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THE PHOSPHORYLATION POTENTIAL GENERATED BY RESPIRING MITOCHONDRIA

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SUMMARY

1. The phosphorylation potential, $\Delta G_P = \Delta G_0' + 1.36 \log ([ATP]/[ADP][P_i])$, where $\Delta G_0'$ is the standard free energy of hydrolysis of ATP at a given pH, and [ATP], [ADP] and $[P_i]$ refer to concentrations in the suspending medium, has been determined in rat-liver mitochondria under various conditions.

2. The ATP/ADP ratio is relatively constant, over a 10-fold range of phosphate concentration. Thus, the phosphate potential is higher at low phosphate concentration. State-4 rat-liver mitochondria in the presence of succinate, oxygen and low concentrations of phosphate in State 4 maintain a phosphorylation potential of 16.1 kcal (67.3 kJ) per mole ATP.

3. High concentrations of ATP inhibit ADP uptake, and it is suggested that this is the reason for the independence of the ATP/ADP ratio on the phosphate concentration. A steady-state ratio is set up dependent upon two processes that are relatively slow compared with State-3 respiration, namely ADP transport and ATP hydrolysis.

4. The phosphorylation potential calculated from the concentrations of total ADP, ATP and P_i within State-4 mitochondria is 4.5 kcal/mole less than that in the suspending medium.

5. It was shown experimentally that the phosphorylation potential cannot be calculated from the ΔG of the redox couple, the respiratory-control ratio and the P:O ratio, as has been suggested in the literature.

6. The measured phosphorylation potential is 83% of that calculated from the span succinate to oxygen, assuming thermodynamic equilibrium, and 95% of that calculated from the span NADH to oxygen.

7. Based on the measurements of the phosphorylation potential and of the redox potentials and redox states of redox components in mitochondria, ubiquinone and cytochrome *b* are found at their expected position at the junction of the phosphorylations at Sites 1 and 2. The iron–sulphur centres 2 and 5 and the iron–sulphur centre of succinate dehydrogenase also probably lie at this junction. Cytochrome a_3 lies at its expected junction between phosphorylation Sites 2 and 3. A number of

Abbreviation: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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electron carriers (cytochromes *c*, c_1, and *a*, the iron–sulphur centre of Complex III and the EPR-detectable copper), however, lie in the 'no-man's land' within Site 2.

8. A phosphorylation potential of 16.1 kcal/mole corresponds to a membrane potential of 350 mV in State 4, on the basis of the chemiosmotic hypothesis.

INTRODUCTION

In the presence of oxidizable substrate and oxygen, mitochondria are able to catalyse the phosphorylation of ADP by P_i. When the concentration of P_i exceeds that of the ADP, the rate of respiration sharply declines as the ADP is consumed[1]. Muraoka and Slater[2] (*cf.* Klingenberg and Schollmeyer[3]) concluded that, in such 'State-4' mitochondria[1], the redox reactions of the respiratory chain and the phosphorylation reactions are near thermodynamic equilibrium. They suggested that in State 4 an equilibrium is reached, that may be expressed by the so-called 'phosphorylation potential', which may be measured by determining the concentrations of ATP, ADP and P_i in the medium in which mitochondria are suspended, after State 4 is reached. The phosphorylation potential (ΔG_P) is given by

$$\Delta G_P = \Delta G_0' + 1.36 \log \frac{[ATP]}{[ADP][P_i]} \quad (1)$$

where $\Delta G_0'$ is the standard free-energy change of the reaction given in Eqn 2.

$$ADP + P_i \rightleftharpoons ATP + H_2O \quad (2)$$

Values for $\Delta G_0'$ for this reaction under various conditions have been recently redetermined by Rosing and Slater[4] and tabulated for various values of pH, ionic strength and Mg^{2+} concentration. We shall refer to the concentration term in Eqn 1, 1.36 log ([ATP]/[ADP][P_i]), as ΔG_c.

Cockrell *et al.*[5] reported a single determination of the phosphorylation potential in State-4 rat-liver mitochondria oxidizing succinate at 25 °C in the presence of rotenone, at pH 7.8, in the absence of added Mg^{2+}. They found that $\Delta G_c = 6.0$ kcal/mole which, combined with a value of 9.6 kcal/mole for $\Delta G_0'$ under these conditions[6], gave 15.6 kcal/mole for the phosphorylation potential. On the basis of the new value for $\Delta G_0'$ under these conditions[4], this value of ΔG_P may now be recalculated as 14.9 kcal/mole. In several lectures and reviews, we have reported values of ΔG_c similar to that found by Cockrell *et al.*[5] in respiring rat-liver mitochondria[7–9] and *Azotobacter* particles[9,10] and in illuminated chloroplasts[9,11]. In the light of the recent redetermination[4] of $\Delta G_0'$, however, the values of ΔG_P calculated from these data must be decreased. This made it desirable to repeat the early measurements, using an improved method of stopping the reaction.

These new determinations are described in this paper, together with other experiments relevant to the question of the nature of the State-3 to State-4 transition in respiring mitochondria. The conclusion in the earlier papers[7–10] that in State 4 Eqn 3 is close to thermodynamic equilibrium is confirmed.

$$NADH + H^+ + \tfrac{1}{2}O_2 + 3ADP + 3P_i \rightleftharpoons NAD^+ + H_2O + 3ATP + 3H_2O \quad (3)$$

RESULTS

The phosphorylation potential in State-4 rat-liver mitochondria

The effects of varying the concentrations of phosphate, ADP and ATP on the phosphorylation potential with succinate as substrate are shown in Table I. The ATP/ADP ratio is relatively constant over a wide range of P_i and ADP concentration, so that the calculated phosphate potential is lower at the high phosphate concen-

TABLE I

DETERMINATION OF PHOSPHORYLATION POTENTIALS AT VARYING CONCENTRATIONS OF ATP, ADP AND P_i WITH SUCCINATE AS SUBSTRATE

Expt 1 was carried out in an Oxygraph and Expt 2 in a vessel in which oxygen was led over the medium which was stirred vigorously by a magnetic stirrer (see Methods). The reaction mixture contained 50 mM KCl, 46 mM Tris–HCl buffer, 2.5 mM EDTA and 7.5 mM succinate (Expt 1) or 40 mM sucrose, 66 mM KCl, 20 mM Tris–HCl buffer, 1.5 mM EDTA and 5 mM succinate (Expt 2), and varying amounts of ATP, ADP and P_i. Temperature 25 °C. pH 7.7. Protein, 1 mg/ml in Expt 1 and 2.5 mg/ml in Expt 2. 0.15 μg rotenone/mg protein (Expt 1) and 1.0 μg rotenone/mg protein (Expt 2).

