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INVESTIGATIONS OF PROPERTIES OF THE AMELANOTIC MELANOMA IN THE HAMSTER

JON MORTON AASE

1962

YALE UNIVERSITY

SCHOOL OF MEDICINE

DEPARTMENT OF PATHOLOGY

Investigations of Properties of the Amelanotic Melanoma in the Hamster

A Thesis Presented in Partial
Fulfillment of the Requirements
for the Degree of Doctor of Medicine

Jon Morton Aase

New Haven, Connecticut, April, 1962

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Terminology

The nomenclature used in this study follows that adopted by the Third Conference on the Biology of Normal and Atypical Pigment Cell Growth in 1951. (43) Definitions are as follows:

Melanoblast: an embryonic cell potentially capable of forming melanin.
Melanocyte: a mature melanin-producing and melanin-containing cell.
Macrophage: a cell containing phagocytized melanin.
Melanophore: a pigment effector cell in lower animals.

Acknowledgements

The work described herein could neither have been begun nor completed without the generous and patient help of many people who gave of their time, talents and facilities. To Dr. H.S.N. Greene, in whose laboratory these experiments were carried out, I am indebted not only for guidance, but for shaping my attitudes toward research. An expression of gratitude to Dr. Martin Salwen seems small reward for his invaluable help and encouragement, especially in the final phases of the research and the preparation of the microphotographs. The dopa reactions were carried out in the laboratories of Dr. Donald King whose personal assistance is gratefully acknowledged. Miss Elizabeth Harvey and Mr. Richard Sutphin were a constant source of strength and help, and are largely responsible for any technical successes achieved. Last, but far from least, are my wife, Barbara, and my sister, Kirsten, on whose capable shoulders was placed the burden of the typing and final assembly of this Thesis.

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Introduction

The melanomata are among the most fascinating, and, at the same time, the most frustrating of the neoplasms. These tumors were among the first to attract the attention of the cancer investigators, for their dramatic appearance, bizarre behavior, and dreadful prognosis in Man present an arresting picture. Since most of the malignant melanomas arise in the skin, they are easily accessible for diagnostic and therapeutic procedures, and, in animals, for experimental manipulation. In addition, their characteristic pigmentation makes identification of the primary tumor and its distant metastases easy, and enables the investigator to detect and observe these neoplasms from the earliest stages in their development. Therefore, the melanomata have been the subject of intensive study, despite their notorious unpredictability--for while the usual course of these tumors is characterized by rapid local growth, early widespread metastasis and relentless progression to inevitable death, yet in rare instances there is only slow local extension, with appearance of metastases only months or years later, and, in some cases, there has been complete spontaneous regression. (1,13,49,64)

Laboratory investigation of these remarkable tumors has been given renewed impetus in recent years with the

development of modern techniques of transplantation, experimental carcinogenesis, tissue culture and biochemical assay. Study of the melanomas themselves, in Man and the lower vertebrates, has been supplemented by investigations of the constituent cell, the melanocyte, in normal and abnormal growth, and the production and properties of its pigment, melanin. Special interest has centered around those neoplasms which, while composed of melanocytes, do not produce melanin pigment--the so-called amelanotic melanomas. Here is a unique opportunity to investigate the basic relationships between cell function and proliferation, for while these tissues retain their fundamental histological similarity, their biological properties are often strikingly different. (2,6)

Problem

The problem which is the topic of this thesis is that of the relation between malignant growth potential and pigment formation in an amelanotic melanoma. Work was begun in 1960 with such a tumor which had been derived by selective transplant from an originally heavily pigmented melanoma which had appeared spontaneously in the Syrian Golden hamster. (25) The amelanotic variant differed markedly from the parent line, being characterized by a rapid rate of general growth and a high degree of malignancy. The possibility that these properties might somehow be related to the tumor cells' inability to produce melanin provided the point of departure for the investigations which followed.

Objectives

If there is, indeed, a relation between pigment production and biological characteristics in this melanoma, a reasonable experimental approach might be to attempt to alter the melanin-producing capacity of the component cells, and determine the effects, if any, on the behavior of the tumor. The observation that the melanotic and amelanotic variants are often morphologically identical with the exception of pigment formation further encourages this hypothesis.

Both melanotic and amelanotic melanomas are known to occur spontaneously in experimental animals (17, 19, 25) and several have been maintained through literally hundreds of serial transplantations. Some workers have found that by selection of the more (or less) pigmented portions of the parent tumor for transplant, a change in the gross pigmentation of the line may be obtained over several generations. (2, 19, 25) The same effect may be achieved by transplantation into different host strains. (2, 26) However, there have not yet been reports of successful experimental transformation of an amelanotic to a melanotic tumor within one host generation. The objective of the work described here was to induce such a change by any of several experimental methods in an unpigmented melanoma in the hamster.

I

General Considerations

In approaching the problem of re-pigmentation of the amelanotic melanoma it is well to begin with an examination of the tissues involved, and how they became pigmented in the first place.

The Melanocyte

In the skin and certain other tissues of vertebrates, there appear highly specialized, branched cells which contain within their cytoplasm dense accumulations of granules made up of brown or black pigment. These are the melanocytes (from the Greek melas, black and -kytos, cell) and their unique appearance and properties have been observed for nearly a century in vertebrates from goldfish to man.

Origin

Embryological studies in amphibia, birds, mice and man have confirmed that the stem cells of the melanocyte series originate in the neural crest. (50) This structure, arising close to the neural tube very early in development, disperses its cells and disappears within a short period in embryogenesis. While the melanocyte precursors or melanoblasts cannot be distinguished from other neural crest cells at this time, it has been shown that their pattern of migra-

tion is from dorsal to ventral and anterior to posterior, following the general scheme of embryological maturation. It is believed that this migration carries the melanoblasts to all parts of the body, and it can be demonstrated that they have reached their ultimate locations in the integument and elsewhere long before there is any sign of pigmentation. (50)

Distribution

Skin.-- In the mammalian fetus, the precursors of the melanocytes appear in all cutaneous regions early in development. In many mammals, and probably in man, they are first distributed as a generalized "bed" of immature pigment cells in the dermal layer of the skin,[1] and only sometime later do they migrate further to their final location at the junction of the dermis and epidermis. (72) Here they form a continuous, loosely connected network which persists through the life of the animal. While the concentration of these potential pigment cells varies from area to area in the skin, their distribution is remarkably constant in a given region from animal to animal, and seems to be independent of the visible pigmentation of the overlying skin and hair. (51, 60)

In their mature form, the melanocytes may be demonstrated by special stains. They are seen to be elaborately

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[1] Many investigators believe certain nevi, and the "Mongolian" spots found in the sacral area in some newborn babies, to be remnants of this primordial dermal layer of melanocytes. (72, 36)

branched dendritic cells interspersed among the basal cells of the epidermis. (Fig. 1.) Their delicate processes are filled with pigment granules, and course among the surrounding epidermal cells, which may be seen to contain similar dark particles. As the basal cells move outward during keratinization, the granules disperse and disintegrate into a fine dust. Occasionally, effete melanocytes will be carried to the surface and sloughed off.

