HISTOCHEMICAL STUDIES ON ALKALINE PHOSPHATASE OF THE SALAMANDER RETINA IN THE LATE EMBRYOGENESIS

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It is well established that alkaline phosphatase increases its activity when the embryo is actively initiating the tissue differentiation (Krugelis, '50, Krugelis *et al.*, '52, Yoshida, '55 b). It is very probable that the alkaline phosphatase synthesized during embryonic development must be served and accumulated for the special functions concerning the histogenesis and organogenesis.

According to the histochemical and biochemical works, alkaline phosphatase is present in the nuclei (Dounce, '50, '52) and its activity corresponds with the rate of phosphorous turnover of desoxyribonucleic acid (Brachet et Jeener, '48).

The interesting result was found by Osawa ('51), that the high activity of alkaline phosphatase obtained in the eye vesicle was decreased when the retina had become functional, and this change of activity was attributed to the utilization of this enzyme, in some way, in the process of retinal histogenesis.

In previous papers, it was shown that the activity of alkaline phosphatase was high as compared with that of acid phosphatase in the isolated nuclei from ox retina (Yoshida, '57 a), though no nuclei were stained positively by the histochemical method (Yoshida, '57 b).

From the above facts, the present study was designed to ascertain whether the positive staining of nuclei depends upon the specific natures of nuclei originated in the used tissues and organs, or not.

MATERIALS AND METHODS

Salamander (*Hynobius dunni*) was used as materials. As the standard staging in embryonic development of this salamander is not yet established, the staging described in this paper is not definite. In order to compare the histogenesis of retina with the sites of alkaline phosphatase, the following materials were chosen: 1. embryos before hatching. 2. larva on 10 days after hatching. 3. larva on 30 days after hatching. 4. adult retina and liver.

The eye ball and liver were excised from the freshly killed adult salamander, and they were immersed into a cold acetone for fixation. Embryos and larva were also fixed with a cold acetone in a refrigerator (2° C.) for 24 hours. After the fixation, materials were left for one hour at room temperature (15° C.), and embedded in paraffin at 53° C. The sections were cut as thin as 15 μ . The usual

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cytological techniques from the deparaffinization to the washing of sections were made in a refrigerator (2° C.), to prevent any loss of the enzyme.

For the demonstration and visualization of the locality indicating the presence of alkaline phosphatase in the used materials, the method modified by Osawa ('51), which was mainly based on the methods by Gomori, Takamatsu and von Kóssa, was applied to compare the results with the data presented by him.

Sodium β -glycerophosphate was used as substrate. Magnesium chloride was added as activator. Before the incubation of slides, pH of the reaction medium was adjusted to 9.4, with veronal buffer solution by means of a glass electrode pH meter. In the control medium, no substrate was added. The reaction temperature was kept at 37° C. The slides were incubated for 3, 18, 69 and 72 hours.

The conversion of calcium phosphate precipitated during incubation to metallic silver, which indicates the sites of enzyme, was made in silver nitrate solution under the exposure to light by an electric lamp (200 W) for one hour. The positive regions of alkaline phosphatase were colored brown or black.

RESULTS

(1) *The localization of alkaline phosphatase in the liver*

Fig. 1 shows the sites of enzyme in the liver cells. The moderate staining was observed in the cytoplasm and bile canaliculi. While the strong staining was found in the cell wall and the nuclei, the nuclear components, such as chromatin and nucleoli, were revealed with the highest intensity of color. Though the attempt to ascertain the adsorption of calcium phosphate to nuclei was not paid to the liver nuclei, all nuclei on sections did not always exhibited the intensity of color in the same extent. With regard to the difference of color intensity obtained in the liver nuclei, there are two main possibilities that these changes depend upon the marginal or inner position of nuclei even in the same slide, or upon the difference in the physiological state of nuclei. These results, however, are in agreement with the one obtained in the regenerating rat liver by Novikoff ('52).

FIG. 1. The localization of alkaline phosphatase in adult salamander liver. Fixed in acetone. Incubated for 3 hours. $\times 330$.

