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ATP ASE-POSITIVE AND METALLOPHILIC CELLS IN THE SKIN OF FROG, *RANA CATESBEIANA*

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ABSTRACT

The skin of bull frog, *Rana catesbeiana* was studied by a zinc iodide osmium method and an ATPase staining method to examine the occurrence of cells equivalent to the mammalian Langerhans cells. It was demonstrated that ATPase-positive and metallophilic Langerhans cells were absent from the skin of bull frogs. Birbeck granules, which are characteristic to the mammalian Langerhans cells, were also not found by electron microscopy. These results suggest that an advanced immunological defense system including the activities of Langerhans cells is not developed in the skin of bull frog, *Rana catesbeiana*. The primitive features of the amphibian immune system were also discussed. In the course of this study, the techniques used to find out the Langerhans cells demonstrated metallophilia as well as ATPase and other nucleotide phosphatase (GTPase and GMPase) activities in the dermal yellow pigment cells, xanthophores, which do not occur in mammals. The metal deposition consisted predominantly of zinc and osmium. The enzyme activities were localized in the pterinosomes of the xanthophore.

Keywords: ATPase, metallophilia, Langerhans cell, xanthophore, frog skin

INTRODUCTION

Langerhans cells in the mammalian skin are migratory dendritic cells located among the epithelial cells of the epidermis. They are believed to play a significant role as antigen presenting cells in the immunological defense system,[1-7] and also to play some important but unidentified role in the process of keratinization.[8,9] Despite the increasing knowledge about the function of Langerhans cells, there has been little knowledge of whether or not cells identical with or similar to mammalian Langerhans cells are present in lower vertebrates, which are considered to have a primitive immune system.[10]

The zinc iodide osmium method and enzyme histochemical techniques for the demonstration of ATPase activity have been generally used to identify the Langerhans cells in mammalian skin.[11-14] Electron microscopy has also been used to demonstrate rod- or racket-shaped Birbeck granules, which are characteristic to the Langerhans cells.[9] These techniques were applied in the present study to find out the Langerhans cells in the amphibian species. As an example of this species, bull frog *Rana catesbeiana* was used in the present study because its epidermis is weakly keratinized.

It was also reported as a finding of this study, that the above techniques demonstrated metallophilia and positive ATPase reactions specifically in the yellow pigment cells, xanthophores (which do not occur in mammalian species), in the dermis of *Rana catesbeiana*.

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MATERIALS AND METHODS

Six adult bull frogs, *Rana catesbeiana* were obtained from a local animal dealer in Nagoya, Japan, during the summer. Small skin fragments were dissected out from the dorsal head region and also from the ventral area near the hind limb joint and fixed in the following fixatives:

a) cold (below 4°C) 10% formalin (with or without 1% $CaCl_2$) for 30 min to 1 hr for enzyme studies or for 24 hr for general histology.

b) cold (below 4°C) 2.5% glutaraldehyde-2% paraformaldehyde mixture in 0.1M phosphate buffer (pH 7.4) for 3 hr followed by post fixation in 1% OsO_4 in 0.1M phosphate buffer for routine plastic embedding.

c) cold (below 4°C) zinc iodide osmium (ZIO) for 16 hr prepared after Rodriguez and Caorsi[12]: 12g of zinc powder and 5g of iodine bisublimate thoroughly mixed and dissolved in 200 ml of 0.2M Veronal-HCl buffer at pH 7.4. This mixture was shaken for 10 min and immediately filtered. The filtrate was then mixed with 2% aqueous OsO_4 solution at a ratio of 3:1 to get fixation solution for immediate use.

The tissues fixed in solutions (b) and (c) were washed thoroughly with their respective diluted buffers, followed by dehydration in a series of ethanol, and embedded in paraffin or Quetol. For light microscopic observations, 5 μm thick paraffin and 1.0 μm thick plastic sections were cut and studied with or without staining with toluidine blue. For electron microscopic observations, unstained or double stained (with uranyl acetate and lead citrate) ultrathin sections were utilized. Sites of metal deposition were analyzed by an X-ray microanalyzer, Kevex EDX, attached to a Hitachi H 800 electron microscope.