Hair.-- With the development of the hair primordia and their penetration into the deeper layers of the skin, melanoblasts are carried down from the epidermal network to assume positions among the matrix cells of the hair bulb and in a small zone in the nearby dermis. (50,51) With the beginning of hair growth, the melanoblasts mature into functioning melanocytes and release pigment which is incorporated into the hair-forming cells and is carried outward with their growth. When a follicle enters its resting (telogen) stage, the melanocytes of the hair bulb degenerate, and, with the beginning of the next cycle of growth, are replaced from a "reservoir" of precursor pigment cells in the dermal portion of the papilla. (29, 50, 61) (Fig. 1.) Recent work has shown that, after the first few days of life, there is no further migration of potential pigment cells into the follicular structures, and if the mature and immature melanocytes present are selectively destroyed, they are not replaced from epidermal or other sources, and the follicle involved will henceforth produce

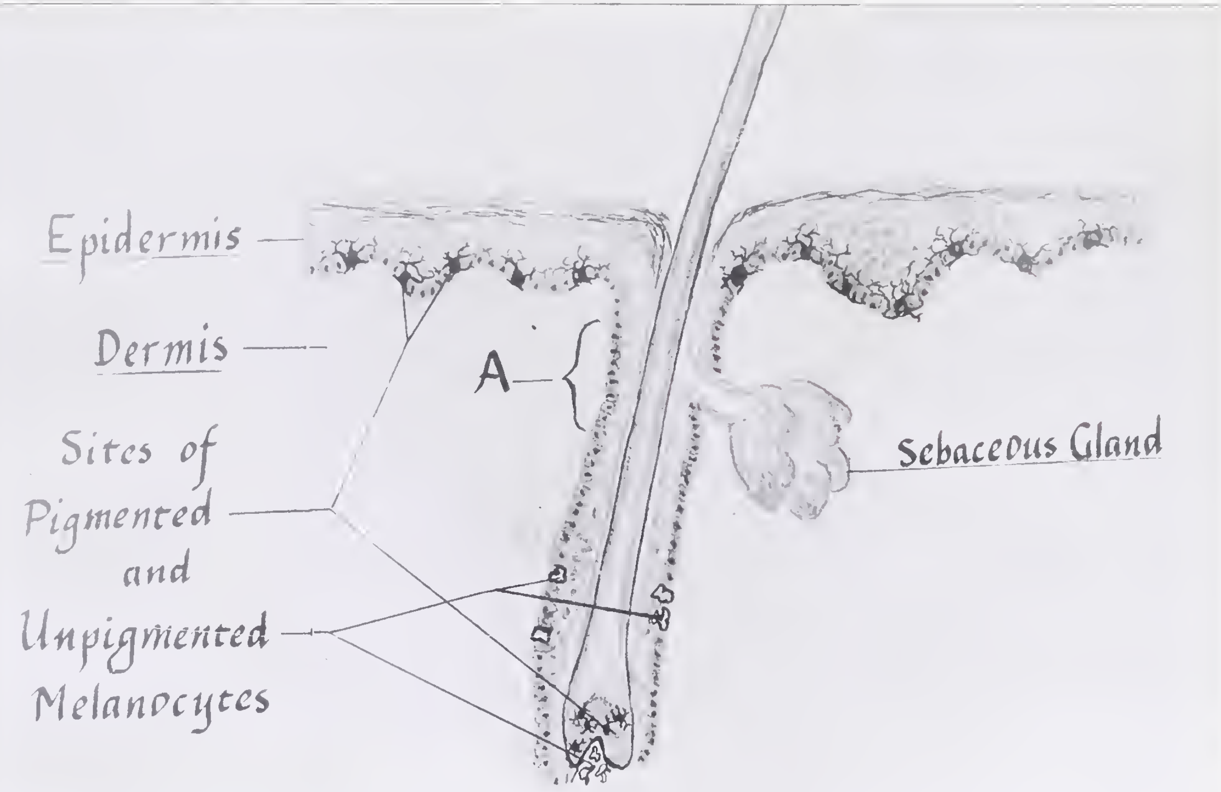

Fig. 1.- Distribution of melanocytes in the skin and hair follicle.

only white hairs. (9)

On the other hand, if the melanocytes at the dermal-epidermal junction are destroyed (e.g. by dermabrasion) repigmentation of the skin occurs by migration of the unpigmented "resting" melanocytes from their location deep in the follicular sheath up and out into the adjacent regenerating epithelium. (61, 62) While the mechanism of this movement appears to be passive transport of the potential melanocytes along with the outward growth of the follicular epidermal cells, there is some evidence to point to the active mobilization and migration of individual unpigmented precursor cells, and their marked proliferation can be demonstrated. (61, 62)

Leptomeninges.-- In the central nervous system, melanocytes are found at the junction of the pia and arachnoid. They are especially numerous near penetrating blood vessels and on the ventral surface of the medulla.

Eye.-- In the mammalian eye, the uveal tract and the outer (pigment) layer of the retina are sites of melanin production. While the melanocytes of the choroid and iris are thought to be of neural crest origin, those of the retinal pigment epithelium may have their embryologic source in the outer layer of the optic cup.[2]

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[2] Fitzpatrick and Kukita have shown that these cells possess measurable tyrosinase activity only during a limited phase of embryogenesis, and that no activity can be demonstrated in adult retinal pigment epithelium in the fowl,

Function

In all its locations in the body, the melanocyte acts solely to produce the dark-colored pigment, melanin. In the lower animals, body and hair coloration may be of great importance for camouflage, identification or imitative disguise. In the retina and in the hairless areas of the skin, the protective properties of pigmentation are primarily those of sparing delicate underlying structures from the full effects of visible and ultraviolet radiation. (A good example in man is sun-tanning.) However, increased melanin production is the characteristic response of the melanocyte to a number of non-specific stimuli: thermal, chemical or X-ray burns, chronic irritation, and superficial infection will all produce hyperpigmentation of the skin.

Melanin Production

Biochemistry

The elaboration of melanin within the melanocyte takes place through a well-defined series of steps which has been clearly elucidated in recent years. The essential starting material is the amino acid tyrosine, available as such from dietary sources, or by the enzymic hydroxylation of phenylalanine.

The first step of the series is the conversion of tyrosine to dihydroxyphenylalanine (dopa), mediated by the

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mouse or human. (15)

copper-containing enzyme tyrosinase. This same enzyme then further catalyzes the formation of dopa quinone from dopa. (Fig. 2.) Both these reactions require molecular oxygen, and the first seems also to be dependent upon the presence of a trace of pre-existing dopa to act as an electron donor. (33, 37) Further oxidation leads to a series of rather unstable quinone intermediates, any of which may polymerize with its own kind or other members of the group.[3] (Fig. 2.) The end product (so-called "natural" melanin) is the result of the attachment of these polymeric units to a protein matrix, yielding a highly insoluble, deeply colored substance, which, under different circumstances, may be responsible for gross pigmentation in shades of brown, black, buff or even blue (through the Tyndall effect).

While the limiting factors of this complex pathway have not yet been determined, at least three substances must be present in adequate quantities to initiate the process:

1. A suitable substrate -- tyrosine or dopa
2. The enzyme tyrosinase
3. Molecular oxygen.

The interaction of these substances may be affected by several modifying factors, including temperature, pH, electrolyte concentration and the presence of certain catalysts,

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[3] Although the final product had long been thought to be the result of polymerization of indole 5,6 - quinone, it has been shown that melanin is not "homogeneous with respect to the repeating unit" but contains many of the intermediate products shown in Fig. 2. (40)

