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Topical Review

Regulation of Cellular Energy Metabolism

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Introduction

One of the characteristic features of living matter is its ability to generate metabolic energy which is required for growth and the maintenance of multiple cellular functions. The major pathways whereby eukaryotic organisms produce energy are glycolysis and mitochondrial oxidative phosphorylation; the latter process, which involves complete combustion of glucose to carbon dioxide and water, yields approximately 17 times as much useful energy as does anaerobic production of lactic acid (glycolysis). Owing to its high energy yield, mitochondrial oxidative phosphorylation is responsible for supplying over 95% of the total ATP requirement in eukaryotic cells.

All living organisms are steady-state systems in which the rate of energy production (ATP synthesis) equals the rate of energy utilization (except in rapid transient conditions in which this may not necessarily be true). This means that precise dynamic balance is maintained between the reactions which produce ATP and those which utilize it in which the concentration of ATP in the cell remains at an essentially constant level. Any decline in the usage of ATP causes an immediate decrease in the rate of its synthesis and *vice versa*. The range of activities over which mitochondrial oxidative phosphorylation operates *in vivo* is best illustrated by the observation that in man the rate of ATP synthesis varies from 0.4 g ATP/min/kg body weight at rest to 9.0 g ATP/min/kg body weight during strenuous exercise. This high flux through the ATP synthesizing pathway and its large dynamic range ensures that oxidative phosphorylation plays a paramount role in cellular homeostasis.

In eukaryotic cells, the enzymes of oxidative phosphorylation are intimately associated with or form an integral part of the mitochondrial inner membrane (for review *see* Lehninger, 1966; Wainio, 1970). The site of ATP synthesis (ATP synthase) is located on the internal surface of the inner membrane and communicates with the matrix space. It is well known that the mitochondrial matrix houses but a few reactions which require ATP whereas the cytosol is the main site of ATP utilization. Mechanisms must, therefore, be present which allow the mitochondrial matrix to sense the need of the cytosol for an appropriate rate of ATP generation. Moreover, ATP has to be supplied under conditions for which its hydrolysis is sufficiently exergonic to do the required metabolic work (i.e., its hydrolysis has to provide a negative free energy change of sufficient magnitude). Since the maximum work that ATP hydrolysis can do is dependent on the [ATP]/[ADP] $[P_i]$, variations in the cellular concentrations of ATP, ADP, and inorganic phosphate provide a very sensitive means through which homeostatic mechanisms can regulate energy production.

The mitochondrial respiratory chain is a multienzyme complex which accepts reducing equivalents from NADH and $FADH_2$ and transfers them in a sequence of oxidation-reduction reactions to molecular oxygen with the formation of water and concomitant synthesis of ATP. In cells *in vivo*, reducing equivalents are provided through dehydrogenation of various substrates which are generated by the metabolic feeder pathways such as the tricarboxylic acid cycle or fatty acid oxidation system. Since mitochondrial dehydrogenases utilize either NAD or FAD as reducible coenzymes the amounts of NADH and $FADH_2$ formed by the particular tissue depend on relative activities of these pathways, but in most cells NADH is the

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Table 1. Mitochondrial oxidative phosphorylation *in vivo*

Experimental material (author)	Cytochrome *c*		$[P_i]$	$\frac{[ATP]}{[ADP]}$	$\frac{[ATP]}{[ADP][P_i]}$	$\frac{[NAD^+]}{[NADH]}$	ΔG_{ATP} (kcal/mole (kJ/mole))
	Reduction (%)	Turnover number (e^-/sec)	(mM)		(M^{-1})		
Isolated liver cells (Wilson et al., 1974*a*)	18	5.7	2.93	3.84	1.3×10^3	410	11.8 (49.3)
Cultured kidney cells (Wilson et al., 1977*a*)	25	5.4	5.5	11	2.0×10^3	180	12.1 (50.6)
Isolated liver cells (Erecińska et al., 1977)	19.5	5.1	3.91	4.1	1.1×10^3	86	11.7 (48.9)
	18.6	4.8	0.88	1.0	1.1×10^3	69.7	11.7 (48.9)
Neuroblastoma C-1300 (Kilpatrick-Smith et al., 1981)	40.0	6.6	4.91	9.4	1.9×10^3	9.05	12.1 (50.6)
Rat brain synaptosomes (Rafalowska et al., 1980)	–	3.3	2.5	9.6	3.8×10^3	10	12.5 (52.3)
Perfused rat heart (Nishiki et al., 1978*a*)							
80 cm H_2O		13.9	3.07	23.5	7.6×10^3	2.44	12.9 (53.9)
120 cm H_2O		26.2	4.49	11.7	2.6×10^3	5.59	12.2 (51.0)
epinephrine		29.4	4.75	12.0	2.5×10^3	4.74	12.2 (51.0)
20 mM K^+		3.9	2.57	33.7	1.3×10^4	0.67	13.2 (55.2)
Ca^{2+} free		5.8	1.47	76.0	5.1×10^4	4.72	14.0 (58.5)
Tetrahymena pyriformis (Erecińska et al., 1978*b*)	30	13.9	3.82	1.57	4.11×10^2	230	11.2 (46.8)
Candida utilis (Erecińska et al., 1977)	70	52	20.4	23.41	1.1×10^3	–	11.7 (48.9)
	64	50	6.9	9.25	1.3×10^3	–	11.8 (49.3)
Paracoccus denitrificans (Erecińska et al., 1978*a*)	14.6	13.5	4.02	7.57	1.9×10^3	397	12.1 (50.6)

$\Delta G_{ATP} = \Delta G'_o + 1.36 \log \frac{[ADP][P_i]}{[ATP]}$, where $\Delta G'_o$ was taken to be 7.6 kcal/mole (31.77 kJ/mole) (Guynn & Veech, 1973). This assumes that intracellular pH is 7.0 and $[Mg^{2+}]_{free}$ in the cytosol is about 1 mM

main source of the reducing power for the respiratory chain.

Energetics of Mitochondrial Oxidative Phosphorylation

The overall reactions of mitochondrial oxidative phosphorylation are summarized in the equation:

$$NADH + H^+ + 3\ ADP + 3\ P_i + 1/2 O_2 \rightarrow NAD^+ + 3\ ATP + H_2O$$

Measurements of the concentrations of the individual reactants allow calculations of the energetics of this reaction (Table 1). In liver tissue, for example, the intramitochondrial $[NAD^+]/[NADH]$ is approximately 30, whereas the cytosolic $[ATP]/[ADP]\ [P_i]$ is about $3 \times 10^3\ M^{-1}$. Thus the total free energy change for the transfer of two reducing equivalents from NADH to oxygen is -50.3 kcal (210 kJ) and approximately 36.9 kcal (3×12.3 kcal/mole) are recovered in ATP synthesis, which gives an overall efficiency of 73%. In beating rat heart perfused with glucose, the intramitochondrial $[NAD^+]/[NADH]$ is 2.4 and the cytosolic $[ATP]/[ADP]\ [P_i]$ is $7.9 \times 10^3\ M^{-1}$. The total free energy change for the transfer of two reducing equivalents from NADH to oxygen is -51.8 kcal (217 kJ) and approximately 38.7 kcal (162 kJ) (3×12.90 kcal/mole) are recovered which gives again an overall efficiency of over 75%. The problem then arisis of how this highly efficient system is regulated in cells *in vivo*.

