CHAPTER 4

LIPASE ACTIVITY IN THE HEPATOPANCREAS

It is a well established fact that the long term demands for energy in various tissues are met through the oxidation of lipids. The enzyme lipase catalyses the hydrolysis of fat into fatty acids and glycerol and also brings about the esterification of fatty acid with fat. High concentrations of lipase have been shown by several workers in the various vertebrate and invertebrate muscles and muscular organs indulging in sustained activity. Lipase activity in the developing chick liver was studied by Iype (1961). Vertebrate pancreatic lipase was extensively studied by Desnuelle (1961). The presence of lipolytic enzyme activity in blood, liver, heart and many other tissues in vertebrates and invertebrates is well known. However, very little is known on the lipase activity of the crustacean hepatopancreas. The formation of fatty acids from olive oil by the digestive juice of Astacus, was demonstrated by Hoppe-Seyler in 1877. But Kruger and Graetz (1928), showed that the enzyme concerned is an esterase rather than a lipase since the esters of lower alcohols and lower fatty acids are hydrolysed much more rapidly than fats. In contrast in Homarus gammarus and Palinurus elephas, digestive

juices have a stronger action on fats than on esters of short chain fatty acids. Yonge (1924), studied lipase which acts on olive oil in Nephrops. Lipolytic action of the digestive juice in Daphnia, was studied by Hasler (1935,1937) and Von Dehn (1931). In the present investigation, experiments were carried out to demonstrate the presence of a 'true lipase' in the hepatopancreas of the freshwater crab using a long-chain fatty acid ester, triolein as the substrate.

MATERIALS AND METHODS

The common freshwater crabs, Paratelphusa jacquemontii, were collected locally and were used in the present investigation. The crabs were dissected out and the entire hepatopancreas was removed. Small pieces were fixed in 6% neutral formalin for histochemical observations. The remaining tissue was immediately weighed and frozen in an ice-acetone bath and was used for the quantitative estimation. The fixed tissues were washed and embedded in gelatine. Frozen sections were cut at 10μ thickness on a freezing microtome. The sections were thoroughly washed in distilled water and treated for the histochemical demonstration of lipase employing the Tween method of Gomori (1953). Tween 85 (polyoxyethelene sorbitan trioleate) which was shown to be a specific substrate for true lipase (George and Ambadkar, 1963) was used as a substrate. Boiled section served as controls.

QUANTITATIVE ESTIMATION OF LIPASE ACTIVITY:

Preparation of the enzyme material:

For the quantitative determination of lipase activity the frozen hepatopancreas was cut into small pieces, crushed and ground to a fine pulp in a mortar which was previously cooled to 0°C and kept in an ice bath. The extraction medium used was 70% glycerine which was also cooled to 0°C. Glycerine was slowly added to the pulp, well shaken and allowed to stand for a while. To prevent putrification a few drops of toluene were added to the glycerine solution, which was then kept at 0°C. By this method a considerable part of the enzyme was extracted, since glycerine is a good solvent for lipase. This crude glycerine extract was used as the enzyme material. In all the experiments glycerine extracts with a concentration of 2 gms per 20 ml (w/v) of glycerine were used.

Substrate:

To determine the lipase activity of the extract two substrates were employed - tributyrin, a short chain fatty acid ester and triolein, a long chain fatty acid ester. The method of Fiore and Nord (1949) using polyvinyl alcohol was adapted. Boiled extract was used as the control with all the other conditions remaining the same. The reaction mixtures were incubated at 40°C and the pH was maintained at 7 in all the experiments. Polyvinyl alcohol was found to be superior to gum arabic and gum tragacanth in catalytic work (Rampino

and Nord, 1941) and therefore it was used throughout the present studies. However, polyvinyl alcohol was not a very efficient emulsifying medium for tributyrin but becomes a stable emulsion upon the addition of a single drop of "Tween 80". It should be mentioned that although Tweens themselves are considered to be substrates for lipase, there is no detectable activity at a low concentration such as one drop (0.1 ml) in 100 ml of polyvinyl alcohol.

