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Refractoriness of PET mouse integument to the nerve growth factor

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TO THE NERVE GROWTH FACTOR

BY

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ABSTRACT

This paper deals with the refractoriness of PET mouse integument to the subcutaneous administration of nerve growth factor. Newborn mice were injected once daily with NGF through ten injections. Gross and histological examination showed that the skin of the experimental animals was completely unresponsive to this agent. No alterations in the hair growth cycle or pattern were observed.

INTRODUCTION

From the extensive tissue grafting work by Rawles in 1947, it was determined that the pigmentary system of mammals is derived from the fold of tissue capping the neural tube, known as the neural crest. This rather inconspicuous system of cells gives rise to many other structures, among them the spinal and sympathetic ganglia.

It was reported in 1948 by Bueker, that the implantation of mouse sarcoma 180 tissue into the body wall of 3-day chick embryos produced an excessive enlargement of the spinal ganglia. This work was carried out to test the theory held at that time which stated that the size of a ganglion and its neurons was dependent, to some degree, upon the milieu of the peripheral field of innervation. The real significance of this work was unappreciated until 1951 when Levi-Montalcini and Hamburger, while performing similar experiments, observed an enlargement in not only the sensory but also the sympathetic neurons. Further investigation revealed that a nerve growth factor (NGF) was associated with such neoplastic tissue (Cohen, et al., 1954). A similar factor was also found in snake venom (Cohen and Levi-

Montalcini, 1956) and could be extracted from the submaxillary glands of adult mice (Cohen, 1960).

An investigation by Satterlee (1967) was carried out to determine the effects of NGF upon the melanocyte population in newborn mice. It was reported that minor alterations in the pigmentary system resulted from subcutaneous administration of the nerve growth factor. The pilary system of the experimental animals also exhibited an anomalous condition previously unassociated with this factor. The hair growth at the site of injection was modified producing pigment deficient hairs of normal length and structure, short curled hairs, and normal but minute hairs arranged in a distinct spot.

The purpose of the present investigation was to determine the level of action at which NGF operates to alter hair growth. NGF could be influential in the growth cycle of the minute and curly hairs as a modifier of hair growth, which would support the theory purported by Chase and Eaton (1959) that an inhibitor substance controls the growth of hair in waves. Conversely, the factor could act as an accelerator substance which induces the follicle to by-pass the late anagen and catagen stages to early telogen with the resulting hair being incompletely developed. This would support the theory advanced by Davis (1962) that the activity of a stimulator regulates

the growth of hair in waves. The precise action of NGF can be inferred from mitotic counts of the matrix cells in the affected hair follicles. An increased mitotic count would indicate the action of a stimulator in the environment and a decreased count could be interpreted conversely as an inhibitory action.

METHODS AND MATERIALS

The NGF was isolated from the submaxillary salivary glands of mice and the final purification of the protein involves the use of cellulose ion-exchange columns. Essentially two different methods of isolation were employed. The initial procedure followed that of Satterlee (1967) and the second method followed a similar procedure but was modified to conform with the original technique as described by Cohen (1960).

Isolation of NGF

For each isolation, one hundred adult male mice were sacrificed with chloroform and the submaxillary salivary glands were dissected free. The glands were frozen upon excision until all of the glands were recovered. The combined glands were homogenized in a Virtis "23" homogenizer for 3 minutes at maximum speed in 50 ml of cold distilled water. The homogenate was centrifuged for 10 minutes. All centrifugations were run at 0° C at a relative centrifugal force of 15,000 X g. The supernatant fluid was decanted and the residue was stirred with 22 ml of cold distilled water and recentrifuged.

To 9 volumes of the combined supernatant fluids was added one volume of a stock streptomycin solution (0.2 M

streptomycin sulfate adjusted to pH 7.8 with concentrated NaOH). The final pH of the mixture was 7.0. The mixture was allowed to stand in the refrigerator for 3 hours and was then centrifuged for 5 minutes. The clear red supernatant, referred to later as the original volume (personal communication with Cohen), was retained, and the pellet, consisting of precipitated nucleoproteins, was discarded.

Absolute alcohol was added in a ratio of 0.07 ml per ml of supernatant fluid. The mixture was allowed to stand for 15 minutes at 0° C and was then centrifuged for 10 minutes. The small precipitate was discarded and absolute alcohol was added to the supernatant in a ratio of 0.33 ml for each ml of original solution. The mixture was allowed to stand at -5° C for 45-60 minutes and was then centrifuged for 10 minutes. The supernatant fluid was discarded. The precipitate contained most of the red pigment and the growth factor. To the precipitate was added 25 ml of cold distilled water, and the pasty residue was stirred on a Magnestir in an ice-bath until uniformly dispersed. At this point, Cohen found it convenient to freeze and store the material overnight, but the isolation was continued in the present study by centrifuging the mixture for 10 minutes and then discarding the small precipitate.

The supernatant was fractionated by the addition of

a filtered saturated ammonium sulfate solution, pH 7.3. Five milliliter quantities of saturated ammonium sulfate were added to the supernatant until the mixture was 52.5 percent saturated. A time interval of 15 minutes at 0° C was allowed for precipitation after each addition of the ammonium sulfate. When the mixture was 52.5 percent saturated, it was centrifuged for 5 minutes at 0° C at 12,000 X g and the residue was discarded. The nerve growth factor was precipitated out by continued additions of saturated ammonium sulfate to the supernatant. When the mixture was 71.0 percent saturated, it was centrifuged for 10 minutes at 0° C at 15,000 X g, and the pellet, containing the active material, was retained and dissolved in 25 ml of distilled water to form a clear red solution. The solution was dialyzed with constant stirring in a refrigerator for 36 hours against repeated changes of distilled water. The mixture was centrifuged for 10 minutes and the slight precipitate was discarded.

In the initial isolation procedure, which was performed three times and followed the method of Satterlee (1967), dry cellulose powder was added to the chromatography tubes, which were also arid, and no effort was made to maintain the integrity of the columns. Wash solutions were poured directly onto the cellulose without regard to the consequences of such a disruptive act. In no case

was the technique of backwashing employed. Such an operation creates a homogeneously packed column by ridding the column of air bubbles which could hinder its exchange capacity. The second method of isolation, performed twice, involved a more refined technique of handling of the delicate and sensitive cellulose columns. The ion exchange columns were prepared according to Peterson and Sober (1956) and Stock and Rice (1963) by making a slurry from the prescribed weight of powder and a column volume of the initial wash solution. The slurry was decanted into glass tubes, fitted with sintered glass filters, which had been previously moistened with a column volume of the initial wash solution. The material was allowed to settle under flow conditions induced by gravity alone. Occasionally it was necessary to backwash when air bubbles were observed in the column. In all cases, the cellulose was of such a fine mesh that the column did not run dry. All columns prepared possessed the ability to hold the buffer level at the top of the column against the pull of gravity. It is of the utmost importance that the integrity of the column is maintained. In such an effort, care was taken to protect the top of the column by placing a circle of filter paper, cut accurately to the size of the column, on the top of the cellulose powder when the isolation demanded that the

material be dropped directly on the top of the cellulose. Such a procedure minimized the agitation of the top layer of cellulose.

