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Some Properties of Mitochondria Isolated from the Flight Muscle of the Periodical Cicada, *Magicicada septendecim*

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Mitochondria from the flight muscle of the periodical cicada oxidize pyruvate and D-glycerol 1-phosphate at rates comparable with those obtained with flight-muscle mitochondria from other insects. The oxidation of D-glycerol 1-phosphate is greatly stimulated by low concentrations of Ca^{2+}. However, oxidative phosphorylation with this substrate is optimum over only a narrow range of Ca^{2+} concentration, because of the ability of these mitochondria actively to accumulate Ca^{2+} present at micromolar concentrations. The oxidation of pyruvate via the complete tricarboxylic acid cycle is enhanced by high concentrations of phosphate. When both pyruvate and D-glycerol 1-phosphate are present simultaneously, there is no simple summation of the rates obtained with the substrates singly. Acetyl-L-carnitine, palmitoyl-L-carnitine, glutamate and 2-oxoglutarate are oxidized at rates similar to those obtained with mammalian mitochondria, though lower than those obtained with the two prime substrates. However, no other tricarboxylic acid-cycle intermediates added to the medium were oxidized. From these and other observations it has been concluded that these mitochondria possess a previously undescribed combination of substrate-anion permeases.

In recent years there has been a considerable broadening of our understanding of the oxidative metabolism of insect flight muscle. Some insects utilize carbohydrate during flight, and their flight-muscle mitochondria accordingly oxidize pyruvate and D-glycerol 1-phosphate, the end-products of glycolysis (Zebe & McShan, 1957; Chefurka, 1958). This pattern has been observed in the housefly (Van den Bergh & Slater, 1962), the blowfly (Chance & Sacktor, 1958; Childress & Sacktor, 1966) and the honey bee (Balboni, 1967). Other insects depend very largely on the oxidation of lipids, and possess mitochondria capable of oxidizing fatty acids, as is true of the moths (Stevenson, 1966, 1968*a*,*b*). Still others derive energy from both sources, and have mitochondria capable of oxidizing the carnitine esters of long-chain fatty acids, as well as pyruvate and D-glycerol 1-phosphate. An example is the locust (Beenakkers, 1963; Bode & Klingenberg, 1965; Klingenberg & Bücher, 1959, 1961). However, there are still great gaps in our knowledge. For instance, there is very little information about the oxidative metabolism of the plant-bugs or *Hemipteroidea*, many of which fly. The study described in this paper was designed to gain some information about one insect in this group, the periodical cicada *Magicicada septendecim*. This insect is large, flies moderately well, and is available in great abundance in the south-eastern United States, but only for a few weeks in the early summer of widely spaced years. The insects used in this particular study belonged to Brood X, the most populous of the broods of the 17-year cicada (Marlatt, 1907).

The results reported in the present paper show that mitochondria from the flight muscle of the cicada oxidize pyruvate and D-glycerol 1-phosphate at high rates, providing inferential evidence that these are the products of glycolysis in this tissue. Palmitoyl-L-carnitine was also oxidized, but at a low rate, which appears to rule out a major role in supporting flight. The rapid oxidation of 2-oxoglutarate and glutamate by these mitochondria, but the failure to oxidize other tricarboxylic acid-cycle intermediates, was striking, and is discussed both in terms of the permeability of the mitochondrial membrane and of flight-muscle physiology.

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MATERIALS AND METHODS

All the insects were collected within the confines of Baltimore, Md., during the period 3–23 June 1970. They were of both sexes.

For each mitochondrial preparation 12–18 insects were used. The thoraces were removed and the flight muscle expressed; lipid material was cleaned away by wiping with tissue. The flight muscles were homogenized in 30ml of 0.25M-sucrose–5.0mM-tris–HCl–1.0mM-ethanedioxybis(ethylamine)tetra-acetate, final pH7.7 at 0°C, containing 3mg of the proteolytic enzyme Nagarse (Serva Feinbiochemica G.m.b.H., Heidelberg, Germany), added to facilitate release of the mitochondria from the muscle fibrils. Frequent light homogenizations were made during 10min of digestion at 0°C. The resulting suspension was then filtered through washed cheesecloth and rapidly diluted to 70ml with the above preparation medium supplemented with bovine serum albumin (2mg/ml). The suspension was then centrifuged at 8700*g* for 3min. The supernatant medium was decanted and an upper lighter-coloured layer removed. The deep-red pellet that remained was resuspended and made up to 40ml with preparation medium. The centrifugation was then repeated and the final pellet resuspended to give a volume of 2ml. The yield was 30–40mg of protein. Preparations made without the proteolytic enzyme yielded only a very small amount of mitochondrial protein, and were used only to check the results of experiments on the stimulation of respiration by Ca^{2+}.

Oxygen consumption was followed by using an oxygen electrode, essentially as described by Chappell (1961). The cell was entirely closed during the measurements, except for an aperture just large enough to admit a microlitre syringe. The temperature was controlled at 25°C throughout.

Light-scattering of mitochondrial suspensions was followed by monitoring the E_{700} in a recording Beckman–Gilford spectrophotometer.

