STUDIES ON FINE STRUCTURE AND CYTOCHEMICAL PROPERTIES OF ERYTHROPHORES IN SWORDTAIL, *XIPHOPHORUS HELLERI*, WITH SPECIAL REFERENCE TO THEIR PIGMENT GRANULES (PTERINOSOMES)

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ABSTRACT

The fine structure and the composition of pteridine pigments of erythrophores in adults of the swordtail fish, *Xiphophorus helleri*, were studied by means of cytochemistry, paper chromatography, ionophoresis, centrifugal fractionation, and electron microscopy. It was found that water-soluble pigments of erythrophores consisted exclusively of pteridine derivatives including large amounts of drosopterin, isodrosopterin, neodrosopterin, and moderate amounts of sepiapterin. While these substances were responsible for red pigmentation, moderate quantities of colorless pteridines, biopterin, Rana-chrome 3, xanthopterin, isoxanthopterin, and others, were also detectable. The ultrastructure of the erythrophore is characterized by numerous pigment granules and a well developed tubular endoplasmic reticulum. The former consist of a three-layered limiting membrane and inner lamellae which appear to be whorl-like due to a concentric arrangement of parallel membranes. All of the mentioned pteridines are primarily contained in this organelle which is designated, accordingly, "pterinosome." The possible functions of erythrophores and pterinosomes are discussed in the light of their structure and pigmentary constitution.

INTRODUCTION

It has been shown that the pteridines found in fresh-water fish, amphibians, and reptiles are found characteristically in high concentrations in cells known as xanthophores or erythrophores (1–7). The bright color of these cells has been shown actually to be imparted by pteridines either solely or together with other kinds of pigments such as carotenoids (1–8).

Goodrich and his associates (1) reported, as early as 1941, that the pigments in erythrophores of the swordtail consisted of two compounds, one miscible with organic solvents (carotenoids) and the other immiscible with organic solvents (pteridines). The chemical structure of the latter was identified as erythropterin by the same authors (1). Thereafter, Kauffmann (9) and Ziegler (10) independently have indicated that red colored pigments in swordtail skins are almost identical to drosopterin, isodrosopterin, and neodrosopterin which were found originally in the eyes of *Drosophila*.

Notwithstanding these observations, little information is available as to whether these or other kinds of pteridines are actually localized in erythrophores. Moreover, little is known either about the specific intracellular location of pteridines or in general about the fine structure of xanthophores and erythrophores. While melano-

cytes of mammalian skin have been the subject of several ultrastructure studies (11–13), the bright-colored pigment cells have escaped attention in this respect.

During the development of cyprinid fishes and amphibians, pteridine formation occurs simultaneously with the differentiation of xanthophores and melanophores (3, 5, 6, 14). Moreover, in cyprinid fishes, the color of chromatophores is in definite accordance with the pteridine pattern of these cells (3); presumably, a large share of their pigmentation is attributable to pteridines. Probably these pteridines are synthesized and localized in certain intracellular organelles. The characterization of these organelles would be of considerable interest. First of all, it may provide some clue to the elucidation of the problem of chromatophore differentiation and, moreover, it may contribute to the complete clarification of the role of pteridines in chromatophores.

Accordingly, in this investigation, the fine structure of erythrophores in the swordtail was studied with special reference to both the distribution of pteridines and the participation of pteridines as pigments. Considerable attention is given to pigment granules which appear to be the site of pteridine accumulation.

EXPERIMENTAL PROCEDURE

Materials

The swordtail fish, *Xiphophorus helleri*, used in this experiment was of the inbred homozygous red-scaled type. Homozygous fish were segregated in the author's laboratory by mating red-scaled individuals of commercial origin. Usually, adult fish ranging from 3 to 6 cm in total body length and 5 to 7 months old were used.

Microscopy

Small pieces of dorsal skin of trunk regions or from fins were dissected with sharp-pointed forceps and a razor blade. Microscopic observations on living erythrophores were made on samples kept in Ringer's solution for *Oryzias latipes* (15). The solubility of erythrophore pigment was determined cytochemically: After careful blotting, tissue fragments were treated with organic solvents (alcohol, aceton, chloroform, benzene, petroleum ether, ether, and ethylene chloride) for 2 to 12 hours, or with inorganic solvents (aqueous ammonia, 3 per cent ammonium acetate) for ½ to 3 hours at room temperature with two changes in each. The presence of coloring matter was observed light microscopically without further treatment or after clarification with benzene. The existence of carotenoids in erythrophores was determined by applying concentrated sulfuric acid directly to the samples. In this test, carotenoids are revealed by the appearance of blue coloration (16). For sectioning, specimens were fixed with chloroform-alcohol (1:2 v/v) mixture for 1 hour or more, run through benzene, and embedded in styrene resin (17). After treatment with chloroform to remove the resin, each section was examined with or without staining. For general histological study, Mallory's staining was used.

For electron microscopy, fin webs of dorsal and tail fins were employed exclusively. Immediately after excision, fins were rinsed and trimmed in a chilled solution of 1 per cent osmium tetroxide buffered with Veronal-acetate (pH 7.5) and containing 0.25 M sucrose (18). Specimens were subsequently fixed in this medium at 3°C for 2 hours, with two or three changes. They were dehydrated in an alcohol series and embedded in Epon resin according to Luft's or Kushida's procedure (19, 20), or in styrene according to Kushida (17). To avoid the dissolution of pteridines, the aqueous alcohol series were accomplished as rapidly as possible. Sections were cut on an Hitachi ultramicrotome (type UM-2), and examined in a Hitachi electron microscope (type HS-7). Staining was done with uranyl acetate (21) and/or lead hydroxide (22).

