Thermodynamic Relationships between the Mitochondrial Oxidation–Reduction Reactions and Cellular ATP Levels in Ascites Tumor Cells and Perfused Rat Liver†

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ABSTRACT: Measurements in intact cells of the reactants in eq 1, where the NADH and NAD^+ are the intramitochondrial concentrations of the free coenzyme and the ATP, ADP, and P_i are the cytoplasmic concentrations, gave a mass action ratio within experimental error of the equilibrium constant. The mass action ratios obtained were 1.2×10^7 M^{-2} in ascites tumor cells, 3.9×10^7 M^{-2} in isolated liver cells, and 5.5×10^6 M^{-2} in perfused livers, as compared to the equilibrium constant of 4.4×10^7 M^{-2}. These data extend the previous results for isolated liver cells [D. F. Wilson *et al.* (1974), *Biochem. J. 140,* 57–64] and provide further support for the near-equilibrium model of the mitochondrial oxidative phosphorylation in which the respiration is dependent on the cytoplasmic $[ATP]/[ADP][P_i]$. A description of the mitochondrial respiratory rate is postulated to be provided by a model which assumes near-equilibrium from NAD to cytochrome a_3 (all three phosphorylation sites) coupled with the kinetic expression for the reoxidation of reduced cytochrome a_3 by molecular oxygen.

Previous work has shown that, in suspensions of isolated pigeon heart mitochondria respiring in the presence of substrate and oxygen (Erecińska *et al.*, 1974), a near-equilibrium exists in the multistep reaction described by

$$NADH + 2\text{cytochrome } c^{3+} + 2ADP + 2P_i^{\dagger} \rightleftharpoons NAD^+ + 2\text{cytochrome } c^{2+} + 2ATP \quad (1)$$

The equilibrium constant for this reaction is defined by

$$K_{eq} = \frac{[NAD^+]}{[NADH]} \frac{[\text{cyt } c^{2+}]^2}{[\text{cyt } c^{3+}]^2} \frac{[ATP]^2}{[ADP]^2[P_i]^2} \quad (2)$$

where $[NAD^+]$ and [NADH] represent the intramitochondrial concentrations of the free coenzymes and [ATP], [ADP] and $[P_i]$ represent the extramitochondrial concentrations of the adenine nucleotides and P_i. The determined mass action ratio (Erecinska *et al.*, 1974) gave a value of 6.8×10^6 M^{-2}, indistinguishable within the limits of the experimental error from the equilibrium constant of 3.2×10^6 M^{-2} calculated from the $E_{m7.0}$ values of the NAD couple [−0.320 V (Burton, 1957)] and cytochrome *c* [0.235 V (Dutton *et al.*, 1970)] and the $\Delta G_0'$ for ATP hydrolysis of −8.4 kcal/mol at the low Mg^{2+} concentrations used in the experiments (Benzinger *et al.*, 1959; Guynn and Veech, 1973).

Similarly, the measured concentrations of ATP, ADP, and P_i (which represent cytoplasmic values; Krebs and Veech, 1969), the measured mitochondrial $[NAD^+]/[NADH]$ ratio, and the mitochondrial cytochrome c^{2+}/cytochrome c^{3+} ratio show that reaction 1 is also at near-equilibrium in isolated liver cells (Wilson *et al.*, 1974). In this paper, results are presented which demonstrate that near-equilibrium exists in suspensions of ascites tumor cells and in perfused liver.

Experimental Section

Animals. Female Holtzman or Wistar strain rats (200 g) were starved for 48 hr before being sacrificed. White Swiss female mice were used for culturing the ascites cells.

Reagents. Analytical grade reagents were obtained commercially. Enzymes were the products of either Sigma Chemical Co., St. Louis, Mo. (lactic dehydrogenase, type VI; pyruvate kinase, type II; L-glutamic dehydrogenase, type II, hexokinase/glucose-6-P dehydrogenase and collagenase, type I) or Boehringer Corp. Ltd. (3-hydroxybutyrate dehydrogenase, type II; hyaluronidase). Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was a gift from Dr. P. G. Heytler, E.I. du Pont de Nemours & Co. (Inc.) Central Research Department, Experimental Station, Wilmington, Del.