(2) *The localization of alkaline phosphatase in embryonic retina*

The retina of embryo used was not completely differentiated, in which there was observed a large number of nuclei dividing actively. The localization of alkaline phosphatase in this undifferentiated retina is shown in fig. 2 a. In order to compare the distribution of the enzyme with the one of desoxyribonucleic

FIG. 2. *a*: The localization of alkaline phosphatase in the embryonic retina. $\times 67$. *b*: The localization of desoxyribonucleic acid in the embryonic retina. $\times 80$.

acid, Feulgen nuclear reaction was applied. Fig. 2*b* gives the distribution of desoxyribonucleic acid in the retina.

The diameter of the eye was about $602\ \mu$ in the long axis, and $344\ \mu$ in the short axis. The thickness of the retina was measured at the central area as $109\ \mu$, pigment layer as $4.5\ \mu$. The diameter of the lens was about $105\ \mu$. Though the thickness of the retina is not so much differed from that of the adult, many nuclei at the front side on the pigment layer are prevailing with cell division for the formation of the receptor cells.

The whole region of retina was strongly stained, which suggested that alkaline phosphatase was extremely active. However, it was observed microscopically that the nuclear figure stained with Gomori reaction was distinctly differed from that obtained in the liver nuclei, in which chromatin and nucleoli were stained deeply, in the point that no nuclear granules were utterly stained in the retina. It may be suggested that the wide distribution of enzyme was caused by the staining of the fibrous structure of nervous elements, which scattered

FIG. 3. *a*: The localization of alkaline phosphatase in the lens nuclei. Fixed in acetone. Incubated for 18 hours. $\times 380$.

b: The localization of desoxyribonucleic acid. Fixed in Carnoy's solution. Hydrolysed in $1\ N\ HCl$ ($60^\circ C.$, 20 min.). Stained by Feulgen nuclear technique. $\times 380$.

throughout the retina and originated from the primitive nulloepithelium in the eye cup.

The nuclei of lens were Feulgen positive (fig. 3*b*). There was observed that the nuclei at the posterior region of the lens were elongating forward into fibre shaped nuclei. The mitotic figures of nuclei were found in the anterior region of the lens. The nucleoli were deeply stained like a black spot, in the nucleus being stained pale brown (fig. 3*a*).

(3) *The localization of alkaline phosphatase in the retina of embryo on 10 days after hatching*

Fig. 4*a* shows the distribution of alkaline phosphatase in the retina used. The morphological structures of the retina is shown in fig. 4*b*.

FIG. 4. *a*: The localization of alkaline phosphatase in the retina of embryo on 10 days after hatching. $\times 100$. *b*: The morphological structure of the retina on 10 days after hatching. Stained with haematoxylin and eosin. $\times 80$.

In the retina used, the inner plexiform layer between the layer including ganglion cells and the inner nuclear layer, together with the outer plexiform layer between the inner nuclear layer and the receptor cell layer, were more differentiated, and those layers were appeared as two bands. The outer segments of receptor cells seem to be growing at this embryonic stage.

The inner and outer plexiform layers, which were not stained or reacted with haematoxylin and Schiff's reagent, were positive for the test of alkaline phosphatase as shown in fig. 4*a*. On the contrary to this distribution, retinal nuclei were not stained in the short incubation. After a comparatively long incubation (18 hours), the nuclei and outer segments of receptor cells were stained yellowish brown, less positive than two plexiform layers.

(4) *The localization of alkaline phosphatase in the retina from larva on 30 days after hatching*

The more differentiated retina was used in this experiment. The essential differences in the establishment of retinal elements were not observed between the used retina and adult one.

The prolonged incubation was made to ascertain the effect of adsorption on the retinal elements.

a) Three hours incubation
(fig. 5 *a*).

The sites of alkaline phosphatase in the used retina are limited to the inner and outer plexiform layers noted in the previous experiment. The moderate staining, which is less positive than the intensity of color observed in the plexiform layers, was obtained throughout the retina.

Notwithstanding the fact that the nuclei of bone (*trabecular bar*), which protects the forebrain, were stained in the 3 hours incubation, no nuclei of retina were stained at all.

b) Eighteen hours incubation
(fig. 5 *b*).