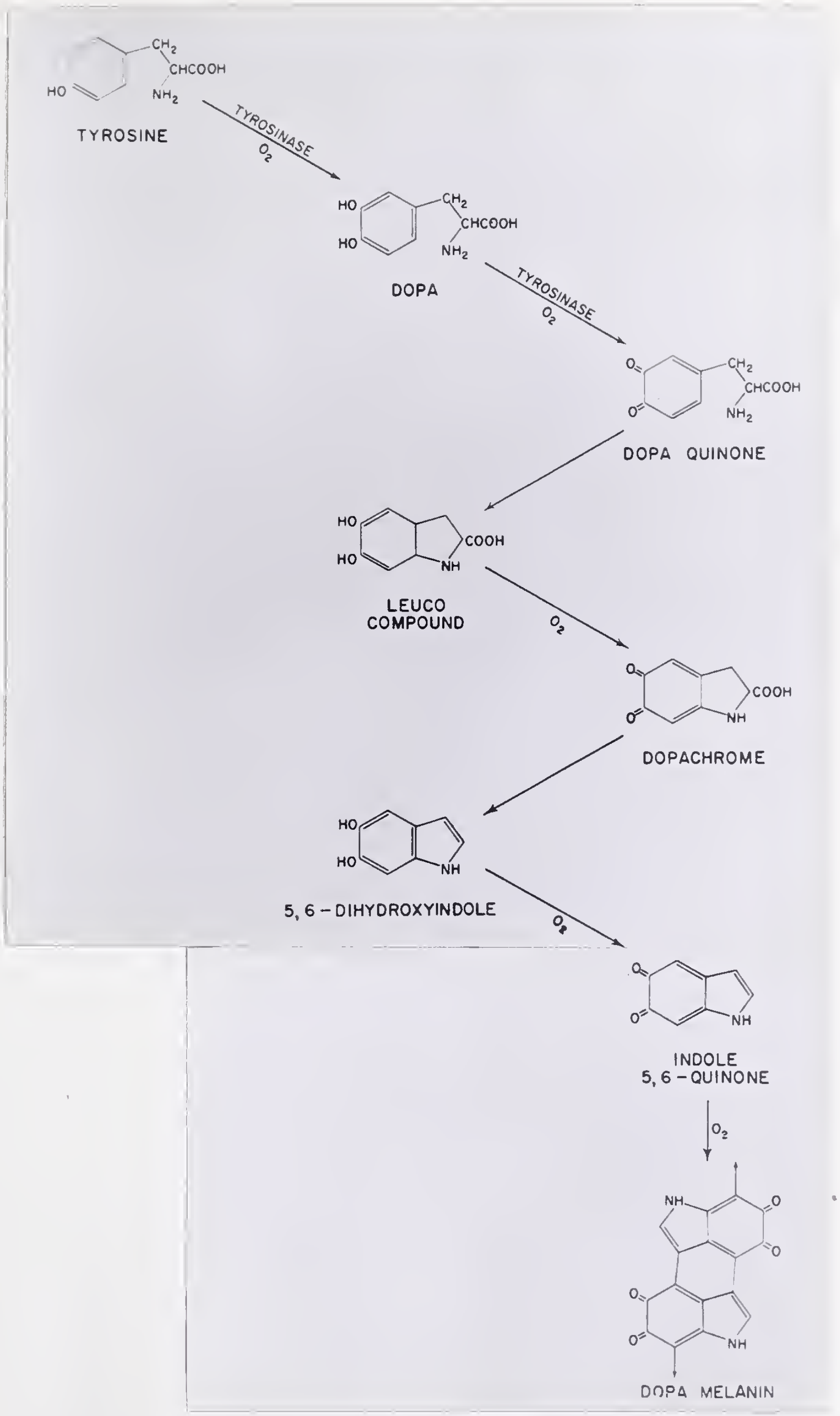

Fig. 2.- Enzymic oxidation of tyrosine to melanin (from Aaron B. Lerner and James D. Case, "Pigment Cell Regulatory Factors", *The Journal of Investigative Dermatology*, *XXXII* (February, 1959), p. 213).

including dopa itself. An important consideration is the presence of chemical groups which can inhibit such copper-containing enzymes as tyrosinase. These include sulfhydryl groups, thiol compounds, heavy metals, and many others. (33)

Site

The locus of action of tyrosinase within the melanocyte was long thought to be in the mitochondria. However, recent studies using ultracentrifugation and electron microscopy have confirmed the presence of specific melanotic organelles within the cytoplasm of the pigment cell. These tiny bodies, called melanosomes, are distinct from the mitochondria, and seem to possess all the tyrosinase activity of the melanocyte. (5,12,54) They are thought to be formed as colorless vesicles in the Golgi material, and as they mature, to become progressively more heavily pigmented as melanin is formed and deposited within them. Eventually, these melanosomes become so loaded with melanin that both internal structure and tyrosinase activity are "masked", resulting in inert melanin granules (15,16), which are distributed throughout the cytoplasm, especially in the dendritic processes. The pigmented particles may then be incorporated into adjacent cells, probably through a mechanism of "pinching off" and phagocytosis of the granule-filled dendrites by neighboring epithelial cells. (16,71)

Hormonal Control

One of the most exciting advances in pigment cell

biology has been the recent investigation into the role of pituitary hormones in the regulation of melanin production and distribution. As early as 1916 it had been reported that ablation of the pituitary gland led to a loss of skin color in tadpoles, while topical application of pituitary extracts had an opposite effect. With the development of more elaborate and precise separation techniques, the active factor was isolated from the crude extract, and found to be an extremely potent biological substance, producing a marked increase in skin pigmentation in the human with dosages of only a few micrograms. (34) In addition, existing nevi were darkened, and new nevi appeared, supporting the hypothesis that this hormone has a general stimulating effect on melanocytes, whatever their location. In 1954, Lerner (34) proposed the appropriate name *Melanocyte-stimulating Hormone* (*MSH*) for this substance. Since that time, he and his group have isolated two types of this hormone, α- and β-MSH from the pituitary glands of hogs, cows, sheep, horses, monkeys and man. (31,32,39) The structure of both types has been determined, and α-MSH has been produced synthetically.[4]

The mechanism of hormone action at the cellular level has been well demonstrated in amphibian melanocytes, where it can be shown that the initial increase in visible pigmentation is largely the result of a change in the distribution

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[4] While β-MSH apparently exists in several different forms in different species, α-MSH, the more active type, seems to have the same structure in all mammals, and is identical to the first thirteen amino acids of Corticotropin A (Hog ACTH). (37,39)

of melanin granules within the cytoplasm, rather than an increase in melanin production per se. (35) Administration of MSH brings about a dispersal of the pigment granules, causing the cell to appear darker. Many substances will inhibit or reverse this action, causing a clumping of the granules close to the nucleus. (35,37,38) This distribution factor may also play a part in the actual synthesis of melanin, however, for when the active melanosomes are dispersed throughout the cytoplasm, there is theoretically more intimate contact with substrate materials, as well as greater surface area for tyrosinase activity. (37)

In this connection, MSH has been shown to increase the absolute amount of melanin present in a melanoma in the hamster. (21) Although little work has yet been done in mammals, it seems evident that this ubiquitous hormone has a profound effect on melanocyte function in all species.

II

Materials and Methods

For the purpose of this discussion it will be convenient to divide the experimental protocol into three Parts, representing the different avenues of approach utilized in attempting to induce melanin production in the amelanotic melanoma. First there will be described the experimental animals, the transplantable tumor which was used, and the method of transplantation, all of which were common to more than one Part.

Animals

The laboratory animals used in all three Parts of this study were Syrian Golden Hamsters (Cricetus auratus) obtained from commercial suppliers. They were brought to the animal room of the laboratory when about two or three months old and were kept separated by age and sex. Diet consisted of Purina Laboratory Chow and tap water, ad libitum (except as noted below). In Part One the animals were kept in separate cages after transplantation. In Parts Two and Three, up to twelve hamsters were kept in a single large cage. The general health of all animals was good at the beginning of the experiment, and the only exogenous illness which occurred during the course of the work was an epidemic of enteritis which

affected the hamsters used in Part Two.

All animals were sacrificed in a chloroform killing jar, and complete autopsies were done except in the few cases of animals lost to cannibalism or disease.

Tumor

The tumor line used in Parts One and Three was derived from a spontaneously occuring melanotic melanoma carried in serial transplant in the laboratory of Dr. H.S.N. Greene.(25) This original tumor was heavily pigmented, but light-colored areas began to appear in early transplant generations, and by selection, a completely amelanotic line was established. It was this strain which was used in the present study.

