CHAPTER II

LIPOLYTIC ACTIVITY AND ITS POSSIBLE SIGNIFICANCE IN LIPID METABOLISM IN THE FIELD CRAB

Paratelogusa hydrodromus
That lipids especially, the neutral lipids are important source of energy during development, molting and reproduction in crustacea has been very well recognized (for review see Gilberg and O’Conner, 1970). Crucial to an understanding of lipid transport mechanisms in animals is the part played by lipases. Several workers using variety of enzymatic assay methods have been able to demonstrate lipase activity in several arthropodan aps. (Fiore and Nord, 1949; George and Eapen, 1959; George, 1964; Gilbert et al. 1965; Wlodawer and Jagwinski, 1967; Stevenson, 1972; Dutkowski and Sarzala Drabikowska, 1973; Downer and Steel, 1973 and Price, 1975). The major lipid in crustacea is stored in the hepatopancreas, which is usually in the form of triacylglycerol. There is no evidence that neutral lipid as such could directly be oxidized. If neutral lipid to be utilized for energy, it has first of all to be hydrolyzed to fatty acids and glycerol; the fatty acids released can undergo β-oxidation in the mitochondria. The glycerol can enter into glycolytic cycle. The presence of enzyme lipase which mediates the hydrolysis of lipids to fatty acids and glycerol has been demonstrated in several crustacea. However, most of these studies have dealt with the role of this enzyme in the process of digestion and absorption of
lipid (for review see Vanweel, 1970). As far as the author is aware there is not much information in crustacea regarding the activity of extra-digestive lipase. Considerable ambiguity concerning the definition of a lipase coupled with unexplainable experimental procedures present to draw absolute conclusions.

The field crab *P. hydrodromus* collected during the months of June and July demonstrated highest amount of lipid in the hepatopancreas. Studies on other crustaceans have revealed that early and mid-premolt hepatopancreas and its level gets depleted during the late premolt activity. Two types of muscle fibres have been identified in the coxal muscles of the field crab. It has been suggested that m.flexor is designed for oxidative metabolism utilizing lipid as chief source, whereas m.extensor is adapted more for anaerobic activity utilizing crabohydrate as chief source of fuel.

An attempt is made therefore in the present investigation to demonstrate lipolytic activity in the hepatopancreas and coxal muscles of the field crab *Paratelphusa hydrodromus*. 
MATERIAL AND METHODS

Animals

The males of *P. hydrodromus* were collected from the paddy fields during the months of June and July. The animals were housed in the laboratory aquaria at 26°C for one week before experimentation. Crabs were fed *ad libitum* with paddy seedlings, earthworms and egg shells. However, crabs were starved for 5 days prior to experiments.

Tissue Preparation for Enzyme Assay

The hepatopancreas was exposed by cutting open the carapace. The tissue was removed from the animal body immediately and blotted on whatman filter paper and stored at 0°C. The coxal muscles were exposed from the ventral side and removed carefully. *M. flexor* and *m. extensor* were carefully separated under binocular and stored at 0°C until used. All these operations could be finished within 2 min. In the case of muscle tissues, *m. flexor* and *m. extensor* were pooled from eight walking legs (four on each side) and used. Each tissue was separately homogenized in appropriate volume of crab chel water saline in potter Elvehjem glass homogenizer with teflon
pestle at slow speed. All these operations are carried at 0°C to 4°C. After homogenization the homogenate was transferred to test tube kept in ice. The thick fat pad formed on the top of the hepatopancreas homogenate was carefully removed by spatula. The homogenates were used almost immediately for the lipase assay.

**Enzyme Assays**

Two methods have been followed to determine lipolytic activity - (i) Titrmetric, and (ii) Radioassay.

