

Electron-microscopic Observation on the Dermal Chromatophores of Normal Frogs and Three Kinds of Color Variants in *Rhacophorus schlegelii*

By

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(With 16 Text-figures and 1 Plate)

INTRODUCTION

Rhacophorus schlegelii is a small arboreal species, being nearly the same in size as *Hyla arborea japonica*. This species is distributed in Honshu, Shikoku and Kyushu, Japan and most abundantly found in hilly areas near mountains. The dorsal surface is usually bright yellowish-green and very similar to that of *Hyla arborea japonica*, when the latter reveals a green color on a smooth green leaf. In contrast to the tree-frog, *Rhacophorus schlegelii* can not change the dorsal color into other than dark green.

The changeable coloration of the dorsal surface of tree-frogs or pond frogs is exhibited by the dermal chromatophore units consisting of xanthophores, iridophores and melanophores (BAGNARA, TAYLOR and HADLEY, 1968). Of these chromatophores, the melanophores are the most ubiquitous. They are found in every site of the body and appear to be indispensable to formation of the black background of iridophores. However, the present authors have learned that another kind of chromatophores takes the place of the melanophores in the dorsal surface of *Rhacophorus schlegelii*. This is a layer of violeophores derived from xanthophores.

Three kinds of color variants have been discovered in this species. They differ in dorsal color from similar variants found in the other anuran groups, as the dermal melanophores are lacking and substituted by violeophores in the wild-type frogs.

The development and minute structure of the dermal chromatophores of *Rhacophorus schlegelii* will be reported in this paper together with the three color variants.

MATERIALS AND METHODS

A yellow variant of *Rhacophorus schlegelii* GÜNTHER was discovered on April 19, 1972 at Mr. YOSHIMURA's rice field located in Toyohira-cho, Yamagata-gun, Hiroshima Prefecture. This variant was brought at once to our laboratory by a reporter of the Chugoku Newspaper. On June 18, 1976, many albino tadpoles of

the same species were found at Mr. SUEOKA's rice field located in Mitsui, Hikari, Yamaguchi Prefecture. Eight of them were collected and brought to our laboratory by Mr. M. USHIMI, a teacher of biology at the Hikari High School. All these tadpoles metamorphosed normally and attained sexual maturity in the breeding season of the following year. Seven of them were males and the remainder was a female. On June 18, 1979, five more albino tadpoles were discovered at Mr. NAGASE's rice field located in Daito-cho, Ohhara-gun, Shimane Prefecture. They were brought to our laboratory by Mr. T. NONOMURA, an officer of Daito-cho and reared until sexual maturity. On June 24, 1983, five other albino tadpoles collected from Wakayama Prefecture were given to the present authors by Mr. T. KUBO, an officer of Wakayama branch of Japan Radio Broadcasting Station. Four of the five albino tadpoles metamorphosed normally and attained sexual maturity.

On July 23, 1976, many tadpoles of the black-eyed mutation were found at a rice field located in Okuyama, Ashiya, Hyogo Prefecture. Forty-two of them were collected and brought to our laboratory by Mr. S. WAKANA, an undergraduate at the Kwansei Gakuin University. Although they were reared very carefully, only thirteen tadpoles completed metamorphosis and eventually eight frogs attained sexual maturity in the following year. Of these frogs, seven were females and one was a male.

Mature and juvenile normal *Rhacophorus schlegelii* were collected from the suburbs of Hiroshima in June, 1978.

Before making electron-microscopic sections of the skin, mature wild-type females, albinos and black-eyed mutants were adapted for about 24 hours to a light environment. Pieces of the dorsal and lateral skins and those of the eyeball removed from each of these frogs were cut into minute pieces in cold 0.1 M phosphate buffer (pH 7.4) containing 4% glutaraldehyde and kept in the same solution for 2 hours after renewal of the fluid. The minute pieces were then postfixed in 0.1 M phosphate buffer (pH 7.4) containing 2% osmic acid for 2 hours. These procedures of fixation were performed at 2~4°C. The fixed samples were dehydrated in an ethanol series and embedded in Epon 812. Sections were made on a Porter-Blum MT-1 ultramicrotome with a glass knife at the thickness of silver or silver-gold and double stained with saturated uranyl acetate and alkaline lead citrate. Observation was made under a Hitachi Hs-8 electron microscope.

The developmental stages reported in this paper follow those of *Rana pipiens* established by TAYLOR and KOLLROS (1946) for convenience sake.

OBSERVATION

I. Wild-type frogs

1. Dermal chromatophores of the dorsal skin

The dorsal surface of mature *Rhacophorus schlegelii* collected from the field is usually bright yellowish-green (Plate I, 1). In contrast to *Hyla arborea japonica*,

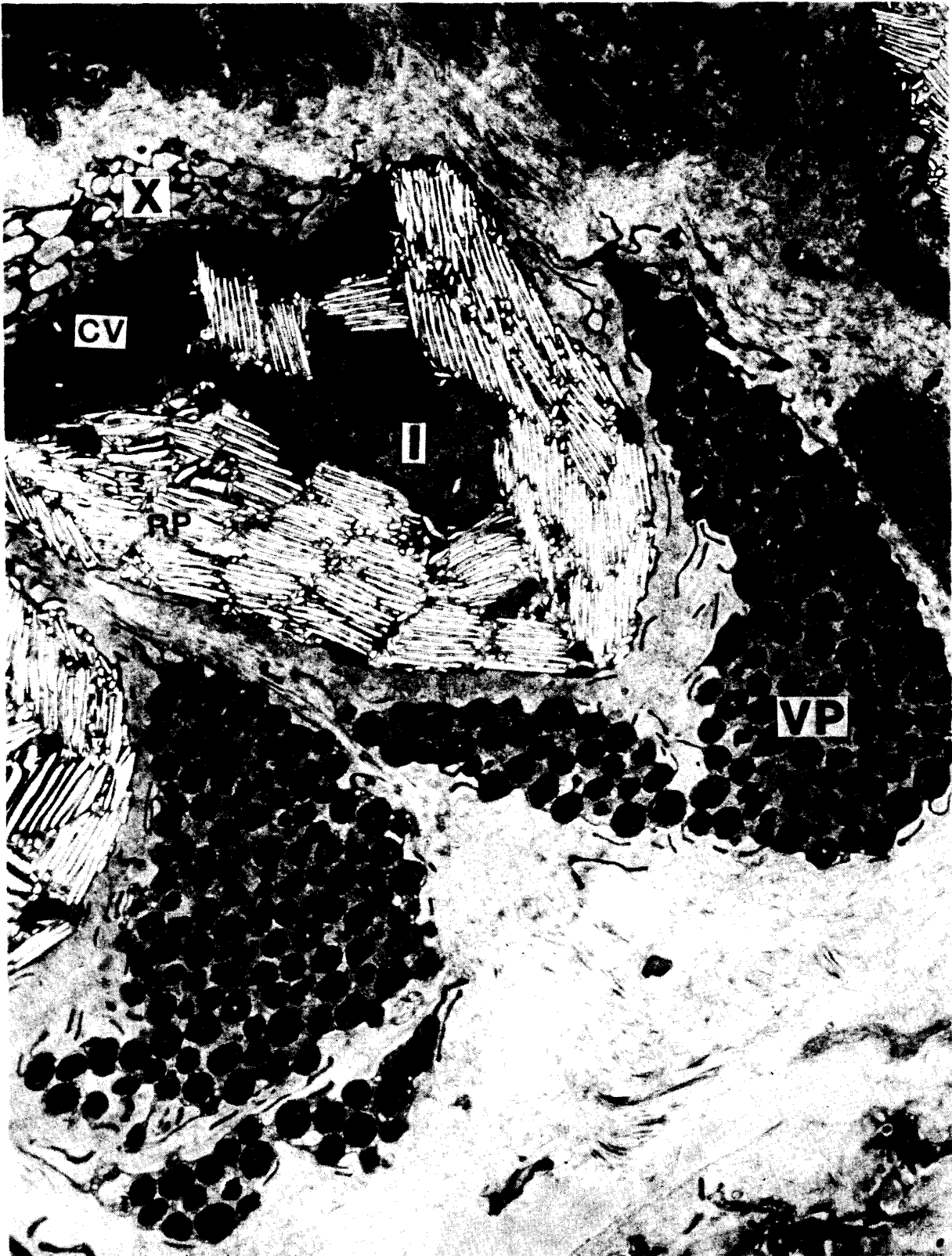


Fig. 1. Electron microphotograph of dermal chromatophores in the dorsal skin of a mature wild-type *Rhacophorus schlegelii*. × 7000

X, xanthophore I, iridophore VP, violephore CV, carotenoid vesicles
RP, reflecting platelets

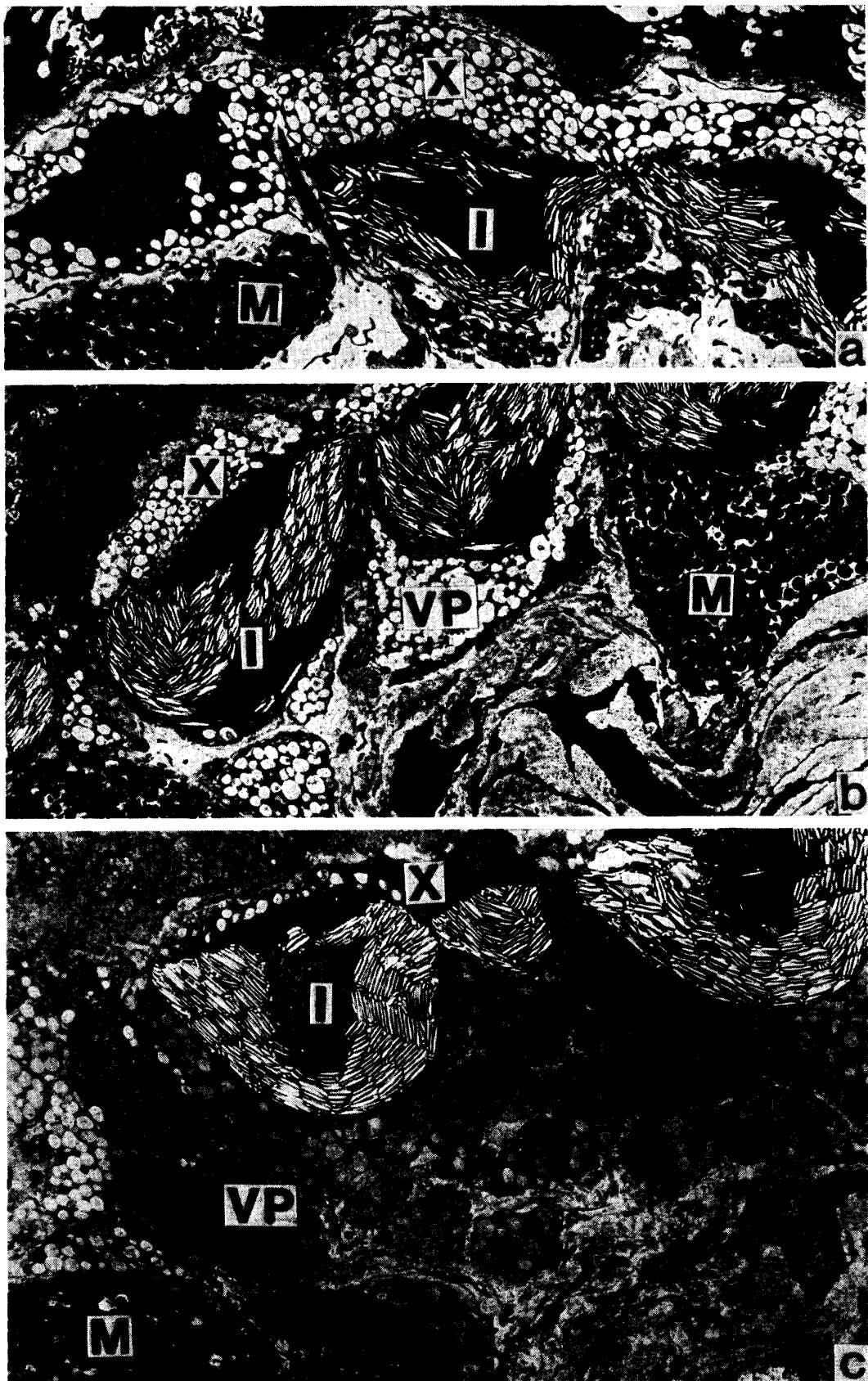


Fig. 2.

they can not change into other than dark green that occurs after adaptation for a long time to a dark environment. However, when the dermal chromatophores are examined under an electron microscope, they are similar to those of *Hyla arborea japonica* in that they form dermal chromatophore units coined by BAGNARA, TAYLOR and HADLEY (1968). Each of these units consists of a xanthophore, an iridophore and a violeophore in place of a melanophore (Fig. 1).