Previously, the two variants had been found to differ markedly in behavior. For example, vascular invasion had been found to occur by as early as the nineteenth day of growth in the amelanotic, as against the ninetieth day in the melanotic line. This extreme difference in malignancy was not originally reflected in any change in morphology other than that concerned with pigment production.

Another characteristic of both variants was a marked propensity for sarcomatous transformation, inducing tremendous overgrowth of normal connective tissue of the host animal to serve as stroma for the tumor parenchyma. It is thought that this tendency on the part of the amelanotic melanoma resulted in its eventual conversion to a fibrosarcoma, since the later transfer line differs greatly from the original tumor in cellular morphology, although the biological characteristics remain quite similar. For this reason, it

must be noted that the "amelanotic" tumor used in Part Three, while directly descendant from the original melanoma, is apparently made up of an entirely different cell type, and the validity of attempts to bring about melanin formation in this tumor remain in doubt.

When the transplanted neoplasms were allowed to grow for more than two weeks, extensive necrosis developed in the center of the tumor mass. This sometimes resulted in breakdown of the overlying skin, with ulceration and, often, cannibalization of the exposed tissue.

Transplantation Techniques

Transplantation of the "amelanotic" tumor in Parts One and Three was carried out by the classic trocar method. A hamster from the stock line carrying the neoplasm subcutaneously was sacrificed after three to four weeks of growth, the skin was shaved and sterilized with alcohol, and the tumor excised. A section was taken from the most peripheral (least necrotic) portion of the growth and transferred to a Petri dish containing sterile normal saline. It was then cut with scissors into fragments about two millimeters on a side. Using sterile technique, these were loaded one at a time into a #15 blunt-pointed trocar. The skin of the right flank of the host animal was sterilized with alcohol, and a stab wound was made through the skin with a sharp-pointed scalpel. The loaded trocar was introduced into the wound and forced subcutaneously high into the right axilla, after which the tumor fragment was ejected with the stylet, and the trocar

withdrawn. The hamster was then returned to its cage. No apparent infection of the puncture wound or subcutaneous tract was encountered.

Dopa Reaction

Before beginning Part One of the experiment, and again at its conclusion, specimens of the transplanted tumor from the stock line were subjected to incubation with dihydroxyphenylalanine in an effort to determine their capability for pigment formation. Frozen sections of twenty to forty micra were fixed in cold acetone for two hours, then placed in a solution of dopa in phosphate buffer (pH 6.8). After two hours in this preparation, half of the tissue specimens were removed and placed in plain buffer solution as controls. Both groups were protected from light and incubated overnight (sixteen hours) at 37° C. The degree of pigmentation was estimated grossly and by microscopic examination of wet mounts of the thick sections.

Part One

Administration of Tyrosine and Melanocyte-stimulating Hormone (MSH)

Twenty-four young male hamsters were divided into three groups of eight each. Portions of the amelanotic melanoma were successfully transplanted into the right axilla of each animal, and they were placed in individual cages. The first group was treated with daily injections of Melanocyte-stimu-

ulating Hormone (MSH), the second with a diet high in tyrosine, and the third with both MSH and tyrosine. The stock line of tumor-bearing hamsters served as controls.

Tyrosine.-- Administrations of high levels of tyrosine in the diet was accomplished by substitution of a saturated solution of this amino acid for the animals' drinking water. For this purpose, 5.26 gm. of L-tyrosine, (Nutritional Biochemicals Corporation) was dissolved in five liters of tap water at approximately 80° C. Although this amount of tyrosine should produce a saturated solution at 50° C., cooling to room temperature did not cause supersaturation. This solution was mixed and stored in a five-liter flask, from which it was dispensed to the individual drinking bottles of the second and third groups of hamsters. The preparation was taken surprisingly well in lieu of water, and no adverse effects were noted. Drinking bottles were checked and refilled daily, and the stock solution replenished as needed. Records were kept of approximate daily consumption by each animal, and from these values, the estimated average intake of tyrosine was judged to be from 15 to 20 mg. per day.

MSH.-- The dosage of MSH was calculated by extrapolation from the experimental human dosage of 4 mg. per day. (39) For a 100-gm. hamster, this works out to about 6×10^{-3} mg. per day. Accordingly, approximately 1.5 mg. of α-MSH, supplied from the laboratory of Dr. A.B. Lerner, was dissolved in 125 ml. of sterile normal saline, and stored in a sealed

vacuum bottle under refrigeration. Each day an 8-ml. aliquot of this solution was removed, and 0.5 cc. injected subcutaneously into each of the sixteen animals of the first and third groups. Injection was made under the skin of the flanks, alternating sides each day. This treatment was tolerated well, and no abscesses or other complications developed.

When it was decided during the course of the experiment to carry on the MSH administration for two additional weeks, no further supply of the hormone was available. Therefore, the remaining solution was diluted to a concentration of approximately 4×10^{-3}mg./cc. and the injections were continued as before. The total amounts of MSH administered to each animal during the three-week course of treatment was approximately 9×10^{-2} mg.

Part Two

Application of DMBA

In this portion of the study, an attempt was made to reproduce the work of Dr. Shubik and his group (14,48,57,58) involving the induction of transplantable melanotic tumors by topical application of certain carcinogens. The most potent of these substances seems to be 9,10 dimethyl- 1,2 benzanthracene (DMBA), which consistently produces pigmented lesions in the skin of the hamster. (58)

<u>DMBA</u>.-- A 1% solution of DMBA was prepared by dissolving

0.50 gm. of the powdered compound in 50 gm. of Mineral Oil, U.S.P.

Administration.-- Using electric clippers, the hair was shaved from the lower back of nineteen young male hamsters. Eight received a single drop (approximately 0.05 cc.) of the DMBA solution topically in the shaved area. Four others were similarly treated with mineral oil alone.

A second group of four hamsters received injections of 0.05 cc. of DMBA solution subcutaneously in the shaved area, utilizing a tuberculin syringe and 23-ga. needle. The three remaining animals were similarly injected with mineral oil alone. Although the injected substances were not sterile, abscess formation did not occur.

The animals were segregated according to the treatment received, and allowed regular hamster diet and water ad libitum. An epidemic of enteritis struck the colony during the course of this part of the experiment, and several animals were lost to autopsy through cannibalism.

Part Three

DMBA Treatment of Amelanotic Tumors

As a natural outgrowth of the preceding two parts, it was decided to attempt to determine the effect of DMBA upon the pigmentary and biological characteristics of an amelanotic melanoma. The only suitable tumor line being carried in serial passage at the time of this part of the study was

the "amelanotic" growth described previously (*supra*, p. 16). While this neoplasm was thought to have undergone transformation to a fibrosarcoma, a further attempt to induce pigment production was felt to be justified on the basis of its direct descent from, and biological similarity to, the original amelanotic melanoma.

Consequently, fragments of the tumor were successfully transplanted into the right axillae of twelve large male hamsters. Two weeks later, when the explants had reached approximately 1 cm. in diameter, 0.1 cc. of 1% DMBA solution (supra, p. 20-21) was injected directly into the tumor mass in eight of the animals. The remaining four received identical injections of mineral oil alone.

The tumors were allowed to grow for six more weeks. At that time, all twelve animals were sacrificed and autopsied, the tumors were weighed and examined, and portions were sent for microscopic sections.

III

Results

This section of the study is also divided into three Parts, to correspond to those of Materials and Methods.

Part One

Dopa Reaction

This reaction was carried out on sections of the transplanted tumor as described above (p. 18). Initially, the sections left in the dopa solution became grossly jet black, in contrast to the control group, which showed only light gray pigmentation. Four weeks later, however, "amelanotic" tumors both from the experiment in progress and from the stock passage line showed the same degree of darkening in the dopa solution as did a leiomyoma used as a control. It is thought that the increase in pigmentation observed on both occasions might have been largely the result of auto-oxidation of dopa, rather than the action of an intact melanin-synthesizing system.

