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Studies on the Change of Isozyme Patterns of Lactate and Malate Dehydrogenases During Embryonic Development of Some Amphibians

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兩棲類 胚發生에 따른 Lactate Dehydrogenase 및 Malate Dehydrogenase의 Isozyme 변화에 관하여

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要 約

개구리 2種 (*Rana nigromaculata*와 *Rana plancyi chosenica*)과 도롱뇽 (*Hynobius leechii*)의 胚發生에 따른 lactate dehydrogenase (LDH)와 malate dehydrogenase (MDH)의 isozyme組成變化를 polyacrylamide 電氣泳動法으로 조사 분석하고 이를 成體의 몇 器官과 비교하였다.

*R. nigromaculata*에서 LDH의 B subunit의 合成을 지배하는 유전자는 heterozygous이고 *H. leechii*에서는 A subunit의 合成을 지배하는 유전자가 heterozygous라고 추정된다.

위의 3種의 兩棲類의 胚에서 發生初期에는 LDH-1 (심장형)의 活性이 높으나 發生이 진행됨에 따라 LDH-5 (근육형)의 活性도 점차 증가한다.

MDH의 경우 發生初期부터 MDH-m과 MDH-s가 존재하고 발생 全段階를 통하여 그 組成에는 변화가 없으나 MDH-m의 活性이 점차 증가하는 경향을 보인다.

INTRODUCTION

The mechanism controlling the synthesis of new proteins associated with differentiation which appear at a particular time and place in the developing organism is a fundamental problem in developmental biology. Many investigations have

been focussed on this problem by correlating the appearance of new proteins with specific morphological events. It was first demonstrated by Flickinger and Nace (1952) that a significant alteration in the total protein content of developing *Rana temporaria* embryo took place at the neural fold and tailbud stages. In urodele embryos, Denis (1961) observed the appearance of tissue-specific differences in protein components as early as the onset of gastrulation. Other experiments(Spiegel *et al.*, 1970; Malacinski, 1971; 1972) raised the possibility that qualitative changes in basic proteins of amphibian developing embryos might appear as early in development as the cleavage or blastula stage.

The use of protein, particularly enzymes which reflect sensitively the state of cellular differentiation is experimentally advantageous since changes can be easily assayed. Another advantage in using enzymes is that most of them exist in multiple molecular forms. In connection with the problem of cellular differentiation, the ontogeny of isozymes of several amphibian dehydrogenases such as lactate dehydrogenase (LDH), malate dehydrogenase (MDH), isocitrate dehydrogenase (IDH) and 6-phosphogluconate dehydrogenase (6-PGD) has attracted the interest of many investigators.

Studies of this kind have been carried out with several amphibia including *Rana* (Adams and Finnegan, 1965; Chen, 1968; Wright and Moyer, 1966, 1968; Wright and Subtelny, 1971; Johnson and Chapman, 1971), *Amblystoma* (Adams and Finnegan, 1965), *Xenopus* (Claycomb and Villee, 1971; Wall and Blackler, 1974) and *Callula* (Park,1976), and the patterns of these isozymes are known to be species- and tissue-specific. Changes observed during the development of these tissue-specific isozymic patterns are thought to indicate the relative activity of the genes controlling the synthesis of the individual isozyme.

In the present study, the isozymic patterns of LDH and MDH in the developing embryos of *Rana nigromaculata, Rana plancyi chosenica* and *Hynobius leechii* were examined by polycrylamide gel electrophoresis and the patterns were compared to those of adult organs.

MATERIALS AND METHODS

Adults and embryos of the urodele *Hynobius leechii* and adults of the anurans *Rana nigromaculata* and *Rana plancyi chosenica* were collected during the breeding season in Kongju area, and maintained in the laboratory at room temperature. The techniques of artificial ovulation and fertilization of *Rana* were essentially those described by Rugh (1962). Eggs from a single female were striped into several glass dishes and fertilized with sperm suspension prepared by crushing the testes of a single male in 10 ml 1 : 10 Holtfreter's solution, and embryos were raised in filtered spring water at 20—22°C.