Two hypotheses are entertained currently which try to explain regulation of mitochondrial oxidative phosphorylation. The first, which for the sake of simplicity will hence forward be referred to as the near equilibrium hypothesis (Wilson et al., 1974*b*), postulates that the first two sites of mitochondrial oxidative phosphorylation are at near equilibrium, i.e., the amount of energy generated during transfer of two reducing equivalents from intramitochondrial NADH to cytochrome *c* is equal, within experimental error, to that required for the synthesis of 2 moles of ATP in the cytosol. The overall regulation of such a system is exerted through the essentially irreversible reaction between reduced cytochrome *c* and molecular oxygen, catalyzed by the enzyme cytochrome *c* oxidase. However, since ATP is synthesized within the mitochondrial matrix, the translocation of adenine nucleotides across the mitochondrial inner membrane is, according to this concept, an obligatory intermediate step in the reaction sequence and, as such, must also be at near equilibrium. This means that if the transport of ATP, ADP, or P_i is coupled to ion and/or electrical gradients across the mitochondrial membrane, this coupling must occur without loss of free energy ($\Delta G \rightarrow 0$).

Table 2. Phosphorylation values of intra- and extramitochondrial adenine nucleotides during oxidative phosphorylation by isolated mitochondria

Author (substrate)	Mg_e^{2+}	$[P_i]_e$ (mM)	$\frac{[ATP]_i}{[ADP]_i}$	$\frac{[ATP]_e}{[ADP]_e}$	$\frac{[ATP]_e}{[ADP]_e[P_i]_e}$ (M^{-1})	ΔG_{ATP} (kcal/mole)	Respiratory rate (natoms O_2/min/mg prot)
Slater et al., 1973[a] (Succ. + Rot.)	–	1.45	2.8 (6.8)	191	1.3×10^5	15.7	State 4
Wanders et al., 1981 (Succ. + Rot.)	+	10	4	100	1.0×10^4	13.0	40
Letko et al., 1980 (Glut. + Mal.)	+	5	4.8	50	1.0×10^4	13.0	State 4
Brawand et al., 1980 (Glut. + Mal.)	+	12	2.7	245	2.0×10^4	13.46	17
		2	1.28	193	9.6×10^4	14.38	
Davis & Lumeng, 1975 (Glut. + Mal.)	+	2	4	60	3.0×10^4	13.69	17
Heldt et al., 1972 (Succ. + Rot.)	+	0.48	3.9	29	6.0×10^4	14.10	–
Goldstein & Aprille, 1982 No substrate		0	1.65	–	–	–	–
Erecińska & Wilson (*unpublished*) (Glut. + Mal.)	–	2–4	8–12	–	–	–	State 4
Kunz et al., 1981 (Succ. + Rot.)	+	1.8	–	100	5.5×10^4	14.05	25
		9.4	–	80	8.5×10^3	12.94	30
		29.0	–	100	3.4×10^3	12.40	24–30
Davis & Davis-van Thienen, 1978 (Glut. + Mal.)	+	3	–	205	6.8×10^4	14.18	20
		9	–	190	2.1×10^4	13.48	
		27	–	180	6.7×10^4	12.80	
Holian et al., 1977[b] (Glut. + Mal.)	–	1.6	–	130	8.1×10^4	15.08	35.7
		1.6	–	77	4.8×10^4	14.76	56
		3.2	–	149	4.7×10^4	14.75	56
		8.5	–	338	4.0×10^4	14.66	56
Holian & Wilson, 1980 (Glut. + Mal.)	–	0.5	–	112	2.2×10^5	15.68	7.6

[a] Results for experiments at pH 7.7. ΔG_{ATP} was calculated using a ΔG_o at pH 7.7 of 8.7 kcal/mole (36.4 kJ/mole). The two values for $[ATP]_i/[ADP]_i$ are in the presence and absence, respectively, of extramitochondrial adenine nucleotides.

[b] Values for dog heart mitochondria which contain 4–6 times as much cytochrome per mg of protein as do rat liver mitochondria. The latter were used in all of the other experiments in this Table. ΔG_{ATP} (*see* Table 1) was calculated using values for $\Delta G'_o$ of 7.6 kcal/mole (31.77 kJ/mole) for experiments with $[Mg^{2+}]_e$ and 8.4 kcal/mole (35.11 kJ/mole for experiments without added magnesium (Guynn & Veech, 1973). Calculations were made for pH 7.0, although the experimental values ranged from pH 7.0 to 7.4.

The second hypothesis, hereafter referred to as the translocase hypothesis, postulates that the adenine nucleotide translocase reaction (for review *see* Klingenberg, 1980), which exchanges ATP^{4-} for ADP^{3-} across the mitochondrial membrane, regulates the overall rate of oxidative phosphorylation. According to this concept, the adenine nucleotide translocator is far displaced from equilibrium and serves as the detector system by which mitochondria sense changes in extramitochondrial [ATP]/[ADP]. It is postulated therefore, that respiration coupled to ATP synthesis is controlled by the [ATP]/[ADP] and not the [ATP]/[ADP] $[P_i]$ (Davis & Lumeng, 1975; Küster, Bohnensack & Kunz, 1976; Letko, Küster, Duszynski & Kunz, Brawand, Folly & Walter, 1980; Kunz et al., 1981). Since it has been reported that the [ATP]/[ADP] ratios measured in the mitochondrial matrix and in the extramitochondrial environment are not the same (*see* Table 2 for summary), disagreement exists among the proponents of the translocase concept as to which of the two is the regulatory parameter. Whereas it was originally proposed that respiration responds exclusively to changes in $[ATP]_e/[ADP]_e$ (Davis & Lumeng, 1975), recent experiments in some laboratories have given rise to the suggestion that respiratory activity is related to the intramitochondrial [ATP]/[ADP] (Kunz et al., 1981; Wanders, Groen, Meijer & Tager, 1981). It should be pointed out that maintaining $[ATP]_e/[ADP]_e$ greater than $[ATP]_i/[ADP]_i$ requires metabolic energy. The source of the necessary energy

is commonly considered to be the membrane potential generated by the mitochondrial respiratory chain coupled to an exchange of ATP^{4-} for ADP^{3-} (*see* Klingenberg, 1980, for summary). Such a mechanism implies that as long as the translocation of adenine nucleotides requires energy, the transport of ADP_{in} and ATP_{out} has to be driven by energy taken out from respiration and, as a consequence, the energy produced by the respiratory chain is partially utilized for phosphorylation of internal ADP and partially for translocation of ADP and ATP.

