CHAPTER 4

STUDIES ON DEVELOPING AVIAN LIVER 4. THE CORRELATION OF LIPOGENIC FUNCTION OF THE LIVER WITH THYROID ACTIVITY IN PIGEON DURING POST-HATCHING DEVELOPMENT

Functional maturity of the liver of pigeon during post-hatching period is found to take place at a time when the parental care becomes terminal (Chapters 1, 2, and 3) which was around 20th day. From the data on the metabolite contents (Chapter 1), the activity of acetylcholinesterase and concentration of electrolytes (Chapter 2) and ascorbic acid content (Chapter 3), it was concluded that the liver attains the full complements of metabolic machinery by 20th day. During post-hatching development, the metabolic adaptations in the liver are influenced by the diet instinctively provided by the parents. During first five days after hatching the squab is fed with only crop milk which has more protein and less fat and no carbohydrates. Hence, gluconeogenesis is the most adaptive

feature in the first five days. Gradually the crop milk is withdrawn and the nestling is fed only with grains by 15th day onwards. The increased influx of carbohydrates necessitates the inhibition of gluconeogenesis and activation of lipogenic pathways (Chapter 1). Since the variation$ in the diet is almost genetically predetermined, the liver of the young ones could easily anticipate the forthcoming dietary changes. This could be possible by activating the synthesis of rate limiting enzymes by hormones. The gluconeogenesis and the fatty acid (FA) synthesis could be activated by an interplay of glucagon, insulin and thyroxine (Goodridge, 1975). The glucagon inhibits FA synthesis by increasing the concentration of c-AMP (Goodridge, 1973), while insulin stimulates FA synthesis (Goodridge, 1975). Thyroxine stimulates the fatty acid synthesis through the action of lipogenic enzymes such as 'malic' enzyme (ME) and Glucose-6-phosphate dehydrogenase (G-6-PDH) (Tepperman and Tepperman, 1964; Goodridge, 1975). According to Goodridge et al. (1974), in the liver cells of birds, thyroxine, glucagon and one or more serum factors combine to determine the concentration of the enzymes associated with fatty acid synthesis.

Since long term adaptations in the biosynthesis of fatty acids are regulated by hormones, similar hormonal

control may also be necessary for the development of metabolic adaptations in the liver after hatching. To test this contention, the lipid content, the lipid components and the lipogenic enzymes such as G-6-PDH and ~~NADP~~-'malic' enzyme in the liver as well as total and fractional lipids in the serum were studied quantitatively in pigeons along with the cytometric study of thyroid gland.

MATERIALS AND METHODS

The growing as well as adult domestic pigeons were collected from the open aviary maintained by the department. The young ones of 1, 5, 10, 15, 20, 25, 30 days and adults were brought to the laboratory and sacrificed by decapitation immediately after collecting blood from jugular vein for serum. The liver was collected immediately after they were sacrificed and was processed for the estimation of fat and the activities of G-6-PDH and 'malic' enzyme. Thyroid glands were fixed in Bouin's fixative and 5 μ thick paraffin sections were prepared for histological study.

ENZYME ASSAYS:

A piece of liver was homogenized in 0.25 M sucrose and homogenate was centrifuged at 20,000 g for 15 minutes.

Supernatant was used for assaying the activities of enzymes. Glucose-6-phosphate dehydrogenase (E.C.1.1.1.49) was assayed employing the method of Kornberg and Horecker (1955) with modifications as described by Marks (1966). NADP-malate dehydrogenase (E.C.1.1.1.40) was assayed by method described by Hsu and Lardy (1969). The protein content of the homogenate was estimated by Biuret method (Layne, 1957) and enzyme activities are expressed as μ mole $NADPH_2$ formed/mg protein/min. at 30°C.

LIPIDS:

Total lipid content was estimated employing the method of Folch et al., (1957) using the mixture of chloroform and methanol in 2:1 (v/v) as extractant and measured gravimetrically and expressed as g/100 g fresh liver.

Lipid fractions were separated by the thin layer chromatography. Glass plates (20 x 20 cms size) were coated with silica gel G (500 μ thick) and activated at 110°C for one hour (Stahl, 1965). The plates were predeveloped over night in ether and after drying each plate was marked in six 3 cms lanes and was reactivated. Known quantity of the liver and serum lipid extracts were spotted in the four lanes, a mixture of authentic standards in the fifth lane

and remaining lane was considered as blank. The plates were then developed ~~in~~ unidirectionally using two solvent systems of Freeman and West (1966). The plate was allowed to run up to 13 cms from the point of spotting using solvent-1 containing Diethyl ether, Benzene, Ethanol and Acetic acid in a ratio of 40:50:2:0.2 (v/v) respectively. The plate was dried in air and was developed in the second solvent system, a mixture of Diethyl ether and Hexane in 6:94 (v/v), which was allowed to run upto 17 cms from the point of origin. The plates were then dried in the oven for 30 minutes at 60°C and were kept in the chamber saturated with iodine vapour to render the spots visible. Corresponding areas marking the spots of different lipid fractions from sample, standard and blank were separated and taken into separate test tubes and processed for the quantitative estimations employing the method described by Marzo *et al.* (1971). The concentration of different lipid fractions were expressed as percentage of total lipid in the fresh liver or serum (mg/100 mg lipid).

