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Studies on Alkaline Phosphatase Activity in Amphibians II. Changes in Alkaline Phosphatase Activity during Development in *Rana catesbeiana*

By

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ABSTRACT

The changes in alkaline phosphatase activity during development in *Rana catesbeiana* were examined by electrophoresis and spectrophotometry in embryos and hatching and hatched tadpoles, and in various visceral organs, such as the liver, pancreas, kidney and intestine of tadpoles and froglets. Alkaline phosphatase first appeared at the hatching tadpole stage. The liver showed the first band of alkaline phosphatase at st. III. This band migrated at 2.3~2.4 cm from the origin. A new band having a mobility of 2.1 cm appeared at one month after st. XXV. The activity of this enzyme became low at st. XIX, and then increased. In the pancreas, a band having the same mobility as the first band of the liver appeared at st. III and remained in all subsequent stages. Activity was high at st. XIX and after st. XXIII. In the kidney, a band migrating at 1.3~1.4 cm appeared at st. III and existed in all the subsequent stages. Activity was high at st. XIX and again at one month after st. XXV. In the intestine, the first band of 2.0~2.1 cm in mobility appeared at st. 24~25 and was replaced by the second band of 1.6 cm in mobility which appeared at st. XXIV or XXV. Activity was high at st. XIX and also at st. XXIV or XXV.

INTRODUCTION

According to PIATKA and GIBLEY (1967), the alkaline phosphatase in the developing pronephros of *Rana pipiens* is important in the reabsorbing and possibly secretory function of the tubules proper. BROWN and MILLIGNTON (1968) made histochemical studies of alkaline phosphatase in the small intestine of *Rana temporaria* during the larval development and metamorphosis.

In a previous paper, the present author has reported on the changes in electrophoretic pattern of alkaline phosphatase during development from cleavage to completion of metamorphosis in *Rana japonica* and *Rana nigromaculata* (KASHIWAGI, 1990). The electrophoretic patterns of alkaline phosphatase at the tadpole stages differed from those observed at the embryonic stages. The bands appearing at the early tadpole stages diffused and increased in density when development proceeded toward the late tadpole stage, and then disappeared at the stage before the completion of metamorphosis. New bands appeared when tadpoles com-

pleted metamorphosis. In this case, no observations of alkaline phosphatase have been made in each of the visceral organs of the two frog species because of the small size of these organs at the tadpole and froglet stages. Thus, in the present study, the tadpoles and froglets of *Rana catesbeiana* which are remarkably large as compared with those of the foregoing species were used.

The purpose of the present study is to examine the changes of alkaline phosphatase activity during development in the liver, pancreas, kidney and intestine of *Rana catesbeiana*.

MATERIALS AND METHODS

An egg mass of *Rana catesbeiana* SHAW was shipped on 5 June 1986 from Nishikawa-cho, Kitakatsushika-gun, Saitama Prefecture through a biological supply company to our laboratory. Nearly all the eggs cleaved and developed normally under laboratory condition. The tadpoles were transferred to cement tanks, 91 cm×65 cm×20 cm, placed in the open air. They were fed on boiled spinach, and froglets were fed on crickets. Developmental stages of the tadpoles and froglets follow those of *Rana pipiens* established by SHUMWAY (1940) and TAYLOR and KOLLROS (1946), respectively, for convenience' sake.

The materials used for electrophoretic analyses of alkaline phosphatase in the present study were those of 23 developmental stages from gastrulation to one month after the completion of metamorphosis.

Crude extracts of alkaline phosphatase for electrophoresis were prepared by homogenizing entire embryos and tadpoles at the earlier stage, as well as various organs obtained from tadpoles and froglets. Homogenization was made after adding one or two volumes of distilled water in a glass homogenizer cooled with ice-water.

