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THE EFFECTS OF SINGLE-DOSE X-IRRADIATION ON MELANOCYTES IN THE LEG MUSCULATURE AND SKIN OF THE PET MOUSE

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INTRODUCTION

Many workers have dealt with the problems involved in pigmentation of the skin, and some have made use of x-rays as a tool for these investigations. That x-rays can cause hyperpigmentation in skin has been known for a long time (Quevedo and Grahn, '58). That x-rays, paradoxically, can cause depigmentation is also known (Chase, '49; Cohen, '63). However, the exact nature of the mechanisms involved in the relationship between x-rays and pigment changes has not been determined with any certainty. It has been variously proposed that induced hyperpigmentation is due to: 1, an increase in pigment cell numbers by division of existing pigment cells (Becker, et al., '52); 2, an increase in the rate of melanin synthesis in each pigment cell in situ (Staricco, '63; Rovee and Reams, '64); 3, a migration of wandering amoeboid pigment cells into stimulated areas (Staricco, '63; Silvers, '58); or 4, an indirect, chemical response elicited by adjacent epithelial cells (Quevedo and McTague, '63; Cohen, '59; Markert and Silvers, '56).

While most investigations have been limited to the study of pigment cells in the epidermis and hair follicles, this work embraces pigmentation of the leg muscles and dermis as well. To this end, PET mice were used which

have an abundance of pigment cells in the leg muscles, but which show restriction of integumentary melanocytes to the dermis and hair. This characteristic provided the unique opportunity of comparing isolated systems of pigment cells in their response to identical stimuli of x-rays. Because pigment cells originate in the neural crest early in development (Rawles, '47) and migrate throughout the embryo, all pigment cells theoretically possess the same genetic potential for proliferation and melanogenesis. However, all pigment cell populations do not react equally to identical stimuli. Differences, therefore, must be dependent upon the cellular environment of the melanoblast (Markert and Silvers, '56).

Because terminology used to describe the various kinds of pigment cells recently has been changed (Fitzpatrick, et al., '66), the following will be employed in this paper.

Pigment cell. A general term for any cell in the melanocyte series.

Melanoblast. An immature cell in the melanocyte series, not yet containing melanosomes nor undergoing melanogenesis, but which has the potential to become, and at maturity normally does become melanotic.

Amelanotic melanocyte. A mature cell in the

melanocyte series, having melanosomes devoid of melanin, and not undergoing melanogenesis due either to cell milieu or sulfhydryl inhibition. It is capable of melanogenesis upon proper degree of stimulation, but under normal conditions it remains dormant.

Melanocyte. A mature, differentiated pigment cell that contains melanin within structured melanosomes.

MATERIALS AND METHODS

Mice of the PET strain are unusual in that melanocytes are found in significant numbers in the leg musculature and dermis. The initials PET stand for Pigmented Extraepidermal Tissue. The strain was established in 1958 at the Medical College of Virginia and was found to have a nearly ubiquitous distribution of extraepidermal melanocytes.

Chase ('49) has shown that 200-250 r of x-irradiation would provoke threshold pigment cell activity in hair, and Bloom ('54) reports that exposure to 500-800 r results in threshold pigmentation in human skin. However, as the optimum dosage level needed to stimulate pigment cell activity in muscle and dermis was unknown, a spectrum ranging from 200 to 7000 r was used in the work presented

here. The radiation levels, in roentgens, were 200, 400, 500, 600, 750, 900, 1000, 2000, 3000, 4000, 5000, 6000, and 7000.

Different doses of x-rays were given to the right hind limb of litter mates in order to eliminate the possibility that any one litter might show an atypical response at a given dosage level. The left hind limb, not irradiated, served as a control. Additionally, one or two mice of each litter were not irradiated and also served as controls. Mice were irradiated at one to four days of age, and were sacrificed ten or eleven days after irradiation.

During the x-raying procedure, mice were separated from their mother for several hours, and mounted with adhesive strips on individual polystyrene platforms. Polystyrene was chosen because its radiation absorption rate is close to that of living tissue and does not scatter the radiation. To facilitate irradiation, the mice were positioned so that the right hind limb was extended. The platforms were arranged so that the x-ray beam would impinge only upon the mouse limb and polystyrene.

The x-ray machine used was a General Electric 1000 KVP Maxitron, at the Medical College of Virginia (Figs. 4 and 5). At 1000 KVP and with a current of three milliamperes, the radiation intensity obtained at one cm distance

was 500 r per minute, HVL of 3.7 mm of lead. An x-ray beam of 16 mm diameter was used. The area to be irradiated was visibly delimited by light prior to x-raying for accurate positioning of the limb. The irradiated area included the entire limb except the proximal portion of the thigh. The x-ray machine contains inherent filtration consisting of a brass-water-brass jacket surrounding the x-ray tube. Additional filters are unnecessary because the soft x-rays are unable to pass through the inherent filtration (Fig. 3). Only high energy x-rays of practically uniform intensity pass through to the specimen, thus assuring nearly equal radiation intensity in skin and muscle, and eliminating the usual superficial erythema by machines less effectively filtered.

Immediately after irradiation, mice were identified with a toe clipping code. Ten days after irradiation the mice were killed in ether and a chemical depilatory was applied to the hind limbs of those not already epilated due to irradiation. The limbs were amputated and the skin was stripped back to the ankle. Following fixation in 5% formol-saline, the specimens were dehydrated in increasing concentrations of alcohol and cleared in methyl salicylate.

The lateral and medial heads of the gastrocnemius, and the attached soleus and plantaris muscles were teased

apart so the entire muscle group would lie flat. The left and right muscles of a mouse were mounted in methyl salicylate on the same slide. Using an ocular grid and a hand tally counter, counts were made of the number of melanocytes in the left and right muscles of each mouse. If preservation of the muscle for later reference was desired it was transferred from methyl salicylate to absolute alcohol, cleared in xylene and mounted in balsam.

Because the age of mice at sacrifice ranged from 11-16 days, it was important to determine whether the age differences caused significant variations in melanocyte counts. One entire litter, consisting of nine mice, was not irradiated, and was used as an age control. Three mice were killed each day on the 12th, 13th and 14th days. Standard procedure was followed as for the irradiated mice.

In addition to the above procedure, one litter of irradiated mice had their muscles subjected to L-dihydroxyphenylalanine (dopa). Dopa is an amino acid precursor of dopa melanin, which is typically found in mammalian melanocytes. Tyrosinase, (an enzyme found in melanocytes on the verge of melanogenesis and in melanocytes actively proliferating melanin) catalyses the oxidation of dopa to dopaquinone. Ring closure takes place spontaneously to form indole-5,6-quinone, which then polymerizes to form melanin (Fig. 2).

This complex series of reactions (known as the Raper scheme of melanogenesis, and oversimplified above), is catalyzed entirely by tyrosinase (Raper, '27; Seiji, et al., '63; Swan, '63). Therefore, dopa is converted to melanin in any cell which either produces or contains tyrosinase. Hence dopa effectively and selectively darkens potential melanocytes and makes them visible.

Buffered dopa solution consists of 20 parts M/10 KH_2PO_4 to 80 parts M/10 Na_2HPO_4, with dopa added at the rate of one mg dopa per ml buffer. In the procedure used (Becker, '48), the skin was stripped back, exposing the gastrocnemius muscle, and the leg was fixed in 5% formol-saline for one hour. The leg was then rinsed several times in saline and placed in buffered dopa solution for one hour at 38° C or until the solution turned red. The old solution was then replaced with fresh dopa and incubation was continued for another five hours. Thereafter, the leg was returned to formol-saline to complete the fixing process. The fixed leg was mounted for microscopic examination.

GROSS OBSERVATIONS

Irradiation effects in mice sacrificed ten to eleven days after irradiation included changes in total leg size,

pigmentation of the plantar skin and skin overlying the gastrocnemius, greying of hair and epilation.

The irradiated right limbs of animals that received dosages in excess of 1000 r were observably smaller than the control limb at the time of sacrifice. The toe-to-heel length and the length of the entire limb diminished progressively as the dosage level increased. With dosages of 5000 r or more the limbs were atrophied to half the normal size, and there was an incrustation with a scabrous cover to the extent that the animal could not use the limb for walking. When such limbs were cleared and observed under the dissecting microscope the long bones were seen to be diminished in size correspondingly. Also, the gastrocnemius muscle was approximately half normal size. That radiation exceeding several hundred r causes the retardation of growth of epiphyses of immature bones has been reported in The Biological Effects of Atomic Radiation (National Academy of Science, '60). The nature of the damage to muscle cells could not be ascertained since study of whole mounts and histological sections revealed no visible change. Previous reports have shown that the apparent radiosensitivity of muscle is of a low order (Bloom, '54). Muscle is relatively unaffected by doses of radiation which produce damage in other tissues, such as various connective tissues and

epithelia. Some of the difference in sensitivity can be explained by the fact that cells not actively dividing may not show the effects of damage until they divide. For example, in the epidermis there are few cells dividing at any one moment, and the full effects of radiation do not become manifest until many of the resting cells divide. Hepatic epithelium is normally untouched by relatively large amounts of radiation; however, if a large part of the liver is removed, the remaining part will regenerate with great numbers of mitoses and irradiation will affect the organ (Bloom, '54).

EFFECTS IN SKIN

Radiation-induced changes occur in skin at dosages much lower than those causing changes in muscle. The high apparent radiosensitivity of skin probably is due to the relatively high mitotic rate of skin. Analysis of the plantar surface revealed a slight increase in the pigmentation of the right footpad at dosage levels as low as 200 r, and at 400-750 r there was pronounced darkening, easily observable with a dissecting microscope. Between 900 and 1000 r the reaction was one of slight darkening, while at 2000 r no darkening could be detected. There was a marked

depigmentation from normal at 3000 r, probably due to radiation-induced destruction of melanocytes (Table I).

