CELL STRUCTURE AND THE METABOLISM OF INSECT FLIGHT MUSCLE

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The biochemical properties of housefly flight muscle have recently been investigated to ascertain the mechanisms whereby energy is made available for the contractile processes (Sacktor, 1952, 1953, *a* and *b*, 1954). These observations demonstrated the complete oxidation of some citric acid cycle intermediates in the sarcosomes (mitochondria) of these muscles, and showed that this oxidation could be coupled with synthesis of the energy-rich phosphate bonds in ATP.[1] Notwithstanding the multitude of enzymes in the mitochondrion, it was reported that isolated sarcosomes were unable to oxidize glycogen and glucose (Levenbook, 1953, and Sacktor, 1954). Since several experimenters had shown that these compounds are the main substances utilized during flight of flies (*cf.* Chadwick, 1953), it appeared that extrasarcosomal enzymatic activities, too, were concerned with flight metabolism. This viewpoint was also inferred from previous results (Sacktor, 1953, *a* and *b*), which demonstrated a parallel in the distribution of certain enzymatic properties and the three prime morphological particulars of the flight muscle; namely, fibrils, sarcosomes, and sarcoplasm (the matrix that engulfs both fibrils and sarcosomes). Additional evidence for the localization of enzymes within these morphological entities and the relationship between them in the over-all metabolism of the flight muscle is provided in the present study.

EXPERIMENTAL

Fractionation Procedures

Houseflies, *Musca domestica*, of mixed sexes were used. These were reared and maintained by the procedure described previously (Sacktor, 1950).

Flight muscle homogenates were prepared by grinding isolated thoraces in cold 0.9 per cent KCl with a Potter-Elvehjem homogenizer for 1 minute. The brei was then filtered through several layers of cheese-cloth to remove pieces of cuticle. The volume of the filtrate was adjusted with additional KCl so that the final suspension contained the equivalent of 25 thoraces per ml. (10 to 15 mg. dry weight per ml.).

The soluble and particulate fractions were obtained by centrifuging muscle homogenates, in the cold, for 10 minutes at about 10,000 *g*. The first supernatant was collected and used as

[1] The following abbreviations will be used: ATP, adenosinetriphosphate; ADP, adenosinediphosphate; AMP, adenosinemonophosphate; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide.

the sarcoplasm fraction. The residue was washed in KCl and recentrifuged for 5 minutes. The precipitate was then resuspended in a volume of KCl to yield the equivalent of 25 thoraces per ml. As previously reported (Sacktor, 1953, *a*), this fraction consisted of muscle fibrils and some intact, but mostly fragmented, mitochondria.

Sarcosomes were isolated by the technique already detailed (Sacktor, 1953, *a*, 1954).

Materials

ATP was obtained from Pabst Laboratories and ADP, AMP, DPN, TPN, and cytochrome *c* were from Sigma Chemical Company. All substrates were commercial preparations and were used without further purification. All solutions were adjusted to approximately pH 7.4.

Methods

Oxygen consumption measurements were made in a conventional Warburg apparatus. The temperature was 25°C. and the gas phase was air. All constituents of the reaction mixture were localized in the main compartment of the flasks. As soon as the enzyme suspensions were prepared, aliquots were added and the flasks were mounted on the water bath. After a 10 minute equilibration period the stop-cocks were closed and oxygen consumption readings were taken at 10 minute intervals for 1 hour.

The dry weight of homogenates was determined by drying an aliquot of the preparation in an oven at 110°C. for 24 hours. The weights were corrected for salt concentration. Protein, in mitochondrial suspensions, was determined by the method of Lowry *et al.* (1951). Crystalline bovine serum albumin was used as the standard protein.

Glycogen was determined by a modification of the procedure of Good *et al.* (1933).

RESULTS

1. The Endogenous Respiration of Flight Muscle Homogenates

Preliminary attempts to demonstrate oxidation of glucose by flight muscle homogenates were inconclusive since respiration was not stimulated upon addition of this substrate. Similar results were noted by Graham (1946) with muscle preparations from larvae of the codling moth and by Barron and Tahmisian (1948) with cockroach muscle. In the housefly, and probably with these other species, this negative result was apparently due to an extremely high endogenous respiration (QO_2, expressed as mm^3O_2/hour/mg. dry weight, was about 7.5). Earlier efforts to reduce this respiration by dialysis or by incubating homogenates so as to reduce their endogenous substrates were unsuccessful owing to loss of enzymatic activity. A satisfactory technique for overcoming this disheartening situation was found by using flies which had been previously starved. The effect of starvation on the endogenous respiration of flight muscle homogenates is shown in Table I.

As seen from this table, the endogenous respiration was diminished 50 per cent after a starvation period of 2½ days. Further starvation resulted in death of the flies. When 30 μM of glucose was added, the oxygen uptakes of homogenates were essentially the same irrespective of the number of days the flies went unfed. It was also observed that best results were obtained by initiating the starvation period immediately upon adult emergence rather than after the

flies had once been permitted to feed. In addition, it was found that these starvation effects were more rapid at higher temperatures and that starved males died before females similarly handled. The sexes were not different, however, in their rate of glucose oxidation.

