Some Properties of Pyruvate and 2-Oxoglutarate Oxidation by Blowfly Flight-Muscle Mitochondria

By R. G. HANSFORD

Department of Biochemistry, University College, Cathays Park, Cardiff CF1 1XL, U.K.

(Received 12 October 1971)

1. High rates of state 3 pyruvate oxidation are dependent on high concentrations of inorganic phosphate and a predominance of ADP in the intramitochondrial pool of adenine nucleotides. The latter requirement is most marked at alkaline pH values, where ATP is profoundly inhibitory. 2. Addition of CaCl_2 during state 4, state 3 (Chance & Williams, 1955) or uncoupled pyruvate oxidation causes a marked inhibition in the rate of oxygen uptake when low concentrations of mitochondria are employed, but may lead to an enhancement of state 4 oxygen uptake when very high concentrations of mitochondria are used. 3. These properties are consistent with the kinetics of the NAD-linked isocitrate dehydrogenase (EC 1.1.1.41) from this tissue, which is activated by isocitrate, citrate, ADP, phosphate and H^+ ions, and inhibited by ATP, NADH and Ca^{2+} . 4. Studies of the redox state of NAD and cytochrome *c* show that addition of ADP during pyruvate oxidation causes a slight reduction, whereas addition during glycerol phosphate oxidation causes a 'classical' oxidation. Nevertheless, it is concluded that pyruvate oxidation is probably limited by the respiratory chain in state 4 and by the NAD-linked isocitrate dehydrogenase in state 3. 5. The oxidation of 2-oxoglutarate by swollen mitochondria is also stimulated by high concentrations of ADP and phosphate, and is not uncoupled by arsenate.

Fly flight-muscle mitochondria oxidize the products of glycolysis in this tissue, pyruvate and glycerol phosphate (Zebe & McShan, 1957; Chefurka, 1958), both with great activity and with a high degree of metabolic control (see Sacktor, 1970, for a review). Because of this the oxidation of these substances at the level of isolated mitochondria has attracted considerable attention. Van den Bergh (1964) first realized that the oxidation of pyruvate by housefly flight-muscle mitochondria was singular in that higher concentrations of phosphate and ADP were required for optimum activity than were needed for the process of phosphorylation. He attributed this requirement to succinyl-CoA synthetase (EC 6.2.1.4). Subsequently, Hansford & Chappell (1968) and Hansford (1968) showed that the NAD-linked isocitrate dehydrogenase (EC 1.1.1.41) from blowfly flight-muscle mitochondria in fact required both ADP and a high concentration of phosphate and suggested that this enzyme might be the locus of control. The present paper reports more experiments along those lines, designed specially to test whether the properties of pyruvate oxidation by isolated mitochondria under a variety of experimental conditions are always consistent with the kinetic properties of NAD-isocitrate dehydrogenase. In addition, experiments are reported in which the properties of 2-oxoglutarate oxidation are described and compared,

in an attempt to simplify the experimental system by avoiding the isocitrate dehydrogenase step.

Materials and Methods

Mitochondria were prepared from the flight muscles of *Calliphora erythrocephala* as described by Chappell & Hansford (1969).

Oxygen consumption was followed by using an oxygen electrode of the Clark pattern, essentially as described by Chappell (1961). The incubation cell was closed and the temperature was maintained at the values cited in the text by circulating water through an external water jacket.

Ferricyanide reduction was followed by using a Pye-Unicam SP.800 spectrophotometer at 420 nm and 1 ml cuvettes of light-path 1 cm. At the higher concentrations of mitochondria, it was necessary to use a blank also containing ferricyanide. The spectrophotometric method has the advantages of convenience and economy over the oxygen-electrode technique, in that a 1 ml system can be used. It may be more applicable to fly flight-muscle mitochondria than to mitochondria from other tissues, in that their high degree of coupling ensures good respiratory control in the absence of phosphorylation linked to electron flow between cytochrome *c* and oxygen, and

their high specific dehydrogenase and respiratory-chain activities ensure a reasonable rate of reduction at low protein concentrations, so that light-scattering is not obtrusive.

The enzymically active fraction used for investigating the properties of NAD-isocitrate dehydrogenase was prepared by suspending mitochondria at a concentration of 4mg/ml in 0.1M-potassium phosphate buffer, pH7.2, containing 2mM-ADP, 2mM-EDTA and 10mM-GSH, and subjecting them to four 15s bursts of ultrasonic vibration in an MSE 60W ultrasonic disintegrator. The composition of the medium is that used by Goebell & Klingenberg (1964). During this treatment the tube containing the mitochondria was surrounded by an NH_4Cl -ice freezing mixture at -10°C . Sonicated particles were sedimented by centrifugation at 100000g for 30min, and the resulting supernatant was used for assays. Rotenone and oligomycin were added to inhibit any NADH dehydrogenase and adenosine triphosphatase activities of mitochondrial origin, but such contamination was normally negligible. Enzyme activity was followed by recording the increase in E_{340} with time caused by NAD reduction.

The redox state of cytochrome *c* was monitored in a dual-wavelength spectrophotometer constructed by Professor J. B. Chappell, on the principle laid down by Chance & Legallais (1951), and that of NAD by using a fluorimeter attachment to the same instrument.

Atractyloside was a gift from Professor A. L. Lehninger. All other chemicals were obtained commercially and were of the highest purity available. Solutions, including those used for mitochondrial

preparations, were made up in water de-ionized with an Elgastat.

Results

'Priming' of pyruvate oxidation

Mitochondria prepared by using the proteolytic enzyme Nagarse have a low complement of endogenous intermediate, and pyruvate oxidation is sub-optimum in the absence of an added 'primer'. This may either be proline (Childress & Sacktor, 1966; Sacktor & Childress, 1967), which gives rise to intramitochondrial glutamate and hence oxaloacetate, or may be generated within the mitochondrion in the presence of pyruvate, ATP and HCO_3^- (Hansford, 1968). The different applicability of these two methods became apparent during this study. Thus, priming in the presence of pyruvate, ATP and HCO_3^- occurs only in ionic incubation media in which the cation is a penetrant, albeit a poor one (Table 1). It is less effective in iso-osmotic choline chloride than in potassium chloride, and less effective still in sucrose. However, the addition of proline to these media elicits a high rate of respiration, suggesting that no process other than the priming is impaired. The oxidation of proline effectively primes pyruvate oxidation in all media, with the qualification that the process is very slow in MgCl_2 . Osmotic studies (Hansford & Lehninger, 1972) have shown that the membrane of blowfly flight-muscle mitochondria has a finite permeability to cations when the organelles are respiring. The order of permeability is $\text{K}^+ > \text{choline}^+ > \text{Mg}^{2+}$. It may be the cation itself that is

Table 1. *Priming of pyruvate oxidation in ionic and non-ionic media*

Rates of oxygen consumption were measured with an oxygen electrode, at 25°C . Mitochondria (1.6mg of protein) were added to each of the media detailed below, giving a total volume of 4.2ml. In all incubations 2.5mM-pyruvate and 24mM-phosphate were present; 5mM-proline, 0.24mM-ATP and 2.5mM- NaHCO_3 were present where indicated. The pH was adjusted to 7.2 in each case. The first state 3 rate, (1), was elicited by the addition of ADP, to 0.5mM, 5min after the beginning of the incubation; the second, (2), by adding an equal amount of ADP after 3.5 min of state 4 respiration. Omission of ATP and bicarbonate did not decrease the rate obtained in the tris-citrate medium. The choline chloride experiments utilized a different mitochondrial preparation.