**Titrmetric Method**

The method of Piore and Nord (1949), using glycerol tributyrate and purified triolein emulsified in polyvinyl alcohol was followed to estimate the rate of hydrolysis by different tissue preparations. The incubation mixture consisted of 1.0 ml emulsion, 1.0 ml Phosphate buffer of pH 7.6 (0.1 M and 1.0 ml enzyme source. The control preparations were run with each experiment by adding 2 ml alcohol acetone mixture (1:1) to the incubation mixture before the enzyme source was added to it. The mixture was incubated at 30°C for 1 to 4 hours with a constant shaking. At the end of incubation period the reaction in the experimental flasks was terminated by adding 2 ml alcohol-acetone
mixture (1:1). The liberated acid moiety was titrated using microburette against aqueous 0.05 N NaOH with phenolphthalein as an indicator. The enzyme units were expressed as the amount of NaOH (0.05 N) required to neutralize the fatty acid produced in the reaction under above mentioned conditions.

Radioassay

The method followed for radioassay of enzyme activity was essentially similar to that described by Gilbert et al. (1965) with little modifications. In this procedure the radioactive substrates are prepared as follows:

**PREPARATION OF RADIOACTIVE SUBSTRASES**

1-\(^{14}\)C Triacylglycerol

Triacylglycerol uniformly labelled in the carboxyl position with \(^{14}\)C was obtained by germinating soyabeanse in a medium containing \(^{14}\)C acetate. About 50 gms of soyabeanse were allowed to soak in running tap water for about two hours. The beans were then transferred to a beaker containing 250 milicuries of \(^{14}\)C acetate dissolved in 10 ml of boiled distilled
water. They were left at room temperature for about 8 hours, with occasionally stirring. The labelled acetate was completely absorbed by the seeds during this time. They were then spread over moistened thick blotting paper and allowed to germinate in the dark at 30°C for 48 hours. The seedlings were then homogenized in a waring blender with 400 ml of methanol and lipids were extracted according to the method of Bligh and Dyer (1957). The lipid extracts was concentrated and loaded on to an alumina column prepared in distilled chloroform. The neutral lipid fraction was eluted with one litre of chloroform. After concentrating the elutes the neutral lipid fraction which consists major portion of triacylglycerol was further purified by subjecting it to an alumina column and eluted with 2 per cent acetone in petrol. The purity of the labelled triacylglycerol was checked on tlc plate with a standard triglyceride (Sigma Co. U.S.A.). The tlc plate showed only one spot corresponding to the standard triglyceride.

1 - \(^{14}C\) Diacylglycerol

Labelled diacylglycerol was prepared by hydrolyzing \(^{14}C\)-lecithin with phospholipase C. The incubation mixture consisted of \(^{14}C\)-lecithin : 2.0 ml tris maleat buffer
(pH 7.4), 3.5 mg phospholipase C (Sigma U.S.A.) prepared in 5 ml of 1% bovine serum albumin and 1 ml of 0.5 M CaCl$_2$. The incubation was carried at 37°C for 60 min. with a constant shaking. The reaction was terminated by adding 25 ml methanol and lipid was extracted according to the method of Bligh and Dyer (1957). The extracted lipid was analysed on preparative silica gel tlc plate along with standard diacylglycerol. After exposing the plate in an iodine chamber, the diacylglycerol band formed was scrapped out and transferred to a small chromatographic column and eluted with chloroform. The chloroform was evaporated in a flask evaporator and labelled diacylglycerol was stored in cool.

$^{14}$C Monoacylglycerol

Labelled monoacylglycerol was obtained by hydrolysing purified labelled triglyceride with an acetone dried powder of goat pancreas. The reaction mixture consisted of 3.0 ml 1 M tris HCl buffer (pH 8) : 0.2 ml (w/v) CaCl$_2$ : 0.5 ml 0.1% sodium taurocholate : labelled triglyceride ($2 \times 10^9$ cpm/min) and 25 mg pancrease powder. After 3 hour of incubation at 40°C with a constant shaking, the reaction was terminated by adding 10 ml methanol. The lipid fraction was extracted according
to the method of Bligh and Dyer (1957). Labelled monoacylglycerol was separated on preparative tlc plate as described above.

**Substrate Preparation**

The labelled substrates obtained as a result of the procedure described above were emulsified in gum acacia sol (1 gm/4 ml distilled water). Purified mono, dia- and triolein were used as carrier substrates. Radiolabelled and carrier substrates were sonicated for 2 min before use.

