Chapter - VII

Alkaline Phosphatase Activity in Differentiating Liver of Chick: A histochemical and quantitative study
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INTRODUCTION

Evidences have been accumulating that alkaline phosphatase rises rapidly to high levels during those embryonic periods in which differentiation is taking place, as demonstrated in amphibia by Krugelis (1950) and Lovtrup (1955). It has been shown by Lindeman (1949) for chick retina and specially by Moog (1950, 51) for chick and mouse duodenum that the phosphatase level of a given organ rapidly rises before the onset of function for that organ, and may therefore be looked upon as a biochemical differentiation prior to function. Karczmar and Berg (1951) found high alkaline phosphatase activity immediately preceding histogenesis in embryonic and regenerating Amblystoma limbs. The possible role of phosphatases in protein synthesis, specially fibrous proteins, is suggested by Bradfield (1947) and supported by Davidson (1949). The time of sharp rise of alkaline phosphatase approximately corresponds to the timing of morphological differentiation, and of most rapid synthesis of RNA, protein and phospholipids (Rogers et al., 1960). Unlike many enzymes appearing in the early part of differentiation, alkaline phosphatase shows high activity in the early period of epigenesis but it becomes decreased in the later phase of development (Flexner et al., 1956). It has also been suggested that alkaline phosphatase has been associated
with cell proliferation in the embryo (Willmer, 1942; Moog, 1943; Hamburger, 1948).

Several works have been done on the role of alkaline and acid phosphatases on the morphological differentiation of the brain and spinal cord of chick and other animals (Moog, 1944; Chiquoine, 1954; Mulnard, 1965; Mcalpine, 1959; Rossi, PeScetto and Reale, 1951,57; Yoshida, 1958; Kundu, 1972). But few works have been done on the role of alkaline phosphatase in the differentiation of organs, other than the CNS. The role of alkaline phosphatase in the differentiation of kidney and intestine has been studied by Rogers (1933). In the present work it was decided to study the alkaline phosphatase activity, both by histochemical and quantitative biochemical methods, at various ontogenic stages of liver in chick, and to seek whether there is any correlation of the phosphatase activity with the growth and differentiation of liver.

**MATERIALS AND METHODS**

The material of the experiment include livers of white Leg Horn chick embryos incubated at a constant temperature of 38°C. On and from the 6th day of incubation, embryos were taken out every alternate day up to 20th day.

For quantitative analysis, the method followed for determination of alkaline phosphatase was that of Ohmori (1937).
The entire livers were quickly dissected out, weighed and kept in a cold moist chamber to avoid desiccation. The tissues were homogenized in 1.0 ml of chilled distilled water and then transferred to the reaction mixture consisting of 1.0 ml of carbonate bicarbonate buffer (pH 10.4) and 1.0 ml of 0.2% sodium p-nitrophenol phosphate solution. Then the mixture was incubated for one hour at 37°C. After incubation the enzyme action was terminated by the addition of 1.0 ml of 20% trichloroacetic acid and kept for 12 hours to precipitate the protein followed by centrifugation at 3000 r.p.m. for 15 minutes. To 1.2 ml of the supernatant, 1.8 ml of saturated sodium carbonate solution was added. A control was run along with the experimentals with all the constituents of the reaction mixture and 1.0 ml of glass distilled water, instead of the tissue extract. The quantity of p-nitrophenol in the aliquot was measured at 420 mμ, using a Bausch and Lomb spectronic 20 spectrophotometer. Phosphatase activity is expressed as the μg of p-nitrophenol liberated in one hour/100 mg of tissue at 37°C. As sufficient amount of p-nitrophenol phosphate was present in the system, its concentration was thus not rate limiting. Each experiment was repeated at least five times and the mean value with standard error was recorded.

For histochemical detection of alkaline phosphatase Gomori's modified technique, using 80% chilled alcohol as fixative and sodium β-glycerophosphate as substrate has been used (Gomori, 1952). The tissues incubated at pH 9. The liver of the embryos at various developmental stages was
dissected out, as soon as possible, and were fixed in 80% chilled alcohol. After the routine process of embedding in paraffin, serial sections were made at 10 μ followed by the routine process for the alkaline phosphatase reactions. On and from 4th day of incubation, histochemical studies were made at every alternate day up to 20th day.

