CHAPTER 2

HISTOCHEMISTRY OF THE AVIAN ADIPOSE TISSUE

I The Adipose Tissue of the Pigeon:

The adipose tissue of mammals especially rats have been the subject of extensive studies in recent years. It has been shown that the rat adipose tissue is metabolically active, capable of actively synthesizing fatty acids from precursors like glucose and acetate (Feller,1954; Hausberger et al,1954). Active synthesis of glycogen in the adipose tissue (especially in the brown adipose tissue) is suggested from the work of various authors (as reviewed by Lever, 1957). Of the two types of adipose tissue present in certain mammals, the brown variety is reported to be more active. In the pigeon, however, only the yellow adipose tissue seems to be present and it is profusely supplied with blood (Chapter 1). The adipose tissue in birds is of especial interest, because migratory birds are known to store large amounts of fat in this tissue, prior to migration (Odum and Perkinson,1951; McGreal and Farner,1956). Since it has been shown that fat is the chief source of energy for long and sustained muscular activity (Weis-Fogh,1952; George and Jyoti,1957), it seems logical to think that the adipose tissue in such animals should play a vital role in metabolism, especially with regard to lipids. The pigeon is known to utilize mainly fat as fuel during prolonged muscular exercise (George and Jyoti,1957), and a histophysiological study on the adipose tissue of this animal was therefore considered worthwhile. Histochemical reactions for the following were studied: lipase, alkaline phosphatase, acid phosphatase, adenosine triphosphatase (ATPase), succinic, lactic,

malic, glycerophosphate, glutamic and D-glucose dehydrogenases, glycogen, fat, phospholipids, cholesterol, sulphydryl groups and water-insoluble aldehydes and ketones.

Material and Methods

The material used in the present study was the visceral adipose tissue, covering the coils of the intestine on the ventral side, of the blue rock pigeon (Columba livia). It was our experience that when pigeons were well-fed in the laboratory for about two weeks, the above mentioned visceral adipose tissue was observed to grow into enormous bulk and provided excellent material for such study. The tissue was removed after the bird was decapitated and bled to ensure that most of the blood was drained off.

Lipase activity was studied employing the "Tween" method of Gomori using "Tween 80" (Atlas Powder Co. U.S.A.) as substrate. Fresh frozen sections were mounted on clean dry slides without any adhesive, and allowed to remain at room temperature for about 15 minutes to ensure adherence. This procedure was employed in all the other enzyme studies also unless otherwise stated. Before fixation, sections were deprived of fat by soaking in ethyl ether for 10 minutes. This modification was found necessary because sections not treated in this way did not yield good results. The same procedure was adopted for all other enzymes except the dehydrogenases. Sections were fixed in cold (4°C) 6% neutral formalin for 16 hours. After fixation the sections were washed in running water for 1 hour and then rinsed in distilled water and incubated at 40°C for 8 hours. The incubation mixture contained 5 ml 0.1 M bicarbonate buffer of pH 8.4, 2 ml 10% calcium chloride, 2 ml 5% "Tween 80",

40 ml distilled water and a crystal of thymol as preservative (George and Scaria,1958). This mixture was preincubated at 40°C overnight so as to precipitate all free fatty acids, if present. This was then filtered through Whatman No. 1 filter paper and used for incubation of sections. The rest of the procedure was the same as described by Pearse (1954). The sections were not counterstained. Sections boiled in distilled water before incubation and those incubated in substrate blank served as controls.

Alkaline phosphatase was studied according to the revised method of Gomori, sodium glycerophosphate being used as the substrate. Defatted sections were fixed in cold 10% neutral formalin for 1 hour, washed in running water for 30 minutes, rinsed with distilled water and incubated in the substrate medium (Pearse,1954), at 40°C for 24 hours. The rest of the procedure was the same as described by Pearse (1954). Sections which were kept in boiling water for 10 minutes prior to incubation was used as control. Another control employed was incubating the sections in the incubation medium devoid of the substrate.