The carboxymethyl cellulose (CM-cellulose) cation exchanger powder and the diethylaminoethyl cellulose (DEAE-cellulose) anion exchanger powder were purchased from Nutritional Biochemicals Corporation.

A short column of CM-cellulose was prepared as follows: 1 gm of the powder, in a column of 2 cm diameter and 40 cm length, was washed with a mixture of one part 0.5 M NaOH and one part 0.5 M NaCl, and then with 0.005 M NaCl until the eluate was slightly acidic. The dialyzed ammonium sulfate fraction was then passed through the column at a flow rate of 3-4 drops per minute. Under these conditions the active material was not absorbed. The cellulose was washed with 2 column volumes of distilled water and the water clear eluates were combined and designated as the CM-1 fraction. All of the red pigment remained adsorbed to the column.

A column of DEAE-cellulose was prepared. 3 gm of the powder, in a 2 cm diameter column, were successively washed with solutions containing (a) 0.5 M NaOH, 0.5 M NaCl, (b) 0.1 M NaCl, (c) 0.1 M phosphate buffer, pH 6.0 (0.0942 moles of NaH_2PO_4 and 0.0058 moles of Na_2HPO_4 to one liter of water), and (d) 0.005 M NaCl. The CM-1

fraction was then passed through the column followed by 80 ml of 0.01 M NaCl. The eluates were discarded. The active material was then eluted with 70 ml of 0.12 M NaCl (DEAE-fraction).

A second column of CM-cellulose was prepared. 3.5 gm of the powder, in a 2 cm diameter column, were successively washed with solutions containing (a) 0.5 M NaOH, 0.5 M NaCl, (b) 0.01 M NaCl, (c) 0.1 M sodium acetate buffer, pH 4.38 (0.0675 moles of acetic acid and 0.0325 moles of sodium acetate to 1 liter of water), (d) 0.01 M NaCl. The DEAE-fraction was then passed through the column. The column was washed with 40 ml of 0.1 M NaCl and then with 100 ml of 0.3 M NaCl. All the eluates thus obtained were discarded. The active material was then eluted with 70 ml of 0.75 M NaCl.(CM-2 fraction). The CM-2 fraction was dialyzed overnight against 0.1 M NaCl.

A third column of CM-cellulose was prepared as follows: 500 mg of the powder, in a 1 cm diameter and 30 cm long column, were washed as directed above for the CM-2 fractionation, except that the final wash was made with 0.1 M NaCl instead of 0.01 M NaCl. The dialyzed CM-2 fraction was passed through the column. The column was then washed successively with 15 ml of 0.1 M NaCl, 15 ml of 0.3 M NaCl, and 20 ml of 0.35 M NaCl. These eluates were discarded. The active material was then eluted with

15 ml of 0.75 M NaCl (CM-3 fraction). The material was kept frozen and under such conditions was stable for several weeks.

Preparation of Control Solution

A control solution was prepared in which 70 ml of 0.75 M NaCl was dialyzed overnight against 0.1 M NaCl and passed through a column which had been prepared identically to that of the third CM-cellulose column. The final eluate was designated as the CM-3 carrier.

Assay of the Nerve Growth Factor

An ultraviolet absorption spectrum was run on the active material from runs 1, 4, and 5 using 0.75 M NaCl as a standard. The operation was performed using a recording spectrophotometer and the CM-3 fraction from the frist isolation showed no appreciable absorption at any wave length. The active material from runs 4 and 5 showed a peak at 280 mμ and a trough at 250 mμ.

The biological assay which was utilized to determine the nerve growth promoting activity of the CM-3 fraction was the hanging drop tissue culture technique according to Cohen, et al., (1954) and Cohen (1958;1959). Three or four sensory ganglia from 8-day chick embryos were explanted in each culture. The medium consisted of one

part rooster plasma, and two parts Eagle's 199 medium with the material to be tested in a final volume of 0.075 ml per culture. The cultures were observed after 18 hours of incubation at 37° C and the presence of fiber outgrowth served as an index of NGF.

Injection Procedure

In the first isolation procedure, a 1 cm diameter column, rather than a 2 cm diameter column was utilized for the CM-1 column. The product of this run was injected intracutaneously into newborn PET mice in a volume of 0.05 ml per injection. The first injection was administered from 24-36 hours after birth and single daily injections were made subsequently for fourteen days. At various time intervals, two mice were sacrificed by decapitation and skin from the injection site was recovered. In the second isolation, the animals were injected subcutaneously with 0.05 ml per injection, and in the third run, the animals were injected subcutaneously with 0.1 ml per injection. In both of these runs, two animals were sacrificed and the skin was recovered at varying intervals post primary injection time.

After the final two isolations, newborns were given single subcutaneous dosages of 0.1 ml of the solution to be tested for ten days. Every twelve hours after the first

injection, two experimental animals were sacrificed. Every 24 hours after the first injection two control animals were sacrificed.

In all animals, the point of injection was slightly anterior to the pelvis and lateral to the middorsal line. The point of the needle (27 gauge, 10 mm) was carried rostrally to the area slightly caudal to the small fat pad which covers the scapula. It was intended that the syringe needle discharge its solution in approximately the same area upon each injection. After sacrificing the animal, the skin was removed from over the injection site. To insure a relatively flat piece of skin with which to work, the sample was placed with the dermal side down on a slip of filter paper. This greatly facilitated its inspection as a whole mount and its sectioning. The tissue was fixed in Bouins solution for 24 hours, dehydrated, cleared in methyl salicylate, and examined in whole mount. The tissue was then cleared in xylene, embedded in paraplast, sectioned at 7 μ, stained with Delafield's hematoxylin, and examined histologically.

A total of five isolations were performed and a total of 64 mice were injected with the CM-3 fraction in the first three experimental series. A total of 10 control animals were simultaneously run in conjuction with the three isolations. After the final two isolation

procedures, a total of 34 mice were injected with the nerve growth factor while 20 control animals were employed.

RESULTS

Run number one

A total of fourteen mice were injected with 0.05 ml/ injection of the CM-3 fraction, and skin samples were taken at 72, 120, 168, 192, 216, 264, 312, and 336 hours after the first injection. Only one animal exhibited the distinct spot which was reported (Satterlee, 1967) as a result of NGF administration. The spot appeared as a recognizable entity at 6 days of age and was identified by a characteristic circular area, 6 mm in diameter, of unpigmented and partially pigmented hairs (Fig. 1). Upon whole mount inspection, several abnormalities were evident. Many fully formed but unpigmented hairs covered the entire spot. Scattered throughout the center of the spot, a collection of short, curly, stunted hairs were observed (Figs. 2 and 3). Histological examination of skin samples from experimentals as early as 72 hours revealed a curling of the hair in the follicles (Fig. 4) and a depigmentation of the hair in other follicles (Figs. 5 and 6). Although abnormalities in hair growth were obvious, the follicles displayed many mitotic figures which are typical of normal anagen hair. The height of the skin appeared increased with the major difference in the dermis (Fig. 7). This seemed a consequence of hypertrophy and

hyperplasia of the sebaceous glands (Fig. 4). Although only one animal manifested the distinct spot, tissue recovered at all ages showed one or more of the abnormalities cited above. These results were surprising as a very vital part of the isolation procedure was not performed according to Cohen. All control skin appeared normal in whole mount and histologic observation.