**Enzyme Assay**

The standard reaction mixture contained in a final volume of 2 ml, 0.5 ml enzyme source and 0.5 ml emulsified substrate containing 25 to 30 micromoles of tri-, di- and monoacylglycerol equivalent to 2 x 10^6 cts/min in each case, 0.7 ml tris maleate buffer (pH 8) and 0.3 ml CaCl_2 (0.1 M). The incubation was carried out at 30°C for 1 hour to 4 hour. Control preparations were run with each experiment by adding appropriate vol of methanol to the incubation mixture before the addition of homogenate and incubated along with the samples. The reaction in the experimental tubes was terminated
by the addition of 2 ml of methanol, and the lipid portion was extracted following the method of Bligh and Dyer (1957). The reaction tubes and control tubes were centrifuged for 10 min at 3,500 rpm. A known vol. of chloroform layer containing lipid was removed, evaporated and redissolved in known volume of chloroform. The free fatty acid content of the lipid sample was separated on silica gel plate using the solvent system of n-hexane: solvent ether : acetic acid (90:18:1.5 v/v/v). With this solvent system monoacylglycerol and phospholipids remained at the origin, while diacylglycerol and triacylglycerol and free fatty acids were identified with their respective standards after exposing the plate to iodine vapour. The free fatty acid spot was scrapped out from the plate and transferred to a counting vial containing liquid scintillation solution (12 mg ppo/ 5 ml toluene). The radio-activity in free fatty acid was measured on Beckman's scintillation counter.

**Enzyme Units**

Except in radio-activity there is no differences between the unlabelled and labelled substrates, since both were prepared in the same way. When this condition is met, and the amount of the substrates (μ moles) as
well as the radio-activity of the sample and free fatty acid fraction are known the enzyme activity is calculated as usual following the formula given below.

\[
\frac{\text{Cpm in total sample}}{\text{Substrate (\text{mole}) \times ester bond in sample}} = \text{Specific activity of the fatty acid}
\]

\[
\frac{\text{Cpm obtained in FFA}}{\text{Specific activity of fatty acid}} = \text{Micromoles of fatty acid released}
\]

Preparation of Pancreatic Oil

The hepatopancreatic oil was prepared by extracting the lipid from freshly collected hepatopancreas (10 gm) with chloroform : methanol mixture (2:1 v/v, Folch et al. 1957). The chloroform was completely removed under reduced pressure at 50°C and stored under stream of nitrogen until used.

pH Optima

The effect of pH on the hydrolysis of different substrates was estimated by adjusting the reaction mixture to the various pH levels prior to the addition of enzyme source.
Time Course Hydrolysis

The rate of hydrolysis of different substrates against incubation time was determined incubating the standard reaction mixture at 30°C with a constant shaking. The tubes were removed at desired intervals of time for free fatty acid analysis.

Determination of Dry Weight of Tissue

The dry weight of the different tissues was determined by drying 1 ml duplicate sample of the homogenate for 24 hours at 100°C and kept in vacuum desicator till constant weight was obtained.

RESULTS

The results of the titrimetric determination on the hydrolysis of tributyrin, triolein and hepatopancreatic oil by the hepatopancreas and leg muscle preparations are summarised in Table 1. All tissue preparations hydrolysed tributyrin at a greater rate than that of triolein and hepatopancreatic oil. Among the three tissues hepatopancreas exhibited highest rate of hydrolysis with all the three substrates, next was m.flexor and least was found with m.extensor. It is
Table 1: Summary of the Hydrolities of Triolein and Tributyrin by the
Hepatopancreas, M. Flexor, and M. Extensor of the Field Crab.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Hepatopancreas</th>
<th>M. Flexor</th>
<th>M. Extensor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tributyrin</td>
<td>6.5 ± 0.15</td>
<td>1.2 ± 0.01</td>
<td>1.5 ± 0.01</td>
</tr>
<tr>
<td>Triolein</td>
<td>2.9 ± 0.09</td>
<td>0.8 ± 0.09</td>
<td>0.2 ± 0.02</td>
</tr>
</tbody>
</table>

Results indicate mean ± S.D. of five experiments for each substrate. One enzyme unit = amount of 0.05 N NaOH required to neutralize the fatty acid produced in the reaction.

