# MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF GOLDFISH ERYTHROPHORES AND THEIR PTERINOSOMES

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## ABSTRACT

The fine structure of integumental erythrophores and the intracellular location of pteridine and carotenoid pigments in adult goldfish, *Carassius auratus*, were studied by means of cytochemistry, paper and thin-layer chromatography, ionophoresis, density-gradient centrifugal fractionation, and electron microscopy. The ultrastructure of erythrophores is characterized by large numbers of somewhat ellipsoidal pigment granules and a well-developed system of tubules which resembles endoplasmic reticulum. The combined morphological and biochemical approaches show that pteridine pigments of erythrophores are located characteristically in pigment granules and are the primary yellow pigments of these organelles. Accordingly, this organelle is considered to be the "pterinosome" which was originally found in swordtail erythrophores. Major pteridines obtainable from goldfish pterinosomes are sepiapterin, 7-hydroxybiopterin, isoxanthopterin, and 6-carboxyisoxanthopterin. Density-gradient fractions indicate that carotenoids are mostly associated with the endoplasmic reticulum. Both tyrosinase and possibly a tyrosinase inhibitor containing sulfhydryl groups are present in the pterinosome. The possible existence of a tyrosinase inhibitor is suggested by the marked increase of tyrosinase activity upon the addition of iodoacetamide or *p*-chloromercuribenzoic acid. In the light of their fine structure, pigmentary composition, and enzymatic properties, the erythrophores and pterinosomes are discussed with respect to their probable functions and their relationship to melanophores.

## INTRODUCTION

It has been clearly shown that pteridine derivatives prevalent in the skins of lower vertebrates are specifically located in bright-colored yellow and red pigment cells called xanthophores and erythrophores, respectively (3, 17–19, 27, 28, 33, 34, 45). Some of these compounds are known to function as cell pigments. Typical examples are the participation of yellow sepiapterins as larval xanthophore pigments in a wide variety of species of cyprinid fish and amphibians and the participation of red drosopterins as erythrophore pigments in xiphophorin fishes and amphibians (27, 28, 33, 34). With regard to the subcellular distribution of pteridines in such chromatophores, it has been clearly shown in recent studies of swordtail erythrophores that drosopterins and sepiapterins, together with other colorless pteridine derivatives, are located characteristically in a cytoplasmic organelle which has been named the pterinosome (28). Morphological properties, such as the pres-

ence of a limiting membrane and of a characteristic intragranular lamellar framework, indicate that the pterinosome is a discrete cytoplasmic organelle.

As for the mode of pigmentation of goldfish xanthophores and erythrophores, it has also been shown that, even though carotenoid pigments are primarily responsible for their coloration, pteridine derivatives exist abundantly and characteristically in these cells and are concerned with pigmentation (27). The principal pteridine derivatives are sepiapterins, 7-hydroxybiopterin, isoxanthopterin, and 6-carboxyisoxanthopterin (27, 31). Among these, sepiapterins are found to be the exclusive yellow pigments of postembryonic larval xanthophores (27). Colorless 7-hydroxybiopterin and isoxanthopterin are both present in extremely large amounts in adult erythrophores, and presumably they act as pigments either *in situ* in conjugation with other cellular materials or in polymerized form (1, 31, 43). Cytochemical analysis has emphasized that these pteridines, both colored and colorless, are closely associated with pterinosomes in their location (27). These findings have led us to consider that in goldfish erythrophores there exists a particular organelle which is associated with pteridine deposition and which is comparable to the pterinosome found in swordtail erythrophores.

In mammalian melanocytes, the enzyme tyrosinase is localized on a subcellular organelle called a premelanosome and is responsible for the synthesis of melanin (42). It has been demonstrated in xanthic goldfish that melanization can occur in xanthophores. Thus, Loud and Mishima have suggested strongly the possible transformation of xanthophores into melanophores (25). Since melanization seems to occur in these xanthophores, it is reasonable that tyrosinase should be present. It is interesting that both melanophores and xanthophores have the same origin (13, 21) and that their pigment organelles, premelanosomes and pterinosomes, are similar in appearance. Thus, if tyrosinase is present in xanthophores, it would logically be localized on the pterinosome.

This investigation was performed to determine the subcellular localization of pteridines in adult erythrophores of the goldfish. The possible presence of tyrosinase in the pterinosome also was investigated. The data are discussed in relation to the similarities between pterinosomes of erythrophores and melanosomes of melanophores.

## MATERIALS AND METHODS

### *Materials*

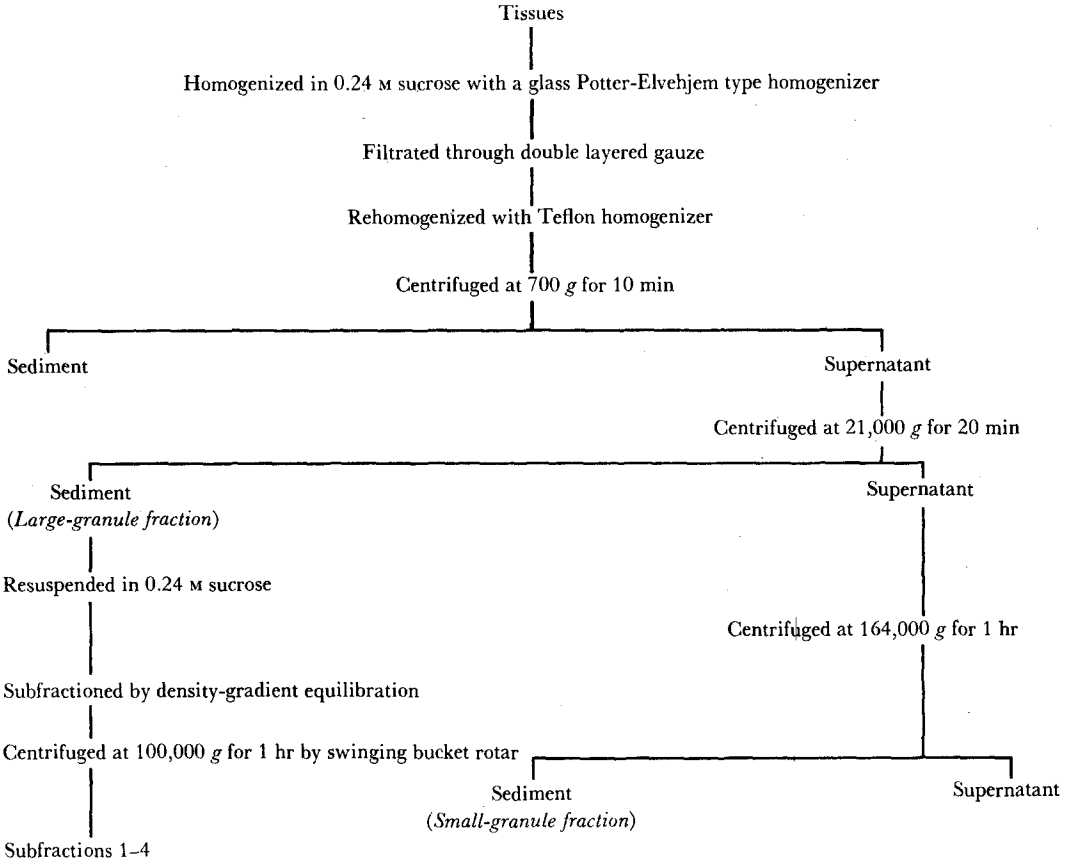
The goldfish, *Carassius auratus*, used in these experiments were of the homogeneously orange-colored, fan-tailed variety (*Ryukin*) obtained from commercial hatcheries. Fish from 10-months-old to 2-years-old with standard body lengths of 2.5–4.0 cm were used.