**OBSERVATIONS**

A. **Quantitative study**: Different aspects of the study, viz. percentages of p-nitrophenol liberated, and a graph showing the trend in the changes of liberation of p-nitrophenol by alkaline phosphatase, at various epigenetic stages of liver in chick, are given in Table-I and Fig.1.

**Table - I**

<table>
<thead>
<tr>
<th>Age in days</th>
<th>6 days</th>
<th>8 days</th>
<th>10 days</th>
<th>12 days</th>
<th>14 days</th>
<th>16 days</th>
<th>18 days</th>
<th>20 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>W</td>
<td>198.54</td>
<td>218.91</td>
<td>188.24</td>
<td>175.72</td>
<td>149.61</td>
<td>157.56</td>
<td>219.33</td>
<td>183</td>
</tr>
<tr>
<td></td>
<td>+5.55</td>
<td>+3.55</td>
<td>+4.60</td>
<td>+4.21</td>
<td>+1.13</td>
<td>+2.07</td>
<td>+5.5</td>
<td>+4.</td>
</tr>
<tr>
<td>D</td>
<td>1297.58</td>
<td>1107.83</td>
<td>810.33</td>
<td>729.67</td>
<td>546.22</td>
<td>559.11</td>
<td>735.37</td>
<td>530</td>
</tr>
</tbody>
</table>

* Activity expressed by the amount in μg of p-nitrophenol liberated by 100mg of tissue in one hour at 37°C at pH 10.6. 

W = Activity determined on fresh tissue weight basis. 
D = Activity determined on dry tissue weight basis.
AMOUNT OF P-NITROPHENOL LIBERATED (mg/100mg OF TISSUE)

Fig.-1

CHANGES IN THE CONCENTRATION OF ALKALINE PHOSPHATASE IN THE DIVERGENT LIVER OF CHICK, DRY TISSUE WEIGHT BASIS
From the experimental data it has been found that the activity of alkaline phosphatase remains higher during 6 to 8 days of incubation. From fresh tissue weight basis it has been observed that the enzyme activity at 8 days is higher than that of 6 days. From 8 days there is gradual decrease of the phosphatase activity up to 14 days of incubation. After 14 days the alkaline phosphatase activity again becomes higher up to 18 days, and then shows its downward trend.

A more or less similar trend in the changes of alkaline phosphatase activity can be observed if the values are compared on dry tissue weight basis, except at 8 days of incubation, where the enzyme activity is lower than 6 days of incubation.

On fresh tissue weight basis, the trend of alkaline phosphatase activity is characterized by two more or less equal peaks - one at 8 days and the other at 18 days of incubation. But on dry tissue weight basis the trend of phosphatase activity is provided with two unequal peaks - the first and highest peak remains during 6-8 days, and the second comparatively lower peak situated at about 18 days of incubation (Fig.-1). The changes in the concentrations of the enzyme alkaline phosphatase throughout the whole period of incubation are statistically significant (Table-II).
Table - II

Changes in the concentration of alkaline phosphatase in developing liver of chick and their test of significance.

<table>
<thead>
<tr>
<th>Days</th>
<th>t</th>
<th>df at</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>6(X) and 8(Y)</td>
<td>23.867</td>
<td>6 at 5% = 2.45</td>
<td>H.Sig.</td>
</tr>
<tr>
<td>8(X) and 10(Y)</td>
<td>54.99</td>
<td>6 at 5% = 2.45</td>
<td>H.Sig.</td>
</tr>
<tr>
<td>10(X) and 12(Y)</td>
<td>24.22</td>
<td>7 at 5% = 2.37</td>
<td>H.Sig.</td>
</tr>
<tr>
<td>12(X) and 14(Y)</td>
<td>69.22</td>
<td>8 at 5% = 2.31</td>
<td>H.Sig.</td>
</tr>
<tr>
<td>14(X) and 16(Y)</td>
<td>3.41</td>
<td>8 at 5% = 2.31</td>
<td>Sig.</td>
</tr>
<tr>
<td>16(X) and 18(Y)</td>
<td>30.38</td>
<td>7 at 5% = 2.37</td>
<td>H.Sig.</td>
</tr>
<tr>
<td>18(X) and 20(Y)</td>
<td>37.71</td>
<td>7 at 5% = 2.37</td>
<td>H.Sig.</td>
</tr>
</tbody>
</table>