For sampling, the developmental stage of the urodele embryo was determined according to Harrison's table for *Amblystoma punctatum*, and that of the anuran embryo according to Shumway's table for *R. pipiens* (cited in Rugh, 1962). The heart, skeletal muscle, stomach, and brain from adults were homogenized in a volume of 1 : 10 Holtfreter's solution in a ratio of 1 : 3, W/V.

The *Hynobius* embryos were dissected out individually from the jelly coats and the *Rana* eggs and embryos were freed of their jelly membranes by treatment with a solution of 2% cysteine-HCl and 0.1% papain at pH 7.8 for 3—5 minutes. All dejellied embryos were washed several times with 1 : 10 Holtfreter's solution. About 300 eggs or embryos at each of the selected stages, were homogenized in 10 μl 1 : 10 Holtfreter's solution per embryo with a glass homogenizer on ice. The organ, egg and embryo homogenates were immediately centrifuged at 10,000xG at 0°C for 20 minutes. The relatively clear supernatant located between the superficial light fatty layer and the dark precipitate in the tube was pipetted out carefully and used directly for electrophoresis or stored at −20°C. The total protein content of each sample was determined by the method of Lowry *et al* (1951). The discontinuous polyacrylamide gel electrophoresis was performed essentially according to Davis (1964) using a 2.5% upper gel and a 7% lower gel. The amount of protein loaded was 200 μg for each sample. Electrophoresis was carried out at 20—30 mA per slab (80—200V) for 6 hours or at 3 mA per tube for 2 hours at 5°C in 0.01 M tris-0.2 M glycine buffer system (pH 8.3). Bands of LDH and MDH were visualized by incubating the gels at 37°C in dark in the staining mixture formulated by Shaw and Prasad (1970). Control gels were also incubated as above, but in the absence of substrate (sodium malate or sodium lactate) (Shaw and Koen, 1965).

RESULTS

1. Isozymes of LDH and MDH in Adult Organs.

The electrophoretic patterns of lactate dehydrogenase from different organs of *Rana nigromaculata*, *R. plancyi chosenica* and *Hynobius leechii* are shown in Fig. 1. In *R. nigromaculata*, seven or eight bands were revealed in the heart, five bands in the stomach and two bands in the skeletal muscle (Fig. 1, a). The fast migrating three bands in the heart are believed to be subbands of LDH-1 as discussed below. On the other hand, skeletal muscle had a typically active cathodic band (LDH-5) and heart had a predominent anodic band (LDH-1), which is a pattern found in most vertebrate species.

A similar pattern was found in *R. plancyi chosenica* (Fig. 1, b). In this species, there was a faster migration of all bands but LDH-5 which kept the same position as that of *R. nigromaculata*, and there was no rapidly-migrating subbands in the position of LDH-1 in the heart. The band of LDH-3 of both frogs usually appeared

as a very faint one.

In *H. leechii*, the stomach gave four bands and the skeletal muscle seven bands (Fig. 1, c) in contrast to the patterns observed in two species of *Rana*. The three slower migrating bands in the *Hynobius* muscle is believed to be subbands of LDH-5 and seems to be one of the most striking characteristics of LDH isozyme pattern of this species.

Fig. 2 shows the electrophoretic patterns of malate dehydrogenase from several organs of two species of *Rana*. In both species there were three bands each in the heart, stomach and brain and two bands in the skeletal muscle. The most anodally migrating one (in muscle) and two (in others) bands were found in the supernatant fraction (MDH-m). It was also noted that the MDH-m and the slower MDH-s of *R. nigromaculata* (Fig. 2, a) migrated much further than those of *R. plancyi chosenica* (Fig. 2, b). Therefore, when the mixture of extracts from the same tissue of two species was applied to electrophoresis, the MDH-m of *R. nigromaculata* and the slower MDH-s of *R. plancyi* often appeared as a single broad band in the middle position. In two species, the fast migrating MDH-s usually appeared as a faint band.