Results of irradiation on ventral and dorsal leg skin are in sharp contrast to those on plantar skin. In skin covering the dorsal portion of the PET leg, unirradiated melanocyte populations are usually 60/mm^2, while unirradiated ventral populations are usually 200/mm^2 (Rovee and Reams, '64). Below 900 r no change could be detected in these numbers. With dosages greater than 900 r the dorsal population increased to 200/mm^2, a population density equal to that of the ventral. The ventral skin population remained constant. An increase in dosage did not produce the characteristic progressive increase in melanocyte numbers found in plantar skin, or decrease as found in muscle. Rather, the melanocytes remained at the 200/mm^2 density even with 7000 r, a level well above that which results in the destruction of melanocytes in the plantar skin and muscle. It is believed that a particular tissue is able to accomodate a melanocyte population up to a specified maximum, this number being determined genetically (Reams, '66). If the maximum number is present, no amount of stimulation by irradiation, surgical trauma, or other means, will produce a further increase in the melanocyte population. It may be that skin in the leg region is able

to carry a maximum of 200 melanocytes/mm^2. This would account for the dorsal increase from 60 to 200, the lack of any increase in the ventral population, and the final equality in population. It is most likely that the apparent increase in melanocyte numbers comes not from cell proliferation or migration, but from stimulation of amelanotic melanocytes already established at the site but not undergoing melanogenesis (Rovee and Reams, '64; Quevedo and Smith, '63; Reynolds, '54). Why the melanocytes are able to persist in this region, actually in a healthy condition even after such high dosage levels as 7000 r, is not known.

The phenomenon of x-ray-induced depigmentation has been reported in hair and hair follicles (Chase, '49; Chase, et al., '63), in feathers, and mammalian skin (Cohen, '63). Cohen x-irradiated the ears of guinea pigs and noted a loss of epidermal pigmentation following dosages of 2000 r or more. He proposes that x-ray-induced depigmentation is mediated via the epidermis concerned. This conceivably could be a mechanism for the depigmentation observed in the plantar skin of the PET mouse at 3000 r, but it does not account for the depigmentation of the muscle, which is isolated from epidermis. In light of this observation it seems more reasonable to propose that depigmentation in both instances is due to the destruction of melanocytes as

a result of the radiation.

Cross-sectional studies of skin irradiated with 3000 r and above show a denuded dermis covered with a thick incrustation. In regions of skin along the irradiated border there is present what appears to be a highly proliferative epithelium growing on the dermis and pushing along the line between living and destroyed skin (Figs. 27 and 28). The destroyed skin is being extruded as a scab as the new epidermis moves along. Bloom ('54) states that after complete degeneration of the epidermis due to destruction of the cells of the germinative layer, regeneration occurs by ingrowth of epithelium from the surrounding uninjured epidermis. At these radiation levels the dermis of the PET mouse becomes thinner than normal. The hypodermis consists almost entirely of adipose tissue lacking hair follicles. This contrasts with normal hypodermis which has hair follicles penetrating the adipose tissue (Figs. 25 and 26). Degenerating hair follicles can be seen in some instances.

There is a very noticeable increase in melanocytes in the superficial dermis as seen in cross-section. They appear healthy and undoubtedly were stimulated by radiation. The PET strain of mice is unique in that the normal melanocyte population in the skin is located in the dermis rather

than in the epidermis. Even the normally heavy pigmentation of the ventral leg skin, when subjected to the sodium bromide skin splitting technique (Staricco and Pincus, '57), shows that all melanocytes reside in the dermis, while the epidermal sheet is devoid of melanocytes. Most reports on skin pigmentation have dealt with epidermal melanocytes, no doubt because in mammals these are the most common.

With a dosage of 3000 r, melanocytes have been observed in epidermis in the PET mouse, an area normally free of melanocytes. Their presence is particularly significant because it raises the question of their origin. Whether they are of dermal origin and have migrated into the damaged epithelium or whether they have been stimulated into melanogenesis from a previously amelanotic condition is not known.

In discussing skin damage where the epidermis and superficial dermis are destroyed by burning, Ham and Leeson ('61) note that epithelial cells from the deeper parts of the hair follicles survive and grow out to form a new epidermis. Although Bloom ('54) describes the same phenomenon for hair follicles not completely destroyed by radiation, no evidence was obtained in this study to indicate that a comparable effect occurs after severe radiation damage.

All regeneration seems to be directed from the periphery into the damaged area. The absence of dermal ridges or papillae at the junction of the regenerated dermis and epidermis is worthy of note. The rete pegs (epidermal equivalent of dermal papillae and germinative centers), are also absent.

EFFECTS ON HAIR

It is known generally that irradiation of the skin causes destruction of the hair follicles, greying and the falling out of hair. As mentioned above, in cross-sections of skin given 3000 r or more, hair follicles are mostly absent, there being only a few scattered disintegrating follicles left. At 2000 r most follicles remain but are in a state of disorganization, and no hairs are seen in the follicles. Bloom ('54) states that laboratory mammals require about 2000 r or more of x-rays to produce permanent epilation. In the PET mouse, 2000 r seems to be the dosage required for permanent epilation. Below this dosage hairs remained in the follicle.

Bloom ('54) also says that the phenomenon of temporary epilation occurs with lower dosages after about three weeks. However, most of our animals were sacrificed only ten days

after treatment, an insufficient time for temporary epilation to occur. At dosages between 400 and 1000 r, the presence, in PET mice, of acuminate hairs which taper toward the follicle and there become grey (Fig. 22) indicates that the follicles were entering the catagen phase (the transition stage between an actively growing and a resting follicle) in the hair growth cycle. Therefore, temporary epilation might soon have followed had the animals lived.

Montagna ('62), in discussing epilation, says that most of the cells of the matrix are destroyed 24 hours after exposure to x-rays. One week later the bulb is lost and the lower part of the follicle is reduced to a thin cord of cells composed mostly of the outer root sheath. Since the growth of hair is the result of cell proliferation in the matrix, and it is these cells that are destroyed upon irradiation, there are not enough cells to form a hair of full diameter, and so the hair becomes acuminate and falls out. The acuminate hairs should not be confused with club hairs, which form in normal active follicles just before the follicle enters the catagen phase. Club hairs are normally lost when the follicle enters the telogen, or resting phase in the hair growth cycle, as are the acuminate hairs. Radiation is said to precipitate and abbreviate the catagen phase by destroying cells within the matrix (Montagna,

'62). Thus, radiation-induced temporary epilation is really a modification of a normally occurring phenomenon, while permanent epilation is the result of complete destruction of the matrix.

Some greying of hair was observed in irradiated areas but no relationship between degree of greying and dosage could be detected. The occurrence of grey hairs was noted after dosages of 200 to 900 r. X-ray induced greying of hair may be caused by destruction or inactivation of the melanocyte population of each follicle, which supplies the matrix cells of the follicle with pigment granules (Chase, '50). Chase was able to obtain a ratio between x-ray dosage and greying, and found increased greying with higher dosages. He notes that variable effects of x-rays on greying are due to the stage of hair growth at the time of treatment. By plucking hairs from a region of mouse skin, he was able to synchronize the hair growth cycle in follicles of that area. Treatment of such areas resulted in a maximum greying effect in inactive follicles (telogen phase). Active follicles at the time of x-raying (anagen phase) later produced hair with a minimum of greying. The irregular greying response in the PET mouse was probably due to lack of synchronization of follicles.

When newborn mice are exposed to a particular dosage

of x-ray they will develop less greying than normally would be expected for that dose. At this stage new follicles are still being laid down and are incorporating their initial melanoblast supplies (Chase, '50). Therefore, a minimal greying response in the newborn PET mouse is not surprising.

EFFECTS IN MUSCLE

Counts were made of the total number of melanocytes in the left and right gastrocnemius muscles of 64 experimental animals. Among 20 unirradiated control animals, total melanocyte numbers in the left gastrocnemius ranged from 422 to 1225, and in the right from 393 to 1339. Due to this wide range of variability, direct comparisons could not be made between melanocyte numbers in either the right or left gastrocnemius. However, a definite relationship has been observed in melanocyte numbers between the left and right gastrocnemius in the same individual. In 20 unirradiated animals the total melanocyte number in the right gastrocnemius averaged 70 more than in the left. An index reference number was obtained by subtracting the number of melanocytes in the left muscle from the number of melanocytes in the right. If the subtraction of the control from irrad-

iated melanocyte numbers gave a positive index, then an increase in melanocyte numbers in the right irradiated gastrocnemius was indicated. A negative index indicated a decrease.

Counts of melanocyte numbers in the gastrocnemius of the age controls indicated no significant variation in the index from day 12 to 14, and small changes observed in the index were probably due to normal biological variability. There was a definite tendency for the total melanocyte number in both the left and right gastrocnemius to increase progressively, but since the rate of increase is approximately the same in both muscles, the index is not seriously affected. Therefore, the index retains its significance, and it can be compared among animals of different ages (Table 2).

Among irradiated animals there is an inverse relationship between the melanocyte index and dosage. As shown in figure 1, as dosage increases from 200 to 7000 r, the melanocyte number decreases. It is interesting to note that Chase ('49, '50) observed that a similar pattern exists between dosage and number of melanocytes destroyed in hair follicles. Contrary to our expectations, there is no indication of melanocyte stimulation in muscle at any dosage level. At 7000 r, for example, the index is -995, with 1315 melanocytes in the left gastrocnemius, and 320 in the

right. That x-rays caused the destruction of melanocytes is indicated by the presence in irradiated muscle of atypical melanocytes showing signs of disintegration. In many cases, melanin granules are seen outside the boundary of the melanocyte (Figs. 12 and 13).

Radiation has been shown not only to increase melanogenic activity within active melanocytes in skin, but also to activate amelanotic melanocytes (Quevedo and Szabo, '65). Clearly, x-irradiation failed to provoke pigment cell activity in the musculature of the PET mouse. Legs irradiated with from 1000 to 5000 r, and subsequently treated with dopa, also failed to show pigment cell activity, and had melanocyte numbers comparable to those in animals not subjected to dopa. If amelanotic melanocytes are present in muscle an increase in total numbers of visible melanocytes in irradiated and dopa treated muscles would be expected. Therefore, it can be assumed that the number of melanocytes observed represents the total pigment cell population, with no amelanotic melanocytes available for activation.