The Near Equilibrium Hypothesis

It has been known since the early studies of Chance and Hollunger (1961) and Klingenberg and Scholl-meyer (1961) that the redox reactions between the NAD and cytochrome *c* couples can be reversed by addition of ATP. Systematic studies carried out in the middle 1970s showed that in suspensions both of isolated mitochondria (Erecińska, Veech & Wilson, 1974) and intact cells of various types, and in perfused organs respiring in the presence of substrate and oxygen (Wilson et al., 1974*a*, *b*; Hassinen & Hiltunen, 1975; Reed, 1976; Wilson et al., 1977*a*; Erecińska, Kula & Wilson, 1978*a*; Erecińska, Wilson & Nishiki, 1978*b*; Nishiki, Erecińska & Wilson, 1978*a*), the reaction

$$NADH_m + 2\,c^{3+} + 2\,ADP_c + 2\,P_{i_c} \leftrightarrow NAD^+_m + 2\,c^{2+} + 2\,ATP_c$$

where *m* and *c* signify the intramitochondrial and cytosolic compartments, respectively, is associated with a free energy change of approximately zero (Table 1).

As stated above, the existence of near equilibrium in the first two sites of oxidative phosphorylation means that regulation of cellular energy production must originate at the third phosphorylation site, i.e., in the cytochrome *c* oxidase reaction. The rate of this reaction depends on the concentrations of its two substrates: reduced cytochrome *c* and molecular oxygen. Changes in the concentration of reduced cytochrome *c* are determined by the near equilibrium state in the first two phosphorylation sites and are linked, therefore, to alterations in the redox level of the intramitochondrial NAD couple (i.e., the intramitochondrial $[NAD^+]/[NADH]$) and the cytosolic [ATP]/[ADP] $[P_i]$. Moreover, since cytochrome oxidase is a part of the coupling mechanism at site III its activity is also dependent on the [ATP]/[ADP] $[P_i]$. Thus the rate of mitochondrial respiration is governed by the interplay of four factors:

1. concentration of the respiratory chain proteins
2. concentration of molecular oxygen
3. intramitochondrial substrate levels and metabolic state which determine the intramitochondrial $[NAD^+]/[NADH]$
4. the rate of cellular ATP utilization which determines the cytosolic [ATP]/[ADP] $[P_i]$

Concentration of Respiratory Chain Proteins

The first and perhaps the simplest of the regulatory parameters of mitochondrial oxidative phosphorylation is the concentration of the respiratory chain proteins. It is a matter of common observation that mitochondria that contain more redox carrier per mg protein, such as heart mitochondria, exhibit high respiratory activity. However, this is determined by genetic factors since decreases and increases in protein concentration require utilization of protein synthesizing machinery which take hours or days to manifest themselves. Nevertheless, under conditions of a prolonged load imposed on cellular ATP generating systems such as exercise or chronic administration of thyroxine, increases in the amount of mitochondrial respiratory chain carriers have been observed (Nishiki, Erecińska, Wilson & Cooper, 1978*b*; Terjung, Winder, Baldwin & Holloszy, 1973).

Intramitochondrial $[NAD^+]/[NADH]$

It is evident that under most experimental conditions of short term regulation the concentrations of the respiratory chain proteins remain essentially constant and compensatory changes which follow an altered metabolic state are confined to the remaining three variables. In order to simplify our discussion we shall assume initially that the concentration of oxygen is maintained high and constant. Under such conditions analysis of factors which regulate oxidative phosphorylation is limited to intramitochondrial $[NAD]^+/[NADH]$ and the cytosolic [ATP]/[ADP] $[P_i]$. It is worth pointing out that changes in these two parameters are very closely interconnected: whereas the cytosolic [ATP]/[ADP] $[P_i]$ serves as the primary sensor of an altered need for energy production, the mitochondrial $[NAD^+]/[NADH]$ allows its rate to change at constant [ATP]/[ADP] $[P_i]$. An interesting early illustration for this statement is afforded by the now classic observations of David Keilin (1925) on the flight muscle in which stimulation of activity was accompanied by a large reduction of cytochrome *c*, which is now known to be due

in part to an increase in the supply of reducing substrates.

It is generally observed that at a constant [ATP]/[ADP] [P_i] respiration is inversely related to the intramitochondrial [NAD^+]/[NADH]. This dependence of respiratory activity on the redox state of the NAD couple has been demonstrated in suspensions of mitochondria (Holian, Owen & Wilson, 1977; Wilson, Owen & Holian, 1977*b*) and in a number of cells in vivo (Wilson et al., 1974*a, b*; Erecińska et al., 1978*b*; Erecińska & Wilson 1978; Nishiki et al., 1978*a*), including bacteria (Erecińska, Davis & Wilson, 1979; Erecińska, Deutsch & Davis, 1981). Inspection of these results (Table 1) shows that cells maintain a much higher [NAD^+]/[NADH] than is generally observed in isolated mitochondria. Liver cells, for example, operate at an [NAD^+_m]/[$NADH_m$] of 30 to 100 and perfused rat heart of 1 to 10, whereas in isolated mitochondria values of 0.1 to 0.01 are commonly encountered. This rather crucial difference has often been overlooked when extrapolations are made from *in vitro* studies on isolated mitochondria to *in vivo* systems.

Cytosolic [ATP]/[ADP] [P_i]

The responsiveness of cellular respiration to changes in the rate of ATP utilization has long been known (Fisher 1931; Chance, 1957). In most cells the concentrations of adenine nucleotides and inorganic phosphate are maintained at reasonably constant values and do not differ substantially from tissue to tissue (Table 1). Total adenine nucleotides are between 3–8 mM and inorganic phosphate about 1–4 mM. On the other hand, the cytosolic [ATP]/[ADP] [P_i] at which different tissues operate can vary from 5×10^4 M^{-1} in resting skeletal muscle (Veech, Lawson, Cornell & Krebs, 1979) to 7×10^2 M^{-1} in constantly "mobile" *Tetrahymena pyriformis* (Erecińska et al., 1978*b*). Similar variations in [ATP]/[ADP][P_i] can occur in transition from low to high activity. For example, when a physiological work load is imposed on skeletal muscle it causes a rapid decrease in the [ATP]/[ADP] [P_i] which can be as much as 50-fold and yet still not compromise normal muscular activity.

The near equilibrium concept explicitly postulates that respiration responds to changes in the [ATP]/[ADP] [P_i] as a whole and not to alterations in the concentrations of individual reactants. This means that changes in all three of these parameters contribute to the regulation of respiration and if all of them change in such a way that the [ATP]/[ADP] [P_i] remains constant, at an unaltered [NAD^+]/[NADH], respiration also remains constant. In agreement with that, it has been shown that in suspensions of liver cells, decrease in P_i from 4 to about 1 mM induced by incubation with either fructose or glycerol is accompanied by a parallel decline in cellular [ATP]/[ADP] such that respiration remains constant (Erecińska et al., 1977). Although it has been argued that 1 mM P_i may limit activity of the mitochondrial phosphate carrier kinetically (Davis & Davis-van Thienen, 1978) similar changes have been observed in the yeast *Candida utilis* where alterations in inorganic phosphate occur in the much higher range of 10 to 30 mM (Erecińska et al., 1977). Moreover, the small contribution from the mitochondria to the total adenine nucleotide content in this organism ensures that the compensatory decline in the [ATP]/[ADP] when phosphate is lowered is confined predominantly to the cytosolic compartment. Our unpublished results on a bacterium *Paracoccus denitrificans* show that increase in intracellular phosphate from 4 to 8 mM causes an approximate twofold increase in the [ATP]/[ADP] without alteration in respiratory activity.