Rat Liver Cells. The liver cells were prepared by the method of Berry and Friend (1969), incorporating the modifications described by Cornell *et al.* (1973) and Krebs *et al.* (1974). Incubations were carried out as described previously (Wilson *et al.*, 1974).

Ascites Tumor Cells. Strain L.12.10 was grown for 8–10 days in the peritoneal cavity of Swiss female mice. The cells were washed with 0.154 M NaCl–0.006 M KCl–0.011 M phosphate (pH 7.4) buffer, or Krebs–Henseleit (1932) saline, thereby removing any blood present, and were suspended in the same buffer as used for the washing at a concentration of about 20 mg wet wt/ml. The ascites cells were incubated and treated in a similar manner to the liver cells (Wilson *et al.*, 1974). The dry weight of the cell suspension and of the buffer was determined for each preparation. A factor of 3.7 was used to convert dry weight into wet weight of cells.

Liver Perfusion. The technique of liver perfusion was essentially the same as reported by Scholz *et al.* (1969), except that the perfusion medium was Krebs–Henseleit saline (1932) without albumin. Flow rates were in a range of 28–

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† Abbreviation used is: P_i, pyrophosphate.

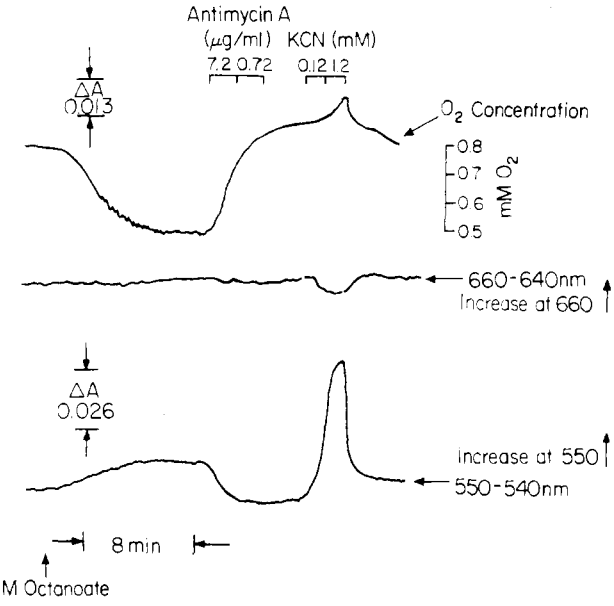

FIGURE 1: The measurement of cytochrome *c* reduction and respiration in perfused rat liver. The liver was removed from a 48-hr starved 200-g female Wistar rat and perfused with Krebs–Henseleit saline equilibrated with a 95% O_2–5% CO_2 gas mixture. A lobe of the liver was placed between two light pipes, one serving as the light source and the other carrying transmitted light to a photomultiplier. The light source was modulated to give repeating sequential pulses of light at 550, 540, 660, and 640 nm. The photomultiplier output was then electrically processed to measure the absorbance changes at 660 nm minus 640 nm and 550 nm minus 540 nm. The oxygen concentration of the perfusate was measured at the outlet catheter. The perfusate was made 1 mM in octanoate at the arrow and this was maintained throughout the experiment. The antimycin A and KCN were added to the perfusion fluid for the indicated times, beginning at the first mark, changing concentration at the second mark, and ending (zero concentration) at the final mark.

32 ml/min and the temperature at the liver surface during perfusion was maintained at 30 ± 1°. Livers in which metabolites were to be assayed were freeze-clamped according to the method of Wollenberger *et al.* (1960) and the tissue was treated further as described by Williamson *et al.* (1967).

Measurement of the Redox State of the Cytochromes in Cell Suspensions. A sample of the incubated cell suspensions was added to a cuvet for measurement of the redox state of the cytochromes in an Aminco-Chance or Johnson Foundation dual wavelength spectrophotometer, using the wavelength pair 550 nm − 540 nm for reduced cytochrome *c*. The half-bandwidth of the measuring light was 1.6 nm. In order to prevent sedimentation, the suspension of the cells in the cuvet was continuously mixed with a vibrating stirrer. After the initial spectrophotometric reading had become stable, 5 μl of a 10-mg/ml solution of antimycin A was added (in 70% dimethylformamide) or 20 μM rotenone and 10 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) to cause complete oxidation of cytochrome *c*. Antimycin A or rotenone plus FCCP gave identical results. The observed decrease in the concentration of reduced cytochrome *c* was taken to correspond to 100% oxidation of the cytochrome (Wilson *et al.*, 1974). After complete oxidation, 40 μM cyanide was added. The resulting small rapid increase in absorbance observed in the suspension of liver cells is caused by the formation of catalase-CN compound (Sies *et al.*, 1973) and was taken as the catalase interference at this wavelength pair. Addition of 2 mM cyanide ensured over 95% reduction of cytochrome *c* without the interference from the oxy–deoxyhemoglobin transition which would occur if anaerobiosis were used to attain complete reduction of cytochrome *c*.

