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The Respiratory Activity and Permeability of Housefly Sarcosomes

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Studies of the respiratory metabolism of the thoracic muscles of flying insects have in recent years led to rather conflicting reports. There are three main problems.

(1) *Respiratory activity.* The very high rate of oxygen consumption of insects in flight has been pointed out by Chadwick (1953). Since the number of mitochondria (sarcosomes) in the insect thorax (Levenbook & Williams, 1956) and the protein content of the sarcosomes (Watanabe & Williams, 1951) are known, the respiratory capacity per milligram of sarcosome protein may be estimated. These and similar calculations (Chance & Sacktor, 1958) for the housefly gave minimum values for Q_{O_2} (μl. of oxygen/mg. of sarcosome protein/hr.) *in vivo* of about 500. This is very much higher than that reported for isolated housefly sarcosomes. The highest value measured manometrically is reported by Gregg, Heisler & Remmert (1960), who found, with pyruvate plus fumarate as substrates, Q_{O_2} 114 at 25°. Sacktor & Cochran (1958), in manometric studies, obtained the highest respiratory rates when α-glycerophosphate was the substrate (Q_{O_2} 55). Using a vibrating platinum electrode, Chance & Sacktor (1958) measured Q_{O_2} 215 for the oxidation of α-glycerophosphate, a value that could be increased up to 580 by the addition of the uncoupling agent dibromophenol, thus approaching, in isolated sarcosomes, the probable value for oxygen uptake in the intact flight muscle. The fact that the rate of α-glycerophosphate oxidation exceeded the rate obtained with succinate by a factor of 10–20 and the rate with other Krebs-cycle intermediates by factors as large as 50–100, suggested to these authors that α-glycerophosphate is the principal substrate for the activation of the respiratory chain in flight, and that the Krebs cycle does not mediate a significant portion of the flight-muscle metabolism.

(2) *Respiratory control.* Although it is known (Chadwick, 1953) that in the intact insect the increase of respiratory rate upon initiation of flight can be as high as 100-fold, it has proved difficult to elucidate the physiological control mechanism involved. The lack of a measurable response of respiration of their isolated housefly sarcosomes to the addition of phosphate (Sacktor, 1954) or adenosine diphosphate (Sacktor & Cochran, 1958) led Chance & Sacktor (1958) and Estabrook & Sacktor (1958) to the proposals that the respiratory rate is controlled by the substrate concentration or by selective inhibition of certain oxidase systems. However, dependence of the rate of respiration of isolated insect sarcosomes on the concentration of adenosine diphosphate has now been firmly established (Gonda, Traub & Avi-Dor, 1957; Klingenberg & Bücher, 1959; Gregg *et al.* 1960).

(3) *Oxidative phosphorylation.* Originally, no phosphorylation coupled to the oxidation of foodstuffs was found in sarcosomes from insect sources (Sacktor, 1953). But after Lewis & Slater (1953) had demonstrated oxidative phosphorylation in sarcosomes from the blowfly, their results were confirmed for sarcosomes from the locust (Rees, 1954), housefly (Sacktor, 1954), mosquito (Gonda *et al.* 1957), wax-moth larva (Wojtczak & Wojtczak, 1959), cockroach (Cochran & King, 1960) and blowfly larva (Newburgh, Potter & Cheldelin, 1960). In most cases the addition of serum albumin to the reaction medium had a pronounced stimulatory effect on phosphate esterification. Whereas in some cases the requirement for serum albumin appears to be absolute (Sacktor, 1954; Sacktor, O'Neill & Cochran, 1958; Wojtczak & Wojtczak, 1960), other authors report high P:O ratios without added serum albumin (Gregg *et al.* 1960; Newburgh *et al.* 1960).

Van den Bergh & Slater (1960) reported that the addition of a supernatant fraction from the thoracic muscle of the housefly to the reaction medium in which the sarcosomes were tested had three effects: the respiratory rates increased 10- to 20-fold and approached those found *in vivo*; respiratory control by the concentration of adenosine diphosphate was established and high P:O ratios could be obtained in the absence of serum albumin.

In the present paper it is shown that this supernatant effect is due to the continuous formation in the supernatant fraction of rapidly oxidizable substrates and that the low respiratory rates, the lack of respiratory control and the low P:O ratios found with most substrates are caused by permeability barriers for these substrates in the sarcosomes.

METHODS AND MATERIALS

Isolation of sarcosomes. Sarcosomes were isolated from the thoracic muscle of the housefly, *Musca domestica*, essentially by the technique of Watanabe & Williams (1951). The flies were immobilized by cooling below 4° for 30 min. and placed on an ice-cooled glass plate in the cold room. The heads and abdomens were removed and 200 thoraces were gently pounded in a mortar with 5 ml. of isolation medium. The resulting brei was filtered by suction through two layers of muslin, previously saturated with isolation medium, into a tube immersed in an ice bath. The filtrate was centrifuged at 4° for 3 min. at 150*g* and the supernatant again centrifuged for 8 min. at 3000*g*. The sedimented sarcosome pellet was rinsed with two portions of isolation medium and then suspended in 4 ml. of isolation medium with the help of a plastic pestle fitting closely into the centrifuge tube. This procedure gave sarcosomal suspensions containing 6–8 mg. of protein/ml. The last supernatant fraction, containing 2·5–3·5 mg. of protein/ml., was used in some experiments. Except in the experiments given in Table 10, 0·25M-sucrose–1 mM-EDTA, pH 7·4, was used as isolation medium.

