The Control of Tricarboxylate-Cycle Oxidations in Blowfly Flight Muscle

THE OXIDIZED AND REDUCED NICOTINAMIDE-ADENINE DINUCLEOTIDE CONTENT OF FLIGHT MUSCLE AND ISOLATED MITOCHONDRIA, THE ADENOSINE TRIPHOSPHATE AND ADENOSINE DIPHOSPHATE CONTENT OF MITOCHONDRIA, AND THE ENERGY STATUS OF THE MITOCHONDRIA DURING CONTROLLED RESPIRATION

By RICHARD G. HANSFORD

*Gerontology Research Center, National Institute of Child Health and Human Development,
National Institutes of Health, Baltimore City Hospitals, Baltimore, Md. 21224, U.S.A.*

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1. A study is presented of the mitochondrial NADH content during controlled (state 4) and active (state 3) pyruvate oxidation by blowfly flight-muscle mitochondria. The results confirm and extend those of an earlier study (Hansford, 1972) which indicated an increased reduction in state 3. Nicotinamide nucleotide is normally highly oxidized during state 4; however, there can be substantial reduction in the presence of carnitine or high concentrations of proline, or on lengthy incubation in the presence of either of the systems used to generate intramitochondrial tricarboxylate-cycle intermediate. 2. Omission of phosphate leads to substantial reduction and this can be reversed by adding phosphate or acetate. 3. Estimations of NAD^+ and NADH in fly thoraces show a marked increase in NADH on flight, tending to corroborate the results of mitochondrial experiments and testifying to the importance of dehydrogenase activation in this tissue. 4. Determination of intramitochondrial adenine nucleotides reveals a total of 4–5 nmol/mg of protein, and an ADP content of less than 0.1 nmol/mg during state 4 oxidation of pyruvate and proline. ATP content is found to increase slowly during state 4 and this is attributed to the net phosphorylation of AMP. 5. The uncoupling agent carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone leads to hydrolysis of some, but not all, of the mitochondrial ATP. Studies of mitochondrial ATPase (adenosine triphosphatase), measured by external pH change, show that it is inactive unless the mitochondria are allowed to respire for several minutes in state 4 in the presence of phosphate before the addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. It is suggested that phosphate uptake is essential for maximal ATPase activity. 6. Studies of the fluorescence of the fluorochrome 8-anilino-1-naphthalenesulphonic acid suggest that the energy status of the mitochondrion is high during state 4 pyruvate oxidation, and decreases slightly in state 3. The implications of these findings are discussed.

This paper concerns itself with the redox state of nicotinamide nucleotide and the energy charge of adenine nucleotide (in the nomenclature of Atkinson, 1968) during the oxidation of pyruvate by blowfly flight-muscle mitochondria. These parameters are of interest for two reasons. First, they influence the activities of the various enzymes involved in the complete oxidation of pyruvate. For this reason, they must be determined before a rational explanation can be given of the steady-state concentrations of tricarboxylate-cycle intermediates presented in the preceding paper (Johnson & Hansford, 1975). At present there is no literature on the phosphorylation state of adenine nucleotides in isolated insect flight-muscle mitochondria respiring under defined conditions, and very little on the redox state of NAD in

mitochondria from the blowfly (Hansford, 1972). Secondly, the unusual response of nicotinamide nucleotide to the addition of ADP (Hansford, 1972), involving a reduction rather than the oxidation commonly observed with other mitochondria (Chance & Williams, 1956a), raises the question of the energy status of fly mitochondria during controlled (state 4; Chance & Williams, 1956a) pyruvate oxidation, and this can be approached by a measurement of ATP and ADP. These results are presented, together with those from the fluorochrome technique which gives some information on the presence and magnitude of the mitochondrial membrane potential. Finally, determinations of NAD^+ and NADH in rested and flying flies are presented, and they tend to vindicate the use of isolated mitochondria as a model system.

Materials and Methods

Mitochondria were prepared from the flight muscle of *Phormia regina*, as described by Hansford (1974).

Mitochondrial NADH was monitored fluorimetrically, by using a Farrand A3 fluorimeter, primary filter Farrand no. 7-37 and secondary filters nos. 4-70 and 3-73. Output was measured on a Beckman 1 mV recorder, with 25 mV of backing-off voltage. Although the fluorescence of NADPH would be measured under these conditions, it does not contribute appreciably, as NADP(H) is very low in fly flight-muscle mitochondria (Price & Lewis, 1959; Birt, 1961). In the experiments involving 8-anilino-1-naphthalenesulphonic acid fluorescence, the secondary filters were replaced by filter no. 3-71 and the sensitivity decreased 30 times. Controls indicated no appreciable changes in fluorescence in the absence of the fluorochrome. All mitochondrial experiments reported in this paper were performed between 21° and 22°C, and used media saturated with O₂.

