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Effect of Bromocriptine on the Larval Skin of the Green Toad, *Bufo viridis viridis* Leurenti

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In the premetamorphic larval green toad, *B. viridis viridis*, as in other anurans, the skin is made up of a fibrous dermis and an epidermis of stratified epithelium. The effects of bromocriptine, an antiprolactin drug, on the premetamorphic skin of *B. viridis viridis* was examined. Bromocriptine, dissolved in rearing water at four different concentrations, induced a number of changes in the skin of treated tadpoles. In rough sequence of appearance, these changes include: retraction of the melanocyte dendrites, synchronous burst of the apical vesicles of the superficial epithelial cells, gradual disappearance of the melanosomes from the epithelial cells and widening of the intercellular spaces. In addition, macrophages appeared in the superficial dermis amongst the retracted melanocytes. White crystals were observed on the skin surface and similar crystals were ingested by the macrophages. Prolonged treatment with bromocriptine resulted in hypertrophy and extraction of some epidermal cells. Deep melanocytes of the mesenteries were not affected by bromocriptine-treatment indicating that the drug did not penetrate deep into the tadpole tissue. Whether the macrophages observed in the dermis were recruited from deeper tissues or were converted melanocytes is another issue in need of study.

Key words: Anurans, antiprolactin, bromocriptine, skin, MSH.

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The skin is an extremely vital structure to both larval and adult amphibians. In addition to its prime role in sensation and protection against both physical and chemical agents, the amphibian skin performs a number of other functions. These include respiration, ion exchange (STIFFLER 1994, 1996; SORENSEN & LARSEN 1996) and water balance (JORGENSEN 1993, 1997).

The early anuran larval (premetamorphic) skin is composed of an inner dermis and an outer epidermis (WARBURG *et al.* 1994; AMICHE *et al.* 2000). The dermis is highly acellular. It is made up of a loose, deep layer and a dense, superficial layer composed of orthogonally arranged lamellae of collagen fibers. The epidermis, on the other hand, is made up of a stratified epithelium composed of a variety of cells (FOX 1985, 1986; QAR & AL-ADHAMI 2003).

The premetamorphic stage is characterized by low levels of thyroid hormones (TH). The stage is further stabilized by relatively large amounts of prolactin (PRL) secreted by the pituitary. The antagonistic actions of these two sets of hormones on the amphibian larval tissues are well known (RABELO & TATA 1997).

Bromocriptine (2-bromo-12′-hydroxy-2′-5′-ergotaman-3′,6′,18-trione) is an ergot derivative used as dopamine receptor agonist (WELCH 1993). It has been used in treating hyperprolactinemia and some diseases associated with dopamine neuron degeneration such as certain menstrual problems and Parkinson's disease (JARVIK *et al.* 2000). It is also known to block the release of the growth hormone and PRL. The latter, PRL, acts as a juvenile and growth hormone in anuran amphibians (TATA *et al.* 1991; BAKER & TATA 1992; TAKADA *et al.* 1996).

The present work aims at investigating the structural effects of bromocriptine on the skin of the premetamorphic green toad, *Bufo viridis viridis*. Such a study may be valuable in exploring some of the unknown consequences of the use of this drug.

Material and Methods

Larvae of the toad, *B. viridis viridis,* were collected in spring 2004 from Ziglab dam in Alghor Alshamali, Jordan. In the laboratory, they were

reared in aquaria containing dechlorinated water, fed boiled lettuce and kept under laboratory conditions. The premetamorphic larval stages selected were equivalent to stage 50-52 of NIEUWKOOP and FABER (1956) proposed for the South African toad, *Xenopus laevis*.

Bromocriptine treatment

An aqueous, 0.25% stock solution of bromocriptine was prepared by dissolving one tablet of Parlodel (2.5 mg bromocriptine) in 10 ml of dechlorinated water. From this stock, different dilutions were made. These dilutions were: 0.025%, 0.025‰, 0.0025‰. The tadpoles were then divided into four groups of twenty animals each. Each group was then raised in one of the previous bromocriptine solutions in addition to one control group.

Light microscopy

The tadpoles were sacrificed by an overdose of tricane methane sulphonate (MS222). Young tadpoles were fixed *in toto*, whereas pieces of flank skin were removed from the elder individuals. Fixation was done in Bouin's fluid for a minimum of 24 hours. Samples were then washed in 70% ethanol, dehydrated in upgraded ethanol, cleared in xylene and embedded in paraffin wax. The 7 μm sections were stained with hematoxylin and eosin or Mallory's triple stain. They were examined and photographed using a Wild MPS 51 microscope.