For some experiments the sarcosomes were treated in a MSE sonic disintegrator (output, 60 W; frequency, 60 kHz). During treatment, the preparation was immersed in an ice bath.

Measurement of respiration and oxidative phosphorylation. Oxygen consumption was measured in differential manometers with narrow capillaries and gas volumes of 6–7 ml. The standard reaction medium used in all experiments contained 15 mM-KCl, 2 mM-EDTA, 5 mM-$MgCl_2$, 50 mM-tris, 30 mM-potassium phosphate buffer (pH 7·5), 1 mM-ADP, 30 mM-glucose, 150–180 Cori units of hexokinase and substrate as indicated. The pH of the reaction mixture was brought to 7·5 by the addition of HCl (unless otherwise stated), the temperature was 25°, the reaction volume 1 ml. and the reaction period 30 min. The centre well contained a piece of filter paper dipping into 0·1 ml. of 2M-KOH.

Phosphorylation was usually determined from the disappearance of inorganic phosphate, determined by Sumner's (1944) modification of the Fiske–Subbarow method in the reaction medium deproteinized with trichloroacetic acid. However, when phosphorylation was very small, it was determined by the enzymic method of Slater (1953). Both methods appeared to be consistent within the limits of experimental error.

Respiratory control was measured in the standard reaction medium, but with 0·1 mM-ATP in place of ADP, glucose and hexokinase. When the uptake of O_2 was constant for 6 min., 3–6 μmoles of ADP were added from the side arm. The respiratory-control index is the ratio of the respiratory rates before and after the addition of ADP.

Analytical procedures. Protein determinations were made by the biuret method of Gornall, Bardawill & David (1949), as modified by Cleland & Slater (1953).

Pyruvate was determined spectrophotometrically with DPNH and lactate dehydrogenase by measuring the decrease of extinction at 340 mμ.

For the determination of α-glycerophosphate, samples were incubated for 90 min. with sufficient sarcosomes for oxidation of all the α-glycerophosphate, and the increase in dihydroxyacetone phosphate during this treatment was estimated. In our hands, this procedure gave more reproducible results than the direct methods described by Bublitz & Kennedy (1954) and by Hohorst, Kreutz & Bücher (1959). In experiments with added α-glycerophosphate recovery was between 96 and 99%.

Dihydroxyacetone phosphate was determined spectrophotometrically at 340 mμ with DPNH and an enzyme fraction from rabbit muscle, described by Racker (1947) as 'α-glycerophosphate dehydrogenase and aldolase'. In these determinations excess of EDTA was added to prevent the phosphorylation of hexose monophosphate by phosphohexokinase present in the enzyme preparation.

Glucose was determined by a procedure based on a method described by Teller (1956). In this method the enzymic oxidation of glucose with glucose oxidase is coupled to the peroxidation of the colourless *o*-dianisidine to an orange–red dye. The increase in extinction at 420 mμ is followed until the reaction is finished (45–60 min.). If glucose had to be determined in the presence of trehalose, a correction was made for the simultaneous oxidation of trehalose by the enzyme. This was done by extrapolation of the reaction rate between 60 and 120 min. back to the start of the experiment. If the amount of trehalose present was greater than the amount of glucose to be determined, this method gave very satisfactory results. The recovery of added glucose was 94–102%.

Trehalose was determined by a modified procedure of Wyatt & Kalf (1957) as the difference in glucose content between a sample hydrolysed for 45 min. at 100° at pH 1·5 and a sample that had been hydrolysed for 90 min. at 100° at pH 0 (pH values measured with a glass electrode). In experiments with added trehalose recovery was 83–95%.

Paper chromatography of carbohydrates was performed for 24 hr. at room temperature on Whatman no. 1 paper by the descending technique with propanol–ethyl acetate–water (7:1:2, by vol.) as developing solvent (Baar & Bull, 1953). Sugars were detected by the method of Trevelyan, Procter & Harrison (1950).

Materials. Chemicals were obtained from the following sources: ADP, ATP, tris, DPN, TPN, DL-α-glycerophosphate, α-oxoglutarate, L-proline, coenzyme A and DL-isocitric lactone, Sigma Chemical Co.; L-malate, Nutritional Biochemicals Corp.; trehalose, Brocades, Amsterdam; succinate, pyruvate, glutathione, glucose 6-phosphate and fructose diphosphate, C. F. Boehringer und Soehne; serum albumin, Armour; Amytal, Amsterdamsche Chinine Fabriek; thiamine pyrophosphate, Hoffmann–La Roche and Co. Ltd. Glass-redistilled water was used in preparing all solutions. A solution of isocitrate was prepared from the lactone by alkaline hydrolysis for 1 hr. at 100°.

Lactate dehydrogenase, alcohol dehydrogenase, α-glycerophosphate dehydrogenase, glucose 6-phosphate dehydrogenase, glucose oxidase and peroxidase were obtained from C. F. Boehringer und Soehne. Hexokinase was prepared from yeast according to the procedure of Darrow & Colowick (1961), the final crystallization step being omitted.