The intensity of color indicating the activity of enzyme was more increased in the 18 hours incubation. The positive reaction was obtained in the same sites noted in the 3 hours incubation. The connection of the two plexiform layers with the optic nerves, which were similarly stained brown, was observed as shown in fig. 5 *b*. There was observed that the nuclei of retina remained unstained even in this long incubation; on the other hand, the nuclei of bone stained in the short incubation were positive with the high intensity of color.

c) Seventy-two hours incubation
(fig. 5 *c*).

The precise location indicating the activity of alkaline

FIG. 5. The localization of alkaline phosphatase in the larval retina on 30 days after hatching.

a: Three hours incubation. $\times 50$.

b: Eighteen hours incubation. $\times 73$.

c: Seventy-two hours incubation. $\times 63$.

FIG. 6. *a*: The morphological structure of adult salamander retina. Fixed in Carnoy's solution. Stained with haematoxylin and eosin. $\times 600$.

b: The localization of alkaline phosphatase in adult retina. The outer segments, inner segments (*i*) and the outer plexiform layer (*s*) were intensely positive. Fixed in acetone. Incubated for 18 hours. $\times 600$.

c and *d*: The positive regions were more intensive and extended. The inner nuclear layer was negative.

c: The inner plexiform layer and the ganglion fibre (*s*) adjacent to the ganglion cells were extremely positive. The gradual moderate staining was observed in the receptor nuclei. Incubated for 72 hours. $\times 600$.

d: The outer segments were intensely positive. Fixed in acetone. Incubated for 72 hours. $\times 600$.

phosphatase is not detectable with the result obtained here, in which the most highly intensity of color is found throughout the retina and forebrains, as the result of the prolonged incubation. In fact, the main elements, namely the inner and outer plexiform layers, colored in the short incubation as indicated above, were not distinguished owing to the strong staining of the inner nuclear layers. These results might be accounted for by the artifact due to the diffusion of enzyme and calcium phosphate from the inner and outer plexiform layers which were first stained.

(5) *The localization of alkaline phosphatase in adult retina*

The morphological structure of adult retina is shown in fig. 6a. The nuclei distributed in three layers were basophilic. Three nuclear layers consist of the ganglion nuclear layer, the inner nuclear layer including bipolar, amacrian and horizontal nuclei, and the receptor nuclear layer.

The outer segments of receptor cells, which bind acid dyes, are comparatively large and their length are about 46μ . The width of retina, the inner plexiform and outer plexiform layers are approximately 155μ , 16μ and 6.5μ respectively.

a) *Eighteen hours incubation* (fig. 6b)

The similar pattern of staining in larval retina was observed in adult one. In addition to the inner and outer plexiform layers, which were found as the main essential sites indicating the activity of enzyme in previous experiment, the moderate staining was noted in the inner and outer segments of receptor cells. However, no nuclei were positive even with prolonged incubation.

b) *Seventy-two hours incubation* (fig. 6c and d)

The increment of color intensity was obtained in the regions which were moderately stained in the 18 hours incubation. However, in addition to the increment of color intensity, the positive regions were more extended as shown in fig. 6c and d. The ellipsoid which was not so much distinct in the 18 hours incubation was stained black. As shown in fig. 6d, the inner plexiform layer as well as the nuclear part of receptor cells were stained.

The nuclei of ganglion cells were positive to some extent, though there was no such a tendency observed in the nuclei in the inner nuclear layer, which was not stained at all even in the same experimental condition under the prolonged incubation. The intensely positive staining was also limited to the ganglion fibre adjacent to the ganglion cells in the most part of the retina (fig. 6c).

DISCUSSION

As indicated in the results, the specific localization of alkaline phosphatase was obtained in salamander retina. The similar pattern of the distribution of the enzyme was observed in both adult and larva. In general, the positive staining due to the test for the demonstration of alkaline phosphatase was limited to the inner and outer plexiform layers, which connect through the optic nerves with the forebrain, and to the inner and outer segments of receptor cells. Such a distribution of alkaline phosphatase was first observed in the retina of embryos

before and after hatching, in which it seemed that the retina had practically become to be functional in view of its development of morphological structures. On the contrary to these distribution obtained in the functional retina, the wide and intense distribution of enzyme was noted throughout the retina of the used embryo. Osawa ('51) found in his histochemical work the fact that some kinds of organ (intestine, kidney and heart) showed the highest grade of intensity for the test of alkaline phosphatase, while others (cornea, lens, notochord and retina) the lowest, in the swimming frog larva.

