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Equilibrium Relations between the Oxidation–Reduction Reactions and the Adenosine Triphosphate Synthesis in Suspensions of Isolated Liver Cells

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1. The redox state of cytochrome *c*, cytochrome *a* and the mitochondrial NAD couple, and the phosphorylation state of the adenine nucleotides, were measured in suspensions of isolated rat liver cells. 2. The ΔG for the transfer of two electrons from the mitochondrial NAD to the cytochrome *c* couple is calculated to be 104kJ (24.8kcal). 3. The ΔG associated with the synthesis of ATP at the measured phosphorylation state is calculated to be 95kJ (22.7kcal)/2mol of ATP. 4. The near equality of ΔG of the electron-transport process and ΔG required for ATP synthesis indicates near-equilibrium between the mitochondrial respiratory chain and the extramitochondrial phosphorylation state. 5. The existence of near-equilibrium in the coupled reactions implies that the respiratory activity depends on the ratio [ATP]/[ADP][P_i] and not on the concentrations of the individual reactants. 6. If the overall system of oxidative phosphorylation is at near-equilibrium, all intermediary reactions must also be at equilibrium. Hence if the intramitochondrial and extramitochondrial phosphorylation states are indeed different, it follows that any differences in the activities of ATP, ADP and P_i must be coupled to ion gradients and/or potentials across the inner mitochondrial membrane in such a way that translocation occurs without loss of free energy. 7. The metabolic state of the mitochondria in the cell can be defined by the turnover number of the cytochromes, the cytoplasmic phosphorylation state, and the oxidation–reduction potential of the NAD couple, rather than by the availability of ADP, substrate and O_2.

Previous work on the properties of the mitochondrial respiratory chain (Erecińska *et al.*, 1974); Wilson & Erecińska, 1972) has shown that the oxidation–reduction components between the NAD couple and cytochrome a_3 form near-equilibrium systems in intact mitochondria. These systems are of two types. The first consists of components of the same redox potential (E_h), referred to as isopotential groups. Group I includes NAD, iron–sulphur proteins and the flavoprotein of NADH dehydrogenase. The E_h of this group is approx. −0.30V. Group II includes ubiquinone, cytochrome b_{560} (cytochrome b_k of Sato *et al.*, 1971), iron–sulphur proteins and the flavoprotein of succinate dehydrogenase. The E_h of this group is approx. 0.01V. Group III includes cytochromes c_1, *c* and *a*, the iron–sulphur protein of Rieske *et al.* (1964) and copper protein. The E_h of this group is approx. 0.31V. Group IV includes cytochrome a_3 and possibly a copper protein. The E_h of this group is approx. 0.62V.

The second type of equilibrium system concerns the relationships between ATP synthesis and electron transport. These relationships may be described by the following type of equation:

$$\begin{array}{c}\text{Reduced carrier (Group I)} + \text{oxidized carrier (Group II)} + \text{ADP} + P_i \\ \Downarrow\Uparrow \\ \text{Oxidized carrier (Group I)} + \text{reduced carrier (Group II)} + \text{ATP}\end{array} \quad (1)$$

There are three reactions of this type, involving interactions between Groups (I) and (II), Groups (II) and (III) and Groups (III) and (IV). The overall result of the reactions between Groups I and III is

$$\text{NADH} + 2\text{cytochrome } c^{3+} + 2\text{ADP} + 2P_i \rightleftharpoons \text{NAD}^+ + 2\text{cytochrome } c^{2+} + 2\text{ATP} \quad (2)$$

The equilibrium constant *K* of this reaction is

$$K = \frac{[\text{NAD}^+]}{[\text{NADH}]} \times \frac{[\text{cytochrome } c^{2+}]^2}{[\text{cytochrome } c^{3+}]^2} \times \frac{[\text{ATP}]^2}{[\text{ADP}]^2[P_i]^2} \quad (3)$$

These relationships hold in suspensions of isolated mitochondria under a variety of conditions when the [NAD^+]/[NADH] ratio of the mitochondrial matrix and the extramitochondrial value of [ATP]/[ADP][P_i] are inserted (Wilson & Erecińska, 1972; Erecińska *et al.*, 1974). This implies that the components of eqn. (3) are at near-equilibrium. It must be emphasized that in eqn. (3) the [NAD^+]/[NADH] ratio refers to the free, as opposed to the protein-bound, nicotinamide–adenine dinucleotides, and this ratio can be calculated from the measured concentration of acetoacetate and 3-hydroxybutyrate by the method of Williamson *et al.* (1967). The [cytochrome c^{2+}]/[cytochrome c^{3+}] ratio can be ascertained spectrophotometrically and the concentrations of ATP, ADP and P_i can be determined by standard methods (see below).

