Chapter-VIII

Histochemical and Biochemical Study on Acid Phosphatase Activity in Differentiating Liver of Chick
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INTRODUCTION

The development of liver, like many other organs, is accompanied by several anabolic events, such as cell proliferation, development of various tissues and synthesis of numerous chemical compounds, viz., proteins, carbohydrates, lipids etc. but the catabolic processes such as degeneration of cell and cellular constituents and break down of biochemical compounds are also important in the differentiation of liver. It is generally regarded that these anabolic and catabolic processes are coordinated and integrated with great precision during morphogenesis of an organ. The lysosomal hydrolytic enzymes, which predominantly consist of acid phosphatases, play a great role in the catabolic processes of cells and tissues.

The role of acid phosphatase in the development of central nervous system has been studied by several workers, such as, according to Mulnard (1955), acid phosphatase activity appears very early in the faetal nervous system in rat and also in that of human embryo specially in relation to the ependymal cells, in the migratory neuroblasts and later in the nerve cells (McKay et al., 1955). In guineapig a constant high level of acid phosphatase activity is seen in the brain and in the neuroblasts at all stages of development (Flexner and Flexner, 1948). In twenty four hours chick embryo, high acid phosphatase
activity is observed, but it becomes decreased later (Moog, 1943, 1944). Bertolini and Pons (1961) have described the histochemical distribution of acid phosphatase in late, as well as early, stages of development of the chick brain. They consider that the results in the late developmental period for this enzyme support the view that phosphatase is concerned with differentiation processes at this time.

The relation of acid phosphatase in the development and regression of tail in anurans has been studied by Robinson (1970). He observed that increases in acid phosphatase activity correspond in time to periods of active tissue regression. He also stated the presence of two distinct forms of acid phosphatase during both development and metamorphosis of anuran amphibia. The amount of one form relative to the other remains essentially constant during late development and early metamorphosis. But in the late regressing tail this ratio is significantly changed and characterized by the increase in the amount of one form with some concurrent decrease in the other.

Donitova and Rokhlenko (1966) demonstrated the presence of acid phosphatase in the lysosomes of embryonic chick liver cells, i.e. in the same cellular organelles which are found in the liver of mature animals and in many other tissues. The role of lysosomal enzymes, which mainly consist of acid phosphatases, in cytolysis and autolysis has been studied by various workers (Novikoff, 1963; Duve, Ch. De and Wattiaux, 1966).
Often the view has also been advocated that acid phosphatase activity is associated with cell death during morphogenesis (Glucksmann, 1957; Saunders, 1966). Thus the evidences are in favour of the view that the lysosomal acid phosphatase participates in cell degeneration, regression and catabolic processes during embryonic development of an organ. So, during development the intensity of acid phosphatase activity in an organ at a given time, may roughly be taken as the index of cellular degeneration and catabolic processes at that particular time; although in many cases these tissue degeneration and catabolic processes might be prerequisites for synthesis of new tissues and biochemical compounds.

With a view to the facts stated above an examination has been made both by histochemical and quantitative biochemical methods, of the activity of acid phosphatase at various developmental stages of chick liver, with the aim of getting some information about the time and intensity of cellular degeneration and catabolic processes, and its apparent relation with the growth and differentiation of liver.