RESULTS

Stimulatory action of the supernatant fraction

Insect sarcosomes can be isolated by a relatively mild procedure. Yet in many respects they resemble partly damaged mitochondria from mammalian sources (for review see Slater, 1960). To determine

Table 1. *Comparison of properties of housefly sarcosomes at different stages in the isolation procedure*

'Filtrate' is the suspension obtained by filtering the muscle brei through muslin. Substrate concentrations were 60 mM-succinate, 20 mM-glutamate and 20 mM-α-oxoglutarate + 20 mM-malonate. In these experiments the standard reaction medium was used, except that the pH was adjusted with acetic acid, which gave for the isolated sarcosomes slightly higher P:O ratios with succinate than were found in later experiments when the pH was adjusted with HCl. The values given are means with the number of experiments in parentheses.

	Q_{O_2}*		P:O		Respiratory-control index	
Substrate	Filtrate	Sarcosomes	Filtrate	Sarcosomes	Filtrate	Sarcosomes
Succinate	257 (10)	52 (11)	2·03 (10)	0·60 (7)	3·6 (2)	1·0 (5)
Glutamate	232 (3)	25 (2)	2·30 (3)	0·88 (2)	3·2 (2)	1·0 (2)
α-Oxoglutarate + malonate	190 (3)	28 (2)	2·27 (3)	1·19 (2)	3·3 (5)	1·0 (4)

* Q_{O_2} of the filtrate is expressed per mg. of total protein.

Table 2. *Effect of repeated centrifuging on the properties of housefly sarcosomes*

The suspension obtained by filtering the muscle brei through muslin was centrifuged (8 min. at 3000*g*) and the sediment then again suspended in its own supernatant. This treatment was repeated a number of times. After each treatment a sample was withdrawn. Substrate: 20 mM-α-oxoglutarate + 20 mM-malonate.

No. of treatments	Q_{O_2}*	P:O	Respiratory-control index
0	190	2·22	3·08
1	146	2·00	2·52
2	134	1·89	2·25
3	89	1·68	1·96

* Q_{O_2} is expressed per mg. of total protein.

whether this represents a real property of the sarcosomes *in vivo*, or is caused by damage during the isolation procedure, we investigated the properties of the sarcosomes at different stages in the isolation procedure. In Table 1 the properties of the suspension obtained by filtering the muscle brei through muslin are compared with those of the isolated sarcosomes. It is clear that the filtered muscle brei gave much higher respiratory activity, P:O ratios and better respiratory control than the isolated sarcosomes.

For the striking differences between these preparations two possible explanations appeared to be open. Either the sarcosomes were severely damaged during the rest of the isolation procedure, or a soluble factor, necessary for a good operation of the sarcosomes, was lost in the supernatant fraction. Mechanical damage to the sarcosomes was indeed shown to occur during centrifuging and suspension (see Table 2), but not to the extent necessary to explain the data of Table 1. Further experiments provided support for the second explanation. When the supernatant fraction was added to the medium in which the isolated sarcosomes were tested large stimulations of respiration, phosphorylation and respiratory control were observed.

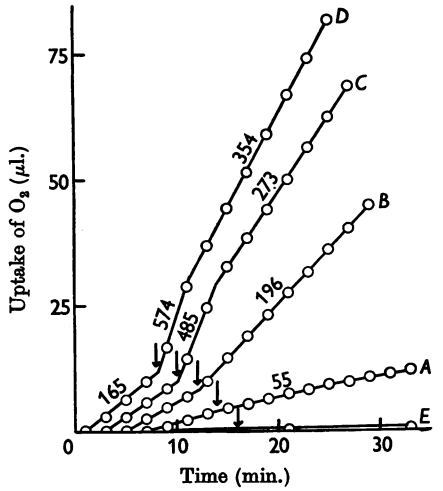

Fig. 1. Effect of the addition of increasing amounts of supernatant fraction on the respiration of housefly sarcosomes. At the arrow, 3 μmoles of ADP were added from the side arm. The flasks contained 60 mM-succinate and 0·63 mg. of sarcosome protein. The supernatant fraction contained 3·34 mg. of protein/ml. *A*, No supernatant fraction added; *B*, *C* and *D*, with 0·1, 0·2 and 0·3 ml. of supernatant fraction respectively; *E*, with 0·3 ml. of supernatant fraction without sarcosomes. Figures above the straight parts of the curves are Q_{O_2} values. In this experiment the pH of the standard reaction medium was adjusted with acetic acid instead of HCl, which was used in most other experiments.

A typical experiment with succinate as substrate is shown in Fig. 1 and average values are given in Table 3.

As it was clear now that the isolated sarcosomes were capable of a high respiratory activity, approaching the values calculated for the flying insect and much higher than those previously reported for manometric experiments, we tested the sarcosomes with a number of substrates. It became clear (Table 4) that these can be divided

Table 3. *Effect of addition of increasing amounts of supernatant fraction on the properties of isolated housefly sarcosomes*

Substrate: 60 mM-succinate. Values given are means with the number of experiments in parentheses. The pH of the standard reaction medium was adjusted with acetic acid (cf. Table 1).

