Chapter - III

Histochemical and Quantitative Study of Carbohydrates in the Differentiating Liver of Chick
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INTRODUCTION

It is well known that the storage and breakdown of glycogen is one of the most important functions of liver. Glycogen occurs in greatest concentration in the liver and helps to maintain the blood sugar level. It subserves a function both of carbohydrate storage and of energy source for this gland (Dukes, 1955). Thus the liver acts as a glucostatic mechanism by storing carbohydrates, when they are in excess in the body, and by supplying carbohydrates from its reserve when they are deficient in the body. As carbohydrate metabolism is of utmost importance for morphogenesis (Spratt, 1955,58) it is reasonable to expect that carbohydrates are also essential for the liver's own development. According to Dalton (1937) glycogen first becomes evident in the hepatic cells of 7 day embryos, and increase in the utilization of carbohydrates as an energy source occurs during the period from 10th to 13th day of incubation, Conklin (1964) stated that prominent glycogen inclusions first appeared on the 12th day of development, becomes most prominent by the 18th day and declined slightly after hatching. Rinaudo (1961) noticed that parallel to the increase of fructose-1-6-diphosphatase activity, there is also an increase of the contents of the hepatic glycogen in different developmental stages starting from the 12th day. He suggested the possibility
that in liver of chick embryo, the glucose and glycogen synthesis proceeds from compounds with 3 atoms of carbon, in a manner similar to a reversal of anaerobic glycolysis. From the study of enzymes obligatory for glyconeogenesis Ballard and Oliver (1963) suggested the development of glyconeogenetic ability of liver as early as the 11th day of incubation, and this ability reached maximum on 16th and 17th day. Their study support the contention that during the period of active glycogen formation 3 carbon compounds are more important substrates than glucose. Guha and Wegmann (1961) found that up to the 12th day, chick embryo liver contains phosphorylase only in one form i.e. in active form. It is only from the 12th or 13th day that phosphorylase appears in two forms (with inactive form predominating) and the enzyme systems which maintain the equilibrium between the two forms (inactive and active) also appear at this period. It is known that phosphorylase plays a prominent role in the anabolism and catabolism of glycogen. Thus, so far as the carbohydrate metabolism is concerned the differentiation of liver reached a new phase from the 12th day of incubation.

In the present investigation it was decided to study quantitatively the contents of carbohydrates, and also histo-chemically the localization and accumulation of glycogen at different stages of the ontogeny of liver in chick. The study may reveal if there is any correlation between the amount of carbohydrates present at a particular stage of development and normal processes of growth, differentiation and histogenesis of liver at that particular time.
MATeRIALS AND METHODS

The experiment was performed on pure bred white Leghorn eggs, obtained from the Govt. Poultry Farm, Burdwan, incubated at a constant temperature of 38°C with 75% relative humidity in the incubator on an average.

A. Histochemical method:

For histochemical study of glycogen the livers of the embryos, starting from 4 days of incubation and successively with one day’s interval, were dissected out and fixed in alcoholic Bouin’s fixative. After the routine process of embedding in paraffin, serial sections were made at 10 μ. Cytochemically demonstrable glycogen were studied by PAS technique (Glegg et al., 1952). The histological sections were oxidised in periodic acid solution for ten minutes and stained in Schiff’s reagent for thirty minutes at room temperature. Then the sections were washed in sulphite wash water, dehydrated and mounted in Canada balsam. At least five embryos were considered for each set of observation.

Few slides of each stage were treated with 2% diastase solution for one hour at 37°C and then stained with Schiff’s reagent. Very faint reaction indicates that the stained materials, in the untreated sections, were mainly glycogen. Some slides of each stage of incubation were also stained with Mason’s Trichrome to study the rate of deposition of collagen during the whole incubation period.
B. Quantitative method:

The quantitative estimation of carbohydrates was done by following the anthrone method (9-oxyanthracene) of Umbreit et al. (1964). The livers of the embryos, starting from 6 days of incubation and successively with one day's interval, were dissected out separately and weighed carefully. The weighed tissues were then homogenized with a measured quantity of glass distilled water and then centrifuged for 10 minutes at 2000 RPM. 1 ml of the clear supernatant was transferred in a tube containing 2 ml glass distilled water and then 6 ml of the color reagent (0.4 gm of anthrone was dissolved in 200 ml of 9.3 M sulphuric acid) were added slowly along the wall of the tube and mixed well. Then the mixture is heated in boiling water bath for three minutes and then quickly transferred to cold water. The intensity of the color developed in the solution was determined at 620 m\mu in a Bosch and Lomb Spectronic - 20 spectrophotometer. The readings were compared with the standard curve drawn with pure glucose in an identical way and the quantity of the entire tissue was thus calculated. Each observation was repeated at least four times.