Enzyme histochemistry Cryostat sections of fresh unfixed or briefly fixed tissues were stained for the following sets of enzymes:

a) ATPase activity by Wachstein and Meisel technique[15] with or without addition of calcium and magnesium salts in the fixatives or incubating solutions.

b) GTPase activity modified from Wachstein and Meisel technique for ATPase by replacing the substrate ATP with GTP.

c) GMPase activity modified from Wachstein and Meisel technique for AMPase[15] by replacing the substrate AMP with GMP.

RESULTS

Light microscopy

The bull frog epidermis was a stratified squamous epithelium consisting of several layers of epithelial cells with a thin superficial layer of cornified cells. There was no granular layer such as seen in mammals. Where the skin was dark, dendritic melanophores were present among the epithelial cells. Melanosome granules were also present within these epithelial cells (Fig. 1).

With the ZIO method, no cell identical with or equivalent to the Langerhans cell in the mammalian epidermis could be identified within the frog skin. Some of the epithelial cells, especially those in the germinal layer, showed fine granular metal deposition. The xanthophores lying close beneath the epidermis demonstrated varying degrees of heavy metal deposition in the form of brown to black granules (Fig. 1). In routine formaldehyde-fixed and unstained sections, it was, however, difficult to identify the xanthophores. In ZIO-fixed tissues, the metal deposition made the cells easily identifiable.

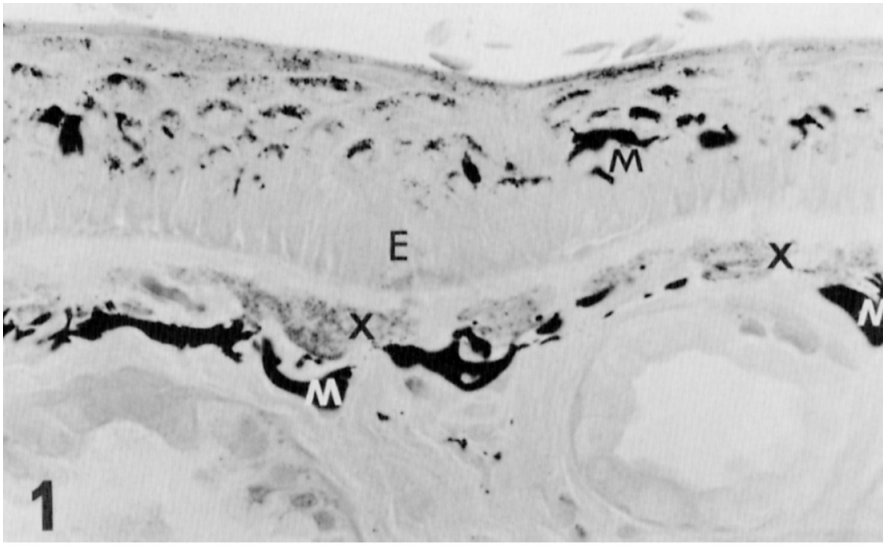

Fig. 1 Unstained semithin section of the skin treated with ZIO method. A layer of xanthophores (X) is present below the epidermis (E). Dark dendritic cells in the epidermis and below the layer of xanthophores are melanophores (M). The epithelial cells in the upper layer of the epidermis contain small granular melanosomes. ×400.