Several organs such as kidney, liver and ovary from an adult toad frog showed the high activities of this enzyme compared with that of acid phosphatase (Yoshida, '55 a). These results may be suggested that alkaline phosphatase must be served and also accumulated for the future physiological functions in organs such as liver and kidney. Osawa ('51) reported that the activity of alkaline phosphatase obtained in the eye vesicle had a tendency to decrease its activity through the succeeding retinal histogenesis. The same phenomena was observed in this experiment. However, it seems to be dangerous to conclude that the decrease of activity of enzyme is only attributed to the decrease of the area reacted, with reference to the difference in the localities between the undifferentiated and functional retina.

The evidence that ribonucleic acid located in the receptor nuclei and the inner segments of receptor cells in several kinds of adult frogs seems to explain the role of ribonucleic acid in the functional retina (Yoshida, unpublished). According to Takata ('52), the regenerating eye contains a large amount of ribonucleic acid, which is abundant, in general, in the rapidly growing tissues. Therefore, both substances, ribonucleic acid and alkaline phosphatase, would be participated in the retinal histogenesis.

In the prolonged incubation, the artifact was occurred in some regions of both adult and larval retinae. From these figures, the precise location indicating the presence of alkaline phosphatase would not be discriminated and concluded. These artifacts might be resulted from the diffusion of enzyme and calcium phosphate, as reported in rat liver nuclei (Novikoff '52).

In the case of retina, the diffusion is not only limited to the nuclei, but also is found in the cytoplasmic structures. However, the adsorption of calcium phosphate to the nuclei was not observed in the retinal nuclei under the comparatively shorter incubation. As to the nuclei, the specific difference for the test of alkaline phosphatase was noted between the nervous tissues such as forebrains and retina, and others. Accordingly, there exist *two types of nuclei* with regard to the results obtained here.

The content of desoxyribonucleic acid of an individual nucleus in the larval retina of salamander was decreased in spite of the establishment of their histogenesis (Yoshida, '57 c). This decrease of desoxyribonucleic acid may suggest some relations between the role of desoxyribonucleic acid and the activity of alkaline phosphatase in the nuclei. According to Novikoff ('52), calcium phosphate produced during incubation is adsorbed by the cellular structures containing desoxyribonucleic acid such as the nuclei of rat liver and the bands of the *Drosophila* chromosomes. However, negative result was obtained in the case of

retinal nuclei, at least, from the present histochemical test of alkaline phosphatase. The new technique recommended by Novikoff ('55) seems to be supported by the data obtained in the isolated nuclei in non-aqueous solutions (Allfrey *et al.* '55). Nevertheless, the activity and locality of alkaline phosphatase could not be determined only by the histochemical method, because the nuclear fractions isolated from ox retinae showed an intensively high activity of alkaline phosphatase in the biochemical method (Yoshida, '57a). It seems to be inevitable that there exists a discrepancy between the histochemical and the biochemical result for the demonstration of alkaline phosphatase in the nuclei of nervous tissues.

SUMMARY

The localizations of alkaline phosphatase in the embryonic, larval and adult retinae of salamanders were determined by the histochemical method. The main results are summarized as follows:

1) The localization of alkaline phosphatase was observed throughout the retina in the embryonic stage with the intensity of color exhibiting the high activity of enzyme there.

2) The sites indicating the activity of alkaline phosphatase were found in the inner and outer plexiform layers in both larval and adult retinae. The positive sites were observed in the inner and outer segments and the ellipsoid of receptor cells. These sites are not essentially different from those obtained in mammals.

3) The intensity of color observed in the inner segments is more distinct than that in the outer segments of receptor cells.

4) Alkaline phosphatase seemed to participate in the lens formation, owing to the marked intensity in the nucleoli of lens nuclei.

5) A close relationship between alkaline phosphatase and desoxyribonucleic acid was not detected from the view of the localization of both substances in the retina.

6) Two types of nuclei were recognized between the liver and nervous tissues by the present histochemical method.

7) As to the nuclear phosphatase, the present histochemical results are not in agreement with the biochemical data previously obtained in ox retina.

8) The artifact was observed in both nuclei and cytoplasmic structures under the prolonged incubation.

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