In contrast to the clearly visible effect of changes in concentration of inorganic phosphate on respiration in intact cells, its role in regulation of respiration in isolated mitochondria is much more controversial. Whereas some authors consider that [P_i] is as important in determining the respiratory rate as are [ATP] and [ADP] (Owen & Wilson, 1974; Holian et al., 1977; Wilson et al., 1977*b*; Lemasters & Sowers, 1979), others report that respiration is essentially independent of phosphate concentration (Davis & Lumeng, 1975; Davis & Davis-van Thienen, 1978; Kunz et al., 1981). However, experiments which led to these diametrically opposed conclusions were carried out under different conditions. While Holian et al. (1977) used phosphate concentrations in the range 1–8.5 mM and very low external magnesium, Kunz et al. (1981) and Davis and Davis-van Thienen (1978) worked at three [P_i] in the range 1–27 mM (1–3, 9, and 27–29 mM) and with high magnesium in the medium. One should bear in mind that the control of respiration by [ATP]/[ADP] [P_i] is only observed when the first two sites of oxidative phosphorylation are at near equilibrium. It follows that at any given [ATP]/[ADP] [P_i], a larger [ATP]/[ADP] must be generated when a higher concentration of P_i is present and/or when there is high [Mg^{2+}] in the medium. (The presence of high Mg^{2+} has two effects of consequence: firstly, it severely depresses the external levels of unchelated ADP and ATP, the substrates for translocation;

secondly, it decreases by 0.8 kcal/mole the free energy of ATP synthesis which means that for any given $[P_i]_e$ the same ΔG_{ATP} is reached at 3.9-fold higher $[ATP]_e/[ADP]_e$.) However, increase in [ATP]/[ADP] above certain value may decrease the translocase activity to a level at which it starts to limit the rate of oxidative phosphorylation (*see* Appendix for details). An examination of the results of Holian et al. (1977) and Holian and Wilson (1980) (Table 2) shows that under the conditions they used significantly more energy was conserved by ATP synthesis than in those used by other authors (>15 kcal/mole *vs.* 13–14 kcal/mole). It is likely, therefore, that near equilibrium was attained in the former experiments and hence respiration was dependent on $[P_i]_e$. By contrast, in the latter studies near equilibrium was presumably not reached and the adenine nucleotide translocator may indeed have been rate-limiting. It should be noted that *in vivo*, the free concentration of magnesium is 0.1–1.0 mM but the much lower [ATP]/[ADP] $[P_i]$, high [ATP]+[ADP] and modest $[P_i]$ values make it unlikely that translocation is substrate limited.

Oxygen Dependence of Cellular Energy Metabolism

In all considerations presented above it was assumed that concentration of molecular oxygen which is the second substrate for the oxidase reaction, is high and constant. However, in a number of pathological and even certain physiological situations this assumption is not true. One would expect that lowering the concentration of oxygen compromises the capacity of the respiratory chain to synthesize ATP. Yet measurements from a number of laboratories have demonstrated that in suspensions of intact cells (*see*, e.g., Warburg & Kubowitz, 1929; Longmuir, 1957; Wilson, Erecińska, Drown & Silver, 1979*a*; Wilson, Owen & Erecińska, 1979*b*) as well as in isolated mitochondria (Degn & Wohlrab, 1971; Petersen, Nicholls & Degn, 1974; Oshino, Sugano, Oshino & Chance, 1974; Sugano, Oshino & Chance, 1974) the rate of respiration remains essentially independent of oxygen tension down to very low values (the apparent K_m for oxygen is less than 1 μM) which seems to imply that cellular metabolism is rather insensitive to changes in O_2 concentration. On the other hand, recent studies show that changes in the redox state of cytochrome *c* and in cytosolic [ATP]/[ADP] $[P_i]$ and increase in lactate production and in the lactate/pyruvate occur at oxygen concentrations even above 100 μM (Wilson, Erecińska, Drown & Silver, 1977*a*; Wilson et al., 1979*a*, *b*; Jones & Mason, 1978). This has been interpreted as evidence that oxidative phosphorylation is, in fact, dependent on oxygen concentrations at values much higher than those which affect the respiratory activity. This apparent paradox disappears when one realizes that the rate of respiration (i.e., the rate of energy production) is determined by the rate of ATP utilizing reactions which are independent of oxygen tension. Thus although a fall in oxygen tension does cause a decrease in the rate of ATP synthesis (respiration), it does not affect the rate of ATP utilization. However, such a situation leads to an immediate decline in the [ATP]/[ADP] $[P_i]$ which induces, through the near equilibrium relations in the first two sites of oxidative phosphorylation, an increase in reduction of cytochrome *c* and activation of cytochrome *c* oxidase. The consequent enhancement of respiration proceeds to the point at which the rate of ATP synthesis again matches the rate of ATP utilization. Since the latter had remained the same as at the higher oxygen tension, the original rate of respiration is restored although at a lower cytosolic [ATP]/[ADP] $[P_i]$ and a new, more reduced redox state of cytochrome *c*. These compensatory changes are so rapid that the initial transient decreases in respiration are not discernable experimentally and gradual lowering of oxygen tension is reflected in progressive reduction of cytochrome *c* and decline in the [ATP]/[ADP] $[P_i]$.

The dependence of the apparent K_m for oxygen on the level of reduction of cytochrome *c* and the cytosolic [ATP]/[ADP] $[P_i]$ means that the oxygen reaction cannot be described in terms of simple Michaelis-Menten kinetics with a single K_m value but that the apparent K_m can change over a wide range as the metabolic parameters are varied. This may explain the differences in K_m reported from various laboratories as well as the fact that values obtained from cells in suspensions are higher than those calculated for isolated mitochondria. An alternative explanation for these findings invokes the existence of steep oxygen gradients between the cytosol and the mitochondria and large differences in oxygen tension in the two compartments (Chance, 1976; Jones & Mason, 1978). In support of this suggestion observations are quoted that in perfused rat heart deoxygenation of myoglobin and the reduction of mitochondrial respiratory chain components occur in parallel despite very different in vitro oxygen dependencies for the two (Tamura, Oshino, Chance & Silver, 1978). It should be remembered, however, that in isolated mitochondria the $[NAD^+]/[NADH]$ is usually much more reduced (0.1 to 0.01) in the steady-state