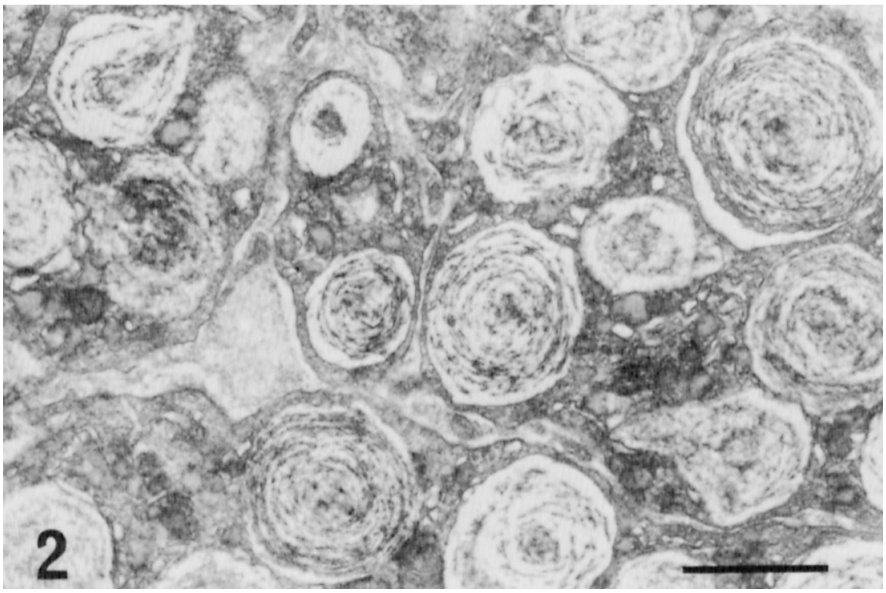

Fig. 2. An electron micrograph of xanthophores (fixed with GA and OsO_4). The cytoplasm contains many pterinosomes in which concentric lamellar structures are present. ×19,000.

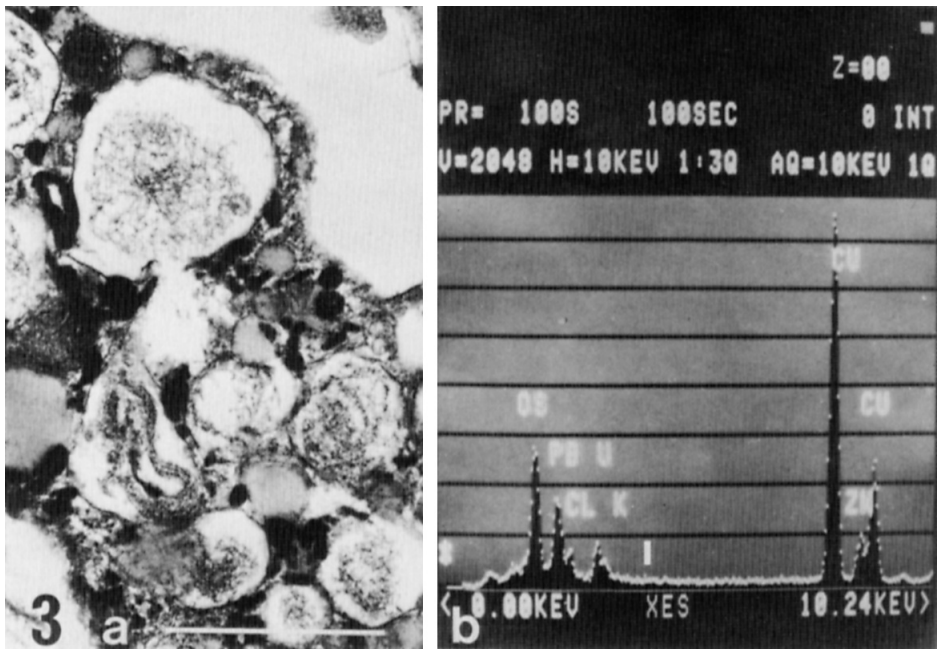

Fig. 3. a) An electron micrograph of a xanthophore treated with ZIO method and stained with uranyl acetate and lead citrate. Metal deposition is seen as large granules in the cytoplasm and as fine granules on the membranous structures in the pterinosomes. ×29,000.
b) EDX analysis of a large granule in a ultrathin section of xanthophore treated with ZIO method and stained with uranyl acetate and lead citrate. There is a peak for Zn but none for I. Peaks of Os, Pb, U, Cl, K, and Cu can be seen in GA-OsO_4 fixed, and U-Pb stained samples.

Electron microscopy

Cells with characteristic Birbeck granules (Langerhans cell granules) could not be found in either the epidermis or dermis. There were occasional lymphocyte-like migrating cells among the epithelial cells of the epidermis, but the Birbeck granules could not be found in these cells. The epithelial cells were connected with each other by desmosomes. Their cytoplasm was abundant in tonofilaments, but keratohyaline granules or lamellar granules such as seen in the mammalian epidermis were not observed. Melanophores with dendritic profile were present in the epidermis as well as in the dermis beneath the layer of xanthophores. The fine structure of the xanthophore has been studied.[16–20] The xanthophores of the bull frog are typically similar to those of any frog or fish species. The pterinosome granules of the xanthophore consisted of lamellar structures bounded by a limiting membrane (Fig. 2). Metal deposition was observed mostly as dense granules of varying sizes up to 200 nm in diameter, randomly distributed in the cytoplasm, and also as fine granular deposits on the lamellar structure of the pterinosomes (Fig. 3a). X-ray microanalysis of the metal depositions showed the presence of zinc and osmium predominantly (Fig. 3b).

