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Hemoglobin Transition from Larval to Adult Types in Normally Metamorphosing, Metamorphosed and Metamorphosis-Arrested *Hynobius retardatus*

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ABSTRACT—A transition of hemoglobin subunits during ontogeny of a hynobiid salamander, *Hynobius retardatus,* was studied using SDS-PAGE, AUT-PAGE and two-dimentaional electrophoresis in conbination of the two. Hemoglobins from normally metamorphosing and metamorphosed animals reared at different temperatures and from metamorphosis-arrested larvae, which were reared in aqueous solution of thiourea and sodium perchlorate, were analyzed. Larval type hemoglobin was composed of 4 electrophoretically distictive subunits, named HL1, HL2, HL3 and HL4. Similarly, adult hemoglobin was composed of 4 subunits, all of which were different from the larval subunits. Progress of the larval developmental stages basically depended on the temperature: the lower temperature caused retarded metamorphosis (10°C) or arrested metamorphosis (4°C). Transition of the hemoglobin in those animals also depended on the temperatures, but not on the ages or body sizes of them. Although no indication of metamorphosis was observed at external morphological level in the larvae reared at 4°C, adult type subunits of hemoglobin were detected in them. In metamorphosis-arrested larvae reared at room temperature, the transition of hemoglobins occurred in the same time schedule as in the controls, suggesting that the transition was completely independent from the morphological metamorphosis.

INTRODUCTION

A changeover from a larval or foetal form of hemoglobin to an adult form is a frequent phenomenon in vertebrates [12]. In anuran amphibians, a switch in hemoglobin synthesis is reported to occur at metamorphosis, resulting in the replacement of the larval globin subunits by a set of distinct adult ones [3, 7]. This event, therefore, provides a useful model for investigating the developmental control of gene expression [1, 2, 22, 26].

In urodelan amphibians, however, a completely different, biologically important phenomenon which never happens in anuran amphibians, is known: many salamanders are able to retain their larval form throughout their lives, becoming sexually mature without undergoing metemorphosis [14, 23]. This phenomenon, neoteny, provides different possibility for investigating the developmental control of gene expression. It has been reported that the axolotl, a famous neotenic form in *Ambystoma mexicanum,* is not biochemically neoteneous: they show the transition of hemoglobins from larval to adult types without any indication of anatomical metamorphosis [4, 10]. Contrary to this, in *A. tigrinum nebulosum* no hemoglobin changes have been reported with metamorphosis [28]. Thus, it is important to investigate biochemical changes during the ontogeny in neotenic species. Although *Hynobius retardatus,* a hynobiid salamander inhabited in Hokkaido, Japan is a very unique species to have been reported to show neoteny [13, 21, 22], little is known on biochemical changes before, during and after the metamorphosis in this species. In order to light these problems the transition of hemoglobins from larval to adult types was analyzed at first.

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MATERIALS AND METHODS

Animals

Fertilized eggs of *Hynobius retardutus,* were collected from several ponds or small streams in the vicinity of Sapporo in the breeding season. Newly hatched larvae were reared at 4 different temperatures such as 4°C, 10°C, 16°C and 22°C. Others were reared at a room temperature either in aqueous solution of 0.02% thiourea (TU) and 0.02% sodium perchlorate (SPC) to arrest the metamorphosis, or goitrogens-free medium as controls. They were fed with live *Tubifex*. After they metamorphosed, they were transferred to a terrarium, and fed with live *Tubifex* every two days. Developmental stages were determined according to the normal table for *Hynobius nigrescens* [8].

Preparation of hemolysates

Larvae, metamorphosed juveniles and sexually mature adults were lightly anesthetized by immersing them in aqueous solution of MS222 (Sandoz). Smaller larvae were bled by punctuating heart in ice-chilled 50% PBS (PBS: 72.7 mM NaCl, 18 mM KH_2PO_4, 57 mM Na_2HPO_4) with 10 mM EDTA. Larger animals were bled by cutting tails, and blood was suspended in ice-chilled 70% PBS with 10 mM EDTA. The blood cells were washed by repeated centrifugation at 1000 rpm, followed by counting blood cell number. They were then lyzed with lysophosphatydil cholin (0.05%, 5 min at room temperature), and centrifuged. The supernatants after 10000 rpm centrifugation for 10 min were served as major erythrocyte proteins. After the amount of protein was determined using BCA Protein Assay Reagent (Pierce Chemical Co.), the samples were electrophoresed or frozen at −80°C. Samples from very small larvae (such as younger ones at 4°C) were combined together because the amount of hemolysate from one larva was too small.