Isolation of Pigment Granules

In one part of this study, it seemed advantageous to study isolated pigment granules. For these experiments, approximately 5 gm of fresh skin per test were peeled from the fish and were washed with cold physiological solution. All subsequent procedures were carried out below 3°C. Samples were homogenized in 3 to 5 parts of 0.25 M sucrose, at first lightly in a Potter-Elvehjem glass homogenizer, and then in a Teflon homogenizer, after filtration through double-layered gauze. The homogenates were centrifuged at 300 *g* for 5 minutes. 0.25 M sucrose was usually employed as the centrifuging medium. The supernatant obtained was layered on the top of a centrifuge tube containing one to two parts of the medium and centrifuged at 700 *g* for 10 minutes. After repeated centrifugation by the same procedure, the supernatant was centrifuged at 5000 *g* for 20 minutes. The sediments were resuspended in the sucrose, layered over the medium which contained about 3 times the volume of the suspension, and centrifuged again at 5000 *g* for 20 minutes. Almost all of the red granules were precipitated in this fraction. In order to compare these particles with other intracellular organelles of skin cells, the supernatant obtained at 5000 *g* was successively centrifuged by a similar procedure at 15,000 *g* for 20 minutes, then 24,000 *g* for 30 minutes, and finally 105,000 *g* for 60 minutes. Half of each

sediment was prepared for electron microscopy with the fixatives described above. The remaining halves were utilized for chromatographic assay.

Determination of Pteridines

For this purpose, paper chromatography (ascending method) and paper ionophoresis (horizontal strip method) were employed. Tissue samples were squashed directly on filter paper or subjected to extraction according to the partial modification (ammoniacal aqueous alcohol in place of aqueous alcohol) of Awapara's method (23) and then chromatographed. Solvents employed were *n*-propanol–1 per cent ammonia (2:1 v/v), isopropanol–2 per cent ammonium acetate (2:1 v/v), isopropanol–water (7:3 v/v), water-saturated *n*-butanol, *n*-butanol–acetic acid–water (4:1:1 v/v), 2,4-lutidine–*n*-butanol–water (4:1:1 v/v), 5 per cent acetic acid, 3 per cent ammonium chloride, and 3 per cent sodium acetate. Ionophoresis was carried out in 0.05 M formic acid (pH 2.0), 0.05 M ammonium acetate (pH 3.4), 0.05 M acetate buffer (pH 4.0–5.5), 0.05 M phosphate buffer (pH 6.0–7.5), and 0.05 M Veronal buffer (pH 8.0–9.0). As a routine procedure, papers were developed twice with *n*-propanol–1 per cent ammonium and then isopropanol–2 per cent ammonium acetate in the first dimension. In the second dimension, ionophoresis was performed with 0.05 M formic acid.

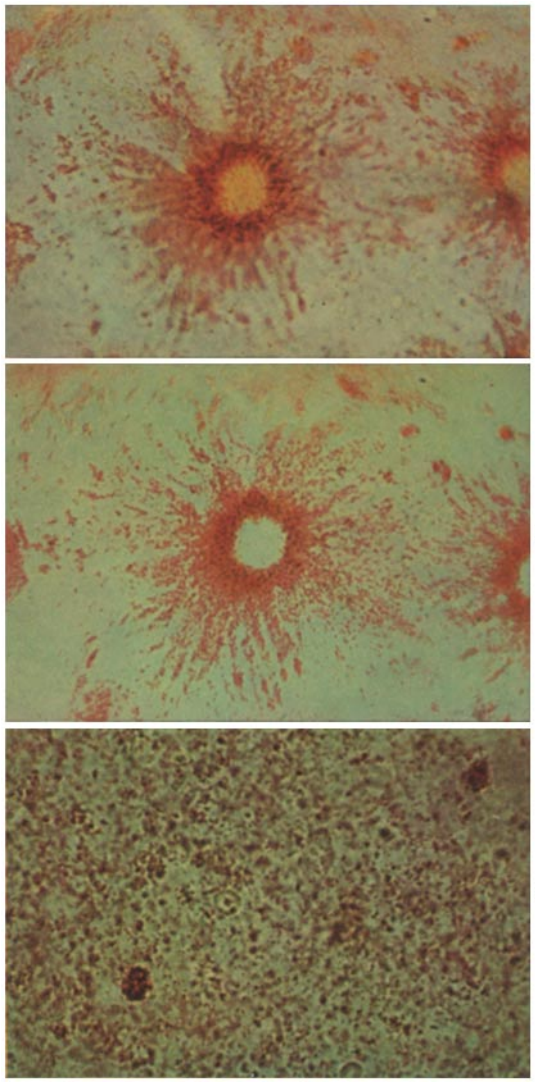
Pteridines were detected by their emission of fluorescence under UV irradiation of 255 mμ or 365 mμ. Identification of pteridines was made by comparing the R_f of each spot with that of authentic materials. Drosopterin, isodrosopterin, and neodrosopterin used as the controls were from a crude extract obtained from the eyes of *Drosophila melanogaster* "oregon." Authentic sepiapterin was obtained in pure form from *Drosophila*. Dihydroxanthopterin was prepared by reducing xanthopterin according to Nawa's procedure (24). The quantity of pteridines were estimated grossly on the basis of size, shape and fluorescent intensity of each spot on the paper chromatogram.

RESULTS

Cytochemical Properties of Erythrophores

In the homozygous red-scaled swordtail, the integumental coloration was imparted exclusively by erythrophores.[1] These cells varied considerably with respect to size, appearance of cytoplasmic processes, degree of pigmentation, and mode of

[1] Throughout this paper, preference is given to the term erythrophore instead of erythro-xanthophore, for reasons of simplicity in description and of difficulty in discriminating the two cell types in this species.



The embedding and staining processes are indicated throughout the legends by the following abbreviations: Ep, Epon 812 after Luft; St. styrene after Kushida; LH, lead hydroxide; UA, uranyl acetate.

FIGURE 1 Photomicrographs of erythrophores from adult skins of the swordtail fish. Fig. 1 *a* Living. Photographed in a physiological salt solution. Fig. 1 *b* The same cell after treatment with a chloroform-alcohol mixture for 2 hours. Yellow pigments disappear completely. Fine red pigment granules are distinctly observable. × 500.

FIGURE 2 Photomicrograph of pigment granules (pterinosomes) isolated from erythrophores by the centrifugation method. Aggregates of the granules are seen. Photographed in 0.25 M sucrose. × 1125.