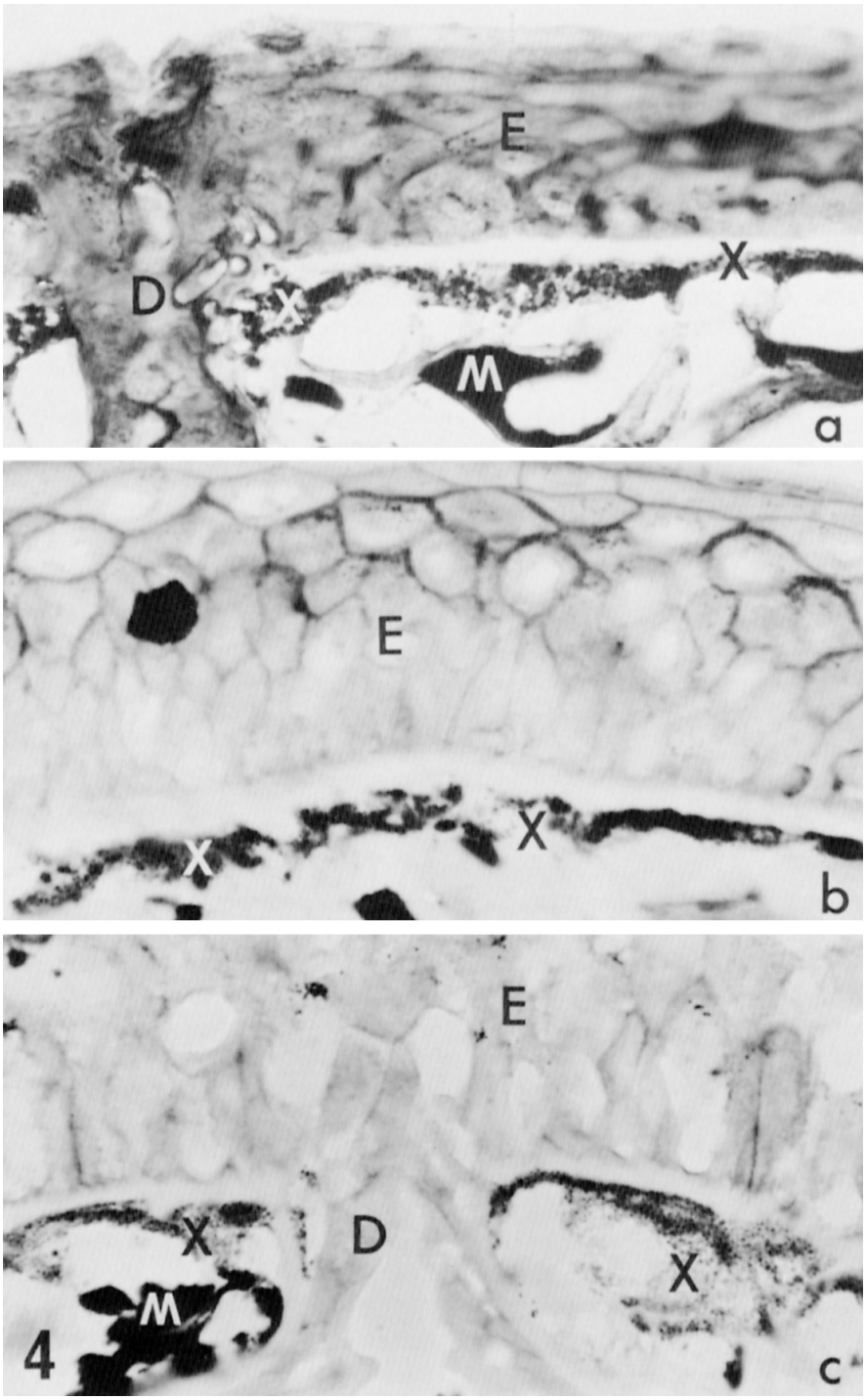

Fig. 4. a) Frozen section, ATPase reaction. Cytoplasmic granules of xanthophores (X) give strong reaction for ATPase.
b) Frozen section, GTPase reaction. The granules of xanthophores (X) give strong reaction for GTPase. Cell membranes of epithelial cells located in the middle layers of the epidermis are also GTPase-positive.
c) Frozen section, GMPase reaction. The granules of xanthophores (X) also give strong reaction for GMPase. E: epidermis; M: melanophore; D: excretory duct of granular gland. ×900.