Electron microscopy

Small pieces of the flank skin were fixed in 2.5% gluteraldehyde in 0.1% M sodium cacodylate buffer (pH 7.4) for two hours, washed and minced under a dissecting microscope. Postfixation was made in 1% osmium tetroxide in the same buffer for three hours. Sectioning was made with the use of a LKB ultratome. Semithin sections were stained with methylene blue, while ultrathin sections were stained with uranyl acetate and lead citrate and examined under a Zeiss EM 10 CR.

Results

Tadpoles of the green toad, *B. viridis viridis,* at NF stage 52 are homogeneous black in color with no spots or ornamentation (Fig. 1a). The abdominal skin, however, is a homogeneous white. The skin is made up of an inner dermis and an outer epidermis with a basal lamina in between (Fig. 2). The dermis, in turn, is made up of a deep, loose layer and an outer, dense, fibrous layer. The black color of the tadpoles is due to the evenly dispersed melanocytes (Fig. 3a). The ultrastructural picture (Fig. 4), too, shows a clear conformity with the previous description (QAR & AL-ADHAMI 2003).

Experiment (bromocriptine-treatment)

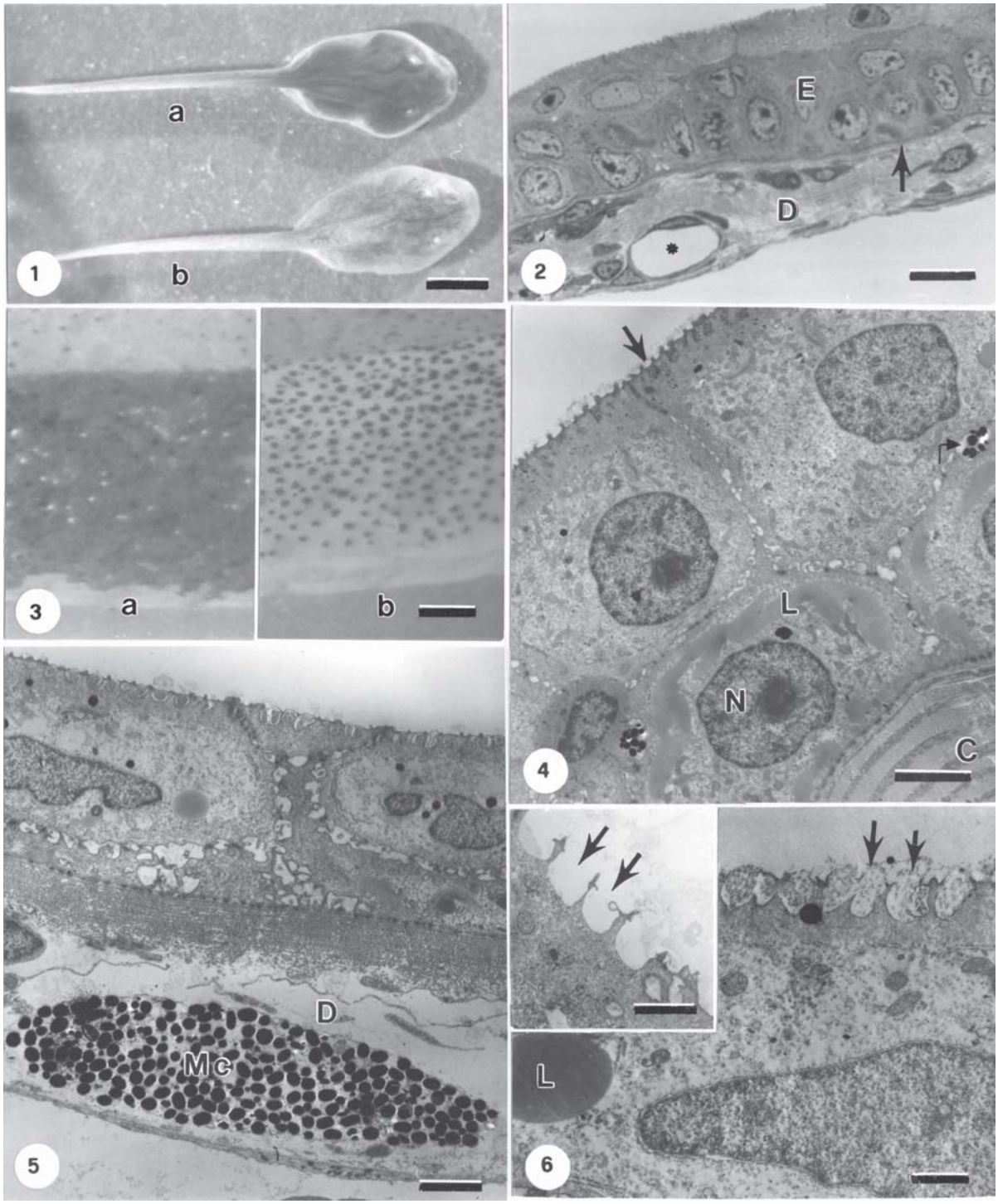
Bromocriptine treated tadpoles showed a number of changes during the course of the experiment. These changes are described and discussed here in the sequence of their appearance.