In order to convert the hemoglobins to the stable cyanmethemoglobin, 0.2 volumes of 2% $K_3Fe(CN)_6$, 0.5% KCN, 0.1% $NaHCO_3$ were added to the blood cells suspended in 70% PBS. After the cells were lyzed and centrifuged, the clear red supernatant was analyzed with a spectrophotometer. From the maximal absorbance at 540 nm, it was concluded that the major hemolysate proteins were hemoglobins.

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [9] using 15% separating gels. Acid/urea/Triton X 100-polyacrylamide gel electrophoresis (AUT-PAGE) was done according to Zweidlar [29]. Two-dimentional electrophoresis in conbination of the two was conducted according to Ohsumi and Katagiri [18]. All electrophoresed gels were stained with Coomassie Brilliant Blue.

RESULTS

Identification of hemoglobin subunits

Fig. 1 shows electrophoretic profiles of hemoglobin subunits from larval and adult *Hynobius retardatus*. In SDS-PAGE (Fig. 1, Panel A), two distinct bands in larval hemoglobins were detected, and tentatively named L1 and L2. Approximate molecular weights of them were 13700 and 15000, estimated from the mobility on the SDS-PAGE. Adult hemoglobin was shown to be composed of 3 fractions, from A1 to A3 whose molecular weights were estimated as 14000, 15000 and 15500, respectively.

In AUT-PAGE (Fig. 1, Panel B), hemolysates of typical larval blood were fractionated into 4 major bands and 1 faint band, which was considered to be some polypeptide other than hemoglobin. Adult hemoglobins were also fractionated into 4 distinct bands, three of which migrated to more cathodal direction than the larval bands.

Two-dimentional electrophoresis showed clearly 4 different spots in larval and adult hemolysates (Fig. 2). They were named as HL1, HL2, HL3, and HL4 in larval samples (Fig. 2, Panel A), and HA1, HA2, HA3 and HA4 in adult samples (Fig. 2, Panel B), respectively. From the electrophoretic mobility in SDS-PAGE, L1 band seemed to be fractioned into HL1, HL2 and HL3, and L2 corresponded to HL4. Similarly, A1 were composed of HA1 and HA2, and A2 corresponded to HA3, and A3 correponded to HA4. When the hemolysates