Addition	Q_{O_2}	P:O	Respiratory-control index
None	52 (11)	0·60 (7)	1·0 (5)
Serum albumin (2%)	49 (4)	1·21 (2)	1·0 (1)
Supernatant fraction (0·1 ml.)	244 (4)	1·79 (4)	1·87 (3)
Supernatant fraction (0·2 ml.)	518 (4)	2·23 (4)	3·83 (3)
Supernatant fraction (0·3 ml.)	660 (7)	2·25 (6)	3·51 (5)

Table 4. *Respiratory activity, respiratory control and oxidative phosphorylation with sarcosomes isolated from housefly thoracic muscle*

Substrate concentrations: 60 mM-DL-α-glycerophosphate; 20 mM-pyruvate + 10 mM-L-malate; 20 mM-citrate; 18 mM-DL-isocitrate; 20 mM-L-glutamate; 20 mM-α-oxoglutarate with and without 20 mM-malonate; 60 mM-succinate; 20 mM-L-malate; 20 mM-L-proline; 20 mM-DL-β-hydroxybutyrate; 30 mM-glucose; 20 mM-glucose 6-phosphate; 20 mM-fructose diphosphate; 30 mM-trehalose; 100 mM-sucrose. When DPNH was the substrate, 1 mM-DPN^+ was added + 2·4% of ethanol + alcohol dehydrogenase. For TPNH, 1 mM-TPN^+ + 20 mM-glucose 6-phosphate + glucose 6-phosphate dehydrogenase were used. In both cases 50 mM-nicotinamide was added. Values given are means (range of values in parentheses) and number of experiments.

Substrate	Q_{O_2}	P:O	Respiratory-control index
α-Glycerophosphate	673 (540–862) 11	1·38 (1·12–1·52) 4	1·67 (1·37–1·83) 4
Pyruvate + malate	245 (173–290) 9	2·23 (1·97–2·52) 7	8·76 (7·26–9·98) 3
Citrate	13 (11–16) 3	—	—
Isocitrate	20 (14–25) 3	0·28 (0·24–0·31) 2	—
Glutamate	20 (14–27) 4	0·84 (0·75–0·99) 4	1·0
α-Oxoglutarate	31 (24–35) 8	0·79 (0·58–1·02) 4	1·0
α-Oxoglutarate + malonate	25 (21–28) 3	1·14 (1·00–1·40) 3	1·0
Succinate	46 (33–65) 18	0·40 (0·28–0·68) 9	1·0
Malate	23 (16–29) 3	0·22 (0·18–0·30) 3	1·0
Proline	51 (46–56) 2	0·78 (0·63–0·93) 2	1·0
β-Hydroxybutyrate	2	—	—
DPNH	45 (31–63) 4	0·28 (0·17–0·38) 3	1·0
TPNH	6 (4–7) 5	—	—
Glucose*	0	—	—
Glucose 6-phosphate*	0	—	—
Fructose diphosphate*	0	—	—
Trehalose*	0	—	—
Sucrose*	0	—	—
Endogenous substrate*	0	—	—

* To test these substrates 10 mM-ADP was added to the standard reaction medium in place of glucose–hexokinase.

into two classes: the rapidly oxidizable substrates (α-glycerophosphate and pyruvate), the oxidation of which is accompanied by high P:O ratios and is under control of the ADP concentration, and the slowly oxidizable substrates (e.g. the Krebs-cycle intermediates), that give low P:O ratios and show no measurable respiratory control. The supernatant fraction, as prepared, did not contain appreciable amounts of α-glycerophosphate or pyruvate, but these substrates were formed in equimolecular amounts on incubation at room temperature after addition of ADP, ATP, phosphate and magnesium (Table 5). The same results were obtained with a supernatant fraction obtained by centrifuging for 1 hr. at 100000*g*.

Since, then, the formation in the supernatant fraction of α-glycerophosphate and pyruvate, known to be the end products of glycolysis in insect

Table 5. *Formation of pyruvate and α-glycerophosphate in the supernatant fraction of housefly thoracic muscle*

Three portions (1·5 ml.) of freshly prepared supernatant fraction were incubated at room temperature with 15 mM-KCl, 2 mM-EDTA, 50 mM-tris–HCl buffer, 9 mM-$MgCl_2$, 2·5 mM-ADP, 1·5 mM-ATP and 30 mM-potassium phosphate buffer. Final volume, 2 ml.; pH 7·5. At the times indicated reactions were stopped by heating at 100° for 2 min. After the protein was removed by centrifuging, the mixtures were assayed for α-glycerophosphate and pyruvate. The values given are μmoles/ml. of supernatant fraction.

Incubation period (min.) ...		0	10	20
Pyruvate	found	0·24	2·60	6·06
	formed	—	2·36	5·82
α-Glycerophosphate	found	0·22	2·52	5·57
	formed	—	2·30	5·35

flight muscle (Kubista, 1957), could provide a satisfactory explanation for the stimulatory action of this fraction, the effect of the glycolytic inhibitor iodoacetic acid was studied. The data presented in Table 6 show that glycolysis can be nearly completely inhibited by 1 mM-iodoacetate (cf. discussion following Chefurka, 1958). Table 7 shows that this concentration of iodoacetate could abolish the stimulatory action of the supernatant fraction. Thus the effect of the supernatant fraction is adequately explained by the formation of pyruvate and α-glycerophosphate from endogenous carbohydrate in this fraction, catalysed by glycolytic enzymes also present in the supernatant.

Permeability of housefly sarcosomes

As has already been pointed out (Slater, 1960), three possible explanations appear to be open for the discrepancy between the high oxygen consumption of the fly, *in vivo*, and the low rates of oxygen uptake with isolated sarcosomes, when oxidizing Krebs-cycle intermediates: (*a*) the respiratory-enzyme systems of the isolated sarcosomes are damaged during isolation; (*b*) the isolated sarcosomes are not readily permeable to intermediates of the Krebs cycle (Lewis & Slater, 1954); (*c*) a large part of the respiration of insect sarcosomes, *in vivo*, occurs by a pathway different from that of the Krebs cycle. As shown above, it is now no longer necessary to invoke the first explanation, since the isolated sarcosomes are able rapidly to oxidize pyruvate, the substrate of the Krebs cycle. The third explanation will be discussed in the following section. The existence of a permeability barrier was demonstrated by treating the sarcosomes in a sonic disintegrator. Respiratory rates with Krebs-cycle intermediates increased as a result of this treatment, sometimes well over tenfold (see Fig. 2 and Table 8).