THYROID GLAND:

For the histocytometric study of the thyroid gland, 5 μ thick wax sections were stained with Haematoxylin-Eosin

and height of the cells were measured using high power (45 X) objective and occular eyepiece. The height of the follicular cells are expressed in microns (μ).

RESULTS

From the data (Table 1) it is clear that the lipogenic enzymes like 'malic' enzyme and G-6-PDH (both of which are capable of supplying enough $NADPH_2$ required for lipid synthesis) showed increased activity in the liver by the 20th day of post-hatching development. Of the two enzymes, 'malic' enzyme showed a tremendous increase (from 5.9 units on 1st day to 239.80 units on 20th day). This peak level of activity (Fig. 1) on the 20th day coincided with maximum deposition of lipid in the liver (13.53%). Of the various lipid components, the triglycerides and cholesterol only showed similar trends of increase on 20th day. Phospholipid fraction and the free fatty acids (FFA) registered a fall on the same period. In fact phospholipid and cholesterol showed an inverse relationship. The serum total lipid components (Table 2b) did not show any cognizable trends of decrease or increase.

Thyroid gland became more and more active as the development proceeded, as indicated by the increase of

CHAPTER 4

EXPLANATION FOR FIGURE

Fig. 1. Graph showing quantitative analyses of activities of glucose-6-phosphate dehydrogenase and 'malic' enzyme and lipid content of the liver along with height of the follicular cells of thyroid gland of pigeon during post-hatching development.

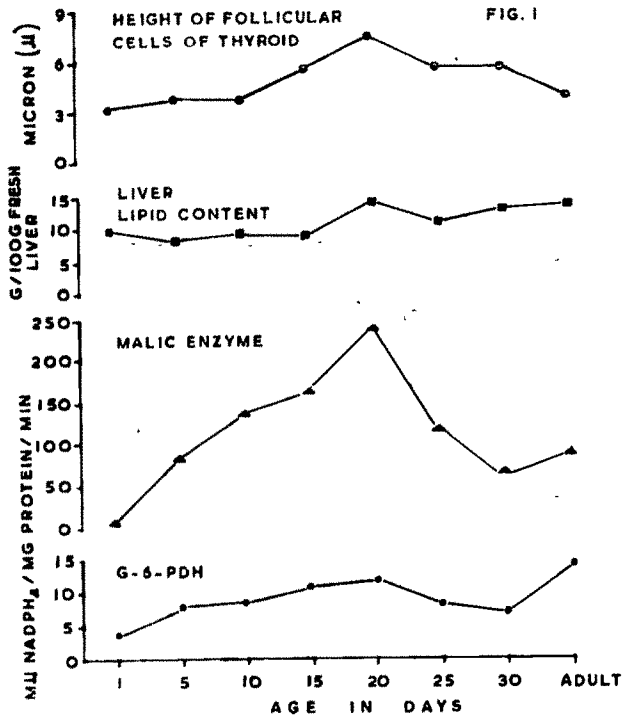
HEIGHT OF FOLLICULAR CELLS OF THYROID
MICRON (μ)
9
6
3
0
LIVER LIPID CONTENT
G/100G FRESH LIVER
15
10
5
0
MALIC ENZYME
250
200
150
100
50
0
G-6-PDH
MM NADPH2 / MG PROTEIN / MIN
15
10
5
0
1
5
10
15
20
25
30
ADULT
AGE IN DAYS

FIG. 1

TABLE 1

Activities of Glucose-6-phosphate dehydrogenase and 'malic' enzyme, lipid content of liver and height of the thyroid follicular cells of pigeon during post-hatching development. Mean ± S.D.

