

DEVELOPMENT OF CATABOLIC PATHWAYS IN INSECT FLIGHT MUSCLES. A COMPARATIVE STUDY

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Abstract—Activities of enzymes representative of glycolytic and β -oxidative pathways and citric acid and glycerophosphate cycles were measured in the developing flight muscles of three species: *Calliphora erythrocephala*, *Locusta migratoria*, and *Philosamia cynthia*. The activities were measured *in vitro* under optimal conditions.

The enzyme pattern of young flight muscles is quite different from the adult pattern. In the second half of the developmental period final differentiation towards the adult metabolic pattern takes place, in *Calliphora* leading to exclusively carbohydrate-oxidizing capacities, in *Locusta* to properties enabling both aerobic glycolytic and β -oxidative processes, whereas *Philosamia* becomes oriented to fatty acid oxidation. This differentiation starts after a temporary rise of lactate dehydrogenase activity, a phenomenon that seems to be connected with invagination of tracheoblasts into the muscle fibres. This tracheolization might be necessary for differentiation towards the species specific metabolic properties of the adult flight muscle.

Theoretical aspects of the enzyme activities, as they were measured in the *in vitro* assays, are discussed and related to the physiological qualities of the flight muscles of the three species investigated.

INTRODUCTION

CARBOHYDRATES and fatty acids play a predominant rôle in the generation of energy for prolonged flights in insects (BEENAKKERS, 1969; SACKTOR, 1970). Participation of amino acids in catabolic pathways of flight muscles has been demonstrated in some insect species (SACKTOR and CHILDRESS, 1967; STEVENSON, 1968; DE KORT *et al.*, 1973), but it has not been proved that they contribute directly to the energy supply for continued flight; proline, for instance, might enhance the rate of pyruvate metabolism in flight muscles of *Phormia regina* by raising the mitochondrial concentration of citric acid cycle intermediates (SACKTOR and CHILDRESS, 1967).

It is generally agreed that three physiological types of insect flight muscles can be distinguished: carbohydrate utilizers, lipid utilizers, and combination utilizers (BEENAKKERS, 1969). BEENAKKERS (1969) investigated the catabolic properties of

the dorsal longitudinal flight muscles of nine insect species belonging to four different orders by measuring enzyme activities of the glycolytic chain, the β -oxidative pathway, and the citric acid cycle and showed among others that the flight muscles of *Apis mellifica* (Hym.) are specialized for carbohydrate oxidation, those of *Philosamia cynthia* (Lep.) for fatty acid oxidation, whereas the flight muscles of *Locusta migratoria* (Orth.) can use both carbohydrates and fatty acids. As far as investigated, most Diptera are dependent on carbohydrates during flight (*Drosophila*, WIGGLESWORTH, 1949; *Phaenicia*, YURKIEWICZ and MATHUR, 1969; *Aedes*, NAYAR and VAN HANDEL, 1971).

The final characteristics of the adult flight muscles must be obtained during the structural and functional development of the growing muscles. BEENAKKERS (1963, 1964) measured the activities of some catabolic enzymes during the development of locust flight muscles. In an elaborate study BROSEMER *et al.* (1963) investigated the development of subcellular structures together with enzymatic properties in the flight muscles of the same insect.

In endopterygote insects data about muscle development are mainly restricted to structural studies. HEROLD (1965) investigated sarcosomal development in the honey-bee flight muscle; NÜESCH (1965) connected structural development of flight muscles of *Antheraea* with the functional appearance of actomyosine. Electron microscopic studies of flight muscle development in *Calliphora* were made by AUBER (1969) and in *Lucilia* by PERISTIANIS and GREGORY (1971).

The present study deals with the differentiation of enzyme activities in developing flight muscles of *Philosamia cynthia*, and *Calliphora erythrocephala*, (which in preliminary experiments turned out to be a carbohydrate utilizer) in comparison with *Locusta migratoria*.

MATERIALS AND METHODS

Insects

Migratory locusts, *L. migratoria*, were reared in our laboratory under crowded conditions. Within 24 hr after the fourth ecdysis the larvae were collected and kept under crowded conditions at 30°C, r.h. of about 40%, and with a photoperiod of 12 hr. They were fed with reed or with equal parts of wheat and rye-leaves, supplemented with rolled oats. Under these conditions the fifth instar period lasts 9 days.

