CHAPTER 6

LIPASE ACTIVITY AND RATE OF OXYGEN UPTAKE DURING METAMORPHOSIS AND SOME OBSERVATIONS ON THE RESPIRATORY QUOTIENT AND THE CAPACITY FOR FATTY ACID OXIADATION OF THE LARVA

The hydrolysis of triglycerides into fatty acids and glycerol is catalysed by the enzyme lipase. A high lipase activity has been demonstrated both histochemically and quantitatively in the fat metabolizing tissues such as the flight muscles of birds (George and Scaria, 1956; George and Scaria, 1958; Vallyathan, 1963), bats (George, Susheela and Scaria, 1958), and insects (George, Vallyathan and Scaria, 1958; George and Bhakthan, 1960a,b&c) and the vertebrate heart (George and Iype, 1959, 1963) and a direct relationship between the activity of this enzyme and the extent of fat utilization has been established. Whether fat is used for energy purposes or for the synthesis of other substances like glycogen, conversion to fatty acids and glycerol is the first step in the chain of reactions that follow. A reduction in the fat content during the transformation of the larva to adult in Anthrenus has already been discussed (Chapter 5). It was of interest, therefore, to follow the concurrent changes in the activity of lipase during metamorphosis.

The occurrence of lipase in insects has been demonstrated histochemically or quantitatively in the fat bodies of Rhodnius (Wigglesworth, 1958), Schistocerca (George and Eapen, 1959a&b) and a number of other Orthopteran insects viz. Periplaneta americana, Gryllus, Grillotalpa africanus, Mecapoda elongata, Acrida exaltata, Aeolopus affinis, Epacromia dorsalis and Poecilocera

picta (Hegdekar, 1963) and the flight muscles of dragon fly, bumble bee (George, Vallyathan and Scaria, 1958) and a number of Orthopteran insects (George and Bhakthan, 1960a&b). The properties of the Cockroach fat body lipase was studied by Hegdekar (1963) and the enzyme concerned was shown to be a true lipase. A lipase system which hydrolyses triacetin, tributyrin, tri-n-valerin, tri-isovalerin and tricaprylin with tributyrin being the most susceptible, was also reported in the gut and other tissues of the house fly (Baker and Peretsky, 1958). The enzyme which acts on tributyrin at pH 7.4 is considered to represent a true lipase which is responsible for the hydrolysis of neutral triglycerides (Martin and Peers, 1953). This enzyme activity was determined in the present investigations employing a manometric technique. Histochemically, the enzyme could be demonstrated in the larval fat body of Anthrenus employing the classical lead nitrate technique of Gomori. But some difficulties were encountered in the interpretation of the results since it was observed that some substance present in the tissues, probably uric acid, interfered with the reaction by forming insoluble lead compounds with the final production of the coloured lead sulphide. This aspect is being investigated more critically at present and the results are not sufficiently conclusive to be presented here.

Since the present studies showed a high lipase activity in the larva, it was thought that it would possess an active system for the oxidation of fatty acids. Therefore the capacity of the whole body homogenate of the larva to oxidise Sodium butyrate under in vitro conditions was examined.

The rate of oxygen uptake during metamorphosis was also studied. Observations were also made on the respiratory quotient.

MATERIAL AND METHODS

The insects were obtained from laboratory cultures maintained at 32 ± 1°C on a diet of dried and crushed pigeon breast muscle supplanted with 5% Brewer's yeast.

Lipase activity: The lipase activity was measured by a manometric method adapted from Martin and Peers (1953) using a bicarbonate carbon-dioxide buffer system of pH 7.4 at 37°C in the Warburg respirometer (George, Valltathan and Scaria, 1958). Five insects were used for each determination. The insects were homogenized in 3 ml of ice cold distilled water in chilled mortars, centrifuged at 3000 r.p.m. for five minutes and 1 ml of the supernatant carefully pipetted avoiding the layer of fat on the surface and used as the enzyme material. The reaction flask contained 1.5 ml of 0.025M bicarbonate buffer solution and 1.0 ml of the enzyme solution in the main chamber and 0.5 ml of tributyrin (emulsified by shaking 4% (v/v) tributyrin in 0.0148M bicarbonate with a drop of 'Tween 80') in the side arm. The flasks and the manometers were gassed with a mixture of 95% nitrogen and 5% carbon dioxide for 3 minutes. After an equilibration time of 10 min in the constant temperature bath, the substrate was tipped and after another equilibration of 3min the levels were adjusted and the readings recorded. Further readings were taken at half hour intervals for one hour. The lipase activity is expressed as microlitres (µl) of CO_2 evolved per hour per insect and also as µl CO_2 per hour per 10mg wet weight of the insect.