Some insight into the nature of this endogenous substrate was found by concurrent analyses of the thorax of the starving fly for glycogen. These results are shown in Table I, from which it is apparent that there was a parallel in the decrease in glycogen content and the endogenous respiration of the muscle. Even up to death (2½ days), when some endogenous oxidation still remained, this compound was found. Allied to these observations, Wigglesworth (1949) determined by histochemical means the distribution of glycogen in *Drosophila* and its utilization during starvation. He found that, next to the fat body, the most conspicuous deposits of this carbohydrate were in the halteres and

TABLE I

Effect of Starvation on the Endogenous Respiration of Flight Muscle

Flies starved	Endogenous respiration*	Glycogen‡
days	*per cent*	*μg./thorax*
0	100	14
½	—	8
1	81	5
1½	73	5
2	65	3
2½	51	2

* Relative amount of O_2 uptake with glucose as substrate set at 100.

‡ Measured as glucose equivalents.

flight muscles, and that during starvation glycogen gradually disappeared from the halteres but persisted in the thoracic muscles even upon death. Glycogen from both these sources would be in the homogenates used in the present studies. Thus, all the evidence indicates that glycogen was the main substrate for the endogenous respiration of flight muscle.

2. The Effects of Cofactors on the Oxygen Uptake of Flight Muscle Homogenates

To obtain the maximal rate of oxidation with glucose as substrate, the flight muscle homogenate had to be fortified with cofactors. As shown in Table II oxygen consumption was stimulated when the enzyme preparation was augmented with inorganic phosphate, Mg ions, cytochrome *c*, and ATP. In contrast, additional DPN had no effect. In view of the possible aerobic oxidation of glucose *via* a TPN-requiring system, this coenzyme was also tried. It was found here that TPN, by itself or in combination with DPN, was not necessary for maximal respiration. It was noted that neither AMP nor ADP could substi-

tute fully for ATP. ADP, however, was better than the monophosphate. Various combinations of nucleotides, such as ATP plus ADP or AMP, were not as effective as ATP alone at the same total nucleotide concentration.

3. Respiratory Substrates for Flight Muscle Homogenates

Additional knowledge of the biochemical properties of whole muscle was a prerequisite for further studies on the metabolic role of each morphological

TABLE II

Requirements for Maximal Glucose Oxidation

Experiment No.	Test system	O_2 uptake
		mm.³/hr.
19–20	Complete	112
	" less phosphate	67
	" " cytochrome *c*	96
	" " Mg	90
	" " ATP	74
	" " DPN	115
	" " homogenate	0
	Endogenous	42
20–22	Complete	118
	" less DPN	122
	" " " , plus TPN	113
	" plus TPN	120
21–25	Complete	118
	" less ATP, plus AMP	57
	" " " , plus ADP	87
23–25	Complete, (ATP, 10 μM)	108
	" plus ADP	95

The complete reaction mixture contained: 30 μM phosphate buffer, pH 7.4; 20 μM $MgCl_2$; 5 μM ATP; 30 μM glucose; 0.57×10^{-4} μM cytochrome *c*; 0.3×10^{-2} μM DPN; 1.0 ml. enzyme preparation; and 0.9 per cent KCl to a final volume of 2.3 ml. When indicated, 5 μM AMP or ADP, and 0.6 mg. TPN were added. Flies starved 2 to 2½ days.

entity. To obtain such information, approximately 70 compounds of various types were selected and evaluated as possible respiratory substrates. For expedience, a standard experimental procedure for all substrates was adopted. This was based on the conditions found optimal for glucose oxidation, as described in the preceding sections. The oxygen uptake of each homogenate, fortified with cofactors but in the absence of an added substrate, was tested simultaneously. A compound was considered utilized only when the respiratory rate in its presence was greater than this endogenous oxygen uptake. In Table III the substrates which were oxidized by flight muscle homogenates are shown.

As shown in this table, housefly flight muscle was able to oxidize a variety of compounds. This included all the Krebs citric acid cycle substrates, the intermediates of the Meyerhof-Embden glycolytic scheme, some sugars, and several amino acids. Although many substrates were utilized, the muscle demonstrated

TABLE III

Respiratory Substrates for Flight Muscle Homogenates

Substrate	Q_{O_2}	Substrate	Q_{O_2}
	mm.³ O_2/hr./ mg. dry weight		*mm.³ O_2/hr./ mg. dry weight*
No substrate	4.0	Fructose-6-phosphate	8.1
Hexoses		Fructose-1,6-diphosphate	10.0
d-Glucose	7.8	3-Phosphoglycerate	5.3
d-Fructose	7.8	Phosphopyruvate	4.7
d-Mannose	7.6	α-Glycerophosphate	6.3
Galactose	5.3	Glycerol	4.6
Disaccharides		Acetate	5.1
Sucrose	7.0	Krebs cycle intermediates	
d-Trehalose	8.0	Citrate	7.5
Maltose	7.0	Cis aconitate	7.8
d-Turanose	5.0	Isocitrate	6.7
Trisaccharides		α-Ketoglutarate	7.3
Melezitose	5.6	Succinate	6.9
Polysaccharides		Fumarate	7.4
Glycogen	8.2	Malate	7.2
Dextrin	6.3	Oxaloacetate	5.9
Glucosides		Pyruvate	5.7
α-Methyl glucoside	4.5	Amino acids	
Glycolytic intermediates		*l*-Proline	5.3
Glucose-1-phosphate	8.0	*l*-Glutamic	5.5
Glucose-6-phosphate	8.6	*l*-Cysteine	4.7
Ribose-5-phosphate	6.3	Glutathione	4.8