Age in days	G-6-PDH[1]	'Malic'[2] enzyme	Total[3] lipid	Height of the thyroid[4] follicular cells
1	3.977 ± 2.042	5.998 ± 3.162	9.970 ± 0.224	3.371 ±0.072
5	7.761 ± 1.070	83.626 ± 3.168	8.427 ± 0.722	3.704 ±0.123
10	8.753 ± 0.101	139.570 ± 40.740	8.853 ± 1.063	3.704 ±0.146
15	10.680 ± 4.039	163.890 ± 29.990	8.235 ± 1.293	5.556 ±0.237
20	11.728 ± 1.027	239.813 ± 38.720	13.523 ± 1.813	7.403 ±0.343
25	8.263 ± 2.705	115.320 ± 33.790	10.770 ± 2.209	5.556 ±0.127
30	7.318 ± 1.817	65.757 ± 12.220	12.590 ± 0.283	5.556 ±0.209
Adult	15.693 ± 2.190	86.050 ± 9.376	12.125 ± 1.029	3.704 ±0.117
*Significant at the level	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$

*p values refer to differences between 1 day and 20 day stages. The Student's 't' test was used to analyze differences in means.

1 and 2 - Activities expressed as mμ mole $NADPH_2$ formed/mg protein/min.; 3 - G lipid/100 G liver; 4 - Micron (μ).

TABLE 2a

Lipid composition in liver of pigeon during post-hatching development. Lipid fractions expressed as mg/100 mg lipid. Mean ± S.D.

Age in days	TG	DG	MG	FFA	PHL	CH	CHE	CH/PHL ratio
1	24.00 ± 4.00	3.63 ±1.13	4.30 ±0.29	3.50 ± 0.76	32.55 ± 5.95	1.71 ±0.04	35.30 ± 8.50	0.05
5	34.10 ± 4.25	4.40 ±1.91	5.70 ±1.09	10.90 ± 1.63	40.65 ± 2.55	1.16 ±0.06	4.90 ± 0.18	0.03
10	23.23 ± 2.60	2.20 ±0.10	2.20 ±0.40	3.70 ± 0.30	39.40 ± 1.90	2.39 ±0.01	1.80 ± 0.10	0.06
15	47.70 ± 6.84	2.44 ±0.22	1.90 ±0.06	15.10 ± 0.12	36.00 ± 1.81	2.99 ±0.12	1.70 ± 0.13	0.08
20	57.40 ± 8.01	4.20 ±0.70	1.40 ±1.01	4.00 ± 1.01	24.00 ± 3.54	4.28 ±0.90	1.80 ± 0.65	0.18
25	39.10 ± 9.21	4.30 ±1.21	3.40 ±1.15	11.80 ± 3.60	33.94 ± 9.00	2.15 ±0.51	2.40 ± 0.80	0.06
30	30.60 ± 5.30	2.80 ±0.69	4.50 ±1.56	18.60 ± 5.03	36.50 ± 3.65	2.10 ±0.36	2.30 ± 0.41	0.06
Adult	6.70 ± 0.30	8.50 ±0.72	6.70 ±0.41	26.70 ± 2.37	41.10 ± 2.12	1.50 ±0.11	2.40 ± 0.10	0.04

CH = Cholesterol, CHE = Cholesterol esters, DG = Diglycerides, FFA = Free fatty acids, MG = Monoglycerides, PHL = Phospholipids, TG = Triglycerides.

TABLE 2b

Total lipid content and lipid composition in serum of pigeon during post-hatching development. Lipid fractions are expressed as mg/100 mg lipid and total lipid as mg/ml serum. Mean ± S.D.

Age in days	Lipid in serum	TG	DG	MG	FFA	PHL	CH	CHE	CH/PHL ratio
1	2.10 ±0.60	22.70 ± 2.67	10.40 ± 0.42	7.60 ± 2.41	2.30 ± 0.28	20.30 ± 1.04	1.11 ±0.10	2.70 ± 0.60	0.06
5	0.86 ±0.31	28.39 ± 6.25	3.89 ± 0.85	6.40 ± 1.47	8.60 ± 2.41	46.10 ± 1.89	4.60 ±1.59	7.70 ± 3.20	0.10
10	1.60 ±0.12	25.90 ± 2.64	4.17 ± 0.80	4.70 ± 0.80	7.69 ± 0.24	31.50 ± 1.85	3.60 ±0.12	6.70 ± 1.16	0.11
15	1.20 ±0.20	25.40 ± 2.34	6.50 ± 0.81	6.80 ± 1.53	9.30 ± 0.83	37.30 ± 1.61	2.90 ±0.10	3.50 ± 0.17	0.08
20	1.80 ±0.12	25.50 ± 2.43	2.90 ± 0.41	2.70 ± 1.38	2.40 ± 0.90	31.80 ± 1.67	3.40 ±0.16	10.30 ± 2.43	0.10
25	1.70 ±0.31	24.90 ± 2.43	4.80 ± 1.71	7.30 ± 1.67	9.01 ± 1.36	35.00 ± 3.22	2.30 ±0.63	6.20 ± 1.40	0.06
30	1.70 ±0.43	15.10 ± 2.19	6.10 ± 1.54	5.46 ± 1.39	8.50 ± 0.14	33.14 ± 2.40	2.40 ±0.40	7.97 ± 1.06	0.07
Adult	0.85 ±0.14	26.50 ± 1.89	13.20 ± 1.06	12.60 ± 1.56	11.50 ± 1.83	54.10 ± 2.43	2.90 ±0.37	8.50 ± 1.13	0.05

CH = Cholesterol, CHE = Cholesterol ester, DG = Diglycerides, FFA = Free fatty acids, MG = Monoglycerides, PHL = Phospholipids, TG = Triglycerides.

height of the follicular cells. The height of the cells reached maximum on 20th day denoting that the maximum production of thyroxine was around 20th day (Fig. 1).