For the determination of mitochondrial ATP, ADP and AMP, 2 ml portions of mitochondrial suspensions were ejected into 1.3 ml portions of stirred 16% (v/v) HClO₄ in 40% (v/v) ethanol. After removal of precipitated protein by centrifugation, the extracts were neutralized to pH 7 with 3M-KOH, after the addition of *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (Tes buffer) to a final concentration of 50 mM. KClO₄ was removed by centrifugation at 0°C. The nucleotide content of the extracts was determined fluorimetrically, by the procedures of Williamson & Corkey (1969). The NADH used in the determination of AMP and ADP was cat. no. 6500 from P-L Biochemicals, Milwaukee, Wis., U.S.A.

ATPase* activity was followed indirectly by recording pH change in a lightly buffered medium, by using a Beckman Expandomatic pH-meter on the expanded scale, and a Sargent recorder, full scale 2.5 mV. Quantification was achieved by a titration over the same pH range by using standard HCl.

Flies were flown and frozen as described by Hansford (1974). At least eight, but usually ten flies, were used per extract. For NAD⁺ determinations the thoraces were extracted with 8% (v/v) HClO₄ in 40% (v/v) ethanol, and the extracts were adjusted to pH 5.8 with 1M-KOH in the presence of 50 mM-2-(*N*-morpholino)ethanesulphonic acid (Mes buffer) (final concentration). For NADH determinations, extraction was with ethanolic KOH, as described by Williamson & Corkey (1969). The recovery was 85%, judged by the addition of standard NADH before the crushing of the frozen thoraces. Values have not been corrected to allow for this 15% loss. NAD⁺ and NADH were assayed fluorimetrically, by the use of

*Abbreviation: ATPase, adenosine triphosphatase.

alcohol dehydrogenase and lactate dehydrogenase respectively, as described by Williamson & Corkey (1969). NADH was always assayed as soon after the preparation of the extract as possible.

Results

Redox state of mitochondrial nicotinamide nucleotide

The addition of ADP to a suspension of fly flight-muscle mitochondria oxidizing pyruvate in the presence of ATP and HCO₃⁻ gives rise to a reduction of mitochondrial nicotinamide nucleotide (Hansford, 1972). This unique response to ADP involves an almost total oxidation of nucleotide during state 4 respiration. The experiment shown in Fig. 1 was carried out in an attempt to describe the response to ADP more quantitatively, and to achieve a higher degree of reduction by partially inhibiting cytochrome oxidase activity. This was done by using progressively increasing concentrations of azide. It is seen that the reduction achieved in state 4 is strikingly small until the respiratory-chain activity falls to less than 10% of the uninhibited activity, judged from the

Fig. 1. Effect of azide on the rate of state 3 pyruvate oxidation and on the percentage reduction of mitochondrial NAD in states 3 and 4

Mitochondria were incubated in a medium comprising 0.12M-KCl-20mM-potassium Tes buffer-10mM-potassium phosphate-2mM-pyruvate-2.5mM-KHCO₃-0.5mM-ATP. The pH was 7.2, the temperature 21°C and the final protein concentration 0.6mg/ml. After 4.5 min, azide was added to the concentration indicated, and the state 4 NADH content was determined fluorimetrically, as described in the Materials and Methods section. ADP was added to 1.5mM 1.5 min later and the state 3 content was determined. For calculation of percentages, 0% reduced is defined by the state before azide addition (which differs less than 2% from that obtained on extensive substrate depletion) and 100% reduced by the state of anaerobiosis. The rates of state 3 respiration were recorded with an O₂ electrode, under the same conditions. The state 4 rate was obtained in the absence of azide. ●, State 4 reduction; ○, state 3 reduction; △, state 3 O₂ uptake.

rate of O₂ consumption. At this point there is a rapid increase in reduction with further azide; 50% reduction is achieved when respiratory-chain activity, as measured by the state 3 rate of O₂ uptake, is about twofold the activity needed to account for state 4 O₂ consumption. By contrast, inhibition of state 3 respiration is progressive, giving a linear plot of percentage reduction against percentage inhibition (not shown) as expected if respiratory-chain activity is limiting, or nearly limiting, in that state. The striking difference between these results and those obtained with pigeon heart mitochondria by Chance & Williams (1956*b*), in which nicotinamide nucleotide was 100% reduced in state 4 and 70% reduced in state 3 in the presence of sufficient azide to cause a large (state 3) reduction of cytochrome *c*, can be attributed to a tight dehydrogenase control in fly state 4 respiration.