While microscopic interpretation of the thick sections was difficult, the pigment appeared to have a diffuse rather than a granular distribution. It is known that many oxidizable substances will produce such diffuse pigmentation

under the conditions of this reaction (42, p. 524). Therefore, the presence of potentially functioning melanocytes in this tumor was not verified. However, their absence is not confirmed either, as will be discussed later.

Tumor Characteristics

Gross Appearance.-- Of the twenty-four hamsters used in this Part, all but two survived through the entire twenty-five-day course of the experiment.[5] At necropsy, findings were the same regardless of the type of treatment received. The tumor mass was found to be extremely large and nodular with a rubbery consistency and a fairly extensive vascular supply. The average dimensions were approximately 3 X 5 cm., and their gross appearance was similar to that shown in Figs. 13 and 14. The color was pinkish-white, and in no case was there any visible evidence of darkening or pigment production.

On cross section, the tumors presented an appearance similar to that shown in Fig. 15. In all instances there was a great deal of central necrosis, with only a thin (2-4 mm.) peripheral shell of firm, pink tissue, surrounding a large amount of slightly yellow, caseous material. Again, there was no visible dark pigmentation in any of the specimens.

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[5] One of these animals died, apparently of pneumonitis, on the 11th day of the experiment, the other of unknown causes on the 21st day. Necropsy findings in both these animals were identical with those described for the rest of the group, except that the tumors were smaller, with less central necrosis.

Microscopic Appearance.-- The microscopic appearance of the tumor is shown in Fig. 3. It consists of sheets of irregularly polygonal to rounded cells with foamy, acidophilic cytoplasm. The nuclei are round to oval, vacuolated, and contain one or more prominent nucleoli. Mitoses are relatively scarce, averaging two to three per high field, but mitotic activity varies considerably from one field to another. Rare multinucleated cells can be seen. In many sections there are seen zones of focal necrosis, in which the cells have lost their sharp cytoplasmic outlines, and the nuclei appear pynknotic.

Interspersed among the loosely arranged sheets of tumor cells can be seen spindle-shaped cells with small dark-staining muclei and a moderate amount of acidophilic cytoplasm. In some sections these can be seen to represent the remnants of fibrous tissue and striated muscle which has been invaded and destroyed by the tumor.

Fig. 4 shows a discrete nodule, made up of typical tumor cells, lying at some distance from the main mass of the growth. Here again may be seen the invasion of adjacent tissues in what is thought to be a metastatic site.

Special stains ("Kernechtrot" technique) were used in addition to hematoxylin and eosin in an attempt to demonstrate the presence of melanin. In none of the sections examined was there any microscopic evidence of intracellular pigment production.

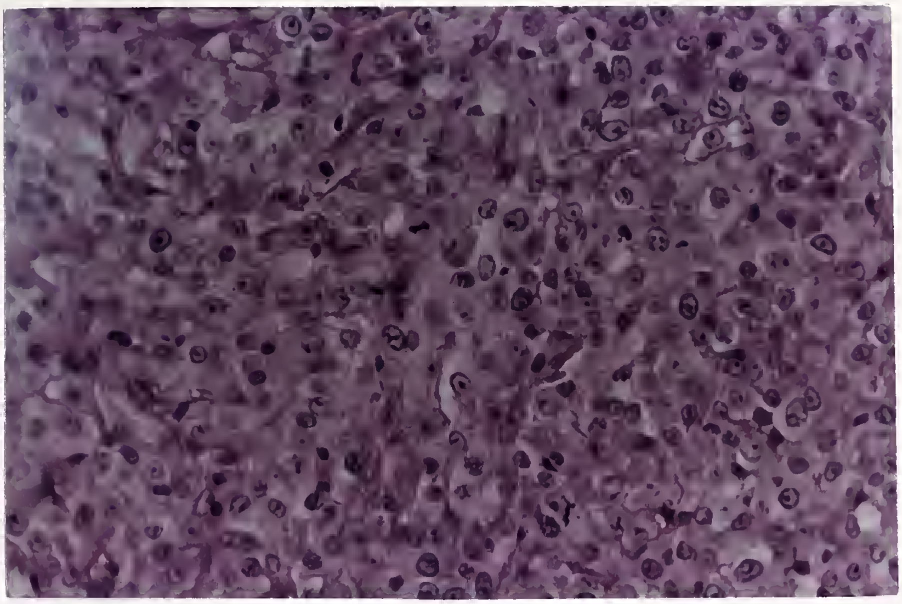

Fig. 3.- "Amelanotic melanoma", H & E, X250.

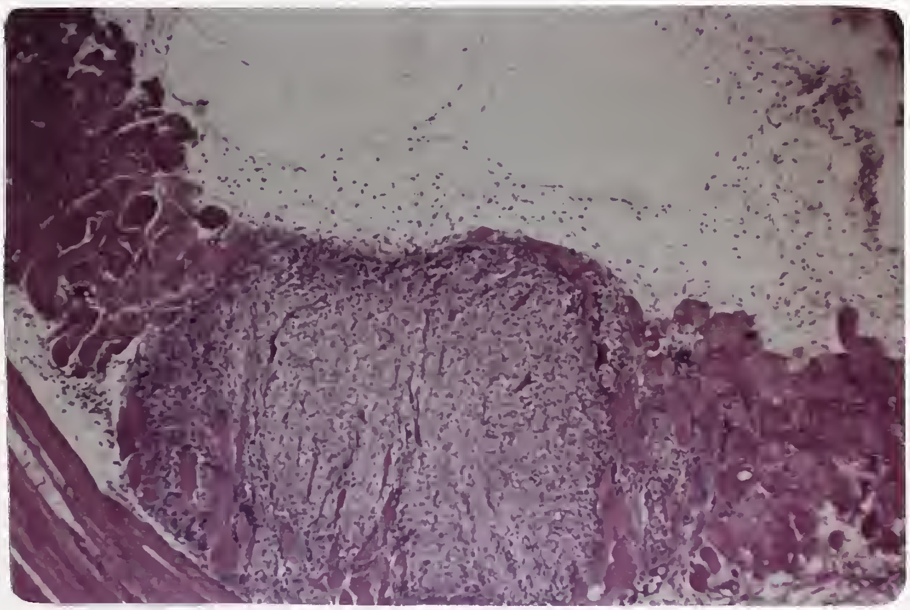

Fig. 4.- Tumor nodule, H & E, X25.

Metastases.-- Gross examination of the viscera revealed no detectable metastases. Separate records of lymph node involvement were not made, but in several instances, it was evident that the axillary nodes were enlarged.

Representative specimens of liver and lungs were obtained and sent for histological preparations. Microscopic examination of the sections confirmed the absence of metastases in these organs. Kernechtrot stains also failed to demonstrate the presence of melanin pigment in any of these sites.

Part Two

During the seven-month course of this Part of the study, an epidemic of enteritis was responsible for the death of eleven of the nineteen animals. The proportion of deaths from this cause was almost identical in the experimental (seven of twelve) and the control (four of seven) groups.

Gross Appearance

All the surviving animals of this Part are shown in Fig. 5. The most striking characteristic to be noted is the region of extensive depigmentation of hair which is present over the lower back of all but one of the hamsters treated with the DMBA solution. This area corresponds to the site of application of the carcinogen, and is most extensive and clearly delineated in those animals which received the solution topically. The three control animals (seen in the lower left-hand corner of Fig. 5) showed no evidence of

Fig. 5.- Appearance of hamsters used in Part II.

similar depigmentation.

