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Studies on Alkaline Phosphatase Activity in Amphibians I. Alkaline Phosphatase Activity during Development in *Rana japonica* and *Rana nigromaculata*

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ABSTRACT

Electrophoretic patterns of alkaline phosphatase and its activities at 20 developmental stages from cleavage to completion of metamorphosis and in 18 organs and plasma of adults were compared with one another in *Rana japonica* and *Rana nigromaculata*. The bands of alkaline phosphatase first appeared at the tail-bud stage in *Rana japonica*, while they appeared at the hatching tadpole stage in *Rana nigromaculata*. In each species, a distinct change in electrophoretic pattern occurred at an early tadpole stage, then at a late tadpole stage, and finally at the stage of complete metamorphosis. Ten kinds of isozymes of alkaline phosphatase were found in nine organs of *Rana japonica*, while seven kinds of isozymes were observed in eight organs and plasma of adult *Rana nigromaculata*.

INTRODUCTION

Although alkaline phosphatase has long been subject of biochemical and histochemical studies in mammals, only a few reports have been made on the biological roles of these enzymes in amphibians. PIATKA and GIBLEY (1967) have assumed that alkaline phosphatase plays a role of morphogenesis in the developing pronephros as well as reabsorption and possibly secretory function in the proximal tubules of the kidney in *Rana pipiens*. BROWN and MILLIGNTON (1968) have suggested that alkaline phosphatase is associated with dietary change in the developing intestine of *Rana temporaria*.

In mammals, alkaline phosphatase has been found to be located on the brush borders of convoluted tubules of the kidney (BURSTONE, 1958; WACHSTEIN and BRADSHAW, 1965) and on the striated borders of the intestine (WATANABE and FISHMAN, 1964). A correlation of alkaline phosphatase to fat absorption in the intestine has been reported by MADSEN and TUBA (1952), INGLIS, KRANT and FISHMAN (1967) and WARNOCK (1968). MOOG (1951) has reported that alkaline phosphatase of the duodenum in the mouse begins to appear at about 14 days after birth and reaches the maximum at the age of 18 days. MOOG *et al.* (1966, 1968, 1969) have later observed in the duodenum of mice the appearance of a new type

of alkaline phosphatase in addition to the presence of three types of this enzyme at the age of 20 days. By this age, the microvilli change their shape from short and wide to long and narrow, without changing their volume (OVERTON, 1965). These results seem to indicate that the change in alkaline phosphatase of the duodenum in mice reflects the differentiation of the microvilli.

In two Japanese anuran species, *Rana japonica* and *Rana nigromaculata,* the present author studied the changes of alkaline phosphatase activity in the whole body and various organs during development from cleavage to completion of metamorphosis in order to elucidate the relationship between the development of these frog species and alkaline phosphatase activity.

MATERIALS AND METHODS

Adult specimens of *Rana japonica* GÜNTHER and *Rana nigromaculata* HALLOWELL were collected during the breeding season from the suburbs of Hiroshima City. Ovulation was induced by injecting the pituitaries of *Rana catesbeiana* SHAW into the body cavity of mature females. Eggs were fertilized with sperm suspended in Cl-free water. Egg masses in which more than 90% of the eggs cleaved normally were utilized. Cleaving eggs and embryos were all kept in PETRI dishes, 17.5 cm in diameter, and maintained at room temperature. Tadpoles were raised in enameled pans, 46.5×31.0×11.0 cm in size, at room temperature. Water was changed on alternate days. Tadpoles were fed on boiled spinach.

Cleaving eggs, embryos, tadpoles and metamorphosed frogs at the following 20 developmental stages were used for analysis: SHUMWAY's stages 7, 9, 12, 15, 17, 18, 19, 20, 23, 25, and TAYLOR and KOLLROS' stages I, II, III, IV, X, XIV, XVI, XVIII, XXI and XXV in *Rana japonica*; SHUMWAY's stages 7, 12, 15, 17, 19, 20, 22, 23, 25, and TAYLOR and KOLLROS' stages I, II, III, V, VIII, X, XIV, XVI, XVIII, XXI and XXV in *Rana nigromaculata.* Blood and 18 kinds of organs were obtained from both males and females of the two species.