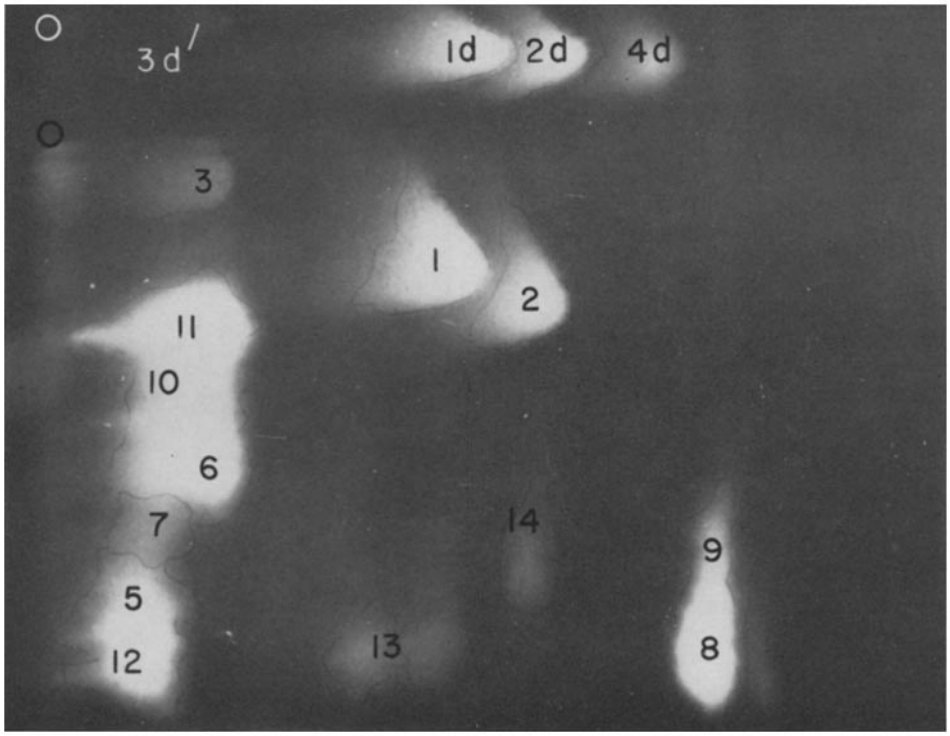


FIGURE 3 Ionophoreogram showing the composition of pteridines in pterinosomes. In the first dimension, paper chromatography was done twice, using *n*-propanol–1 per cent ammonia and then iso-propanol–2 per cent ammonium acetate. In the second, ionophoresis was done with 0.05 M formic acid (0.14 mA/cm for 7 hours at 5°C). Abbreviations in the figure are as follows: *d*, Control drosopterins obtained from *Drosophila*; black and white circles, starting position of the samples to be examined and of the control applied in the second development, respectively.

Spot number in figure	Identification	Visible color	Fluorescence color
1	Drosopterin	orange	yellowish orange
2	Isodrosopterin	orange	orange
3	Neodrosopterin	red	red
4	Drosopterin D	orange	orange
5	Sepiapterin	yellow	yellow
6	Xanthopterin	colorless	yellow
7	Dihydroxanthopterin or its decomposed product	colorless	green
8	Biopterin	colorless	blue
9	Rana-chrome 3	colorless	blue
10	2-Amino-4-hydroxy-6-carboxypteridine	colorless	blue
11	Isoxanthopterin	colorless	violet
12	Unknown (Labile pteridine)	colorless	blue
13	Unknown	colorless	blue
14	Unknown	colorless	violet