Acetyl-L-carnitine was synthesized as described by Fraenkel & Friedman (1957); palmitoyl-L-carnitine was a gift from Dr M. D. Lane.

State 3 and state 4 respiration are respiration in the presence of ADP and after its phosphorylation, respectively, as defined by Chance & Williams (1955).

RESULTS AND DISCUSSION

Oxidation of pyruvate and D-*glycerol* 1-*phosphate.* Table 1 shows that the rate of D-glycerol 1-phosphate oxidation by cicada mitochondria was as high as any previously described for other insect mitochondria (Chance & Sacktor, 1958; Van den Bergh & Slater, 1962; Klingenberg & Bücher, 1959; Stevenson, 1968*a*). The rate of oxygen consumption with pyruvate as substrate was somewhat lower. When D-glycerol 1-phosphate oxidation was taking place at a maximal rate with the dehydrogenase essentially saturated with 6.6mM-DL-glycerol phosphate and 0.5μM-Ca^{2+}, an addition of pyruvate resulted in an increase in respiratory rate considerably less than the rate of oxygen uptake with pyruvate alone. Such non-additivity is somewhat puzzling, as the operation of the D-glycerol 1-phosphate shuttle (Zebe, Delbrück & Bücher, 1957; Bücher & Klingenberg, 1958; Sacktor & Dick, 1962) for the transfer of reducing power into the mitochondrion would demand that pyruvate and D-glycerol 1-phosphate be utilized at the same rate as they are produced, i.e. that the total rate of oxygen consumption with pyruvate must be five times that with D-glycerol 1-phosphate, and that these rates should summate. Such a summation is found with flight-muscle mitochondria from the blowfly (Hansford & Sacktor, 1971). The answer to the paradox may lie in our ignorance of the concentration of D-glycerol 1-phosphate present *in vivo* in the cicada; it is possible that the dehydrogenase is never more than partially saturated, even in the presence of Ca^{2+}.

Table 1. *Rates of oxidation of* D-*glycerol* 1-*phosphate and pyruvate*

Mitochondria (0.7–1.5mg of protein) were added to a system comprising 0.12M-KCl, 10mM-potassium HEPES [2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid] buffer (pH7.1), 10mM-tris phosphate (pH7.1), and 1mg of bovine serum albumin/ml. DL-Glycerol phosphate was added to give the concentrations indicated; sodium pyruvate was added to 2.0mM. In addition, a calcium–EGTA [ethanedioxybis(ethylamine)tetra-acetate] buffer stabilizing 0.5μM-Ca^{2+} was present at an EGTA concentration of 1mM when DL-glycerol phosphate was a substrate. Malate was present at 3.3mM when the substrate was pyruvate. ADP was added to 0.67mM 2min after the mitochondria when the substrate was pyruvate–malate, to allow 'sparking' of pyruvate oxidation by oxaloacetate. The final volume was 3ml and the temperature was 25°C.

	Rates of oxygen uptake (μg-atoms of O_2/min per mg of protein)		
Substrate Preparation no. ...	1	2	3
Pyruvate	0.57	0.53	0.43
3.3mM-DL-Glycerol phosphate	—	0.68	0.66
Pyruvate+3.3mM-DL-glycerol phosphate	—	1.10	0.74
6.6mM-DL-Glycerol phosphate	0.94	0.83	—
Pyruvate+6.6mM-DL-glycerol phosphate	0.91	1.08	—

Isolated cicada mitochondria show tightly coupled respiration. Thus, the mean respiratory-control ratio obtained with pyruvate was 12 (five preparations) and that with D-glycerol 1-phosphate 3.4. The best values obtained were 20 and 4.6 respectively, with P/O ratios of 2.9 and 1.7. Typical polarographic traces are shown in Fig. 1. There is no discrepancy between the respiratory-control ratios with these two substrates, such as is found with mitochondrial preparations from flies and which has been attributed to the presence of some broken mitochondria (Chappell & Hansford, 1969). High acceptor control ratios with D-glycerol 1-