To find out how much of the lipolytic activity was

due to the alimentary canal lipase, the alimentary canals from 5 larvae each were dissected out in ice cold conditions, homogenised in 1 ml of cold distilled water and the activity determined as above.

Fatty acid oxidation: The oxidation of butyric acid was studied by measuring the oxygen uptake in a standard manometric system. The method followed was the same as that adopted by George and Bhakthan (1963) who studied the oxidation of butyrate in honey bee flight muscles. Malate was used as a "sparker", since the presence of a citric acid cycle intermediate is known to be essential for the fatty acid oxidation system. The experiments were conducted at 37°C. The gas phase in the manometric system was atmospheric air. Each reaction flask contained 0.6 ml of 0.1M phosphate buffer of pH 7.4, 0.15 ml 0.15M $MgCl_2$, 0.2 ml 0.0225M ATP, 0.15 ml 0.00225M cytochrome C, 0.2 ml 0.015M DPN, 0.2 ml 0.0075M coenzyme A, 0.2 ml 0.15M Sodium malate (neutralized with KOH) and 0.8 ml of the homogenate. In addition, the samples (complete system) contained 0.5 ml of 0.03M Sodium butyrate which was substituted by 0.5 ml of distilled water in the blanks (incomplete system), thus making a total volume of 3 ml in both the flasks. This gave a final concentration of 20 µM phosphate buffer, 7.5 µM $MgCl_2$, 1.5 µM ATP, 0.1125 µM cytochrome C, 1 µM DPN, 0.5 µM coenzyme A, and 10 µM Sodium malate in each flask. The final concentration of Sodium butyrate was 5 µM in the samples. The central well of the reaction flask contained 0.2 ml of 20% KOH and a roll of filter paper to absorb the CO_2 evolved.

Homogenates were prepared by grinding 20 insects in 2 ml of ice cold distilled water in chilled mortars. Samples of 0.8 ml each from the same homogenate (this contained more than 50 mg of fresh tissue) was added in the complete and incomplete systems. The homogenate was added last and the flasks were placed on ice slabs until they were mounted on the manometers. The manometers were shaken at 100 oscillations per minute at an amplitude of 4.5 cm.

Oxygen uptake: The oxygen uptake was measured at 32°C using the Warburg respirometer. The central well of the flask contained 0.2 ml of 20% KOH and a Whatman filter paper of 2 cm square to absorb the CO_2 produced. Twenty insects were used for each determination and the oxygen uptake is expressed as µl O_2 per 10 mg of live weight per hour.

The respiratory quotient was determined by the direct method of Warburg (Umbreit et al., 1957) at 32°C.

RESULTS

Data obtained on the lipase activity are presented in Table I and Fig. I. The absolute activity per insect was maximum at the larval stage. Only a small part of this was contributed by the alimentary canal lipase which amounted to 14.16 ± 2.47 µl CO_2/ hour out of the 98.20 ± 12.8 µl CO_2/ hour liberated by the entire larva. The prepupa also showed a high lipolytic activity. The activity then decreased progressively till the 7th day pupa beyond which there was an increase. In the later life of the emerged beetle there was a definite increase in lipase activity. The absolute activity of the late adult (ie. activity per one insect) was lesser than that of the larva,

TABLE I

LIPASE ACTIVITY AND OXYGEN UPTAKE DURING METAMORPHOSIS

Stage of development	Lipase activity		Oxygen uptake
	µl CO_2/insect/hr	µl CO_2/10 mg body wt./hr	µl O_2/10 mg body weight/hour
Larva (full grown)	98.20 ± 12.80	145.40 ± 19.85	12.35 ± 1.12
Prepupa	69.20 ± 2.82	116.57 ± 6.00	4.91 ± 0.38
1st day pupa	40.47 ± 8.61	75.20 ± 15.03	3.79 ± 0.73
3rd day pupa	39.53 ± 7.58	76.18 ± 17.15	3.57 ± 0.82
5th day pupa	38.88 ± 6.95	69.62 ± 14.13	4.62 ± 0.76
7th day pupa	24.40 ± 5.57	45.87 ± 7.09	5.38 ± 0.34
1st day pr. adult	38.21 ± 4.68	71.95 ± 9.36	7.22 ± 0.79
3rd day pr. adult	31.72 ± 15.19	56.56 ± 21.55	9.01 ± 0.13
1st day emerged adult	34.94 ± 9.94	68.46 ± 19.11	11.62 ± 0.44
Late adult collected at random	62.21 ± 12.03	173.83 ± 33.57	-