The reaction mixture was the same as the complete system shown in Table II with the following exceptions: DPN omitted; ATP, 15 μM; all substrates, 30 μM.

marked substrate specificity. This specificity was illustrated with the hexoses. Here, glucose, fructose, and mannose were rapidly oxidized, galactose only moderately so, but sorbose was not metabolized. This same discrimination was shown with the disaccharides, for although four substrates of this type were oxidized (Table III), three others, lactose, melibiose, and cellobiose were not. Also, the trisaccharide, melezitose, served as a substrate, yet raffinose did not. Of interest was the observation that, although starch was not oxidized, the partially hydrolyzed starch, dextrin, was utilized. Even though some amino acids were oxidized; others, *l*-leucine, *l*-histidine, *l*-aspartic, glycine, *dl*-α-alanine, *l*-phenylalanine, and *l*-hydroxyproline were not used as respiratory

substrates. Distinctions between isomers were also found, for α-glycerophosphate was oxidized whereas β-glycerophosphate was not.

In addition to the specificity shown towards substances structurally related, there were other types of compounds from which no member was utilized. Flight muscle homogenates did not oxidize the following pentoses: *d*-ribose, *l*-arabinose, *l*-fucose, *d*- or *l*-xylose, and *l*-rhamnose. It should be noted, as shown in Table III, that the phosphorylated pentose, ribose-5-phosphate, was oxidized. None of the polyhydric alcohols, mannitol, dulcitol, sorbitol, or *i*-inositol served as substrates. Of considerable interest was the finding that fatty acids were not oxidized. Those tested included propionic, butyric, β-hydroxybutyric, valeric, caproic, caprylic, and capric acids. The glycolytic end-products, lactic acid, ethanol, and acetaldehyde, were not utilized. The last compound, as well as dihydroxyacetone and glyceraldehyde, caused marked inhibition of the endogenous respiration.

4. The Localization of Enzymes within the Muscle

(*a*) *The Separation of Soluble from Particulate Fraction.*—In the preceding section the behavior of whole flight muscle homogenates towards various substrates has been described. The properties of the muscle were examined further by separating these homogenates into two components, the soluble and particulate fractions. The oxygen uptake of these fractions was then determined with selected representatives of those substrates which were oxidized by the entire muscle. It was found that, regardless of the substrate used, there was no appreciable oxygen consumption with the soluble fraction (sarcoplasm). In contrast, with some substrates the particulate fraction accounted for most of the respiration noted with the whole homogenate. These results are shown in Table IV.

As shown in this table, the particulate fraction of flight muscle oxidized all the Krebs citric acid cycle intermediates, α-glycerophosphate, and the amino acids, glutamic, cysteine, and proline. This cellular component, however, was not able to metabolize glycogen, the hexoses, ribose-5-phosphate, glycerol, acetate, nor any substrate in the Meyerhof-Embden glycolytic scheme. A notable exception to the absence of glycolytic enzymes was the demonstration that the particulate fraction actively utilized phosphopyruvate.

(*b*) *The Recombination of Particulate and Soluble Fractions.*—It had been found that little oxygen was consumed when either the particulate fraction or the sarcoplasm was incubated with such substrates as glycogen or the hexoses. Yet, it was also known that the whole muscle was capable of oxidizing these substrates completely. For these reasons, it was apparent that each of the cellular components contributed part of the necessary enzymatic complex. This hypothesis was substantiated by the experiments reported in Table V, wherein the data demonstrate that both the particulate fraction and the sar-

TABLE IV

Enzymatic Activity of the Particulate Fraction of Flight Muscle Compound with Comparison of That of Whole Homogenate and Isolated Sarcosomes

Substrate	Whole homogenate	Particulate fraction	Isolated sarcosomes
	mm.³ O_2/hr./25 thoraces		QO_2
No substrate	57	10	1.1
Citrate	118	128	9.3
Cis aconitate	105	104	9.4
Isocitrate	91	132	25.5
α-Ketoglutarate	109	50	17.2
Succinate	102	66	11.0
Fumarate	91	104	11.7
Malate	89	90	6.0
Oxaloacetate	66	24	10.9
Pyruvate	68	24	21.5
Glycogen	99	8	
Glucose	122	4	0.8
Fructose	130	6	
Mannose	118	8	
Glucose-1-phosphate	101	10	
Glucose-6-phosphate	129	8	
Fructose-6-phosphate	135	14	
Fructose-1,6-diphosphate	153	14	0.9
3-Phosphoglycerate	95	8	
Phosphopyruvate	76	36	10.5
α-Glycerophosphate	128	106	12.0
Glycerol	64	6	
Acetate	69	10	1.2
Ribose-5-phosphate	94	8	
l-Proline	90	90	13.1
l-Glutamic	95	44	7.7
l-Cysteine	72	114	15.6
Glutathione	84	34	2.3

