OXIDATIVE ENZYMES IN THE THORACIC MUSCLES OF THE WOODROACH LEUCOPHAEA MADERAE [1]

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Our present knowledge of cellular metabolism is based largely on results obtained from studies using mammalian tissues. The mechanisms of many of the enzyme-catalyzed reactions of respiration and glycolysis have been elucidated during the past twenty years. During recent years the methods developed for the study of mammalian tissues have been used to study some of the oxidative enzymes in insect tissues, with the result that considerable information has accumulated concerning certain of the oxidative enzymes in various insect tissues.

In this connection Barron and Tahmisian (1948) found that the oxygen consumption of muscle from male cockroaches, *Periplaneta americana,* is double that from female roaches. Sacktor and Bodenstein (1952) reported on the cytochrome oxidase activity of various tissues of the American cockroach, and Harvey and Beck (1953) studied in considerable detail the succinoxidase and cytochrome oxidase systems in the leg muscle of this form. They found that the succinoxidase activity of muscle from the male cockroach is three times that of muscle from the female. Spirtes (1951) demonstrated the presence of Krebs cycle enzymes such as aconitase, isocitric, malic and succinic dehydrogenases, fumarase and condensing enzyme, and also cytochrome oxidase and lactic dehydrogenase in the tissues of *Drosophila melanogaster;* and Bodenstein and Sacktor (1952) studied the cytochrome oxidase during metamorphosis of *Drosophila virilis;* Sacktor (1951a, 1951b, 1952) reported on the cytochrome oxidase activity of normal and DDT resistant house flies, *Musca domestica;* Sanborn and Williams (1950) studied the cytochrome system in the tissues of the Cecropia silkworm; Watanabe and Williams (1951) showed that succinic, α-glycerophosphate, malic and pyruvic dehydrogenases and cytochrome oxidase are present in the sarcosomes of insect muscles; and Collias, McShan and Lilly (1952) reported results of studies on the succinoxidase and cytochrome oxidase systems in the tissues of the large milkweed bug, *Oncopeltus fasciatus.* Bodine, Lu and West (1952) found marked differences in the succinoxidase activity in mitotically active and blocked cells of the developing embryo of the grasshopper, *Melanoplus differentialis.*

Investigations of this kind serve to clarify further our knowledge of the relationship of the cellular metabolic reactions of insect tissues to those already known for mammalian tissues. Furthermore, information obtained for insects and other lower forms is of value from the comparative standpoint and may provide the basis for an insight into the mechanism by which energy is provided for certain specialized behavior patterns in insects such as the cockroach.

[1] This investigation was supported in part by a grant from funds supplied by the Wisconsin Alumni Research Foundation.