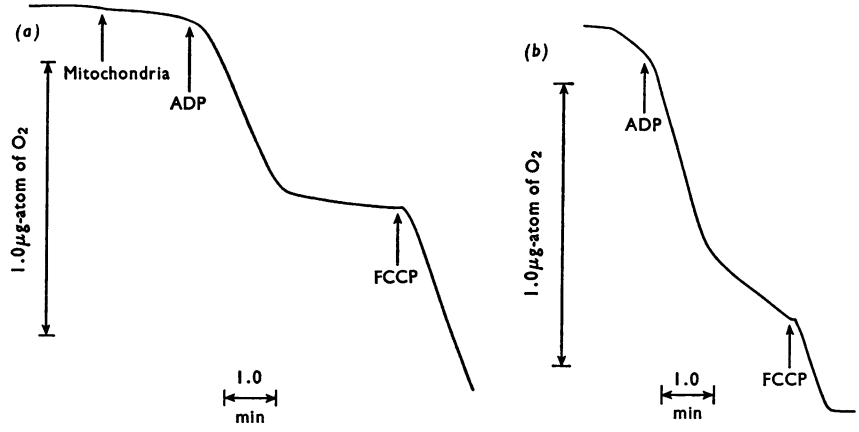

Fig. 1. Comparison of the respiratory control obtained with (*a*) pyruvate and (*b*) DL-glycerol phosphate as substrates. Oxygen consumption was measured at 25°C in a system comprising 0.12M-KCl, 10mM-potassium HEPES buffer (pH7.1) and 1mg of bovine serum albumin/ml. In (*a*) the substrate was 3.3mM-pyruvate and 3.3mM-malate. The phosphate concentration was 10mM. In (*b*), the substrate was 6.7mM-DL-glycerol phosphate in the presence of 1mM-EGTA buffering Ca^{2+} at 0.5μM; the phosphate concentration was 3.3mM. Approx. 2.5 and 1.5μmol of ADP was added in (*a*) and (*b*) respectively. Where indicated, trifluoromethoxycarbonyl cyanide phenylhydrazone (FCCP) was added, to 3μM. The total volume was 3ml, and the amount of mitochondrial protein 0.73mg. The preparation was made on 16 June 1970.

phosphate as substrate are uncommon in preparations of insect mitochondria (Chance & Sacktor, 1958; Gregg, Heisler & Remmert, 1960; Van den Bergh & Slater, 1962; Stevenson, 1968*b*); but cf. Klingenberg & Bücher, 1961; Hansford & Chappell, (1967).

The finding that very low concentrations of Ca^{2+} activate the mitochondrial glycerol phosphate dehydrogenase (EC 1.1.99.5) of blowfly flight muscle (Hansford & Chappell, 1967) has been subsequently extended to insects from a variety of orders (Donnellan & Beechey, 1969; Klingenberg & Buchholz, 1970). Here it is extended to another (Fig. 2). Although the concentration of Ca^{2+} required for maximal activation (0.5μM-Ca^{2+}) is very much the same, cicada flight-muscle mitochondria differ from those of the blowfly in that an excess of Ca^{2+} leads to an inhibition. This suggested that these mitochondria might, unlike those from the blowfly (Carafoli, Hansford, Sacktor & Lehninger, 1971) accumulate Ca^{2+} at micromolar concentrations in the medium and subsequently become damaged by extensive deposition of calcium phosphate. This possibility is discussed further below.

The addition of pyruvate alone to these mitochondria usually failed to elicit an appreciable oxygen uptake, even when the preparation was fresh. Addition of proline facilitated a high rate of pyruvate oxidation (Table 2), as first described for the blowfly by Childress & Sacktor (1966). Notably, exogenous 2-oxoglutarate did not [and this is discussed later in terms of an obligatory antiport of 2-oxoglutarate and malate across the membrane, in the terminology of Mitchell (1967)], nor did

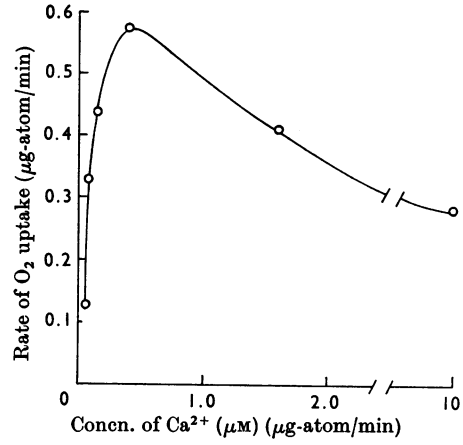

Fig. 2. Dependence of state 3 oxidation of D-glycerol 1-phosphate on the concentration of free Ca^{2+} present. Rates of oxygen uptake were measured in a medium containing 0.12M-KCl, 10mM-potassium HEPES buffer, 7.6mM-tris phosphate, 2.75mM-DL-glycerol phosphate, 1mg of bovine serum albumin/ml and the concentration of Ca^{2+} indicated, stabilized by the appropriate Ca^{2+} buffer, present at 1mM-EGTA. The pH of each incubation mixture was 7.1. The respiratory rate shown was achieved in the presence of 1.7mM-ADP. The amount of mitochondrial protein added was 0.95mg, in a total of 3ml.

the addition of pyruvate, ATP and bicarbonate, implying the absence of pyruvate carboxylase activity, which is present in blowfly muscle (Hansford & Sacktor, 1970). The total failure of succinate to 'spark' pyruvate oxidation and the slowness with which malate acted suggests that the dicarboxylate anion permease (Chappell & Haarhoff, 1967) is lacking or of very low activity in the cicada flight-muscle mitochondria, as described below.

