SECTION — B

FUEL UTILIZATION BY THE LEG MUSCLES OF THE CYBISTER CONFUSUS DURING INDUCED SWIMMING ACTIVITY
I. Evidences for Carbohydrate Utilization by the Leg Muscles

1. Glycogen Content of the Leg Muscles as well as of the Fat Body and its Reduction During Swimming Exercise:

It is widely recognized now that the metabolic energy in the working muscles is provided by the oxidation of fuel stuffs by atmospheric $O_2$ with concomitant production of $CO_2$. The RQ has been of value by virtue of inference to judge the kind of substance undergoing oxidation. Such determinations have indicated that most of Hymenoptera and Diptera have the RQ during flight equal to unity, suggesting the combustion of carbohydrates (Chadwick and Gilmour, 1940; Chadwick, 1947; Williams et al., 1943; Wigglesworth, 1949; Hocking, 1953). Quantitative measurements on the changes in the total body glycogen produced by sustained flight have been made by Williams, et al., (1943) using Drosophila funebris and Lucilia sericata and by Hudson (1958) using Phormia regina, and in each case a progressive fall in glycogen was found during flight. A similar reduction in glycogen content of mosquitoes has been observed by Rowley (1970), and Nayar and Van Handle (1971). Wigglesworth (1949) reported that during continuous flight of Drosophila there was a general utilization of glycogen from all depots,
including muscles and fat body. Examination of *Culex pipiens* after flight to exhaustion also revealed an almost complete disappearance of glycogen from flight muscles and fat body (Clements, 1955). There are other data, however, which indicate that the utilization of glycogen takes precedence over the sugars held in the gastrointestinal tract (Hudson, 1958). From these results it seems that in Hymenoptera and Diptera during intense muscular activity the glycogen reserves and sugars from many sources of the insect body are mobilized and directed to the working muscles to meet the increasing energy demand.

The aquatic dytiscid *Cybister confusus* has an efficient leg muscle system. The whitish leg muscles by their rapid contractions enable the beetle not only to swim fast but also to take quick turns in the aquatic medium. It should be expected that the energy transformation should proceed vigorously during swimming. It is possible therefore, that the leg muscles have an efficient biochemical machinery to meet the exigencies of energy demands for all its spasmodic actions. George and Nene (1965) and Parkar and George (1972) have reported that the white fibres of pigeon breast muscle indulge only in short bursts of activity such as for the bird's quick take-off or rapid movements, using glycogen as the chief
fuel, though in sustained flight fat is utilized by the red fibres. It is therefore anticipated that the Cybister beetle may be utilizing its glycogen reserves during its all acrobatic movements. The present investigation is therefore aimed at ascertaining, if a preferential utilization of glycogen pool of the muscles to begin with and later of the fat body takes place during induced swimming performance. The present opportunity was also availed of to see whether, the sugars held in the gastrointestinal tract would contribute towards such a muscular activity.

MATERIAL AND METHODS

Adult Cybister confusus beetles were selected from a number of them kept in the laboratory.

Induced Swimming Activity:

15 to 20 male beetles were chosen for the experiment. The fresh weight of each beetle selected fell within a range of 3.63 gm. to 3.69 gm. with an average of 3.67 gm. They were starved for 24 hr. before they were induced to swimming activity.

In order to make beetles swim vigorously they were introduced into an aquarium designed for this purpose (Fig. II).
They were kept under continuous swimming by letting a forceful water jet from one side. At the end of each 10 min. interval of exercise they were killed by decapitation. The leg muscles, fat body and gastrointestinal tract were removed separately and stored at 0°C until used. Two sets of controls were used, one set of beetles was killed almost immediately at the onset of the experiment and the other at the regular intervals of time along with the experimental ones.

Extraction and Estimation of Glycogen:

A known quantity of tissue taken in a centrifuge tube was digested with 20% alcoholic KOH in boiling water. After complete digestion of the tissue an equal volume of 95% ethanol was added to each extract, and left overnight at 4°C to precipitate the glycogen. The tubes were later centrifuged and the supernatant poured off. The washed residue was dissolved in a known volume of distilled water. A known aliquot in each case was analysed for glycogen content by the anthrone method (Seifter, et al., 1950). The intensity of the colour was read on "Spectronic 20" at 620 mp. The water-soluble carbohydrates of the gastrointestinal tract were determined as follows:

The complete tract was homogenized with a known volume
of distilled water in Potter and Elvejem glass homogenizer. The homogenate was centrifuged at 3,500 rpm. The total sugar content in the supernatant was determined by the anthrone method. Glucose was used as standard and 1.11 as conversion factor for glycogen. Unless otherwise stated the results obtained for carbohydrates were calculated as glucose equivalent. All the analyses mentioned hitherto were performed in triplicate.

RESULTS

The results obtained in the present study are summarized in tables II and III. The changes in the concentrations of glycogen in the leg muscles as well as in the fat body are indicated in Fig. III. The reduction in glycogen content of the leg muscles was quite marked after the 20 min. of exercise; while it remained practically unchanged during this period in the fat body. In subsequent periods of exercise, however, a progressive reduction of glycogen occurred in the fat body (Fig. III) at a rate of 1 mg./min./gm. wet wt. of tissue. After 40 min. of continuous swimming activity the glycogen content of the leg muscles dropped to 25%, compared to that of the control of 87%. For the first twenty minutes the rate of glycogen reduction was 21 µg./beetle/min. (calculated on the basis of the total wt. of the leg muscles (490 mg.) of a beetle), and was reduced to 17 µg./beetle/min. for the next 20 min.
Table II. Effect of vigorous swimming activity on the glycogen content of the leg muscles as well as fat body of the beetle Cybister confusus

<table>
<thead>
<tr>
<th>Duration of exercise in minutes</th>
<th>mg. glycogen/gm. wet wt. of tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LEG MUSCLE</td>
</tr>
<tr>
<td></td>
<td>Unexercised</td>
</tr>
<tr>
<td>00 (4)</td>
<td>0.59 ± 0.01</td>
</tr>
<tr>
<td>10 (4)</td>
<td>0.63 ± 0.003</td>
</tr>
<tr>
<td>20 (4)</td>
<td>0.64 ± 0.28</td>
</tr>
<tr>
<td>30 (4)</td>
<td>0.62 ± 0.01</td>
</tr>
<tr>
<td>40 (4)</td>
<td>0.54 ± 0.02</td>
</tr>
<tr>
<td>50 (4)</td>
<td>0.59 ± 0.01</td>
</tr>
<tr>
<td>90 (4)</td>
<td>0.59 ± 0.01</td>
</tr>
</tbody>
</table>

The number of insects used in the experiments are indicated in the parenthesis.

The results are mean ± standard error.
Fig. III EFFECT OF VIGOROUS SWINNING ACTIVITY ON THE GLYCOGEN CONTENT OF THE LEG MUSCLES AS WELL AS THE FAT BODY OF THE CYBISTER BEETLE.
Table III. Total sugar content of the gastrointestinal tract during swimming activity of the *Cybister confusus*

<table>
<thead>
<tr>
<th>Duration of exercise in minutes</th>
<th>Microgram sugar/beetle</th>
</tr>
</thead>
<tbody>
<tr>
<td>00 (4)</td>
<td>21.00 ± 4.4</td>
</tr>
<tr>
<td>10 (4)</td>
<td>19.20 ± 1.9</td>
</tr>
<tr>
<td>20 (4)</td>
<td>20.00 ± 1.6</td>
</tr>
<tr>
<td>30 (4)</td>
<td>17.00 ± 1.09</td>
</tr>
<tr>
<td>40 (4)</td>
<td>24.00 ± 1.6</td>
</tr>
<tr>
<td>50 (4)</td>
<td>20.00 ± 0.92</td>
</tr>
<tr>
<td>90 (4)</td>
<td>14.50 ± 1.7</td>
</tr>
</tbody>
</table>

The number of insects used are presented in the parenthesis. The results are mean ± standard error.
Table III indicates the amount of total sugars in the gastrointestinal tract and its changes during exercise of the Cybister beetle. There seems to be no appreciable reduction in the sugar content of the gastrointestinal tract of the exercised beetle.