DISCUSSION

The results of previous work suggest that in many cases of melanocyte stimulation by means of irritants,

trauma, UV or x-irradiation, hyperpigmentation is primarily the result of activation of amelanotic melanocytes (Rovee and Reams, '64; Quevedo and Smith, '63; Reynolds, '54). Much has been said of these cells, and some investigators have seen cells which they profess to be amelanotic melanocytes (Breathnach, et al., '63; Staricco, '63). Yet amelanotic melanocytes frequently elude positive identification and their presence is known mainly through indirect evidence. For example, PET embryonic tissue devoid of visible melanocytes will develop melanocytes when isolated as a graft in the chick coelom (Mayer and Reams, '62).

There is no question that melanoblasts exist, migrate through the embryo from the neural crest, and upon reaching a suitable cellular environment differentiate and initiate melanogenesis (Rawles, '47). However, the number of melanoblasts persisting essentially in the embryonic condition in the adult animal has never been determined. It is true that melanoblasts are potentially melanotic cells, and as such one might believe that they would react with dopa to give visible proof of their existence. If they possess tyrosinase in the amelanotic stage it might be counterbalanced by minute traces of sulfhydryl-containing compounds. The path of melanin synthesis would then be blocked, causing the cells to appear dopa-negative (Flesch and Rothman, '48).

Extracts of human epidermis have been found to inhibit the oxidation of tyrosine and dopa, both intermediates in the synthesis of melanin, thus preventing melanogenesis. The inhibition has been found to be in direct proportion to the concentration of -SH.

Grafting of embryonic tissue into the chick coelom has been the usual method to determine whether a population of amelanotic melanocytes exists at a particular embryonic stage. The same technique might be employed advantageously for adult skin, as all traces of -SH could be eliminated by first washing the skin before placement into the chick coelom. Sulfhydryl inhibition could be eliminated, thus making possible the activation of amelanotic melanocytes. In vivo studies (Flesch and Rothman, '48) show that an increase in melanin synthesis, as in tanning by UV, is preceeded by a decrease in -SH content of skin. It has been suggested that melanin-stimulating factors such as UV and x-ray act indirectly by eliminating the -SH inhibition, thus allowing the enzymatic oxidation of pigment precursors which form melanin. Such a mechanism might be proposed for the activation of melanoblasts as well as amelanotic melanocytes.

There seems to be a great variation in degree of -SH inhibition in different regions of the same mouse and in different strains of mice, producing variations in sensitivity

to radiation. Melanocytes in the ear skin of most strains of mice are dermal. When this region was irradiated (Quevedo and Grahn, '58) epidermal melanocytes were activated, indicating release from -SH inhibition in the epidermis but not the dermis. The PET strain normally has melanocytes in the dermis but they are absent from the epidermis in every region. Radiation-induced hyperpigmentation in PET skin was due to a further increase in dermal melanocyte numbers, but not to activation of epidermal melanocytes until high dosages of x-radiation were used. The extreme resistance to activation of epidermal melanocytes could be attributed to a greatly increased sensitivity to normal levels of -SH, producing an intense -SH inhibition. Only after dosages of 3000 r or more are melanocytes present in the epidermis of the PET strain. Apparently, amelanotic melanocytes are present in the epidermis and are capable of being activated, although they have an extremely high activation threshold.

Amelanotic melanocytes differ from melanocytes in their greater sensitivity to -SH groups, and melanogenesis may be completely inhibited by scarcely detectable traces of -SH. PET amelanotic melanocytes in plantar skin are activated in direct proportion to increase in radiation (decreasing -SH levels), indicating that these cells have

varying sensitivities. As the -SH level drops, individual cells are triggered into melanogenic activity as their particular threshold is reached. Amelanotic melanocytes of the dermis seem to have a narrow range for activation, as their threshold is reached at about 900 r, when they suddenly become melanotic. Cells of the epidermis apparently have greater sensitivity to -SH groups than other populations, and extreme stimulation is required to remove all traces of -SH.

It is proposed that amelanotic melanocytes, essentially melanoblasts that persist into the adult stage, are ubiquitous in skin and other potentially melanocyte-bearing tissues except in special cases. In addition, results in this study suggest that the number of pigment cells in the PET skin is rigidly determined. It is conceivable that the maximum number of pigment cells is always present, the total numbers of amelanotic melanocytes and melanocytes equaling the genetic maximum. When maximum numbers of melanocytes are not present, amelanotic melanocytes are there, and the proper stimulus will cause them to undergo melanogenesis. In special cases, as in muscle and irradiated skin of the dorsal leg, only melanocytes are present. All pigment cells are then active, and the application of pigment-producing stimuli cannot further increase pigmentation. In such cases the genetic maximum is seen.

If, indeed, the above is true, the ratio between amelanotic melanocytes and melanocytes would be inversely proportional, thus an increase in melanocytes would be accompanied by a corresponding decrease in amelanotic melanocytes. Montagna ('66), working with rhesis monkey skin has described such a phenomenon wherein an inverse ratio exists between melanocyte numbers and numbers of Langerhans cells. Langerhans cells are non-pigmented cells believed to be in the melanocyte series. They are highly controversial entities and are currently the subject of intensive investigations. It is conceivable that Langerhans cells are really amelanotic melanocytes, thus offering an explanation for Montagna's otherwise unexplainable ratio.

Mayer and Reams ('62) demonstrated that in PET mice melanoblasts are migrating through the leg musculature at 15 days of development. At 19 days, still amelanotic, they have essentially reached their full complement and no longer migrate or proliferate. The increase in numbers of melanocytes in leg musculature observed by Mayer and Reams during the first week postnatal is merely the normal activation of amelanotic melanocytes. In grafting bits of embryonic gastrocnemius muscle into chick coelom, Reams ('66) demonstrated that the proliferative ability of melanoblasts in muscle is progressively diminished from 15 to 19 days of

development, and after 19 days proliferation ceases. It has been assumed in grafts of this type that all melanoblasts present come to produce melanin, possibly due to the lack of -SH inhibition. As grafts of 19 day muscle show melanocyte densities comparable to those in intact muscle, this suggests that there are no residual melanoblasts in intact muscle. The fact that no hyperpigmentation was evoked in muscle with x-rays supports the above hypothesis and also indicates that -SH inhibition is not involved with melanocytes in muscle. Without inhibition, all amelanotic melanocytes are activated during the first week postnatal. Thus, no further increase in melanocyte numbers would be possible from any type of stimulation, regardless of intensity.

SUMMARY

An investigation was carried out to determine the effects of x-irradiation on isolated pigment cell populations in the leg musculature and skin of the PET strain of mouse. Ten days after subjecting the right hind limbs to single x-ray dosages ranging from 200 to 7000 r the following results were obtained:

1, Melanocyte numbers in irradiated muscle decreased progressively as the x-ray dosage increased.

2, Ventral leg skin, which contains a melanocyte

density of 200/mm^2 when unirradiated, maintained this density through the entire dosage range.

3, Dorsal leg skin, which contains a melanocyte density of 60/mm^2 when unirradiated, showed a density of 200/mm^2 at 900 r, maintaining this population through 7000 r.

4, Plantar skin showed a progressive increase in pigmentation at dosages from 200 to 750 r, a progressive decrease from 750 to 2000 r, and a complete absence of pigment above 2000 r.

5, A variable greying response was noted in hair with dosages from 200 to 900 r, and at 2000 r epilation began.

In interpreting these results it is proposed that:

1, The total number of amelanotic melanocytes and melanocytes in a tissue equals a genetically determined maximum.

2, All amelanotic melanocytes are capable of being activated when the proper stimulus is applied to overcome the -SH inhibition.

3, Increases in melanocyte numbers after irradiation are primarily due to activation of amelanotic melanocytes.

4, Decreases in melanocyte numbers after irradiation (depigmentation) are due to destruction of pigment cells.

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TABLE I. EFFECTS OF X-RAY DOSAGES ON THE LEG OF PET MICE

DOSE IN ROENTGENS	INDEX NO. MUSCLE	NUMBER MELANOCYTES		RELATIVE RESPONSE[1]		
		VENTRAL SKIN	DORSAL SKIN	PLANTAR SKIN [2]	GREYING OF HAIR	EPILATION
Control		240/mm^2	60/mm^2			
200	-110	200/mm^2	60/mm^2	1 +	1 +	0 —
400	-189	"	"	3 + + +	1 +	0 —
750	-364	"	"	4 + + + +	1 +	0 —
900	-280	"	200/mm^2	2 + +	1 +	0 —
1000	-205	"	"	1 +	0	0
2000	-411	"	"	0 —	0	4
3000	-357	"	"	depig.	0	4
4000	-446	"	"	"	0	4
5000	-591	"	"	"	0	4
6000	-622	"	"	"	0	4
7000	-995	"	"	"	0	4

[1] Scale ranges from 0 (no reaction), to 4 (maximum reaction)

[2] Reaction in plantar skin is hyperpigmentation.

TABLE 2

NUMBER OF MELANOCYTES IN THE GASTROCNEMIUS MUSCLE AS AFFECTED BY THE AGE OF MICE

Data were collected from nine mice, of the same litter.

AGE	INDEX	AVERAGE NO. MELANOCYTES		
		LEFT	RIGHT	L + R
12 days	60.6	889	947	1836
13 days	-5.0	1012	1007	2019
14 days	106.0	1055	1161	2216

Figure 1. The influence of x-irradiation on melanocyte numbers within the gastrocnemius muscle of the PET mouse. Counts of melanocyte numbers have been converted to index reference numbers.

