AN ABSTRACT OF THE THESIS OF

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In mammals, all epidermal melanocytes react to exposure by ultraviolet (UV) light by increased activity and population. Yun and Montagna (Anat. Rec., 1966. 154, 161), for example, reported that after UV irradiation DOPA-positive dendritic cells in the rhesus monkey appeared at the dermoepidermal junction where they are not normally seen. With repeated irradiation, more melanocytes appeared until peak numbers were reached, after which regression occurred.

The experiments reported here were designed to reconfirm Yun and Montagna's findings and to determine 1) the long-term effects of UV light on pigment cells, 2) whether stimulated pigment cells can be restimulated after a period of rest, 3) whether tyrosinase activity increases and remains at a high level with UV stimulation or decreases once it reaches this peak, and 4) how ultrastructural features relate to DOPA positivity in melanocytes.

The hair of six adult rhesus monkeys was removed, and thoracic, abdominal, and back skin was exposed 5 times per week to 185-350mμ UV light. Skin biopsies were prepared for routine histological and electron microscopic observation, the histochemical demonstration of DOPA, and the biochemical assay of melanin synthesis.

With sequential irradiation, the number of histochemically demonstrable DOPA-positive melanocytes increased slowly during the first 15 irradiations, rapidly increased to a peak at 30 dosages,

then declined steadily to a basal level which was maintained for the duration of the experiment (216 exposures or 43 weeks). Whether the pigment cells could be restimulated was determined by shading one-half the irradiated area, then after 3 months reirradiating for 11 weeks. During the shading period, the number of DOPA-positive cells decreased to 0, but with restimulation they again increased and subsequently declined to a basal level.

Ultrastructural studies confirmed the histological observations that indeterminate and Langerhans cells but no epidermal melanocytes were present in nonirradiated animals. The melanosomes produced by UV stimulation differed morphologically from developing melanosomes (stages I & II) of other species in that they lacked an organized internal filamentous matrix. Melanization occurred by a deposition of flocculent material within this structure, but often melanization was not completed.

At their numerical peak (after 30 exposures), the melanocytes contained mostly fully developed melanosomes, and a small number of melanosomes were transferred to the adjacent keratinocytes. With subsequent irradiation, the number of melanosomes in the melanocytes and the melanocytes themselves decreased. Melanogenic activity, identified by the incorporation of labelled DOPA or tyrosine, paralleled the pattern seen histochemically in the DOPA-positive cells.

The epidermis of the rhesus monkey had a pool of indeterminate cells which were stimulated by UV light to form melanocytes.

Some conclusions can be drawn about why the melanocytes suddenly become inactive despite continued irradiation. Since no melanosomes passed into the dermis and virtually none remained in the epidermal melanocytes, they may have been transferred to keratinocytes at such a slow rate that the synthesis of new ones was inhibited. The mechanism involved was probably keratinocyte injury since after an appropriate rest period, reirradiation caused DOPA-positive melanocytes to reappear. In addition, high threshold levels of irradiation were required to stimulate melanogenesis.

EFFECTS OF ULTRAVIOLET IRRADIATION ON THE PIGMENTARY SYSTEM OF THE RHESUS MONKEY (MACACA MULATTA)

by

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INTRODUCTION

For thousands of years, man has realized that sunlight is an environmental force which affects all living things. Only in recent times, however, has he begun to understand the action of solar radiation, one profound effect of which is skin color. Because pigmentation accounts for one of the most obvious differences in man and animals, the photobiology of pigmentation constitutes an important area of scientific investigation.

I. The Melanocyte, A Historical Review

Among earliest investigators of the pigmentary system was Weidenrich (1900) who theorized that pigmentation in vertebrates consists of four "envelopes": perineural, perivascular, pericelomic, and peridermal. Thus he thought that pigment-forming cells were embryologically related to the nervous system. After Weidenreich, a Swiss dermatologist, B. Block (1917), investigated the function of pigment cells. Using the histochemical technique known as the DOPA reaction, he demonstrated melanin-producing cells in the epidermis which he believed to be variants of basal epidermal cells that were formed as the result of solar radiation. Despite some erroneous conclusions, Block did show that an enzyme is involved in the formation of melanin and that melanocytes are present in Caucasian skin.

In the early 1900s, biochemists began to study melanin formation in plants and lower animals. Their work provided a basis for understanding melanin in mammalian systems. Some early investigators (Bourquelot and Bertrand, 1895) found that the toadstool, Russula nigricans, contained tyrosinase. Another investigator, W. Biedermann (1892) demonstrated that oxygen is necessary for melanin formation in the mealworm beetle, Tenebrio molitor. Later, using plant tyrosinase, Raper (1926, 1928, 1932) worked out a biosynthetic pathway, demonstrating that tyrosine is the necessary precursor for melanin formation and that DOPA is the second product in the biosynthetic pathway. This scheme was later confirmed and modified by Mason (1948).

The presence of tyrosinase in vertebrates was first demonstrated by Hogeboom and Adams (1942). Using mouse melanoma, they separated a monophenolase, which was involved in the conversion of tyrosine, from a diphenolase, which was involved in the oxidation of DOPA to DOPA-quinone. Working with the same problem, another group of investigators (Lerner et al., 1949) were not able to separate tyrosinase activity from DOPA-oxidase activity. Their explanation of one activity instead of two was based on the observation that the induction period of tyrosinase was so long that it appeared as if two enzymes were present. With small amounts of DOPA as catalyst, they shortened the

induction period and thus demonstrated in mammalian tissue that DOPA may act as catalyst in the oxidation of tyrosine to DOPA.

While biochemical evidence was accumulating which characterized the pigment cells, histochemical studies of human skin (Becker, 1927, 1930, 1942; Boyd, 1949; Laidlaw, 1932; Radaeli, 1953; Staricco and Pinkus, 1957) established that melanocytes are localized at the dermoepidermal junction in the skin of every body region. Work by Rawles (1940, 1947) and DuShane (1943) established the origin of melanocytes as the neural crest. Masson (1948) used the term melanoblast to describe a glandular cell whose product was excreted into other cells. He believed that this excretory process should be given a special name, cytocrine. Billingham (1948a, 1948b) extended Masson's findings and observed that melanogenesis occurred only in the cytoplasm of dendritic cells and that dendritic cells secreted pigment into other epidermal cells, keratinocytes. A few years later, the highly branched pigment cell was called a melanocyte (Fitzpatrick and Lerner, 1953). Today the term melanocyte is used to define a cell with a rounded body and one to several dendritic processes. The cytoplasm is somewhat clearer than the surrounding keratinocytes because the melanocyte lacks tonofilaments. Moreover, these cells lack desmosomes but are capable of synthesizing tyrosinase which is incorporated into special organelles, the melanosomes.

Other cells often associated with pigment production and pigment-producing cells are the melanophores and melanoblast. The former is a pigment cell which functions with other chromatophores to cause a rapid color change by the intercellular movement of melanosomes, i.e., by aggregation and dispersion (Fitzpatrick et al., 1966). A melanoblast is a precursor of a melanocyte or melanophore.

II. Location of Melanin Biosynthesis

Pigment granules in the melanocyte were first described by Smith (1925). A few years later, Russell (1946) noted that the hair color of different strains of mice was due to a single type of distinctive pigment granule. Using centrifugal fractionation of ciliary bodies from bovine eyes, Herrmann and Boss (1945) found that the isolated granules contained DOPA-oxidase, cytochrome oxidase, and succinic dehydrogenase. Thus, they thought that pigment granules were metabolically active and not inactive end products of pigment metabolism. Using mouse melanoma in a differential centrifugation study, Lerner et al. (1949) found tyrosinase activity associated with particulates and regarded these units as microsomes or particles of the same size as microsomes.

One of the earliest ultrastructural studies of pigment granules was done by Mason and his associates (1947).

A few years later another group (Birbeck, Mercer, and Barnicot, 1956) showed that melanocytes had particles with an internal structure and that these contained a membranous system. They also stated that the pigment granules showed variation in development. Although some earlier investigators (DuBuy et al., 1949) thought that pigment granules were modified mitochondria, Backer et al. (1960) later showed that melanin granules and mitochondria were separate organelles of the melanocyte, each with a typical enzyme system, and demonstrated this by monitoring the biochemically derived fractions with electron microscopic studies.

Using mouse melanoma, a group of investigators (Seiji et al., 1961, 1963; Seiji and Iwashita, 1963a, 1963b) determined the morphological and biochemical characteristics of pigment granules. They hypothesized that tyrosinase was synthesized on ribosomes and transferred through the endoplasmic reticulum to the pigment granules (melanosomes) where the enzyme was stored. (In 1963, Seiji proposed the term melanosome instead of melanin granule). They observed that pigment granules were melanized until they became uniformly dense and passed to the surrounding cell. Tyrosinase activity was shown not only in the melanosomes but also in the smooth surfaced membranes, rough surfaced membranes, and on ribosomes (Seiji et

al., 1961). Most labeled DOPA was incorporated into melanosomes (Seiji and Iwashita, 1965b). The exact amount of DOPA incorporation was difficult to determine because some DOPA was already present in the melanosome (Takahashi and Fitzpatrick, 1966). Thus, the site of incorporation of C^{14}-DOPA (Nakai and Shubik, 1964) or C^{14}-tyrosine (Moyer, 1966) was demonstrated; both labels incorporated into the same site, the melanosome. Another group (Zelickson, Hirsch, and Hartmann, 1964), however, found that C^{14}-DOPA incorporated into the rough surfaced membranes; therefore, they hypothesized that the endoplasmic reticulum was the primary site of tyrosine and DOPA reactions.

Hirsch (1939) and Masson (1948) originally proposed that melanosomes arose from the Golgi complex. This hypothesis has been substantiated by several electron microscopic studies (Barnicot, Birbeck, and Cuckow, 1955; Dalton and Felix, 1953; Hu, Swedo, and Watson, 1967; Hunter, Mottaz, and Zelickson, 1970; Novikoff, Albola, and Biempica, 1968; Seiji et al., 1961, 1963; Toshima, Moore, and Sandberg, 1968; Wellings and Siegel, 1963; Zelickson et al., 1964, 1965). Generally, these studies determined that early stage melanosomes arose from vesicles which pinched off from the cisternae of the Golgi apparatus, enlarged, and formed a protein matrix for the deposition of melanin.

Other evidence indicates that most of the early stage melanosomes (premelanosomes) are contained in and develop within the smooth endoplasmic reticulum which was connected to the Golgi apparatus during melanogenesis (Maul, 1969).

Thus, the current view of melanosome development is that tyrosinase is synthesized on ribosomes and transferred by the endoplasmic reticulum to the Golgi apparatus where the enzyme is assembled into units. These units are surrounded by a limiting membrane forming a vesicle. This is the first of four stages as defined by Toda, Hori, and Fitzpatrick (1968) and therefore called stage I melanosome. As shown by electron microscopy and cytochemistry, this vesicle is spherical or has filaments with distinct periodicity. In stage II, the melanosome is oval and displays numerous membranous filaments, some crosslinked and maintaining a distinct periodicity. Stage II was previously called the premelanosome. During stage III, melanin is deposited and partially obscures the characteristic periodicity. Finally, in stage IV, the melanosome becomes a uniformly dense particle without a discernible internal structure (Toda, Hori, Fitzpatrick, 1968). In general, melanosome structure in man is similar but less uniform in other mammals. In mice, structure varies with the genotype (Quevedo, 1973).

Eumelanin melanosomes are oval and 0.15 x 0.37 μ (Breathnach, 1971). The internal structure consists of compound fibers, 9 mμ in diameter, which appear in longitudinal section as hollow cylinders with a lumen 3.5 mμ in diameter and a wall 3.5 mμ thick. The compound fibers, which are composed of unit fibers 3.5 mμ in diameter, appear as a loosely coiled helix. The compound fibers arranged parallel exhibit a 6-8 mμ periodicity and appear lattice-like because of cross linking between fibers. These cross-linking fibers are 3.5 mμ in diameter and spaced 4.5 mμ apart (Moyer, 1966). With melanization, the spacing becomes obscured and the melanosome exhibits a cross striated appearance. Melanization continues until the matrix is electron opaque. Whether the matrix is made of a protein structure on which tyrosinase is deposited or whether tyrosinase acts as both structural protein and enzyme has not been conclusively shown. Toda and Fitzpatrick (1971), however, did observe that tyrosinase is located on the outer membrane of the melanosome.

Melanosomes appear to have acid phosphatase activity associated with them. Seiji and Iwashita (1965a) found acid phosphatase in melanosomal fractions of melanoma. Their preparations, however, contained melanosomes from both

melanocytes and phagocytes. Hori et al. (1968) ultrastructurally demonstrated the presence of acid phosphatase activity in melanosome complexes of keratinocytes. Other investigators (Gazzolo and Prunieras, 1968; Olson, Nordquist, and Everett, 1970; Seiji and Kikuchi, 1969) have demonstrated acid phosphatase-positive vesicles containing melanin in keratinocytes. Once inside keratinocytes, the melanosomes with associated lysosomal enzymes undergo a gradual degradation and become fragmented (Oktaki and Seiji, 1971).

III. The Langerhans cell and the Indeterminate Cell

Despite the development of extensive histochemical methods, the electron microscope is the best means of identifying epidermal components. Because of ultrastructural features, a positive means for separating melanocytes, Langerhans cells, and indeterminate cells is possible.

The Langerhans cells were first seen in the upper epidermis by Langerhans (1868). They are a distinctive unit in the mammalian epidermis and dermis, often in the past considered as part of the melanocyte system. They have also been found in other stratified squamous epithelia such as oral mucosa, gingiva, and vagina (Montagna and Parakkal, 1974). Langerhans cells are

probably of mesodermal origin. They contain distinctive organelles, the Langerhans granules, which set the cells apart from any other cell. Unlike the melanosomes, this racquet- or rod-shaped organelle in the cytoplasm has no formative stages. Whether the granules originate from the plasma membrane and move into the cell or develop from the Golgi apparatus and then migrate outward is still a question (Tarnowski and Hashimoto, 1967; Zelickson, 1965, 1966). Despite some doubt about their function, Langerhans cells are an independent cell population in the epidermis. After irradiation they incorporate H^3-thymidine, and divide (Giacometti and Montagna, 1967). Langerhans cells and melanocytes are dissimilar in many other ways: in response to melanogenic stimuli, in location within the epidermis, and in regional distribution. The enzyme content, too, is different. For example, the ATPase reaction in guinea pig epidermis is specific for Langerhans cells and not melanocytes (Wolff and Winkelmann, 1967a). Despite extensive work, the function and origin of these cells remain unknown.

The indeterminate cell is a nonkeratinocyte, dendritic cell which lacks melanosomes, Langerhans granules, and desmosomes. Breathnach (1971) believes there is no evidence to support the theory that indeterminate cells are a separate cell population. Many other investigators, however, have observed indeterminate

cells in the epidermis (Swanson, Wayte, and Helwig, 1968; Zelickson and Mottaz, 1968; Zelickson, Mottaz, and Hunter, 1972), in senile white hair (Sato, Kukita, and Jimbow, 1973), and in oral epithelium (Hutchens, Sagebiel, and Clarke, 1971). Although their function has not been conclusively established, the experiments described here may provide some clues.

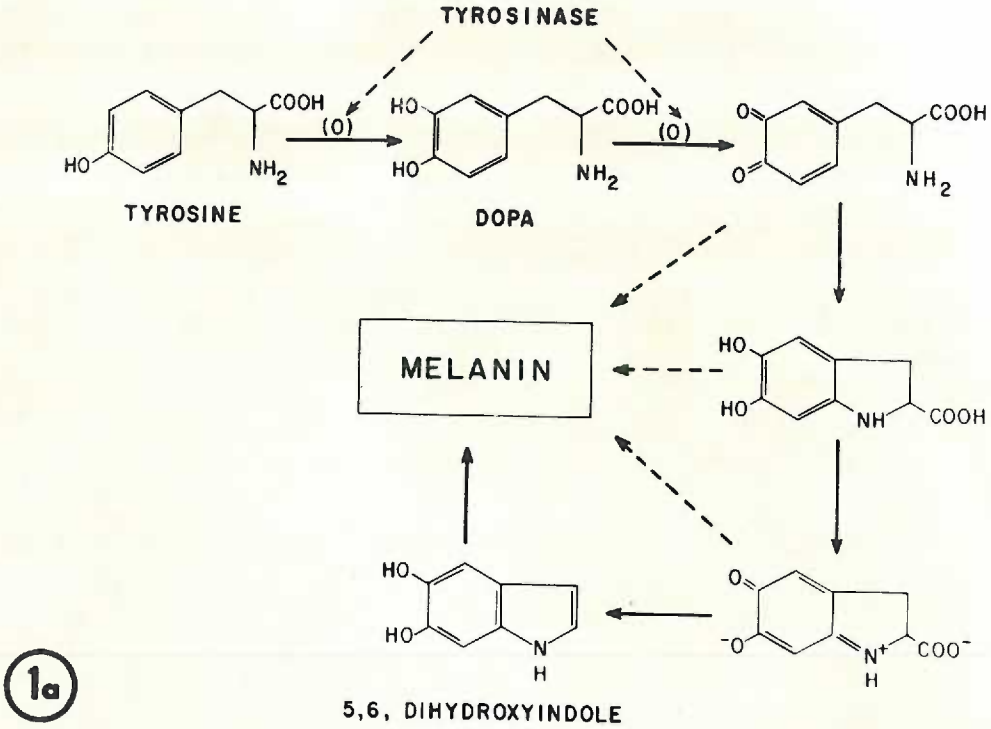
IV. Metabolic Pathway of Melanin

Melanin formation involves the conversion of an amino acid, tyrosine, to a polymer, melanin. In mammalian tissue this reaction is carried out by tyrosinase; it is the only well-demonstrated enzyme in the pathway. In the work described by Raper (1928) and modified by Mason (1948, 1955) (Fig. 1b), tyrosine is oxidized to DOPA in the presence of tyrosinase and molecular oxygen. Initially slow, this reaction rate increases rapidly after an induction period. The DOPA formed is oxidized enzymatically in a reversible reaction to DOPA-quinone. The remaining steps proceed rapidly without tyrosinase although its presence increases the rates. Indole-5,6-quinone polymerizes to melanin through an intermediate of polyindole quinones.

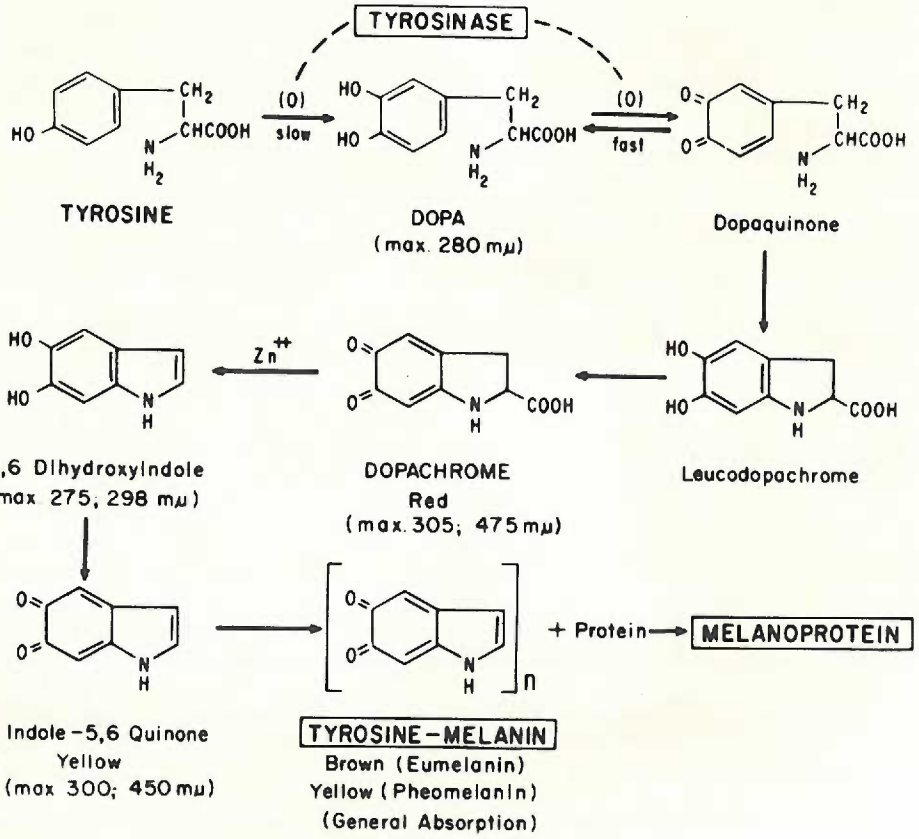
Tyrosinase is an enzyme consisting of a copper-protein complex. The presence or absence of inhibitors which normally affect any enzymatic reaction will naturally affect tyrosinase.

Figure 1a A modification of the Raper-Mason biosynthetic pathway based on work of Hempel, Swan, and Nicolaus (Duchon, Fitzpatrick, and Seiji, 1968).

Figure 1b Raper-Mason biosynthetic pathway of tyrosine to melanin (Mason, 1948, 1955).



1a



1b

Indole-5,6 Quinone
Yellow
(max 300; 450 mμ)

General factors causing inhibition are temperature, pH, and concentration of substrate. One specific factor is any agent which binds copper; this bonding inactivates the enzyme (cf Hu, 1968). A reactive sulfhydryl group, for example, will inhibit the enzyme reaction. Rothman et al. (1946) have reported that plant tyrosinase is inhibited by a sulfhydryl-containing compound in the human epidermis. These sulfhydryl compounds are thought to inhibit melanin formation by combining with tyrosinase-bound copper or by forming complexes with intermediate compounds during melanin biosynthesis (Roston, 1960; Mason and Peterson, 1965; Seiji, 1967). Halprin and Ohkawara (1967) have demonstrated reduced glutathione in epidermal extracts and they believed that this compound inhibits melanin formation, since less reduced glutathione and glutathione reductase occurred in Negro skin than in Caucasian skin. Another specific factor is the presence of a reducing agent such as ascorbic acid. The conversion of tyrosine to DOPA is oxidative, and a strong reducing agent decreases this reaction.

Because of the difficulty of isolating natural sources of melanin, most early conclusions were based on model experiments in vitro. With these, the Raper-Mason pathway assumes

that melanins are polymers of indole-5, 6-quinone. Using labeled precursors, Swan (1963) and Robson and Swan (1966) have shown that the Raper-Mason pathway may be oversimplified. Instead of all indole-5, 6-quinone units, melanin is a combination of monomer units; these units are DOPA-chrome and hydrogenated 2, 3-dihydroxyindole besides the indole-5, 6-quinone units.

Some of the recent work on the structure of natural melanin was done by Nicolaus and Piattelli (Nicolaus, 1962; Nicolaus, Piattelli, and Fattorusso, 1964; Nicolaus and Piattelli, 1965) on melanins from vertebrates. They found that animal melanins were "indole" in type (composed of indole-5, 6-quinone units) whereas plant melanins were "catechol" in type. The molecular weight, however, was not determined.

Using radioactive DOPA, Hempel (1966) determined that the structure of melanin was similar to that found by Swan. Considering the new findings, the Raper-Mason scheme might be more accurately depicted as shown in Figure 1a.

V. Pigmentation in Mammals

Melanoblasts first invade the epidermis in the ten-week human fetus. By the 14th week these cells are quite numerous. The differentiation of melanoblasts into melanocytes is affected by genetic factors as well as the environment into which they are

incorporated. Studies on human genetics are relatively few, but those on the mouse have clarified in considerable detail the genetic control of pigmentation. Genetic factors probably influence the fine structure of the melanosome, the level of tyrosinase activity, and the length and size of melanocytic dendrites.

Just as the structure of the epidermis varies slightly from region to region, so does the number of epidermal melanocytes in human adults (Staricco and Pinkus, 1957; Szabo, 1954). Regional differences, absent in the fetus, develop shortly after birth. There are, however, no significant differences in melanocyte population between symmetrically opposite sides of the same person (Szabo, 1959) or other animals (Billingham and Medawar, 1953). Apparently there is a peak number of melanocytes postnatally which decreases about 10% of the total population for each ten years in man (Quevedo et al., 1965). With age melanocytes progressively cease melanogenesis, transfer melanosomes into surrounding keratinocytes, and remain in an amelanotic form, lacking any characteristic organelles (Quevedo, Szabo, and Virks, 1969).

An age-related decline also occurs in the number of melanocytes of the dorsal trunk epidermis of pigmented mice. DOPA-positive melanocytes reach a maximum number two days

after birth, then decline until at two weeks only very few remain (Quevedo et al., 1966). Species, however, vary widely in the number of active epidermal melanocytes.

In a survey of the epidermal pigmentation of 49 sub-human primates, Machida and Perkins (1967) reported that about one-third of the animals had slight to intense epidermal pigmentation. In the adult rhesus monkey, no melanocytes were found in the general body epidermis. Visible melanocytes were present only in the epidermis of face, eyelids, and friction surfaces (Montagna, Yun, and Machida, 1964), the external lips and oral mucosa (Hutchens et al., 1971). By 50 days of development, however, melanocytes actively producing early stage melanosomes were found in the rhesus fetus. But after 125 days, no melanocytes were present (Bell, 1973). Pigmentation varies greatly in the dermis. The skin has large and small, dermal and follicular, usually bluish, pigmented areas surrounded by pink skin.

VI. Effects of Ultraviolet Light on the Pigmentary System

Although the number of epidermal melanocytes varies in mammalian systems, the reaction to ultraviolet light is fairly consistent. In man, after a single irradiation, Pathak, Sinesi, and Szabo (1965) found no significant increase in the number of

melanocytes. Morphological changes, however, indicate an increased functional activity. After 10 to 15 exposures, Quevedo et al. (1965) reported an increase in the number of DOPA-reactive melanocytes present at the dermoepidermal junction. This increase occurred in all ages, the number of melanocytes in irradiated skin exhibiting an age-dependent decline paralleling that in non-irradiated skin. After prolonged solar irradiation, Mitchell (1963), however, found that age had no effect on the number of melanocytes.