P. cynthia was reared at 24°C, r.h. of 50%, and with a 12 hr photoperiod. Egg development lasted 12 days; then after about 30 days the larvae started spinning the cocoon and generally 4 days later larval-pupal ecdysis took place. Eighteen days after the ecdysis the adult emerged. The larvae were fed with privet leaves.

The blowfly *C. erythrocephala* was reared at 25°C and a daily illumination of 16 hr. Oviposition took place on rumpsteak, and egg development was completed within 24 hr. After a developmental period of 6 to 7 days on rumpsteak, the larvae were starved for the next 2 days; then pupariation followed. One day after pupariation the animals were collected. Further development until adult emergence lasted 10 to 11 days. The adults were fed with water and a mixture of sucrose and yeast flakes (3 : 1, w/w).

Preparation of the flight muscles

In the locust, already on the day following the last larval ecdysis distinct flight muscles can be distinguished. On several days during fifth instar and adult development the dorsal longitudinal flight muscles were dissected out and placed in 3 ml 0.1 M potassium phosphate buffer, containing 2 mM sodium EDTA (pH 7.3), kept on ice. In each experiment about 100 mg muscle (fresh weight) was used.

On several days between the larval-pupal and pupal-adult ecdysis and after adult emergence, flight muscles of the mesothoracic segment of *Philosamia* were collected. Particularly during the first days after larval-pupal ecdysis the dorsal flight muscles cannot be exactly separated from the fat body and other tissues in the thorax, and therefore contamination with non-muscular material occurs. About 60 to 80 mg of the collected material was placed in the buffer mentioned above.

In *Calliphora* dissection of the flight muscles was practically impossible, therefore about 20 thoraces were collected on several days after pupariation up to and including the first day after emergence of the adult. With a double scalpel the thoracic part of the insect was cut out. After adult ecdysis, the thoracic appendages and the head were cut off and the abdomen and gut were removed. Particularly during pupal and pharate adult development the flight muscles were contaminated with non-muscular material.

The collected muscles were disrupted with an ultra-Turrax disintegrator (Janke-Kunkel). The suspension was stirred for 30 minutes with a magnetic stirrer and then centrifuged (20,000 *g* for 15 min). The residue was resuspended in 3 ml of fresh K-phosphate buffer. The extract and the resuspended sediment were separately assayed for enzyme activities. All manipulations with the dissected muscles until measurement of the enzyme activities were carried out at 0 to 2°C.

Estimation of enzyme activities

Activities were estimated photometrically at 25°C, using a photometer with automatic recording of absorption against time. The following activities were measured: glyceraldehydephosphate dehydrogenase (E.C. 1.2.1.12; GAPDH), lactate dehydrogenase (E.C. 1.1.1.27; LDH), glycerophosphate dehydrogenase (E.C. 1.1.1.8; GDH), 3-hydroxyacyl-CoA dehydrogenase (E.C. 1.1.1.35; HOAD), citrate synthase (E.C. 4.1.3.7; CS), and succinate dehydrogenase (E.C. 1.3.99.1; SDH). The assay mixtures are described in BEENAKKERS (1969).

Protein determinations

Protein was determined according to LOWRY *et al.* (1951), using a bovine albumin solution as a standard.

RESULTS

As discussed earlier (BEENAKKERS, 1969), it is possible to evaluate the rôle of a particular enzyme in the metabolic system by comparing the activities of that enzyme in different tissues, for instance various muscles or, in the scope of the present investigation, on different developmental days of a certain organ. The

enzymes were chosen in such a way that participation of the main catabolic processes, important for energy generation, could be estimated. Moreover by comparing the ratios of enzymes belonging to different pathways, information about their quantitative significance for metabolism in various muscles can be obtained (PETTE, 1965).

TABLE 1—RELATION OF ENZYME ACTIVITIES IN THE DEVELOPING FLIGHT MUSCLES OF THE BLOWFLY, *C. erythrocephala**

	Age					Day after adult emergence
	Days after pupariation					
	2	4	6	8	10	1
Glycolytic chain						
GAPDH	46,100	31,400	46,900	69,500	114,400	194,500
LDH	13,900	7400	9700	4500	2800	< 50
Glycerophosphate cycle						
GDH	70	< 50	< 50	340	11,900	48,300
β -oxidative pathway						
HOAD	4800	2300	4000	4200	2200	< 50
Citric acid cycle						
CS	2200	1700	2350	9900	23,800	45,200
Ratio:						
GAPDH/GDH				205	10	4
GAPDH/HOAD	10	14	12	16	52	> 1000
GAPDH/CS	21	18	20	7	5	4
HOAD/CS	2.2	1.3	1.7	0.4	0.1	→ 0

* Activities expressed as μ moles/g tissue protein per hour. Each value represents the mean of a least 5 experiments.