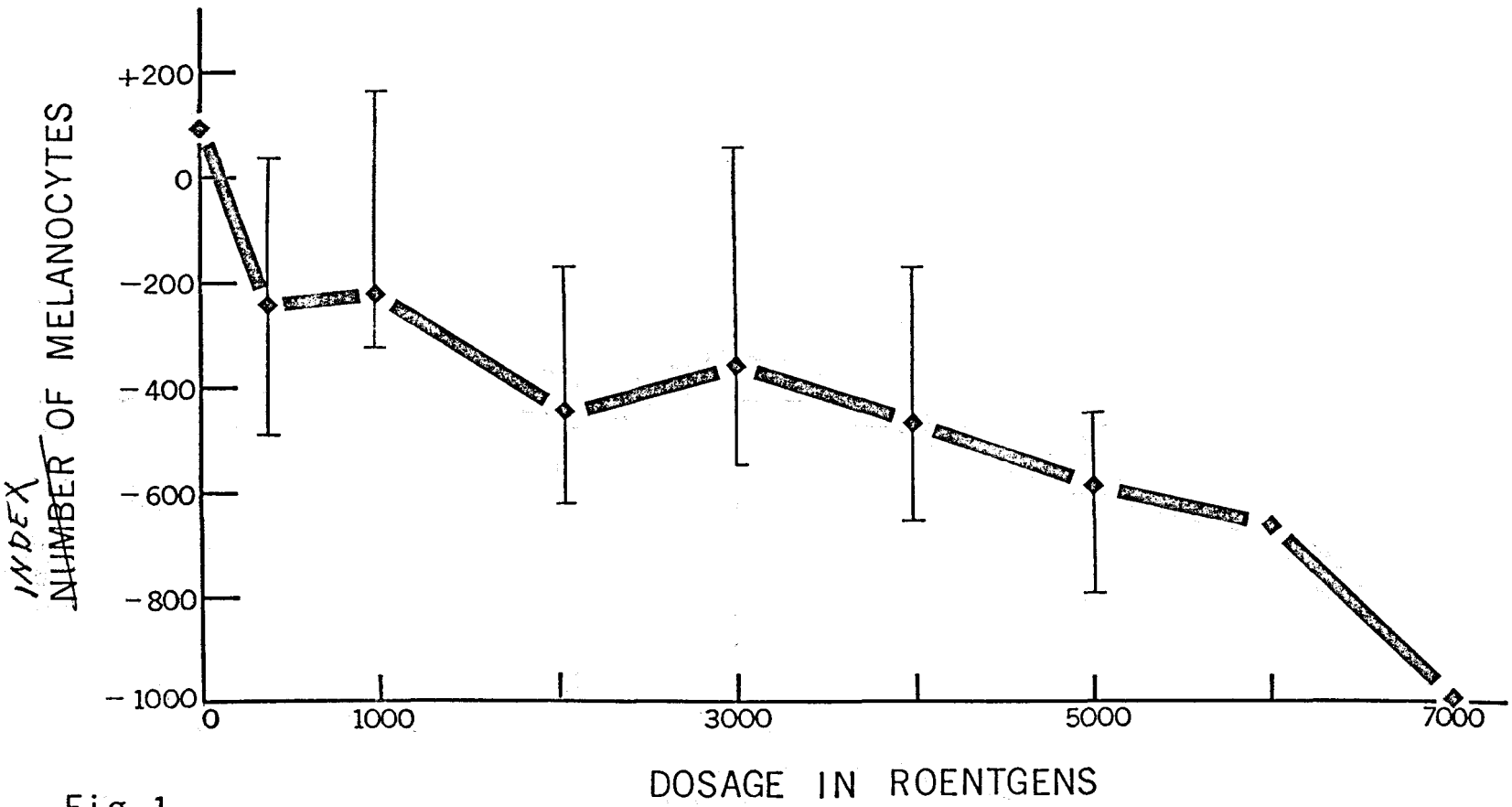

+200
0
−200
−400
−600
−800
−1000
INDEX NUMBER OF MELANOCYTES
0
1000
3000
5000
7000
DOSAGE IN ROENTGENS

Fig. 1

Figure 2. Proposed scheme of melanogenesis.

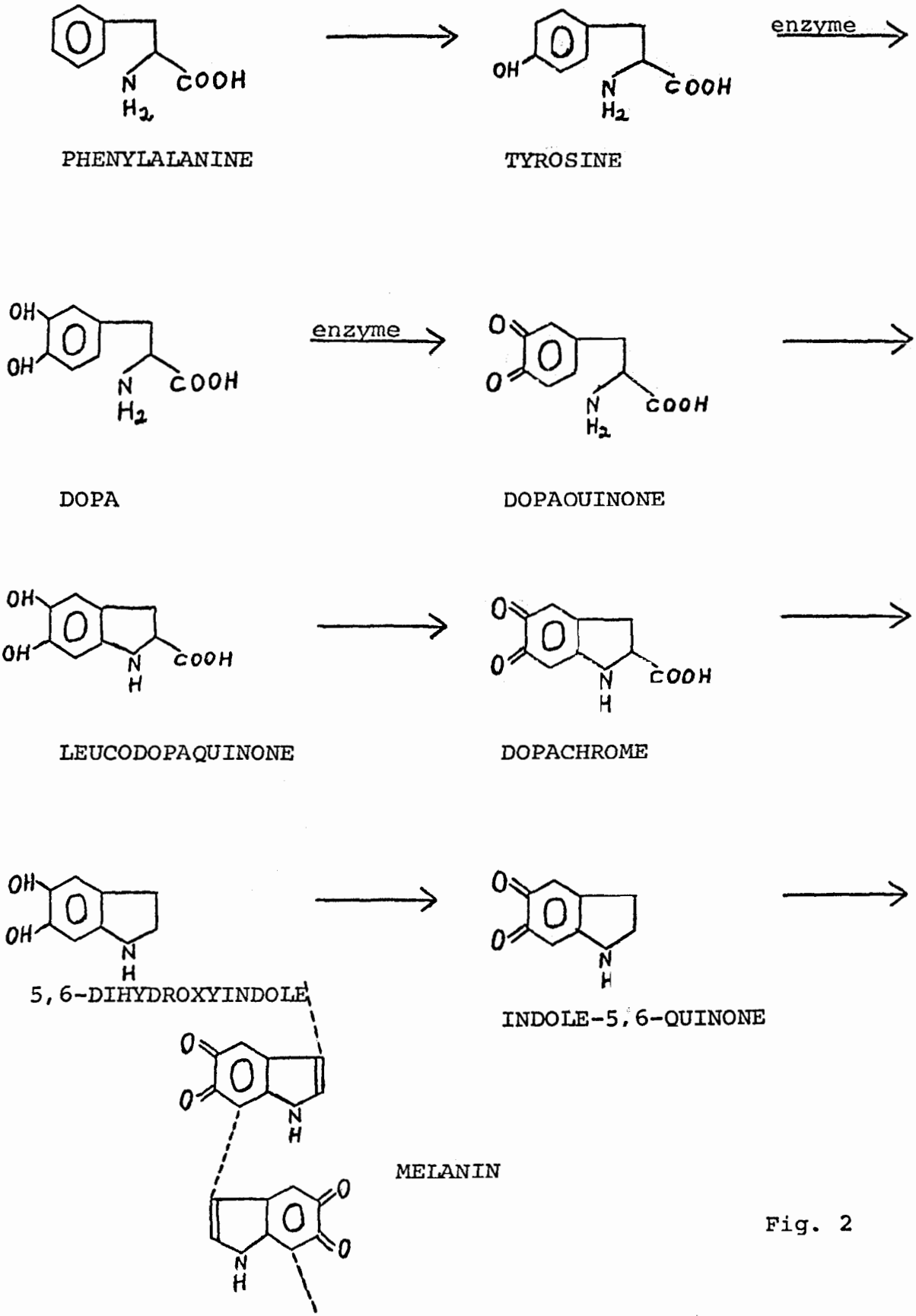

enzyme
PHENYLALANINE
TYROSINE
enzyme
DOPA
DOPAQUINONE
LEUCODOPAQUINONE
DOPACHROME
5,6-DIHYDROXYINDOLE
INDOLE-5,6-QUINONE
MELANIN

Fig. 2

Figure 3. Diagram of General Electric 1000 KVP Maxitron, showing modification of cone to give a collimated x-ray beam.