The present experiments were designed to test whether the equilibrium in system (3) also exists in mitochondria in their natural environment, i.e. within the intact cell. The experiments were therefore carried out on isolated liver cells. When the [NAD^+]/[NADH] ratio was modified by changing [3-hydroxybutyrate]/[acetoacetate] or when [ATP]/[ADP] was modified by addition of adenosine, the other factors of eqn. (3) changed so that K remained constant.

Experimental

Rats

Female rats, of the Wistar strain, starved for 48h, weighing about 200g were used.

Reagents

Standard analytical-grade reagents were obtained from British Drug Houses Ltd., Poole, Dorset, U.K. All enzymes were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was a gift from Dr. P. G. Heytler, E. I. Du Pont de Nemours and Co. (Inc.), Central Research Department, Experimental Station, Wilmington, Del., U.S.A.

Rat liver cells

The liver cells were prepared by the method of Berry & Friend (1969) incorporating the modifications described by Cornell *et al.* (1973) and Krebs *et al.* (1973). The cells were suspended in Krebs–Henseleit (1932) saline containing 2.5% dialysed bovine serum albumin at a concentration of about 40mg wet wt./ml and were either used immediately or were incubated, with rapid shaking, at 25°C or 37°C. Incubations were in 25ml Erlenmeyer flasks containing 2ml of suspension (about 80mg of cells) and 1ml of additions (substrates and medium containing albumin). The gas space was filled with O_2+CO_2 (95:5) and sealed with rubber stoppers. $HClO_4$ (0.3ml of 20%, v/v) was added to the flask at the end of the incubation period when metabolites other than cytochromes were to be determined. The dry weight of the cell suspension and of the medium containing albumin was determined for each cell preparation. A factor of 3.7 was used to convert dry weight into wet weight of cells.

Measurement of the redox state of the cytochromes

A sample (3ml) of the incubated suspension (or, for controls, 2ml of cells plus 1ml of medium containing albumin) was added to a cuvette for measurement of the redox state of the cytochromes in an Aminco–Chance dual-wavelength spectrophotometer. The wavelength pairs 550–540nm for reduced cytochrome *c* and 445–460nm for reduced cytochrome *a* were used. The half-band width of the measuring light was 2.7nm for the α region and 4.5nm for the Soret region. The first measurement indicates the concentration of reduced cytochrome in the sample. By then causing complete oxidation, and afterwards complete reduction, of the cytochromes, the percentage reduction in the original sample can be calculated.

To prevent sedimentation, the suspension of the cells in the cuvette was continuously mixed with a vibrating stirrer and the additions were made with the aid of a Hamilton micro-syringe. After the initial spectrophotometric reading had become stable 20μM-rotenone and 10μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone were added to cause complete oxidation of the cytochromes, and the decrease in the concentration of reduced cytochrome was recorded. This reading was taken to correspond to 100% oxidation of the cytochrome because rotenone inhibits the transfer of electrons between NADH dehydrogenase and ubiquinone (Groups I and II). Thus the carriers on the O_2 side of the rotenone block become completely oxidized. As endogenous substrates (e.g. succinate, fatty acids) could still supply electrons to the respiratory chain, the uncoupler was added to ensure complete oxidation. In fact very little further oxidation was observed on the addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. However, as carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone itself absorbs at 445–460nm it was omitted when measurements were made at these wavelengths and the oxidation caused by rotenone was assumed to represent 100% oxidation. A relatively high concentration of inhibitor and uncoupler were necessary to overcome their binding to bovine serum albumin in the medium.

The fully reduced state of the cytochrome was taken to be that after the addition of 2mM-cyanide.

The use of cyanide is preferable to anaerobic conditions because suspensions contain traces of haemoglobin and the oxy–deoxy-haemoglobin transition interferes with the spectrophotometric measurements.