Data presented represent the average of 5 or more determinations
± = Standard deviation

though it appears higher when based on unit body weight. Lipase

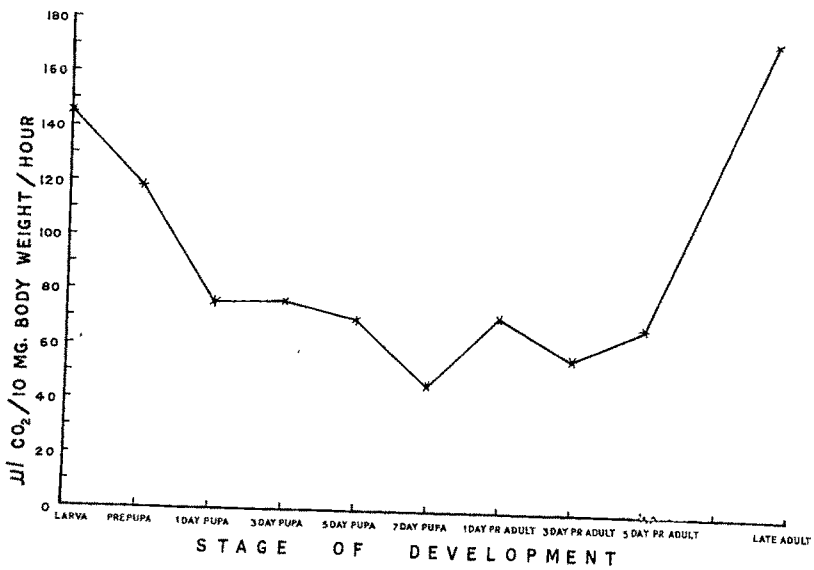

Fig. I Showing the changes in lipase activity during metamorphosis

thus showed a U shaped activity curve during metamorphosis. The range of variation was more in the pre-emergent and adult periods.

Results of the experiments on the oxidation of Sodium butyrate by whole body homogenates of the larva is presented in Table II. It could be noted that there was no increase in the oxygen uptake in the system which contained butyrate compared to that of the control (containing malate alone). This shows that the homogenate of the larva was incapable of oxidising Sodium butyrate under <u>in vitro</u> conditions.

TABLE II

RESULTS OF THE EXPERIMENTS ON THE OXIDATION OF SODIUM BUTYRATE BY WHOLE BODY HOMOGENATES OF THE LARVA

No. of experiments	Oxygen uptake by the incomplete system (with malate alone) µl O_2/10mg body wt./hr	Oxygen uptake by the complete system (with malate and butyrate) µl O_2/10mg body wt./hr
1.	3.311	2.209
2.	4.315	1.850

The oxygen uptake during the different stages of metamorphosis is shown in Table I and Fig. II. Oxygen uptake