Table 1 summarizes data on the activities of the enzymes measured in the blowfly. During the first 6 days of development flight muscles show relatively low CS activities and high LDH activities. In the second half-period metabolism becomes highly aerobic, as CS activity increases considerably and the activity of LDH drops to very low values. Together with a rise in glycolytic capacity the glycerophosphate cycle becomes very important, as shown by an enormous increase in GDH activity (*cf.* the ratio GAPDH/GDH). As particularly in experiments with animals shortly after pupariation contamination with other thoracic tissues will be substantial, it is not clear whether the muscles themselves ever had

β -oxidative capacity. But at any rate the decreasing activities of HOAD indicate a decrease in fatty acid oxidation. In the adult, flight muscle metabolism is confined to aerobic breakdown of carbohydrate; the ratio GAPDH/CS is four and the ratio HOAD/CS approximates zero.

TABLE 2—RELATION OF ENZYME ACTIVITIES IN THE DEVELOPING FLIGHT MUSCLES OF THE MOTH, *P. cynthia**

	Developmental day after						
	Larval-pupal ecdysis					Adult emergence	
	4	7	10	12	17	3	5
Glycolytic chain							
GAPDH	25,100	15,000	14,900	16,000	19,700	17,600	18,100
LDH	1590	2400	4100	6000	2800	1300	600
Glycerophosphate cycle							
GDH	7600	3700	2300	2100	4500	11,100	12,740
β -oxidative pathway							
HOAD	3800	3600		10,300	66,000	82,000	98,000
Citric acid cycle							
CS	1600	1800		3700	43,000	71,000	80,500
SDH	<50	<50		170	1900	3600	4200
Ratio:							
GAPDH/GDH	3.3	4.1	6.5	7.6	4.4	1.6	1.4
GAPDH/HOAD	6.6	4.2		1.6	0.3	0.2	0.2
GAPDH/CS	1.6	8.3		4.3	0.5	0.2	0.2
HOAD/CS	2.4	2.0		2.8	1.5	1.1	1.2
CS/SDH				22	23	20	19

* Activities expressed as μ moles/g protein per hour. Each value represents the mean of at least 5 experiments.

Table 2 gives the activity data in the flight muscles of *P. cynthia*. With respect to our experimental data the following phenomena during development are most important. GAPDH activity remains practically unchanged; the initial higher value on the fourth day after larval-pupal ecdysis could be caused by contamination with non-muscular material. During the first developmental period the activity of HOAD is low, but around day 12 its specific activity increases considerably which is continued until day 5 of the adult life. The activity of CS follows the same trend. LDH activity is increasing up to the twelfth day after larval-pupal ecdysis and then decreases considerably. GDH activity is acting in the opposite

TABLE 3—RELATION OF ENZYME ACTIVITIES IN THE DEVELOPING FLIGHT MUSCLES OF THE LOCUST, *L. migratoria**

	Developmental day after									
	Fifth larval ecdysis					Larval/adult ecdysis				
	2	5	8	2	4	6	8	12		
Glycolytic chain										
GAPDH	23,000	36,500	41,000	45,800	52,500	75,5000	69,200	68,800		
LDH	1600	1600	1830	690	590	320	300	310		
Glycerophosphate cycle										
GDH	800	1300	1700	22,700	29,200	37,400	33,800	32,600		
β -oxidative pathway										
HOAD	4800	9000	15,000	24,400	43,100	56,000	62,700	66,300		
Citric acid cycle										
CS	8000	14,000	18,700	22,800	34,100	44,300	52,000	56,500		
Ratio:										
GAPDH/GDH	29	28	24	2.0	1.8	2.0	2.0	2.1		
GAPDH/HOAH	4.8	4.1	2.7	1.9	1.2	1.3	1.1	1.0		
GAPDH/CS	2.9	2.6	2.2	2.0	1.5	1.7	1.3	1.2		
HOAD/CS	0.6	0.6	0.8	1.0	1.3	1.3	1.2	1.2		