Run number two

A total of nineteen mice received injections of the CM-3 fraction in a volume of 0.05 ml/ injection. Skin samples were taken at 72, 96, 120, 168, 216, 264, 288, and 336 hours after the first injection. Although this isolation procedure had been performed precisely as indicated by Satterlee, the abnormalities he observed did not appear. Only minor alterations in the integument were observed. A slight dimunition in the number of follicles at the injection site and the appearance of a number of small incompletely formed hairs from a sample recovered after 72 hours were the only abnormalities observed (Figs. 8 and 9).

Run number three

It was hypothesized that the volume of the CM-3 fraction administered in run two was below the minimum

required to produce the spotting effect, so a volume of 0.1 ml/ injection was administered to 31 mice in run three. Skin was recovered at periods of 12, 24, 36, 48, 60, 72, 84, 96, 144, 192, 240, 288, and 336 hours after the first injection. In all cases, the skin recovered at this time appeared completely normal without any of the aberrations attributed to NGF administration. The skin from the six control animals injected with 0.1 ml/ injection of CM-3 carrier and carried through 10 injections was normal.

Runs four and five

At this point in the research Dr. Stanley Cohen of Vanderbilt University was contacted, and through his generous guidance, alterations were made in the Satterlee procedure to confrom with the Cohen procedure. The biological assay utilizing tissue culture techniques to definitively confirm the presence of NGF was undertaken at his suggestion. The CM-3 fractions of runs four and five were assayed for nerve growth factor, and in both cases, the tissue culturing of chick spinal ganglia in the presence of the CM-3 fraction resulted in extensive neurite outgrowth (Figs. 10 and 11). Ganglia in control cultures were completely unreactive (Fig. 12). The absorption spectra of the CM-3 fractions showed an identical pattern to that observed by Cohen (1960) confirming the

presence of the growth factor (Fig. 13). A total of 34 mice were injected with the nerve growth factor and skin samples were recovered at intervals of 12, 24, 36, 48, 60, 72, 84, 96, 108, 120, 132, 144, 156, 168, 192, 216, and 240 hours after the first injection. In all cases the tissue appeared completely normal and undistinguishable from the control tissue injected with the CM-3 carrier.

DISCUSSION

The neural crest has been proven to be the progenitor of cells which contribute to the formation of many structures. Extensive research utilizing vital staining and extirpation procedures, much of which was summarized by Horstadius (1950), has given embryologists an indication of the many strucutres which this transitory embryonic tissue produces. Weston (1963) using labled neural crest and radioautographic techniques, unquestionably confirmed much of the previous research which had been performed. He showed that the spinal and sympathetic ganglia, the chromaffin cells of the suprarenal medulla and the majority of the pigment cell population are all derived from this common embryological site.

Levi-Montalcini and her co-workers have shown that many tissues derived from the neural crest are particularly reactive to the nerve growth factor, which was first isolated from mouse sarcoma tumors (Cohen, et al., 1954; and Cohen and Levi-Montalcini, 1957) and later isolated from snake venom (Cohen and Levi-Montalcini, 1956; and Cohen, 1959) and the mouse submaxillary salivary gland (Cohen, 1960). NGF markedly stimulates the growth of embryonic sensory and embryonic and mature sympathetic nerve cells in a variety of vertebrates, including man

(Levi-Montalcini and Cohen, 1960; and Levi-Montalcini and Booker, 1960). The ganglion of Remak as well as the chromaffin cells also show a responsiveness to the nerve growth factor (Levi-Montalcini and Hamburger, 1951; Levi-Montalcini, 1952; and Levi-Montalcini and Hamburger, 1953). The submucosal and intramural plexuses (of Meissner and Auerbach) of the enteric system and the parasympathetics, however, are refractory to the nerve growth factor (Levi-Montalcini and Hamburger, 1951; and Levi-Montalcini and Hamburger, 1953). The ventro-lateral cells of the embryonic spinal ganglia likewise exhibit a lack of response to the NGF while the dorso-medial cells are extremely sensitive (Levi-Montalcini and Hamburger, 1951).

To analyze the effect of this protein upon the epidermal and dermal pigment cell population, Satterlee (1967) subcutaneously injected a solution, which was thought to contain the nerve growth material, in daily administrations to 1-4 day-old PET mice for ten days. Grossly, the effects of this material upon the integument were profound. At the 7th day of treatment, a whitish spot averaging 8-10 mm in diameter appeared over the injection site. At the termination of the injection period, the skin was cleared and examination in whole mount revealed that the nonpigmented and partially pigmented hairs had masked, upon gross observation, an

area of integument which contained abnormally structured appendages. At the core of the spot, approximately 2-4 mm in diameter, was a dense zone of minute black normally structured hairs. Centrifugal to this region was a ring of short, black, curly hairs and an associated area containing a reduced number of integumentary melanocytes.

Histological examination supported the whole mount observations. The short black hairs at the hub of the spot appeared small but well formed. Their anatomical position within the integument appeared normal with the base of the follicle stationed a short distance above the panniculus carnosus. The growth pattern of the dwarf hair was highly irregular due to a pronounced curling of the hair follicles. The follicles possessed good structural integrity although restricted to the dermis. The fully developed white and gray hairs which ringed the periphery were easily distinguishable from their pigmented neighbors.

The needle's penetration path through the subcutaneous connective tissue, and the region of injection with the resulting disruption of the subcutaneous tissue, could be identified in serial sections.

The objective of the present study was to duplicate the above experiment and examine more critically the alterations which occur in the hair growth cycle as a

result of NGF administration. The fact that I observed similar results to those listed above only in the first isolation and injection procedure, which was performed incorrectly and in which the spectrophotometrically determined absorption peaks were undetectable, indicates that purified nerve growth factor is not responsible for the anomalies. In all animals injected with NGF, as confirmed by the tissue culture and absorption spectrum assays, there were no alterations in the hair growth nor in any of the other skin appendages. It can be concluded that PET mouse integument is refractory to the administration of nerve growth factor.

Possibly some material other than nerve growth factor is responsible for the abnormalities recorded by the previous investigator. It is conceivable that the refinement in technique, enabling the isolation of a highly purified NGF, removed such a hypothetical impurity. It is highly unlikely that the impurity, if one exists, was the epidermal growth factor (EGF) which also was isolated from the submaxillary gland of mice (Cohen, 1962). Levi-Montalcini and Cohen (1960) reported that the injection of the CM-1 fraction, which now is known to contain EGF, produced somatic abnormalities unassociated with injection of purified NGF. Injection of this factor resulted in the following changes: (1) loss of body weight, which was

readily apparent on the third day after birth and became more severe in the following days, and a general stunting of growth. At the end of the fourth week, the mice were barely larger than at the end of the first week; (2) failure of hair growth was apparent at the end of the first week and even at the end of a full month the mice were still naked; (3) a precocious opening of the lids, which normally takes place between the twelfth and fourteenth days, occurred between the fifth and seventh days; (4) eruption of the upper and lower incisors and the first signs of their calcification preceeded by four or five days these processes in controls. The previous investigator did not record any sign of these deviations in growth pattern.