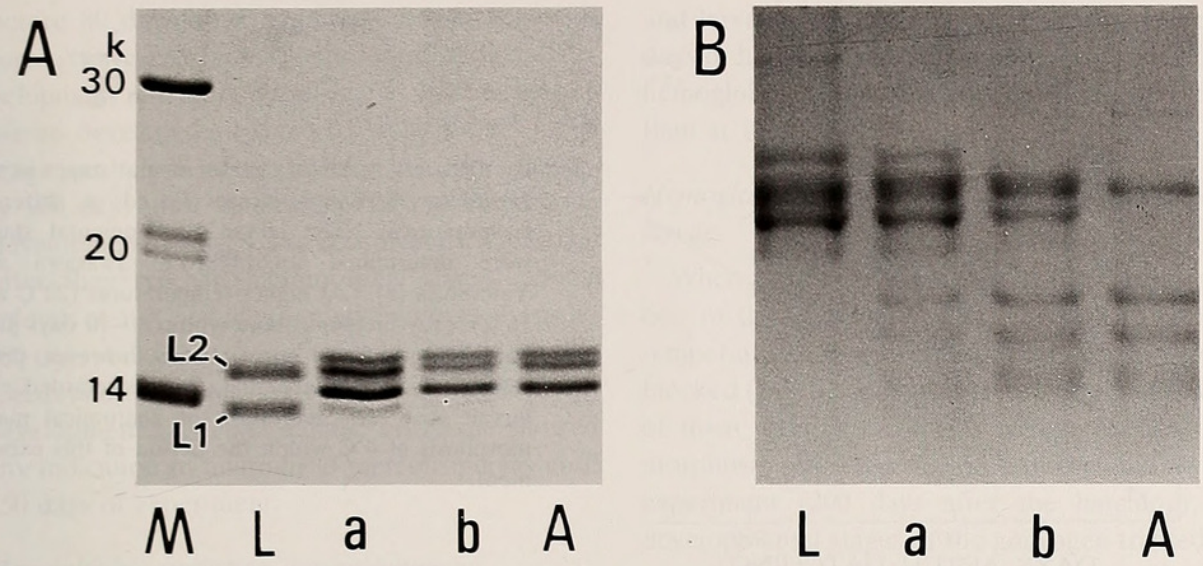
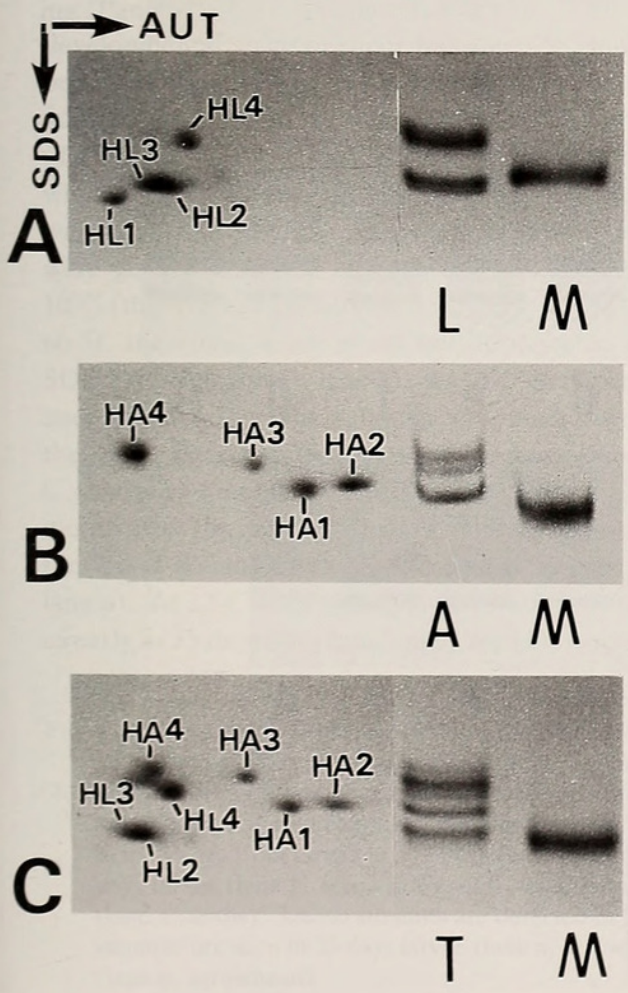


FIG. 1. Typical electrophoregrams of hemoglobins from larval and adult *Hynobius retardatus*. Lane M, molecular marker; L, typical larval hemolysate (from larvae 3 days after hatching); a, hemolysate from metamorphosing larva; b, hemolysate from metamorphosed juvenile; A, typical adult hemolysate from sexually mature male. Panel A, SDS-PAGE. Panel B, AUT-PAGE. The direction of electrophoresis was from top (+) to bottom (−).



from metamorphosing larvae were analyzed on the two-dimentional electrophoresis, 8 spots (from HL1 to HL4 and from HA1 to HA4) were usually detected (Fig. 2, Panel C, in this photograph HL1 was accidendally missed), suggesting convincingly that all subunits were electrophoretically different.

Larval development at different temperatures

Fig. 3 shows time courses of development in *Hynobius retardatus* which were reared in different temperatures. Progress of larval development and metamorphosis was completely depending on the water temperatures. All animals reared at 22°C completed metamorphosis by 70 days after hatching. The larvae at 16°C developed a little slowly compared with those at 22°C, and metamorphosed

FIG. 2. Electrophoretic profiles of larval and adult hemoglobins on two-dimentional PAGE using AUT (initially) and SDS (secondary). Lane M, molecular marker on SDS-PAGE (14 K); L, typical larval hemolysate on SDS-PAGE; A, typical adult hemolysate on SDS-PAGE; T, hemolysate from metamorphosing larva showing transition from larval to adult hemoglobins. Panel A, typical larval hemoglobin (from larvae 3 days after hatching). Panel B, typical adult hemoglobin (from sexually mature male). Panel C, hemoglobin from metamorphosing larva.