The redox potential (E_h) of the cytochromes was calculated according to the equation

$$E_h = E_{m7.0} + \frac{2.3RT}{nF} \log \left(\frac{[\text{oxidized carrier}]}{[\text{reduced carrier}]} \right) \quad (4)$$

where $E_{m7.0}$ is the half-reduction potential at pH 7.0, R is the gas constant, T is absolute temperature, n is the number of electrons and F is the Faraday constant (23.06 kcal/equiv. per V), 2.3 RT/nF being for cytochrome 0.06 V at 25°C.

Cytochrome *c* and cytochrome *a* are members of the same isopotential group and therefore their E_h values are virtually the same. In a series of measurements it was shown that if an $E_{m7.0}$ of 0.245 V was used for cytochrome *a*, then the same E_h value was found for cytochrome *c* as for cytochrome *a*. An $E_{m7.0}$ value of 0.245 V lies within the range 0.210–0.255 V (Lindsay & Wilson, 1972; Wilson & Brocklehurst, 1973) which is found in isolated mitochrondria at various phosphorylation states of the adenine nucleotide system.

Since the signal-to-noise ratio for cytochrome *a* was more than twofold higher than that for cytochrome *c*, mainly because of the greater absorbance changes, only the redox state of cytochrome *a* was measured in some experiments. However, because the E_m for cytochrome *c* (in contrast with that for cytochrome *a*), is more accurately known (+0.235 V ±0.005 V), is pH-independent and is unaffected by the phosphorylation state of the adenine nucleotides (Dutton *et al.*, 1970) the $E_{h(cyt.)}$ values in Tables 3 and 4 refer to cytochrome *c*.

Measurements of the mitochrondial [NAD⁺]/[NADH] ratio

[3-Hydroxybutyrate] and [acetoacetate] were measured by the method of Williamson *et al.* (1962) and the [NAD⁺]/[NADH] ratio was calculated by the method of Williamson *et al.* (1967). The E_h of the mitochondrial NAD couple was calculated by using the [3-hydroxybutyrate]/[acetoacetate] ratio according to eqn. (4); $E_{m7.0}$ was taken to be −0.266 (Krebs *et al.*, 1962).

Measurements of ATP, ADP and P_i

Tests showed that the ATP (Lamprecht & Trautschold, 1963) and ADP (Adam, 1963) measured were intracellular; neither was present in the supernatant. The bulk of these adenine nucleotides is assumed to be in the cytoplasmic space (see Krebs & Veech, 1969, 1970).

Measurement of P_i in the liver cells presents a special problem because, unlike the ketone bodies, the distribution of P_i between medium and cells is not even. In intact liver the [P_i] is about 4 mM (Stubbs *et al.*, 1972) whereas in circulating plasma it is only about 2 mM. The liver cells were prepared and suspended in a medium containing 1 mM-P_i and at the concentration of cells used (approx. 40 mg wet wt./ml) it is not possible to measure, with sufficient accuracy, the P_i content of the cells, as techniques for separating the cells, without disturbing the distribution of P_i, are not reliable. When [P_i] was measured by the method of Martin & Doty (1949), after washing and resuspending the cells in a P_i-free saline, values of 10 μmol/g wet wt. were obtained. When the livers were perfused with a medium containing 0.1 mM-P_i the isolated cells contained about 6 μmol of P_i/g wet wt. These findings indicate that the isolated cells accumulated P_i during the preparation. The two values (10 μmol/g wet. wt and 6 μmol/g wet wt.) have been used in the calculations of [ATP]/[ADP][P_i].

Thermodynamic symbols and conventions

The conventions and symbols used throughout this paper are those of Lewis & Randall (1923) except that ΔG is used instead of ΔF. In agreement with Clark (1960) the oxidation–reduction potential relative to the standard hydrogen electrode is designated E_h. The potential at 50% reduction at pH 7.0 is designated $E_{m7.0}$ (midpoint potential or half-reduction potential) in preference to the E_0' symbol (for justification see Clark, 1960).

Results

O_2 uptake

Under the conditions used the liver cells respire at a steady O_2 consumption rate of 1.3 μmol/min per g wet wt. at 25°C and 2.5 μmol/min per g wet wt. at 37°C in the absence of added substrate. In the presence of 10 mM-lactate and 10 mM-ethanol the O_2-consumption rates are 1.2 μmol/min per g wet wt. at 25°C and 2.2 μmol/min per g wet wt. at 37°C. This respiratory rate is slow enough to ensure that the cells remain aerobic during the course of the experiments.