The samples of skin which showed reactivity to the CM-3 fraction, in all cases, were injected in the region just above the panniculus carnosus and below the base of the follicles (Fig. 14). Possibly the CM-3 fraction isolated by the previous investigator contained the NGF, although no assays were run to determine this, and his method of injection played a role in the effect of the CM-3 eluate. As I repeated the injection procedure, the ability to inject at a specifically desired level was acquired, and in the latter runs an effort was made to inject below the panniculus carnosus (Figs. 15 and 16).

This procedure was undertaken to minimize the disruption and possible damaging of follicles by intracutaneous injection. But, conversley, the panniculus carnosus may in such cases have acted as a barrier to the purified NGF.

It is inconceivable that the stage of hair growth was influential in the effects of the isolate upon the skin. During normal hair development, a hair goes through three phases in each generation of growth. Dry (1926) divided the growth cycle into the anagen, catagen, and telogen stages. Chase (1954) divided the anagen stage, which is the phase of active growth, into six substages. Anagen I-IV encompasses the formation of the follicle as the epidermal 'germ' region grows down and surrounds the dermal papilla. The strucutral integrity of the appendage is created during these stages, the mitotic activity in the matrix is initiated, and the hair starts forming. In anagen V the hair grows to about the level of the epidermis. In anagen VI the hair emerges beyond the surface of the skin. In the mouse, anagen VI lasts for 8 to 9 days and the hair will grow at the rate of nearly 1 mm/ day.

Catagen is a relatively brief transitional stage during which the follicle is converted from an active to a resting unit. Mitotic activity in the matrix ceases and the club and capsule of the resting hair are formed.

The telogen stage is the resting stage in which there is no activity.

Dry (1926) stated that the first generation of hair growth was completed on the sacrum of the mouse by the 19th day after birth. This would mean that all of the hair considered in the present study and that of Satterlee (1967) was in the anagen phase. It has been shown repeatedly that the stage in which a hair exists is extremely important in relation to its reactivity to stimuli. Chase (1965) noted that wounds, including grafting, chemical damage, and plucking could induce a resting follicle to go into anagen, but the effect of these same treatments on follicles already active was varied, from little for some to pronounced regressions for others. The fact that all hair examined in the present and the previous experiment was in the same phase of hair growth rules out the possibility that a common stimulus (NGF) produced strikingly different and distinctly characteristic effects during specific stages of the growth cycle.

While Satterlee's results could not be duplicated, even while employing identical isolation techniques, some substance isolated from the submaxillary gland produced the aberrations which he observed. Although it is impossible to state with any authority the nature

of the material producing the abnormalities, it can be concluded that the effects were not a result of the administration of highly purified nerve growth factor.

SUMMARY

It was found that PET mouse integument is refractory to NGF. When newborn mice were injected with purified nerve growth factor, no alterations nor abnormalities were observed in the hair growth, skin appendages, nor any other feature of the experimental animal's development. Unanswered is the question as to the nature of the material which produced the aberrations in PET mouse skin as reported by Satterlee (1967).

Fig. 1

14 day old mouse injected with CM-3 fraction from run 1. 4 X

Fig. 2

Tissue from over injection site recovered after 264 hours. Animal injected with CM-3 fraction from run 1. 40 X


