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THE EFFECTS OF A NERVE GROWTH-PROMOTING PROTEIN ON THE INTEGUMENT OF PET MICE

BY

CRAIG BAUMAN SATTERLEE

APPROVED:

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COMMITTEE CHAIRMAN

EXAMINING COMMITTEE

_____ _____

COMMITTEE MEMBER

_____ _____

COMMITTEE MEMBER

_____ _____

THE EFFECTS OF A NERVE GROWTH-PROMOTING PROTEIN ON THE INTEGUMENT OF PET MICE

A THESIS
PRESENTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF RICHMOND
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS OF THE
DEGREE OF MASTER OF SCIENCE IN BIOLOGY

BY
CRAIG BAUMAN SATTERLEE
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INTRODUCTION

A factor which specifically stimulates the growth of sensory and sympathetic nerve cells has been under investigation for a number of years. This factor, originally isolated in 1951 from mouse sarcomas 37 and 180, (Levi-Montalcini et al., '51) was named the "nerve-growth factor" (NGF) (Cohen, '58). Its action was first shown *in vivo* by inoculating the sarcoma into chick embryos (Levi-Montalcini, '52; Levi-Montalcini et al., '51) and subsequently *in vitro* by culturing chick ganglia and mouse sarcoma together (Levi-Montalcini et al., '54). In the years immediately following the initial discovery, much more potent sources of NGF were found in the salivary glands and salivary secretions of a variety of species. These include rattle snake venom (Cohen, '58), the venom of the Gila monster (Cohen et al., '56), and the saliva and salivary glands of the mouse (Cohen, '60, '62).

It was not until a partially purified fraction of NGF from the mouse submaxillary gland was injected into newborn mice that non-neural effects were observed. Among the non-neural changes noted was a failure of hair to grow although the purified NGF did not produce this effect (Cohen, '62). Cohen showed that the non-neural effects were due to an "epidermal growth

factor", later renamed "epithelial growth factor" by Jones ('60), which was different from the NGF though present in the same starting material.

Since the purified NGF has its most pronounced effect on the sensory and sympathetic ganglia cells which are embryonically derived from the neural crest (Horstadius, '50; Hamburger, '52; Yntema, '47) it was thought that the NGF may effect other neural crest derivatives as well. As above mentioned, hair growth was effected by the partially purified NGF and hair is pigmented by melanocytes which are neural crest derivatives (Rawles, '47; Weston, '63). It was the purpose of this research to qualitatively explore the effects of purified NGF on the integument and specifically on pigmentation by melanocytes in mice of the PET strain.

MATERIALS AND METHODS

A. Preparation of the nerve-growth factor.

The procedure for the isolation of the NGF followed that of Cohen ('60) and was performed twice. The submaxillary glands of eighty adult male mice (22 gm body weight or over) were excised and put in a freezer (-5°C) for 24 hr. The frozen glands were homogenized for 3 min at maximum speed in a Virtis homogenizer after addition of 50 ml of cold glass-distilled water. The homogenate was centrifuged in an International Centrifuge (Model HR-1) for 10 min. All centrifugations were performed at 0-2°C at 15,000 x g. The supernatant was decanted and the residue suspended in 22 ml of cold glass-distilled water. After centrifugation for 10 min the second supernatant was decanted and combined with the initial one.

To nine volumes of the combined supernatant fluids was added one volume of stock streptomycin solution (0.2 M streptomycin sulfate adjusted to pH 7.8 with concentrated NaOH). The mixture was refrigerated for 3 hr and was then centrifuged for 5 min. The clear pink supernatant was retained and the residue discarded.

Absolute ethyl alcohol was added to the supernatant in a ratio of 0.07 ml per ml of supernatant. The mixture

stood for 15 min at 0°C and then was centrifuged for 10 min. The small precipitate was discarded. To the supernatant was added absolute alcohol in a ratio of 0.33 ml per ml of original solution. The mixture was allowed to stand for 60 min at -5°C, and then was centrifuged for 10 min. The supernatant was discarded and the precipitate was dissolved in 25 ml of cold distilled water. At this point the mixture was frozen (-5°C) for 36 hr.

The frozen mixture was thawed and centrifuged; the small precipitate was discarded. The supernatant was fractionated by the addition of a saturated ammonium sulfate solution. The fraction precipitating between 52.5 and 71% supernatant saturation was retained (a time interval of 15 min at 0°C was allowed for the precipitation after each addition of ammonium sulfate solution). The active precipitate was dissolved in 25 ml of distilled water to form a clear pink solution. This solution was dialyzed in tubing with a wall thickness of 0.0010 in, with stirring, for 36 hr against repeated changes of distilled water. The mixture was then centrifuged and the slight precipitate discarded.

A column of CM-cellulose was prepared as follows: 1 gm of carboxymethyl cellulose powder (Nutritional Biochemicals Co.) was put into a 2 cm diameter column

40 cm in length and washed with a mixture of 0.5 M NaOH and 0.5 M NaCl (0.5 moles of NaOH and 0.5 moles of NaCl to 1 liter of distilled water) and then with 0.005 M NaCl until the eluate was slightly acidic. The pH of the eluate was checked by holding pH paper strips at the bottom of the column. The dialyzed ammonium sulfate fraction was then passed through the column at a flow rate of 3-4 drops per min. The flow rate was regulated by placing a titration tube above the column and adjusting the stopcock. The cellulose was washed with 2 column volumes of distilled water (approx. 300 ml). The distilled water eluate was combined with the ammonium sulfate fraction eluate. This solution was designated as the CM-1 fraction.