In hairless mice, repeated daily exposure to ultraviolet light inhibited the loss of DOPA-positive melanocytes from the dorsal trunk of neonatal epidermis (Quevedo et al., 1966). Ultraviolet light also elicited the appearance of numerous DOPA-reactive epidermal melanocytes in maturing and adult mice (Quevedo et al., 1966). The differences in the tanning potential of plantar skin and hairy skin appeared to be regulated by genetic influences on the melanocyte. In mice the tanning response varied with the phenotype (Hadley and Quevedo, 1966). Snell (1963) found that after repeated irradiation of guinea pig epidermis, the number of melanocytes increased significantly.

Yun and Montagna (1966) found that after ultraviolet irradiation, DOPA-positive dendritic cells appeared at the

dermoepidermal junction of rhesus epidermis. With sequential irradiation, the number of these cells increased until peak numbers were reached; with additional irradiation, the number decreased. Thus, they described the activity of a unique pigmentary system, but left many questions unanswered.

VII. Ultraviolet Light

A. Biophysical action

Ultraviolet light is that wavelength which is below the visible spectrum at the violet end (380 mμ). Although ultraviolet of very short wavelengths can be produced artificially under optimal conditions, the shortest wavelengths to reach the earth are 285-290 mμ (Freeman et al., 1966; Sliney, 1972). The amount and kind of solar radiation reaching the earth's surface depends upon time of day, latitude, season, geographic altitude, ozone layer, and regional atmospheric variations such as cloudiness, haze, smoke, dust, fog, and humidity. Intensity of sunlight is also related to the angle and distance of the sun to the earth.

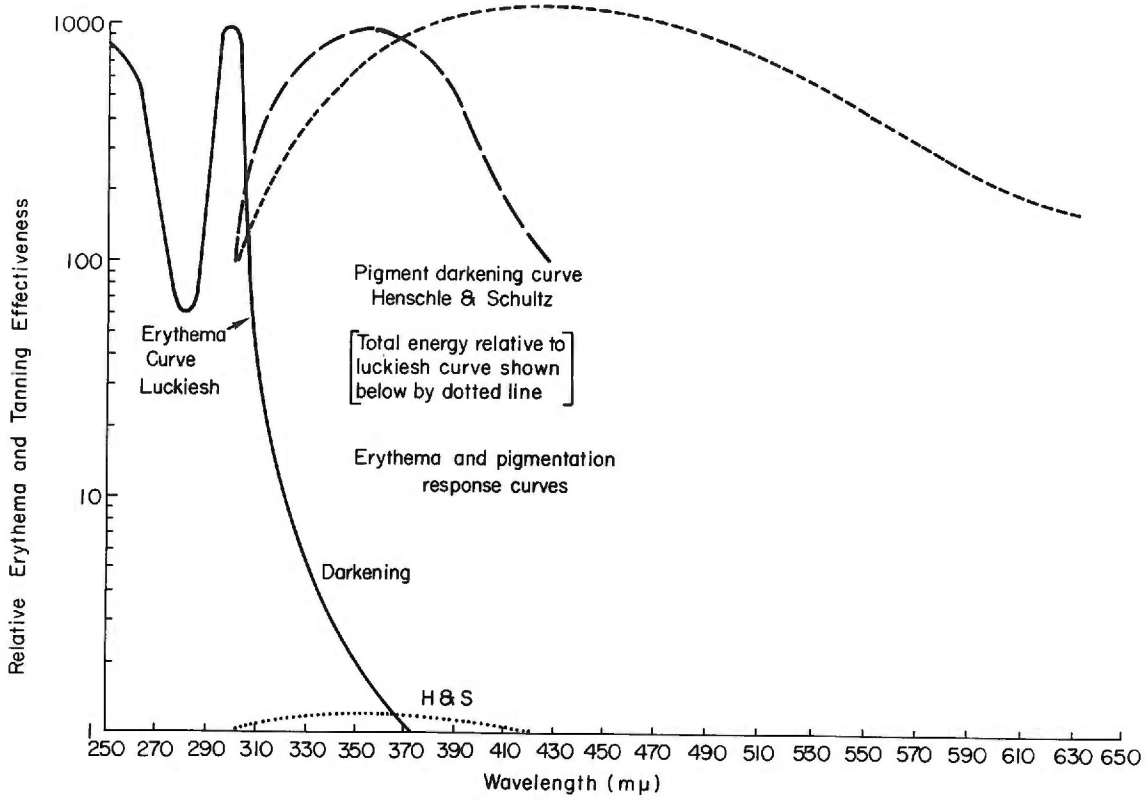
About 75% of the sun's energy is in the 300-700 mμ range. Skin can utilize a large part of this solar energy without extensive injury. Some wavelengths (290-400 mμ), however, are capable of inducing sunburn, carcinogenesis, phototoxic and photoallergic reactions, and many other abnormal modifications.

Human skin reflects most visible light but very little ultraviolet light. The ability to scatter light, therefore, protects skin against actinic damage. In human skin, the stratum corneum is quite effective in scattering ultraviolet irradiation. Melanosomes and melanoprotein also absorb, scatter, and attenuate ultraviolet light and protect the melanocyte against inactivation or death (Pathak, 1967). Absorption of radiant energy and dissipation as heat protect against cell injury.

B. Responses of the skin to ultraviolet light

Over 100 years ago, physicists recognized that sunburn was caused by an invisible portion of the sun's rays. Some time later the action spectrum for erythema was determined and reported by Hauser and Vahle (1922). Using monochromatic light, Luckiesh (1946) found a similar spectrum (Fig. 2) with a primary peak at 297 mμ with minimum values at 280 and 320 mμ. With high-intensity monochromatic radiation, Urbach (1969) eliminated stray light which significantly contributed to inaccuracy. He found a maximum response at 297 mμ and a secondary response at 257 to 260 mμ. Freeman et al. (1966) found that maximum erythema was produced at 305-308 mμ. Including radiation from artificial sources, the erythema producing spectrum is generally considered those wavelengths between 250 and 320 mμ.

Figure 2 Action spectrum for erythema (solid line) (Luckiesh, 1946) and immediate pigment darkening (long broken line, Henschke and Schulte, 1939) (short broken line, Pathak, 1967).


1000
100
10
1
Relative Erythema and Tanning Effectiveness
Erythema
Curve
Luckiesh
Pigment darkening curve
Henschle & Schultz
Total energy relative to luckiesh curve shown below by dotted line
Erythema and pigmentation response curves
Darkening
H & S
250 270 290 310 330 350 370 390 410 430 450 470 490 510 530 550 570 590 610 630 650
Wavelength (mμ)

Erythema initially appears during exposure and disappears shortly after termination of irradiation. Erythema then reappears two to four hours later and reaches maximum intensity in fourteen to twenty hours (Cotran and Pathak, 1968). Responses after ultraviolet irradiation vary, depending upon the dose of ultraviolet, amount of preexisting pigment, and thickness of the epidermis. Ultraviolet light has two distinct erythema effects on the skin: one on the epidermis and the other on the dermis (Van der Leun, 1965a, 1965b). Histologically, the vascular response of erythema is characterized by vasodilatation, increased permeability, and exudation of plasma proteins and leukocytes. Leukocytosis is visible throughout the upper dermis; the number of white blood cells present in the dermis reaches a maximum by twenty-four hours. Degenerative changes of the epidermis are present sixty minutes after a single exposure. Epidermal cells become more rounded; the prickle cells gradually become separated. The epidermis exhibits occasional necrosis and damage which is shown by vacuolated cells with shrunken cytoplasm and pyknotic nuclei. After 48 hours, epidermal cell disorganization and regeneration is apparent. At the same time, arborization of dendrites and hypertrophy occur within the melanocytes, if any are present, in the skin. Seventy-two hours after exposure, epidermal cells exhibit

regeneration, and desquamation of damaged cells occurs. After 96 hours almost the entire epidermis is in a regenerative phase with an increased number of DOPA-positive cells present.

Melanogenesis, like sunburn, is closely related to irradiation by those wavelengths which produce erythema. Changes causing erythema can influence pigmentation. In human epidermis two distinct processes take place. The first is immediate pigment darkening. It begins without any latent period; after termination of irradiation, pigmentation fades rapidly at first, then more slowly (Pathak, Riley, and Fitzpatrick, 1962). Immediate pigment darkening consists of oxidation of melanin and transfer of existing melanosomes in the keratinocyte. Oxidation produces unstable, free radicals of the melanin (Pathak, 1967). Henschke and Schulze (1939) report the spectrum for immediate pigment darkening as 300 to 430 mμ, whereas Pathak (1967) believes it extends from 300 to 700 mμ (Fig. 2).

The second process is melanogenesis--the production, transfer, and distribution of melanosomes in five stages. First, the number of active melanocytes increases by either proliferation or activation of amelanotic (dormant) melanocytes or both. Second, the number of melanosomes increases as the result of augmented synthesis. Melanocytes and keratinocytes contain more stage IV

melanosomes, and the melanocytes also contain increased numbers of early stage melanosomes. Third, tyrosinase activity increases as a result of additional new synthesis in active melanocytes. Fourth, tyrosinase is activated by ultraviolet-inhibiting sulfhydryl compounds in the epidermis. Fifth, the number of melanosomes transferred is increased (Pathak et al., 1971).

VIII. Purpose

The experiments reported here were designed to reconfirm Yun and Montagna's (1966) findings and to determine: 1) the long-term effect of ultraviolet light on pigment cells, 2) whether pigment cells can be restimulated after initial stimulation and an adequate rest period, 3) whether tyrosinase activity increases with ultraviolet light stimulation and remains at a high level or whether it decreases after once reaching this high level, and 4) how ultrastructural features relate to DOPA-positivity.

MATERIALS AND METHODS

I. Animals and Their Care

Eight 7 to 10 year old male rhesus monkeys (Macaca mulatta) were used for these experiments. Six animals were irradiated with ultraviolet light and two controls were not. Hair was clipped from the side, thorax, and abdomen of each animal, and the areas to be irradiated were tattooed. All animals were then clipped weekly for the duration of the experiments.

II. Irradiation Methods

Experimental animals were irradiated with one of two different ultraviolet sources five times each week (Monday through Friday). One source consisted of two Westinghouse FS20T12 fluorescent sun lamps with an aluminum reflector unit; the spectral output ranged from 275 mμ to 350 mμ, but most was in the middle ultraviolet range (Appendix I). The other source was a Hanovia Aero-kromayer ultraviolet light. The ultraviolet intensity for the spectral region 185 mμ to 313 mμ* on contact with the front window of the lamp was 7.0×10^4 μw/cm^2.

Energy output was calibrated weekly with a Rentschler click meter (Model SM-200) equipped with a phototube (Model WL767); the instrument was precalibrated against a lamp standardized

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*Hanovia Lamp Company would not supply a spectral output curve as Westinghouse did.

at the United States Bureau of Standards. After calibration, exposure time was readjusted so that doses given were consistent.

Each animal irradiated with the Westinghouse lamps was held in place by a press cage. Specific areas were exposed at a distance of 25 centimeters; the rest of the body was shielded with a cloth. In the first experiment (R-I), the left side of the animal was exposed 57 times (over 83 days) to 6.2×10^4 $\mu w/cm^2$ (Kechijian, 1965). In the second experiment (R-II), the right side was irradiated with 6.4×10^5 $\mu w/cm^2$.

After 95 exposures (over 134 days), half of the side area that had received ultraviolet was covered with a cloth. This area was shaded for 92 days; then the cover was removed and the whole side again irradiated. Thus, half of the right side received 216 dosages (over 318 days) and the other half received 95 dosages (over 134 days), 92 days shade, and 56 subsequent dosages (over 82 days).

In the third experiment (R-III), the animals irradiated with the Hanovia lamp were tied down supinely. With the front window of the ultraviolet lamp placed in contact with the skin of the central abdomen and thorax, the animals were exposed to 7.0×10^5 $\mu w/cm^2$ of ultraviolet light for 83 times (over 126 days).

III. Processing of Tissues

A. Sampling

Full-thickness samples of skin 1 cm x 3 cm were removed from animals under general anesthesia. Biopsy specimens were selected randomly from both irradiated and nonirradiated areas; no scar tissue surrounding previous biopsy sites was included. In the first experiment (R-I), two specimens from one animal of a group of six were removed after 3, 8, 11, 21, 26, 41, and 57 irradiations (see Table I). In the second experiment (R-II), biopsies from one to six different animals of the group were removed after 4, 9, 16, 21, 26, 31, 37, 52, 67, 72, 79, 93, 98, 103, 108, 111, 116, 121, 140, 157, 166, 172, 183, 188, 192, 199, 206, and 216 irradiations (see Table II). In the same experiment, biopsies from one animal of the group of six were removed from the shaded area after 5, 12, 19, 26, 33, 40, 47, 68, and 90 days (see Table III). In addition, specimens from two to six animals were removed from the reirradiated area after 6, 12, 19, 23, 28, 32, 39, 46, and 56 exposures (see Table IV). In the third experiment (R-III), three biopsies from at least three animals were removed after 15, 21, 25, 30, 34, 41, 48, 58, 72, 78, and 83 irradiation periods. In this same experiment three biopsies were removed from six animals after 4, 37, and 67 exposures.

The six irradiated animals were randomly selected for periodic biopsies.

B. Histological studies

A small portion of each biopsy was fixed for 24 hours in 10% buffered neutral formalin (Appendix II), embedded in paraffin, and processed according to routine procedures for light microscopy. Paraffin sections were cut at 7, 10, and 20 micra, floated on a water bath, and mounted on slides. The following histological staining procedures were used: Harris's hematoxylin and eosin (Lillie, 1965); toluidine blue, buffered to pH = 4.5 (Montagna, Chase, and Melaragno, 1951); periodic acid-Schiff method (Montagna, Chase, and Lobitz, 1953); and acid orcein-Verhoeff (Roman et al., 1967).

C. Histochemical studies

A second portion of each biopsy was treated by the "split skin" technique (modification of Staricco and Pinkus, 1957). After the hair and subcutaneous tissue was removed, the samples were pinned to a flat cork with the epidermal side up and incubated in 2N sodium bromide for two hours at 56° C. The specimens were then removed from the halide and submerged in warm tap water in which the epidermis was separated from the dermis. Epidermal and dermal sheets were washed in distilled water and

processed for either the DOPA (dihydroxyphenylalanine oxidase) reaction (modification of Laidlaw and Blackberg, 1932; Rappaport, 1955) or for adenosine triphosphatase (ATPase) (modification of Wolff and Winkelmann, 1967a) for demonstration of Langerhans cells (Appendix III).

D. Light microscopy

Microscopic observations and measurements were made with a Carl Zeiss GFL standard microscope equipped with 2.5, 10, 40, and 100 power objectives, Kpl 10 x wide angle eyepieces, and ocular reticule. The ocular reticule was calibrated with a Carl Zeiss stage micrometer.

All DOPA-positive dendritic melanocytes were counted in a 1 mm^2 area of the epidermis at a magnification of 100 x. The number of these areas counted per biopsy varied between 10 and 50 depending on the size of the tissue.

E. Electron microscopy

Small blocks of tissue, 1 to 2 mm^2, were fixed either directly in 1% OsO_4 in veronal acetate buffer for two hours (Palade, 1952) or overnight in a modified Karnovsky's fixative (Russell, 1972), washed in two 15 minute changes of Millonig's phosphate buffer, then postfixed in 1% OsO_4 in phosphate buffer for two hours (Appendix IV). Tissues were dehydrated through

graded ethanol to propylene oxide and embedded according to Spurr (1969) (Appendix V). All fixation and dehydration procedures were performed at 4° C. Tissues for light microscopic comparison were sectioned at 1 μ on a Porter-Blum MT-2B ultramicrotome, dried on slides, and stained with toluidine blue. Adjacent sections were dried on slides and inspected, unstained, for the presence of pigment or portions of melanocytes. When these features were found, the ensuing portions were cut (80-110 μm) with a diamond knife for electron microscopic observation, placed on uncoated 200 mesh grids, and stained triply with lead citrate (Reynolds, 1963), uranyl acetate (Watson, 1958), and again lead citrate (Appendix VI). Sections were viewed with a Philips 200 electron microscope operating at 60 KV.

F. Biochemical studies

Biochemical studies were conducted on specimens from the eight animals; six were irradiated 5 times per week as previously described for R-II, and two received none. Four 1 x 3 cm elliptical biopsies were taken after 10, 13, 18, 23, 28, 32, 38, 43, 53, and 68 exposures; two animals were biopsied at each interval, a non-irradiated control and an irradiated animal. The latter were randomly selected for the periodic biopsies. Samples were removed and processed immediately.

For the measurement of tyrosinase activity, a modification of the technique described by Kitano and Hu (1971) for determining melanin synthesis was used. Epidermal sheets were incubated in C^{14}-DOPA or C^{14}-tyrosine, then in nonlabeled DOPA or tyrosine, homogenized, and collected on millipore filters. The filters were placed in scintillation cocktails and counted with a liquid scintillation spectrometer. Puromycin was added with the tyrosine to inhibit general protein synthesis. Phenylthiourea was added to both DOPA and tyrosine to inhibit tyrosinase activity. Details of the procedure are given in Appendix VII.

The rate of incorporation of C^{14}-DOPA or C^{14}-tyrosine + puromycin into ultraviolet stimulated epidermis was compared with that of controls (the above label plus phenylthiourea). The difference is reported as the rate of melanin synthesis.

IV. Statistical Analyses

Mean and standard error of the mean were computed for all interval data on a calculator (Canola 167P). To statistically validate the data from the first two experiments (R-I and R-II), polynomial regression analysis was done on the histochemical data from experiment R-III. This analysis was done on a Scientific Data System Computer, programmed to compute 3rd to 7th order polynomial regression models and plot the results.

OBSERVATIONS AND RESULTS

I. Epidermal Pigmentary System

A. Histological and histochemical study

1. Long-term effects

To confirm Yun and Montagna's (1966) findings that activation of nonmelanotic melanocytes occurred after ultraviolet irradiation, six adult rhesus monkeys were irradiated with the same dosage (6.2×10^4 $\mu w/cm^2$) and type of lamp (Westinghouse). With this regimen, no visibly detectable erythema appeared during the first 96 hours after initial irradiation; redness did not develop at any stage during the experiment. Macroscopically, no pigment areas could be seen at any time; the irradiated skin appeared similar to that of the nonirradiated controls.

In split skin preparations, no DOPA-positive melanocytes were seen in the general body epidermis of the control animals and the nonirradiated areas of treated animals. Melanogenesis was minimally stimulated with daily sequential exposure to 6.2×10^4 $\mu w/cm^2$ ultraviolet light. In only three of the nine samples, melanocytes displayed sporadic DOPA activity (see Table I; Fig. 3). These melanocytes appeared very small and faintly reactive with DOPA.

In a second experiment, the animals were irradiated with

Figure 3 Melanocyte population in the epidermis after sequential exposure to 6.2×10^4 $\mu w/cm^2$ ultraviolet light. Brackets represent standard error of the mean.

DOPA-Positive Melanocyte Population in Skin Irradiated With Westinghouse Lamp FS20

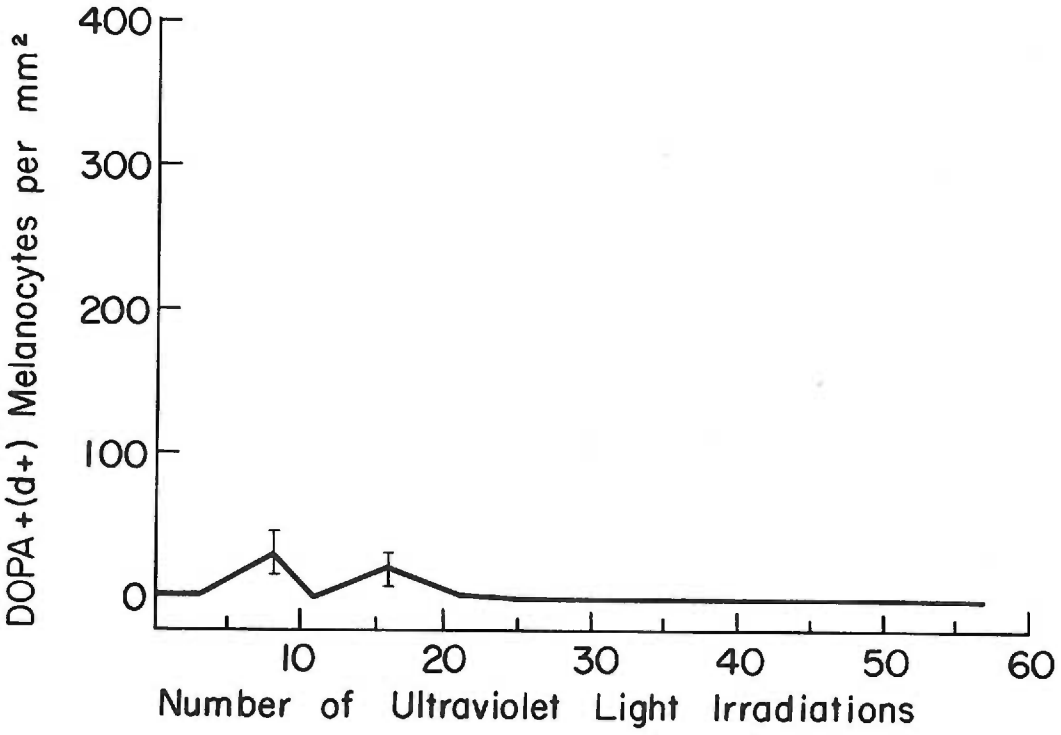


Table I The DOPA-positive melanocyte population in split skin preparations of normal and irradiated epidermis (Westinghouse lamp, $6.2 \times 10^4 \mu w/cm^2$)

Animal Number	Biopsy	Number of UV Light irradiations per time period	Melanocyte population per mm^2 left side $S_{\bar{x}}$ *
1-8	B-1	0	0
4	B-1	3 (over 5 days)	0
	B-2		0
5	B-1	8 (over 12 days)	17 ± 5.0
	B-2		44 ± 9.7
6	B-1	11 (over 19 days)	0
	B-2		0
2	B-1	16 (over 26 days)	20 ± 11.5
	B-2		0
4	B-1	21 (over 33 days)	3 ± 0.9
	B-2		0
2	B-1	26 (over 40 days)	0
	B-2		0
3	B-1	41 (over 61 days)	0
	B-2		0
7	B-1	57 (over 83 days)	0
	B-2		0

* $S_{\bar{x}}$ = standard error of the mean

6.4×10^5 $\mu w/cm^2$ ultraviolet light, a 10-fold increase in the amount previously administered. A perceptible erythema which developed 24 hours after the initial irradiation was intensified after two days of irradiation and by four days was very deep and dark. It continued in some animals for up to nine exposures, by which time it had become spotty and diffusely spread. During this development, some animals showed peeling and scaling. After about ten days, the redness changed to a light brown which gradually disappeared.

With sequential irradiation, the number of DOPA-positive dendritic cells increased, slowly at first (up to 16 irradiation periods), then more rapidly until a peak was reached after 31 irradiations. Then with continued irradiation, the number of DOPA-positive cells declined sharply and remained at a basal level for the duration of the experiment (216 exposures or 43 weeks) (Fig. 4). Numerous secondary peaks were evident after 52, 79, 103, 183, and 192 exposures, but compared with the primary peak, they were relatively small and deviated little from each other. The minor basal fluctuations were due to individual animal variation; since each animal had its own threshold of stimulation, each responded to the same dosage at different levels.

Histologically, no DOPA-positive melanocytes were found

Figure 4 Melanocyte population in the epidermis after exposure to 6.4×10^5 $\mu w/cm^2$ ultraviolet light. The broken line represents the number of melanocytes present during shading and reirradiation. Brackets are standard error of the mean.