Histochemistry

ATPase activity. Except for the cornified cells of the most superficial layer, the membranes of the epithelial cells gave weak ATPase reaction. No dendritic or specialized cells reacting with the ATPase staining technique could be identified in the epidermis. In the dermis, the pterinosome granules of xanthophores gave strong reaction for ATPase (Fig. 4a). Without addition of calcium and magnesium salts, the pterinosomes failed to show ATPase activity. The activity was also inhibited by heat treatment (60°C) of the tissue sections. The reactions at most of the other ATPase-positive sites of the skin were also retarded or inhibited by heat treatment or by withdrawing Ca and Mg ions from the substrate solution.

GTPase activity. Cell membranes in the epidermis, except for those in the basal layer, gave strong GTPase reaction. The xanthophores gave very strong reaction in their pterinosome granules (Fig. 4b).

GMPase activity. The sites of GMPase activity were almost the same as those of GTPase activity (Fig. 4c).

DISCUSSION

The present study did not demonstrate the presence of Langerhans cells in the skin of frog, *Rana catesbeiana* either by ZIO and ATPase staining methods or by electron microscopy. It is usually difficult to make a conclusion on the absence of any migratory cell types from a tissue by observations on limited numbers of samples; however, since the epidermal structure of the bull frog was almost uniform in different areas and since the Langerhans cells, if present, are supposed to distribute evenly in the epidermis, it can be concluded that cells identical with the Langerhans cells in mammals are absent from the skin of *Rana catesbeiana*.

Among the vertebrates, mammals possess the most advanced immunological defense system, in which the thymus, spleen, and lymph nodes are fully developed.[10] The mammalian Langerhans cells have been shown to travel from the skin to regional lymph nodes,[6,21] where they are believed to mediate immune responses by close contact with immunologically competent lymphocytes. In the immune system of amphibians, on the other hand, such skin-associated lymph nodes are undeveloped.[10] The absence of Langerhans cells in the skin of the bull frog seems also to characterize the primitive form of the amphibian immune system. The Langerhans cells may be specific constituents of the skin-associated lymph node system. A primitive form of skin-associated lymph nodes is developed phylogenetically first in avian species.[10,22] Therefore, it is interesting, and will be the subject of our further study, to find out whether Langerhans cells are present in the skin of these species.

Mammalian Langerhans cells are distributed in the epidermis and in some other epithelial tissues consisting of keratinizing cells.[9] Experiments on epidermoid metaplasia induced by vitamin A deficiency demonstrated that Langerhans cells appeared in the transformed epithelia in the trachea and urinary bladder.[8] These results have suggested that the mammalian Langerhans cells have some functional relationship with the process of keratinization. The absence of Langerhans cells from the weakly keratinized epidermis of the bull frog would suggest that the mechanism of keratinization in amphibian skin may be different from that in mammalian skin. However, more extensive studies on the Langerhans cells in the skin of different groups of vertebrates are needed to specify the precise role of the Langerhans cells in epidermal keratinization.

The present study has also shown that the techniques used to find out the mammalian

Langerhans cells, demonstrated xanthophores in the dermis of the bull frog. The reactions for ATPase and other nucleotide phosphatases were strongly positive in their pterinosome granules. There may be two possible explanations for these enzyme activities. One is that the ATPase activity in the pterinosome granule might be correlated with the movement of the granule within the xanthophore as suggested in the case of melanophore.[23] The other is that the activities of the purine nucleotide phosphatases may be associated with some steps of the formation of pigment pteridines, which are synthesized from purines or from their nucleosides and nucleotides within the xanthophore.[17,24]

X-ray microanalysis of ultrathin sections from ZIO-treated tissues has demonstrated that the metal depositions in the xanthophore contained predominantly zinc and osmium. The osmiophilia is rather common to the cell structures, but it is pointed out that zinc is preferentially deposited on the cytoplasmic structures of xanthophores from ZIO solution. Although the significance of the zinc deposition is not understood, the ZIO method is useful as a selective stain for xanthophores of amphibian skin.