In Figs. 6 and 8 the whitened areas are seen to better advantage. The appearance of the shaved skin of the animals is shown in Figs. 7 and 9. Here it may be seen that the underlying skin also appears hypopigmented. Furthermore, the illustrations show the numerous deeply pigmented lesions which appeared in all the DMBA-treated hamsters. These ranged from black macules less than 1 mm. in diameter to blue-black nodules 6 mm. in diameter. Their distribution extended from the lower back, close to the site of DMBA application, far forward to the dorsal skin of the forehead. None were found in the abdominal skin of any animals. The normally pigmented costo-vertebral spots of the hamster may be seen on either side of the back, and most of the melanotic lesions seemed to lie cephalad from these landmarks. It is particularly notable that the lesions were rare in the immediate vicinity of the areas of depigmentation; that is, in the area to which the DMBA was directly applied.

In none of the animals was there any evidence of metastases to lymph nodes or viscera.

Macules.-- The macular type was the more numerous with an average of seventeen present on the back of each animal. Only those lesions which measured more than 1 mm. in diameter were counted, and the largest found was about 2 mm. across. The skin of these areas was intact, and there was no evidence of inflammation or other change in nearby tissues.

Fig. 6.- Appearance of depigmented area, hamster DMBA-2.

Fig. 7.- Appearance of shaved skin, hamster DMBA-2, showing normal costo-vertebral spots and numerous melanotic lesions.

Fig. 8.- Appearance of depigmented area, hamster DMBAS-3.

Fig. 9.- Appearance of shaved skin, hamster DMBAS-3, showing wide distribution of pigmented lesions.

Nodules.-- The dark blue-black nodular lesions were fewer in number, but larger, ranging from 1 to 6 mm. in diameter. Their distribution was similar to that of the macular type, but the largest examples were found close to the midline at about the level of the costo-vertebral spots. On palpation they were found to have a firm consistency and to be attached to the skin, but not to underlying tissues. Again, there was no evidence of local inflammation or ulceration of the skin. Upon dissection, these lesions, as well as the macular type, were found to be confined entirely to the skin with no extension to the subcutaneous structures.

Microscopic Appearance

Representative specimens of both types of melanotic lesions were sent for histologic sectioning and staining. The macular and the nodular varieties (Fig. 10) showed essentially the same cellular types and structural relationships, varying only in a quantitative sense.

The chief histological feature of these lesions was the presence of large numbers of pigment-containing cells in the dermal layers of the skin. These accumulations were seen to be entirely separated from the dermal-epidermal junction by a clear zone of dermal connective tissue. No proliferative activity in the junctional region was noted in any of the sections, and the overlying skin appeared entirely normal. There was no evidence of invasion of blood vessels or other surrounding tissues. This microscopic appearance exactly corresponds to that of the "cellular blue

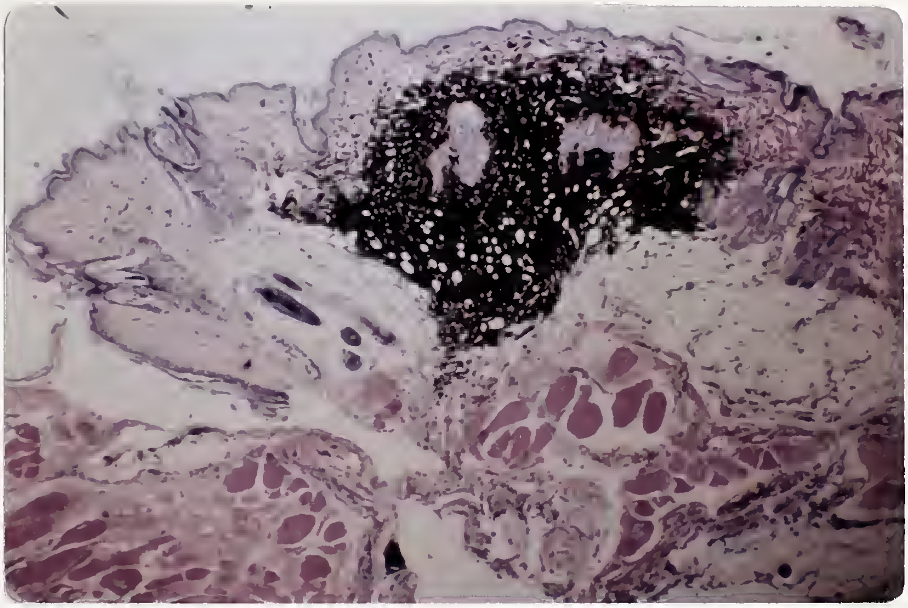

Fig. 10.- Macular pigmented lesion, H & E, X25

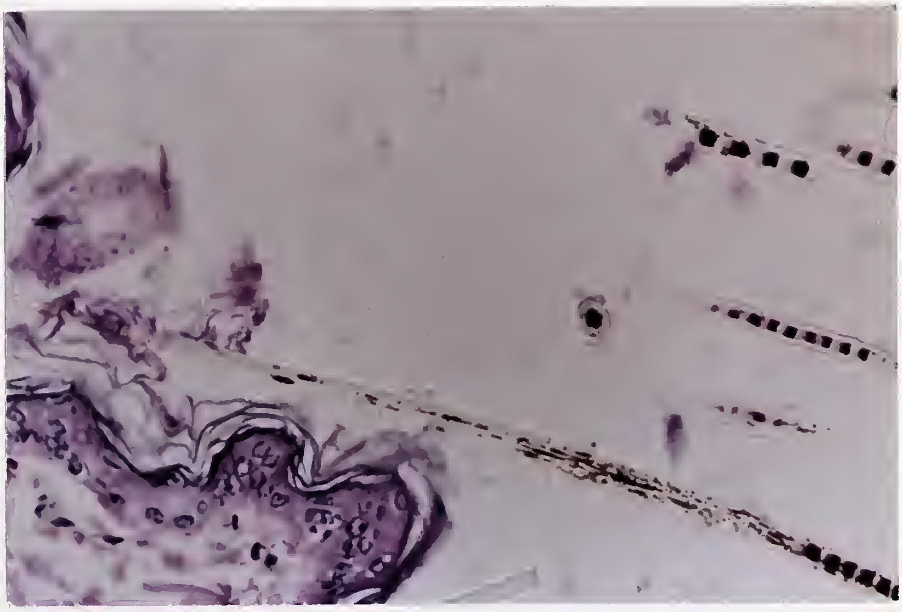

Fig. 11.- Periphery of nodular pigmented lesion, showing depigmented hair shaft. H & E, X250

nevus" of man, as noted by Shubik and his group in their original work. (14)

Closer examination of these nevus cells (Fig. 12) reveals the majority to be polyhedral to round in shape, giving the appearance of being actually distended by their contained pigment (presumably melanin), which completely obscures nuclear detail. Less heavily pigmented spindle-shaped cells are also seen, especially at the periphery of the lesion. Here, the cytoplasmic pigment can be seen to consist of brownish granules. Nuclei are basophilic, oval in shape and nucleoli are not seen. Mitoses are extremely rare.

Hair follicles lying close to the nevi were structurally intact, but in several sections the hair issuing from them was seen to be unpigmented. (Fig. 11)

Part Three

All twelve hamsters used in this Part survived the entire eight-week course of the experiment. One tumor in both the control and experimental groups became severely ulcerated, with loss of most of the tumor substance. These two cases were excluded from the weight figures given below, but were included in the necropsy study.