2. Ontogeny of LDH and MDH in the Developing Embryos.

A) Ontogeny of Lactate Dehydrogenase.

The embryonic pattern of LDH isozymes in *R. nigromaculata* during development is presented in Fig. 3A. The unfertilized eggs and early developing embryos were characterized by a very strong band of LDH activity which migrated rapidly toward the anode and by an extremely faint band which moved slowly and was usually difficult to see in photographs because of its weak activity. The former band seemed to be identical to LDH-1 and the latter to LDH-5 when compared with the LDH pattern of the adult heart. These two bands persisted until stage 20 (gill circulation) after which the activities of the bands increased gradually. At stage 24 (right out-gill disappear), there was a rapid increase in the LDH-5 activity and an abrupt appearance of a new band between LDH-1 and LDH-5. This new pattern remained without change until feeding stage.

The electrophoretic pattern of LDH during development of *R. plancyi chosenica* embryo is shown in Fig. 3B. The pattern is similar to that of *R. nigromaculata* except the rapid increase of LDH-5 activity at stage 19 (heart beat) which presumably means activation of LDH-A and LDH-B genes earlier than in *R. nigromaculata*. Both species did not give complete five bands until stage 25.

The developmental pattern of LDH in *Hynobius leechii* embryo is presented in Fig. 3C. The pattern is strikingly different from those of the above two species of frog. Five molecular forms of LDH are present in unfertilized egg and early

embryos. The most cathodic band among them usually appeared as a faint band indicating lower enzyme activity. This pattern persisted until stage 20 (closed neural folds) with the gradual increase in the activity of the most cathodic band. At stage 25 (ten somite), there appeared two additional faint bands with slow electrophoretic mobility. This new pattern remained until stage 36 (s-flexture), when six more new bands appeared making a total of eleven bands. Thereafter, the activities of these isozymes increased gradually until stage 40 (free swimming).

B) Ontogeny of Malate Dehydrogenase.

The electrophoretic pattern of MDH in *R. nigromaculata* is shown in Fig. 4A. All embryonic stages examined showed consistently only two molecular forms of MDH. The less mobile isozyme MDH-m was present as a faint band at stage 7, but increased remarkably in the activity during development. On the other hand, of two MDH-s presented in the adult tissues, the slower one did not appear until feeding stage.

Similar pattern was found in embryos of *H. leechii* (Fig. 4C). The pattern, however, was more compact, and the anodally migrating band (MDH-s) was predominent with constant activity throughout all the stages examined. The cathodic band, MDH-m, appeared as a very faint band at earlier stages, but increased slowly as the development proceeded.

The developmental pattern of MDH in *R. plancyi chosenica* embryo is presented in Fig. 4B. All embryonic stages showed consistently three molecular forms of MDH. The pattern was identical with that of adult tissues (Fig. 2, b). At stage 25, the cathodic band, MDH-m, increased repidly.

None of the LDH- and MDH-bands illustrated above was seen when the gels were incubated in the absence of substrate (sodium lactate or sodium malate).

DISCUSSION

Isozymes of lactate dehydrogenase have long been examined in a variety of anuran species. Chen (1968) reported that LDH existed in three molecular forms in extracts of adult tissues and developing embryos of *Bombina variegata*. Nine species of *Rana* were reported to contain 6—8 different isozymes of LDH (Moyer *et al.*, 1968). *R. sylvatica* and *R. virgatipes* were reported to have as many as 18 to 25 LDH isozymes (Moyer *et al.*, 1968). Claycomb and Villee (1971) have found an isozyme pattern of up to 9 bands in extracts of several tissues and developing embryos of *Xenopus laevis*. In the previous paper (Park, 1976), *Callula tornieri* were found to contain three molecular forms of LDH. On the other hand, Adams and Finnegan (1965) reported that LDH existed in twelve molecular forms in urodele *Amblystoma gracile*. In another newt, the genus *Taricha*, Moyer *et al.* (1968) have found 6—8 isozymes in the same position observed in frogs. Thus there is a great