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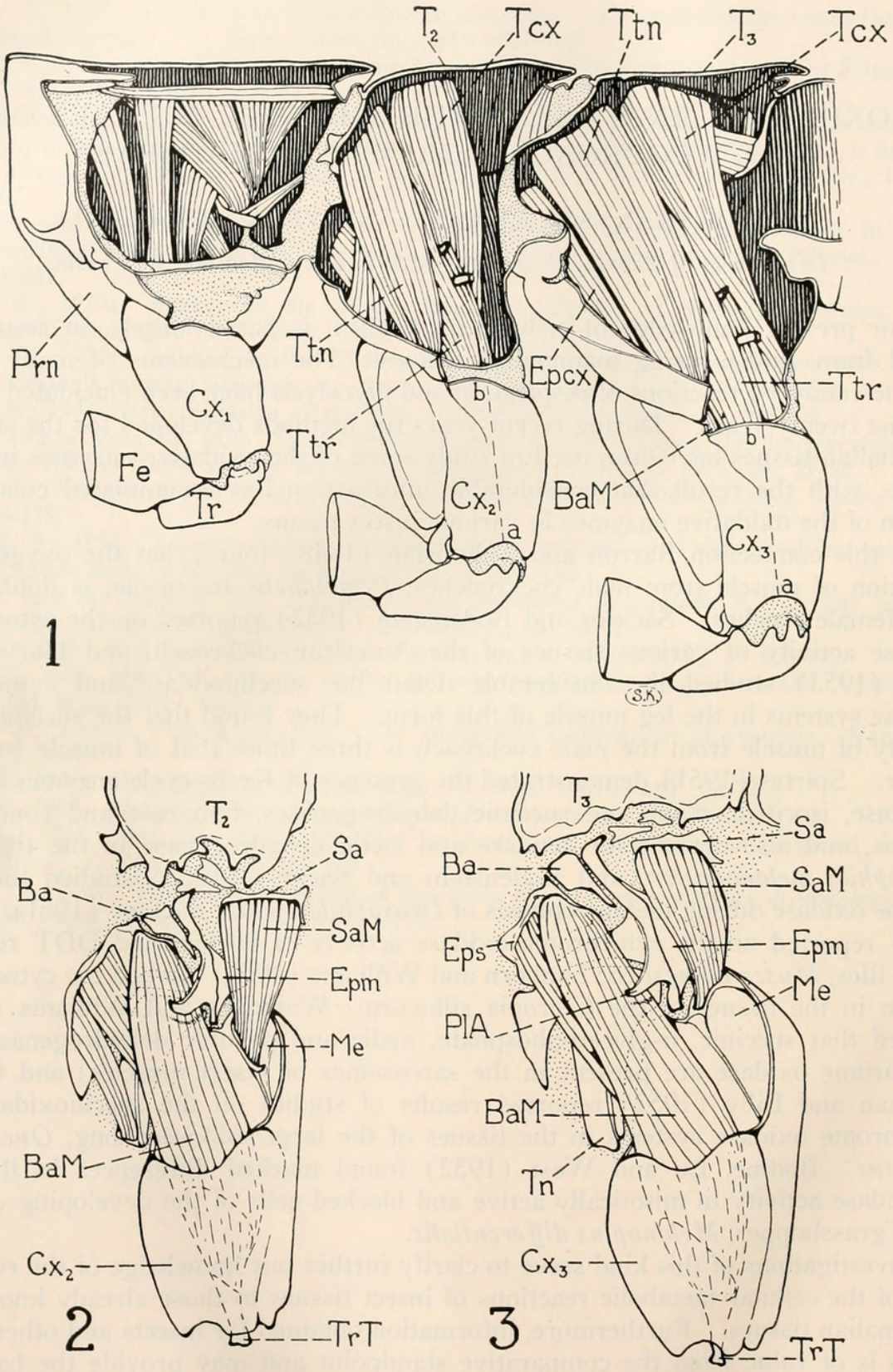

FIGURE 1. Mesal view of the right half of the thorax of the woodroach, *Leucophaea maderae*, showing some of the pigmented mesothoracic and metathoracic muscles (*BaM*, basalar muscle; *Tcx*, tergo-coxal muscle; *Ttn*, tergo-trochantinal muscle; *Ttr*, tergo-trochanteral apodeme muscle) used in the preparation of "leg muscle" homogenate. *a-b*, line of incision along coxae made to expose the entire muscles prior to removal; *Cx*, coxa; *Epcx*, episternalcoxal

The present paper reports results of a study of the succinoxidase, cytochrome oxidase and fatty acid oxidase of thoracic pigmented muscle from the woodroach, *Leucophaea maderae*. The mechanism of action and optimum conditions for these systems have been studied extensively in some mammalian tissues by Keilin and Hartree (1949), Slater (1949a, 1949b), Chance (1952), Lehninger (1946), and Lehninger and Kennedy (1948).

Materials and Methods

The woodroaches used in this study were isolated soon after metamorphosis and kept in dated containers so that muscle tissue could be obtained from roaches of known age. In certain of the earlier experiments, however, adult roaches of unknown age were used. Males and females were kept separately. All roaches were fed on the same constant dog pellet diet and ample food and water were always available to them.