Relative importance of the Krebs cycle in oxygen consumption

The third suggestion (*c* above) still had to be looked into. The most likely possibility for an oxidative pathway different from that of the Krebs cycle is certainly the oxidation of α-glycerophosphate, so persistently emphasized by Sacktor (1958, 1961), Chance & Sacktor (1958), Estabrook & Sacktor (1958) and Sacktor & Packer (1961) as the physiological substrate for the activated sarcosomes. To compare the relative importance of the α-glycerophosphate oxidase and the Krebs cycle in sarcosome respiration, we incubated sarcosomes plus supernatant fraction in the standard reaction medium without added substrate. After the oxygen uptake had been recorded for 30 min. the reaction was stopped and the reaction mixture assayed for pyruvate and α-glycerophosphate. Assuming (*a*) that no glycolytic intermediates between triose phosphate and pyruvate accumulate, (*b*) that the soluble α-glycerophosphate dehydrogenase can compete successfully with the sarcosomes for all the DPNH formed outside the particles, (*c*) that no substrates other than α-glycerophosphate and pyruvate are available for the sarcosomes and (*d*) that 5 atoms of oxygen are taken up for the oxidation of 1 molecule of pyruvate (i.e. that no Krebs-cycle intermediates accumulate), we can calculate the percentage of the total oxygen uptake mediated by the Krebs cycle. The results of such an experiment are shown in Table 9. It should be noted that if assumption (*b*) is only partly correct, the relative importance of the Krebs cycle is underestimated in Table 9. Assumption (*d*) was proved correct in preliminary experiments.

Carbohydrate in the supernatant fraction

To investigate the nature of the carbohydrate in the supernatant fraction responsible for the formation of α-glycerophosphate and pyruvate, an

Table 6. *Inhibition by iodoacetic acid of the formation of α-glycerophosphate and pyruvate in the supernatant fraction from housefly thoracic muscle*

For incubation conditions see Table 5. Incubation time, 20 min.

Iodoacetate (mM)	Pyruvate formed (μmoles/ml. of supernatant fraction)	α-Glycerophosphate formed (μmoles/ml. of supernatant fraction)
0	5·92	5·79
0·01	6·18	5·87
0·1	3·35	3·15
1	0·32	0·47
10	0·15	<0·01

Table 7. *Abolition of the stimulatory action of the supernatant fraction by addition of iodoacetic acid*

Substrate: 60 mM-succinate.

Supernatant (0·3 ml.)	Iodoacetate (1 mM)	Q_{O_2}	P:O	Respiratory-control index
−	−	46	0·40	1·0
+	−	672	2·04	3·74
−	+	47	0·21	1·0
+	+	47	0·21	1·0

isolation medium without sucrose (0·154 M-KCl–1 mM-EDTA, pH 7·4, see below) was used. Paper chromatography of the supernatant fraction obtained in this isolation medium revealed the presence of at least eight different carbohydrate fractions. The four main spots on the chromatogram could be identified by cochromatography as glucose, trehalose, sucrose and fructose.

Quantitative glucose determinations indicated that the fully hydrolysed (90 min. at 100° at pH 0) supernatant fraction contained 25 mM-glucose (average of three different supernatant fractions, range 24·6–25·6), of which 3·0–3·4 mM was present in the supernatant fraction as free glucose, 10·5–13·0 mM was derived from trehalose and 9·6–10·9 mM was derived from other carbohydrates. It could be shown that this last fraction is not metabolized under the conditions of our experiments, whereas trehalose is readily hydrolysed, even leading to an increase in the concentration of free glucose. In other experiments, in which excess of sarcosomes were added to prevent the accumulation of oxidizable substrates, we found that for every glucose unit that disappeared from the reaction medium 12 oxygen atoms were consumed (the average of seven experiments was 11·9; range, 10·2–13·0). This strongly suggested that glucose is the only monosaccharide metabolized under the conditions of our experiments.

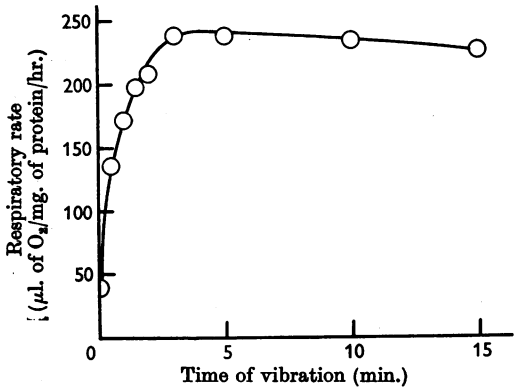

Fig. 2. Effect of ultrasonic vibration on the respiratory rate of housefly sarcosomes with succinate as substrate. A portion (8 ml.) of a sarcosome suspension of 3·06 mg. of protein/ml. was treated in a sonic disintegrator (see Methods and Materials section). At least once every 2·5 min. the probe of the instrument was removed and chilled in ice. At the times indicated a sample (0·4 ml.) was withdrawn and tested immediately. Substrate concentration, 60 mM.

