The Nature of Controlled Respiration and its Relationship to Protonmotive Force and Proton Conductance in Blowfly Flight-Muscle Mitochondria

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1. To determine whether controlled (State 4) pyruvate oxidation can support a high energy state, measurements of the redox span NAD–cytochrome *c*, phosphorylation potential and protonmotive force (the gradient in electrochemical activity of protons across the mitochondrial inner membrane) were made as indices of energy status. For comparison, these three measurements were also made with glycerol 3-phosphate, an alternative substrate. The two substrates gave essentially identical values for the redox span NAD–cytochrome *c* in State 4, and the phosphorylation potential was of sufficient magnitude to be considered in equilibrium with the redox span over the first two phosphorylation sites. The magnitude of the protonmotive force in State 4 was much less and the implications of this finding are discussed. 2. Measurements made during the controlled (State 4) to active (State 3) transition indicated that with glycerol 3-phosphate as substrate, both the redox span NAD–cytochrome *c* and the protonmotive force were diminished; the State 4 → State 3 transition with pyruvate as substrate was accompanied by an increase in the redox span but a decrease in protonmotive force. The contrary behaviour of these two energetic parameters in the presence of pyruvate was ascribed to a transient excess in the flux of protons through the adenosine triphosphatase relative to the proton-pumping respiratory chain, in spite of the increased dehydrogenase activity. 3. The lower protonmotive force seen in State 3 relative to State 4 with pyruvate as substrate was due to a diminution of both the electrical ($\Delta\psi$) and the chemical (ΔpH) components; with glycerol 3-phosphate, the magnitude of the decrease in protonmotive force during the State 4 → State 3 transition was similar to that seen with pyruvate, but was due to a large decrease in the electrical component ($\Delta\psi$) and a small rise in the chemical component (ΔpH). The reason for the difference seen in the behaviour of the components of the protonmotive force was investigated but not established. 4. In the presence of oligomycin and ADP, oxidation of pyruvate, but not of glycerol 3-phosphate, supported a greater protonmotive force than in State 4, in keeping with the dehydrogenase activation and increased redox span NAD–cytochrome *c* found under these conditions. 5. Experiments involving the use of uncoupling agent to stimulate respiration are compared with those in which limiting concentrations of ADP were used. Estimates of the proton conductance of the inner membrane indicate a similar non-linear dependence on uncoupler concentration with the two substrates. 6. A model is proposed as an explanation of the high rates of controlled glycerol 3-phosphate oxidation. The model relies on a high permeability of the inner membrane to protons and other ions being induced by glycerol 3-phosphate oxidation in State 4.

The nature of controlled respiration (State 4 in the nomenclature of Chance & Williams, 1955) in isolated blowfly flight-muscle mitochondria when pyruvate is the principal substrate is particularly interesting because the respiratory-chain components, NAD and cytochrome *c*, are highly oxidized in this state (Hansford, 1972). Furthermore on addition of ADP (State 3 of Chance & Williams,

1955) both NAD and cytochrome *c* become more reduced (Hansford & Chappell, 1968; Hansford, 1972), quite the opposite of what is observed with mammalian mitochondria (Chance & Williams, 1955), but consistent with both the behaviour of NAD in the flight muscle *in vivo*, when a resting fly takes to flight (Hansford, 1975), and the early observations of Keilin (1925) on a wax moth. The oxidized nature of

the respiratory-chain components in State 4 has been attributed to an adenine nucleotide control at the dehydrogenase level (Hansford, 1972, 1975), and isocitrate dehydrogenase (NAD^+) has been identified as the principal locus of control (Hansford, 1974; Johnson & Hansford, 1975); the higher degree of reduction in State 3 is then due to the greater stimulation of dehydrogenase activity relative to the activation of electron flow down the respiratory chain. However, the question arises as to whether the energy of oxidoreduction in State 4 is sufficient to poise the generated ATP/ADP ratio (Danks & Chappell, 1974; Hansford, 1975) or whether State 4 in these mitochondria is energy-poor. Some experiments designed to evaluate the latter possibility have been reported (Hansford, 1975) and, in particular, the measurement of the ATP/ADP ratio indicated an energized State 4, although the mitochondrial ATPase* was inactive under certain conditions, conceivably allowing a dissociation of redox events and phosphorylation.

In the present work the question of the energy status during State 4 pyruvate oxidation has been investigated directly by measuring the redox span NAD -cytochrome *c*, phosphorylation potential and protonmotive force [Mitchell's (1966) terminology for the electrochemical gradient of protons across the mitochondrial inner membrane] under similar conditions. In addition an alternative substrate, glycerol 3-phosphate, has been used for comparison, since it induces a demonstrably energized State 4: NAD is reduced by reversed electron transport (Chance & Hollunger, 1961; Klingenberg & Bücher, 1961) in State 4 and becomes more oxidized in State 3, in this way resembling the behaviour of mammalian mitochondria.

The results indicate that by the three criteria considered, pyruvate oxidation, like glycerol 3-phosphate oxidation, supports an energized State 4. Furthermore they allow comment on (i) the relationship between redox span and phosphorylation potential (see Wilson *et al.*, 1973; Erecinska *et al.*, 1974; Slater *et al.*, 1973), (ii) the nature of controlled respiration and (iii) the magnitude and behaviour of the protonmotive force under conditions of measured redox span and phosphorylation potential. All three parameters have not been measured in previous work (Mitchell & Moyle, 1969a; Nicholls, 1974a,b; Rottenberg, 1975; Wiechmann *et al.*, 1975) and a knowledge of their respective magnitudes under identical experimental conditions is important in the discussion of the adequacy of protonmotive force as an intermediate of oxidative phosphorylation. In addition, the present work extends the measurement of protonmotive force to a system in which effective

* Abbreviations: ATPase, adenosine triphosphatase; Tes, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]aminoethanesulphonic acid.

control at the level of the dehydrogenases has been demonstrated.

A part of this work has been communicated in preliminary form (Johnson & Hansford, 1976).

Experimental

Methods

Preparation of mitochondria. Flight-muscle mitochondria were prepared from blowflies (7–9-day-old *Phormia regina* of either sex) by the method of Chappell & Hansford (1972) incorporating the minor modifications of Hansford (1974). The final wash and resuspension of the final pellet was in 0.25M-sucrose/10mM-Tes, pH 7.2.

Incubation of mitochondria. A basal medium of 0.12M-NaCl/20mM-Tes, pH 7.2, was used throughout. Unless stated otherwise, it was saturated with O_2 at 25°C, the temperature used for the incubations, and O_2 was blown at the surface of the medium in experiments conducted in an open cell. When the principal substrate was pyruvate, the medium was supplemented with 2.5mM-sodium pyruvate, 0.5mM-ATP (sodium salt), 2.5mM- NaHCO_3 , 20mM-Tris/sodium phosphate, 10 μM -RbCl and 0.5 μM -valinomycin. When the principal substrate was glycerol 3-phosphate, the medium was supplemented with 10mM-DL-glycerol 3-phosphate (sodium salt), Ca^{2+} buffered to 0.3 μM with 0.75mM- CaCl_2 plus 1mM-EGTA at pH 7.2, 5mM-Tris/sodium phosphate, 10 μM -RbCl and 0.5 μM -valinomycin. For the determination of protonmotive force, $^{86}\text{RbCl}$ was used (approx. 10nCi/ml, increased to 0.1 μCi /ml when the membrane potential component approached 0mV) and the medium contained in addition 0.2mM-KCl, 0.1mM-[^3H]acetate (approx. 0.25 μCi /ml), 10 μM - KS^{14}CN (approx. 0.25 μCi /ml), 30mg of Dextran T-40/ml and 2.5mM-sodium aspartate (pyruvate incubations only). All concentrations given are final and the pH of solutions added to the basal medium was adjusted to 7.2 where practicable.

Measurement of O_2 tension. A Clark-type O_2 electrode monitored the O_2 tension, either in a thermostatically controlled closed cell in control experiments or in an open cell in experiments where samples were withdrawn. In the latter situation, where O_2 was blown at the surface of the medium, it was necessary to turn off the O_2 supply in order to observe State 4 $\rightarrow$ 3 $\rightarrow$ 4 transitions with pyruvate as substrate, although the initial phase of the experiment (the first 5 min) was conducted under O_2 . The O_2 recording from open-cell experiments was not used for quantitative purposes.