All treated tadpoles became pale in color within several hours of treatment even with the lowest concentration used (Fig. 1b). Examination under the dissecting microscope showed that the melanocytes became rounded (Fig. 3b) and that the animal was lightly covered by sparse, tiny, whitish crystals that stuck to the outer body surface, including the eyes. Sectioned material of animals treated for twelve hours showed that the melanocytes withdrew their dendrites to attain an oval outline into which the melanosomes aggregated (Fig. 5). The epidermis at this stage of treatment showed a slight widening of the intercellular spaces.

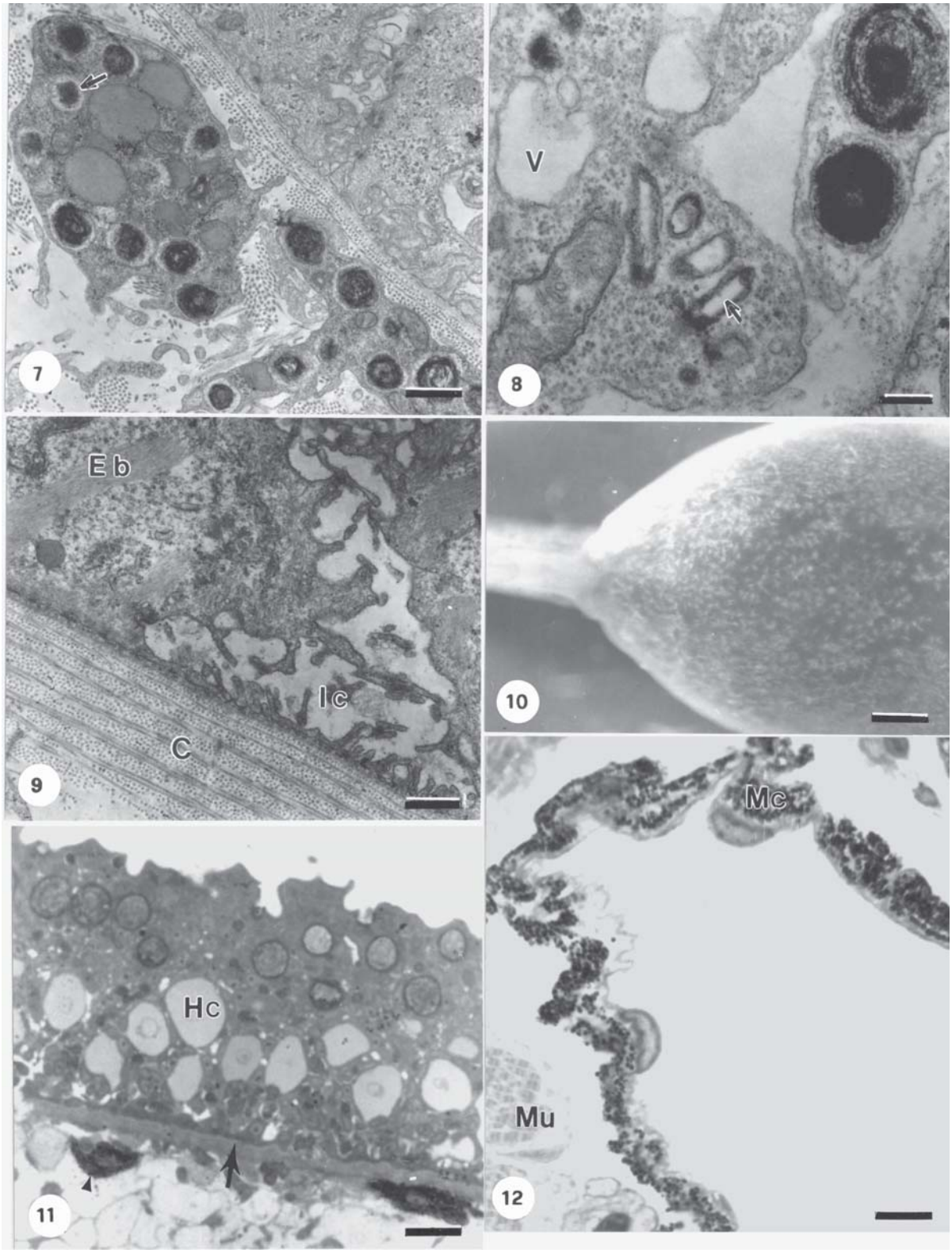
Material fixed after 24 hours of treatment showed that almost all the apical secretory vesicles of the epidermis were erupted and their contents, mostly mucus, was discharged and formed a thin protective film coating the animal (Fig. 6, inset). This event was followed by the gradual disappearance of melanosomes from the epidermis.