Sarcosomes isolated in potassium chloride–EDTA medium

Since an isolation medium without sucrose was needed for the experiments described in the preceding section, we examined the properties of the sarcosomes isolated in various media. It was found

Table 8. *Effect of ultrasonic vibration on the respiratory rate of housefly sarcosomes*

A portion (6 ml.) of a sarcosome suspension (3·14 mg. of protein/ml.) was treated in a sonic disintegrator for 2·5 min. For substrate concentrations see Table 4.

Substrate	Q_{O_2} Untreated sarcosomes	Q_{O_2} Disintegrated sarcosomes
α-Glycerophosphate	634	464
Pyruvate + malate	263	244*
Succinate	36	257
DPNH	31	381
α-Oxoglutarate	25	131*
Isocitrate	21	298†
Glutamate	23	53*
Malate	24	68‡

* 1 mM-DPN^+, 1 mM-thiamine pyrophosphate, 0·3 mM-coenzyme A, 50 mM-nicotinamide and 3mM-glutathione were added.

† 1 mM-TPN^+ + 1 mM-DPN^+ + 50 mM-nicotinamide were added. This is the initial rate; after 7–9 min. it sharply decreased.

‡ 1 mM-DPN^+ + 50 mM-nicotinamide were added.

Table 9. *Accumulation of α-glycerophosphate and pyruvate during the simultaneous formation and oxidation of these substrates in homogenates of housefly thoracic muscle*

Different amounts of sarcosomes and supernatant fraction were incubated together in the standard reaction medium without added substrate. After the uptake of O_2 had been recorded for 30 min., the reaction was stopped and the reaction mixture assayed for α-glycerophosphate and pyruvate. The supernatant fraction contained 3·25 mg. of protein/ml. In the last column (contribution of Krebs cycle) the percentage of the total consumption of O_2 that is mediated by the Krebs cycle is calculated.

Supernatant fraction (ml.)	Sarcosomes (mg. of protein)	Q_{O_2}	Uptake of oxygen (µg.atoms)	Pyruvate accumulated (µmoles)	α-Glycerophosphate accumulated (µmoles)	Contribution of Krebs cycle (%)
0·60	0·46	609	11·72	18·42	13·32	47
0·30	0·46	558	10·09	10·00	10·66	84
0·30	0·92	477	17·48	6·65	4·48	73
0·30	1·39	343	19·73	5·99	2·09	67
0·15	1·85	256	17·41	0·94	0	79

Table 10. *Properties of housefly sarcosomes isolated in potassium chloride–EDTA medium*

0·154M-Potassium chloride–1 mM-EDTA, pH 7·4, was used as isolation medium. For substrate concentrations see Table 4. Values given are means (range of values in parentheses) and number of experiments.

Substrate	Q_{O_2}	P:O	Respiratory-control index
α-Glycerophosphate	754 (635–886) 13	1·33 (1·16–1·50) 11	1·7 (1·64–1·73) 2
Pyruvate + malate	647 (522–766) 18	2·40 (2·16–2·60) 13	8·9 (8·42–9·34) 2
Succinate	18 (13–22) 10	0·48 (0·45–0·53) 4	1·0
DPNH	10 (7–11) 3	—	—

that sarcosomes isolated in 0·154M-KCl–1 mM-EDTA, pH 7·4, had higher respiratory activities with α-glycerophosphate and pyruvate + malate, and lower activities with succinate and DPNH, than those isolated in sucrose–EDTA (see Table 10; cf. Table 4). It should be noted that with these sarcosomes the respiratory rate for pyruvate + malate closely approaches the rate for α-glycerophosphate, both being high enough to account for the oxygen uptake of the living housefly in flight.

When we repeated with these sarcosomes the experiments described in Table 9, the figures calculated for 'contribution of Krebs cycle' were significantly higher, lying between 79 and 85%. The more rapid oxidation of pyruvate and the slower oxidation of exogenous DPNH may account for these higher figures calculated for the relative importance of the Krebs cycle in oxygen consumption.

Age of the flies

From the very beginning of the study of oxidative phosphorylation in insects, the age of the animals has been regarded as an important factor with an undeniable, though not understood, influence on the properties of the isolated sarcosomes. Lewis & Slater (1954) found lower P:O ratios with sarcosomes isolated from blowflies younger than 10 days. Klingenberg & Bücher (1959) could demonstrate respiratory control with α-glycerophosphate as substrate only when the sarcosomes were isolated from locusts 10–15 days old. These reports made us decide to use flies older than 9 days in all experiments. No systematic differences were found between preparations from houseflies from 10 to 27 days old. However, to see whether the differences between our sarcosome preparations and those of Sacktor (1954), who used houseflies 4–7 days old, and Gregg, Heisler & Remmert (1959), who used houseflies about 4 days old, might be attributed to differences in age, we tested sarcosomes from houseflies within 24 hr. after emergence. Although the yield of sarcosome protein was only 40% of our standard preparations (cf. Levenbook & Williams, 1956), we found respiratory rates, P:O ratios and respiratory-control indices comparable with those of our standard preparations for all substrates tested.