The muscle tissue used was dissected from the meso- and metathoracic segments immediately after the roaches were killed by severing the head and abdomen. A mid-ventral incision through the thorax divided it into two halves. Remnants of the gut, large tracheal tubes and fat body were quickly cleaned away, and the large bundles of thoracic muscles were exposed as shown in Figure 1. Incisions along the meso- and metathoracic coxae along dotted lines *a-b* made it possible to separate these muscles in bulk with a few ventral and dorsal incisions. The tissue was weighed and placed in ground glass homogenizing tubes contained in an ice bath, and homogenized within 8 minutes after the roaches were killed. Sufficient water was added to give a 2.5 per cent homogenate which was used for the succinoxidase determinations. It was necessary to prepare a 0.5 per cent homogenate for the cytochrome oxidase and a 10 per cent homogenate for the determinations of fatty acid oxidase.

These muscles are sometimes referred to as the "leg muscles," and most of these muscles are in fact concerned with leg function. Roaches, such as the cockroach, *Periplaneta americana* as well as the woodroach, *Leucophaea maderae* and others, although comparatively weak flyers, can and do fly. Woodroaches in particular were observed on rare occasions to fly distances of 10–12 feet in slow, labored flight in the insectary. Further, Roth and Willis (1952) have shown that the wings of male *Periplaneta americana* and male *Blatta orientalis* are vibrated actively prior to copulation.

It is clear, then, that some muscles in roaches must function in flight and wing vibration. Carbonell (1947) in a detailed study of the thoracic musculature of the cockroach *Periplaneta americana* noted that these muscles bore little resemblance to

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muscle; *Fe,* femur; *Prn,* pronotum; T_2, mesothoracic tergum; T_3, metathoracic tergum; *Tr,* trochanter.

Figure 2. Mesal view of the mesothoracic flight muscles (*BaM,* basalar muscle; *SaM,* subalar muscle) which lie among the leg muscles and also included in the homogenate preparations. *Ba,* basalare; *Epm,* epimeron; *Me,* meron; *Sa,* subalare; *TrT,* trochanteral tendon. Other abbreviations as above.

Figure 3. Similar view of metathoracic flight muscles included in the homogenate preparations. *Eps,* episternum; *PlA,* pleural apodeme; *Tn,* trochatin. Other abbreviations as above.

those of other insects, and that in the musculature of the wings the cockroach thorax differs widely from the normal scheme of wing-bearing segments as given by Snodgrass (1935). The size of the basalar muscles (pronator-extensor of the wings) and the subalar muscles (depressor-extensor of the wings), which lie in the midst of the large leg muscles, led Carbonell to conclude that they must play an important role in flight.

Dissection of the woodroach revealed that prominent basalar muscles (BaM) and subalar muscles (SaM) are present in both the mesothoracic (Fig. 2) and metathoracic segments (Fig. 3). In fact, the basalar muscles [3] in both segments are the largest and longest of all the individual muscles present. These large flight muscles, together with the large leg muscles, are pigmented pink, in contrast to the smaller ventral longitudinal and certain smaller oblique muscles which are a translucent white color—and it was these pink pigmented muscles as a group which were used for the preparation of homogenates. Further, each homogenate represents not a mixture of muscles from several insects, but a preparation from the muscles of one roach of known age.

The enzyme determinations were made by use of the conventional Warburg apparatus. The homogenates were prepared by use of sharp-pointed, ground-glass homogenizers. The homogenates used for the study of succinoxidase and cytochrome oxidase were made with water and those for fatty acid oxidase with 0.154 *M* KCl. The proper amount of homogenate was placed in the flasks with the required cofactors for each of the enzyme systems studied. The flasks were placed in the bath at 38° C., and ten minutes were allowed for equilibration in the case of succinoxidase and cytochrome oxidase, and 6 minutes for fatty acid oxidase. Readings of oxygen consumption were taken at 10-minute intervals for at least 40 minutes. The average value for the number of 10-minute periods during which the oxygen consumption was constant, which was usually four periods, was used as a basis for calculating the Q_{O_2} values.

The methods used for the determination of succinoxidase and cytochrome oxidase were those reported by Schneider and Potter (1943). The optimum concentrations of required factors, and other conditions for maximum succinoxidase activity of thoracic muscle of the woodroach were determined. The concentrations of factors used for the cytochrome oxidase determinations were the same as those that have been reported for mammalian tissues. The fatty acid oxidase determinations were done by the method reported by Lehninger and Kennedy (1948). Final flask concentrations for the different enzyme systems are given in the footnotes to the tables.