In juvenile frogs (stage XXV) immediately after metamorphosis, the dorsal surface is dull green. The dermal chromatophores consist of xanthophores, iridophores and melanophores which are arranged into three layers, upper, middle and lower, as those of *Rana nigromaculata* and *Hyla arborea japonica* (Fig. 2a). Thereafter, a part of xanthophores begin to fall into the spaces between iridophores and melanophores and are separated from the upper xanthophores which are still situated in their original place. With the development of lower xanthophores, the melanophores begin to degenerate. At the same time, the pterinosomes contained in the upper xanthophores become ill-developed. In froglets which are one month after metamorphosis and about 20 mm in body length, the dermal chromatophores of the dorsal skin are divided into four kinds, xanthophores, iridophores, mother cells of violeophores and melanophores (Fig. 2b). Two months later when the froglets are about 30 mm in body length, the upper xanthophores are very thin and the melanophores are mostly degenerated. The remnants of degenerating melanophores are found here and there. In the lower xanthophores which have already developed into a thick layer covering the undersurface of each iridophore, the pterinosomes begin to develop a lamellar structure (Fig. 2c). These xanthophores become to appear light violet under a light microscope. When the frogs grow larger, the dermal melanophores completely degenerate, while the lamellar structure of the pterinosomes is gradually developed and increases in electron density. The lower xanthophores eventually become violeophores like those found in adult frogs (Plate I, 7, 8).

a. Xanthophore

The xanthophores of *Rhacophorus schlegelii* are flat cells, being about 10 μ in width and 3 μ in thickness. They are remarkably thin as compared with those of *Rana nigromaculata*, *Hyla arborea japonica* and *Bombina orientalis*. Each xanthophore contains a very few carotenoid vesicles which are almost spherical and about 0.3 μ in the largest diameter. Pterinosomes are spheroidal and about 0.6 μ in the largest diameter. The inside of most pterinosomes is of a faint concentric, lamellar structure, while no lamellar structure is observable in some pterinosomes.

Fig. 2. Electron microphotographs of dermal chromatophores in the dorsal skins of immature wild-type *Rhacophorus schlegelii*. × 3000

- a. Froglet (stage XXV) immediately after metamorphosis.
 - b. Froglet, being about 20 mm in body length. One month after metamorphosis.
 - c. Froglet, being about 30 mm in body length. Two months after metamorphosis.
- X, xanthophore I, iridophore M, melanophore VP, violeophore

Pterinosomes are evenly distributed in xanthophores.

b. Iridophore

The iridophores of this species are truncated cones in shape, being about 14μ in width and 9μ in thickness. They are located immediately beneath the xanthophores and adjoin the latter with their wide surfaces. They are aligned at intervals of $0.5 \sim 4 \mu$. In the center of each iridophore, there is a large nucleus of irregular shape. The nucleus is surrounded with numerous bundles of reflecting platelets. Each bundle is principally arranged in parallel with the wall of the iridophore. Reflecting platelets are usually rods in shape, being about 1.6μ in length and 0.08μ in width, and aligned at intervals of about $0.05 \sim 1.0 \mu$. In addition to these rod-shaped reflecting platelets, there are a few small cuboidal or irregularly shaped ones.

While the cytoplasm of each iridophore is mostly filled with reflecting platelets, its upper part along the undersurface of a xanthophore is almost exclusively occupied by a distinct layer of carotenoid vesicles which is usually $2 \sim 2.5 \mu$ in thickness. This layer is developed upon the reflecting platelets arranged regularly in each iridophore and compensates for a defective function of xanthophores, in contrast to the situation found in the frogs such as *Rana nigromaculata*, *Rana brevipoda* and *Hyla arborea japonica*. The carotenoid vesicles are somewhat larger

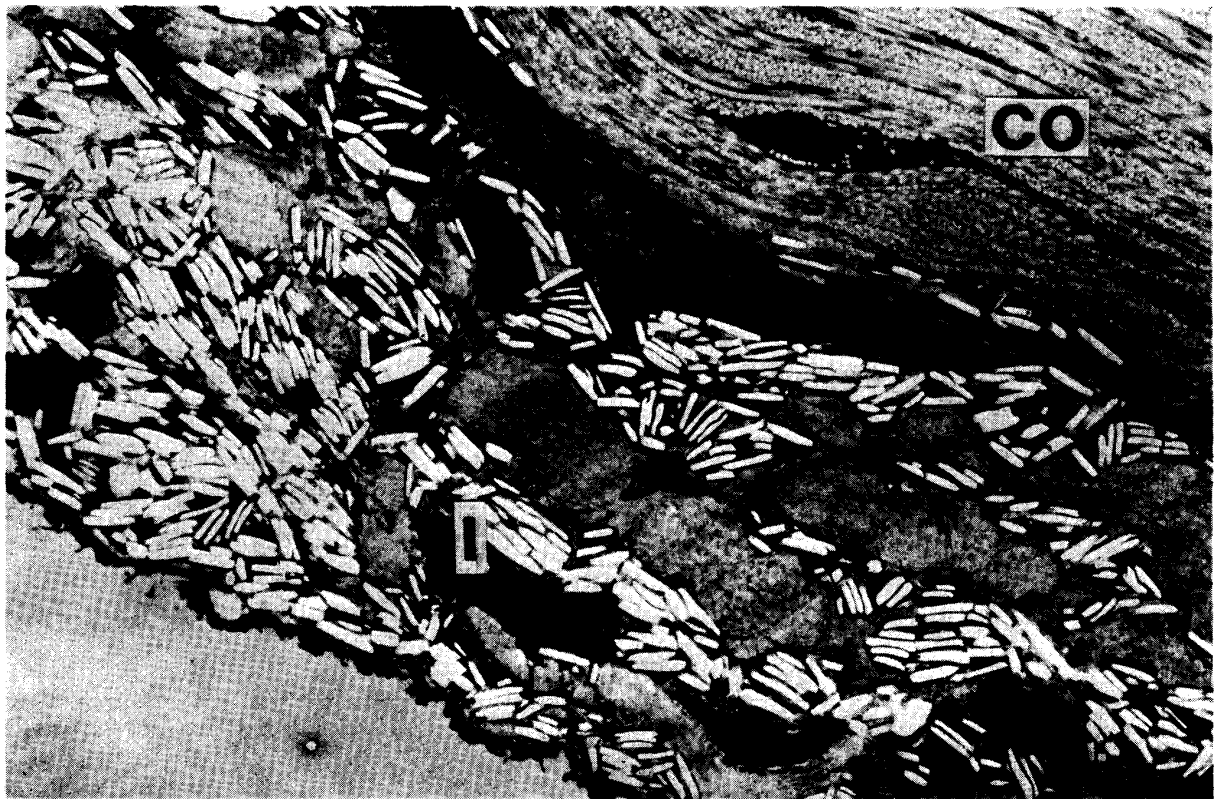


Fig. 3. Electron microphotograph of subcutaneous iridophores located beneath the dorsal skin in a wild-type *Rhacophorus schlegelii*. × 6000

CO, collagenous fibers

I, iridophore

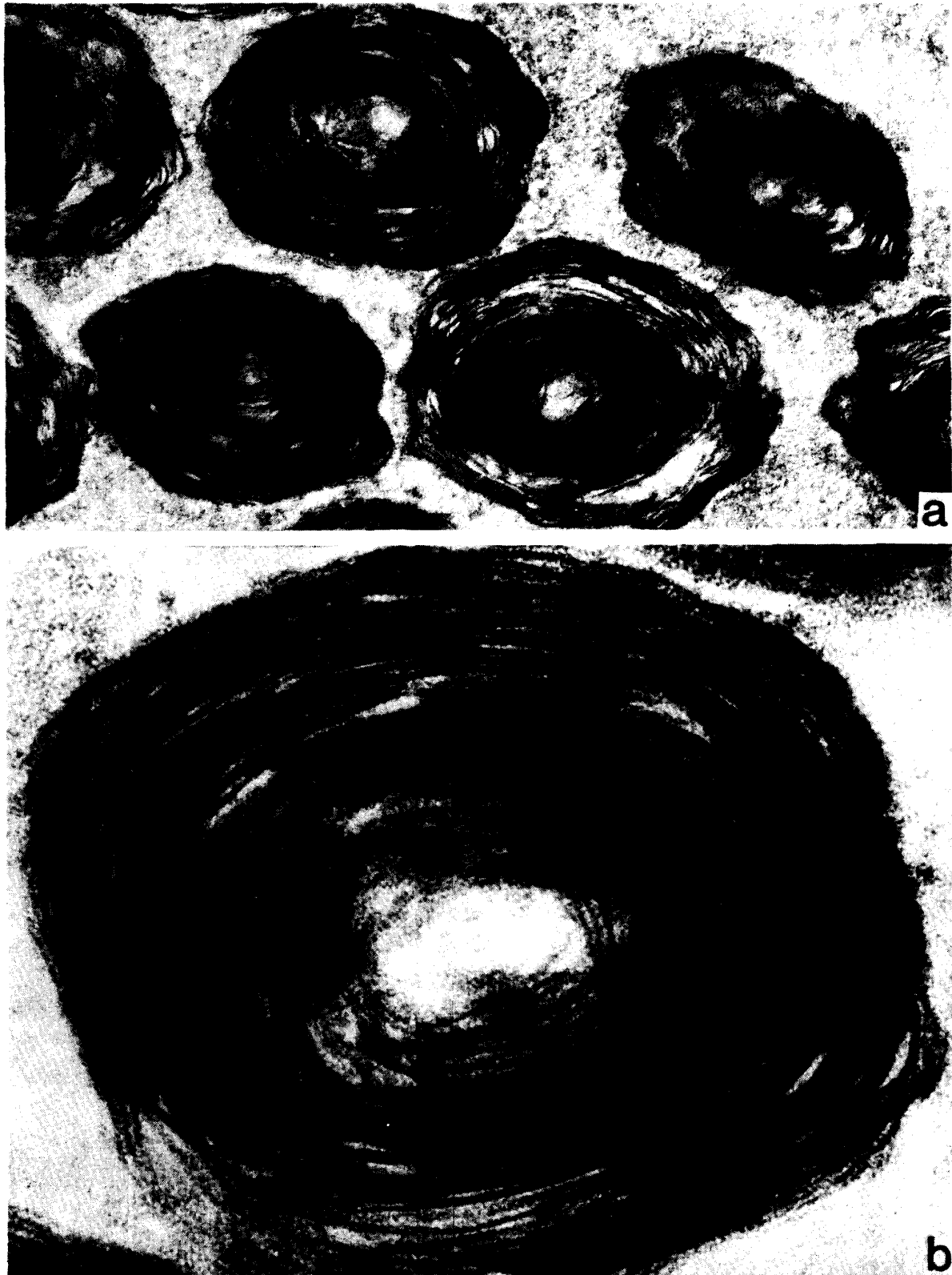


Fig. 4. Electron microphotographs of violeopterinosomes contained in a violeophore in the dorsal skin of a mature wild-type *Rhacophorus schlegelii*.

a. Violeopterinosomes.

× 64000

b. Violeopterinosome.

× 192000

than those contained in xanthophores; they are about 0.5μ in diameter.

The subcutaneous tissue located beneath the dorsal skin has another type of iridophores. These iridophores differ in shape and arrangement from the dermal iridophores described above. They are ramified flat cells filled with reflecting platelets. In contrast to the reflecting platelets contained in dermal iridophores, those of subcutaneous iridophores are very irregular in length and remarkably thick. They are about $0.4 \sim 2.2 \mu$ in length and usually about 0.3μ in width. They are not so regularly arranged as those found in the dermal iridophores; they scarcely form regular bundles. In subcutaneous tissue, three or four rows of iridophores constitute a reflecting layer, being about $12 \sim 25 \mu$ in thickness. These iridophores appear to be held between branches of loose collagenous tissue. A similar reflecting layer of subcutaneous iridophores is also found under the ventral skin (Fig. 3).

c. Melanophore

The dermal melanophores of *Rhacophorus schlegelii* are situated under the iridophores in froglets immediately after metamorphosis (stage XXV), and filled with completed melanosomes, just as those of *Rana nigromaculata* and *Hyla arborea japonica*. With the growth of the froglets, the melanophores gradually degenerate. In froglets, 20~30 mm in body length, degenerating melanophores still surround the iridophores. In adult frogs, the melanophores have completely degenerated. The normal melanophores of the froglets immediately after metamorphosis as well as the degenerating melanophores of the froglets at later stages are all filled with accomplished melanosomes and contain no premelanosomes (Fig. 2a).

d. Violeophore

While melanophores have completely degenerated among the dermal chromatophores of adult *Rhacophorus schlegelii*, violeophores which are filled with dark-violet granules are arranged at the situation where melanophores are found in *Rana nigromaculata* or *Hyla arborea japonica*. They are substituted for melanophores in the green species of the genus *Rhacophorus*.