The revised method of Gomori was successfully employed on fresh frozen sections of the adipose tissue to demonstrate acid phosphatase activity (Glick,1949). After extraction with ether, sections were fixed in cold (4°C) 10% formalin for 1 hour, washed in running water for 1 hour, rinsed in distilled water and incubated for 6 hours at 37°C. The incubation mixture was prepared as described by Glick (1949). The stock solution was filtered and diluted with twice the amount of distilled water before use. Incubated sections were rinsed well in distilled water and kept for a few minutes

in 2% acetic acid. After rinsing again in distilled water the sections were placed in dilute yellow ammonium sulphide and then washed in distilled water and mounted in glycerine jelly. Boiled sections were used as control.

ATPase was detected by the procedure of Pearse and Reis (Pearse,1954), making use of adenosine triphosphate as substrate. Sections were fixed in cold 10% neutral formalin for 1 hour, washed in running water for the same time, rinsed in distilled water and incubated for 4 hours at 37°C. The incubation medium used was the same as described by Pearse (1954). After incubation, sections were washed with 2% calcium nitrate, rinsed in distilled water and treated with 2% cobalt nitrate for 5 minutes. Sections were thoroughly washed in running water and then in distilled water and treated with dilute yellow ammonium sulphide. After washing, the sections were mounted in glycerine jelly. A few sections were incubated as for alkaline phosphatase at pH 9.2 and for the same enzyme at pH 7.5, and in a similar medium as for ATPase containing distilled water in place of the substrate (Pearse,1954). In addition, sections boiled in distilled water before incubation were also used as control.

Succinic dehydrogenase activity was studied by the method of Straus et al (Pearse,1954), using 2:3:5 triphenyl tetrazolium chloride (TTC) as the hydrogen acceptor. The same method was extended to the study of lactic, malic, glycerophosphate, glutamic and D-glucose dehydrogenases. Fresh frozen sections were dropped as they were cut, into cold 0.1 M phosphate buffer of pH 7.2. After being kept in the buffer for 15 minutes to ensure that all

the endogenous activity was lost, they were transferred into the respective incubation mixtures in cuvettes and incubated for 15 minutes at 37°C. Incubation mixture in each case contained 1.5 ml of phosphate buffer of pH 7.2, 1 ml of 0.1 M solution of the substrate, 7.5 mg of TTC and 0.625 mg of diphosphopyridine nucleotide (DPN) in a total fluid volume of 2.5 ml. DPN was omitted from the incubation mixture for succinic dehydrogenase because this enzyme does not require the co-enzyme (Baldwin,1957). A few sections were incubated in incubation media not containing the respective substrates. These served as controls.

Paraffin sections of adipose tissue treated with Rossman's fluid were stained for glycogen by the Best's carmine method (Pearse, 1954). Sections treated with saliva for the removal of glycogen and then stained for glycogen served as control.

For the staining of fat, the tissue pieces were fixed in Baker's calcium-formol (Pearse,1954) and Ciaccio's fluid. Gelatin sections of calcium-formol fixed tissue were cut at 15 μ. The tissue pieces fixed in Ciaccio's fluid were subjected to prolonged postchromation and paraffin sections of this were cut at 15 μ (Chou,1957). Both gelatin and paraffin sections were stained with saturated Sudan Black B in 70% ethanol. Cain's method (Pearse,1954) for the demonstration of neutral and acidic lipids were also employed.

The acid haematein method of Baker was employed for the detection of phospholipids. Baker's pyridine extraction test was applied as control (Pearse,1954). For the study of cholesterol, the Schultz method (Pearse,1954) was employed. Sulphydryl groups

were detected by Bourne's nitroprusside test (Glick,1949), and water-insoluble aldehydes and ketones by the Albert and Leblond reaction (Glick,1949).

Results

Figure 1 is the photomicrograph of the adipose tissue section treated to show lipase activity. The sites of enzyme activity are represented by a brownish precipitate. The abundance of the precipitate is due to the high lipase activity.