The inhibitors were prepared in stock solutions which were in most cases 0.001 *M*. The solution of diethylstilbestrol was prepared by the procedure reported by McShan and Meyer (1946). The dry weight determinations of flight muscle were done by weighing the fresh tissue, placing it in a weighed tube and drying at 75° C. for 24 hours, after which the dry tissue was weighed and the weight used for calculating the percentage dry weight in terms of fresh weight.

Cytochrome *c* used for the determinations of succinoxidase and cytochrome oxidase was prepared by a modification of the method of Keilin and Hartree (1937),

[3] The basalar muscle actually arises from a tendon at the margin of the episternum adjacent to the basalare in each segment, but Crampton (1927) regards this margin of the episternum as an anterior portion of the basalare in the cockroach, *Periplaneta americana*.

or was obtained from the Sigma Chemical Company. Analytical reagent grade chemicals were used.

Results and Discussion

The succinoxidase activity of homogenates of thoracic muscle from female roaches of different ages was determined with different concentrations of succinate, phosphate buffer, calcium chloride, aluminum chloride and cytochrome *c*. The

Table I

Determination of the optimum concentrations of constituents required for maximum activity of succinoxidase in homogenates of woodroach thoracic muscle

Constituents			Concentrations of variable constituents and Q_{O_2} values**								
Absent	Present	Final *M* in flask									
$AlCl_3$	Phosphate pH 7.3	variable	*M**	0.0	0.0083	0.017	0.033	*0.050*	0.066	0.100	0.133
$CaCl_2$	Sod. succ. Cyto. *c*	0.1 2×10^{-5}	Q_{O_2}	37.0	40.0	69.0	83.0	*132.0*	115.0	115.0	98.0
$AlCl_3$	$CaCl_2$	variable	*M*	0.0	0.0004	0.0008	0.0012	*0.0016*	0.0020		
	Sod. succ. Cyto. *c* Phosphate	0.1 2×10^{-5} 0.05	Q_{O_2}	107.0	135.0	158.0	193.0	*208.0*	199.0		
$CaCl_2$	$AlCl_3$	variable	*M*	0.0	0.0004	0.0008	0.0012	*0.0016*	0.0020		
	Sod. succ. Cyto. *c* Phosphate	0.1 2×10^{-5} 0.05	Q_{O_2}	126.0	175.0	177.0	166.0	*186.0*	177.0		
None	Sod. succ.	variable	*M*	0.025	0.050	0.075	0.100	0.125	0.150	0.175	*0.200*
	Cyto. *c* Phosphate $CaCl_2+AlCl_3$ each	2×10^{-5} 0.05 1.6×10^{-3}	Q_{O_2}	99.0	125.0	142.0	160.0	174.0	189.0	199.0	*199.0*
None	Cyto. *c* ($\times 10^{-5}$ *M*)	variable	*M*	0.5	1.0	1.5	*2.0*	2.5	3.0		
	Sod. succ. Phosphate $CaCl_2+AlCl_3$ each	0.1 0.05 4×10^{-4}	Q_{O_2}	94.0	113.0	128.0	*131.0*	115.0	103.0		
None	pH	variable	pH	6.38	6.76	7.17	*7.3*	7.59	7.91		
	Sod. succ. Phosphate $CaCl_2+AlCl_2$ each Cyto. *c*	0.1 0.05 4×10^{-4} 2×10^{-5}	Q_{O_2}	94.0	112.0	128.0	*131.0*	115.0	103.0		

* Final molarity in flask.

** Q_{O_2} values are based on a dry weight content of 18.2 per cent and are averages of 2 to 5 runs using 0.1 ml. of 2.5 per cent homogenate except 0.2 ml. was used when cytochrome *c* and the pH were varied.

results italicized in Table I indicate the concentrations which gave maximum activity are 0.2 *M* succinate, 0.05 *M* phosphate buffer of pH 7.3, 1.6×10^{-3} *M* of each calcium and aluminum chlorides, and 2×10^{-5} *M* cytochrome *c*. Results of runs made at different pH values show that maximum activity was obtained at pH 7.3.