as compared to mitochondria in cells *in situ* (1 to 100). Moreover, the metabolic "buffering" of the intramitochondrial pyridine nucleotides in isolated mitochondria is very small as compared to that in cells, and thus the $[NAD^+]/[NADH]$ can freely adjust to maintain a constant respiratory rate and give a lower apparent K_m for oxygen. Several other lines of evidence argue against the existence of large intracellular oxygen gradients. First, when mitochondria are placed in a metabolic state similar to that *in situ* with high [ATP]/[ADP] $[P_i]$, the oxygen dependence of cytochrome *c* reduction is similar to that of intact cells (Wilson et al., 1979*b*). Second, direct measurements of oxygenation of yeast hemoglobin in cells in situ or added to external medium give no indication of measurable oxygen gradients despite the fact that these cells have high mitochondrial activity (Oshino et al., 1972). Third, calculations of Boag (1969) and ourselves (Wilson, 1982) demonstrate that liver mitochondria consume oxygen at a velocity equal to about 1% of the diffusion-limited rate even when the oxygen pressure is approximately 1 mm Hg. On the other hand, perfusions with low oxygen capacity, hemoglobin-free media such as those reported by Tamura et al. (1978) and Sies (1978) could result, especially at low oxygen tensions, in large differences in PO_2 between the arterial and venous ends of the capillaries and lead to longitudinal oxygen gradients. This would give heterogeneous normoxic and hypoxic areas of the tissue which could be responsible for the observations of the above authors.

All these considerations suggest that diffusion of oxygen is rapid enough to prevent formation of steep intracellular oxygen gradients. The information available to date indicates that the apparent insensitivity of respiration to lowered oxygen concentration is the consequence of beneficial changes in regulatory parameters of cellular energy metabolism which maintain ATP production at an undiminished level down to very low oxygen tensions.

Concerted Responses of Cellular Regulatory Parameters

It follows from the discussion above that a cell, in order to regulate its energy production, has at its disposal four variables. In principle, each of them is independently regulated by cellular metabolism; in practice, homeostatic mechanisms oppose drastic alterations in the individual variables and under most conditions two or three factors change in concert to obtain the final effect. The best illustration for this statement is the sequence of events which occur upon a sudden increase in cellular ATP utilization.

Initially, there is a rapid decrease in cytosolic [ATP]/[ADP] $[P_i]$ which leads to stimulation of respiration. Under most conditions, the near equilibrium relations which link the redox reactions between the NAD and cytochrome *c* couples and the [ATP]/[ADP] $[P_i]$ bring about an increase in the $[NAD_m^+]/[NADH_m]$, i.e., oxidation of the intramitochondrial pyridine nucleotides. This in turn stimulates directly and/or indirectly the NAD-dependent dehydrogenases and leads to a more rapid generation of NADH. The magnitude of changes in [ATP]/[ADP] $[P_i]$ and the subtle balance between the various reactions may lead to a situation in which the increase in respiration occurs with lowered, unaltered, or even increased $[NAD_m^+]/[NADH_m]$.

The Translocase Hypothesis

The translocase hypothesis postulates that the exchange of adenine nucleotides across the mitochondrial inner membrane determines the overall rate of respiration. Experimental support for this hypothesis comes from a number of observation which will be discussed briefly below.

Inequality of Intra- and Extramitochondrial [ATP]/[ADP] under Steady-State Conditions both in Isolated Mitochondria in Vitro and in Mitochondria in Situ.

Differences in extra- and intramitochondrial [ATP]/[ADP] were observed in the early experiments of Klingenberg and coworkers (Klingenberg, 1970; Heldt, Klingenberg & Milovancev, 1972) and have since been confirmed in a number of laboratories (*see* Table 2). The intramitochondrial [ATP]/[ADP] ratios in state 4 (i.e., in mitochondria respiring at maximum external [ATP]/[ADP]) are reported to be between 1 and 12, whereas the extramitochondrial values are found to be between 50 and 150. If these values do indeed reflect the ratios of *free* adenine nucleotides, then the energy required to tansport one mole of ADP in and one mole of ATP out of the mitochondrial matrix can be calculated to be 1.37–2.32 kcal/mole (5.7–9.7 KJ/mole). It is generally considered that this energy is provided by electrogenic coupling of translocation to the membrane potential (Klingenberg, 1980).

Measurements of the intramitochondrial [ATP]/[ADP] *in vivo* have been made both by digi-

tonin treatment of cells (Siess & Wieland, 1976; Akerboom et al. 1978) and by fractionation of tissues in nonaqueous solvents (Soboll, Scholz & Heldt 1978; Schwenke, Soboll, Seitz & Sies, 1981). Both methods give values of approximately 1 but serious reservations concerning the techniques (*see*, e.g., Veech et al., 1979) leave the real value open to question.

Electrogenic Character of the Translocase

Evidence for the electrogenic character of the translocase comes from two lines of observations. One relates to the difference in steady-state distribution of adenine nucleotides across the mitochondrial membrane discussed in the preceding section. The second relies on measurements of ions cotransported during adenine nucleotide exchange. Initially, Wulf, Kaltstein and Klingenberg (1978) reported that some proton movements could be measured concomitant with the [ATP]/[ADP] exchange and concluded that the reaction was partially electroneutral. Subsequent reports (LaNoue, Mizani & Klingenberg, 1978) have indicated, however, that no proton (or potassium, magnesium, or calcium) movements accompany this process. It was postulated, therefore, that the exchange of adenine nucleotides is fully electrogenic. If this is true, then the transmembrane electrical potential (negative inside) generated by mitochondrial electron transfer must favor transport of ATP_{out} and ADP_{in} and the translocase itself becomes subject to control by transmembrane electrical gradients. In agreement with this, Klingenberg and Rottenberg (1977) reported that when the external [ATP]/ADP] was kept constant at a value of 2, the intramitochondrial [ATP]/[ADP] varied from 0.3 to 0.01 as the function of membrane potential in the range 60 to 150 mV (as measured by rubidium distribution in the presence of valinomycin). It is surprising that the authors chose to work at such unusually low external and internal values of [ATP]/[ADP], and a question arises whether the same relations would be observed if the external ratio had been 50–100, a situation more commonly imposed on isolated mitochondria. Experiments with the translocator reconstituted in the lipid membranes (Krämer & Klingenberg, 1980) are less convincing because the response of the exchange to membrane potential is much smaller than in intact mitochondria (less than 20-fold change in the ratio of ATP to ADP uptake rates for 190 mV change in membrane potential).

However, if one accepts that the translocation is indeed fully electrogenic and dependent on membrane potential one can calculate whether this reaction occurs with any loss in free energy, i.e., how far it is displaced from equilibrium. The total amount of free energy available in translocation equals the sum of the transmembrane potential and $2.3\,RT\log\frac{[ATP]_e/[ADP]_e}{[ATP]_i/[ADP]_i}$. It has been reported by a number of laboratories that transmembrane electrical potential in state 4 is about 150 mV (14.5 KJ/mole) (Nicholls, 1974; Azzone, Bragadin, Pozzan & Dell'Antone, 1976; Holian & Wilson, 1980); the commonly reported difference between the external and internal [ATP]/[ADP] is about 10–50 (5.7–9.7 KJ/mole). This gives the loss in free energy during translocation of adenine nucleotides in state 4 of 8.8–4.8 KJ/mole, which suggests that the reaction is only slightly reversible and might be kinetically limiting in oxidative phosphorylation.