Measurement of the redox and phosphorylation states

The data necessary for the calculation of the equilibrium relationships between the mitochondrial respiratory chain and the cytoplasmic phosphorylation state are shown in Tables 1 and 2. The ratio [3-hydroxybutyrate]/[acetoacetate] is low in the liver cells (approx. 0.35), in contrast with a value of 2.6 found in freeze-clamped liver (Stubbs *et al.*, 1972) and a ratio of between 0.99 and 1.36 found in the medium of a liver perfused without added substrate (Krebs *et al.*, 1969). This is due to the

Table 1. *Measurement of the oxidation–reduction states of cytochrome c and cytochrome a, the redox state of the NAD couple and the phosphorylation state of the adenine nucleotide system in suspensions of isolated liver cells*

Liver cells [25.9mg wet wt./ml (A) and 19.9mg wet wt./ml (B)] were incubated at 25°C for 15min. Measurements were made as described in the text. [ATP], [ADP], [P_i] and [cytochromes] are expressed in μmol/g wet wt. [3-Hydroxybutyrate] and [acetoacetate] are expressed in μmol/ml of incubation mixture. P_i was measured in a separate experiment (see the text). For details of the calculation of the ratio [cytochrome *a* oxidized]/[cytochrome *a* reduced] see the Experimental section. In Expt. A perfusion of the liver and incubation of the cells was with 1mM-P_i; in Expt. B the P_i concentration was 0.1mM.

	Concentration or ratio of metabolites			
	Expt. A		Expt. B	
Reactant or reactant ratio	1 No additions	2 10mM-Lactate 10mM-ethanol	1 No additions	2 10mM-Lactate 10mM-ethanol
3-Hydroxybutyrate	0.077	0.115	0.092	0.268
Acetoacetate	0.218	0.108	0.270	0.160
[3-Hydroxybutyrate]/[Acetoacetate]	0.35	1.05	0.34	2.13
Cytochrome *c* oxidized	0.013	0.012	—	—
Cytochrome *c* reduced	0.0025	0.0041	—	—
[Cytochrome *c* oxidized]/[Cytochrome *c* reduced]	5.2	2.93	6.55	6.55
Cytochrome *a* oxidized	0.0070	0.0067	0.0081	0.0081
Cytochrome *a* reduced	0.0021	0.0024	0.0018	0.0018
[Cytochrome *a* oxidized]/[Cytochrome *a* reduced]	3.33	2.79	4.5	4.5
ATP	1.65	1.60	2.29	2.02
ADP	0.32	0.27	0.48	0.42
P_i	10	10	6	6
[ATP]/[ADP][P_i]	516M^{-1}	593M^{-1}	795M^{-1}	802M^{-1}

Table 2. *Measurements of the oxidation–reduction states of cytochrome c and cytochrome a, the redox state of the NAD couple and the phosphorylation state of the adenine nucleotide system in suspensions of isolated liver cells in the presence and absence of adenosine*

Liver cells (21.7mg wet wt./ml) were incubated at 37°C for 60min in the presence of 10mM-lactate and 1mM-oleate. See Table 1 for other details.

	Concentration or ratio of metabolites	
Reactant or reactant ratio	Expt. C_1 No additions	Expt. C_2 +0.5mM-Adenosine
3-Hydroxybutyrate	0.489	0.705
Acetoacetate	0.532	0.406
[3-Hydroxybutyrate]/[Acetoacetate]	0.92	1.74
[Cytochrome *c* oxidized]/[Cytochrome *c* reduced]	5.2	3.7
Cytochrome *a* oxidized	0.0059	0.0054
Cytochrome *a* reduced	0.0016	0.0021
[Cytochrome *a* oxidized]/[Cytochrome *a* reduced]	3.69	2.57
ATP	1.84	4.42
ADP	0.42	0.71
P_i	10	10
[ADP]/[ADP][P_i]	438M^{-1}	623M^{-1}

absence of an external supply of fatty acid in the isolated cells. Addition of lactate and ethanol or lactate and oleate increased the [3-hydroxybutyrate]/[acetoacetate] ratio.