Medium	Rate of oxygen uptake ($\mu\text{g-atom/min per mg of protein}$)					
	Pyruvate, proline			Pyruvate, ATP, bicarbonate		
	State 3 (1)	State 3 (2)	State 4	State 3 (1)	State 3 (2)	State 4
0.13M-KCl	0.655	—	0.043	0.653	0.800	0.024
0.13M-NaCl	0.620	—	0.034	0.412	0.544	0.017
0.13M-Choline chloride	0.400	0.514	0.079	0.100	0.208	0.051
0.087M- MgCl_2	0.108	0.280	0.026	0.011	—	—
0.07M-Tris-citrate	—	—	—	0.408	0.370	0.019
0.26M-Sucrose	0.332	0.400	0.089	0.075	—	—

required for the carboxylation process, or it may be an accompanying anion, e.g. phosphate. Other studies (not shown) revealed that proline is an ineffective primer at acid pH values (below 6.5), ion whereas the carboxylation reaction becomes progressively less effective at alkaline pH values. The latter effect can be countered by using higher concentrations of HCO_3^- , and possibly reflects the fact that CO_2 is the species which penetrates the mitochondrion.

The fact that no primer was required in an iso-osmotic tris-citrate medium shows that the citrate ion can enter these mitochondria, provided that the concentration gradient is large enough. At more physiological concentrations it is a very poor penetrant (Van den Bergh & Slater, 1962). The lowered state 3* and elevated state 4 rate of pyruvate oxidation obtained in the experiment using a sucrose medium and proline to allow optimum priming (Table 1) is a

* Abbreviations: state 3 and 4 respiration, respiration during and after the phosphorylation of ADP respectively; EGTA, ethanedioxybis(ethylamine)tetra-acetate.

general finding. This diminished capacity for oxidative phosphorylation appears to correlate with a low intramitochondrial salt content, in that it becomes progressively more marked if a mitochondrial preparation is repeatedly washed. This has the effect of lowering the endogenous K^+ content (matrix) from approx. 100nequiv./mg of protein to as low as 10nequiv./mg. A detailed study of the effect of K^+ on oxidative phosphorylation in liver mitochondria (Gomez-Puyou *et al.*, 1970) suggested that a variety of cations were effective in restoring competent oxidative phosphorylation in K^+ -depleted mitochondria. The results presented here are similar, with the marked exception that phosphorylation is optimum in iso-osmotic NaCl, whereas high concentrations of Na^+ are grossly deleterious to liver mitochondria.

Effect of pH on pyruvate oxidation

A study of the effect of pH on pyruvate oxidation (Fig. 1) showed that there is a steep decline in activity with increasing alkalinity, such that at pH 7.6 the rate

Fig. 1. Dependence of pyruvate oxidation on pH

(a) Oxygen consumption was followed, at 25°C. The medium comprised 0.12M-KCl, 10mM-tris-HCl, 25mM-potassium phosphate, 5mM-sodium pyruvate, 6mM-proline, 0.25mM-ATP and 2mM- NaHCO_3 . ADP was added to 0.6mM 3min after the mitochondria, to elicit the rate of oxidation plotted. (b) Ferricyanide reduction was followed at 25°C. The incubation was begun by adding mitochondria to a medium comprising 70mM-tris-citrate, 21.5mM-tris-phosphate, 2mM-KCl, 1.3mM- NaCN , 2mM-ferricyanide, 1mg of bovine serum albumin/ml, 0.4mM-ADP, 15ng of valinomycin/ml and 1-3 μM -carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide, depending on the pH. The rate plotted is the highest achieved. The pH of each medium was adjusted before the experiment with HCl or tris base.

Fig. 2. Dependence of pyruvate oxidation (a) on [ADP] at pH 6.8 and 7.4 and (b) on pH at two different ADP/ATP ratios

(a) Oxygen consumption was measured at 25°C in a medium comprising 70 mM-tris-citrate, 2 mM-KCl, 25 mM-tris-phosphate, 2.5 mM-pyruvate, 1 mg of bovine serum albumin/ml, 0.25 μg of oligomycin/ml, 10 ng of valinomycin/ml and 2 or 4 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine, depending on the pH. The pH was either 6.8 or 7.4. At 1 min after the addition of mitochondria to this medium, adenine nucleotides were added to a total concentration of 1.2 mM, and the rate of oxygen consumption was recorded. ○, pH 6.8; □, pH 7.4. (b) The system was the same as that used in (a), except that the pH of each incubation was adjusted with HCl or tris base before the addition of mitochondria, to give the value indicated. ○, 50% ADP; □, 100% ADP.

achieved is not more than half the maximal rate. This is in contrast with the pH-dependence of glycerol phosphate oxidation, which shows a plateau from pH 6.6 to 8.3 (not shown) and is consistent with the progressive inhibition of one of the tricarboxylic acid-cycle enzymes with increasing pH. In the experiments corresponding to Fig. 1(a), the incubation medium included both proline and ATP-HCO₃⁻, and 3 min was allowed in each experiment for priming before the addition of ADP to elicit respiration. There are two possible objections to this procedure. First, the priming processes may be pH-dependent. This objection is largely overcome by the demonstration that the rates achieved after a longer preincubation period are essentially the same. Secondly, energy-linked accumulation of penetrant cation and phosphate during this preincubation phase will be dependent on pH, and will affect the internal buffering power of the mitochondrion. For this reason the experiments shown in Fig. 1(b) omitted the coupled preincubation period and involved the addition of mitochondria to an iso-osmotic tris-citrate medium containing 2 mM-KCl, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine and valinomycin. Basically, the shape of the curve in Fig. 1(b) resembles that in Fig. 1(a), although the loss of activity at pH values more alkaline than 7 is more marked.