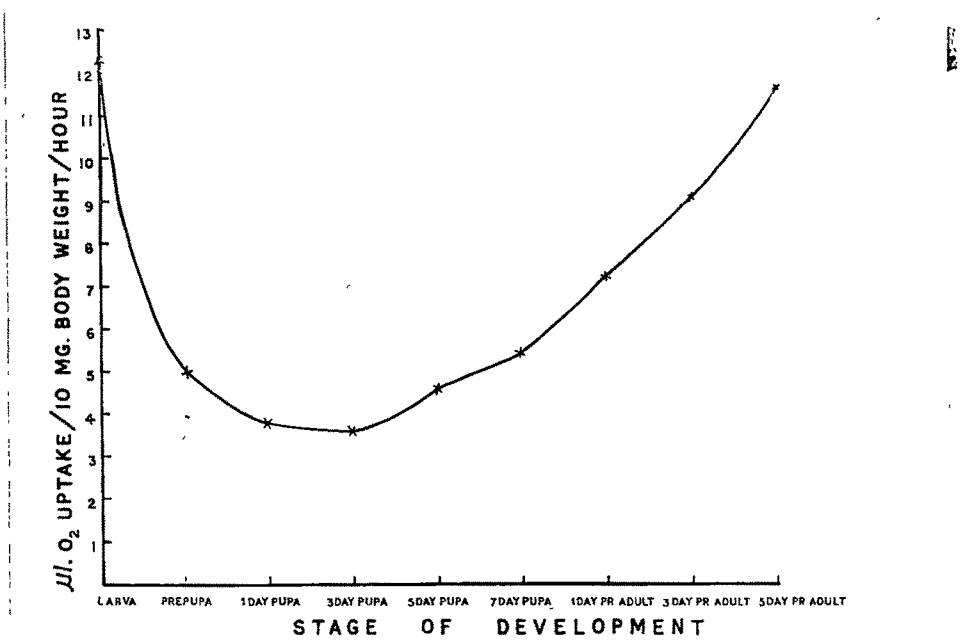

Fig. II Graph showing the oxygen uptake during metamorphosis

followed a U - shaped curve during metamorphosis, the lowest level being in the period between the 1st day and the 3rd day pupa.

The respiratory quotient of the larval stage was found to be 0.827 ± 0.071. In some of the pupal stages viz. the prepupa and the late pupa the CO_2 production was found to be erratic without any definite rhythm, though the O_2 uptake was uniform and therefore the respiratory quotient could not be calculated with sufficient degree of precision.

DISCUSSION

Fat metabolism has been extensively studied in higher animals and many of the synthetic and degradative pathways have been well established. Relatively little is known about the metabolism of fat in insects. Though the synthesis of fat by cell free preparations of the fat body (Zebe and McShan, 1959; Tietz, 1961) and its utilization at different phases of insect life such as pupation, inanition and sustained flight have been demonstrated our understanding of the various enzymatic reactions involved in the synthesis as well as break-down of fat in insects is far from complete.

Lipase has been demonstrated in many insects of different Orders, mainly in the fat body and the flight muscles of efficient fliers. In Anthrenus larva since only a small part of the activity was contributed by the alimentary canal lipase the major part of this enzyme could be thought to exist in the tissues, particularly in the fat body. The high rate of lipase activity could denote a high rate of hydrolysis of fat. It was

expected therefore that the fatty acids liberated by the hydrolytic action of lipase may be oxidised by the larva for energy purposes. But the present experiments failed to demonstrate the oxidation of butyrate. No explanation can be provided for this at present. Though the *in vitro* oxidation of fatty acids was demonstrated in the thoracic tissues of *Schistocerca* (Meyer *et al.*, 1960) and *Apis* (George and Bhakthan, 1963), it could not be demonstrated in the thoracic tissues of *Locusta* or in the whole body homogenate of mosquitoes (Gilmour, 1961). As Gilmour (1961) has pointed out it is possible that the right experimental conditions have not yet been established for the study of fatty acid oxidation in insect preparations. Though the presence, in insect tissues of the enzyme lipase which catalyses the hydrolysis of fat into fatty acids and glycerol is now well established, regarding the further metabolism of fatty acids and the enzymes involved we have little knowledge at present. Perhaps its metabolic fate and the site where it occurs may vary in different insects and at different stages of development in the same insect.

Since the larva is mainly concerned with the synthesis of large amounts of fat, the question may be raised here whether the high lipase activity of the larva indicates the esterification of fatty acids with glycerol under *in vivo* conditions. Lipase was once thought to catalyse both the hydrolytic as well as the esterifying reactions (Baldwin, 1957). But there is increasing evidence at present to show that the synthesis of triglycerides

takes place not by the reaction between fatty acids and glycerol but by the reaction between fatty acyl coenzyme A and glycerophosphate (Hübscher, 1963). The formation of these high energy compounds is an endergonic process and therefore the synthesis of fat by the reversal of lipase action has been questioned (Jedekin and Weinhouse, 1954; Tietz and Shapiro, 1956). However, it remains to be explained whether the same enzyme or enzymes could help in the synthetic process under in vivo conditions in the presence of ATP and coenzyme A, and catalyse the breakdown of fat in the absence of these. If that is so, the high enzyme activity obtained in the larva could be explained as an index of the esterification of fatty acids in the physiological conditions existing in the living insect. The same enzyme may also catalyse the hydrolysis of fat under in vitro conditions as is seen here. It may be mentioned here that in the chick embryo there is a high lipase activity both in the yolk sac as well as the liver by the 16th day of incubation, when the fat level in the yolk sac shows a decline and the liver fat shows a steady increase (George and Iype, 1962). These observations led the authors to infer that while lipase is actively breaking up fat into fatty acids in the yolk sac the lipase in the liver is building up the liver fat from the fatty acids.

