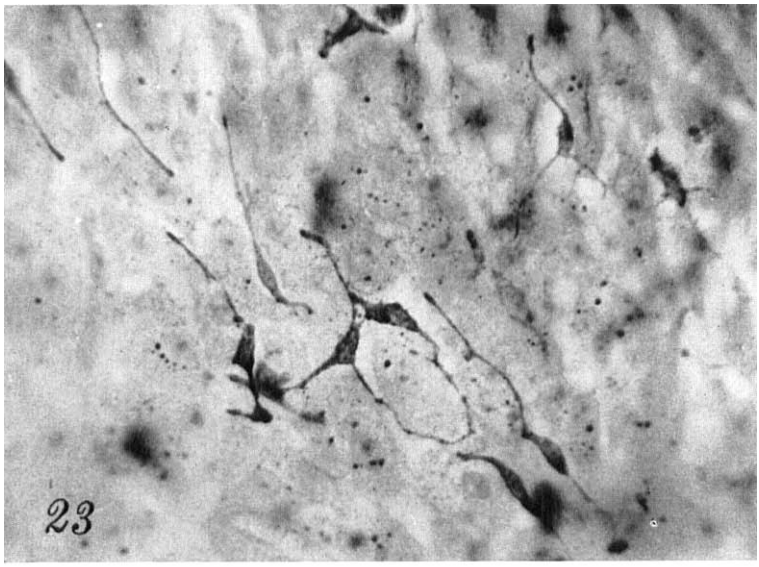
FIG. 17. Melanocytes in the outgrowing sheet of Negro foreskin cultures. Note the short processes of the bipolar cells. The light area in the cell body is the nucleus. A few large cells not darkened by dopa are epithelial cells.

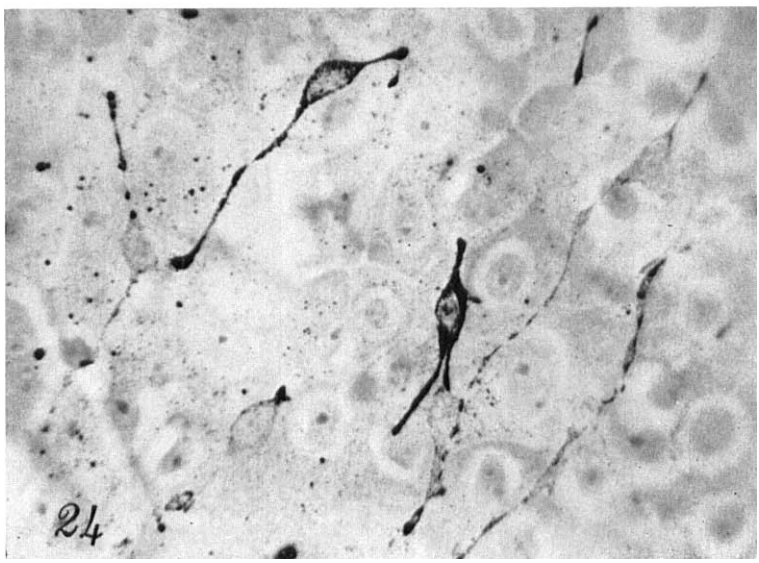
FIGS. 18, 19 & 20. Melanocytes of pigmented nevi. Note the wide variations in sizes and shapes. Some cells are not much larger than the pigment cells seen in Fig. 17 with more or less rounded contour (Fig. 18); the majority are larger cells with long and branched processes. Nodular swellings are present in some of the processes (Fig. 19 & 20).





FIGS. 21–22. Melanocytes in the dermal portion of nevus explants. Dopa reaction. 430×. Phase contrast microscopy. Nevus cells of various sizes and shapes; some are strongly dopa positive, some, only weakly positive. All are definitely granular.