### *Microscopy*

Materials for light and electron microscopy were dissected either from webs of dorsal and tail fins or from middorsal head skins devoid of scales. For light microscopic examination of water-miscible cell pigments, small pieces of tissue were treated with ethanol, acetone, or chloroform-ethanol (1:2 v/v) mixtures alone or with the addition of ether, petroleum ether, benzene, or xylene for more than 2 hr with several changes. The tissues were embedded via ethanol into styrene resin according to Kushida's procedure (23). Sections were examined either without further treatment or after treatment with chloroform in order to remove resins. The localization of fat-soluble coloring pigments was ascertained by the use of frozen sections of fresh materials. Carotenoids were detected by the development of blue color after treatment with concentrated sulfuric acid (24). For electron microscopy, as a routine method, tissues were trimmed immediately after excision to a size of 1–2 $mm^2$ and were fixed for 1–2 hr, below 1°C, in 1 or 2% $OsO_4$ in either acetate-Veronal buffer (pH 7.6) containing 0.24 M sucrose (9) or in phosphate buffer (pH 7.6) (30). In some cases, materials from the same individuals were fixed in 2.5% glutaraldehyde either in 0.1 M cacodylate (pH 7.6) or 0.065 M *s*-collidine (pH 7.6) buffer for 1–3 hr, followed by postfixation for 1 hr in $OsO_4$ dissolved in the same buffer as in the prefixation (7, 41). Some specimens were fixed in chloroform-ethanol (2:1 v/v) mixture for approximately 2 hr with several changes. After processing through a graded series of ethanol, specimens were embedded in Epon resin according to Luft's procedure (26) or in styrene according to Kushida's method (23). Thin sections were cut serially, usually at the cross-sectional orientation for the skin plane, with glass knives on a Porter-Blum MT1 ultramicrotome. Staining was done with uranyl acetate (44) and/or lead hydroxide (29). Occasionally, specimens were stained with uranyl acetate in their blocks before dehydration according to the procedure of Farquhar and Palade (14). Sections from these blocks were examined either without further contrasting or by staining with lead acetate. They were examined in a Hitachi electron microscope (Model HS-7). For comparison of electron microscopic observations with light microscopic observations, sections of 1–4 $\mu$ were

TABLE I

*Summarized Procedure for Isolation of Pterinosomes from Goldfish Skins*




cut from the same block. In the case of styrene embedding, sections were stained according to Mallory's procedure (39) after removal of the resin.

## *Fractionation of Pigment Cell Components*

Centrifugal fractionation of skin and fin tissues was done by the sucrose-density gradient method (42). Animals were sacrificed by totally bleeding them after the heart had been severed. Skins and fins were detached quickly and were washed with cold, Ringer's solution. They were then immersed in ice-cold 0.24 M sucrose solution and were washed by several changes of the medium. Thereafter, 0.24 M sucrose was used routinely as the suspending medium, unless otherwise specified. All subsequent procedures were carried out below 3°C. After the tissue had been weighed, it was homogenized lightly in approximately 3–5 parts sucrose in a Potter-Elvehjem type glass homogenizer and filtered through double-layered gauze. The crude homogenate thus prepared was rehomogenized in a Teflon homogenizer until the materials were homogeneously dispersed. The resulting orange-colored homogenates were centrifuged first at 700 *g* for 10 min in order to exclude nuclei and cell debris (Table I). The supernatant was centrifuged again at 21,000 *g* for 20 min and yielded a reddish sediment. This sediment was considered to be the "large-granule fraction" described by Seiji et al. (42). The large-granule fraction was further subjected to subfractionation by equilibrating in a sucrose-density gradient. The density gradient was prepared in tubes of a Hitachi swinging-bucket rotor (RSP-40) by layering serially 0.5 ml of nine different concentrations of sucrose solution. The tubes were allowed to stand for 18–20 hr before use at 7°C. The most successful combination of sucrose concentrations for the isolation of pigment granules was 2.6, 2.4, 2.2, 2.0, 1.8, 1.6, 1.4, 1.2, and 1.0 M in sequence from the bottom to the top of the tube (Fig. 1). As a rule, sediments of the large granule fraction were resuspended in about 3 ml of sucrose, and then each 1 ml of the suspension was layered carefully on the top of the tube. Usually, either three or six identical tubes were prepared simultaneously

and centrifuged at 100,000 *g* for 60 min with the Hitachi Model 55P preparative ultracentrifuge and its swinging rotor. After centrifugation, the subfractions appearing in the gradient were recorded and separated by the use of a tube cutter. For the purpose of washing and subsequently making a pellet, the identical subfractions obtained were combined and resuspended in an adequate amount of sucrose solution slightly lower in concentration than the medium in which they were equilibrated in the gradient. For biochemical analysis, portions of the suspensions were centrifuged at 136,000 *g* for 1 hr and were collected as pellets. For morphological examination, the remaining suspensions were fixed in about 10 parts of freshly prepared, ice-cold fixative solutions containing 1% $OsO_4$, 0.1 M acetate-Veronal buffer, and sucrose, the concentration of which either was equal to that of the sucrose of the suspensions or was 1.5 M when sucrose concentrations of the suspensions exceeded this value. The fixed particles were collected by centrifugation at 164,000 *g* for 40 min. In order to study the pigmentary constituents of smaller particulates, the supernatant obtained at 21,000 *g* was subjected to further centrifugation at 164,000 *g* for 1 hr. The resulting pellet was washed once by resuspending it in 0.24 M sucrose and was recentrifuged at the same speed. For convenience, the particulates thus obtained were termed the "small-granule fraction."

### *Chemical Determination of Pteridines and Carotenoid Pigments*

Detection and identification of pteridines in isolated particles were accomplished by means of paper chromatography and ionophoresis. The detailed technique used was essentially the same as that described in preceding reports (27, 28, 31). The qualitative analysis of carotenoids was done by thin-layer and paper partition chromatography. In many cases, samples to be examined were squashed directly either on the thin film [silica gel (Wakogel-BO Wakô Pure Chemical Industries, LTD, Osaka, Japan)-calcium hydroxide (6:1 w/w)] or on filter papers (Whatman No. 1) and developed with benzene-petroleum ether-acetone (10:10:1 v/v) or toluene-methanol (4:1 v/v) mixtures. In some cases, samples were subjected to extraction repeatedly with a chloroform-ethanol (1:2 v/v) mixture of 5–10 parts of their wet weight for 10 min at 50°C. The extracts were combined and centrifuged at 1500 *g* for 10 min in order to remove proteins and other insoluble components. Part of the supernant was utilized for a partition test between 90% methanol and petroleum ether in which the combined carotenoid content was determined collectively (12). For chromatography, the extract evaporated to dryness *in vacuo*, was dissolved in a small amount of ether or acetone, and then applied either to silica gel (thin layer) or to filter paper. Carotenoids were detected by their visible color or by their emission of fluorescence under UV irradiation of 255 or 365 mμ. Identification was made by comparing the $R_f$ value of each spot with that of authentic materials. The β-carotene and xanthophyll used as the control were the commercially available products, but in some cases they were purified by chromatography. Quantitative determination of pigments in the isolated particulates was made as follows. Each pellet was treated twice with 5 ml of chloroform-70% ethanol (3:1 v/v) mixture at 50°C for 5 min. The extracts collected by centrifugation at 1500 *g* for 10 min were combined and then mixed with the equal volume of chloroform and 3 ml of distilled water. Upon vigorous shaking and subsequent low speed centrifugation for 20 min, the mixture was separated into two layers. The pteridines were concentrated in the epiphasic aqueous layer, whereas the carotenoids were concentrated in the hypophasic chloroform-ethanol layer. The residual proteins in the mixture were precipitated as a thin, white film between the two layers. Each layer was pipetted up separately and adjusted to volume with distilled water or a chloroform-ethanol mixture. In order to minimize loss of unextracted pteridines, every residue was subjected to reextraction with 5 ml of 70% ethanol containing 1% ammonia at 50°C for 20 min. The extracts obtained were thereafter treated according to Awapara's method (2). The pteridine contents in the extracts were estimated by means of fluorimetry designed for 7-hydroxybiopterin and isoxanthopterin (27). The detailed technique used in this estimation was essentially the same as that described in a preceding report, except that actual measurement was made on the mixture without isolation of each specific pteridine. For this reason, yellow sepiapterine which has a different spectrum is essentially excluded from the estimates obtained by this method. The total pteridine content was calculated for each particulate fraction in terms of micrograms of quinine. The carotenoids were quantitated colorimetrically; the absorbance of each extract was determined by wavelengths of 400, 450, and 500 mμ by use of a Hitachi Model EPU-2 spectrophotometer. For quantitative comparisons, the total content of each particulate fraction was expressed in terms of absorbance when concentrated to 1 ml of a chloroform-ethanol (3:1 v/v) mixture.