Measurement of redox components. The oxidized and reduced forms of NAD were assayed enzymically by the methods of Williamson & Corkey (1969) after extraction from mitochondrial incubations as described by Hansford & Johnson (1975). The reduced

form of cytochrome *c* was measured by dual-wavelength spectrophotometry by using the wavelength pair 551–540 nm (Chance & Sacktor, 1958). Total cytochrome *c* was estimated as follows: since the mitochondria as prepared are essentially devoid of endogenous substrate, the fully oxidized position was determined in O₂-saturated basal medium containing 5 mM-Tris/sodium phosphate and the fully reduced position was given on addition of freshly prepared Na₂S₂O₄ solution to 5 mM; a further addition of glycerol 3-phosphate to 5 mM gave a more stable record and a more easily determined end point for the fully reduced determination. Pyruvate incubations were monitored at the same wavelengths and the degree of cytochrome *c* reduction was assessed relative to the fully reduced position (achieved by addition of 5 mM-Na₂S₂O₄) and by reference to the full-scale calibration described above. Incubations with glycerol 3-phosphate as substrate were assessed relative to the fully oxidized position (before addition of substrate), since the fully reduced position was subject to appreciable drift. State-3 values were measured as the apparent steady-state degree of reduction on addition of 2 mM-ADP for pyruvate incubations and 1 mM-ADP for glycerol 3-phosphate incubations. Redox potentials (E_h) relative to the standard hydrogen electrode were calculated from the relationship:

$$E_h = E_m + \frac{59}{n} \log \left(\frac{[\text{oxidized}]}{[\text{reduced}]} \right) \text{ mV}$$

where E_m is the midpoint (or half-reduction) potential of the couple under the prevailing conditions, n is the number of electrons accepted by the oxidized species in forming the reduced species, and [oxidized] and [reduced] are the activities of these respective species. In this work, the midpoint potential for NAD at pH 7.0 has been corrected so as to be appropriate for a mitochondrial matrix of pH 8.0, consistent with a pH difference of 0.8 unit across the mitochondrial inner membrane (see the Results section). Thus the value of –320 mV has been made 30 mV more negative (see Clark, 1960) giving an E_m value of –350 mV. Furthermore the total amounts of NAD⁺ and NADH, extracted and determined as described above, have been used for [oxidized] and [reduced] respectively, with a value for n of 2. For cytochrome *c*, the assumed midpoint potential of 235 mV (after Dutton *et al.*, 1970) is independent of pH in the neutral range (Rodkey & Ball, 1950) and of membrane potential differences across the mitochondrial inner membrane (Hinkle & Mitchell, 1970); the proportion of oxidized and reduced components was determined as described above and n is 1.

Measurement of phosphorylation potential. Mitochondrial incubations were carried out with [³H]ATP and [³H]ADP of similar specific radioactivity in

pyruvate experiments and with [³H]ADP alone in glycerol 3-phosphate experiments. Samples (200 μl) were withdrawn and expelled into 400 μl of ice-cold 0.5 M-HClO₄. After removal of denatured protein by centrifugation (10000g, 2 min), 100 μl portions of acidified extract were taken for P_i analysis by the method of Martin & Doty (1949). In addition, 400 μl portions were neutralized with a predetermined volume of 3 M-KOH and the adenine nucleotides were separated by the method of Heldt *et al.* (1972). After chromatography, the adenine nucleotide samples were made up to the same aqueous volume (4 ml) and the same acid concentration (approx. 0.7 M with respect to HCl) and counted as gels after the addition of 16 ml of ACS solution. The mean total c.p.m. recovered was used as a measure of the total c.p.m. added to the incubation; by assaying spectrophotometrically the ATP and ADP stock solutions for ATP, ADP and AMP, essentially by the methods of Williamson & Corkey (1969), assuming that the added ³H-labelled tracer nucleotides did not alter these concentrations appreciably and that the labelled and unlabelled nucleotides equilibrated rapidly, it was possible to express the c.p.m. as a concentration of adenine nucleotide. The nominal specific radioactivity of both the [³H]ADP and [³H]ATP solutions was 2 Ci/mol. Phosphorylation potential (ΔG) values were calculated from the equation:

$$\Delta G = \Delta G'_0 + 5.7 \log \left(\frac{[\text{ATP}]}{[\text{ADP}][\text{P}_i]} \right) \text{ kJ/mol}$$

where $\Delta G'_0$ is the standard free-energy change under defined conditions, and [ATP], [ADP] and [P_i] are the activities of these respective compounds external to the mitochondrial matrix. The calculated values include the assumption that activity and concentration are identical and that at pH 7.2, with no externally added Mg²⁺, the standard free-energy change in 34.2 kJ/mol (Rosing & Slater, 1972).

Measurement of protonmotive force. The ion-distribution method of Nicholls (1974b) was used with the following modifications. Mitochondria were separated rapidly from the incubation medium by one of two centrifugation procedures: most commonly used was a silicone method (see LaNoue *et al.*, 1973) in which 300 μl samples of incubation fluid were layered on to approx. 35 μl of silicone mixture (LaNoue *et al.*, 1973), below which was a more dense layer comprising 60 μl of 0.12 M-NaCl/20 mM-Tes, pH 7.2, 17% (w/v) Ficoll and approx. 0.05% (w/v) Blue Dextran, contained in Beckman 400 μl centrifuge tubes. The silicone was sufficient to give depth of 2 mm within the centrifuge tube and the incubation medium contained 30 mg of Dextran T-40/ml. In the second method, the silicone layer was omitted, as was the Dextran T-40 from the medium, so that samples were layered directly on to

the dense Ficoll layer. In both cases centrifugation was for 1 min in a Beckman microcentrifuge (model 152); the uppermost layer was removed as completely as possible, which included wiping the surface of the silicone with a tissue when that method was used, the pellet was resuspended and the bottom phase, identified by the Blue Dextran, was added to a vial containing 0.5 ml of water. After adding 10 ml of Aquasol the vials were analysed for ^{86}Rb , ^{14}C and ^3H by liquid-scintillation counting.

A second modification of Nicholls' (1974b) method was the inclusion of S^{14}CN^- rather than ^{14}C methylamine, since under conditions approaching zero protonmotive force the membrane-potential component reversed, making estimation by $^{86}\text{Rb}^+$ distribution inaccurate. SCN^- is a rapid penetrant of the flight-muscle mitochondrial inner membrane, as in liver mitochondria (Mitchell & Moyle, 1969b), so that S^{14}CN^- could be used as an index of internally positive membrane potentials, as found with bacterial chromatophores (Schuldiner *et al.*, 1974), or as a measure of extramatrix space (see below). As described by Nicholls (1974b), 40 μl samples of incubation medium, removed before addition of mitochondria, were counted for radioactivity under the conditions used for the mitochondrial measurements, i.e. in 0.5 ml of water to which had been added 60 μl of the dense Ficoll solution, together with 10 ml of Aquasol. Counts from mitochondrial samples, after correction for background and cross-contamination, were then compared with the 40 μl standard values so as to give an equivalent volume or space within the sample. Adopting Nicholls' (1974b) approach, let the spaces for ^{86}Rb , ^{14}C and ^3H be r , c and h respectively. The membrane-potential component, $\Delta\psi$, is first calculated by computing $\frac{r-c}{m}$ = N , where m is the matrix volume. For values of N equal to or greater than 10, which is usually the case, $\Delta\psi = 59 \log N$, since c is a very good approximation of the extramatrix space, v . For values of N less than 10, c is a less accurate measure of v and a new factor, α , is calculated from:

$$\alpha = \frac{[\text{Rb}^+]_{\text{matrix}}}{[\text{Rb}^+]_{\text{medium}}} = \frac{N + \sqrt{N^2 + 4}}{2}$$

whereon $\Delta\psi = 59 \log \alpha$. When $\frac{r-c}{m}$ is negative, the magnitude alone is used to calculate N and α , and $\Delta\psi$ is then $-59 \log \alpha$. The pH component is given by the degree of ^3H acetate accumulation, i.e. $\frac{h-c}{m}$

for values of N greater than 10, and $\frac{h-v}{m}$ for values of N less than 10, v being given accurately by $r - \alpha m$ (when $r > c$) or $c - \alpha m$ (when $c > r$). Thus $-\Delta\text{pH} = \log \frac{h-v}{m}$ and $-59 \Delta\text{pH} = 59 \log \frac{h-v}{m}$. Δp , the proton-

motive force, is the arithmetic sum of the individual components, $= \Delta\psi - 59 \Delta\text{pH}$. The values of m used were 2.0 $\mu\text{l}/\text{mg}$ of protein and 1.0 $\mu\text{l}/\text{mg}$ of protein for glycerol 3-phosphate-energized and de-energized mitochondria respectively (Hansford & Lehninger, 1972), and 1.3 $\mu\text{l}/\text{mg}$ of protein for pyruvate-energized mitochondria (R. G. Hansford, unpublished observations). Each value was corrected for the osmotic dead-space of 0.4 $\mu\text{l}/\text{mg}$ of protein (Hansford & Lehninger, 1972), giving final values of 1.6 $\mu\text{l}/\text{mg}$ for glycerol 3-phosphate-energized, 0.9 $\mu\text{l}/\text{mg}$ for pyruvate-energized and 0.6 $\mu\text{l}/\text{mg}$ for de-energized mitochondria in the absence of substrate. The study of Hansford & Lehninger (1972) is not strictly comparable in that none of the space determination was made in an experimental system containing valinomycin. However, it is noted that blowfly mitochondria show an active K^+ uniport when energized in the absence of valinomycin (Hansford & Lehninger, 1972) and that the concentration of valinomycin-transportable cation used in the present work was limited to 0.21 mM, so that volume changes of large amplitude were avoided. Admittedly any space determination on energized mitochondria is open to error owing to anaerobiosis on separation of the mitochondria from the suspending medium. However, it should be emphasized that a large error in the estimation of m leads to a relatively small inaccuracy in the calculation of Δp (e.g. a 2-fold change in the value of m produces an alteration in Δp of not more than 35 mV).