The data of Table II (Experiments 1 to 3) show that under the above conditions the oxygen consumption was directly proportional to the amount of tissue reacting

for 0.05, 0.10 and 0.15 ml. of 2.5 per cent homogenate but not for 0.20 and 0.25 ml. The results from Experiments 1, 2 and 3 are shown graphically in Figure 4. When 0.175 *M* succinate was used (Experiment 4, Table II) there was not quite a direct proportionality between the oxygen consumption and the amount of tissue reacting. These results indicate that a much higher concentration of succinate is required for optimum activity of the succinoxidase system of woodroach muscle than for this system of other tissues such as rat liver which requires only 0.05 *M* (Schneider and Potter, 1943). Harvey and Beck (1953) found 0.11 *M* succinate optimum for American cockroach muscle.

Essentially no oxygen was consumed when the succinoxidase system was run without substrate, and without tissue. When the cytochrome *c* was left out of the

TABLE II

Relation of oxygen consumption to amount of woodroach thoracic muscle used in the succinoxidase system

Amount of tissue* ml. 2.5% homogenate	Experiment No.							
	1(3)**		2(3)**		3(1)**		4(1)**	
	Oxygen consumption							
	Cmm. per 10 min.	Q_{O_2}	Cmm. per 10 min.	Q_{O_2}	Cmm. per 10 min.	Q_{O_2}	Cmm. per 10 min.	Q_{O_2}
0.05	7.6	201.2	6.1	160.8			8.0	210.9
0.10	15.0	197.3	12.1	160.0	19.5	257.2	15.5	204.0
0.15	22.4	196.9	18.4	161.3	28.6	252.0	21.9	192.0
0.20			23.4	154.1	34.6	228.0		
0.25					39.0	205.7		

* Flask concentrations of the constituents used in the system were sodium succinate 0.2 *M* except it was 0.175 *M* for experiment 4, phosphate buffer of pH 7.3 0.05 *M*, aluminum and calcium chlorides each 1.6×10^{-3} *M*, cytochrome *c* 2×10^{-5} *M*. The muscle used in these experiments was taken from adult females of unknown age.

** Number of runs with two flasks per each amount of tissue. The Q_{O_2} values are based on the average oxygen consumption for the first four 10-minute periods and a dry weight content of 18.2 per cent.

system the average oxygen consumption per 10 minutes was 2.9, 5.3 and 7.5 $mm.^3$, respectively, for 0.05, 0.10 and 0.15 ml. of 2.5 per cent homogenate. This was presumably due to the presence of cytochrome *c* in the flight muscle since this cytochrome has been shown to be present in cockroach muscle (Barron and Tahmisian, 1948; Harvey and Beck, 1953). Homogenates of woodroach muscle were therefore treated with sodium hydrosulfite to reduce the cytochromes and examined by use of a Hartridge Reversion Spectroscope. Absorption bands at the proper wave-lengths for cytochromes *a, b* and *c* were found, indicating the presence of these cytochromes in the woodroach muscle. On the basis of this evidence it appears that the activity of the system without added cytochrome *c* was caused by the presence of this component in the muscle homogenate.

The Q_{O_2} values of the different amounts of muscle homogenate used in Ex-

periments 1, 2 and 3 (Table II) were essentially constant and the average values were, respectively, 198.5, 161.0 and 254.6. The over-all average Q_{O_2} was 205. Adult female woodroaches of various ages were used for these experiments. These Q_{O_2} values are based on a dry weight of 18.2 per cent. The dry weights given in Table III, which range from 21.5 to 29.8 per cent, were done at different times than were those on which the above 18.2 per cent is based. The reason for this difference in the dry weights of these two series of experiments is not apparent.