Effect of ADP/ATP ratio and pH on pyruvate oxidation

Fig. 2(a) shows the results of an experiment in which the composition of a mixture of ADP and ATP was varied, at two different pH values, and the rate of pyruvate oxidation was measured. Respiration was stimulated by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine, and oligomycin was present before the addition of adenine nucleotides, to prevent the hydrolysis of ATP. Under these conditions, the composition of the internal pool of adenine nucleotides should reflect that of the exogenous nucleotide, as there is no membrane potential to favour the entry of ADP (Pfaff & Klingenberg, 1968). A sole proviso is that the size of the exchangeable pool is not known, as internal AMP (not measured) will not exchange (Pfaff & Klingenberg, 1968). Under these conditions it is apparent that high rates of respiration are much less dependent on a high intramitochondrial ADP concentration at pH 6.8 than at pH 7.4. When 100% of the internal exchangeable nucleotide is ADP, then respiration is less pH-sensitive. This is the condition that presumably would have obtained in the experiment in Fig. 1(b), in the presence of an uncoupling agent. The equilibrium of pH across the membrane was again of prime concern in these experiments. To this end, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine and valinomycin were present, and the

priming of pyruvate oxidation was accomplished by using an iso-osmotic citrate medium.

This dependence of the effect of adenine nucleotides on the pH suggested that the experiments of Fig. 1 should yield different results when repeated at different ADP/ATP values. This was found to be so (Fig. 2b). Thus when only 50% of the adenine nucleotide was ADP there was a considerable sharpening of the pH profile, with a more pronounced inhibition at alkaline pH values. The effect of phosphate on oxidations in different mixtures of adenine nucleotides was investigated (Fig. 3), as phosphate is known to stimulate state 3 pyruvate oxidation in the housefly (Van den Bergh, 1964) and blowfly (Hansford & Chappell, 1968) and it was thought that activation by H^+ and by phosphate might be cumulative. Phosphate activated respiration throughout the range of adenine nucleotide composition, but activation was most marked at pH 7.4, in the presence of 100% ADP. Studies at pH 6.9 and 7.4 with proline and pyruvate-ATP- HCO_3^- as primers gave essentially similar results (not shown). In a sense this is surprising, as these experiments required a coupled phase before the addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. Under these circumstances valinomycin cannot be used, as it

leads to a severe inhibition in rate throughout the range of K^+ concentrations (2–150 mM). For this reason the ionophore was not included. Presumably the lengthy time-scale of these experiments was enough still to allow essentially complete equilibration of H^+ across the membrane.

Effect of phosphate on pyruvate oxidation

ADP-stimulated pyruvate oxidation is optimum at phosphate concentrations in excess of 20 mM (Van den Bergh, 1964; Hansford, 1968). The 'phosphate swelling' of mammalian mitochondria described by other authors (e.g. Bartley & Enser, 1964; Harris & Van Dam, 1968) occurs in the sense that there is an uptake of phosphate and whatever cation is present, with a consequent expansion of the matrix compartment to about 2.5 μ l/mg of protein (Hansford & Lehninger, 1972). However, this is in no way deleterious to oxidative phosphorylation as respiratory-control ratios are maximal under these conditions. Further experiments showed that high concentrations of phosphate are only necessary under alkaline conditions. Fig. 4 shows a reciprocal plot of rates of ADP-stimulated respiration at pH 6.8 and 7.4, yielding K_m values for phosphate of 1.6 and 5.3 mM respectively.

Fig. 3. Dependence of pyruvate oxidation on [ADP] at pH 6.9 and 7.4, at 5 mM- and 25 mM-phosphate

Ferricyanide reduction was measured in the presence of 2 mM-KCN. Media were made up containing 70 mM-tris-citrate, 5 mM- or 25 mM-tris-phosphate, 2 mM-KCl, 1.6 mM-ferricyanide, 4 mM-pyruvate, 1 mg of bovine serum albumin/ml, 0.4 μ g of oligomycin/ml, of overall pH 6.9 or 7.4. At 2 min after the addition of mitochondria, ADP and ATP to total 2.5 mM and valinomycin (to 10 ng/ml) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (to 1.5 or 3 μ M) were added. $\circ$, 25 mM-phosphate, pH 6.9; $\square$, 5 mM-phosphate, pH 6.9; $\bullet$, 25 mM-phosphate, pH 7.4; $\blacksquare$, 5 mM-phosphate, pH 7.4.

Vol. 127

Fig. 4. Stimulation by phosphate of state 3 pyruvate oxidation at pH 6.8 and 7.4

Mitochondria (1.0 mg of protein) were added to a medium comprising 0.12 M-KCl, 10 mM-tris-HCl, 2 mM-pyruvate, 6 mM-proline, 5 mM- $NaHCO_3$, 0.25 mM-ATP, 0.6 mg of bovine serum albumin/ml and the concentration of phosphate indicated. The total volume was 4 ml and the temperature 25°C. The pH was adjusted to 6.8 or 7.4 with HCl or tris base, as appropriate. Then 3.5 min later, ADP was added, to 1 mM, and the rate of oxygen consumption measured. The inverse of this rate is plotted versus the inverse of the phosphate concentration. $\circ$, pH 6.8; $\square$, pH 7.4.

Fig. 5. Dependence of the rate of pyruvate oxidation on [pyruvate]

Mitochondria were added to a medium comprising 70mM-potassium citrate, 25mM-potassium phosphate, 1.6mM-ferricyanide, 1.6mM-potassium cyanide, 1mg of bovine serum albumin/ml and 1mM-ADP. The reduction of ferricyanide was initiated by the addition of pyruvate to give the concentrations indicated. The temperature was 25°C and the pH 7.1.

When carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine was used to stimulate respiration there was essentially no requirement for high concentrations of phosphate at either pH value, but such a requirement did become apparent at pH 7.7. These results may be explained either on the basis that H⁺ and phosphate are both activators of a limiting dehydrogenase, or that only phosphate is such an effector, and the intramitochondrial concentration of phosphate reflects the pH gradient across the mitochondrial membrane. In the presence of an artificially imposed pH gradient, alkaline within the matrix, phosphate would be concentrated within the mitochondrion (Palmieri *et al.*, 1970). It is difficult to distinguish between these two hypotheses.