Dependence of Respiration on $[ATP]_e/[ADP]_e$ and $[P_i]_e$

Although the rate of adenine nucleotide exchange is dependent on the extramitochondrial [ATP]/[ADP] and independent of phosphate concentration (Duée & Vignais, 1969), the relationships between respiration and $[ATP]_e/[ADP]_e$ are more complex. It is generally agreed that at a constant $[P_i]$ respiration of isolated mitochondria is dependent on $[ATP]_e/[ADP]_e$ (Davis & Lumeng, 1975; Küster et al., 1976; Holian et al., 1977). Yet when the concentration of inorganic phosphate in the external medium is varied between 1.5 and 8.5 mM the rate of respiration is dependent on the [ATP]/[ADP] $[P_i]$ and not on the [ATP]/[ADP] (Owen & Wilson, 1974; Holian et al., 1977; Lemasters & Sowers, 1979). At higher concentrations of P_i, it is generally agreed that in isolated mitochondria respiration becomes limited by the translocase reaction and shows no dependence on phosphate concentration (for discussion *see* the appendix). (Most investigators have found that respiration is independent of total adenine nucleotide concentration in the range 1–8 mM (Küster et al., 1976; Holian et al., 1977) but reports to the contrary have also appeared in the literature (Davis & Lumeng, 1975).)

It was concluded by Davis and Lumeng (1975) that respiration was dependent on external [ATP]/[ADP] but independent of internal [ATP]/[ADP], which remained constant when the respiratory activity was varied. In later studies, Brawand et al.

(1980) argued that respiration was also dependent on the internal [ATP]/[ADP], although the data presented in support of this contention showed that the internal [ATP]/[ADP] only declined from 1.28 to 0.83 when respiration increased by a factor of 6–8. Although this change could hardly prove the authors' point, Letko et al. (1980) reported a much better correlation between the decrease in $[ATP]_i/[ADP]_i$ and increase in oxygen consumption. Following this line of investigation, claims have been made recently (Kunz et al., 1981; Wanders et al., 1981) that the rate of respiration is directly related to intramitochondrial [ATP]/[ADP] and "that there is no direct, unequivocal control of respiration by the extramitochondrial [ATP]/[ADP] ratio" (Wanders et al., 1981). The arguments for this suggestion are based on experiments carried out on liver mitochondria, which are capable of high rates of intramitochondrial ATP utilization for citrulline synthesis (Duszyński et al., 1981; Kunz et al., 1981; Wanders et al., 1981). It was observed that the intramitochondrial [ATP]/[ADP] remained the same at any one particular rate of respiration and was independent of the site(s) of ATP usage. On the other hand, the extramitochondrial [ATP]/[ADP] was, at the same rate of respiration, higher in the presence of citrulline synthesis than its absence.

Effect of Translocase Inhibitors

The fourth argument in favor the rate-limiting role of adenine nucleotide exchange is the effect of atractyloside and carboxyatractyloside on respiration and cellular metabolism (Akerboom, Bookelman & Tager, 1977; Stubbs, Vignais & Krebs, 1978; Lemasters & Sowers, 1979; Kunz et al., 1981). It was argued that if translocase were at near equilibrium, at a given concentration of the inhibitor the degree of inhibition should be higher at higher rates of mitochondrial respiration and lower at lower rates. By contrast, it has been observed both in suspensions of isolated mitochondria and in suspensions of liver cells that when the rates of respiration were altered, the degrees of inhibition of respiration and translocation by a given concentration of the atractylosides were the same. Although there is a tendency towards a sigmoid character of the inhibitor titration curves in some of the results (e.g., Lemasters & Sowers, 1979), and Stubbs et al. (1978) argued that inhibition of a complex series of reactions by atractyloside does not necessarily indicate that the translocase is a rate-limiting step in that sequence, the inhibitory pattern which is observed has been considered strong support for the translocase concept.

Assumptions and Weaknesses of the Two Hypotheses

The Near Equilibrium Concept

The crucial assumption of the near equilibrium concept is the stoichiometry between electron transfer and ATP synthesis. It has been assumed in all evaluations of near equilibrium relations in the first two sites of oxidative phosphorylation that a precise stoichiometry of 2 molecules of ATP per 2 electrons transferred from the NAD to the cytochrome *c* couples is maintained. This assumption rests on a large body of experimental observation, old and new (for summary *see* Lehninger, 1966; Wainio, 1970; and recently, LaNoue et al., 1978), that phosphorylation of NADH by external ADP and inorganic phosphate occurs with an ATP/O ratio of 3. (It should be mentioned that claims of Hinkle and Yu (1979) of lower P/O values measured in rat liver mitochondria have received little experimental support from other laboratories.) An independent confirmation for the stoichiometry of 2 ATP formed per 2 electrons transferred from NADH to cytochrome *c* comes from measurements of the apparent equilibrium constant between the redox reactions and ATP synthesis in the near equilibrium segment of the respiratory chain. These results show that the value for K_{app} is essentially the same whether measured in the forward (i.e., when respiration drives ATP synthesis) or in the reverse (i.e., when ATP hydrolysis establishes the redox states of the NAD and cytochrome *c* couples) direction and equal, within the limits of experimental error, to the expected values calculated from the equilibrium constants for the partial reactions. As long as the forward and reversed reactions occur by the same pathway, these results provide strong support for the stoichiometry used in our analysis (Forman & Wilson, 1982).

The second point that deserves discussion concerns the accuracy of the measurements of the intramitochondrial $[NAD^+]/[NADH]$ and the cytosolic $[ATP]/[ADP][P_i]$. Determination of the values for these parameters provides the basis for the analysis of the energetics of electron transfer and ATP synthesis and substantial errors in the measurements could weaken experimental support for the concept.

Recent results obtained with the NMR technique suggest that the concentration of free cyto-

solic inorganic phosphate in tissues such as the heart may be by a factor of 2 lower than that measured by analytical procedures (Burt, Cohen & Bárány, 1979; Radda & Seeley, 1979; Meyer, Kushmerick & Brown, 1982). Moreover, Veech et al. (1979) have argued on the basis of the calculations of the apparent value of free [ATP]/[ADP] from other enzyme reactions that the cytosolic $[ADP]_{free}$ may be substantially lower than that obtained from analysis of the total tissue concentrations. Assuming that there is no error in the direct extrapolation of the values for the relevant K_{eq} measured under *in vitro* conditions to the *in vivo* situations and that the assessment of the cytosolic pH is correct, the combined effects of these estimates and the NMR results would increase the cytosolic [ATP]/[ADP] $[P_i]$ values by a factor of 6–8 as compared to those used by us.