A column of DEAE-cellulose was prepared: 3 gm of diethylaminoethyl cellulose powder (Nutritional Biochemicals Co.) were put into a 2 cm diameter column 40 cm in length and were successively washed with the following solutions: (a) 0.5 M NaOH and 0.5 M NaCl mixture, (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 and designated as the DEAE fraction.

A second column of CM-cellulose was prepared: 3.5 gm of the powder were put into a 2 cm diameter column 40 cm in length and were successively washed with solutions containing (a) 0.5 M NaOH and 0.5 M NaCl mixture, (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), and (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 of the eluates thus obtained were discarded. The active material was then eluted with 70 ml of 0.75 M NaCl and designated as the CM-2 fraction.

A third column of CM-cellulose was prepared: 500 mg of the powder were put into a 1 cm diameter column 30 cm in length and were washed with solutions containing (a) 0.5 M NaOH and 0.5 M NaCl mixture, (b) 0.01 M NaCl, (c) 0.1 M sodium acetate buffer, pH 4.38, and (d) 0.1 M NaCl. The CM-2 fraction was dialyzed for 24 hr against changes of 0.1 M NaCl and 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 and designated as the CM-3 fraction. This fraction contained the isolated nerve-growth factor and was stored in a freezer at -5°C.

B. Preparation of CM-3 control.

Since the active protein of the CM-3 fraction was eluted in 0.75 M NaCl, a control solution without protein was prepared as follows: 70 ml of 0.75 M NaCl were designated as a CM-2 control. This solution was dialyzed, as was the active CM-2 fraction, and passed through a 1 cm diameter column 30 cm long with the procedure used to elute the CM-3 active fraction. The resulting solution was designated as the CM-3 control.

C. Preparation of inactive CM-3 fraction.

Since the NGF has been found to be heat labile (Cohen, '60), 10 ml of the active CM-3 fraction were boiled for 15 seconds to inactivate the protein. This solution was designated as boiled NGF.

D. Animals and experiments.

Newborn PET mice were given a series of subcutaneous injections, one per day for 10 consecutive days. The types of injections were (a) an empty #27 gauge needle insertion which acted as a control for trauma from needle penetration, (b) 0.9% NaCl which acted as a chemical reaction control, (c) CM-3 control fraction

which acted as a second chemical reaction control, (d) boiled NGF which acted as chemical control for the heat labile NGF, and (e) CM-3 active NGF fraction which acted as the only injectant containing active NGF. Mice receiving no injection were also used as controls. All injections were administered in a dosage of 0.1 ml, except for the CM-3 active fraction which was administered in dosages of both 0.05 ml and 0.1 ml. The point of entry of the needle was the sacral area on the dorsal back region of newborn PET mice. In order to avoid possible confusion between test effects and effects of trauma at the point of needle penetration, the needle was extended anteriorly (subcutaneously) the length of the needle shaft (10 mm) before injection was made. The point of furthest penetration of the needle will be referred to as the injection site. An effort was made to inject in approximately the same place each time. Generally, female mice accepted and maintained their litters during the experimental procedure. Occasionally, however, entire litters or individual pups were destroyed by their mothers. In these cases the entire injection procedure was reenacted with pups from other mothers. Twenty-four hours after the final injection the mice were sacrificed under chloroform anesthesia and the skin was removed from over the injection site.

E. Tissue preparation.

Immediately following excision the skin was fixed in Bouin's solution, dehydrated, and cleared in methyl salicylate. The samples were shaved with a razor blade and examined as whole mounts. Photographic records were made of selected whole mounts prior to their being sectioned. For histological examination fixed samples were embedded in paraffin, serially sectioned at 11 μ and stained with Delafield's hematoxylin.

RESULTS

A. CM-3 active NGF fraction.

Upon gross examination of the two mice receiving 0.05 ml of the CM-3 active fraction no abnormalities in hair growth pattern and hair color were observed. The whole mount preparation of the skin from the injection site, however, showed a few white and gray hairs interspersed throughout the growth of normal black hairs. A diminution in pigment cell numbers in areas of the dermis at the injection site was evident. The hair growth pattern was the same as that of the uninjected controls (figs. 1, 2, 13).

Eleven mice received injections of 0.1 ml of the CM-3 active fraction. Of these, nine mice displayed changes in the integument when observed in each of the three stages of examination. Grossly, an area 8-10 mm in diameter of unpigmented and partially pigmented hairs appeared at the injection site at day 6 and 7 of injection. All other integumental features appeared normal. Examination of the whole mount preparations revealed several abnormalities (figs. 5-8). In general three integumental discrepancies were observed: (a) a disappearance of pigmentation in hair at the site of injection, (b) a reduction of pigment cell numbers in the dermis,

and (c) a definite modification of the hair growth pattern. The area of unpigmented hairs encompassed a small dense area of abnormally short pigmented hairs that had been masked by the longer unpigmented hairs. The area of dense black hair was circular (2-4 mm in diameter). The growth pattern of the dwarf hair was highly irregular due to a pronounced curling of the hair follicles. In the surrounding area a marked decrease in pigment was evident in many hairs while others were completely devoid of pigmentation. The number of hair follicles was also reduced in this unpigmented region. Peripheral to the unpigmented area the skin appeared normal. Histological examination supported the whole mount observations and showed no further abnormalities (figs. 10-13).