TABLE V

The Effect on Oxidation of Recombination of the Sarcoplasm and the Particulate Fraction

Substrate	Particulate alone	Sarcoplasm alone	Particulate and sarcoplasm
	mm.³ O_2/hr.	*mm.³ O_2/hr.*	*mm.³ O_2/hr.*
No substrate	5	8	22
Glycogen	4	14	48
Glucose	2	9	56
Fructose	3	16	67
Mannose	4	17	63
Glucose-1-phosphate	5	18	67
Glucose-6-phosphate	4	15	61
Fructose-6-phosphate	7	16	51
Fructose-1,6-diphosphate	7	15	87
3-Phosphoglycerate	4	9	42
Glycerol	3	10	38
Acetate	5	9	28
Ribose-5-phosphate	4	9	54

coplasm were required for the oxidation of glycogen, the hexoses, glycerol, acetate, ribose-5-phosphate, and all the phosphorylated intermediates of glycolysis, except phosphopyruvate. It will be recalled that this substrate, as well as members of the citric acid cycle and some amino acids, required only the particulate fraction for their oxidation. With these substrates, the respiration of the recombination of the two components was approximately equal to the oxidation of the particulate fraction alone plus the small oxygen consumption observed with the soluble fraction. The traces of oxygen uptake noted with the soluble fraction were due to minute mitochondrial fragments which failed to sediment in the preparation of the sarcoplasm (*cf.* Sacktor, 1953, *a*).

(*c*) *Comparison of Particulate Fraction with Isolated Sarcosomes.*—As previously reported (Sacktor, 1953, *a*), the particulate fraction of muscle homogenates consists of fibrils and sarcosomes (mitochondria). Although prelimi-

TABLE VI

Replacement of Particulate Fraction by Isolated Sarcosomes

Morphological entity	Oxygen uptake	
	Glucose	Fructose-1,6-diphosphate
	*mm.*³ O_2/*hr.*	*mm.*³ O_2/*hr.*
Particulate fraction	3	4
Sarcosome	3	4
Sarcoplasm	18	19
Particulate + sarcoplasm	84	90
Sarcosome + sarcoplasm	106	95

nary attempts to separate a pure preparation of fibrils from sarcosomes were unsuccessful, insight into the biochemical properties of each component could be obtained by a study comparing the particulate fraction with isolated sarcosomes, prepared independently. In this manner, it was demonstrated that isolated mitochondria oxidized every substrate which was metabolized by the particulate fraction (Table IV).

Moreover, as pointed out in Table VI, despite the multitude of enzymes required for the oxidation of hexoses, the sarcosome could substitute for the particulate fraction. As seen here, only the isolated muscle mitochondria in conjunction with the sarcoplasm were needed for glucose and fructose diphosphate utilization.

DISCUSSION

The data presented define some of the biochemical mechanisms whereby energy is provided for the contractile processes in the flight muscle of the housefly. These results also show that a wide variety of substances can be converted

to supply this energy, but, at the same time, they point to the marked substrate specificity of this muscle. It should be reemphasized that these results were obtained with a standard experimental procedure. This procedure, which was based on optimal conditions for glucose oxidation, need not represent the ideal situation for other compounds. Despite such limitations, certain aspects of the mechanisms concerned in fly metabolism have now been clarified.

The Nature of the Substances Utilized during Fly Flight

The utilization of glycogen during flight of flies was suggested by Chadwick and Gilmour (1940) and Chadwick (1947) from experiments in which oxygen uptake and carbon dioxide output were measured during *Drosophila* flight. This supposition was substantiated further by Williams *et al.* (1943) and Wigglesworth (1949) who demonstrated that the flies' glycogen reserves were depleted in flight. It was found in the present studies that the depletion in these reserves during starvation paralleled the decrease in endogenous respiration. In addition, it was shown that fly flight muscle oxidized glycogen. The present results thus contribute evidence favorable to the postulate that, in flies, glycogen provides the major vehicle for storage of flight energy, and that it can be rapidly mobilized to meet the metabolic requirements of the active flight muscle.