H.Sig. = Highly significant ; Sig. = Significant

B. Histochemical study:

The changes in the intensity of alkaline phosphatase activity as evidenced from histochemical study, runs more or less parallel with the quantitative biochemical study. Here the level of alkaline phosphatase activity becomes higher from 4 to 8 days, then gradually becomes lower up to 12 days and again becomes higher up to 18 days of incubation. After 18 days the enzyme activity becomes lower.

When comparison of the phosphatase activity is made between liver parenchymal cells and perivascular connective tissue or endothelial lining of the blood sinusoids, it is observed that the enzyme reaction remains higher in the parenchymal cells during 4 to 6 days, but at 8 days of incubation
the phosphatase reaction becomes higher in perivascular connective tissue than in the liver parenchyma. At 10 days of incubation there is practically no difference in the phosphatase reaction between the above two cell types. From 12 days the phosphatase reaction again becomes higher in perivascular connective tissue up to 14 days and then gradually becomes less (but higher than parenchymal cells) up to 16 days, and at about 18 days the phosphatase reaction becomes more or less equal with the parenchymal cells. At 20 days of incubation the reaction again becomes higher in perivascular connective tissue.

The alkaline phosphatase activity becomes higher along the connective tissue fibres from 16 days and the reaction is more intensified during 18 and 20 days of incubation. It is of interest to note that higher reaction of the phosphatase activity, along connective tissue fibres, starts from the periphery and then gradually extends towards the central portion of the liver.

Comparison of the phosphatase reaction between the nuclei and the cytoplasm of the parenchymal cells shows that, during 4 to 8 days, the phosphatase activity is higher in nuclei than in the cytoplasm. At 10 days of incubation the activity shows no difference between the nuclei and the cytoplasm. From 12 days the phosphatase reaction again becomes higher in nuclei and this condition persists up to 16 days. From 18 days the reaction becomes higher in the cytoplasm than in the nuclei of the parenchymal cells.
DISCUSSION

Both from the histochemical and biochemical observations, it is quite apparent that the activity of the enzyme gradually becomes higher from 4 to 8 days, slower from 8 to 12 or 14 days, and again becomes higher up to 18 days of incubation; after which the activity shows the downward trend. Thus the general pattern of the activity of alkaline phosphatase shows two peaks during the whole embryonic period, one between 6 to 8 days and the other between 16 to 18 days of incubation.

The development of liver, from 4 to 8 days, shows the upward trends of mitotic index, glycogen, (Chapter-II and III), bile secretion, peptidase activity, elaboration of lipids etc. The cells of the liver parenchyma undergo a marked increase in size between the 4th to 6th days of incubation. Development of the connective tissue reticulum, the cells of which differentiate to form blood cells, starts at 7 days of incubation (Romanoff, 1960). All these observations lead to suggest that growth and differentiation of liver are rapid during this period. The activity of alkaline phosphatase also shows its upward trend at this time.

From 8 to 12 days, the rate of cell proliferation falls from 3.22 to 1.67 (Chapter II) and also there is fall of glycogen. The disappearance of the hematopoietic connective tissue reticulum occurs at about 9th day. The activity of the alkaline phosphatase shows its downward trend in this period.
After 12 days there is a slight increase of mitotic index, increase in the parenchymal cell size and vigorous development of hematopoietic connective tissue at about 14th day. The differentiation of the mesenchymal portion of the biliary ducts is found in its initial stages in chick embryos of 12 to 15 days of incubation. After 14th day mitotic index and hematopoiesis become lower, but the elaboration of biochemical compounds such as carbohydrates, lipids etc. becomes increased. Activity of alkaline phosphatase also runs parallel with the synthesis and accumulation of other chemicals of the liver. This later increase of alkaline phosphatase from 14 to 18 days has some relationship to differentiating processes in preparation for function.