Treatment and presentation of data. Where possible the data presented are from more than one mitochondrial preparation. However, when single experiments are used to illustrate a particular point, similar experiments have been performed a sufficient number of times (at least twice) to establish the validity of the case. When significant quantitative variations were found between mitochondrial preparations, the data presented are of two types: one drawn from multiple determinations with a single preparation, and the other from multiple preparations using a mean of individually determined values for each preparation. Student's t test was used to establish whether groups of values differed significantly.

Materials

Valinomycin and oligomycin were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was from Pierce Chemical Co., Rockford, IL, U.S.A.; these compounds were added as ethanolic solutions. Hexokinase [from Boehringer (Mannheim) Corp., New York, NY, U.S.A.] was dialysed overnight against 1000 vol. of basal medium before use. Carbonic anhydrase was also from Boehringer (Mannheim) Corp. $^{86}\text{RbCl}$, ^3H acetate and Aquasol

were obtained from New England Nuclear, Boston, MA, U.S.A., and KS^{14}CN and ACS solution were from Amersham-Searle, Arlington Heights, IL, U.S.A. Dextran T-40 and Ficoll were from Pharmacia, Uppsala, Sweden.

Except as noted above, all reagents were dissolved in twice glass-distilled water, and were of the highest grade available.

Results

At the outset, it was a primary objective to measure protonmotive force, since this appears to be a most direct index of energy status (Mitchell & Moyle, 1969a; Nicholls, 1974a,b; Rottenberg, 1975). Of the available methods for estimating membrane potential (see Rottenberg, 1975) it was decided to use a method of measurement which depends on determining the equilibrium distribution of K^+ across the inner membrane in the presence of valinomycin. The use of such a method demands that a medium almost free of K^+ be used to avoid mitochondrial swelling or lysis, and this is a disadvantage for flight-muscle mitochondria, since pyruvate oxidation is optimal in KCl media (Hansford, 1972). In the present study NaCl media were used, a brief evaluation of which follows.

Mitochondria incubated with pyruvate in the presence of ATP and HCO_3^- to provide oxaloacetate (see Hansford, 1972) and in NaCl medium (see the Experimental section), gave State-3 rates of respiration approx. 50% of those usually observed in KCl media. Correspondingly, NAD reduction in State 3 observed fluorimetrically (see Hansford, 1975) was less marked in NaCl medium than in KCl medium and light-scattering changes, monitored as an absorbance change at 650nm, were less rapid in NaCl than in KCl medium, although they appeared to be of similar magnitude. It should be emphasized that high respiratory-control ratios were maintained in NaCl media, typical values ranging between 15 and 20. Addition of 10mM-proline to the pyruvate-ATP- HCO_3^- system gave elevated State-4 rates of respiration without significantly increasing State-3 rates and therefore offered no advantage. By contrast, glycerol 3-phosphate oxidation was little affected when NaCl was substituted for KCl: State-3 rates of respiration were identical, and NAD reduction and light-scattering changes in State 4 showed similar quantitative and kinetic characteristics; respiratory-control ratios were characteristically low, usually between 2 and 3.

Redox span NAD-cytochrome c

Initial studies were directed towards establishing the magnitude of available energy from oxidoreduction and this was attempted by estimation of the redox span between NAD and cytochrome c. Ideally, the proportions of free NAD^+ and NADH are

required and this is usually done indirectly (see Williamson *et al.*, 1967). Since no convenient substrate couple is available in flight-muscle mitochondria, direct enzymic estimations of extracted NAD^+ and NADH were performed (Table 1). In confirmation of the results obtained from fluorimetry (Hansford, 1972), with pyruvate as substrate, NAD was highly oxidized in State 4, becoming more reduced in State 3, whereas glycerol 3-phosphate gave considerable NAD reduction in State 4 and almost none in State 3. Furthermore the absolute amounts of NAD recovered ($3.63 \pm 0.18 \text{ nmol/mg}$ of protein) are in good agreement with previously published values (Price & Lewis, 1959; Birt, 1961). The scatter on the values with glycerol 3-phosphate was due principally to the variation in the total NAD pool when expressed on a protein basis (possibly because of varying degrees of contamination from broken mitochondria). When expressed on the basis of percent of total reduction, the values for glycerol 3-phosphate become 65.1 ± 2.0 in State 4 and 1.1 ± 0.6 in State 3.

The proportion of reduced cytochrome c was

Table 1. *Redox state of NAD during States 4 and 3 with pyruvate and with glycerol 3-phosphate as substrate*
The pyruvate incubations were carried out by adding mitochondria (about 26mg of protein) to a basal medium containing 2.5mM-pyruvate, 0.5mM-ATP, 2.5mM- HCO_3^- , 20mM- P_i , 10 μM -RbCl and 0.5 μM -valinomycin, the final volume being 20ml. After 5min, ADP was added to 2.5mM and after initiation of State-3 respiration duplicate double samples were removed and treated as described in the Experimental section. When State-4 respiration was resumed, two more double samples were removed for analysis. The glycerol 3-phosphate incubations were carried out in a similar fashion by adding mitochondria (about 13mg of protein) to a basal medium containing 10mM-DL-glycerol 3-phosphate, Ca^{2+} (buffered to 0.3 μM , final concentration, with 0.75mM- CaCl_2 /1mM-EGTA at pH7.2, 5mM- P_i , 10 μM -RbCl and 0.5 μM -valinomycin, the final volume being 20ml. ADP was added to 1mM after 1min, permitting duplicate double samples to be removed and quenched in State 3; two more double samples were removed in the ensuing State 4. The values shown are means $\pm$ S.E.M. for duplicate samples from separate incubations with different preparations of mitochondria. The mean total NAD content was 3.63 ± 0.18 (16) nmol/mg of protein.

Substrate	State	Concentration (nmol/mg of protein)	
		NAD^+	NADH
Pyruvate	4	3.78 ± 0.09 (4)	0.02 ± 0.01 (4)
Pyruvate	3	3.52 ± 0.05 (4)	0.29 ± 0.06 (4)
Glycerol 3-phosphate	4	1.23 ± 0.19 (4)	2.26 ± 0.23 (4)
Glycerol 3-phosphate	3	3.38 ± 0.64 (4)	0.05 ± 0.03 (4)

estimated under the conditions used for the NAD determinations (Table 2). Again, with pyruvate as substrate an increased reduction was observed on going from State 4 to State 3 respiration, whereas the opposite behaviour was seen with glycerol 3-phosphate as substrate, confirming the results of Hansford (1972).

Having estimated the redox state of NAD and of cytochrome *c*, it was possible to compute the respective redox potentials relative to the hydrogen electrode and then to calculate the redox span over the two phosphorylation sites (see Table 3). The results

of the calculations (Table 3) indicate that the transition from State 4 to State 3 is associated with a rise in the value for the redox span when pyruvate is the principal substrate, and a fall in the magnitude of the redox span when glycerol 3-phosphate is used. The former response can be understood on the basis of the known control by the ratio ATP/ADP at the dehydrogenase level (Johnson & Hansford, 1975), whereas the latter response is characteristic of

Table 2. Redox state of cytochrome *c* during States 4 and 3 with pyruvate and with glycerol 3-phosphate as substrate

Reduced cytochrome *c* was measured as described in the Experimental section using about 1.4 mg of mitochondrial protein in a final volume of 2 ml, but otherwise the incubation conditions of Table 1. All determinations were done in duplicate, including that for the measurement of the 100% value (see the Experimental section), and the values presented are means $\pm$ S.E.M. from different preparations of mitochondria.

Substrate	Reduction (%) in	
	State 4	State 3
Pyruvate	8.7 $\pm$ 0.7 (4)	14.6 $\pm$ 1.8 (4)
Glycerol 3-phosphate	59.0 $\pm$ 2.4 (4)	42.5 $\pm$ 3.8 (4)

Table 3. Redox span NAD–cytochrome *c* during States 4 and 3 with pyruvate and with glycerol 3-phosphate as substrate

The redox potential values of NAD and cytochrome *c*, E_h NAD and E_h cytochrome *c* respectively, were calculated as described in the Experimental section and by using the data of Tables 1 and 2. The redox span, ΔE_h , is defined as E_h cytochrome *c* minus E_h NAD; the error limits on ΔE_h were computed by assuming that the variance of a sum (or difference) is the sum of the individual variances, and are expressed as $\pm$ S.E.M.

Substrate (State)	E_h NAD (mV)	E_h cytochrome <i>c</i> (mV)	ΔE_h (mV)
Pyruvate (4)	-278 $\pm$ 6	296 $\pm$ 2	574 $\pm$ 6
Pyruvate (3)	-317 $\pm$ 3	281 $\pm$ 4	598 $\pm$ 5
Glycerol 3-phosphate (4)	-358 $\pm$ 1	226 $\pm$ 3	584 $\pm$ 3
Glycerol 3-phosphate (3)	-298 $\pm$ 2	243 $\pm$ 4	541 $\pm$ 4

Fig. 1. Phosphorylation of externally added ADP driven by the oxidation of pyruvate and of glycerol 3-phosphate (a) Pyruvate. Mitochondria (about 2.3 mg of protein) were suspended in 5 ml of an air-saturated basal medium containing the additions noted in Table 1 except that ADP was added to approx. 0.9 mM. Sampling as described in the Experimental section was carried out at the times indicated. Adenine nucleotides were separated and their concentrations determined as described in the Experimental section. (b) Glycerol 3-phosphate. Mitochondria (about 1.8 mg of protein) were suspended in 5 ml of O_2 -saturated basal medium containing the additions noted in Table 1, except that ADP was added to approx. 0.9 mM. Sampling and treatment of samples was as described in (a). The adenine nucleotides are: $\circ$, ATP; Δ , ADP; $\square$, AMP.

phosphate-acceptor control at the level of the respiratory chain. Nevertheless, the absolute values for the redox span in State 4 are strikingly similar, and this is in spite of the very different redox potential exhibited by the individual components in the two systems.