FIGS. 23–24. Silver reaction. Melanocytes in cultures of pigmented foreskin. 430×. Phase contrast microscopy. Fine silver deposits in the cytoplasm of melanocytes including the dendritic processes. The epithelial sheet is in the background. Scattered silver granules are also seen in the epithelial cells in the cytoplasm surrounding the nucleus. Small amounts of extra cellular silver deposits are also visible. Fig. 23 shows anastomosis of cytoplasmic processes of some of the dendritic cells.

Melanocytes in Nevi

The pigment cells were usually found in cultures grown from the superficial portions of nevi (Figs. 18–20). Two types of melanocytes were observed, the

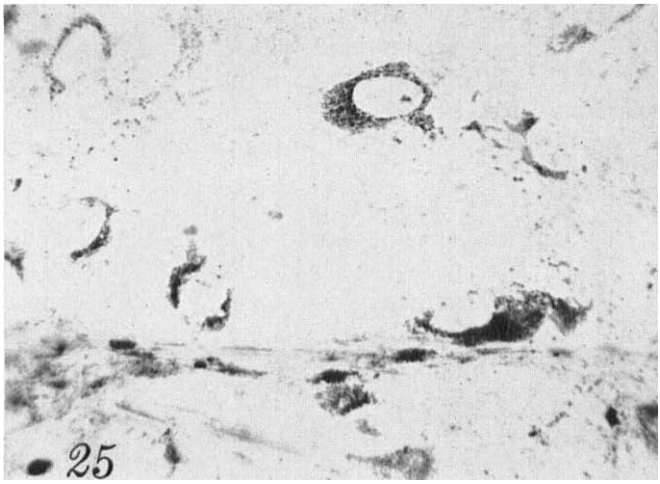
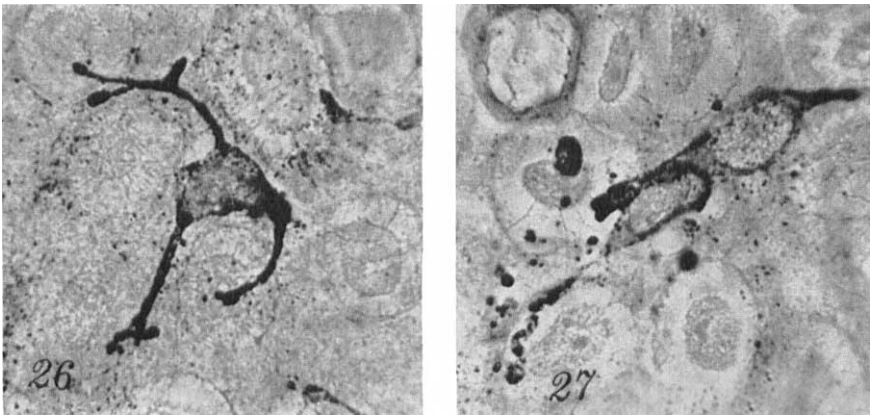


FIG. 25. Silver reaction. 430×. Epithelial outgrowth of normal pigmented foreskin with a few fibrocytes at the lower one-third of the picture. Note the perinuclear deposits of silver in the epithelial cells and fibrocytes. Nuclei and cell outlines are not visible.



FIGS. 26–27. Melanocytes of pigmented foreskin. 970×. Phase contrast microscopy. Note the highly branched melanocytes, with processes either encircling the periphery of the epithelial cells or lying above some of the nuclei. Extracellular clumps of black granules close to the tips of some of the processes of the melanocytes (Fig. 27). This phenomenon probably represents what is known as clasmatosis.

small type as ordinarily seen in normal epidermal outgrowth and a large variety. The latter were at least 2 to 4 times larger than the small type, reacted strongly to dopa reagent and became filled with black granules. Some cells were seen to have nodular swellings along the course of the dentrites, some had "end-organs" in form of "caps" or "end-buttons" as described by Billingham (15). These features, however, except the size, were not characteristics of nevi alone; cells of similar appearance were also seen in some of the normal skin explants.