diversity in the band pattern among species. Most of the amphibia examined have either more or less than the predicted five isozymes and do not always fit the hypothesis of the random aggregation of two subunits A and B (Markert, 1963). To explain these observations several authors have postulated the presence of one or more additional genes coding for one or more new polypeptide subunits (Adams and Finnegan, 1965; Wright and Moyer, 1966, 1968; Koen and Goodman, 1969). Adams and Finnegan (1965) indicated that three polypeptide subunits under control of three nonallelic genes were responsible for complex LDH isozyme pattern of *Amblystoma gracile*, and illustrated the possibility with heterozygosity at one gene locus due to a mutation.

In the present study, the appearance of seven or eight bands in the heart of *R. nigromaculata*, and seven bands in the skeletal muscle and eleven bands in the embryos of *H. leechii* could only be explained by the above assumption. Furthermore, it is thought that a mutation has occurred at "B" gene in case of *R. nigromaculata*, and "A" gene in case of *H. leechii*.

As shown in Fig. 1, LDH-1 is most concentrated in the heart and LDH-5 is predominent in the skeletal muscle. This result is similar to those found in other amphibia (Wright and Moyer, 1966, 1968; Claycomb and Villee, 1971; Wall and Blackler, 1974). If this assignment is correct, it is quite reasonable to conclude here that the aggregation of subunits in amphibia is not random. Furtheremore, the very faint band of LDH-3 indicates that the factors controlling aggregation are very complex.

It has been known that there are two major electrophoretic forms of MDH in amphibia. These isozymes are controlled by separate genetic loci (Shaw, 1969 a,b) and present in different subcellular fraction, one in supernatant (MDH-s) and the other in mitochondria (MDH-m). Multiple subbands of each of the two major forms probably represent conformational isomers (Kitto *et al.*, 1966).

Chen (1968) reported that MDH existed in two molecular forms in extract of adult tissues and developing embryos of *Bombina variegata*. This pattern is identical to that of *H. leechii* (Fig. 4C). Two anuran species, *R. pipiens* and *R. palustris* were found to contain one molecular form of MDH-m and three molecular forms of MDH-s (Wright and Subtelny, 1971; Johnson and Chapman, 1971). In the present study, however, two species of *Rana* had three bands showing MDH activity which were consisted of one band of MDH-m and two bands of MDH-s.

In the present study, LDH isozyme patterns obtained from embryonic extracts of three amphibian species at various stages of development were characterized by a gradual increase in the activity of the slower migrating isozyme (Fig. 3). The first evidence of this increased activity (LDH-s) was observed at around heartbeat stage (Shumway's stage 19) in *H. leechii* and *R. plancyi*. In *R. nigromaculata* the

activity began to increase a little later than the other two species (at around stage 24). This result is in agreement with those in other amphibia; *R. sylvatica*(Wright and Moyer, 1966) and *Xenopus laevis* (Claycomb and Villee. 1971: Wall and Blackler, 1974). This finding indicates that the genes specifying LDH subunits must be turned on approximately at stage 19, or slightly before, and the rapid increase of LDH-5 at this stage is likely due to the differentiation and metabolism of functional muscle cells.

As shown in Fig. 4, the pattern of MDH did not change during the embryonic development but the activity increased gradually as the development proceeded in the three species of the present study. In the hybrid embryos of *Rana*. however, paternal and hybrid forms of MDH were detected at stage 14 (neural fold) (Park, unpublished). It is thought that this result reflects the rapid increase in the oxygen consumption which takes place from gastrula stage (Ha and Park, 1963).

