70. *The Relation between the Pterins and
Chromatophores in the Medaka,
Oryzias latipes*^{*)**)}

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Various kinds of pterin derivatives have been found in the chromatophores of lower vertebrates such as Reptilia, Amphibia and fish.¹⁾ In the medaka (*Oryzias latipes*), the black(wild), blue, orange-red or white color-varieties have been known, and their chromatophores have genetically and physiologically been studied quite adequately.²⁾ Therefore, this fish appears to be an interesting and suitable object for examining the relationship between pterins and chromatophore pigmentation, but no detailed study has been reported yet. This paper concerns itself with the following items: (1) the relation between the body coloration and pterins, (2) the kinds and distribution of pterin, (3) the mode of appearance of pterins during development and their association with chromatophore differentiation.

Materials and methods. The black and orange-red homozygous strains of *Oryzias* were used in this experiment. Pterins were extracted by a mild treatment e.g. with warm 70% alcohol followed by deproteination with chloroform (slightly modified Awapara's method)³⁾ under a photographic "safe light". However, such an extraction method failed to avoid the oxidation or degradation of some easily oxidizable or photolabile pterins. A crush method, therefore, was mainly adopted, viz., fresh materials of whole embryo, stripped adult skin or various kinds of tissues were directly crushed on a filter paper (Whatman No. 1) and chromatographed ascendingly. This method was very simple and was found to be one of the most reliable ones in order to appreciate the real situation of pterins within the cells. Pterins were mainly determined by their fluorescing color and Rf values in two dimensional chromatogram. Solvent systems included, *n*-propanol-1% ammonia (2:1), *n*-butanol-acetic acid-water(4:1:1), water saturated butanol, 5% acetic acid, and 3% urea. As authentic materials, the following were used: biopterin

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(rana-chrome 1, rana-pterin 1, BP), rana-chrome 3 (almost certainly 2-amino-4-hydroxy-6-hydroxymethylpteridine, rana-pterin 3),⁴⁾ 2-amino-4-hydroxypteridine (AHP), 2-amino-4-hydroxy-6-carboxypteridine (AHP-6-COOH), isoxanthopterin (IXP), xanthopterin (XP), dihydroxanthopterin (XP-H₂), and sepiapterin (SP). The wild and *se* strains of *Drosophila* were also used to identify isosepiapterin (ISP), drosopterin (DP), isodrosopterin (IDP), and neodrosopterin (NDP).⁵⁾⁶⁾ AHP was also determined by its conversion into IXP by xanthine dehydrogenase. The approximate amounts of respective pterins were deduced from the intensity and area of fluorescent spots in the chromatogram.

Results. 1. *The relation between the body-coloration and pterins.* A carotenoid test using concentrated sulphuric acid (98%) was positive on fresh xanthophores of adult skin or scales.⁷⁾ The carotenoid and fat granules⁸⁾ of such materials were perfectly extracted with organic solvents e.g. petroleum ether, chloroform, alcohol, benzol or carbon bisulphide. The carotenoid test on the materials treated as such became negative. The histological observation showed the presence of fine granules which were faintly yellowish and fulfilled the cell in both color-varieties. We suggest that these granules should be called "medaka-pterinosome". This pterinosome consists almost certainly of pterins and proteins deducing from the knowledge concerning the purely isolated swordtail-pterinosome.⁹⁾¹⁰⁾ The pterins could be easily eluted from the carotenoid free skin or scales with dilute ammoniacal alcohol. The fine granules remained intact in the xanthophores. All kinds of pterins in adult were demonstrated in the elute. It seems that these pterins have almost unmistakably originated in the pterinosomes as well as in the free state within the cell, since no pterins are found in the residue. No apparent differences in the kinds of pterins were found between the two color-varieties. Thus, body-color difference in both may be ascribed to the amounts of melanin and carotenoid. Medaka-pterinosome color was too faint to influence the body-coloration which differed from that caused by red colored pterinosomes in swordtail.