DISCUSSION

Almost complete disappearance of glycogen occurred in the white tetanic fibres, whereas no change occurred in the tonic red fibres of the pigeon breast muscles after electrical stimulation, has been shown by George and Nene (1965), Parker and George (1972). The authors have concluded that, white fibres indulge only in short bursts of activity such as quick take-off or rapid movements by using glycogen as the chief fuel. The aquatic dytiscid beetle Cybister confusus is a fast swimmer as well as a good diver. It is expected that the powerful coxal muscles by their rapid contractions enable the beetle to have quick movements and also to manoeuvre sudden turns while diving and swimming. It is possible therefore, that glycogen should be the predominant fuel for all such quick actions. From the histochemical detection of fat and glycogen (Sect. A) it is deduced that leg muscles contain considerable amount of the latter.
The large glycogen reserves and its depletion by catabolism during flight in flies (Sacktor, 1955 and 1961) indicate its utilization as the principal source of energy for flight activity. Hudson (1958) estimated *Phormia regina* utilized carbohydrate (expressed as glycogen) at a rate of 14.79 μg./fly/min., while only 3 μg./fly/min. in *Culex tarsalis* by Rowley (1970). The leg muscles of the Cybister beetle was found to contain about 288 μg. of glycogen (calculated value on the basis of the total wt. of the leg muscles (490 mg.) of a beetle). On the basis of the amount of glycogen in the leg muscles left over after 20 min. of exercise the muscles appeared to have utilized at a rate 28 μg./beetle/min. This high rate of glycogen utilization by the beetle during vigorous swimming activity may be attributed to the fact that the beetle is a heavier insect. However, it has been found that the amount of glycogen present in the leg muscle is insufficient to support the swimming performance of the beetle for more than 10 min. It appears therefore that the beetle must turn to a secondary source, the fat body, for its fuel requirement during the subsequent periods of exercise. Using histochemical methods Wigglesworth (1949) and Clements (1955) have demonstrated the depletion of glycogen in the fat body after flight to exhaustion in *Drosophila* and
Similarly, in the present investigation it is found that the fat body glycogen in the Cybister beetle got reduced after 30 min. of exercise. A decrease in leg muscle glycogen during the initial period of exercise and subsequent reduction of it in the fat body of Cybister beetle would therefore suggest that to begin with, the leg muscles seem to have utilized their intracellular glycogen and once this glycogen pool got reduced it was drawn upon from the fat body. This finding thus supports the suggestion of Sacktor (1965) that when the thoracic glycogen pool is depleted mosquitoes must turn to secondary and possibly tertiary carbohydrate sources.

As early as 1949 Wigglesworth had demonstrated that in the glycogen-depleted fly, ingested sugars are accessible almost immediately to support flight. Clements (1955) had shown in mosquitoes that the content of the gastrointestinal tract served as a source of flight energy. Similarly, that the crop sugars in blood sucking Tabanus was a most important source of fuel for flight energy, had been demonstrated by Hocking (1953). In Phormia it had been shown that the utilization of total glycogen takes precedence over the sugars held in the gastrointestinal tract (Hudson, 1958). In the present
study, however, the total sugar content of the gastrointestinal tract at regular intervals of exercise up to 50 minutes, did not reveal much differences (table III) which suggest that the contribution of sugars held in the tract are insignificant under the present experimental conditions.

**SUMMARY**

1. A progressive reduction of glycogen reserves in the leg muscles of the *Cybister* beetle was observed during the first 20 min. of exercise, while it remained practically unchanged in the fat body during this period. In subsequent periods of exercise, however, a reduction of glycogen occurred in the fat body also.

ii. It is suggested therefore that to begin with the leg muscles seem to utilize their intracellular glycogen and once this glycogen got reduced, it was drawn upon from the fat body.
ii. Utilization of Glycogen Fractions During Exercise of Leg Muscles of *Cybister confusus*

Bloom *et al.* (1951) have reported that glycogen of the liver and muscle is separable into acid extractable and residual or hot KOH extractable fractions. That these two glycogen fractions disappear at different rates during muscle stimulation has been recorded by Bloom and Knowlton (1953). Similar fractions into a free one and a fixed one has been demonstrated in the fat body glycogen of *Bombax mori* (Saito, 1963 cited by Clegg, 1965). In the preceding study it has been shown that to begin with the leg muscles of the beetle *Cybister confusus* utilized their glycogen reserves during its continuous swimming activity and later the fat body glycogen. The present work is aimed at ascertaining, whether the glycogen in the leg muscles as well as in the fat body exists in two fractions and if so whether preferential utilization of one fraction or the other takes place during induced swimming activity by the leg muscles of *C. confusus*.

**MATERIAL AND METHODS**

Adult *Cybister* beetles were chosen from the laboratory stock for experiments with respect to the effect of induced swimming performance on the glycogen fractions of the leg.
muscles as well as of the fat body of the beetle. The method described in the preceding part of this study has been followed to exercise the beetles.

**Glycogen Extractions:**

The free glycogen or acid extractable glycogen (AEG) from the leg muscles and fat body was extracted with trichloroacetic acid (TCA) following the method outlined by Kugler and Wilkinson (1959-1960) whereas, the residual glycogen (RG) was extracted with hot 20% KOH as described by Seifter et al. (1950). To extract AEG, a known quantity of (0.3 - 0.35 gm.) each of leg muscles and fat body was homogenized for three min. in 2 ml. 10% TCA. The homogenate was transferred into a centrifuge tube and washed again twice with an additional 2 ml. TCA each time. The RG fraction on the other hand was extracted by digesting the known quantity of each tissue in 2 ml. 20% KOH. The rest of the procedure was as described in the preceding section.

**RESULTS AND DISCUSSION**

Table IV depicts the two glycogen fractions, the acid extractable (AEG) and the residual (RG) and their reduction during induced swimming performance of *C. confusus*.
Table IV. Utilization of glycogen fractions during vigorous swimming performance of the *Cybister confusus*

<table>
<thead>
<tr>
<th>Duration of exercise in minutes</th>
<th>mg. glycogen/gm. wet wt. of tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LEG MUSCLE</td>
</tr>
<tr>
<td></td>
<td>Free (AEG) % Reduction Fixed % Reduction</td>
</tr>
<tr>
<td>00 (3)</td>
<td>0.89 ± 0.07* 57.0 0.67± 0.08 43.0</td>
</tr>
<tr>
<td>10 (3)</td>
<td>0.52 ± 0.01 -- 0.59± 0.03 --</td>
</tr>
<tr>
<td>20 (3)</td>
<td>0.20 ± 0.01 -- 0.28± 0.05 --</td>
</tr>
<tr>
<td>30 (3)</td>
<td>0.07 ± 0.01 4.5 0.113± 0.12 7.3</td>
</tr>
<tr>
<td>40 (3)</td>
<td>--- -- --- --</td>
</tr>
</tbody>
</table>

The number of insects used in each determination is presented in the paranthesis.

* The results are averages of the mean ± standard error.
The AEG fraction (0.89 ± 0.07) is more or less equal to the RG fraction (0.67 ± 0.08) in the leg muscles; in the fat body on the other hand the RG fraction (27.5 ± 3.9) is very much more than the AEG fraction (9.0 ± 1.0) (table IV). After 30 min. of continuous exercise the AEG and RG fractions of the leg muscles dropped respectively from 57% and 43% to 4.5% and 7.3%. The corresponding values in the fat body on the other hand were 24.6% and 73.3% to 8.2% and 36.45% (table IV). During the first 10 min. of exercise the leg muscles seem to have utilized their AEG fraction at a rate of 15 μg./min./beetle (calculated on the basis of total wt. (490 mg.) of the leg muscles), while the RG fraction was affected to a much lower extent (0.67 to 0.59 mg./100 mg. wet wt. of tissue). However, in subsequent intervals of exercise considerable reduction in both AEG and RG fractions of both the leg muscles and fat body was observed. It is concluded from these observations that during vigorous exercise the leg muscles of Cybister confusus preferentially utilize the AEG fraction to begin with; later on as the glycogen store gets depleted in the leg muscles both fractions of the glycogen reserves from the fat body are drawn upon, but preferentially more from the AEG fraction.
SUMMARY

i. The glycogen content of the leg muscles as well as of the fat body of Cybister beetle could be fractionated into free (acid extractable, AEG) and fixed (residual, RG) fractions.

ii. The AEG fraction is more or less equal to the RG fraction in the leg muscles; in the fat body on the other hand the RG fraction is very much more than the AEG fraction.