SUMMARY

Polyacrylamide gel electrophoresis was used to investigate the patterns of LDH and MDH isozymes in the embryo and adult of amphibia: *Rana nigromaculata, Rana plancyi chosenica* and *Hynobius leechii*. *Rana nigromaculata* is considered to be heterozygous for the gene specifying the "B" subunit of LDH, and *Hynobius leechii* to be heterozygous for the gene specifying the "A" subunit of LDH.

The LDH isozyme pattern of embryos of the above three species is characterized by a gradual increase in the activity of LDH-5 (muscular form) during development.

Two or three molecular forms of MDH is present steadily from early embryos and in adult. Of the MDH isozymes, the more cathodic one (MDH-m) appears weakly in early developing stages, but increases slowly in the activity as the embryo develops.

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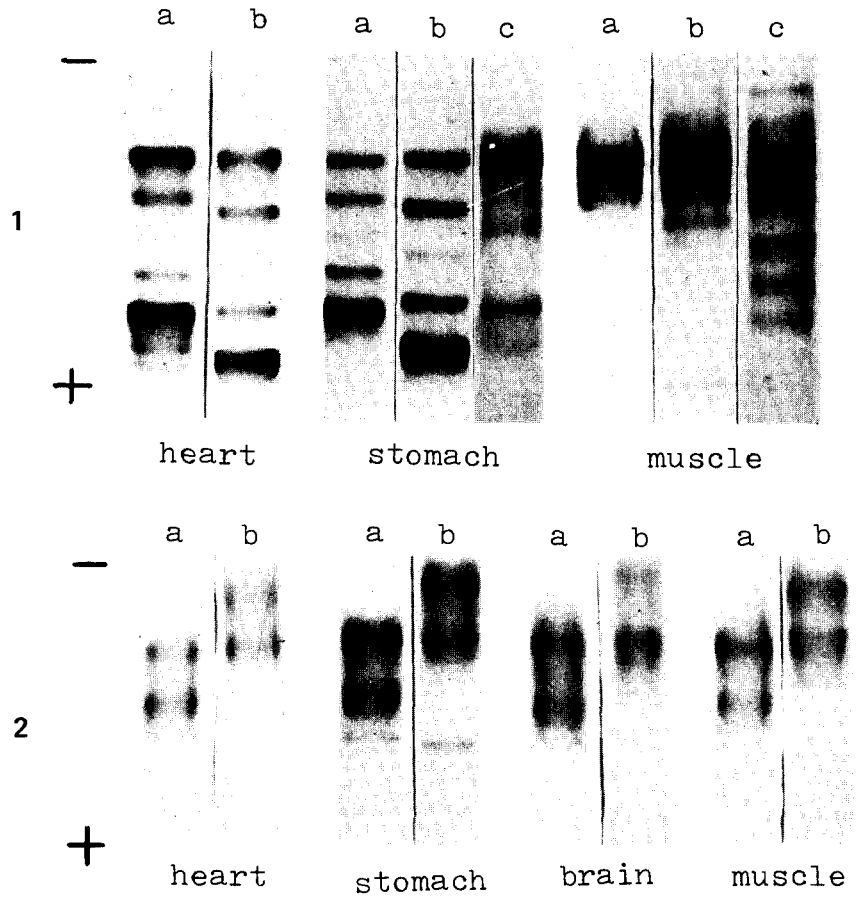

Fig. 1. Distribution of lactate dehydrogenase isozymes in the heart, stomach and skeletal muscle of *Rana nigromaculata* (a), *Rana plancyi chosenica* (b) and *Hynobius leechii* (c).

Fig. 2. Distribution of malate dehydrogenase isozymes in the heart, stomach, brain and skeletal muscle of *Rana nigromaculata* (a) and *Rana plancyi chosenica* (b).