Preparation of polyvinyl alcohol:

Polyvinyl alcohol (2.5 gms) - (Grade 71-30) was shaken in an Erlenmeyer flask with 250 ml distilled water. 1.25 ml of 0.1 N HCl was added and the mixture was heated at 75-85°C for a few minutes, till the solution became clear. Then 25 ml of distilled water was added and heated for a few minutes. The above solution was then cooled and brought to the desired pH (pH 7) by adding 0.1 N NaOH.

Preparation of the emulsion:

To 100 ml of the polyvinyl alcohol solution 3 ml of triolein was added and emulsified in a Waring blender for five minutes and was used. In the case of tributyrin 2.8 ml of tributyrin and a single drop of "Tween 80" were used.

Buffer solution:

McIlvaine buffer of the same pH as that of the polyvinyl alcohol solution was used.

Assay of enzyme activity:

Into a 250 ml Erlenmeyer flask were measured 10 ml of the emulsion, 5 ml of the buffer and 5 ml of the enzyme preparation. The flasks were well shaken and incubated at 40°C for 4 hours with constant shaking. At the end of the incubation period 30 ml of alcohol-acetone mixture (1:1) was added to stop further enzymic reaction. After 10 minutes it was filtered using a fluted Whatman filter paper (No.1) and 25 ml of the clear filtrate was titrated against 0.05 N aqueous NaOH using a microburette. The indicator used was 0.3 ml of a 1% solution of alcoholic phenolphthalein. The value thus obtained was doubled and recorded.

For the measurement of lipase activity the results are expressed as lipase units.

Lipase unit:

The lipase activity required to liberate a quantity of oleic acid equivalent to 1 ml of 0.05 N NaOH, when 10 ml of the substrate consisting of 3 ml of triolein in 100 ml of polyvinyl alcohol solution is allowed to react with 5 ml of McIlvaine buffer of pH 7 at 40°C for 4 hours, the total volume of the mixture being 20 ml.

Lipase value:

The number of lipase units present in 1 gm of wet tissue.

Calculation:

An extract consisting of 0.5 gm of hepatopancreas in 5 ml glycerine liberates oleic acid equivalent to 2.5 ml of 0.05 N NaOH during the early stage of gonadal development. The number of lipase units in 5 ml of the extract is therefore 2.5. Therefore the lipase value of the hepatopancreas during the early period of the gonadal development is 5 per gm wet tissue.

Two controls were tried. In one the enzyme was boiled for 10 minutes and used after cooling. In the second, the fresh enzyme preparation was used after adding the alcohol-acetone mixture to the substrate and thereby breaking up the emulsion and rendering the enzyme completely inactive. The readings obtained for these two types of control experiments were identical. The boiling method was therefore employed throughout the present study.

OBSERVATIONS

Histochemical observations as well as the quantitative estimations show a more or less similar pattern of enzyme activity in both the sexes. Histochemically lipase activity was demonstrated as brownish precipitates at the sites where the enzyme activity was present. Even though, the enzyme was found to be active in all the seasons it was found to be

comparatively higher during the early stages of gonadal development. This was evident from the bigger size and density of precipitate in the cells of the tubules as well as the comparatively higher value obtained in the quantitative estimation.

Moderately uniform enzyme activity was obtained in the tubules as well as in the intertubular connective tissue of the hepatopancreas in both the sexes soon after the aestivation, which is the non-feeding period for the animal (Fig. 1). The connective tissue surrounding the epithelial cells of the secondary secretion duct showed moderately high enzyme activity. Enzyme activity was confined to the storage cells while the secretory cells of the tubules showed no enzyme reaction. The activity in the tubule was more intense towards the tapering distal end where only the developing or embryonic storage cells were seen (Fig. 2). During the early period of gonadal development a sudden but temporary increase in the fat store was noted in the hepatopancreas, due to the active feeding soon after the aestivation (Chapter 3). This was followed by an increase in the lipase activity which was revealed both histochemically and quantitatively. Histochemical observations showed that the enzyme activity was localized only in the tubules, the intertubular connective tissue showing no enzyme activity (Fig. 3). During this period the storage as well as the secretory cells showed