At this stage of treatment, a number of macrophages appeared underneath the epidermis occupying the wide spaces that resulted from the withdrawal of the processes of the melanocytes. These macrophages show secondary lysosomes containing either melanosomes at different stages of degradation (Fig. 7) or rod-like crystals comparable, both in size and shape, to those observed on the body surface of the tadpoles (Fig. 8). They also show features of high activity including the formation of pseudopodia-like processes. They contain slightly lobulated nuclei, mitochondria of variable sizes and shapes, free ribosomes and polysomes. The ER is poorly developed and the ground cytoplasm is highly vacuolated. The dermis showed no profound changes apart from the previously mentioned ones.

The basal epidermal cells showed a dramatic change in shape and were highly attenuated with their oval-spherical nuclei located in the thick central part of the cell. Their peripheral extensions achieved the shape of extremely flattened plates of cytoplasm adhering to the basal lamina through hemidesmosomes or protruding into the intercellular spaces (Fig. 9). Some cells showed intense



Figs 1-6. Fig. 1. Light micrograph of the premetamorphic tadpole of the green toad. a – control, b – experimental. Bar = 2 mm. Fig. 2. Light micrograph of the skin of a premetamorphic tadpole. The epidermis (E) is separated from the dermis (D) by a sharp basal lamina (arrow). Bar = 10 μm. Fig. 3. Light micrograph of the tail region in (a) control and (b) experimental tadpoles. Notice the contracted melanocytes. Bar = 0.1 mm. Fig. 4. Electron micrograph of the premetamorphic skin. Basal cells are characterized by a spherical nucleus (N) and a large liposome (L). Small clusters of melanosomes are seen in the intercellular space (small arrow). The free surface of the epidermis is decorated by short, stubby microvilli intervened by apical vesicles (large arrow) Bar = 2μm. Fig. 5. The skin of an animal treated for twelve hours. The melanocyte (Mc) in the dermis (D) has propelled its processes and becomes oval in shape. Bar = 3 μm. Fig. 6. The skin of an animal treated for thirty-six hrs. The apical vesicles have erupted (arrows). L: liposome; N: nucleus. Bar = 1μm. Inset. The apical vesicles (arrow) have all erupted and discharged their contents. Bar = 0.5 μm.



Figs 7-12. Figs 7 & 8. Macrophages from the dermis of animals treated for thirty-six hours. These macrophages are located just underneath the fibrous dermis and are highly lobated. They are rich with organelles and inclusions. V: vacuole, large arrow: secondary lysosome, small arrow: crystal. Bars = 1μm and 0.3 μm, respectively. Fig. 9. The basal epidermis in animals treated for thirty-six hours. The intercellular spaces (Ic) have become very spacious. Eb: figure of Eberth. Bar = 1 μm. Fig. 10. A light micrograph of the posterior trunk region of a tadpole treated for thirty-six hours showing heavy precipitation of white crystals. Bar = 0.5mm. Fig. 11. Light micrograph of the skin of a tadpole treated for two months. The melanocytes (arrowheads) underlying the fibrous dermis (arrow) are retracted. Most of the epidermal cells are hypertrophied and some show signs of extraction (Hc). Bar = 10 μm. Fig. 12. Light micrograph of a section through the trunk region of a tadpole treated for tow weeks. The melanocytes (Mc) in the peritoneum are not affected by the treatment. Mu: muscle. Bar = 25 μm.

masses of tonofilaments (figures of Eberth) that give rise to smaller bundles terminating on the hemidesmosomes (Fig. 9). Most of the basal intercellular spaces have become extremely spacious, interrupted by thin, irregular and branched cytoplasmic processes of the opposing cells. Apart from the previous features, the basal cells showed fewer and smaller liposomes than those observed in the control.