The embryos up to the tail-bud stage were freed of jelly membranes by treatment with papain, cystein and sodium thioglycollate (SPIEGEL, 1951). The jelly membranes of the embryos at the later stage were removed with iridectomy scissors and watchmaker's forceps. The kidney, liver, pancreas and intestine were cut off from tadpoles and froglets immediately after the animals were pithed, and washed with cold RINGER's solution. Especially, the contents of the intestines were thoroughly washed out with the same solution. After removing water by blotting papers, the above organs were weighed or measured. Eleven to 16 tadpoles at each of the 11 stages from st. III to st. XXIII, 11 individuals at st. XXIV or XXV, six froglets at one week after st. XXV and 10 frogs at one month after st. XXV were used. As the pancreas was too small, 10 samples obtained from 10 specimens were gathered as a lump, and two lumps were weighed at each stage.

The homogenate of each sample was placed in centrifuge tubes, and then frozen and thawed twenty times to separate alkaline phosphatase from insoluble cellular particles. After this treatment, the homogenate was centrifuged at 20,000 *g* at 4°C for 30 minutes. From the supernatant layer, 0.02 ml was sucked every time by a

pipet and poured into a gel slot for electrophoresis.

The butanol extracts of the organs were prepared by MORTON's method (1954). They were used for determination of alkaline phosphatase activity and protein content, both of which were necessary to obtain the specific activity of alkaline phosphatase in each organ.

Electrophoresis was carried out by the method described by OGITA (1965). After completion of electrophresis, the gel was stained by the method of BURSTONE (1958). Ten to 20 embryos at each of the five stages from st. 11 to st. 19, 5~16 tadpoles at each of the 15 stages from st. 20 to st. XXIII, eight individuals at st. XXIV or XXV, eight froglets at one week after st. XXV and 11 frogs at one month after st. XXV were used.

Assay for alkaline phosphatase activity of the liver, pancreas, kidney and intestine was performed by following the method of BESSEY, LOWRY and BROCK (1946). The protein content in each organ was determined by the method of LOWRY, ROSEBROUGH, FARR and RANDALL (1951). The specific activity of alkaline phosphatase in each organ was obtained from the quotient of alkaline phosphatase activity divided by the protein content, and was expressed as μM *p*-nitrophenol liberated(PNP)/mg protein/hour. The numbers of tadpoles and frogs examined were 5~16 at each of the eight tadpole stages between st. III and st. XXIII, six at st. XXIV or XXV and five at one month after st. XXV.

OBSERVATION

I. Changes of liver, pancreas, kidney and intestine in weight or length during development

Figs. 1 and 2 show the changes of weight or length which took place in the liver, pancreas, kidney and intestine during development from st. III to one month after completion of metamorphosis.

The mean weight of the liver attained a maximum of 0.179 gm at st. XIX, and then decreased until st. XXIV or XXV, whereupon it showed a rapid increase. The mean weight of the pancreas decreased from a maximum of 0.032 gm at st. XVII and st. XIX to a minimum of 0.003 gm at st. XXIV or XXV, and then increased again. The mean weight of the kidney attained a maximum of 0.036 gm at st. XIV, remained almost unchanged until st. XXIII, and then decreased before the completion of metamorphosis. The mean length of the intestine sharply decreased (92.3%) from a maximum of 40.2 cm at st. XVII and st. XVIII to a minimum of 3.1 cm at st. XXIV or XXV, whereupon it once again began gradually to increase.

II. Electrophoretic pattern and activity of alkaline phosphatase

The electrophoretic patterns of alkaline phosphatase in the whole bodies of embryos and hatching and hatched tadpoles were examined (Fig. 3A). The