Measurement of the Redox State of Cytochrome c in Perfused Livers. The redox state of cytochrome *c* in the perfused liver was measured at 550–540 nm simultaneously with the absorbancy change at 600–640 nm, using a time-sharing dual wavelength spectrophotometer (Theorell *et al.*, 1972). $\Delta A_{660\text{-}640\ nm}$ reflected steady-state changes of catalase-H_2O_2 compound and catalase-CN compound (Sies *et al.*, 1973) and was used to correct for the catalase-CN compound spectral interference at the measuring wavelength of cytochrome *c*. In addition, comparison of the absorbancy changes at these two wavelength pairs allowed identification of artifacts evoked by movements of the liver which occur in some experiments. Oxygen uptake was measured continuously with a Clark oxygen electrode placed in the outlet catheter of the perfused liver.

After the initial 5 min of the "flow through" perfusion system necessary for the instrumental adjustments, the perfusion system was changed to the "recirculating" one with a total volume of 120 ml of the perfusate. The perfusate was continuously gassed with 95% O_2–5% CO_2 mixture. In about 15–20 min after the start of the perfusion, the state of reduction of cytochrome *c* and the rate of oxygen uptake became constant, indicating that a steady state had been reached. The rate of oxygen uptake at this stage was 1.7 μmol of O_2 per min per g of liver without added substrate and 2.7 μmol of O_2 per min per g of liver with 1 mM octanoate. A sample of the perfusate was withdrawn for the assays of 3-hydroxybutyrate and acetoacetate and the liver was either freeze-clamped (for those experiments in which metabolites were to be determined) or the redox state of cytochrome *c* was determined. For the latter, the perfusion was changed back to a flow-through system and 5 mM antimycin A solution in 70% dimethylformamide was infused for 2 min at a rate of 0.03 ml/min and for a further 2 min at a rate of 0.003 ml/min to give final concentrations in the medium entering the liver of 50 and 5 μM, respectively. A decrease in absorbance was observed (Figure 1) which reached a steady state level in about 4 min. Cyanide was subsequently infused to give a concentration of 0.05 μM for 1 min and then increased in stepwise fashion to 0.2 and 0.5 mM.

The redox state of cytochrome *c* (after correction for the catalase contribution—see above) was estimated from the $\Delta A_{550\text{-}540\ nm}$ in the presence of 0.5 mM cyanide (100% reduced) minus $\Delta A_{550\text{-}540\ nm}$ in the presence of antimycin A (100% oxidized) as described previously (Wilson *et al.*, 1974). The $E_{m7.0}$ of cytochrome *c* at 25° was taken to be 0.235 V (Dutton *et al.*, 1970) and that at 30° as 0.230 V (Erecińska and Vanderkooi, unpublished data).

Measurements of the Mitochondrial [NAD⁺]/[NADH] Ratio. The mitochondrial [NAD⁺]/[NADH] ratio in suspension of liver cells or perfused liver was calculated according to Williamson *et al.* (1967) from the 3-hydroxybutyrate and acetoacetate concentrations measured by the method of Williamson *et al.* (1962). The $E_{m7.0}$ at 25° of [3-hydroxybutyrate]/[acetoacetate] was taken to be −0.266 V (Krebs *et al.*, 1962) and, at 30°, as −0.276 V. This latter value was derived by interpolation from the values for K_{eq} at 25° (Krebs *et al.*, 1962) and 38° (Williamson *et al.*, 1967).