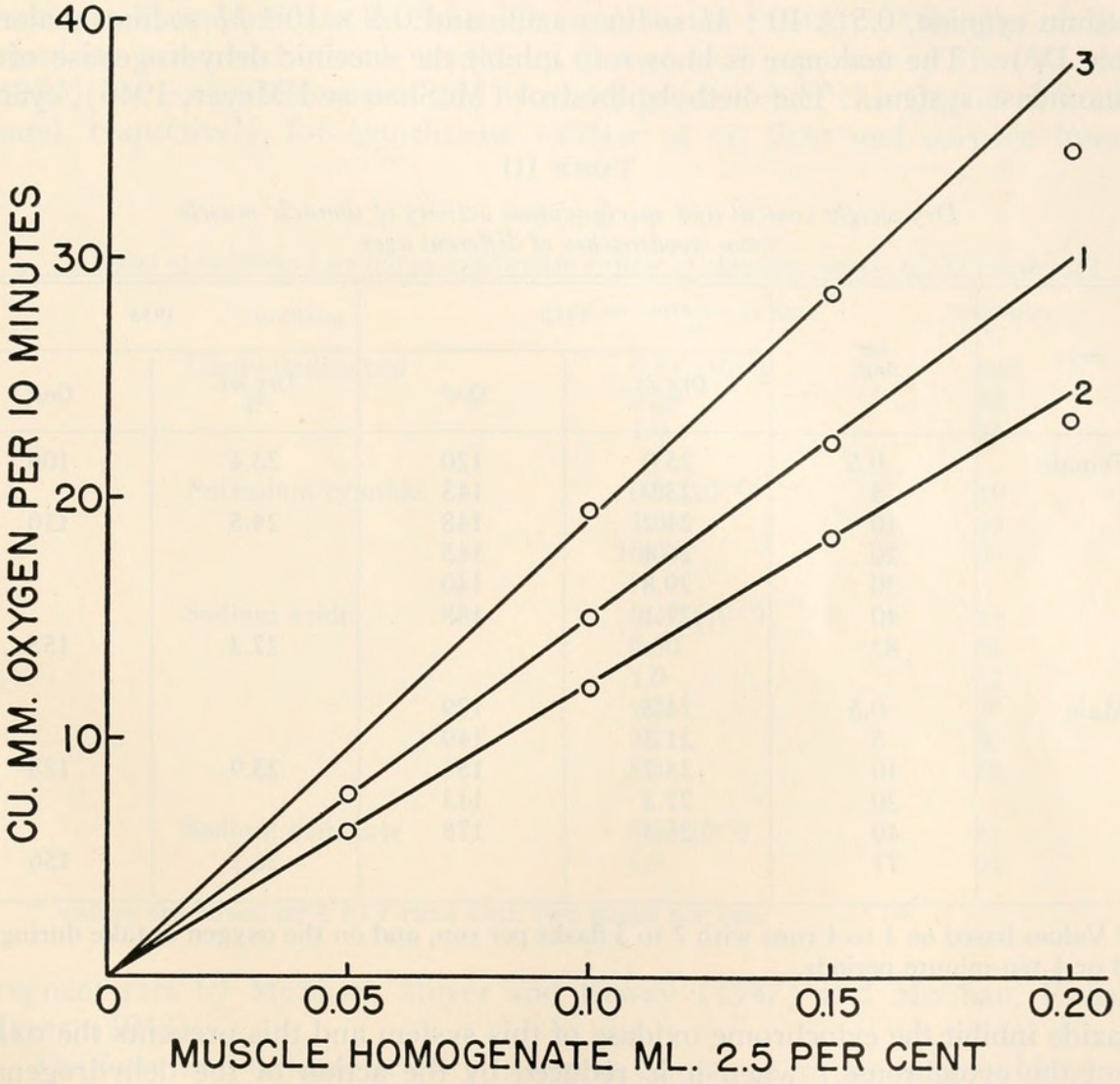

FIGURE 4. Results showing relation of different amounts of enzyme or tissue to oxygen consumption. Tissue concentrations which resulted in directly proportional oxygen uptakes are shown.

The roaches were kept under essentially constant conditions as to water, food and temperature, but factors other than these and the possible relation of dry weight to age may be involved.