The experiments referred to above (Fig. 1) and in Hansford (1972) involved pyruvate oxidation in the presence of ATP and HCO₃⁻. Further experiments designed to characterize this unorthodox response to ADP showed that the inclusion of proline in the reaction mixture may give rise to a large reduction in state 4, and that the subsequent addition of ADP then causes a complex, but essentially oxidized, redox change (Fig. 2). The degree of reduction achieved

Fig. 2. Response of intramitochondrial NAD to the addition of proline

Mitochondria (approx. 1 mg of protein) were added to a medium comprising 0.12*M*-KCl–20*mM*-potassium phosphate (pH 7.2)–2.5*mM*-pyruvate–2.5*mM*-KHCO₃–0.5*mM*-ATP. Intramitochondrial NADH was monitored fluorimetrically, as described in the Materials and Methods section. Where indicated, proline was added to 30*mM*, ADP to 1*mM* and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) to 1 μ *M*. The final volume was 2 ml.

Table 1. Effect of substrate on the reduction of intramitochondrial NAD during state 4, state 3 and uncoupled respiration
Reduction was determined fluorimetrically, as described in the Materials and Methods section and in the legend to Fig. 1. Zero reduction is taken as that obtained on incubation without substrate for 4 min, and 100% reduction that obtained during anaerobiosis. ADP was added to 1 *mM* and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone to 1 μ *M* to give state 3 and uncoupled respiration respectively. Results quoted as *x*–*y* refer to maximum and minimum values of reduction obtained during the complex response to ADP. States 3 (1) and (2) refer to successive additions of ADP

Substrate (and concentration) (mM)	P _i concentration (mM)	% reduction			
		State 4	State 3 (1)	State 3 (2)	Uncoupled
Pyruvate (2.5) ATP (0.5) KHCO ₃ (2.5)	20	0	16	—	15
Pyruvate (2.5) ATP (0.5) KHCO ₃ (2.5) DL-Carnitine (10)	20	41	32–17		
Pyruvate (2.5) Proline (20)	20	35	22–14	29–20	11
Pyruvate (2.5) Proline (20)	0	100			
Proline (20)	20	1.5			
Pyruvate (2.5) Proline (5)	20	0 23	17–7	24–11	
Pyruvate (2.5) DL-Carnitine (10)	20	40	7		
Pyruvate (2.5) Proline (20) DL-Carnitine (10)	20	54	—	—	14

during state 4 depends on the concentration of proline added (Table 1), being 23% at 5mM and 35% at 20mM. Similarly, the addition of carnitine during the state 4 oxidation of pyruvate gave rise to a large reduction (Table 1) even when pyruvate oxidation was already potentiated by the presence of ATP and HCO_3^- , to provide oxaloacetate. The simultaneous presence of both proline and carnitine gave still further reduction. Neither pyruvate nor proline added alone gave appreciable reduction (Table 1), relative to the control in the absence of added substrate. Proline is oxidized slowly by these mitochondria (Sacktor & Childress, 1967; Hansford & Sacktor, 1970), but does serve to supply tricarboxylate-cycle intermediate (Sacktor & Childress, 1967). Carnitine accepts acetyl groups (Childress *et al.*, 1967) and may thus potentiate pyruvate dehydrogenase activity in the absence of an active tricarboxylate cycle. However, incubation of these mitochondria with pyruvate, ATP and HCO_3^- for a period of 5 min, as in the first part of the experiment of Fig. 2, ensures a maximal rate of O_2 consumption on adding ADP (Hansford, 1972). The addition of proline and carnitine in no way increases the rate of O_2 consumption in the presence of ADP, though the increased state 4 reduction in Fig. 2 and Table 1 clearly testifies to the increased activity of proline and pyruvate dehydrogenases. It appears that the inclusion of proline or carnitine circumvents a control of metabolism exerted by adenine nucleotides at the dehydrogenase level. The analysis of the steady-state concentrations of tricarboxylate-cycle intermediates presented in the preceding paper (Johnson & Hansford, 1975) allows the identification of the NAD-isocitrate dehydrogenase (EC 1.1.1.41) as this site of control.

An extremely important factor in determining the redox state of nicotinamide nucleotide during state 4 pyruvate oxidation is the concentration of P_i . Table 1 shows that the omission of phosphate gives a reduction of 100% when both pyruvate and 20mM-proline are present; addition of phosphate to 20mM lowers this reduction to 35%. Lower concentrations of phosphate allow more reduction (not shown). It is suggested that the effect of phosphate is to allow the uptake of the predominant cation present in the incubation medium, and that the energy demand of this uptake is reflected in increased nicotinamide nucleotide oxidation. Consistent with this proposal is the finding that the addition of 10mM-potassium acetate gives a large reoxidation of nucleotide which has been extensively reduced in the absence of phosphate. Both acetate and phosphate have been shown to support the energy-linked uptake of K^+ by these mitochondria (Hansford & Lehninger, 1972).