Effect of pyruvate concentration on the rate of pyruvate oxidation

An iso-osmotic tris-citrate medium was again used to prime pyruvate oxidation. The pyruvate-proline system was considered unsuitable as the pyruvate participates in transamination, and the pyruvate-ATP-HCO₃⁻ system equally unsuitable, as the pyruvate is a presumed substrate for carboxylation. In either case, the *K_m* determined experimentally might reflect a process other than oxidation. Fig. 5 shows that the *K_m* for pyruvate is approx. 0.3mM

Table 2. Effect of temperature on pyruvate oxidation at two different phosphate concentrations

Oxygen consumption was measured at the temperatures indicated. The medium comprised: 0.12M-KCl, 10mM-tris-HCl, 5mM- or 25mM-tris-phosphate, 2.5mM-pyruvate, 2.5mM-NaHCO₃, 0.25mM-ATP, 1mg of bovine serum albumin/ml, and the pH was 7.4. After 3.5min of preincubation, ADP was added to 0.6mM, and the rate measured. For each 1.64mg of mitochondrial protein was used, and the total volume was 4ml.

Temperature (°C)	Phosphate concn. (mM)	Rates of oxygen uptake (μg-atom/min per mg of protein)	
		State 3	State 4
15.0	25	0.220	0.017
15.0	5	0.170	0.022
19.8	25	0.450	0.015
19.8	5	0.268	0.042
24.5	25	0.556	0.044
24.5	5	0.354	0.070
30.0	25	0.754	0.064
30.0	5	0.405	0.081

under these conditions. This is necessarily an overestimate in that maximal rates were not achieved immediately on addition of ADP, and some small proportion of the pyruvate would have been oxidized in the interim. This *K_m* value is similar to the concentration of pyruvate found by assay of blowfly thoraces (Sacktor & Wormser-Shavit, 1966) and rather higher than the *K_m* of purified enzyme complex from other sources (e.g. 2×10^{-5} M for the pig heart enzyme; Scriba & Holzer, 1961). It is possible that this relatively low affinity is a consequence of a limiting permeability to pyruvate. In keeping with this is the finding that fly flight-muscle mitochondria swell extremely slowly in iso-osmotic ammonium pyruvate solutions (Hansford, 1968; Van den Bergh, 1967) or in iso-osmotic sodium pyruvate in the presence of glycerol phosphate as a source of energy. The concept of free permeation of pyruvic acid (Klingenberg, 1970) may not be applicable to mitochondria from this tissue.

Effect of temperature on the rate of oxidation of pyruvate or pyruvate plus glycerol phosphate

Temperature is an important experimental variable in work involving blowfly flight-muscle mitochondria in that blowflies are capable of flight over a range of temperatures. Table 2 shows an experiment designed to test whether one characteristic of pyruvate

oxidation at 25°C, the activation by phosphate, was obtained at higher and lower temperatures. This was so, although the magnitude of the stimulation by phosphate at 25°C shown by this preparation was somewhat smaller than usual. A second experiment involving temperature is shown in Table 3. This concerned the simultaneous oxidation of pyruvate and glycerol phosphate, the end-products of glycolysis in this tissue. When both the substrates were present simultaneously the total rate of oxidation was 85% of the sum of the two rates obtained separately, and this partial summation applied throughout the temperature range studied. The high rate of oxidative phosphorylation ($>4.6 \mu\text{mol}$ of ATP/min per mg of protein at 30°C) observed when both of the products of glycolysis are available to these mitochondria is indeed noteworthy.

Effect of Ca^{2+} on pyruvate oxidation

Possible effects of Ca^{2+} on the functioning of the tricarboxylic acid cycle became of interest with the demonstration by Vaughan & Newsholme (1969) that Ca^{2+} at concentrations as low as $1 \mu\text{M}$ is inhibitory to NAD-isocitrate dehydrogenase from a variety of tissues. It was found that Ca^{2+} -EGTA buffers stabilizing $1 \mu\text{M}$ - Ca^{2+} had no effect on either state 3 or state 4 pyruvate oxidation, suggesting that at this concentration Ca^{2+} does not equilibrate across the mitochondrial inner membrane. This may be rationalized on the basis of the lack of a specific high-affinity carrier system for Ca^{2+} in these mitochondria (Carafoli *et al.*, 1971) and the lack of penetration of the $[\text{CaEGTA}]^{2-}$ chelate itself. Higher concentrations of added CaCl_2 may lead to a dramatic inhibition, a result briefly noted by Carafoli *et al.* (1971), or may lead to an activation of state 4 respiration, presumably by imposing an energy demand for cation uptake. The crucial factor seems

to be the concentration of mitochondrial protein used. Thus (Fig. 6) the same concentration of Ca^{2+} gave an inhibition when 1.3 mg of protein was used, and a stimulation when 5.2 mg was used. The explanation of this protein dependence is probably that at the higher concentrations the organelles sequester enough Ca^{2+} materially to lower the external Ca^{2+} concentration. If this is true, then a corollary is that a certain accumulation of Ca^{2+} is admissible before any inhibition of respiration sets in. Fig. 7 shows experiments in which low concentrations of mitochondrial protein were used, and CaCl_2 was added during uncoupled respiration, leading to a dramatic inhibition. At the moment of addition of CaCl_2 all of the ATP originally present should have been hydrolysed by the carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone-induced adenosine triphosphatase, so that 0.25 mM-ADP was present. It is unfortunately not possible to omit adenine nucleotides entirely, as very low rates are then obtained. The delayed onset of inhibition found in the presence of atractyloside suggests the intriguing possibility that the penetrant species is $[\text{CaADP}]^-$, although it is possible to construct other hypotheses.

Kinetics of NAD-isocitrate dehydrogenase from blowfly flight-muscle mitochondria

A limited kinetic study of this enzyme was undertaken, with a view to explaining aspects of the mitochondrial oxidation of pyruvate discussed in this paper. As with the enzyme from other animal tissues (Chen & Plaut, 1963; Goebell & Klingenberg, 1964), ADP has the effect of lowering the apparent K_m for isocitrate. ATP has a specific inhibitory effect, in addition to inhibiting by chelating Mg^{2+} , and thus there is a very pronounced dependency of rate on the composition of a mixture of ADP and ATP. This experiment (Fig. 8) was performed in an attempt to

Table 3. *Effect of temperature on the oxidation of pyruvate, glycerol phosphate and pyruvate plus glycerol phosphate*

In experiments involving pyruvate oxidation, mitochondria (1.9 mg of protein) were suspended in a medium comprising 0.12 M-KCl, 10 mM-tris-HCl, 10 mM-potassium phosphate, 2.5 mM-pyruvate, 0.25 mM-ATP, 2.5 mM- NaHCO_3 , 1 mg of bovine serum albumin/ml, of total volume 4 ml. After 8 min preincubation, a Ca^{2+} -EGTA buffer stabilizing $0.5 \mu\text{M}$ - Ca^{2+} was added to 1 mM and ADP to 1.25 mM. In incubations involving both substrates, DL-glycerol phosphate was also added, to 6 mM, at this time. The rate of oxygen consumption was recorded. Experiments involving glycerol phosphate only omitted the pyruvate and the preincubation period. The pH was 7.1 in all cases.