Expt	P_i (*mM*)	*ADP* (*mM*)	*ATP* (*mM*)	[*ATP*]/[*ADP*]	ΔG_c	$\Delta G_o'$	ΔG_P
					(*kcal/mole*)		
1	0.8	0.015	3.96	264	7.5	8.7	16.2
	2.2	0.013	4.16	320	7.0	8.7	15.7
	3.8	0.003	1.15	383	6.8	8.7	15.5
	4.3	0.013	3.88	298	6.6	8.7	15.3
	10.8	0.015	3.56	237	5.9	8.7	14.6
2	0.5	0.080	10.2	127	7.4	8.7	16.1
	0.5	0.146	20.0	136	7.4	8.7	16.1
	3.1	0.071	10.1	142	6.3	8.7	15.0

TABLE II

EFFECT OF THE pH ON THE PHOSPHORYLATION POTENTIAL

The reactions were carried out in an Oxygraph. The reaction mixture contained 50 mM sucrose, 50 mM KCl, 50 mM Tris–HCl buffer, 0.4 mM EDTA, 5.5 mM P_i, 6.5 mM succinate, 0.1 μg rotenone/mg protein and 3.6 mM (Expt 3) or 2.6 mM (Expts 1, 2 and 4) ATP. State 3 was initiated by addition of 0.13 mM ADP. Temperature, 25 °C. Protein, 1.5 mg/ml.

pH	P_i	*ADP*	*ATP*	[*ATP*]/[*ADP*]	ΔG_c	$\Delta G_o'$	ΔG_P
	(*mM*)				(*kcal/mole*)		
6.2	5.45	0.0028	2.62	935	7.1	7.4	14.5
7.0	5.33	0.0025	2.92	1144	7.3	7.9	15.2
7.7	5.45	0.0060	3.80	631	6.9	8.7	15.6
8.3	5.43	0.0224	2.77	123	5.8	9.5	15.3

TABLE III

EFFECT OF Mg^{2+} ON THE PHOSPHORYLATION POTENTIAL

The reactions were carried out in an Oxygraph. The reaction mixture contained 50 mM sucrose, 50 mM KCl, 50 mM Tris–HCl buffer, 0.4 mM EDTA and varying amounts of ATP and P_i. State 3 was initiated by addition of 0.17 mM ADP. With succinate as substrate 0.1 μg rotenone/mg protein was present. Temperature, 25 °C. Protein, 1.4 mg/ml. pMg was calculated from the amount of Mg^{2+} added, the concentrations of P_i, ADP and ATP and the binding constants, as detailed in Methods.

Substrate	*pH*	*Mg^{2+} added (mM)*	*pMg*	*P_i (mM)*	*ADP*	*ATP*	*[ATP]/[ADP]*	*ΔG_c (kcal/mole)*	*$\Delta G_o'$*	*ΔG_P*
Succinate (7.5 mM)	7.5	—		0.50	0.027	2.05	76	7.1	8.5	15.6
	7.5	1.7	4.4	0.51	0.011	2.00	182	7.6	8.1	15.7
Succinate (6.8 mM)	7.7	—		5.76	0.0130	5.517	423	6.6	8.7	15.3
	7.7	2.0	5.4	5.54	0.0143	5.536	387	6.6	8.7	15.3
	7.7	4.0	4.6	5.54	0.0117	5.478	468	6.7	8.4	15.1
	7.7	8.0	2.8	5.37	0.0150	5.478	366	6.6	7.5	14.1
β-Hydroxybutyrate (5 mM)	7.0	—		3.25	0.0125	2.32	185	6.5	7.9	14.4
	7.0	3.1	3.5	2.97	0.0038	2.44	642	7.3	7.0	14.3

trations. At low phosphate concentrations the potential is a little more than 16 kcal/mole.

The effect of pH on the phosphorylation potential at constant P_i is shown in Table II. In comparison with the effect on $\Delta G_0'$ (ref. 4) that on ΔG_c is quite small, except above pH 8 where a considerably lower ΔG_c and a somewhat low ΔG_P were found, possibly because of some uncoupling at high pH.

The addition of magnesium causes a decrease in $\Delta G_0'$ (ref. 4), which at high concentrations is not compensated for by an increase in ΔG_c. Up to a pMg value of 3.5, Mg^{2+} has little effect on ΔG_P.

Succinate, β-hydroxybutyrate and malate (in the presence of glutamate) all yield about the same phosphorylation potential (Table IV). The value with ascorbate, in the presence of TMPD, is a little lower. The slower oxidation of glutamate to 2-oxoglutarate maintains a considerably lower potential. This was also found to be the case when the oxidation of succinate was slowed by addition of increasing amounts of malonate (Table V). The rate of oxidation of glutamate is only 16% of that of succinate, and the effect of malonate is only noticeable when the respiratory rate reaches a comparable value.

The difference between exogenous and endogenous phosphorylation potentials

Klingenberg *et al.*[12] reported that the ratio of the concentrations of endogenous ATP and ADP in State-4 mitochondria oxidizing succinate is lower than the ATP/ADP ratio in the suspending medium. Taking into account the higher internal phosphate concentration, the phosphorylation potential of the exogenous ATP, ADP and P_i system was in his experiments 2.8 kcal/mole higher than that of the endogenous system.

TABLE IV

DETERMINATION OF PHOSPHORYLATION POTENTIALS WITH VARIOUS SUBSTRATES

The reactions were carried out in an Oxygraph. The reaction mixture contained 50 mM sucrose, 50 mM KCl, 45 mM Tris–HCl buffer, 1.8 mM EDTA, 4.8 mM P_i, 2.6 mM ATP. State 3 was initiated by addition of 0.25 mM ADP. With glutamate as substrate (in the presence of malonate) no ATP was present at the beginning of the experiment. With succinate 0.11 μg rotenone/mg protein and with TMPD–ascorbate 0.11 μg rotenone and 0.04 μg antimycin/mg protein were present. Temperature, 26 °C. pH 7.7. Protein, 1.33 mg/ml.

Substrate	P_i	*ADP*	*ATP*	[*ATP*]/[*ADP*]	ΔG_c	$\Delta G_o'$	ΔG_P
	(*mM*)				(*kcal/mole*)		
TMPD (56 μM), ascorbate (6.6 mM)	4.76	0.108	2.41	22	5.0	8.7	13.7
Succinate (6.6 mM)	4.72	0.009	2.65	300	6.5	8.7	15.2
β-Hydroxybutyrate (6.6 mM)	4.59	0.015	2.99	200	6.3	8.7	15.0
Malate (6.6 mM), glutamate (6.6 mM)*	4.75	0.022	2.59	120	6.0	8.7	14.7
Glutamate (6.6 mM)**	4.45	0.111	0.15	1.4	3.2	8.7	11.9

* 6.6 mM malonate and 1.6 mM arsenite also present.

** 3.3 mM malonate and 1.6 mM arsenite also present.

TABLE V

EFFECT OF INHIBITION OF THE SUCCINATE OXIDATION ON THE PHOSPHORYLATION POTENTIAL

The experiment was carried out in an Oxygraph. The reaction mixture contained 43 mM Tris–HCl buffer, 43 mM sucrose, 43 mM KCl, 1.7 mM EDTA, 2.4 mM succinate, 4.0 mM ATP, 4.5 mM phosphate and 0.23 μg rotenone/mg protein. State 3 was initiated by addition of 0.19 mM ADP. pH 7.75. Temperature, 25 °C. Protein, 1.3 mg/ml. The respiratory rates in the absence of malonate were 182 and 78 natoms oxygen per min per mg protein in States 3 and 4, respectively.