### *Tyrosinase Assay*

As starting materials for the isolation of these particulate fractions by centrifugation, approximately 10 g of fresh skin per test were utilized. Each pellet obtained by the procedure described in the previous section was suspended in a small amount of distilled water (in most cases, 2 ml) by using a Teflon homogenizer. This process seemed advantageous in in-

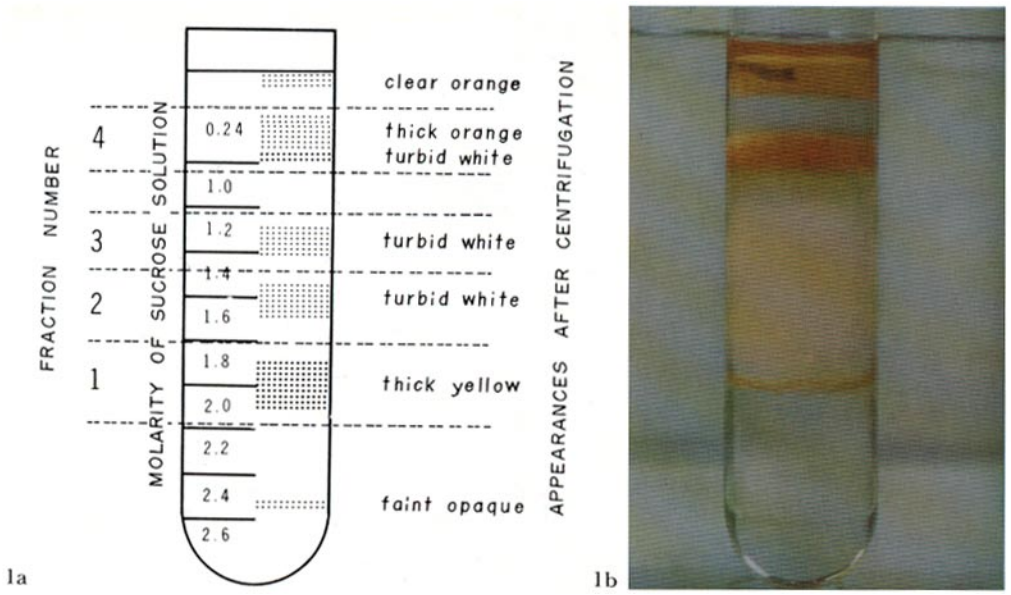


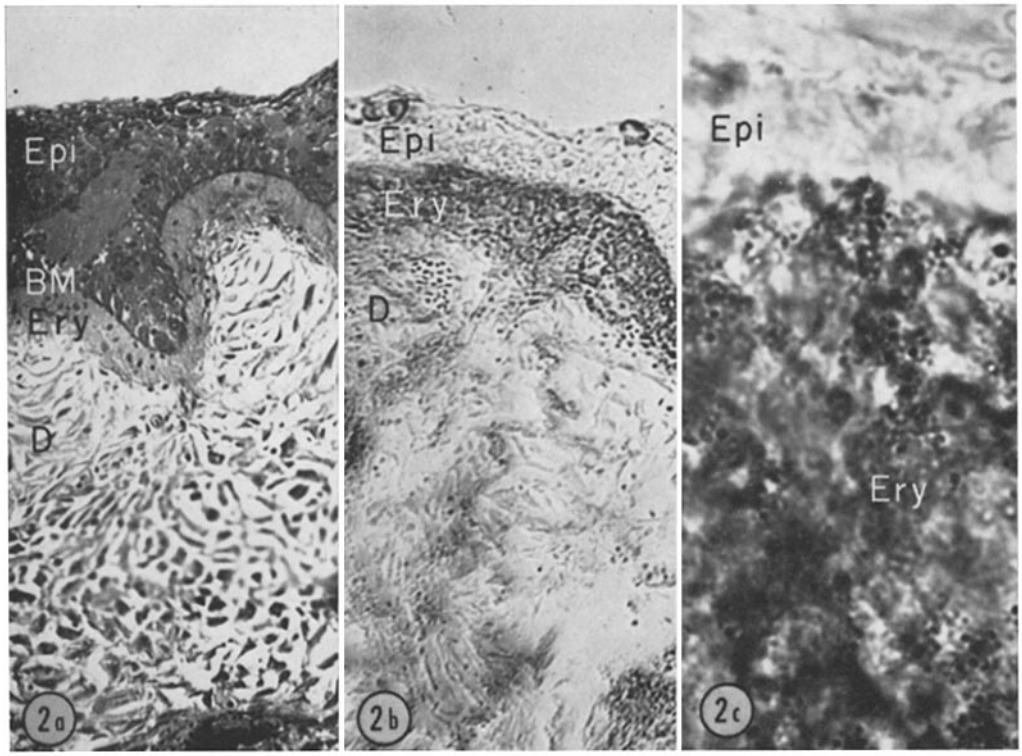

FIGURE 1 *a*, Diagrammatic representation of layering in sucrose-density gradient (left) and resulting sedimentation pattern (right) used for pterinosome isolation from goldfish skins. Broken lines show the places where the tube was cut; the numbers between the lines refer to symbols of fractions thus obtained. *b*, Appearance of the tube after centrifugation; the pterinosome fraction is characterized by thick yellow coloration.

creasing the osmotic breakdown of the particles, thus facilitating the dispersion of enzyme into the suspending medium. Tyrosinase activities were determined colorimetrically by measuring the amount of enzymatically synthesized melanin (6). The assay medium contained $6.4 \times 10^{-4}$ M *l*-tyrosine, $8 \times 10^{-6}$ M DOPA ($\beta$-(3,4-dihydroxyphenyl)-l-alanine), 0.077 M phosphate buffer (pH 6.8) and, 100 IU penicillin per ml. In order to exclude the possible inhibition of tyrosinase activity by sulfhydryl agents as was found in *Orizias latipes* (20), in one part of this assay, iodoacetamide and *p*-chloromercuribenzoic acid were added so as to give a final concentration of $8 \times 10^{-3}$ M and $8 \times 10^{-4}$ M, respectively. As a rule, each 2.1 ml of the assay medium thus prepared was mixed with 0.5 ml of freshly prepared particle suspension. As a control, the particulate suspension was treated in boiling water for 3 min. Assay was also made on a medium lacking *l*-tyrosine, the total volume of which was adjusted to 2.6 ml with distilled water. The reaction mixtures were then incubated at 30°C for 18 hr with occasional shaking. At the end of incubation, each mixture was heated in boiling water for 3 min and to it was added 3 parts of a chloroform-ether mixture (1:2 v/v) to 1 part of the reaction mixture, in order to remove fat-soluble ingredients unfavorable for colorimetry. After vigorous shaking and subsequent centrifugation at 1500 *g*, the black pigments formed were collected as pellets. The pellets were then dryed and stored *in vacuo*. Subsequently, they were dissolved in approximately 2 ml of 0.2 N NaOH solution by heating it at 70°C for 5 min. After adjusting the volume with NaOH solution, the absorbance of each solution was examined at wavelengths of 400, 450, 500, 550, and 600 m$\mu$ with a Hitachi EPU-2 spectrophotometer. A linear relationship has been found to exist between melanin content and absorbance when the above-described method is utilized in examining melanin content obtained from black moor goldfish (*Kurodemekin*) skin (Matsumoto, J., and M. Obika. Unpublished data).

## RESULTS

### *General Morphology of Erythrophores*

Observations with the light-microscope revealed that erythrophores were located in the dermis just beneath the basement membrane where they formed a continuous layer. For orientation, the general organization of a cross-section of the skin is shown in Fig. 2. The erythrophores in this layer were very numerous and contained dendritic processes which were extremely complex in shape, making it difficult to determine the shape of an




In FIGS. 2–9 the embedding and staining processes are indicated throughout the legends by the following abbreviations: Ep, Epon; St, styrene; UA, uranyl acetate; LH, lead hydroxide. Symbols used in this paper are: *BC*, basal cell of epidermis; *BM*, basement membrane; *Co*, collagen fibril; *D*, dermis; *Epi*, epidermis; *Ery*, erythrophore; *er*, endoplasmic reticulum (smooth-surfaced); *g*, Golgi complex; *mit*, mitochondrion; *n*, nucleus; *pg*, pigment granule pterinosome; *pm*, plasma membrane; *pv*, micropinocytic vesicle; *scl*, subepidermal collagenous lamella.

FIGURE 2 Light micrographs of cross-sections of dorsal head skin of orange-colored goldfish. *a*, Fixed in Carnoy's fluid and stained with Mallory's procedure. The general organization of the skin and the location of erythrophores can be seen. St. × 864. *b*, Fixed in chloroform-ethanol (1:2 v/v) mixture. Without staining. Yellow granules can be seen in the erythrophores after complete removal of carotenoids. Yellow coloration was contrasted through a blue filter. St. × 896. *c*, Fresh, frozen section in physiological salt solution. The presence of pigment granules within erythrophores is apparent. × 2000.

individual erythrophore. When observations were made on the fin web where the erythrophore population was reduced, the erythrophore could be seen as a stellate-shaped cell with processes branching out from the nucleated central region. The cytoplasm contained a large number of pigment granules of similar size.