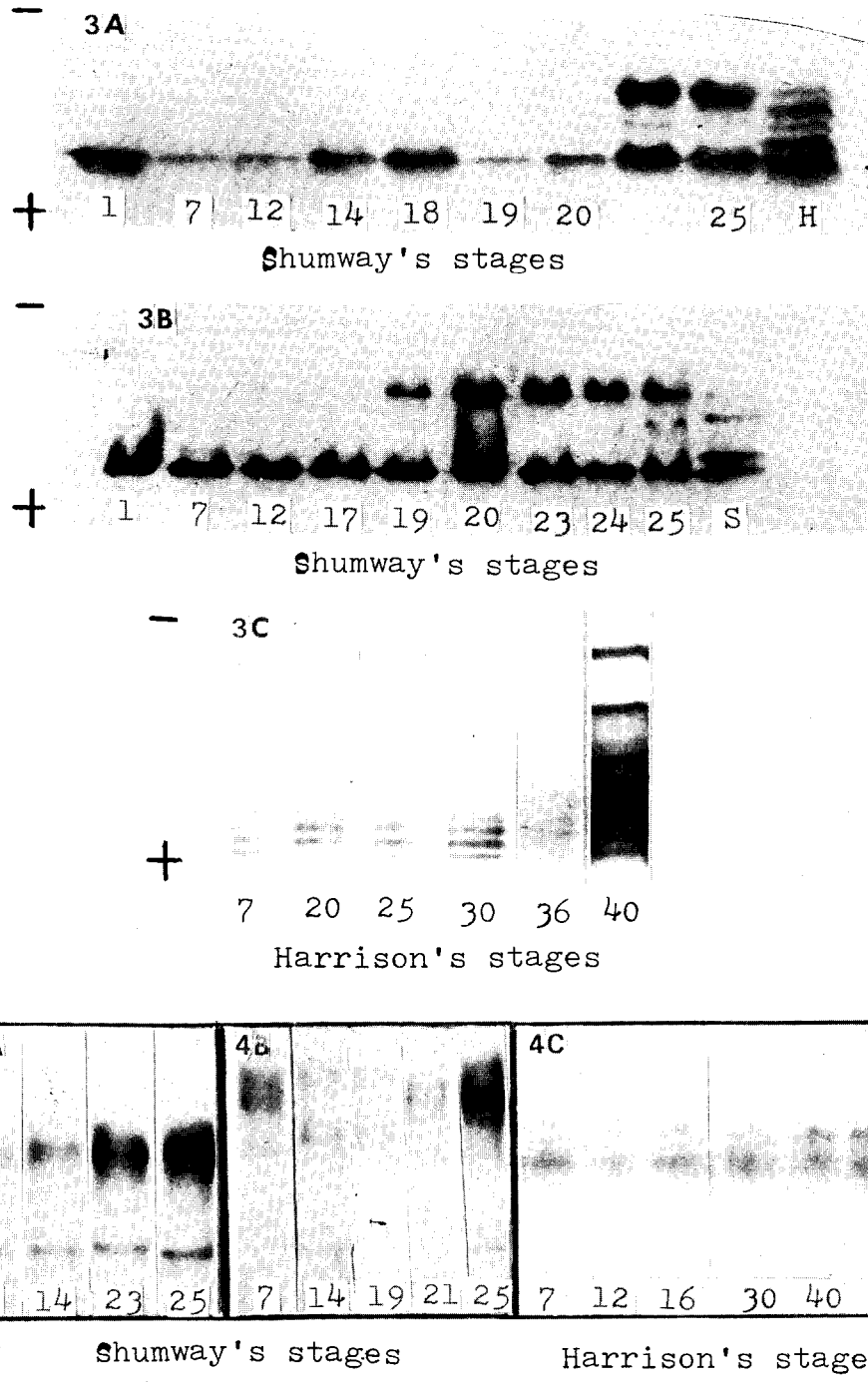

Fig. 3. Changes in the lactate dehydrogenase isozyme patterns of developing embryos of *Rana nigromaculata* (A), *Rana plancyi chosenica* (B) and *Hynobius leechii* (C).

Fig. 4. Changes in the malate dehydrogenase isozyme patterns of developing embryos of *Rana nigromaculata* (A), *Rana plancyi chosenica* (B) and *Hynobius leechii* (C).