iii. It appears that during vigorous exercise the leg muscles of Cybister beetle preferentially utilize the AEG fraction to begin with; later on as the glycogen store gets depleted in the leg muscles both fractions of the glycogen reserves from the fat body are drawn upon, but preferentially more from the AEG fraction.
iii. Trehalose Content of the Haemolymph and its Reduction During Swimming Activity of *Cybister confusus*

In addition to glycogen, it is now well established that the disaccharide trehalose also supports flight muscle activity (Sacktor, 1970). Trehalose was identified as the principal blood sugar in many insect species (Wyatt and Calf, 1956; 1957; Howden and Kilby, 1956) and in the flight muscles of *Locusta* as well (Bücher and Klignenberg, 1958). As early as 1937, Beutler found that the sugar in the blood decreased during flight of the honey bee and, in fact, the duration of flight was limited by the availability of blood sugar. Depletion of trehalose in blood (Evans and Deither, 1957; Polacek and Kubista, 1960; Clagg and Evans, 1961; Srivastava and Rockstein, 1969) and muscle (Bücher and Klignenberg, 1958) during flight has been recorded in good many insects. It appears therefore that the trehalose is an important metabolite. It has been noticed in the previous study (Sect. B) that the glycogen content of the leg muscles as well as of the fat body of *Cybister* beetle remained practically unchanged during the first ten minutes of its vigorous swimming activity. It is therefore anticipated that the free sugars especially the trehalose in the blood must have been directed to the leg muscles during the initial
period of their activity. The present investigation is designed to assess the amount of blood trehalose and its participation during vigorous exercise of the *Oybister confusus* beetle.

**MATERIAL AND METHODS**

The beetles employed and the method to induce vigorous swimming activity in them was as described in the first part (Sect. B. i) of this study.

**Collection of Haemolymph:**

To collect the haemolymph, the head of the exercised and unexercised beetles were removed along with the alimentary canal. The cut end of the insect was held against a pre-cooled centrifuge tube. Most of the haemolymph (0.02 ml./beetle) was collected by gently pressing the abdomen. The body fluid of three beetles was pooled and centrifuged for 5 min. at 400 x g at 4°C to remove cells and fat. 5 μmoles of glutathione/ml. of haemolymph was added to avoid blackening.

**Separation and Estimation of Trehalose:**

The trehalose content of the haemolymph was determined by paper chromatography as described by Putman (1957).
The haemolymph was diluted 10 times with distilled water. A known aliquot of this diluted haemolymph was partially deionized by passing it through Amberlite IR-120 (H form). After drying the deionized extracts at 30°C under reduced pressure, the residue was redissolved in 1 to 1.5 ml. distilled water. An aliquot of the above and a standard trehalose (20 µg.) solutions were applied separately on Watmen No. 1 paper and developed ascendingly at room temperature using a solvent system of n butanol; ethanol; water (52:32:16 v/v/v). After developing, the paper was cut into two strips, the one containing the standard trehalose was treated with ammonical silver nitrate (Trevelgan et al., 1950) to stain trehalose. An area corresponding to standard trehalose of the other strip was cut out and macerated with spatula in 1 ml. of distilled water; trehalose in the supernatant was determined by anthrone method of Carrol et al., (1956). The results are expressed as µ moles of trehalose/ml. of haemolymph.

RESULTS

Table V and Fig. IV present the trehalose level in the haemolymph during twenty minutes of vigorous swimming activity of the Cybister confusus beetle.

A progressive fall in the trehalose level of the haemolymph during the 20 min. of the exercised beetle
Table V. Trehalose content in the haemolymph of the *Cybister confusus* during vigorous swimming activity

<table>
<thead>
<tr>
<th>Duration of exercise in minutes</th>
<th>Micromoles of trehalose/ml. haemolymph</th>
<th>Control</th>
<th>Experimental</th>
<th>Rate of reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>00 (4)</td>
<td>19.00 ± 1.6*</td>
<td>16.70 ± 2.1</td>
<td>2.50</td>
<td></td>
</tr>
<tr>
<td>05 (4)</td>
<td>15.50 ± 1.7</td>
<td>11.65 ± 1.6</td>
<td>3.85</td>
<td></td>
</tr>
<tr>
<td>10 (4)</td>
<td>18.75 ± 1.2</td>
<td>06.34 ± 0.99</td>
<td>12.41</td>
<td></td>
</tr>
<tr>
<td>15 (4)</td>
<td>17.15 ± 1.6</td>
<td>03.10 ± 0.54</td>
<td>14.05</td>
<td></td>
</tr>
<tr>
<td>20 (4)</td>
<td>13.50 ± 1.53</td>
<td>06.00 ± 0.80</td>
<td>7.50</td>
<td></td>
</tr>
</tbody>
</table>

The number of beetles used in each determination is represented in the paranthesis.

* The results are the mean ± standard error.
Fig. IV  
TREHALOSE CONTENT IN THE HAEMOLYMPH OF THE CYBISTER BEETLE DURING VIGOROUS SWIMMING ACTIVITY
is quite evident (table V). The depletion of trehalose during the initial exercise was less; in subsequent periods of activity however, its level was slightly increased (Fig. IV).

DISCUSSION

The trehalose in the blood of Cybister confusus beetle (19 μmoles/ml. haemolymph) appears to be an important metabolite. The marked decrease in the trehalose concentration of the haemolymph from the onset of exercise for 20 min. indicates its utilization by the vigorously acting leg muscles. These results thus support the earlier observations that the blood trehalose acts as a substrate to supply energy to the working muscles (Evans and Deither, 1957; Clegg and Evans, 1961; Polacek and Kubista, 1960; and Sacktor and Wormser-Shavit, 1966). The slight increase in haemolymph trehalose after 15 min. of exercise probably indicates its turnover from the fat body, since carbohydrates are released into the blood mostly as the disaccharide trehalose (Clegg and Evans, 1961).
SUMMARY

i. The trehalose in the blood of Cybister beetle (19 micromoles/ml. haemolymph) appears to be an important metabolite.

ii. The marked decrease in the trehalose concentration of the haemolymph from the onset of exercise for 20 min. indicates its utilization by the vigorously acting leg muscles.
iv. Phosphorylase Activity in the Leg Muscles During Swimming Activity of Cybister confusus

The presence of phosphorylase in insect muscle is implied by Sacktor's (1955) finding that glycogen supported anaerobic respiration in housefly flight muscle homogenates and functioned as efficiently as that free sugars. The histochemical evidence for phosphorylase in sections of locust muscle incubated with glucose-1-phosphate (G-1-P) and AMP with concomitant deposition of glycogen has been provided by Hess and Pearse (1961). Similarly, its activity was demonstrated in Bombax mori larvae (Shigematsu, 1956a and b) fat body (Shigematsu, 1966) and midgut (Ito and Horie, 1959). That insect muscle phosphorylase exists in two forms phosphorylase a and b, similar to those in mammalian skeletal muscle is now very well understood (Childress and Sacktor, 1970). Cori (1956) was the first to demonstrate that the increased rate of glycogen breakdown during electrical stimulation of the muscle was accompanied by the conversion of phosphorylase b to a. A significant increase in the phosphorylase a level has been reported in rainbow trout when subjected to vigorous muscular activity by chasing (Nakano and Tomlinson, 1967). In the blowfly Phormia initiation of flight induced an immediate increase in the
relative amount of the enzyme in the "a" form (Childress and Sacktor, 1970).

Evidence is presented in the preceding section to the effect that the leg muscle glycogen got reduced after 10 min. of vigorous exercise of the Cybister confusus beetle, indicating thereby its breakdown. The present study is undertaken to elucidate the phosphorylase activity in the leg muscles during active movements.

MATERIAL AND METHODS

The adult Cybister beetles used in the present study were chosen from the laboratory stock. The method described in the first part (i) was followed to induce vigorous exercise in the beetles.