TABLE I

Table showing the lipase value of the hepatopancreas during different seasons

Number of experiments	Season	Sex	Substrate	Substrate concentration ml/100 ml PVA*	Enzyme concentration	Lipase units in 5 ml enzyme material	Lipase value
5	Soon after the estivation	Male	Triolein	3.0	2 grams in 20 ml glycerine	1.21	2.42
		Female				1.32	2.64
		Male	Tributyrin	2.8	-do-	5.76	11.52
		Female			-do-	5.39	10.78
6	During the initial stages of gonadal development	Male	Triolein	3.0	-do-	4.76	9.52
		Female			-do-	4.51	9.02
		Male	Tributyrin	2.8	-do-	8.47	16.94
		Female			-do-	9.02	18.04
5	After the gonadal development, before the aestivation	Male	Triolein	3.0	-do-	2.47	4.94
		Female			-do-	2.53	5.06
		Male	Tributyrin	2.8	-do-	5.98	11.96
		Female			-do-	6.32	12.64

*PVA - Poly Vinyl Alcohol solution

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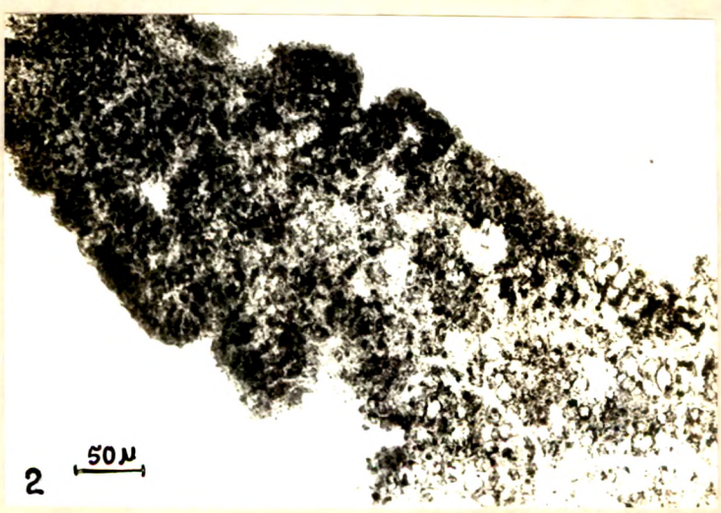
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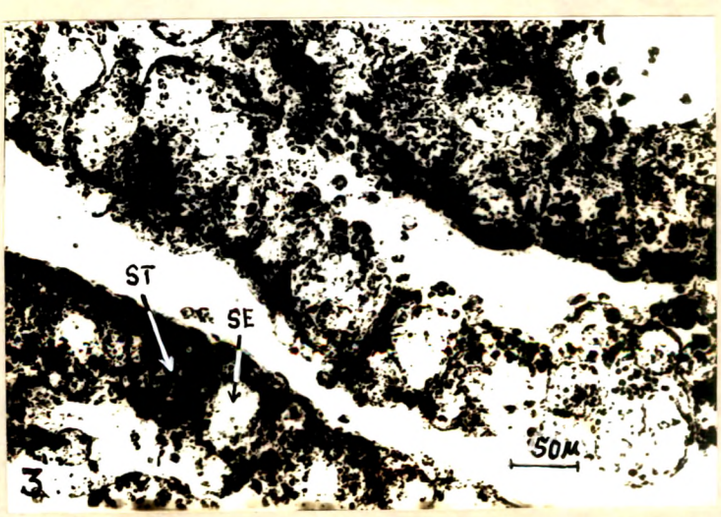
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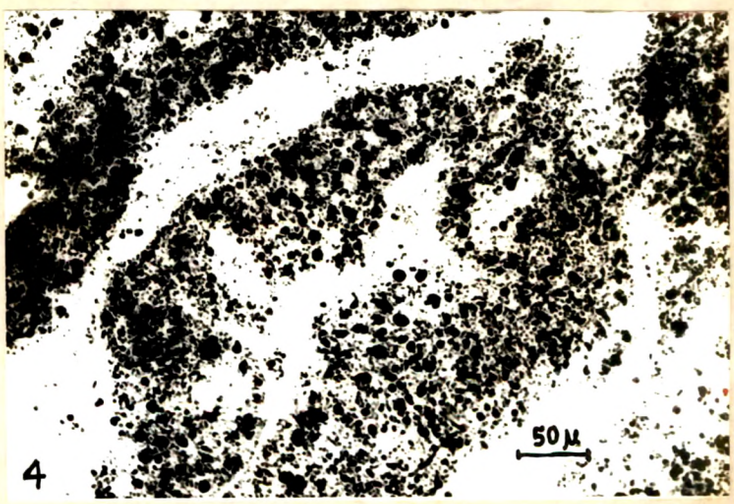
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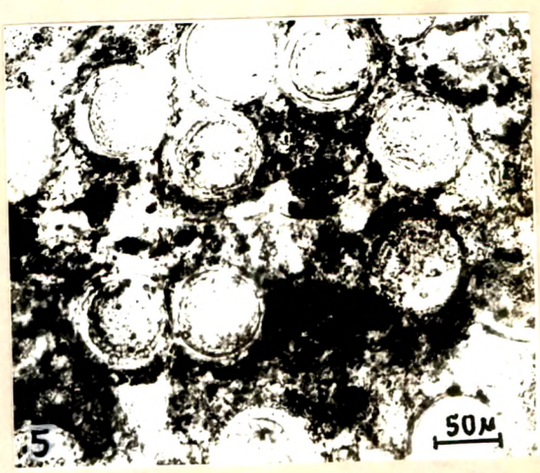
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EXPLANATION TO THE FIGURES