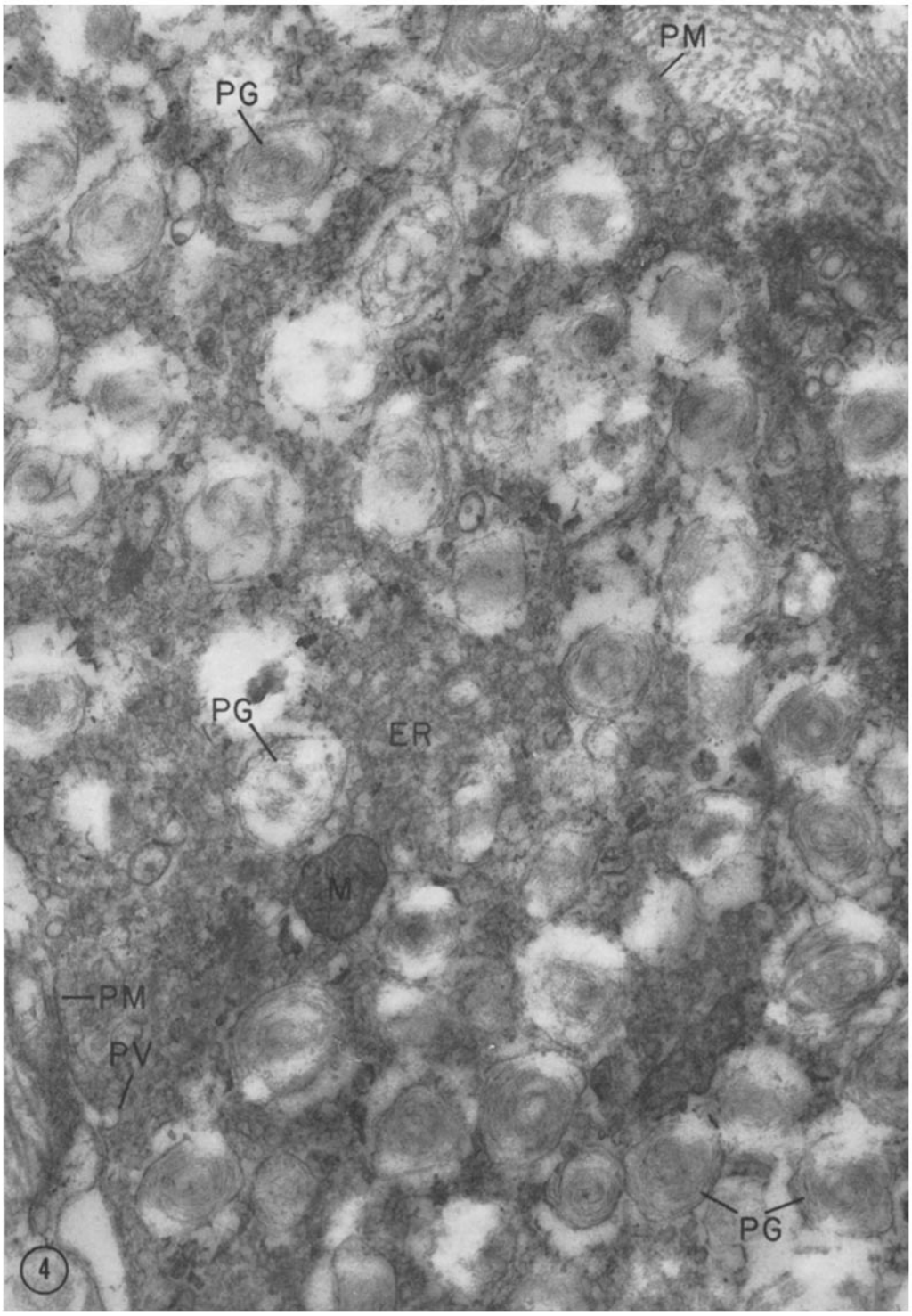


FIGURE 4 Electron micrograph of a portion of cytoplasmic processes of an erythrophore. The cytoplasm is filled with pigment granules (pterinosomes) (*PG*) and tubular smooth-surfaced endoplasmic reticulum (*ER*). RNP particles occur in groups. Mitochondria (*M*) are relatively small in number. Pinocytotic vesicles (*PV*) are seen along the plasma membrane (*PM*). UA, Ep, × 30,000.

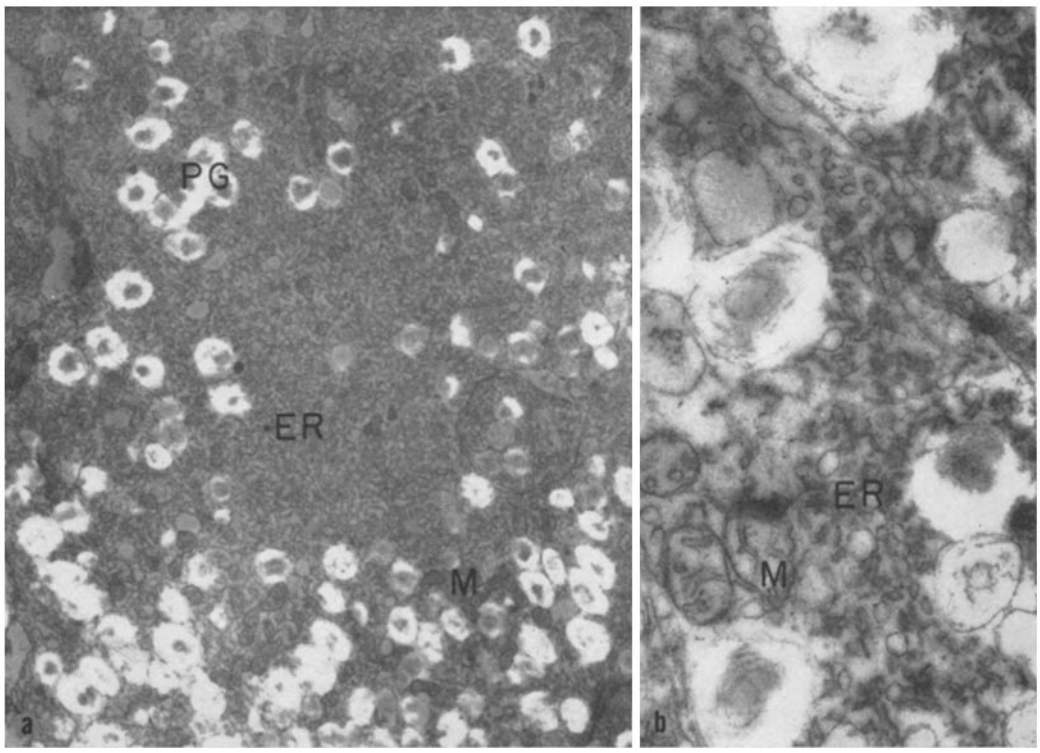


FIGURE 5 Electron micrographs of a portion of the cytoplasm of an erythrophore. Fig. 5 *a* The central portion of the cytoplasm which is occupied with the well developed, smooth-surfaced endoplasmic reticulum (*ER*). Pigment granules (pterinosomes) (*PG*) and mitochondria (*M*) are also numerous. Fig. 5 *b* Profiles of the endoplasmic reticulum enlarged to show the branching, tubular framework of rather constant diameter. Fig. 5 *a*, UA, Ep, × 7500; Fig. 5 *b*, UA, Ep, × 30,000.

deposition of pigment granules. However, little difference was detected in the chemical properties of their pigments. Cytochemical assay showed that erythrophore pigmentation consists of two different substances, a yellow fat-soluble compound and a red water-soluble compound (Fig. 1). The former, giving a typical positive reaction for carotenoid tests, was distributed almost homogeneously over the entire cytoplasm. In some cases, as pointed out by Goodrich and his associates (1), aggregates of this pigment were observed at the central portion of the cell. On the other hand, the red pigment was located specifically in pigment granules, which were densely dispersed within the cytoplasm. With the light microscope, these granules appeared to be homogeneous in size and were a little larger than melanin granules in skin melanophores of the melanophore-laden swordtail. The red pigment disappeared rapidly after treatment with 1 per cent aqueous ammonia or 5 per cent ammonium acetate and more gradually after treatment with distilled water. In effect, this disappearance was an elution of the red coloring materials into the medium. This medium, when concentrated *in vacuo* and examined by means of paper chromatography and ionophoresis, was shown to contain a large amount of both colored and colorless pteridines. The presence of pteridines in the pigment granules of erythrophores was more clearly indicated in the isolation experiments. Red pigment granules isolated by centrifugal fractionation (Fig. 2) contained a large amount of pteridines, identical to those found in erythrophores *in situ*. Moreover, the pteridine content of fractions containing red pigment granules was far greater than that of fractions free of pigment granules. From these facts, it appears that in erythrophores the pteridines are accumulated in intracytoplasmic particles at a high concentration and in this way are