Tissue Preparations:

After exercise (15 min.) the beetles were killed by decapitation. The leg muscles were removed and homogenized in a known vol. of distilled water in Potter and Elvehjem glass homogenizer at 0°C to 4°C. The enzyme source was used almost immediately. Control analyses were made of the leg muscles taken out from the beetle at the onset of the experiment.
Phosphorylase Assay:

The phosphorylase activity was determined in the muscle homogenate as followed by Valyathan and George (1964). The reaction mixture taken in test tube consisted of: 0.2 ml. sodium citrate buffer (0.1 M) of pH 5.9; 0.3 ml. Potassium fluoride (0.154 M); 0.05 ml. Glucose-1-Phosphate (G-1-P) (0.2 M) and of 0.1 ml. freshly prepared muscle homogenate; incubated for 15 min. at 30°C with constant shaking. The reaction was terminated by the addition of 1 ml. 10% trichloroacetic acid (TCA). Control preparations were run with each experiment by adding 1 ml. 10% TCA to the incubation medium. After incubation and the addition of TCA, the tubes were centrifuged at 10°C at 4,000 rpm. The inorganic phosphate liberated from the G-1-P by the enzyme activity was estimated by the method of Fiske and SubbaRow (1925) and the intensity of the colour was read on "Spectronic 20" at 660 μm. The enzyme activity was calculated as ug. phosphorous liberated/mg. dry weight of tissue at 30°C for 15 min. The dry weight of the muscle was determined by drying 1 ml. duplicate samples of the muscle homogenate for 24 hr. at 100°C and kept in a vacuum desiccator till constant weight was obtained.
Table VI. Phosphorylase activity in the exercised and unexercised leg muscles of the beetle *Cybister confusus*

<table>
<thead>
<tr>
<th>Tissues</th>
<th>µg. phosphorous/mg. dry tissue/15 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>i) Unexercised (2)</td>
<td>31.59 ± 1.390</td>
</tr>
<tr>
<td>ii) Exercised  (2)</td>
<td>44.92 ± 2.404</td>
</tr>
<tr>
<td></td>
<td>&quot;†&quot; (test)</td>
</tr>
<tr>
<td></td>
<td>4.8 (significant)</td>
</tr>
</tbody>
</table>

The results are mean ± standard error.

The insects used in each determination is presented in the parenthesis.
RESULTS AND DISCUSSION

The results obtained on the phosphorylase activity of the exercised and unexercised leg muscles of Cybister beetle are presented in the table VI. It is quite evident that the enzyme activity was increased significantly in the exercised leg muscles compared to that of the unexercised one. These results support the earlier observations (Sect. B) that during intense muscular activity the leg muscles of Cybister confusus depend solely on their glycogen reserves. Sacktor and Wormser-Shavit (1966) have reported that an intense rate of glycogenolysis was triggered on initiation of flight in the blowfly Phormia regina. Conversion of inactive phosphorylase b to the active a form during intense muscular activity has been recorded in insects as well as in vertebrates (Cori, 1956; Nakano and Tomlinson, 1967; Childress and Sacktor, 1970). In the present investigation also it appears that the significant increase in the phosphorylase activity during exercise of the Cybister beetle accounts for the possibility of the increased glycogenolysis in the leg muscles during its vigorous exercise.
SUMMARY

Quantitative determination of phosphorylase activity in the exercised and unexercised leg muscles of *Cybister* beetle has shown that a significant increase in the enzyme activity was obtained in the former muscle. This increased enzyme activity in the exercised leg muscles accounts for the possibility of the increased glycogenolysis in them during vigorous swimming activity.
II. Evidences for Lipid Utilization by the Leg Muscles

1. Lipid Content and Lipolytic Activity in the Leg Muscles as well as in the Fat Body During Exercise of *Cybister confusus*

During the swimming activity the leg muscle tissue is metabolically very active. Fuel reserves are mobilized and energy yielding processes marshalled and regulated to provide the increasing energy demands. It is very well recognized now that the fat is a fuel of significant importance during prolonged flight in birds and insects (George and Berger, 1966; Weis-Fogh, 1967). Williams (1945) examined the fat content of two unknown moths and suggested that fat was the chief fuel for their migratory flight. An extensive study by Beall (1948) on the utilization of fat in the migratory monarch butterfly *Danaius plexippus* is also suggestive of the combustion of fat for energy. During flight a low respiratory quotient indicating fat utilization was recorded in some active insects (Krogh and Weis-Fogh, 1951; Zebe, 1954). In *Schistocerca gregaria*, the desert locust, 80 to 85% of total energy expended in the first five hours of migratory flight was derived according to Weis-Fogh (1952) from fat. A similar utilization of fat during prolonged flight of birds has been reported by George and Jyoti (1955) and George (1964).
Domroes and Gilbert (1964) in *Cecropia* moth have found a sudden reduction in total fat in flight, though the amount of fat had remained a constant quantity during metamorphosis. This abrupt reduction has been assumed to be due to flight activity in young adults. There are other data, however, which indicate that those insect species which derive the energy for flight through the oxidation of fat, begin their flight activity with glycogen breakdown. Bücher and Klingenberg (1958) found a decrease in the carbohydrate level during initial flight in the migratory locust. Similarly Cockbain (1961) showed that during the flight of the tethered *Aphis* glycogen was used for the first one hour period of flight, after which fat took its place and was able to sustain flight for as long as 12 hr. This initial utilization of carbohydrates and then fat by the flight muscles of these insects appears to be associated with the ability to increase their thoracis temperature before they could take to continuous or migratory flight (Beenakkers, 1965).

If fat is to be used as fuel, it has first to be hydrolyzed. The lipolytic activity in muscles and its relation to fat utilization during sustained flight of birds as well as some insects has been provided by George
and his school of muscle physiologists (George and Berger, 1966; George, et al., 1958; George and Bhakthan, 1960a; 1961 and 1963; George, 1964). Gilbert et al. (1965) demonstrated an extra digestive lipase in flight muscles as well as in fat body and its significance in lipid transport in the silkworm *Hyalophora cecropia*. Stevenson (1972) has shown that the flight muscles of the *Prodenia* moth contained a very active monoglyceride (MG) lipase, with very low ability to hydrolyze triglyceride (TG) and diglyceride (DG). The fat body lipase on the other hand was able to hydrolyze TG-, DG-, and MG.

It has been demonstrated in our previous study (Sect. B) that the beetle *Cybister confusus* would make use of its haemolymph trehalose, intramuscular glycogen, (25% over the control value of 87%) and finally the fat body glycogen (22.26% over the control value of 90%) respectively, during the first 40 min. of its vigorous swimming activity. In the subsequent periods of exercise, however, the leg muscle (95%) as well as the fat body glycogen (82%) content remained practically unreduced (table II). These results therefore lead us to believe that a substrate other than carbohydrates, possibly the lipids might have been consumed during continuous swimming performance after the first 40 min. exercise. The present study is therefore aimed at
ascertaining whether the leg muscles of C. confusus would make use of their lipid store as well as that from the fat body and if so, whether evidences for its hydrolysis during induced swimming activity are available.

MATERIAL AND METHODS

Induced Swimming Activity:

The method followed to induce vigorous swimming activity of beetles was as described in the previous Section (Sect. B). The fresh weight of each beetle fell within a range of 3.53 gm. to 3.61 gm. At the end of each 10 min. interval of exercise lasting 170 min. the beetles were killed by decapitation. Their leg muscles and fat body were removed separately and stored at 0°C until used.

Determination of Total Lipids:

The total lipid content of the leg muscles as well as of the fat body was determined according to the method of Folch et al. (1957) using chlor form-methanol 2:1 (v/v) as a solvent system. The tissues were dried at 100°C till a constant weight was obtained. A known quantity of this dried tissue was powdered and homogenized with an appropriate vol. of methanol in Potter and Elv hajem glass homogenizer.
The homogenate was then quantitatively transferred to a 50 ml. separatory funnel, then added the chloroform. The two solvents were partitioned by the addition of 0.2 vol. water. After the funnel was shaken, the mixture was allowed to stand overnight, the lower chloroform layer containing lipid was drawn off, the solvent was removed under the reduced pressure and the total lipids were estimated gravimetrically in triplicate. The results are expressed as per cent dry weight of tissues.