According to Table 1 [cytochrome *a*] and [cytochrome *c*] in suspensions of rat liver cells were 7–9nmol/g wet wt. and 13–15nmol/g wet wt. respectively. The [cytochrome *c*]/[cytochrome *a*] ratio was about 1.8 (for mitochrondrial values see Wainio, 1970). As seen in Fig. 1 cytochrome *c* and cytochrome *a* were in a more reduced state in the cell suspension than they were in the experiments with isolated mitochondria of Wilson & Erecińska (1972) and Erecińska *et al.* (1974), the degree of reduction in the cell being between 10 and 30% for cytochrome *c*, cytochrome *a* being slightly more reduced than cytochrome *c* under most conditions (see, for example Table 1).

The measured [ATP]/[ADP][P_i] in the intact cells is in good agreement with the values found in freeze-clamped liver (Veech *et al.*, 1970). [ATP] was approx. 2μmol/g wet wt., which is similar to the values found by Berry & Kun (1972). Addition of adenosine increases the value of [ATP]/[ADP] of the cytoplasmic adenine nucleotides mainly because of an increase in [ATP] (Chagoya de Sanchez *et al.*, 1972).

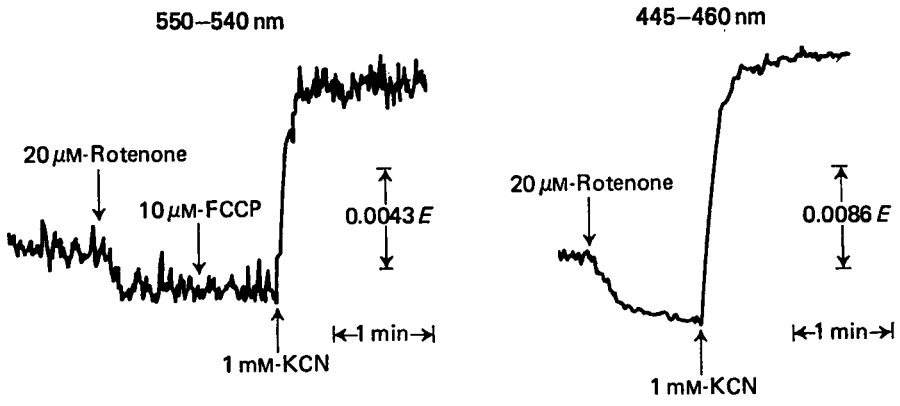

Fig. 1. *Measurement of the redox state of cytochrome c and cytochrome c in suspensions of isolated liver cells*

Isolated liver cells (21.7mg wet wt./ml) were preincubated for 15min at 25°C and placed in a spectrophotometric cuvette in an Aminco–Chance dual-wavelength spectrophotometer. Time proceeds from left to right. Downward deflexion designates oxidation of the cytochromes, upward deflexion designates reduction. For other details see the Experimental section. FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

[ATP]/[ADP] varied relatively little; it was between 4 and 6. When the P_i concentration obtained from representative cell preparations (see the Experimental section) was used the value of [ATP]/[ADP] $[P_i]$ was between $400\,M^{-1}$ and $800\,M^{-1}$.

Free-energy relationships between the oxidation–reduction reactions of the respiratory chain and the phosphorylation state

These results are shown in Table 3. It follows from the E_h values of cytochrome *c* (0.275 ± 0.010V) and the E_h values of the NAD^+ couple (-0.263 ± 0.012V) that the difference in E_h (ΔE) between the two couples is $+0.538\pm0.02$V. Hence the free energy ($\Delta G_{ox.-red.}$) of the reaction

$$NADH + 2\,cyt.\,c^{3+} \rightleftharpoons NAD^+ + 2\,cyt.\,c^{2+} \quad (5)$$

is on average $-nF\times0.538$V. The free-energy relationships between the oxidation–reduction reactions and ATP synthesis is obtained by assuming a stoicheiometry of two ATP molecules synthesized for each two electrons transferred. The ΔG associated with the transfer of two reducing equivalents is calculated to be -104 ± 4.2kJ/2 electrons (−24.8 kcal/2 electrons).

The ΔG associated with the hydrolysis of ATP is obtained from the relationship

$$\Delta G_{ATP} = \Delta G'_{0ATP} + 2.303RT\log\left(\frac{[ADP][P_i]}{[ATP]}\right) \quad (6)$$

where $\Delta G'_{0ATP}$ is the standard free energy of hydrolysis of ATP at pH7.0 and cellular concentrations of free Mg^{2+}. When the measured [ATP]/[ADP][P_i] ratios are used in conjunction with the $\Delta G'_{0ATP}$ of −31.9kJ/mol (−7.6kcal/mol) from Guynn & Veech (1973) the calculated ΔG/2 mol of ATP is −95 kJ/2mol of ATP (−22.7kcal/2mol of ATP).