1

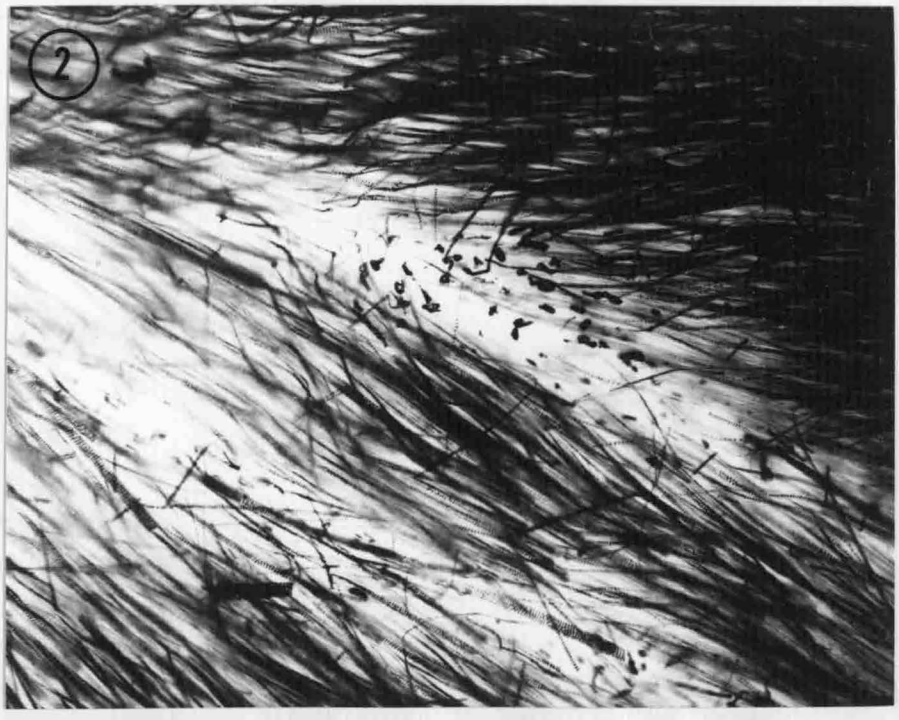

2

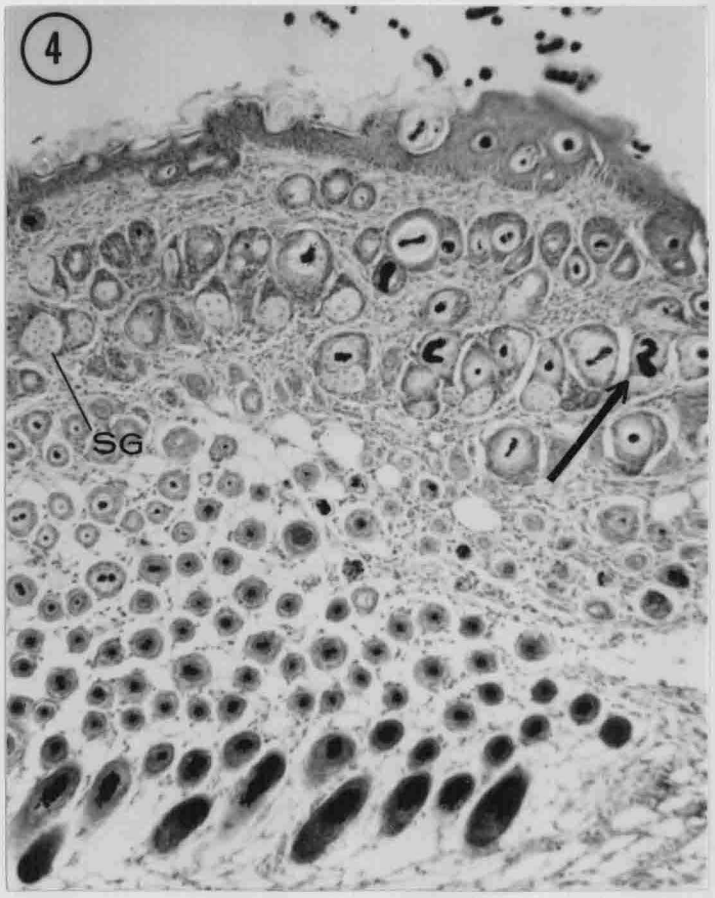
Fig. 3

High power of figure 2. Note short curled hairs at injection site. 125 X

Fig. 4

Skin recovered at 72 hours from run 1. Note curling of hair in the follicle (arrow). Sebaceous glands show hypertrophy and hyperplasia (S. G.). 125 X


3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SG

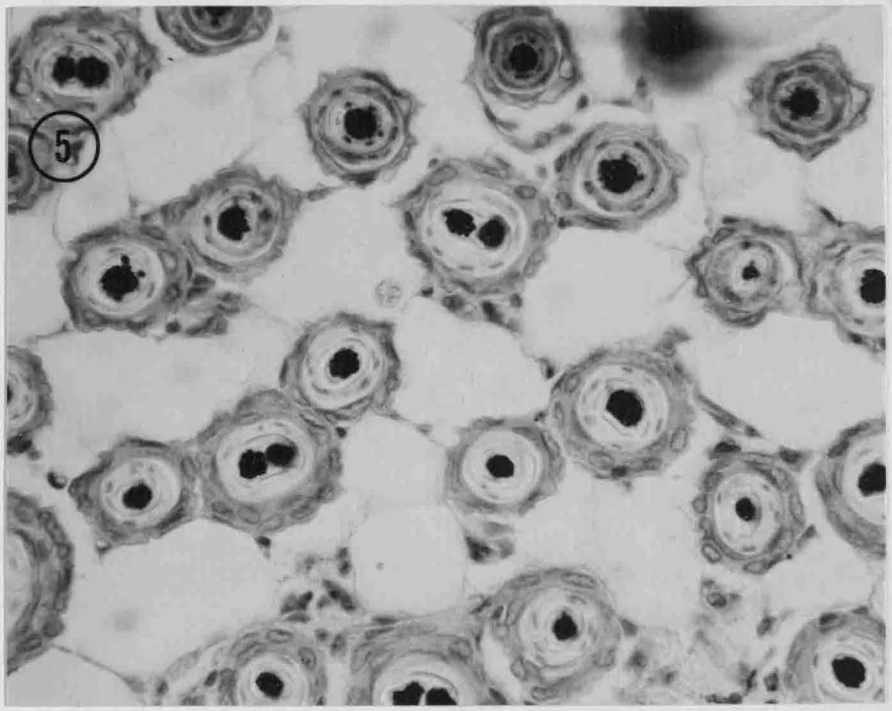
Fig. 5

Cross section of normal hair at 72 hours.

440 X

Fig. 6

Cross section of nonpigmented and partially pigmented hair from skin in figure 4. 440 X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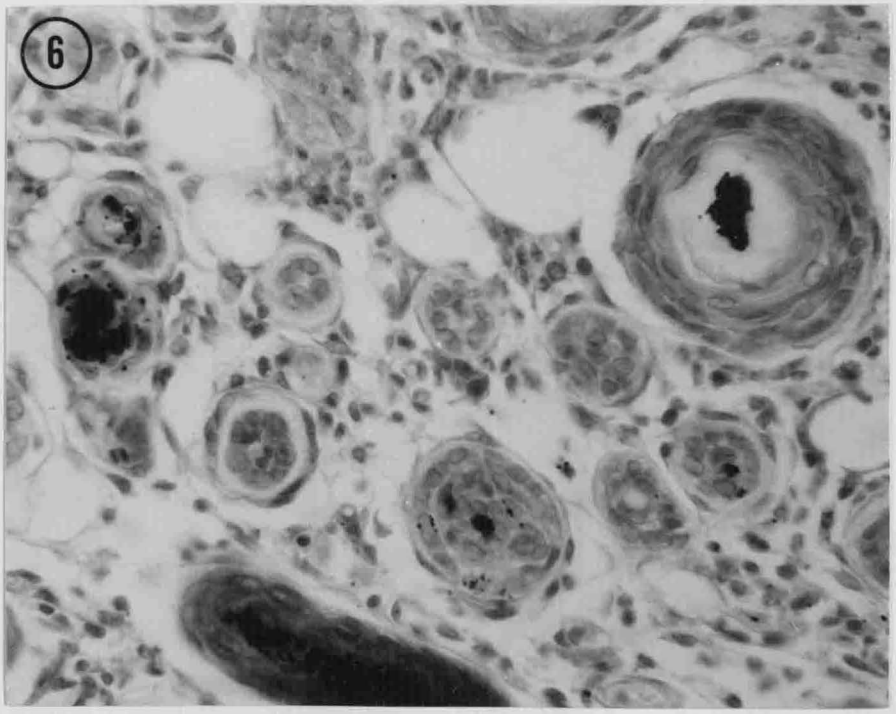

6

Fig. 7

Skin from injection area in run 1 at 72 hours.

Arrow indicates level of skin outside of reactive area.

Note enlargement in sebaceous glands (S. G.).