Temperature (°C)	Rate of oxygen uptake (μg -atoms/min per mg of protein)		
	Glycerol phosphate	Pyruvate	Glycerol phosphate plus pyruvate
15	0.33	0.33	0.54
25	0.64	0.91	1.31
30.5	0.89	1.29	1.83

Fig. 6. Oxygen-electrode traces showing the differing responses to Ca^{2+} obtained at different concentrations of mitochondrial protein

Mitochondria were suspended in 0.12M-KCl, 10mM-tris-Cl, 24mM-potassium phosphate, 5mM-pyruvate, 2.5mM- NaHCO_3 , 4.75mM ATP, 0.6mg of bovine serum albumin/ml, final pH7.2. The total volume was 4.2ml and the temperature 25°C. Where indicated by the arrow, CaCl_2 was added, to 2.4mM. The amount of mitochondrial protein used in each experiment was as follows: (a) 0.37 mg/ml; (b) 0.74 mg/ml; (c) 1.48 mg/ml; (d) 2.22 mg/ml. The solid line represents the trace obtained in the presence of Ca^{2+} , and the broken line the result of a parallel incubation in which no CaCl_2 was added.

Fig. 7. Inhibition of uncoupled pyruvate oxidation by Ca^{2+}

Mitochondria (approx. 0.6mg of protein) were added to a medium comprising 0.12M-KCl, 25mM-potassium phosphate, 2.5mM-pyruvate, 5mM-proline, 2.5mM- NaHCO_3 , 0.25mM-ATP, 0.6mg of bovine serum albumin/ml, final pH7.0. After 4 min carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was added to 1.8 μM , to elicit a high rate of oxygen consumption. Where indicated, CaCl_2 , to 0.25mM, was added. In addition, 0.3mg of atractyloside was added (ATRACT) 20s before the CaCl_2 . The total volume was 4ml.

mimic conditions *in vivo*, in that the mitochondrial pool of adenine nucleotides is fixed in size (Pfaff *et al.*, 1965) and varies in its state of phosphorylation, or energy charge (Pfaff & Klingenberg, 1968; Atkinson, 1968). The total concentration of adenine nucleotides used in these experiments was the content determined by Price & Lewis (1959) divided by the matrix space of these mitochondria, determined by Hansford & Lehninger (1972). An error is introduced in that no AMP is included in the mixture; however, it has no effect on the enzyme, and its concentration, in whole flight muscle, is low (Sacktor & Hurlbut, 1966). There is a requirement for a much higher percentage of ADP at pH7.4 than at pH6.9 to achieve the same rate. There is a striking resemblance between this plot, of results obtained at the enzyme level, and that in Fig. 2, where the experimental material was a suspension of mitochondria. The phosphate concentration in these two studies was deliberately matched, as it was found that phosphate acts as an activator of isocitrate dehydrogenase (Fig. 9), lowering the apparent K_m for isocitrate. Great care was taken in this latter study to ensure that the pH was the same at all phosphate concentrations, in view of the dramatic effect of pH. Ionic strength was also matched.

Fig. 8. Dependence of NAD-isocitrate dehydrogenase activity on the percentage of ADP in a mixture of ADP and ATP at pH 6.9 and 7.4

The enzymically active fraction was added to a medium comprising 0.15M-KCl-50mM-potassium 2-(N-2-hydroxyethylpiperazin-N'-yl)ethanesulphonate, 25mM-potassium phosphate, pH 6.9, or 0.15M-KCl-25mM-potassium phosphate, pH 7.4. These media were calculated to be of approximately equal ionic strength. In addition, each medium contained 0.61 mM-*threo*-D₃L-isocitrate, 2.1 mM-NAD, 10mM-MgCl₂, and ADP plus ATP totalling 5mM. The temperature was 25°C. ○, pH 6.9; □, pH 7.4.

Fig. 9. Activation of NAD-isocitrate dehydrogenase by phosphate

The phosphate concentrations indicated were achieved by mixing 0.1M-potassium phosphate, pH 7.2, and 0.15M-KCl-50mM-potassium 2-(N-2-hydroxyethylpiperazin-N'-yl)ethanesulphonate, pH 7.2, in different proportions. *threo*-D₃L-Isocitrate (0.51 mM), NAD (2.5 mM), ADP (4.5 mM) and MgCl₂ (10 mM) were also added. The reaction was initiated by adding 20 μl of sonicate supernatant to a 1.1 ml system. The temperature was 25°C.

Oxidation of 2-oxoglutarate

Certain properties of pyruvate oxidation, which in this tissue involves the classical operation of the tricarboxylic acid cycle with neither gain nor loss of cycle intermediates, have been described and have been tentatively attributed to NAD-isocitrate dehydrogenase. However, the demonstration of a rather similar nucleotide-dependence of proline oxidation (Hansford & Sacktor, 1970) might suggest control of a later reaction in the tricarboxylic acid cycle, probably that catalysed by 2-oxoglutarate dehydrogenase. With mitochondria from most tissues this hypothesis could easily be tested by experiments involving 2-oxoglutarate oxidation. This is not straightforward with fly flight-muscle mitochondria, however, as 2-oxoglutarate is a very poor penetrant of the mitochondrial inner membrane (Van den Bergh & Slater, 1962). Work by Azzi & Azzone (1967) and Brierley (1970) on mammalian mitochondria has shown that a non-specific permeability to anions appears at alkaline pH, and advantage was taken of this fact to obtain rapid 2-oxoglutarate oxidation (Table 4). A hypo-osmotic medium was used, as the membrane also becomes increasingly permeable to ions on being stretched (Hansford & Lehninger,

1972). It was found that the addition of MgCl₂ was essential for high rates of oxidation, although it was not necessary when working with undamaged mitochondria. Under these conditions, the consumption of oxygen involves both 2-oxoglutarate dehydrogenase and succinyl-CoA synthetase, as CoA has to be regenerated by the latter reaction, and any properties described could be the properties of either enzyme. Further oxidation of the succinate produced was inhibited by the addition of malonate. Table 4 shows that 2.5 mM-ADP gave an appreciably higher rate than 0.25 mM. In this connexion it is noted that though grossly swollen, these mitochondria retained an intrinsic impermeability to adenine nucleotide, as atractyloside still inhibited. Similarly there was a considerable stimulation by phosphate concentrations in excess of those normally required by succinyl-

CoA synthetases from animal sources (K_m 0.15–0.48 mM; Cha, 1969). Care was taken to use potassium phosphate and to add valinomycin, both so that the phosphate could enter freely, and so as not to increase the osmotic pressure of the (hypo-osmotic) medium. However, at least part of the effect of phosphate is that of providing ionic strength within the mitochondrion, in that a potassium acetate solution of the same ionic strength, in the presence of valinomycin, also gave some stimulation. In the presence of both activators the rate of oxygen consumption with 2-oxoglutarate as substrate (0.37 $\mu\text{g-atom}$ of O_2/min per mg of protein) was more than one-fifth of the maximum state 3 rate of pyruvate oxidation (0.94 $\mu\text{g-atom}$ of

O_2/min per mg of protein) and therefore enough to explain the flow rate through the tricarboxylic acid cycle. Arsenate, added in the absence of phosphate, gave very little stimulation of 2-oxoglutarate oxidation (Table 4).