* Activities expressed as μ moles/g tissue protein per hour. Each value represents the mean of 4-7 experiments.

way, emphasizing an anaerobic period in about the middle of the total developmental period. Comparison of the ratios GAPDH/HOAD on the various developmental days reveals that during development the flight muscles become oriented to fatty acid oxidation which is also to be seen from the final ratio GAPDH/CS being 0.2, and the reduction of the ratio HOAD/CS to 1.2 during the second half period of development. From the day that SDH activity was definitely measurable with our assay, a virtually constant proportion to CS activity exists, indicating a concomitant rise in citric acid cycle enzymes. The ratio between these two enzymes is almost identical with that found in the flight muscles of several other insects (BEENAKKERS, 1969).

The enzyme activities in the flight muscles of the locust are shown in Table 3. During the fifth instar, in the beginning of which flight muscle development starts, the metabolic pattern is different from that after adult ecdysis. GDH activity is low but increases considerably during the moulting period; LDH activity, on the contrary, remains relatively high until ecdysis, whereupon its specific activity decreases. In the first week of development the activity of CS is higher than in the muscles of both other insects during a corresponding period; this could mean that the metabolism has more aerobic properties. After adult ecdysis the specific activity of CS increases strongly. This is accompanied by a substantial rise of HOAD activity; its increase exceeds that of CS, as is visible also from the changes in the HOAD/CS ratio. Contrary to the situation in the blowfly GAPDH/CS ratio in the adult locust muscle is only 1.2; the HOAD/CS ratio equals that in the moth.

There is a striking phenomenon, present in the development of the flight muscles of the three species examined: at about the middle of the developmental period, LDH activity reaches a relatively high value. For reasons discussed later, in Fig. 1 the activities of the enzymes on the various developmental days were plotted in relation to the activity of the respective enzymes on the day of maximal LDH activity. It seems quite pertinent that this day is a decisive day in development; from this day on a definite course leading to the ultimate metabolic pattern is taken. Aerobic pathways become more pronounced, not only the citric acid cycle, but also the glycerophosphate cycle, important for reoxidation of cytoplasmic NADH originating from carbohydrate oxidation. In *Calliphora* β -oxidation becomes unimportant. In *Philosamia* the metabolic pattern is definitely driven to fatty acid oxidation; the glycolytic pathway is unaffected. In *Locusta* the activity of this pathway is still increasing after the reference day, and β -oxidation becomes relatively more and more important.

DISCUSSION

In order to evaluate activity changes during the development of the flight muscles and to compare enzyme patterns in the various developmental stages, we must keep in mind that particularly in early development other material than flight muscles will be present in thoracic preparations, at least in the two endopterygote