1. Preparation of alkaline phosphatase

Cleaving eggs and embryos up to the tail-bud stage were freed from jelly membranes by SPIEGEL's method (1951). Jelly membranes of embryos after the tail-bud stage were removed with iridectomy scissors and watchmaker's forceps. Tadpoles were starved for five days before extraction of enzymes to expel ingested substances from the stomach and intestines.

Crude extracts were obtained by grinding cleaving eggs, embryos, tadpoles or adult organs after adding an equal volume of distilled water in a glass homogenizer cooled with ice-water. In each case, about 20~40 cleaving eggs or embryos, 5~10 tadpoles at each stage, or each kind of various organs obtained from more than five adults were used. While homogenization was done by using the whole bodies of *Rana japonica* at all the tadpole stages and those of *Rana nigromaculata* at stages I and II of tadpoles, the bodies of *Rana japonica* at the young frog stage and those of *Rana nigromaculata* at the later tadpole and young frog stages were divided

into two portions, one of which contained only the visceral organs (VO), while the other contained the remaining body mass excluding the visceral organs (BM). The two portions, VO and BM, were separately homogenized. The homogenates were centrifuged at 20,000 *g* at 4°C for 30 minutes. The supernatant was utilized for electrophoresis. Plasma was prepared by centrifuging blood obtained from the hearts of adult frogs at 3,000 r.p.m. at room temperature.

2. Electrophoresis

Thin layer polyacrylamide gel electrophoresis was carried out according to the procedure described by OGITA (1965) and MASUZAWA, KAMATA, SAKOYAMA, CHIBA and OGITA (1973). Extracts from tadpoles of *Rana japonica* at st. I to XXI and those from organs and bloods of adult frogs of the two species were diluted with three or four-fold of distilled water to obtain a better resolution of electrophoretic patterns. Each slot was filled with 0.02 ml of extracts. Electrophoresis was done for about 2.5 hours, when the leading edge moved 8 cm from the origin. Alkaline phosphatases of individuals, organs and plasmas at different stages were compared with one another by calculating their relative mobilities, each of which was the quotient of the migrating distance between the origin and the center of each band divided by the distance between the origin and the leading edge.

OBSERVATION

I. Rana japonica

1. Embryos, tadpoles and froglets

At the cleavage and embryonic stages (st. 7, 9, 12 and 15), no band of alkaline phosphatase was found. A single light band with relative mobility of 0.31 appeared first at stage 17. At the hatching stage (st. 20), another faint band with slow electrophoretic mobility of 0.18 appeared abruptly (Fig. 1A). These two bands increased in width and density at the hatched tadpole stage (st. 23),

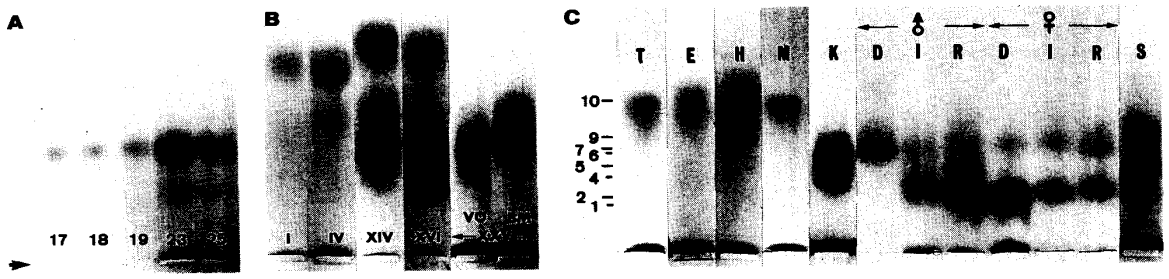

Fig. 1. Electrophoretic patterns of alkaline phosphatase from *Rana japonica*.
A. Embryos at stages 17, 18 and 19 and tadpoles at stages 23 and 25.
B. Tadpoles at stages I to XVI and a young frog at stage XXV.
C. Various organs of adults. T, Tongue. E, Esophagus. H, Heart. M, Skeletal muscle. K, Kidney. D, Duodenum. I, Small intestine. R, Rectum. S, Skin.

although they became thin again at stage 25, and were not observed at the subsequent stages.