Malonate (*mM*)	*Respiratory rate* (*arbitrary units*)		*ATP*	*ADP*	P_i	[*ATP*]/[*ADP*]	ΔG_c	$\Delta G_o'$	ΔG_P
			(*mM*)				(*kcal/mole*)		
	State 3	*State 4*							
0	100	100	4.08	0.0104	4.08	394	6.7	8.8	15.5
0.033	73	94	4.39	0.0106	4.50	413	6.7	8.8	15.5
0.066	63	96	4.26	0.0129	4.26	331	6.6	8.8	15.4
0.099	53	87	4.26	0.0146	4.44	293	6.5	8.8	15.3
0.164	37	79	4.23	0.0199	4.42	213	6.3	8.8	15.1
0.328	22	43	4.26	0.0722	5.14	59	5.5	8.8	14.3
0.660	14	28	4.05	0.1830	4.53	22	5.0	8.8	13.8

TABLE VI

COMPARISON OF EXOGENOUS AND ENDOGENOUS PHOSPHORYLATION POTENTIALS

The reactions were carried out in an Oxygraph. The reaction mixture contained 50 mM sucrose, 50 mM KCl, 45 mM Tris–HCl buffer, 1.5 mM P_i, 1.3 mM EDTA, 0.67 mM ATP, 7.5 mM, succinate and 0.07 μg rotenone/mg protein. State 3 was initiated by addition of 0.2 mM ADP. When State 4 was reached mitochondria were separated by rapid centrifugation through silicone and both the supernatant and the acid layer were analysed for ATP, ADP and P_i. In Expt 2 no exogenous adenine nucleotides were present and the reaction was stopped after 1 min by mixing with phenol–chloroform–isoamylalcohol (see Methods). Protein, 2 mg/ml. pH 7.7. Temperature, 25 °C.

Expt	*Exogenous*				*Endogenous*			
	ATP	*ADP*	P_i	ΔG_P (*kcal/mole*)	*ATP*	*ADP*	P_i	ΔG_P (*kcal/mole*)
	(*mM*)				(*mM*)			
1	0.688	0.0036	1.45	15.7	5.0	1.8	12.0	11.2
2	—	—	—	—	5.4	0.8	11.5	11.8

We have measured endogenous phosphorylation potentials under various conditions with succinate as substrate in the presence of rotenone (Table VI). In calculating the endogenous potentials we assumed (i) all the ATP, ADP and P_i is free, (ii) the internal pH equals the external pH, (iii) a small amount of free magnesium (0.1 mM) is present in the matrix space, and (iv) the ionic strength of the matrix space is equal to 0.2. The last three assumptions are of importance for selecting the

$\Delta G_0'$ to be used for the calculation of the endogenous phosphorylation potential. It is clear that there is indeed a significant difference between the endogenous potential calculated in this way and exogenous potential. The low endogenous phosphorylation potential is probably not caused by hydrolysis of ATP as result of an anaerobic state developing during centrifugation through silicone oil[13], since mitochondria incubated with succinate and phosphate without added adenine nucleotides have the same low endogenous phosphorylation potential when the reaction is stopped directly by the phenol method (Expt 2, Table VI).

According to Klingenberg *et al.*[12] uncoupler brings about an equalization of the exogenous and endogenous ATP/ADP ratios when ATP and ADP are added to mitochondria in the absence of substrate. We have tested the effect of the uncoupler CCCP at high exogenous ratios maintained by phosphoenolpyruvate in the presence of pyruvate kinase. Table VII shows that the internal ATP/ADP ratio was substantially increased by the addition of uncoupler, without any effect on the external ratio, but equilibration was not reached and, in fact, the concentration of endogenous ADP reached was no lower than that reached in respiring mitochondria. This experiment does not, therefore, exclude the possibility previously suggested[7] that some of the mitochondrial ADP is firmly bound so that the phosphorylation potential is seriously underestimated if it is assumed that it is all free.

TABLE VII

EFFECT OF UNCOUPLER ON THE ENDOGENOUS ATP/ADP RATIO

Mitochondria were incubated in a medium containing 40 mM sucrose, 66 mM KCl, 1.3 mM EDTA, 4 mM $MgCl_2$, 33 mM Tris–HCl buffer, 3.5 mM phosphoenolpyruvate, 1 mM ATP and 25 units (μmole/min) pyruvate kinase. Temperature, 25 °C, pH 7.8. After 3 min, two samples were taken, one being deproteinized immediately with the phenol mixture for the determination of the total (ATP+ADP). The mitochondria from the second sample were centrifuged through silicone oil into 15% $HClO_4$ and the acid layer analysed immediately for ATP and ADP derived from the mitochondria. The external concentrations of ATP and ADP were calculated from the difference between the analysis of the two samples. After taking the samples 0.5 μM CCCP was added to the reaction mixture and, after 3 min, samples were again taken and treated as described. Protein, 2 mg/ml.

CCCP (μ*M*)	*Exogenous*			*Endogenous*			
	ATP (*mM*)	*ADP* (*mM*)	*ATP/ADP*	*ATP* (*mM*)	*ADP* (*mM*)	*AMP* (*mM*)	*ATP/ADP*
0*	—	—	—	3.5	12.4	1.9	0.28
0	0.80	<0.006	>130	11.0	11.3	0.9	0.97
0.5	0.76	<0.002	>380	17.0	3.0	0.8	5.66

* No added ATP or pyruvate kinase.

The role of the adenine nucleotide translocator

In contrast to the sharp transition from State 3 to State 4 when a low concentration of ADP (0.2 mM) is consumed by respiring mitochondria (Fig. 1A) in the absence of added ATP, the transition is gradual in the presence of a high concentration of ATP (Fig. 1B). Thus the respiration is inhibited long before all the ADP