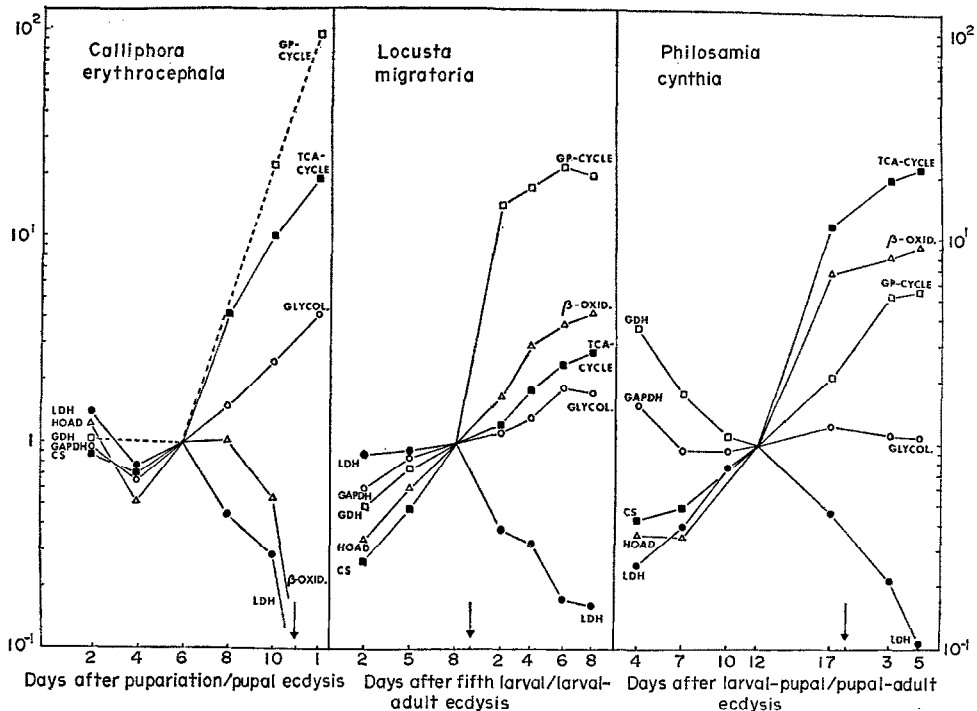


FIG. 1. Development of the main catabolic pathways in the flight muscles of three insect species. The activities of the enzymes at the indicated developmental days are related to the activity of the respective enzyme at the day of maximal lactate dehydrogenase activity. Related activities are plotted in a logarithmic scale. On the left side of each drawing the actually measured enzymes are given, on the right side the pathway they represent. Arrows indicate the moment of adult emergence. (For abbreviations see Materials and Methods; GP cycle, glycerophosphate cycle).

insects. We therefore started our experiments not before the second day after pupariation in *C. erythrocephala* and day 4 after larval-pupal ecdysis in *P. cynthia*. In the latter insect flight muscles were dissected out as well as possible. In another lepidopteran, *Antheraea*, NÜESCH (1965) showed that already at day 3 muscle fibres of the adult muscles are present. In the blowfly *Lucilia cuprina*, PERISTIANIS and GREGORY (1971) demonstrated that at the first day after puparium formation the development of the dorsal longitudinal flight muscles starts by longitudinal cleavage of two sets of three larval muscles in which neighbouring myoblasts are incorporated. In *C. erythrocephala* AUBER (1969) also saw larval muscles taking part in the development of adult flight muscles.

So, although certainly some products of histolytical processes will have been present in early pupal preparations, it seems quite certain that a substantial part of it was muscular tissue, quickly increasing later. These considerations and the foregoing data lead us to state that the various developmental stages of the flight

muscles of the insects investigated show their own characteristic set of enzyme activities. The ultimate enzyme pattern is different from the pattern in earlier stages, at least up to a certain developmental level. Not only the specific activities of the enzymes are different, but also the ratios between the enzymes. In *P. cynthia* the final pattern is reached shortly after imaginal ecdysis and the maximum catabolic activity around day 3 of emerged adult life. In the locust the definite enzyme pattern is present in the 8-day-old emerged adult, although some activity ratios, for instance GAPDH/GDH and HOAD/CS, become 'final' some days earlier. The most profound changes take place in the period between the last day before and 4 days after adult ecdysis. This is in accordance with the findings of BROSEMER *et al.* (1963) that in *Locusta* during the first days of emerged adult life differentiation of the typical adult flight muscle characteristics occurs. In *C. erythrocephala* the enzyme pattern in the flight muscles of the first-day-emerged adult may not be the ultimate pattern. AUBER (1969) showed that the number of filaments in the myofibrils increases till day 10, but any profound change in enzyme ratios is not to be expected, although the specific activities of glycolytic and citric acid cycle enzymes may still increase after day 1. The enzyme ratios in the flight muscles of the adult honey-bee, also a carbohydrate utilizer, are almost identical with those of the blowfly on the first day after adult emergence (BEENAKKERS, 1969). The most pronounced changes are observed 6 days after pupariation; from that day on metabolism is drastically turned over to oxidative carbohydrate breakdown.