Prepupa also shows considerable activity of lipase, which drops gradually following a U shaped curve corresponding to that of oxygen uptake. Since a reduction in fat content occurred during the pupal period, the lipase activity

definitely indicates the hydrolysis of fat. But nothing could be said regarding the fate of the fatty acids thus liberated. They may either be converted to carbohydrates *in toto*, or while fatty acids could be oxidised to produce energy, glycerol could be used for building up carbohydrates simultaneously.

The high lipase activity in the pre-emergent and emerged adults indicates a considerable break-down of fat. Since fat is required for deposition in the developing eggs, fatty acids liberated from the fat body may be transported to the ovary. Transport of fat in the form of fatty acids is known in vertebrates (Gordon, 1957, 1958; Fredrickson and Gordon, 1958; Vallyathan, 1963). Though it was shown that glycerides could be released as such from locust fat body into the haemolymph under *in vitro* conditions (Tietz, 1962), it could be expected that the transport of fat into the developing ovaries would require the breakdown of fat into fatty acids so as to enable its penetration into the ovarian tissue and for its release from the fat body which occurs probably in the form of a mixture of triglycerides, partial glycerides and free fatty acids. A correlation between lipase activity in the fat body and the deposition of triglycerides in the developing oocytes has also been established in the grasshopper, *Poecilocera picta* (Hegdekar, 1963). The high individual variations in the lipase activity during the pre-emergent and adult periods may be a sex difference. The possibility of fat being utilized also as a source of energy

during adult life cannot, however, be ruled out.

No far reaching conclusions could be made from the data obtained on the oxygen uptake or the respiratory quotient. It is well known that the curve of oxygen uptake follows a more or less U shaped course during the pupal period (Wigglesworth, 1950; Karlson and Sekeris, 1964). The present results are in conformity with this general observation. The low oxygen consumption during the initial stages of pupation shows that the aerobic energy metabolism is at a low ebb during this period. Probably the energy comes from anaerobic utilization of glycogen during this period.

The respiratory quotient which represents the ratio of the volume of CO_2 produced to the volume of O_2 consumed often helps to understand the nature of the energy metabolism. If only carbohydrates are burned, one molecule of CO_2 will be produced for each molecule of O_2 consumed and therefore the RQ will be 1. The RQ obtained here for the larval stage is consistently below 1 and shows that carbohydrates do not form the major part of the metabolites oxidised. This is only to be expected since the food consists mainly of protein and since a quantitative analysis of the larval body showed a high percentage of fat and very little of glycogen. The RQ which varied between 0.71 to 0.91 with a mean of 0.82 could be taken to indicate the oxidation of fat and probably of some protein too. In a biological system, none of the substrates are oxidised solely to the exclusion of others at

any time and so probably fats and proteins and to a much lesser extent carbohydrates are also oxidised during the larval stage. Proteins are not usually oxidised to produce energy, but in this insect since the food consists mainly of protein, it is likely that some aminoacids are oxidised to produce energy and metabolic water.

As mentioned earlier the CO_2 production in some of the pupal stages was inconsistent and erratic and the RQ could not be calculated. The oxygen uptake, on the other hand, was continuous in all the stages of metamorphosis. It is interesting to note that the CO_2 production in the larval stage was also continuous. It is known that in some insects the CO_2 is given off discontinuously, in discrete bursts (Buck, 1962). Schniederman and Williams (1955) made a detailed study of this phenomenon in diapausing Cecropia pupae and showed that at low metabolic rates the 'CO_2 bursts' are accentuated. The burst phenomenon tends to disappear when the oxygen uptake is high. This explains why a continuous CO_2 production occurs in the larval stage of Anthrenus as against some periods in the pupal stage. Oxygen uptake was found to be continuous and noncyclic in Cecropia pupa also. The elucidation of the pattern of CO_2 production in the pupal stage of Anthrenus requires further study.