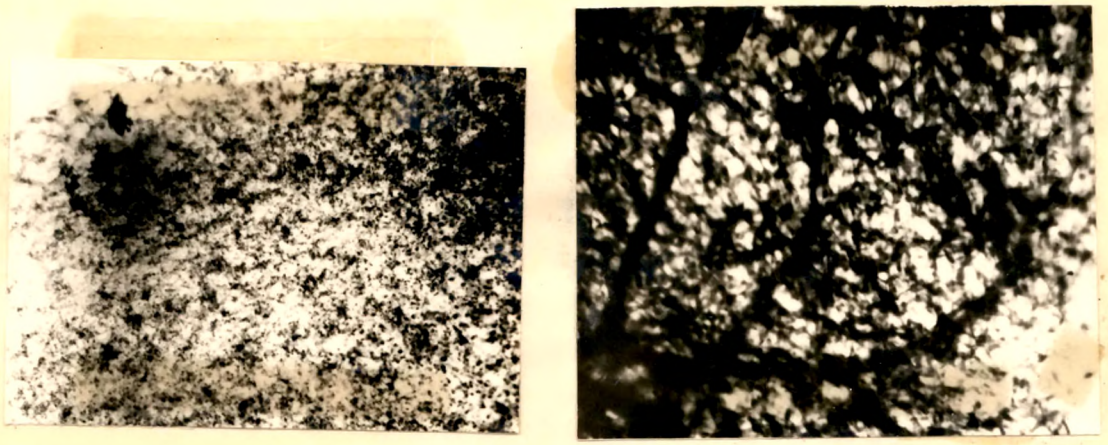


Fig. 1 Fig. 2

Fig. 1 - Section of pigeon adipose tissue treated to demonstrate lipase activity.
Fig. 2 - Section of pigeon adipose tissue treated to show alkaline phosphatase activity.

Sections treated for alkaline phosphatase activity were coloured brown indicating appreciable enzymic activity (Figure 2). Nuclei and capillary endothelium stand out in the extent of activity as was seen by the intensity of the colour. This is in conformity with the observation of Fawcett (1952) on the adipose tissue of the rat. From the intensity of the colour developed it was found that

acid phosphatase and ATPase occur in the cell periphery and the nuclei, in considerable concentrations.

All the dehydrogenases tried were found to be present except D-glucose dehydrogenase. Dehydrogenase activity was detected by the pink colour developed in the cells, due to the reduction of colourless TTC into coloured formazan, by the agency of the dehydrogenases. The formazan thus formed went into solution in the fat which is abundant in the tissue.

Glycogen was not detected in the adipose tissue of the pigeon. The yellow adipose tissue of the rat is reported to be containing only negligible amounts of glycogen unlike in the brown type where there is considerable amount of glycogen (Fawcett,1952). Sections stained with Sudan Black B revealed, that the cell periphery contains only negligible amounts of fat and the inside of the cell is filled with fat. Sections stained with Nile blue (Cain's method) showed a predominant red colouration indicating the abundance of neutral fats. The absence of detectable blue colouration indicated the scarcity of acid lipids. Phospholipids and cholesterol were found only in very low concentrations. Sulphydryl groups and water-insoluble aldehydes and ketones were also not detected in appreciable quantities.

Discussion

For the histochemical study of lipase "Tween 80" was used instead of "Tween 40" and "Tween 60" as substrate. Fawcett (1952) expressed doubt as to the presence of a 'true lipase' in the adipose tissue of the rat after a histochemical study of this enzyme. He used "Tween 40" and "Tween 60" as substrates (Gomori's method).

Since these saturated "Tweens" are not specific substrates for 'true lipase' he proposed that the fat-splitting enzyme in the rat adipose tissue may be an esterase. But it is known that an unsaturated "Tween", like "Tween 80" is attacked specifically by 'true lipase' (Gomori,1953). Since in the present study I have used "Tween 80" as substrate it can be said with some measure of certainty that the lipolytic enzyme of the adipose tissue of the pigeon is a 'true lipase'. This however, does not suggest that some esterases are absent. From figure 1 it is evident that the lipase activity in this tissue of the pigeon is very high and the amount of precipitate deposited is an index of the concentration of the enzyme. This observation is substantiated by the quantitative estimation of this enzyme in this tissue of the pigeon (Chapter 5).