**Estimation of Lipase Activity:**

The lipolytic activity of the leg muscles as well as of the fat body was determined after the 50 min. of continuous swimming activity. The method followed by essentially similar to that described by Gilbert et al. (1965). At the end of 50 min. of exercise the beetles were killed by decapitation. The leg muscles and fat body were homogenized in Potter and Elvehjem glass homogenizer in a known vol. of ice-cold saline water. The reaction mixture contained in a final vol. of 2 ml.; 0.5 milimoles of tris maleate buffer (pH 7.6); 0.5 ml. enzyme source; 1.0 ml. emulsified substrate (by sonication) containing 20 to 25 micromoles of pure tri-, di-, and monolein plus 14C-tri-, di- and monoglyceride equivalent to 50,000 cts/min. in each case. The tubes were incubated with constant shaking at 37°C.
for four hours. Control preparations were run with each experiment by adding 2 ml. of methanol before the addition of the homogenate and incubated along with the samples. At the end of incubation 2 ml. of methanol was added to each of the experimental tubes. The lipid content of each reaction tube was immediately extracted according to the method of Bligh and Dyer (1959). The tubes were then centrifuged at 3,500 r.p.m. for 10 min. to separate the chloroform layer. The chloroform layer from each reaction tube was drawn off carefully and the solvent was removed in vacuo. The lipid residue was redissolved in a known volume of chloroform and a known aliquot of this (100 µl) was analysed on silicagel thin layer chromatogram plate (tlc) for its free fatty acid (FFA) content, using a solvent system of n-hexane: diethyl ether: acetic acid (90:18:1.5 v/v/v). The FFA fraction was identified with its authentic standard (supplied by Dr. F. H. Mattson) after exposing the tlc plate to iodine vapour and was marked under the ultraviolet light. The FFA spot was scraped out and transferred to a counting vial containing liquid scintillation solution (PPO 12 mg./5 ml. of toluene). The radio activity in the FFA was measured in a Liquid Scintillation Counter (Beckman). Quenching was corrected for, by the addition of an internal standard. The activity of
lipase is expressed as micromoles of fatty acids liberated per 100 mg. of dry tissue per hr.

RESULTS

The changes occurred in the lipid content of the leg muscles as well as of the fat body during vigorous exercise of the Cybister are presented in the table VII and fig. V. The amount of the total lipid content of the leg muscles remained almost unchanged during the first 30 min. of exercise. In the subsequent period of exercise, however, (after 40 to 60 min.) the lipid amount from the leg muscles got reduced to 66.67% (over the control value of 0%). Similarly, in the fat body, no appreciable changes in the fat content was observed during the initial swimming activity of 40 min. (table VII). In subsequent periods (60 min.) however, the lipid content of the fat body also got reduced to 38.61%. The quantitative determination of lipase as ascertained by the radioactive counts obtained in the free fatty acid fractions, of the leg muscles with TG, DG and MG as substrates, has revealed that (table VIII) the leg muscles are capable of hydrolyzing all the three substrates tested for. Though all the three substrates are hydrolyzed, there seems to be some variation in the extent of hydrolysis in each case. More MG appears to be hydrolyzed than TG and DG.

The effect of swimming activity on the lipase content in the leg muscles as well as of the fat body using TG as substrate indicated (table IX) that the enzyme activity was only slightly
Fig. V

CHANGES IN THE LIPID CONTENT OF THE
LEG MUSCLES AS WELL AS OF THE FAT BODY
OF THE CYBISTER DURING VIGOROUS
SWINNING ACTIVITY

% FAT BY DRY WEIGHT OF THE TISSUE

FAT BODY

LEG MUSCLES

TIME IN MINUTES
Table VIII. Lipolytic activity in the leg muscles of Cybister confusus with respect to Mono-, Di- and Triglycerides as substrates

<table>
<thead>
<tr>
<th>Substrates</th>
<th>µ. moles of fatty acids liberated/100 mg. dry tissue/hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experimental</td>
</tr>
<tr>
<td>Monoglyceride</td>
<td>(4)</td>
</tr>
<tr>
<td>Diglyceride</td>
<td>(4)</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>(4)</td>
</tr>
</tbody>
</table>

The number of insects used is presented in the parenthesis.
The results are mean ± standard error.
Table IX. Lipolytic activity in the leg muscles as well as fat body of the Cybister confusus after 50 min. of continuous exercise

<table>
<thead>
<tr>
<th>Tissues</th>
<th>FAT BODY</th>
<th>LEG MUSCLES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μ. moles of fatty acids liberated/100 mg. dry tissue/hr.</td>
<td>μ. moles of fatty acids liberated/100 mg. dry tissue/hr.</td>
</tr>
<tr>
<td>Unexercised (4)</td>
<td>20.0 ± 2.58</td>
<td>2.0 ± 0.4</td>
</tr>
<tr>
<td>Exercised (4)</td>
<td>26.0 ± 1.68</td>
<td>2.9 ± 0.41</td>
</tr>
<tr>
<td>&quot;t&quot; (test)</td>
<td>2.0 (Significant)</td>
<td>1.6 (Not significant)</td>
</tr>
</tbody>
</table>

The number of insects used is indicated in parenthesis.

Differences between experimental and control readings was analyzed employing the student's t test.
increased (2.0 ± 0.4 to 2.9 ± 0.41 μ.moles/100 mg. dry tissue/hr.) in the leg muscles, while a significant increase (20.0 to 26.0 μ.moles/100 mg. dry tissue/hr.) of it in the fat body was noticed in the exercised beetle, after an interval of 50 min. of continuous exercise.

DISCUSSION

Utilization of Intramuscular Lipid:

The fact that some insects deplete their reserves of fat during sustained flight indicates that fat can serve as a metabolic fuel for flight muscle contractions. George and Berger (1966) have made it amply clear that the migratory birds utilize chiefly their lipid reserves during prolonged flight. The insect species which are known to utilize chiefly lipids during their prolonged flight have been found to begin their flight activity with carbohydrate metabolism (Weis-Fogh, 1952; Bürcher and Klingenberg, 1958; Cockbain, 1961; and Hofmanova et al., 1966). In the present observations it could be seen (table VII) that the lipid content of the leg muscles of the Cybister beetle after continuous swimming (40 min.) got depleted to 40% (by dry wt. of the tissue) of the control value, suggesting thereby its catabolism by them. A similar reduction in the free lipid content of the liver and breast
muscle of pigeon on electrically stimulating as well as on subjecting the bird to forced flight was noted by George and Jyoti (1953, 1955b). In insects during the rapid transition of muscle tissue from a "resting" state to physiological work, the metabolic rate of the tissue increases as great as 50-100 times (Sacktor, 1970). In the Cybister beetle as in other insects the fuel reserves are known to be distributed between muscles, haemolymph and fat body. It could be expected then that during vigorous muscular activity the leg muscles of Cybister beetle may make use of their lipid reserves from haemolymph and fat body as well.

Contribution of Lipid from the Fat Body during Exercise:

The Cybister beetle has a well-developed fat body in the haemocoel. Moreover, the amount of carbohydrates (0.59 mg./gm. wet wt. of tissue) and lipids (10.0% by dry wt.) present in the leg muscles seems to be inadequate to support the swimming activity for more than 10 min. (table VII). It could be expected then that fat from the fat body might be transported to the active leg muscles through the haemolymph. It is discernable from the results (table VII) that the fat body lipids got reduced (38.6% over the control value of 0.9%) after about 60 min. of continuous exercise. It is suggested therefore that the leg muscles utilize intramuscular lipid
to begin with, and as the exercise continues, lipid material from the fat body is drawn upon to meet the extravagant energy demands of the actively working leg muscles.