2. *The kinds and distribution of pterins.* The materials used are male and female adults and their larvae. Various kinds of tissues and organs in adult, and the whole body of larva were examined. The pterins found were divided into non-colored and colored ones. To the former belong, non-fluorescing hynobius-blue (HB, this substance is transformed very rapidly into AHP-6-COOH by irradiation on chromatogram immediately after development),¹⁾ blue fluorescing BP, rana-pterin 3, AHP and AHP-6-COOH, and violet fluorescing IXP and medaka-F1 1, and greenish fluorescing XP and XP-H₂. Some

of the AHP-6-COOH and XP might be decomposed products of HB, SP, and BP, and XP-H₂ respectively. To the latter belong yellow, yellow fluorescing SP and ISP; respectively orange to red, orange to red fluorescing DP, IDP, and NDP. These pterins were found overwhelmingly in the larvae. No difference in the kinds of pterins was seen between male and female. Concerning the tissues or organs tested, no pterins were found in chromatophore-free skin, leucophore containing white ventral skin, muscle, heart, air bladder, bone, fat body, spleen, liver, and testis. A small amount of pterins was found in kidney and intestine. However, one can recognize the chromatophores or their decomposed product such as granules in the intercellular spaces of these tissues under microscopic observation. Although it is not so easy to remove the chromatophores which invaded the tissues, the more one removes the chromatophores, the less the amount of pterins in the test materials resulted in. Therefore, whether the proper cells of kidney or intestine bear pterins is at present questionable. On the other hand, a very small amount of pterins such as BP, rana-pterin 3, and IXP was proved in the brain and ovarian egg. Most of the pterins were found in the eye, skin and scales, fin and peritoneum. All of these are laden with chromatophores. From the above facts, one may safely conclude that most of pterins are localized in the melanophores, erythrophores or xanthophores.

3. *Developmental changes of pterins.* The kinds and the mode of appearance of pterins during development and their association with chromatophore differentiation were investigated. Three kinds of chromatophores, viz. melanophore, erythrophore and xanthophore appear at approximately the same time before hatching, slight differences existing according to color-variety.⁷⁾ Of the orange-red color variety, the later onset of melanophore melanization and the very poor development of the melanophores are characteristic signs.

Until the chromatophore pigmentation became evident no fluorescent pterins were detected. In the embryos before hatching (Matsui stage 26, ca. 3.5 days after spawning, 22–24°C),¹¹⁾ a small amount of blue fluorescent and colored pterins occurred simultaneously with the appearance of chromatophores. However, the identification of these pterins necessitated the use of a large number of embryos. Colored pterins such as DP and SP were rather clearly demonstrated. In the just hatched 4.5–5.0 mm long larvae (Matsui stage 34, 11 days after spawning, 22–24°C) where the above-mentioned chromatophores are much distributed in the head region, several individuals sufficed to identify the non-colored pterins, that is, HB, BP, AHP, IXP, and XP-H₂. The pterins described above increased their amount with the augmentation of chromatophore population (Fig. 1a).

Fig. 1. a. Larva b. Adult

Figs. 1a and b. Paper chromatograms showing the larval and adult patterns of pterins in the medaka, *Oryzias latipes*. 1st dimension: *n*-propanol-1% ammonia (2:1), 2nd dimension: *n*-butanol-acetic acid-water (4:1:1). a. Five 4.5-5.0 mm long larvae were crushed directly on a filter paper. b. 16 mm² tail fin of adult was crushed directly on a filter paper. Abbreviation AHP: 2-amino-4-hydroxypteridine, AHP-6-COOH: 2-amino-4-hydroxy-6-carboxypteridine, BP: biopterin, DP: drosopterin, HB: hynobius-blue, IDP: isodrosopterin, IXP: isoxanthopterin, NDP: neodrosopterin, SP: sepiapterin, XP: xanthopterin, XP-H₂: dihydro-xanthopterin. AHP-6-COOH* and XP* are decomposed products of HB, SP or BP, and XP-H₂ respectively. Isepiapterin is not shown in the figure. Its R_f value: 0.60 in *n*-propanol-1% ammonia (2:1) and 0.36 in *n*-butanol-acetic acid-water (4:1:1).

However, in the 6-8 mm long larvae, it became rather difficult to detect HB, SP, ISP, DP, IDP, and NDP. On the other hand, rana-pterin 3 and medaka-F1 1 began to appear. With further development, the larval pattern of pterins was transformed gradually into the adult one, the amount of rana-pterin 3 exceeding that of BP. In the 12 mm long young, no difference from the adult was seen in the kinds and amount of pterins (Fig. 1b).⁷⁾ The larval pterin pattern of HB, SP, BP, and AHP at the early phase of chromatophore pigmentation which was recognized in almost all of Amphibia and fishes tested *hitherto* holds for the medaka. This fact is interesting from the phylogenetical standpoint, since the adult pterin pattern is specific to species, that is, some pterins disappear, some continue to exist or some appear newly in the process of development until finally an adult pterin pattern has been established.

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