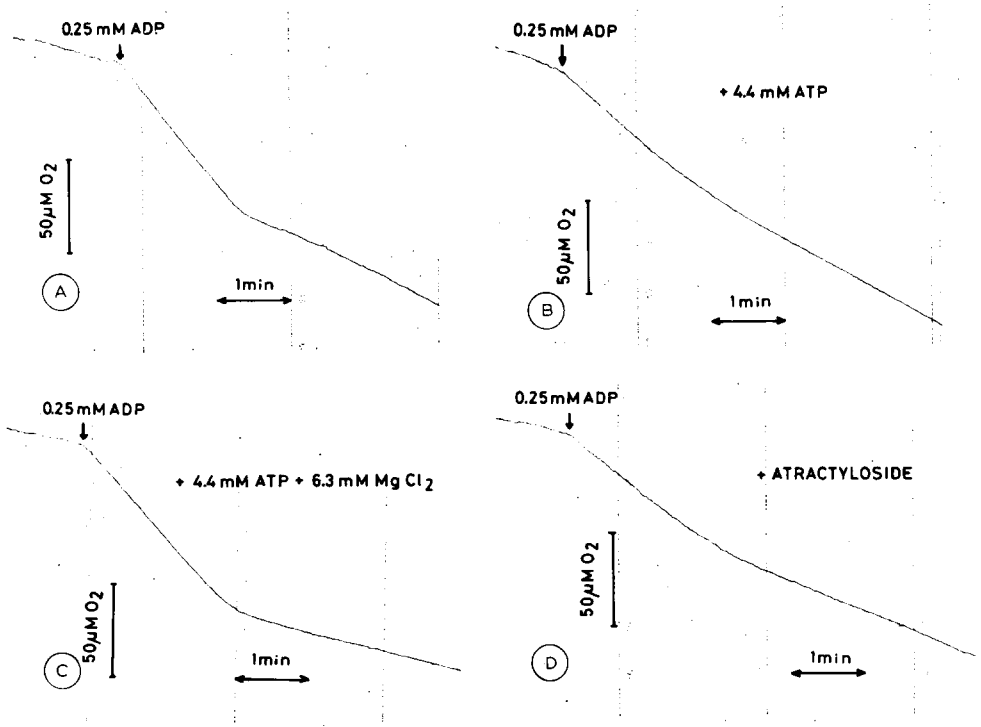

Fig. 1. Inhibitory effect of ATP and atractyloside on rate of oxygen uptake during succinate oxidation in State 3. The reaction was carried out in an Oxygraph. The reaction mixture contained 75 mM sucrose, 20 mM KCl, 30 mM Tris–HCl buffer, 2.5 mM EDTA, 6.3 mM phosphate, 4.7 mM succinate and 0.7 μg rotenone/mg protein in a total volume of 1.5 ml. State 3 was initiated by addition of 0.25 mM ADP. Temperature, 26 °C. pH 7.4. Protein, 0.44 mg/ml. The experiment shown in (B) was carried out in the presence of 4.4 mM ATP, that of (C) in the presence of 4.4 mM ATP and 6.3 mM $MgCl_2$ and that of (D) in the presence of 0.57 μg atractyloside/mg protein.

is consumed. Added Mg^{2+} largely abolishes the inhibitory effect of ATP (Fig. 1C). Addition of atractyloside has the same effect as addition of ATP (Fig. 1D). These results suggest that the rate of respiration is governed by the ATP/ADP ratio, perhaps because of competition between ATP and ADP for entry into the mitochondria which only becomes important at high ATP/ADP ratios. This would also explain the effect of added Mg^{2+} which binds more firmly to ATP than to ADP (*cf.* ref. 14), since free ATP and not the magnesium complex is the substrate of the adenine nucleotide translocator[15].

This suggestion is given strong support by the experiments described in Fig. 2 in which the uptake of [^{3}H]ATP and [^{14}C]ADP by rat-liver mitochondria during State-3 and State-4 respiration at 15 °C were measured. In the experiment described in Fig. 2A, in which no ATP was added, [^{14}C]ADP was transported rapidly into the mitochondria during State 3 while there was only a small uptake of [^{3}H]ATP. However, coincident with the inhibition of the oxygen uptake corresponding to transition to State 4, [^{3}H]ATP was taken up accompanied by a loss of ^{14}C. When ATP was present from the beginning (Fig. 2B), the uptake of [^{3}H]ATP began earlier,

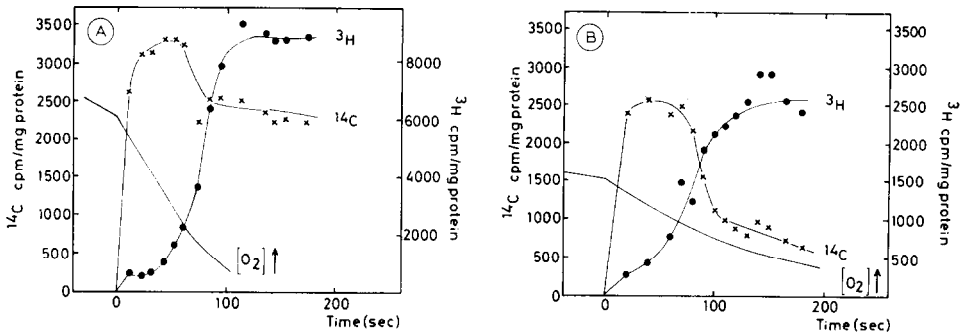

Fig. 2. Uptake of ATP and ADP by rat-liver mitochondria during State 3, State 4 and the State 3–State 4 transition. The reaction was carried out in an Oxygraph. The reaction mixture contained 55 mM KCl, 55 mM Tris–HCl buffer, 2.8 mM EDTA, 8 mM P_i, 7.9 mM succinate and 0.08 μg rotenone/mg protein. State 3 was initiated by addition of 0.21 mM [^{14}C]ADP (237 cpm/nmole) and 0.044 mM [^{3}H]ATP (4050 cpm/nmole). At various times samples were taken and uptake of radioactive label was measured as described in Methods. Temperature, 15 °C. Protein, 1.76 mg/ml. Total volume, 1.85 ml. (A) 0.09 mM ATP. (B) 1.05 mM ATP.

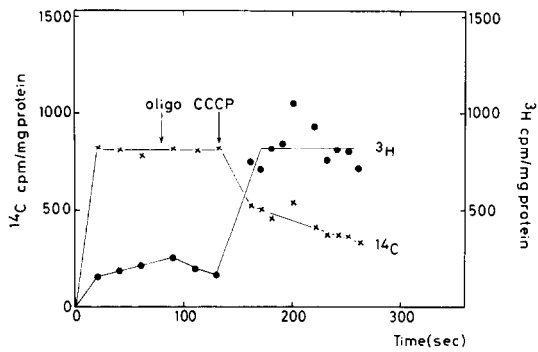

Fig. 3. Effect of the addition of uncoupler on the exchange of endogenous adenine nucleotide against [^{3}H]ATP by rat-liver mitochondria. The experiment was carried out in an Oxygraph. The reaction mixture contained 55 mM KCl, 55 mM Tris–HCl buffer, 2.8 mM EDTA, 8 mM P_i, 1.05 mM ATP, 7.9 mM succinate and 0.08 μg rotenone/mg protein. State 3 was initiated by addition of 0.5 mM [^{14}C]ADP (100 cpm/nmole) and 1 mM [^{3}H]ATP (90 cpm/nmole). After 80 s 6 μg oligomycin/mg protein and after 130 s 0.5 μM CCCP were added. At various times samples were taken and uptake of radioactive label was measured as described in Methods. Temperature, 15 °C. Mitochondrial protein, 1.76 mg/ml. Total volume, 1.85 ml.

at the same time as respiration became inhibited. It may be concluded that ATP can be transported into the inner space of the mitochondrion as soon as a sufficiently high ATP/ADP ratio is built up. Addition of uncoupler + oligomycin in State 3 also leads to [^{3}H]ATP uptake and concomitant loss of ^{14}C label by the mitochondria (Fig. 3), indicating that uncoupler abolishes the preferential uptake of ADP, in agreement with Klingenberg *et al.*[12] and Pfaff and Klingenberg[16].