Prolonged treatment (one-two months) resulted in some other profound alterations in the structure of epidermis. The epidermis in both control and experimental animals became several-cell thick. The bromocriptine-treated animals, however, became whitish because of the lack of melanin and because of the white crystals that intensely cover the whole animal including the eyes (Fig. 10). Other features of this stage of treatment include further widening of the intercellular spaces and excessive hypertrophy and extraction of epidermal cells (Fig. 11).

Deep melanocytes of the internal structures, such as the peritoneum and mesenteries, were not affected by the treatment and had extended processes and dispersed melanosomes (Fig. 12).

Discussion

The histological and ultrastructural pictures of the premetamorphic larval skin of the green toad, *B. viridis viridis,* showed a profound conformity with those given previously for other anurans (ROSENBERG & WARBURG 1992; 1995).

The present study, for the first time to our knowledge, deals with the effect of bromocriptine, a dopamine receptor agonist, on the amphibian larval skin. Bromocriptine-treated tadpoles showed a number of changes during the course of the experiment. These changes are discussed here in the sequence of their appearance.

The tadpoles became pale in color within a few hours of treatment. This change in color is due to two factors. First, the melanocyte dendrites retracted so that the cell attained a roughly oval outline. Under normal conditions, colour change in amphibians does not involve the retraction of these dendrites. Second, melanosomes aggregated in the perinuclear region, a mechanism that is known to underlie colour change in lower vertebrates including amphibians. In these animals, melanocyte stimulating hormone (MSH) secreted by the pituitary gland controls the process of color change including both aggregation and spreading of melanosomes (HEARING 2003).

The mechanism by which this effect was achieved is not clear. One possible mechanism is that bromcriptine, being a dopamine agonist, functions in almost the same way as endogenous dopamine. It binds to MSH receptors on the melanocyte surface, thus preventing the MSH from binding to these cells. This may be the contrivance that elicits the fast response of the melanocytes. Alternatively, bromocriptine blocks the secretion of MSH by the hypophyseal pars intermedia as it does in the case of PRL (FARMER & RUSHEN 1998; FARMER *et al.* 2000) and hence lowers the MSH levels in the blood. This action may be indirect through inhibition of the release of adrenocorticotropic hormone (ACTH) from the neurointermediate lobe of the pituitary (LAURENT *et al.* 2002). ACTH is split in the pituitary intermediate lobe by the prohormone convertase 2 (PC2) to give rise to MSH and corticotropin-like intermediate lobe peptide (CLIP) (BENJANNET *et al.* 1991).

In normal conditions, the melanosomes observed in the epidermal cells usually disintegrate and are replaced by new ones passed to these cells by the underlying melanocytes (HEARING 2003). This process is also hindered by bromocriptine. Melanosomes first disappeared from the basal epithelial cells. The last few melanosomes to be observed in the epidermis were confined to the superficial cells. This observation contributes another factor of color fading of the skin.

The next event was a synchronous burst of the apical vesicles of the outermost epidermal cells and the release of their contents. The released contents, mostly mucus (ROBINSON & HEINTZELMAN 1987), form a thin film that coats the animal, thus protecting it from the deleterious effects of the drug. This explanation is in accordance with the known function of mucus in protecting epithelia and deeper tissues from injurious agents including chemicals (PERES-VILAR & HILL 1999). Explaining the mechanism by which perfluorooctyl bromide (perflubron) stimulates excessive mucus secretion in tracheal segments of the ferret, KISHIOKA *et al.* (1999) suggest that perflubron may induce the release of some mediators that secondarily trigger receptor-mediated secretion. They suggest that the hypersecretion is stimulated by arachidonic acid. Very little is known about the controlling mechanism of mucus secretion in both amphibians and mammals. The released vesicular contents were not readily replaced though the synthesizing machinery, i.e. the Golgi apparatus and endoplasmic reticulum, were apparently not affected. This explains how the drug might have found its way through the skin continuously causing additional effects.

Histological examination of the skin in the experimental animals also showed the presence of macrophages just underneath the fibrous layer of the dermis, in between the retracted melanocytes.

Whether these macrophages were recruited from deeper tissues or were converted melanphores is another issue in need of investigation.

The fact that the deep melanocytes retained their dendrites and their melanosomes remained dispersed in the bromocriptine-treated animals may indicate that the drug had no effect on these cells.

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