* Control values were subtracted from experimental values. ** Control values were subtracted from experimental values. Each value represents the net release of fatty acid value in the reaction mixture.
interesting, however, to note that all the tissue preparations showed higher rate of hydrolysis with pancreatic oil than that of triolein, but comparatively less than tributyrin.

Table 2, indicates the result of hydrolysis of labelled tri-, di- and monoacylglycerol by the different tissue preparations. It is evident from the results that hepatopancreas compared to leg muscle preparations demonstrates higher rate of hydrolysis with all the three labelled substrates. Between the two muscle preparation of m.flexor seems to be better equipped with lipolytic activity than m.extensor. The major neutral lipid fraction in the hepatopancreas is triacylglycerol. Presence of different lipase system (s) operating on mono, di-, and triglycerides has been suggested in Vertebrates. (New Sholme and Statk, 1970). The results of the present results seem to indicate that the hepatopancreas contain different lipase systems with preponderance of triacylglycerol lipase. The hydrolysis of triacylglycerol in the hepatopancreas would result in the production of diacylglycerol and free fatty acids. Although, we have no precise information on the mode of
**TABLE 2**

Hydrolysis of 14C tri-, di- and mono-acylglycerols by the different tissue substrates.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Hepatopancreas</th>
<th>M. flexor</th>
<th>M. plecor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triacylglycerol</td>
<td>1.97 ± 0.002</td>
<td>7.31 ± 0.32</td>
<td>5.09 ± 0.15</td>
</tr>
<tr>
<td>Diacylglycerol</td>
<td>5.21 ± 0.031</td>
<td>5.89 ± 0.15</td>
<td>4.37 ± 0.15</td>
</tr>
<tr>
<td>Monoacylglycerol</td>
<td>1.72 ± 0.001</td>
<td>2.31 ± 0.05</td>
<td>0.59 ± 0.003</td>
</tr>
</tbody>
</table>

*Results indicate mean ± S.D. of six experiments for each substrate.*

Fatty acids of rats and mice liver by the different tissues.

**PART 2: HYDROLYSIS OF 14C TRI- AND MONOACYLGLYCEROLS BY THE DIFFERENT TISSUES**
lipid transport in the field crab, it may be proposed that diacylglycerol and free fatty acids are released into haemolymph and are transported to muscle tissues. Where diacylglycerol presumably be hydrolyzed to free fatty acids for oxidation. The higher diacylglycerol lipase activity in m.flexor compared to the other two glycerides (mono and triacylglycerols) supports this hypothesis.

The effect of pH on the rate of hydrolysis of mono-, di- and triacylglycerol by the hepatopancreas and m.flexor revealed some interesting differences. pH optima for hydrolysis of mono-, di- and triacylglycerol by the hepatopancreas were 7.4 (mono- and di-) and 7.6 to 8.0 for triacylglycerol. It is interesting to note that the hepatopancreas exhibits wide range of pH optima while m.flexor demonstrated only one pH optima (7.6) for mono-, di- and triacylglycerol hydrolysis.

The time course hydrolysis of mono-, di- and triacylglycerol by the hepatopancreas and m.flexor are depicted in Fig. 3 and 4. In the case of m.flexor and hepatopancreas the hydrolysis of all the three glycerol fraction occurred almost after 30 min of incubation and
<table>
<thead>
<tr>
<th>Substrates</th>
<th>Incubation hour</th>
<th>1.0</th>
<th>8.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoacylglycerol</td>
<td>0.6 ± 0.39</td>
<td>2.3 ± 0.16</td>
<td></td>
</tr>
<tr>
<td>Diacylglycerol</td>
<td>0.8 ± 0.6</td>
<td>5.82 ± 0.63</td>
<td></td>
</tr>
<tr>
<td>Triacylglycerol</td>
<td>0.9 ± 0.12</td>
<td>2.2 ± 0.19</td>
<td></td>
</tr>
</tbody>
</table>

Results indicate...
FIG: HYDROLYSIS OF MONO DI AND TRIACYLGLYCEROL BY THE M FLEXOR IN RELATION TO INCUBATION TIME.
TABLE 4 : HYDROLYSIS OF MONO-, DI- AND TRIGLYCERIDES IN RELATION TO INCUBATION TIME