The total rate of oxygen consumption obtained in the presence of pyruvate plus malate was found to be markedly enhanced by high phosphate concentrations (Table 3). This is reminiscent of the effect first seen with fly flight muscle by Van den Bergh & Slater (1962) and later attributed (Hansford & Chappell, 1968) to an allosteric stimulation of the NAD-linked isocitrate dehydrogenase (EC 1.1.1.41). A difference is that with the cicada mitochondria the maximum rate is apparently achieved at a rather lower concentration of phosphate. Table 3 shows that only the state 3 rate is enhanced in the presence of phosphate; the state 4 rate even seems to decline slightly, to yield an increase in the respiratory control ratio at the higher phosphate concentrations. If trifluoromethoxycarbonyl cyanide phenylhydrazone was added during state 4 respiration, then an essentially maximal rate was achieved regardless of phosphate concentration. Since this was not seen in the absence of a prior cycle of ADP phosphorylation, it may be that hydrolysis of ATP in the presence of the uncoupler yields a high intramitochondrial phosphate or ADP concentration in the immediate vicinity of the rate-limiting dehydrogenase and that this is the mechanism of the activation. In this case, a compartmentation over and above a simple distinction between matrix and intermembrane space would have to be invoked, because of the unlikelihood that concentration gradients of the activating ions across the mitochondrial membrane could be maintained in the uncoupled state.

Energy-linked uptake of Ca^{2+} *and its effect on oxidation of* D-*glycerol* 1-*phosphate and pyruvate.* When calcium–ethanedioxybis(ethylamine)tetra-acetate buffers, designed to maintain constant concentrations of free Ca^{2+}, were added to mitochondria phosphorylating ADP with pyruvate as substrate, a marked time-dependent inhibition became apparent (Fig. 3). It is best explained by assuming that these mitochondria accumulate Ca^{2+} from the medium, even while phosphorylating. This is reminiscent of the behaviour of mitochondria from mammalian tissues (Drahota, Carafoli, Rossi

Table 2. *'Sparking' of pyruvate oxidation*

The medium consisted of 0.12M-KCl, 10mM-potassium HEPES buffer (pH7.1), 10mM-tris phosphate (pH7.1), 1mg of bovine serum albumin/ml and 0.33mM-ADP. The substrates shown were added last. The volume was 3ml and the temperature 25°C.

Substrate addition	Maximum rate of oxygen uptake (μg-atoms of O_2/min per mg of protein)	Time required to attain maximum rate (min)
1. 2mM-Pyruvate	0.013	
1mM-ATP, 2mM-$KHCO_3$	0.013	—
6.6mM-Proline	0.350	1.3
2. 3.3mM-Succinate	0.052	
2mM-Pyruvate	0.052	
3.3mM-Malate	0.356	1.4
3. 6.6mM-Proline	0.065	
2mM-Pyruvate	0.540	0.5
4. 2mM-Pyruvate, 4mM-2-oxoglutarate	0.024	
0.84mM-Aspartate	0.360	1.5
5. 2mM-Pyruvate	0.013	
10mM-DL-Carnitine	0.019	

Table 3. *Effect of phosphate concentration on the oxidation of pyruvate*

Mitochondria (1.0–1.5mg of protein) were added to a medium comprising 0.12M-KCl, 10mM-potassium HEPES buffer (pH7.1), 2mM-sodium pyruvate, 3.3mM-tris malate, 1.5mg of bovine serum albumin/ml and the concentration of tris phosphate (pH7.1) indicated. ADP (2.4μmol) was added after 2min in each case. The total volume of the system was 3.0ml, and the temperature 25°C. Where indicated, trifluoromethoxycarbonyl cyanide phenylhydrazone (FCCP) was added to 3μM after the completion of the ADP cycle.

Concn. of phosphate (mM)	Rate of oxygen uptake (μg-atoms of O_2/min per mg of protein)		Respiratory control ratio	FCCP-stimulated respiration (μg-atoms of O_2/min per mg of protein)
	State 3	State 4		
2.0	0.162	0.031	5.3	0.508
4.1	0.258	0.037	6.9	0.552
6.7	0.334	0.030	11.1	—
10.1	0.530	0.026	20.4	—
19.7	0.580	0.025	23.6	0.625

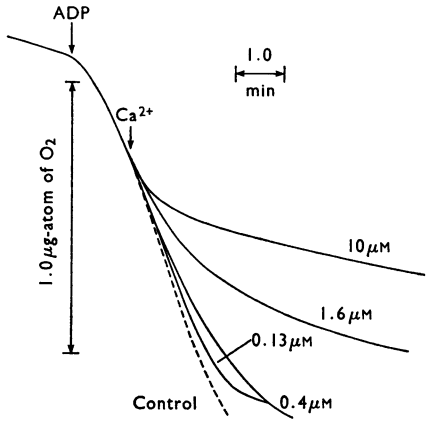

Fig. 3. Inhibition of state 3 pyruvate oxidation on addition of Ca^{2+} buffers. At the point indicated, Ca^{2+} buffers were added to a suspension of mitochondria (0.95mg of protein in 3ml) oxidizing 2.0mM-pyruvate, 3.3mM-malate in a medium of 0.12M-KCl and 10mM-potassium HEPES buffer (pH7.1). The amount of ADP added (5μmol) was enough to assure an essentially linear rate until the anaerobic point, as shown in the control curve. The free Ca^{2+} concentration of each incubation is shown next to the relevant curve.