The DOPA-Positive Melanocyte Population in Skin Irradiated with Westinghouse Lamp FS 20 ($6.4 \times 10^5 \mu w/cm^2$)

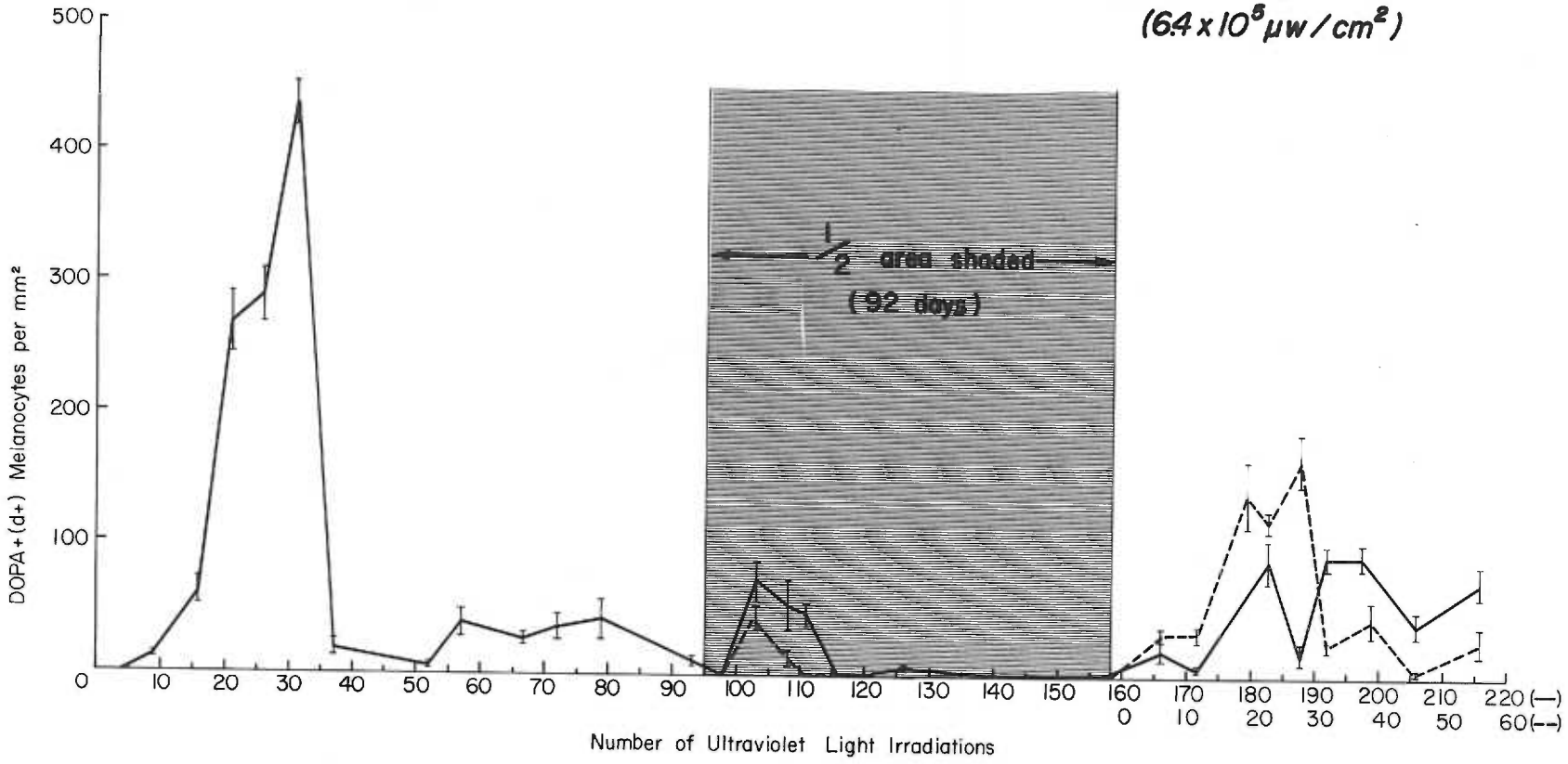


Table II The DOPA-positive melanocyte population in split skin preparations of irradiated epidermis (Westinghouse lamp, $6.4 \times 10^5 \mu w/cm^2$)

Animal number	Biopsy	Number of UV light irradiations per time period	Melanocyte population per mm^2 right side $S_{\bar{x}}$ *
1-8		0	0
2	B-1	4 (over 6 days)	0
	B-2		0
3	B-1	9 (over 13 days)	19 ± 0.4
	B-2		7 ± 1.4
4	B-1	16 (over 22 days)	53 ± 5.7
	B-2		68 ± 10.9
5	B-1	21 (over 29 days)	211 ± 12.5
	B-2		139 ± 11.5
2	B-1		444 ± 39.5
	B-2		276 ± 25.1
6	B-1	26 (over 36 days)	177 ± 17.8
	B-2		350 ± 21.4
	B-3		330 ± 22.2
7	B-1	31 (over 43 days)	523 ± 22.9
	B-2		343 ± 10.9
2	B-1	37 (over 55 days)	10 ± 3.4
	B-2		27 ± 6.2
3	B-1	52 (over 76 days)	0
	B-2		12 ± 2.4
4	B-1	57 (over 83 days)	46 ± 14.0
	B-2		35 ± 9.2
5	B-1	67 (over 97 days)	43 ± 6.6

Table II cont.

	B-2		33 ± 8.5
	B-3		6 ± 1.9
6	B-1	72 (over 104 days)	42 ± 16.9
	B-2		25 ± 8.5
	B-3		56 ± 8.6
	B-4		22 ± 6.1
7	B-1	79 (over 113 days)	64 ± 16.6
	B-2		70 ± 22.2
	B-3		22 ± 12.1
	B-4		13 ± 7.3
2	B-1	93 (over 132 days)	16 ± 4.8
	B-2		5 ± 0.6
	B-3		3 ± 0.7
	B-4		21 ± 7.5
3	B-1	98 (over 139 days)	0
	B-2		0
4	B-1	103 (over 146 days)	70 ± 14.3
	B-2		75 ± 15.6
5	B-1	108 (over 153 days)	43 ± 19.3
	B-2		66 ± 18.7
6	B-1	111 (over 160 days)	56 ± 11.5
	B-2		42 ± 13.6
2	B-1	116 (over 167 days)	0
	B-2		0
4	B-1	121 (over 174 days)	0
	B-2		4 ± 1.6
2	B-1	126 (over 181 days)	8 ± 1.9
	B-2		4 ± 1.9
3	B-1	140 (over 202 days)	0
	B-2		0
2	B-1	157 (over 226 days)	0
	B-2		0

Table II cont.

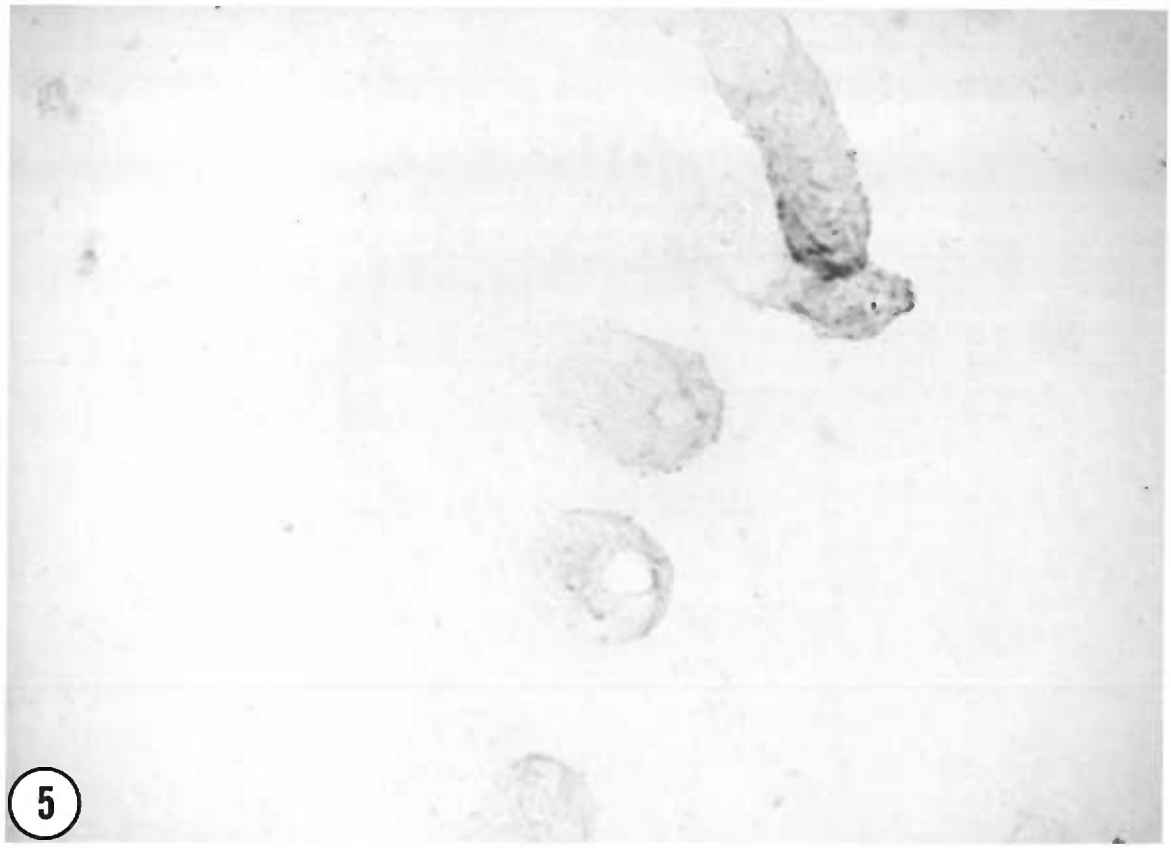
2	B-1	166 (over 239 days)	10 ± 1.6
3	B-1		26 ± 7.6
4	B-1		2 ± 0.9
5	B-1		3 ± 1.0
6	B-1		41 ± 14.2
7	B-1		38 ± 15.7
4	B-1	172 (over 254 days)	6 ± 1.8
2	B-1	183 (over 268 days)	58 ± 9.6
6	B-1		119 ± 23.0
4	B-1	188 (over 275 days)	22 ± 9.7
5	B-1		8 ± 3.1
3	B-1	192 (over 282 days)	46 ± 7.7
7	B-2		137 ± 13.6
2	B-1	199 (over 295 days)	13 ± 3.3
6	B-1		170 ± 19.9
3	B-1	206 (over 304 days)	61 ± 10.6
4	B-1		17 ± 9.4
2	B-1	216 (over 318 days)	127 ± 20.9
7	B-1		17 ± 3.9

$S_{\bar{x}}$ * = Standard error of the mean

either in the nonirradiated animals or in the animals that had been irradiated four times (Fig. 5). After nine exposures, a few DOPA-positive cells appeared (13/mm^2) mainly in the parafollicular areas (Fig. 6). The melanocytes present after irradiation were large (25-30 μ). In all cases, the cell body was found to be strongly DOPA positive. The dendrites, however, were weakly reactive and sometimes difficult to identify. After 16 exposures, the number of melanocytes increased; however, instead of being homogeneously spread, they remained mostly concentrated in the parafollicular areas (Fig. 7). These melanocytes exhibited a definite hypertrophy (Fig. 8). Generally, more than two dendrites, usually extensively branched, were found attached to the perikaryon. After 21 irradiations, the number of DOPA-positive cells increased significantly; the melanocytes appeared fairly homogeneously distributed (Fig. 9) and displayed an increased arborization of the dendrites. After 26 exposures the number of melanocytes increased slightly but their morphological appearance was quite similar to that found after 21 irradiations. The maximum number of active melanocytes was obtained after 31 exposures (Fig. 10). Although the melanocytes seemed to be homogeneously distributed, there was extensive variation between areas. These melanocytes exhibited extensive hypertrophy and hyperplasia (Fig. 11), but the pigment was evenly distributed

Figure 5 Epidermis after four exposures to ultraviolet light. There are no DOPA-positive melanocytes in either nonirradiated or skin irradiated four times. x 165. Figures 5 - 14, split skin and DOPA technique.

Figure 6 Epidermis after nine exposures. A few DOPA-positive melanocytes are found mainly in the parafollicular areas. x 165.



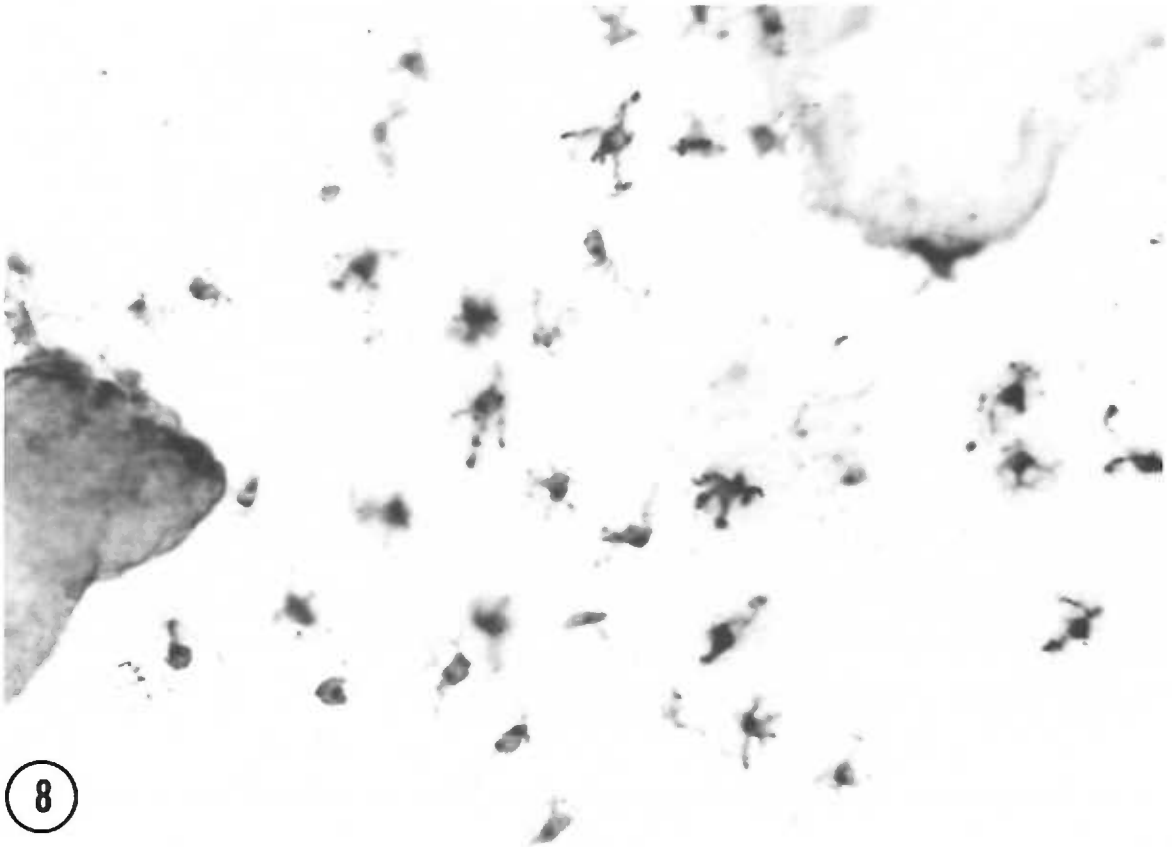
5

6

Figure 7 Epidermis after sixteen exposures. An increased number of DOPA-positive cells are present. x 165.

Figure 8 Higher magnification of Figure 7. Melanocytes exhibit hypertrophy and strongly DOPA-positive dendrites. x 575.

7

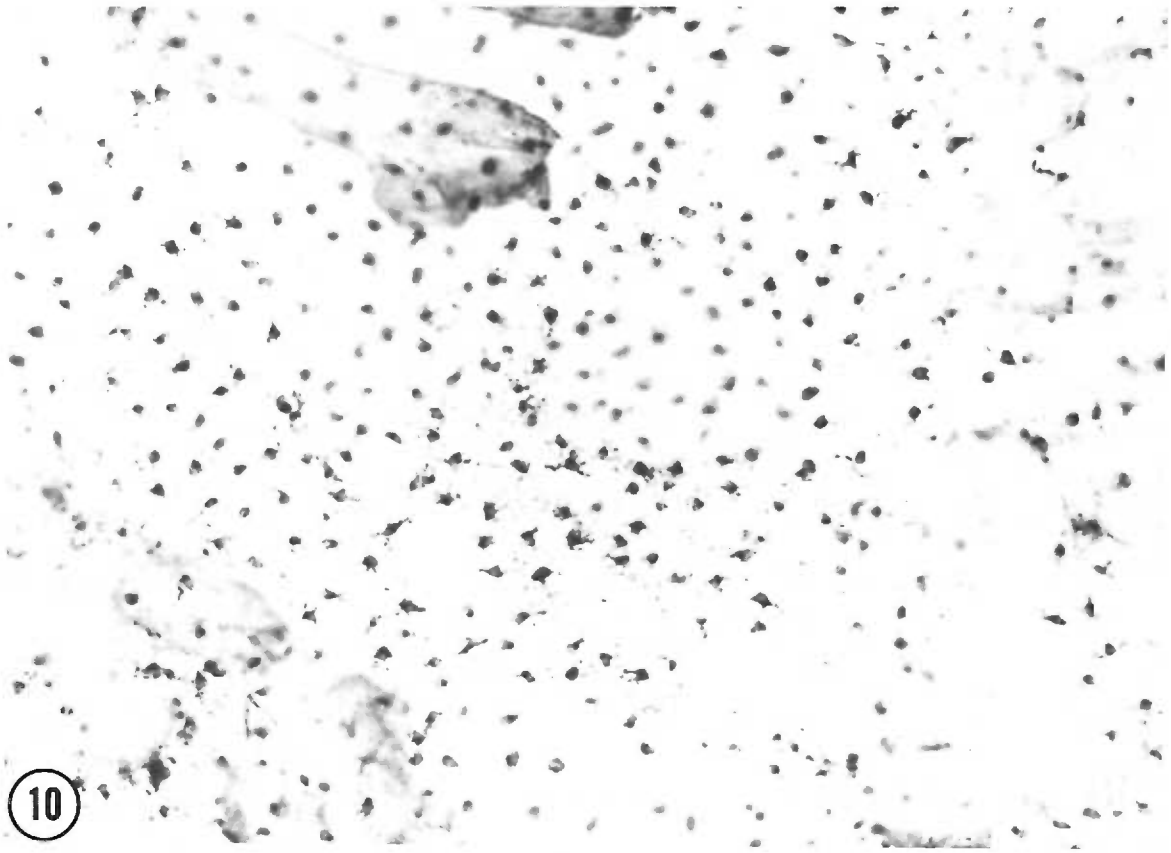


8

Figure 9 Epidermis after 21 irradiations. The melanocytes are fairly homogeneously distributed. x 275.

Figure 10 Epidermis after 31 exposures. The maximum concentration of DOPA-positive melanocytes is evident at this time. x 165.

9



10

between dendrites and the periphery of the cell body. In the skin that had received 37 exposures, the number of DOPA-positive melanocytes dropped significantly and the reactivity also decreased (Fig. 12). The DOPA reaction was spotty; some cells exhibited a strong reaction, others were faintly reactive, and in some areas there were no DOPA-positive cells. After 52 exposures, no strongly DOPA-positive dendritic cells were found (Fig. 13). The few melanocytes present were associated with the outer root sheaths of the hair follicles. After 79 exposures, the histological appearance of skin reactive with DOPA was similar in most sections. A few weakly DOPA reactive cells seemed to be arranged in clusters. These cells were not always indicative of reactivation but sometimes were left over from the original stimulate pool. In most sections faint shell-like outlines of once active melanocytes ("ghosts") could be found which appeared much smaller than earlier activated dendritic cells. Thus, a few DOPA-positive dendritic cells were almost always present but most were "ghosts" (Fig. 14).

The previously described experiment was repeated, this time with a different light source (Hanovia lamp) which delivered about the same amount of ultraviolet energy (7.0×10^5 $\mu w/cm^2$). To statistically validate the previous observations, an increased

Figure 11 Higher magnification of Figure 10. The melanocytes exhibit definite hypertrophy and extensive arborization of the dendrites. x 665.

Figure 12 Epidermis after 37 exposures. The number of DOPA-positive melanocytes significantly decreases and the DOPA reaction is spotty. x 265.

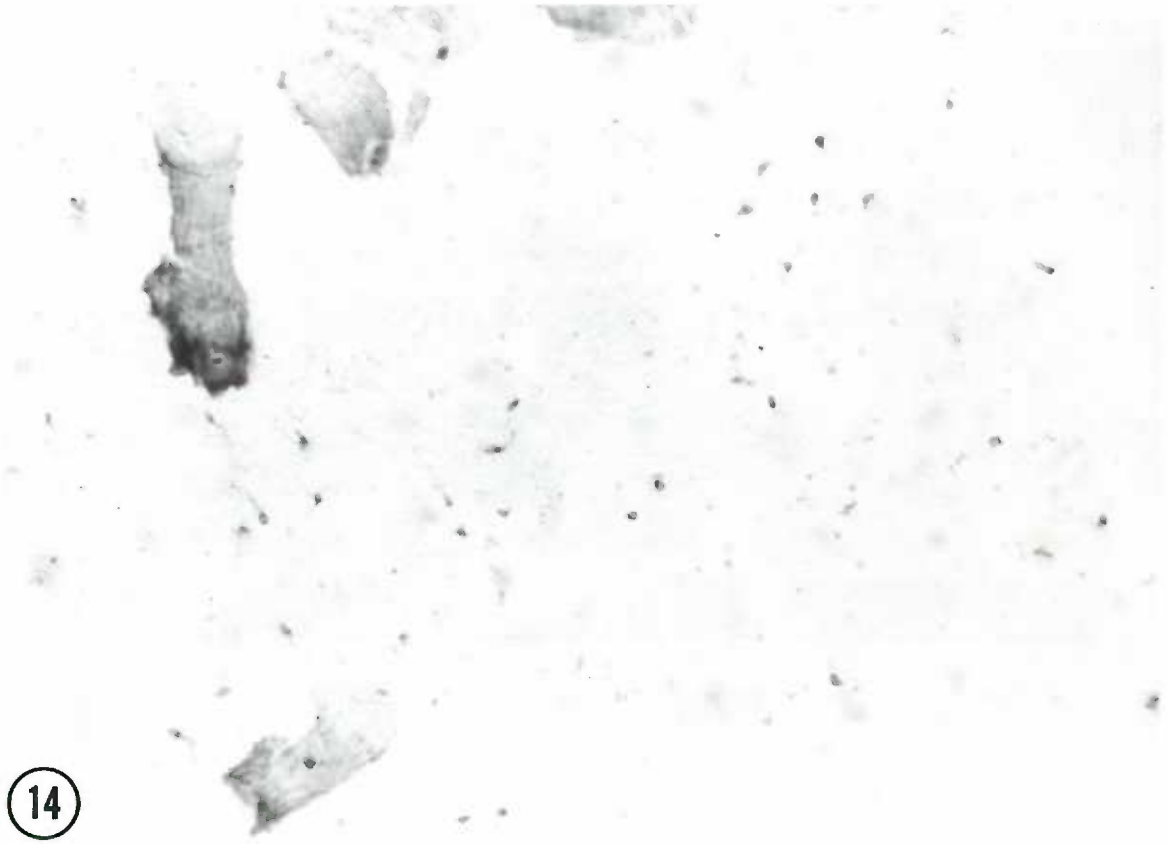
11

12

Figure 13 Epidermis after 52 exposures. No epidermal melanocytes are present except in the root sheath of the hair follicle. x 165.

Figure 14 Epidermis after 79 exposures. A few weakly DOPA-positive cells are in clusters. Most of the detectable cells, however, are "ghosts." x 165.

13



14

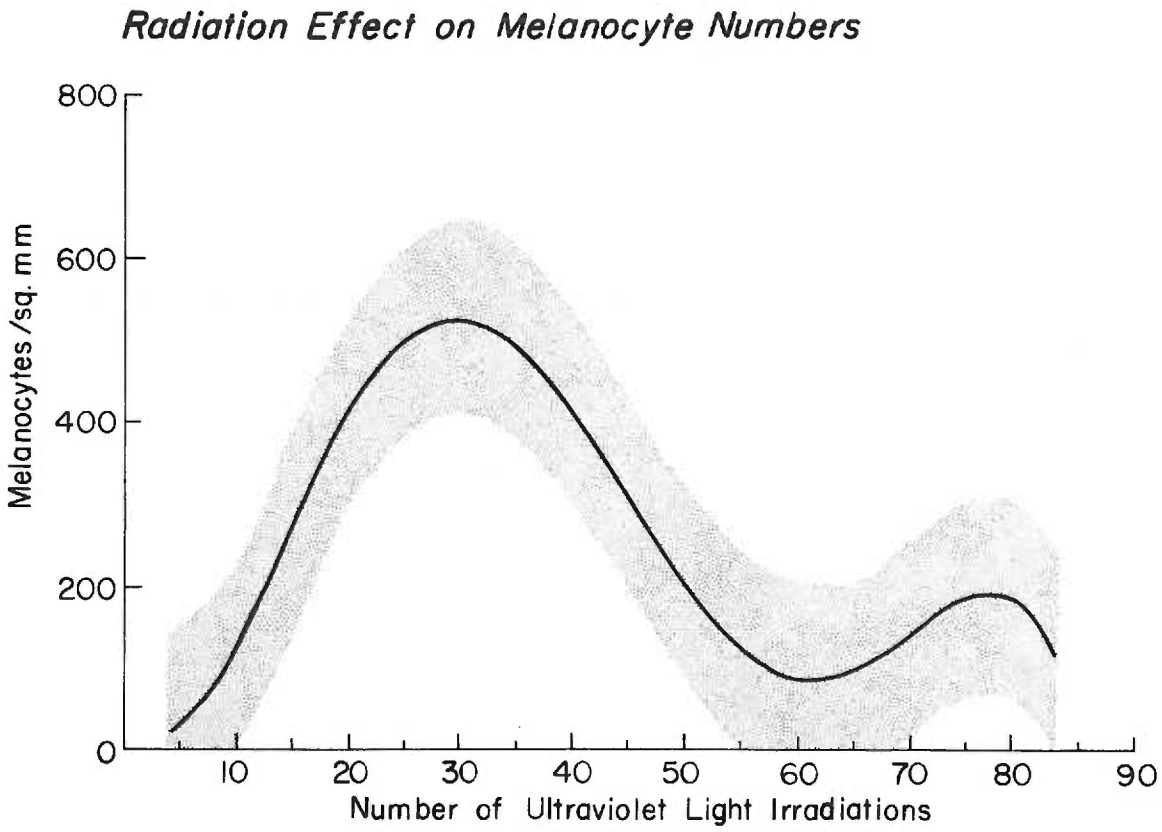
number of biopsies were taken from a multiple number of animals (3 to 6) each time.

Exposed animals developed an erythema 24 hours after irradiation, somewhat earlier than with the Westinghouse lamp. After three days, an intense erythema developed, and the skin started to peel. Within two weeks, the skin stopped peeling, and the irradiated areas became brown.

The number of DOPA-positive melanocytes per mm^2 appearing with sequential irradiation is shown in Figure 15. The curve has fit by polynomial regression analysis. A fifth order polynomial is used to describe the curve because it minimizes the standard error of estimate which is 116. This curve shows the same response as in the second experiment (R-II). Temporally, with sequential irradiation, the number of DOPA-positive melanocytes increased at a rate of 29 per day until a peak was reached after 30 irradiations. With continued irradiation, the number of reactive cells decreased more slowly than they had increased (20 per day) to reach a basal level at about 61 days. In addition, another, much smaller, peak occurred after 78 exposures.

The histological development in this experiment was quite similar to that in the second experiment; only the differences will be explained. The initial appearance of melanocytes after

Figure 15 Melanocyte population in the epidermis after exposure to 7.0×10^5 $\mu w/cm^2$ ultraviolet light. The curve is fit by polynomial regression analysis. Shaded area represents the standard error of estimate.