Generated phosphorylation potential

Phosphorylation potentials generated by pyruvate and by glycerol 3-phosphate oxidation were measured in the total suspension under the conditions used for the redox estimations but at lower protein concentration. The modification was deliberate, since it was desired to measure the phosphorylation potential in the medium rather than in the mitochondrial matrix space, and by lowering the protein concentration the contribution from the matrix component is made negligible. An illustrative experiment in which the concentrations of ATP, ADP and AMP were determined is shown for both pyruvate and glycerol 3-phosphate in Fig. 1. After 3 min of incubation with ATP and HCO_3^- , pyruvate oxidation (Fig. 1*a*) was able to support as high an ATP/ADP ratio as observed at later time points, and in particular the ratio found after the State 3 $\rightarrow$ State 4 transition was no higher than that generated initially. It seems permissible to conclude that during the early time points in Fig. 1(*a*) this pseudo-State 4 was indistinguishable from the true State 4 seen after ADP addition. With glycerol 3-phosphate as substrate (Fig. 1*b*) no such conclusion is possible, since no labelled nucleotide was present before the addition of ADP. A noteworthy feature of both experiments was the finding of a rapid disappearance of AMP on addition of ADP. This is reminiscent of the observation of Danks & Chappell (1974), although there is a crucial difference between the two types of experiment: Danks & Chappell (1974) were observing primarily the behaviour of adenine nucleotides within the matrix compartment, whereas the experimental design used here focuses attention on the extramatrix space. It is probable that the underlying cause of AMP disappearance is the same in both instances and reflects the dual distribution of adenylate kinase on the two sides of the inner membrane.

P_i estimations were performed only on samples from later time points, since an accurate measure of the phosphorylation potential during the net phosphorylation of ADP is not especially informative. Data presented in Table 4 are for State 4 only, as judged from both the O_2 electrode recording and the steady-state phosphorylation-potential values. ΔG values (Table 4) are significantly different for the two substrates and this is due almost entirely to the different final P_i concentrations, the ATP/ADP ratios being very similar. This may be a significant observation, indicating a kinetic limitation by either the

the mitochondrial ATPase or adenine nucleotide translocase, since other experiments (not shown) indicated that with glycerol 3-phosphate as substrate and with a lower P_i concentration, the ΔG value was significantly increased, but the ATP/ADP ratio was maintained between 15 and 20.

Measurement of protonmotive force

The ion-distribution method of Nicholls (1974*b*) was used with the modifications noted in the Experimental section. The two centrifugation methods (see the Experimental section) were preferred over the membrane-filter filtration method of Nicholls (1974*b*), since they gave significantly smaller extramatrix volumes, and therefore less of a correction was required for the calculation of concentration gradients across the inner membrane (see the Experimental section): whereas in our hands the filtration method gave typical extramatrix volumes of 25 μl (with a matrix volume of approx. 0.3 μl), comparable values for the centrifugation methods were 7 μl in the absence of silicone and 0.5 μl in its presence. Clearly, use of the filtration method with even a 10-fold concentration gradient inside-to-out demands a subtraction of 90% of the total and is inaccurate for this reason. Furthermore binding of ^{86}Rb to cellulose nitrate filters has been reported (Schuldiner & Kaback, 1975) which would lead to an overestimation of the membrane-potential component, $\Delta\psi$.

Initial experiments were performed with the centrifugation method in the absence of silicone, in which case it was possible to add substrates and O_2 to the dense lower phase into which the mitochondria were centrifuged. These precautions were designed to delay the time of anaerobiosis and substrate

Table 4. *Measurement of phosphorylation potential in State 4 with pyruvate and with glycerol 3-phosphate as substrate*

The ratio [ATP]/[ADP] is a mean value derived from the last three samples of the appropriate incubation illustrated in Fig. 1 and similarly from the last three samples of a duplicate experiment using a different mitochondrial preparation. These samples were judged to be representative of State 4 from the constancy of the generated ratio and from the O_2 electrode recording. P_i was also estimated in the same samples as described in the Experimental section, giving the mean values shown. ΔG values were calculated from the pooled data as described in the Experimental section and are means $\pm$ S.E.M.

Substrate	[ATP]/ [ADP]	[P_i] (M)	ΔG (KJ/mol)
Pyruvate	16.5	0.0185	50.81 ± 0.29 (6)
Glycerol 3-phosphate	18.7	0.0039	55.07 ± 0.17 (6)

depletion until the mitochondria were well within the lower phase, all of which was taken for scintillation counting. However, the silicone method gave identical results, was technically easier to perform and gave more closely grouped triplicate samples within a given experiment. These advantages were such that most experiments, including all of those reported here, were done by the silicone method. The single disadvantage of the method was that dextran was required in the incubation medium (LaNoue *et al.*, 1973) in order to facilitate passage of the mitochondria through the silicone layer, and at the minimum concentration of dextran for optimum centrifugation (30 mg/ml), pyruvate, but not glycerol 3-phosphate, oxidation was inhibited by approx. 15%. Following a report that aspartate would 'prime' pyruvate oxidation in flight-muscle mitochondria (Danks & Chappell, 1975), the effect of adding aspartate (to 2.5 mM) to the pyruvate-ATP- HCO_3^- system was tested: aspartate counteracted the inhibition due to dextran, elevating State-3 rates of respiration and the degree of NAD reduction (observed fluorimetrically) in State 3 so as to be more comparable with that observed in the absence of dextran. Aspartate was therefore included in all experiments where pyruvate was the principal substrate.

Control experiments (not shown) performed at different protein concentrations demonstrated that the spaces for accumulated markers, after correction for extramatrix space, were linear with added protein (see Nicholls, 1974b). However, when $10 \mu\text{M}$ - $^{86}\text{RbCl}$ alone was used as an indicator of membrane potential, extrapolation of the line to zero protein concentration gave a positive intercept on the ordinate, indicating Rb binding to some component of the system other than mitochondrial protein. It is conceivable that Rb was binding to valinomycin, since the same amount of valinomycin was present at each protein concentration. In any event, this inaccuracy was abolished on addition of KCl to 0.2 mM.

Further control experiments focused on the determination of the protonmotive force developed in the absence of substrate and in the presence of either rotenone (1 $\mu\text{g}/\text{ml}$) or fresh NaCN (2 mM) to block endogenous metabolism, plus 1 μM -carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone to equilibrate the electrochemical activity of protons. This particular measurement was considered to give the best index of binding or non-ideal behaviour of the indicators, since under these circumstances, a protonmotive force of 0 mV is predicted (Mitchell & Moyle, 1969a) and has been verified (Nicholls, 1974a,b). With our experimental conditions, however, the membrane-potential component was negative outside and always less than the pH gradient (acid outside), giving overall a positive value for the protonmotive force. It should be noted that S^{14}CN^- was present so that the reversed polarity of the membrane-

potential component was measured with certainty. The pH component, measured by $[^3\text{H}]$ acetate distribution, may have been overestimated, since it was found that on raising the acetate concentration from 10 to 100 μM , the pH component was apparently diminished. As 5 mM- P_i was also present it is unlikely that the additional acetate was affecting the pH gradient itself; it seems more probable that acetate binding was responsible for the error and was made less significant at the higher acetate concentration. The following typical values were obtained at the protein concentration used for the experiments reported below, namely 0.6 mg/ml; $\Delta\psi$, the membrane-potential component, = -22.3 mV; $-59\Delta\text{pH}$ = 41.2 mV; Δp , = $\Delta\psi - 59\Delta\text{pH}$ = 18.9 mV. This uncertainty in the measurement was felt to be sufficiently small as to justify ignoring its possible contribution to the values reported below. To have raised the acetate concentration further might have influenced significantly the absolute magnitude of the pH gradient, and was not attempted. The final incubation conditions were as described in the Experimental section. The contributions of $\Delta\psi$ and $-59\Delta\text{pH}$ to the protonmotive force determined under non-energized conditions are quite different from those reported by Nicholls (1974b), who invariably found a positive $\Delta\psi$, with mitochondria from rat liver. It is probable that the difference stems from the lower K^+ content of blowfly-flight-muscle mitochondria as prepared.

