CHAPTER IV

NATURE OF THE LIPIDS UTILIZED DURING FLIGHT OF THE DRAGONFLY *PANTALA FLAVESCENS* AND OF THE BEETLE *CYBISTER CONFUSUS*
It is well recognized now that the lipid is the main fuel of quantitative importance during sustained flight in several insects. One aspect of the lipid metabolism that has received increasing attention in recent years is that of lipid release and transport, from the storage tissue to the working muscles. Depot fat of many insects is usually in the form of triglycerides (TG) (Gilmour, 1965). For utilization by the insect during active flight this fat needs to be transported to the working muscles. Several observations on lipid release and transport in insects add credence to the opinion that diglyceride (DG) is the material released from the fat body into haemolymph (Chino and Gilbert, 1964; 1965; Mayer and Candy, 1967; Beenakkers, 1967; Thomas and Gilbert, 1968; Bhakthan and Gilbert, 1968 and 1970; and Beenakkers and Gilbert, 1968). On the other hand Wlodwer and his co-workers (Wlodawer et al., 1966; and Wlodawer and Lagwinska, 1967) obtained major radioactivity in the larvae of the waxmoth *Galleria* in the free fatty acids (FFA) as well as in the TG fraction of the haemolymph. The authors have suggested that the waxmoth larva releases into haemolymph FFA which are partly resynthesized into TG in the haemolymph. Similarly gravimetric, colorimetric and radioisotopic analyses of Cook and Eddington (1967) in the cockroach
Periplaneta americana have shown that FFA are the major lipids released from the TG of the fat body.

Studying the lipolytic activity in the flight muscles of the dragonfly Pantala flavescens and of the beetle Cybister confusus it has been noted (Chapter III) that the enzyme activity in the former insect was almost twice with DG than that of TG, while the flight muscles of the latter insect produced free fatty acids more readily with TG than that of DG. These results lend credence that DG and TG are the lipid fractions utilized preferentially by the flight muscles of Pantala and Cybister respectively. To test this premise, comparative estimate of the loss or gain of various categories of lipid (mono-, di- and triglycerides as well as fatty acids) in the flight muscles of Pantala and of Cybister as well as in the fat body and haemolymph of the latter insect were ascertained before and after flight of both the insects.

MATERIALS AND METHODS

The dragonflies Pantala flavescens used in this investigation were caught in the fields, usually in the evening times. The beetles Cybister confusus were chosen from the laboratory stock. The fresh weight of the beetles and the dragonflies used fell within a range of 3.63 to
to 3.65 gm. and 0.43 to 0.45 gm., respectively. The insects were starved for 12 hr. before they were subjected to induced flight. The procedure followed to induce flight in these insects was similar to that described in the Chapter II. At the end of flight activity lasting upto 120 min., they were killed by decapitation immediately. Control experiments were carried out on the muscles taken out from those which have not been subjected to induced flight.

Preparation of Tissues:

Several attempts made to collect haemocoelic fluid from the dragonfly Pantala failed. In this study analysis of the haemolymph lipid from the Cybister beetle has been carried out. To collect the body fluid the head of the beetle was removed along with the alimentary canal; the blood was sucked into a graduated micropipett (usually about 0.02 ml./beetle). Thus the blood from two beetles was collected in a cold centrifuge tube and centrifuged in a refrigerated centrifuge after which, the lipid from the supernatant was extracted according to the method of Folch, et al., (1957) and the lipid was stored under nitrogen atmosphere at 0°C.
Flight Muscles:

The flight muscles of the *Pantala* (meso- and metathoracic regions) as well as of the *Cybister* (only metathoracic muscles) were removed separately and stored at 0°C until used.

Fat Body:

After removing the flight muscles, the fat body of the *Cybister* beetle was collected mostly from the abdominal region. The fat body was exposed by removing the tergal plates, and it was removed carefully with the help of forceps. It was stored at 0°C until used.

Extraction of Lipid and Its Analysis:

The total lipid was extracted from the flight muscles and fat body following the method of Folch, *et al.*, (1957) using chloroform - methanol 2:1 (v/v) mixture. The detailed extraction procedure has already been described elsewhere (Chapter II).