From the intensity of the stain it was found that alkaline phosphatase, acid phosphatase and ATPase occur in the cell periphery as well as the nuclei in considerable concentrations. In those sections where capillaries were visible, the capillary endothelium was also stained intensely. A similar observation was made by Fawcett (1952) with regard to alkaline phosphatase. The presence of alkaline phosphatase in the adipose tissue appears to be connected with lipid metabolism. It should be pointed out that this enzyme is reported to be present only in the fat-loaded narrow fibres of the pigeon breast muscle and not in the glycogen-loaded broad ones (George, Nair and Scaria, 1958), while the amount of acid phosphatase and ATPase is found to be more in the latter (George and Pishawikar, unpublished). Acid phosphatase

is reported to be involved in the protein synthesis mechanism (Kivalo et al,1958). The role of the ATPase present in considerable concentration in the pigeon adipose tissue cannot be explained within the limitations of the present state of our knowledge. Zimny and Gregory (1958) investigating the composition of the brown adipose tissue of the 13-stripped ground squirrel has reported the concentration of the following phosphate compounds in a decreasing gradation: inorganic phosphate, creatine phosphate and adenosine triphosphate. There are no data available as far as I am aware, on the concentration or even the occurrence of these phosphate compounds in the adipose tissue of the pigeon.

The presence of appreciable concentrations of the enzymes of the Krebs' cycle suggests that the adipose tissue plays an active part in the metabolic activities along with other known active tissues, possibly in the synthesis of fatty acids from carbohydrates which is the main constituent of the animals' diet. The presence of lactic dehydrogenase in the adipose tissue is significant from the fact that normally there is no reason why lactic acid should be present in considerable quantities in the adipose tissue. It is possible that, at least part of the lactic acid produced during muscular exercise is transported to the adipose tissue, through the agency of the blood, where it can be converted to fatty acids via pyruvate, the lactic dehydrogenase operating in the cycle. If so, this should relieve some of the load on the liver, which has a variety of other functions to perform. It should be of interest to note here that when TTC was injected into the live pigeon intravenously as well as intra-

muscularly, the adipose tissue was found to be one of the tissues which showed the greatest dehydrogenase activity, the others being the kidney, liver and the breast muscle.

Fawcett (1952) reported the presence of a considerable quantity of glycogen in the brown adipose tissue of rats and a negligible amount in the yellow. He (1952) further stated that rats which were fasted for 24 hours and then fed showed an increase in the glycogen content in the adipose tissue, when examined after 2 to 12 hours from the commencement of feeding. Similarly I examined a number of pigeons which were fasted for 24 hours and at the end of that period they were fed with a diet consisting of cereals. The birds were sacrificed at different time intervals of 2 hours, 4 hours and 8 hours from the time of termination of the fast and their adipose tissue was examined for glycogen. Negative results were obtained in all the cases. Diurnal variations in the levels of glycogen in the adipose tissue were also noted by Fawcett (1952). Rats which were killed during night hours and examined revealed a greater concentration of glycogen than others taken during day-time. In the present study, all the pigeons used were sacrificed during day-time.

From the results obtained from the study of lipids in the adipose tissue of the pigeon, it appears that they are mainly in the form of neutral fats. This is in conformity with the observations of Fawcett (1952) on the fat in the yellow adipose tissue of the rat.

II The Adipose Tissue of the Fowl and Rosy Pastor:

Having studied some aspects of the enzymic constitution of the pigeon adipose tissue it was thought desirable to extend the same studies to the adipose tissue of two birds exhibiting two divergent modes of activity: the domestic fowl, a nonflying bird and the rosy pastor, a migrant bird capable of sustained flight.

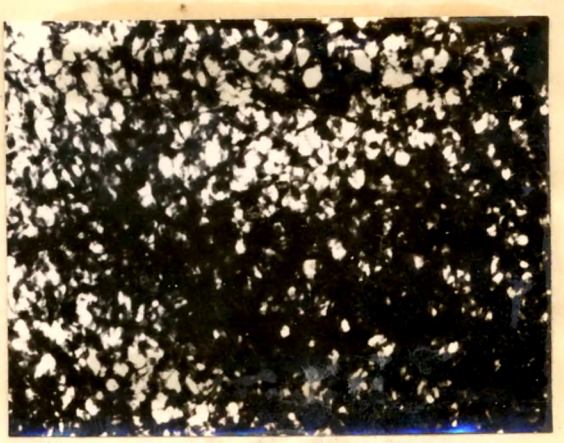
Material and Methods

The fowls used in the present study were the domesticated Indian indigenous ones (Gallus domesticus 'desi'). Only roosters were used in all cases. The rosy pastors (Pastor roseus), were shot and the adipose tissue taken immediately. In both the cases, after decapitation of the birds, the visceral adipose tissue (from the ventral side, covering the coils of the intestine) was taken after most of the blood was drained off. Fresh frozen sections of this tissue was used throughout. Lipase was studied using the "Tween" method of Gomori with "Tween 80" as substrate, alkaline phosphatase by the Gomori method with sodium glycerophosphate as substrate and the succinic dehydrogenase by the method of Straus et al using sodium succinate as the substrate (Pearse,1954). The histochemical procedures were the same as adopted for the pigeon adipose tissue.