Gross Appearance

During the early weeks of this portion of the experiment, frequent inspection of the animals gave the impression

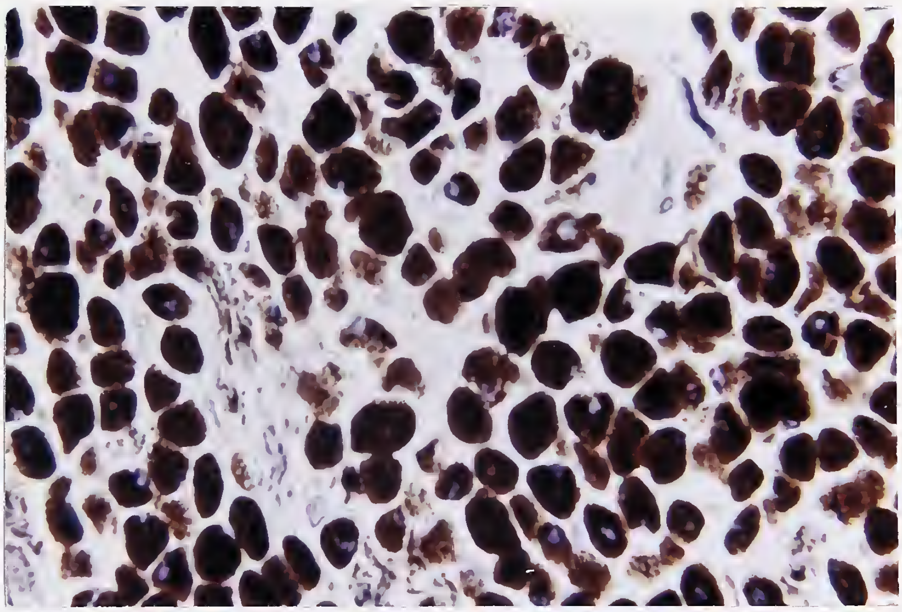

Fig. 12.- Pigment-containing "nevus cells", H & E, X400.

that the DMBA-treated tumors were consistently smaller in size and hence, growing more slowly than those of the control group. At necropsy, however, the differences in size were not striking, and the general appearance of both groups was similar to that of the transplanted amelanotic tumor used in Part One. (Figs. 13 and 14) The overlying skin was ulcerated to some extent in many animals, and in two the largest part of the tumor material was lost, leaving only a wide, shallow crater lined with necrotic tissue.

Palpation revealed a nodular mass with a rubbery consistency which was in some places firmly adherent to the skin. In three of the four control animals, greatly enlarged axillary lymph nodes were also palpable, but none could be felt in the DMBA-treated group.

Control Group.-- The tumors of the control group were dissected out (Fig. 14), and were found to be identical in all respects to those described previously in Part One (*supra*, p. 24). No increase in pigmentation was detected on inspection of the surface or of cut sections. Dissection of the axillary area confirmed the presence of grossly enlarged lymph nodes in three of the four animals. The appearance was grossly similar to that of the main tumor, and these masses were felt to represent regional lymphatic metastases.

Examination of the viscera showed the lungs of all four animals, and the liver of one, to be studded with metastatic nodules. These ranged in size from a pinpoint to 2 mm., and in color from white through pink to a dark brown. Repre-

Fig. 13.- External appearance of tumor-bearing hamster from Part Three.

Fig. 14.- Appearance of tumor *in situ*.

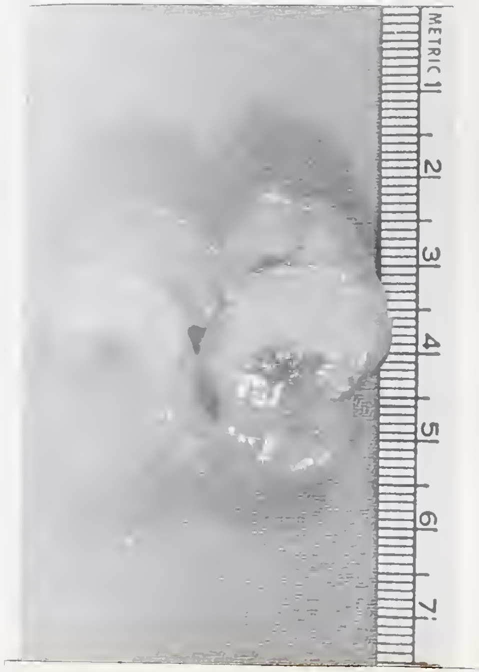

Fig. 15.- Cross section of tumor from Part Three, showing central necrosis.

sentative specimens from affected organs, as well as from the main tumor, were sent for histological sections.

Experimental Group.-- The surface appearance of these tumors was again similar to those of Part One and the control group of this Part, as shown in Fig. 14. However, five of the eight growths were found to be very tightly adherent to the underlying body wall of the hamster by a stout attachment of firm, white tissue. No such connective tissue elements had been found in previous specimens. It seemed almost cartilaginous in nature, but did not extend for any appreciable distance within the tumor mass. This structure is not well demonstrated in Fig. 15, which does, however, show the typical extensive central necrosis seen in all the tumors of Parts One and Three.

There was no evidence of regional lymphatic metastasis in any of the animals of this group. However, all showed metastatic foci in the lungs of the same degree and type seen in the control group. No other organs were found to be involved on gross inspection.

Specimens of lung and tumor were sent for histological section, as was a sample of the white "connective tissue."

Tumor Weights

The tumors from animals of both groups were weighed upon removal. Those of the control group averaged 15 gm. ± 5.3 gm. and those of the experimental group 12 gm. ± 2.6 gm. In view of the small size of the statistical sampling, it

is unlikely that this difference is significant. Moreover, the tumor-free weight of the control animals themselves averaged slightly higher than the DMBA group, indicating that factors of nutrition and general health might play a role in the weight differences.

Microscopic Appearance

Unfortunately, histological sections of the tissue specimens obtained in this Part were not available at the time of this writing.

IV

Discussion

The experimental investigator facing the question of melanization in the melanoma soon begins to feel a bit like Hercules attacking the Hydra. As he nears the solution of one problem, two more rear their heads, and their ramifications often carry him far afield from the original subject. However, the freedom to follow such tempting byways is certainly one of the most appealing (and sometimes, rewarding) aspects of biological research.

In the present study, the first attempts to induce remelanization in an amelanotic melanoma soon led to investigations of many related topics. Among these were experimental carcinogenesis, the biochemistry of melanin production and the effects of various stimuli on the melanocyte itself. In every case, however, there were opportunities to apply new facts and new techniques to the original question:

"What is the relationship between pigment production and cellular proliferation in the melanoma?"

Origin of Amelanotic Neoplasms

The unpigmented cells which make up an amelanotic

melanoma might conceivably be produced in any of several ways.

Biochemical Block

First, there is the possibility that these cells represent originally pigment-producing melanocytes which have undergone regressive change of some kind. It is thought that this change takes place simultaneously with, and as a result of, the onset of malignant growth. This view postulates a biochemical mechanism responsible for the supression of melanin-production. A block of this nature might be found in one or more of the intermediate steps between tyrosine and melanin, in the transport of essential precursors into the cell, in energy-transfer reactions, or in the laying down of the protein "scaffolding" of the melanosome.

Many workers have been able to demonstrate the absence or inhibition of certain enzymes, particularly tyrosinase, in amelanotic melanomas. (8,11,59) The oxygen requirements of functioning melanocytes have also been the subject of precise analysis recently, as have the ultra-microscopic characteristics of the melanocyte, melanosomes and pigment granules. (24,63,71) Unfortunately, the findings of these investigators often have been at variance with one another, (8, 15, 21, 29, 52, 68), and no incontestable data have yet been put forth to show a consistent mechanism to account for the loss of an existing capacity for melanin production.