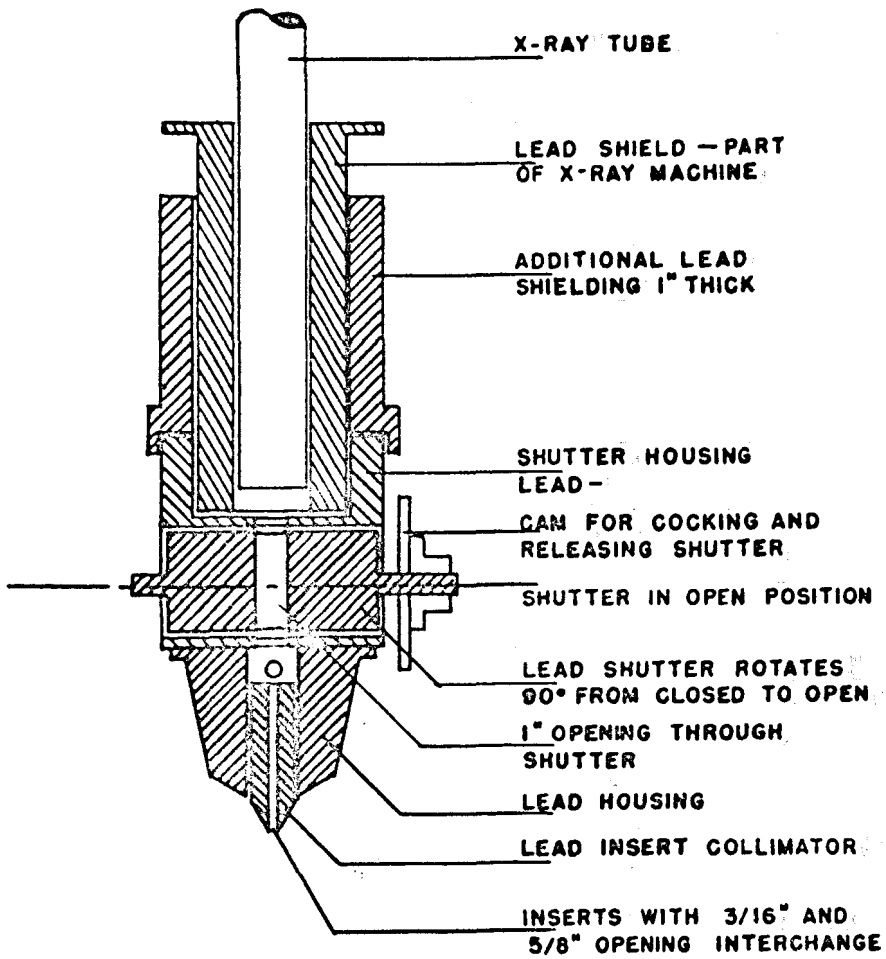
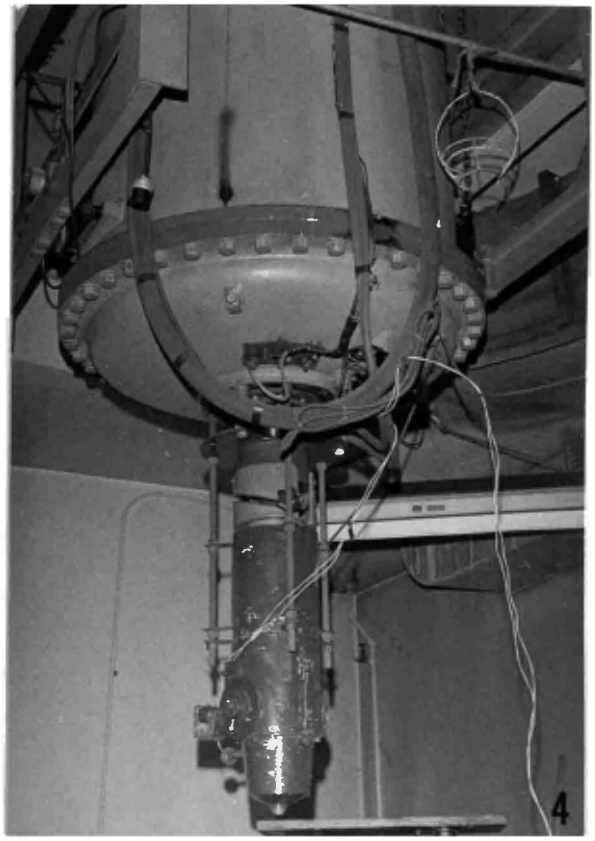

X-RAY TUBE
LEAD SHIELD — PART OF X-RAY MACHINE
ADDITIONAL LEAD SHIELDING 1" THICK
SHUTTER HOUSING LEAD —
CAM FOR COCKING AND RELEASING SHUTTER
SHUTTER IN OPEN POSITION
LEAD SHUTTER ROTATES 90° FROM CLOSED TO OPEN
1" OPENING THROUGH SHUTTER
LEAD HOUSING
LEAD INSERT COLLIMATOR
INSERTS WITH 3/16" AND 5/8" OPENING INTERCHANGE

Fig. 3

Figure 4. General Electric 1000 KVP Maxitron suspended from the ceiling, at the Medical College of Virginia.

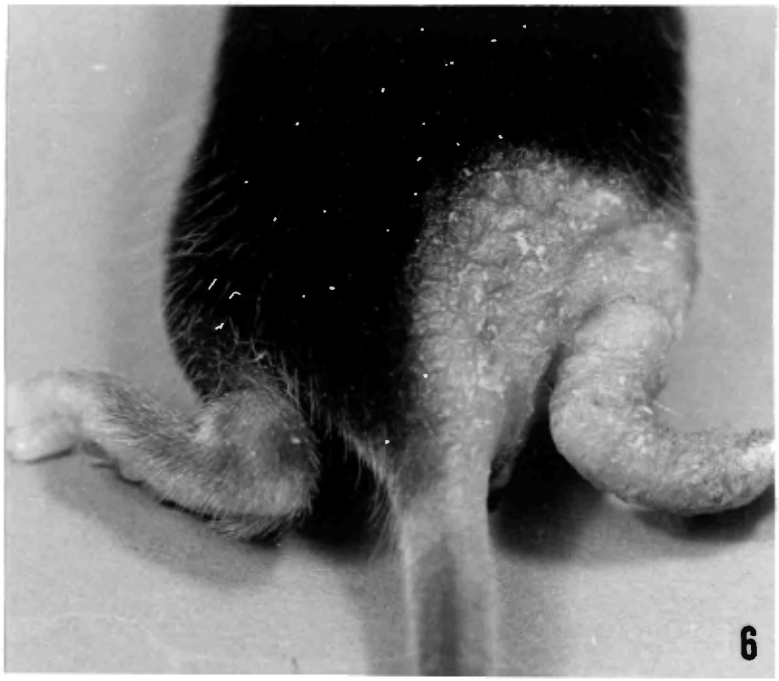
Figure 5. Positioning mouse, mounted on platform, under the Maxitron cone.


4


5

Figure 6. Appearance of right limb ten days after exposure to 4000 r, showing epilation and scaling skin. The sharp boundary between irradiated and unirradiated skin indicates low x-ray scatter.

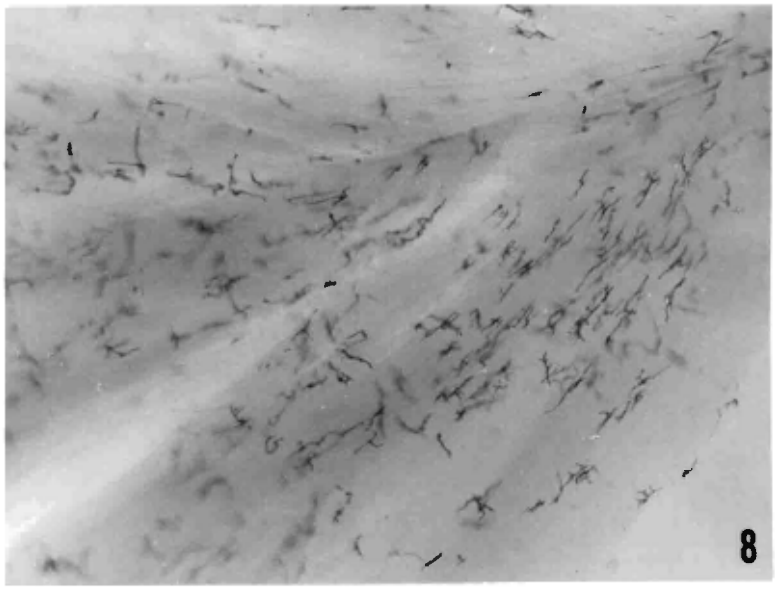
Figure 7. Side view of right limb after exposure to 4000 r. See above.


6


7

Figure 8. Melanocytes within the gastrocnemius muscle of 11 day old control mouse. Whole mount, in methyl salicylate. X 40.

Figure 9. Same as above, X 100.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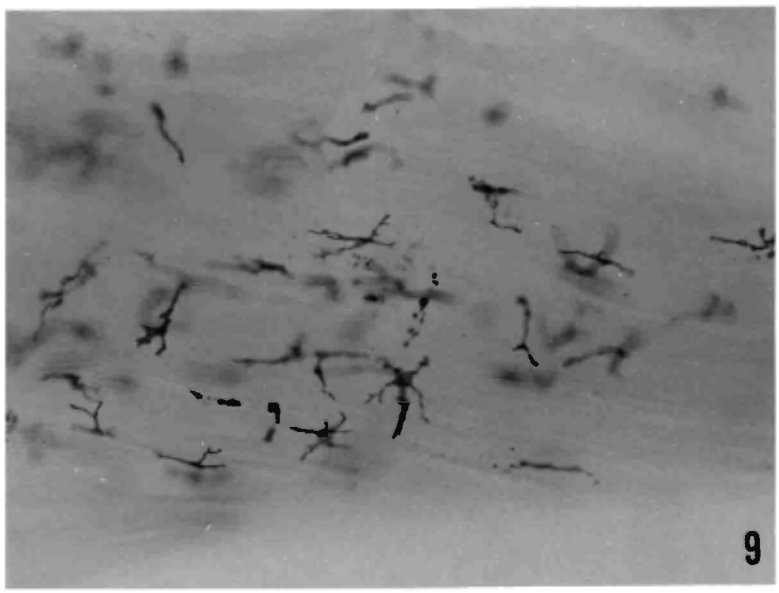
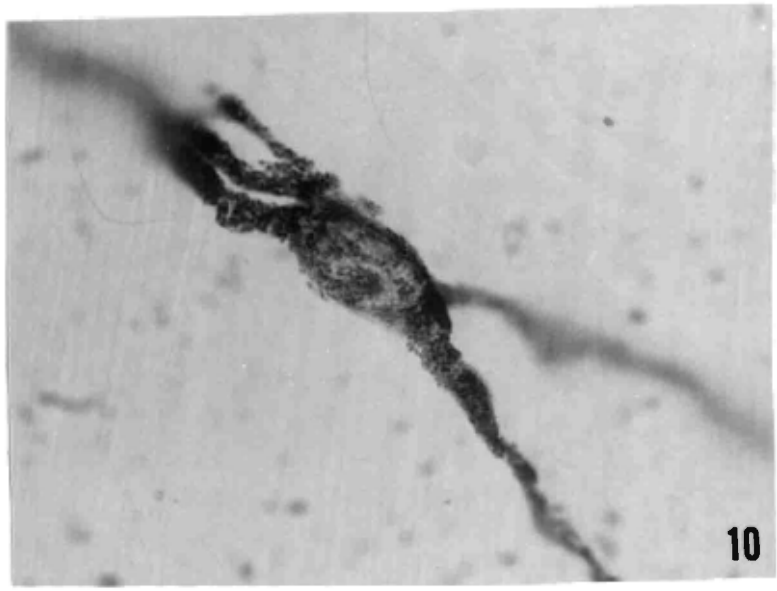

9

Figure 10. Melanocytes in muscle. Note the concentration of melanin granules in branches, and their relative scarcity in nuclear region. ~~Oil Imm.~~ X 1000

Figure 11. Same as figure 10.