Although the evidence above clearly indicates the important role of glycogen in flight metabolism, the present experiments show also that other carbohydrates could provide readily available sources of energy. The three hexoses: fructose, glucose, and mannose, as well as the disaccharides: trehalose, maltose, and sucrose, were oxidized at a rate comparable with that of glycogen. These same six substrates were the only ones which, when fed to exhausted *Drosophila*, supported continuous flight (Wigglesworth, 1949). The results reported here further supplement Wigglesworth's findings. He demonstrated that the following substances had no effect on the duration of flight: sorbose, rhamnose, arabinose, lactose, cellobiose, dulcitol, ethanol, acetic, lactic, and β-hydroxybutyric acids. Our investigations showed that, with the exception of acetate, none of these compounds was oxidized by muscle homogenates. In other experiments, Wigglesworth found that, upon ingestion, some substances, while not capable of supporting continuous flight, had slight beneficial effects on the ability of exhausted *Drosophila* to fly. Of these, galactose, α-methylglucoside, and glycerol have now been found to be metabolized slowly by the muscle, thus, in part, explaining their *in vivo* action. On the other hand, sorbitol, mannitol, inositol, and xylose were also of small benefit in flight, yet these compounds were not oxidized by the muscle. This suggests that these substances were, upon ingestion, converted to utilizable substrates prior to or during transport to the muscle.

The present findings are also in relatively good agreement with the results

of Haslinger (1936), Fraenkel (1940), and Hassett (1948). For the most part, those sugars which are oxidized by fly muscle were found by these investigators to be of nutritional value, as determined by their effect on longevity. Correspondingly, those substances of little or no value in the diet were not metabolized by our preparations. A notable exception to this general agreement is that, as shown here, the intermediates of the Krebs citric acid cycle were rapidly oxidized by muscle preparations, whereas Hassett (1948) reported that these substrates did not support the survival of adult *Drosophila*.

Further examination into the nature of the carbohydrates oxidized by these muscle homogenates reveals additional information. Not including the hexoses, the carbohydrates metabolized were as follows: glycogen, dextrins, sucrose, maltose, trehalose, turanose, melezitose, and α-methylglucoside. All these compounds are α-glucosides. In contrast, the polysaccharides not oxidized were: cellobiose, a β-glucoside; raffinose and melibiose, α-galactosides; and lactose, a β-galactoside. Such evidence indicates that fly muscle possesses an α-glucosidase which hydrolyzed the α-glucosides to utilizable hexoses. Analogously there were no data to suggest a β-glucosidase nor α- or β-galactosidases in the muscle. In contrast to the situation in muscle, in the gut of the adult blowfly, *Calliphora*, an α-galactosidase as well as an α-glucosidase was found, although the enzymes which hydrolyzed the β-forms were absent (Fraenkel, 1940). Hassett *et al.*, (1950) reached similar conclusions from their nutritional studies.

Related to these observations are the recent findings by Myers and Smith (1954) of the detoxification of substituted phenols by conjugation to form β-glucosides in locusts. The locust, like the fly, but in contrast to the American cockroach (Newcomer, 1954), could not hydrolyze β-glucosides. Thus, one may envision the efficiency of such a detoxification mechanism; for, in this situation, the toxicants once conjugated as β-glucosides would be resistant to further metabolism.

A Comparison of the Flight Processes of Flies and Locusts

The nature of the substances utilized during flight of different insect species has attracted particular attention. In general, the results of such experiments lead one to separate insects into two distinct groups. In one such category, to which bees and *Drosophila* belong, only carbohydrates are used. With these species it was found that the respiratory quotients (R.Q.) during flight were equal to unity (Jongbloed and Wiersma, 1934; Chadwick, 1947), and that after flight the insects' reserves of glucose and glycogen were depleted (Beutler, 1936; Williams *et al.*, 1943; Wigglesworth, 1949). In contrast, with the migratory locust, *Schistocerca*, most of the total energy expenditure was apparently derived from fats. Here, during sustained flight, the average R.Q. was about 0.75, and afterwards fat deposits were found to have been consumed (Krogh and Weis-Fogh, 1951; Weis-Fogh, 1952). As in the grasshopper, there were indications that fats were metabolized during the migratory flights of

the leafhopper, *Eutettix* (Fulton and Romney, 1940), and the monarch butterfly, *Danaus* (Beall, 1948). Our data reveal that houseflies are evidently similar to *Drosophila*, since it was demonstrated that, *in vitro*, the flight muscles oxidized only carbohydrates, plus a few amino acids. They were unable to oxidize added fatty acids and β-hydroxybutyrate. It is still possible that oxidation of fatty acids contributed part of the endogenous respiration observed with whole muscle homogenates, but no direct evidence on this point is available.

The flight processes of *Drosophila* and locusts are also contrasted by the diversity in the nature of their oxygen debts. In flies, Chadwick and Gilmour (1940) reported that this debt was exceedingly small, that it was independent of the length of flight, and that it was paid off in 2 minutes after flight had ceased. Unlike *Drosophila*, the locust contracted a definite oxygen debt (Krogh and Weis-Fogh, 1951). Moreover, the recovery period in locusts usually lasted at least an hour, but after prolonged flights as much as 2 hours were needed to return to normal respiration. Since oxygen debt has been associated with the anaerobic formation of lactic acid and products of incomplete fat metabolism, the absence of a significant oxygen debt in flies is not too surprising now that we know that their flight muscles cannot readily oxidize lactic acid nor fats.