B. CM-3 control fraction.

Of the 11 mice injected with the CM-3 control fraction, nine were normal at all stages of observation. However, the tissue from two mice from the first trial, upon whole mount and histological examination, revealed a slight decrease in hair follicle pigmentation. It is believed that this abnormality resulted from needle contamination since in the first few injections in trial 1 the needle was not washed between injections of the CM-3 active fraction and the CM-3 control fraction.

Following this unexpected response, the syringe and needle were carefully washed between each injection.

C. 0.9% NaCl; boiled NGF; empty needle insertion.

All specimens were normal at the three stages of observation.

DISCUSSION

This project has been based on the fact that spinal and sympathetic ganglion cells, as well as the melanocytes which pigment the skin and hair are derived from a common embryonic tissue -- the neural crest. Numerous investigators have used various techniques in an effort to prove this point. However, the work of Rawles ('47) and Weston ('63) offers some of the most convincing evidence for the common neural crest origin of spinal and sympathetic ganglion cells and melanocytes. Rawles, by isolating various portions of mouse embryos and transplanting these segments into the coelom of White Leghorn embryos, noted a definite correlation between the presence of pigment cells in the graft and the phase of development of the neural crest at the time of isolation. She also noted that only those tissues containing neural crest cells were capable of producing melanocytes. Furthermore, she observed skin and hairs, normal in every way, except for a complete lack of pigment when neural crest-free grafts were made. Weston, by labeling neural crest cells with tritiated thymidine and noting the migration pattern and eventual residence of these cells has not only confirmed Rawles' work, but also has resolved many of the discrepancies surrounding the neural crest origin of the spinal and sympathetic

ganglion cells. It is apparent from the research that the spinal ganglia, sympathetic ganglia, and pigment cells commonly evolve from the neural crest.

Since researchers have provoked variations and abnormalities in pigment cells and hair follicles by various physical and chemical agents (Uhr, '57; Van Scott, '58; Chase, '58; Steinmuller, '61), an effort was made to show that the discrepancies observed in the PET mouse integument were due to the NGF itself. Sodium chloride, which was contained in the solution used to extract the CM-3 active NGF fraction, was found to be an insignificant factor in the responses observed as no abnormalities resulted in the integument of mice injected with either CM-3 control solutions or 0.9% NaCl. Although small numbers of hair follicles were destroyed by the trauma of needle penetration the overall effects of needle insertion were consistent in most of the mice injected, regardless of the solution. The fact that the boiled NGF failed to provoke integumental variations demonstrated that the effects were due to a heat labile substance, the NGF. There also appeared to be a possible correlation between the amount of the CM-3 active NGF fraction injected and the degree of response. Only slight variations in the integument were observed in those mice receiving relatively small (0.05 ml) active CM-3 injections. Also, as above mentioned,

trace amounts of the CM-3 active fraction provoked a response in two control mice.

The anagen stage of the hair cycle is the phase of active proliferation and growth; this stage continues for approximately 17 days in newborn mice (Dry, '26). Hair follicles in the anagen stage have been found to be highly susceptible to various chemical and physical influences, whereas those follicles in the telogen (resting) and catagen (senescence) stages generally displayed little or no response (Van Scott, '58; Chase, '58). All injections reported in this experiment were initiated and concluded in the anagen stage. Van Scott ('58) demonstrated that intravenous dosages of methotrexate diminished the diameter of the anagen hair bulb, but not telogen and catagen hair bulbs, in the human scalp and that the degree of constriction in a given patient was proportional to the amount of drug received. Chase ('58) found that x-ray dosages had pronounced effects on hairs at the different stages of development, but that the most devastating effect was on the anagen hairs, often producing epiliation. There was also a direct correlation between the size of the dose (up to 300 r) of radiation received by the hair roots and the percentage of anagen hairs showing morphological changes. Whether or not telogen or catagen hairs would respond

to the NGF as the anagen hairs did remains to be investigated.

Since the NGF has been shown to be a protein (Cohen, '58), it was believed that an immunological response could have been the possible cause of the abnormalities observed. However, Steinmuller ('61) performed heterologous and homologous skin grafts in very young rats and found that total rejection of these grafts did not occur in most cases for a range of 11-22 days, and that the immunological response onset ranged from within 7-17 days after transplantation. Complete rejection in some of the newborn rats was not evident until day 41. Steinmuller concluded that the ability to reject foreign living tissue was absent, or at least deficient in rats at birth, and it didn't become fully operative until sometime later. Furthermore, in an effort to induce hypersensitivity in guinea pigs by means of an antigen, Uhr ('57) found that a foreign protein reaction did not take place for 2-3 weeks after injection. One might assume then, a possible immunological immaturity accompaning the early development of mice, similar to that of other mammals, that would negate the possibility that the NGF could be evoking a foreign protein response. That mice do not respond to foreign protein during the first two post natal weeks gains support from the homoplastic grafting experiments of Rovee and Reams ('64).