The experiments involving pyruvate, ATP and HCO_3^- presented in Figs. 1 and 2 and Table 1 involved incubation for not longer than 8min before the addition of ADP or other effector. It was thought that

if energy-linked uptake of potassium phosphate were material, reduction might occur when this process had reached completion. It was found that this was indeed so. Thus, though the nucleotide is invariably oxidized with pyruvate, ATP, HCO_3^- and phosphate during experiments of normal time-span, it becomes reduced if the mitochondria are left for 20min. Moreover, the effects of carnitine and proline, although marked in the short term (Fig. 2, Table 1), are to expedite the attainment of the steady state, rather than to affect the steady-state redox level. The latter is, however, still affected by phosphate concentration. Thus whereas 23min is required to achieve 23% reduction in the presence of pyruvate, ATP and KHCO_3 , the same steady-state reduction was achieved in 11min when 20mM-proline was added to the incubation, and in 16min in the presence of 10mM-DL-carnitine. It is noteworthy that when the state 4 rate of O_2 consumption is monitored for long periods, it declines from a maximal value at 4min (60ng-atoms of $\text{O} \cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$) to a considerably lesser value (37ng-atoms of $\text{O} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$) at 10min and beyond. Equally, light-scattering changes reveal that the rate of swelling (in the 0.12M-KCl-20mM-potassium phosphate medium used for the O_2 -electrode studies) is maximal at 4min and declines sharply after 10min. The NAD(H) becomes slightly more oxidized from 0 to 4min (to 0% reduced) and begins to show reduction after 10min. This parallelism suggests that nucleotide reduction occurs when salt uptake is complete. Although salt uptake is a factor in the oxidation of nucleotide in state 4, it does not, however, seem a sufficient explanation (see the Discussion section).

Finally, the reduction noted on adding the uncoupling agent carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine to mitochondria oxidizing pyruvate in the presence of ATP, HCO_3^- and phosphate (Table 1 and other, unpublished, work, R. G. Hansford) is unique, and shows that under these conditions the activity of the dehydrogenases has increased relative to that of the (maximally activated) respiratory chain. It is seen in a later section that ATPase is maximal under these conditions, thus providing the ADP necessary for the operation of the tricarboxylate cycle.

Redox state of NAD in the thoraces of resting and flying flies

Experiments involving the freezing in liquid N_2 of flying or rested flies revealed that the nicotinamide nucleotide is highly oxidized during rest, and becomes significantly more reduced in flight ($P < 0.001$, Table 2). There may be a slight reversal of this trend at the longer flight time (2min), though this is on the border of statistical significance ($0.05 < P < 0.1$). Thus a parallel may be drawn between the response of the nicotinamide nucleotide of isolated mitochondria to

Table 2. Thoracic content of NAD⁺ and NADH in rest and in flight

The details of the extraction and assay procedures are given in the Materials and Methods section.

Rest or flight (time, s)	NADH (nmol/g wet wt.)	NAD ⁺ (nmol/g wet wt.)	NAD ⁺ /NADH
Rest	63 ± 4 (7)	645 ± 11 (5)	10
Flight (30)	183 ± 17 (3)	561 ± 8 (4)	3.1
Flight (120)	153 ± 9 (4)	589 ± 10 (4)	3.8

Table 3. ATP, ADP and AMP content of blowfly flight-muscle mitochondria incubated in the presence and absence of pyruvate + proline as substrate

Mitochondrial suspension (2 ml; 31 mg of protein) was added to 12 ml of 0.12 M-KCl-20 mM-potassium phosphate, pH 7.2. Samples (2 ml) were taken at the times indicated and deproteinized as described in the Materials and Methods section. Where indicated pyruvate and proline were added to give concentrations of 2.5 mM and 20 mM respectively.

Time (min, s)	Manipulation	ATP (nmol/mg of protein)	ADP (nmol/mg of protein)	AMP (nmol/mg of protein)
0, 35	Sample	2.06	1.12	1.16
0, 55	Sample	2.01	1.27	1.21
1, 35	Add pyruvate + proline			
2, 09	Sample	2.91	<0.1	—
2, 50	Sample	2.99	<0.1	—
6, 15	Sample	3.46	<0.1	—

the energy demand of ADP phosphorylation and of the flight-muscle nicotinamide nucleotide to the energy demand of flight.

Very similar values for thoracic content of total NAD were reported by Birt (1966) for a blowfly, *Lucilia cuprina*. He formed an estimate of the ratio NADH/NAD⁺ of the thorax by measuring NADH and NAD⁺ in the intact insect, as 75% of the total NAD is thoracic (Birt, 1966). The ratio of 0.16 obtained for the adult fly falls between the values for rest and flight presented here (Table 2). The metabolic status of the flies in the earlier study (Birt, 1966) is not clear, as the rest-to-flight transition was not a prime concern.