& Lehninger, 1965). During the period of inhibition it seems likely that the NAD-linked isocitrate dehydrogenase becomes rate-limiting, in accord with a report of its Ca^{2+}-sensitivity (Vaughan & Newsholme, 1969). An alternative explanation, that the mitochondria were swelling and losing soluble enzymes and cofactors, appears less likely in that the curved phase of the traces shown was followed by a linear phase, in which the rate was a function of the concentration of Ca^{2+} present. That such a non-specific effect must contribute to some extent, however, is apparent from the relatively slight inhibition of coupled D-glycerol 1-phosphate oxidation that occurs at these Ca^{2+} concentrations (Fig. 2). In accord with the idea of an energy-linked uptake of Ca^{2+}, additions of up to 100nmol of calcium chloride/mg of protein, in the presence of 10mM-tris phosphate and 1mM-ATP, gave cycles of enhanced oxygen uptake, as described for mammalian mitochondria by Rossi & Lehninger (1964). The rate elicited was 180–210% of the state 4 rate, which is rather low relative to that achieved in the presence of ADP. The Ca^{2+}/O activation ratio appeared to be close to the values accepted for mitochondria from mammalian sources (see Lehninger, Carafoli & Rossi, 1967, for a review). Under the conditions described, subsequent addition of ADP resulted in a normal cycle. However, a series of successive additions of 100nmol of calcium chloride/mg of protein resulted in an inhibited state; presumably the mitochondria had accumulated large amounts of Ca^{2+}. In this connexion it is noted that addition of a calcium-containing buffer in which the Ca^{2+} concentration is 10μM during state 4 pyruvate oxidation resulted in a stimulation in rate as great as that seen on addition of calcium chloride at 100nmol/mg of protein. These results suggest that the cicada flight-muscle mitochondrion possesses a Ca^{2+}-uptake system with a high affinity but of low activity. The high affinity is indicated by the fact that these mitochondria will accumulate Ca^{2+} from a concentration of only 1.0μM; the low activity is suggested by the result that additions of calcium chloride give a lower rate of oxygen consumption than additions of ADP. Blowfly flight-muscle mitochondria, on the other hand, show no enhancement of oxygen uptake on addition of calcium chloride during oxidation of either pyruvate or D-glycerol 1-phosphate (Carafoli *et al.* 1971); moreover, pyruvate oxidation is totally uninhibited by Ca^{2+} buffered at 10μM. This has been equated with the lack of a Ca^{2+}-carrier in these mitochondria (Carafoli *et al.* 1971). It is suggested that the difference in response to Ca^{2+} of these two types of mitochondria reflects the presence or absence of a Ca^{2+} 'permease' and is related in some way to the different physiology of synchronous and asynchronous flight muscle.

*Oxidation of acetyl-*L*-carnitine and palmitoyl-*L*-carnitine.* The rates of oxidation of palmitoyl-L-carnitine and different concentrations of acetyl-L-carnitine are shown in Table 4. The affinity for palmitoyl-L-carnitine appeared to be very high, as addition of 20nmol of the substrate gave a rate quite linear until it was nearly exhausted. It was not possible to exceed this rate by using higher concentrations of substrate and various amounts of bovine serum albumin. The oxidation of acetyl-L-carnitine, by contrast, was dependent on rather high concentrations of the substrate. No oxygen uptake was obtained on addition of potassium stearate, under the conditions described by Stevenson (1968*a*). It would thus seem that of the insects so far described the cicada most resembles the locust in the details of fatty acid oxidation (Beenakkers, 1963; Bode & Klingenberg, 1965).

Addition of DL-carnitine to mitochondria in the presence of pyruvate failed to stimulate oxygen consumption (Table 2). Such an experiment leads to a considerable oxygen uptake with blowfly flight-muscle mitochondria, in which pyruvate oxidation is limited by lack of tricarboxylic acid-cycle intermediates (R. G. Hansford & B. Sacktor, unpublished work). The failure of carnitine to stimulate pyruvate oxidation by removal of the acetyl moiety is unexpected, in view of the rate of oxidation achieved with acetyl-L-carnitine (Table 4).

Table 4. *Rates of oxidation of acetyl-L-carnitine, palmitoyl-L-carnitine, glutamate, proline and tricarboxylic acid-cycle intermediates*

Mitochondria (1–3 mg of protein) were added to a system comprising 0.12 M-KCl, 10 mM-potassium HEPES buffer (pH 7.1) and the concentrations of substrates and phosphate indicated. Also, 1 mg of bovine serum albumin/ml was present throughout except in the incubation mixtures containing palmitoyl-L-carnitine. The rates indicated were obtained in the presence of ADP (0.3–0.6 mM). The total volume was 3.0 ml and the temperature 25°C.