The dermal violeophores of *Rhacophorus schlegelii* are remarkably ramified cells filled with violet-black granules (violeopterinosomes). They thickly cover the lower parts of iridophores with their processes. The violeopterinosomes are almost spherical. While most of them are about 0.8μ in diameter, the largest is about 1.2μ . The violeopterinosomes reveal a clear-cut concentric structure of thin lamellae (Fig. 4). Each lamella appears to be constructed of innumerable dark corpuscles arranged in a layer. Most of the violeopterinosomes have a pale central space that is about 0.1μ in diameter, while a small number of them consist of concentric lamellae throughout. It is evident that the violeopterinosomes have been derived from pterinosomes of the lower xanthophores.

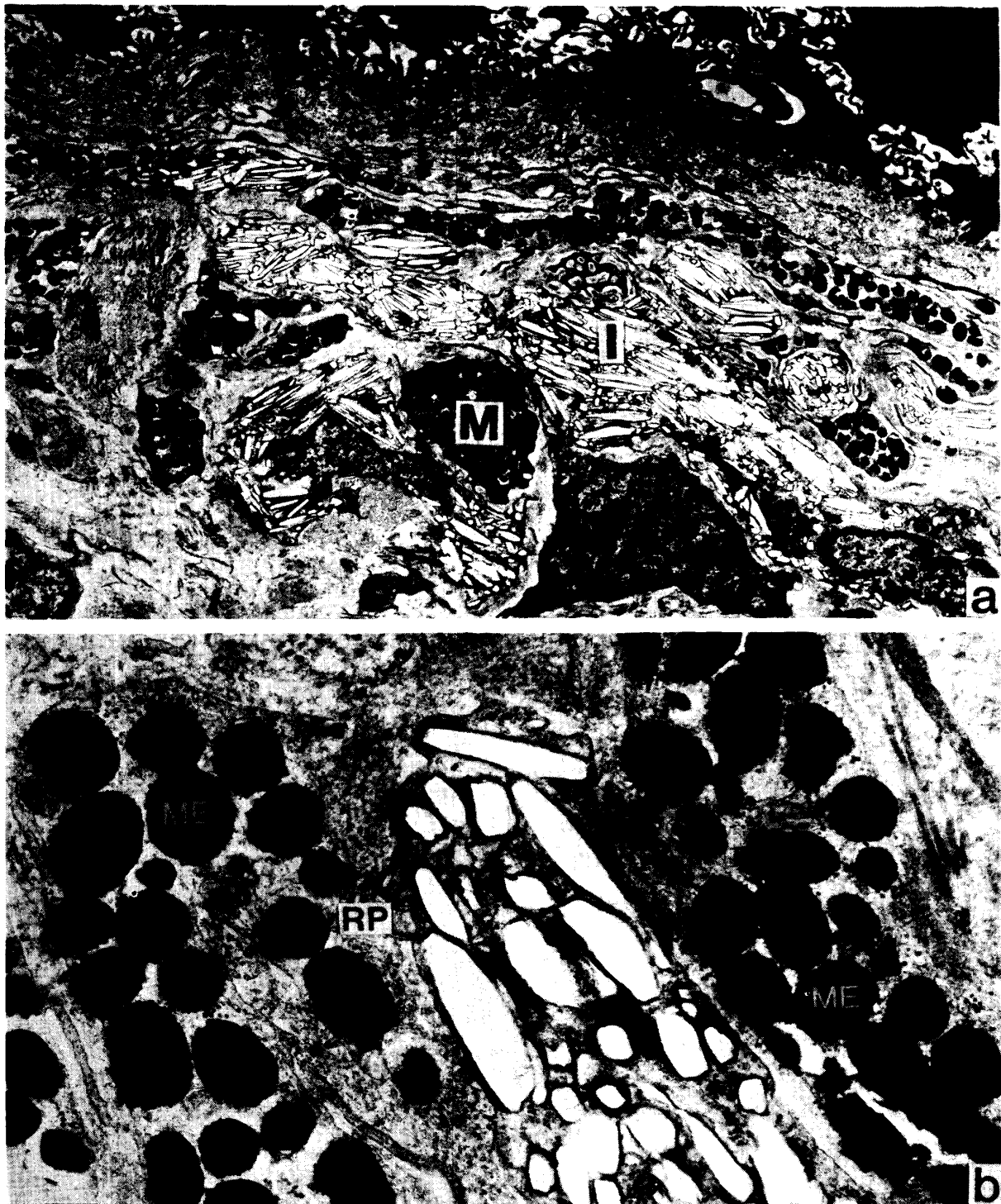


Fig. 5. Electron microphotographs of dermal chromatophores in the flank of a mature wild-type *Rhacophorus schlegelii*.

- a. Cross-section of the side skin. × 4000
b. Two kinds of chromatophores. × 22000
I, iridophore M, melanophore RP, reflecting platelets ME, melanosomes

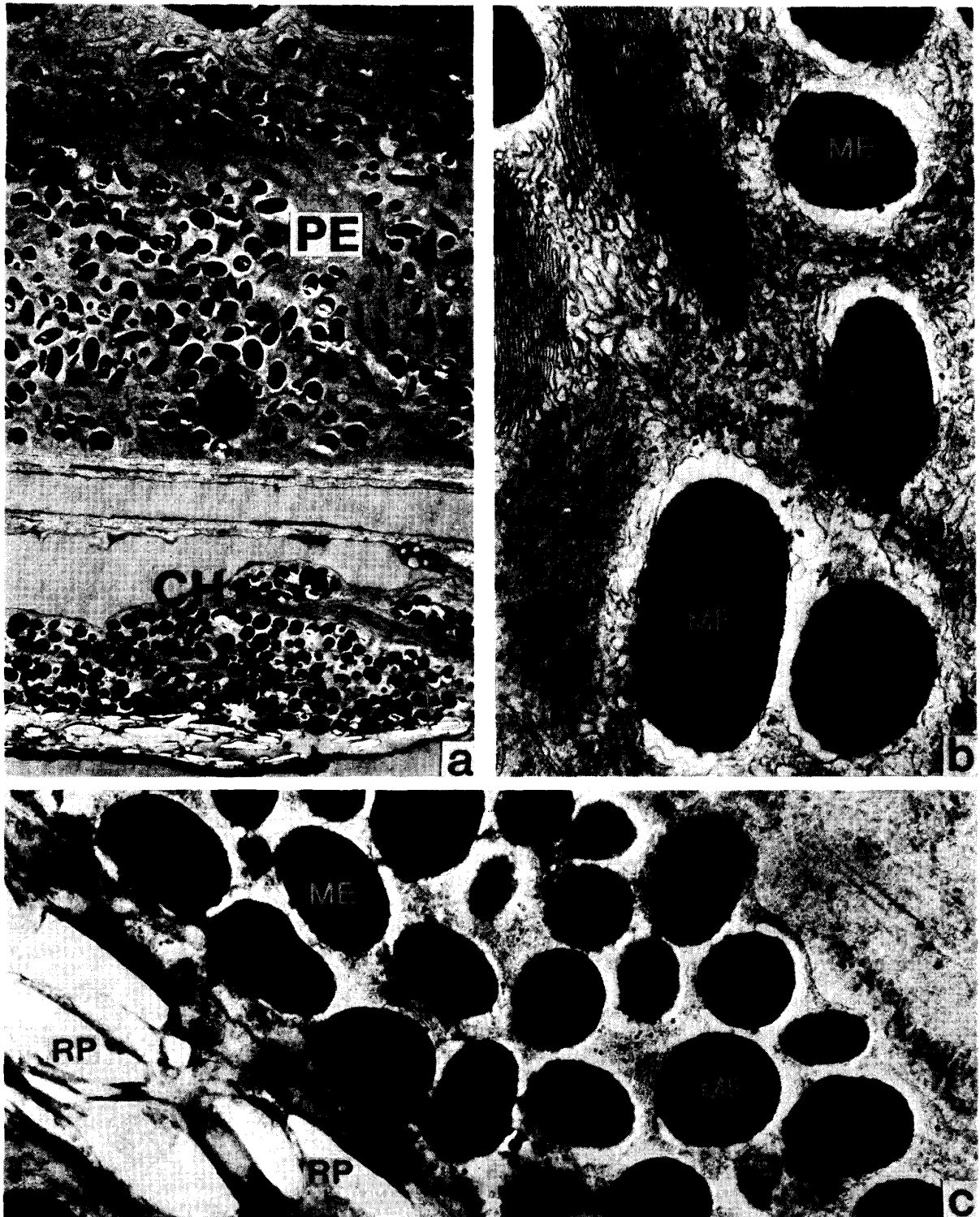


Fig. 6. Electron microphotographs of pigment cells in the eye of a mature wild-type *Rhacophorus schlegelii*.

a. Pigment epithelium and the choroid.

× 3000

b. A part of the pigment epithelium.

× 33000

c. A part of the choroid.

× 22000

PE, pigment epithelium

CH, choroid

ME, melanosomes

RP, reflecting platelets

2. Dermal chromatophores of the flank

On the flank of *Rhacophorus schlegelii*, the green area extending from the back is bordered by a white line, under which there are many irregularly shaped, brown-black spots on a white area. In a cross-section of the skin of the white line, several layers of iridophores with thick dendritic processes are stratified in the uppermost part of the dermis directly under the basal lamina. The reflecting platelets included in these iridophores are irregularly arranged. No carotenoid vesicles are contained in the iridophores.

In a cross-section of a spotted area of the flank, there are many melanophores with long dendritic processes together with iridophore layers. The processes of both kinds of chromatophores are irregularly and complicatedly intertwined with each other. Thus, melanophores are arranged in the uppermost part of the dermis in some cases, while in the other cases iridophores are situated in this part of the dermis. The melanosomes are mostly ellipsoids. When 20 melanosomes were measured, they were 0.55μ and 0.42μ on the average in major and minor axes, respectively (Fig. 5a, b).

In the skin of the flank, there are no violeophores in both white and spotted areas in contrast to the green area of the back. However, there are thick layers of iridophores stretched transversely in the deepest part of the dermis as found in the dorsal skin.

3. Pigment cells in the eyeball

In the pigment epithelial cells of the retina, abundant melanosomes are crowded around the rod cells. Some of the melanosomes are ellipsoids near the spheres. When 15 of them were measured, they were 0.75μ and 0.60μ on the average in major and minor axes, respectively. Some other melanosomes are long ellipsoids. When 15 of them were measured, they were 1.24μ and 0.49μ on the average in major and minor axes, respectively. However, the longest melanosomes attained 2.2μ in major axis (Fig. 6a, b).

In the choroid, melanophores constitute a thick layer in the area near the pigment epithelium. The melanosomes included in these melanophores are all spheroids and uniformly distributed in each cell. When 15 of them were measured, they were 0.65μ and 0.55μ on the average in major and minor axes, respectively. A few iridophores are included together with abundant melanophores in the choroid (Fig. 6a, c).

II. Albino

Albinos are due to a single recessive gene in the homozygous condition. When a female albino was mated with a male albino, the offspring were all albinos. When a female albino was mated with a wild-type male, all the offspring were of the wild-type.

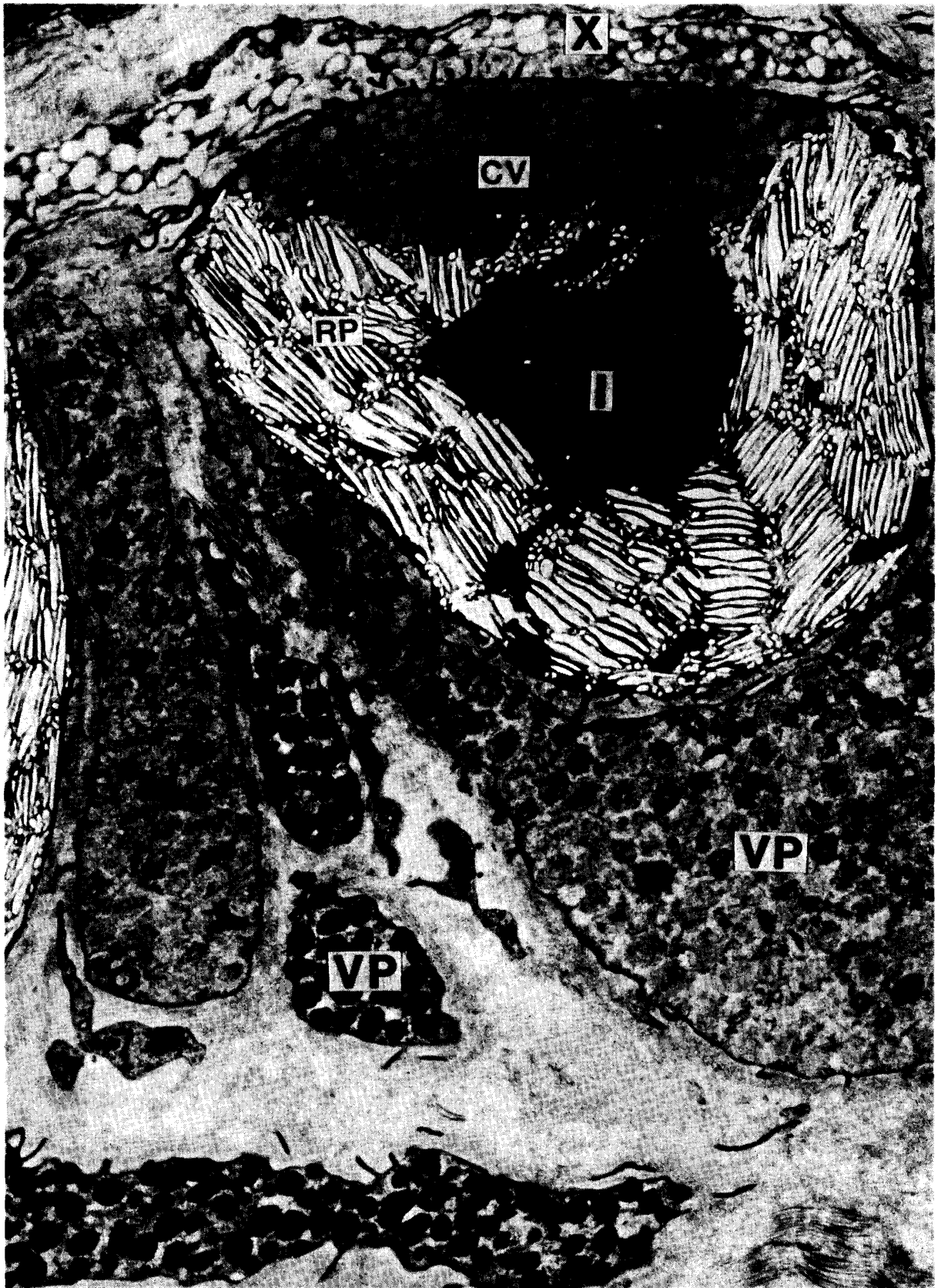


Fig. 7.