There are other data, however, which indicate that the flight processes of flies and locusts may be reconcilable. Wigglesworth (1949) pointed out that *Drosophila* utilize fats and carbohydrates during starvation, and he suggested that both substances were used for muscular activity. The present results also showed that fats could be a source of energy to fly muscle; for, although fatty acids were not oxidized as such, the data (Table III) demonstrate that the products of fat activation, acetate and glycerol, are respiratory substrates. Correspondingly, Weis-Fogh (1952) reported that glycogen as well as fats was consumed in flight of *Schistocerca*. This was particularly marked during the earlier phases of flight.

Wigglesworth (1949) recognized that the distinction between these two insects in the nature of the substrates used for their flight was not as definite as previously supposed. He suggested that fats could be converted into a form which would provide an immediate source of energy for the muscular contraction during flight of *Drosophila*, but that this occurred at an insufficient speed to support continuous flight. Weis-Fogh (1952) objected to this postulate. He pointed out that *Drosophila* muscle did not work at a higher rate than did that of *Schistocerca*, and that therefore the factors which made fat inapplicable in flying flies and of importance in locusts must be qualitative rather than quantitative in nature. He conjectured further that the muscle of the fly was highly specialized and had lost its ability to oxidize fats. The observations reported in this paper tend to support Wigglesworth's argument, but, at the same time, they do not necessarily disagree with the viewpoint of Weis-Fogh.

We have already emphasized that fly muscle does not utilize fatty acids,

but does oxidize the product of fat metabolism, acetate. Moreover, there is no evidence to indicate that the biochemical processes in locust muscle are different from fly muscle in this respect. In fact, just the opposite is the case. As with the fly, in the muscle of *Periplaneta*, a species comparatively closely related to the locust, butyrate was not oxidized appreciably, if at all. But, again in accord with flies, acetate was rapidly metabolized (Barron and Tahmisian, 1948). It seems likely that neither the fly nor the locust muscle can initiate the oxidation of fatty acids. Thus, it follows that fat metabolism must occur in another tissue. A most logical site would be the fat body, where most of the fat is actually stored and where, as has been shown recently (Sacktor and Bodenstein, 1952; Sacktor *et al.*, 1953; Bodenstein, 1953), active enzymatic processes occur. Here, we may imagine, fatty acids can be metabolized to yield acetate, which in turn, may be transported to the muscle where it can undergo further oxidation *via* the Krebs citric acid cycle. Once in close juxtaposition with muscle enzymes, acetate was utilized at approximately the same rate in *Periplaneta* as in the fly. Assuming the same to be true in *Schistocerca*, it is suggested that the significant difference between the flight metabolism of flies and locusts may be found in the processes which limit the production of acetate in the fat body. This argument is supported by the recent studies of Rees (1954) who reported that locust muscle preparations do not oxidize octanoate.

A Comparison of the Intermediate Carbohydrate Metabolism in Insect and Vertebrate Muscle

In the preceding sections some of the biochemical properties of insect muscle have been discussed. From this, an apparent general similarity in the intermediate carbohydrate metabolism of insect and vertebrate muscle is indicated. Yet, from the few detailed studies of these processes in insects certain differences from the accepted vertebrate scheme have been revealed.

(*a*) *Glycolysis.*—In accord with the situation in vertebrates, it has been shown here, as it has also been shown in grasshopper muscle by Humphrey and Siggins (1949), that glycogen, glucose, and the phosphorylated hexoses were suitable substrates for glycolysis. Further, as shown in Table II, the cofactors needed for optimal utilization of glucose in insects are similar to those required in vertebrates. On the other hand, the pathway and particularly the end-products of glycolysis in insects are still very much in question. Gilmour (1941, *a*) reported that, upon recovery from anaerobiosis, grasshopper muscle consumed more oxygen than needed to repay its oxygen debt, if due to lactic acid. He also showed (1941, *b*) that the amount of lactic acid formed was not sufficient to account for all the glycogen used by *Tenebrio* larvae subjected to lack of oxygen. Barron and Tahmisian (1948) also postulated that there were end-products of anaerobiosis other than lactic acid. They found that the lactic

acid produced during glycolysis of cockroach leg muscle was far less than what was to be predicted from their simultaneous determinations of the CO_2 evolution from bicarbonate buffer. Although these workers stated that pyruvic acid was not formed, later Humphrey (1949) found that actually more pyruvate than lactate was produced during glycolysis of roach muscle. This unusual distribution of the two acids was also demonstrated in locust muscle (Humphrey and Siggins, 1949). Another departure in the glycolytic pattern of insect muscle from that of vertebrates, is that iodoacetate inhibits the liberation of CO_2 but has no effect on the production of lactic or pyruvic acids in insect tissues (Barron and Tahmisian, 1948; Humphrey and Siggins, 1949).

Admittedly, our knowledge of the anaerobic phase of carbohydrate breakdown in orthopteran muscle is limited, but even less is known of the situation in the flight muscles of flies. However, in these species too the data signify that lactic acid is not an end-product of glycolysis. It will be recalled that no appreciable oxygen debt was found after prolonged flight, and this may be interpreted to indicate that not much lactic acid was formed even during extensive muscular activity. Also, in view of our finding that muscle preparations were unable to oxidize lactate, it seems doubtful that this compound would be routinely accumulated. Thus, the possibility must be recognized that lactic acid need not be produced in this type of skeletal muscle.