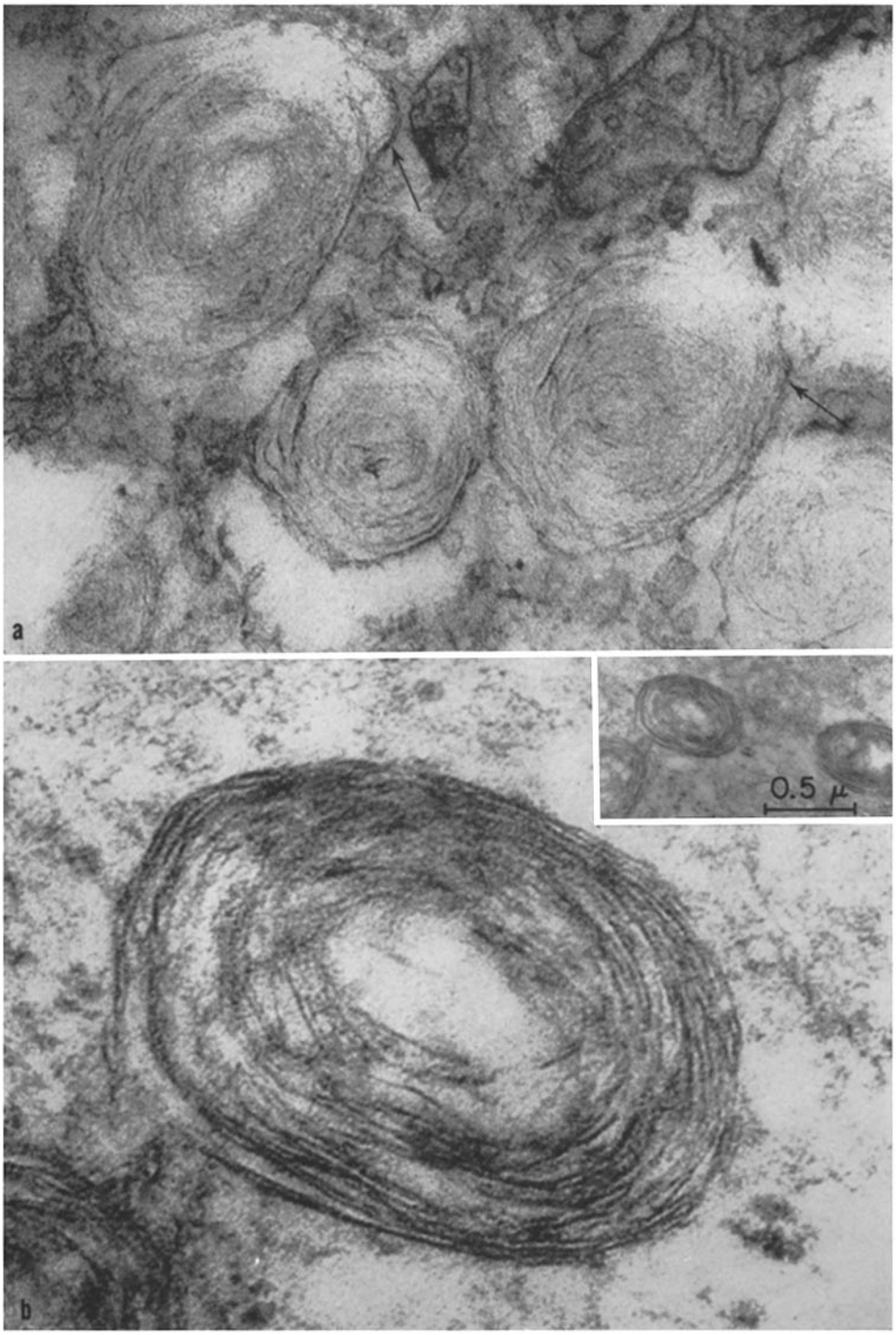


FIGURE 6 Electron micrographs of pterinosomes. Fig. 6 *a* The presence of the three-layered limiting membrane (arrow) and the internal texture is distinctly recognizable. (Enlargement of a part of Fig. 4). Fig. 6 *b* The inner lamella is composed of more than ten layers of parallel membranes in concentric or whorl-like arrangement. The inset shows an image of cytoplasm around the enlarged pterinosome. Fig. 6 *a*, UA, Ep, × 90,000; Fig. 6 *b*, UA and LH, Ep, × 150,000; inset, × 25,000.

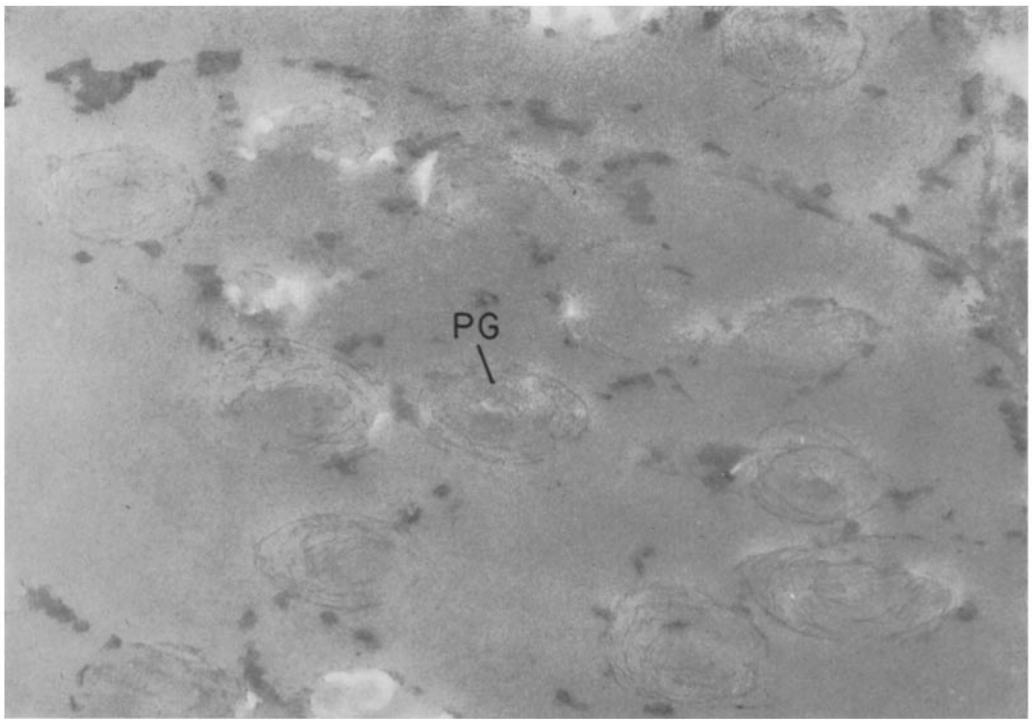


FIGURE 7 Electron micrograph of a part of the erythrophore treated with chloroform-alcohol mixture. Even after complete removal of lipid inclusions with the above mixture, the inner lamellar structure of pterinosomes is present. The majority of the other cellular framework is destroyed or disappears. UA and LH, Ep, × 45,000.

participating in the pigmentation of erythrophores. Because of their distinct and characteristic accumulation of pteridines, these granules will hence forth be designated by the term "pterinosome".