The free-energy release by the interaction of the NAD couple with cytochrome *c*[−104kJ (−24.8 kcal)/2 electrons] is, within the limits of error, equal to the amount required for the synthesis of 2mol of ATP [+95kJ (+22.7kcal)]. This implies an efficiency of nearly 100%, as expected for a system that is readily reversible and is at near-equilibrium.

In Table 4 the values are given for the apparent equilibrium constants when calculated from (*a*) the experimentally determined values of [NAD^+]/[NADH], [cytochrome^{2+}]/[cytochrome^{3+}] and [ATP]/[ADP][P_i] and (*b*) the $E_{m7.0}$ values of the NAD couple (−320V) and cytochrome *c* (0.235V) and the $\Delta G'_0$ for the hydrolysis of ATP [−31.9kJ/mol (−7.6kcal/mol)].

The calculation of the apparent equilibrium constant from (*a*) gives values in the range between 4.4×10^5 and $2.9\times10^6\,M^{-2}$, whereas the independent calculation from (*b*) gives $4.4\times10^7\,M^{-2}$. The difference between the two values ($K_{eq.}$calculated/$K_{eq.}$measured) can be accounted for by experimental shortcomings.

A probable source of error lies in the measurements of the phosphorylation state of the adenine nucleotides. Measurements were made on whole cell suspensions and it is assumed that the values obtained are representative of the cytoplasmic compartment (Krebs & Veech, 1969). However, the work of Klingenberg *et al.* (1969) and Heldt *et al.* (1972) suggests that the [ATP]/[ADP] ratio in the isolated mitochondria may be as much as 10 times smaller than the ratio in the external medium. If this were

Table 3. *Free-energy relationships between the oxidation–reduction reactions of the respiratory chain and the phosphorylation state*

The values for E_h were calculated from the data given in Tables 1 and 2, as described in the Experimental section. E_h is the oxidation–reduction potential with respect to the hydrogen electrode. For details of Expts. A, B and C see Tables 1 and 2. The free-energy relationships were calculated for two phosphorylation sites. The control values are the average of three further experiments where measurements on non-incubated cells were made. For each free energy value the result expressed in kcal is given in parentheses.

$\Delta E = E_h(\text{NAD}^+/\text{NADH}) - E_h(\text{cytochrome } c^{3+}/\text{cytochrome } c^{2+})$ where $E_h = E_m + \frac{2.3RT}{nF}\log\left(\frac{[\text{oxidized carrier}]}{[\text{reduced carrier}]}\right)$;

$\Delta G_{\text{ox.-red.}} = -nF\Delta E$; $\Delta G_{\text{ATP}} = \Delta G'_0 + 1.36\log\left(\frac{[\text{ADP}][P_i]}{[\text{ATP}]}\right)$; $\Delta\Delta G = \Delta G_{\text{ox.-red.}} - \Delta G_{\text{ATP}}$.

Expt.	E_h (cytochrome) (V)	E_h [NAD$^+$]/[NADH] (V)	ΔE (V)	$\Delta G_{\text{ox.-red.}}$ (kJ/2 electrons)	ΔG_{ATP} (kJ/2ATP)	$\Delta\Delta G$ (kJ)
A_1 (Table 1)	0.278	−0.252	0.530	102.5 (24.5)	94.6 (22.6)	8.8 (2.1)
A_2 (Table 1)	0.263	−0.267	0.530	102.5 (24.5)	95.4 (22.8)	7.1 (1.7)
B_1 (Table 1)	0.284	−0.251	0.535	103.3 (24.7)	96.7 (23.1)	6.7 (1.6)
B_2 (Table 1)	0.284	−0.276	0.560	107.9 (25.8)	96.7 (23.1)	11.3 (2.7)
C_1 (Table 2)	0.279	−0.265	0.544	105.0 (25.1)	93.7 (22.4)	11.3 (2.7)
C_2 (Table 2)	0.270	−0.273	0.543	104.6 (25.0)	95.4 (22.8)	9.2 (2.2)
Control	0.265	−0.253	0.518	99.6 (23.8)	92.9 (22.2)	6.7 (1.6)