The deep, purely dermal part did not show active growth, but in some intra-

dermal nevi there was a fair number of melanocytes either on or very close to the explants in the form of aggregates of darkly pigmented, dopa-positive cells (Figs. 21–22). These were distinctly larger cells than those seen in the normal proliferating epidermis; they were either round, fusiform, or irregular in shape. These probably represented nevus cells present in the dermis prior to explantation.

DISCUSSION

Our results clearly indicate the presence in normal human epidermis of two distinct types of cells which differ morphologically and functionally, as well as biochemically. This observation is in agreement with the concepts held by Masson (13), Becker, Sr. (22), and others, and with the findings of Billingham (15), who concluded that the mammalian epidermis is a compound tissue composed of at least two distinct cellular elements, the dendritic cells and the ordinary epithelial cells. Both types of cells are self-sufficient and capable of maintaining themselves in relatively constant numbers in the epidermis and of proliferation following stimulation.

The present study also confirmed both Billingham's (16) and Szabo's (24) findings that, in corresponding sites, white and pigmented skins have approximately equal numbers of melanocytes per unit area, although the population density of melanocytes varies in different regions of the body. The degree of skin color depends upon the pigment-forming capacity of individual melanocytes.

The capacity of pigment production of the melanocytes is determined genetically. Thus, in the Caucasians, the melanocytes are only capable of carrying out this function in a limited fashion; while in colored races, this inherent potentiality is greater. Hyperpigmentation occurs following certain stimuli to which the melanocytes respond with increased activity in pigment synthesis.

It may be mentioned once more that, in our cultures melanin granules were present not only in the dendritic cells, but also in some epithelial cells and fibrocytes. Some or all of the pigment in the outgrowing epidermal cells may have been carried along from the original explant. The presence of melanin in fibrocytes must be attributed to phagocytosis. It is well known that all types of cells exhibit phagocytosis in tissue cultures (25).

In tissue cultures of melanomas of man, mouse, and fish, Grand and Cameron (7) described two types of melanoblasts, small and large. In the small type, the branches of dendrites are very slender and fairly uniform in size, with fine melanin granules dispersed along the dendrites. In the large melanoblasts, the granules are more numerous and vary in size. Irregular swelling is evident along the length of the branching dendrites, giving them a varicose appearance.

The relatively small pigment cells found by us in the outgrowth of normal skin explants resemble the early melanoblasts of fetal life which have been reported by Zimmermann and Cornbleet (26) as having ovoid, fusiform, or stellate cell bodies with relatively short dendritic processes. Although critical comparison of results obtained with different tissues cannot be made, because of the wide variety of technics involved, one is impressed by the similarities in the morphology described. The small bipolar or stellate cells observed in tissue culture of skin and in fetal life, and also the small melanoblasts in cultures of melanomas prob-

ably represent relatively young or embryonic forms of pigment cells. As these cells mature, they slowly increase in size and exhibit a more intense dopa reaction; their processes elongate and arborize. Our views seem to be supported by the observations made by Zimmermann and co-workers (26, 27) in their study of dendritic melanoblasts in early and late fetal Negro skin. Another supporting evidence is the presence of larger cells in the original skin explants than those in the outgrowing sheet. The melanocytes found in the former location naturally are considered to represent the more mature forms.

The presence of larger and more branched melanocytes in pigmented normal skin and the difference in size of the pigment cells derived from normal epidermis and those of nevi grown *in vitro* seem to indicate that the activity of pigment synthesis is also correlated with the size and shape of the cell; also, there seems to be a direct relation between the amount of intracellular pigment and the number and degree of branching of dendrites. The thesis that these changes signify active melanogenesis is supported by the fact that melanocytes are known to be activated by ultra-violet light, thorium X (17, 28), and other forms of radiant energy, and to become larger and more highly branched under these conditions. Personal observations of Staricco and Pinkus (29) confirm that following local application of thorium X, the melanocytes become more strongly dopa-positive, enlarge, and develop more and longer dendrites.