DISCUSSION

Only a few years ago it appeared, from the evidence then available, that isolated sarcosomes from insect sources were different from sarcosomes from mammalian tissues in several significant respects: (1) in their low P:O ratios; (2) in their requirement for serum albumin in the phosphorylation medium; (3) in their lack of respiratory control by the concentration of phosphate and of phosphate acceptor; (4) in their ability to oxidize α-glycerophosphate at many times the rate with pyruvate, glutamate or Krebs-cycle intermediates; (5) in their low respiratory rates as compared with the oxygen consumption of the flying insect.

During the last few years, however, it has been shown that most of these points are no longer tenable. With serum albumin in the reaction medium P:O ratios were obtained approaching those found with mammalian preparations (Sacktor & Cochran, 1958; Cochran & King, 1960). Further, sarcosomes have been isolated from different insect species that exhibit respiratory control in the absence of ADP (Gonda *et al.* 1957; Klingenberg & Bücher, 1959; Gregg *et al.* 1960). Finally, it was shown that high P:O ratios could be obtained without addition of serum albumin and that pyruvate could be oxidized even more rapidly than α-glycerophosphate (Gregg *et al.* 1960).

The investigations reported in this paper show that none of the differences mentioned above is real, but that the sarcosomes from insect thoracic muscles are truly different from mammalian preparations, both in their exceptionally high respiratory rates with their physiological substrates pyruvate and α-glycerophosphate and in their impermeability towards other substrates.

Oxidative phosphorylation

We confirmed the observations of Gregg *et al.* (1960) that the oxidation of α-glycerophosphate and pyruvate is accompanied by high P:O ratios without the addition of serum albumin to the reaction medium. Although these authors do not state so explicitly, we assume that α-glycerophosphate and pyruvate were the only substrates

giving them these satisfactory results, as in our experiments. With all other substrates tested we found low P:O ratios. These could be substantially increased by the addition of serum albumin, approaching then the values reported for mammalian preparations, with the exception of that for α-oxoglutarate. In agreement with other investigators (Sacktor & Cochran, 1958; Cochran & King, 1960), we did not find P:O ratios exceeding 2·5 for this substrate in the presence of serum albumin. Mainly from the work of Wojtczak & Wojtczak (1960) we know now that this effect of serum albumin may be attributed to its capacity to bind the fatty acids that form the active component (Hülsmann, Elliott & Slater, 1960) of the endogenous uncoupling agent, enzymically (Lewis & Fowler, 1960) generated in the sarcosomes (cf. Hülsmann, 1958). In unpublished experiments we found that the properties of the adenosine triphosphatase, induced by this endogenous uncoupler, in its dependence on the pH and on the concentration of uncoupler, closely resembled that induced by pure oleic acid (Borst & Loos, 1959). This strongly suggests that the endogenous uncoupler from housefly sarcosomes also consists of a mixture of unsaturated fatty acids.

Respiratory control

The respiratory-control indices obtained with pyruvate indicate that respiration in insect sarcosomes is to a large extent under control of the ADP concentration. The much lower respiratory-control index with α-glycerophosphate is in full agreement with the results of Klingenberg & Bücher (1959) and of Gregg *et al.* (1960). However, both systems used by Gregg *et al.* (1960) to measure respiratory control are different from ours and are likely to give higher control ratios. Gregg *et al.* either compared oxygen uptake in flasks with and without added adenine nucleotide or they added ADP from the side arm to a reaction medium containing serum albumin and 100-fold excess of EDTA over Mg^{2+} ions. In our system, as described by Holton, Hülsmann, Myers & Slater (1957), no serum albumin is present, 5 mM-magnesium chloride is used (compared with 2 mM-EDTA), and from the beginning 0·1 mM-ATP is present. We did not use the high sucrose concentrations indicated by Klingenberg & Bücher (1959) as an absolute requirement for the demonstration of respiratory control. In fact, the respiratory-control indices were unaffected by varying the sucrose concentration between zero and 0·3 M. Respiratory control by the phosphate concentration could also be demonstrated. The control ratios with pyruvate + malate in the absence of added phosphate ranged from 17 to 48 with an average of 24 in seven experiments.

Respiratory capacity

For the first time, respiratory rates have been measured with pyruvate + malate and manometrically with α-glycerophosphate that can account for the oxygen consumption of the housefly in flight. As it was not inconceivable that the protein determination of Cleland & Slater (1953) was unsuitable for housefly sarcosomes and therefore responsible for the exceptionally high Q_{O_2} values, we determined the nitrogen content of two sarcosomal preparations by the Kjeldahl method. By comparing the nitrogen content with the protein concentration, determined by the normal procedure, we found that the protein contained 14·7–15·2% of nitrogen, which does not suggest that the protein determination underestimates the true value.

From the available data, it is impossible to decide why our sarcosome preparations, which in many respects closely resemble those of Gregg *et al.* (1960), exhibit a much higher respiratory activity. It seems unlikely that this is due to differences in reaction conditions, as the respiratory rates were very little affected by relatively large changes in the reaction conditions, such as variations in the concentration of sucrose (zero–0·3 M), phosphate (10–50 mM), potassium chloride (15–115 mM) and tris (zero–0·1 M).

The isolation procedure and the isolation medium are probably more important factors in determining the properties of the isolated sarcosomes. The observed difference in the rate at which pyruvate is oxidized by sarcosomes isolated in sucrose–EDTA and potassium chloride–EDTA (Q_{O_2} 245, as against 647) is a strong indication in this direction. However, according to Newburgh *et al.* (1960) little difference in activity was observed when sarcosomes from blowfly larvae were isolated in these media.