OBSERVATION

A. Quantitative study:

The amount of free carbohydrate at various stages of development of chick liver has been shown in Table - I.
Table - I

Amount of carbohydrates (mg/100 mg of tissue) in differentiating liver of chick with standard error

<table>
<thead>
<tr>
<th></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>Wet tissue</td>
<td>0.4529</td>
<td>1.5934</td>
<td>1.8636</td>
<td>1.5677</td>
<td>0.8662</td>
<td>3.3226</td>
<td>12.4698</td>
<td>4.3331</td>
</tr>
<tr>
<td>Weight basis</td>
<td>±0.0579</td>
<td>±0.0767</td>
<td>±0.0693</td>
<td>±0.0093</td>
<td>±0.1115</td>
<td>±0.0930</td>
<td>±0.0370</td>
<td>±0.0640</td>
</tr>
<tr>
<td>Dry tissue</td>
<td>3.01</td>
<td>8.06</td>
<td>8.02</td>
<td>6.38</td>
<td>3.16</td>
<td>11.79</td>
<td>41.70</td>
<td>12.15</td>
</tr>
<tr>
<td>Weight basis</td>
<td>±0.269</td>
<td>±0.212</td>
<td>±0.304</td>
<td>±0.027</td>
<td>±0.040</td>
<td>±0.330</td>
<td>±0.087</td>
<td>±0.930</td>
</tr>
</tbody>
</table>
It may be observed from the results that the amount of free carbohydrates increased up to 10 days and then gradually declined up to 14 days of incubation. From 14 days there is a sharp and marked increase of the carbohydrate content up to 18 days, when the value reached maximum i.e. 12.46 mg/100 mg of tissue. From 18 to 20 days of incubation, the carbohydrate quantity falls from 12.46% to 4.33% (Table - I). Thus the amount of carbohydrates in chick liver increased at two periods during embryonic development, one between 6 to 10 days and another between 14 to 18 days of incubation. The increase in the amount of carbohydrates, between 6 to 10 days, remains comparatively less than that of the period ranging between 14 to 18 days of incubation.

More or less same trends in the concentration of carbohydrates were observed when the values were compared on dry tissue weight basis, except between 8 to 10 days of incubation. When the amount remains more or less constant.

Table - II
Changes in the concentration of carbohydrates in the liver of chick at various phases of development and the 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>13.9502</td>
<td>7 at 5% = 2.37</td>
<td>H.Sig.</td>
</tr>
<tr>
<td>8(X) and 10(Y)</td>
<td>0.0997</td>
<td>8 at 5% = 2.31</td>
<td>Insig.</td>
</tr>
<tr>
<td>10(X) and 12(Y)</td>
<td>2.014</td>
<td>7 at 5% = 2.37</td>
<td>Insig.</td>
</tr>
<tr>
<td>12(X) and 14(Y)</td>
<td>67.083</td>
<td>7 at 5% = 2.37</td>
<td>H. Sig.</td>
</tr>
<tr>
<td>14(X) and 16(Y)</td>
<td>25.993</td>
<td>8 at 5% = 2.31</td>
<td>H. Sig.</td>
</tr>
<tr>
<td>16(X) and 18(Y)</td>
<td>87.712</td>
<td>8 at 5% = 2.31</td>
<td>H. Sig.</td>
</tr>
<tr>
<td>18(X) and 20(Y)</td>
<td>31.638</td>
<td>8 at 5% = 2.31</td>
<td>H. Sig.</td>
</tr>
</tbody>
</table>

Insig. = Insignificant ; H.Sig. = Highly significant
B. Histochemical study:

Histochemical study was made starting from the 4th day of incubation, and then on every alternate days up to 20 days of incubation.

Concentration of glycogen in 4 to 8 day embryos:

Localization of glycogen is not demonstrated in the cells of the 4th day liver. On the 6th day, glycogen begins to appear and it increases up to the 8th day. In each stage, the localization is prominent around the blood vessels or in the perivascular connective tissue, than in other regions of the sections. High content of glycogen is found in the nuclei of developing blood cells on the 8th day.

Concentration of glycogen in 8 to 12 day embryos:

On the 10th day, the concentration of glycogen is slightly lower than on the 8th day, both in the parenchymal and nonparenchymal cells of the liver. But here also the localization is higher in the nonparenchymal cells, which occupy mainly around the blood vessels. The condition on the 12th day is similar but the concentration is lower than in the 10 day liver.

Concentration of glycogen in 12 to 18 day embryos:

On the 14th day, the glycogen concentration is higher than on the 12th day, both in the parenchymal and connective tissues, more so in the perivascular connective tissue. An appreciable amount of glycogen is also found in the developing blood cells.
The concentration of glycogen in the liver on the 16th to 18th days is highest in the parenchymal and nonparenchymal cells. During this period there is practically no difference in concentration between the parenchymal and the perivascular connective tissues. From the Mason's trichrome stained sections it has been observed that the deposition of collagen is higher during 16 to 18 day period in the nonparenchymal zone of the liver.

Concentration of glycogen after 18th day:

This period is characterized by decrease in the localization of glycogen specially on the 20th day, and increase in its concentration in the perivascular connective tissue.