1. Dermal chromatophores of the dorsal skin

This kind of color mutants is orange yellow in dorsal coloration and has red pupils from the tadpole stage through completion of metamorphosis, like usual albinos. However, this dorsal color becomes gradually tinged with green with the growth of froglets and eventually changes into yellowish green. When this color is compared with that of the wild-type frogs at the mature stage, it is a little more yellowish than the latter. Moreover, there are a few small yellow areas on the back (Plate I, 3, 4). The pupils are always red. When this color mutant is adapted to a dark environment for a long time, the dorsal color becomes dull yellowish-green.

When the dorsal skin of the albinic frogs is observed under an electron microscope, the dermal xanthophores and iridophores as well as the subcutaneous iridophores do not differ from those of wild-type frogs (Fig. 7).

Small and thin melanophores are found in the dermis of froglets until one to two months after metamorphosis, being about 30 mm in body length. In these melanophores, a few small premelanosomes at the earliest stage of differentiation are included. They are 0.3μ and 0.2μ on the average in major and minor axes, respectively. No completed melanosomes are found anywhere. The melanophores gradually degenerate with the growth of froglets. In mature frogs, there are no dermal melanophores in the dorsal skin (Figs. 8, 9).

Violeophores having violet-black pigment granules (violeopterinosomes) are not observable in froglets immediately after metamorphosis. With the growth of froglets, the dermal xanthophores gradually increase and enter into the area between iridophores and melanophores. When froglets attain a body length of about 30 mm, the pterinosomes contained in the xanthophores situated now under the iridophores begin to reveal a lamellar structure consisting of electron-dense granules and gradually become violeopterinosomes. The orange yellow of the dorsal surface of froglets is gradually tinged with green. In proportion to the degree of green tinge, the violeopterinosomes having an electron-dense lamellar structure increase in number.

The dorsal green color of mature albinos is paler than that of the mature wild-type frogs. The dermal violeophores in the latter are almost filled with completed violeopterinosomes, all of which reveal an electron-dense lamellar structure. In contrast, about half the number of the dermal violeophores in mature albinos are incomplete in differentiation, while the others are almost the same as those found in the wild-type frogs. The incomplete violeophores are similar to those found in one- to two-month-old wild-type froglets, being 20~30 mm in body length.

Fig. 7. Electron microphotograph of dermal chromatophores in the dorsal skin of a mature albino.

× 7000

X, xanthophore I, iridophore VP, violeophore CV, carotenoid vesicles
 RP, reflecting platelets

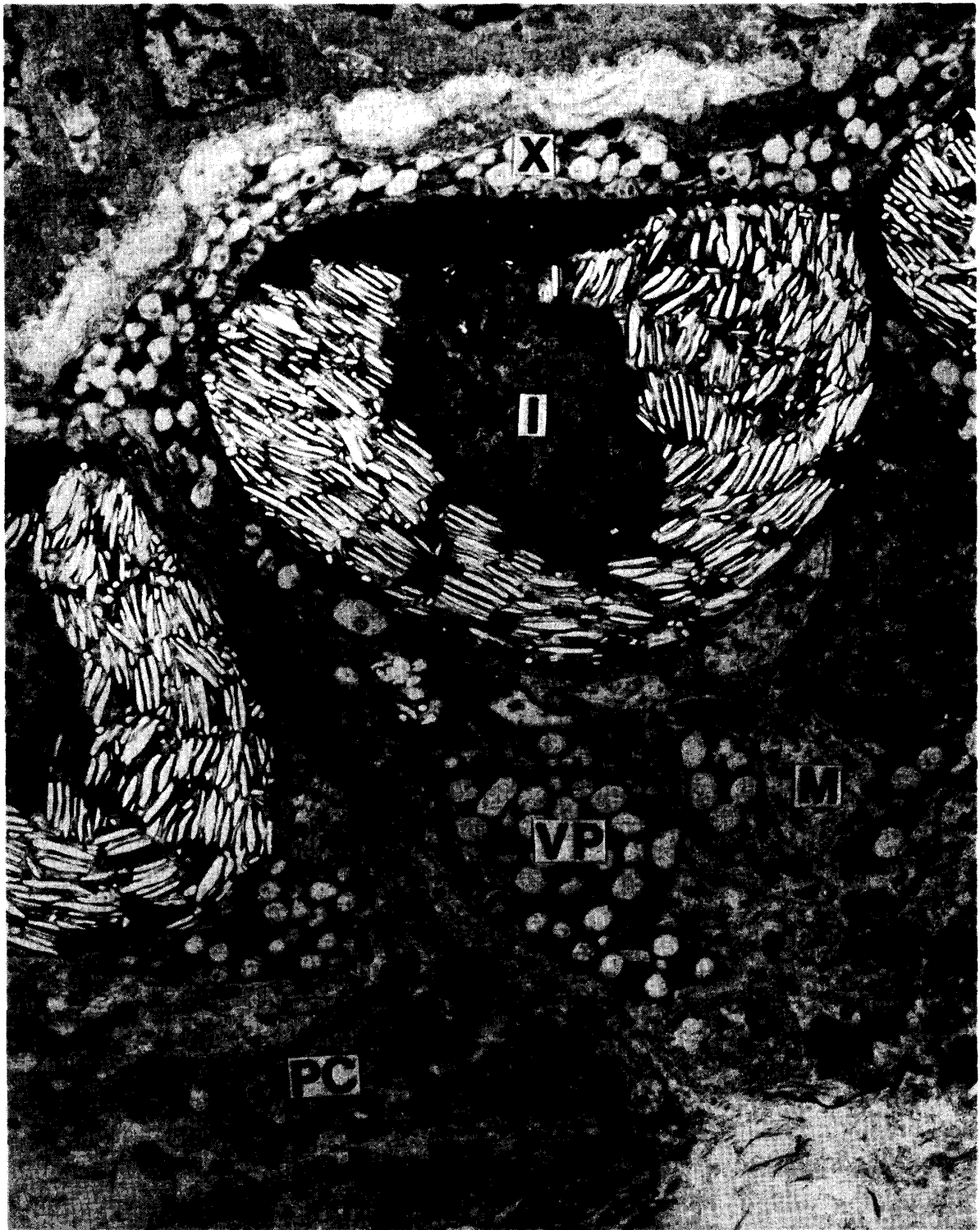


Fig. 8. Electron microphotograph of dermal chromatophores in the dorsal skin of an albinic froglet, being about 30 mm in body length. Two months after metamorphosis. × 7000

X, xanthophore I, iridophore VP, violeophore M, melanophore PC, phagocyte

The violeophores of albinos show very characteristic features in formation of violeopterinosomes. As stated above, there are two types of dermal violeophores, complete and incomplete. The former type is filled with complete violeopteri-

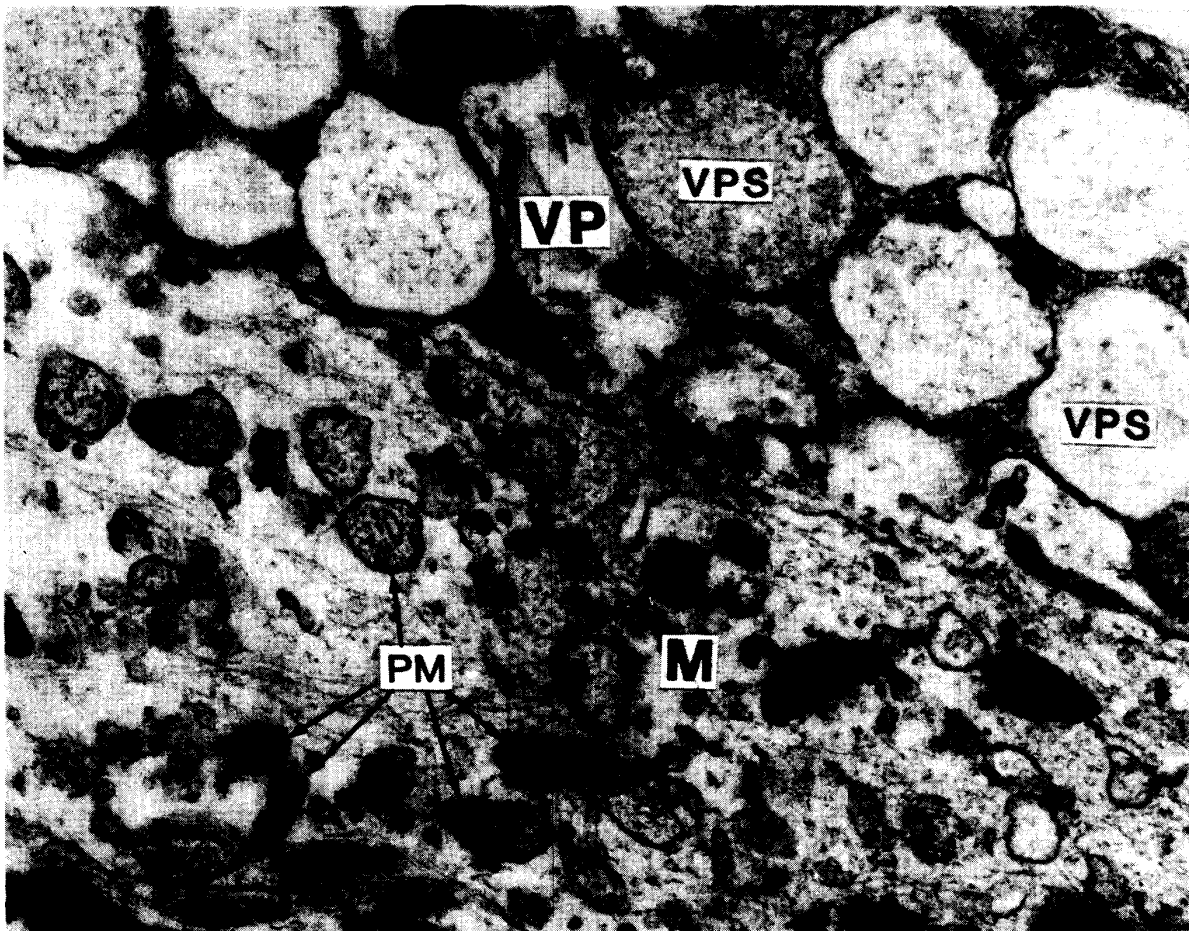


Fig. 9. Electron microphotograph of dermal chromatophores in the dorsal skin of the same albino as shown in Fig. 8. × 33000

VP, violephore M, melanophore VPS, violepterinosomes PM, premelanosomes

nosomes, while the latter contains numerous pterinosomes at various differentiation stages in addition to a few incomplete violepterinosomes. The dermal iridophores are surrounded by arms of the incomplete violephores. The complete violephores are always located under the incomplete ones.

In the incomplete violephores, the transforming process of violepterinosomes from pterinosomes is observable. This process is divided into four stages for convenience sake.

Stage I. Pterinosomes at this earliest stage are equal to the complete violepterinosomes in size and shape and stained paler than the surrounding stroma. Their inside consists of a loose reticular structure and is enveloped by a limiting membrane. There is no indication of the lamellar or concentric structure as found in complete violepterinosomes (Fig. 10a).