Adenine nucleotide content of blowfly flight-muscle mitochondria

Both the discussion of the data obtained on the relative activities of the various enzymes involved in the oxidation of pyruvate (Johnson & Hansford, 1975), and the discussion of the energy status of state 4 prompted by the finding of highly oxidized nicotinamide nucleotide, required the determination of the energy charge of the adenylate system in these mitochondria. This information is not available in the literature, as previous work (Price & Lewis, 1959) involved extraction of the mitochondria as prepared, in an ill-defined metabolic state. Table 3 presents the results of an experiment in which a sample of a mito-

chondrial suspension in a buffered air-saturated medium was added directly to HClO₄. This technique was suggested by the failure in control experiments to maintain aerobiosis during separation of these highly active mitochondria from the incubation medium. It should give a faithful measure of energy charge during controlled respiration, but clearly precludes experiments in which adenine nucleotide is added, and therefore state 3 respiration. Table 3 shows that these mitochondria retain 2 nmol of ATP/mg of protein during preparation, which was unexpected in view of the lack of endogenous substrate suggested by the negligible rate of O₂ uptake in the absence of added substrate (less than 7 ng-atoms of O · min⁻¹ · mg of protein⁻¹).

Addition of pyruvate plus 20 mM-proline gave a significant increase in ATP within the next 30 s, and a concomitant fall in ADP. This confirms the ability of the mitochondria to phosphorylate under these conditions. There is then a further slow increase in ATP content, which is thought to represent phosphorylation of the AMP, in view of the lack of ADP. This would presumably involve a nucleoside monophosphate kinase in the matrix compartment. Such a localization has indeed been suggested for a portion of the adenylate kinase activity found in flight-muscle mitochondria (Danks & Chappell, 1974).

AMP determinations were found to be impossible in the presence of pyruvate, as the highly purified

NADH used at high concentration in this assay contains a large amount of AMP. The incubation in the absence of pyruvate allows an accurate estimate of the total nucleotide to be made. This is 4.4 nmol/mg of protein, or approx. 1.8 mM during state 4 respiration [the matrix volume determined by Hansford & Lehninger (1972) being used]. These mitochondria (Table 3) were sedimented and resuspended immediately before the experiment. When this was not done (Table 4) a slightly higher total nucleotide content was found, and some of this had presumably leaked from the mitochondria on standing.

Price & Lewis (1959) found substantially more ADP+ATP using an isotopic method, and a very high AMP content. The experiment of Table 4 confirms the negligible ADP content in the presence of pyruvate and 20 mM-proline, and illustrates moreover the hydrolysis of intramitochondrial ATP in the presence of the uncoupling agent carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine. The fact that 2 nmol of ATP/mg of protein is found 2 min after the additions of the uncoupling agent is surprising, but tends to correlate with the finding of the same amount of ATP in the freshly prepared (and substrate-depleted) mitochondria (Table 3). This could reflect an inhibition of the uncoupler-stimulated ATPase, as discussed, or a compartmentation of adenine nucleotide. Some of the ATP in heart mitochondria also persists in the presence of uncoupling agents (LaNoue *et al.*, 1972). The addition of oligomycin (Table 4) allows the phosphorylation of ADP by succinyl-CoA synthetase, an event inferred to occur in housefly mitochondria (Van den Bergh, 1964) and directly demonstrated by assay of nucleotides with

Table 4. Effect of adding carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine and oligomycin on the ATP and ADP content of mitochondria oxidizing pyruvate + proline

Mitochondrial suspension (1.2 ml; 16 mg of protein) was added at time zero to 14.5 ml of a medium comprising 0.12 M-KCl-20 mM-potassium phosphate (pH 7.2)-2.5 mM-pyruvate-20 mM-proline. At the times indicated, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine (FCCP) was added to 1 μ M and oligomycin to a concentration of 3 μ g/ml. Samples were withdrawn as shown.

Time (min, s)	Manipulation	ATP (nmol/mg of protein)	ADP (nmol/mg of protein)
5, 00	Sample	5.5	0
5, 23	Sample	5.4	0
5, 34	Add FCCP		
7, 00	Sample	1.88	2.18
7, 48	Sample	1.95	2.91
7, 58	Add oligomycin		
9, 23	Sample	4.1	0.45
10, 02	Sample	4.8	0

mitochondria from mammalian tissues (see, e.g. LaNoue *et al.*, 1972).

Significantly, an experiment with pyruvate and 5 mM-proline as substrate showed phosphorylation and no further increase in ATP content on increasing the proline concentration to 20 mM (not shown). This was despite an increased NAD⁺ reduction at higher proline concentrations (Table 1) and an increase in the rate of O₂ consumption from 22 to 33 ng-atoms of O $\cdot$ min⁻¹·mg of protein⁻¹. Thus there is no good correlation between O₂ consumption, NAD⁺ reduction and ADP phosphorylation during state 4 respiration.