Electron microscopy of erythrophores revealed that their cytoplasm was occupied with a large number of round, membrane-bounded organelles and a well-developed tubular endoplasmic reticulum (Figs. 3 and 4). When these observations were compared with light microscopic observations, the membrane-bounded organelles were readily identified as pigment granules characteristic of this cell and most commonly had a diameter of 0.4–0.6 $\mu$. The tubular endoplasmic reticulum was composed of smooth-surfaced membranes. Frequently the elements of the reticulum were connected with one another, forming a meandering network. These tubules had an average diameter of 110 A when sectioned nearly transversely. In addition to these organelles, Golgi complexes were commonly observed which occasionally appeared in a typical arrangement of an array of parallel membranes enclosing a number of vesicles of

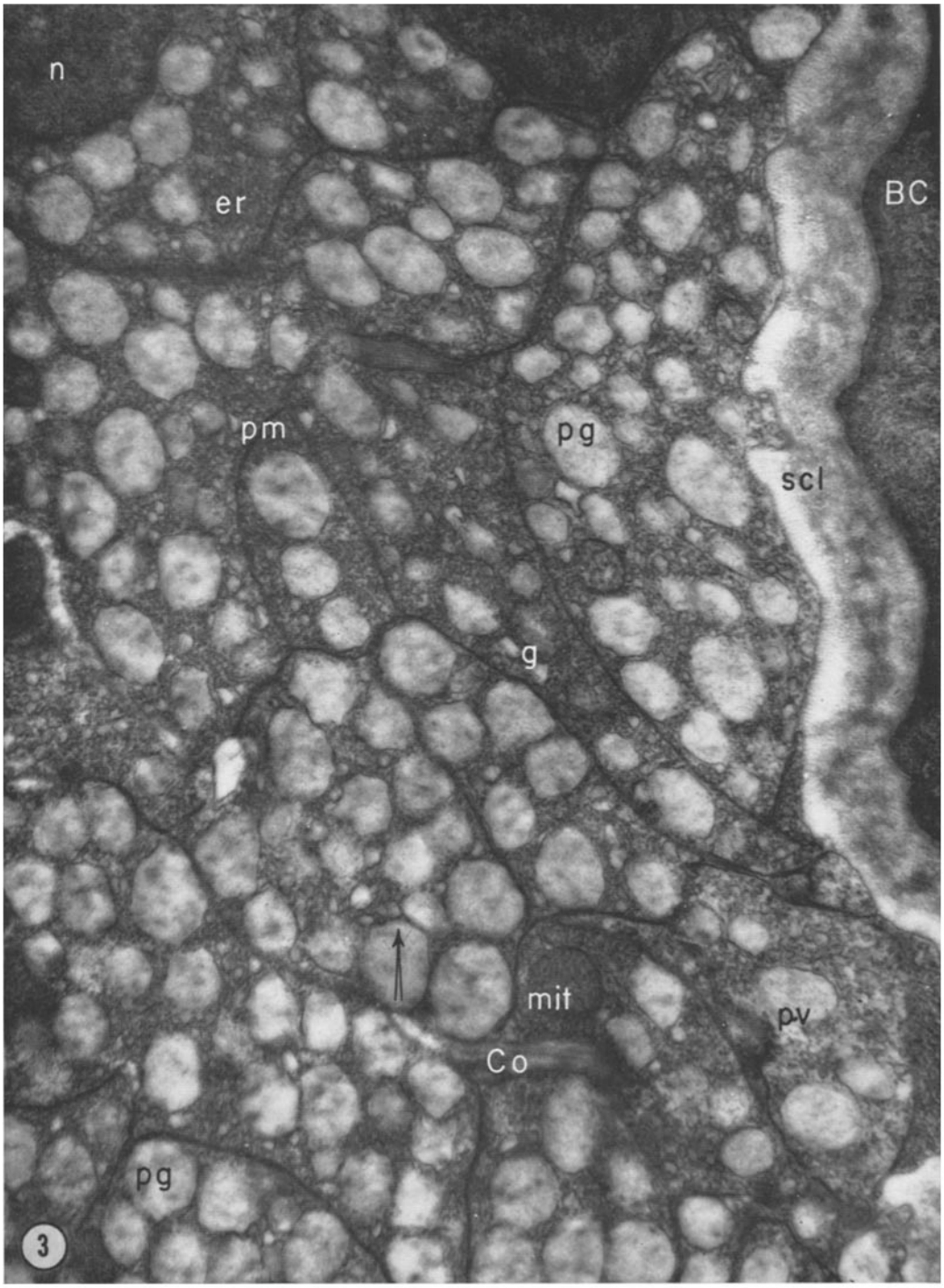



FIGURE 3 Electron micrograph of the dorsal head skin showing the location of erythrophores. Double-stemmed arrow as for Figure 4 b. Ep, UA, and LH. × 20,000.

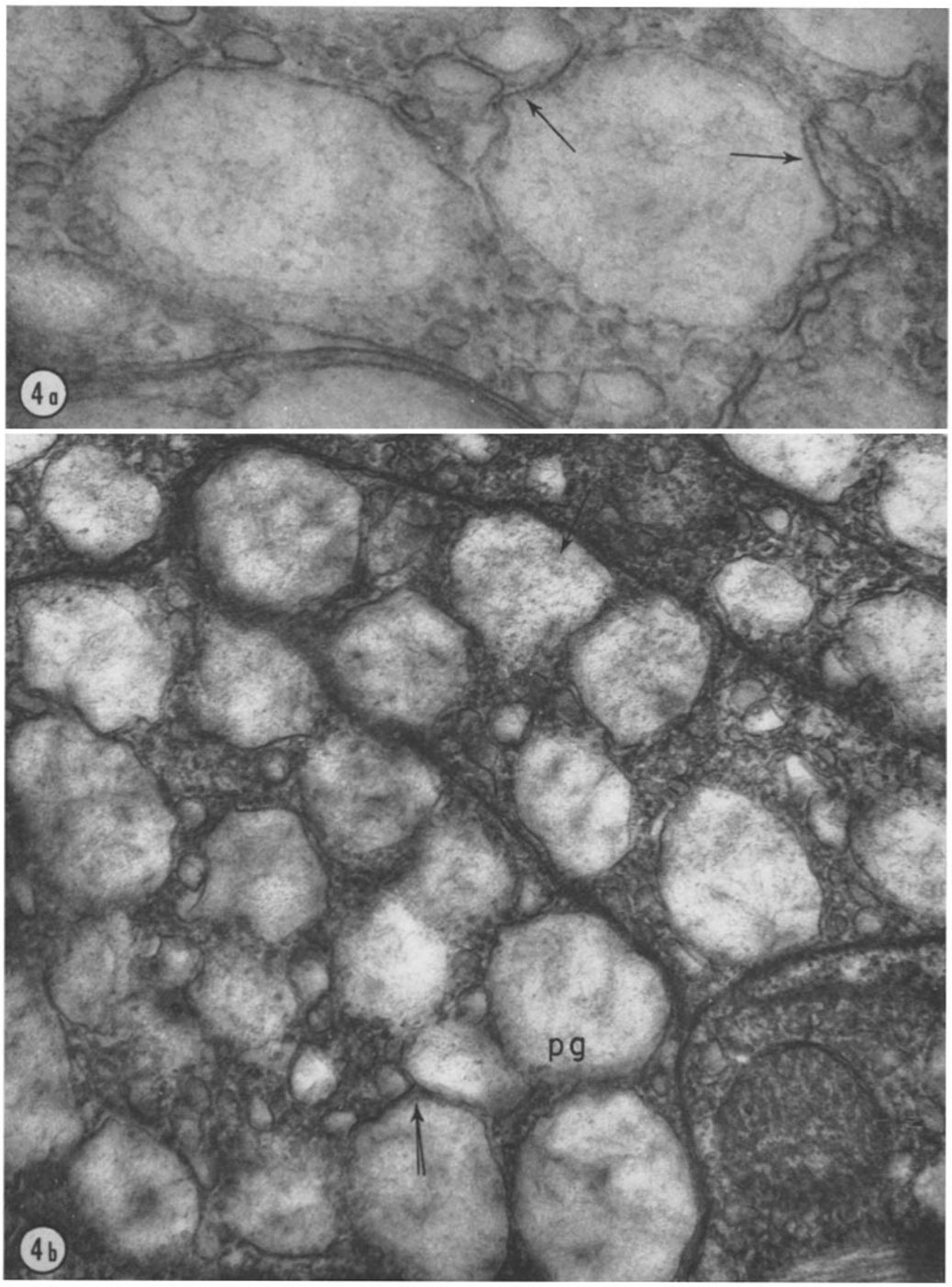
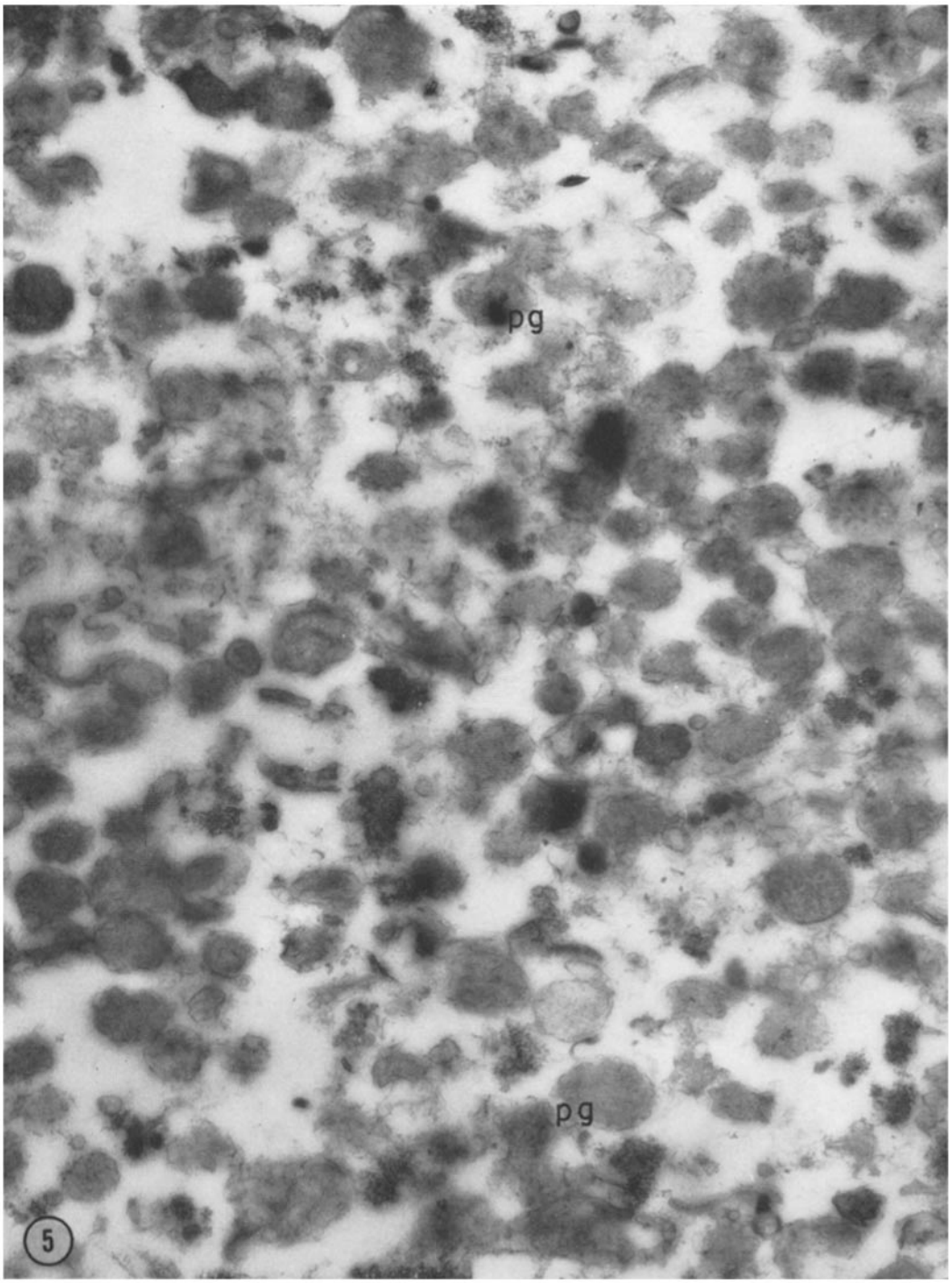