Date of mitochondrial preparation	Substrate and concentration	Phosphate concentration (mM)	Rate of O_2 uptake (μg-atoms/min per mg of protein)
1. 12 June 1970	6 μM-Palmitoyl-L-carnitine + 3.3 mM-malate	10	0.069
	2.3 mM-Acetyl-L-carnitine + 3.3 mM-malate	10	0.046
	4.6 mM-Acetyl-L-carnitine + 3.3 mM-malate	10	0.061
	9.2 mM-Acetyl-L-carnitine + 3.3 mM-malate	10	0.078
	3.3 mM-Malate	10	0.010
2. 15 June	8 mM-Glutamate	2	0.133
	8 mM-Glutamate	8.6	0.138
	8 mM-Glutamate + 3.3 mM-malate	10	0.146
	4.2 mM-2-Oxoglutarate	10	0.017
	4.2 mM-2-Oxoglutarate + 1.7 mM-malate	10	0.137
	4.2 mM-2-Oxoglutarate + 1.7 mM-malate	2	0.071
3. 23 June	4.2 mM-2-Oxoglutarate	10	0.064
	4.2 mM-2-Oxoglutarate + 3.3 mM-malate	10	0.188
	4.2 mM-2-Oxoglutarate + 3.3 mM-maleate	10	0.182
	4.2 mM-2-Oxoglutarate + 3.3 mM-malonate	10	0.123
	2.6 mM-Citrate + 3.3 mM-malate	10	0.010
	3.3 mM-Malate	10	0.010
	6.7 mM-Glutamate	1.7	0.060
	6.7 mM-Glutamate + 4 mM-arsenite	1.7	0.060
4. 24 June	10 mM-Glutamate	10	0.045
	10 mM-Glutamate + 4.2 mM-2-oxoglutarate	10	0.164
	4.2 mM-2-Oxoglutarate + 3.3 mM-malate	10	0.094
	4.2 mM-2-Oxoglutarate + 3.3 mM-malate + 10 mM-glutamate	10	0.149
	3.3 mM-Malate	10	0.009
	5 mM-Proline	10	0.020
	10 mM-Proline	10	0.020
	8 mM-Succinate	3.3	0.015
	2 mM-Pyruvate + 3.3 mM-malate	10	0.174
	6.6 mM-DL-Glycerol phosphate + 0.5 μM-Ca^{2+}	3.3	0.264

It must be questioned whether the rates of oxidation of palmitoyl-L-carnitine described here could contribute significantly to the total provision of energy during flight. The possibility that this oxidation is due to the presence of mitochondria from another tissue of the cicada was considered, but was thought unlikely in that the percentage contamination would have to be very high to account for these rates. No heterogeneity of size was seen in the electron microscope (D. Beck, unpublished work).

Oxidation of tricarboxylic acid-cycle intermediates and amino acids. There was no oxidation of added citrate (Table 4) with or without malate to facilitate its entrance (Chappell & Haarhoff, 1967). Addition of succinate, in the presence or absence of rotenone, gave rise to a low rate of oxygen uptake, which was not enhanced by further additions of succinate or diminished by addition of phosphate (cf. Tulp & Van Dam, 1969). Addition of an equimolar amount of malonate gave a large and immediate inhibition, whereas addition of the same amount of malonate to mitochondria oxidizing pyruvate gave a relatively slight inhibition, which moreover did not become apparent until 1 min had elapsed. On these grounds it is suggested that oxidation of external succinate may be due to variable amounts of damaged mitochondria in the preparation. It was not stimulated by ADP, in keeping with the fact that it was below the state-4 rate found with other substrates. In their failure to oxidize citrate and succinate these mitochondria resemble those from the fly (Van den Bergh & Slater, 1962; but see Tulp & Van Dam, 1969). They

differ, however, in oxidizing glutamate and 2-oxoglutarate very competently and with a high degree of coupling. In this respect, they resemble no other preparation of flight-muscle mitochondria so far described. The glutamate oxidation (Table 4) was insensitive to arsenite and was not stimulated by high concentrations of phosphate, as 2-oxoglutarate oxidation was. This suggests that 2-oxoglutarate normally leaves the mitochondrion during glutamate oxidation. The oxidation of 2-oxoglutarate was greatly stimulated by malate, malonate or maleate. This probably represents a facilitation of the penetration of 2-oxoglutarate as described by Meijer & Tager (1966) and Chappell, Henderson, McGivan & Robinson (1968). The fact that the rate of oxidation of added 2-oxoglutarate finally achieved in the presence of malonate was lower than that found with malate (Table 4) may reflect a gradual inhibition of succinate dehydrogenase by malonate, rather than a differential effect on the permease. At first sight the rapid oxidation of 2-oxoglutarate and the poor oxidation of succinate and malate are contradictory, in that 2-oxoglutarate traverses the mitochondrial membrane in antiport with malate (Papa, D'Aloya, Meijer, Tager & Quagliariello, 1969). However, only the initial phase of 2-oxoglutarate oxidation need be limited by the relative impermeability to malate, as the product of the subsequent oxidation of 2-oxoglutarate is probably malate. In fact, the activation by malate or maleate is slow (Fig. 4); there is a lag of some 30s before the rate of 2-oxoglutarate oxidation becomes maximal. When malonate is the activator, this lag is longer. Fig. 4 also shows that the oxidation of 2-oxoglutarate is tightly coupled, which makes it even less likely that the permeability to this anion is an artifact. The enhanced rate of 2-oxoglutarate oxidation seen at the higher phosphate concentrations could reflect a requirement of phosphate by the substrate-level phosphorylation step or by isocitrate dehydrogenase, if there is some oxaloacetate decarboxylase activity. Although a similar effect of phosphate on the oxidation of pyruvate by blowfly mitochondria has been attributed to the isocitrate dehydrogenase (Hansford & Chappell, 1968), the results here with 2-oxoglutarate make the first enzyme perhaps a more likely candidate. Surprisingly, the rates of oxidation of glutamate and 2-oxoglutarate are additive (Table 4). This result is easily explicable only if 2-oxoglutarate leaves the mitochondrion as glutamate is oxidized.