Stage II. Pterinosomes are stained almost similarly to the surrounding stroma. The loose reticular structure found at stage I begins to change into a concentric structure. While some of them have a somewhat compact and dark center, the others have an almost

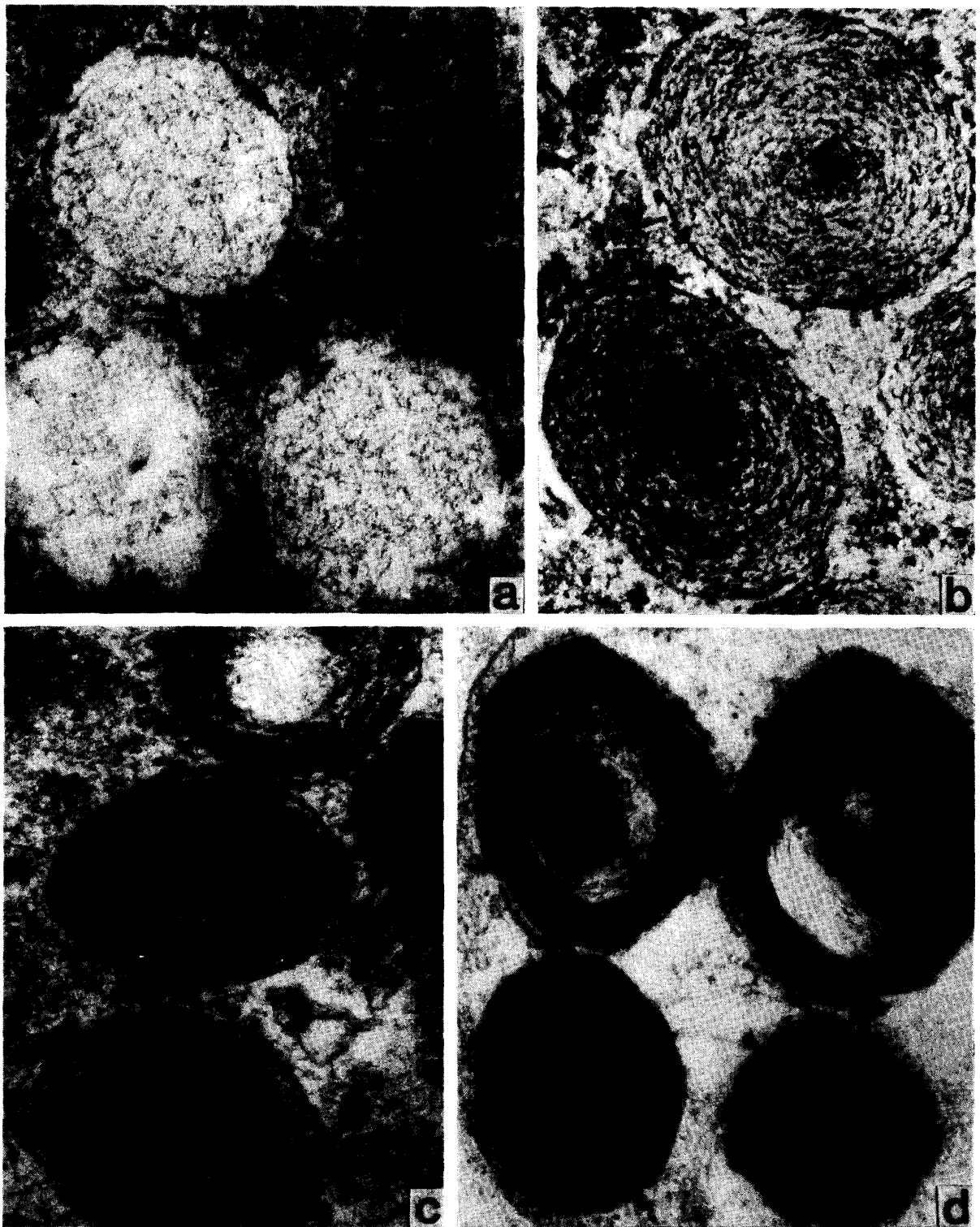


Fig. 10. Developmental stages of violeopterinosomes.

× 64000

a. Stage I.

b. Stage II.

c. Stage III.

d. Stage IV.

pale center without any special structure (Fig. 10b).

Stage III. In the concentric structure of pterinosomes, compact lamellae begin to appear. At the same time, the pterinosomes begin to

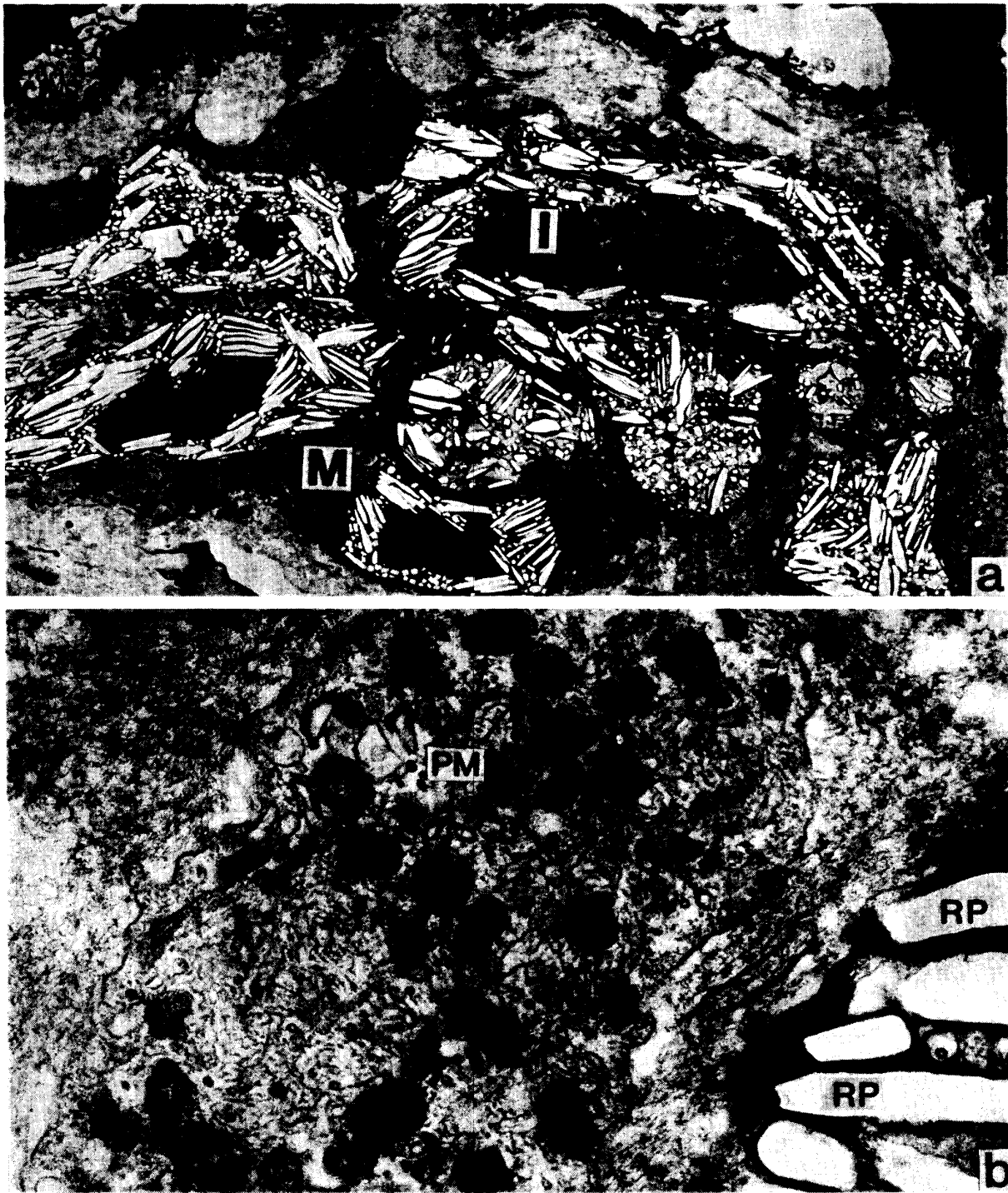


Fig. 11. Electron microphotographs of dermal chromatophores in the flank of a mature albino.

a. Cross-section of the side skin.

× 4000

b. Two kinds of chromatophores.

× 33000

I, iridophore M, melanophore RP, reflecting platelets PM, premelanosomes

change into incomplete violeopterinosomes (Fig. 10c).

Stage IV. Violeopterinosomes contained in complete violeophores are the same as those of the wild-type frogs in shape, size and structure (Fig. 10d).

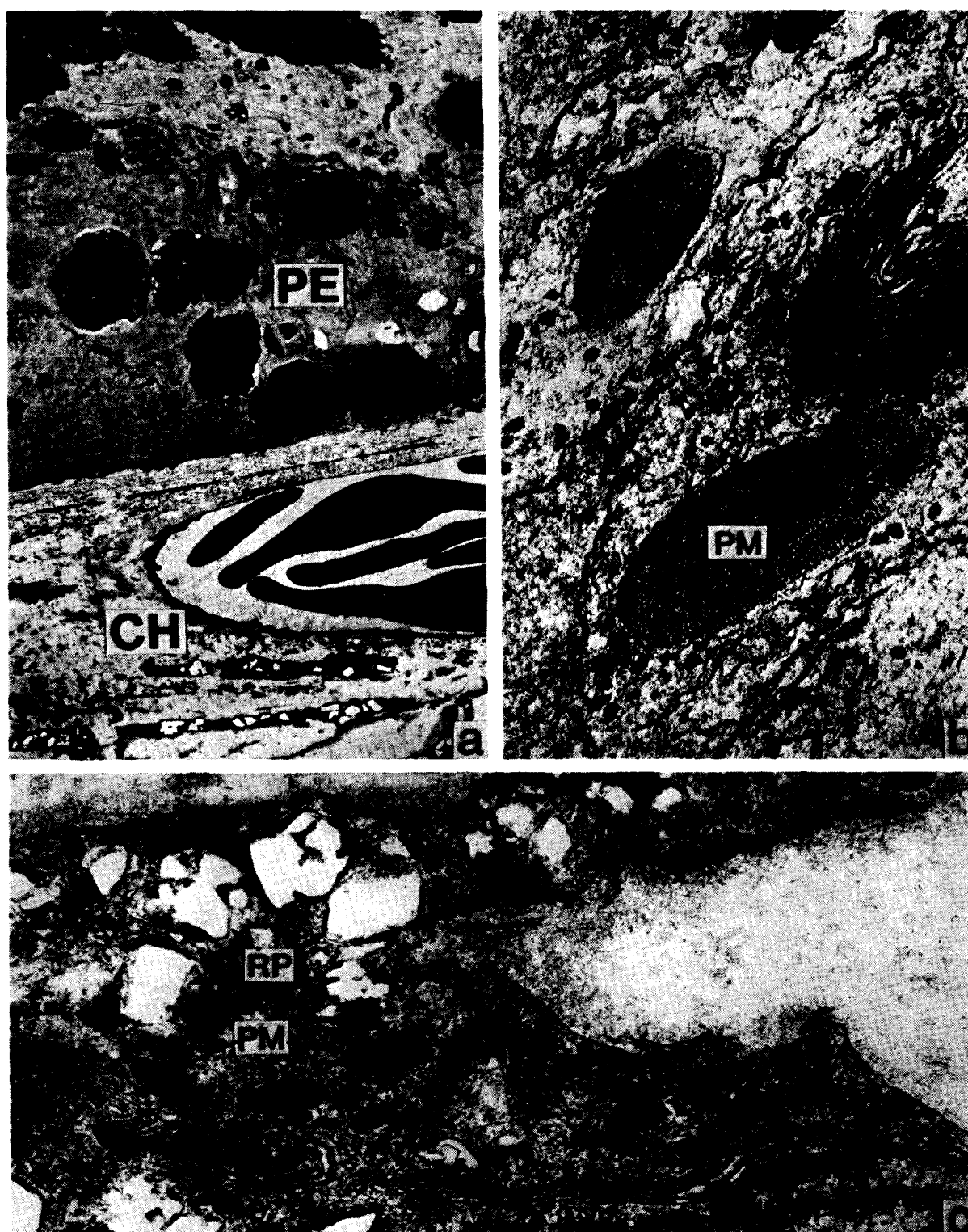


Fig. 12. Electron microphotographs of pigment cells in the eye of a mature albino.

- | | |
|--|---------|
| a. Pigment epithelium and the choroid. | × 3000 |
| b. A part of the pigment epithelium. | × 66000 |
| c. A part of the choroid. | × 22000 |
- PE, pigment epithelium CH, choroid PM, premelanosomes RP, reflecting platelets

2. Dermal chromatophores of the flank

The flank of mature albinic *Rhacophorus schlegelii* has no brown-black spots, although there is a white area bordering on the upper green area. When observed under an electron microscope, the kind of chromatophores and their arrangement in the white area are the same as those of the wild-type frogs (Fig. 11a). In the position where melanophores are situated in a cross-section of the spotted skin of the wild-type frogs, there are abnormal melanophores containing premelanosomes. The dendritic processes of these abnormal melanophores are always found among dendritic processes of iridophores. When 18 premelanosomes were measured, they were 0.26μ and 0.15μ on the average in major and minor axes, respectively. It was found that they were less than half the size of the melanosomes found in the wild-type frogs (Fig. 11b).