A new band having a greater relative mobility of 0.53~0.56 than those observed previously appeared at stage I. This band gradually increased in density, remained until stage XVIII and then disappeared (Fig. 1B). In addition to this band, another faint band with relative mobility of 0.35~0.39 appeared at st. IV. This faint band gradually diffused to the cathodal direction and increased in density from st. X to XVIII. These two bands disappeared at st. XXI, and two new bands appeared at st. XXV, one of which was present in the visceral organs (VO) of young frogs and was 0.28~0.29 in relative mobility, while the other was present in the body mass (BM) and was 0.31~0.35 in relative mobility.

2. Adult frogs

Electrophoresis of alkaline phosphatase was performed in 18 kinds of organs and plasma of adult frogs. However, electrophoretic patterns appeared in nine kinds of organs, the tongue, esophagus, heart, skeletal muscle, kidney, duodenum, small intestine, rectum and skin, while no patterns were recognized in the other nine kinds of organs and plasma. In the electrophoretic patterns of the former nine organs, 10 bands were discriminated from one another. They were designated as band 1 to band 10 in the order of increasing electrophoretic mobility (Fig. 1C). The alkaline phosphatase patterns of the tongue, esophagus, heart and skeletal muscle revealed band 10, which showed the fastest migration of 0.38 (Fig. 1C; Table 1). The electrophoretic pattern of the kidney revealed band 5 which had a relative mobility of 0.25 and was usually diffuse and dark, indicating a high activity.

The electrophoretic patterns of the duodenum, small intestine and rectum were not simple. While the duodenum in one (No. 1) of the three males examined had band 2 of 0.16 and band 6 of 0.28 in relative mobility, respectively, that of another male (No. 2) had a single band 6 of 0.28. The duodenum of the remaining male

TABLE 1
Relative mobility of the bands of alkaline phosphatases from nine organs of *Rana japonica*

Organ	Relative mobility									
Tongue										0.38
Esophagus										0.38
Heart										0.38
Skeletal muscle										0.38
Kidney					0.25					
Duodenum	0.15	0.16				0.28	0.29	0.30		
Small intestine	0.15	0.16				0.28	0.29	0.30		
Rectum		0.16	0.20			0.28	0.29			
Skin				0.21					0.34	
Band	1	2	3	4	5	6	7	8	9	10

(No. 3) possessed two bands, one of which was band 2 of 0.16 and the other was band 7 of 0.29 in relative mobility. Band 2 was somewhat darker than band 7. The duodenum of one (No. 4) of the two females examined possessed bands 1 and 7 which were 0.15 and 0.29 in relative mobility, respectively. Band 1 was distinctly darker than band 7. The duodenum of the other female (No. 5) had band 2 of 0.16 and band 8 of 0.30 in relative mobility. Band 8 was somewhat darker than band 2. The small intestine of male No. 1 possessed bands 1 and 8 which were 0.15 and 0.30 in relative mobility, respectively. Band 1 was somewhat darker than band 8. Each of the small intestines of males Nos. 2 and 3 showed two bands. While male No. 2 had band 2 of 0.16 and band 6 of 0.28 in relative mobility, male No. 3 had band 1 of 0.15 and band 6 of 0.28 in relative mobility. In male No. 2, band 2 was somewhat darker than band 6, while in male No. 3, band 6 was somewhat darker than band 1. The small intestine in one (No. 4) of the two females examined had band 7 of 0.29 and band 1 of 0.15 in relative mobility, while that of the other female (No. 5) had band 2 of 0.16 and band 7 of 0.29 in relative mobility. While band 1 was slightly darker than band 7 in female No. 4, band 7 was somewhat darker than band 2 in female No. 5. The rectum of male No. 1 had one band (band 3) which was 0.20 in relative mobility, while each of the other two males (Nos. 2 and 3) possessed two bands. In male No. 2, the two bands (bands 2 and 7) were 0.16 and 0.29 in relative mobility, while in male No. 3, the two bands (bands 2 and 6) were 0.16 and 0.28 in relative mobility, respectively. In the two males, band 2 was distinctly darker than the other band. The rectum of female No. 5 possessed band 3 which was 0.20 in relative mobility, while that of the other female (No. 4) had bands 2 and 6, which were 0.16 and 0.28 in relative mobility, respectively. These two bands were similar to each other in density.