DISCUSSION

The changes in the carbohydrate level in the chick liver at various epigenetic phases may have some correlation with the growth and differentiation of that organ. As some amount of carbohydrate is essential for the development (Spratt, 1955, 1958), and as carbohydrate metabolism is one of the most important functions of liver, it is expected that the level of carbohydrates in the developing liver at a particular stage of differentiation, may be influenced by these two factors. During the first phase of liver development, i.e. between 4th to 8th day of incubation, the growth and differentiation are higher, as evidenced by the increase of mitotic index (Chapter - II) by the beginning of the synthesis of lipids, secretion of bile etc. (Romanoff, 1960).
Concentration of glycogen and carbohydrate contents also become higher in this period, particularly on 8th and 10th day. Extensive hematopoiesis occurs in the perivascular connective tissue on the 8th day; histochemical study shows that in this region the glycogen is higher than in other regions of the liver.

Between the 8th and 12th day, growth of liver is slower, when there is a decrease in the mitotic index, hematopoiesis and also in carbohydrate concentration. On the 14th day there is intensive hematopoiesis in the liver and the mitotic index is slightly increased. Although the quantitative method shows the lower level of carbohydrates on the 14th day than on the 12th day, but histochemical study clearly indicates higher glycogen reaction than on the 12th day, particularly in the perivascular connective tissue and developing blood cells.

From 14th to 18th day, carbohydrate contents and glycogen concentration rapidly increase and they become highest on the 18th day, as evidenced both from cytochemical and biochemical methods. It has been found that the collagen, a specific cell protein of connective tissue, undergoes a significant increase in the developing liver on the 18th day (Herrmann and Barry, 1955). The highest quantity of carbohydrates between 16th and 18th days may be accounted for by the extensive development of collagen and the efficient storing capacity of the parenchymal cells of liver on this period.
Fig. 1.
Thus up to the 14th day, the concentration of carbohydrates follows a typical growth curve. The mitotic index and hematopoiesis are higher on the 8th day, lower on the 12th day and again becomes higher on the 14th day (Chapter - II; Romanoff, 1960). In each of these stages, the carbohydrate concentration follows the same course. But after 14th day, it does not follow the growth curve, and the relation between the carbohydrate concentration and mitotic index and hematopoiesis is reversed, that is, there is an increase in carbohydrates and decrease in mitosis and hematopoiesis. The growth and differentiation of the liver nearly reach completion by the 16th day, after which the functional activity of the liver is increased and the cells are very efficient to store carbohydrates, lipids, etc; so it may be concluded that the synthesis and accumulation of carbohydrates up to the 14th day, is mainly associated with the growth and differentiation of the liver cell themselves, and that the synthesis and storage of carbohydrates after 14th day, is principally associated with the functional activity of the liver and the development of collagen during this period.

From the studies of several investigators (Dalton, 1937; Rinaudo, 1961; Guha and Wegmann, 1961; Ballard and Oliver, 1963; Conklin, 1964) it is quite apparent that, so far as the carbohydrate metabolism is concerned, the development of chick liver reached a new phase from the 12th day of incubation. The present findings also support the above view, as the synthesis and accumulation of free carbohydrates and glycogen rapidly increased to the highest level after 12th day of incubation.
After 18th day there is a sudden fall of carbohydrate at about 20th day (Table - I and Fig.-1). In birds the physical activities and movements of the embryo become high immediately before hatching, and thereby resulting higher metabolic activities. As yolk contents become less in this time, the stored carbohydrates in the liver might be the source of energy for the higher metabolism. Moreover, the liver in bird is a hematopoietic organ during the embryonic and immediate post embryonic periods (Farner, 1960). Though opinions differ regarding the relation of the rate of cell division and glycolysis, it has been found that the later is increased in the cell before their division and decreased when the cells are in dividing stages (Brachet, 1947; Erickson, 1947; Stern and Kirk, 1948).

The fall of carbohydrates on the 20th day might be due to enhanced glycolysis, which may be correlated with the higher metabolic activities prior to hatching and to the immediate post embryonic hematopoiesis. The higher concentration of carbohydrates on the 8th and 14th days, when the mitotic rate and hematopoiesis are higher may be correlated with the lower rate of glycolysis in these periods.

SUMMARY

Concentration of glycogen and contents of free carbohydrates, at different stages of ontogeny of liver in chick, have been made by cytochemical and quantitative biochemical methods respectively. It has been found that concentration is gradually increased from 6th to 8th day of incubation and it decreases
up to 12th day and again rises from 14th day to reach its maximum between 16th and 18th days. Subsequently, concentration falls on about 20th day. Localization of glycogen is definitely higher in perivascular connective tissue throughout the period of development of liver, except between 16th and 18th days, when it is more or less equal in both parenchymal and nonparenchymal perivascular connective tissues. Up to the 14th day, concentration of glycogen and free carbohydrates run more or less parallel with mitotic index and hematopoiesis, after which their relation is just reverse i.e. cell division is decreased but glycogen concentration is increased considering the synthesis and accumulation of carbohydrates, the study also indicates that the differentiation of liver reached a new phase after 12th day of incubation. The implications of these observations have been discussed.

REFERENCES