Separation of Neutral and Phospholipids:

A known amount of lipid (usually 50 to 60 mg.) from both the muscles and fat body was subjected to silicic acid (Mallinckrodt, U.S.A.; 100 mesh) column chromatography for their neutral and phospholipid separation. The neutral
lipid along with the free fatty acids and phospholipids were eluted with 150 ml. of dry (peroxide free) diethyl ether and distilled methanol respectively. Further separation of neutral lipid into individual glycerides and FFA was effected on thin layer chromatographic (tlo) plate.

Thin Layer Chromatography:

A slurry of silicagel (National Chemical Laboratory, India) and plaster of paris (34:6 wt/wt) in 80 ml. of distilled water was spread on glass plates (20 cm. x 20 cm.) of 500 micron thick. The plates were dried in an oven at 100°C and cooled to room temperature. A suitable aliquot (100 µl) of the mixture of neutral lipid and FFA from each tissue as well as of the total lipid from the haemolymph in chloroform was applied on separate plates, and the lipids were resolved into triglyceride (TG) free fatty acids (FFA), 1,3 diglyceride (1,3 DG) 1,2 diglyceride (1,2 DG) and monoglyceride (MG) by developing with n-hexane: diethyl ether:acetic acid (90:18:1.5 v/v/v). A standard mixture of glycerides and fatty acids. (Generously supplied by Dr.F.H.Mattson) was run each time along with the tissue samples. After development the spots were made visible by exposing the plates to iodine vapour and were marked under the
ultraviolet light. Individual spots were scraped out and were transferred into small chromatographic tubes from which individual glycerides and FFA were eluted with 50 ml. of peroxide free diethyl ether.

**Estimation of Glycerides:**

Individual glycerides were estimated according to the method described by Raghven and Ganguly (1967). After evaporating the diethyl ether, the dried tlc samples were saponified with 1 ml. of 2% alcoholic KOH at 60°C to 70°C for 30 min., after which 1 ml. of 8% HCl was added. The liberated glycerol was treated with 0.1 ml. sodium metaperiodate (0.05 M) for 15 min. in a semi-dark chamber and the colour was developed by the addition of 0.5 ml. phenylhydrozene hydrochloride reagent (0.125 M). At the end of 10 min. 0.2 ml. of potassium ferricyanide solution (0.137 M) was added, followed by 2.5 ml. of concentrated HCl. The volume was made upto 10 ml. with distilled water and the colour was immediately read on "Specol" photoelectric colorimeter at 540 mp. The amount of individual glycerides were calculated from a standard graph prepared from the glycerol. The results are expressed as µg glycerol released per 100 mg. wet wt. of tissue.
Fig. VIII

STANDARD CURVE FOR GLYCEROL

OPTICAL DENSITY

MICROGRAM GLYCEROL
Determination of Fatty Acids:

The total fatty acids (TFA) and free fatty acid (FFA) content of the different tissue samples were estimated colorimetrically following the method of Lauwerys (1969) with some modifications. The TFA content of the tissue was estimated as follows: A known quantity of lipid thus extracted from the tissue was saponified with alcoholic KOH at 60°C for 60 min. After removing the non-saponifiable lipid, the aqueous layer was acidified with a few ml. of concentrated HCl; the liberated TFA was then extracted with solvent ether. The ether layer containing TFA was thoroughly washed with distilled water to remove the HCl. The washed ether layer was removed under reduced pressure and TFA was redisolved in known vol. of chloroform. A known aliquot in triplicate was assayed for TFA content of the different tissues. To estimate the FFA, the spot from the tlc plate was collected as described above. The dried samples of TFA and FFA collected in test tubes were vigorously shaken with 3 ml. petrol – chloroform mixture (1:1) plus 2.5 ml. of copper reagent consisting of: 7 ml. triethanolamine; 0.3 ml. glacial acetic acid; 3.25 gm. Cu(NO₃)₂; 6.25 gm. of K₂SO₄; 17 gm. Na₂SO₄ and water to give a final volume of 100 ml. The density of this solution was greater than petrol. The tubes were then centrifuged at 3,000 rpm for
Fig. IX

STANDARD CURVE FOR FREE FATTY ACIDS USING PALMITIC ACID
10 minutes. A known aliquot of the petroleum layer was taken and then added 0.5 ml. of 0.1% (w/v) sodium diethyl dithiocarbamate prepared in n-butanol. After mixing, the density of the colour was read at 440 mu against a reference solution of 3 ml. petrol - chloroform mixture (1:1) and 0.5 ml. sodium diethyl dithiocarbamate reagent. The results obtained were corrected with reference to a blank and were calculated from a standard curve obtained for palmitic acid (Fig. IX).