3. Pigment cells in the eyeball

Melanosomes are found neither in the pigment epithelium of the retina nor in the choroid of the eyeballs of albinic *Rhacophorus schlegelii*. In the pigment epithelial cells and the choroidal melanophores, there are premelanosomes whose inside seems to be of stripe or fretwork structure (Fig. 12a).

Although a very few premelanosomes are contained in the pigment epithelial cells of the retina, they are always found in the cytoplasm bordering on the rod cells, as in the wild-type frogs. While some of them are nearly spheres, the others are long ellipsoids. When 12 of the former premelanosomes were measured, they were 0.49μ and 0.40μ on the average in major and minor axes, respectively. When 12 of the latter premelanosomes were measured, they were 0.68μ and 0.25μ in major and minor axes, respectively. It was found that these premelanosomes are distinctly smaller than the melanosomes contained in the pigment epithelial cells of the wild-type frogs (Fig. 12b).

A comparatively numerous premelanosomes are found in the melanophores of the choroid. They are usually ellipsoids. When 15 of them were measured, they were 0.38μ and 0.24μ on the average in major and minor axes, respectively. They were less than half the size of melanosomes in the choroid of the wild-type frogs. In the cytoplasm of choroidal melanophores, there are sometimes irregularly shaped holes which seem to be the same as those which lost reflecting platelets in iridophores (Fig. 12c).

III. Black-eyed mutant

Of the eight mature black-eyed variants, seven were females and one was a male, as previously stated. A mating experiment was conducted between the single male and one of the females. It was found that 14 fertilized eggs became feeding tadpoles and 13 of the latter metamorphosed normally and attained sexual maturity. All these offspring were black-eyed. It is evident that the black-eyed variants are mutants.

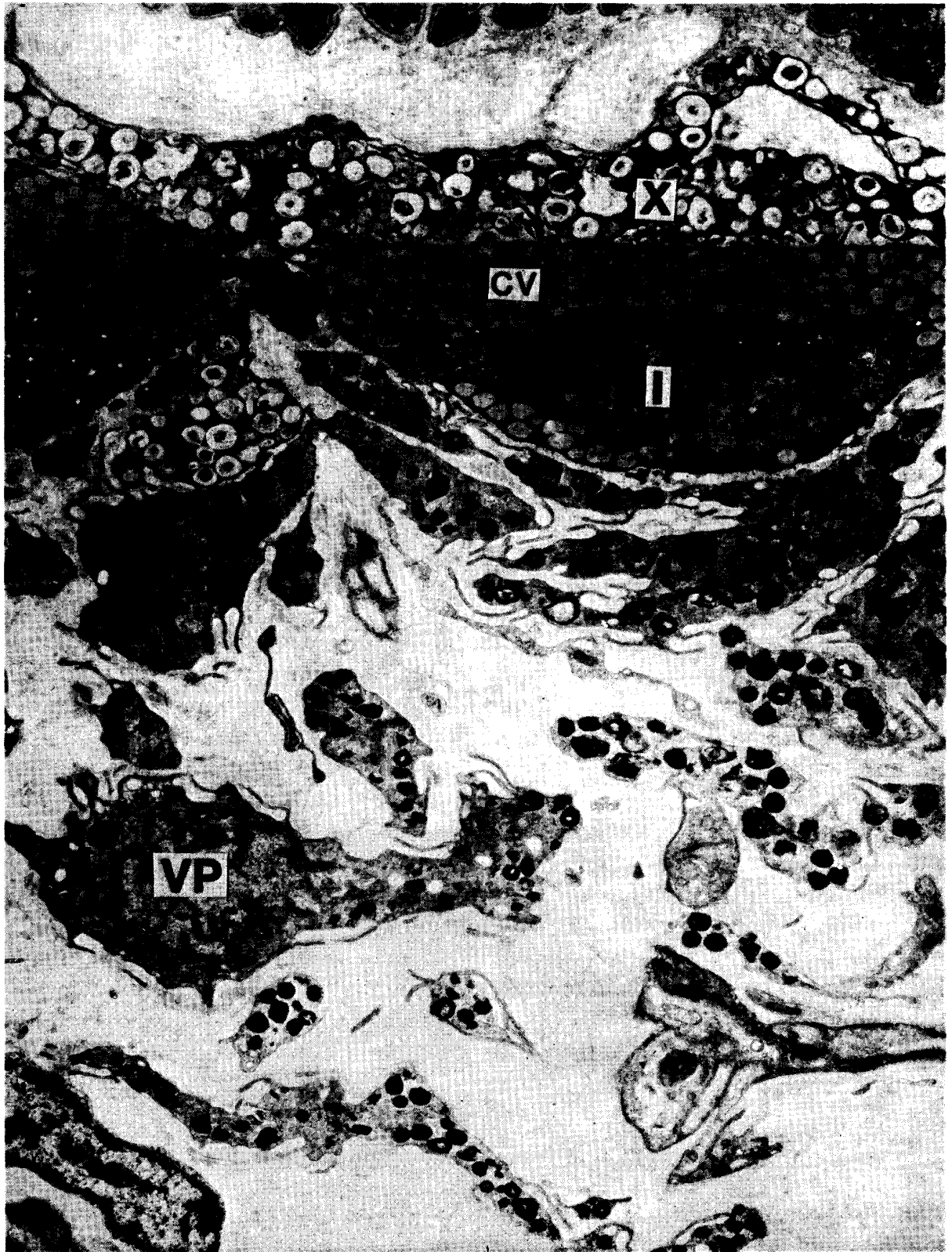


Fig. 13. Electron microphotograph of dermal chromatophores in the dorsal skin of a mature black-eyed mutant. × 7000

X, xanthophore I, iridophore CV, carotenoid vesicles VP, violcophore

This kind of mutants is very similar in appearance to the melanoid variant in *Rana pipiens* (RICHARDS, TARTOF and NACE, 1969) and the black-eyed variant in *Rana nigromaculata* (NISHIOKA, 1977). At the early tadpole stage, the whole body is dark and semitransparent. The skin has no metallic luster owing to abnormal iridophores. After metamorphosis the dorsal surface does not become green, but is usually dark brown even at the stage of sexual maturity, although those of the maternal frog and one of the 13 offspring have dull-yellow areas. As the iris is brownish black, it is not contrasted with the black pupil. The black-eyed mutants do not make color change. The visceral organs such as liver, intestine, oviducts and fat bodies are seen through the semitransparent ventral wall (Plate I, 5, 6). When the dorsal skin of these black-eyed mutants at the sexually mature stage is examined under an electron microscope, it is found that the dermal chromatophores consist of xanthophores, violeophores and very abnormal iridophores (Fig. 13). In the subcutaneous tissue, there is a layer of abnormal iridophores.

a. Xanthophore

The xanthophores of the black-eyed mutants are very similar to those of the above albinos and the wild-type frogs. They are about 12μ wide and 3μ thick. In each xanthophore, there are a mixture of carotenoid vesicles and pterinosomes, which are nearly the same in shape, size and number as those of the wild-type frogs. However, there are some pterinosomes which reveal a concentric structure more distinct than those of the wild-type frogs. In some cases, dendritic processes of xanthophores are extended under iridophores and laid among the processes of violeophores. It is remarkable that a few violeopterinosomes are contained together with many pterinosomes in these processes of xanthophores.

b. Iridophore

While iridophores are located beneath the xanthophores, they contain no reflecting platelets (Figs. 13, 14b). They are about 14μ wide and 6μ thick, being about two-thirds of the normal iridophores in thickness. The nucleus is elliptical in cross-section. The upper part of each iridophore is occupied with well-developed carotenoid vesicles as found in the iridophores of the above albinos and the wild-type frogs. In many iridophores, some carotenoid vesicles are also found in the lower part of each cell. In the areas where reflecting platelets should exist, some darkly stained granules, $0.06 \sim 0.14 \mu$ in diameter, and small vacuoles are found together with mitochondria and some other organelles. These darkly stained granules and small vacuoles seem to be abortive reflecting platelets (Fig. 14b). However, the dull-yellow areas in the dorsal surfaces of two mature black-eyed mutants seem to indicate the existence of iridophores containing reflecting platelets, just as the dull-yellow spots or flecks in the dorsal surfaces of black-eyed mutants of the Yc stock in *Hyla arborea japonica* (NISHIOKA and UEDA, 1985b).

No iridophores containing reflecting platelets are also found in the subcutaneous layers under the dorsal skin (Fig. 15).

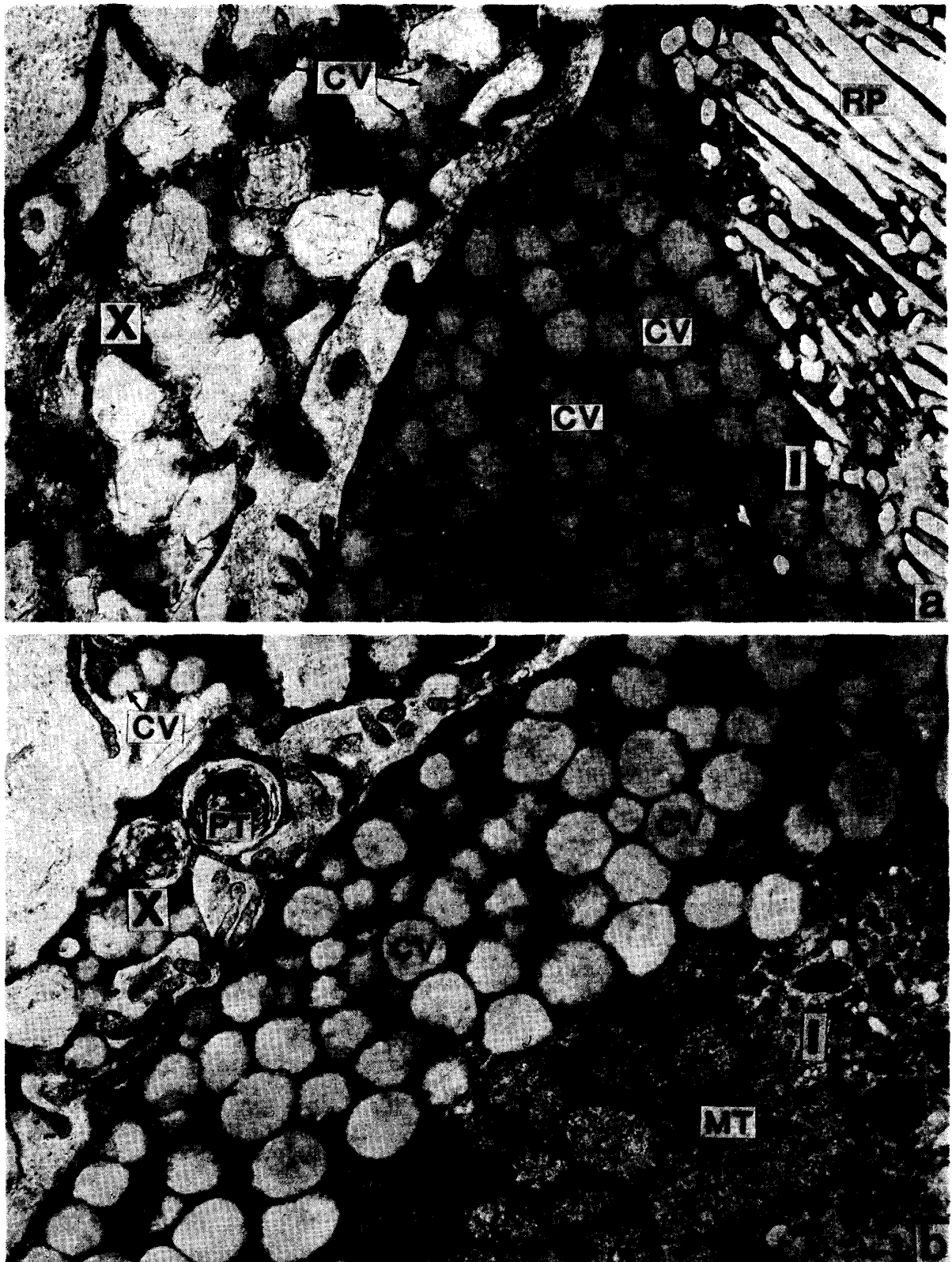


Fig. 14. Electron microphotographs of two kinds of dermal chromatophores in the dorsal skins of a wild-type frog and a black-eyed mutant. × 22000

a. Wild-type frog.

b. Black-eyed mutant.