Proline is only poorly oxidized compared with the corresponding activity in fly flight-muscle mitochondria (Sacktor & Childress, 1967), grasshopper flight muscle (Brosemer & Veerabhadrappa, 1965), and tsetse-fly flight muscle (Bursell, 1963). Nevertheless, this rate is adequate to provide the oxaloacetate necessary for maximal pyruvate oxidation (Table 2).

Inhibition of 2-oxoglutarate oxidation by alanine. Van den Bergh (1964) described a relatively high rate of oxidation of a mixture of 2-oxoglutarate and alanine by fly flight-muscle mitochondria, although the oxygen uptake with each substrate alone was slight. This he attributed to a vectorial transaminase, yielding intramitochondrial pyruvate. The same experiment with cicada flight-muscle mitochondria showed that oxidation of 2-oxoglutarate is inhibited by alanine (Fig. 5). There was

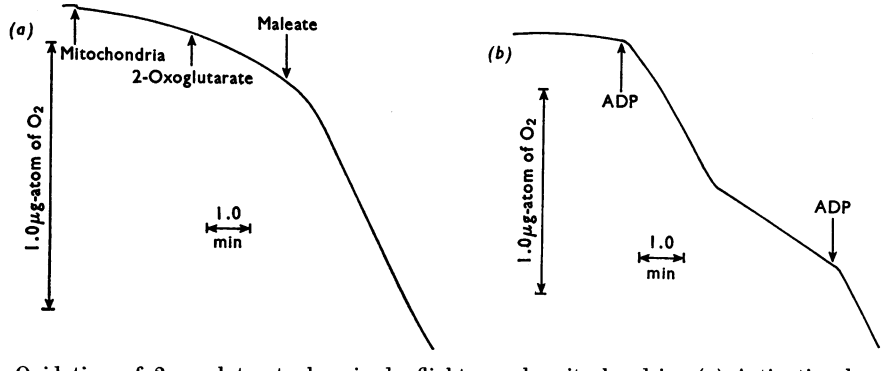

Fig. 4. Oxidation of 2-oxoglutarate by cicada flight-muscle mitochondria. (*a*) Activation by maleate. Mitochondrial protein (1.46mg) was added to a medium comprising 0.12M-KCl, 10mM-potassium HEPES buffer, 10mM-tris phosphate, 1mg of bovine serum albumin/ml and 1.5mM-ADP (pH7.1). Where indicated 12.5µmol of 2-oxoglutarate and 10µmol of maleate were added. The final volume was 3ml. (*b*) Respiratory control of 2-oxoglutarate oxidation. The medium comprised 0.12M-KCl, 10mM-potassium HEPES buffer (pH7.1), 10mM-tris phosphate (pH7.1), 1mg of bovine serum albumin/ml, 4.2mM-oxoglutarate and 3.3mM-malate. Where indicated, mitochondria (1.46mg of protein) and ADP (approx. 2µmol) were added.

no indication of any competition between alanine and 2-oxoglutarate, but, at a constant alanine concentration, the effect was more marked at low malate concentrations. Oxidation of glutamate was not inhibited by alanine, either in the presence or absence of arsenite. Two possible mechanisms were considered. In one it is assumed that alanine transaminates with 2-oxoglutarate. If this is the case, then neither of the products of the transamination can be accessible to their respective dehydrogenases, as both pyruvate and glutamate are good substrates. In addition, the differential effect at various malate concentrations is difficult to explain on this model. In the other model, alanine inhibits the entry of 2-oxoglutarate. In some ways this is the more attractive hypothesis. Thus, alanine does cause a slight diminution in swelling in ammonium 2-oxoglutarate solutions. Care should be taken in interpreting this, however, as transaminations may be occurring in these experiments, and the swelling is not pronounced in any case. Moreover, it is difficult to explain on this theory the finding that alanine inhibits state 3 and state 4 oxidation almost equally. If permeation were absolutely limiting, then the percentage inhibition of the state-3 rate would be the more severe. In addition, egress of 2-oxoglutarate is clearly not affected, as alanine does not inhibit glutamate oxidation in the presence of arsenite.