**MATERIALS AND METHODS**

Livers of White Leghorn chick embryos were the materials used in this study. Freshly laid eggs were incubated at a constant temperature of 38°C, with 75% relative humidity in the incubator.
On and from 6th day of incubation, livers were taken out of the embryos at every alternate days up to 20th day. For quantitative study, on each specific day, the entire livers were quickly dissected out and kept in a cold moist chamber to prevent desiccation. The tissues were then weighed and the acid phosphatase activity was determined according to the method of Ohmori (1937). Each tissue sample was homogenised with 1.0 ml of chilled glass distilled water and transferred to a clean dry test tube. To this tube the reaction mixture consisting of 1.0 ml of acetate buffer of pH 4.8 (prepared by adding 80.0 ml of 0.1(N) acetic acid to 120 ml of 0.1(N) sodium acetate), and 1.0 ml of 0.2% sodium p-nitrophenol phosphate was added. The enzymatic action was continued for one hour at a constant temperature of 37°C. After the stipulated period the reaction was stopped by the addition of 1.0 ml of 20% trichloroacetic acid into each tube. The tubes were allowed to stand for 12 hours for complete precipitation of the protein, centrifuged at 3000 r.p.m. for 15 minutes and the supernatant was collected. To 1.2 ml of this supernatant 1.8 ml of saturated solution of sodium carbonate was added. A control was simultaneously run which included all the constituents of the reaction mixture and 1.0 ml of glass distilled water instead of the tissue extract. The quantity of liberated p-nitrophenol in the aliquot was measured at a wave length of 420 mµ in a Bausch and Lomb Spectronic-20 Spectrophotometer. Phosphatase activity is expressed as the mg of p-nitrophenol liberated in one hour.
by 100 mg of tissue at 37°C at pH 4.8. Each experiment was
repeated at least four times and the mean value with standard
error was recorded.

For histochemical study of acid phosphatase Gomori's
technique (Gomori, 1952), using chilled formo-aceto-alchol
as fixative and Sodium /3-glycerophosphate as substrate has
been used. The livers of the embryos of various developmental
stages were dissected out, as soon as possible, and were fixed
in formo-aceto-alchol. Then the tissues were dehydrated,embe­
ded in paraffin and serial sections were made at 10μ. Acid
phosphatase reaction was made at pH 5. On and from 4th day of
incubation, histochemical study was made at every alternate
days up to 20th day.

OBSERVATIONS

A. Quantitative study:

The acid phosphatase activity expressed in milligram of
p-nitrophenol liberated in one hour by 100 mg of tissue cal­
culated both on dry and on fresh tissue weight basis has been
shown in Table - I. It may be seen that the activity of acid
phosphatase does not remain constant, but undergoes changes at
different phases of epigenesis. From the table - I and Fig.-1,
it has been found that the activity of the enzyme \( \frac{h}{x} \)
becomes higher and lower throughout the whole period of incuba­
tion, such as it shows comparatively lower activities during
6,10,14 and 18 days, and higher activities during 8,12,16 and
20 days of incubation.
Table - I

*Changes in the activity of acid phosphatase in differentiating liver of chick.

<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>0.821</td>
<td>1.140</td>
<td>0.825</td>
<td>0.922</td>
<td>0.400</td>
<td>0.812</td>
<td>0.656</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>±0.092</td>
<td>±0.087</td>
<td>±0.015</td>
<td>±0.012</td>
<td>±0.085</td>
<td>±0.005</td>
<td>±0.005</td>
<td>±0.0</td>
</tr>
<tr>
<td>(D)</td>
<td>5.368</td>
<td>5.770</td>
<td>3.550</td>
<td>3.742</td>
<td>1.459</td>
<td>2.882</td>
<td>2.195</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>±0.037</td>
<td>±0.078</td>
<td>±0.030</td>
<td>±0.010</td>
<td>±0.017</td>
<td>±0.002</td>
<td>±0.002</td>
<td>±0.0</td>
</tr>
</tbody>
</table>

* Activity expressed by the amount in milligram of p-nitrophenol liberated by 100 mg of tissue in one hour at 37°C at pH 4.8.

(W) = Activity determined on fresh tissue weight basis.

(D) = Activity determined on dry tissue weight basis.

Again the quantitative data shows the higher level of acid phosphatase activity from 6 to 12 days with the highest activity at 8 days of incubation, when the amount of p-nitrophenol liberated on fresh tissue weight basis is 1.140. This period is followed by a marked drop in the activity at 14 days, when the enzyme shows its lowest activity. After 14 days the phosphatase activity again becomes increased at 16 days and 20 days with a slight drop at 18 days of incubation.