Biological Block

The second theory for the existence of amelanotic melanomas postulates that with the onset of rapid proliferation of the melanocytes, the energy supply and metabolic activity of the cell is diverted to growth and division, at the expense of the melanin-producing system. Supporting this view is the common observation that the amelanotic tumors tend to be more rapidly growing and invasive of the two types. (10,17,18,19,52) Furthermore, darkly pigmented primaries will often produce amelanotic metastases. The unpigmented tumors are also generally found to possess greater mitotic activity and a higher degree of anaplasia. (19,27,53)

Following this line of reasoning, however, it is difficult to explain the occurance of melanotic metastases from amelanotic primaries (6,49), and the occasional observation of pigmented and unpigmented lesions arising simultaneously and having identical growth characteristics. (59) Finally, this hypothesis would not apply to those very rapidly growing melanomas which are, at the same time, heavily pigmented. (18,49)

Promelanocyte Theory

There is a third rationale which may account for the existence of the amelanotic melanoma. This is the theory that, in the normal animal, there are both pigmented and unpigmented melanocytes which are capable of undergoing malignant change. The unpigmented cells, which will be called "promelanocytes", have been shown to be present in many sites

in mammalian skin. (51,61) It is thought that they form a "reservoir" of potential melanocytes, which replace those lost by normal attrition in the hair follicles, and perhaps, at the dermal-epidermal junction. (29, 45, 61) Some of the loci in which they have been found are shown in Fig. 1, p. 7.

It has been demonstrated that this "promelanocyte" is capable of fairly extensive migration, presumably at least in part by ameboid movement. (23,62) Once it arrives in its ultimate site of residence, however, it assumes all the morphological and functional characteristics of a mature melanocyte. These cells may be compared in some respects to the embryonic melanoblasts, but their constant presence in the adult skin precludes the use of that term.

Another capability of the promelanocyte is proliferation. This may be assumed to take place in the normal course of melanocyte replacement in the hair follicle, and it has been shown to be a factor in the repigmentation of denuded areas of skin. (62) Both these characteristics, then, set these cells apart from the mature melanocyte, which apparently is not capable of either active movement or, in the normal state, rapid cell division.

The existence of the promelanocyte forms an attractive hypothesis to account for the appearance of amelanotic melanomas. A tumor made up entirely of these cells would be completely amelanotic. Growth would be rapid, mitoses frequent, and, at least theoretically, a higher degree of anaplasia might be expected, since these cells appear to be

more closely related to the multipotential stem cell than are the pigmented melanocytes. If there were also an admixture of neoplastic cells of the more mature type, a tumor of mixed characteristics and mixed pigmentation would result, with the possibility of metastasis of either (or both) varieties.

Of course, this theory in no way explains the etiology of the malignant change in the melanocyte, nor is it meant to. However, the very existence of "mixed" tumors made up of both pigmented and unpigmented cells would point very strongly to the simultaneous reaction of cells of both types to a common stimulus.

Experimental Correlations

How do the experiments described in this paper relate to this hypothesis?

In the first place, the transplantable amelanotic melanoma with which work was begun proved frustrating in several respects, the major one being its tendency to sarcomatous transformation. When implanted in the host animal, the tumor engendered neoplastic change in the adjacent normal connective tissue, leading to the formation of a fibrosarcoma. (25) There are strong indications that this secondary tumor overgrew the original melanoma and thereafter was carried as the main constituent of the transplant line. If this is the case, the results of Part One and Three of this study are of questionable validity.

Part One

The first attempts at inducing re-melanization in this tumor were actually based on two separate lines of reasoning:

1. If hypofunction of the tyrosine-melanin pathway is responsible for the depigmentation, a great excess of one of the substrate constituents (conceivable tyrosine) might serve to drive the reaction towards completion.

2. If the melanocytes actually retain their melanin-producing capacity, but in a masked or inhibited form, perhaps the administration of large amounts of their naturally-occurring controlling hormone would promote a return to normal function.

In both cases, results were negative, with no pigment detectable grossly or microscopically, and an equivocal dopa reaction.

In terms of the "promelanocyte" theory, this approach has several drawbacks. If the tumor is composed of these cells whose pigment-producing mechanisms presumably are not yet present, immature, or inhibited, a simple excess of substrate would not be expected to "bridge a gap" which might be present anywhere in the complex biochemical pathway. In the same way, a hormone whose only known action is upon the mature melanocyte need not be expected to have a similar

effect on its precursor cell. As a matter of fact, MSH has been proven <u>not</u> to promote an increase in the number of melanocytes, as might be expected if pro-melanocytes were stimulated to melanogenic activity. (37)

Parts Two and Three

The second and third Parts of this study, involving the administration of the carcinogen 9,10 dimethyl- 1,2 benzanthracene (DMBA), were intended to investigate the previously demonstrated ability of this substance, in common with others, to produce melanocyte proliferation. (7,14,41, 46, 48,67) Again, the results obtained in Part Three are difficult to interpret because of the questionable identity of the transplanted tumor. The absence of regional lymphatic metastases in the treated animals is intriquing, however, and it is hoped that further work may be done along these lines in the future.

The experimental results of the second Part are essentially identical with those obtained by Shubik, et. al. in the original study. (14,58) One point of difference was a shorter latent period in the development of the melanotic lesions. These were found to be present by the 29th week after treatment, as against 38 to 41 weeks in the original report. The absence of neoplastic changes other than the typical "blue nevi" after a single application of DMBA has been confirmed by others. (14,58)

One observation, however, apparently has never before been reported. That is the appearance of areas of persistent

depigmentation of hair over the site of DMBA application. Many workers have kept the animals close clipped throughout the course of the experiment, which might account for the omission.

In this connection, Ghadially and Barker have shown that one of the first sites of action of topically applied carcinogens is in the upper hair follicle. Often the pilo-sebaceous apparatus is destroyed by the substance before neoplastic changes begin. (22) In the hamster, in contrast to other laboratory animals, there are accumulations of melanocytes lying close to the pilo-sebaceous structures of some follicles. (Fig. 1, Zone "A") It is apparently these groups of cells which are induced to undergo proliferation by the action of DMBA, with the formation of intra-dermal nevi. (22,66) In genetically white (but not albino) animals, these accumulations are made up of amelanotic, or, if you will, promelanocytes. In these animals too, DMBA will produce proliferative lesions. (28,46) However, in this case, very lightly pigmented "nevi" are the rule, although they are histologically similar to those induced in the dark-coated hamsters. Here is strong evidence that pigmented or unpigmented melanocytes will "breed true" when undergoing at least mild neoplastic change.

Thus, the results of the experiments described here would indicate the following relationships:

1. The response of hamster skin to high concentrations of DMBA in single application is the

permanent destruction of follicular melanocytes, with sparing of other hair and skin structures.

2. In slightly lower concentrations, this substance, as well as some others, stimulates rather rapid proliferation of perifollicular melanocytes and/or promelanocytes. (28,47) In the case of melanocytes, the proliferation is associated with increased melanin production and its phagocytosis by macrophages.

V

Summary

An attempt has been made to arrive at some sort of unified hypothesis to account for the differently pigmented lesions which the proliferation of melanocytes can produce. The following summarizes the author's conclusions:

1. The skin of many mammals contains simultaneously pigmented (mature) and unpigmented (precursor) melanocytes (promelanocytes).
2. Under normal conditions, only the latter cell type has the capacity for migration and proliferation, the mature melanocyte serving only as a stationary pigment factory.
3. Both cell types are capable of undergoing neoplastic change, in some cases simultaneously, apparently in response to a common unknown stimulus. The macroscopic pigmentation of the resultant tumor and its metastases will depend upon the proportions of these constituent cells.
4. While the promelanocytes retain the ability to undergo maturation into pigment-producing forms, the opposite transformation has not been proved; that is, there is no incontrovertable evidence

for dedifferentiation in the melanoma.

It is hoped that further work, including several definitive experiments in the hamster, can be carried out in the future.

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