The electrophoretic pattern of alkaline phosphatase in the skin showed bands 4 and 9, which were 0.21 and 0.34 in relative mobility, respectively. These two bands were similar to each other in density.

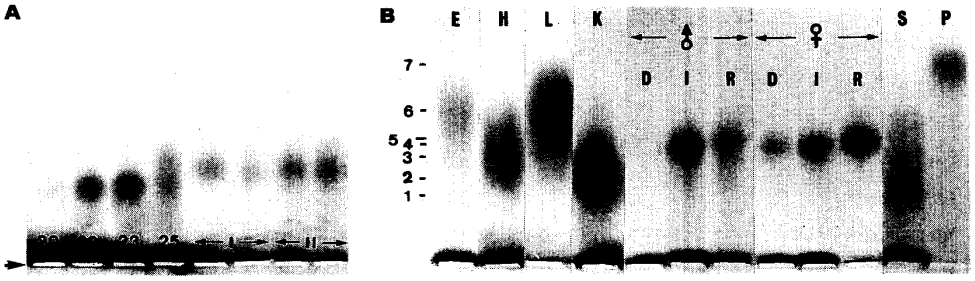

Fig. 2. Electrophoretic patterns of alkaline phosphatase from *Rana nigromaculata*.
A. Tadpoles at stages 20, 22, 23, 25, I and II.
B. Various organs and plasma of adults. E, Esophagus. H, Heart. L, Liver. K, Kidney. D, Duodenum. I, Small intestine. R, Rectum. S, Skin. P, Plasma.

II. Rana nigromaculata

1. Embryos, tadpoles and froglets

No alkaline phosphatase band appeared in embryos until stage 19. A single band with relative mobility of 0.21 or 0.22 appeared at the hatching stage (st. 20), which remained until stage 25 (Fig. 2A). However, this band disappeared at the subsequent stages.

Another band having a mobility of 0.27 or 0.28 appeared in tadpoles at stages I and II. This band was found only in the portion of visceral organs (VO) at the later stages, although it was gradually diffused and increased in density. This band disappeared at st. XXI. However, a new band with a relative mobility of 0.33 appeared at st. XXV in the portion of visceral organs. This band was surrounded by fainter areas. In the body mass (BM), no distinct band was found at all the tadpole stages, although there was a wide stain of alkaline phosphatase at st. X to XXV.

2. Adult frogs

Electrophoresis of alkaline phosphatase was performed in 18 kinds of organs and plasma of adult frogs. However, electrophoretic patterns appeared in eight kinds of organs, the esophagus, heart, liver, kidney, duodenum, small intestine, rectum and skin, and plasma, while no patterns were recognized in the other 10 kinds of organs. In the electrophoretic patterns of the former eight kinds of organs and plasma, seven bands could be discriminated from one another. They were designated as band 1 to band 7 according to increasing electrophoretic mobility (Fig. 2B). The alkaline phosphatase patterns of the esophagus and liver revealed band 6 of 0.38 in relative mobility (Fig. 2B; Table 2). The electrophoretic pattern of the heart revealed band 3 of 0.30 in relative mobility. The pattern of the kidney showed band 2 which was 0.24 in relative mobility. All these four bands were diffused and three of them except that of the esophagus were distinctly

TABLE 2

Relative mobility of the bands of alkaline phosphatases from nine various organs and blood plasma of *Rana nigromaculata*

Organ and plasma	Relative mobility						
Esophagus						0.38	
Heart			0.30				
Liver						0.38	
Kidney		0.24					
Duodenum				0.33			
Small intestine				0.33			
Rectum					0.34		
Skin	0.20						
Plasma							0.56
Band	1	2	3	4	5	6	7

dark. The duodenums of all the three females and one of the two males examined possessed band 4 which was well defined and 0.33 in relative mobility, while the remaining male had no band. The small intestines of all the females and males showed band 4 which was 0.33 in mobility. The rectums of all these females and males had band 5 which was 0.34 in mobility. The electrophoretic pattern of alkaline phosphatase in the skin showed band 1 which was 0.20 in relative mobility and was located in a widely diffused stain. The plasma revealed band 7 which was 0.56 in relative mobility.