Table 4. *Equilibrium constant ($K_{eq.}$) for the first two sites of mitochondrial oxidative phosphorylation as calculated from the experimental values of Tables 1 and 2 and from the thermodynamic constants*

The [NAD$^+$]/[NADH] ratio was calculated as described in the Experimental section. The calculated equilibrium constant was calculated from the half-reduction potentials of cytochrome *c* and NAD (0.235V and −0.320V respectively) and a $\Delta G'_0$ for ATP hydrolysis of −31.9kJ/mol (−7.6kcal/mol). The resulting value is $4.4\times10^7\,\text{M}^{-2}$.

Expt.	[NAD$^+$]/[NADH]	$\left(\frac{[\text{Cytochrome } c^{2+}]}{[\text{cytochrome } c^{3+}]}\right)^2$	$\left(\frac{[\text{ATP}]}{[\text{ADP}][P_i]}\right)^2(\text{M}^{-2})$	$K_{eq.}(\text{M}^{-2})$	$\frac{K_{eq.}\text{ calculated}}{K_{eq.}\text{ measured}}$
A_1 (Table 1)	1.84×10^2	3.6×10^{-2}	2.7×10^5	1.7×10^6	26
A_2 (Table 1)	5.84×10	11.6×10^{-2}	3.4×10^5	2.3×10^6	19
B_1 (Table 1)	1.99×10^2	2.3×10^{-2}	6.32×10^5	2.9×10^6	15
B_2 (Table 1)	2.93×10	2.3×10^{-2}	6.43×10^5	4.3×10^5	102
C_1 (Table 2)	6.8×10	3.4×10^{-2}	1.92×10^5	4.4×10^5	100
C_2 (Table 2)	3.67×10	6.8×10^{-2}	3.88×10^5	9.6×10^5	45

true for the whole cell, then a significant fraction of the ADP measured could be in the mitochondria. This would lead to a systematically low value for [ATP]/[ADP][P_i]. Further, ADP is a substrate of many cellular enzymes and the total [ADP] measured is only 0.5mM. Therefore there may be a significant amount of bound ADP. Since free ADP is the reactant, binding of ADP would cause the measured value of the cytoplasmic [ATP]/[ADP][P_i] to be too low.

Another possible source of error is in the values used for [P_i]. Owing to technical difficulties, the concentration of P_i was not measured under exactly the same experimental conditions as those under which the concentrations of the other reactants were determined (see the Experimental Section for details).

The spectrophotometric measurements of the cytochromes also contain a possible systematic error because rotenone inhibits only the oxidation of the NAD-linked substrates and not substrates of flavoprotein-linked dehydrogenases such as succinate dehydrogenase or 3-glycerol phosphate dehydrogenase. Thus the cytochromes may still be partially reduced in the rotenone-inhibited steady state because of the presence of endogenous substrates, the oxidation of which is not inhibited under the test conditions.

The trend of these errors would all be in the same direction and would cause a decrease in the difference between the free-energy changes of the coupled reactions. This supports the conclusions that, in the intact cells, as in isolated mitochondria, the reactants are at near-equilibrium.

Discussion

It is a striking finding that a state of near-equilibrium exists between the difference in the redox state across two mitochondrial phosphorylation sites on the one hand and the extramitochondrial

phosphorylation state on the other. This has previously been demonstrated (Erecińska *et al.*, 1974) for suspensions of isolated pigeon heart mitochondria and is now confirmed for isolated liver cells. Thus, within the limits of experimental error, the energy derived from the transfer of 2 mole equivalents of electrons from the mitochondrial NAD couple to the isopotential carriers of Group III (including cytochrome *c*) is equal to the energy conserved in the synthesis of 2 mol of ATP from ADP and P_i. It should be pointed out that establishing the existence of equilibria does not bear on the nature of molecular mechanisms of oxidative phosphorylation.