When the activity of the enzyme is considered on dry tissue weight basis it has been found that the trend of activity is more or less similar with the fresh tissue weight basis.
CHANGES OF THE CONCENTRATION OF THE ENZYME ACID PHOSPHATASE IN THE DIFFERENTIATING LIVER OF CHICK.

Fig. - 1
But on dry tissue weight basis the peaks of the activity at early stages of development are comparatively higher than the peaks at later stages of development. The changes in the activity of enzymes are statistically significant throughout the whole incubation period (Table-II).

<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>8.206</td>
<td>8 at 5% = 2.31</td>
<td>Sig.</td>
</tr>
<tr>
<td>8(X) and 10(Y)</td>
<td>4.625</td>
<td>8 at 5% = 2.31</td>
<td>Sig.</td>
</tr>
<tr>
<td>10(X) and 12(Y)</td>
<td>10.66</td>
<td>8 at 5% = 2.31</td>
<td>H.Sig.</td>
</tr>
<tr>
<td>12(X) and 14(Y)</td>
<td>223.3</td>
<td>7 at 5% = 2.37</td>
<td>H.Sig.</td>
</tr>
<tr>
<td>14(X) and 16(Y)</td>
<td>142.3</td>
<td>8 at 5% = 2.31</td>
<td>H.Sig.</td>
</tr>
<tr>
<td>16(X) and 18(Y)</td>
<td>687.0</td>
<td>6 at 5% = 2.45</td>
<td>H.Sig.</td>
</tr>
<tr>
<td>18(X) and 20(Y)</td>
<td>221.0</td>
<td>8 at 5% = 2.31</td>
<td>H.Sig.</td>
</tr>
</tbody>
</table>

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

B. Histochemical study:

As regards the trend of acid phosphatase activity the results of histochemical study are more or less similar with the results of quantitative biochemical study. Here also the phosphatase activity remained higher during early periods of development, particularly between 4 and 8 days of incubation. This period is followed by a fall of the enzyme activity at 14 days. After 14 days the phosphatase activity again becomes higher.
Comparative study of the phosphatase activity between liver parenchyma and connective tissue shows no difference during 4 to 6 days of incubation. But at 8 days of incubation the phosphatase reaction is higher in perivascular and intertubular connective tissue and lower in parenchymal cells. This difference is very marked at 12 days of incubation. Although this condition of higher phosphatase activity in connective tissue and lower activity in parenchymal cells persists up to 20 days of incubation, but after 14 days the phosphatase activity becomes slightly higher in parenchymal cells. At about 20 days of incubation high level of acid phosphatase activity are found at the periphery of the liver.

It is of interest to note that the acid phosphatase activity is high in the nuclei of early developing red blood corpuscles, but in advanced stage of R.B.C. the phosphatase activity is very high in the cytoplasm, and it is practically absent in the nuclei.

DISCUSSION

From the quantitative study it may be observed that the trend of phosphatase activity follows a rhythm of higher and lower activities; but comparatively higher activities are found in early epigenetic periods extending up to 8th day, followed by a marked fall at 14th day of incubation and again becomes higher at later periods of development.
From the histochemical study it may be observed that in differentiating chick liver, there occurs two patterns in the activity of the acid phosphatase:

(a) In the parenchymal cells the phosphatase activity is high during 4 to 6 days and then becomes lower up to 14 days and again becomes higher in the later phases of prenatal development.

(b) In the connective tissue of liver, high activity of acid phosphatase persists up to 12 days of incubation and then the activity slightly becomes less during later periods of development.

The first phase of liver development, which starts from the liver primordium and extends up to 8th or 9th day of incubation, shows higher rates of growth and differentiation. During early period of this phase the developing liver tissues are in a state of vigorous reorganization. Endodermal outgrowths are proliferated from the liver plates on all sides of the ductus venosus and radially into the surrounding connective tissue. The proliferation of liver trabeculae is accompanied by the sprouting of capillaries from the ductus venosus, which interdigitate with the endodermal proliferations in a more complex fashion. Soon trabeculae of tissue derived from both cranial and caudal liver plates fuse with one another and form anastomoses. In chick, the first anastomoses may occur
before the end of the 3rd day or early in the 4th day (Romanoff, 1960). Besides this, the developing liver exhibits the increase of mitotic index from 4 to 8 days (Chapter-II), increase of cell size of liver parenchyma, beginning of secretion of bile, storing of glycogen and the appearance of lipid granules in this phase of development (Romanoff, 1960). High levels of acid phosphatase activity are also found in this period.