Radiation Effect on Melanocyte Numbers
800
600
400
200
0
Melanocytes /sq. mm
10
20
30
40
50
60
70
80
90
Number of Ultraviolet Light Irradiations

ultraviolet irradiation occurred much sooner in the third experiment than in the second. The rate of increase and maximum density in R-III was greater. After 25 irradiations, very few of the cells looked as if they were declining in DOPA activity; they still appeared to be stimulated. After 30 irradiations, some samples had very large DOPA-positive cells; dendrites were sometimes twice as long as the cell body and were so plentiful that they completely occluded the areas and made the skin appear dark even though very little pigment was detected in the surrounding keratinocytes. At this stage, there were portions of each biopsy in which only dendrites were present. After 37 irradiations and stages with even more irradiation, the melanocytes appeared very small and washed out. Occasionally, only the dendrites contained a few melanosomes, whereas in earlier stages the entire cell contained melanosomes.

2. Restimulation

To determine whether melanocytes could be restimulated, I modified the second experiment so that part of the area that had been originally irradiated was shaded. During this three-month period, the number of discernible melanocytes decreased from the basal level to zero in less than one month and remained in this state as long as the area received no irradiation.

Table III The DOPA-positive melanocyte population in split skin preparations of shaded epidermis after 95 exposures of ultraviolet light (Westinghouse lamp, 6.4×10^5 $\mu w/cm^2$)

Animal number	Number of days shade	Melanocyte population per mm^2 $S_{\bar{x}}$ *
3	5	0
4	12	44 ± 8.5
5	19	13 ± 5.7
6	26	0
2	33	0
4	40	0
2	47	0
4	68	0
3	90	0

* $S_{\bar{x}}$ = standard error of the mean

Table IV The DOPA-positive melanocyte population in split skin preparations of epidermis after 95 exposures of ultraviolet light and 92 days of shade

Animal number	Number of UV light reirradiations per time period	Melanocyte population per mm^2 right side $S_{\bar{x}}$ *
2	6 (over 11 days)	38 ± 9.5
3		47 ± 5.0
4		0
5		92 ± 10.0
6		18 ± 3.6
7		0
4	13 (over 22 days)	32 ± 6.4
6		32 ± 9.6
5	19 (over 30 days)	153 ± 14.8
7		125 ± 7.0
6	23 (over 36 days)	134 ± 13.5
2		103 ± 9.6
5	28 (over 43 days)	215 ± 17.6
4		116 ± 13.4
7	32 (over 51 days)	47 ± 6.0
3		2 ± 1.0
2	39 (over 60 days)	6 ± 1.5
6		80 ± 24.9
4	46 (over 69 days)	0
3		9 ± 3.0
7	56 (over 83 days)	34 ± 14.8
2		22 ± 7.5

* $S_{\bar{x}}$ = standard error of the mean

Histologically, no DOPA-positive cells or even ghosts could be found after one month of shade. With reirradiation, the number of DOPA-positive cells increased slowly during the first twelve exposures, and the number of reactive cells increased rapidly to a peak (165 per mm^2). With continuing irradiation, the number rapidly decreased to a basal level and remained there for the duration of the restimulation period (Fig. 4). The total number of DOPA-reactive cells was much less after restimulation than after initial stimulation.

Histologically, all signs of previous melanogenic activity had disappeared by 28 days of shading. For the duration of the shading period, no perikaryons, dendrites, or cell "ghosts" were found. With reirradiation, DOPA-positive cells appeared earlier than after the initial stimulation. The few DOPA-reactive cells tended to cluster in groups; most of them exhibited a weak reaction similar to cells starting to synthesize melanin. After 12 exposures, the reaction was distributed throughout the melanocyte; both perikaryon and dendrites contained a similar number of melanin granules. After 19 restimulation exposures, the melanocytes were homogeneously distributed. They appeared morphologically similar to those produced in response to the initial irradiation: large cells with an extended cell body and extensive arborization

of the dendrites. In most areas, some pigment, more than after initial irradiation, was transferred to the surrounding keratinocytes. After 32 exposures and after peak activity, the melanocytes still contained some pigment; most, however, seemed to be transferred. Thus, cells appeared to be stimulated within a definite period of time. Although the DOPA-positive cells appeared more rapidly after ultraviolet stimulation, they also disappeared faster. By 37 exposures, there were very few DOPA-reactive cells, and these seemed to be marginally reactive. With continued reirradiation, the histological appearance continued to be stable with only a very few faintly reactive cells.

B. Biochemical study

To determine whether tyrosinase activity increases after ultraviolet stimulation and either remains at a high level or decreases, I adapted Kitano and Hu's technique (1971). The rate of C^{14}-DOPA or C^{14}-tyrosine plus puromycin uptake by melanosomes indicated the rate of melanin formation. In Figure 16, data reported as counts per minute per milligram of tissue (wet weight) vs. number of ultraviolet irradiations are shown. Data using both labels and inhibitors are included on the graph. The general pattern of activity of both labels is similar: with sequential irradiation, tyrosinase activity increases to a peak; with continued

Figure 16 Uptake of labeled melanin precursors in the epidermis after exposure to 6.4×10^5 $\mu w/cm^2$ ultraviolet light. Brackets indicate standard error of the mean. Ph. = phenlythiourea, Pu. = puromycin.

Melanin Synthesis in Melanocytes of Irradiated Skin

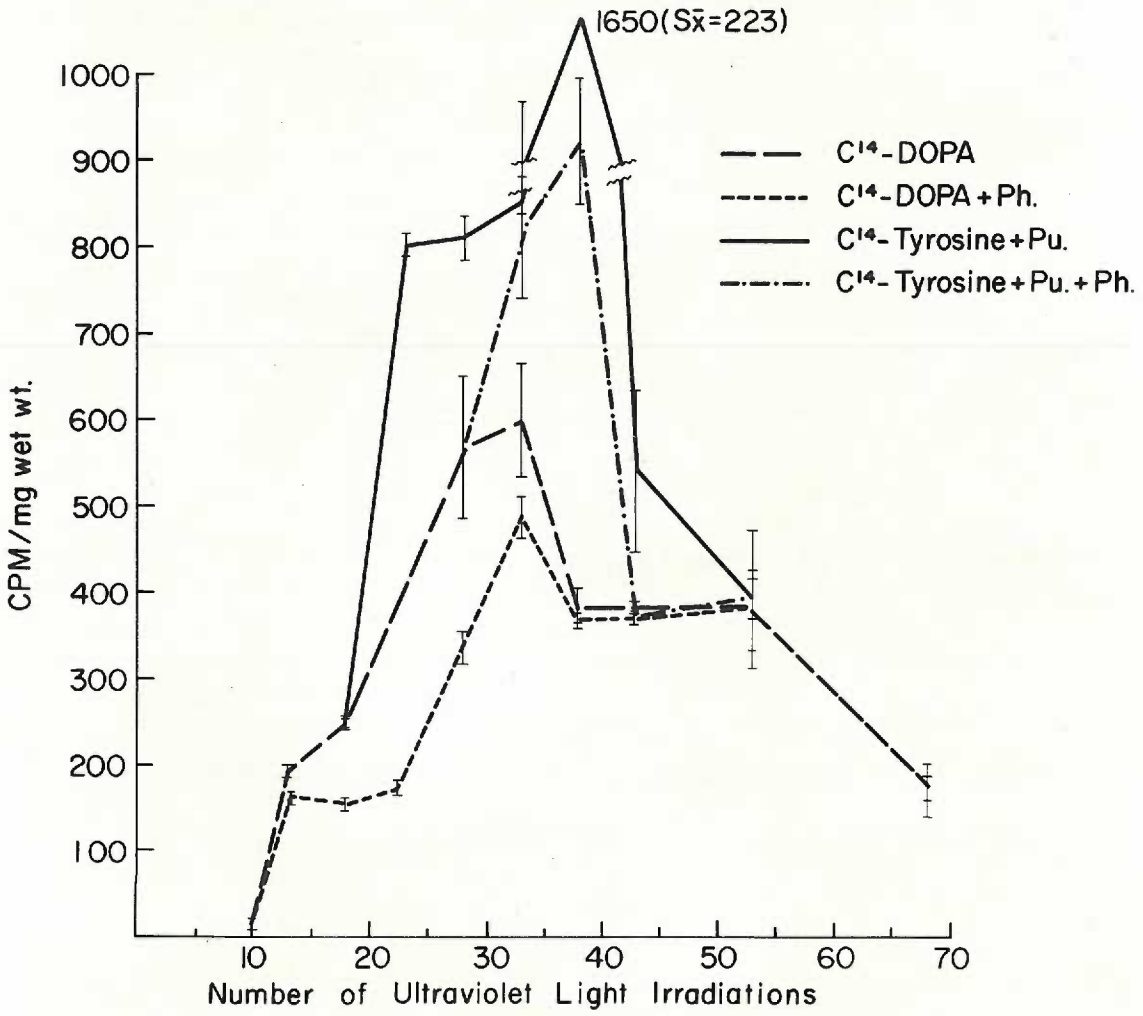
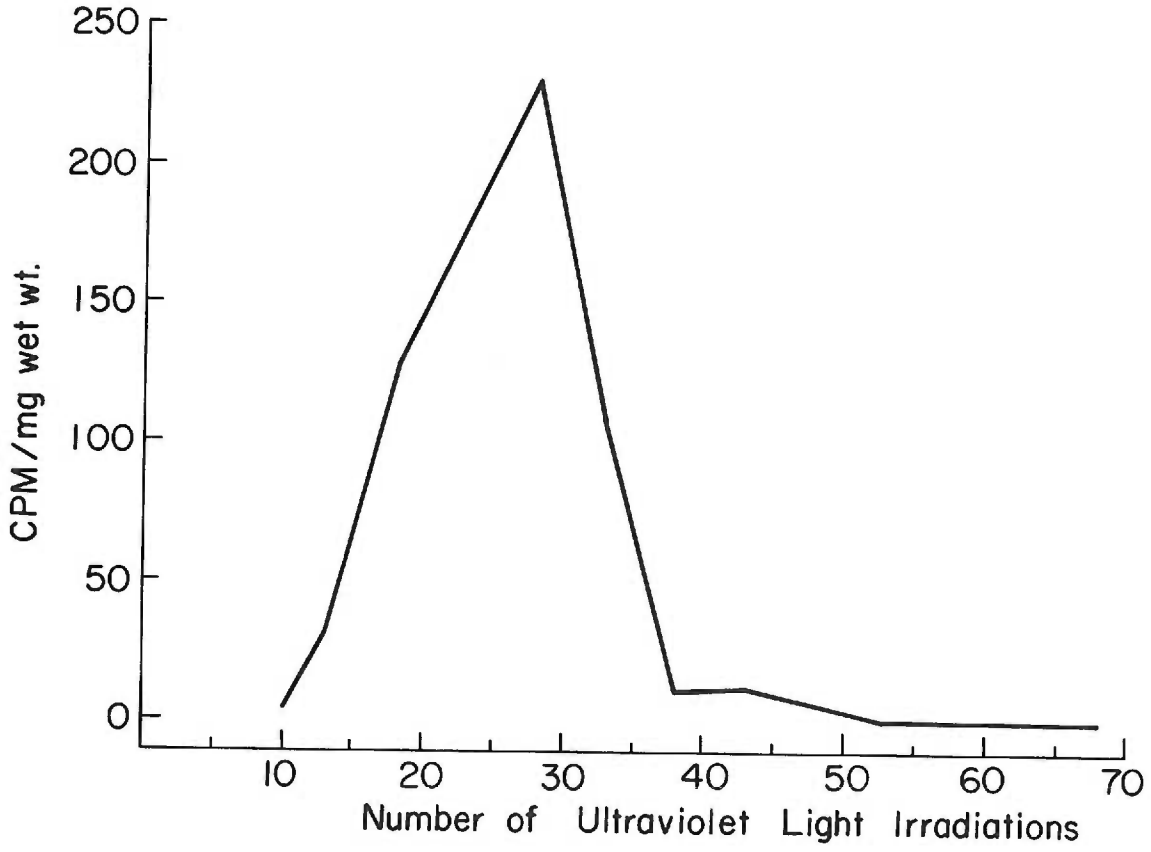


Figure 17 Rate of melanin synthesis after sequential daily irradiation. Data is extrapolated from Figure 16.

Melanin Synthesis in Melanocytes of Irradiated Skin (C^{14}-DOPA)-(C^{14}-DOPA + Ph.)



irradiation, it decreases to a basal level. Although the exact peak of both labels is different, both have a single peak. In addition, phenylthiourea does not completely inhibit melanin synthesis. The best graphic display of the rate of melanin biosynthesis is shown in Figure 17. This indicates the rate of melanin synthesis only, which has been determined by the rate of labeling of C^{14}-DOPA, minus C^{14}-DOPA plus phenylthiourea. This again demonstrates the previous pattern, i.e., an increase to peak activity followed by an equally rapid decrease.

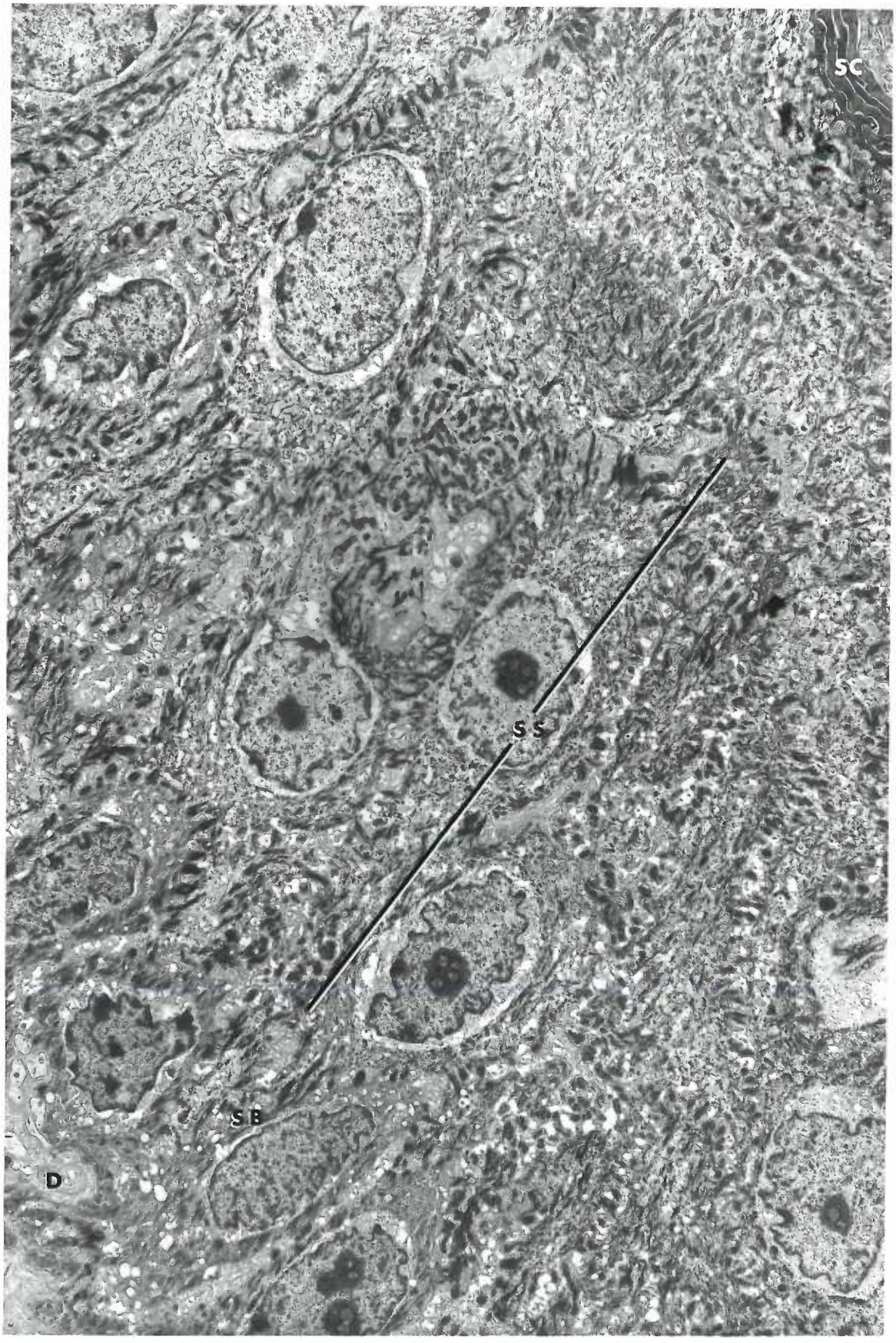
C. Ultrastructural study

How melanocyte ultrastructure related to DOPA positivity was examined.

In the general body epidermis of nonirradiated adult rhesus monkeys, no melanocytes are present. The rhesus does, however, have a moderately thick epidermis with a well-developed and well-defined basal layer, a spinous layer always at least two cells thick, a continous granular layer, and a compact cornified layer (Fig. 18). Although most epidermal cells are keratinocytes, there are two types of dendritic cells present: the Langerhans cell and the indeterminate cell.

Langerhans cells, like all dendritic cells, are distinguished from the surrounding keratinocytes by the lack of tonofilaments or

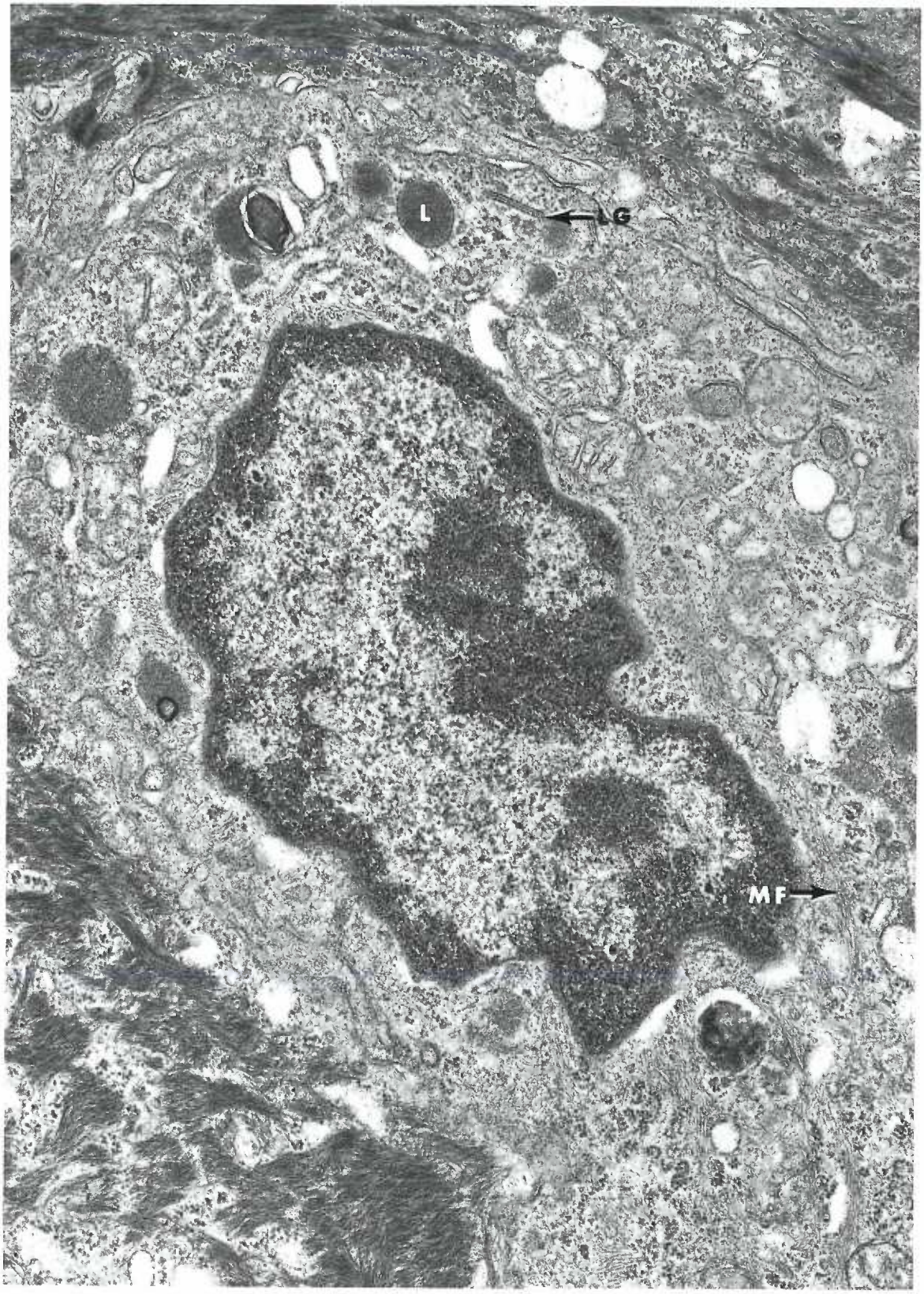
Figure 18 Skin of nonirradiated adult rhesus monkey. The epidermis, bordering the dermis (D), contains a well developed basal layer (SB) and spinous layer (SS) besides a compact stratum corneum (SC). Note the lack of dendritic cells in the basal layer. x 5,200.


5C
S S
S B
D

tonofibrils. Instead they possess microfilaments or cytofilaments. These cells are found in all layers of the Malpighian layer but most frequently in the spinous layer. Most have irregular, indented nuclei and characteristic rod-shaped or racket-shaped organelles, the Langerhans granules. These structures have an outer limiting membrane and a central core that has periodically spaced particles. The racket-shaped structures have a similar morphology except that the limiting membrane is expanded at one end into a vesicle. The Langerhans granules are randomly dispersed (Fig. 19). In addition, these cells have electron-opaque bodies in which some authors have demonstrated (Breathnach, 1971; Rowden, 1967; Wolff, 1967) acid phosphatase activity. Therefore, these structures may be lysosomes; some of them, however, may be myelin bodies. Of all types of dendritic cells in normal epidermis, this type of cell seems to be the most common.

The indeterminate cell, like all dendritic cells, lacks desmosomes or maculae adherentes. They possess all the features of dendritic cells, e.g., microfilaments which do not aggregate into bundles, but lack defining cytoplasmic organelles--Langerhans granules or melanosomes. These cells usually have an indented nucleus but lack the typical electron-opaque bodies that are seen in Langerhans cells. Often they are found in the basal layer but

Figure 19 A Langerhans cell in the epidermis of non-irradiated skin. The surrounding keratinocytes contain tonofibrils whereas the Langerhans cell has distinct microfilaments (MF). Characteristic of this cell is the Langerhans granules (LG). Numerous lysosomes (L) (Rowden, 1967; Sagebiel, 1972; Wolff, 1967) or electron opaque bodies are present. x 39,600.


L
G
MF

sometimes in the spinous layer. Vesicles are occasionally found in the cytoplasm. Though less common than Langerhans cells, these cells nevertheless constitute a sizable component of the cells in the normal rhesus epidermis (Fig. 20).

After four irradiations, the response of the pigment cell ultrastructure varied greatly. Some sites contained very active dendritic melanocytes whereas others contained none. In active melanocytes, all stages of melanosome formation were found (Fig. 21). The early developing melanosomes (stages I and II) were difficult to recognize because most lacked an organized internal filamentous network. Stages I and II appeared to be membrane-limited vesicles with variable amounts of internal opacity. A few melanosomes contained concentric rings (Fig. 22). Melanin, however, was not uniformly deposited on these concentric structures; melanization seemed to occur, rather by the deposition of flocculent material from a central core until the membrane-limited vesicle was completely filled and uniformly electron opaque (Fig. 22). Sometimes, however, melanin deposition stopped before melanization was complete. After four irradiations, very few fully developed melanosomes (stage IV) were found. One feature of the melanosomes which helped to identify the early stages was the fairly consistent size of the vesicles. During this period, very few melanosomes

Figure 20 The indeterminate cell in the epidermis of nonirradiated skin. There are neither Langerhans granules or melanosomes present. The structure shown by the arrow is not a melanosome but an atypical dense body. This cell lacks the typical electron opaque bodies as shown in the previous figure. x 22,300.

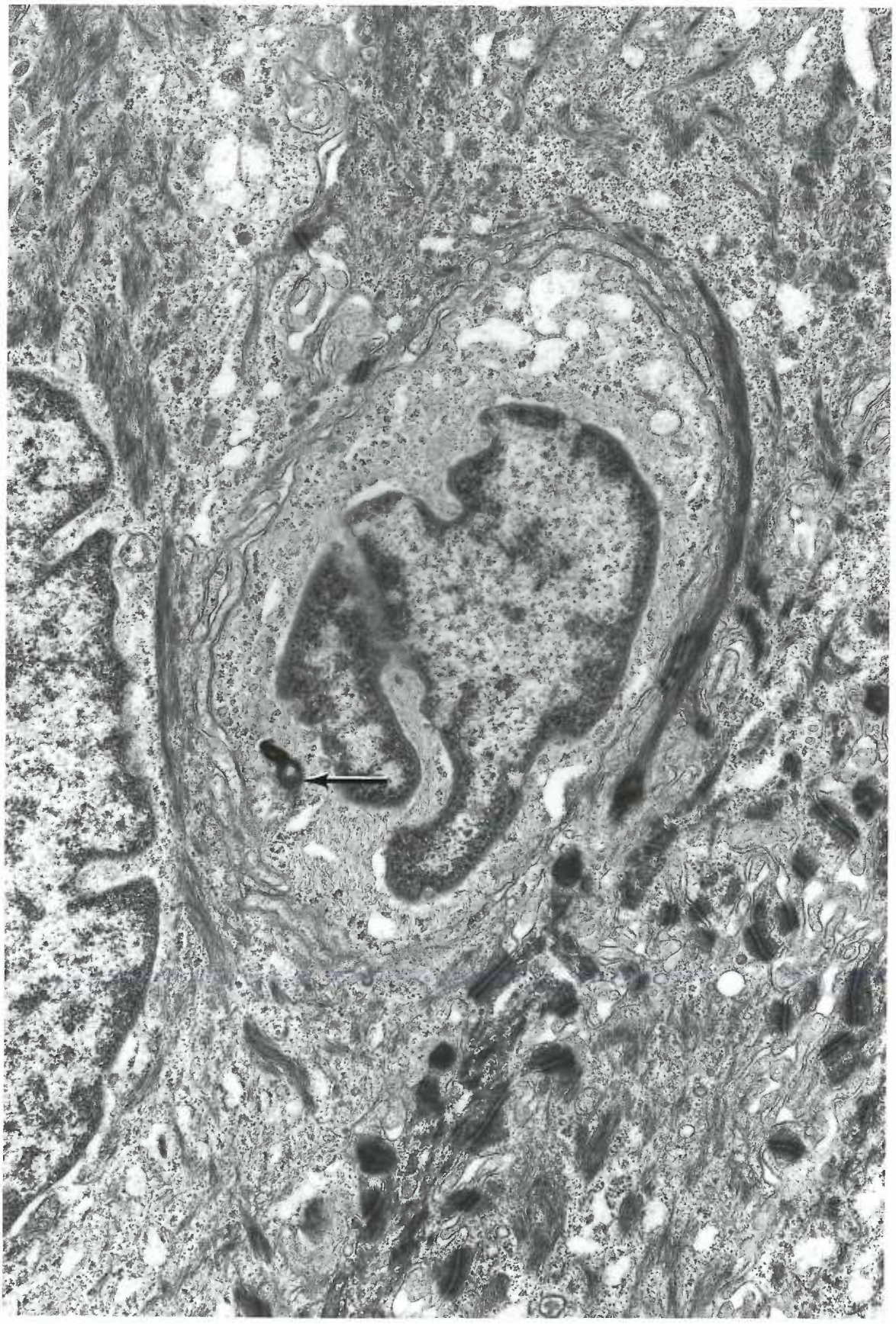


Figure 21 A melanocyte and surrounding keratinocytes after four irradiations. Several stages of melanosomes (II-IV) are present. x 21,100.