Protonmotive force in State 3, State 4 and intermediate states

Fig. 2 illustrates typical experiments in which the magnitude of the protonmotive force, Δp , was estimated with pyruvate (Fig. 2a) and with glycerol 3-phosphate (Fig. 2b) as substrate. Qualitatively the behaviour of the protonmotive force on addition of ADP was very similar for the two substrates and changes noted in State 3 were completely reversed in the ensuing State 4. With pyruvate as substrate, the protonmotive force in State 4 was apparently greater than with glycerol 3-phosphate and was decreased in State 3 by a decrease in the magnitude of both components. With glycerol 3-phosphate as substrate, the decrease in protonmotive force in State 3 was of similar magnitude to that with pyruvate, but was the result of a large decrease in the membrane-potential component and a slight increase in $-59\Delta\text{pH}$. The latter behaviour has not been observed previously in any system during State 4 $\rightarrow$ State 3 transitions (see Rottenberg, 1975). It is worth noting that although the experiments reported in Fig. 2 were designed to give comparable estimates of the protonmotive force with the two substrates, other preparations of mitochondria yielded somewhat different values for the protonmotive force, particularly with pyruvate as substrate. This variability was reflected in the State-3 rates of respiration with pyruvate as substrate, in

Fig. 2. Measurement of protonmotive force during States 4 and 3 with pyruvate and with glycerol 3-phosphate as substrate. Mitochondria (4.8 mg of protein) were added to 8 ml of a basal medium containing the additions noted in Table 1 plus dextran and radioactive markers as explained in the Experimental section. At the points indicated, 300 μl samples were removed and centrifuged as described in the Experimental section, the times of sampling corresponding to the starting of the centrifuge. The values of the membrane-potential component, $\Delta\psi$ (●), the chemical component, $-59 \Delta\text{pH}$ (▲), and their arithmetic sum, Δp (■), were derived as explained in the Experimental section. ADP was added where indicated to 2.5 mM in the pyruvate experiment (a) and to 1.5 mM in the glycerol 3-phosphate experiment (b).

that it was consistently observed that the higher rates of respiration were associated with the higher values for protonmotive force. Additional representative values are to be found in other experiments (e.g. Figs. 3 and 5, and Table 6) and are summarized in Table 5.

The continuous sampling of a single incubation as illustrated in Fig. 2 is limited in its time-resolution and, in particular, it is difficult to compare the behaviour of the protonmotive force with respiratory activity. This problem may be surmounted by measuring the protonmotive force under steady-state conditions of respiration generated by ATP and controlled amounts of externally added ATPase (see Davis & Lumeng, 1975), which has the added advantage of making experimentally accessible the more physiological states between State 4 and State 3. Unfortunately, we were unable to prepare a soluble ATPase free of $(\text{NH}_4)_2\text{SO}_4$ that was able to maintain steady rates of respiration. Accordingly, hexokinase in the presence of glucose was used, which was less desirable in that P_i was removed continuously as glucose 6-phosphate, but it gave steady rates of respiration and increments in respiratory activity which were directly proportional to the amount of hexokinase added. P_i removal was minimized by performing a separate incubation for each particular respiratory state, and at its maximum the loss of P_i was no greater than 1.5 mM for the course of the

incubation with either substrate. The results of such an experiment are shown in Fig. 3. With pyruvate as substrate (Fig. 3a), the protonmotive force fell throughout the range of respiratory rates, and it appeared that $-59 \Delta\text{pH}$ was more responsible for this diminution than was the membrane-potential component. There was an apparent discontinuity in the curve describing protonmotive force on going from almost 90% of the State-3 rate to 100%, i.e. on going from a situation of limiting ADP to one of excess ADP. Presumably this reflects a shift from rate limitation by the ATP synthetase to limitation by the flow of hydrogen from substrate to water, at high ADP availability and consequent high matrix ADP concentrations. The influence of the dehydrogenase activation by ADP may be appreciated when Figs. 3(a) and 3(b) are compared: with glycerol 3-phosphate as substrate (Fig. 3b) there is a much more direct relationship between protonmotive force and respiration rate at the higher levels of respiratory activity. In fact, except for the single incubation done with MgCl_2 as the sole addition, the points lie close to a straight line (see Nicholls, 1974a). However, the displacement of this one point from the line was a reproducible finding and is not understood. It is noteworthy that the behaviour of the individual components, and in particular the rise in the pH component on activating respiration with glycerol 3-phosphate as substrate, was

Fig. 3. Protonmotive force as a function of respiration rate as varied with limiting ADP concentrations with pyruvate and with glycerol 3-phosphate as substrate

(a) Pyruvate. Incubations were carried out under the conditions of Table 1 with the following modifications: the medium contained dextran and radioactive markers (as explained in the Experimental section) plus 10mM-glucose; the final volume was 2ml, and 1.2mg of mitochondrial protein was added at time zero. After incubating for 4min the ATP concentration was raised to 2mM and at 6min, three samples were removed for centrifugation as in Fig. 2. Elevated rates of respiration were obtained in five separate incubations by adding MgCl_2 to 2mM (incubation 2), MgCl_2 plus increasing quantities of a dialysed hexokinase solution (see the Experimental section; incubations 3–5), and finally MgCl_2 plus excess hexokinase (twice that required to give a State-3 rate of respiration) plus 2mM-ADP in which case no ATP addition was made at 4min (incubation 6). Elevated rates of respiration were initiated at 5min, allowing 1min to elapse before removing the first of the three samples. O_2 electrode recordings from air-saturated medium were made immediately before the incubations were carried out. (b) Glycerol 3-phosphate. The design of the experiment was similar to (a) except that 2mM-ATP (when added) was present at the start of the incubation and samples were withdrawn after 90s. Additions of MgCl_2 , MgCl_2 and hexokinase etc., were made at 30s. O_2 electrode recordings were made from control experiments using O_2 -saturated medium. The points shown are means of the triplicate samples and s.e.m. is given when greater than 1mV. $\Delta\psi$ (●), $-59 \Delta\text{pH}$ (▲) and Δp (■) were derived as explained in the Experimental section.

entirely consistent with that observed previously in the single incubations (Fig. 2). Moreover, the small size of the errors on the triplicate samples removed at different times indicates that equilibration of the radioactive markers was complete by the earliest sampling time, 1min after stimulation of respiration.

Having obtained information on the respective magnitudes of the redox span, phosphorylation potential and protonmotive force, a comparison of these quantities may be made, as shown in Table 5. The data indicate that with either substrate in State 4, the energy of oxidoreduction (ΔE_h) and of phosphorylation (ΔE_{ATP}) are of extremely similar magnitude, whereas the measured protonmotive force is very much smaller. The possible implications of these

findings are considered in the Discussion section. Table 5 also shows that with pyruvate as substrate, the redox span increased in going from State 4 to State 3, whereas the protonmotive force fell significantly. The latter change may be more readily appreciated from the $\Delta\Delta p$ column in Table 5, where the State 4 $\rightarrow$ State 3 difference in protonmotive force is presented, the scatter on the absolute values being due to differences between individual mitochondrial preparations, as noted above. This apparent dissociation of the redox span and protonmotive force has not been observed before, primarily because in mammalian systems provision of ADP causes a decrease in each of the three measured parameters. Advantage has been taken of the insect system to explore this particular point more fully.

Table 5. *Comparison of redox span, phosphorylation potential and protonmotive force in States 4 and 3 with pyruvate and with glycerol 3-phosphate as substrate*

ΔE_h is the redox span NAD–cytochrome *c* (from Table 3) divided by 2 to give the redox span per phosphorylation site. ΔE_{ATP} is derived from the phosphorylation-potential data of Table 4 converted from kJ/mol into mV by dividing the values in Table 4 by $2F$ (considering ATP formation as a 2-electron process; F , the faraday); E_{ATP} was not determined in State 3 (n.d.). Δp is the protonmotive force expressed as a mean value $\pm$ s.e.m. with the numbers of mitochondrial preparations given in parentheses; each value taken for statistical analysis was itself the mean of at least three determinations on each preparation. $\Delta\Delta p$ is the mean difference in Δp between States 4 and 3 $\pm$ s.e.m. with the numbers of mitochondrial preparations given in parentheses. Other details are given in Tables 3 and 4.

Substrate (State)	ΔE_h (mV/site)	ΔE_{ATP} (mV)	Δp (mV)	$\Delta\Delta p$ (mV)
Pyruvate (4)	287 ± 3 (4)	264 ± 2 (6)	180 ± 6 (7)	
Pyruvate (3)	299 ± 3 (4)	n.d.	159 ± 13 (3)	30 ± 3 (3)
Glycerol 3-phosphate (4)	292 ± 2 (4)	286 ± 1 (6)	169 ± 3 (6)	
Glycerol 3-phosphate (3)	271 ± 2 (4)	n.d.	140 ± 4 (4)	31 ± 2 (4)

Table 6. *Effect of oligomycin and ADP on protonmotive force with pyruvate as substrate*

One incubation was performed using the conditions of Fig. 2(a). Mitochondria (4.8 mg of protein) were added to a final volume of medium of 8 ml to initiate the reaction. After 5 min, three samples were withdrawn and centrifuged as described in the Experimental section; at approx. 6 min 30 s, oligomycin was added to give 1.2 μ g/mg of mitochondrial protein and three more samples were taken (between 7 min 20 s and 8 min 20 s); finally, ADP was added to a concentration of 2 mM at 9 min and three samples were removed (between 9 min 30 s and 10 min 30 s). The three sets of triplicate samples are identified in the Table by the respective additions to the mitochondrial incubation. The components of Δp were calculated as explained in the Experimental section. The values are means $\pm$ s.e.m. for the triplicate samples.