Measurement of the fluorescence of 8-anilino-naphthalene-1-sulphonic acid as an indicator of mitochondrial energy status

The intensely oxidized nicotinamide nucleotide seen when blowfly flight-muscle mitochondria oxidize pyruvate in the presence of ATP and HCO₃⁻

Fig. 3. Response of the fluorochrome 8-anilino-naphthalene-1-sulphonic acid to the energization of the mitochondrion with pyruvate and proline and to the generation of state 3 respiration

Mitochondria (1 mg of protein) were added to 2 ml of medium comprising 0.12 M-KCl-20 mM-potassium Tes buffer (pH 7.2)-10 mM-potassium phosphate (pH 7.2)-30 μ M-8-anilino-naphthalene-1-sulphonic acid. Fluorescence was monitored as described in the Materials and Methods section. Pyruvate, proline, ADP, oligomycin (Oligo) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine (FCCP) were added at the times indicated giving concentrations of 2.5 mM, 5 mM, 1 mM, 0.25 μ g/ml and 1 μ M respectively. The final volume was 2.2 ml. At the time of pyruvate addition the mitochondria had been incubated for 11 min.

Fig. 4. Response of 8-anilino-naphthalene-1-sulphonic acid fluorescence to the addition of ADP and phosphate to a suspension of mitochondria oxidizing pyruvate and proline

Mitochondria were added to a medium comprising 0.12M-KCl-20mM-potassium Tes buffer (pH7.2) - 2.5mM-pyruvate-5mM-proline - 30 μ M-8-anilino-naphthalene-1-sulphonic acid. At the points indicated, ADP was added to 0.5mM, phosphate (pH7.2) to 5mM and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) to 1 μ M.

has called into question the nature of controlled respiration in these mitochondria (Hansford, 1972). In mammalian mitochondria state 4 $\rightarrow$ state 3 transitions are associated with increased oxidation of NADH (Chance & Williams, 1956a) and a drop in the mitochondrial energy status, as measured by the sum of the membrane potential and transmembrane pH gradient (Mitchell & Moyle, 1969; Padan & Rottenberg, 1973). It seemed possible that the converse is seen in the fly. This question was approached by studying the fluorescence of the probe 8-anilino-naphthalene-1-sulphonic acid, which was first applied to mitochondrial systems by Azzi *et al.* (1969). The basis of this approach is that the fluorescence of this compound added to a mitochondrial suspension has been shown to be affected by the magnitude of the mitochondrial membrane potential (Azzi *et al.*, 1971), and the membrane potential has been suggested to be

the major component of the total protonmotive force, under normal state 3 and 4 conditions (Mitchell & Moyle, 1969). Incubation of mitochondria in the presence of only phosphate gave substrate depletion, and a slow increase in fluorescence, indicating de-energization (Fig. 3). Addition of pyruvate led to a slight energization, and this was much enhanced by the addition of proline. The phosphorylation of ADP gave a small apparent de-energization (Fig. 3) but so did the addition of ADP in the presence of oligomycin. The uncoupling agent carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone gave a rapid de-energization, followed by a considerable reversal. When the reaction was initiated with pyruvate and proline, in the absence of phosphate, ADP gave a slight increase in energy status (Fig. 4), consistent with dehydrogenase activation, as did a subsequent addition of phosphate. However, in this experiment, too, state 3 was of lower energy status than the subsequent state 4. These experiments therefore provide no evidence for an 'energy-poor' state 4. The argument is unaffected by the exact nature of the energy state to which the probe is responding. However, it can be shown that a membrane potential does affect the fluorescence, as the creation of a diffusion potential by adding valinomycin in the presence of pre-existing gradients in K⁺ ion concentration gave rise to a fluorescence change, and a linear relationship between this change and the logarithm of the K⁺ gradient (not shown) as described by Azzi *et al.* (1971). Unfortunately, it was not possible to obtain in this fashion a fluorescence change as large as that obtained with oxidizable substrate. Extrapolation, assuming linearity, indicated a membrane potential of 100-120mV during state 4 pyruvate oxidation. This value is much smaller than that found by Mitchell & Moyle (1969) in a more direct determination, but quite similar to that determined by Padan & Rottenberg (1973) in rat liver mitochondria.