Discussion of the oxidation of tricarboxylic acid-cycle intermediates and glutamate. Some of the evidence presented here suggests that mitochondria from the flight muscle of the 17-year cicada are permeable to glutamate and 2-oxoglutarate, but not readily permeable to other members of the tricarboxylic acid cycle. In this context the low rate of malate oxidation observed may be insufficient to rule out permeability to malate, as there was no means of removing oxaloacetate in the experiments carried out. However, the slowness of the activation of pyruvate and 2-oxoglutarate oxidations by malate, the very slight swelling in iso-osmotic ammonium malate solutions, and the fact that in other tissues both malate and succinate are probably handled by the same carrier (Chappell & Haarhoff, 1967) and that succinate is poorly oxidized by these mitochondria, all point to lack of the dicarboxylate carrier. By contrast, 2-oxoglutarate oxidation is rapid and is activated by the same dicarboxylate anions, both physiological and non-physiological, that are required in mammalian systems (Chappell *et al.* 1968). This high rate of 2-oxoglutarate oxidation, yet low rate of penetration of exogenous succinate and malate, suggests that malate leaves the mitochondrion during 2-oxoglutarate oxidation. This is consistent with the ability of exogenous proline and the inability of exogenous 2-oxoglutarate to 'spark' the oxidation of pyruvate (Table 2). Although there is probably no requirement for malate to enter the mitochondrion during steady-state oxidation of 2-oxoglutarate, such a penetration is required for swelling in an ammonium 2-oxoglutarate solution. This arises because only those anions capable of yielding protons inside the mitochondrion will support swelling (Chappell & Crofts, 1966). This is only true for the 2-oxoglutarate anion if its movement is coupled via 2-oxoglutarate/malate, malate/phosphate and phosphate/OH^- antiporters to the movement of OH^- ion. Thus the low rate of swelling observed is consistent with the absence of the malate/phosphate antiporter that was postulated above. It has been suggested that permeabilities to 2-oxoglutarate, glutamate, aspartate, and malate are required for the operation of a shuttle present in mammalian tissues for moving reducing power between the mitochondrial and cytoplasmic compartments (Borst, 1962; Chappell *et al.* 1968). In this connexion it is noted that an active carrier system for malate (and succinate) has been found in every mammalian tissue investigated. Almost certainly, this shuttle is not needed in cicada flight muscle, as D-glycerol 1-phosphate is oxidized avidly, making it likely that the glycerol phosphate shuttle proposed by Zebe *et al.* (1957) is operative, as in flight muscle of other insects (Sacktor & Dick, 1962). The low malate permeability in this tissue, then, is entirely consistent with its primarily respiratory function. The rapid coupled oxidations of 2-oxoglutarate and glutamate clearly distinguish the cicada mitochondria from all other insect flight-muscle mitochondria that have been described.

What is the purpose of the specific permeability of the cicada mitochondria to glutamate and

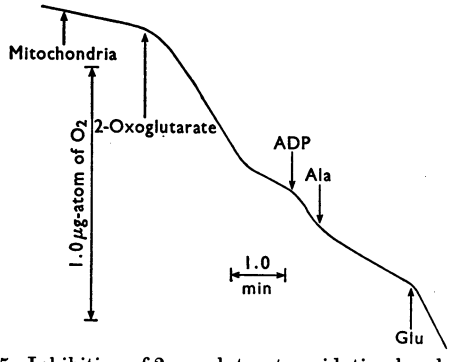

Fig. 5. Inhibition of 2-oxoglutarate oxidation by alanine. Mitochondria were added to a medium comprising 0.12 M-KCl, 10 mM-potassium HEPES, 10 mM-tris phosphate, 3.3 mM-malate, 0.57 mM-ADP and 1 mg of bovine serum albumin/ml. Where indicated 10 μmol of alanine (Ala) and 20 μmol of glutamate (Glu) were added, to a total volume of 3 ml. The pH was 7.1.

2-oxoglutarate? The first possibility is that the cicada may derive some energy for flight from the oxidation of glutamate or 2-oxoglutarate obtained from plant sap. It seems unlikely that they could be a prime source of energy, in that their rates of oxidation are low relative to those obtained with most flight-muscle mitochondria in the presence of D-glycerol 1-phosphate or pyruvate. Moreover, addition of glutamate to cicada mitochondria oxidizing pyruvate leads to no further increase in rate, possibly because the segment of the respiratory chain from NADH to ubiquinone is rate-limiting. However, this does not exclude a role in the sparing of pyruvate from oxidation. A second possibility is suggested by the fact that the highest rate of 2-oxoglutarate oxidation, relative to pyruvate and D-glycerol 1-phosphate oxidations, was obtained with the last preparation made (Table 4). Late in the life of the cicada there appears to be a diminution in the mass of flight muscle and a significant decrease in flight activity. Autolysis of flight muscle to support egg-laying is a known phenomenon in another insect of this superorder, the aphid (Johnson, 1959). It is therefore conceivable that at this time the mitochondria are assisting in the process of flight-muscle breakdown, and that the glutamate and 2-oxoglutarate permeabilities may be related to this. Each group of insects used in this particular study would have been heterogeneous with respect to age, as the life of the individual cicada is less than the three weeks during which the insects were available. It is probably fair to say, however, that the insects that gave the highest rate of 2-oxoglutarate oxidation (Table 4) were essentially all old.

Whatever the role of these activities, the cicada possesses a unique combination of substrate-anion permeabilities, a finding that tends to support the thesis that specific membrane carriers or permeases are genetically determined and functionally related to the physiology of the tissue.

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