DISCUSSION

Since both the activity of the lipogenic enzymes and the concentration of lipids were maximum in the liver at 20th day, it is possible to conclude that the lipogenesis was maximum around this period. This increased lipogenesis could be in a way, considered as an adaptive mechanism. The young one receives large quantity of carbohydrate rich food (grains) by 20th day which could easily trigger off lipid synthesis. However, the increase in the activity of lipogenic enzymes such as 'malic' enzyme is not found to be related to this enhanced carbohydrate rich food alone. In fact, the increase in the enzyme activity is gradual reaching the peak level by 20th day. The triglyceride concentration in the liver also started increasing by 15th day. In other words the lipogenic capacity of the liver develops gradually as the enzyme induction and synthesis are activated. Initially, the increase in the activity of the enzyme did not result in an increased lipid deposition in the liver. The only

explanation to this increase in the activities of the enzymes is that they are being activated by hormones. Thyroid gland is found to become more and more active, judging from the height of follicular cells. This elevation of thyroidal activity could adaptively increase the lipogenic capacity of the liver, by the time parents provide maximum carbohydrate rich food enabling the young ones to convert the excess carbohydrate into fat and store. The maximum deposition of the stored energy is imperative at this stage because after 20th day the parental care ceases to exist and the young ones are forced to leave the nest. The sudden decrease in the food availability decreased the lipogenic activity and not only the lipid deposition registered a fall by 25th day but ~~also~~ the activities of the enzymes also showed a reduction. By 30th day however, lipid concentration showed a more or less adult level indicating that the lipogenesis, lipid mobilization and utilization have reached a stabilized pattern. By this time the young one has thoroughly learned to procure enough food by itself.

Of the two $NADPH_2$ generating enzymes studied, 'malic' enzyme was more active and at the same time showed a 50 fold

increase by 20th day, while G-6-PDH showed only a 3 fold increase. In the liver of birds, 'malic' enzyme is reported to be more involved in lipogenesis (O'Hea and Leveille, 1969; Patel, 1976).

The action of thyroxine on the 'malic' enzyme synthesis has long been proved (Tepperman and Tepperman, 1964; Wise and Ball, 1964; Young, 1968; Chandrabose and Bensadoun, 1971; Goodridge et al., 1974). Thyroxine by activating the enzyme synthesis (Colton et al., 1972) paves the way for lipogenic adaptation in the liver by 20th day. There are some reports suggesting a secondary role of insulin in the regulation of carbohydrate metabolism in adult birds. Maraud et al. (1965) have reported that insulin stimulates the iodine uptake by thyroid gland in chick embryo. It is interesting to note here that around this time maximum production of insulin could be also taking place as islet tissues increase in the pancreas (Chapter 7). Thus insulin could also play a significant role in lipogenesis both directly as well as through its stimulatory action over the thyroid gland. Hence around 20th day, all the conditions necessary for lipogenesis viz., maximum activities of lipogenic enzymes, insulin and thyroxine production and abundance of

carbohydrate rich food are met with, and the lipid synthesis gets maximum momentum.

Thus, in all probability the metabolic adaptations in the liver, which is an essential part of the functional maturity of the liver during post-hatching development, is induced by hormones such as thyroxine.

From the data on the concentrations of various classes of lipids in the developing liver (Table 2a) it is seen that the triglyceride fraction increased gradually till 20th day. This gradual increase coincided with that of lipogenic enzymes thereby indicating the role of 'malic' enzyme and perhaps that of G-6-PDH. Another noticeable tendency is in the cholesterol$_{(CH)}$ to phospholipid$_{(PHL)}$ ratio. The ratio is quite high in the liver between 15th and 20th day and in the serum between 10th and 20th day. The mobilization of lipid from the liver and its transport in the serum is believed to be influenced by CH/PHL ratio. A high ratio in some way is correlated with decreased lipid mobilization while low ratio facilitates rapid mobilization. The low ratio, which indicates that phospholipid fraction is relatively more could in one way or other help the formation of lipoproteins thereby increasing the mobilization and transport of lipid especially triglycerides.