For all three muscle types examined it is clear that essential changes in the metabolic pattern of the developing flight muscles take place after the temporary rise in LDH activity, i.e. at day 6 after puparium formation in *Calliphora*, 12 days after larval-pupal ecdysis in *Philosamia* and at the day of larval adult ecdysis in the locust. For the last-named insect BROSEMER *et al.* (1963) have correlated the rise in LDH activity with the invagination of tracheoblasts into the muscle fibre, a phenomenon consequently taking place after larval-adult apolysis. Differentiation of locust flight muscles succeeds this tracheolization. Also in the two endopterygote insects investigated, the moment of maximum LDH activity seems to be some kind of turning point, a decisive point for further development towards the adult metabolic pattern. As was visualized in Fig. 1 from that moment on the definite course to final metabolic properties is taken, both with respect to the aerobic nature as to the kind of substrate(s) used for energetic purpose. If it is true that also in *Calliphora* and *Philosamia* the temporary rise in LDH activity reflects tracheoblast invagination, it might be assumed that this phenomenon necessarily precedes final development of the flight muscle. As far as we are aware, no data are available to confirm this in Lepidoptera. In another blowfly, *Lucilia*, in which adult emergence takes place $7\frac{1}{2}$ days after pupariation, PERISTIANIS and GREGORY (1971) demonstrated penetration of tracheoblasts into the muscle fibres at day $3\frac{1}{2}$ to $4\frac{1}{2}$. These time relations are in good agreement with our enzymatic findings in *Calliphora*. WOLFE (1954) stated that in *C. erythrocephala* pupal-adult apolysis takes place at *ca.* 80 hr after pupariation, which indicates that also in this insect tracheolization occurs after adult apolysis as in the locust. Of course, our findings do not allow any

causal relation to be predicted between these time-sequential processes; it should be merely used as a working hypothesis.

There is another point that ought to receive some attention. PETTE (1965) discussed the significance of enzyme ratios, as presented in the tables, in giving information about the quantitative importance of a certain pathway in the specific metabolism of a particular tissue (the so-called correlation of systems). Table 4

TABLE 4—SYSTEM CORRELATIONS IN THE ENZYME PATTERN OF THE FLIGHT MUSCLE OF THE INSECTS

	<i>C. erythrocephala</i>	<i>L. migratoria</i>	<i>P. cynthia</i>
$\frac{\text{Glycolysis}}{\beta\text{-Oxidation}} : \text{GAPDH/HOAD}$	> 1000	1.0	0.2
$\frac{\text{Glycolysis}}{\text{Citric acid cycle}} : \text{GAPDH/CS}$	4	1.2	0.2
$\frac{\beta\text{-Oxidation}}{\text{Citric acid cycle}} : \text{HOAD/CS}$	→0	1.2	1.2

summarizes the main system correlations in the adult flight muscles of the three insects investigated. It must be emphasized that we are dealing here with obligatory aerobic muscles. As already discussed, the GAPDH/HOAD ratio indicates the relative importance of the two major substrates for energy generation.

We may assume that the activity of the citric acid cycle is related to the energy demand of the adult flight muscle. This implies that for optimal use of this cycle the activities of the preceding pathways, glycolysis and β -oxidation, must meet its activity. In normal *in vivo* situations a 1 : 1 ratio between each of the supplying pathways and the Krebs cycle is supposed. In our *in vitro* measurements, under optimal activity conditions, a deviating value could be expected. As the blowfly is an exclusively carbohydrate utilizer the GAPDH/CS ratio will be representative for an optimal relation between the pathways to which they belong. Hence it follows (Table 4) that in order to obtain a maximum of energy from the oxidation of carbohydrates, i.e. to ensure an efficient supply of acetyl-CoA to the citric acid cycle, under the conditions used in the *in vitro* assays the enzyme activity of GAPDH must be four times that of CS. This value is in good accordance with the GAPDH/CS ratio of 4.5 in the brain tissue of the rat (PETTE, 1965). If in some tissue fatty acid oxidation is the exclusive pathway, as in *P. cynthia* flight muscles during continued flight, the data in Table 4 predict the activity of HOAD to be 1.2 times that of CS. If we consider the enzyme ratios from locust flight muscles in the light of these figures it is evident that fatty acid oxidation can meet the energy demands of intense flight, but carbohydrate oxidation, however, cannot, which

supports the idea that carbohydrates are used in raising thoracic temperature, during first flight activities or in periods of relatively low wing-beat frequencies.

The GAPDH/CS ratio of 0.2 in *Philosamia* suggests that glycolysis, although being of little, if any, value for direct energy supply, might have some significance for flight. This suggestion is supported by the fact that in the second half of development the activity of the glycerophosphate cycle, represented by GDH, increases. It seems possible that glycolytic activities are meant for the supply of citric acid cycle intermediates, a suggestion that was made earlier by STEVENSON (1968) in discussing the results of experiments on carbohydrate metabolism in *Prodenia eridania*.

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