Pteridines in Pterinosomes

Water-soluble pigments obtained from pterinosomes consist of the pteridines shown in Fig. 3: Two orange and orange-fluorescing, one red and red-fluorescing, and one yellow and yellow-fluorescing substances, found in abundance, were identified, respectively, as drosopterin, isodrosopterin, neodrosopterin (hence drosopterin group references 25, 26), and sepiapterin (27); two blue, one green, and one violet-fluorescing, found in large quantity, proved to be biopterin, Ranachrome 3 (presumably, 2-amino-4-hydroxy-6-hydroxymethylpteridine) (28), xanthopterin, and isoxanthopterin; one blue and one green-fluorescing, found in smaller amounts, were identified as 2-amino-4-hydroxy-6-carboxypteridine and, presumably, dihydroxanthopterin (24). Besides these substances, three unidentified fluorescent substances were detected: one, found in large quantity, was extremely unstable and easily decomposed to 2-amino-4-hydroxy-6-carboxypteridine, while the others were unknown with respect to chemical structure. Drosopterin D (29), found together with the drosopterin group in *Drosophila*, was hardly detectable. Further details on the chemical properties of the above pteridines will appear in a forthcoming paper.

Ultrastructure of Erythrophores

Erythrophores are easily identified by their position along the basement membrane and by their characteristic red pigment granules. These chromatophores display an irregular satellite or amoeboid shape, stretching their cytoplasmic processes in various directions. Inside the cell there is one nucleus which appears to be elliptical, kidney-like, or, in most cases, irregularly shrunken in form. Erythrophore cytoplasm contains a large number of round or oval organelles and a dense

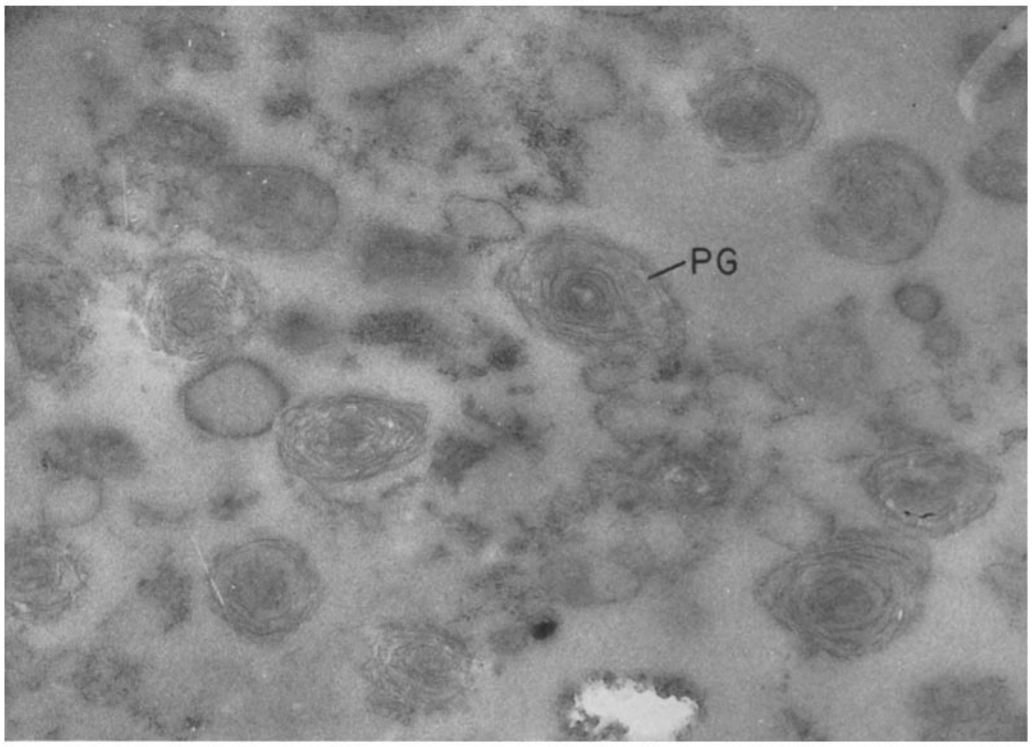


FIGURE 8 Electron micrograph of pterinosomes isolated by the centrifugation method. The profile of the isolated granules is quite similar to that found in the cell *in situ*. UA, St, × 30,000.

tubular endoplasmic reticulum. The former are pigment granules and most prominent in the cytoplasmic processes. Most commonly they have a diameter of 0.7 to 0.5 μ (Fig. 4). The endoplasmic reticulum of erythrophores is a network of branching tubules of relatively constant diameter, apparently belonging to the smooth-surfaced membranous system (Fig. 5) (30). In some cases, these tubules were found to communicate with the plasma membrane. Mitochondria with characteristic longitudinal cristae are also numerous. Clusters of ribonucleoprotein particles (RNP) occur in moderate number. The rough-surfaced endoplasmic reticulum is poorly developed; however, the Golgi complex in typical form is frequently seen. Around the plasma membrane, a considerable number of small pinocytotic vesicles of various sizes appears.

Morphological Properties of Pterinosomes

As shown in Fig. 6, the pterinosomes are limited by a thin membrane which exhibits a triple-layered structure (Fig. 6 *a*). Inside the particles, a whorl-like or concentric arrangement of parallel membranes can be observed. This membranous system, when satisfactorily fixed, consists of thirteen to twenty layers of parallel membranes. From the profiles observable in sections, pterinosomes seem to be composed of a concentric series of small spheres or spheroids. In areas where the membranes are closely and regularly packed, the intermembrane space is of the order of 120 A. However, in other areas it is generally greater than this and is highly variable. At higher magnification, each inner membrane can be resolved into two lines, each approximately 45 A wide (Fig. 6 *b*). In general, it was observed that the swelling or bursting of the lamellae resulted in a widening of the intermembrane spaces. Judging from the micrographs, it was apparent that the limiting membrane is thicker than the intragranular membranes. This difference holds with respect to the width of each of their paired submembranes. The estimates of width of each membrane are depicted in Fig. 9. The difference in thickness of outer and inner membranes may indicate differ-