Control at the dehydrogenase level versus that at the level of the respiratory chain

Discussion of the control properties of component enzymes of the tricarboxylic acid cycle should not obscure the fact that it is generally thought that mitochondrial oxidations are controlled at the level of the respiratory chain, via the interaction of ADP with the

Table 4. Oxidation of 2-oxoglutarate by swollen fly flight-muscle mitochondria at alkaline pH values

(a) Dependence on [ADP]. Mitochondria (2.3 mg of protein) were added to a medium comprising 1.25 mM-potassium phosphate, 3.2 mM-potassium 2-oxoglutarate, 1.25 mM-NAD, 0.08 mg of cytochrome *c*/ml, 5 mM-MgCl₂, 1 μM -carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and 6 ng of valinomycin/ml. The pH was adjusted to 7.8. Oxygen consumption was measured by using an oxygen electrode, at 25°C. The total volume was 4 ml. (b) Dependence on [phosphate]. The conditions were the same as given above, except that 2.5 mM-malonate was present and that [phosphate] and [ADP] were varied as shown. A different mitochondrial preparation was used.

(a)

Concn. of ADP (mM)	Rate of oxygen uptake ($\mu\text{g-atom}/\text{min}$ per mg of protein)
0.25	0.054
0.62	0.070
1.25	0.111
1.87	0.131
2.50	0.135
1.87 plus 0.18 mg of atractyloside/ml	0.049

(b)

Concn. of phosphate, arsenate or acetate (mM)	Rate of oxygen uptake ($\mu\text{g-atom}/\text{min}$ per mg)	
	At 0.61 mM-ADP	At 2.4 mM-ADP
Phosphate (1.2)	0.087	0.11
Phosphate (2.4)	0.12	0.17
Phosphate (6.1)	0.18	0.29
Phosphate (12.0)	0.23	—
Phosphate (23.8)	0.29	0.37
Arsonate (12.0)	0.064	—
Phosphate (1.2) + acetate (72)	0.138	—

Fig. 10. Response of NAD and cytochrome *c* to the addition of ADP during oxidation of (a) glycerol phosphate and (b) pyruvate

Reduced cytochrome *c* was measured by dual-wavelength spectrophotometry, by using the wavelength pair 550–540 nm. NADH was measured by fluorimetry. Mitochondria were suspended in 0.1 M-KCl–10 mM-tris–HCl–25 mM-potassium phosphate, pH 7.1. Medium (a) contained in addition 0.5 μM -Ca²⁺, stabilized by a Ca²⁺–EGTA buffer, of EGTA concentration 1 mM. Medium (b) contained 2 mM-pyruvate, 2 mM-NaHCO₃ and 1 mM-ATP. The total volume was 2.5 ml. DL-Glycerol phosphate and ADP were added as indicated. The temperature was 25°C. The two experiments employed different mitochondrial suspensions.

Table 5. *Effect of phosphate concentration and pH on state 4 pyruvate oxidation*

(a) Effect of phosphate. Mitochondria were incubated in a medium comprising 0.12M-KCl, 2.5mM-pyruvate, 0.25mM-ATP, 2.5mM-NaHCO₃, 0.6mg of bovine serum albumin/ml and the concentration of phosphate indicated. The pH was adjusted to 7.4. Oxygen consumption was measured, at 25°C. ADP was added to a concentration of 0.6mM 6min after the beginning of the experiment. The rate recorded is the rate of oxidation following phosphorylation of this ADP. (b) Effect of pH. The medium comprised 0.12M-KCl, 25mM-potassium phosphate, 2.5mM-pyruvate, 0.6mM-ATP, 2.5mM-NaHCO₃ and 1.2mg of bovine serum albumin/ml. The pH of each incubation was adjusted with HCl or tris base to give the value indicated before the addition of the mitochondria. The rate recorded is that achieved after the phosphorylation of 0.5mM-ADP. The mitochondrial suspensions used in the two experiments were different, but in each case a high concentration of mitochondria (approx. 3.7mg of protein in 4ml) was used to ensure adequate rates. The temperature was 25°C.

(a)	
Phosphate (mM)	Rate of oxygen consumption (μ g-atom/min per mg)
2.4	0.041
4.8	0.036
9.8	0.032
24.1	0.027
(b)	
pH	Rate of oxygen consumption (μ g-atom/min per mg)
6.53	0.051
6.80	0.052
7.00	0.041
7.24	0.037
7.51	0.022
7.80	0.016

adenosine triphosphatase system (Chance & Williams, 1955). Although control can be exerted at both levels, one must be absolutely limiting in the resting insect. To try to decide between these two possible loci of control, redox studies of respiratory-chain components were undertaken. It was found that whereas addition of ADP to mitochondria oxidizing glycerol phosphate resulted in an oxidation of NADH and cytochrome *c*, in the classical fashion (Chance & Williams, 1955), addition of ADP to mitochondria oxidizing pyruvate resulted in very little change in redox state of these components, even though a

20–30-fold facilitation of respiration was occurring. When a change was detectable it was in the converse direction, i.e. NAD and cytochrome *c* became more reduced on addition of ADP (Fig. 10). A change of this sign was observed especially when there was already ATP present in the medium. Clearly, ADP activates both the reduction and the oxidation of these components, to almost the same degree. When oligomycin was present, the addition of ADP resulted in a larger reduction (not shown); under these conditions activation of the respiratory chain is ruled out. This unusual response to ADP was noted by Hansford & Chappell (1968) and Hansford (1968), and its occurrence in housefly mitochondria was confirmed by Tulp & Van Berkel (1970).

Table 5 shows the response of state 4 pyruvate oxidation to pH and the slight diminution that is obtained with increasing phosphate concentration. These results are pertinent to the consideration of whether isocitrate dehydrogenase limits state 4 oxidation (see the Discussion section).