Because the activity of 3-hydroxybutyrate dehydrogenase was less than 3 μmol per g wet wt per hr at 25°, the [NAD⁺]/

TABLE I: Measurements of the Redox States of Cytochrome *c* and the Mitochondrial NAD Couple and the [ATP]/[ADP][P_i] Ratio in Perfused Rat Liver.[a]

Reactant or Reactant Ratio	Expt A (2)	Expt B (2)	
3-Hydroxybutyrate	0.115	0.55	*0.41
Acetoacetate	0.509	0.32	*0.25
[3-Hydroxybutyrate]/[acetoacetate]	0.24	1.72	*1.64
ATP	1.43	2.07	
ADP	0.72	0.96	
P_i	3.85	3.08	
[ATP]/[P_i]	516 M^{-1}	703 M^{-1}	
	Expt C (2)	Expt D (3)	
[3-Hydroxybutyrate]/[acetoacetate]	*0.39	*1.82	
[Cytochrome *c* oxidized]/[cytochrome *c* reduced]	2.43	2.10	

[a] The perfusions were carried out as described in the Experimental Section. In experiments A and B, the livers were freeze-clamped at the time which corresponded to the addition of antimycin A in parallel experiments (C and D) when the redox state of cytochrome *c* was determined. Reactants are expressed in μmol/g fresh wt or in the perfusate in μmol/ml. * Indicates that 3-hydroxybutyrate and acetoacetate were measured in perfusate samples taken immediately before freeze-clamping or addition of antimycin A. The values are the average of two or three experiments.

[NADH] ratio in suspension of ascites tumor cells was calculated from the concentrations of 2-oxoglutarate, NH_4^+, and glutamate and the equilibrium constant for the glutamate dehydrogenase system (Engel and Dalziel, 1967). Glutamate was determined by the method of Bernt and Bergmeyer (1965), 2-oxoglutarate by the method of Bergmeyer and Bernt (1965), and NH_4^+ by the method of Kirsten *et al.* (1963). K_{eq} at 25° and pH 7.0 was taken to be 1.33×10^{-6} M (Engel and Dalziel, 1967), and the $E_{m7.0}$ of [NAD^+]/[NADH] was −0.320 V. It was further taken into account that 2-oxoglutarate was intracellular, while NH_4^+ and glutamate were uniformly distributed between the ascites cells and the suspending medium (see Table III). The measured activity of glutamate dehydrogenase (measured by the method of Schmidt, 1965) at 25° was 278 μmol of glutamate formed per g wet wt per hr.

Measurements of ATP, ADP, and P_i. ATP was determined by the method of Lamprecht and Trautschold (1963), ADP by the method of Adam (1963), and P_i by the method of either Martin and Doty (1949) for liver cells, or Lowry and Lopez (1946) for freeze-clamped liver and ascites cells. [P_i] in ascites tumor cells was measured in parallel experiments in which the cells were centrifuged down, washed, and resuspended in P_i-free medium. [P_i] in isolated liver cells was measured after rapid separation from the suspending medium by the method of Hems, Lund, and Krebs (in preparation). The cellular [P_i] obtained by this method was about 3 mM, somewhat lower than the values (6–10 mM) obtained previously by simply washing the cells in P_i-free medium prior to assay (Wilson *et al.*, 1974).

Thermodynamic symbols and conventions are those used previously (Wilson *et al.*, 1974). The $E_{m7.0}$ of −0.320 V at 25° and −0.327 V at 30° for the NAD couple are used consistently in this paper, although Clark (1960) indicates that a value of −0.315 V at 25° would be preferred.

Results

Tables I, II, and IV summarize the experimental results necessary for the calculation of the equilibrium relationships between the mitochondrial respiratory chain and the cytoplasmic phosphorylation state in three different systems: in perfused rat liver, in suspensions of ascites tumor cells, and, for the sake of comparison, in suspensions of rat liver cells [the latter results supplement the experiments of Wilson *et al.* (1974) in that they include improved intracellular phosphate determinations].