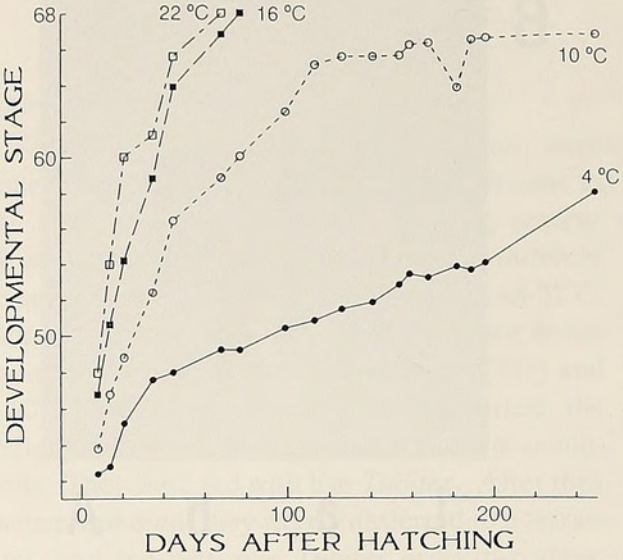
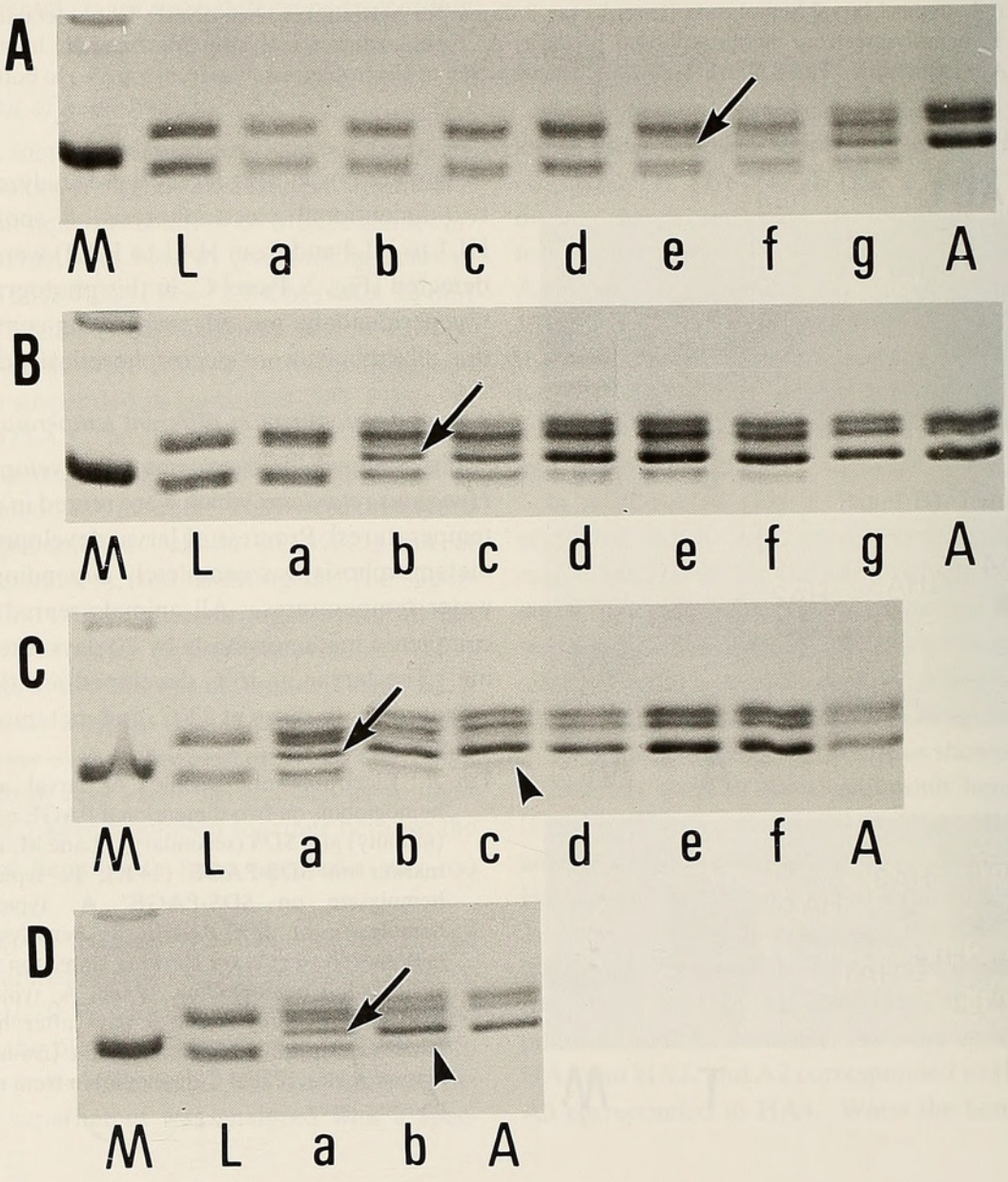