Though it does appear that the NGF is the cause of the integumental discrepancies observed, the actual reason for the abnormalities is left for further experimentation. Whether there has been an increase or loss of normal homeostatic inhibitors or whether stimulated metabolites were released from damaged cells is total conjecture at this point.

SUMMARY

1. As a protein isolated from mouse submaxillary glands has been found to stimulate hypertrophy and hyperplasia of two neural crest derivatives (the spinal and sympathetic ganglia) of numerous vertebrates, an effort was made to ascertain NGF action on a third neural crest derivative -- the pigment cell.

2. Newborn PET mice were given a series of ten injections of an active NGF solution. Other newborns were injected with one of several types of control solutions. Only those mice receiving active NGF injections displayed integumental abnormalities.

3. Three types of abnormal responses were observed at the injection site in mice receiving NGF injections:

a. a marked diminution in pigment cell numbers in the dermis.

b. a disappearance of pigmentation in hair

c. a definite modification in the hair growth pattern.

4. The data in conjunction with the literature support the fact that these responses were not due to a foreign protein response, physical trauma, or an adverse chemical reaction, but to the NGF itself.

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TABLE 1

Summary of Response of Mice to Experimental Procedures.

			Type of observation		
Injectant	Dosage (ml)	Total no. of mice injected	Gross	Whole mount	Histological
Active CM-3	0.05	2	0	2	2
Active CM-3	0.1	11	9	9	9
CM-3 control	0.1	11	2	2	2
Boiled NGF	0.1	6	0	0	0
0.9 % NaCl	0.1	6	0	0	0
Needle	---	2	0	0	0

Figure 1. Unstained whole mount of dorsal trunk skin of normal 14 day PET mouse. Low low power.

Figure 2. 13 day normal skin, 11 u section, Delafield's hematoxylin. Low power.

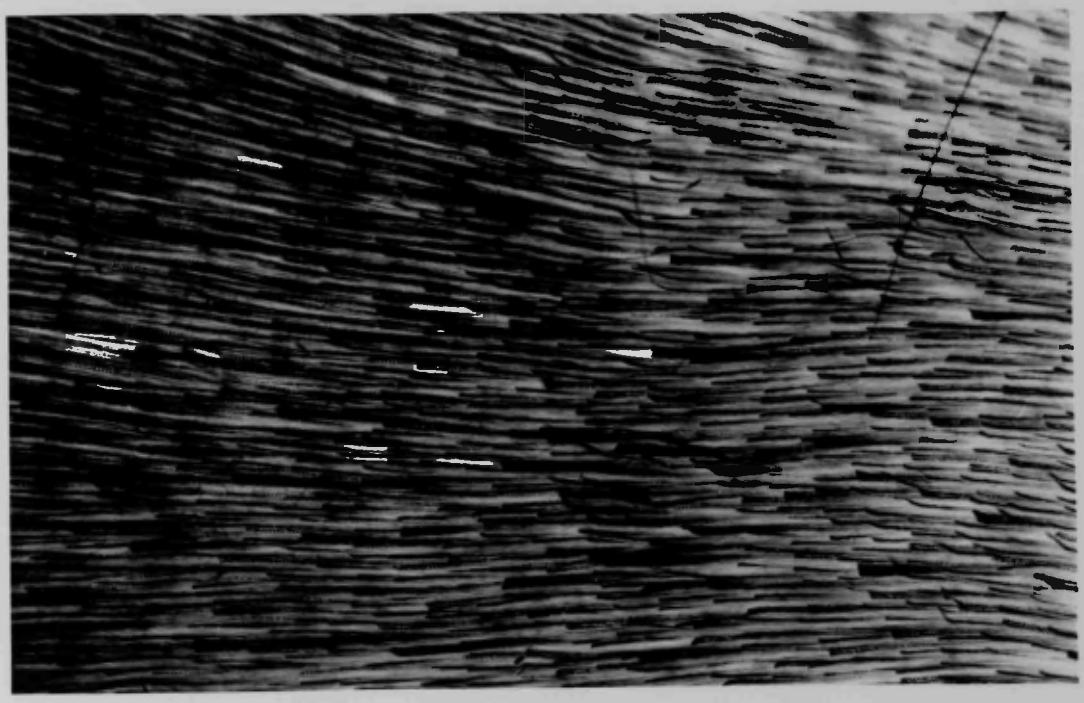
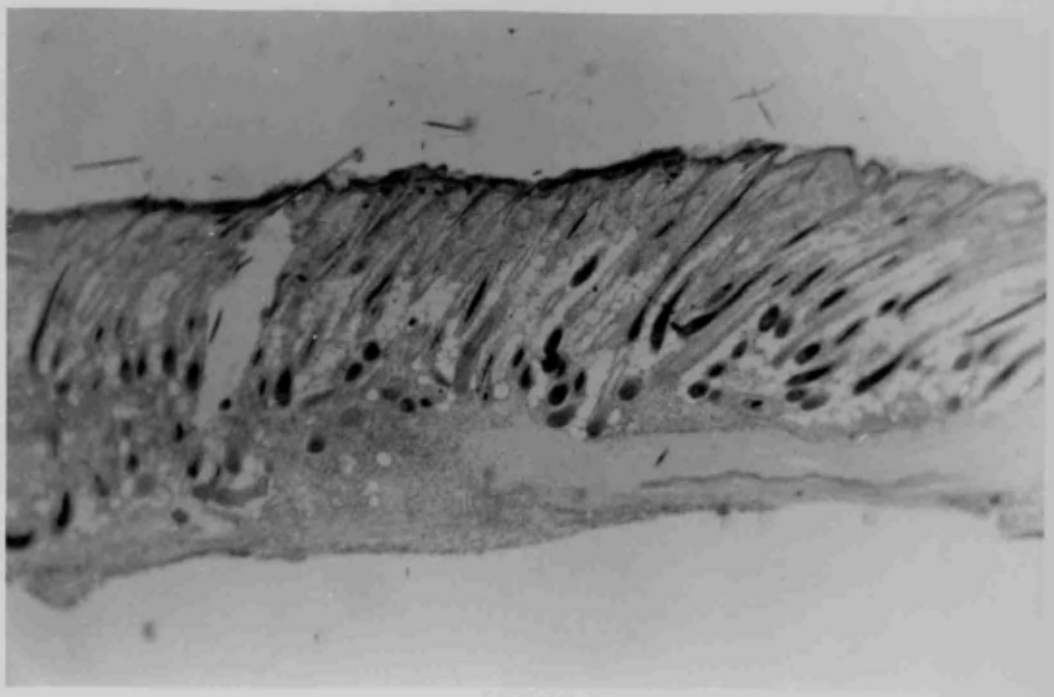