Hence from the above discussion, a particular attention should be given to the argument that the first phase of rapid rises in levels of alkaline phosphatase, between 4 to 8 days, is associated with higher rate of growth and differentiation of liver in this period, and the later phase of the height of the enzyme activity between 14 to 18 days may be associated with the differentiation at later epigenetic periods, is related with the differentiation processes in preparation for function of the liver. This finding supports the Roger's hypothesis that the alkaline phosphatase plays a role in differentiation, not only of central nervous system, but also in the differentiation of organs other than the central nervous system, such as, in our study the liver.
Half (1914) suggested that there are two distinct embryonic periods during which blood is formed in the liver of the chick. The first begins on the 7th day of incubation and extends up to 9th day, and the second period begins on the 11th day, the hematopoietic rate becomes highest on 14th or 15th day, and then the process gradually ceases before the end of incubation. Hematopoietic activity is exhibited by a loomeshed connective tissue reticulum that develops from the endothelial and peritoneal cells during this period. From the present histochemical study it has been observed that the alkaline phosphatase activity becomes higher, even than the parenchymal cells, during the development of hematopoietic perivascular connective tissue reticulum, and thus leading to the suggestion that alkaline phosphatase has some role in the formation of blood. The higher phosphatase reaction in perivascular connective tissue at 20th day might be due to the beginning of the development of hematopoietic tissue for immediate post embryonic hematopoiesis. The present observation also suggests that alkaline phosphatase participates in the development of connective tissue fibres, which starts at the periphery of the liver at about 16th day and become intensified during 18th and 20th day of incubation.

Histochemical observations shows higher reaction of the phosphatase activity in the nuclei than in the cytoplasm of parenchymal cells during early stages of development. But in later stages, i.e. at about 18 and 20 days of incubation, the phosphatase reaction is higher in the cytoplasm. Although it
has been suggested that the nuclear staining observed with the Gomori technique is mainly due to artifact or due to diffusion of enzyme into the nuclei, after death, from sites in which it is normally present, but convincing evidences are also present regarding the presence of definite nuclear phosphatase (Pearse, 1968). For this reason it would be unwise to consider definitely the observed phosphatase reaction in the nuclei as true nuclear phosphatases. But the possibility of higher metabolic activity leading to the increased phosphatase reaction in nuclei of early stages of liver development can not be ruled out. Similarly higher cytoplasmic phosphatase reaction, at later stages of incubation, might be due to higher metabolic activity in the cytoplasm.

**SUMMARY**

1. Activity of alkaline phosphatase at different ontogenic stages of liver in chick has been studied both by histochemical and quantitative biochemical methods.

2. It has been observed that the activity of the enzyme gradually becomes higher from 4 to 8 days, lower from 8 to 12 or 14 days and then becomes higher up to 18 days of incubation; after which it shows the downward trend. Thus the general trend of the activity of alkaline phosphatase shows two peaks during the whole period of incubation, one between 6 to 8 days and the other between 16 to 18 days.
3. From 4 to 8 days of incubation, the growth and differentiation of liver is high. During this phase the enzyme activity is more. Growth and differentiation of liver is slow from 8 to 12 days; alkaline phosphatase activity is less in this period. After 12 days the liver undergoes secondary differentiation and is dominated by its functional development; the enzyme activity is high in this period.

4. High activity of alkaline phosphatase has also been observed during the development of hematopoietic perivascular connective tissue reticulum, and during the formation of connective tissue fibres in the liver.

5. It has also been observed that, during early stages of liver development, the phosphatase reaction is higher in nuclei, but at later stages the enzyme reaction is higher in the cytoplasm of the liver cells. This might be due to higher metabolic activity in the nuclei during early stages and in the cytoplasm during later stages of development of liver.

REFERENCES