Fig. 3. Progress of larval developmental stages in the *Hynobius retardatus* larvae reared at different temperatures. The larval developmental stages were determined according to Iwasawa and Yamashita [8]. At higher temperatures (22°C and 16°C), they metamorphose within 80–70 days after hatching. At lower temperatures, however, progress of larval developmental stages is retarded. No larvae show any indication of anatomical metamorphosis at 4°C within the period of this experiment.



before 80 days after hatching. Lower temperatures, however, caused retardation of larval development and metamorphosis: at 10°C almost all larvae developed to stage 63, fully grown larval stage just before metamorphosis [8], approximately 100 days after hatching. The progress of the developmental stages was conspicuously retarded after they reached metamorphic stages. Some animals at 10°C could complete metamorphosis by 180 days, but others could not complete even after 250 days of hatching. At 4°C retardation in larval development was conspicuous: no larvae showed any indication of anatomical metamrophosis until 250 days of experiment.

Hemoglogbin transition during ontogeny

Fig. 4 shows typical elecrophoretic profiles of the hemolysates of animals before, during and after metamorphosis reared at different temperatures on SDS-PAGE. In 4°C-reared larvae, adult type subunits were detected 159 days after hatching (Panel A, arrow on lane e), when the average developmental stage of them was stage 58, much before the morphological metamorphosis. The adult type subunits were also detected in the larvae of 68 days at 10°C (Panel B, arrow on lane b), when the average developmental stage of them was stage 58.8. Inversely, the larval type bands were getting faint. In the larvae of 249 days at 10°C (the average developmental stage was stage 66.5), the larval bands could not be detected on SDS-PAGE (Panel B, lane g). At 16°C an appearance of the adult type subunits was much faster: they were detected 35 days after hatching (Panel C, arrow on lane a). After 126 days of rearing (a month after the completion of the metamorphosis) the larval subunits were not detected (Panel C, lane d). At 22°C adult type subunits were detected as early as 35 days after hatching (Panel D, lane a), and larval subunits were hardly detected after 68 days of hatching, showing more rapid transition of hemoglobin subunits from larval to adult types than at 16°C.

Hemoglobin transition in metamorphosis-arrested larvae

When the larvae were reared in aqueous solution of thiourea and sodium perchlorate at room temperature, metamorphic events were basically blocked (Fig. 5). An average developmental stage of them when the controls completed the metamorphosis was stage 63.5. At the end of this experiment (200 days after the hatching), the developmental stages of the goitrogen-treated lar-

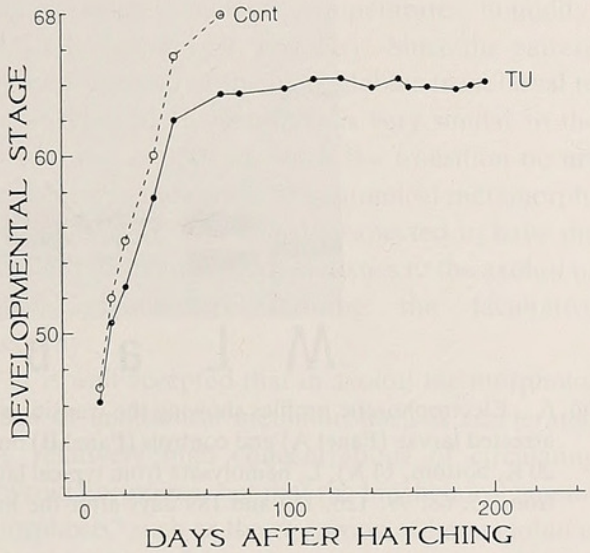


Fig. 5. Progress of larval developmental stages in the larvae reared in goitrogens (0.02% thiourea and 0.02% sodium perchlorate) at room temperature (TU, solid line). Although all controls (cont, broken line) metamorphose 70 days after hatching, proceeding of the metamorphosis in the goitrogen-treated larvae is arrested or extremely retarded. At the end of experiment, the average developmental stage in goitrogen-treated larvae is stage 64.