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Incubation hour</th>
<th>1.0</th>
<th>8.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoacylglycerol</td>
<td></td>
<td>2.3 ± 0.32</td>
<td>6.3 ± 0.16</td>
</tr>
<tr>
<td>Diacylglycerol</td>
<td></td>
<td>1.84 ± 0.23</td>
<td>6.6 ± 0.13</td>
</tr>
<tr>
<td>Triacylglycerol</td>
<td></td>
<td>0.952 ± 0.13</td>
<td>5.82 ± 0.29</td>
</tr>
</tbody>
</table>

Results represent...
FIG: HYDROLYSIS OF MONO-DI-AND TRIACYLGlyCEROL BY THE
HEPATOPANCREAS IN RELATION TO INCUBATION TIME

![Graph showing hydrolysis of mono-di- and triacylglycerol by the hepatopancreas in relation to incubation time.](image-url)
FIG: HYDROLYSIS OF MONO-DI-AND TRIACYLGLYCEROLS BY THE M.FLEXOR AT DIFFERENT pH.
FIG: HYDROLYSIS OF MONO-DI-AND TRIACYLGLYCEROL BY THE HEPATOPANCREAS AT DIFFERENT pH.
was fairly linear up to 5 hours. The hydrolysis of triacylglycerol by the hepatopancreas was linear up to eight hours of incubation, while the hydrolysis of mono- and diacylglycerol was reduced gradually after four hours. The decline in the hydrolysis of the two latter substrates cannot be explained with these preliminary experiments. It may be due to the saturation of the incubation medium with free fatty acids and/or may also be due to the insufficient supply of substrates to the active diacyl- and monoacylglycerol lipases present in the muscle.

**DISCUSSION**

The evolutionary success of the class Crustacea has been facilitated by the animal's ability to store large quantities of fat and use this substrate as a source of energy for various life processes and as a source of metabolic water to supplement strategies of water conservation. Fat is stored in the hepatopancreas in the form of triacylglycerol and ester hydrolysis is a necessary prelude to oxidation of the fatty acids. The hydrolysis of lipid is of particular significance in the hepatopancreas when one considers the role of this tissue in lipid metabolism. Unfortunately qualitative
nature of lipid release and mode of transport in crustacea under different physiological conditions, especially with reference to molt cycle has not been clearly defined. There are reports to show that 65% of the total lipid present in the haemolymph is in the form of phospholipid which seems to be characteristic of crustaceans. (Allen, 1972; Bligh and Scott, 1966; Huggins and Munday, 1968; Lee and Puppione, 1978; Brichon et al. 1980). Recent reports have demonstrated the presence of lipoproteins in the haemolymph of decapod crustaceans. (Allen, 1972; Fielder et al. 1971; Lee and Puppione, 1978). It is worthwhile to mention in this context that in the field crab also the neutral lipid content is about 40%. (Chapter 7).

It has been well recognized in majority of organisms investigated in respect of role of lipid in energy metabolism that neutral lipid constitutes the chief source of energy for all vital processes. (George and Berger, 1966; Gilbert, 1967; Drummond, 1970; Fest, 1970; Gilbert and O'Conner, 1970; Lawrence, 1976 and Downer, 1978).

In vitro and in vivo experiments conducted by numerous workers on insect fat body, analogous tissue of
hepatopancreas, have shown that specific lipids are released from fat body in response to factor in the haemolymph probably hormonal in nature, (Beenakkers, 1969; Mayer and Candy, 1969; Goldsworthy et al. 1972, and Spencer and Candy, 1976), based on insect species. (Chino and Gilbert, 1965b; Tietz, 1967; Wlodayer and Lagwinska, 1967, Chang and Friedman, 1969 and Gilbert and Chino, 1974) depending on physiological exercise (Kallapur, 1978). Crucial to understanding of lipid transport is the part played by lipase(s).

The major areas in which lipolytic activity in crustacea has been dealt with, is found in relation to digestion and absorption. (For review see Van Weel, 1970). The main tissue in which extra digestive lipase activity would be expected to be found in hepatopancreas, muscle and haemolymph.