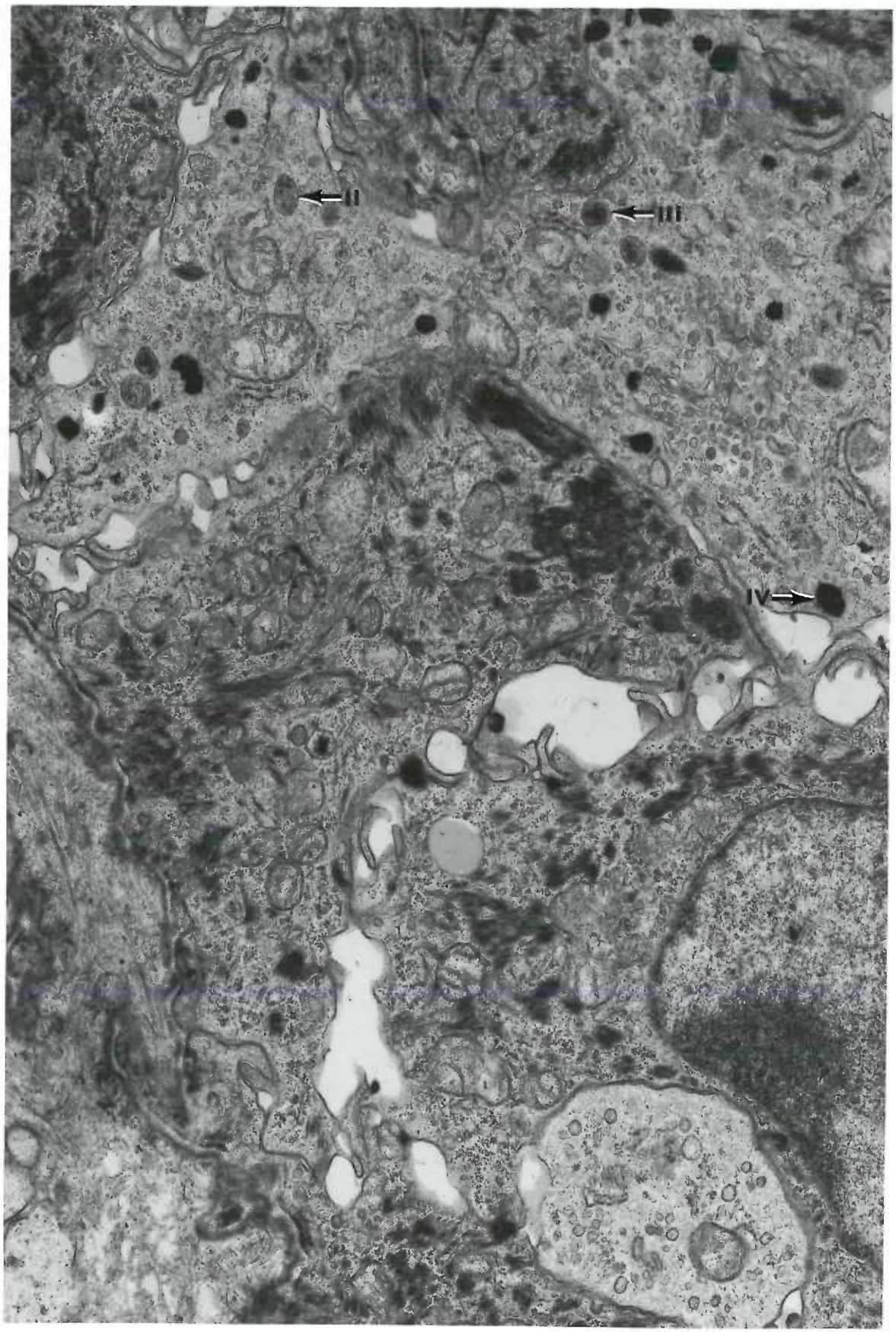
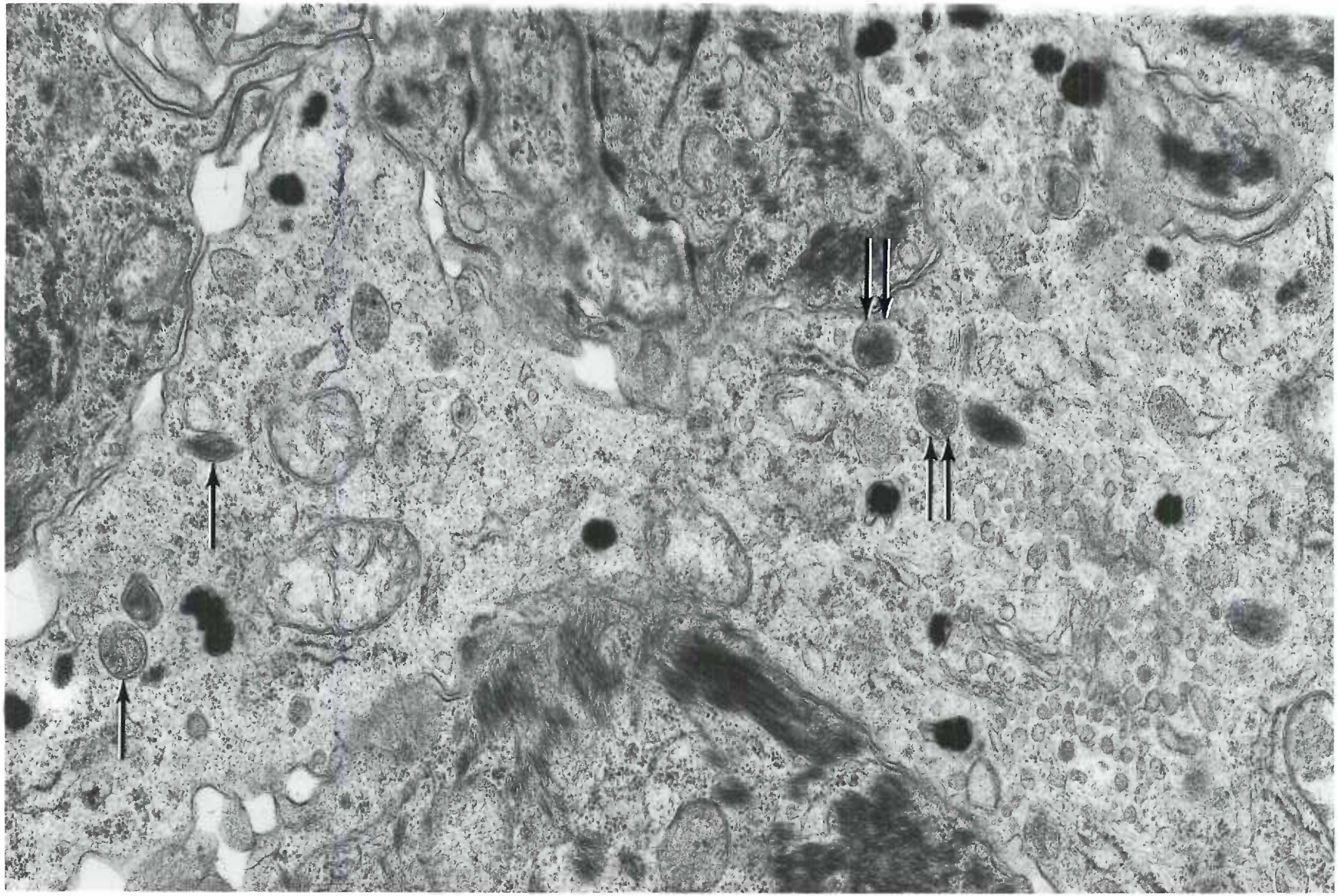

II
III
IV

Figure 22 A higher magnification of Figure 21. The early stage melanosomes generally lack internal filamentous network although occasional concentric structures are seen (single arrow). Melanization appears to be a deposition of a flocculent material (double arrows). A few completely developed melanosomes (IV) are found within the melanocyte. x 33,500.



were transferred to the surrounding keratinocytes. When melanosomes were transferred, they occurred as single units rather than as membrane-bound complexes in the keratinocyte. Stimulated melanocytes had an active Golgi apparatus and extensive rough endoplasmic reticulum. Numerous small vesicles (50-80 mμ) were also dispersed throughout the cytoplasm.

Mitotic figures were found in the keratinocytes of the basal layer but not in developing melanocytes or indeterminate cells.

After 15 exposures, the population of melanocytes increased. These cells contained both early and late stages of melanosome formation; developing melanosomes, however, were less numerous than after four exposures (Fig. 23), and many lacked a completely melanized internal structure; these cells also showed both active Golgi zones and rough endoplasmic reticulum. At this stage, a small group of cells, presumably indeterminate cells, remained hard to characterize; they had active Golgi zones and many small vesicles (40-150 mμ) (Fig. 24).

After 21 irradiations, a still larger population of active melanocytes was found. By this time, most of the melanosomes were completely melanized; very few developing melanosomes were found. Melanosomes, however, often exhibited incomplete melanization with some clumping or aggregating of smaller

Figure 23 A melanocyte and surrounding keratinocytes after fifteen exposures. The number of early stage melanosomes is reduced. The melanocyte contains both active Golgi (G) and rough endoplasmic reticulum (RER). x 19,300.

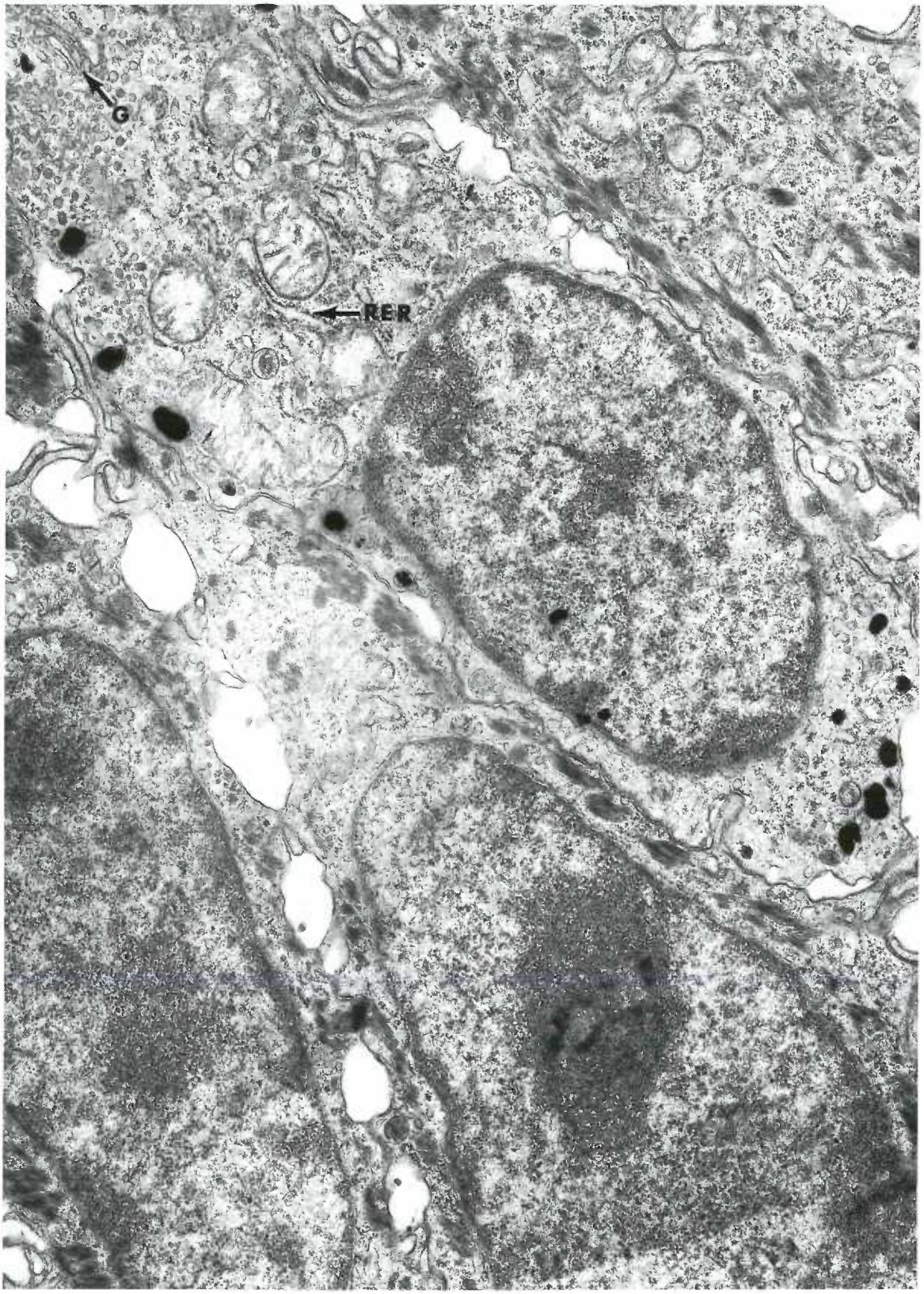
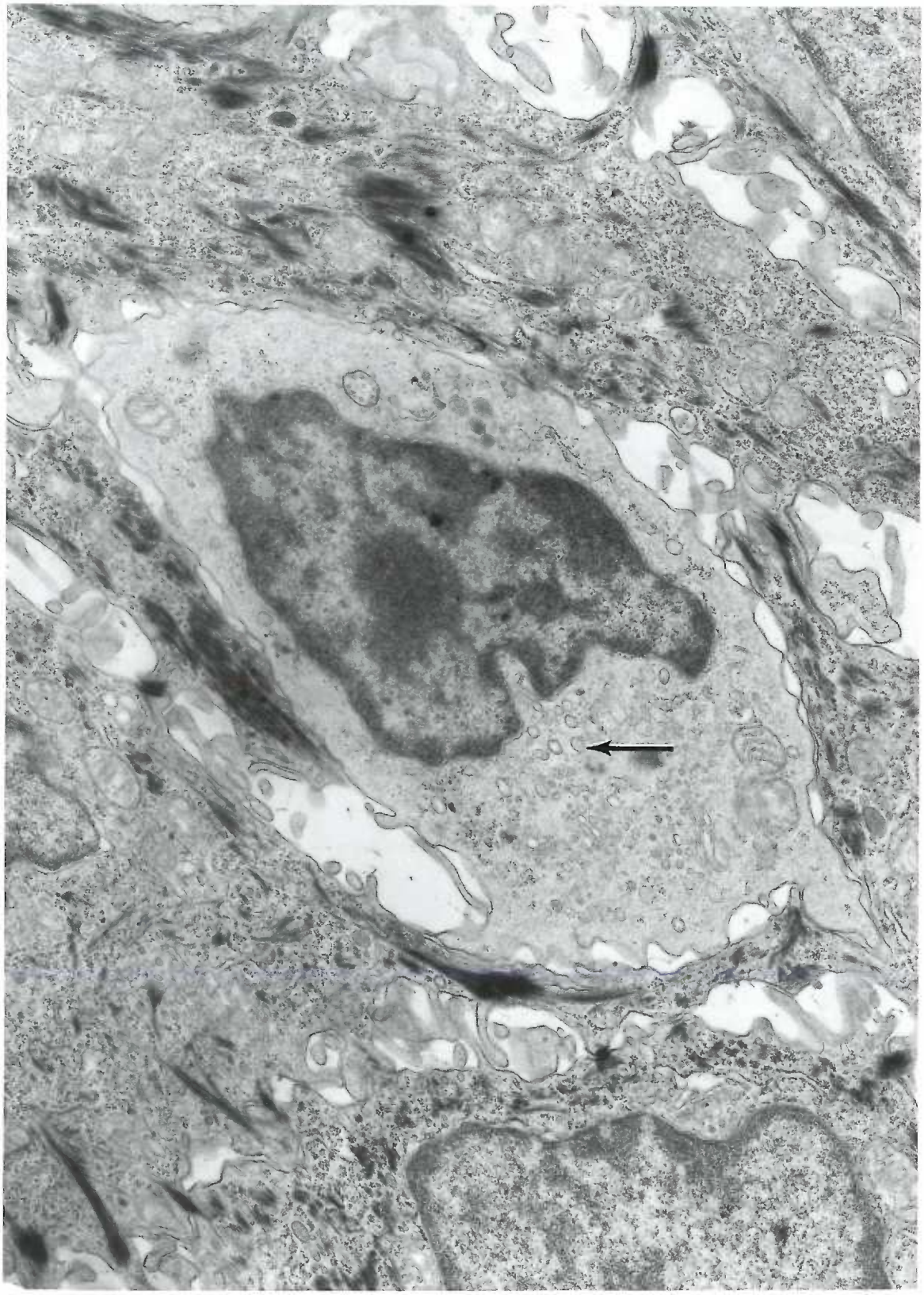

G
RER

Figure 24 Indeterminate cell after fifteen exposures. Note the presence of numerous vesicles (arrow). x 19,700.



melanosome-like particles similar to phaeomelanin (Fig. 25). In addition, very little pigment was transferred, most remaining in the dendrites of the melanocytes (Fig. 25).

After 25 exposures, a few melanosomes were transferred to the surrounding keratinocytes, but most remained at the periphery of the melanocytes (Fig. 26). At peak activity (30 exposures), the melanocytes contained fully developed melanosomes (stage IV) with no distinctive early-stage melanosomes (stages I and II). The rough endoplasmic reticulum did not appear to be as active as at previous intervals and often very few or no Golgi-associated vesicles were found (Fig. 27).

After 37 exposures, the melanocytes contained only a few melanosomes (Fig. 28) which showed no signs of extensive activity but also no signs of damage. At this stage also, the population of indeterminate cells appeared to be increasing in specimens receiving additional irradiation (Fig. 29). These cells were quite similar to those in untreated epidermis, but they appeared to have more cytofilaments (7-8 mμ).

II. Dermal Pigmentary System

The amount of pigmentation in the dermis varied. The pigmented areas, both large and small, appeared to be bluish and to be surrounded by a pink area where melanocytes were not

Figure 25 An epidermal melanocyte after 21 exposures. Some melanosomes (arrows), which appear morphologically similar to phaeomelanin, exhibit incomplete melanization. x 18,600.

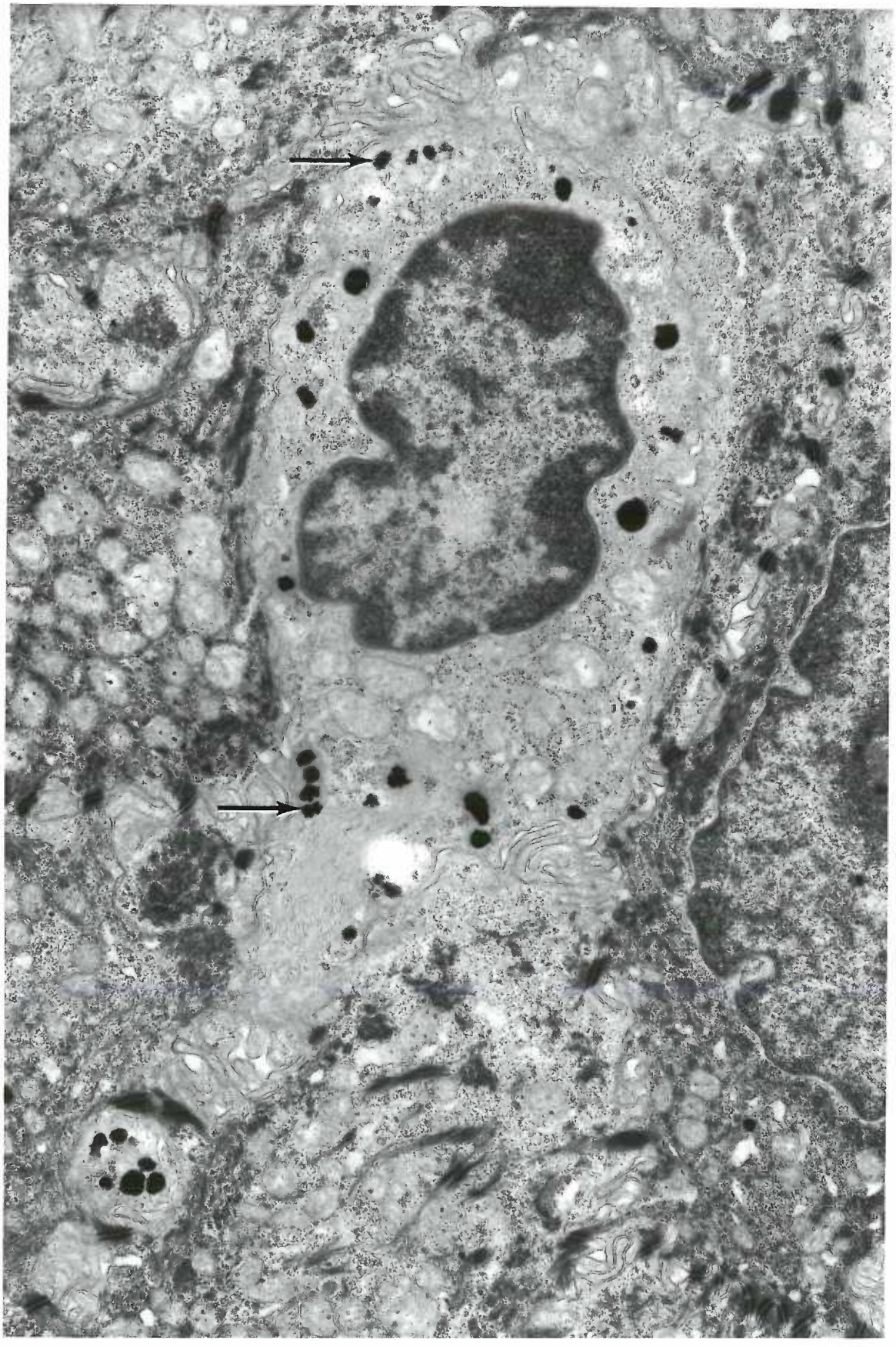


Figure 26 The epidermis after 25 exposures. Although a few melanosomes (arrows) are transferred to the surrounding keratinocytes, none are transferred in the immediate area of the dendrite (DE). x 6,000.

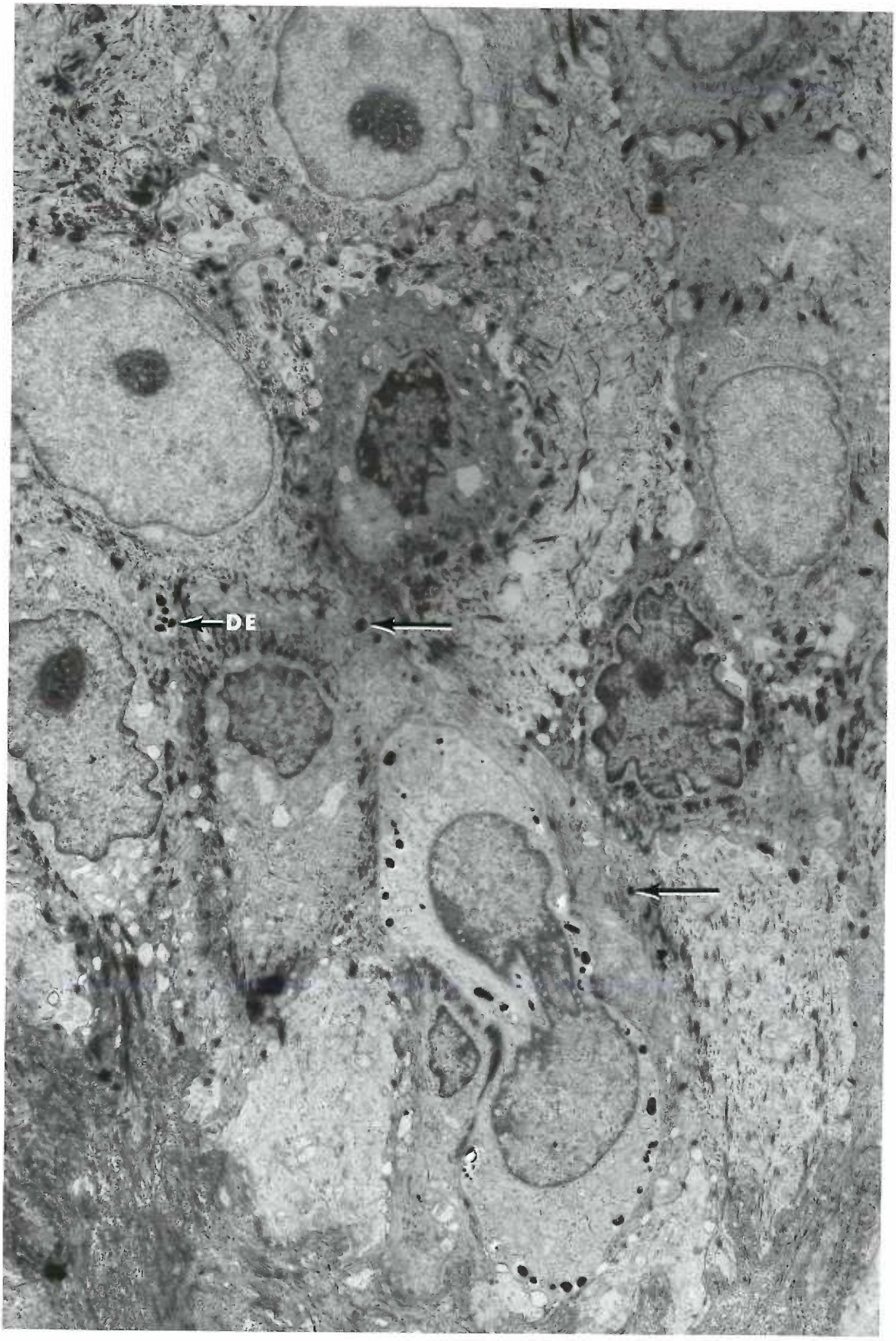

DE

Figure 27 A melanocyte with the surrounding keratinocytes after 30 exposures. The melanosomes are fully developed. Although two stage III melanosomes are present (arrows), no earlier forms are evident. x 18,600.