The reversal of the oxidation–reduction reactions between the NAD and cytochrome *c* couples on addition of ATP has been demonstrated for isolated mitochondria by Chance & Hollunger (1961) and Klingenberg & Schollmeyer (1961). A reversal indicated that, in principle, equilibrium can exist between the two redox couples and the adenine nucleotide system. Klingenberg & Schollmeyer (1961) discussed the reversal in terms of equilibrium and stressed that it is the ratio of [ATP]/[ADP][P_i] rather than [ATP] that is relevant in this context. The possibility of establishing equilibrium, however, does not mean that equilibrium actually exists. For example, glucose 6-phosphate dehydrogenase or hexokinase do not establish equilibrium (although the reactions they catalyse are reversible and the potential activities of the enzymes are high) because of the allosteric inhibition of the enzymes by NADPH or glucose 6-phosphate respectively (see Krebs, 1974). Further evidence for the existence of near-equilibrium in isolated mitochondria was provided by the demonstration (Klingenberg & Schollmeyer, 1961; Wilson *et al.*, 1973*a*,*b*) that the respiratory rate can depend on [ATP]/[ADP][P_i].

If the overall system of oxidative phosphorylation between the two sites is at near-equilibrium, then all intermediary reactions must also be at near-equilibrium. This implies that, if ATP, which has been synthesized, appears primarily within the mitochondrial matrix, as is commonly assumed, then the exchange of adenine nucleotides and P_i across the inner mitochondrial membrane must also be at near-equilibrium. This does not necessarily mean that the phosphorylation states within and without the mitochondrion are identical (see Klingenberg, 1970); rather it means that any differences in the activities of ATP, ADP and P_i are coupled to ion gradients and/or the potential across the inner mitochondrial membrane in such a way that translocation occurs without loss of free energy.

The basis of this discussion is that a precise stoicheiometry of 2 molecules of ATP per 2 electrons carried from the NAD couple to the cytochrome *c* couple is maintained. Such stoicheiometry was verified for suspensions of isolated mitochondria (Erecińska *et al.*, 1974). Lower values, such as those reported by Klingenberg (1970), may be caused by ATPase* activity, as his experimental system (high [ATP], low respiratory rate) was particularly sensitive to this enzymic activity.

Since the [ATP]/[ADP][P_i] ratio is common to all three phosphorylation sites, it follows that the free-energy change between Group III and Group IV (the cytochrome a_3 couple) is the same as that between Groups I and II and II and III. It further follows that the rate of oxidative phosphorylation is controlled by the phosphorylation state of the cytoplasmic adenine nucleotides and not by the individual concentrations of ADP and P_i. The control expresses itself by changing the state of reduction of cytochrome a_3, thereby determining the rate of O_2 consumption (Wilson *et al.*, 1973*a*,*b*); thus the rate of O_2 consumption is directly proportional to the concentration of reduced cytochrome a_3. Since the reaction between reduced cytochrome a_3 and O_2 is practically irreversible, changes in the redox state of this carrier determine the net respiratory flux. It is also noteworthy that the value of the phosphorylation state of the adenine nucleotides of the external medium in experiments with isolated mitochondria (Cockrell *et al.*, 1966; Erecińska *et al.*, 1974; Slater *et al.*, 1973) can be 50–100 times higher than that observed for the liver cells. This is possible because under the test conditions the NAD couple was more oxidized and cytochrome *c* was in most cases more reduced in the liver cells than in the isolated mitochondria. Thus the free-energy change for the coupled reactions approaches zero in both systems.

The behaviour of suspensions of isolated mitochondria has been descriptively characterized by states 1–5 (Chance & Williams, 1956). The states were defined by whether substrate supply, [ADP] or [O_2] limits the rate of O_2 consumption. Although this description proved useful in discussing suspensions of isolated mitochondria, mitochondria in intact cells are always in the presence of substrate, ADP, ATP and P_i. Thus *in vivo* the concept of these states is of limited use, since mitochondria *in vivo* are always in states corresponding to the region between states 3 and 4 of isolated mitochondria. The metabolic state of the mitochondria in the cell is adequately defined by the turnover number of the cytochromes (rate of O_2 consumption/cytochrome), the cytoplasmic phosphorylation state and the oxidation–reduction potential of the NAD couple. In principle, this information permits the calculation of the oxidation–reduction potentials of the isopotential groups of components.

In mammalian tissues, under physiological conditions, it is unlikely that the mitochondria are ever limited by the concentrations of ADP or P_i. Under 'abnormal' conditions (in the presence of added

* Abbreviation: ATPase, adenosine triphosphatase.

inhibitors or uncouplers of oxidative phosphorylation), disequilibrium can occur, resulting in respiratory control by other reactants.

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