The results given in Table III show the relation of age and sex to the dry weight and succinoxidase activity of woodroach muscle. There seems to be some tendency for the dry weight of muscle of both sexes to increase with increase in age after 5 days of adulthood. These results also show that there was an increase in

the succinoxidase activity of the muscle with increase in age of the roaches. Although most of these experiments were done during the spring of 1952, they were repeated for certain ages in the spring of 1953 with similar results, Table III). Further work, however, is necessary to clarify the variation in dry weight content of roach muscle.

The inhibition of the succinoxidase system of woodroach thoracic muscle was studied by use of diethylstilbestrol, cyanide, azide and malonate. Forty to 60 per cent inhibition was obtained with 0.27×10^{-4} *M* diethylstilbestrol, 0.5×10^{-4} *M* potassium cyanide, 0.5×10^{-3} *M* sodium azide and 0.5×10^{-2} *M* sodium malonate (Table IV). The malonate is known to inhibit the succinic dehydrogenase of the succinoxidase system. The diethylstilbestrol (McShan and Meyer, 1946), cyanide

TABLE III

Dry weight content and succinoxidase activity of thoracic muscle from woodroaches of different ages

Sex	Age days	1952		1953	
		Dry wt. %	Q_{O_2}*	Dry wt. %	Q_{O_2}*
Female	0.5	25.0	120	23.4	106
	5	23.9	143		
	10	24.2	148	24.5	110
	20	26.4	145		
	30	29.8	140		
	40	27.1	188		
	82			27.3	155
Male	0.5	24.3	129		
	5	21.5	149		
	10	23.7	154	23.9	128
	20	27.3	143		
	40	25.3	178		
	77			26.2	156

* Values based on 1 to 4 runs with 2 to 3 flasks per run, and on the oxygen uptake during the first 3 or 4 ten-minute periods.

and azide inhibit the cytochrome oxidase of this system and this prevents the oxidation of the cytochrome *c* when it is reduced by the action of the dehydrogenase. These inhibitors appear to affect the succinoxidase system of woodroach muscle in the same way as they affect this system in mammalian tissues.

Experiments were done to determine directly the succinic dehydrogenase activity of woodroach muscle by using brilliant cresyl blue (BCB) in the system as the mediator of hydrogen transport in place of the cytochrome system. When BCB was used in the system a Q_{O_2} of 76.5 was obtained as compared with 194 and 173, respectively, when cytochrome *c*, and BCB plus cytochrome *c* were present in the system. Similar results were obtained with rat liver which was run as a control. When cyanide was used in the system with BCB there was an increase of 83 per cent in the Q_{O_2} of woodroach muscle (Q_{O_2} of 76.5 for BCB alone) but under the same conditions cyanide did not cause an increase in the activity of rat liver. This in-

creased oxygen consumption when cyanide is added to the BCB system has been reported previously for leg muscle of the American cockroach (Harvey and Beck, 1953).

A Q_{O_2} of 1770 was obtained for the cytochrome oxidase of muscle from female roaches 30 days of age when 0.05 ml. and 0.1 ml. of 0.5 per cent homogenate were used per flask. Each flask also contained final concentrations of 0.033 *M* phosphate buffer of pH 7.3, 0.0114 *M* ascorbic acid, 4×10^{-5} *M* aluminum chloride, and 8.7×10^{-5} *M* cytochrome *c* which are essentially the amounts of these factors used for rat liver cytochrome oxidase by Schneider and Potter (1943). The Q_{O_2} of 1770 obtained for woodroach muscle is close to that of 1520 reported by Harvey and Beck (1953) for cockroach muscle but is much greater than the Q_{O_2} of 377 and 387 found, respectively, for cytochrome oxidase of rat liver and corpora lutea from

TABLE IV

Effect of inhibitors on the succinoxidase system of thoracic muscle of the woodroach

Inhibitor	Concentration in flask *M*	Inhibition %
Diethylstilbestrol	0.27 (10^{-4})	59*
	0.50	92
	1.0	96
Potassium cyanide	0.5 (10^{-3})	40
	1.0	93
	10.0	97
Sodium azide	0.27 (10^{-3})	44
	0.50	56
	1.0	61
	1.3	79
	2.7	80
	5.8	78
Sodium malonate	0.5 (10^{-2})	41
	1.0	60

* Values are based on 4 to 7 runs with two flasks per run.

pregnant rats by McShan, Meyer and Erway (1947) and McShan, Erway and Meyer (1948).