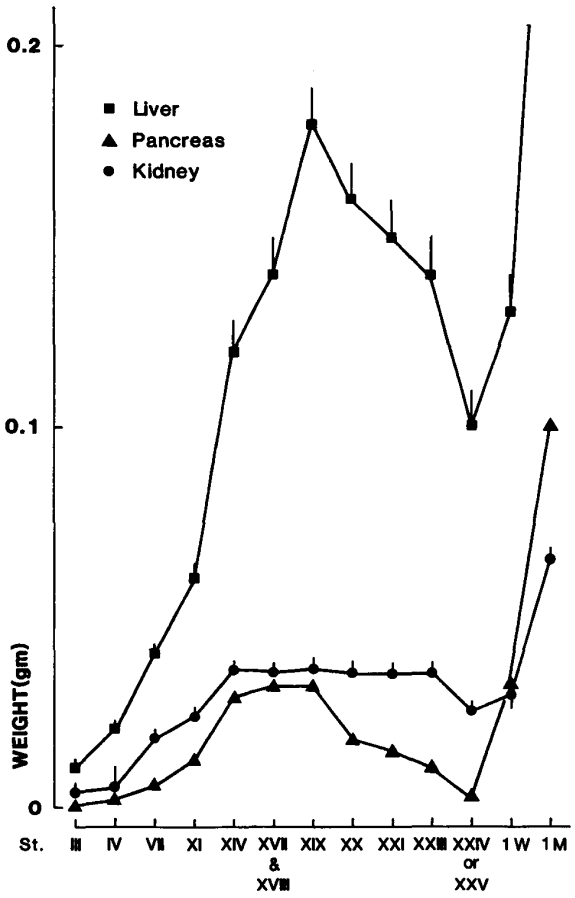

Fig. 1. Changes in weight of the liver, pancreas and kidney during development. Vertical bar in each point shows the standard error of the mean.

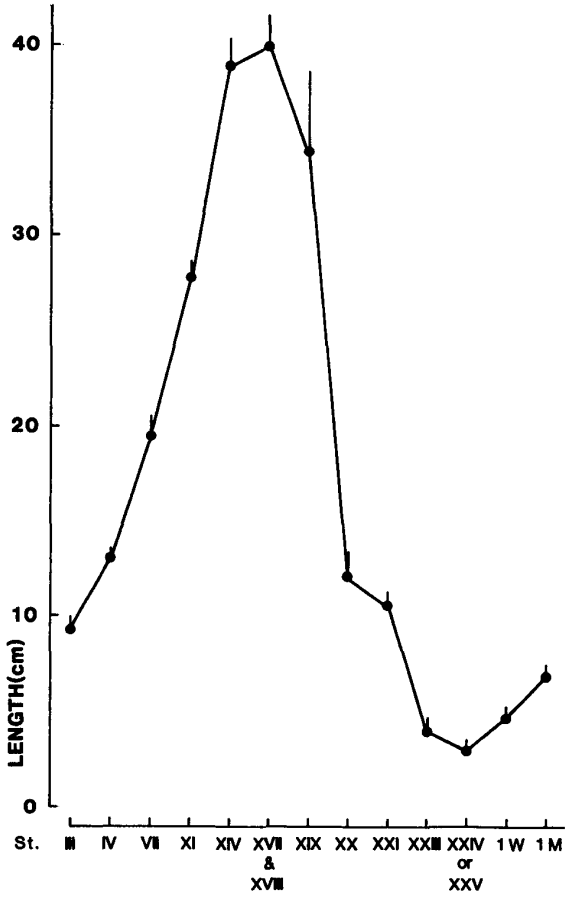

Fig. 2. Change in length of the intestine during development. Vertical bar in each point shows the standard error of the mean.

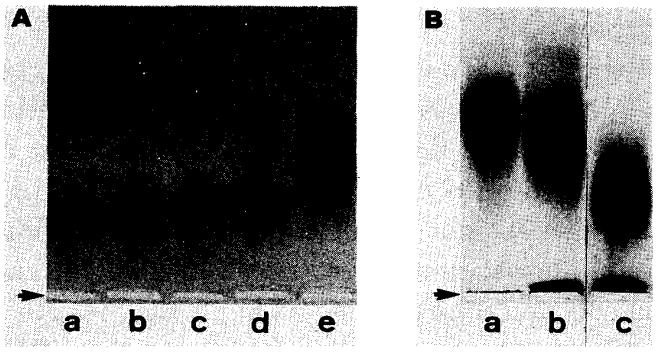

Fig. 3. Electrophoretic patterns of alkaline phosphatase.
A. Tadpoles. a. St. 20. b. St. 21. c. St. 22. d. St. 23. e. St. 24~25.
B. The liver at st. XIV (a) and one month after st. XXV (b), and kidney at st. XIX (c).

embryos at st. 11, 12, 14, 17 and 19 showed no trace of alkaline phosphatase activity. In the hatching tadpoles at st. 20, a single band appeared at 1.0~1.2 cm in the anodal direction from the origin. This band gradually intensified in