Measurement of uncoupler-stimulated ATPase

The finding of some residual ATP after treating the mitochondria with carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (Table 4) and a consideration of the unusual presence of a high ATP/ADP but low NADH/NAD ratio during controlled pyruvate oxidation (Tables 1 and 3) prompted an investigation of the mitochondrial ATPase. This was done indirectly by following the pH change associated with the synthesis or hydrolysis of ATP (Mitchell & Moyle, 1968). It was found that whereas uncoupler added after an ADP cycle (Fig. 5) elicited a rate of pH change in excess of that associated with phosphorylation, addition of uncoupler to mitochondria incubated with ATP alone gave no hydrolysis. This was true even when the uncoupling agent was added after preincubating mitochondria with ATP for more

Fig. 5. Measurement of uncoupler-stimulated ATPase by the recording of pH change

(a) Mitochondria (1.5–2 mg of protein) were added to 4 ml of a medium comprising 0.13 M-KCl–5 mM-potassium phosphate–2 mM-pyruvate–2.5 mM-KHCO₃–0.4 mM-ATP; the pH was 7.2. Changes in pH were recorded by using a glass electrode, as described in the Materials and Methods section. At the points indicated ADP was added to 0.62 mM, and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) to 1.5 μ M. (b) Mitochondria were added to a medium comprising 0.13 M-KCl–5 mM-potassium phosphate–2.5 mM-KHCO₃–1.6 mM-ATP and 5 μ M-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. The pH was 7.2 and the buffering power in the medium was determined to be essentially the same as that in (a).

than 20 min. By contrast, incubation with pyruvate, ATP and HCO₃[−] potentiated an active hydrolysis, the exact rate and extent of the hydrolysis depending on the time of preincubation (Fig. 6). It was noticed that omission of phosphate inhibited the development of an uncoupler-stimulated ATPase. This is shown in Fig. 7. The experiment involves the use of glycerol 3-phosphate as substrate, as this allows for more rapid salt uptake. Acetate is ineffective as a replacement for phosphate, although light-scattering controls (not shown) indicated greater swelling with 25 mM-acetate than with 4 mM-phosphate. Thus the uptake of K⁺ and a penetrant anion is not sufficient: there is a specific requirement for phosphate. Gomez-Puyou *et al.* (1972) have shown a dependency on K⁺ ion uptake of ATPase in K⁺-depleted liver mitochondria, and Jung & Hanson (1973) have shown a requirement for respiration and for phosphate in the development of ATPase in cauliflower mitochondria. In both cases, an effect on the entry of ATP into the

mitochondria is invoked. This seems not to be a complete explanation of the results with the fly, as control experiments have shown that the ATPase of sonic particles is also enhanced by phosphate. Mitchell & Moyle (1970) have reported a similar finding with the ATPase of particles from liver mitochondria.

Discussion

Chance & Williams (1956a) proposed a model for the control of oxidative metabolism in which the activity of respiration was dependent on the concentration of phosphate acceptor. In the absence of acceptor (ADP) the transfer of electrons along the respiratory chain was inhibited, and the NAD became reduced (state 4), with a consequent inhibition of dehydrogenase activity. This model has been found to apply not only to mitochondria from diverse sources, but also to intact tissues. Thus Chance & Connelly (1957) observed that the nicotinamide nucleotide in frog skeletal muscle became oxidized during a muscle twitch, and Chance *et al.* (1965) observed that the nicotinamide nucleotide in rat heart became oxidized during each contraction. That the model has to be modified before it can be applied to blowfly flight muscle, a highly energetic tissue, is apparent from Table 3. The significant increase in NAD⁺ reduction on the initiation of flight suggests a very great activation of the dehydrogenases, and an increased activity relative to that of the respiratory chain. When mitochondria are isolated and the redox state of nicotinamide nucleotide is studied during pyruvate oxidation, then the same reduction may be observed on the facilitation of respiration by ADP (Table 1). When comparisons are made between these two sorts of experiments, it has to be remembered that the thoracic NAD(H) represents both mitochondria and cytosol, and that the mitochondria *in situ* oxidize both pyruvate and glycerol 3-phosphate [see Sacktor (1970) for a review]. However, it seems unlikely that the redox state of the cytosol changes much on flight, as the ratio of glycerol 3-phosphate/dihydroxyacetone phosphate remains constant (Sacktor & Wormser-Shavit, 1966) and this ratio has been used as an index of the redox state of the cytosol (Bücher & Klingenberg, 1958), lactate dehydrogenase being inactive in this tissue. It further seems unlikely that glycerol 3-phosphate is an active reductant of NAD⁺ during flight, as reversed electron transfer from glycerol 3-phosphate to NAD⁺ is greatly inhibited by the phosphorylation of ADP (Hansford, 1972). It thus seems that the increased reduction of NAD⁺ on flight can be largely attributed to a facilitation of the oxidation of pyruvate, the other product of glycolysis in this exclusively carbohydrate-utilizing tissue. It is demonstrated in the preceding paper (Johnson & Hansford, 1975) that the main locus of

Fig. 6. Effect of preincubation with substrate on the development of uncoupler-stimulated ATPase

Mitochondria were incubated in a medium comprising 0.13 M-KCl-5 mM-potassium phosphate-2.5 mM-pyruvate-0.5 mM-ATP-2.5 mM-KHCO₃; the pH was 7.2 and the volume 4 ml. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was added to give a concentration of 1 μ M either before the mitochondrial addition (a) or after 2 min (b), 4 min (c) or 6 min (d) of incubation. ATPase was followed indirectly by pH change and the system was calibrated by the addition of 0.6 μ equivalent of HCl.

control of pyruvate oxidation is NAD-isocitrate dehydrogenase, and that the most significant activator of this enzyme *in situ* in the mitochondrion is ADP. Thus the Chance & Williams (1956a) model has to be modified in the sense that ADP, produced by the activity of the myofibrillar ATPase, acts not

only as phosphate acceptor, but also as an allosteric effector of a rate-limiting enzyme.