Figure 3. 13 day skin, injected with 0.75 M NaCl. Note hemorrhage channel indicating needle penetration. Low low power.

Figure 4. 12 day skin injected with boiled NGF. Compare to figure 2. Low power.



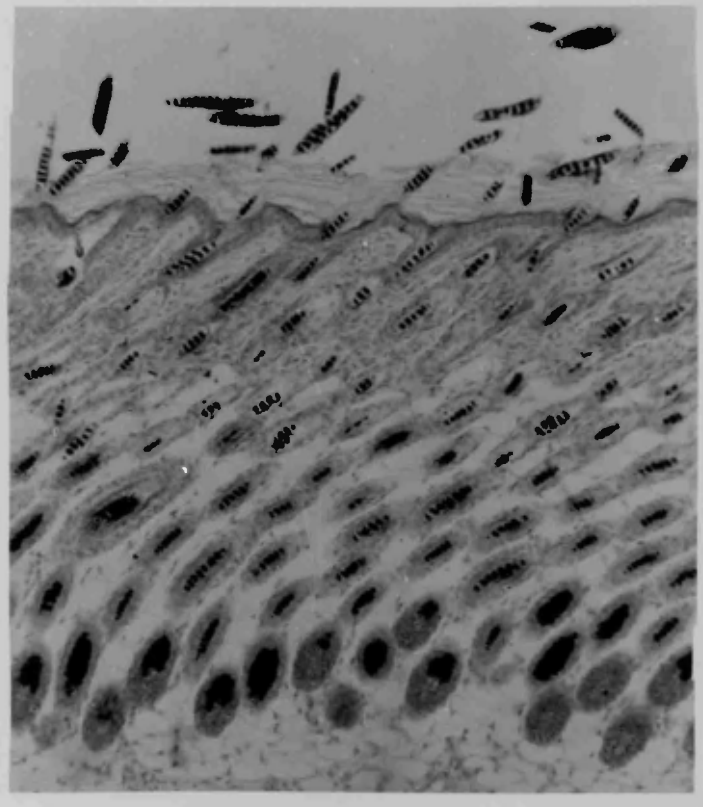
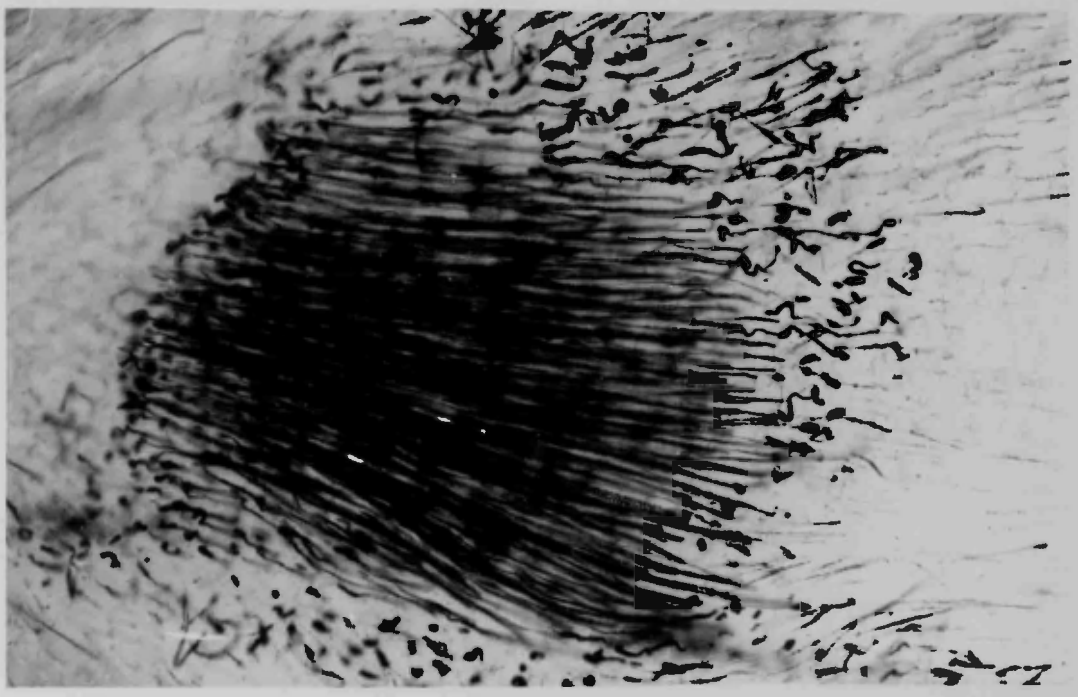