Fig. 1. T. S. of the hepatopancreas (July) showing moderately uniform lipase activity in the tubules as well as in the connective tissue.

Fig. 2. L. S. of a tubule of hepatopancreas showing intense enzyme activity towards the tapering distal end of the tubule where only embryonic storage cells are seen.

Fig. 3. L. S. of hepatopancreas during the early stage of gonadal development showing the enzyme activity in both secretory (SE) and storage (ST) cells. Note the absence of enzyme activity in the inter-tubular connective tissue.

Fig. 4. T. S. of hepatopancreas showing comparatively less enzyme activity towards summer when tubule get filled with neutral fat.

Fig. 5. Numerous fat globules in the interstitial connective tissue of the hepatopancreas showing peripheral enzyme activity.

enzyme activity. In all the other seasons the secretory cells were completely negative. Again when the tubules were filled with fat during summer, a considerable decrease in the enzyme activity occurs (Fig. 4). A large number of fat globules with slight peripheral activity were seen in the storage cells of the tubules (Fig. 5). The connective tissue showed very low enzyme activity.

Table I shows the results of the quantitative estimations of lipase activity in the hepatopancreas during the different seasons. It may be seen that comparatively high enzyme activity was obtained in the hepatopancreas during the early stage of the gonadal development supporting the histochemical findings. A higher rate of hydrolysis was noticed in the case of tributyrin than triolein.

DISCUSSION

A high lipase activity in the adipose tissue of certain vertebrates was reported by George and Eapen (1958, 1959). High lipase activity has also been shown in the fat loaded fibres of the pigeon breast muscle (George and Scaria, 1958) and in the fat body of the desert locust Schistocera gregaria, (Weis-Fogh, 1952; George and Eapen, 1959). Weis-Fogh (1952) showed that in the locust Schistocera gregaria, at least two-thirds of the energy liberated during flight was derived from fat. Fat bodies of insects are often compared with

mammalian liver, since a number of metabolic reactions are known to be occurring in them. Similarly, the crustacean hepatopancreas, which is known to perform the functions of both liver and pancreas of higher animals is a metabolically active tissue and the main store in this tissue is fat. It has been shown that the increase of fat takes place in this tissue prior to gonadal development and before aestivation in summer (Chapter 3). The storage of fat in certain animals for the utilization in the production of eggs and sperms and also for hibernation and aestivation is well known (George and Desai, 1954), and it is also well established that the long term demands for energy is met through the oxidation of lipids (George and Jyoti, 1957; Weis and Fogh, 1952). It necessarily follows that tissues having a high fat store such as the hepatopancreas should play a vital role in the general metabolism of such animals. The presence of a high storage of fat naturally leads to the expectation of a fairly high activity of lipolytic enzymes at the sites of the fat store. The lipid hydrolyzing enzymes are mostly esterases. Lipase is essentially an esterase, but is distinguished from other esterases by the fact that it readily hydrolyzes lipids, which are esters of organic acids with alcohols, especially triglycerides in preference to any other substrate (Baldwin, 1953; Fruton and Simmonds, 1953; Sumner and Somers, 1953). In the present study in order to fulfill the specificity