Fig. 4. Electrophoretic profiles on SDS-PAGE showing time courses in the transition of hemoglobins from larval to adult types at different temperatures. Lane M, molecular marker (top, 20 K, bottom, 14 K); L, hemolysate from typical larvae (3 days after hatching); lanes from a to g, hemolysates from 35, 68, 99, 126, 159, 189 and 249 days animals after the hatching; A, hemolysate from typical adult. Panel A, 4°C. Adult type subunits are seen in hemolysates from larvae at 159 days (lane e, arrow). Panel B, 10°C. Adult type subunits are clearly seen in 68 days larvae (lane b, arrow). Panel C, 16°C larvae. Adult type subunits are already detected in 35 days larvae (lane a, arrow). Larval subunits are detected in 99 days larvae (lane c, arrwohead). Panel D, 22°C. Adult type subunits are seen in 35 days larvae (lane a, arrow). Larval subunits are getting faint in 68 days after the hatching (lane b, arrowhead).

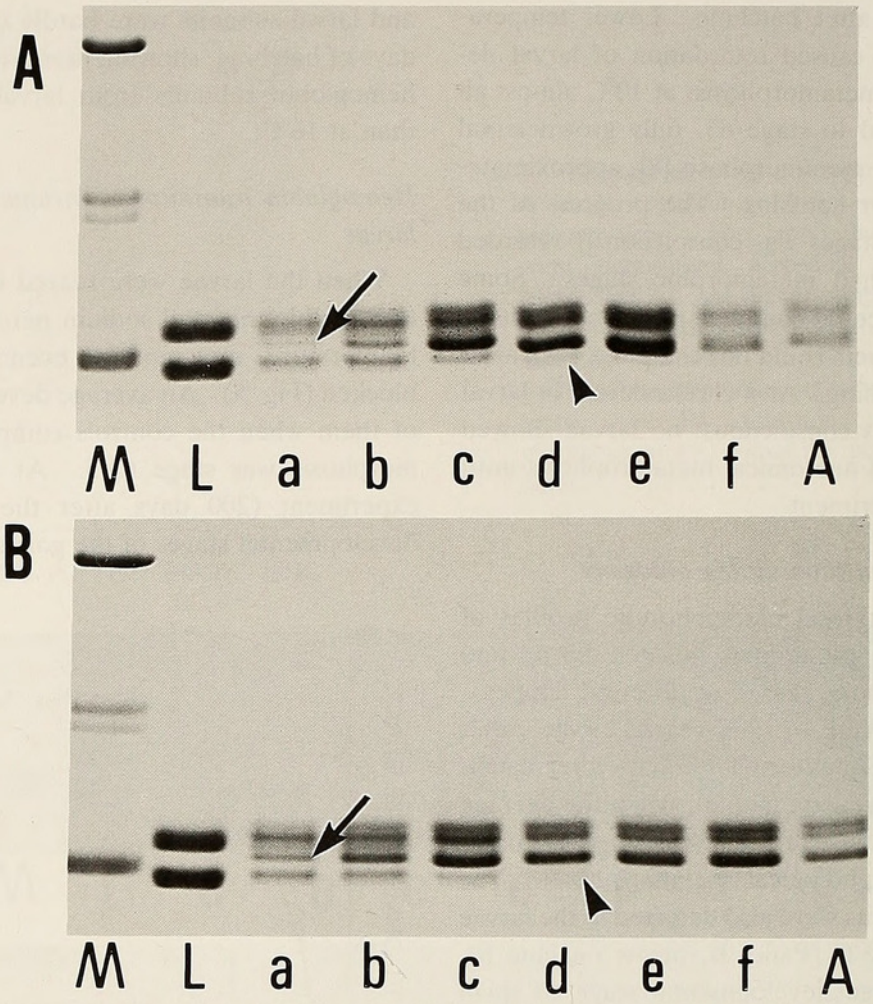


Fig. 6. Electrophoretic profiles showing the transition of hemoglobins from larval to adult types in metamorphosis-arrested larvae (Panel A) and controls (Panel B) on SDS-PAGE. Lane M, molecular marker (top, 30 K, middle, 20 K, bottom, 14 K); L, hemolysate from typical larvae (3 days after the hatching); lanes from a to f, hemolysates from 35, 68, 99, 126, 159 and 189 days after the hatching; A, hemolysate from typical adult.

vae were varied from stage 63 (just before the initiation of the metamorphosis) to stage 65 (initiation of the absorption of external gills), showing individual differences.