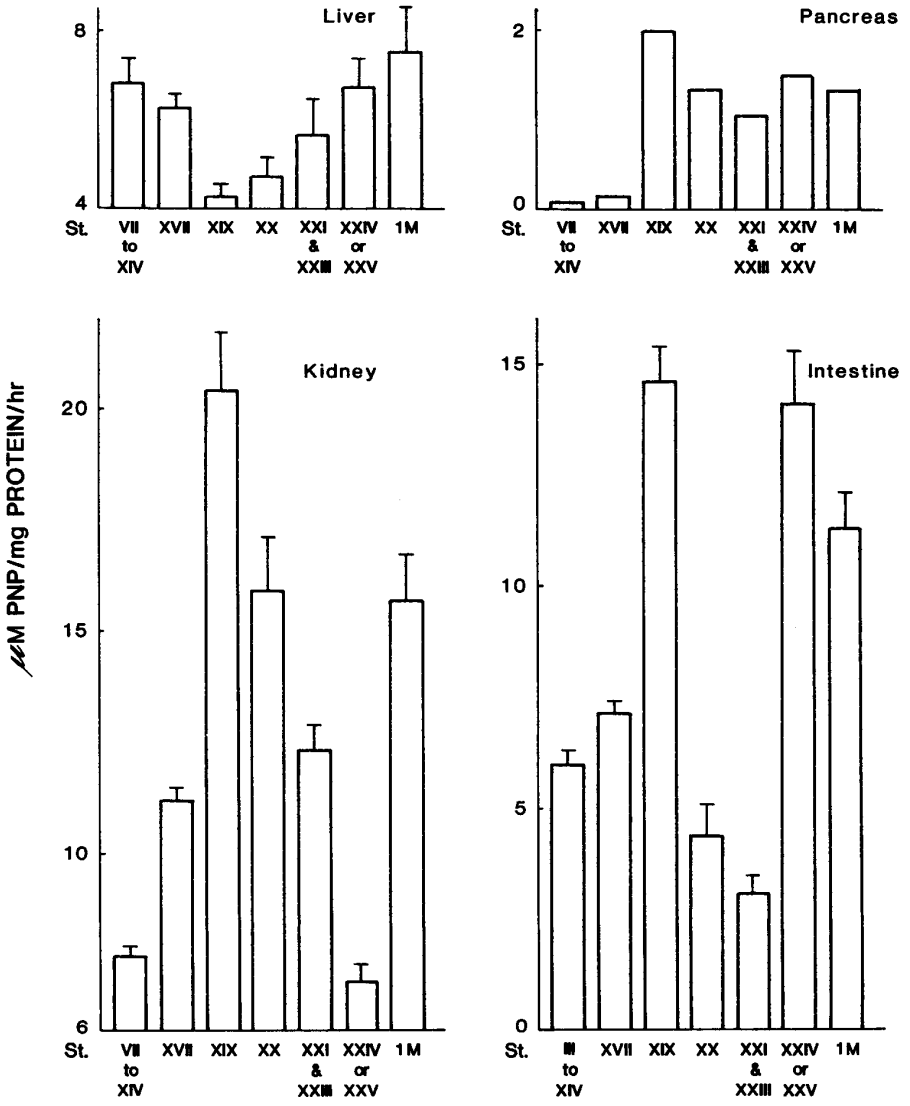

Fig. 4. Changes of alkaline phosphatase activities in developing liver, pancreas, kidney and intestine. Vertical bar in each column shows the standard error of the mean.

activity up to st. 23 of hatched tadpoles, and then became very thin at st. 24~25 just before the commencement of feeding. However, at this stage a second, fast-migrating band, being 2.0~2.1 cm in mobility, appeared in addition. This additional band came from the intestine.