In the second phase of liver development, which comprises between 8th to 14th day, the growth is comparatively lower as evidenced by the fall of growth rate at 11th, fall of mitotic index at 12th day and decrease of glycogen from 9th to 12th day of incubation. Activity of the enzyme is comparatively lower in this period and lowest at 14th day of incubation.

From 14 to 20 days, which constitutes the third phase of liver development, the acid phosphatase activity again shows higher levels. The later increase of phosphatase activity may be associated with the differentiation of liver as well as with the specific functions of the cell types in which they are localised. The observations and discussion stated above tempt to suggest that there is some sort of relation of acid phosphatase activity with the growth and differentiation of liver. Although the probable association of acid phosphatase activities with the process of differentiation has been suggested by various authors (Moog, 1943; Flexner, 1948; Bertolini
and Pons, 1961; Kundu, 1972), but still the exact role played by this enzyme in events of morphogenesis and differentiation is obscure (Torrey, 1965). Forceful arguments have been offered regarding the association of the acid phosphatase with the intracellular digestion and tissue degeneration during morphogenesis (Glucksmann, 1957; Saunders, 1966; Robinson, 1970). Since acid phosphatase are present in the lysosomes of embryonic chick liver cells (Donitova and Rokhlenko, 1966) and since all major classes of cell constituents are shown to be hydrolizable by lysosomal enzymes, it may be expected that the lysosomal acid phosphatase is associated with the cell death and catabolic processes during chick liver morphogenesis.

During 4 to 6 days, the developing liver tissues are in a state of vigorous reorganization and large amount of tissue degeneration occurs in this period, both in the proliferative endodermal cords and in the connective tissues. At about 8th day of incubation, the liver appears as a compact organ and phagocytic kupffer's cells are definitely found in the endothelial lining of the sinusoids (Romanoff, 1960). Histochemical study shows a higher acid phosphatase activity during 4 to 6 days in the endodermal cords as well as in the connective tissues, and at 8 days onwards the enzyme activity is definitely higher in the connective tissues and endothelial linings. The high concentration of acid phosphatase in the cytoplasm of the advanced developing R.B.C. may be due to the degeneration of cellular organelles, which are practically absent in matured R.B.C., by the lysosomal enzymes. These findings further support
the view that the lysosomal acid phosphatase participates in intracellular digestion and tissue degeneration during organogenesis of liver. Again the rhythm of higher and lower activities of acid phosphatase may be due to the rhythmical pattern of degeneration of cell and cellular constituents during morphogenesis of liver. Rhythmical pattern of the acid phosphatase activities has also been observed during differentiation of C.N.S. in chick by Kundu (1972).

**SUMMARY**

1. Acid phosphatase activity 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 acid phosphatase does not remain constant, but undergoes changes at different phases of epigenesis. The activity rhythmically becomes higher and lower throughout the whole period of epigenesis. Comparatively higher activities have been found up to 8 days of incubation, and then the activities become lower up to 14 days and again increased at later periods of development.

3. Higher acid phosphatase activity has been observed in the perivascular connective tissue than the parenchymal cells from the 8th to the 20th day of incubation.
4. The acid phosphatase activity is high in the nuclei of early developing red blood corpuscles, but in advanced stage of R.B.C. the phosphatase activity is very high in the cytoplasm, and it is practically absent in the nuclei.

5. The activity of acid phosphatase may have some relation with the cell death and catabolic processes during chick liver morphogenesis. The relation of the phosphatase activity with the growth and differentiation of the liver has been discussed.

REFERENCES