DISCUSSION

The appearance of alkaline phosphatase in amphibians was first reported by BRACHET (1946). KRUGELIS (1950) and LØVTRUP (1955) have confirmed the existence of alkaline phosphatase in gastrulae of *Xenopus laevis* and *Ambystoma mexicanum* by means of a spectrophotometer. By the same method, O'DAY and FINNEGAN (1970) have also elucidated the presence of alkaline phosphatase in gastrulae of *Ambystoma gracile* and *Taricha torosa*. PIATKA and GIBLEY (1967) have observed a high activity of alkaline phosphatase in the pronephros of embryos at the neural tube stage by a histochemical technique in *Rana pipiens*. On the other hand, BROWN and MILLIGNTON (1968) have examined alkaline phosphatase in the intestine of *Rana temporaria* by electron microscopy and have confirmed that they appear first at the hatched tadpole stage. HAH (1974) has reported histochemically that alkaline phosphatase is first observable in the pronephros of tail-bud embryos in *Bombina orientalis*. MANWELL (1966) has observed a difference between the electrophoretic mobilities of tadpole and adult *Rana catesbeiana* livers.

In the present study, alkaline phosphatase was examined by electrophoresis in embryos, tadpoles, young frogs and various organs and plasma of adult frogs of *Rana japonica* and *Rana nigromaculata*. In *Rana japonica,* one or two bands of alkaline phosphatase were observed at the embryonic stages and also at the hatching and hatched tadpole stages. Thereafter, they were replaced by a new band having a greater mobility at stage I. This band gradually intensified in density as development proceeded, persisted until st. XVIII, and eventually disappeared at st. XXI. An additional band of slower migration appeared at st. IV, gradually increased in density and then disappeared at st. XXI. Two new bands appeared at st. XXV. In *Rana nigromaculata,* no band of alkaline phosphatase was found at the embryonic stages. A single band first appeared at the hatching and hatched tadpole stages. This band was followed by another band having a greater mobility at st. I and II, and at later stages in the portion of visceral organs. This new band gradually diffused and increased in density in later stages, and then disappeared at st. XXI. At st. XXV a new band appeared. There was only a diffuse stain of alkaline phosphatase in the body mass other than the visceral organs.

The organs of adult *Rana japonica* possessed 10 molecular forms of alkaline phosphatase. The tongue, esophagus, heart, skeletal muscle and kidney showed

one band, while the skin showed two bands. In the duodenum, small intestine and rectum, there were usually two bands, one of which showed a higher activity than the other. On the other hand, the organs and plasmas of adult *Rana nigromaculata* had seven molecular forms of alkaline phosphatase. The esophagus, heart, liver, kidney, duodenum, small intestine, rectum, skin and plasma all showed one band.

In the human, BOYER (1961) has clarified the existence of five bands in alkaline phosphatase of the plasma. In addition, he (1963) has reported that the alkaline phosphatases of the liver, bone, intestine, kidney and placenta are electrophoretically distinguished from one another. SMITH, LIGHTSTONE and PERRY (1968) have reported that five bands are discriminated from one another in the electrophoretic patterns of the liver, bone, kidney, intestine and lung as well as plasma in the human. MULIVOR, HANNIG and HARRIS (1978) performed electrophoresis on the extracts from fetal and adult intestines. According to their findings, the fetal alkaline phosphatase band differs in mobility from the adult one, and the latter appears between 28 and 32 weeks of gestation.

It is necessary to investigate the changes in alkaline phosphatase which occur in various organs during development from tadpoles to frogs in order to clarify the relationship between alkaline phosphatase and differentiation of various amphibian organs.

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