The electrophoretic patterns and activity of alkaline phosphatase in the liver, pancreas, kidney and intestine of tadpoles and froglets were as follows. In the liver, a wide band having a greater mobility (2.3~2.4 cm) than either of the two bands observed at st. 24~25 appeared at st. III and persisted until one week after completion of metamorphosis (Fig. 3B). A new diffuse band situated more cathodal and migrating at 2.1 cm appeared at one month after completion of metamorphosis. A medium enzyme activity was observed in this organ, although this activity decreased from st. VII to st. XVII and became low at st. XIX. After this stage there was some increase in activity, which lasted until one month

after completion of metamorphosis (Fig. 4).

The electrophoretic patterns of alkaline phosphatase in the pancreas showed a wide light band, having the same mobility as that of the first band of the liver. It appeared at st. III and remained in all the subsequent stages. The enzyme activity in this organ was very low until st. XVII. This activity increased sharply and attained a peak at st. XIX, but was followed by a decrease toward st. XXI and st. XXIII. After these stages, the activity rose again (Fig. 4).

In the kidney, a single band migrating at 1.3~1.4 cm appeared at st. III and remained in the subsequent stages (Fig. 3B). A very high enzyme activity was observed in this organ. This activity attained a peak at st. XIX. The activity became low at st. XXIV or XXV, but it was followed again by a rapid increase (Fig. 4).

As stated earlier, the first band in the intestine made its appearance at st. 24~25, just before the onset of feeding. While this band was observed at st. III as a single major band of 1.9~2.3 cm in mobility, it was gradually diffused in the anodal direction as development proceeded, and became thin or disappeared at st. XX (Fig. 5). A minor band with fast mobility of 4.4~4.7 cm appeared abruptly

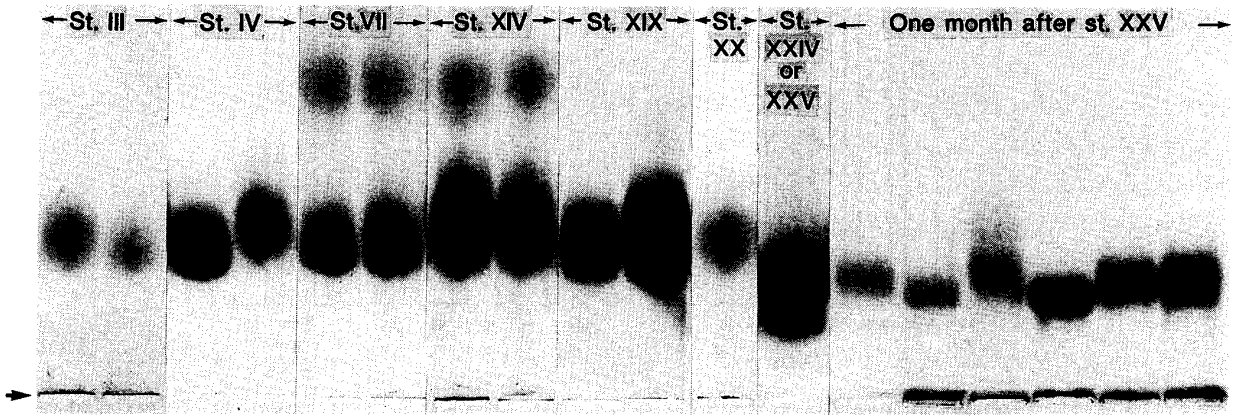

Fig. 5. Electrophoretic patterns of alkaline phosphatase in developing intestines.

at st. VII. This band became wider until st. XIV and soon thereafter disappeared. Neither major nor minor bands was detected between st. XXI and st. XXIII. At st. XXIV or XXV a compact major band of 1.6 cm in mobility appeared, being often accompanied by an additional minor band near the slot inserted with the sample. A narrow and sharply demarcated band migrating in the major band region at st. XXIV or XXV was observed at one month after completion of metamorphosis. The alkaline phosphatase activity increased slowly between st. III and st. XVII and attained a peak at st. XIX (Fig. 4). Thereafter, it rapidly decreased until st. XX and attained the lowest at st. XXI and st. XXIII. After these stages there was a sharp increase up to a peak at st. XXIV or XXV. A high enzyme activity was detected in this organ.