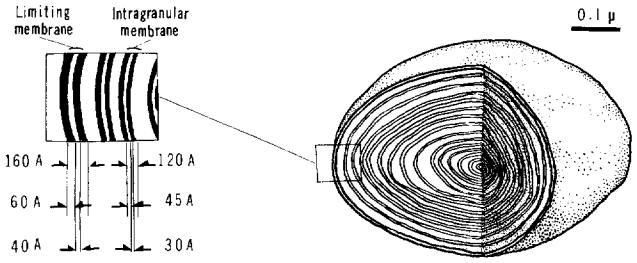


FIGURE 9 Diagrammatic representation of the possible texture of pterinosomes. The pterinosome is spherical or more possibly spheroid in shape, its diameters along the longer and shorter axis being estimated as approximately 0.7 and 0.5 μ, respectively. The three-layered limiting membrane envelops an internal membranous system of more than ten concentrically organized spheres or spheroids. The spacing between intragranular membranes *in situ* is assumed to be homogeneous and regular. The widths of the dense and opaque parts of the limiting and intragranular membranes are indicated in the figure.

ences in chemical properties and thereby possibly in their origin. When treated with organic solvents such as chloroform-alcohol or petroleum ether to remove lipid inclusions, only the internal structure of the particles is retained. Even this, however, is somewhat fragmentary (Fig. 7). Similarly, the lamellar structure just described was also recognized in pterinosomes isolated by the centrifugation method (Fig. 8). Since abundant pteridines were present in these particles, it is possible that the location of pteridines is in the internal membranes. An attempt was made to elute pteridines from the granules with aqueous ammonia without disintegrating the internal texture of the granules. This experiment, however, was not successful because the pterinosomes were damaged beyond recognition. On the other hand, the pteridines in the isolated granules were hardly extractable by mild washing with isotonic saline or with sucrose solutions.

DISCUSSION

In general, it has been well established that yellow sepiapterin and isosepiapterin (hence sepiapterin group) are utilized as pigments in the larval skin xanthophores in a wide variety of amphibian and cyprinid species (2–6), whereas the red drosopterin group, usually together with the sepiapterin group, takes part in pigmentation of the adult skin erythrophores of a rather few reptiles, amphibians and fresh-water fish (5, 7, 10). However, little is known about the specific sites of pteridine location at the subcellular level. In fact, relatively little is known about the general morphological and cytochemical characteristics of either xanthophores or erythrophores.

Evidence reported in this paper clearly shows that in the swordtail the colored pteridines such as the drosopterins and sepiapterin of erythrophore are specifically localized in its cytoplasmic particles, the pterinosomes. It seems reasonable, therefore, that these compounds are primarily acting as cell pigments. In addition, it is also shown that the majority of colorless pteridines such as biopterin, xanthopterin, isoxanthopterin, and others also exist in these particles. As for their possible participation in pigmentation, it is supposed that they either do not act as pigments or do act in a conjugated form either with each other or with protein as is strongly suggested in goldfish erythrophores (3). Since almost all of the above-described pteridines are closely related to one another with respect to structure, degradation, and general metabolism (24, 26–28, 31–33), it appears reasonable to surmise that they are interconverted. Whether enzymatic or not, it would appear that this interconversion occurs in the pterinosomes and that, as a result, erythrophore pigmentation is subjected to modification, depending upon physiological conditions. As a matter of interest in relation to the physiological role of erythrophores and pterinosomes, it should be pointed out that the majority of pteridines obtained from erythrophores is photolabile. Recently, the characteristic distribution of pteridines in pterinosomes has been established more precisely by the use of density gradient centrifugation (unpublished data).

Electron microscopy of erythrophores had indicated that their pigment granules are discrete cytoplasmic organelles. Cross-sections reveal that the pterinosome has a three-layered limiting

membrane and a series of concentric lamellae internally. Because the full complement of pteridines is present together with an intact internal lamella even after various cytochemical treatments, and because the elution of pteridines from the granules never occurs without disintegration of the internal lamellae, it is presumed that the major part of pteridines present in the granules is bound to the thin lamellar membranes. In this connection, it has been shown that in xanthophores of the yellow-scaled and the wild type swordtails, the carotenoid pigments are mostly responsible for pigmentation (1), while pteridines rarely function in this respect. The assortment of pteridines in xanthophores is considerably different from that of erythrophores. Not only do these cells lack bright-colored pteridines, but their content of colorless pteridines is also different both in quantity and quality. Within these xanthophores, granular structures are also present, but these granules lack the concentric membranous texture typical of the erythrophore pterinosome (unpublished data). This observation also supports the concept of a correlation between colored pteridines and the intragranular lamella. On the other hand, the fine structure of pigment granules as reported in this paper has been discovered in xanthophores of *Rana* and *Bufo* which are laden with a large amount of colorless pteridines (unpublished data). This would suggest that perhaps most intracellular pteridines are related to the lamellar structures.

With regard to cytoplasmic organelles other than pigment granules, it was shown that the smooth-surfaced endoplasmic reticulum was extremely abundant, whereas the rough-surfaced reticulum was scarce in swordtail erythrophores. In this respect, profiles of the tubular endoplasmic reticulum of the erythrophore are quite comparable to those of the socalled chloride cell in the teleost gill (34). Possibly this signifies that erythrophores are engaged in some function such as osmoregulation.

During the early phases of amphibian development, the autonomy of biosynthesis of pteridines is endowed in chromatophores (6). It would be important to know whether the pteridine-forming enzyme system is distributed in the pterinosomes. Such an approach would make it possible to establish the cytochemical properties of the pigment granules.

In view of what is known about the process of melanin granule formation in mammalian melanocytes (35), it seems possible that the pterinosomes of swordtail erythrophores are also closely related in their origin to the smooth-surfaced membranes. In fact, the limiting membrane of the pterinosome is similar in profile to the smooth-surfaced membrane, and occasionally a communication was observed between the two membranes. Elucidation of the origin and structural changes of pterinosomes in the course of erythrophore differentiation may cast some light on the problem of cytodifferentiation of chromatophores. Experiments along this line are in progress.