RESULTS

The results of the present investigation are summarized in the table IX and X. The total lipid content in the flight muscles of the Pantala was about 9% (by fresh wt.) of which, neutral lipid constituted 66% (of the total lipid), whereas the corresponding values in the flight muscles of the Cybister were 7% and 64% (of the total lipid). The total glycerides and total fatty acid content in the flight muscles of the Pantala respectively before and after flight were 7.68 mg. and 6.6 mg./100 mg. wet wt. of muscle, whereas its counterpart in the Cybister beetle were 5.66 mg. and 5.19 mg./100 mg. wet wt. of muscle. The FFA content in the flight muscles of the Pantala before flight constituted 12.7% and was increased to 17.58% after flight, while the increase
Table IX. Changes in the glycerides and fatty acid levels in the flight muscles of the dragonfly *Pantala flavescens* after a flight period of 120 min.

<table>
<thead>
<tr>
<th></th>
<th>Unflown</th>
<th>%</th>
<th>Flown</th>
<th>%</th>
<th>Difference</th>
<th>Increase/decrease over the unflown %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoglyceride</td>
<td>(7)</td>
<td>450 ± 49.30</td>
<td>5.8</td>
<td>600 ± 65.00</td>
<td>7.8</td>
<td>150</td>
</tr>
<tr>
<td>1,2 diglyceride</td>
<td>(7)</td>
<td>1100 ± 79.00</td>
<td>14.32</td>
<td>230 ± 32.26</td>
<td>2.3</td>
<td>870</td>
</tr>
<tr>
<td>1,3 diglyceride</td>
<td>(7)</td>
<td>1000 ± 38.00</td>
<td>13.02</td>
<td>300 ± 12.60</td>
<td>3.9</td>
<td>700</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>(7)</td>
<td>1350 ± 117.5</td>
<td>17.99</td>
<td>1017 ± 113.0</td>
<td>13.29</td>
<td>333</td>
</tr>
<tr>
<td>Free fatty acid</td>
<td>(7)</td>
<td>980 ± 62.5</td>
<td>12.76</td>
<td>1350 ± 77.5</td>
<td>17.58</td>
<td>370</td>
</tr>
<tr>
<td>Total fatty acid</td>
<td>(4)</td>
<td>2800 ± 220.0</td>
<td>32.55</td>
<td>3100 ± 460.0</td>
<td>40.56</td>
<td>300</td>
</tr>
<tr>
<td>Total neutral lipids and fatty acid content</td>
<td>7.68(mg.)</td>
<td>6.6(mg.)</td>
<td>1.08(mg.)</td>
<td>-14.06</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The values indicate mean ± standard error.

The number of insects used is indicated in parenthesis.
in the FFA content in the flight muscles of the Cybister beetle before and after flight was 12.72 to 17.5%. The total fatty acid content of the flight muscles of both the insects was slightly increased after flight (table IX and X).

It is seen from the table IX that the 1,2 and 1,3 DG content in the flight muscles of the Pantala after flight were considerably reduced (79.3%), whereas TG fraction got depleted to a very less extent (24.67%). In the Cybister beetle on the other hand after flight, the flight muscles lipid revealed no such significant changes in the 1,2 DG (16%) and 1,3 DG (13.79%), while TG content got reduced considerably (table X).

The total glycerides and fatty acids content in the fat body of the Cybister beetle was almost double than that of its counterpart in the flight muscles (13.5 mg./100 mg. wet wt. of tissue) of the same insect. The analysis of the fat body neutral lipid exhibited a significant decrease in 1,2 DG (52.3%) and FFA (49.34%) content but only a moderate reduction in TG (21%) after flight. The total lipid analysis of the haemolymph of the same insect has indicated a considerable increase in the FFA (66.6%) content as well as a slight increase in the TG (88.00%) after flight, while 1,2 and 1,3 DG remained unaffected. The total flight muscle weight of the Cybister beetle and of the Pantala respectively
Fig. X: Oxidation of butyrate, octanoate, stearate, and palmitate by the flight muscle homogenate of the dragonfly Pantala flavescens.