TABLE II: The Redox State of Cytochrome *c* and the Mitochondrial NAD Couple, and the [ATP]/[ADP][P_i] Ratio in Suspensions of Isolated Ascites Tumor Cells.[a]

		Experiment					
		A	B	C	D	E	F
Reactant or Reactant Ratio	mg dry wt/ml	24.6	18.8	19.4	23.1	24	30
	pH	7.2	7.5	7.4	7.4	7.3	7.1
Glutamate		0.35	0.30	0.37	0.40	0.42	0.36
NH_4^+		0.185	0.30	0.20	0.25	0.19	0.18
2-Oxoglutarate		0.06	0.055	0.21	0.17	0.087	0.14
[NAD^+]/[NADH]		23.8	41.9	85	80	30	52
[cyt. *c* oxidized]/[cyt. *c* reduced]		2.83	2.37	2.38	2.42	2.35	3.61
ATP		4.69	5.09	5.11	4.96	5.26	4.39
ADP		0.65	0.43	0.50	0.50	0.48	0.38
P_i		8.0	8.0	8.0	8.0	8.0	8.0
[ATP]/[ADP][P_i]		903 M^{-1}	1480 M^{-1}	1013 M^{-1}	1240 M^{-1}	1370 M^{-1}	1440 M^{-1}

[a] Ascites tumor cells were obtained as described in the text. ATP, ADP, P_i, and 2-oxoglutarate are expressed in μmoles/g wet weight. NH_4^+ and glutamate are expressed in μmoles/ml of incubation mixture. The incubation was carried out at 25° for 15 min in KCl–NaCl–P_i medium (A, C, E) or in Krebs-Henseleit saline (B, D, F). The cells were aerated during incubation with O_2 (A, C, E) or 95% O_2–5% CO_2 mixture (B, D, F).

TABLE III: Distribution of Glutamate, NH_4^+, and 2-Oxoglutarate between the Ascites Tumor Cells and the Suspending Medium.[a]

	Total Suspension	Supernatant
Glutamate	0.39	0.37
NH_4^+	0.23	0.20
2-Oxoglutarate	0.015	<0.001

[a] The cells, after 10-min incubation, were divided in equal volumes: one-half of the cell suspension was centrifuged at 8000*g* for 8 min; the supernatant was deproteinized by the addition of perchloric acid (Wilson *et al.*, 1974) and after neutralization used for the determination of glutamate, NH_4^+, and 2-oxoglutarate. The other half was deproteinized immediately and further treated as described above (Wilson *et al.*, 1974). The values are expressed in μmoles/ml of incubation mixture. The values are the averages of two experiments.

The ratio of [3-hydroxybutyrate]/[acetoacetate] was always below 1.0 (0.2–0.8) in isolated liver cells and in the liver perfused without added substrate. Addition of 1 mM octanoate to the liver perfusate raised the [3-hydroxybutyrate]/[acetoacetate] ratio to about 1.8. Thus, the free $[NAD^+]/[NADH]$ in the mitochondria varied by a factor of 9 (see Stubbs *et al.*, 1972).

The 3-hydroxybutyrate dehydrogenase activity of ascites tumor cells is rather low (<3 μmol per g per hr at 25°) and, thus, this enzyme is not a suitable indicator of the mitochondrial NAD couple. However, the glutamate dehydrogenase activity is 278 μmol per g per hr at 25° and thus was sufficient to be used as an indicator for this redox couple. For the calculation of the redox state of the NAD couple, it is relevant that the NH_4^+ and glutamate were equally distributed between cells and medium, whereas the 2-oxoglutarate was intracellular (Table III). The $[NAD^+]/[NADH]$ ratio in ascites tumor cells was lower than in liver cell suspensions or perfused liver. Similar differences were observed between hepatomas and "control" livers by Weber *et al.* (1971).

The [ATP]/[ADP] ratios in the isolated liver cells found in these experiments were in good agreement with previous results (Wilson *et al.*, 1974). They were close to those found in liver freeze-clamped after perfusion in the absence of added substrate (Table I and Veech *et al.*, 1970). The measured [ATP]/[ADP] ratios were higher in ascites cells (8–15) than in liver cell suspensions (4–6) or perfused liver (2–3). [ATP] was between 4–5 μmol per g wet wt of ascites tumor cells and 2–3 μmol/g wet wt in the liver cell suspensions and perfused liver.

However, in contrast to our previous findings (Wilson *et al.*, 1974), $[P_i]$ was similar in perfused liver and in isolated liver cells (2–4 mM) as determined by parallel experiments in which the cells were separated from the phosphate-containing medium (see Experimental Section). The $[ATP]/[ADP][P_i]$ ratio was between 0.9×10^3 and 2.6×10^3 M^{-1} for liver cell suspensions and for ascites tumor cell suspensions.