Calculation of phosphorylation potential from steady-state rates

Rottenberg *et al.*[17] propose that oxidative phosphorylation is regulated by two flows and forces, *viz.* the oxidation flow J_O and the phosphorylation flow J_P with

the corresponding forces A_O and A_P. In the terminology of this paper, $A_O = -\Delta G_O$ of the redox reaction (ΔG_{redox}) and $A_P = -\Delta G_P$. The P:O ratio is equal to $(J_O)_3 : (J_P)_3$, and the respiratory-control index (r) by $(J_O)_3 : (J_O)_4$, where the suffices refer to State 3 and State 4, respectively. The flows are related to the forces by Eqns 4 and 5

$$(A_P)_3 = R_P \cdot (J_P)_3 + R_{PO}(J_O)_3 \tag{4}$$

$$(A_O)_3 = R_O \cdot (J_O)_3 + R_{OP}(J_P)_3 \tag{5}$$

where the R terms are phenomenological coefficients, related to the kinetic parameters of the system. Assuming that the matrix of the phenomenological coefficients is symmetrical (Onsager relation), the phenomenological relations are linear and the coefficients are constant and equal in State 3 and State 4, Rottenberg derived Eqn 6.

$$\Delta G_P = \frac{\Delta G_{redox}(1 - r)}{r \cdot (P{:}O)} \tag{6}$$

We have tested this relationship with various substrates and under various conditions, by determining the respiratory-control index from an Oxygraph trace, calculating the P:O ratio from the amount of oxygen necessary to phosphorylate the ADP added and determining the phosphate potential by direct analysis. Table VIII shows that, in our experiments, little correlation was found between the phosphorylation potential calculated by Rottenberg's relationship and that directly measured.

TABLE VIII

COMPARISON OF MEASURED PHOSPHORYLATION POTENTIALS AND POTENTIALS CALCULATED ACCORDING TO THE METHOD OF ROTTENBERG *et al.*[17]

The reactions were carried out in an Oxygraph. The reaction mixture contained 50 mM sucrose, 50 mM KCl, 45 mM Tris–HCl buffer, 0.4 mM EDTA and various amounts of ATP, P_i and Mg^{2+}. State 3 was initiated by addition of 0.27 mM ADP. With TMPD–ascorbate 0.12 μg rotenone and 0.05 μg antimycin/mg protein and with succinate as substrate 0.12 μg rotenone/mg protein were present. Temperature, 25–26 °C. Protein, 1.2 mg/ml. The following ΔG_{redox} values were used for the calculation: succinate, −38 kcal; ascorbate, −34 kcal; β-hydroxybutyrate, −52 kcal.

pH	*Substrate*	$[Mg^{2+}_{free}]$ (*mM*)	*P:O*	*r*	ΔG_P	
					(*a*)*	(*b*)**
6.2	Succinate (7 mM)	—	1.49	3.2	17.5	14.5
7.0	Succinate (7 mM)	—	1.49	5.2	20.6	15.2
7.7	Succinate (7 mM)	—	1.62	4.4	18.1	15.9
7.7	Succinate (7 mM)	—	1.45	1.18	4.0	15.7
8.3	Succinate (7 mM)	—	1.05	1.57	13.1	15.3
7.7	Succinate (7 mM)	4.5	1.43	5.4	21.6	14.0
7.7	TMPD (5.6 μM), ascorbate (6.6 mM)	—	1.00	1.11	3.4	14.3
7.7	β-Hydroxybutyrate (6.6 mM)	—	1.71	1.51	10.3	15.1
7.7	β-Hydroxybutyrate (6.6 mM)	—	1.72	1.13	3.5	14.8

* Calculated from Eqn 6.
** Calculated from Eqn 1.

Rottenberg *et al.*[17] suggest further that the phenomenological coefficients R_P and R_{PO} may be calculated by measuring $(A_P)_3$ at different values of $(J_P)_3$ and applying Eqn 4. Table IX shows, however, that during the increase of $(A_P)_3$ that occurs during the phosphorylation of ADP, the two flows are either constant in the absence of ATP (Expt 1, Table IX) or change proportionally in the presence of ATP (Expt 2, Table IX). This leads to the conclusion that the phenomenological equations are not linear and should be described by terms of a higher order.

TABLE IX

DETERMINATION OF $(J_P)_3$, $(J_O)_3$ AND $(A_P{}^{ex})_3$ DURING STATE 3

The experiment was carried out in an Oxygraph. At various times in State 3 a sample was taken and deproteinized by the phenol mixture. The reaction mixture contained 50 mM KCl, 46 mM Tris–HCl buffer, 2.5 mM EDTA, 7.5 mM succinate, 7.5 mM P_i (Expt 1) or 5.6 mM P_i (Expt 2) and 0.3 μg rotenone/mg protein (Expt 1) or 0.17 μg/mg protein (Expt 2). Expt 2 was carried out in the presence of 7.5 mM ATP. State 3 was initiated by addition of 0.176 mM (Expt 1) or 0.380 mM ADP (Expt 2). $(Jo^{ex})_3$ (natoms O/s) was calculated from the Oxygraph trace and $(J_P{}^{ex})_3$ (nmoles/s) from the amount of ADP that was phosphorylated between zero time and the time indicated. pH 7.6. Temperature, 25 °C. Protein, 0.7 mg/ml in Expt 1 and 1.1 mg/ml in Expt 2.

Expt	*Time* (*s*)	*ADP* (*mM*)	*ATP* (*mM*)	P_i (*mM*)	$(J_P)_3$	$(J_O)_3$	$(J_P)_3:(J_O)_3$	ΔG_P
1	0	0.176	0	7.5	—	—	—	—
	20	0.123	0.025	7.4	2.7	1.6	1.69	10.6
	25	0.109	—	7.4	2.7	1.6	1.69	10.9
	30	0.097	—	7.4	2.6	1.6	1.63	11.1
	32	0.094	0.053	7.4	2.6	1.6	1.63	11.2
	45	0.061	0.080	7.4	2.6	1.6	1.63	11.7
	55	0.032	0.100	7.3	2.6	1.6	1.63	12.2
	67	0.011	0.151	7.3	2.5	1.6	1.56	13.1
	75	0.002	0.171	7.3	State 4			13.8
	150	0.005	0.164	7.3	State 4			13.8
2	0	0.380	7.63	5.60	—	—	—	—
	42	0.254	7.63	5.47	2.15	1.81	1.2	13.5
	60	0.246	7.66	5.47	1.87	1.71	1.1	13.6
	72	0.217	7.71	5.44	1.94	1.62	1.2	13.7
	109	0.170	7.76	5.39	1.55	1.37	1.1	13.8
	136	0.120	7.82	5.34	1.25	1.18	1.06	14.0
	173	0.056	7.85	5.28	0.83	0.88	0.94	14.6
	270	0.020	7.86	5.24	State 4			15.2

DISCUSSION

On the basis of the experiments described in this paper, the following description of the events that occur on the transition of rat-liver mitochondria from State 3 to State 4 is possible. During State-3 oxidation of a rapidly oxidizable substrate, such as succinate, the rates of electron transfer through the respiratory chain and phosphorylation of ADP by P_i, coupled with the electron transfer, greatly exceed those

of side reactions such as energy-requiring ion movements or the adenylate kinase reaction. According to Kemp *et al.*[18], a rate-limiting step in State-3 respiration is the transport of ADP into and of ATP out of the mitochondria, a process catalysed by the adenine nucleotide translocator. Fig. 2 shows that the translocator preferentially transports ADP into the mitochondria until State 4 is approached, when as shown by the results in Table I the ATP/ADP ratio exceeds 100. The transport of ADP into the mitochondria now becomes so slow that the rate of the side reactions is no longer negligible. In consequence, the ATP/ADP ratio reaches a steady-state value, governed by two processes that are both relatively slow compared with State-3 respiration, *viz.* (i) the transport of ADP in the presence of a high concentration of ATP and (ii) energy-dissipating reactions that lead to the conversion of ATP to ADP.