O$_2$ uptake (μl/mg protein/30 minutes)

- Endogenous
- Butyrate
- Octanoate
- Stearate
- Palmitate
- Palmitate + Carnitine
Fig. XI  OXIDATION OF BUTYRATE, OCTANOATE, STEARATE AND PALMITATE BY THE FLIGHT MUSCLE HOMOGENATE OF THE CYBISTER BEETLE
is 580 mg. and 120 mg. (on average). On the basis of the reduction in the neutral lipids and fatty acids of the flight muscles, it can be calculated that, the flight muscles of the Cybister and of the Pantala have utilized neutral lipids and fatty acids approximately at a rate of 23.0 µg. and 10.7 µg./insect/min. respectively. It could be expected then than the amount of neutral lipids and fatty acids of the flight muscles in each case would support the flight more than 8 hr. in both the cases.

DISCUSSION

During the flight of the migratory locust considerable increase in total fatty acids content was demonstrated in the haemolymph and a slight one in the flight muscles indicating their transport from the storage organ to the muscles (Beenakkers, 1965). The author concludes that the amount of total body fat would support locust flight for about 5 hr. and it will be longer than that if carbohydrate reserves is also included. The lipid content in the flight muscles of the dragonfly Pantala flavescens as well as in the Cybister confusus were found to be respectively 9% and 7% (by fresh wt.). The FFA content in the flight muscles of both insects increased considerably, while the total fatty acid content on the other hand was moderately increased, after a flight period of 120 min. (table IX and X). While
discussing the lipolytic activity in the flight muscles of Pantala as well as of Cybister and in the fat body of the latter insect, it has been found that the lipase activity of the fat body of the Cybister beetle was as great as seven times of its flight muscles. It is possible therefore that the significant increase in FFA content of the flight muscles of the Cybister beetle may perhaps be due to the transport of FFA from the fat body resulted from the hydrolysis of its glycerides as well as the glycerides of the flight muscles. In Pantala on the other hand the significant increase in the FFA content appears to be due to the hydrolysis of glycerides of the flight muscles alone, since this insect has no distinct fat body in the haemocoel. It can be calculated from the rate of reduction after flight in the neutral lipid and fatty acid content of the flight muscles that the amount of muscle lipid alone would support flight activity for about 6 to 8 hr. in both the insects. However, a good portion of lipid in the muscles may be present as a structural component. It has already been shown (Chapter II) that in the Cybister beetle the major fuel during flight is contributed by the fat body, while in Pantala it was assumed that the fuel reserves is distributed between the muscles and blood.
Utilization of Glycerides During Flight:

In *Cecropia* flight muscles Gilbert *et al.* (1965) found greater lipolytic activity towards DG than towards TG. The authors have suggested that the DG enters the flight muscles whereupon the fatty acids are cleaved from the glycerol ester and rapidly oxidized in the flight muscles. In *Pantala* after a flight exercise lasting for 120 min, there are significant decrease in DG in the flight muscles. The decreases in TG is nonsignificant; so also the increase in MG. The possible sources from which fat is utilized for flight in this insect are the haemolymph and the muscle tissue itself. Since some portion of the FFA, DG and MG met with in the muscles during exercise are breakdown products of TG. It is presumed that they are thus produced in the muscle tissue itself and are transported from the haemolymph. Sufficient quantity of haemolymph were not available for analysis from this insect and as such the amount derived from each tissue could not be separately assessed. The lipase activity in the flight muscles of the *Pantala* was five times more towards DG than towards TG (Chapter III). These results thus lead to believe that the flight muscles of *Pantala* preferentially utilize DG during its sustained flight as was found in *Cecropia* (Gilbert *et al.*, 1965) and in *Locusta migratoria* (Crabtree and Newsholme, 1972).
In the *Cybister* beetle the amount of fat in the form of glycerides and FFA in the flight muscles is less than that of the *Pantala*. The lipid analysis of the flight muscles of *Cybister* after the flight activity (90 min.) revealed that the TG fraction of the flight muscle lipid got reduced, while DG remained unchanged (Table X). This certainly leads to believe that TG must have been used up during its flight activity. It has been found in the preceding study that (Chapter III) the lipase activity in the flight muscles of this insect with TG as substrate was more active than DG as substrate.