Hydrolysis of Lipids during Intense Muscular Activity:

The lipolytic activity in a tissue is an index of its capacity to utilize lipid. George and Scaria, (1956) and George (1964) have drawn a relation between lipase content and fat utilization during sustained muscular activity in some birds as well as in insects. George et al., (1958) obtained greater lipase activity in the flight muscles of the dragonfly Pantala flavescens and the locust Locusta migratoria compared to that of the bumblebee Xylocopa. This difference between these insects seem to be correlated with the genetic capacity of the dragonfly and locust to depend on fat as the chief fuel during a prolonged flight, whereas the hymenopteran bumblebee like the honey bee is a honey gatherer and depends exclusively on carbohydrates for energy during flight. It can be seen from the present investigation (table VIII) that the leg muscles of Cybister beetle are able to hydrolyze TG, DG and MG, although MG was comparatively more susceptible for enzyme action than TG and DG. The fat body on the other
hand was able to hydrolyze all the three substrates equally well (Chapter III). These observations thus support the findings of Stevenson (1972), that the flight muscles of Prodenia moth hydrolyze MG at a faster rate than TG and DG, while the fat body lipase could hydrolyze all the three substrates. George and Bhakthan (1960) found that the meso- and metathoracic muscles of the beetle Heliocopris bucephalus contained more amount of lipase than the prothoracic muscles; an indication of the activity of this enzyme in the flight of the beetle. Again a higher lipolytic activity in the slow-contracting muscles compared to the fast-contracting ones, among the leg muscles of the cockroach Periplaneta americana has been observed by George and Bhakthan (1961). In the Locusta migratoria on the other hand the total fatty acid contents in the flight muscles was increased by 30% after 6.5 hr. of flight. This was believed to have come from fat break-down in the fat body by fat body lipase (Beenakkers, 1963, 1965). In this context the observations of George and Eapen (1959) that the fat body lipase of the Schistocerca gregaria was more than that present in the adipose tissue of the pigeon and its thoracic muscles is of considerable significance, as it throws light on the flying capacity of this insect. It is discernible from the present study that
the lipase activity in the exercised leg muscles was slightly increased. The activity of the enzyme in the fat body on the other hand was about 15 times that in the leg muscles with TG as substrate (Table IX). As this significant increase in the fat body lipase was obtained during induced swimming performance of the Cybister confusus it could be expected then that the fat body TG (which constitutes the major lipid fraction of the fat body lipid) is hydrolyzed by its lipase and the free fatty acids thus liberated are carried to the leg muscles for its further oxidation.

SUMMARY

i. In the Cybister beetle as in other insects the fat reserves are known to be distributed between muscles, haemolymph and fat body. Depletion of lipid content of the leg muscles as well as of the fat body has been observed during continuous swimming performance of the beetle.

ii. The leg muscles appear to utilize their intramuscular lipid store to begin with, and once this store got reduced critically, the lipids from the fat body are drawn upon.
iii. Lipolytic activity of the leg muscles as well as of the fat body suggests that, the former tissue hydrolyzes MG at a faster rate than DG and TG, whereas the fat body tissue could hydrolyze MG, DG and TG equally well.

iv. A significant increase in the lipase activity was obtained in the fat body with TG as substrate during vigorous swimming activity of the beetle.

v. It is concluded from these results that the fat body TG (which constitutes the major fraction of the fat body lipid) is hydrolyzed by its active lipase/lipases and the free fatty acids liberated are carried to the leg muscles for oxidation.
ii. Possibility of Lipid Transport to the Leg Muscles from the Haemolymph and Fat Body During Exercise of Cybister confusus

In the migratory locust much of the energy for continuous muscular work is derived by oxidation of lipids (Weis-Fogh, 1952) and the lipids are transported from the storage organ, the fat body to the working muscles via the haemolymph. However, opinion differs as regards the nature of lipid transport from the storage organ to the working muscles. Gilbert and his associates have shown in some insects that diglyceride (DG) is the material released into haemolymph from the fat body (Chino and Gilbert, 1964 and 1965; Bhakthan and Gilbert, 1968; and 1970; Beenakkers and Gilbert, 1968; Thomas and Gilbert, 1968). On the other hand Wlodawer and his co-workers (Wlodawer et al., 1966; Wlodawer and Langwinska, 1967) in the larva of the waxmoth Galleria found major radioactivity in the free fatty acids (FFA) as well as in the triglycerides (TG) fractions of the haemolymph. These findings prompted the authors to state that the waxmoth larva releases into the haemolymph FFA which are partly resynthesised into TG. Also FFA are partly transported to the site of utilization bound to protein. Similarly gravimetric and radioisotopic analyses of Cook and Eddington (1967) in the cockroach Periplaneta americana have suggested that FFA and TG are
the major lipids released from the fat body. During locust flight Beenakkers (1965) found a considerable increase in the total fatty acid content of the haemolymph and therefore the author suggested the transport of fatty acids to the flight muscles. Stevenson (1972) found an active lipase in the fat body of the Galleria moth which could hydrolyze TG, DG and monoglyceride (MG) very efficiently and the author is of the opinion that FFA resulting from the complete hydrolysis of TG of the fat body are transported to the flight muscles.

It has been suggested in the preceding part of this study that in the Cybister confusus beetle during its continuous swimming performance the leg muscles utilize carbohydrates to begin with, and then switch on to lipid reserves of its own as well as those from its fat body. Thus the present opportunity was availed of to find out the organic forms in which lipids are transported from the fat body during induced swimming activity of the Cybister confusus beetle.

MATERIAL AND METHODS

The adult Cybister beetles were chosen for experiments from the stock maintained in the laboratory. The fresh
weight of each beetle was ascertained before subjecting them to vigorous swimming exercise. Their weights were within a range of 3.71 to 3.73 gm.

**Induced Swimming Activity:**

The method followed to induce continuous swimming activity in the beetles has already been described in the previous part of this study (Sect. B i). The collection of the haemolymph and the separation of tissues from the beetle were effected as described elsewhere (Sect. B ii).

**Extraction of Total Lipids from the Different Tissues:**

The total lipid from the leg muscles as well as fat body and haemolymph was extracted according to the method of Polch et al. (1957) using chloroform:methanol 2:1 (v/v).

**Separation of Individual Glycerides, Fatty Acids and their Estimation:**

A known quantity of the extracted lipid was separated into neutral and phospholipids on column chromatography using silicic acid (Mallinkrodt, 100 mesh). Neutral and phospholipids were eluted with peroxide-free diethyl ether and pure methanol respectively. The neutral lipid fraction was further separated into individual MG, 1,2 IG, 1,3 DG,
FFA and TG on thin layer chromatogram (tlc) plate using hexane, ether, acetic acid as solvent system (90:18:1.5 \(v/v/v\)). The individual glycerides were estimated colorimetrically as described by Raghavan and Ganguly (1967). The FFA fraction of the tlc plate and total fatty acid (TFA) content of the total lipid was estimated according to the method described by Lauwerys (1969). The total fatty acid content of the three tissues (leg muscles, haemolymph and fat body) was estimated after the total lipid from each was saponified with alcoholic KOH and hydrolyzed with 1N HCl.

**RESULTS**

The changes in the glyceride content as well as fatty acid level after the vigorous exercise of the *Cybister* beetle are recorded in the tables, X, XI and XII. The total neutral lipid content of the exercised leg muscles got reduced to 17.97% after a period of 90 min. of continuous swimming exercise. Of the various glyceride fractions of the exercised leg muscles, considerable reduction occurred in MG (43%), while DG (28.31%) and TG (6.7%) to a less extent (table X). The fat body on the other hand exhibited more depletion in 1,2 DG (37%), and less in TG (6.4%) and
The longitudinal section of the leg muscle of the *Cybister* beetle as revealed by electronmicrograph (Urenyl acetate lead citrate).

Fewer and small-sized mitochondria (Mt) and tracheoles (Tr) are seen in between the myofibrils (M). X 16,300 (Print magnification).
PLATE-10

The longitudinal section of the flight muscle of the Cybister beetle as revealed by electronmicrograph. (Urenyl acetate lead citrate) Myofibrills (M) are seen close to tracheoles (Tr). Aggregation of mitochondria (Mt) and fat droplets (F) are seen in close association with tracheoles. X 6,300 (Print magnification).
TABLE XI. Effect of exercise (50 to 90 min.) on the free fatty acid (FFA) levels in the various tissues of the *Cybister* beetle

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Control beetles (μg./100 mg. dry tissue)</th>
<th>Experimental beetles (μg./100 mg. dry tissue)</th>
<th>Difference</th>
<th>Increase/decrease over the control %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leg Muscle</td>
<td>1.9 ± 0.35</td>
<td>2.9 ± 0.33</td>
<td>1.0</td>
<td>52.62</td>
</tr>
<tr>
<td>Fat Body</td>
<td>9.0 ± 0.82</td>
<td>5.9 ± 0.42</td>
<td>-3.1</td>
<td>-34.45</td>
</tr>
<tr>
<td>Haemolymph</td>
<td>1.2 ± 0.35 (μg./ml.)</td>
<td>2.7 ± 0.8 (μg./ml.)</td>
<td>1.5</td>
<td>125.00</td>
</tr>
</tbody>
</table>

The FFA values indicate mean and standard error.