Much emphasis has recently been placed on the use of well-buffered isolation media (Klingenberg & Bücher, 1959) containing oxidizable substrates (Gregg *et al.* 1960). Our isolation media were not buffered (except for the 1 mM-EDTA present) and no substrates were added to it. It seems not unreasonable, however, to speculate that the substrates, formed in the soluble part of the thoracic extract, form a superior substitute for the added substrates.

We are unable to explain the differences between our preparations and those of Sacktor and colleagues (Sacktor, 1953, 1954; Sacktor *et al.* 1958; Sacktor & Cochran, 1958; Chance & Sacktor, 1958; Estabrook & Sacktor, 1958). Whereas their sarcosomes oxidize pyruvate very slowly, and moreover have all the characteristics of loosely coupled oxidative phosphorylation, our preparations oxidize pyruvate very rapidly and are tightly coupled.

Impermeability of the sarcosomes

By breaking up the particles, the oxidation rate for succinate and other substrates can be greatly increased. This shows clearly that the oxidase systems concerned are present in abundance, but that the sarcosomes are not readily permeable to these substrates. Permeability barriers towards external DPNH have been recognized in rat-liver mitochondria (Lehninger, 1955) and in blowfly sarcosomes (Sacktor, 1961), and recently it was shown (Singer & Lusty, 1960) that undamaged mitochondria are not readily permeable to phenazine methosulphate, a dyestuff often used in assays of succinic dehydrogenase. But permeability barriers in mitochondria towards such simple ions as succinate have never been demonstrated before. Yet the only alternative explanation, namely that the slow oxidation of succinate with the intact sarcosomes was due to inhibition by oxaloacetate formed from the succinate (cf. Greengard, Minnaert, Slater & Betel, 1959; Azzone, Eeg-Olofsson, Ernster, Luft & Szabolcsi, 1961; Slater & Hülsmann, 1961), could be eliminated by studying the effect of Amytal, which prevents the formation of oxaloacetate. The presence of 1·75 mM-Amytal, sufficient to inhibit completely the oxidation of malate, either had no effect or slightly inhibited the oxidation of succinate by the sarcosomes.

Another argument for the abundant presence of all the Krebs-cycle enzymes is found in the observation, already mentioned, that no Krebs-cycle intermediates accumulate during the oxidation of pyruvate.

In view of the apparent permeability barrier, two possible explanations appear to account for the total absence of respiratory control with all substrates except α-glycerophosphate and pyruvate. First it may be assumed that the penetration of the substrate molecules to their dehydrogenases in the sarcosomes forms the rate-limiting step in the oxidation of these substrates. Addition of ADP cannot stimulate respiration because the availability of ADP is not rate-limiting. The hydrolytic side reactions of oxidative phosphorylation, promoted by the endogenous uncoupler, can fully abolish the need for ADP during these slow oxidations. But with the rapid oxidations of α-glycerophosphate and pyruvate the rate of hydrolysis of energy-rich intermediates, which is independent of the rate of the oxidation reaction, becomes slower than the formation of these intermediates, so that the supply of ADP becomes rate-limiting.

According to the second explanation, intact sarcosomes are completely impermeable to all substrates except pyruvate and α-glycerophosphate. The slow oxidation observed with all these substrates is brought about by a relatively small number of partly damaged sarcosomes. These are no longer tightly coupled and therefore exhibit no respiratory control. This second explanation is the more attractive one, since it can also explain the low P:O ratios that accompany the slow oxidations. The seeming contradiction that sarcosomes isolated in a potassium chloride isolation medium give higher respiratory rates with α-glycerophosphate and pyruvate and lower rates with succinate and DPNH than do sarcosomes isolated in the normal sucrose medium also gains a greater significance.

Physiological substrates for the sarcosomes

In view of the observations reported here, it seems unnecessary to retain an exceptional position for α-glycerophosphate as the only physiological substrate for flight-muscle activity. On the contrary, it has become clear that pyruvate as well as α-glycerophosphate can be oxidized at the required rate and therefore that pyruvate oxidation is likely to be the more important in flight muscle metabolism (five times on an oxygen basis and 7·5 times on an energy basis). Moreover, every system in which α-glycerophosphate is oxidized preferentially leads to an accumulation or excretion of pyruvate, phenomena that have never been observed in living insects.

In sharp contrast with the high respiratory rates observed with α-glycerophosphate and pyruvate are the low oxidation rates for all other external substrates. However, α-glycerophosphate and pyruvate are the only products of the sarcoplasmic metabolism *in vivo* that need to penetrate rapidly into the sarcosomes.

SUMMARY

1. Sarcosomes isolated from the thoracic muscle of the housefly can oxidize α-glycerophosphate and pyruvate at rates comparable with those calculated for the flying insect. This rapid oxidation is accompanied by high P:O ratios in the absence of serum albumin and is to a large extent under control of the adenosine diphosphate concentration.

2. Glutamate, reduced diphosphopyridine nucleotide and Krebs-cycle intermediates are oxidized at very much lower rates, accompanied by poor phosphorylation and by no measurable respiratory control. It is shown that this is due to permeability barriers for these substrates in the sarcosome.

3. The addition of a supernatant fraction to sarcosomes which are oxidizing one of the latter substrates has a stimulatory effect on respiration, phosphorylation and respiratory control. It is shown that this stimulation is caused by the

continuous formation of equimolecular amounts of α-glycerophosphate and pyruvate from carbohydrate sources in the supernatant fraction.

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