This provides a simple explanation for the finding (see Table I) that the ATP/ADP ratio is largely independent of the phosphate concentration, which would not be the case if the chemical reaction Eqn 3 reached true thermodynamic equilibrium in State 4. The well-known fact that the respiratory rate in State 4, although much lower than in State 3, is not zero also shows that true thermodynamic equilibrium is not reached. The question at issue is: how close can the system – substrate, O_2, ADP, ATP, P_i – come to thermodynamic equilibrium in State 4? In this respect, only the maximum values of the phosphorylation potential are of interest. The lower values found with high phosphate concentrations (Table I) are now explained by the fact that the higher ATP/ADP ratios to be expected with higher P_i concentrations cannot be reached because of the limitations on ADP transport at these high ratios, as explained above. The lower values of the phosphorylation potential at low pH (Table II) can be explained in a similar way. Because of the lower $\Delta G_0'$ at low pH, ΔG_c (and, therefore, at constant P_i, the ATP/ADP ratio) must be higher in order that ΔG_P be constant. Even though the ATP/ADP ratio exceeded 1000 at pH 7.0 in the experiment given in Table II, the calculated phosphorylation potential was no greater than 15.2 kcal/mole ATP. The effect of Mg^{2+} in high concentration (Table III) can be explained in a similar way.

The low phosphorylation potentials measured with slowly respiring substrates are presumably due to the fact that the side reactions become relatively more important than the rate of phosphorylation coupled with electron transport. The lower values found with ascorbate–TMPD (Table IV) agree with the experience that the respiratory-control index is lower with this substrate pair than with succinate. A commonly advanced explanation for this difference, however, namely that the energy-dissipating 'leaks' are greater in Site 3 than in the other phosphorylating sites, appears now to be improbable, since these leaks would also operate with succinate as substrate. It is more likely that TMPD is actively accumulated as a cation, at the expense of the phosphate potential, as suggested by J. L. Howland (personal communication).

The maximum values of the phosphate potential reached with succinate as substrate and at low phosphate concentrations were a little more than 16 kcal. Under the conditions reached in State 4 in these experiments (succinate, 7.5 mM; fumarate, 0.25 μM; pO_2, 0.1 atm; pH 7.7), the $\Delta G'$ of the redox reaction

$$\text{Succinate} + \tfrac{1}{2}\,O_2 \rightleftharpoons \text{fumarate} + H_2O$$

is -38.8 kcal/mole (the E_0' for the succinate–fumarate couple used in this calculation

is $24-0.7\cdot59=-19$ mV (*cf.* ref. 19)). If thermodynamic equilibrium were reached in the reaction given by Eqn 7,

$$\text{Succinate} + \tfrac{1}{2}O_2 + 2ADP + 2P_i \rightleftharpoons \text{fumarate} + H_2O + 2ATP + 2H_2O \qquad (7)$$

then $\Delta G'$ of the phosphorylation reaction

$$2ADP + 2P_i \rightleftharpoons 2ATP + 2H_2O$$

must be equal to 19.4 kcal/mole ATP. The ratio $16.1/19.4=0.83$ is a measure of how close thermodynamic equilibrium may be reached in State 4.

With succinate as substrate, the same value for the phosphorylation potential was found in the absence of rotenone as in its presence. Under these conditions, the succinate–fumarate couple reacts with the NADH–NAD^+ couple by reversal of the respiratory chain, and it becomes possible to calculate the phosphorylation potential to be expected from the span NADH to oxygen, if the whole system were in equilibrium. In State 4 under the conditions of these experiments (low P_i concentration), the NADH/NAD^+ ratio was found to be 1.6. If it is assumed that this approximates the relative concentrations of unbound NADH and NAD^+ (see, however, Hoek[20]), it may be calculated that the $\Delta G'$ of the redox reaction

$$NADH + H^+ + \tfrac{1}{2}O_2 \rightleftharpoons NAD^+ + H_2O$$

is -50.8 kcal/mole. If this free energy is utilized for the synthesis of 3 moles of ATP per mole NADH, without any side reaction, a phosphorylation potential of 16.9 kcal/mole ATP would be expected. The experimental value is 95% of that expected.

The fact that mitochondria can still make ATP against a phosphorylation potential of 16.1 kcal/mole ATP has important implications for the mechanism of oxidative phosphorylation. In the first place, it means that the redox-potential gap between the redox components of the respiratory chain on the substrate and oxygen sides of a phosphorylation site must differ by 350 mV for a 2-electron transfer. Secondly, it implies that the redox potentials of all redox components of the respiratory chain, in equilibrium with substrate, O_2 and the ADP, ATP, P_i system must be grouped equally between NADH and oxygen. A gap of 350 mV between phosphorylation sites is consistent with the respiratory-chain carriers being grouped at -350 mV (NADH), -0 to 50 mV, 350–400 mV and 750 mV (O_2). In this context the redox potentials referred to are not the standard or mid-point potentials, but the actual potentials given by the equation

$$E' = E'_0 + \frac{59}{n}\log\frac{[ox]}{[red]}$$

at 25 °C, where n is the number of electron equivalents.

In Fig. 4, the redox potentials of redox components of mitochondria where the redox state has been measured in State 4[2], are given. Since it was impossible to resolve spectrophotometrically the contributions of the three *b* components (*b*-562 *b*-558 and *b*-566) identified in rat-liver mitochondria[30] which differ in redox potential by 90 mV[24], the unresolved value for the potential, calculated from the data of Wilson and Dutton[24] has been used. Mid-point potentials are known for a number of redox

carriers present in mitochondria whose redox state has not been measured in these experiments. These are shown in the upper part of Fig. 4. For purposes of calculation, it has been assumed that these carriers are 50% reduced.