FIGURE 4 Electron micrograph of cytoplasmic processes of erythrophores. *a*, Pigment granule (pterinosome) is surrounded by a unit membrane (arrow). Ep, UA, and LH. × 70,000. Taken with JOEL Model JEM-7A electron microscope. *b*, Each pterinosome is equipped with an irregularly arranged framework of thin fibrils. Cross-sectional profiles of thin fibrils (arrow) indicate that the material of their internal texture is not fragment of membrane. Enlarged part (double-stemmed arrow) of Fig. 3. Ep, UA, and LH. × 40,000.




Figs. 5–9 are electron micrographs of particles isolated by centrifugation.

FIGURE 5 Large granule, fraction 1. It is composed almost exclusively of pterinosomes. Most of the pterinosomes have lost their limiting membrane partially or entirely, presumably as a result of osmotic damage. Frequently, the aggregates of the internal filaments of these particles can be seen. St, UA. × 21,000.

varying sizes. Mitochondria with well-developed tubular cristae, oval or elongated in shape, were also numerous. With regard to the distribution of the above-described organelles, it was observed that frequently pigment granules were more numerous in the processes or in the distal portion of the cell, whereas the Golgi complex was found peripheral to the nucleus. The mitochondria were dispersed somewhat randomly throughout the cytoplasm. Ribonucleoprotein particles occurred in groups in moderate number. The rough-surfaced endoplasmic reticulum was observed rarely. Along the plasma membrane, cytoplasmic pits and membrane-bounded vesicles were seen.

### *Cytochemistry of Erythrophore Pigments*

With regard to the pigmentary materials of the erythrophores, it was shown cytochemically in a preceding paper (27) that they are composed of two different groups of compounds: orange-colored, organic solvent-miscible carotenoids and yellow-colored, water-miscible pteridines. This finding was confirmed again in detail in this investigation. When fresh skins were treated with ethanol, acetone, or chloroform-ethanol mixture alone or even after the repeated treatment with ether, petroleum ether, benzene, or xylene, the cytoplasmic pigment granules of the erythrophores, as mentioned above, remained faintly yellow (Fig. 2 *b*). This pigment disappeared readily after treatment with 1% aqueous ammonia which resulted in the elution of pteridines into the medium. This medium, when concentrated *in vacuo* and examined by means of paper chromatography, was found to contain a large amount of both colored and colorless pteridines. The former were identified as sepiapterin and cyprino-rouge (27) whose nature remains unsettled, whereas the latter were identified as 7-hydroxybiopterin, isoxanthopterin, and 6-carboxyisoxanthopterin.

### *Intracellular Location of Pteridines*

When the large-granule fraction of the skin homogenates was fractionated by centrifugation with the density-gradient technique, six particulate preparations were isolated. Among them, five preparations, which were present in large enough quantities to be analyzed, were divided into four fractions numbered in series from 1 to 4 from the bottom to the top of the tube as shown in Fig. 1. Fraction 1, appearing as a compact, rather narrow band at a zone corresponding to approximately 1.9 M sucrose, was composed of a suspension of yellow particles. Usually, this band of particles appeared as a cluster which exhibited a yellow, flaky appearance. Both fractions 2 and 3 were colorless but were recognized by their turbidity at zones corresponding to about 1.5 M and 1.3 M sucrose concentrations, respectively. Fraction 4, seen as a moderately narrow band at the top of the gradient, consisted mostly of a thick orange material with a flaky appearance. In addition, a white, cloudy, thin band was observed directly beneath fraction 4. This band was usually included in fraction 4. At the top of the gradient appeared a clear orange region which consisted exclusively of carotenoid pigments presumably dissociated from the particles during the treatments. The faint yellow materials that appeared near the bottom of the tube were so slight in amount that they were neglected in this study. The equilibration pattern of each fraction in the density gradient used was quite reproducible in repeated experiments, with the exception of a minor shift in the position of fraction 1. Morphological examination with the electron microscope indicated that, as illustrated in a series of micrographs (Figs. 5–9), fraction 1 consisted of an almost homogeneous population of large particles. The profile of these particles was quite similar to that of pigment granules found in erythrophores *in situ* (Fig. 5). Occasionally, a few other types of particles, such as mitochondria, were contained in this fraction. However, isolated pterinosomes were so fragile that it was practically impossible for them to retain their characteristic ultrastructure during further purification. The representative particles of the populations in fractions 2 and 3 were mitochondria, whatever their origin may have been (Figs. 6 and 7). The particles in fraction 2 appeared to be heavy whereas those in fraction 3 were lighter. Fraction 2 contained various kinds of particles in addition to mitochondria. Elements of rough-surfaced endoplasmic reticulum were frequently seen; they probably had their origin in cells other than erythrophores, since rough-surfaced endoplasmic reticulum was scarce in differentiated erythrophores. In fraction 4, membrane-bounded vesicles, possibly elements of smooth-surfaced reticulum (40) were predominant (Fig. 8). Further, the small-granule fraction was shown to be composed of membranous fragments and ribosome-like particles (Fig. 9). Consequently, it was apparent that the pigment granules as found in erythrophores *in situ* were rarely detectable in the preparations other than fraction 1.