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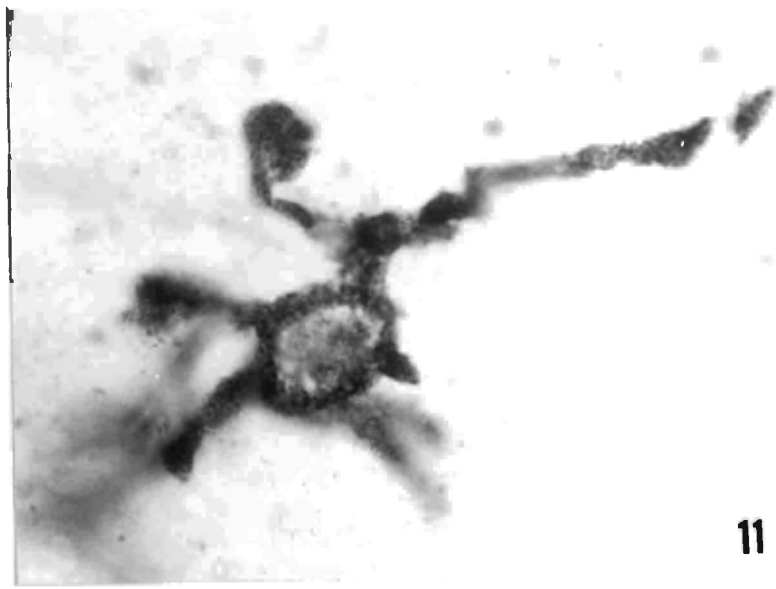
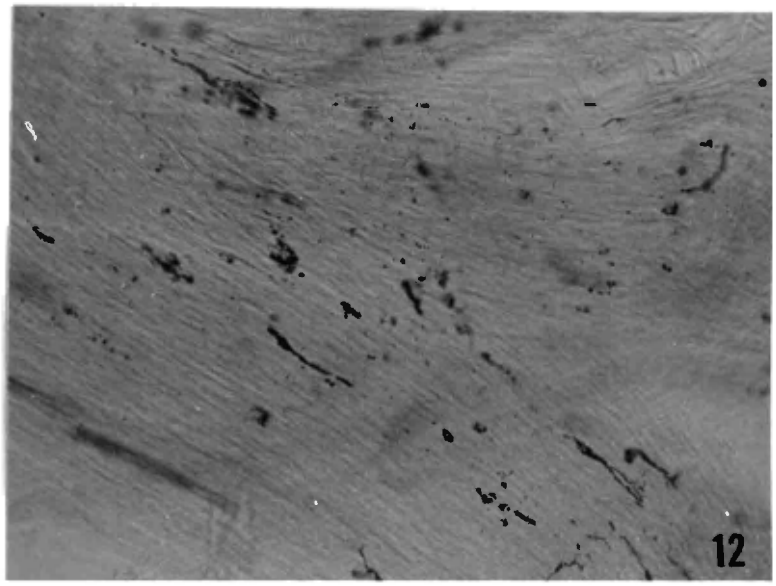


11

Figure 12. Melanocytes within gastrocnemius muscle, ten days after dosage of 4000 r. Loose melanin granules imply melanocytes are disintegrating. X 100.

Figure 13. Same as figure 12, X ~~450~~ 1000.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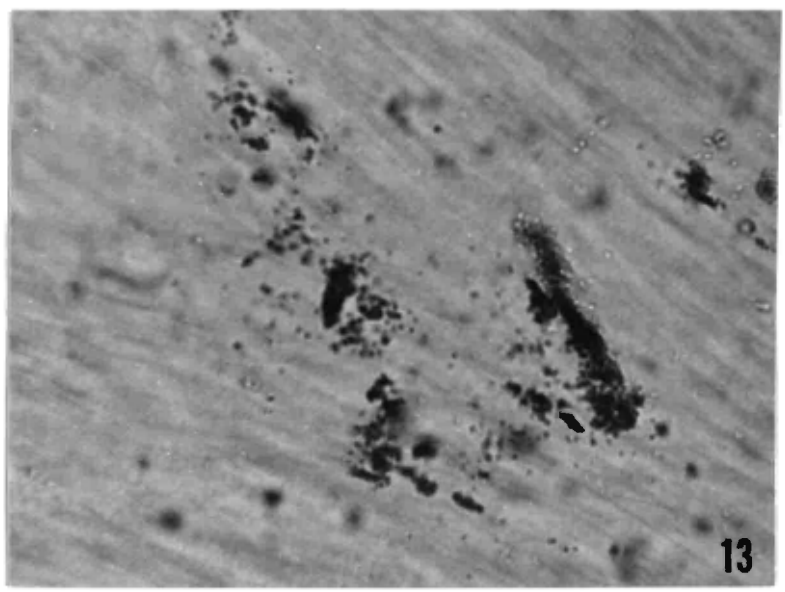

13

Figure 14. Unirradiated ventral skin. Note dense population of melanocytes. X 100.

Figure 15. Unirradiated dorsal skin. Note sparce population of melanocytes. Melanocytes in dorsal skin are larger than those in ventral skin. X 100.


14

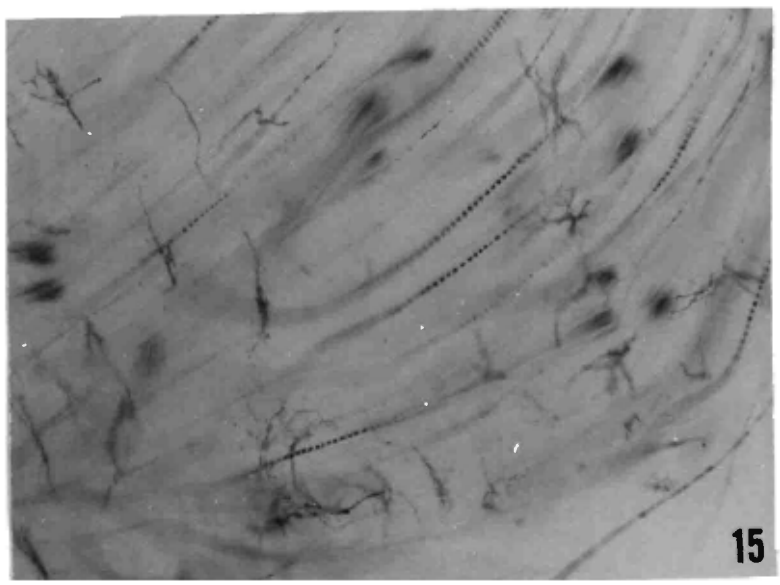
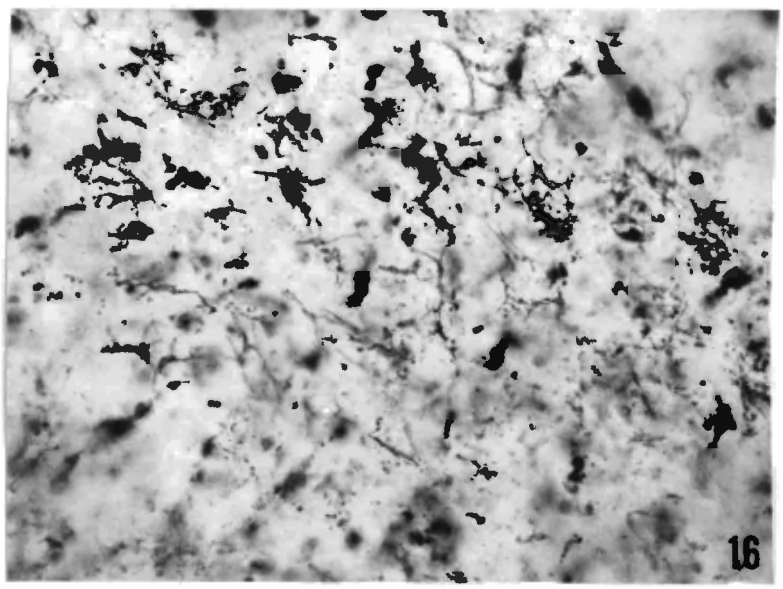

15

Figure 16. Dorsal skin after dosage of 5000 r. Epilation is complete, although a few atrophied follicles remain. X 100.