125 X

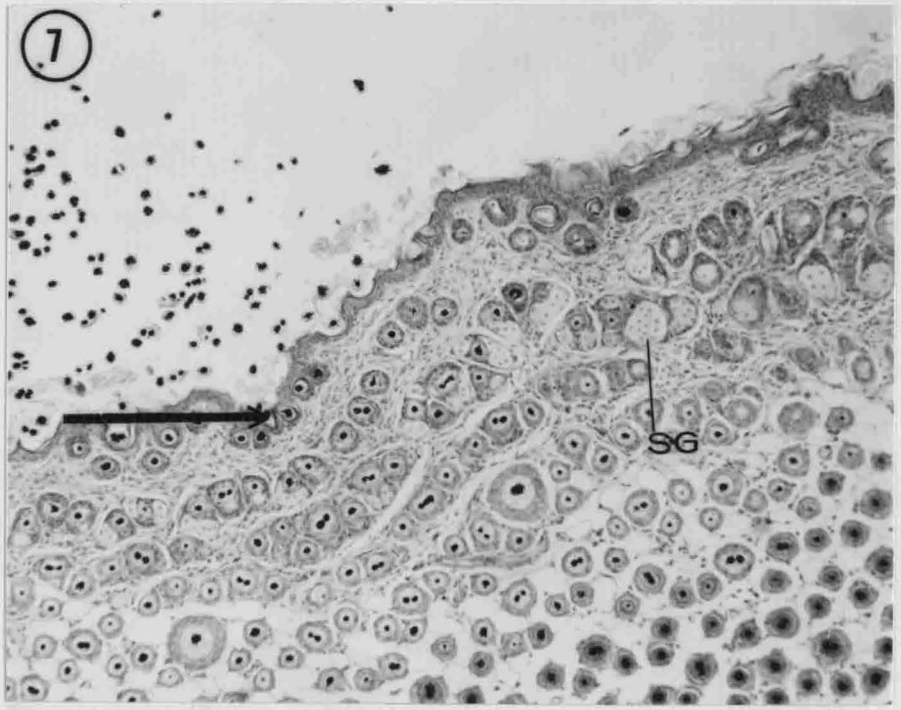

7
SG

Fig. 8

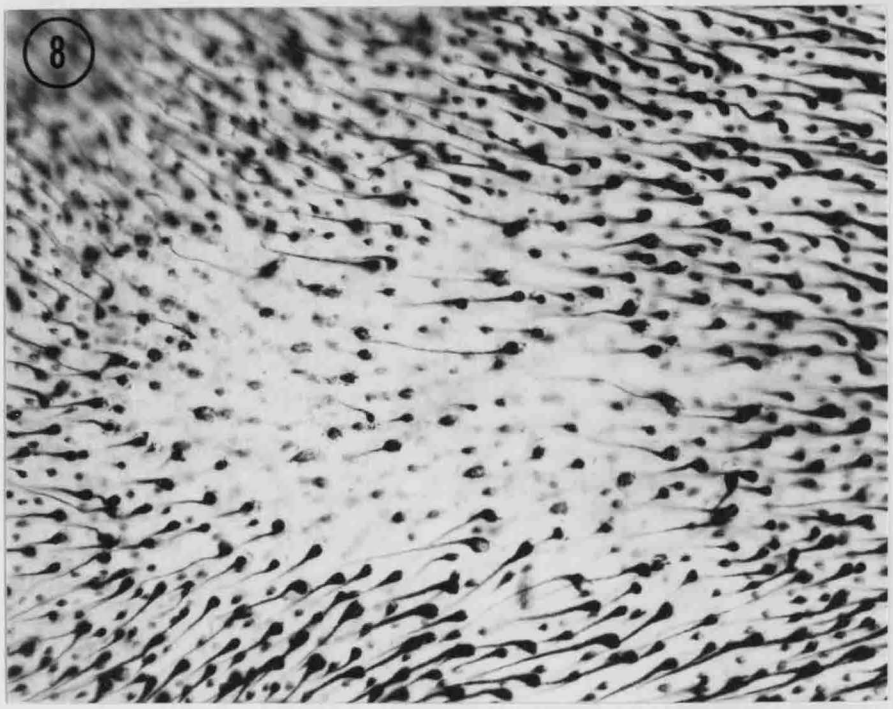
Skin from injection site in run 2 at 72 hours.

40 X

Fig. 9

High power of figure 8.

100 X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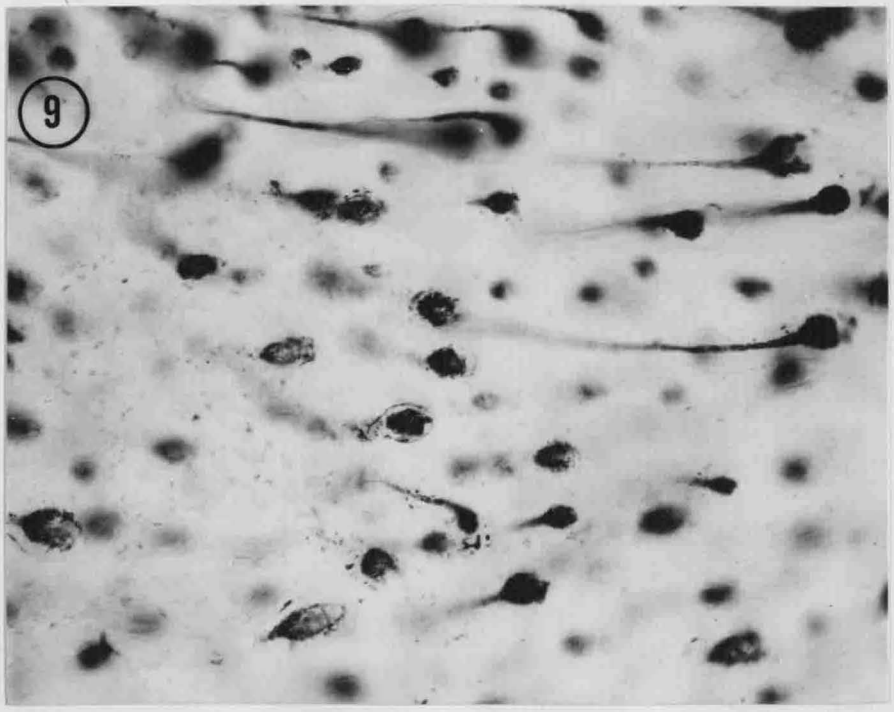
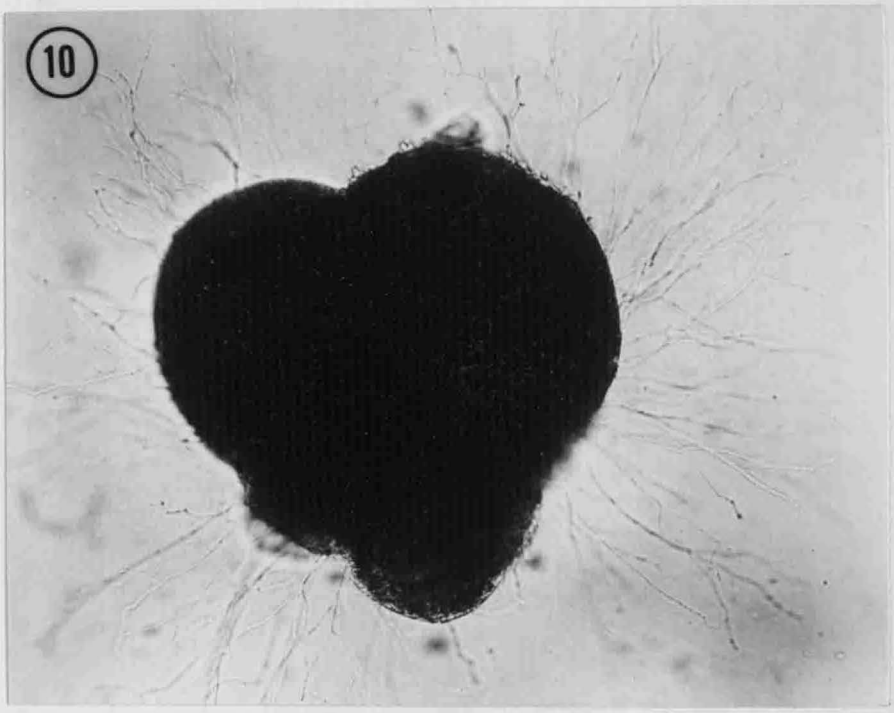