**Release of Glycerides from the Fat Body During Flight of the Beetle *Cybister confusus***:

The fat body which is the main storage organ for most insects not only takes up and accumulates lipids, but also releases them into the haemolymph where they were transported to various sites of utilization. During flight of *Locusta migratoria* the amount of glycerides as well as FFA increased in the haemolymph (Weis-Fogh, 1965). Beenakkers (1965) in the same insect found a considerable increase in the total fatty acid content in the haemolymph, while, FFA remained unchanged after flight. Tietz (1962) found that glycerides were released from the locust fat body when incubated in
haemolymph. Later on Chino and Gilbert (1965) confirmed this finding for the fat body of Cecropia and proved that the glycerides released into the haemolymph were mostly DG suggesting thereby DG as a means of lipid transport. Similar observations of increased DG in the haemolymph has been reported by Beenakkers (1969) in locust and Bhakthan and Gilbert (1970) in Manduca sexta. In the Cybister beetle unlike Pantala there is distinct fat body present in the haemocoel. It has already been pointed out that during sustained flight the major fuel reserves is contributed by the fat body (Chapter II). It is seen from the table X that 1,2 DG and FFA content of the fat body got depleted significantly with concomitant increase in the FFA content of the haemolymph. The lipid analysis of the haemolymph on the other hand revealed a slight increase in the TG (20%), whereas DG remained practically unaffected. Mostly DG is hydrolytic product of TG. The depletion of DG in the fat body without a concomitant increase of it in the haemolymph would therefore indicate that DG fraction of the fat body probably must have been further hydrolyzed and resulting fatty acids are released into the haemolymph, since fat body lipase is very active towards DG also. In this context the observations of Wlodawer and his associates (1966 and 1967) that in the larvae of the waxmoth Galleria pre-labeled...
fat body released FFA into the haemolymph and they are subsequently incorporated into TG by an active lipase in the blood are of considerable interest. Similarly In vitro experiments of Cook and Eddington (1967) with analytical as well as isotopic methods have found that the TG and FFA are the one released into the haemolymph from the fat body of Periplaneta americana. The results obtained on the Cybister beetle with respect to release of glycerides from the fat body into the haemolymph appear to support the findings of Woldawer and co-workers (1965 and 1967) in that the first stage of hydrolysis of glycerides takes place in the fat body and resulting FFA are released into haemolymph which are partly transported to the working muscles and partly resynthesized into TG.

**SUMMARY**

1. In *Cybister confusus* the amount of fat in the form of glycerides and fatty acids (5.66 mg./100 mg. wet wt. of tissue) in the flight muscles is less than that of *Pantala flavescens* (7.68 mg./100 mg. wet wt. of tissue). But the fat body in this insect is a very rich source of fat having almost double that of the muscles (13.5 mg./100 mg. wet wt. of tissue). The haemolymph also contains a lot of fat (4.4 mg./ml.). After a flight of 120 min. the
fat body loses more than 25% of its fat, which is more than what the flight muscles lose. The haemolymph on the other hand gains total fat reflecting thereby that its gain of fat is from the fat body.

ii. The levels of the various neutral lipid fractions as well as of the fatty acid content in the flight muscles of both insects after a flight period of 120 min. is indicated below:

<table>
<thead>
<tr>
<th>Individual glycerides</th>
<th>Pantala</th>
<th></th>
<th>Cybister</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before flight</td>
<td>After flight</td>
<td>Before flight</td>
<td>After flight</td>
</tr>
<tr>
<td>Monoglyceride</td>
<td>5.85</td>
<td>7.8</td>
<td>8.1</td>
<td>7.06</td>
</tr>
<tr>
<td>1,2 diglyceride</td>
<td>14.32</td>
<td>2.3</td>
<td>4.24</td>
<td>3.53</td>
</tr>
<tr>
<td>1,3 diglyceride</td>
<td>13.02</td>
<td>3.9</td>
<td>10.24</td>
<td>8.83</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>17.99</td>
<td>13.29</td>
<td>31.10</td>
<td>17.67</td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>12.76</td>
<td>17.58</td>
<td>12.72</td>
<td>17.49</td>
</tr>
<tr>
<td>Total fatty acids</td>
<td>32.55</td>
<td>40.36</td>
<td>33.57</td>
<td>37.10</td>
</tr>
</tbody>
</table>

iii. With regard to the form of fat utilized in flight, the preference goes to the fatty acids, since they are increased in amount in the flight muscles of both the insects after a flight of 120 min. In the Cybister beetle they are increased in the haemolymph also. These increases are presumed to be for the sake of energy production in the muscles. There is no conclusive indication to the effect that glyceride as such can be utilized during flight by the insect.