Results

Figures 1 and 2 show the photomicrographs of the sections of the adipose tissue of the fowl and rosy pastor respectively treated to demonstrate lipase activity. The adipose tissue of the

rosy pastor showed a much higher activity of this enzyme as was evident from the more abundant deposit of the brownish precipitate.



100μ

Fig. 1 Fig. 2

Fig. 1 and 2 - The adipose tissue of the fowl and rosy pastor respectively treated for lipase activity.

The adipose tissue of the rosy pastor showed a much higher alkaline phosphatase activity than the same tissue of the fowl (Fig. 3 and 4).

Fig. 3 100μ Fig. 4

Fig. 3 and 4 - Photomicrographs of sections of the adipose tissue of the fowl and rosy pastor respectively treated for alkaline phosphatase activity.

The cell periphery is conspicuous by its dark colour indicating considerable enzyme activity. The cytoplasm of the cells is confined to the cell periphery while the inside is filled with fat. In the case of the rosy pastor the capillary endothelium too stands out dark due to the high concentration of the enzyme. As far as the fowl is concerned such a pronounced activity was not detected. This is probably because the overall activity of this enzyme in the fowl adipose tissue might be much less.

Succinic dehydrogenase was found to be occurring in almost the same concentration in the adipose tissue of these two birds, or perhaps a little more in the rosy pastor.

Discussion

The adipose tissue of birds and many other animals appears to play a major role in lipid metabolism. It is well known that enormous quantities of fat are stored in the adipose tissue of migratory birds prior to migration. Considerable part of this fat is used up during the migratory flight. It should be pointed out that the energy released by combustion and utilization of unit weight of fat is more than that obtained from that weight of protein and carbohydrate put together. And storage of fat in the form of neutral fat, is a much easier process than that of either protein or carbohydrate. Yet another advantage that of storing as fat is the yield of the much-needed metabolic water made available in larger amounts especially to such birds, which indulge in non-stop flight during migration. As mentioned earlier the adipose tissue is capable of active fatty acid synthesis as is evident from the work of Feller (1954) and

Hausberger et al (1954) on the rat adipose tissue. Similarly the adipose tissue of birds also should be capable of actively synthesizing fatty acids. However, there appear to be some differences in the relative capacities for fat synthesis. A fuller discussion on this aspect is reserved for a later chapter (Chapter 5). The fatty acids synthesized within the adipose tissue has to be converted into fats for which the presence of lipase is essential. Taking this into consideration it seems logical to assume that the concentration of lipase in the adipose tissue should be directly proportional to its ability to synthesize fat. If so, the amount of fat synthesized in the adipose tissue of the rosy pastor will be much more than that in the fowl, the lipase activity in the former being very high. Along with lipase alkaline phosphatase was also found to be more in the adipose tissue of the rosy pastor. This appears to support my earlier contention that alkaline phosphatase has some important role in lipid metabolism. Thus the higher concentration of both lipase and alkaline phosphatase in the rosy pastor adipose tissue appears to be an indication of a higher lipid metabolism. This is of special significance because, rosy pastor is a migrant; migrating from India to Europe by the end of April or beginning of May and returning by August. These birds used in the present study were procured approximately two weeks before they disappeared from Baroda. At that time the adipose tissue is very active and large quantities of fat accumulate in them. There was a marked increase in the amount of fat deposition in the adipose tissue of these birds from about 30 days prior to

their disappearence from this locality. By the time they begin to leave, the adipose tissue throughout the body was found to be virtually filled with fat. In sharp contrast, the fowl is known to be a very poor flier and consequently the general body metabolism is also low.

The presence of almost equal concentration of succinic dehydrogenase in the fowl and rosy pastor adipose tissue suggests that the oxidative metabolism in this tissue in the two birds do not vary considerably. In conclusion it can be said that the overall activity of the adipose tissue of the rosy pastor during the later part of the premigratory phase is considerably higher than that of the same tissue in the fowl.