9

Figs. 10 and 11

Spinal ganglia from 8-day old chicks which were explanted into tissue cultures containing NGF from runs 4 and 5. Note the fiber outgrowth. 100 X


10

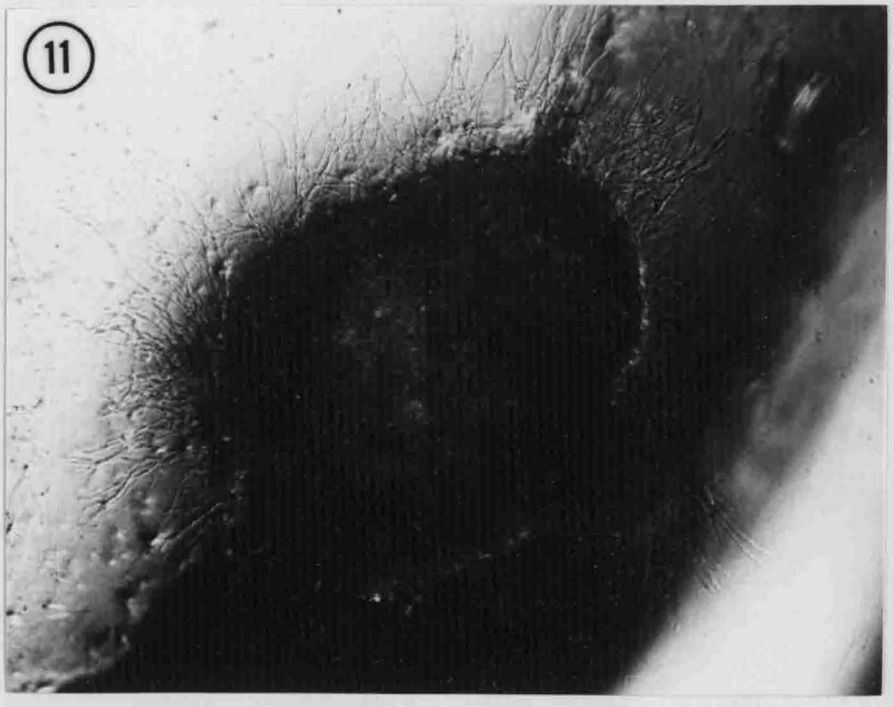

11

Fig. 12

Spinal ganglion from 8-day old chick embryo cultured in control medium. 100 X

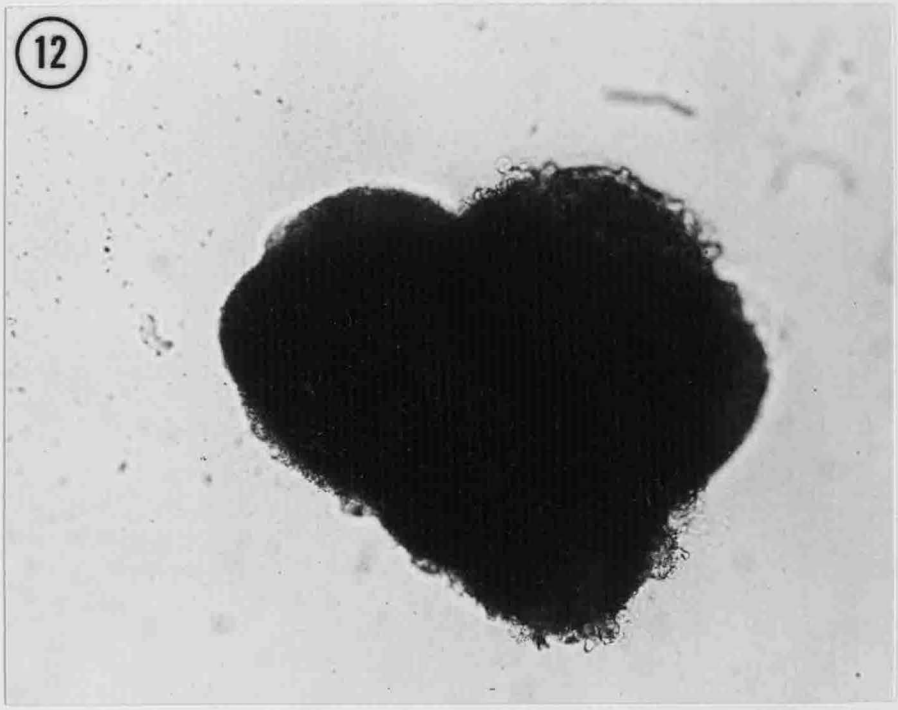

12

Fig. 13

Absorption spectrum of CM-3 fractions from runs 4 and 5.