Addition	$\Delta\psi$ (mV)	$-59 \Delta pH$ (mV)	Δp (mV)
None	118 ± 1.2	54 ± 0.3	172 ± 1.2
Oligomycin	$137 \pm 0.7^*$	54 ± 0.7	$190 \pm 0.7^*$
Oligomycin + ADP	$146 \pm 0.7^{*\dagger}$	53 ± 1.2	$199 \pm 1.7^{*\ddagger}$

* The value is significantly different from that with no addition, $P < 0.001$.

† The value is significantly different from that with oligomycin alone, $P < 0.001$.

‡ The value is significantly different from that with oligomycin alone, $P < 0.01$.

Behaviour of the redox span and protonmotive force with pyruvate as substrate

The results so far are consistent with the protonmotive force responding qualitatively to the behaviour of the phosphorylation potential. However, the chemiosmotic hypothesis (Mitchell, 1966) predicts that the protonmotive force should also be affected by the flow of reducing equivalents down the respiratory chain and ultimately by dehydrogenase

activity. In the State 4 $\rightarrow$ State 3 transition with pyruvate as substrate, ADP acts as an activator of isocitrate dehydrogenase, the primary locus of adenine nucleotide control in the tricarboxylate cycle (Johnson & Hansford, 1975), and as a substrate for phosphorylation. These two actions of ADP may be separated by the inclusion of oligomycin to inhibit the mitochondrial ATPase, in which case only the dehydrogenase activation is observed (Johnson & Hansford, 1975). An experiment in which protonmotive force was measured in the presence of oligomycin and ADP is presented in Table 6. Addition of sufficient oligomycin to inhibit completely ADP-stimulated respiration (see Fig. 4, below) increased the protonmotive force significantly above the State-4 value, and a further addition of ADP gave a significant increase over the value found with oligomycin alone. In both instances, the increase in protonmotive force was due solely to an increase in the membrane-potential component ($\Delta\psi$) with no significant change in $-59 \Delta pH$. The reason for the increase in protonmotive force with oligomycin alone may be related to the presence of extramitochondrial ATPase of myofibrillar origin. In any event, such behaviour is not unexpected, since addition of oligomycin alone under these conditions frequently leads to enhanced NAD reduction (observed fluorimetrically), indicating dehydrogenase activation. Although the redox span was not measured accurately under these conditions, it was certainly increased on addition of oligomycin and ADP: cytochrome *c* (measured as described in the Experimental section) went from 9% reduced in State 4 to 21% reduced with oligomycin plus ADP, a modest change, whereas NAD (estimated fluorimetrically) appeared to be almost totally reduced by oligomycin and ADP. Hence (and cf. Tables 1 and 2), the redox potential of NAD was made negative to a far greater extent than was that of cytochrome *c*, and the redox span NAD–cytochrome *c* was increased. None of these changes was observed with glycerol 3-phosphate

as substrate, indicating an insensitivity of the glycerol 3-phosphate dehydrogenase to ADP.

Having established that the protonmotive force was indeed sensitive to dehydrogenase activity, it remained to be answered why the protonmotive force was decreased on transition from State 4 to State 3, while the redox span NAD–cytochrome *c* was increased (Table 3). Evidently ADP potentiates ATP synthetase activity, with the tendency to diminish protonmotive force, more than it activates at the dehydrogenase level. This suggests that there is an excess activity of ATP synthetase (and the adenine nucleotide translocase) in State 3. Some indication that this may be the case is seen in Fig. 4 which illustrates an experiment examining the oligomycin-sensitivity of ADP-stimulated pyruvate oxidation. The marked sigmoidicity of the inhibition curve is reminiscent of that observed previously with rat liver mitochondria (Ernster *et al.*, 1963; Lardy *et al.*, 1964) and is similarly attributed to an excess activity of ATPase over that required for State-3 pyruvate oxidation. It should be remembered, however, that NaCl media give suboptimal State-3 rates of pyruvate oxidation and so this conclusion may not necessarily apply to fly mitochondria *in situ*.

Effect of uncoupling agent on the measured protonmotive force

As a further means of assessing the applicability of the measurements of protonmotive force to energy

Fig. 4. Inhibition of ADP-stimulated pyruvate oxidation by oligomycin

Mitochondria (1.2 mg of protein) were incubated in 2 ml of an air-saturated basal medium containing the additions noted in Table 1. Oligomycin, when present, was added 5 min after the mitochondria and ADP (to 2 mM, an excess) was added 1 min later. Rates of respiration before and after ADP addition were measured, allowing the data to be presented as an inhibition of the ADP-stimulated rate.

conservation in flight-muscle mitochondria, the effect of the uncoupling agent carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was examined. With pyruvate as substrate (Fig. 5a), the concentration of uncoupling agent was restricted to a range (0–0.25 μM) in which respiratory rates were linear; at higher concentrations, a progressive inhibition of respiration was observed. With glycerol 3-phosphate as substrate (Fig. 5b) no such inhibition was found, permitting a wider range of uncoupler concentration to be used. The qualitative behaviour of protonmotive force was similar to that found when respiration was stimulated by ADP (Figs. 2 and 3): with pyruvate as substrate (Fig. 5a), protonmotive force fell throughout, owing to a progressive decrease in both of the individual components, except at the highest uncoupler concentration, when the pH component was increased. Thus the components measured at the highest uncoupler concentration were similar to those found when no substrate was present (see above). This was also true when glycerol 3-phosphate was used as substrate (Fig. 5b), although in this case there was no discontinuity in the curve describing –59 ΔpH, since this component increased throughout, similarly to its behaviour in the presence of ADP (Figs. 2 and 3).

On plotting the data from the experiment of Fig. 5 as a function of respiration rate, the curves shown in Fig. 6 emerge. The two curves show broadly similar characteristics (the curve obtained with glycerol 3-phosphate could also be resolved approximately into two intersecting straight lines) and, as discussed by Nicholls (1974a), indicate a dependence between the measured protonmotive force and respiratory rate up to a limiting rate of O₂ consumption determined by the maximum activities of the dehydrogenases and/or respiratory chain.

Finally, if it is assumed that a constant number of protons is extruded per loop of the respiratory chain (Mitchell, 1966) or per phosphorylation site, then, at steady state, a given respiration rate corresponds to a given rate of proton leakage across the mitochondrial inner membrane. By using the appropriate protonmotive-force value from Fig. 5 a membrane conductance to protons may be derived for each concentration of uncoupling agent. For the purposes of this calculation, a value of 3H⁺ translocated per phosphorylation site has been used. This is based on the determinations of Brand *et al.* (1976) rather than those of Mitchell & Moyle (1967) and is consistent with the thermodynamic data of this study, presented in Table 5 (see the Discussion section). Assumption of a different value would yield the same conclusions. The results (Fig. 7) indicate a similar non-linear dependence of the conductance on the uncoupler concentration for the two substrates. The similarity of the two curves is noteworthy, since it indicates that the uncoupler is acting by a common

Fig. 5. Protonmotive force measured as a function of uncoupling agent concentration with pyruvate and with glycerol 3-phosphate as substrate

(a) Pyruvate. Individual incubations were performed for each uncoupling agent concentration, each containing 1.2 mg of mitochondrial protein in a final volume of 2 ml. The medium was that of Table 1 with the additions noted for the same Table plus dextran and radioactive markers as explained in the Experimental section. Uncoupling agent, when present, was added 5 min after the mitochondria and triplicate samples were removed 1 min later. Each incubation was preceded by a control experiment using air-saturated medium in which an O_2 electrode monitored the course of the reaction. (b) Glycerol 3-phosphate. The incubations were similar to (a), additions to the basal medium being as described for Table 1 and in the Experimental section. A different mitochondrial preparation was used but the protein concentration was as in (a). Samples were removed 90 s after the addition of mitochondria and 1 min after addition of uncoupling agent, when present. Control experiments using an O_2 electrode were done in O_2 -saturated medium, but were related to air-saturated conditions by repeating the incubation lacking uncoupling agent in medium saturated with air. $\Delta\psi$ (●), $-59\Delta\text{pH}$ (▲) and Δp (■) were calculated as described in the Experimental section and are mean values. S.E.M. is presented when greater than 1 mV. The uncoupler was carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

mechanism in the two systems, consistent with the prediction (Mitchell, 1966) that uncouplers act by increasing the proton permeability of the mitochondrial inner membrane. The curvature of the lines in Fig. 7 is marked and possibly minimized by the over-estimation of Δp values close to 0 mV (see above). Corresponding log-log plots of the data gave gradients of 1.75 for pyruvate and 1.77 for glycerol 3-phosphate, possibly indicating the interaction of two uncoupler molecules in promoting proton permeability.