Figure 17. Ventral skin after dosage of 5000 r. Note that the melanocyte population density is approximately equal to that of the dorsal skin. X 100.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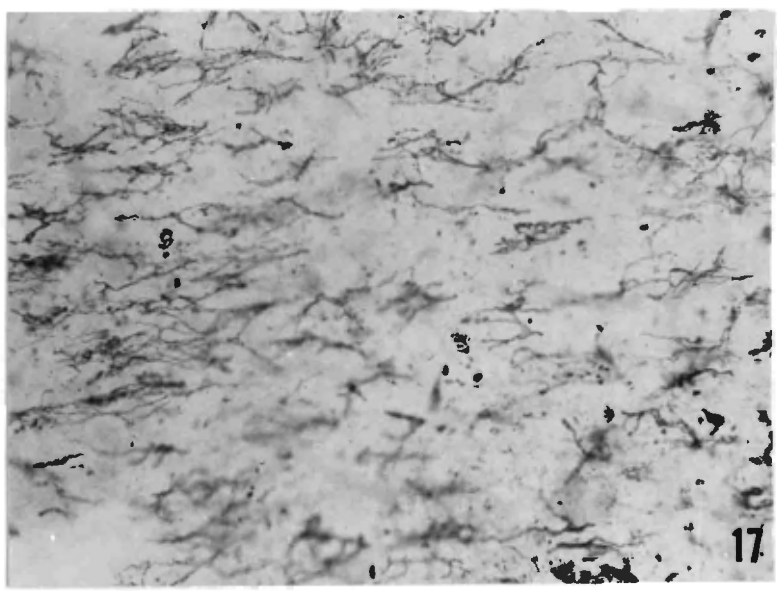
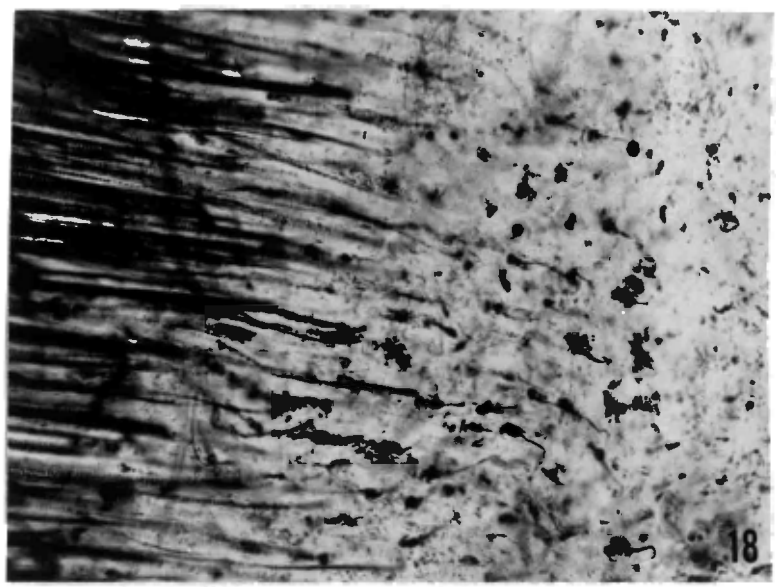

17

Figure 18. Border of x-rayed area subjected to 7000 r. Note the gradation from haired area on left, to one of atrophic follicles in the center of photo. On the right an increase in melanocyte numbers can be seen. X 40.

Figure 19. Magnification of central portion of figure 18, showing atrophic follicles and melanocytes. X 100.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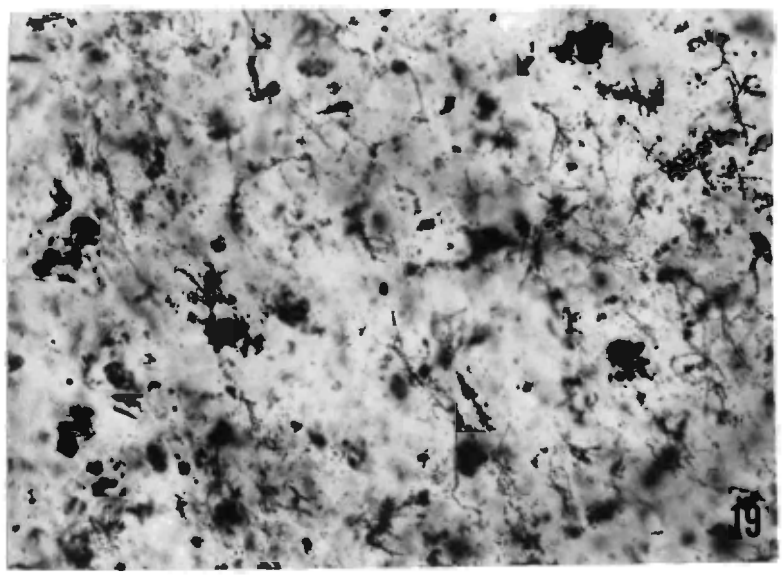
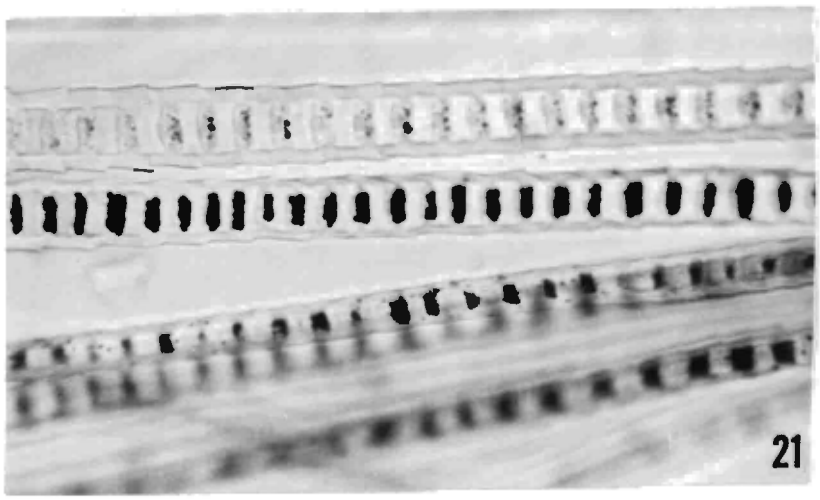

19

Figure 20. Grey hair and melanocytes in skin subjected to 400 r. X 100.

Figure 21. Contrasting grey and pigmented hair. 400 r. X 450.

Figure 22. Acuminate hairs tapering toward the follicle. This phenomenon, caused by radiation death of some of the matrix cells, is followed by temporary epilation. 400 r. X 100.


20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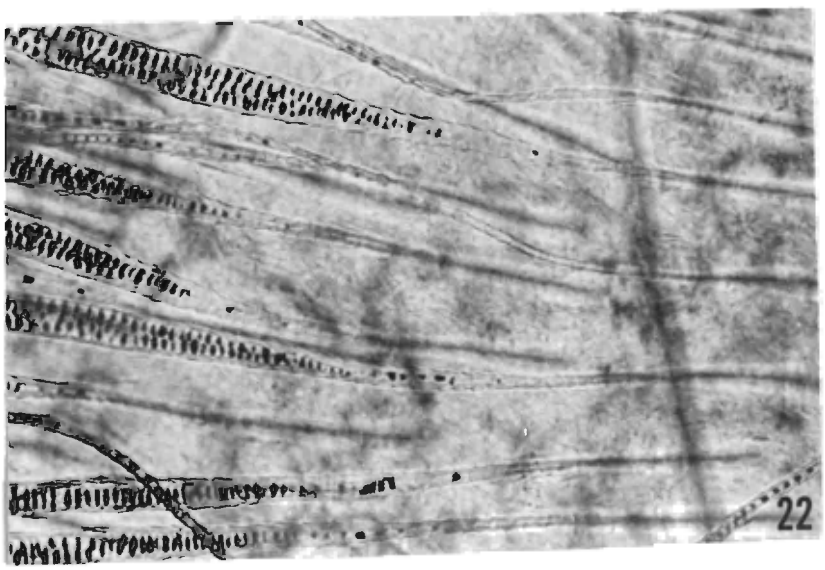
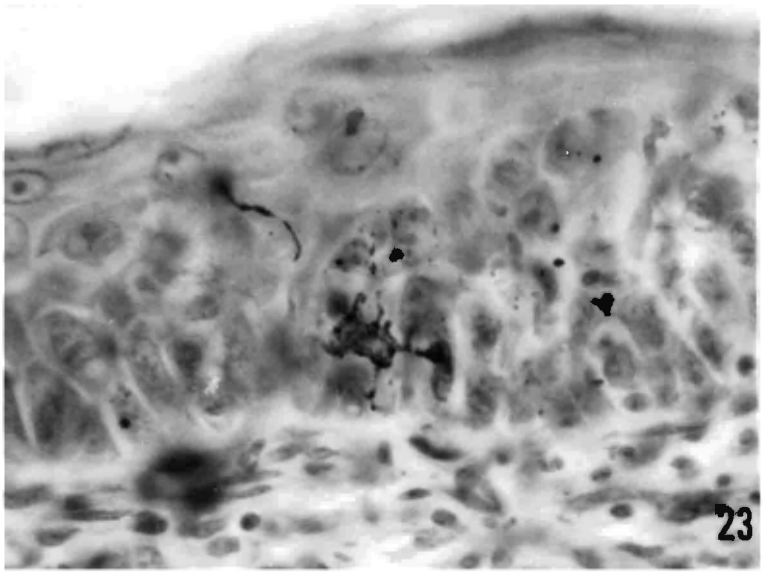

22

Figure 23. Epidermis of PET mouse, normally devoid of melanocytes, has a healthy melanocyte population after exposure to 3000 r. X 450.

Figure 24. Interfollicular melanocytes in skin subjected to 5000 r. X 450.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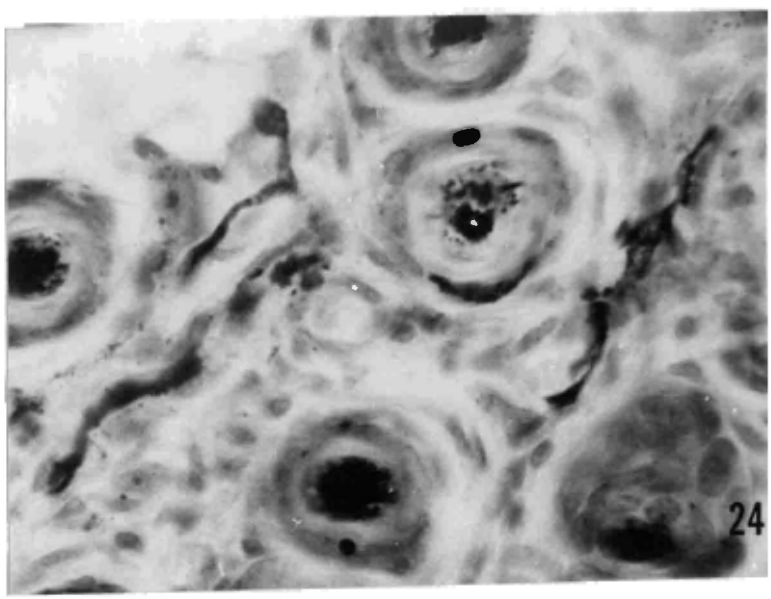
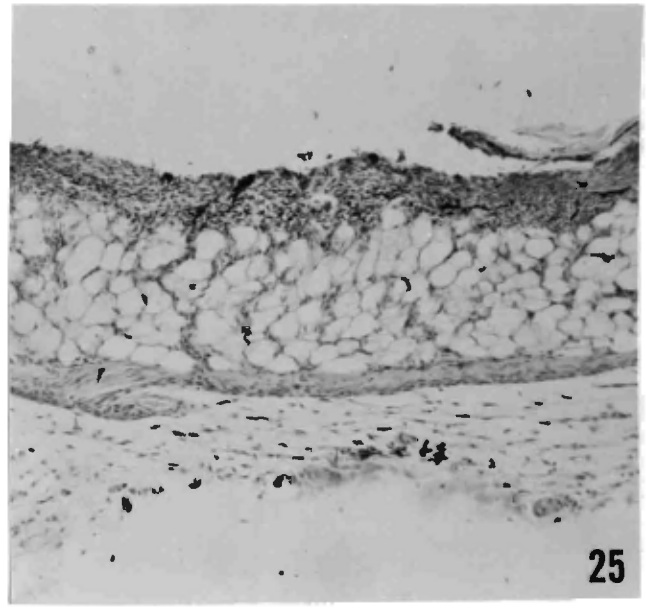

24

Figure 25. Cross-section of PET skin 10 days after dosage of 7000 r. Note that epidermis and follicles are lacking, and that the dermis is unusually thin. Melanocytes are greatly increased in number. X 100.