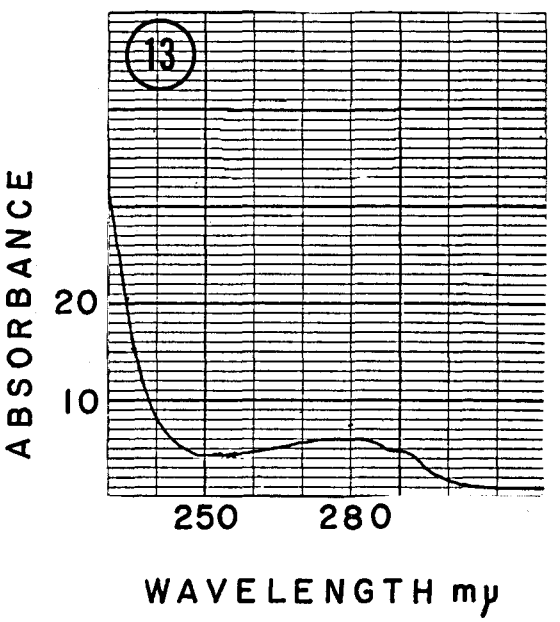

13
ABSORBANCE
20
10
250
280
WAVELENGTH mμ

Fig. 14

Same as figure 4. Note intracutaneous penetration path of needle (arrow). 125 X

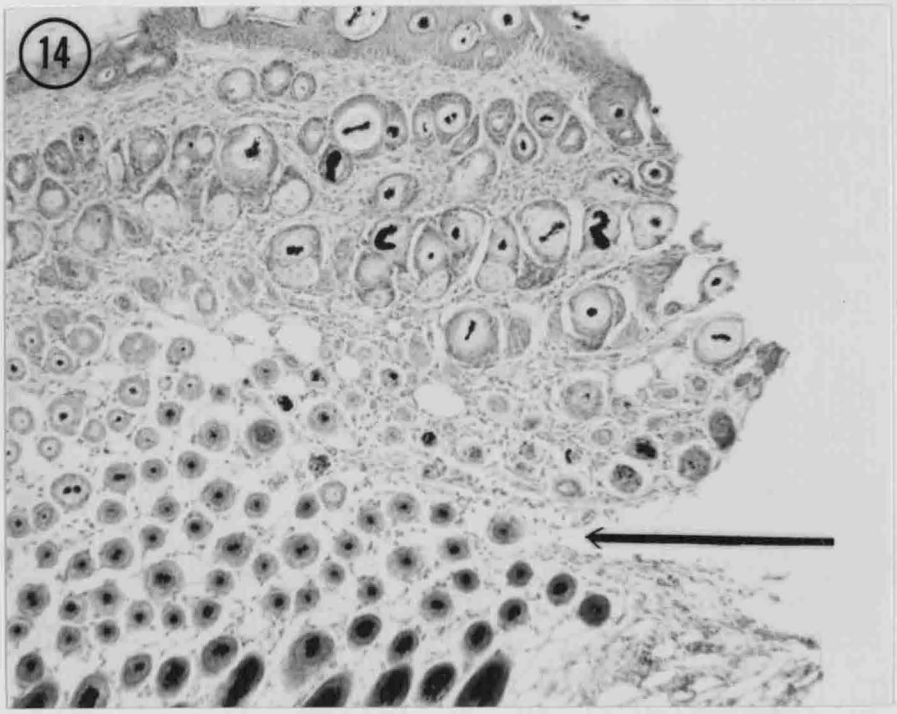
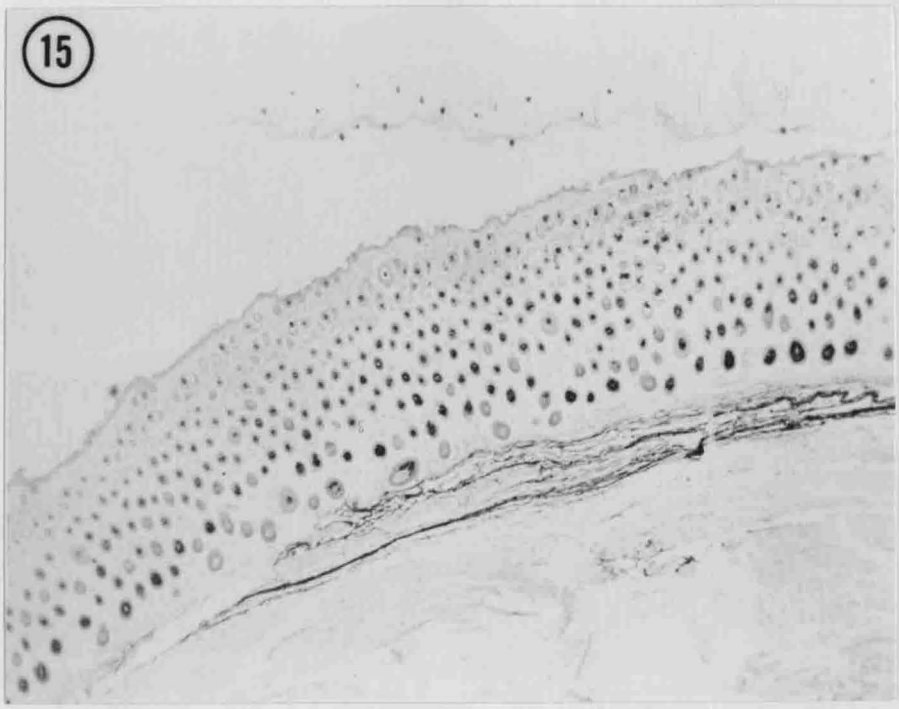

14

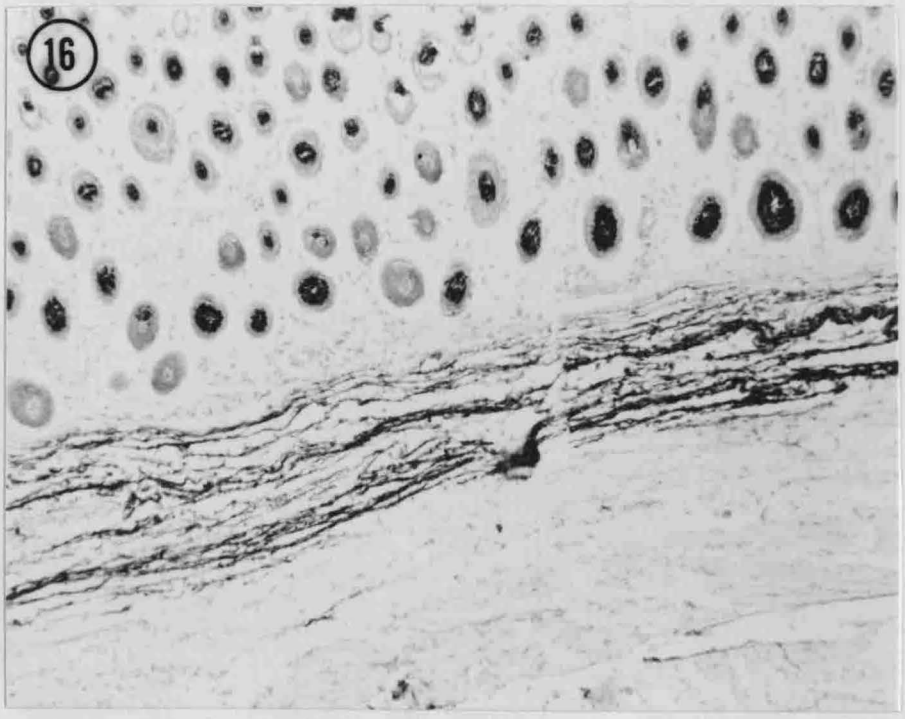
Fig. 15

Four day old PET mouse injected with India ink to illustrate the penetration path of the needle. Note that the majority of the ink is concentrated below the panniculus carnosus. 40 X

Fig. 16

High power of figure 15. 100 X


15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VITA

Walter Hollifield Dorman was born in Baltimore, Maryland on August 13, 1944. He attended grade schools in that city and graduated from McDonogh School, McDonogh, Maryland in 1962. In the fall of 1962 he entered Wake Forest College and majored in Biology. While at Wake Forest he was elected to the position of president of the Inter-Fraternity Council during his junior year and president of the Gamma Omicron chapter of Theta Chi fraternity during his senior year. He graduated from Wake Forest in 1966 with the B. S. degree.

In the fall of 1966 he entered the Master's program in the Deaprtment of Biology at the University of Richmond. While in this program he was elected president of Beta Theta chapter of Beta Beta Beta national honorary biological society and assisted in the General Biology and Comparative Anatomy laboratories. He attended numerous scientific meetings including the 1967 and 1968 meetings of the Association of Southeastern Biologists and the 1968 meeting of the Virginia Academy of Science. He co-authored a paper entitled "The Refractoriness of Mouse Dermal Melanocytes to Hormones" which he presented at the 1968 ASB meeting and which was published in volume 50 of the Journal of Investigative Dermatology. He received the Master of Science degree in August of 1968 and will enter the University of Maryland School of Medicine in September of 1968.