requirement for lipase it was felt desirable to use as substrate, a triglyceride with long-chain fatty acid triolein, and thus facilitate the separation of lipase from any other esterase that might be present. It is well known that the esterase does not hydrolyze triolein and appears only to hydrolyze substrates in solution. Moreover, it has been stated that lipase activity is assayed by estimating the free fatty acids liberated on incubation of the enzyme with a pure triglyceride preferably triolein in a medium suitably buffered (pH 7-9) and usually containing an emulsifying agent (Desnuelle, 1951). The kinetics of the formation of mono- and diglycerides during the hydrolysis of triolein by pancreatic lipase was reported by Desnuelle et al. (1948, 1951). Since tributyrin has a shorter chain length than triolein, the enzyme splits shorter chains more quickly than the longer ones. This accounts for the comparatively high lipase value obtained for tributyrin, the whole of which may not be pure lipase. Esterases may also act on the substrates having shorter chain length. But as for triolein, having the longer chain length, the lipase value obtained is much less when compared to the lipase value of tributyrin. Since it is well known that the esterase does not act upon triolein, the lipase value obtained in the case of triolein may be regarded as a 'true lipase'. A moderate increase in the lipase value with triolein, was obtained in the hepatopancreas, in the early months of gonadal development. Prior to this a sudden and temporary increase in the fat content

also was noticed, which gets reduced in the following months (Chapter 3). These observations regarding the fat content and its reduction in the preceding months, together with a comparatively high lipase value parallel to that, tend to show a relationship between the enzyme lipase and the substrate fat and its utilization during these months for the gonadal development.

Histochemical observations using "Tween 85" as the substrate also showed a remarkable increase in the enzyme activity, in the tubules of the hepatopancreas at the stage of gonadal development. Recently, even though there is a controversy regarding the substrate specificity of Tweens, since they are water soluble esters of fatty acids, the results obtained in the present investigation using Tween 85 and triolein are comparable. However, triolein is the widely accepted natural substrate for true lipase and the histochemical observations with Tween 85 are comparable to the results obtained with triolein. Therefore it is tempting to make a concluding remark that the activity obtained with Tween 85 in the hepatopancreas, also may be regarded as a 'true lipase'. This increase in the enzyme activity was seen only in the cells of the tubules while the intertubular connective tissue remained negative to the reaction. The increased fat observed in the tubules during this period was the neutral fat or triglyceride, whereas the connective tissue

contained only acidic lipids. The high enzyme activity observed in the tubules is suggestive of the breakdown and utilization of the triglycerides for the development of the gonads. Before and after the spermatogenesis and oogenesis, the tubules as well as the connective tissue showed the presence of a uniformly distributed, low amount of precipitate indicating a much reduced activity. In the later stage i.e. towards summer even though an increased storage of fat was seen in the cells of the tubules, a decreased enzyme activity was noticed. This may be due to the fact that during this period fat is not being utilized by the animal but is being stored up in the cells of the tubules for the various metabolic requirements that the animal will have to face during the aestivation period. This is accounted for the progressive reduction of the fat store from the hepatopancreas soon after the aestivation. In the present investigation lipase activity was not studied during the process of aestivation because of the non-availability of enough specimens. Nevertheless, lipolytic enzymes are essential for the progressive reduction of stored fat and its utilization for energy needs. It has been suggested by Baldwin (1953), when he wrote "It has usually been assumed, though never proved that fats are hydrolytically split into glycerol and free fatty acids before any oxidation takes place. This is not entirely an unreasonable supposition, for cells of most kinds seem to be furnished with lipolytic enzymes,

the action of which is freely reversible". So the noticeable reduction of fat in the tubules of the hepatopancreas soon after the aestivation suggests the possibility of fat being utilized during the non-feeding time or the aestivation period. This again indicates the presence of appreciable lipase activity during that period.