The fatty acid oxidase activity is low as compared to the succinoxidase activity of woodroach muscle, and there does not appear to be a significant change in activity with increase in age of the roach (Table V).

The results of this study show that succinoxidase and cytochrome oxidase systems are present in the thoracic pigmented muscle of the woodroach, *Leucophaea maderae*. In this muscle, however, the succinoxidase is more than twice as active and the cytochrome oxidase more than four times as active as in rat liver. On the other hand the fatty acid oxidase of rat liver is about ten times that of the woodroach muscle. Perhaps this is to be expected since the liver is known to be the locus for fatty acid metabolism.

Optimum conditions were determined for eliciting the maximum succinoxidase activity of woodroach muscle and it was found that this muscle requires four times

the concentration of succinate as does rat liver. In this connection Harvey and Beck (1953) found that the succinoxidase of leg muscle from the American cockroach requires 0.11 *M* succinate which is more than double that required by rat liver (Schneider and Potter, 1943). These results suggest that tissues high in succinoxidase, such as roach muscle, require higher concentrations of succinate for maximum activity than do tissues which contain a lower concentration of this system.

The results obtained with the BCB system, inhibitors and the required cofactors indicate that the mechanism of action of woodroach muscle succinoxidase is similar to that of mammalian tissues.

The increase in the succinoxidase activity of woodroach muscle with increase in age and the possible trend toward an increase in dry weight with increase in age may have physiological significance which is not apparent at present. In this connection Sacktor (1951b) showed that the cytochrome oxidase activity of normal and DDT-resistant house flies changes during pupal development, and Watanabe and Williams (1951) have reported differences in the cytochrome oxidase activity

TABLE V

Fatty acid oxidase activity of thoracic muscle from female woodroaches

Age in days	Q_{O_2}**
10*	3.1
20	5.8
40	3.0
60	4.5
Adult	3.7
Ave. for all ages	4.0

* When water was used for homogenizing muscle from a roach 10 days old a Q_{O_2} of 2.3 was obtained as compared to 3.1 for 0.154 *M* KCl.

** The amount of muscle tissue used per flask was 0.25 ml. of 10 per cent homogenate made with 0.154 *M* KCl. The final flask concentrations of reagents were 0.033 *M* KH_2PO_4-K_2HPO_4 of pH 7.4, 0.002 *M* potassium octonoate, 0.07 *M* KCl, 0.013 *M* $MgSO_4$, and 6.6×10^{-4} *M* KATP. The Q_{O_2} values are based on a dry weight content of 18.2 per cent.

of sarcosomes of Phormia isolated from insects of different ages. Further, Harvey and Beck (1953) found that succinoxidase is three times as active in the thoracic muscle of the male as in the female American cockroach, *Periplaneta americana.* These results for *Periplaneta* have been confirmed in our laboratory. It is therefore of interest that the succinoxidase activity in the thoracic muscle of the woodroach, *Leucophaea maderae,* is essentially the same in both sexes.

SUMMARY

1. The thoracic muscle of the woodroach, *Leucophaea maderae,* was shown to contain high concentrations of succinoxidase and cytochrome oxidase and a low concentration of fatty acid oxidase as compared to rat liver.

2. The conditions required for optimum activity of the succinoxidase system were determined and it was found that this system requires four times the concentration of succinate as does succinoxidase of rat liver.

3. Succinoxidase activity of thoracic pigmented muscle in the woodroach is essentially the same in both sexes, whereas in the American cockroach, *Periplaneta*

americana, the activity is three times as great in the muscle of the male as in that of the female. These latter results with *P. americana* (Harvey and Beck, 1953) have been confirmed in our laboratory.

4. Results were obtained which indicate that the succinoxidase activity of woodroach thoracic muscle increases with increase in the age of the roach.

5. The results of studies with cofactors, inhibitors and the brilliant cresyl blue system indicate that the mechanism of action of the succinoxidase of woodroach muscle is similar to that of mammalian tissues.

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