X, xanthophore

I, iridophore

CV, carotenoid vesicles

RP, reflecting platelets

PT, pterinosomes

MT, mitochondria

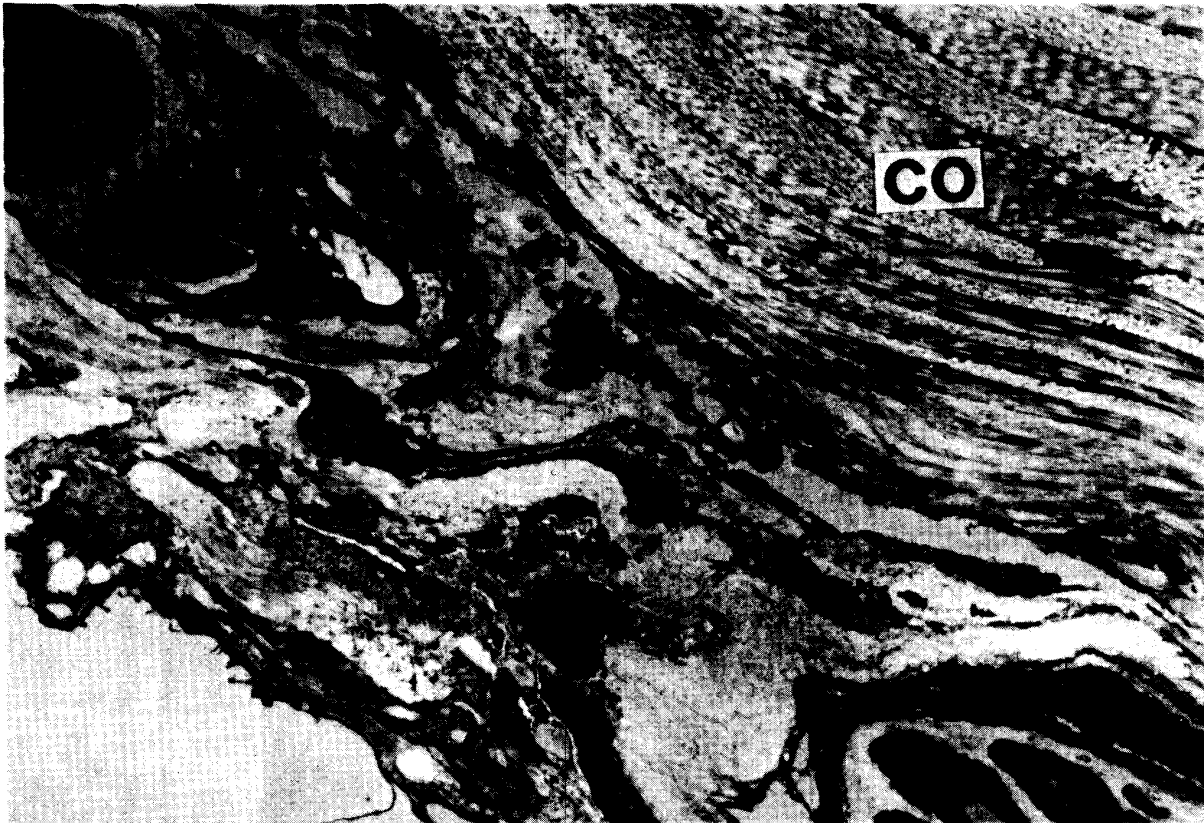


Fig. 15. Electron microphotograph of the subcutaneous layers under the dorsal skin of a black-eyed mutant. × 6000
CO, collagenous fibers



Fig. 16. Electron microphotograph of violepterinosomes contained in a violephore in the dorsal skin of a mature black-eyed mutant. × 64000

c. Melanophore

The melanophores in the black-eyed mutants at the juvenile frog stage are normal and the same as those in the wild-type frogs. In the dorsal skin, dermal melanophores which abundantly contain complete melanosomes are observable until two to three months after metamorphosis. However, all these melanophores have degenerated without leaving any trace in mature frogs.

In contrast, the dermal melanophores with complete melanosomes in the spotted area of the flank always exist as in the normal frogs. The pigment epithelial cells in the retina and the choroidal melanophores in the eyeball have also complete melanosomes as those of the wild-type frogs.

d. Violeophore

The violeophores of the black-eyed mutants have long dendritic processes extending horizontally in the range of 7~20 μ and cover the undersurfaces of iridophores, as those of the wild-type frogs. Although the violeopterinosomes are, roughly speaking, the same as those of the wild-type frogs, there is a comparatively large variety in size and structure. When their sizes are averaged, they are somewhat smaller than those of the wild-type frogs, being about 0.5 μ in diameter. While some violeopterinosomes are spherical, the others are distorted to various degrees. While many violeopterinosomes have a pale wide space in the center, some others have a small concentric mass in such a space. In a few cases, two or three violeopterinosomes are united to form a small mass. In most violeophores, there are usually many vacuoles which are considered to be the traces of degenerated violeopterinosomes (Figs. 13, 16).

IV. *Yellow variant*

The single yellow variant collected in 1972 was a mature female. The dorsal surface of this variant is bright reddish-yellow and has several small dark spots. The edges of the dorsal color on the lateral sides are bordered with a white line. On the flank under this white line, there are irregularly shaped, brown-black spots, as found in the wild-type frogs. In this variant, there are also similar spots on the undersurface of the lower jaw. The pupils are black and the irises are of golden color.

When the dorsal skin of the body was examined under a light microscope, no violeophores were observable, while the xanthophores and iridophores were normal. The presence of incomplete violeophores with colorless previoleopterinosomes was not determined, as the dermal chromatophores were not examined under an electron microscope. However, it seems evident that this variant could not become green for lack of the normal violeophores (Plate I, 2). There is no doubt that the irregularly shaped brown-black spots on the flanks consist of dermal melanophores.

The yellow female variant was mated with a wild-type male. It was found that 83 juvenile frogs produced from this mating were all of the wild-type. As the

present authors failed in obtaining the F₂ offspring, it was indeterminable whether the yellow variation is a genetic trait or not. It was only evident that this yellow variation is not due to a dominant gene. However, on the basis of the lack of one kind of dermal chromatophores, it is probable that this yellow variation is due to a single recessive gene in the homozygous condition.

V. Chemical nature of violet-black pigment

Violeophores are a kind of chromatophores which have originated from dermal xanthophores lying upon iridophores and taken the dermal melanophore's place after locomoting to the site under iridophores. Violeopterinosomes are considered to have changed in quality from pterinosomes, that is, the lamellar structure of the latter was developed further, increased in electron density and became violet.

Refrigerated sections of the dorsal skin with dermal chromatophores were made at 15 μ . A part of them were fixed in 10% formalin, while the other part were not fixed. When these sections were examined under a light microscope after addition of 1% NaOH solution, the violet-black pigment of violeophores was dissolved and the solution became red. When they were examined after addition of 2% NaOH solution, the pigment was dissolved within one hour. When peracetic acid was added to the sections, the pigment faded within four hours. The pigment was dissolved at once by addition of 32 N H₂SO₄. However, the violet-black pigment did not change within 24 hours by addition with 1% NH₄OH, 1 N HCl, 97% ethanol, acetone, ether, chloroform and toluene.

Recently, the violet-black pigment contained in the dorsal skin of *Rhacophorus arboreus*, a closely allied species of *Rhacophorus schlegelii*, has been examined by Dr. T. SUGA, Professor of Chemistry at the Department of Chemistry, Hiroshima University (private communication). According to him, this pigment is probably a new compound which is principally constituted from pterin-6-carboxylic acid. Thus, it is also probable that the violet-black pigment of *Rhacophorus schlegelii* is a similar compound.

DISCUSSION

1. Dermal chromatophores of wild-type frogs

The structure and function of dermal chromatophores in amphibians have been described in detail by BAGNARA (1966, 1976), BAGNARA, TAYLOR and HADLEY (1968) and BAGNARA and FERRIS (1974). BAGNARA, TAYLOR and HADLEY have insisted that three kinds of dermal chromatophores, xanthophores, iridophores and melanophores, in the dorsal skin of adult amphibians construct the dermal chromatophore units for rapid color changes. The light- and electron-microscopic structures of the dermal chromatophores in the dorsal skins of wild-type Japanese frogs, *Rana nigromaculata*, *Rana brevipoda porosa* and *Hyla arborea japonica* have been reported by the present authors (1977a, b, 1985a, b, c).

Of the dermal chromatophores, the xanthophores containing pterinosomes and carotenoid vesicles are located immediately below the basal lamina. Under the xanthophores, there are iridophores filled with reflecting platelets. The melanophores including melanosomes occupy the undermost site and extend their long dendritic processes upward and horizontally. HAMA and FUKUDA (1964) have found that the kind of pteridines in the adult xanthophores differs from species to species and that a striking change in chromatophore and pteridine pattern occurs at the time of metamorphosis.

In *Rhacophorus schlegelii*, *Rana japonica* and *Rana temporaria*, the pteridines contained in xanthophores of adults are the same as those of larvae (HAMA and FUKUDA, 1964). In the latter two species, erythrophores including red pigment granules are situated irregularly beneath the xanthophores. According to them, the red pigment is drosopterin. OBIKA and MATSUMOTO (1968) have confirmed that the erythrophores are very similar in structure to the xanthophores situated closely above them in the dorsal skin of *Rana japonica*. YASUTOMI and HAMA (1973) identified the red pigment granules in the erythrophores of the same species with drosopterinosomes which had been named by HAMA (1966) in a fresh-water fish. While the erythrophores contain red drosopterinosomes, the xanthophores have both yellow sepiapterinosomes and yellow carotenoid vesicles.

On the other hand, the existence of distinctive melanophores have been reported in the skins of the leaf frog subfamily, Phyllomedusinae, by TAYLOR and BAGNARA (1969), BAGNARA, TAYLOR and PROTA (1973), BAGNARA and FERRIS (1974) and BAGNARA, FERRIS and TAYLOR (1976). In contrast to the usual melanophores found in *Rana* and *Hyla*, they are wine red in color and contain very large melanosomes which are composed of an inner kernel of eumelanin and an outer fibrous matrix filled with a red pigment. According to BAGNARA, FERRIS, TURNER and TAYLOR (1978), this pigment is pterorhodin, a pterin dimer.

In the present paper, a new type of the dermal chromatophore unit found in the dorsal skin of *Rhacophorus schlegelii* is reported. The dermal chromatophores of juvenile frogs immediately after metamorphosis are very similar to those in the dorsal skins of mature *Rana nigromaculata* and *Hyla arborea japonica*; they consist of xanthophores, iridophores and melanophores. Thereafter, a part of xanthophores is separated from the remaining part and falls into the spaces between iridophores and melanophores. These lower xanthophores become violet-black violeophores. The melanophores degenerate and are completely replaced by the violeophores. The latter are filled with violet-black granules (violeopterinosomes), which reveal a clear-cut concentric structure of thin lamellae. While the upper xanthophores are remarkably thin and contain a very few carotenoid vesicles, the upper part of each iridophore along the undersurface of a xanthophore is almost exclusively occupied by a distinct layer of carotenoid vesicles. Thus, the yellowish-green color of the dorsal surface resembling that of *Hyla arborea japonica* seems to be principally brought out by cooperation of the upper layer of carotenoid vesicles and the lower part of regularly arranged reflecting platelets of each iridophore and violet-black violeopterinosomes contained

in each violeophore which was derived from a xanthophore. The existence of such a thick layer of carotenoid vesicles in each iridophore of the dorsal skin has not yet been observed in any other kind of frogs.

The violet-black pigment contained in the dorsal skin of *Rhacophorus arboreus*, a closely allied species of *Rhacophorus schlegelii*, was recently investigated by Dr. T. SUGA. According to him, this pigment is probably a new compound which is principally constituted from pterin-6-carboxylic acid. It is also probable that the violet-black pigment of *Rhacophorus schlegelii* is a similar compound. This situation seems to show that the violet-black pigment obviously differs from the red pigment, pterorhodin, found in the skins of the leaf frog subfamily by BAGNARA, FERRIS, TURNER and TAYLOR (1978), although both kinds of pigments belong to the pterin group.

2. Three kinds of mutants

a. Albino

In anurans, numerous albinos have been discovered by many investigators (HENSLEY, 1959; GILBOR and DOWLING, 1974). The findings that the albinos examined genetically are always due to a recessive gene in the homozygous condition as those in the other groups of vertebrates have been reported by EALES (1933) and SMALLCOMBE (1949) in *Rana temporaria*, by TOKUNAGA (1949) in parthenogenetically developed *Rana nigromaculata*, by BROWDER (1972) and SMITH-GILL, RICHARDS and NACE (1970, 1972) in *Rana pipiens*, by HOPERSKAYA (1975) and BLUEMINK and HOPERSKAYA (1975) in *Xenopus laevis*, by NISHIOKA (1977) in *Rana nigromaculata* and by the present authors (1977b, 1985a) in *Hyla arborea japonica*, *Rana nigromaculata* and *Rana brevipoda*. In *Hyla arborea japonica*, they reported that ten stocks of albinos collected from ten different places are divided into three groups, which differ from one another in the locus of albino genes. The present authors (1985a) have also reported in the *Rana nigromaculata* group that 13 albino stocks including three obtained from irradiated gametes, nine *Rana nigromaculata* and one *Rana brevipoda* collected from the field are divided into five groups having different loci.