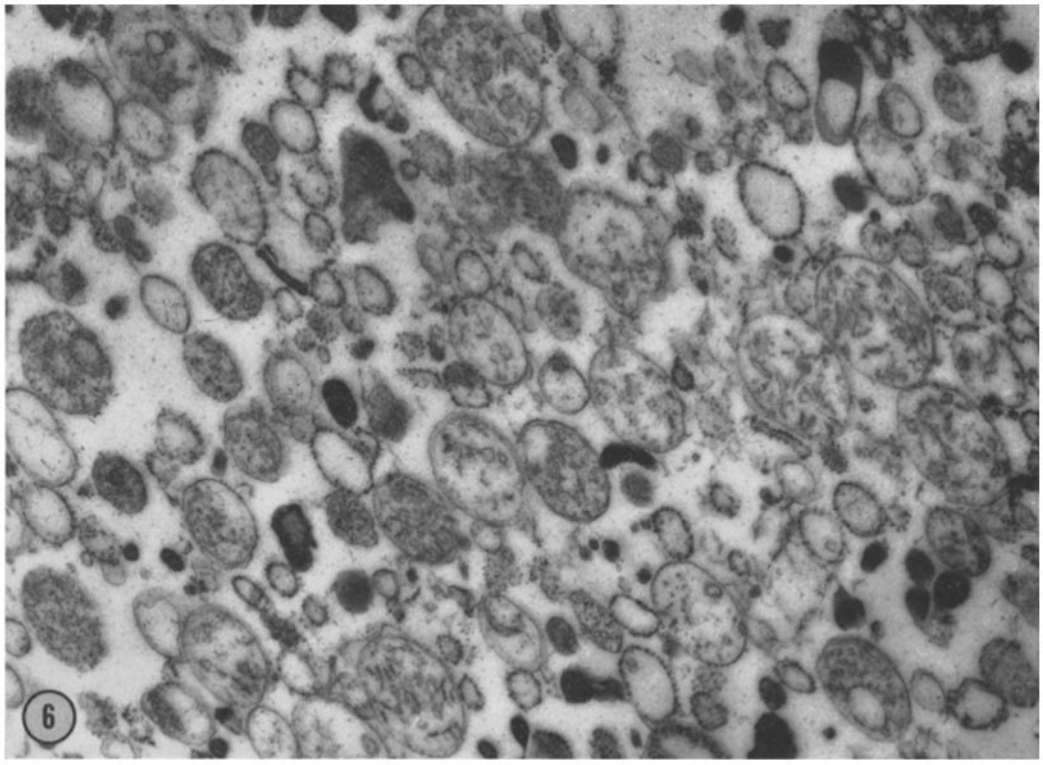



FIGURE 6 Large granule, fraction 2. The population of this fraction consists of various kinds of particles including granular endoplasmic reticulum which probably has its origin from cells other than erythrophores. Mitochondria are considered as the representative particulates of this fraction. No pterinosomes are recognizable. St, UA. × 23,000.

Quantitative analysis of the pigmentary composition of isolated particles indicated that pteridines were present in a particularly high concentration in fraction 1 but were present in small or negligible amounts in other fractions. Representative data on the quantitative distribution of pteridines in the various fractions are given in Table II. In both fraction 4 and the small-granule fraction, small to moderate amounts of pteridines were found. With regard to carotenoids, it was shown that major parts of this pigment obtainable from erythrophores were detectable in fraction 4 and in the small-granule fraction. From these findings, it is reasonably deduced that in erythrophores the pteridines are accumulated in pigment granules at a high concentration and that they differ from carotenoids with respect to their intracellular location. Because of their distinct and characteristic accumulation of pteridines, the particles found in fraction 1 are regarded as one type of pterinosome.

### *Pigmentary Composition of Pterinosomes*

Paper chromatography showed that the types and relative amounts of pteridines found in pterinosomes were essentially comparable to those found in erythrophores *in situ*. One yellow-colored and yellow-fluorescing derivative, found in a moderate amount, was identified as sepiapterin; two violet-fluorescing substances, found in extremely large quantities, proved to be 7-hydroxybiopterin and isoxanthopterin; one violet- and two blue-fluorescing substances, found in smaller amounts, were identified as 6-carboxyisoxanthopterin, biopterin, and 2-amino-4-hydroxy-6-carboxypteridine, respectively. Isolated pterinosomes, when treated with organic solvents such as acetone or chloroform-ethanol, retained their yellow color. The gradual disappearance of this pigmentation, however, after treatment with 1% aqueous ammonia was accompanied by the dissolution of the above-described pteridines. Apart from pteridines,

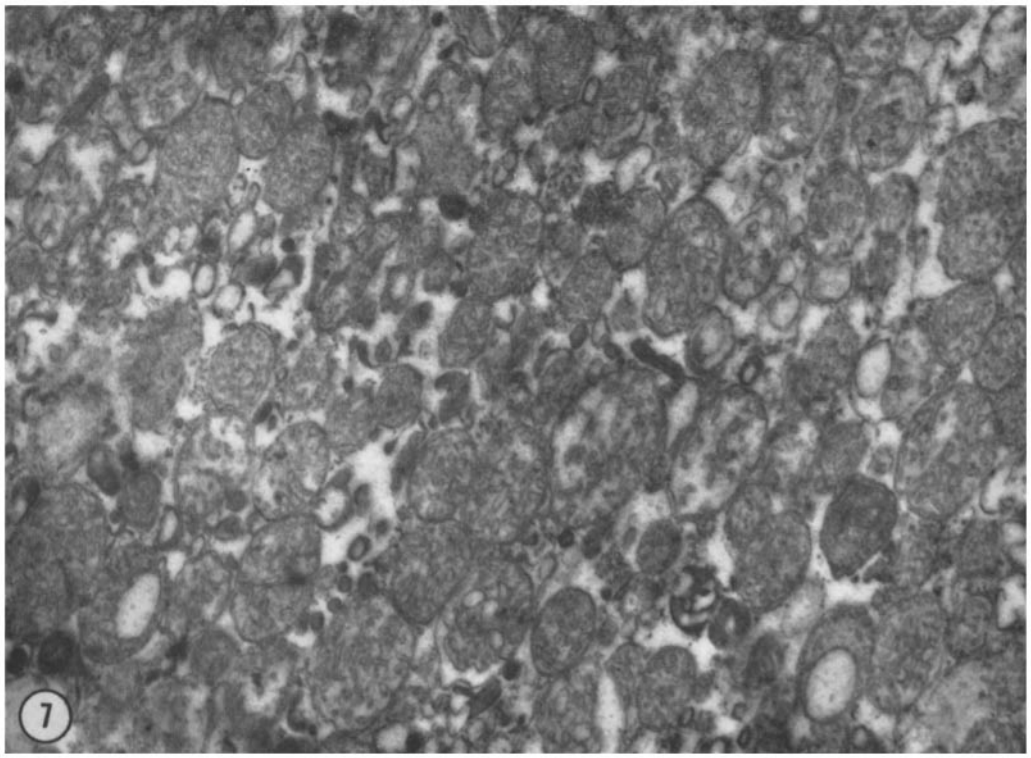


FIGURE 7 Large granule, fraction 3. Mitochondria are dominant. St, UA. × 23,000.

it was found by means of thin-layer chromatography that only an extremely small amount of carotenoids is contained in pterinosomes. It is concluded that the yellow pigments in pterinosomes are, for the most part, ascribable to pteridines.

### *Tyrosinase Activity in Pterinosomes*

To find out the possible correlation of pterinosomes with melanosomes in melanophores, the tyrosinase activity of pterinosomes was examined in parallel with the above-mentioned studies. Accordingly, the distribution of this enzyme in various types of cytoplasmic particles obtainable from skin tissues also was examined. Representative data are given in Table III. It was apparent that tyrosinase activity does occur in pterinosomes. Furthermore, it was also shown that the addition of sulfhydryl-blocking agents such as iodoacetamide or *p*-chloromercuribenzoic acid resulted in a marked increase of tyrosinase activity; the activity was enhanced to as much as six times the normal level when the iodoacetamide concentration was $8 \times 10^{-3}$ M. From these results, it appears that tyrosinase activity in pterinosomes may be masked by sulfhydryl agents under ordinary physiological conditions. Thus, when the activity of this enzyme in pterinosomes is unmasked, it is found to be high enough to be detected by the assay procedure employed in this study. When a similar assay was carried out on other types of particles, a weak activity was observed in fraction 4 and in the small-particle fraction, and a negligible activity was found in mitochondrial fractions. As judged from morphological observations made in parallel with the enzyme assay, it seems unreasonable to attribute the high activity of tyrosinase to other types of particles which are occasionally found in an extremely small amount in the pterinosome fraction.