Behaviour of the component $-59\Delta\text{pH}$ on stimulation of respiration

It has been noted above (Figs. 1, 2 and 5) that whenever respiration was stimulated with pyruvate as

substrate, the protonmotive force diminished, owing to a fall in both the membrane-potential component, $\Delta\psi$, and $-59\Delta\text{pH}$, as in mammalian mitochondria (Mitchell & Moyle, 1969a; Nicholls, 1974b; Rottenberg, 1975). However, when glycerol 3-phosphate was oxidized, stimulated respiration was associated with a large drop in the electrical component, $\Delta\psi$, and a small rise in $-59\Delta\text{pH}$. The reason for the unusual behaviour with glycerol 3-phosphate was possibly due to the collapse of an ion gradient not reproduced in the pyruvate system. Accordingly the 0.12M-NaCl normally used was replaced in separate experiments by 0.25M-sucrose, 0.08M- Na_2SO_4 and 0.12M-Tris/HCl. However, in no case was there a significant decrease in $-59\Delta\text{pH}$ on

Fig. 6. Relationship between protonmotive force and respiration rate as varied with uncoupling agent

The data of Fig. 5 have been replotted against the respiration rates obtained in the control experiments described in the legend to that Figure. Δ represents Δp values with pyruvate as substrate (see Fig. 5a and $\square$ represents Δp values with glycerol 3-phosphate as substrate (see Fig. 5b).

stimulating glycerol 3-phosphate oxidation with $0.2 \mu\text{M}$ -carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine.

The possibility that the pyruvate system was aberrant was also considered, since the conditions used here are quite different from those used by Mitchell & Moyle (1969a) and by Nicholls (1974b). In particular, pyruvate oxidation produces 3 mol of CO_2 /5g-atoms of O consumed. Hence, if in states of elevated respiration, CO_2 equilibrates with the matrix water more rapidly than it does with the medium, presumably owing to carbonic anhydrase in the matrix compartment, an asymmetric acidification of the matrix space and consequent lowering of $-59 \Delta\text{pH}$ might result. This possibility was tested in separate incubations, (i) by the inclusion of 5-acetamido-1,3,4-thiadiazole-2-sulphonamide (Diamox) to 0.5 mM (to inhibit the presumed mitochondrial carbonic anhydrase) and (ii) by adding externally 0.5 mg of carbonic anhydrase (to ensure rapid equilibration of CO_2 with the medium). In neither case was the diminution of the chemical component, $-59 \Delta\text{pH}$, on stimulation of respiration reversed, so that an easy solution to this problem was not forthcoming.

Fig. 7. Membrane conductance to H^+ as a function of uncoupling agent concentration

Membrane conductance to H^+ was derived as follows: by multiplying the rates of respiration in Fig. 6 by the number of protons assumed to be translocated per loop (3) and by the number of loops assumed to be required as judged from P/O ratios [three for pyruvate (Δ) and two for glycerol 3-phosphate ($\square$)], a proton current in nequiv./min per mg of protein is obtained; the proton current was divided by the appropriate Δp value in Fig. 5 giving the membrane conductance to H^+ at a given concentration of uncoupler (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine).

Discussion

Energy status of the mitochondria during controlled pyruvate oxidation

The principal reason for undertaking this work was to determine whether pyruvate oxidation, characterized by a high degree of respiratory control, an adenine nucleotide control at the dehydrogenase level, and oxidized respiratory-chain components in the controlled state, was sufficient to maintain a high-energy state in State 4. The answer is unambiguous: by comparison with glycerol 3-phosphate oxidation (which is not subject to adenine nucleotide control at the dehydrogenase level and gives rise to relatively reduced respiratory-chain components in State 4) and by reference to three indices of mitochondrial energy potential, namely the respective magnitudes of the redox span NAD-cytochrome *c*, phosphorylation potential and protonmotive force, controlled pyruvate oxidation preserves a high energy state. An examination follows of the details relating to this claim.

The determination of the redox span NAD-

cytochrome *c* was based on measurements of total NAD⁺ and total NADH extracted from mitochondria and a midpoint potential assumed to be appropriate for mitochondrial matrix conditions. As discussed by Williamson *et al.* (1967), the use of total nucleotide estimations may lead to the NAD couple being made more negative than is actually the case, but this problem is not easily surmounted in these mitochondria owing to the absence of an appropriate substrate couple. There is also some doubt about the correctness of the standard value for the NAD couple (−320 mV at pH 7.0) as it relates to intramitochondrial conditions. Erecinska *et al.* (1974), determined that the midpoint potential for the NAD⁺/NADH couple in the matrix of pigeon heart mitochondria was −296 mV, but they assumed that the intramitochondrial pH was that of the suspending medium and that NADH fluorescence was linearly related to NADH free within the matrix space. In view of these unsubstantiated assumptions, there appears to be no good reason for departing from the value used here.

A comparison of the redox span NAD–cytochrome *c* with pyruvate and with glycerol 3-phosphate as substrate (Table 3) indicated a striking similarity between the two conditions in State 4, in spite of the very different redox states of the individual components. Since glycerol 3-phosphate causes NAD reduction by reversed electron transfer, an energy-dependent process (Chance & Hollunger, 1961; Klingenberg & Bücher, 1961), there is no reason for assuming a de-energized mitochondrion on the basis of the oxidized nature of the respiratory-chain components when pyruvate is the substrate.

The same conclusion is reached when the generated phosphorylation potentials are examined. Although glycerol 3-phosphate appeared to be capable of producing somewhat higher phosphorylation potentials, evidence was cited indicating that this was due to a kinetic limitation dependent on the ratio ATP/ADP together with a lower P_i concentration used in the glycerol 3-phosphate experiments. In any event, the phosphorylation potential values in State 4 with the two substrates were sufficiently close to the redox-potential spans discussed above for the two processes of electron transfer and ATP formation in the region NAD–cytochrome *c* to be considered close to equilibrium (see Rolleston, 1972), in general agreement with the behaviour of other mitochondria (Wilson *et al.*, 1973; Erecinska *et al.*, 1974; Slater *et al.*, 1973).

Estimates of protonmotive force in State 4 also support the argument favouring a high energy state during controlled pyruvate oxidation. The values for protonmotive force are at least as high with pyruvate as substrate as with glycerol 3-phosphate, and on imposing an energy demand by presenting ADP, for example, the protonmotive force was decreased, as

found with liver mitochondria (Mitchell & Moyle, 1969a; Nicholls, 1974b). In every respect examined, therefore, pyruvate oxidation in State 4 is sufficient to maintain a high energy state.

Thermodynamic sufficiency of the measured protonmotive force

The data presented in Table 5 in which the redox-potential span, phosphorylation potential and protonmotive force are compared immediately suggest that protonmotive force is inadequate to account for its postulated role (Mitchell, 1966) as an intermediate between energy of oxidoreduction and of phosphorylation. Implicit in this are two assumptions, namely that the measurements themselves are not subject to systematic error and that the proton stoichiometry (the number of protons translocated per 2 electrons per phosphorylation site) is 2 as found by Mitchell & Moyle (1967). Some discussion of these points follows.

The most likely cause of systematic error in this work is that the mitochondria were becoming anaerobic at the medium–silicone interface, so that a significant collapse of the ion-gradients occurred before entry into the silicone layer. Experiments designed to eliminate this potential problem by omitting the silicone were described above (see the Results section) and they indicated that anaerobiosis was not a significant factor. Some uncertainty was also noted in the measurement of the chemical component, −59 ΔpH, by acetate distribution. However, if, as seemed probable, acetate binding was the cause of the error, it would have made a negligible difference to −59 ΔpH when ΔpH was approx. 1 pH unit, and only assumed significance at values of ΔpH of less than 0.5. In any case, error of this type would have led −59 ΔpH, and hence protonmotive force, to be overestimated, contrary to what is required of an explanation of the low values. In summary, the low values of protonmotive force relative to redox-potential span and phosphorylation potential are not easily explained on the basis of error. Indeed, taken together with the information of Nicholls (1974b) and Wiechmann *et al.* (1975), on rat liver mitochondria, it seems to be generally true that the measured protonmotive force is numerically smaller than the phosphorylation potential, and, it may be assumed, the redox span per site. The easiest solution to this problem would be to adopt the formulation of Slater (1971) among others, and place the proton-pumping activity on a side pathway not necessarily in equilibrium with a more central high-energy intermediate. However, the experiments of Brand *et al.* (1976) have called into question the original proton stoichiometry of Mitchell & Moyle (1967). If a value of 3 H⁺ translocated per site (Brand *et al.*, 1976) is used, there is good agreement between the measured protonmotive force and that of approx. 190 mV expected

on theoretical grounds (see Mitchell, 1966). Proton-motive force, therefore, may be quantitatively adequate to account for its proposed function as a high-energy intermediate in oxidative phosphorylation, but only if this amended stoichiometry applies.

Behaviour of the protonmotive force and its components during experimental manipulations

Under conditions of respiratory inhibition and in the presence of uncoupling agent the protonmotive force tended to zero, having a positive chemical component ($-59 \Delta\text{pH}$ greater than zero) balanced by a negative electrical component. This situation is unlike that found in liver mitochondria where it is the chemical component that is reversed (Nicholls, 1974*b*), i.e. the matrix space is acidic relative to the medium. This different behaviour is probably a consequence of the respective ion contents of the mitochondria and the suspending medium: liver mitochondria, high in endogenous K^+ , suspended in a sucrose medium low in K^+ , will, in the presence of valinomycin, generate a membrane-potential positive outside, so that on addition of uncoupling agent the matrix becomes acidic; fly mitochondria washed in K^+ -free medium contain very little endogenous K^+ (R. G. Hansford, unpublished work), so that the dominant ion movement on suspension in NaCl media is probably a Na^+/H^+ exchange (see Mitchell & Moyle, 1969*b*). Such an exchange would tend to alkalize the matrix space and give rise to a reversed electrical component in the presence of uncoupling agent, as observed.