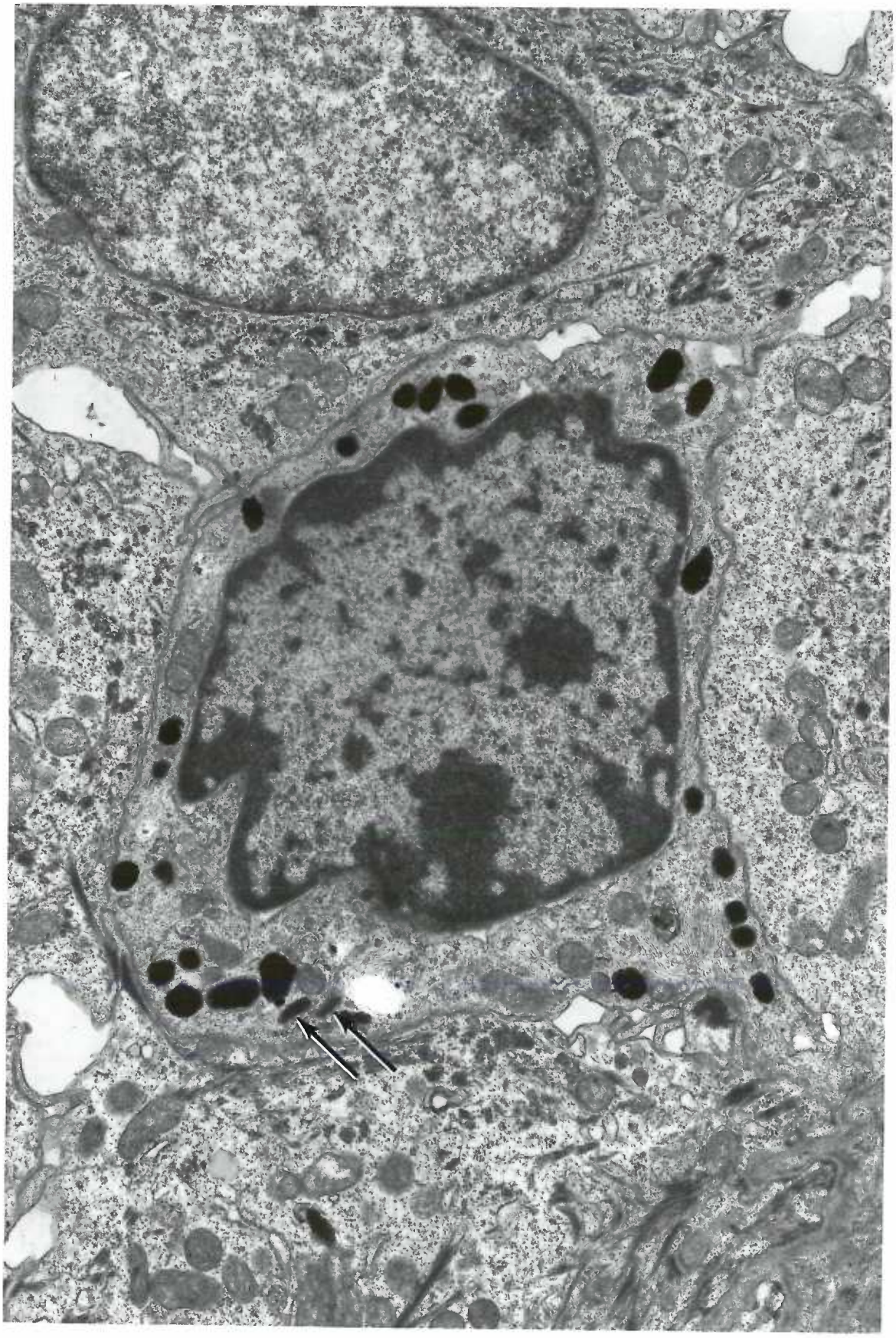


Figure 28 A melanocyte in the basal layer of the epidermis after 37 exposures. Only a few melanosomes are present (arrow). x 25,600.

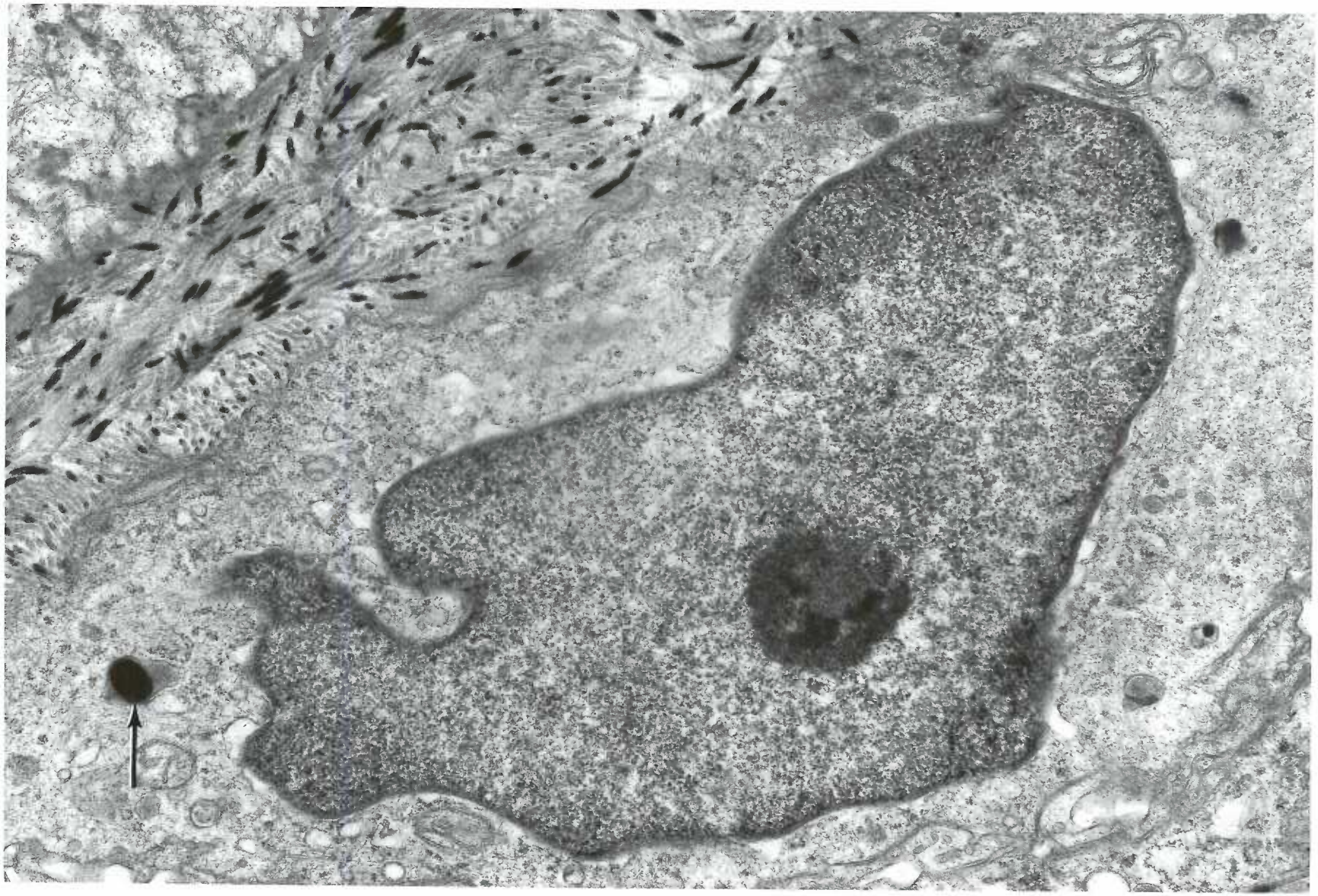
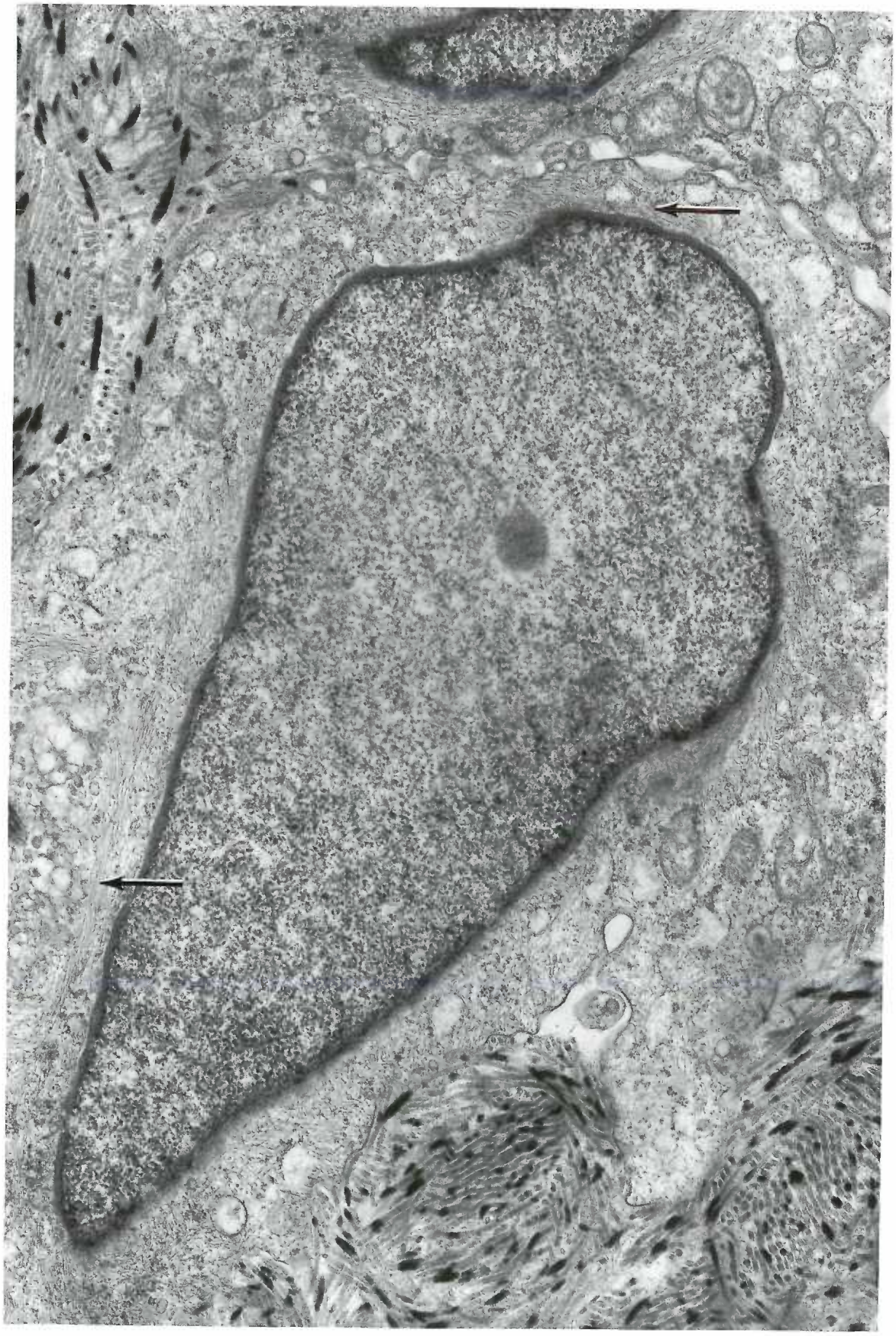


Figure 29 An indeterminate cell in an epidermal ridge after 37 irradiations. Note the well defined microfilaments (arrows). x 25,600.



present. The concentration of pigment in these cells varied. Most of the pigment cells were melanophages, that is, histocytes which had phagocytized melanin. The pigment cells, therefore, are not melanocytes since they do not contain developing melanosomes (stages I to III), but only a few fully melanized structures. DOPA-positivity was difficult to assess in the dermal melanophages because melanization was complete or almost so in the melanosomes. Melanophages found in the reticular part of the dermis appeared long and spindle shaped with usually more than two dendrites; the cell body looked similar in size to the dendrites (Fig. 30).

Sequential ultraviolet light irradiation produced no changes in size or shape in pigment-containing cells of the dermis. In addition, the uptake of C^{14}-DOPA was not significantly different from that of C^{14}-DOPA plus phenylthiourea when the dermis was incubated in either solution.

III. Changes in Skin (Other than Pigmentary) with Ultraviolet Light

Many studies have reported the early changes in the skin after exposure to ultraviolet light. Hence, only the changes effected by multiple long-term irradiation will be described.

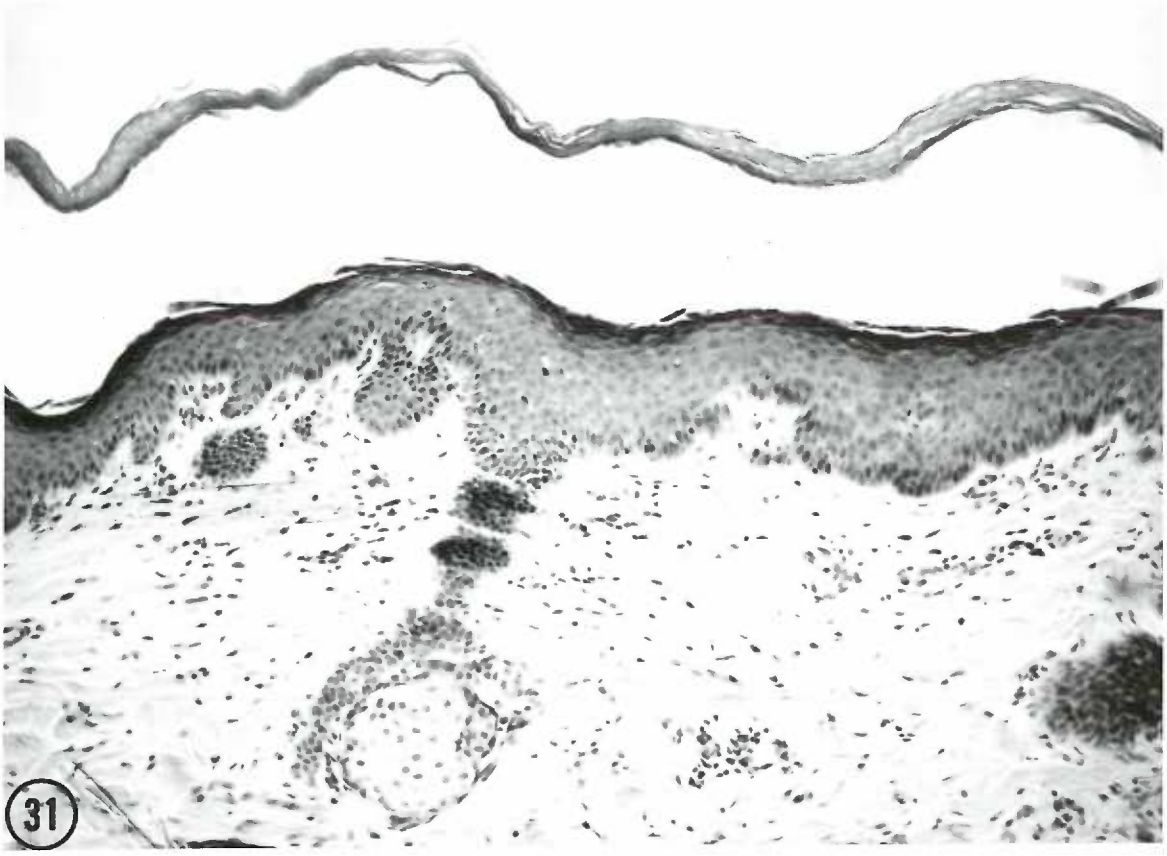
After four exposures, the only change was a small amount of hyperkeratosis with the thickness of the stratum granulosum and Malpighii remaining about the same as in nonirradiated skin. Cells

Figure 30 Melanophages of the dermis. Split skin technique. x 165.

of the basal layer contained an increased number of tonofibrils which, like the nonirradiated controls, were oriented mainly perpendicular to the dermal-epidermal junction. There were, however, no dyskeratotic cells in the lower epidermis of most irradiated animals. The epidermis of only one animal (in a group of six) biopsied after six irradiations contained dyskeratotic cells. After nine exposures, the stratum corneum was slightly thicker than after four exposures but did not thicken appreciably with additional irradiation. The stratum granulosum was well defined and several layers thick. In the Malpighian layer, hydropic swelling of the nucleus with displacement of chromatin to one side of the nuclear membrane indicated areas of focal degeneration. In the basal layer, the keratinocytes were pycnotic and the cytoplasm was vacuolated. The vessels of the dermis, mainly the superficial capillaries, exhibited vasodilatation with hypermia and cellular exudation. The prevailing leukocytosis was closely confined to the areas surrounding the superficial capillaries; but with continued irradiation, this vascular injury continued so that the dermis displayed a chronic inflammatory infiltrate (Fig. 31). After 15 or 16 exposures, the stratum spinosum had increased to four or five layers of often rounded cells, with their somewhat eosinophilic cytoplasm. The normal cytoplasm is basophilic. There was

Figure 31 Skin after nine exposures. The superficial capillaries exhibit vasodilatation and cellular exudation. All layers of the epidermis show varying degrees of thickening. H & E. x 165.

Figure 32 Skin after 15 exposures. The stratum Malpighii contains clusters of dyskeratotic cells. The cytoplasm of some of the keratinocytes appears eosinophilic. H & E. x 370.



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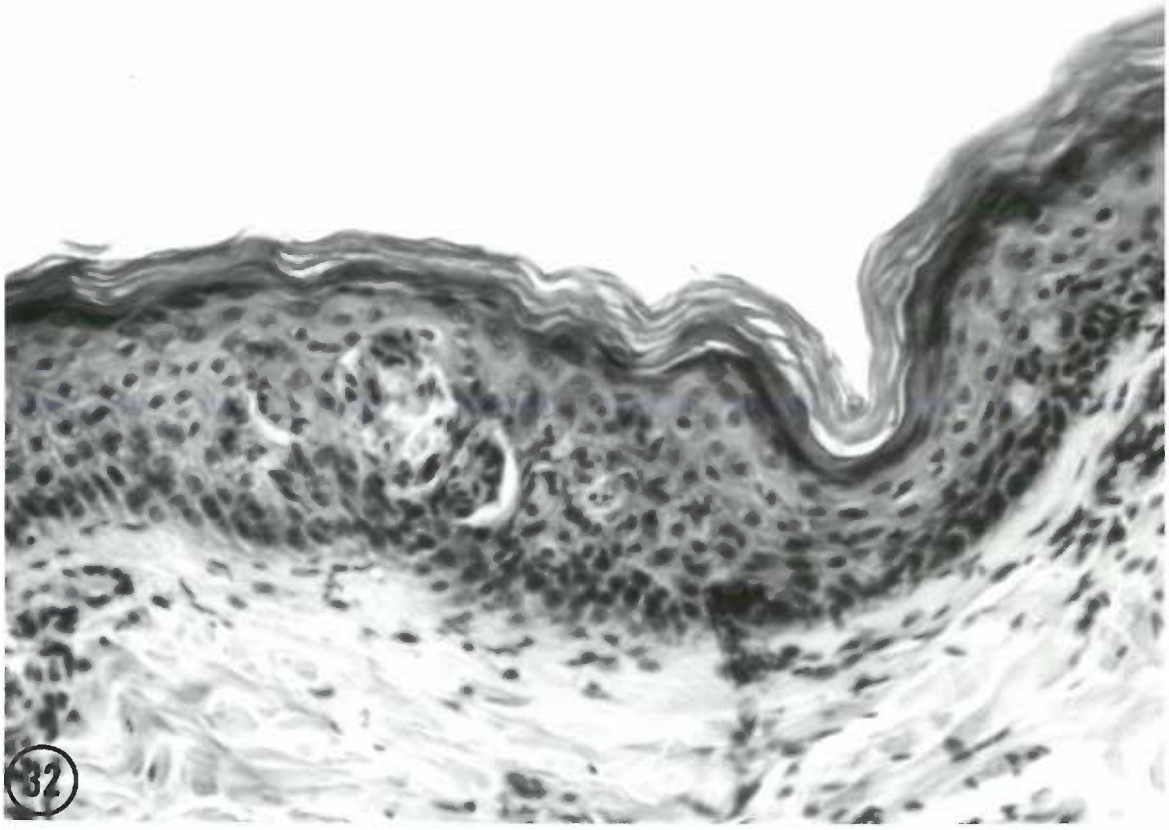


32

epidermal cell disorganization, with single or groups of dyskeratotic cells found in all parts of the stratum Malpighii (Fig. 32). In many cells, the tonofibrils were so tightly aggregated that it was impossible to distinguish the individual components. These cells also contained increased amounts of glycogen either in single particles or in rosettes. One animal showed leukocytic infiltration of the epidermis (Fig. 33). After 21 exposures, an additional change was a general epidermal disruption with some eosinophilic accumulation in the upper Malpighian layer. After 25 or 26 exposures, an increased thickness was quite variable from one area to another. The formation of a subepidermal bulla was found only at this stage. Processes from the degenerating cytoplasm of keratinocytes extended from the lower ends of detached basal cells into the cavity of the bulla. These processes appeared to have separated from their interdigitations with the dermis (Fig. 34). Thus, the bulla seemed to form as a result of damage to both the basal cell and the surrounding components of the upper dermis. In addition, there was evidence of cellular exudation from injured capillaries in the upper dermis. After 34 exposures there was extensive hyperplasia of all layers of the epidermis. The Malpighian layer was seven to nine layers thick, and the granular layer three to five layers thick (Fig. 35). With continued irradiation (up to

Figure 33 Leukocytic infiltration of the epidermis after 15 exposures. x 30,700.