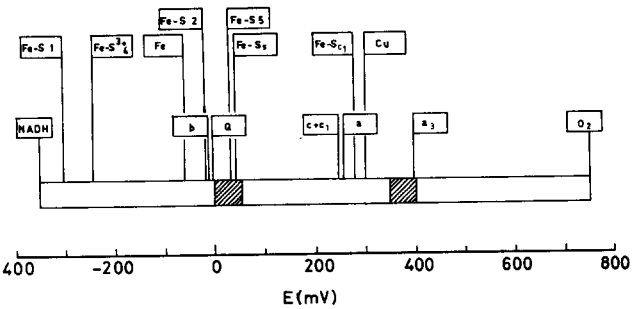

Fig. 4. Redox potentials of redox components of mitochondria in State 4 at pH 7.7, 25 °C. The following mid-point potentials (E_0') have been used: NADH, −340 mV at pH 7.7 (ref. 21); iron–sulphur centres 1, 2, 3+4 and 5 (indicated by Fe–S 1, *etc.*) −305, −20, −245 and 40 mV at pH 7.2 (ref. 22); high-spin iron (indicated by Fe), −60 mV at pH 7.2 (ref. 22); ubiquinone, 66 mV at pH 7.0 (ref. 23); cytochrome *b*, 0 at pH 7.3 (ref. 24); iron–sulphur centre of succinate dehydrogenase (indicated by Fe–S6), 30 mV at pH 7.0 (ref. 25, *cf.* ref. 22); iron–sulphur centre in Complex III (indicated by Fe−Sc1), 280 mV at pH 7.0 (ref. 26); cytochrome $c+c_1$, 225 mV at pH 7.2 (ref. 27); cytochrome *a*, 220 mV at pH 7.2 (ref. 28); cytochrome a_3, 380 mV at pH 7.2 (ref. 28); EPR-detectable copper of cytochrome *c* oxidase (indicated by Cu), 250 mV at pH 7.0 (ref. 26); oxygen, 810 mV at pH 7.0. The E_0' values for pH 7.7 were calculated on the basis of known relationships [for NADH (ref. 21), Q (ref. 23), *b* (ref. 23), *c* (ref. 29), *a* (ref. 28), a_3 (ref. 28) and oxygen] or assumed to be the same as at the measured pH (iron–sulphur centres, high-spin iron, copper). On the lower row of components, the redox states are those determined by Muraoka and Slater[2] in State-4 mitochondria except for NADH where a value of 62% reduction, determined under the conditions of the measurements of the phosphate potential (no added Mg^{2+}, low P_i) was used. For the upper row of components, it has been assumed, in the absence of any experimental information, that these are 50% reduced in State 4. The shaded areas on the lower block indicate the region of uncertainty in consequence of the fact that the experimentally determined phosphorylation potential is 95% of the calculated.

The following conclusions may be drawn (*cf.* ref. 7):

(1) Site 1 lies between NADH (−346 mV) and ubiquinone (−4 mV) and cytochrome *b* (−14 mV).

(2) Site 2 lies between the ubiquinone–cytochrome *b* region (about −10 mV) and cytochrome a_3 (396 mV).

(3) Site 3 lies between cytochrome a_3 (396 mV) and oxygen (753 mV).

(4) Iron–sulphur centres 2 and 5 (ref. 22) and the iron–sulphur centre of succinate dehydrogenase measured by Wilson *et al.*[25] all lie, together with cytochrome *b* and Q, at the junction between Site-1 and Site-2 phosphorylation.

(5) If their mid-point potentials and the redox states used in these calculations are correct, the potentials of cytochromes *c*, c_1 and *a* lie in the "no-man's land" between the junctions between Site-1 and Site-2, and Site-2 and Site-3 phosphorylations, respectively. This would seem to indicate that these components are not in equilibrium with the rest of the respiratory chain or with the ADP, ATP, P_i system in State-4 mitochondria. It seems likely that the same conclusion can be drawn with respect to the EPR-visible copper of cytochrome *c* oxidase and the Fe–S protein

associated with Complex III, but this is less certain since the redox state of these components in State-4 mitochondria is not known. In any case, this block of 5 electron carriers, lying between 250 and 300 mV is difficult to reconcile with current concepts of the phosphorylating respiratory chain.

(6) In view of the uncertainty on the redox situation and also with respect to the measurement of the midpoint potentials ($n < 1$)[22], not too much attention can be given to the fact that the iron–sulphur centres 1 and 3+4 of NADH dehydrogenase and the high-spin iron seem also to lie in "no-man's land" within Site 1.

It should be noted that the potentials used in these calculations are those of the uncoupled system, and that the calculations are not valid if the potentials change on energization, as claimed by Wilson and Dutton[24]. There is, however, no evidence for more than a small change in the real (as opposed to the apparent[31]) redox potential on energization[31,32].

According to the chemiosmotic hypothesis of Mitchell[33], the transfer of electrons and hydrogen atoms along the respiratory chain is coupled with the transfer of protons from inside to outside the inner mitochondrial membrane. This leads to a higher concentration of H^+ outside than inside the membrane and to the development of an electrical potential due to the transport of positively charged particles to the outside. According to this hypothesis a certain protonmotive force may be built up by the respiratory chain. The magnitude of the protonmotive force may be given by the expression

$$\Delta p = \Delta\psi - 59\Delta\text{pH} \text{ mV}$$

where $\Delta\psi$ mV is the membrane potential. The transfer of protons will stop when the protonmotive force equals the work necessary for the proton transfer. The volume of the mitochondrion is so small that, in the absence of side reactions, the movement of a very small number of protons will lead to a potential of several hundred millivolts. Under these conditions, then

$$\Delta p \approx \Delta\psi$$

Since, according to the chemiosmotic hypothesis, the protonmotive force may be utilized to drive the synthesis of ATP from ADP and P_i, its magnitude is equal to that of the phosphorylation potential. Taking into account the stoicheiometry of $2\ H^+ = 1$ ATP, assumed by the chemiosmotic hypothesis, a phosphate potential of 16.1 kcal corresponds to a membrane potential ($\Delta\psi$) of 350 mV. Mitchell and Moyle[34] have calculated the value of $\Delta\psi$ by measuring the H^+ and K^+ gradients induced by adding valinomycin in the presence of K^+. According to the Mitchell hypothesis, the membrane potential is thereby collapsed, since it is utilized to drive K^+ into the mitochondria. This enables more protons to be driven out of the mitochondria by the operation of the respiratory chain, so that Δp is now equal to 59 ($\Delta\text{pK}^+ - \Delta\text{pH}$) mV where $\Delta\text{pK}^+ = -\log([K^+]_o/[K^+]_i)$, the suffices, o and i referring to outside and inside, respectively. The value of the protonmotive force calculated in this way is 227 mV. If it is assumed that Δp is the same in the presence of valinomycin as in its absence, it follows that $\Delta\psi$ in the absence of valinomycin also equals 227 mV, corresponding to 10.5 kcal/2*e* or a phosphate potential of 10.5 kcal/mole ATP.

The simplest explanation for the difference between this value and the 16.1

kcal/mole found in this paper is that not all of the energy that can be conserved by respiring mitochondria is utilized for proton and potassium transport in the presence of valinomycin. In terms of the chemiosmotic hypothesis, one might imagine that the membrane potential is not completely collapsed and that the true expression for the protonmotive force in the presence of valinomycin is given by the equation $\Delta p = \Delta\psi + 59\ (\Delta pK^+ - pH)$ and that $\Delta\psi$, under these conditions equals $355 - 227$ mV $= 123$ mV. Alternatively, it might be expected that 'leak' reactions are relatively more important under the conditions of the valinomycin experiment than when the phosphate potential is measured. In any case, even in the presence of valinomycin and under the conditions of the experiments of Mitchell and Moyle[34], high ATP/ADP ratios were found in our hands and relatively high phosphate potentials were calculated, even though the P_i concentrations were high (Table X). It is clear that, at the concentrations used by Mitchell and Moyle[34], valinomycin competes very poorly with the phosphorylation system.

A phosphate potential of 16.1 kcal/mole implies, then, that, if the Mitchell chemiosmotic hypothesis is valid, a membrane potential of 350 mV exists in State 4. This is an exceptionally high potential for a biological membrane. However, Mitchell[35] has suggested that the stoichiometry of the ATPase in State 4 is greater than $2\ H^+$:ATP.