A point of particular interest in the flight-muscle mitochondria was the State 4 $\rightarrow$ State 3 transition with pyruvate as substrate, since it is on addition of ADP that the oxidation of pyruvate is accompanied by an activation of dehydrogenases and relatively reduced respiratory-chain carriers (Hansford, 1972; Tables 1 and 2 of this paper). The components of protonmotive force in State 4 were not unlike those found in liver mitochondria (Mitchell & Moyle, 1969*a*; Nicholls, 1974*b*), the major component being electrical. In the presence of ADP a fall in protonmotive force was observed during pyruvate oxidation due to a diminution in both electrical and chemical components. This was somewhat surprising, since the redox span NAD-cytochrome *c* was apparently increased under these conditions, and the protonmotive force and redox span are intimately related according to the chemiosmotic hypothesis (Mitchell, 1966). Although the midpoint potential of the NAD couple is less negative in State 3 owing to the lower ΔpH (approx. 0.3 unit) and consequent acidification of the matrix space, this can account for a change of not more than 10 mV in the redox poise of NAD (see, Clark, 1960), whereas the increase in the span is 24 mV. Furthermore the estimates of NAD^+ and NADH are total amounts, whereas it is the free species

that are required for the correct calculation of the redox potential. If the binding of NADH is of high affinity, it is possible that the redox state of NAD may be more negative in State 3 relative to State 4 than the measurements in Table 1 suggest. The increase in the redox span on going from State 4 to State 3 with pyruvate as substrate appears to be substantiated, therefore.

The contrary behaviour of the protonmotive force and redox span in the pyruvate system was attributed to an excess of ATPase activity over that required for State-3 pyruvate oxidation, implying that, before the establishment of the steady-state, dissipation of the proton gradient was more rapid than its rate of formation, in spite of the dehydrogenase activation. On titrating the ATPase activity with oligomycin, it is predicted that the dehydrogenase activation should exert a more pronounced effect on the protonmotive force. The limiting case is presented in Table 6 in which the protonmotive force was observed to increase on ADP addition, as expected. A limitation at the level of the adenine nucleotide translocase has been discounted by a similar sigmoid titration curve obtained with the inhibitor atractyloside (R. G. Hansford, unpublished work). Such an excess of activity is in keeping with the pronounced changes expected in the matrix adenine nucleotide content when effecting dehydrogenase activation.

Some indication of dehydrogenase activation may be discerned from the behaviour of the protonmotive force when the two substrates are compared. Whereas increasing rates of glycerol 3-phosphate oxidation and decreasing protonmotive force are simply related (Figs. 3*b* and 6), in agreement with Nicholls' (1974*a*) observations on brown-adipose-tissue mitochondria, this is not necessarily the case with pyruvate as substrate: when pyruvate oxidation was stimulated by limiting concentrations of ADP (Fig. 3*a*), the protonmotive force was not decreased in a regular fashion, the curve describing protonmotive force exhibiting a marked discontinuity. However, when pyruvate oxidation was stimulated by $0.05 \mu\text{M}$ - and $0.1 \mu\text{M}$ -uncoupling agent (Fig. 6) the protonmotive force fell to a greater extent and in a more simple manner than when ADP was used. The apparent linearity seen in Fig. 6 can be understood in terms of the ATP hydrolysis which must occur in order for pyruvate oxidation to be stimulated, since elevated rates of respiration depend absolutely on ADP provision with pyruvate as substrate (Van den Bergh, 1964); the greater decrease in protonmotive force at low uncoupler concentrations with pyruvate relative to glycerol 3-phosphate (Fig. 5) is more in keeping with the greater respiratory stimulation observed with pyruvate, and probably indicates the extent to which dehydrogenase activation contributed to the protonmotive force when ADP was provided externally (cf. Figs. 3*a* and 6).

The experiments conducted with uncoupling agent also allowed an estimation of the proton conductance of the mitochondrial inner membrane by use of the measurements of protonmotive force and by making an assumption with regard to the proton current (see the Results section). Although substituted phenylhydrazones generally give rise to a linear relationship between membrane conductance to protons and total uncoupler concentration (Liberman & Topaly, 1968; LeBlanc, 1971; Nicholls, 1974a), a pronounced non-linearity was found in the present work (Fig. 7), more in keeping with a square law. As an explanation of this behaviour, it is proposed that the unusual lipid composition of the flight-muscle mitochondrial membranes (Guarnieri *et al.*, 1976) favours the mobility of a dimeric form of the uncoupler anion (see Finkelstein, 1970), since membrane composition is an important determinant of uncoupler function (Hopper *et al.*, 1970). Another more trivial explanation of the sigmoid behaviour might be a non-specific binding of uncoupling agent, quantitatively more significant at the lower concentration, decreasing the amount available to act as a proton carrier within the hydrophobic phase of the inner membrane. Necessarily such binding would have to be of high affinity.

Nature of controlled respiration in flight-muscle mitochondria

Although it has been established that controlled respiration in flight-muscle mitochondria is associated with a high energy state, there are certain features of State 4 which demand further inspection. When analysed in terms of generated redox span, protonmotive force and phosphorylation potential, the oxidations of pyruvate and glycerol 3-phosphate are almost identical. However, the respiratory-control ratios exhibited by these two substrates are markedly different owing to the high rate of respiration seen with glycerol 3-phosphate in the controlled state. A possible explanation of this finding is that disrupted mitochondria can oxidize glycerol 3-phosphate, but not pyruvate owing to loss of cofactors. However, a discrepant finding is that the matrix volume in State 4 varied with the substrate being oxidized: with glycerol 3-phosphate, the volume is 2.0 $\mu\text{l}/\text{mg}$ of mitochondrial protein, whereas with pyruvate it is significantly smaller, being only 1.3 μl of mitochondrial protein (see the Experimental section). Taken by themselves these observations suggest a greater accumulation of salt (see Hansford & Lehninger, 1972) when glycerol 3-phosphate is oxidized and this in turn would depend on a greater availability of energy with this substrate. However, the three indices of energy potential do not support this proposition.

Although of limited physiological significance [since in the thorax of the resting insect, NAD is highly oxidized (Hansford, 1975), consistent with a rate

of glycerol 3-phosphate oxidation insufficient to generate a high energy state], the following model is proposed as a means of explaining these experimental observations. It is suggested that glycerol 3-phosphate can energize isolated flight-muscle mitochondria to an extent greater than that possible with pyruvate as substrate, possibly owing to lack of control at the dehydrogenase level. The greater energization with glycerol 3-phosphate results in a greater accumulation of salt and a larger matrix volume than with pyruvate as substrate. However, the greater swelling with glycerol 3-phosphate leads to greater stretching of the mitochondrial membrane and consequent increase in proton permeability, or dielectric breakdown (see Nicholls, 1974b). The increased proton permeability is then responsible for the elevated rate of State-4 respiration seen with glycerol 3-phosphate. According to this model the three indices of energy potential do not register the initially higher energy state with glycerol 3-phosphate, because the measurements are made in the steady state, at a later time. Thus the increased proton flux with glycerol 3-phosphate leads to a lowering of the protonmotive force and other energetic parameters to a point where, fortuitously, they are similar to those observed with pyruvate. It is suggested that the high matrix volume, and consequent membrane permeability, can be maintained during steady-state glycerol 3-phosphate oxidation because entry of Na^+ and Cl^- has occurred during the initial phase of rapid swelling. This leads to a failure fully to reverse swelling when the mitochondria are de-energized and has been documented by Hansford & Lehninger (1972). Thus the proton permeability need not necessarily decline with the protonmotive force.

There is some experimental evidence which may be taken to support this scheme; on examining fluorimetrically the energy-dependent reduction of NAD^+ by glycerol 3-phosphate, a spike of increased fluorescence followed by a decay phase to the steady state is observed (R. N. Johnson & R. G. Hansford, unpublished work) consistent with an initially higher degree of NAD reduction and an initially higher energy potential than found in the steady state. Furthermore a closer examination of the data presented in Fig. 7 is more in keeping with the model described above than with the explanation depending on the presence of broken mitochondria. As the concentration of uncoupling agent was increased over the range 0–0.15 μM , the conductance to protons (Fig. 7) increased to a much greater extent with pyruvate than with glycerol 3-phosphate as substrate, in part reflecting the sigmoid increase in glycerol 3-phosphate oxidation with increasing uncoupler concentration (not shown), and partly the relative insensitivity of the protonmotive force to uncoupling agent in the presence of glycerol 3-phosphate (cf. Figs. 5a and 5b). Whereas the explanation of

broken mitochondria being responsible for the lack of respiratory control would predict an identical uncoupler sensitivity of the protonmotive force and proton conductance with the two substrates, the model proposed here can account for the observed behaviour if it is assumed that the uncoupling agent promotes a partial loss of accumulated salt and decrease in matrix volume. In this way, the addition of uncoupling agent would promote the loss of the proton channel due to membrane stretching, but maintain proton conductance due to the introduction of proton ionophore. Thus the net effect on respiration, protonmotive force and membrane conductance to protons would be relatively slight, as observed.

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