It is evident from the present results that hepatopancreas as well as muscle preparations are capable of hydrolyzing tributyrin at a faster rate than triolein and hepatopancreatic oil. However, considerable ambiguity concerning the use of tributyrin (a short-chain fatty acid ester) as a substrate for the study of lipolysis exists. Lipid hydrolysis (lipolysis) is effected in the
presence of lipase(s), a class of hydrolytic enzymes that has been defined in terms of specificity for long chain fatty acylglycerol esters (Gilbert, 1967) and capacity to hydrolyze the esters of emulsified glycerides at an oil water interface (Jensen, 1971). These definition serve to distinguish lipases from the more general group of hydrolytic enzymes, the esterases, which catalyze the hydrolysis of ester linkages irrespective of chain length or solubility. In the light of these observations, the use of emulsified tributyrin as a substrate for the study of lipolysis does not satisfy the criterion of chain length specificity. According to Gilbert (1967) tributyrin is not a natural substrate hence it has little physiological significance and is not usually present in the animal tissue although the rate of this ester may be an indicator of general metabolic activity. In this context the findings of Van Aspren (1959) who demonstrated the presence of potent aliesterase activity in various body regions of Musca domestica and found 22% of esterase activity responsible for its hydrolysis of tributyrin to be localized in the head region of the insect was of considerable interest. Okuda and Fugi (1967), on the other hand have demonstrated that rat liver lipase could easily be converted into
esterase by acetone treatment or by pancreatic lipase. The authors have suggested a single enzyme that catalyzes the hydrolysis of both short and long chain lipids. Different lipase (s) systems that are responsible for the hydrolysis of long and short chain fatty acid glycerides, one of which has been named as "tributyrinase" and has specificity for short chain glycerol ester such as tributyrin. The result of the present investigation have also indicated that hepatopancreas as well as muscle preparations are capable of hydrolyzing triolein (ester of long chain fatty acid) and hepatopancreatic oil (Table 1) to a lesser degree. It is of great interest, however, to note that between the two substrates hepatopancreatic oil was hydrolyzed to a greater extent by both tissues. This difference in the hydrolytic rate may reflect the choice of natural (hepatopancreatic oil) than artificial substrate (triolein) employed in this investigation or may also suggest that the hepatopancreatic oil may contain larger proportion of short-chain fatty acids, thereby acted upon by both esterases and lipases.

The hydrolysis of labelled tri-, di- and monoacylglycerols by different tissue preparations also revealed some interesting differences (Table 2).
Considerable variations in the levels of lipase activity is noticed in the two tissues of the field crab *P. hydrodromus*. However, these *in vitro* studies may not reflect the *in vivo* activity of the enzyme. In this study the hepatopancreas and leg muscles (*m. flexor* and *m. extensor*) preferentially hydrolyzed labelled triacylglycerol and diacylglycerol respectively at a faster rate. It may be proposed from these studies that the hydrolysis of triacylglycerol in the hepatopancreas results into diacylglycerol and free fatty acids. The appreciable level of diacylglycerol lipase activity in *m. flexor* and *m. extensor* lends credence to the idea that diacylglycerol are preferentially hydrolyzed in order to liberate free fatty acids for muscular energy. The results also serve to indicate that both hepatopancreas and muscle contain more than one lipase species.

With the realization that precise characterization of the enzyme from a quantitative point of view cannot be obtained, with a crude enzyme preparation as used in the present study; the pH optima and time course hydrolysis were nevertheless determined. Lipase from hepatopancreas showed wide range of pH optima (7.0 to 8.0) whereas the *m. flexor* demonstrated fairly narrow range of pH optima (7.4 to 8.0). The hydrolysis of different glycerides as a
function of time by hepatopancreas as well as m. flexor also showed the superiority of former tissue over the latter one.

The lipolytic activity and lipid transport appear to be integral part of the general lipid metabolism of the animal under study. Previous studies (see Gilbert and O'Connor, 1970) have identified the need for precise definition of physiological conditions of the test animal prior to experiments. This phenomenon is of particular importance in the study of hepatopancreas which shows considerable fluctuations in its lipid content. As can be deduced from the present preliminary investigations much more work is necessary to clarify the role of lipase in crustaceans, which would have a profound effect on lipid mobilization.