Figure 5. Whole mount of injection site of NGF. 15 day skin. Low low power.

Figure 6. Whole mount of caudal portion of injection site of Figure 5. Low power.



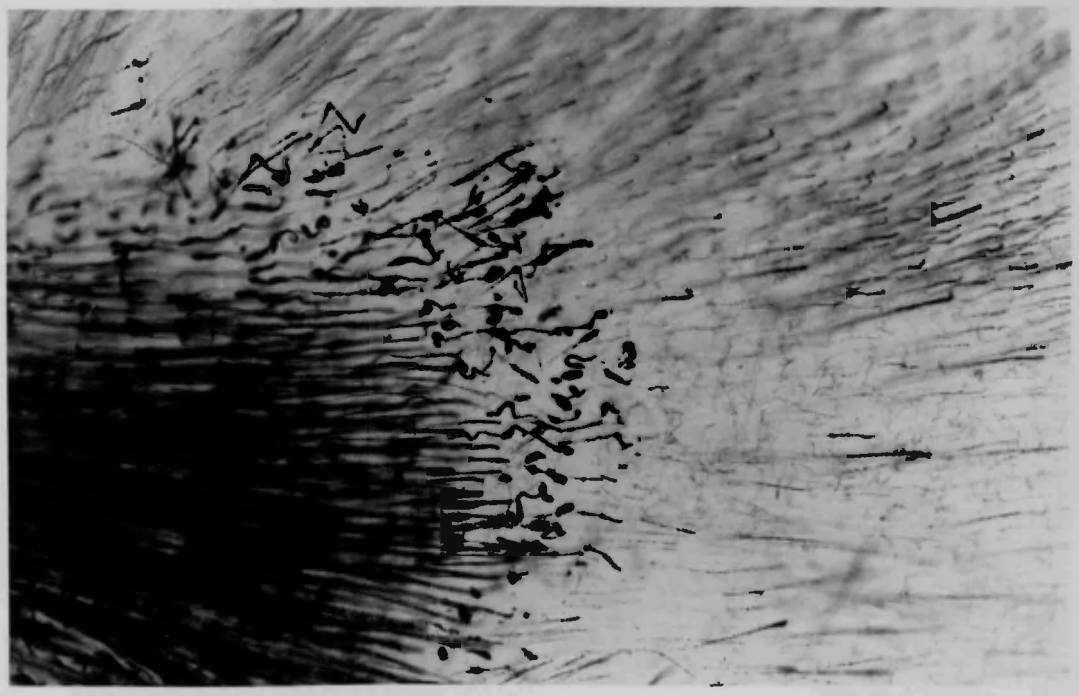


Figure 7. Whole mount of 15 day skin showing peripheral zone of NGF injection site. Low power.

Figure 8. Area adjacent to central tuft of hair in NGF injection site. Low power.