REFERENCES

1) Silberberg, I., The role of Langerhans cells in allergic contact hypersensitivity. A review of findings in man and guinea pigs. *J. Invest. Dermatol.*, **66**, 210—217, 1976.
2) Shelley, W.B. and Juhlin, L., Langerhans cells form a reticuloepithelial trap for external contact antigens. *Nature*, **261**, 46—47, 1976.
3) Rowden, G., Lewis, M.G. and Sullivan, A.K., Ia antigen expression on human epidermal Langerhans cells. *Nature*, **268**, 247—248, 1977.
4) Stingl, G., Katz, S.I., Shevach, E.M. et al., Analogous functions of macrophages and Langerhans cells in the initiation of the immune response. *J. Invest. Dermatol.*, **71**, 59—64, 1978.
5) Kobayashi, M. and Hoshino, T., Cytological and functional differences between Birbeck granule-containing cells (Langerhans cells) and dermal macrophages in the mouse. *J. Electron Microsc.*, **28**, 285—294, 1979.
6) ————, Distribution of cored tubule-containing Langerhans cells in the skin and lymph nodes of mice. *J. Electron Microsc.*, **32**, 197—206, 1983.
7) Wolff, K. and Stingl, G., The Langerhans cell. *J. Invest. Dermatol.*, **80**, 17s—21s, 1983.
8) Wong, Y.C. and Buck R.C., Langerhans cells in epidermoid metaplasia. *J. Invest. Dermatol.*, **56**, 10—17, 1971.
9) Hoshino, T. and Kobayashi, M., Langerhans cells in immunity (Japanese). *The Cell*, **12**, 132—140, 1980.
10) Good, R.A. and Fisher, D.W., Immunobiology. Sinauer Associates, Stamford, 1971.
11) Wolff, K. and Winkelmann, R.K., Quantitative studies on the Langerhans cell population of guinea-pig epidermis. *J. Invest. Dermatol.*, **48**, 504—513, 1967.
12) Niebauer, G., Krauczyk, W.S., Kidd, R.L. et al., Osmium zinc iodide reactive sites in the epidermal Langerhans cell. *J. Cell Biol.*, **43**, 80—89, 1969.
13) Rodriguez, E.M. and Caorsi, I., A second look at the ultrastructure of the Langerhans cell of the human epidermis. *J. Ultrastr. Res.*, **65**, 279—295, 1978.
14) Baker, K.W. and Habowsky, J.E.J., EDTA separation and ATPase Langerhans cell staining in the mouse epidermis. *J. Invest. Dermatol.*, **80**, 104—107, 1983.
15) Pearse, A.G.E., Histochemistry. Theoretical and Applied (3rd ed.), Churchill, London, 1968.
16) Matsumoto, J., Studies on fine structure and cytochemical properties of erythrophores in swordtail, *Xiphophorus helleri*, with special reference to their pigment granules (pterinosomes). *J. Cell Biol.*, **27**, 493—504, 1965.
17) Bagnara, J.T., Cytology and cytophysiology of non-melanophore pigment cells. *Int. Rev. Cytol.*, **20**, 173—205, 1966.
18) Kamei-Takeuchi, I. and Hama, T., Structural change of pterinosome (pteridine pigment granule) during the xanthophore differentiation of oryzias fish. *J. Ultrast. Res.*, **34**, 452—463, 1971.
19) Yasutomi, M. and Hama, T., Ultramicroscopic study of the developmental change of the xanthophore in the frog, *Rana japonica* with special reference to pterinosomes. *Develop. Growth Differentiation*, **13**, 141—149, 1971.

20) ————, Electron microscopic study on the xanthophore differentiation in *Xenopus laevis*, with special reference to their pterinosomes. *J. Ultrastr. Res.*, **38**, 421—432, 1972.
21) Kelly, R.H., Balfour, B.M., Armstrong, J.A. et al., Functional anatomy of lymph nodes. II. Peripheral lymph-born mononuclear cells. *Anat. Rec.*, **190**, 5—22, 1978.
22) Hellman, T., Lymphgefässe, Lymphknötchen und Lymphknoten. In Handbuch der Mikroskopischen Anatomie des Menschen, pp.173—261, Edited by W.V. Möllendorff. Springer, Berlin, 1943.
23) Fujii, R., Pigment Cell (Japanese). Tokyo Univ. Press, Tokyo, 1976.
24) Levy, C.C., Pteridine metabolism in the skin of the tadpole, *Rana catesbeiana*. *J. Biol. Chem.*, **239**, 560—565, 1963.