The number of insects used is indicated in parenthesis.
The TEA values indicate mean and standard error.
The number of insects used is indicated in parenthesis.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Control beetles (µg./100 mg. dry tissue)</th>
<th>Experimental beetles (µg./100 mg. dry tissue)</th>
<th>Difference</th>
<th>Increase/decrease over the control %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leg Muscle</td>
<td>3.6 ± 0.55</td>
<td>4.3 ± 0.52</td>
<td>0.7</td>
<td>19.45</td>
</tr>
<tr>
<td>Fat Body</td>
<td>37.0 ± 4.50</td>
<td>29.8 ± 3.8</td>
<td>-7.2</td>
<td>-19.41</td>
</tr>
<tr>
<td>Haemolymph</td>
<td>2.8 ± 0.58</td>
<td>3.1 ± 0.56</td>
<td>0.3</td>
<td>11.00</td>
</tr>
</tbody>
</table>
MG (3.16%). However, the fat body showed more reduction (23%) in its glyceride content than that of the leg muscles (17.97%) after a continuous swimming performance (90 min.). The total neutral lipid level in the haemolymph on the other hand got increased to 6.25% (table X). Of considerable interest is the finding that the FFA content of the exercised leg muscles as well as of the haemolymph got increased to 52.62% and 125% respectively (table XI) with concomitant reduction (34.45%) of it in the fat body. The TFA level of the leg muscles as well as of the blood got increased slightly, with concurrent reduction of it in the fat body (table XII).

DISCUSSION

The amount of fuel reserves glycogen (0.6 mg./gm. wet wt. of tissue) and lipid (10% by dry wt. of tissue) present in the leg muscles of the Cybister beetle is apparently insufficient to support continuous muscular work during its induced vigorous swimming activity (table VII). It could be expected then that the fuel reserves from the storage organ, the fat body, are mobilized and diverted to the working muscles. During the last few years considerable evidences have accrued to the effect that the glycerides
in the form of DG are released from the fat body into the haemolymph which are then carried to the site of utilization (Tietz, 1962; Chino and Gilbert, 1964 and 1965; Bhakthan and Gilbert, 1968 and 1970). In the Cybister beetle after a continuous swimming activity lasting for 90 min. there is considerable decrease in MG (43.06%) in the leg muscles. The decrease in TG (6.7%) and DG (28.31%) is comparatively less. The lipase activity in the leg muscles of the Cybister beetle was considerably more towards MG than towards DG and TG (table VIII). These results suggest therefore that the leg muscles of the Cybister beetle use preferentially MG as chief substrate for energy during their intense muscular activity as was found in the flight muscles of Prodenia (Stevenson, 1972).

The fat body of the Cybister beetle exhibited 23% reduction in its neutral lipid content (table X) which was more than what the leg muscles have lost after a continuous swimming activity. The lipid analysis of the fat body after the swimming exercise (90 min.) has further shown reduction both in the 1,2 DG (37%) and TG (6.4%). The lipid analysis of the haemolymph of the exercised beetle on the other hand revealed only a slight increase in 1,2 DG (16.6%), while a significant increase (125%) in the FFA
level. The net gain in the neutral lipid content of the haemolymph after the swimming activity (90 min.) is only 6.25%, which is considerably less than what the fat body loses. Surprisingly the TFA level of the exercised leg muscles and haemolymph was slightly increased with concomitant decrease of it in the fat body (table XII). The conclusion that could be drawn from these findings is that, during vigorous exercise the fat body of the Cybister beetle releases FFA into the haemolymph which are then transported to the actively working leg muscles. In this context the findings that lipolytic activity of the fat body of the exercised Cybister beetle was significantly increased appears to be of some significance in the release of FFA (Sect. B ii). It is also interesting to note the observations of Stevenson (1972) who found that the flight muscles of the Prodenia moth contain lipases capable of hydrolyzing only MG, while the fat body is capable of hydrolyzing MG, DG and TG equally well. The author has suggested that the complete hydrolysis of the glyceride (TG) takes place in the fat body; resulting FFA are then transported to the flight muscles to provide energy.

The lipolytic activity and lipid transport appear to be an integral part of the general lipid metabolism of the
insect under study. It has been shown in the preceding study that the leg muscle lipase of the *Cybister* beetle compared to its counter part in the fat body seems to be inefficient in hydrolyzing MG, DG and TG as substrates to cope up with increasing energy demands of the vigorously working leg muscles (Sect. B II). It is therefore suggested that hydrolysis of glycerides is effected in the fat body first and the resulting FFA are transported to the leg muscles through the haemolymph.

**SUMMARY**

i. Analysis of the lipids from the leg muscles, fat body and haemolymph before and after continuous swimming activity of *Cybister confusus* indicated that the MG of the leg muscles got reduced to a greater extent (43.06%), compared to DG (28.31%) and TG (6.7%). The fat body of the exercised beetle on the other hand exhibited considerable reductions in 1,2 DG (37%) and slight one in TG (6.4%). No measurable changes occurred in the glyceride content of the haemolymph, while a considerable increase in FFA (125%) content was noticed.
ii. The exercised leg muscles exhibited a significant increase in FFA (52.62%) content with only a slight increase in TFA (19.45%).

iii. With regards to the nature of lipid fractions utilized during vigorous exercise, the preference goes to the FFA, since they are increased significantly both in the leg muscles and haemolymph. It appears that the fat body glycerides of the Cybister beetle are hydrolyzed by its active lipase and resulting FFA are released into haemolymph, which are then transported to its leg muscles.
iii. Experimental Evidence for Fatty Acid Oxidation in the Leg Muscles during Exercise of Cybister confusus

Based on the RQ obtained during flight (Zebe, 1954; and Beenakkers, 1963; 1965) as well as the reduction of lipid store during flight (Weis-Fogh, 1952; Bücher and Klingenberg, 1958; Polacek and Kubista, 1961), it has been more or less established that some insects derive their flight energy through oxidation of fat also. However, it still remained unclear, whether the flight muscles can themselves oxidize the fatty acids. Some maintain that acetate but not butyrate or octanoate is mainly oxidized by the flight muscles of the cockroach (Barron and Tehmisian, 1948) and by mitochondrial preparation from the thoracic muscles of the desert locust (Rees, 1954). In the same year MoShan et al., (1954) reporting on the oxidative enzymes in the thoracic muscles of the wood roach Leucophaea maderae have recorded that butyrate and octanoate were oxidized in addition to acetate, though the former two only to a slight extent. In this context the suggestion of Sacktor (1955) that the degradation of fatty acids takes place in the fat body, and that the resulting acetate is transported to the flight muscles via haemolymph appears to be quite relevant.
The first positive evidence of the oxidation of long chain fatty acids was provided by Meyer et al. (1960), who showed it in particulate fractions of the flight muscles of the desert locust. Two sets of particulate fractions were used by them; one derived from locusts reared at 35°C and the other from those reared at 45°C. The former fraction was found to be capable of oxidizing butyrate completely, but not the higher chain fatty acids. On the other hand the muscle preparations from the locust reared at 45°C could oxidize the higher fatty acids from C₈ to C₁₆. George and Bhakthan (1963) observed that the flight muscle homogenate of the honey bee Apis dorsetta was capable of oxidizing butyrate and extent of oxidation was found to be highest during the hours when the bee was most active. Beenakkers (1963) found a high activity of the fatty acid oxidizing enzymes indicating the significance of fatty acid oxidation in the flight muscles of desert locust during flight.

That added carnitine stimulates fatty acid oxidation is now very well recognized (Fritz and McEwen, 1959; Fritz and Yue, 1963; Beenakkers and Klingenberg, 1964; and Bode and Klingenberg, 1964; Kallapur and George, 1973).

It is ascertained from the preceding section (Sect. B ii) that during vigorous swimming activity the total
lipids in the leg muscles (3.6 over the control value of 10.8%) as well as the fat body (26.5% over the control value of 55.5%) of the *Cybister confusus* got reduced. However, the fatty acid content of the leg muscles was increased (1.9 to 2.9 µg./100 mg. dry tissue) after induced swimming movements of the beetle (Sect. B, ii). These results lead us to believe that the leg muscles are capable of utilizing lipid as their fuel under exceptional conditions. The present experiment was designed therefore to test whether, the leg muscles are capable of oxidizing fatty acids of different chain length in its normal movements as well as after vigorous swimming exercise.

**MATERIAL AND METHODS**

The beetles were selected from the laboratory stock and were exercised as described elsewhere (Sect. B, i).