### *Ultrastructure of Pterinosomes*

As determined from numerous sections of pterinosomes cut at various orientations, the pterinosome is considered to be an ellipsoidal

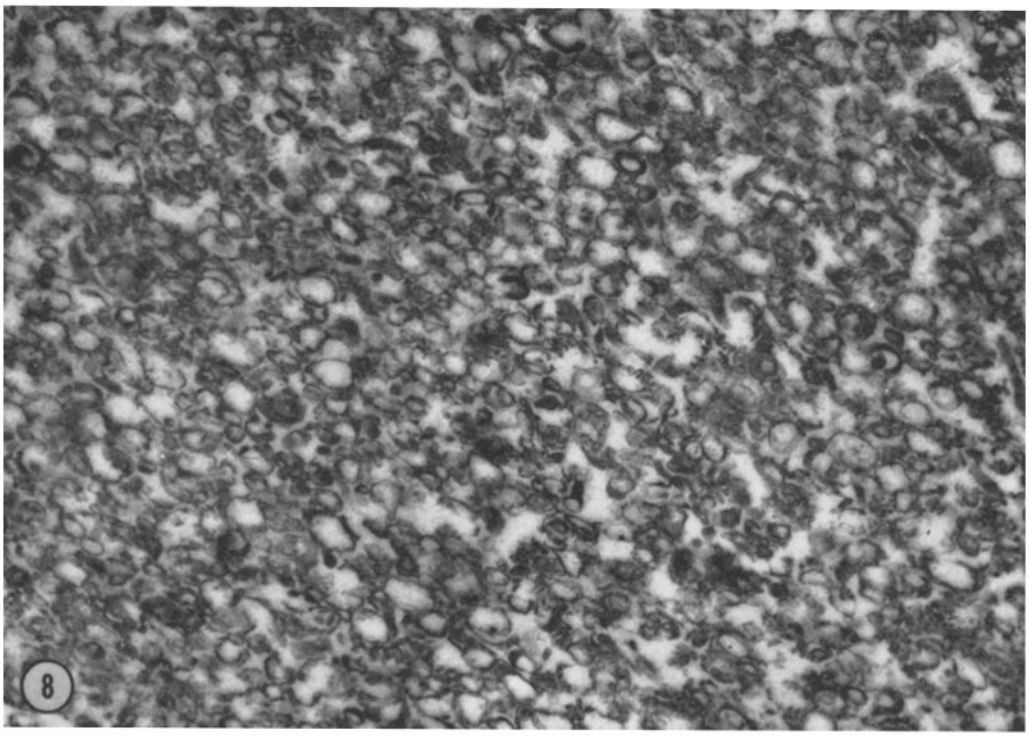


FIGURE 8 Large granule, fraction 4. Membranous elements are arranged in meshlike networks. St, UA. × 23,000.

body, the long and short diameters of which are approximately 0.4 and 0.6 $\mu$, respectively. These bodies are limited by a thin membrane which exhibits a triple-layered structure (Fig. 4 *a*). Within the pterinosome, an irregular arrangement of thin filaments can be observed (Fig. 4 *b*). The thickness of these filaments is approximately 40 A. After isolation of pterinosomes by centrifugation, these filaments are frequently separated from their limiting membranes and form large aggregates. The inner structure of pterinosomes obtained from goldfish erythrophores apparently differs from that of swordtail pterinosomes which exhibit a pronounced lamellar arrangement.

## DISCUSSION

The various types of pigment cells commonly have been designated on the basis of the pigments they contain. With regard to the chemical identification of these pigments, considerable information has been accumulated (4, 15, 16). In this respect, the xanthophores and erythrophores prevalent in the integuments of cold-blooded vertebrates have been characterized as being pteridine-laden pigment cells, the so-called pterinophores (3, 18, 19, 27, 28, 33, 34, 45). Little information is available, however, concerning the intracytoplasmic location of the pigments in the xanthophore and erythrophore. In contrast, however, it has been established that the melanin pigments of the mammalian melanocytes are contained in an organelle, the melanosome (42). As to the distribution of pteridine pigments within erythrophores, it has been shown in a previous study with the swordtail that pteridines exist specifically in distinct cytoplasmic organelles termed pterinosomes (28). The cytochemistry of pterinosomes isolated by means of differential centrifugation indicated that a group of colored pteridines consisting of drosopterin, isodrosopterin, neodrosopterin, and sepiapterins apparently function as cell pigments typifying the erythrophore. With regard to the goldfish erythrophore, it has been shown that the major share of pteridines abundant in the skins is characteristically located in this cell. The main representatives of these compounds are shown to be sepiapterin,

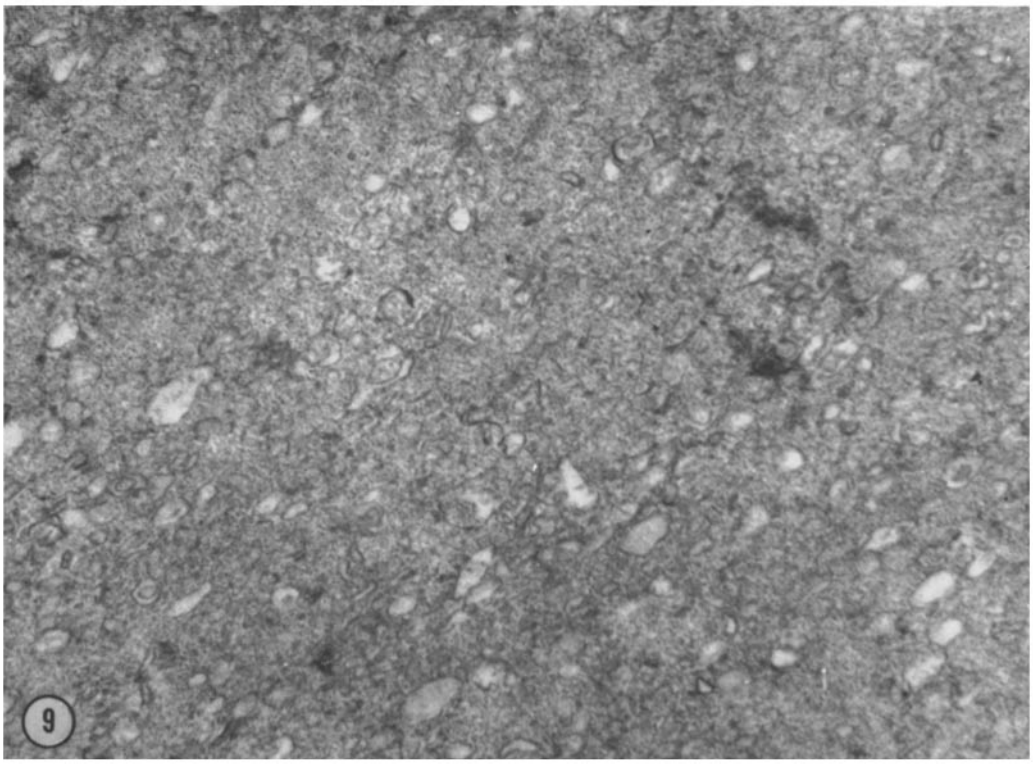



FIGURE 9 Small-granule fraction. This fraction contains membranous fragments and ribosome-like particles. St, UA. × 23,000.

7-hydroxybiopterin, and isoxanthopterin. Cytochemically, it was shown that these pteridines take part in the cell pigmentation as water-soluble yellow pigments, whereas carotenoid pigments are predominantly responsible for the reddish cell coloration. Moreover, it was revealed with the light microscope that the erythrophores have an intracytoplasmic granular framework which is usually associated with pteridines. From these findings, the possibility has been suggested that the pteridine pigments in goldfish erythrophores are also contained in some discrete cytoplasmic particles comparable to swordtail pterinosomes. Evidence provided herein by the application of the density-gradient technique of centrifugation has clearly demonstrated the presence of such an organelle; pteridines found in erythrophores are specifically distributed in a high concentration in this organelle which has been shown to be identical with the pigment granules seen by electron microscopy. This ellipsoidal cytoplasmic organelle consists of a limiting membrane enclosing an irregularly arranged internal matrix of thin fibrils. On the basis of these observations, it is reasonable to conclude that the pigment granules in goldfish erythrophores are one type of pterinosome, analogous to that of the swordtail. With respect to the internal structure and the complement of pteridine pigments, however, the two types of pterinosomes are considerably different. In swordtail pterinosomes, a concentric arrangement of thin lamellae seems to be involved in the deposition of drosopterins, sepiapterins, and colorless pteridines composed mainly of derivatives of 2-amino-4-hydroxy-pteridine; whereas, in goldfish pterinosomes, irregularly arranged thin fibrils appear to be associated with the location of sepiapterin and colorless pteridines composed predominantly of the derivatives of 2-amino-4,7-dihydroxypteridine. In the goldfish, it was shown also that carotenoid pigments, in extremely small quantities, are present in the pterinosome fraction and, therefore, may be present in pterinosomes. These circumstances make it necessary to establish some general