Discussion

Interpretation of the experiments involving variation of pH

The assumption behind the experiments of Figs. 1(b), 2(a) and 3 is that the pH in the region of the enzyme limiting pyruvate oxidation is the same as that of the medium, measured by the glass electrode. This involves equilibration of H⁺ ions across the inner mitochondrial membrane. In an attempt to achieve this, carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone and valinomycin were present in all of these studies. It was feared that under these conditions a pre-existing gradient in K⁺ concentration across the membrane might become converted into a pH gradient, but this does not seem to have interfered to any extent in that repetition of the experiment in Fig. 2(a) with 141mM-K⁺ instead of 2mM gave a similar, though not identical curve. The endogenous K⁺ present within these mitochondria varies with the preparation and is not known in this instance. There is no reason to expect an exact correspondence in the pH-dependence of ADP-stimulated and uncoupled pyruvate oxidation (Figs. 1a and 1b, respectively) as a transmembrane pH gradient, alkaline inside, probably exists in the former case (Mitchell & Moyle, 1969). This would be expected to move curve Fig. (1a) to lower pH values, relative to curve Fig. (1b). Only the acid portion of the curve in fact shows this displacement. The possibility that phosphorylation rather than oxidation was limiting in Fig. 1(a) and that this explains the slightly different pH optimum is unlikely in that the phosphorylation system is active enough to support simultaneous state 3 pyruvate and glycerol phosphate oxidation (Table 3). The conclusion of these pH experiments to

be emphasized is the marked loss of activity at pH values above neutrality, and the activation by higher concentrations of ADP under these conditions.

Control of state 3 pyruvate oxidation

Mitochondria from blowfly flight muscle are in many ways an ideal subject for a study on the functioning and control of the tricarboxylic acid cycle, as respiratory rates are exceedingly high and respiratory control correspondingly tight. In addition, the oxidation of pyruvate by these mitochondria shows several interesting peculiarities, which it was thought should assist in assigning a limiting role to one of the tricarboxylic acid-cycle enzymes involved. That such an enzyme does limit state 3 pyruvate oxidation is shown by the essential summation of state 3 rates of glycerol phosphate and pyruvate oxidation (Table 3), which makes it unlikely that the respiratory chain is limiting in the oxidation of pyruvate alone. It is maintained that the dependency of ADP-stimulated pyruvate oxidation on phosphate concentration (Fig. 4), the differential effects of adenine nucleotides at different pH values (Figs. 2a, 3) and the inhibition by Ca^{2+} (Fig. 7) are all consistent with the idea that the limiting enzyme is NAD-isocitrate dehydrogenase. It is of course possible that another enzyme shows all of these properties, and indeed 2-oxoglutarate oxidation does also show a dependence on high concentrations of ADP and phosphate (Table 4). It would be worth investigating the properties of 2-oxoglutarate dehydrogenase in the cuvette, as this enzyme catalyses a reaction with a large negative standard-free-energy change, and is a plausible site for metabolic regulation.

Control of pyruvate oxidation by adenine nucleotides at the level of pyruvate dehydrogenase appears possible with the results of Linn *et al.* (1969) and Wieland & Siess (1970) that the mammalian enzyme exists in phosphorylated and dephosphorylated forms. However, such a mechanism does not seem to underly the present results in that experiments involving pyruvate oxidation with carnitine as acceptor of acetyl groups showed no effect of ADP or ATP (R. G. Hansford, unpublished work). This result would also counter the suggestion that the effect of adenine nucleotide is at the level of pyruvate penetration into the mitochondrion. Some control of pyruvate dehydrogenase remains necessary in view of the pyruvate concentration in the thorax of the resting fly (Sacktor & Wormser-Shavit, 1966) and the large negative standard-free-energy change of the reaction catalysed. One might speculate on an inhibition by acetyl-CoA (Garland & Randle, 1964).

Control of the tricarboxylic acid cycle by citrate synthase has been demonstrated by Randle *et al.* (1970) for the perfused rat heart. Citrate synthase has not been investigated in the present work.

Control of state 4 pyruvate oxidation

Under conditions of ADP deficiency, i.e. state 4 respiration corresponding to the resting state in the fly, the possibilities are that the limiting activity is isocitrate dehydrogenase, another tricarboxylic acid-cycle enzyme, or the respiratory chain. The redox experiment (Fig. 10) would suggest a limitation at the dehydrogenase level, as the carriers became more reduced on adding ADP. This conclusion is difficult to accept, however, on the grounds that were a dehydrogenase limiting, state 4 would be an energy-poor state, depleted of high-energy chemical intermediate (Slater, 1953, 1958) or protonmotive force (Mitchell, 1966). If this were so, the mitochondria should be incapable of energy-linked functions, and this is shown not to be the case by the stimulation of pyruvate oxidation by Ca^{2+} shown in Fig. 6. Other energy-linked parameters associated with state 4 pyruvate oxidation are swelling in an iso-osmotic potassium phosphate medium (Hansford & Lehninger, 1972), even when atractyloside is added to prevent swelling being driven by hydrolysis of ATP synthesized during a preceding state, and a decreased fluorescence of the fluorescent probe Auramine-O (R. G. Hansford & W. X. Balcavage, unpublished work), which is sensitive to the energization of the mitochondrion (Azzi, 1969). Experiments designed to investigate K^+ accumulation in the presence of valinomycin have, however, consistently failed to show any stimulation of state 4 respiration. This is related to the fact that no respiratory stimulation of glycerol phosphate oxidation is obtained in the presence of K^+ , valinomycin and phosphate, although such a stimulation is apparent in the absence of phosphate. Since phosphate is necessary for pyruvate oxidation, the appropriate experiment cannot therefore be done. In addition, state 4 rates should reflect the properties of the limiting dehydrogenase, if indeed limited at the dehydrogenase level, and such rates are slightly decreased rather than enhanced at higher phosphate concentrations, making it unlikely that they reflect isocitrate dehydrogenase activity, though they are admittedly sensitive to pH (Table 5) and to temperature (Table 2). Finally, it is difficult to conceive of an energy-poor state 4 in that ATP would be hydrolysed, thereby providing ADP and activating the limiting dehydrogenase, if the latter required ADP as an allosteric modifier. The discrepancy between this reasoning and the most obvious explanation of Fig. 10 remains to be explained. Certainly the exactitude with which the activities of both dehydrogenases and the respiratory chain are matched is a most marked characteristic of fly flight muscle.

Tulp & Van Berkel (1970) have commented on the slow transition from state 3 to state 4 in the presence of high concentrations of ATP found with housefly mitochondria oxidizing pyruvate, and inferred that isocitrate dehydrogenase limits oxygen consumption.

Although this inference may well be correct as an explanation of the slowly diminishing state 3 rate, it seems unlikely to hold for the subsequent state 4, which is unaffected by ATP concentration in *C. erythrocephala* mitochondria. The distinction between the control of the two states is one which they fail to emphasize.