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REFERENCES

1. GOODRICH, H. B., HILL, G. A., and ARRICK, M. S., The chemical identification of gene-controlled pigments in *Platypoecilus* and *Xiphophorus* and comparison with other tropical fish, *Genetics*, 1941, **26**, 533.
2. ZIEGLER-GÜNDER, I., Untersuchungen über die Purin- und Pterin-Pigmente in der Haut und in den Augen der Weissfische, *Z. vergleich. Physiol.*, 1956, **39**, 163.
3. MATSUMOTO, J., Role of pteridines in the pigmentation of chromatophores in cyprinid fish, *Japan. J. Zool.*, 1965, **14**, 45.
4. GÜNDER, I., Nachweis und Lokalization von Pterinen und Riboflavin in der Haut von Amphibien und Reptilien, *Z. vergleich. Physiol.*, 1954, **36**, 78.
5. HAMA, T., The relation between the chromatophores and pterin compounds, *Ann. New York Acad. Sc.*, 1963, **100**, 977.
6. OBIKA, M., Association of pteridines with am-

phibian larval pigmentation and their biosynthesis in developing chromatophores, *Develop. Biol.*, 1963, **6**, 99.

7. OBIKA, M., and BAGNARA, J. T., Pteridines as pigments in amphibians, *Science*, 1964, **143**, 485.
8. ORTIZ, E., and WILLIAMS-ASHMAN, H. G., Identification of skin pteridines in the pasture lizard *Anolis pulchellus*, *Comp. Biochem. and Physiol.*, 1963, **10**, 181.
9. KAUFFMANN, T., Untersuchungen über die fluoreszierenden Pigmente in der Haut von Zierfischen, *Z. Naturforsch.*, 1959, **14**, 358.
10. ZIEGLER, I., Genetic aspects of ommochrome and pterin pigments, *Advances Genet.*, 1961, **10**, 349.
11. CHARLES, A., and INGRAM, J. T., Electron microscope observations of the melanocytes of the human epidermis, *J. Biophysic. and Biochem. Cytol.*, 1959, **6**, 41.
12. DROCHMANS, P., Electron microscope studies of epidermal melanocytes, and the fine structure of melanin granules, *J. Biophysic. and Biochem. Cytol.*, 1960, **8**, 165.
13. BIRBECK, M. S. C., Electron microscopy of melanocytes: the fine structure of hair-bulb premelanosomes, *Ann. New York Acad. Sc.*, 1963, **100**, 540.
14. MATSUMOTO, J., KAJISHIMA, T., and HAMA, T., Relation between the pigmentation and pterin derivatives of chromatophores during development in the normal black and transparent scaled types of goldfish (*Carassius auratus*), *Genetics*, 1960, **45**, 1177.
15. YAMAMOTO, T., Changes of the cortical layer of the egg of *Oryzias latipes* at the time of fertilization, *Proc. Imp. Acad. (Tokyo)*, 1939, **15**, 269.
16. LISON, L., Histochimie Animale, Paris, Gauthier-Villars, 1963.
17. KUSHIDA, H., A styrene-methacrylate resin embedding method for ultrathin sectioning, *J. Electronmicroscopy (Tokyo)*, 1961, **10**, 16.
18. CAULFIELD, J. B., Effects of varying the vehicle for OsO_4 in tissue fixation, *J. Biophysic. and Biochem. Cytol.*, 1957, **3**, 827.
19. LUFT, J. H., Improvements in epoxy resin embedding methods, *J. Biophysic. and Biochem. Cytol.*, 1961, **9**, 409.
20. KUSHIDA, H., On the handling of epoxy resins and polyester resins as embedding media for electron microscopy, *J. Electronmicroscopy (Tokyo)*, 1960, **9**, 157.
21. WATSON, M. L., Staining of tissue sections for electron microscopy with heavy metals, *J. Biophysic. and Biochem. Cytol.*, **4**, 475.
22. MILLONIG, G., A modified procedure for lead staining of thin sections, *J. Biophysic. and Biochem. Cytol.*, 1961, **11**, 736.
23. AWAPARA, J., Application of paper chromatography to the estimation of free amino acids in tissues, *Arch. Biochem.*, 1948, **19**, 172.
24. NAWA, S., The structure of the yellow pigment from *Drosophila*, *Bull. Chem. Soc. Japan*, 1960, **33**, 1555.
25. LEDERER, E. Les pigments des invertébrés (à l'exception des pigments respiratoires), *Biol. Rev. Cambridge Phil. Soc.*, 1939, **15**, 273.
26. VISCONTINI, M., Hadorn, E., and KARRER, P., Fluoreszierende Stoffe aus *Drosophila melanogaster*: die roten Augenfarbstoffe, 5. Mitteilung, *Helv. Chim. Acta*, 1957, **40**, 579.
27. FORREST, H. S., and MITCHELL, H. K., Pteridines from *Drosophila*. I. Isolation of a yellow pigment. *J. Am. Chem. Soc.*, 1954, **76**, 5656.
28. GOTO, T., Isolation and characterization of some fluorescent substances in the skin of the frog, *Rana nigromaculata* (Japanese), *Zool. Mag.*, Tokyo, 1959, **68**, 286,
29. THROCKMORTON, L. H., The use of biochemical characteristics for the study of problems of taxonomy and evolution in the genus *Drosophila*, *in* Studies in Genetics, *Texas, Univ. Publ., 6205*, 1962, **2**, 415.
30. DALTON, A. J., and FELIX, M. D., Cytological and cytochemical characteristics of the Golgi substance of epithelial cells of the epididymis, *in situ*, in homogenates and after isolation, *Am. J. Anat.*, 1954, **94**, 171.
31. VISCONTINI, M., Fluoreszierende Stoffe aus *Drosophila melanogaster*; Beitrag zur Konstitutions-aufklärung der Drosopterine, *Helv. Chem. Acta*, 1958, **41**, 1299.
32. TAIRA, T., The metabolism of sepiapterin in *Drosophila melanogaster*; emphasizing its tetrahydro-form, *Japan. J. Genetics*, 1961, **36**, 244.
33. FORREST, H. S., and MITCHELL, H. K., Pteridines from *Drosophila*. III. Isolation and identification of three more pteridines, *J. Am. Chem. Soc.*, 1955, **77**, 4865.
34. DOYLE, W. L., and GORECKI, D., The so-called chloride cell of the fish gill, *Physiol. Zool.*, 1961, **34**, 81.
35. SEIJI, M., SHIMAO, K., BIRBECK, M. S. C., and FITZPATRICK, T. B., Subcellular localization of melanin biosynthesis, *Ann. New York Acad. Sc.*, 1963, **100**, 497.