As pointed out by Klingenberg *et al.*[12], the phosphorylation potential calculated from the concentrations of P_i, ADP and ATP within the mitochondria is much (4.5 kcal/mole ATP, in our hands) less than that calculated from concentrations in the suspending medium. Klingenberg *et al.*[12] consider that this difference is due to a membrane potential, negative inside, that hinders the electrogenic exchange of endogenous ADP^{3-} for external ATP^{4-} (*cf.* ref. 35). An alternative explanation is that much of the endogenous ADP is bound[7]. However, even if the explanation of Klingenberg is correct, it does not invalidate our conclusion that the membrane potential in State 4, calculated on the basis of the assumptions of the chemiosmotic

TABLE X

PHOSPHORYLATION POTENTIAL IN PRESENCE OF VALINOMYCIN

The experiment was carried out in an Oxygraph. The reaction mixture contained 250 mM sucrose, 3.3 mM glycylglycine, 10 mM choline chloride, 5 mM β-hydroxybutyrate, 5 mM $MgCl_2$, 4.5 mM choline phosphate, 2.3 mM ATP and 200 ng valinomycin in Expt 1 and 40 mM sucrose, 67 mM KCl, 1.3 mM EDTA, 34 mM Tris–HCl buffer, 4 mM $MgCl_2$, 5 mM β-hydroxybutyrate, 3 mM P_i and 2.5 mM ATP in Expt 2. State 3 was initiated by addition of 0.2 mM ADP. Total volume 1.8 ml. pH 7.0. Temperature, 25 °C. Protein, 0.77 mg/ml.

Expt	P_i	*ADP*	*ATP*	*ATP/ADP*	ΔG_c	$\Delta G_o'$	ΔG_P
	(*mM*)				(*kcal/mole*)		
1	4.63	0.0108	1.80	167	6.2	6.8	13.0
	4.52	0.0065	2.36	363	6.7	6.8	13.5
2	2.90	0.0050	2.70	540	7.2	6.8	14.0
	2.97	0.0038	2.44	642	7.3	6.8	14.1

hypothesis, should be based on the phosphate potential generated outside the mitochondria. Indeed, whatever the explanation of the difference between the two phosphate potentials, if it is real, the fact remains that the energy pressure generated by respiring mitochondria must be at least as high as 16.1 kcal/mole ATP, since the mitochondria are able to catalyse the synthesis of ATP against an energy pressure of this magnitude. The same conclusion applies to *Azotobacter* particles, in which there is no barrier for ADP or ATP[10].

METHODS

Rat-liver mitochondria were prepared by the method of Hogeboom, exactly as described by Myers and Slater[36]. Protein was determined by the method of Cleland and Slater[37].

Phosphate was determined by the method of Summer[38].

ATP was determined with hexokinase (EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49), ADP with pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27) as described by Bergmeyer[39]. A Zeiss spectrophotometer was used except for the determination of low concentrations of ADP when the more sensitive Aminco–Chance dual-wavelength spectrophotometer was used.

For the measurement of phosphorylation potentials two methods were used:

(a) The reactions were carried out in an Oxygraph vessel of total volume 1.6–1.8 ml filled as given in the legends to the tables and figures. The oxygen concentration was followed with a Clark-type electrode. State 3 was initiated by addition of ADP (0.1–0.4 mM). When the oxygen trace showed that State 4 had been reached, a sample of 0.5–1 ml of the suspension was removed and added to 2 ml of a mixture containing phenol–chloroform–isoamylalcohol (38:24:1). After mixing thoroughly, protein was centrifuged down and the upper layer was analysed for ATP, ADP and P_i. The amounts were corrected for the contribution of endogenous ATP, ADP and P_i determined in a separate experiment in which nucleotides were not added. This correction was, however, very small because of the small amounts of mitochondrial protein used.

(b) Mitochondria were added to a reaction mixture already containing a high ATP/ADP ratio. To avoid anaerobiosis during the incubation air was led over the reaction mixture which was stirred vigorously. At various times the reactions were stopped by removing the mitochondria by rapid centrifugation through silicone oil into 15% (w/v) $HClO_4$. A sample of the supernatant was treated according to the phenol method described above. Endogenous phosphorylation potentials were calculated from the amounts of ATP, ADP and P_i in the acid layer. In calculating the endogenous potentials it was assumed that the volume of the matrix space was 1 μl/mg protein and that the amount of adherent water was 3 μl/mg protein[40]. Deviations from these figures have a rather small effect on the resulting endogenous phosphorylation potentials.

Control experiments showed that ATP and ADP, in concentrations usually present in State 4, when treated according to the phenol-stop method, were recovered quantitatively.

The concentration of free Mg^{2+} was calculated from the equation

$$[Mg_{total}] = [Mg^{2+}] + [ATP]_{total} \frac{[Mg^{2+}](K_1 + K_2[H^+]/K_3)}{1 + K_1[Mg^{2+}] + [H^+]/K_3 + K_2[H^+][Mg^{2+}]/K_3}$$

$$+ [ADP]_{total} \frac{[Mg^{2+}](K_4 + K_5[H^+]/K_6)}{1 + K_4[Mg^{2+}] + [H^+]/K_6 + K_5[H^+][Mg^{2+}]/K_6}$$

$$+ [P_i]_{total} \frac{K_7[Mg^{2+}]}{1 + K_7[Mg^{2+}] + [H^+]/K_8}$$

where $[Mg^{2+}]$ is the concentration of free Mg^{2+} and K_{1-8} are ionization or stability constants of the reactions given in Table XI. $[Mg^{2+}]$ was calculated with the help of an Olivetti desk computer type P 102. The terms on the right-hand side of the equation were calculated for different values of $[Mg^{2+}]$ and added together until a value was found equal to $[Mg_{total}]$. The value for $\Delta G_0'$ corresponding to this concentration of Mg^{2+} was read off Fig. 2 in ref. 4.

In experiments carried out to measure the uptake of [^{3}H]ATP and [^{14}C]ADP by rat-liver mitochondria, the rapid-filtration technique using membrane filters of pore size 0.45 μm was used to separate the mitochondria from the supernatant[44]. The mitochondria were washed with 8% NaCl. The filters were dried and counted in a liquid scintillation counter Type Isocap 300 of Nuclear Chicago.

The P:O ratio was measured as ΔADP:ΔO, where ΔADP is the difference between the initial concentration of ADP and that found after State 4 was reached, and ΔO refers to the total oxygen uptake during State 3.

TABLE XI

IONIZATION AND STABILITY CONSTANTS USED FOR CALCULATION OF $[Mg^{2+}]$

Equilibrium constant	*Reaction*	*Ref.*
K_1	$ATP^{4-} + Mg^{2+} \rightleftharpoons ATPMg^{2-}$	41
K_2	$ATPH^{3-} + Mg^{2+} \rightleftharpoons ATPHMg^{-}$	41
K_3	$ATPH^{3-} \rightleftharpoons ATP^{4-} + H^+$	41
K_4	$ADP^{3-} + Mg^{2+} \rightleftharpoons ADPMg^{-}$	41
K_5	$ADPH^{2-} + Mg^{2+} \rightleftharpoons ADPHMg$	41
K_6	$ADPH^{2-} \rightleftharpoons ADP^{3-} + H^+$	41
K_7	$HPO_4^{2-} + Mg^{2+} \rightleftharpoons MgHPO_4$	42
K_8	$H_2PO_4^{-} \rightleftharpoons HPO_4^{2-} + H^+$	43

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