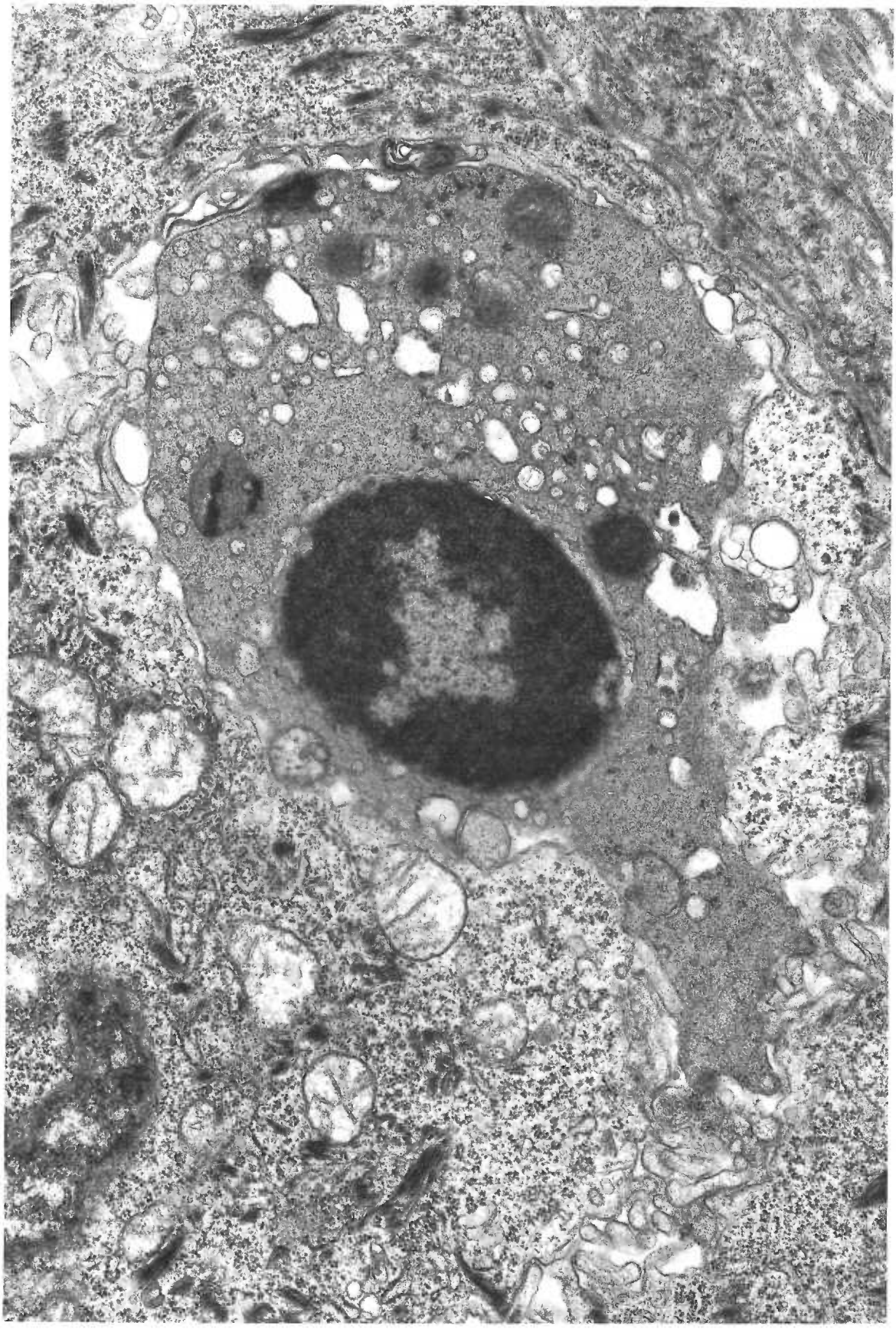
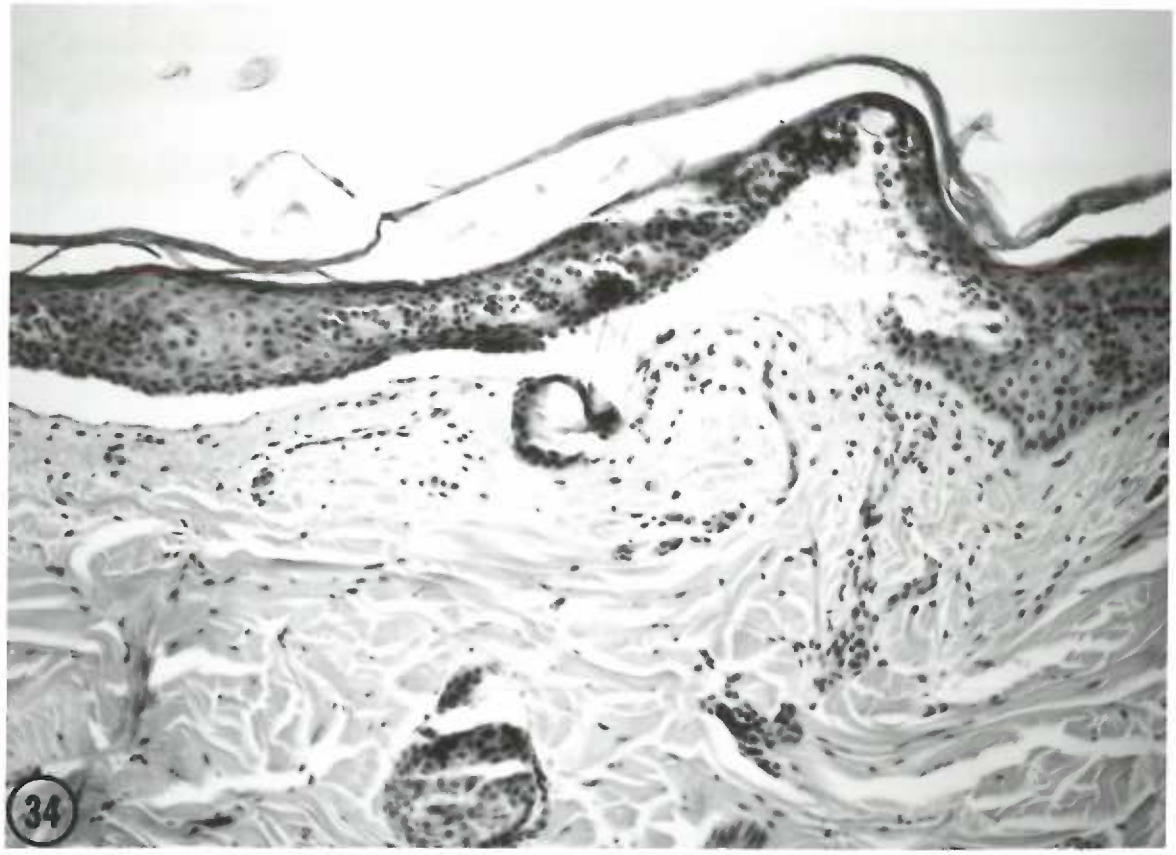
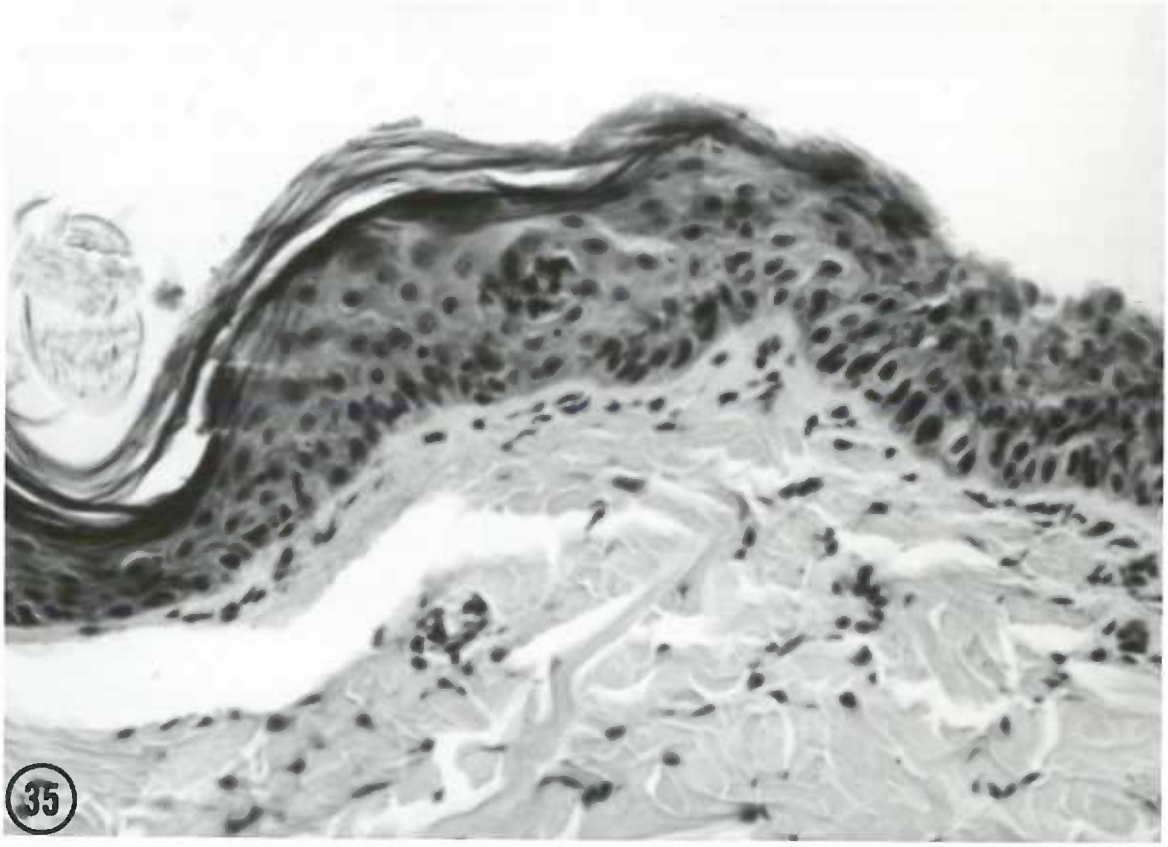


Figure 34 Skin after 25 irradiations. A subepidermal bulla is present which seems to have formed as a result of damage to the basal cells and the surrounding dermal components. H & E. X 165.

Figure 35 Skin after 34 exposures. The epidermis is quite thickened and occasional dyskeratotic cells are present. x 370.

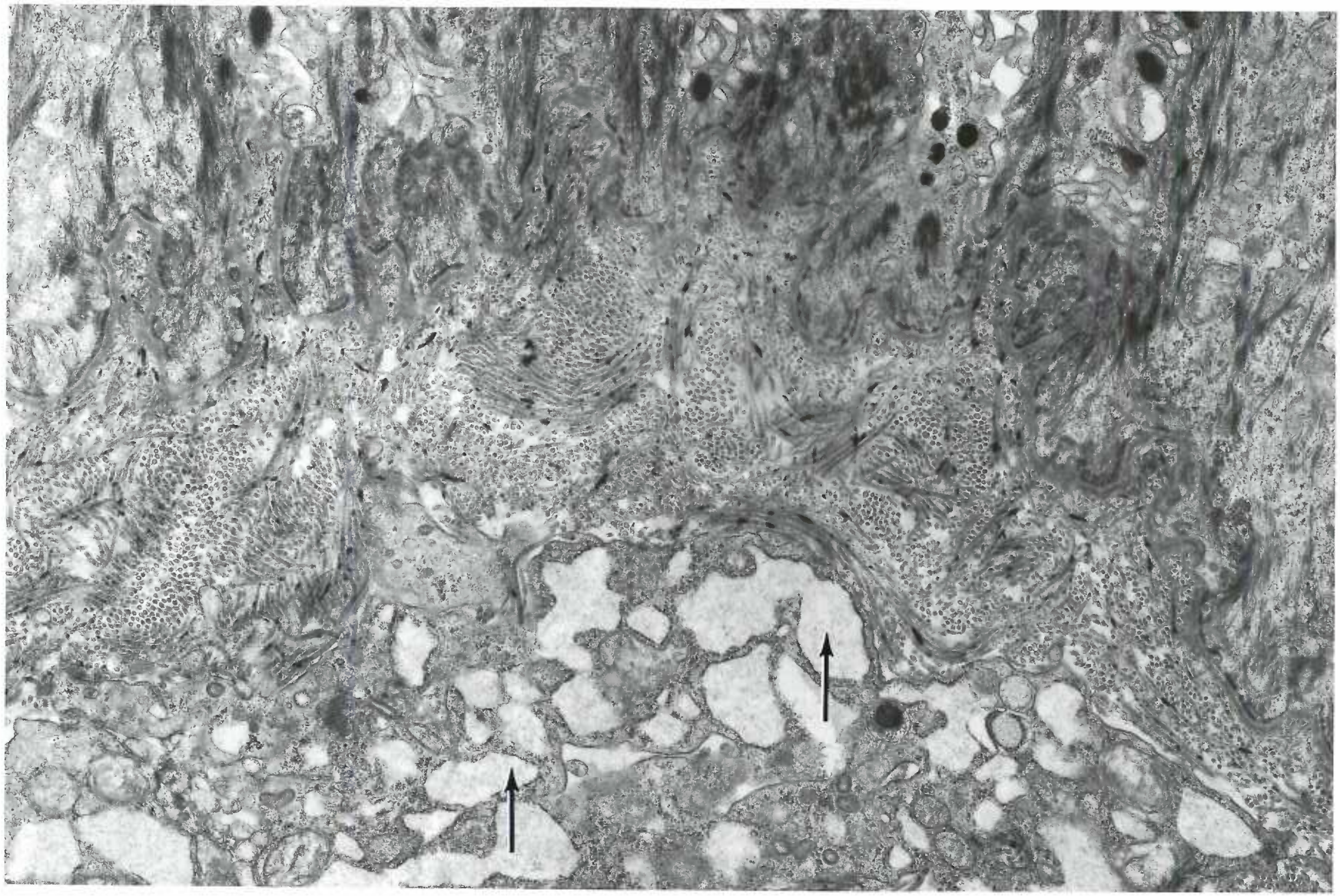

34


35

216 exposures), the morphological features of the skin change very little. A slightly thickened stratum corneum, a continous but small granular layer, a slightly thickened (one to two layers) Malpighian layer, and occasional dyskeratotic cells were typical characteristics. The dermis showed a mild degree of leukocytic infiltration around chronically inflamed superficial capillaries. There was no conspicuous change in the connective tissue, except for an occasional activated fibroblast (Fig. 36). The extensive endoplasmic reticulum characteristic of an active fibroblast contained a slightly electron-opaque, flocculent material. The distended cisternal membranes had ribosomes attached to their cytoplasmic surfaces.

During the entire period of ultraviolet irradiation, the Langerhans cells showed no structural or enzymatic changes. Morphologically, neither the pattern of distribution of these cells nor the intensity of intracellular localization of ATPase activity changed.

Figure 36 Skin of the rhesus after 25 exposures. The active fibroblast in the dermis contains distended endoplasmic reticulum (arrows) with flocculent material inside. x 18,600.



DISCUSSION

The results of these experiments support the original findings of Yun and Montagna (1966) that activation and inactivation of DOPA-positive melanocytes occurs in the rhesus epidermis, and that in the pattern of melanogenic activity, an initial slow increase is followed by a fast increase. In addition, there are some important differences. One is the amount of ultraviolet energy necessary to activate the melanocytes. With the dosage used by these investigators [6.2 x 10^4 $\mu w/cm^2$ (less than one minimum erythema dose)] very few cells are stimulated. A 10-fold increase in ultraviolet light (6.4 x 10^5 $\mu w/cm^2$ in R-II and 7.0 x 10^5 $\mu w/cm^2$ in R-III), however, produces similar results, e.g., erythema, a thickened stratum corneum, and morphological appearance of melanocytes.

The best representation of the true melanogenic activity in the rhesus population is reflected by the polynomial regression curve. When all of the experimental animals were examined simultaneously, they showed variability. Thus all animals receiving the same dose for the same length of time displayed different responses such as the rate of increase of melanogenesis, rate of decrease, and point of peak activity as well as total number of melanocytes. This variability is within the standard error of the estimate for the polynomial regression model. Thus, the small deviations shown in Figure 4 after 52, 79, 103,

and 192 exposures do not indicate reactivation but rather the variations between one animal and another and between the specimens removed from adjacent areas of the same animal. When all animals of the experimental group are compared, these variations in the fluctuation of the basal level are insignificant. The standard error of the mean (shown by the brackets in Figure 4) also substantiates the conclusion that the number of DOPA-positive melanocytes does not vary significantly. For similar reasons, a peak number of DOPA-positive cells occur in the shaded area of Figure 4. This peak does not represent reactivation or continued proliferation of DOPA-positive melanocytes during the shading period, but rather one of six animals in which DOPA-positive melanocytes were observed at the start of the shading period. More important, however, with no irradiation all melanogenic activity disappears.

We do not know why the proliferating melanocytes of irradiated rhesus epidermis are larger than those found normally in other primates and why they tend at first to be localized around the parafollicular areas. A similar situation of hyperplasia and hypertrophy of pigment cells is often seen after inflammation (Papa and Kligman, 1965). Therefore, rhesus melanocytes probably undergo a hypertropic reaction similar to other pathologically damaged tissue, because at first these cells contain no pigment to protect them from cellular

damage. Nevertheless, the developing melanocytes do not appear irreparably damaged. Until peak numbers are reached, extensive branching and arborization indicate pronounced activity. This type of reaction, however, does not occur in all animals. Quevedo and Smith (1963) found that after ultraviolet irradiation the melanocytes in the plantar skin of "dd" genotype mice produced only a few fine dendritic processes. They hypothesized that the areas did not become hyperpigmented because of the poor development of dendrites and the failure to transfer melanosomes to epidermal cells.

The presence of very few DOPA-positive melanocytes and some faintly reactive ones after peak stimulation can be explained in one of two ways. First, with continuing irradiation, most cells are inactivated and only a small population remains; thus, their division results in very few DOPA-positive active melanocytes. Second, not all cells are stimulated simultaneously; therefore, those that are initially activated later are inactivated later. Nevertheless, after peak activity, fairly similar numbers of "ghosts" or shells are found in most sections.

The intensive erythema that developed after exposure to the Hanovia lamp is probably a result of the spectral output (185-313 mμ); it incorporates both a primary erythema peak (297-308 mμ) and a secondary erythema peak (257-260 mμ). The Westinghouse lamp,

however, has wavelengths in the primary erythema range as well as a long wave range. Although long wavelength ultraviolet has a augmentative effect on sunburn damage (Willis, Kligman, and Epstein, 1972), the amount contributing to sunburn is insignificant. Over 100 times more energy is needed with 320-420 mμ light than with the 290-320 mμ range. The levels of erythema are also different. Since the time course of its production varies with the wavelength, that induced by short wavelength (250-260 mμ) appears and fades more quickly than that produced by a sunburn spectrum (290-320 mμ) (Breit and Kligman, 1969). Why the number of cells exposed to two periods of ultraviolet stimulation is always less than the number of cells stimulated during the initial period, and whether the same cells are activated both times are not known. It appears that functioning melanocytes have a definite period during which they are active. Probably the original population of melanocytes is not reactivated but instead a new population arises with reirradiation. Current techniques, however, do not allow this problem to be resolved.

One of the main criteria for developing a technique accurate enough to measure changing tyrosinase levels is that it be sensitive enough to measure such small amounts of enzyme activity as when only 12 melanocytes per mm^2 are present. The established techniques use either mushrooms (Bouchilloux, McMahill, and Mason, 1963;

Kertesz and Zito, 1965), populations predominantly of pigment cells such as melanoma (Adachi and Halprin, 1967; Comstock and Wynne, 1957; Kitano and Hu, 1969, 1970, 1971; Pomerantz, 1964, 1966), or goldfish skin (Chen and Chavin, 1965). Another criterion is that full-thickness skin biopsies be used.

Since tyrosinase is the only known enzyme in the conversion of tyrosine to melanin, the use of radiolabeled DOPA or tyrosine should make it possible to determine tyrosinase activity. Experimental samples, however, must be postincubated with a nonlabeled solution of DOPA or tyrosine to ensure the complete incorporation of DOPA or tyrosine into melanin. Hence the technique actually determines the rate of melanin synthesis. It is nevertheless, sensitive enough for the determination of very small amounts of enzyme. One problem inherent in using this technique, however, is that phenylthiourea does not completely inhibit tyrosinase activity even though Lerner and his co-workers (1950) have found that it thus far is the most effective inhibitor available. In their experimental system, they reported that 10^{-4}M inhibited 90% of the activity. They suggested that the mechanism of inhibition cannot be represented by any simple enzyme-inhibitor reaction, and that phenylthiourea may inhibit tyrosinase other than by binding to copper. Kitano and Hu (1971) also reported that it is virtually impossible to completely abolish tyrosinase

activity by inhibitors without simultaneously impairing protein synthesis. With 10^{-3}M phenylthiourea, they inhibited 51.2% of the C^{14}-DOPA activity, and with puromycin plus 10^{-4}M phenylthiourea 56.9% of the C^{14}-tyrosinase activity. Puromycin, an inhibitor of protein synthesis, is believed not to inhibit melanoprotein synthesis. Also, the cessation of protein synthesis does not affect tyrosinase activity (Kitano and Hu, 1971). In my experiments, the level of inhibition by phenylthiourea after a different number of ultraviolet exposures varied, but the activity was never completely inhibited. This level of inhibition was of the same magnitude as that reported by Kitano and Hu.

The pattern of biochemical activity parallels that of histochemical data; tyrosinase activity increases with sequential irradiation to a peak and then decreases. Why the peak incorporation of C^{14}-DOPA or C^{14}-DOPA plus inhibitors occurs at a different time than the difference in the incorporation of the two is probably due to a variation in technique. This part of the studies demonstrates that there is no compensation on the part of enzyme activity; when the number of morphological units decreases, so does the activity.

Several hypotheses have been advanced concerning the inhibition of tyrosinase and how it is affected by ultraviolet light. Chain and Wilgram (1967) demonstrated the existence of a tyrosinase

inhibitor which was inactivated by ultraviolet light. They, therefore, speculated that tanning after exposure to ultraviolet light occured because of inactivation of the inhibitor, thus synthesis of tyrosinase and the number of melanosomes increased. Pathak (1971) suggested that the levels of sulfhydryl-containing compounds, such as glutathione and cysteine, may not only regulate the functional state of tyrosine but also repress the genes that regulate the synthesis of tyrosinase. If the repressor were destroyed or modified through a process such as oxidation, the number of melanocytes engaged in synthesis of tyrosinase could be increased.

In experiments like those described here, the origin of the DOPA-positive cells is often debated. Does the number of DOPA-positive melanocytes increase by activation of amelanogenic melanocytes, does mitosis increase, or do the cells migrate from the dermis? After irradiating several species of vertebrates, some investigators (Sato and Kawanda, 1972a, 1972b; Snell, 1963; Wolff and Winkelmann, 1967b) concluded that the DOPA-negative dendritic cells situated at the dermoepidermal junction are amelanotic melanocytes and that the increase in DOPA-positivity is due in part to the activation of these normally inactive cells. Sato and Kawanda (1972b) based their conclusion on an experiment in which 1.1% of the DOPA-

positive cells were labeled with H^3-thymidine during ultraviolet irradiation. On the basis of division figures and incorporation of H^3-thymidine within the melanocytes, Quevedo, Brenner, and Kechijian (1963), however, believed that division accounts for at least part of the population increase in the melanocytes of irradiated pedal skin. Mishima and Widlan (1967) substantiate the latter hypothesis.

In the present experiments, the only reasonable conclusion is that a pool of indeterminate cells in the epidermis of the hairy skin of the rhesus monkey is probably stimulated to form melanocytes after exposure to ultraviolet light. This conclusion is based on the observation that significant numbers of indeterminate cells are found in nonirradiated skin and skin after peak melanogenic activity, but virtually none at the peak of melanogenic activity. Unfortunately, no observations were made on serial sections of nonirradiated skin and of skin at the peak point of melanogenic activity. This kind of data on the number of indeterminate cells, melanocytes, and Langerhans cells might help to better elucidate the previous conclusion.

Other authors have reached similar hypotheses. Zelickson and Mottaz (1968) stated that indeterminate cells could represent either a form of premelanocyte in which melanin synthesis can be induced or an effete melanocyte which has ceased to function. If the

latter is correct, indeterminate cells should also be found in upper layers of the epidermis but they are predominantly in the lower layers as shown in these studies. They also thought that indeterminate cells may be undifferentiated cells which give rise to Langerhans cells, melanocytes, or a completely unrelated cell. Later they (Zelickson and Mottaz, 1970; Zelickson, Mottaz, and Hunter, 1972) reported that the number of indeterminate cells changed very little after ultraviolet light but a decrease in indeterminate cell numbers as well as an increase in the mitotic activity of existing melanocytes accounted for the increase in melanocyte numbers. After two weeks of daily ultraviolet irradiation no Langerhans or indeterminate cells were observed. After the application of 7, 12-dimethylbenzanthracene (DMBA), another group of investigators (Tsuji, Sujai, and Saito, 1969) observed that the number of indeterminate cells remained constant whereas the number of Langerhans cells were inversely proportional to the number of melanocytes over a period of active melanogenesis, e. g., after application of DMBA, the number of Langerhans cells decreased whereas the number of melanocytes increased; after two weeks this situation was reversed. Thus, these authors hypothesized that indeterminate cells are undifferentiated cells which may give rise to another epidermal dendritic type cell or that melanocytes and

Langerhans cells can transform into each other through indeterminate cells. Although the indeterminate cell may represent a form of premelanocyte in which melanin synthesis can be induced, it is doubtful that any Langerhans cell-melanocyte transformation could occur. On the basis of morphological and experimental data, the latter two are believed to be separate cell populations in the epidermis (Breathnach et al., 1968); therefore, the transformation hypothesis is not cogent.

After a few exposures to ultraviolet light, the sparse DOPA-positive melanocytes present usually contain all stages (I-IV) of melanosomes. Since the early melanosomes (stages I-II) are different from normal eumelanin melanosomes and lack the typical internal filamentous matrix that displays periodicity, melanization seems to proceed without the formation of an organized structure. In addition, the fully melanized melanosomes look different from the normal stage IV formation. Morphologically, these are often aggregated like phaeomelanin and melanization is sometimes not complete. There is, however, no evidence that rhesus skin contains phaeomelanin. Although these experiments do not explain what happens to the formed melanosomes and why the melanocytes suddenly stop forming them, some conclusions can be drawn. A close relationship exists between melanocytes, melanosomes, and keratinocytes

(Fitzpatrick and Breathnach, 1963; Hadley and Quevedo, 1966, 1967; Pinkus et al., 1959; Quevedo, 1969, 1972; Toda et al., 1973). As in human hair (Mottaz and Zelickson, 1967), keratinocytes appear to phagocytize portions of the melanin-laden dendrites of melanocytes (Cruickshank and Harcourt, 1964; Fitzpatrick and Breathnach, 1963). Thus, the rate of melanin synthesis within melanocytes may be regulated by a feedback mechanism which depends upon the rate at which melanosomes are removed by keratinocytes. Tanning of human skin is associated with an increased population of keratinocytes (Quevedo, 1963). The ultraviolet-stimulated production of keratinocytes provides more vehicles for the removal of melanosomes and may stimulate melanogenic activity within the melanocyte. Several other observations tend to support this theory. Silver and Hu (1968) observed that the irradiation of melanocytes in culture failed to increase the production of melanin. They found that pigment cells receiving ultraviolet irradiation either died outright or multiplied at a temporarily reduced rate. Likewise, the irradiation of pigment cells in culture inhibited cell proliferation but did not stimulate melanin synthesis (Kitano and Hu, 1969); this suggests that melanocytes in vivo require melanogenic stimuli that come from other cells of the epidermis. Thus, the cellular damage inflicted by continued ultraviolet light inhibits repair. This mechanism may function in man,

but it probably does not occur in the rhesus where the melanosomes are not rapidly transferred to the surrounding keratinocytes. Actually, few or no melanosomes are transferred to keratinocytes. (A similar observation was made by Giacometti et al., 1972). This may be a defect in the ability of the keratinocyte to take up the melanosomes.

Mitchell (1963) reported that after prolonged solar irradiation of Caucasian skin, the melanocytes seemed unable to transmit pigment to the keratinocytes, but very dark skin, such as that of aborigines, did not appear to be damaged even by years of exposure to the sun (Mitchell, 1968). Solar degeneration in xeroderma pigmentosum also prevents pigment transfer from occurring normally (Olson et al., 1970). In this condition, some keratinocytes contained little melanin but others showed increased amounts over normal. Similarly, after minor trauma, which caused intercellular edema, the passage of melanosomes to keratinocytes was inhibited, a situation Mottaz, Thorne, and Zelickson (1971) thought to be similar to atopic dermatitis, another situation in which melanosomes cannot be transferred to keratinocytes. Instead, the melanosomes become packaged into complexes within the melanocytes. Prunieras (1969) also hypothesized that the process of melanin transfer may not be dependent on the degree of melanization of melanosomes but rather on the physical contact between the two types of cells. This did not occur

in the irradiated rhesus monkeys despite extensive arborization of the melanocytic dendrites. Why melanosomes are transferred as single units and do not form complexes may be due to the size of the melanosomes. In human skin this size apparently determines the distribution of melanosomes as single granules or as melanosome complexes within the keratinocyte (Konrad and Wolff, 1973; Toda et al., 1972; Wolff and Konrad, 1971). Inside the keratinocyte, melanosomes larger than 0.8 μ are found as single units within a membrane-limited vesicle; smaller ones form complexes with two or more melanosomes in a membrane-limited vesicle. Ultraviolet light induces the formation of larger complexes with more melanosomes (Toda et al., 1972) in Caucasian skin; the size and number of melanosomes are increased from the normal two or three to five to ten within a single membrane.