Figure 26. Unirradiated 11 day PET skin in cross-section. X 100.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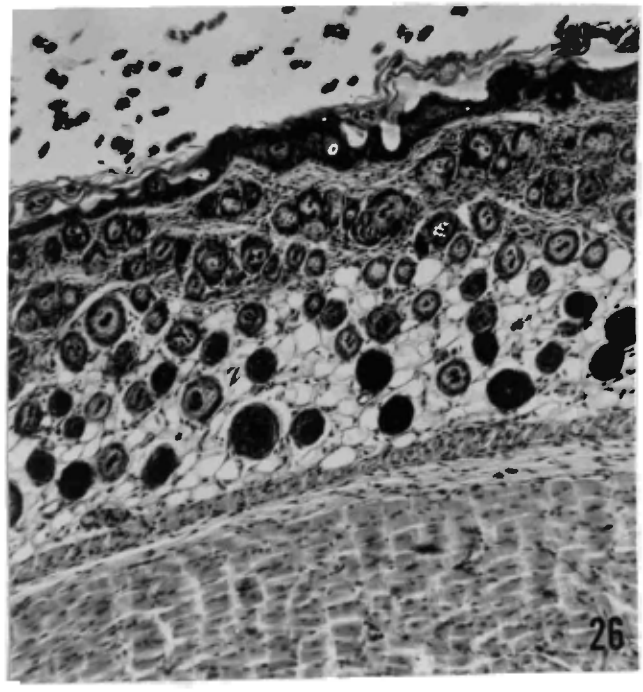
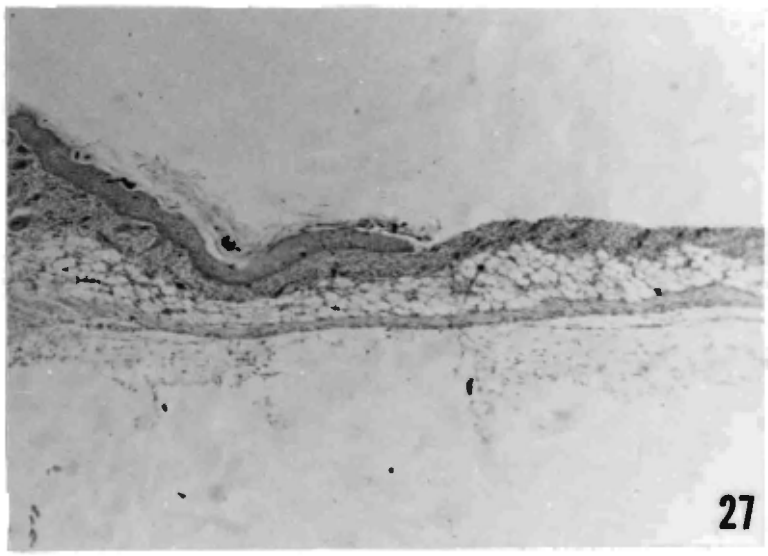

26

Figure 27. Cross-section of PET skin after dosage of 5000 r. Actively proliferating epidermis from periphery of damaged area is growing inward over the denuded dermis. X 40.

Figure 28. Same as figure 27, X 100.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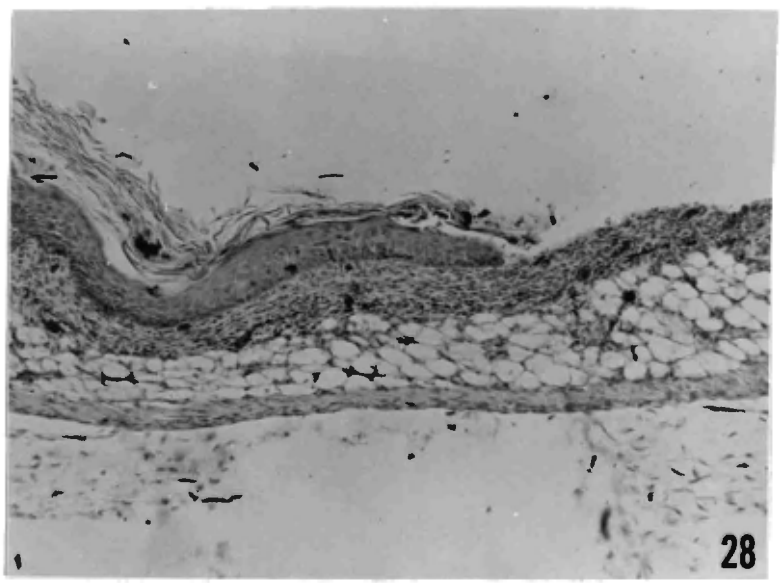
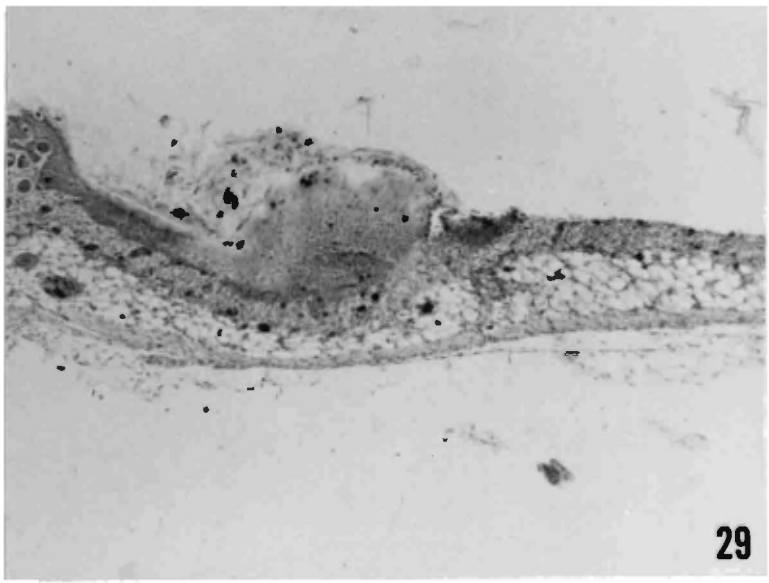

28

Figure 29. Cross-section of PET skin after 5000 r, showing a radiation-induced tumor-like epidermal outgrowth. X 40.

Figure 30. Same as figure 29, X 100.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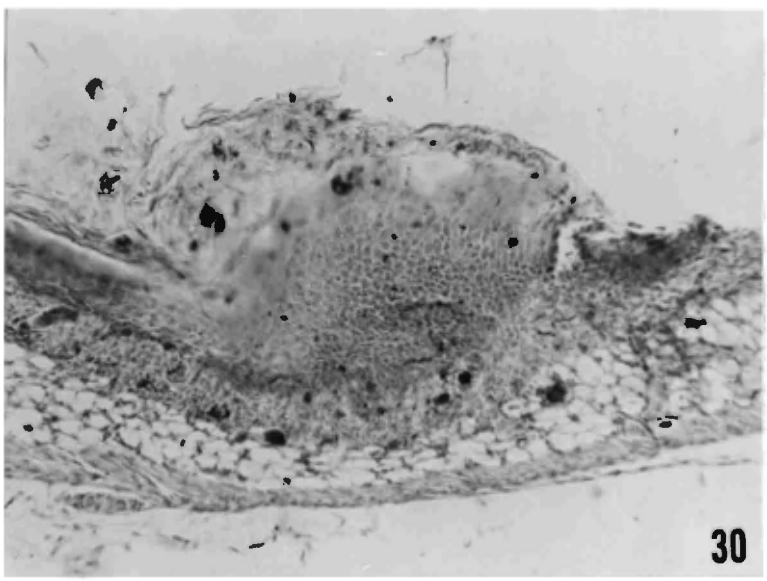
30

VITA

Barry Eckert Schaeffer was born in New York City October 22, 1942. He attended elementary school in Brooklyn, and was graduated from Erasmus Hall High School in 1960. He went on to major in Zoology at the Bronx campus of Hunter College of the City University of New York, there joining the Biology and Fencing clubs. During his four year tenure at Hunter he worked part-time for the Clarendon Medical Labs in Brooklyn, and eventually attained the level of full technologist. In June 1964 he graduated from Hunter with a B.A. degree.

He continued his higher education at the University of Richmond, studying there toward the Master's degree and specializing in Embryology. During this time he managed the greenhouse, and assisted in the General Biology and Genetics laboratories. He was the recipient of a Williams Fellowship granted by the University. He became a member of Beta Beta Beta, the American Society of Zoologists, and the Association of Southeastern Biologists. He attended various Biological conferences during his two year residence at Richmond.

He graduated with the M.A. in Biology in August, 1966, and plans to continue his academic career by studying toward the doctoral degree at New York University.