From the present study, two schemes are suggested which may explain the apparent absence of lactic acid. First, since the necessary lactic dehydrogenase is not found in these muscles, pyruvate immediately enters the tricarboxylic acid cycle. As shown in Table IV, pyruvate was rapidly oxidized by isolated sarcosomes. An alternative mechanism would connote that the later stages of glycolysis as depicted in vertebrate muscle were by-passed in fly muscle. Our data show that one of the intermediates of the anaerobic breakdown of carbohydrates, phosphopyruvic acid, could be directly metabolized, aerobically. Thus this compound could enter the Krebs cycle without first going to pyruvate. This latter hypothesis is supported by our observation that phosphopyruvate was metabolized by isolated insect sarcosomes at a rate comparable to that of some citric acid cycle intermediates (Table IV). It is also in agreement with the conclusions of Barron and Tahmisian (1948). They reconciled the discrepancies between the CO_2 liberation and production of lactic acid in their experiments with roaches by suggesting the accumulation of organic phosphoric acids. Moreover, just recently, the reversal of this proposed scheme, the formation of phosphopyruvate directly from the Krebs cycle compounds, α-ketoglutarate and succinate, has been described in mammalian kidney and liver mitochondria by Bartley (1954) and Neuberg and Mudge (1954). Although this reaction may be found in many tissues, it is questionable whether this bypass exists in vertebrate muscle since, under the same conditions in which we demonstrated the oxidation of phosphopyruvate with insect sarcosomes, iso-

lated rat heart sarcosomes were inactive (Sacktor, unpublished data). If this dissimilar glycolytic scheme in insects can be verified by future experimentation, then a fundamental distinction between insect and vertebrate muscle will have been established. Such a situation would logically make available to the toxicologist definite targets for specific insect inhibitors.

(*b*) *Localization of Enzymes within the Muscle.*—The present experiments have described some of the biochemical activities of insect flight muscle. In addition, since these properties can be apportioned to three morphological entities, the fibrils, sarcosomes, and sarcoplasm, the role played by each of these cellular entities in the over-all contractile mechanism has been examined further. It was already known that each of these fractions possesses a specific mechanism of ATP breakdown, that the sarcoplasm hydrolyzes inorganic pyrophosphate, and that the sarcosomes (mitochondria) contain an adenylate kinase, several dehydrogenases, flavoproteins, cytochrome *c* reductases and oxidase, and couple oxidation with synthesis of high energy phosphate bonds (Watanabe and Williams, 1951; Sacktor, 1953, *a*, *b*, 1954). The data presented here provide additional evidence for the distinct localization of enzymes within insect muscle and, at the same time, point to interesting parallels between this muscle and that of vertebrates.

Although whole muscle homogenates oxidized a number of carbohydrates, no appreciable oxygen uptake was found when the particulate component and the sarcoplasm were prepared from the homogenate and each fraction tested independently. Upon the recombination of these fractions, respiration proceeded. From such observations, it was evident that each of these cellular entities contributes part of the necessary enzyme complex. As shown in Table V, both the sarcoplasm and the particulate component were required for oxygen uptake when the substrates were glycogen, glucose, fructose, or mannose, all the hexose phosphates, or 3-phosphoglycerate. In contrast, only the particulate fraction was needed for the oxidation of phosphoenolpyruvate, pyruvate, and the other members of the tricarboxylic acid cycle (Table IV). This suggests that, as in vertebrate muscle in which the glycolytic enzymes were found to be soluble in aqueous extracts, in the insect flight muscle these glycolytic enzymes were also soluble and localized in the sarcoplasm. On the other hand, the oxidase systems of these muscles were associated with the insoluble fraction. Furthermore, we already knew that this fraction consisted of fibrils and sarcosomes (Sacktor, 1953, *a*). Then, since isolated mitochondria accounted for all the enzymatic properties of the particulate component (Tables IV and VI), it was apparent that the oxidase systems of the insoluble fraction were localized in the sarcosomes.

From the distribution of enzymes in the cytological entities it is evident that, in these flight muscles as in the vertebrate muscle, the complete oxidation of carbohydrates can be divided into the two classical phases: the first, an anaero-

bic phase, found in the sarcoplasm, in which the hexoses are transformed into two trioses; and the second, an aerobic phase, whereby the triose undergoes further metabolism by means of the citric acid cycle located in the mitochondria of these muscles.

The existence of this scheme in insect flight muscle is also supported by other observations not reported as yet. In preliminary experiments on the mechanism of glycolysis in this tissue, it was found that glucose, when incubated with the sarcoplasm, was metabolized, anaerobically, beyond the hexose phosphate step. Also suggestive was the observation that glycogen, as determined by chemical analysis, is localized in the sarcoplasm. Furthermore, it was found that the oxidation of fructose diphosphate was inhibited by malonate and fluoroacetate, and in view of the known action of these inhibitors, it was apparent that the complete oxidation of carbohydrates proceeded through the tricarboxylic acid cycle in insect flight muscle. Thus we have presented evidence to indicate that the metabolic pathways for carbohydrate oxidation in vertebrate muscle have their counterpart in the insect flight muscle. But, perhaps even more important, it has now been shown that these enzymological observations can be related to definite structural entities of the muscle cell.