On the other hand, we have assumed in all the calculations of intramitochondrial $[NAD^+]$/[NADH] that the pH of the mitochondrial matrix is equal to that of the cytosol. This assumption may be incorrect since recent studies indicate that a pH gradient of about 0.5 pH units, alkaline inside, exists across the inner membrane *in vitro* (Azzone et al., 1976; Holian & Wilson, 1981; Ogawa, Chen & Castillo, 1980) and *in vivo* (Sies, Akerboom & Tager, 1977). The values of the intramitochondrial $[NAD^+]$/[NADH] were calculated from the near equilibrium in either the 3-OH-butyrate or glutamate dehydrogenase reaction for which the equilibrium constants are pH dependent (Krebs, Mellanby & Williamson, 1962; Engel & Dalziel, 1967). Thus the intramitochondrial $[NAD^+]$/[NADH] values would be underestimated by a factor of 3–4 which gives an E_h of the NAD couple more negative by approximately 30 mV as compared to that estimated by us. This translates into an equivalent free energy change of 1.38 kcal for 2 equivalents, sufficient to increase the [ATP]/[ADP] $[P_i]$ by a factor of 3.2. Since the possible errors in the cytosolic [ATP]/[ADP] $[P_i]$ measurements tend to cancel those in the $[NAD^+]$/[NADH] estimates, it appears that the maximum difference between the free energy change in the redox reactions and that in the cytosolic ATP synthesis in the first two phosphorylation sites cannot be larger than 1 kcal, within the current limits of experimental error.

Translocase Hypothesis

The challenge to this concept comes from observations which are difficult to accommodate within its current framework.

a) The rate of translocation of adenine nucleotides is essentially independent of phosphate concentration (Duée & Vignais, 1969) in contrast to respiration in intact cells (Erecińska et al., 1977) and isolated mitochondria (Holian et al., 1977), although this is true in the latter only under conditions which do not a priori limit the translocase activity (*see* Appendix).

b) The capacity of the translocator as measured by exchange of labeled adenine nucleotides in isolated mitochondria is higher than the maximum measured rate of oxidative phosphorylation (for review *see* Vignais, 1976; Klingenberg, 1980; Stubbs, 1981). The K_m value for ADP is between 1 and 10 μM and the K_i for ATP is greater than 100 μM (Vignais, 1976; Klingenberg, 1980). This means that in most physiological situations the translocase could operate at a capacity which well exceeds requirements.

c) The rate of adenine nucleotide translocation is dependent on the [ATP]/[ADP] (Klingenberg, 1970; 1980; Vignais, 1976), but both in suspensions of isolated mitochondria (Holian et al., 1977) and in intact cells (Erecińska et al., 1977) the [ATP]/[ADP] can be decreased or increased with no change in respiratory activity (providing that [ATP]/[ADP] $[P_i]$ is held constant).

d) In the prokaryotic microorganism, *P. denitrificans*, which does not require adenine nucleotide exchange for the formation of ATP by the respiratory chain the dependence of oxidative phosphorylation on the concentrations of ATP, ADP, and P_i is very similar to that seen in mitochondrial oxidative phosphorylation in eukaryotic organisms (Erecińska et al., 1978*a*).

e) ATP is utilized predominantly in the cytosol, whereas a very small amount is consumed by the mitochondrial matrix. This is difficult to reconcile with claims (Kunz et al., 1981; Wanders et al., 1981) that there is no direct relationship between the extramitochondrial [ATP]/[ADP] and the respiratory rate.

f) In surveying the literature it is difficult to understand (*see* Table 2) why reports of the $[ATP]_i/[ADP]_i$ ratios for mitochondria respiring at maximal $[ATP]_e/[ADP]_e$ differ so much from laboratory to laboratory. For $[P_i]_o$ of 3–5 mM some authors report $[ATP]_i/[ADP]_i$ ratios of 1 or less, others find them to be from 4–5, whereas our own results are 9–12, a laboratory-to-laboratory range much wider than could reasonably be expected. (In our hands the sample quenching technique is critical and high values are obtained only when conditions are optimal.) Since the extramitochondrial [ATP]/[ADP] ratios are approximately 100

for the same situations the maximum difference between the external and internal ratios calculated from the latter measurements is 10-fold. It should be noted that the mitochondrial matrix contains 150–500 mg prot/ml and much of the ATP and ADP must by bound to various enzyme active sites. Thus in addition to the quenching problems, preferential binding of ADP over ATP within the matrix could cause the measured $[ATP]_i/[ADP]_i$ to be much lower than the ratio of free nucleotides. Either of these factors alone or in combination could mean that the difference in the thermodynamically relevant terms, $[ATP]_{free}/[ADP]_{free}$, across the mitochondrial membrane is approximately 0.

Conclusions

We have evaluated the two currently "reigning" concepts in the regulation of cellular energy production, pointing out their weaknesses and the assumptions involved. Understandably, we are personally biased towards the near equilibrium hypothesis as being the simpler, more internally consistent and of greater predictive power as well as being able to account for a greater number of experimental observations. There is no doubt in our minds that the translocase reaction could be truly rate limiting under a number of experimental circumstances, especially those *in vitro*. The real issue is, however, whether the exchange of adenine nucleotides limits mitochondrial oxidative phosphorylation in the cell *in vivo* and thus controls the overall rate of cellular energy production. A definite answer to this question must await further experimentation. We would like to finish this review with a brief quotation from Albrecht Dürer which best illustrates our feelings.... "But I shall let the little I have learnt go forth into the day in order that someone better than I may guess the truth, and in his work may prove and rebuke my error. And this I shall rejoice that I was yet a means whereby this truth has come to light...."

After submission of this manuscript, a paper appeared in press (Jacobus et al. (1982) *J. Biol Chem.* **257**:2397–2402) in which the authors present results which show that in suspensions of isolated rat liver mitochondria respiration is, under some conditions, dependent on $[ADP]_{free}$. We agree that in the described conditions the respiratory rate was limited by the availability of ADP since the concentrations used were at or below the K_m value for the translocase. The arguments for such a mechanism in intact liver cells are, however, unconvincing and inconsistent with the bulk of information obtained *in vivo* (*see* text).