DISCUSSION

PIATKA and GIBLEY (1967) histochemically investigated alkaline phosphatase in the pronephros of *Rana pipiens* and clarified that its activity is high in the pronephric anlage in 3 mm (neural tube stage) embryos, decreases gradually in the collecting tubules and pronephric ducts of 6 mm (hatching stage) to 12 mm tadpoles, and then almost completely disappears from these portions in 12 mm to 23 mm (one-month-old) tadpoles. HAH (1974), too, histochemically examined the changes of alkaline phosphatase activity of the developing kidney in *Bombina orientalis*. Alkaline phosphatase is first observed in the pronephros at the tail-bud stage. The activity of alkaline phosphatase increases gradually through the transition from the embryonic to tadpole stages, but decreases as the pronephros begins to degenerate. As the development of the pronephros attains its maximum, the mesonephros begins to develop. Remarkably high alkaline phosphatase activity is seen throughout metamorphosis. Using electron microscopy, BROWN and MILLIGNTON (1968) observed changes in alkaline phosphatase activity taking place in the intestinal epithelium of *Rana temporaria* from the hatching stage until the completion of metamorphosis. Alkaline phosphatase was always found at the absorption sites of cells for foodstuffs. While alkaline phosphatase activity appears first at the hatched tadpole stage, it is no longer noticeable at the metamorphosing stage when tadpoles stop feeding. By using the technique of electrophoresis, KASHIWAGI (1990) confirmed the initial appearance of bands of alkaline phosphatase at the tail-bud stage in *Rana japonica* and at the hatching stage in *Rana nigromaculata*. The present study of electrophoretic analysis in *Rana catesbeiana* revealed that one band was initially observed at the hatching tadpole stage. Thus the alkaline phosphatase in hatching tadpoles of *Rana catesbeiana* appears definitely earlier than in the small intestine of *Rana temporaria* and definitely later than in the pronephros of *Rana pipiens* and *Bombina orientalis* and embryos of *Rana japonica*. Such differences may be attributed not only to differences in species, but also to differences in the method of examination.

In *Rana japonica* and *Rana nigromaculata,* the changes in electrophoretic pattern were first observed at the early tadpole stage, again at the late tadpole stage, and finally at the completion of metamorphosis. A more detailed investigation on the changes in electrophoretic pattern and activity of alkaline phosphatase during development was carried out in the present study by using various developing organs of *Rana catesbeiana*. The liver was found to have two bands of alkaline phosphatase. The first band, migrating at 2.3~2.4 cm from the origin, appeared from st. III up to one week after completion of metamorphosis. The second, more cathodal band of 2.1 cm mobility occurred in frogs one month after completion of metamorphosis. These results are in well accord with those obtained by MANWELL (1966), who has reported that the bands of the liver alkaline phosphatase in the tadpoles and adult frogs of *Rana catesbeiana* show a difference in electrophoretic mobility. On the other hand, each of pancreas and kidney

showed a single band during development from st. III to one month after completion of metamorphosis. By means of spectrophotometric examinations, the alkaline phosphatase activity in the developing organs was found to be arranged from higher to lower in the order of the kidney, intestine, liver and pancreas. This seems to show that enzyme activity exhibits organ specificity. The alkaline phosphatase activity in the liver decreased to a low at st. XIX and then increased at the subsequent stages, while the alkaline phosphatase activities in the pancreas and kidney increased to a high at st. XIX, decreased to a low immediately before completion of metamorphosis, and then increased again at all the subsequent stages. The causes of various changes in alkaline phosphatase activity during development in these organs still remain unexplained. The most remarkable amount of change in electrophoretic pattern and enzyme activity during development was found in the intestine. The bands of alkaline phosphatase were different between tadpoles and frogs. The tadpole type of alkaline phosphatase bands appeared at st. 24~25, just before the time when tadpoles began to take a vegetable diet, and subsequently disappeared at the time of metamorphosis when tadpoles ceased to eat. The frog type of alkaline phosphatase bands appeared at st. XXIV or XXV, immediately before the time when froglets began to take an animal diet. An increase in alkaline phosphatase activity was observed at st. XIX when tadpoles could hardly eat vegetable and at st. XXIV or XXV. This seems to suggest that drastic changes of the intestine in structure and function necessitated a distinct increase of the alkaline phosphatase activity.

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