**Preparation of Muscle Homogenate:**

The leg muscles from 2 to 3 normal and exercised beetles were removed and stored in a pre-cooled petri dish. The tissue was then homogenized with ice-cold saline water in a Potter and Elvehjem glass homogenizer at 0°C. The homogenate was used almost immediately to test for the fatty acid oxidation.
Preparation of the Mitochondrial Fraction:

The method described in the Part I (Chapter V) was followed for the preparation of the mitochondrial fractions of the leg muscles. The mitochondrial preparation was checked on Oxygraph (Gilson model) before use. Succinic dehydrogenase activity was used as the marker enzyme for mitochondrial preparation and was assayed according to the method of Kun and Abood (1949) using triphenyl tetrazolium chloride as electron acceptor and sodium succinate as substrate.

Determination of Protein Content:

The protein content of the muscle homogenate as well as that of the mitochondrial preparation was determined according to the method of Lowry et al., (1951).

Measurements of Oxygen Uptake:

The oxygen consumption by the homogenate as well as of the mitochondrial fraction was assessed by the method of Susheela and George (1964) using conventional Warburg's apparatus at 30°C. The detailed procedure has already been described elsewhere (Chapter V).

The effect of added carnitine on the palmitate oxidation
was tested in the muscle homogenate as well as in the mitochondrial preparation with 0.15 M DL-carnitine. The amount of the homogenate and mitochondrial suspension were adjusted in their respective vessels so that each reaction vessel contained 3.7 mg. of the former and 1.6 mg. of the latter. The concentration of each of the substrates used in the present investigation was 0.01 M. After equilibration of the temperature in the flask, the substrate from the side arm was tipped in and readings were taken at the end of every 10 min. interval.

Sodium salts of butyrate, octanoate, palmitate and stearate were used as substrates for fatty acid oxidation. The oxygen uptake by the muscle homogenate as well as by the mitochondrial preparation was expressed as μl. O₂ uptake/mg. protein per 30 min.

RESULTS

The oxidation of different fatty acid salts by the homogenate as well as mitochondrial preparation of the leg muscles of Cybister are summarised in the table XIII and XIV and fig. VI. The leg muscle homogenate could show O₂ uptake only with butyrate and octanoate and not with palmitate and stearate (table XII). Although,
Table XIII. Oxidation of butyrate, octanoate, palmitate and stearate by the leg muscle homogenates of the Cybister beetle before and after induced swimming activity

<table>
<thead>
<tr>
<th>Substrates</th>
<th>O₂ uptake (µl/mg. protein/30 minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before exercise</td>
</tr>
<tr>
<td>Endogenous</td>
<td>38</td>
</tr>
<tr>
<td>Butyrate</td>
<td>49</td>
</tr>
<tr>
<td>Octanoate</td>
<td>45</td>
</tr>
<tr>
<td>Palmitate</td>
<td>39</td>
</tr>
<tr>
<td>Stearate</td>
<td>37</td>
</tr>
<tr>
<td>Succinate*</td>
<td>70</td>
</tr>
</tbody>
</table>

The results are average of three readings in each case.

* Used for comparison.
Table XIV. Effect of added carnitine on the palmitate oxidation by the leg muscle homogenate and mitochondrial fraction of the Cybister beetle.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>O₂ uptake μl/mg. protein/30 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Homogenate</td>
</tr>
<tr>
<td>Endogenous</td>
<td>39</td>
</tr>
<tr>
<td>Palmitate added</td>
<td>37</td>
</tr>
<tr>
<td>Carnitine added</td>
<td>45</td>
</tr>
<tr>
<td>Carnitine plus Palmitate</td>
<td>47</td>
</tr>
</tbody>
</table>

The results are averages of three determinations in each case.
Fig. VI  OXIDATION OF BUTYRATE OCTANOATE STEARATE AND PALMITATE BY THE EXERCISED LEG MUSCLE HOMOGENATE OF CYBISTER BEETLE.

O2 uptake
µl/mg protein/30 minutes

- ENDogenous
- BUTYRATE
- OCTANOATE
- PALMITATE + CARNITINE
endogenous respiration of the exercised leg muscle homogenate was moderately more than that of the unexercised one, the O₂ uptake was slightly increased only with butyrate and octanoate and not with palmitate or stearate. The oxidation of palmitate by the mitochondrial preparation revealed no increase in the O₂ uptake when palmitate alone was added to the reaction mixture. Increased in O₂ uptake was observed however, when carnitine plus palmitate were added to the Warburg's vessel (table XIV).

**DISCUSSION**

In recent years both the respiratory measurements (Beenakkers, 1963a; Bode and Klingenberg, 1964) and by the activity of the fatty acid oxidizing enzymes (Zebe, 1959; Beenakkers, 1963b; and 1964) it has been substantially proved that in the desert locusts lipid reserves were utilized by the flight muscles and that tissue was capable of fatty acid oxidation. Evidence has been sought in the previous study (Sect. B, II) that during vigorous swimming activity, the leg muscles of *Cybister* beetle utilized in addition to carbohydrates, lipids also. It could be expected that the leg muscles have the necessary biochemical machinery to make use of their lipid reserves.
It is discernible from the results obtained in the present study that the leg muscle homogenate has a capacity of oxidizing only the short chain fatty acids butyrate and octanoate and not the higher fatty acids palmitate and stearate. These results thus support the earlier findings of McShan et al., (1954) who have shown that the thoracic muscles of the wood roach Leucophaea maderae oxidized butyrate and octanoate in addition to acetate. Meyer et al., (1960) have demonstrated that the primer in the oxidation of higher fatty acids by the locust thoracic particulate fraction is butyrate. The same author (1960) has further shown that the particulate fraction from the muscles of desert locusts reared at 45°C was capable of oxidizing completely saturated fatty acids from C_{8} to C_{16} unlike that from those reared at 35°C, which could oxidize only butyrate and not the higher fatty acids. While discussing the fatty acid oxidation in the dragonfly Pantala flavescens Kallapur and George (1973) have suggested that to begin with the flight muscles of this insect oxidize short chain fatty acids and once the body temperature is increased the higher fatty acids appear to be made use of. The endogenous O_{2} uptake of the exercised leg muscle homogenate of the Cybister beetle was more than that obtained with unexercised one (table XIII). The O_{2} uptake however, with added
butyrate, octanoate, palmitate and stearate in the exercised one (table XII). These results seem to indicate that during vigorous exercise though the general metabolism of the leg muscles was increased, it had no effect on the oxidation of higher fatty acids.

It is well recognized now that carnitine stimulates the rate of oxidation of fatty acids (Friedman and Frankel, 1955; Fritz, 1955; Fritz and Marquis, 1965; Beenakkers, 1963b; Kallapur and George, 1973). Beenakkers (1963b) working with desert locust flight muscles has demonstrated that added carnitine markedly stimulates fatty acid oxidation both in the homogenate as well as in the mitochondrial fraction. He has also shown that addition of palmitoyl carnitine resulted the mitochondrial preparation exhibiting 50% more activity than the homogenate. That the $O_2$ consumption by the flight muscle mitochondria of the dragonfly *Pantala flavescens* with palmitate as substrate was considerably increased after the addition of carnitine has been shown by Kallapur and George (1973). In the present study palmitate oxidation by the homogenate as well as mitochondrial preparation was slightly increased after the addition of carnitine (table XIII).
These observations lead us to conclude that the leg muscles of the *Cybister* beetle have the capacity of oxidizing butyrate and octanoate but not the higher fatty acids. The failure to oxidize palmitate and stearate by the exercised muscles even when they are metabolically in a highly active state indicates that the *Cybister* beetle leg muscles lack the requisite biochemical machinery needed. It is generally believed that the amount of SDH in a tissue is an index of its oxidative capacity as well as mitochondrial density. The SDH activity in the leg muscles of the *Cybister* beetle was found to be only one third of its flight muscles as well as of the dragonfly *Pantala flavescens* (Chapter I and Sect. A).

**SUMMARY**

i. Experiments conducted by using muscle homogenate as well as mitochondrial fraction of the leg muscles of the *Cybister confusus* have suggested that they have limited capacity to oxidize butyrate and octanoate but not the higher fatty acids palmitate and stearate.

ii. Added carnitine seems to have stimulated the palmitate oxidation in the mitochondrial preparation.