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Appendix I

This appendix illustrates relations between respiration and translocase activity in isolated mitochondria incubated with 1, 5, and 29 mM P_i, in the presence and absence of 1 mM magnesium (Table 3). Respiratory rates and [ATP]/[ADP] [P_i] values for dog heart mitochondria are taken from Holian et al. (1977). Since these data are for low [Mg^{2+}], the energetically equivalent [ATP]/[ADP] [P_i] for 1 mM [Mg^{2+}]$_e$ are obtained by correcting for the difference in $\Delta G'_o$ values for the two conditions, i.e., multiplying by 3.9. The rate of adenine nucleotide translocation is calculated using a simple competitive inhibition model and kinetic constants reported in the literature [V_{max} for the translocase, 772 μmol/min/g protein for beef heart mitochondria at 22° (Klingenberg, 1980); K_m for ADP, 2 μM and K_i for ATP, 50 μM (Souverijn, Huisman, Rosing & Kemp, 1973)]. Substituting into the equation:

$$1/v = \frac{K_m}{V_m}\left(1+\frac{[I]}{K_i}\right)\frac{1}{[S]}+\frac{1}{V_{max}}$$

gives the rate of ADP translocation at each [ATP]/[ADP] value. For simplicity only four different respiratory rates are given. The ADP concentration is calculated assuming [ATP]$_e$ = 1 mM. The calculations show:

1. At 1 mM P_i, translocase activity is higher than the respiratory rate both in the presence and absence of magnesium.
2. At 5 mM P_i, translocase activity is higher than the respiratory rate in the absence of magnesium but becomes limiting in its presence, especially at low respiratory activity.
3. At 20 mM P_i, the translocase rate is limiting both in the presence and absence of Mg^{2+}.

Table 3. Relations between respiration and translocase activity in isolated mitochondria in the presence and absence of Mg^{2+}

Respiratory rate (natom O/min/mg prot)	$\frac{[ATP]}{[ADP][P_i]}$	$\frac{[ATP]}{[ADP]}$	$[ADP]_e$ (μM)	Translocase rate (nmole/min/mg prot)	ATP synthesis rate (nmole/min/mg prot)
A. Approximately 0 $[Mg^{2+}]_{free}$; 1 mM $[P_i]$					
11.5	89,125	89.1	11.2	163	34.5
20.3	39,810	39.8	25.1	289	60.9
50.7	6,310	6.3	159.0	611	152
67.6	2,512	2.5	400.0	699	203
B. 1 mM $[Mg^{2+}]_{free}$; 1 mM $[P_i]$					
11.5	347,600	347	2.9	49.8	34.5
20.3	155,300	155	6.5	103	60.9
50.7	24,610	24.6	40.6	380	152
67.6	9,797	9.8	102	547	203
C. Approximately 0 $[Mg^{2+}]_{free}$; 5 mM $[P_i]$					
11.5	89,125	446	2.2	38.4	34.5
20.3	39,810	199	5.0	82.1	60.9
50.7	6,310	31.5	31.7	332	152
67.6	2,512	12.5	80.0	506	203
D. 1 mM $[Mg^{2+}]_{free}$; 5 mM $[P_i]$					
11.5	347,600	1,739	0.58	10.5	34.5
20.3	155,300	776	1.29	23.0	60.9
50.7	24,610	123	8.1	125	152
67.6	9,797	49	20	249	203
E. Approximately 0 $[Mg^{2+}]_{free}$; 29 mM $[P_i]$					
11.5	89,125	2,584	0.39	7.1	34.5
20.3	39,810	1,154	0.87	15.7	60.9
50.7	6,310	183	5.5	89.4	152
67.6	2,512	72.5	13.8	191	203

Table 4. Cellular energy parameters measured with ^{31}P NMR and by chemical analysis

Tissue	[CrP]	[Cr]	[CrP]/[Cr]	$[P_i]$	$\frac{[ATP]_f}{[ADP]_f[P_i]}$	Author
Brain						
Chemical analysis						
Total	4.72[a]	6.11[a]	0.77	2.72[a]	29,839	Veech et al. (1979)
Synaptosomes	3.00[b]	3.81[b]	0.79	2.50[b]	33,091	Rafalowska et al. (1980)
^{31}P NMR	5.2[c]	5.6[c]	0.93	1.6[c]	60,974	Shoubridge et al. (1982)
Heart perfused with glucose						
Chemical analysis	6.22[a]	4.12[a]	1.51	3.07[a]	51,666	Nishiki et al. (1978*a*)
	6.28[a]	3.89[a]	1.61	2.57[a]	65,998	Nuutinen et al. (1982)
	14.0[b]	15.63[b]	0.90	3.73[b]	25,230	Kauppinen et al. (1980)
^{31}P NMR	10.7[b]	13.6[d]	0.79	3.6[b]	22,961	Matthews et al. (1982)
Skeletal muscle (cat soleus)						
Chemical analysis	11.7[a]	6.1[a]	1.92	9.2[a]	21,903	Meyer et al. (1982)
^{31}P NMR	13.2[b]	4.6[e]	2.87	6.1[b]	49,424	Meyer et al. (1982)

[a] μmol/g wet wt. [b] mM.

[c] In the original publication the levels of P_i and CrP are expressed as CrP/ATP or P_i/ATP. We have calculated the concentrations from these ratios and the total ATP of 2.59 μmol/g wet wt and CrP + Cr of 10.83 μmol/g wet wt given by Veech et al. (1979).

[d] Calculated from the authors estimate of CrP + Cr (chemical analysis) of 14 μmol/g wet wt corrected for extracellular (20%) and intramitochondrial (20%) volumes as indicated (Matthews et al., 1982).

[e] The [CrP] measured by ^{31}P NMR was subtracted from the total CrP + Cr determined by chemical analysis to estimate this value.

$[ATP]_f/[ADP]_f[P_i]$ was calculated for pH 7.2 using equilibrium constant:
$K_{CPK} = [ATP][Cr]/[ADP][CrP][H^+] = 1.66 \times 10^9\ M^{-1}$ at 38 °C and 1 mM free $[Mg^{2+}]$ (Lawson & Veech, 1979).

The conclusion that can be drawn from this somewhat oversimplified system is that when experiments on isolated mitochondria are carried out in the presence of high $[Mg^{2+}]_e$ and/or high $[P_i]_e$, respiration is limited by the adenine nucleotide translocase and hence independent of P_i concentration. By contrast, when low magnesium and low P_i are present in the incubation medium, respiration is thermodynamically controlled by the [ATP]/[ADP] $[P_i]$ and hence dependent on $[P_i]_e$. This can explain much of the apparently conflicting data which exists in the literature.

Appendix II

Since the application of ^{31}P NMR to measurements of cellular energy parameters (for review *see* Burt et al., 1979; Radda & Seeley, 1979; Meyer et al., 1982), persistent claims have been made by the proponents of this method that in tissues such as heart, brain, and skeletal muscle, phosphorylation potentials calculated from NMR data are substantially higher than those obtained from chemical analysis of the tissue (e.g., Matthews et al., 1982). Comparison of some of the actual experimental measurements are given in Table 4. The difficulty in compiling this Table lies in that the overwhelming majority of the papers which present ^{31}P NMR results offers no quantitation of the measurements, i.e., does not give the concentrations of the measured parameters but solely their ratios. Moreover, some of the parameters which are crucial for the calculations, such as [Cr], are unobtainable from this technique. The comparison (Table 4) shows that measurements of CrP, ATP and P_i by ^{31}P NMR do not differ from those obtained by chemical analysis. Most claims to the contrary arise from differences in the equilibrium constant for creatine phosphokinase reaction utilized in calculations by various authors.