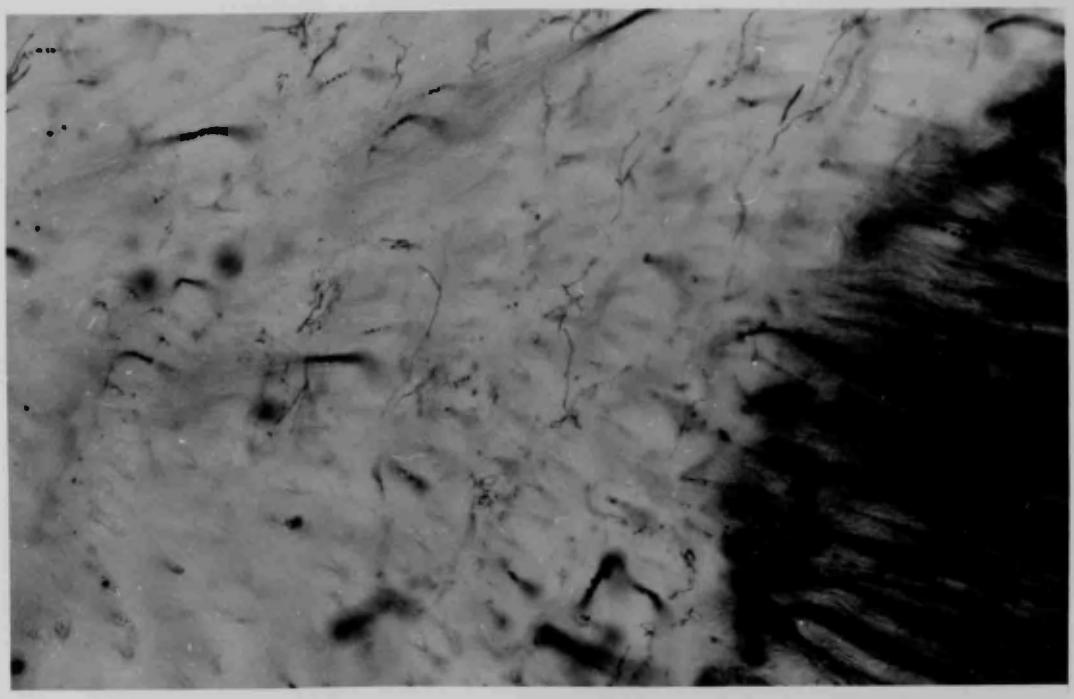
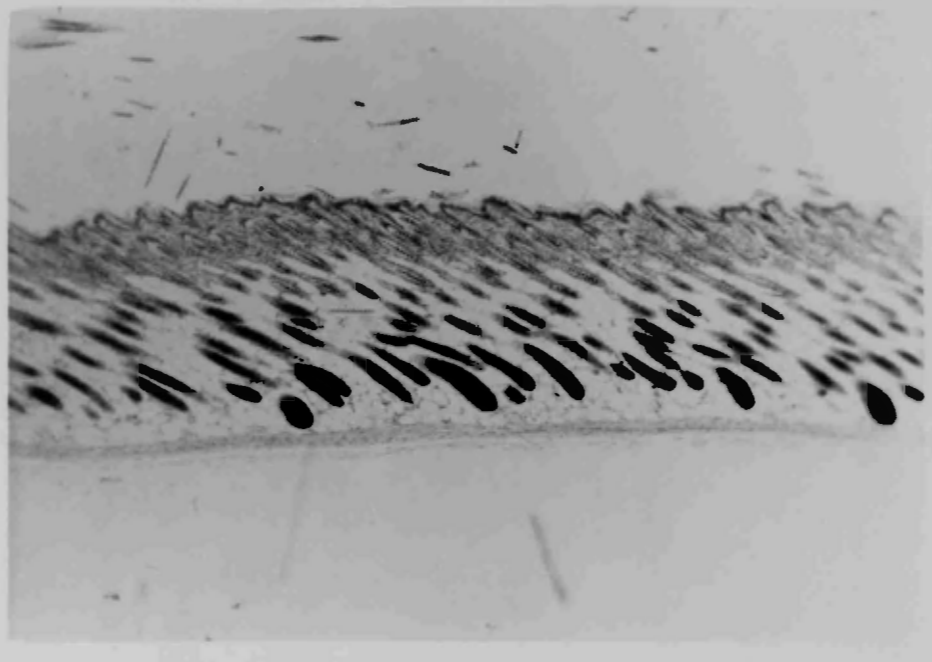


Figure 9. 13 day normal skin. Low low power.

Figure 10. 15 day skin of NGF injected PET mouse. Note absence of pigment in hairs and distortion of some hair follicles. Low low power.

Figure 11. Sample similar to Figure 10. Low low power.