Whereas melanogenic activity increased in some types of dendritic cells, one type, the Langerhans cell, exhibited no structural or histochemical changes after ultraviolet light. Although they may change numerically after continuing ultraviolet irradiation, this was not immediately evident in histological preparations. Wolff and Winkelmann (1967b) made the same observation in ultraviolet-irradiated guinea pig skin.

How pigment functions in the dermis and how it gets there are

questions that remain unanswered. If dermally located, pigment-containing cells are melanocytes, they would have to be observed in the early stages of development to determine if they contain any developing melanosomes. If they are melanophages, changes in their distribution pattern should occur over an extended period of time. A hypothesis concerning the origin of these cells might be formulated from the experimental work of Frenk (1969); painting the skin of guinea pigs with monoethyl ether of hydroquinone resulted in the degradation of epidermal melanocytes and subsequent engulfment by dermal macrophages. Thus, Breathnach (1971) concluded that cells degrading melanin in the dermis are macrophages and since melanocytes are not present in the dermis, melanosomes ingested by dermal macrophages must have originated in the epidermis. Moreover, worn-out melanocytes may be engulfed entirely or in fragmented form by dermal macrophages; this is how they leave the epidermis.

In these experiments, it is doubtful that ultraviolet light had any effect on pigment containing cells in dermal sites. Although some wavelengths penetrated the dermis, the exact amount of ultraviolet exposure could not be determined. Some ultraviolet light must penetrate the papillary dermis since two of the main elements involved in diffusion of ultraviolet rays, melanin and

melanosomes are not present or are present in very small numbers during the first few weeks. This penetration into the dermis is at least partially shown by the activation of fibroblasts as evidenced by distended rough endoplasmic reticulum. Penetration through the reticular layer of the dermis, however, is doubtful. No damage such as solar degeneration is observed in the dermis, possibly because this type of damage is caused by long-wave ultraviolet rays (Stern, 1972); only a very small component of the total energy output is in this range. Also, very little elastic tissue is present in normal rhesus dermis; therefore, no extensive solar degeneration would be expected. But extensive subepidermal bullae do form, and these are histologically similar to ones seen in human burns.

Dermal pigment cells in adult animals, like those in the retina, may represent cells that no longer produce early stage melanosomes. For example, in the rhesus eye, melanosomes develop in a normal progression from stages I to IV. By 60 days of gestation, the pigmented epithelium of the iris, ciliary body, and retina are completely developed (Hu, Endo, and Alexander, 1973). But after this time no melanogenic activity can be observed.

Unlike the many changes in the skin after a single dose of ultraviolet irradiation (Cotran and Pathak, 1968; Nagy, 1970; Nix et al., 1965; Wier, Fukuyama, and Epstein, 1971), those associated

with longer term irradiation are slight. The differences include a more extensive damage, e.g., more dyskeratotic cells and formation of subepidermal bullae, which may be due to the amount of time required for cell repair. Epidermal repair starts at about eight hours after exposure but takes up to 72 hours before epidermal regeneration is clearly visible. Irradiation of skin with heavy doses of ultraviolet can result in significant depression of DNA biosynthesis lasting up to 24 hours; the duration of DNA inhibition depends upon the dose (Kramer et al., 1974). Thus, with daily sequential irradiation, cells may just start DNA repair when they are again irradiated and DNA biosynthesis is suppressed. Since the animals in the present experiment were exposed five times per week, some of the irradiation damage might be slightly reduced with the two-day rest period. During this time, the epidermal cells may be capable of complete repair.

The decrease in the number of DOPA-positive melanocytes cannot be explained by the thickness of the stratum corneum since it thickens only slightly (less than 5 μ) and reaches a maximum thickness after about nine exposures. With continuing irradiation, the layer remains the same thickness. Therefore, the decrease in the number of melanocytes cannot be due to the attenuation of ultraviolet light by a thickening of the stratum corneum. Although extensive glycogen accumulation always results after epidermal

injury, the exact means by which it increases is difficult to explain. Perhaps continuing long-term irradiation causes suppression or modification of the enzymes for glucose metabolism. Ohkawara, Halprin, and Levine (1972) reported a sharp increase in the rate of glycogen formation in the epidermis four hours after ultraviolet irradiation. Im (1969), however, reported a slight decrease in glucose metabolism enzymes in the initial 48 hours after ultraviolet irradiation, but during the next 72 hours this was followed by a two-fold increase.

Studies have shown that the stratum corneum of pigmented skin absorbs more radiation than the stratum corneum of "white" skin and that "white" epidermis transmits more radiation than pigmented epidermis. Therefore, "white" is more susceptible to actinic damage (Pathak, 1967). This is probably the case in the rhesus monkey particularly where the hair is shaved, since there is a general epidermal disruption with some dispersed eosinophilic accumulations in the epidermis. Which cells are damaged? Johnson, Mandell, and Daniels (1972) observed that dyskeratotic cells contained more melanin than neighboring cells and were killed, whereas those containing less or none were undamaged. This may be true in man, but in rhesus monkeys there is extensive dyskeratosis after 15 or 16 exposures, with very little pigment transferred to the

surrounding keratinocytes. Therefore, all cells of the rhesus monkey have the same probability of being damaged.

Cell injury is probably involved here since after initial stimulation and an appropriate rest period, DOPA-positive melanocytes reappear after reirradiation, and since high threshold levels of irradiation must be used to stimulate melanogenesis. Some hypotheses of physical-chemical changes in the epidermis caused by ultraviolet light should be considered. First, damage to the DNA could cause cell death by the formation of thymine dimers. Pathak, Krämer, and Güngerich (1972) found that 285-320 mμ of ultraviolet light produced most of the thymine dimers, 254 mμ of ultraviolet light produced fewer, and 320-400 mμ of ultraviolet produced none. Thus, they proposed that epidermal DNA may be the primary chromophore for absorption of ultraviolet light. Jung et al. (1971) reported that when skin was irradiated with 270-310 mμ of ultraviolet light, thymine dimers formed and damaged DNA portions were excised. As a result, the number of replicating cells decreased. In the present experiments most of these wavelengths were used. Several authors (Cripps et al., 1972; Epstein, Fukuyama, and Epstein, 1968, 1971; Krämer et al., 1974; Tan and Stoughton, 1969) have reported that ultraviolet light stimulates as well as depresses DNA synthesis in mammalian epidermis. This stimulation is reflected

by the post-ultraviolet synthesis of DNA which is repair and semi-conservative replication. Thus, it is possible to eliminate single strand damage; recovery is by the enzymatic process of excision, repair, and recombination. Second, the destruction of amino acids could result in the loss of enzymatic function. In mammalian epidermis, shortly after ultraviolet irradiation, both RNA and protein synthesis are inhibited (Baden and Pearlman, 1964; Epstein, Fukuyama, and Fry, 1970; Fukuyama, Epstein, and Epstein, 1967). How this photochemical change occurs or what damage is caused to the protein units of epidermal enzymes is not completely understood. One of the main problems is that of distinguishing between a primary (one occurring during the absorption of radiant energy) and a secondary effect (one present as the result of cell damage).

Although this work answers some questions about ultraviolet induced appearance and disappearance of DOPA-positive melanocytes in the rhesus epidermis, others remain unanswered. Until better techniques are developed, however, these problems might not be resolved. Nevertheless, the epidermis of the rhesus monkey should be a good model for additional studies of pigmentation, e.g., the effect of other agents (ACTH, MSH, and androgens) on melanogenesis. Because no discernible melanocytes are present in the normal rhesus epidermis, any change in melanogenic activity should be easier to

detect than on other mammalian systems where melanocytes are normally present.

SUMMARY AND CONCLUSIONS

As a result of this study, I have found that 1) with sequential ultraviolet irradiation, the number of DOPA-positive melanocytes increased to a peak number and then declined to a basal level which was maintained for the duration of the experiment (43 weeks); 2) melanocytes were restimulated to a peak number after a three-month rest period; 3) ultraviolet light had no discernible effect on melanophages present in the dermis; 4) developing melanosomes lacked an organized internal filamentous network; 5) early melanosomes (stages I and II) were numerous during the first 15 irradiation periods, but decreased in number so that after 30 exposures only fully developed melanosomes were found; 6) some of these developed melanosomes appeared morphologically similar to phaeomelanin melanosomes; and 7) tyrosinase activity increased with ultraviolet light to a high level and then decreased.

The following hypotheses can be drawn from this study and those of others: 1) The epidermis of the rhesus monkey contains a pool of indeterminate cells which are stimulated to form melanocytes; 2) melanosomes are transferred to keratinocytes at such a slow rate as to probably cause feedback inhibition of additional melanosomal synthesis; 3) the mechanism involved is keratinocyte

injury since after an appropriate rest period reirradiation causes DOPA-positive melanocytes to reappear; 4) keratinocyte injury is probably caused by the high levels of irradiation used to stimulate melanogenesis and results in thymine dimer formation and suppression of DNA repair synthesis. In conjunction with the above, the following observations must be considered: 1) more extensive cellular damage is observed after long-term irradiation than after short term; and 2) the subepidermal bullae which form are histologically similar to those in human burns.

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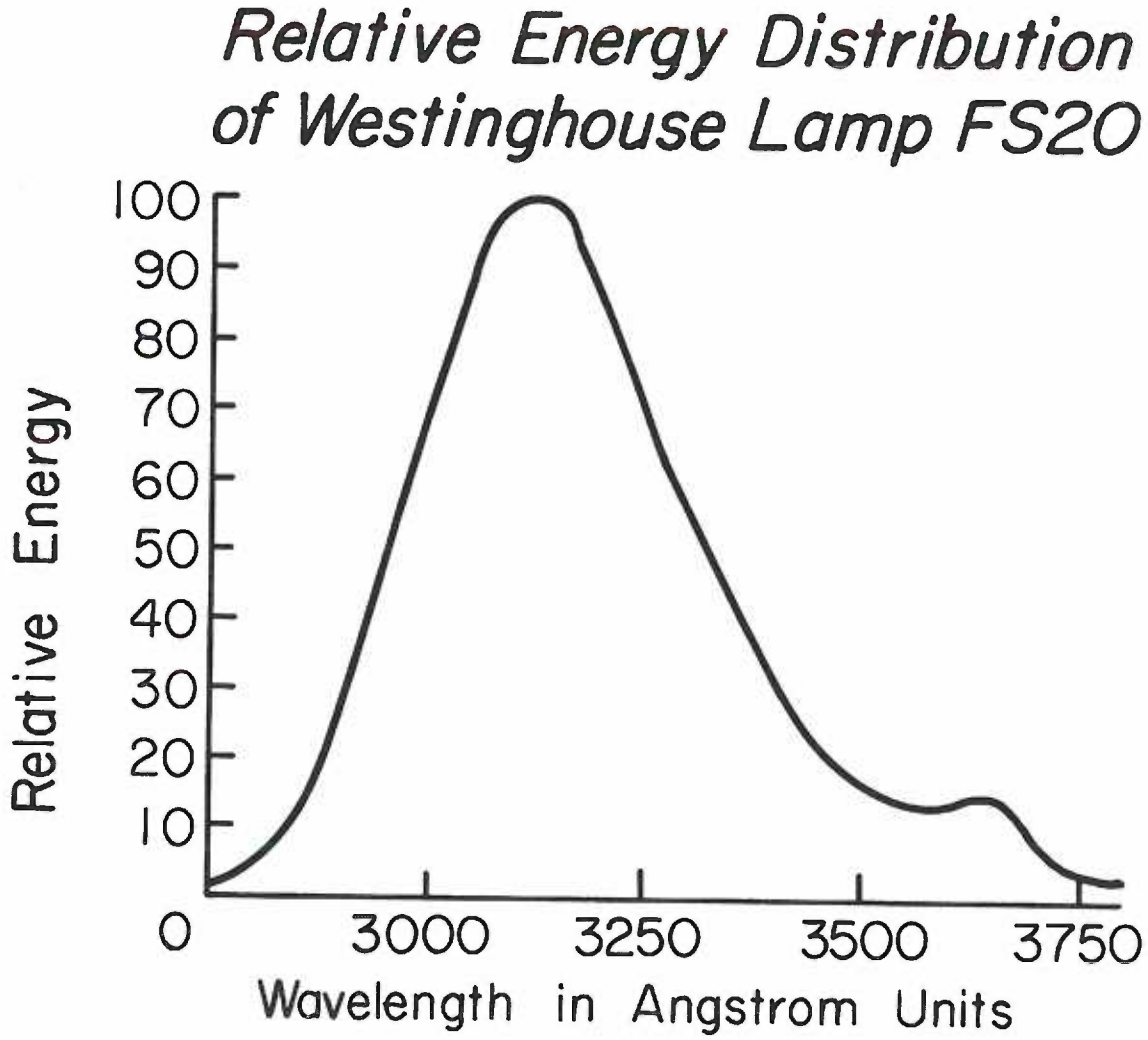
APPENDIX I

Spectral output for Westinghouse lamp.


Relative Energy Distribution
of Westinghouse Lamp FS20
Relative Energy
100
90
80
70
60
50
40
30
20
10
0
3000
3250
3500
3750
Wavelength in Angstrom Units

APPENDIX II

Fixative for histology

Buffered neutral formaldehyde (pH = 7.0)

1.	40% Formaldehyde	100 ml
2.	Distilled water	900 ml
3.	Acid sodium phosphate monohydrate	4.0 gm
4.	Anhydrous disodium phosphate	6.5 gm

APPENDIX III

Histochemical procedures

DOPA reaction (L-β-3, 4-dihydroxyphenylalanine) (modification of Laidlaw and Blackberg, 1932; Rappaport, 1955).

A. Procedure

1. Place epidermal and dermal sheets into 10% buffered neutral formalin at 4° C for 15 minutes.
2. Remove from fixative and wash in six changes of 0.055 M sodium phosphate buffer. pH = 7.4
3. Incubate samples in substrate, 0.1% DOPA (L-3, 4-dihydroxyphenylalanine) (Sigma) in 0.055 M phosphate buffer at 37° C for five hours. (The DOPA solution should be changed every hour during the incubation period.)
4. Remove samples from incubating substrate and wash in six changes of phosphate buffer.
5. Fix with buffered neutral formalin at 4° C overnight.
6. Wash samples in distilled water; dehydrate in graded ethyl alcohols (70%, 80%, 95%, & 100%); clear in xylene, and mount on glass slides with the dermal-epidermal surface facing upward.

B. Control

Same as above except only phosphate buffer is used as a substrate in Step 3.

C. Preparation

Mix a and b below to pH = 7.4

a. 0.055 M Sodium phosphate dibasic

Na_2HPO_4	2.343 gm
H_2O	300 ml

b. 0.055 M Sodium phosphate monobasic

NaH_2PO_4	0.759 gm
H_2O	100 ml

Demonstration of nucleoside triphosphatase using adenosine triphosphate (ATP) as substrate (modification of Wolff and Winkelmann, 1967a).

A. Procedure

1. Fix epidermal sheets in 10% buffered neutral formalin at 4° C for 20 minutes.
2. Rinse tissue in distilled water.
3. Incubate in adenosine triphosphate substrate at 37° C for 1 1/2 hours.
4. Rinse in several changes of distilled water.
5. Rinse very briefly in dilute acetic acid.

6. Develop in dilute ammonium sulfide for 5 minutes.
7. Rinse in distilled water for 2 hours.
8. Fix overnight in 10% buffered neutral formalin at 4° C.
9. Dehydrate in graded ethyl alcohol (70%, 80%, 95%, 100%), clear with xylene, and mount on glass slides with dermal-epidermal surface facing upward.

B. Preparation

1. Incubating substrate mixture

Mix a-e in order as given below

(a) ATP disodium salt (Sigma)

12.5 mg/10.0 ml distilled water 10.0 ml

(b) Tris-maleate buffer (0.2 M, pH = 7.2)

(1) Tris (hydroxymethyl) aminomethane (Sigma)	2.420 gm
(2) Maleic anhydride	1.960 gm
(3) Distilled water to volume	100.0 ml
(4) Add 0.2 M sodium hydroxide to the above (a-c) solution until you reach pH = 7.2	10.0 ml

(c) 0.1 M Magnesium sulfate ($MgSO_4$)	2.5 ml
(d) Distilled water	1.0 ml

(e) 2% lead nitrate $Pb(NO_3)_2$

Add to above solution until cloudy precipitate begins to form (1.2-1.4 ml)

2. Dilute ammonium sulfide solution

1.0 ml/74.0 ml distilled water

3. Dilute acetic acid (CH_3COOH)

0.1 ml glacial acetic acid/1000 ml distilled water

APPENDIX IV

Fixatives for electron microscopy

Palade's Buffered Osmium Tetroxide (Palade, 1952).

A. Mixture

1. Buffer solution	1 volume
2. 0.1 N HCL	1 volume
3. 2% OsO_4	2 volumes

B. Preparation

1. Veronal-Acetate-HCL buffer (store at 4° C)

Stock buffer solution:

Sodium veronal (sodium barbital)	2.89 gm
Sodium acetate (anhydrous)	1.15 gm
Distilled water to make	100.0 ml

Final buffer solution:

Stock buffer solution	2 volumes
0.1 N HCL	2 volumes
Distilled water	1 volume

2. Stock 0.1 N HCL

Concentrated HCL (36%)	8.6 ml
Distilled water to make	1000 ml

3. Osmium tetroxide (store at 4° C)

OsO_4 crystals	1 gm
Distilled water to make	50 ml

Russell's modified Karnovsky's fixative (Russell, 1972).

A. Mixture

1. Distilled water	700 ml
2. Paraformaldehyde	20 gm
3. Glutaraldehyde (25% standard solution)	100 ml
4. Buffer solution	200 ml

B. Preparation

1. Add paraformaldehyde to 300 ml distilled, deionized water and heat to 60° - 70° C while stirring the solution under a hood.
2. Add 4 to 6 drops (or more) of 0.2 N NaOH, drop by drop until clear.
3. Cool to room temperature.
4. Add 100 ml of 25% glutaraldehyde.
5. Add 200 ml sodium phosphate buffer (Millonig's, 0.16 M).

$NaH_2PO_4 \cdot H_2O$ (monobasic sodium phosphate)	
22.6 gm/1000 ml	830 ml
NaOH (12.6 gm/500 ml)	170 ml
Glucose	5.4 gm
1% Calcium chloride	10 ml

6. Add distilled water to make 1000 ml

APPENDIX V

Dehydration and embedding for electron microscopy

A. Dehydration		
1.	30% ethanol	5 min
2.	50% ethanol	5 min
3.	60% ethanol	5 min
4.	70% ethanol	5 min
5.	80% ethanol	10 min
6.	95% ethanol	10 min
7.	100% ethanol	15 min
8.	100% ethanol	15 min
B. Transitional solvent		
1.	100% Propylene oxide	30 min
2.	100% Propylene oxide	30 min
C. Infiltration		
1.	Propylene oxide and embedding (Spurr) mixture (1:1)	30 min
2.	Propylene oxide and embedding (Spurr) mixture (1:3)	30 min
3.	Embedding mixture (Spurr)	Overnight
D. Embedding		
1.	Procedure	

Place tissue with label and fresh embedding mixture (Spurr) into beem capsules and polymerize at 70° C for 8 hours.

2. Preparation

ERL 4206 (vinyl cyclohexene dioxide)	10 gm
DER 736 (diglycidyl ether of poly-propylene glycol)	6 gm
NSA (nonenyl succinic anhydride)	26 gm
S-1 (dimethylaminoethanol)	0.4 gm

APPENDIX VI

Stains for electron microscopy

For thick sections

Toluidine blue

1. Preparation

Toluidine blue	1 gm
Sodium borate ($Na_2B_4O_7 \cdot 10\ H_2O$)	1 gm
Distilled water	100 ml

For thin sections (modification of Reynolds, 1963, and Watson, 1958)

A. Procedure

1. Float grids with tissue onto lead citrate staining solution for 45 seconds.
2. Wash with distilled water, then dry.
3. Float grids on 3% uranyl acetate, 100% ethanol mixture (1:1) for 5 minutes.
4. Wash with distilled water, then dry.
5. Float grids on lead citrate staining solution for 45 seconds.
6. Wash with distilled water, then dry.

B. Preparation

1. Lead citrate

Lead nitrate [$Pb(NO_3)_2$]	1.33 gm

Sodium citrate [$Na_3(C_6H_5O_7)\cdot 2H_2O$] 1.76 gm

Distilled water (CO_2 - free) 30 ml

(a) Add lead nitrate and sodium citrate to water and shake vigorously for about 30 minutes.

(b) To the milky suspension add 8 ml of 1 N NaOH.

(c) Add distilled water to make 50 ml.

2. Uranyl acetate (3%)

3 gm/100 ml distilled water

APPENDIX VII

Procedure for the measurement of melanin synthesis (modification of Kitano and Hu, 1970, 1971; Kitano, 1972; Adachi, 1973)

A. Epidermis labeled with C^{14}-DOPA

1. After removing excess hair and subcutaneous tissue, pin samples to a flat cork, submerge in 2 N sodium bromide, and incubate for 1 1/2 hours at 37° C.
2. Remove the specimen from the halide and submerge in a dish containing warm (35 - 40° C) tap water.
3. Separate the epidermis from the dermis.
4. Wash in distilled water the resulting epidermal and dermal sheets.
5. Divide the epidermal sheet into necessary sample size (about 0.5 cm x 1 cm), blot dry, and weigh.
6. Place samples into TC Hanks solution and incubate for 30 minutes at 37° C. For a control add 10-3 M 1-phenyl-2-thiourea. (Sigma) (an inhibitor of tyrosinase)
7. To the solution add 0.5 μCi/ml, DL-3, 4-dihydroxy-phenylalanine-2-C^{14} (DOPA) (New England Nuclear, spec. act. 9.31 mCi/mM) and incubate at 37° C for 2 hours.
8. After removing excess label solution, wash samples in several changes of phosphate buffer and incubate in a

0.1% DOPA solution (made in phosphate buffer) for 15 minutes at 37° C.

9. Wash epidermis in several changes of distilled water.
10. Place samples in a blender, add 0.5 ml distilled water and make a homogenate.
11. Add trichloroacetic acid (TCA) to the homogenate so that the final TCA concentration is 5% and place the samples overnight in an ice bath.
12. Collect the resulting precipitates on millipore filters (HAWP25) supported on a borosilicate glass filter holder and wash with cold 5% TCA.
13. Wash filters with absolute isopropanol.
14. Air dry filters, then place them in glass scintillation vials which contain 10 ml of toluene and omniflour (New England Nuclear).
15. Count in a liquid scintillation spectrometer.

B. Dermis labeled with C^{14}-DOPA

1. Process dermis in the same manner as described above for steps 1-8.
2. Place dermis into liquid nitrogen. After it is frozen, pulverize in cold stainless-steel cylinder and piston by application of large amounts of force.

3. Put the crushed material into cold 5% TCA overnight and process as described above, steps 12-15.

C. Epidermis labeled with C^{14}-tyrosine

1. Place biopsies (0.5 cm x 1 cm) directly into TC Hanks solution and incubate for 30 minutes at 37° C. For controls, add 1-phenyl-2-thiourea and/or 100 mg/ml puromycin (Sigma).
2. To sample, add 0.5 μCi/ml L-tyrosine-1-C^{14} (New England Nuclear, spec. act. 53.5 mCi/mM) and incubate for 2 hours at 37° C.
3. Remove excess label and wash with distilled water.
4. Incubate samples in 0.1% aqueous solution of tyrosine for 15 minutes at 37° C.
5. Wash samples with distilled water and incubate in 2 N sodium bromide for 2 hours at 56° C.
6. Remove samples, separate the dermis from epidermis, wash, and process as described above, steps 10-15.

D. Treatments

1. Epidermis
   (a) C^{14}-DOPA
   (b) C^{14}-DOPA + 10^{-3} M Phenylthiourea
   (c) C^{14}-Tyrosine + 100 mg/ml Puromycin

(d) C^{14}-Tyrosine + 10^{-3} M Puromycin + 100 mg/ml Phenylthiourea

2. Dermis

(a) C^{14}-DOPA

(b) C^{14}-DOPA + 10^{-3} M Phenylthiourea

E. Reagents

1. 2 N sodium bromide

206 gm/1000 ml distilled water

2. Phosphate buffer

see appendix II section c

3. TC Hanks Solution (Difco Labs.)

Dextrose	1 gm
Sodium Chloride	8 gm
Potassium Chloride	0.4 gm
Calcium Chloride	0.14 gm
Magnesium Sulfate	0.1 gm
Magnesium Chloride	0.1 gm
Monopotassium Phosphate	0.06 gm
Disodium Phosphate	0.06 gm
Sodium Bicarbonate	0.35 gm
Phenol Red	0.02 gm
Triple Distilled Water	1000 ml

pH = 7.2-7.4

4. Trichloroacetic acid

stock solution

75 mg TCA/ 100 ml distilled water

working solution

5 mg TCA/ 100 ml distilled water

5. Scintillation cocktail

4 gm omniflour/ 1000 ml toluene

6. Radioactive labels

(a) C^{14}-DOPA (specific activity = 9.31 mCi/mM)

220 μgm/10 ml phosphate buffer

(b) C^{14}-Tyrosine (specific activity = 53.5 mCi/ mM)

Dilute 50 mCi/0.50 ml to 50 mCi/5.0 ml with sterile distilled water

7. Inhibitors

stock solution:

(a) 1-phenyl-2-thiourea 10^{-2} M

(b) Puromycin 2 mg/ml distilled water

working solution:

(a) 1-phenyl-2-thiourea 100λ/ml

(b) Puromycin 50λ/ml