As the albinos are devoid of melanin pigment, their pupils are red and their dorsal surface reveals the colors of xanthophores and iridophores. While the xanthophores contain yellow or orange-yellow carotenoid vesicles and pale-yellow or colorless pterinosomes, the iridophores usually contain colorless reflecting platelets alone which can not reflect blue color without the black background formed by melanophores. Thus, the dorsal surface of albinos at the tadpole and adult stages is generally yellow or orange-yellow, although the color somewhat differs from strain to strain or from species to species.

The albinos of *Rhacophorus schlegelii* are orange-yellow in dorsal coloration from the tadpole stage through completion of metamorphosis, as those of *Rana* and *Hyla*. However, this dorsal color becomes gradually tinged with green and eventually changes into yellowish green owing to development of violet-black violeophores under the iridophores. In this respect, the albinos of *Rhacophorus*

schlegelii remarkably differ from those of *Rana* and *Hyla*. However, it is noteworthy that the dorsal green color of mature albinos is paler than that of the mature wild-type frogs. This is attributable to the situation that about half the number of the dermal violeophores are incomplete in differentiation, while the others are almost the same as those found in the wild-type frogs. Among various stocks of albinos of *Rana nigromaculata* and *Hyla arborea japonica* observed by NISHIOKA and UEDA (1977b, 1985a), the xanthophores are always normal in contrast to the findings in the albinos of *Rhacophorus schlegelii*.

b. Black-eyed mutant

The melanoid mutants have been reported in the axolotl by HUMPHREY and BAGNARA (1967), BENJAMIN (1970) and DUNSON (1974), in *Pleurodeles waltl* by LACROIX and CAPURON (1970) and in *Rana pipiens* by RICHARDS, TARTOF and NACE (1969). These mutants were confirmed to be due to a single recessive gene in the homozygous condition and to have no iridophores. In this respect, the black-eyed mutants of *Rhacophorus schlegelii* reported here are the same as the above melanoid mutants, since the reflecting platelets are completely lacking in their iridophores. However, the effects of the black-eyed gene on the dermal chromatophores other than iridophores are not always the same. In the melanoid axolotls there is reduction in number of xanthophores and no effect on melanophores (HUMPHREY and BAGNARA, 1967) or there is production of more melanophores and fewer xanthophores than in the wild-type axolotl (BENJAMIN, 1970). In the melanoid mutants of *Rana pipiens*, both iridophores and xanthophores are lacking (RICHARDS, TARTOF and NACE, 1969). In those of *Pleurodeles waltl*, the melanophores increase in number and density.

In contrast to the above melanoid mutants, the xanthophores and violeophores in the black-eyed mutants of *Rhacophorus schlegelii* are very similar to those of the wild-type frogs. Although the reflecting platelets are usually lacking, the iridophores are evidently observable, as the upper part of each iridophore is occupied with well-developed carotenoid vesicles. The dorsal surface of this mutant appears dark brown owing to cooperation of xanthophores, carotenoid vesicles of iridophores and violeophores.

The complete lack of iridophores is also confirmed in the most part of the dorsal skin of the Yc black-eyed stock of *Hyla arborea japonica*, while the iridophores of the Hs black-eyed stock of the same species contain reflecting platelets which are very abnormal in number, size and shape (NISHIOKA and UEDA, 1985b). The iridophores of the black-eyed mutants produced by irradiation of gametes in *Rana nigromaculata* also contain small, abnormally shaped reflecting platelets (NISHIOKA, 1977). In this kind of black-eyed mutant, the melanophores are distinctly expanded and filled with very large melanosomes, while the xanthophores are usually normal. The various kinds of olive mutants of *Rana nigromaculata* obtained by NISHIOKA (1977) from irradiated gametes have semitransparent skin, owing to abnormality of iridophores. The present authors (1977a) have confirmed that the reflecting platelets contained in these iridophores are

remarkably fewer and smaller than those of the wild-type frogs. The xanthophores and melanophores in these olive mutants are completely normal. On the other hand, the present authors (1985a) have observed that in the albinos of the Km stock belonging to the fourth group in *Rana nigromaculata*, both melanophores and iridophores are abnormal, while the xanthophores are normal. The reflecting platelets in each iridophore are remarkably fewer and smaller than those of the wild-type frogs and very irregular in shape. As the abnormal iridophores do not reflect light, the skin of this albino stock is semitransparent.

In the above stated melanoid, black-eyed and olive mutants, complete lack or a severe abnormality of reflecting platelets in the iridophores is a common characteristic, and is always due to a single recessive gene in the homozygous condition, except a very few non-inheritable variants reported by NISHIOKA and UEDA (1985c). The lack or abnormality of iridophores occurs independently or accompanied by that of the other dermal chromatophores. The latter case seems to be pleiotropy of the black-eyed or melanoid gene.

c. Yellow variant

This is an only variant in which the dorsal surface is bright reddish-yellow. It is evident that this color is attributable to the lack of normal violeophores. The xanthophores and iridophores are normal. Although no genetic experiments have been made, it is probable that this yellow variant is due to a single recessive gene in the homozygous condition like the albinos. As this color variant is a very strange one, the existence of such a variant was described here briefly. It is very desirable to obtain this variant again and to examine its morphology and genetics.

SUMMARY

1. The dorsal surface of mature wild-type *Rhacophorus schlegelii* GÜNTHER is usually bright yellowish-green. The dermal chromatophore unit (BAGNARA *et al.*, 1978) in the dorsal skin consists of a xanthophore, an iridophore and a violet-black pigment cell (violeophore) in place of a melanophore. In froglets (stage XXV) immediately after metamorphosis, the dermal chromatophores consist of xanthophores, iridophores and melanophores. Thereafter, a part of xanthophores sink down into the spaces between iridophores and melanophores, and become violet-black violeophores. The melanophores degenerate and are completely replaced by the violeophores. The latter are filled with violet-black granules (violeopterinosomes), which have derived from pterinosomes of the lower xanthophores, and reveal a clear-cut concentric structure of thin lamellae.

Mature frogs have many irregularly shaped, brown-black spots on the white flanks. In these spotted areas, there are many melanophores with long dendritic processes and layers of iridophores. In the pigment epithelial cells of the retina, abundant melanosomes are crowded around the rod cells. In the choroid, melanophores constitute a thick layer in the area near the pigment epithelium.

2. There is a thick layer of carotenoid vesicles upon the reflecting platelets

arranged regularly in each iridophore of the dorsal skin. This layer compensates for a defective function of xanthophores lying just under the basal lamina of the epidermis.

3. A yellow mature variant of this species was discovered in 1972 at a rice field in Hiroshima Prefecture and brought to our laboratory at once. In 1976, 1979 and 1983, several albino tadpoles were collected from Yamaguchi, Shimane and Wakayama Prefectures, respectively. Many tadpoles of the black-eyed mutants were collected in 1976 from Hyogo Prefecture. Most of the albinos and a small number of the black-eyed mutants completed metamorphosis and attained sexual maturity.

4. The albinos at the tadpole stage are orange yellow in dorsal color and have red pupils. After metamorphosis, they are gradually tinged with green and eventually become yellowish green. The green color of the dorsal surface of the mature albinos is paler than that of the mature wild-type frogs. About half the number of the dermal violeophores in the mature albinos are incomplete in differentiation in contrast to those of the wild-type frogs. The dermal iridophores are surrounded by arms of the incomplete violeophores.

The flanks of the mature albinos have no brown-black spots. In the position where melanophores are located in the wild-type frogs, there are abnormal melanophores containing premelanosomes. No melanosomes are found in the pigment epithelium of the retina and in the melanophores of the choroid.

5. Black-eyed mutants are dark and semitransparent at the early tadpole stage. After metamorphosis the dorsal skin is usually dark brown even at the stage of sexual maturity, although there are a few black-eyed mutants having dull-yellow areas. The irises are brownish black. The black-eyed mutants do not make any color change. A part of the visceral organs is seen through the semitransparent ventral wall.

The dermal chromatophores in the dorsal skin of mature black-eyed mutants consist of xanthophores, iridophores and violeophores. The xanthophores are very similar to those of the wild-type frogs, although their dendritic processes extending under iridophores contain a few violeopterinosomes together with many pterinosomes. The iridophores contain no reflecting platelets. The dermal melanophores found at the juvenile stage have completely degenerated at the mature stage as those of the wild-type frogs. The violeophores have long dendritic processes and cover the undersurfaces of iridophores.

6. A single yellow variant was collected from the field. The dorsal surface of this variant is bright reddish-yellow and has several small black spots. There are also irregularly shaped brown-black spots on the flank. In this variant, the violeophores are lacking, while xanthophores, iridophores and melanophores are normal, when observed under a light microscope.

7. The violet-black pigment of *Rhacophorus schlegelii* may be a new compound which is principally constituted from pterin-6-carboxylic acid in common with that of *Rhacophorus arboreus*.

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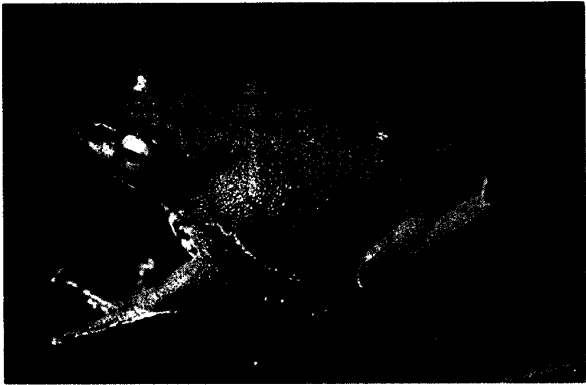
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EXPLANATION OF PLATE

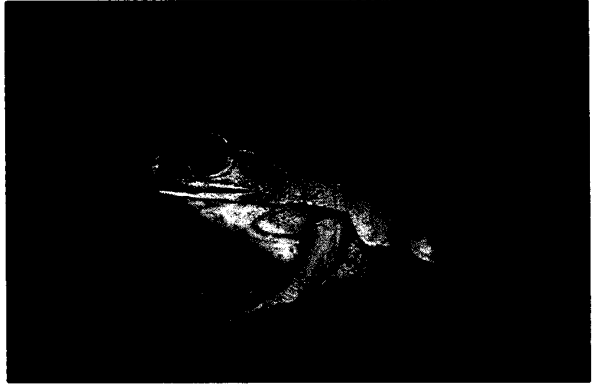
PLATE I

Three kinds of color variants and a wild-type frog in *Rhacophorus schlegelii*.

1. Wild-type frog (♀). ×0.8
2. Yellow variant (♀). ×0.7
3. Albinos immediately after metamorphosis. ×0.8
4. Albino (♀) at the sexually mature stage. ×0.8
5. Black-eyed mutant No. 1. ×0.8
6. Black-eyed mutant No. 2, having dull-yellow areas on the dorsal surface. ×0.8
7. Microphotograph of dermal chromatophores in the dorsal skin of a wild-type frog. Viewed from the upper surface. ×540
8. Microphotograph of a cross-section of dermal chromatophores in the dorsal skin of a wild-type frog. ×450



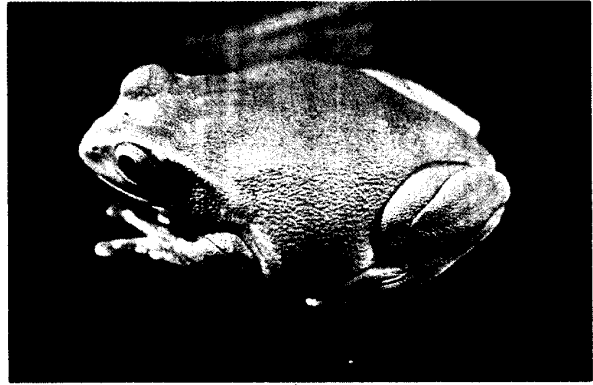
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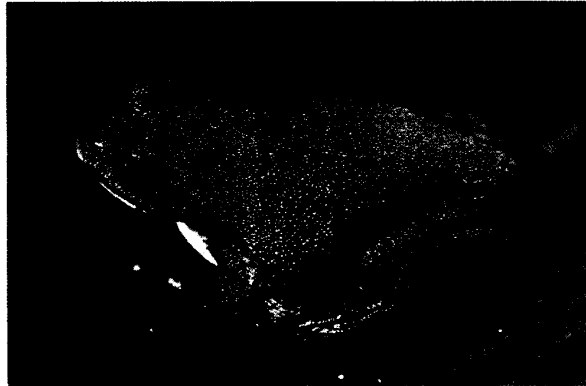
2



3



4



5



6



7



8