Although the external morphological metamorphosis was basically arrested, the hemoglobin transition occurred in the same time schedule as in the controls. Fig. 6 shows typical electrophoretic profiles of hemolysates from metamorphosis-arrested larvae and the controls at the same ages. No differences were detected between the two. Adult type subunits were detected in the metamorphosis-arrested larvae at 35 days after hatching (Panel A, arrow on lane a), when the average developmental stage of them was stage 57.6. Larval type subunits disappeared at 126 days of hatching (lane d, arrowhead), when the average developmental stage of them was 64.3, showing the completion of the transition of hemoglobins from larval to adult types occurred even in the metamorphosis-arrested larvae in the same time schedule as in the controls (Panel B, arrow on lane a; arrowhead on lane d).

DISCUSSION

Hemoglobin subunits

Approximate molecular weights of the hemoglobin subunits in *Hynobius retardatus* estimated by their mobility on the SDS-PAGE are similar to those in *Ambystoma mexicanum* [4], and to those

known in almost all vertebrates, in general. Cathodal migration of adult hemoglobin subunits on AUT-PAGE (Fig. 2, panel B) was consistent with those of other urodele, *Discamptodon ensatus* [27]. Electrophoretically distinct 4 subunits of hemoglobins were detected in both larval and adult samples on the two-dimentional gels. Because the larval subunits are distict from adult ones (Fig. 2, Panel C), it is assumed that they must be different polypeptides encoded by different genes which are developmentally regulated in the similar ways to other vertebrates [1, 2, 25, 26].

Transition from larval to adult types

Transition of the hemoglobin subunits from larval to adult types depends on the temperatures and the developmental stages of the larvae, but not on the ages, in normally metamorphosing and metamorphosed animals. Contrary to this, when the metamorphosis was arrested by goitrogens and reared at room temperature, the transition occurs strictly depending on the ages of animals, but not on the degrees of the anatomical metamorphosis.

By comparing the initial appearance of the adult type subunits in metamorphosing and metamorphosed animals reared at different temperatures (Fig. 4), it is clear that the transition occurs at the similar developmental stages (stage 58–62), in all animal groups. Although the transition occurs earlier in the animals whcih were reared at higher temperatures, and it occurs later in the animals at lower temperatures, the developmental stages when the transitions occur are basically identical. This suggests that the initial step of the transition of the hemoglobins in *H. retardatus* is triggered at the certain developmental stages before the metamorphosis. Since the thyroid glands in *H. retardatus* reared at 4°C are reported to be completely inactive [15], and thyroid hormones are believed to be insensitive at the lower temperature such as of 4°C [16], it is assumed that the transiton of hemoglobin in 4°C-larvae is governed by a different mechanism from the thyroid gland activity. It is possible that the transition is triggered automatically when the larvae attain a specific developmental stage. This is consistent with an earlier observation to show that the hemoglobin transition in *Xenopus laevis* was determined more by chronological age, or size, or some other independent factors, rather than the hormonal control by thyroxine [12].

Relationship to neoteny

According to the degree to which metemorphosis occurs or to the different levels of blockage of metamorphosis, neoteny in salamander species is roughly classified into three categories [5]: (1) Permanent neoteny, in which the target tissues have lost their capacity to respond to thyroid hormones [6] (*Necturus, Siren*). (2) Mexican axolotl, which does not undergo metamorphosis because of shortage of thyroid stimulating hormone (TSH) [19, 20, 24] (*Ambrystoma mexicanum*). (3) Facultative neoteny, they metamorphose depending on the environments (temperatures, humidity) [17] (*A. tigrinum, A. gracilus*). Since the pattern of the transition of the hemoglobins from larval to adult types in *H. retardatus* is very similar to the one in the axolotl, in which the transition occurs without any indication of anatomical metamorphosis [4, 10], *H. retardatus* is expected to have the similar biochemical characteristics to the axolotl or other salamanders showing the facultative neoteny.

It is well accepted that in axolotl the morphological or anatomical metamorphosis is accelerated by relatively high concentrations of circulating thyroid hormones, but that the biochemical "metamorphosis" such as the transition of hemoglobin is regulated by very low concentrations of thyroid hormones [4, 10]. In *H. retardatus,* the similar mechanism in the transition of hemoglobins to the axolotl is also possible. Experimental analysis using thyroid hormones seems need for further elucidation.

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