TABLE II

*Quantitative Distribution of Pteridines and Carotenoids in Particulate Fractions Obtained from Orange-Colored Goldfish Skin by Means of Density Gradient and Differential Centrifugation*

| Fraction* | Representative of population | Contents | | | |
|---|---|---|---|---|---|
| | | Pteridines | | Carotenoids‡ | |
| | | quinine§ sulfate | Relative per cent | Absorbance‖ at 485 mμ | Relative per cent |
| | | μg | | | |
| Large granule, fraction 1 | Pterinosome | 118.7 | (83.1 ) | 0.63 | ( 6.3) |
| fractions 2 and 3 | Mitochondria | 10.0 | ( 7.0 ) | 0.60 | ( 6.1) |
| fraction 4 | Smooth membrane-bounded vesicle | 7.1 | ( 4.95) | 5.55 | (55.9) |
| Small-granule fraction | Ribosome and membrane debris | 7.1 | ( 4.95) | 3.15 | (31.7) |

* Symbols of the fractions as for figures and text.

‡ After centrifugation, approximately 10% of the total amount of particle carotenoids appeared at the top of the gradient as a clear orange layer in which pteridines were negligible.

§ See text for explanation of the unit.

‖ Content is expressed in absorbance when dissolved in 1 ml of chloroform-ethanol (3:1 v/v) mixture.

TABLE III

*Tyrosinase Activities in Particulate Fractions Obtained from Orange-Colored Goldfish Skin by Density Gradient and Differential Centrifugation*

| Fraction | Assay condition* | Relative melanin content‡ |
|---|---|---|
| | | Absorbance at 500 mμ |
| Large granule, fraction 1 | Standard | 1.80 |
| | Standard plus iodoacetamide | 10.20 |
| | Standard plus *p*-chloromercuri-benzoic acid | 8.22 |
| | Standard minus substrate | 0.03 |
| Large granule, fractions 2 and 3 | Standard | 0.18 |
| | Standard plus iodoacetamide | 1.17 |
| | Standard minus substrate | 0.03 |
| Large granule, fraction 4 and small-granule fraction | Standard | 0.57 |
| | Standard plus iodoacetamide | 2.10 |
| | Standard minus substrate | 0.03 |

* The standard assay medium consists of $6.4 \times 10^{-4}$ M *l*-tyrosine, $8 \times 10^{-6}$ M DOPA, 0.077 M phosphate buffer (pH 6.8), and 100 IU penicillin. Iodoacetamide and *p*-chloromercuribenzoic acid are added to give a final concentration of $8 \times 10^{-3}$ M and $8 \times 10^{-4}$ M, respectively.

‡ Relative melanin content is expressed in absorbance when dissolved in 1 ml of 0.2 N NaOH.

criteria for defining pterinosomes. To simplify matters for the sake of understanding, we wish to propose herein that the term pterinosome be adopted to designate any intracellular particle that contains a large amount of pteridine derivatives, irrespective of its morphological properties, coloration, developmental origin, or specific pteridine content. The presence of other pigments in the organelle should not alter its designation as a pterinosome.

Very recently, particles identical in biochemical and morphological characteristics with swordtail pterinosomes have been found in erythrophores and xanthophores of a ranid (35). Such particles also have been observed in other anurans (5) and reptiles (8; Taylor, J. D., Personal communication). In view of these facts, it may generally be accepted that widely occurring pteridine compounds found in the integuments of cold-blooded vertebrates are contained in a specific cytoplasmic organelle, the pterinosome. As to the intracellular location of carotenoid pigments, the present study indicates that they occur mostly in the smooth-surfaced reticulum. This indicates that in their intracellular distribution the two types of xanthophore pigments are apparently different. As judged from the fact that many of the pteridines present in pterinosomes are photolabile (27, 31), it can be presumed that the function of this organelle might be concerned with photochemical events.

The presence of tyrosinase activity was demonstrated to be another biochemical characteristic of goldfish pterinosomes. Furthermore, indications were obtained that in pterinosomes sulfhydryl agents, the properties of which remain unknown, function as a prevalent inhibitor of melanin synthesis. In reference to tyrosinase activity in goldfish xanthophores, Loud and Mishima (25) observed by electron microscopic cytochemistry that melanin granules appeared within xanthophores when xanthic skin was cultured in vitro in the presence of ACTH. Those authors indicate that melanin formation within xanthophores occurs in large bodies which are cytoplasmic organelles characteristic of this cell. With regard to the activation of tyrosinase, they also interpreted that tyrosinase exists in the cytoplasmic sap, possibly in a solubilized form, and comes into association with the large bodies by an appropriate stimulation, such as ACTH administration, and that by such means these bodies, devoid of the enzyme under normal physiological conditions, become pigmented. Thus, they regard the interference of the association of tyrosinase with the large bodies as the real mechanism for the blocking of melanization in the xanthic goldfish. As judged from their morphological properties, these large bodies seem to be pterinosomes. If this is true, the findings presented in this paper appear to be inconsistent with Loud and Mishima's concept, because of the presence of tyrosinase activity in isolated pterinosomes and because of the marked enhancement of tyrosinase activity by the addition of sulfhydryl-blocking agents. In this connection, problems are raised concerning the specific intragranular location of tyrosinase and its inhibitor. In order to solve these problems, experiments are already in progress.

According to Kaufman (22), sepiapterin and its hydrated derivatives act as a cofactor in the hydroxylation of phenylalanin to tyrosine. The knowledge of the connection of such an enzyme with its cofactor present in pterinosomes may provide a basic clue for understanding the inhibitory mechanism of xanthophore melanization.

In the melanophores of goldfish, small amounts of biopterin, xanthopterin, and isoxanthopterin are present (27). This pteridine pattern is slightly different than that of the erythrophores of goldfish. Biochemical assay with the use of differential centrifugation indicates that these compounds are primarily present in the melanosome fraction (27). Since the pteridines that are detected in melanosomes and pterinosomes are closely related to each other with regard to chemical structure and metabolism, it may be surmised that the pigment organelles of melanophores and xanthophores are similar not only in their capacities for melanogenesis but are similar in their general biochemical nature as well. This may suggest that, in the goldfish, erythrophores (xanthophores) are in a labile state ready to be transformed into melanophores. Such a situation, however, does not necessarily mean that the pterinosome is a structural precursor of the melanosome in either normal or induced melanogenesis. Concerning the possibility of the transformation of xanthophores into melanocytes, Chavin and Chen (10) suggest that xanthophores are not melanin-synthesizing units on the grounds that there are no differences in the integumental tyrosinase activities between the xanthophore-laden and the xanthophore-lacking color-varieties of goldfish. In pterinosomes obtained from swordtail and *Rana* erythrophores, tyrosinase activities are hardly detectable even in the presence of

sulfhydryl-blocking agents (35; Matsumoto, J. and M., Obika. Data in preparation). In view of the structure of goldfish pterinosomes, it seems possible that the activities of tyrosinase are related to the arrangement of the intragranular framework. In fact, premelanosomes, precursor organelles of melanosomes, which occur in developing mammalian melanocytes resemble goldfish pterinosomes with respect to their profile of intragranular structure (32). However, the problem of whether the intragranular structure of pterinosomes provides the framework for the support of specific enzymes such as tyrosinase or whether these granules represent functionally inert cell products merely accumulated within vacuoles should be resolved.

Because of the frequent occurrence of Golgi complexes in goldfish erythrophores, it seems plausible, by analogy with melanosomes, that pterinosomes arise from these organelles or from vesicles associated with them. An investigation of the ontogeny of pterinosomes is in progress.

As a matter of interest in relation to the fundamental ultrastructure of erythrophores, apart from pterinosomes, it should be noticed that these cells contain an extensive system of smooth-surfaced tubules. The same observation has been made with swordtail erythrophores. This agranular reticulum has been recognized in various types of cells having different functions, such as chloride cells of teleost gills (37), absorptive epithelial cells of intestine (36), interstitial cells of testis (11), and striated muscle cells (38). However, the major functions of these cells are concerned, more or less, with transport along or across the membrane. This may be the case with erythrophores, since carotenoids may be transported and stored in these tubules. Also, from these observations, inferences can be drawn that erythrophores in general are engaged in functions related, in some way, to osmoregulation or molecular transport. Attempts to elucidate such a possibility may cast some light on the understanding of the physiological significance of pigment cells.

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