It is apparent from the reduction of NAD⁺ which may be observed when ADP (Table 1), carnitine (Table 1) and proline (Fig. 2) are added during state 4 pyruvate oxidation, that there is no simple relation-

Fig. 7. Requirement for P_i in the development of ATPase

Mitochondria were incubated in a medium comprising 0.13 M-KCl-1 mM-ATP-5 mM-DL-glycerol phosphate-7.5 μ M-CaCl₂-12.5 μ M-EGTA [ethanedioxybis(ethylamine)tetra-acetate] (added with the mitochondrial suspension). The pH was 7.1 and the volume 4 ml. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was added 2 min after the mitochondria, to elicit ATPase activity. (a) No addition to basal medium; (b) basal medium + 4 mM-potassium phosphate, pH 7.1; (c) basal medium + 25 mM-potassium acetate, pH 7.1. Buffering power was estimated in each case by the addition of 1 μ equivalent of HCl.

ship between the redox state of NAD(H) and phosphate potential (cf. Erecinska *et al.*, 1974). Thus phosphate potential is demonstrated to be high in state 4 (Table 3), cannot rise on adding carnitine as the ADP content is already vanishingly small, and may be expected to fall in state 3. A reservation is that the present work involves the measurement of both free and bound nicotinamide nucleotide. The oxidized state of the nicotinamide nucleotide during state 4 respiration strongly suggests a disequilibrium between the $\text{NAD}^+/\text{NADH}+\text{H}^+$ couple, and the more reducing of the substrate couples involved in the tricarboxylate cycle, e.g. the isocitrate/2-oxoglutarate+CO₂ couple ($E_0' = -0.38$ V). It seems reasonable to suggest that this disequilibrium stems from an appreciable energy leak, and largely inactivated dehydrogenases. The leak may be attributed to the uptake of potassium phosphate, and it is pointed out in the text that when salt uptake is complete as evidenced by a constancy in light-scattering, there is a gradual reduction of nicotinamide nucleotide. The cause of the dehydrogenase inhibition is less apparent, as it might be thought that the energy leak should lead to a reactivation of NAD-isocitrate dehydrogenase, the mechanism being the activation of mitochondrial ATPase by the fall in $X\sim I$ content (Slater, 1953) or protonmotive force (Mitchell, 1966) and therefore

the generation of the dehydrogenase activator, ADP.

Such reasoning led to a study of the energy status of fly mitochondria during controlled pyruvate oxidation. The high ATP content, and lack of ADP (Tables 3 and 4) strongly suggest a normal state 4, with a high $X\sim I$ content or protonmotive force, despite the oxidized nucleotide. This presupposes that the mitochondrial ATPase is active and mediates between phosphate potential and protonmotive force. Direct studies (Figs. 5, 6 and 7) indicate that addition of uncoupling agent elicits an active ATPase when the mitochondria have respired for several minutes in state 4. Under the conditions of the experiment the nicotinamide nucleotide nevertheless remains oxidized. There is no net ATPase activity during state 4 respiration, and therefore a study of that state based on pH change is not possible, and all that can be said is that the potential for ATPase exists. The second parameter which suggests an energized state 4 is the low fluorescence of the fluorochrome 8-anilinonaphthalene-1-sulphonic acid which is seen in that state (Figs. 3 and 4). In this context the exact nature of the energized state to which the fluorochrome responds (see Brocklehurst *et al.*, 1970) is not material, though membrane potential certainly forms a large component, as described by Azzi *et al.* (1971). The slight increase in

fluorescence on adding ADP (Fig. 3) would suggest de-energization, though a control addition in the presence of oligomycin also gives an increase. This may be related to the intense reduction of respiratory carriers in that state (Johnson & Hansford, 1975), as this appears to have some effect on the fluorescence of the fluorochrome (R. G. Hansford, unpublished work).

After the preparation of this paper the attention of the author was drawn to another study in which the energy charge of fly mitochondrial adenine nucleotide was measured under specified metabolic conditions (Danks & Chappell, 1974). The slow phosphorylation of AMP inferred to occur from the data of Table 3 was demonstrated directly by these authors. An important difference between these two studies is the finding by Danks & Chappell (1974) of a large amount (25% of the total adenine nucleotide) of ADP during controlled pyruvate + proline oxidation. They do not confront the issue that this raises about the nature of controlled respiration.

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