The experiments in which the composition of the internal adenine nucleotide pool was manipulated in the presence of oligomycin, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and valinomycin (Figs. 2 and 3) represent an artificial state 4 (high ATP), state 3 (high ADP) and a gradation of intermediate states. They differ from the situation *in vivo* in that the redox state of the nicotinamide nucleotide cannot be varied, but is presumably always oxidized. *In vivo*, NAD-isocitrate dehydrogenase would be less active, and therefore more likely to be limiting, in that NADH is a potent inhibitor, to the extent that when NADH/NAD is 1:5 the velocity is some 50% of maximal, at 2.5 mM-NAD(H) and 2.4 mM-threo-D,L-isocitrate (Hansford, 1968).

Effect of Ca^{2+}

The experiments involving inhibition on addition of CaCl_2 are equivocal in the sense that NAD-isocitrate dehydrogenase may become limiting when severely inhibited by Ca^{2+} , though not in its absence. However, the experiments that show an enhanced oxygen uptake on adding CaCl_2 are noteworthy in that previous work has emphatically denied such a stimulation (Carafoli *et al.*, 1971). These studies deliberately used an excess of ATP, such that most of the Ca^{2+} was in the form of the chelate $[\text{CaATP}]^{2-}$. This must be true of the fly, where the ATP concentration is high both during flight and at rest (Sacktor & Hurlbut, 1966). It is tempting to speculate that entry of Ca^{2+} as $[\text{CaATP}]^{2-}$ may play a role in mitochondria that lack a Ca^{2+} -carrier system.

I thank Professor J. B. Chappell for his encouragement and for his help with the earlier portion of this work, especially the dual-wavelength spectrophotometry.

References

- Atkinson, D. E. (1968) *Biochemistry* **7**, 4030
 Azzi, A. (1969) *Biochem. Biophys. Res. Commun.* **37**, 254
 Azzi, A. & Azzone, G. F. (1967) *Biochim. Biophys. Acta* **131**, 468
 Bartley, W. & Enser, M. B. (1964) *Biochem. J.* **93**, 322
 Brierley, G. P. (1970) *Biochemistry* **9**, 697
 Carafoli, E., Hansford, R. G., Sacktor, B. & Lehninger, A. L. (1971) *J. Biol. Chem.* **246**, 964
 Cha, S. (1969) *Methods Enzymol.* **13**, 62
 Chance, B. & Legallais, V. (1951) *Rev. Sci. Instrum.* **22**, 627
 Chance, B. & Williams, G. R. (1955) *J. Biol. Chem.* **217**, 409
 Chappell, J. B. (1961) *Biol. Struct. Funct., Proc. IUB/IUBS Int. Symp. Ist.* **2**, 11
 Chappell, J. B. & Hansford, R. G. (1969) in *Subcellular Components* (Birnie, G. D. & Fox, S. M., eds.), p. 43, Butterworths, London
 Chefurka, W. (1958) *Biochim. Biophys. Acta* **28**, 660
 Chen, R. F. & Plaut, G. W. E. (1963) *Biochemistry* **2**, 1023
 Childress, C. C. & Sacktor, B. (1966) *Science* **154**, 268
 Garland, P. B. & Randle, P. J. (1964) *Biochem. J.* **91**, 6c
 Goebell, H. & Klingenberg, M. (1964) *Biochem. Z.* **340**, 441
 Gomez-Puyou, A., Sandoval, F., Chavez, E. & Tuena, M. (1970) *J. Biol. Chem.* **245**, 5247
 Hansford, R. G. (1968) Ph.D. Dissertation, University of Bristol
 Hansford, R. G. & Chappell, J. B. (1968) *Biochem. Biophys. Res. Commun.* **27**, 686
 Hansford, R. G. & Lehninger, A. L. (1972) *Biochem. J.* **126**, 689
 Hansford, R. G. & Sacktor, B. (1970) *J. Biol. Chem.* **245**, 991
 Harris, E. J. & Van Dam, K. (1968) *Biochem. J.* **106**, 759
 Klingenberg, M. (1970) *Essays Biochem.* **6**, 119
 Linn, T. C., Pettit, F. H., Hucho, F. & Reed, L. J. (1969) *Proc. Nat. Acad. Sci. U.S.A.* **64**, 227
 Mitchell, P. (1966) *Biol. Rev. Cambridge Phil. Soc.* **41**, 445
 Mitchell, P. & Moyle, J. (1969) *Eur. J. Biochem.* **7**, 471
 Palmieri, F., Quagliariello, E. & Klingenberg, M. (1970) *Eur. J. Biochem.* **17**, 230
 Pfaff, E. & Klingenberg, M. (1968) *Eur. J. Biochem.* **6**, 66
 Pfaff, E., Klingenberg, M. & Heldt, H. W. (1965) *Biochim. Biophys. Acta* **104**, 312
 Price, G. M. & Lewis, S. E. (1959) *Biochem. J.* **71**, 176
 Randle, P. J., England, P. J. & Denton, R. M. (1970) *Biochem. J.* **117**, 677
 Sacktor, B. (1970) *Advan. Insect Physiol.* **7**, 268
 Sacktor, B. & Childress, C. C. (1967) *Arch. Biochem. Biophys.* **120**, 583
 Sacktor, B. & Hurlbut, E. C. (1966) *J. Biol. Chem.* **241**, 632
 Sacktor, B. & Wormser-Shavit, E. (1966) *J. Biol. Chem.* **241**, 624
 Scriba, P. & Holzer, H. (1961) *Biochem. Z.* **334**, 473
 Slater, E. C. (1953) *Nature (London)* **172**, 975
 Slater, E. C. (1958) *Rev. Pure Appl. Chem.* **8**, 221
 Tulp, A. & Van Berkel, Th. J. C. (1970) *Abstr. Energy Transduction in Respiration and Photosynthesis, Pugnochiuso*, p. 21
 Van den Bergh, S. G. (1964) *Biochem. J.* **93**, 128
 Van den Bergh, S. G. (1967) in *Mitochondrial Structure and Compartmentation* (Quagliariello, E., Papa, S., Slater, E. C. & Tager, J. M., eds.), p. 203, Adriatica Editrice, Bari
 Van den Bergh, S. G. & Slater, E. C. (1962) *Biochem. J.* **82**, 362
 Vaughan, H. & Newsholme, E. A. (1969) *FEBS Lett.* **5**, 124
 Wieland, O. & Siess, E. (1970) *Proc. Nat. Acad. Sci. U.S.A.* **65**, 947
 Zebe, E. C. & McShan, W. H. (1957) *J. Gen. Physiol.* **40**, 779