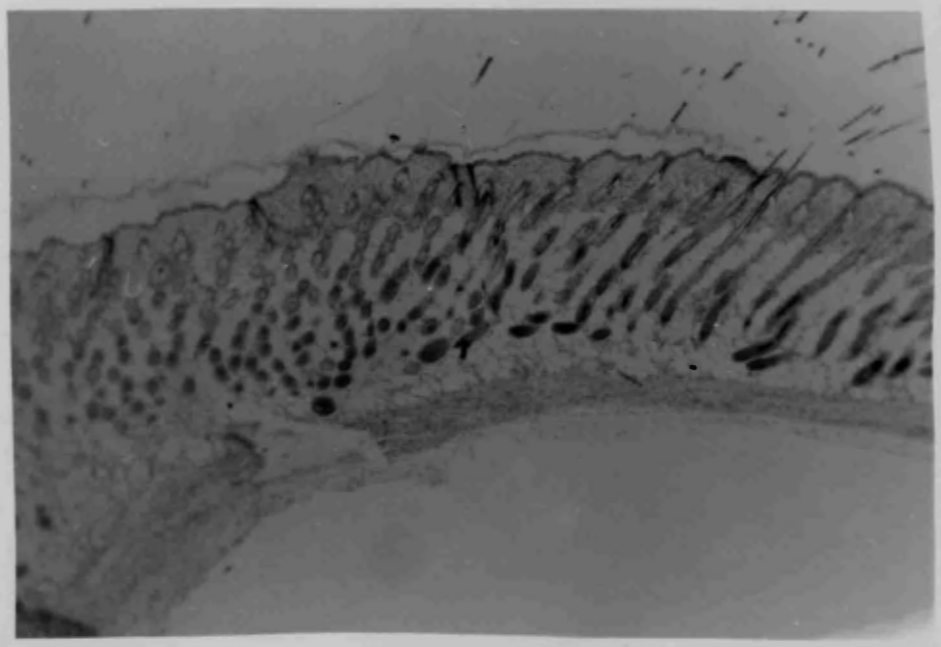
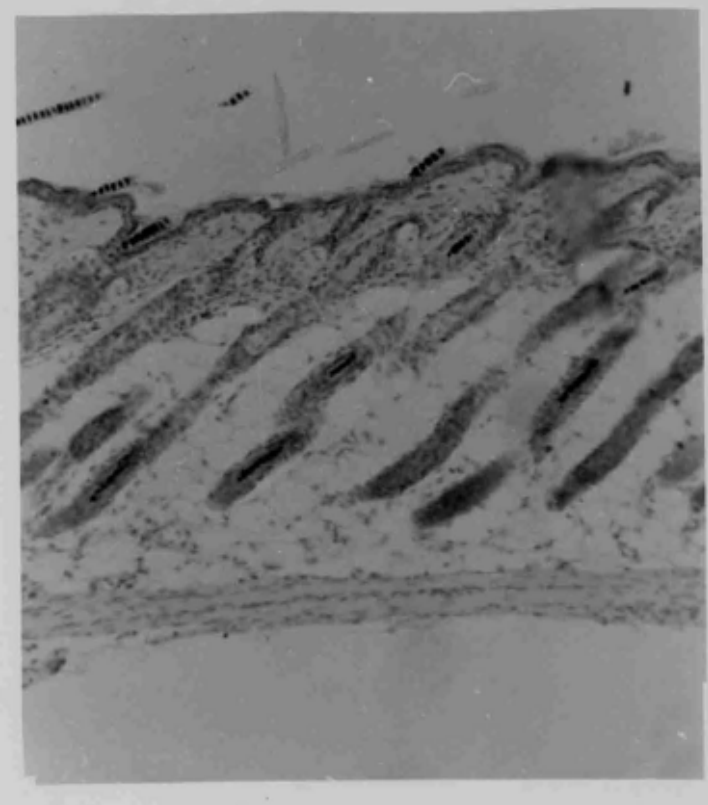
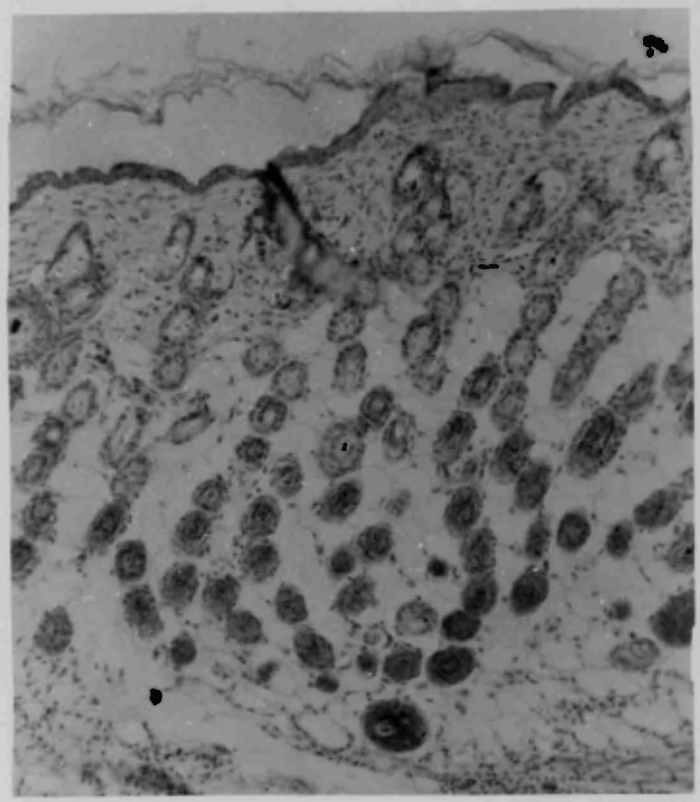


Figure 12. Low power view of Figure 10.

Figure 13. Low power view of Figure 11.





VITA

Craig Bauman Satterlee was born in Millville, New Jersey on July 2, 1943. He attended elementary and secondary schools in Millville and was graduated from Millville Memorial High School in 1961. The following September he entered Juniata College in Huntingdon, Pennsylvania where he served as class treasurer, chairman of underclassmen, and participated in varsity track. He received the B.S. in Biology in June 1965. In September 1965 he began his graduate work at the University of Richmond and received the M.S. in Biology in June 1967.

During his tenure at the University of Richmond he served as laboratory assistant in the General Biology and General Physiology laboratories. He was also employed in the research department of A. H. Robins Pharmaceutical Company.

At Richmond he became a member of Beta Beta Beta, an honorary biological society, where he served as president for one year. While completing his thesis he assumed a full-time teaching position in the physics department of Hermitage High School, Richmond, Virginia.