(*c*) *Mitochondria.*—Although we have pointed to specific differences between insect and mammalian mitochondria, the results of the present study emphasize the many resemblances of the two organelles. As with mammalian mitochondria, insect sarcosomes have now been shown to oxidize all members of the tricarboxylic acid cycle. In addition, the amino acids, proline and glutamic, were oxidized. This is of particular interest since glutamic oxidase, although present in mammalian mitochondria (Kielley and Schneider, 1950), was previously reported missing from insect sarcosomes (Watanabe and Williams, 1951). The oxidation of proline is noteworthy because it was shown by Taggart and Krakauer (1949) to be converted to glutamic acid. Furthermore, as pointed out by Lindberg and Ernster (1954), glutamate was the only amino acid that could be oxidatively deaminated to α-ketoglutarate in the absence of other metabolites. With this in mind, the inability of our preparations to oxidize aspartic acid and alanine, which also enter the Krebs cycle in mammalian mitochondria, may be understandable. Our results could be due not only to the lack of transaminating enzymes, but if these enzymes were present, to the unavailability of suitable acceptors for the amino groups.

It is well known that the enzymatic behavior of mitochondria is dependent on their structural organization. The present results offer examples which have bearing on this concept. It will be recalled that the sarcosomes in the particulate fraction, as prepared, were mostly damaged. At the same time, sarcosomes isolated independently were well preserved. Thus, the effects of physical organization can be examined by comparing the biochemical activity of these two preparations.

It is evident (Table IV) that the enzymic response to structural integrity was not always the same. For, whereas the maximal rates of oxidation of phosphopyruvate, pyruvate, and oxaloacetate were contingent upon intact mitochondria, the rate of isocitrate oxidation seemed independent of damage. Furthermore, the data indicate that the utilization of malic acid was even enhanced by the partial disruption in normal configuration. From these observations, it is quite clear that the understanding of these complex relationships must await additional investigations on the many facets of the problem.

In a previous paper (Sacktor, 1954), it was reported that there was a considerable oxygen uptake with mitochondrial suspensions even in the absence of added substrate. Since we found here (Table IV) that isolated sarcosomes had little endogenous respiration, a partial explanation of this apparent discrepancy is appropriate at this time. Our former experiments were concerned with oxidative phosphorylation in insect mitochondria and this necessitated a reaction mixture differing from the current one by the presence there of serum albumin, glucose, and hexokinase. As reported earlier, it was found again that whenever hexokinase was added to the experimental medium and substrate omitted, the sarcosomes ruptured and concurrently increased their respiratory rate. These events did not occur when the substrate, α-ketoglutarate, was added. It was also found that serum albumin and/or glucose were not concerned with this phenomenon. Moreover, it was soon discovered that these effects of hexokinase were limited to our preparation of this enzyme, for several commercial samples, prepared in a different manner, were inactive in this respect. Without further speculation, it will suffice to state that these experiences have provided an interesting lead for future experiments.

SUMMARY

The biochemical properties of insect flight muscle were investigated to ascertain the mechanisms whereby energy is made available for the contractile processes. It was found:

1. The endogenous respiration of muscle homogenates was diminished by starving the flies. The substrate for this respiration was probably glycogen.

2. To obtain the maximal rate of oxidation of glucose, the homogenate had to be fortified with inorganic phosphate, Mg ions, ATP, and cytochrome *c*. The nucleotides, AMP and ADP, were not as effective as ATP. The addition of DPN or TPN was not necessary for this system.

3. Flight muscle homogenates oxidized glycogen, some sugars, and amino acids, as well as the intermediates of the glycolytic and tricarboxylic acid cycles. Other evidence demonstrated the substrate specificity of the muscle.

4. By centrifugation, the muscle homogenate was divided into two fractions: one, a soluble fraction representing the sarcoplasm; the other, the particulate fraction which contained the fibrils and the sarcosomes.

5. The particulate fraction, alone, oxidized all the citric acid cycle intermediates, α-glycerophosphate, phosphopyruvate, and the amino acids, glutamic, proline, and cysteine. Regardless of the substrate, no oxygen uptake was found with the sarcoplasm by itself.

6. A recombination of the sarcoplasm and the particulate component was required for the oxidation of glycogen, the hexoses, and all the phosphorylated intermediates of glycolysis, except phosphopyruvate.

7. Isolated mitochondria accounted for all the enzymatic activity of the particulate fraction.

These results demonstrate that the enzymes of intermediate metabolism are localized in the sarcoplasm or sarcosomes. The third cytological entity, the myofibrils, plays no role in the energy-providing scheme. From a functional viewpoint, the sarcoplasm and the mitochondria, in combination, furnish the energy for the actomyosin contraction.

The results are discussed in relation to analogous findings in other insects and vertebrates.

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