The opinion that nevi are simply an excessive accumulation of normal melanocytes does not explain the wide differences in sizes of these cells. If the thesis that capacity of melanogenesis is correlated with the size of pigment cells proves to be true, the increase in number and size of melanocytes in nevi may be merely an expression of increased functional activity of these cells as a result of some unknown stimulation. It is also possible that nevus cells may represent the transformation of normal pigment cells into a variety of tumor cells similar to the change of epidermal cells into benign epitheliomas. In our cultures, the dermal element of nevi was not capable of active proliferation. This finding seems to be in keeping with the view that intradermal nevi are composed of relatively quiescent, inactive melanocytes which gather in the corium after dropping down from the epidermodermal junction.

Available data are still too preliminary to make definite conclusions. This is only the beginning of our effort to acquire a better understanding of the pigment-forming cells. Further studies are in progress. The success of cultivation of melanocytes *in vitro* offers a method to study these cells in uniform environment which is unobtainable in the complex intact organism. It is our hope that future pursuits in pigmentary studies utilizing this technic will prove rewarding.

SUMMARY

An attempt was made to study human melanocytes of benign pigmented nevi and of foreskin of white and Negro infants by the tissue culture method.

Dendritic cells giving positive dopa reactions, containing argentaffine pigment granules and presenting other characteristic morphological features, were observed in many of these cultures and were identified as melanocytes.

These cells appeared to represent a distinct type. During several weeks of

observation of the two cellular types in the epidermis (epithelial cells and melanocytes), no transformation of one cell type into the other was observed. Each cell apparently gave rise to daughter cells of its own specific type.

In our study of melanocytes of normal skin, there appeared to be a direct relationship between pigment-producing capacity and cellular size and complexity.

There were definite differences in size and other characteristics between the dopa-positive cells derived from normal skin and those from benign nevi.

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DISCUSSION

DR. SAMUEL BECKER, JR. (Chicago, Ill.): I have nothing to add but I think the presenters should be congratulated for this very beautiful presentation. The use of this tissue culture technic may be one way to settle problems which have seemed unsolvable by routine sectioning.

DR. AARON B. LERNER (New Haven, Conn.): The authors presented beautiful photomicrographs. It would be good to know the effect of different hormones on the movement of pigment granules within the melanocyte. Such studies have never been done with human pigment cells. It might be relatively easy to determine whether or not the pigment granules of human melanocytes can move under the influence of hormones as can those of fish pigment cells.

It would also be good to use pigment cells from patients with vitiligo. The duration of vitiligo might affect the behavior of the pigment cells. Several years ago I said that albinos have a genetic absence of melanocytes. That was not correct. These individual have the pigment cells, but they lack the enzyme tyrosinase needed for the formation of melanin. Recently some investigators showed that melanocytes were not present in the depigmented areas of spotted animals. It may be worthwhile to extend this study to human partial albinos to find out whether or not they differ from the complete albino by actually having a lack of melanocytes.

DR. FUNAN HU (in closing): I wish to thank the discussants for their comments. We have done some time-lapse cinematographic studies of human melanocytes. As mentioned in my presentation, the differentiation between melanocytes, fibrocytes and macrophages in the living unstained preparations is often very difficult. No conclusive information has been obtained so far. Further work is in progress. The present study serves to establish a base line knowledge of human melanocytes in tissue culture.

Dr. W. Chavin of Wayne University and I have observed melanogenesis *in vitro* in tissue cultures of caudal fin of gold fish following introduction of pituitary

hormones in the culture media. This work will be reported in the near future. Similar studies concerning the effects of hormones on human melanocytes in tissue culture are also in progress.

We have made some and plan to make more studies of melanocytes, utilizing tissue culture method in various conditions associated with pigmentary disturbance, such as melanomas, seborrheic keratosis, pigmented basal cell epitheliomas, vitiligo, albinism, and others.

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