The Free Energy Relationships between the Oxidation-Reduction Reactions of the Respiratory Chain and the $[ATP]/[ADP][P_i]$. From the data presented in Tables I, II, and IV, the free energy changes associated with the transfer of 2 mol of electrons from the NAD couple to cytochrome *c* and the free energy change for ATP synthesis can be calculated. It follows from the E_h values of cytochrome *c* and the NAD couple that the differences of E_h (ΔE) between the two couples are 0.514–0.529 V for suspensions of liver cells, 0.530 V for ascites tumor cells, and 0.516–0.531 V for perfused rat liver. The corresponding free energy changes for 2 mol of electrons ($\Delta G_{ox\text{-}red} = -nF\Delta E$) are 23.7–24.4, 24.4, and 23.8–24.5 kcal, respectively (Table V).

The Gibbs free energy change associated with the hydrolysis of ATP can be obtained from the relationship

$$\Delta G_{ATP} = \Delta G'_{oATP} + 2.303RT \log ([ADP][P_i]/[ATP]) \quad (3)$$

where $\Delta G'_{oATP}$ is the Gibbs standard free energy change of hydrolysis of ATP at pH 7.0 and 1 mM free Mg^{2+} [−7.6 kcal/mol (Guynn and Veech, 1973)]. When the measured $[ATP]/[ADP][P_i]$ ratios are used in conjunction with this value for $\Delta G'_{oATP}$, the calculated $\Delta G/2$ mol of ATP is 23.8–24.2 kcal for suspensions of liver cells, 23.6 kcal for suspensions of ascites tumor cells, and 23.0–23.6 kcal for

TABLE IV: The Redox State of Cytochrome *c* and the Mitochondrial $[NAD^+]/[NADH]$ Couple, and the $[ATP]/[ADP][P_i]$ Ratio in Suspensions of Isolated Liver Cells.[a]

Reactant or Reactant Ratio	Expt A No Added Substrate		Expt B 10 mM Lactate, 10 mM Ethanol	
	15 min	30 min	15 min	30 min
[3-Hydroxybutyrate]/[acetoacetate]	0.14	0.17	0.45	0.78
[Cyt. *c* oxidized]/[cyt. *c* reduced]	4.75	4.43	4.15	3.21
ATP	2.34	2.36	2.16	2.08
ADP	0.61	0.64	0.56	0.46
P_i	2.93	2.16	3.03	1.74
$[ATP]/[ADP][P_i]$	1.3×10^3 M^{-1}	1.71×10^3 M^{-1}	1.27×10^3 M^{-1}	2.6×10^3 M^{-1}

[a] Liver cells [17.2 mg wet wt/ml (A), 20.5 mg wet wt/ml (B)] were incubated at 25° for the time indicated. Other conditions are as described in the text. ATP, ADP, and P_i are expressed as μmoles/g wet wt of cells. 3-Hydroxybutyrate and acetoacetate are expressed as μmoles/ml of incubation mixture. The values are the average of two experiments where duplicate incubations were made.

TABLE V: Free Energy Relationships between the Oxidation–Reduction Reactions of the Respiratory Chain and the Phosphorylation State.[a]

Type of material	E_h cytochrome c (V)	E_h ([NAD$^+$]/[NADH]) (V)	ΔE (V)	$\Delta G_{ox\text{-}red}$ (kcal/2 electrons)	ΔG_{ATP} (kcal/2ATP)	$\Delta\Delta G$ (kcal)
Perfused liver no substrate	0.253 0.252–0.254	−0.263 −0.258–0.268	0.516	23.8	23.6	1.2
Perfused liver 1 mM octanoate	0.248 0.239–0.259	−0.263 −0.258–0.268	0.531	24.5	23.0	1.5
Ascites tumor cells	0.260 0.257–0.268	−0.270 −0.262–0.279	0.530	24.4	23.6	0.8
Liver cells no substrate	0.272	−0.242	0.514	23.7	23.8	−0.1
Liver cells 10 mM lactate + 10 mM ethanol	0.269	−0.260	0.529	24.4	24.2	0.2

[a] The E_h values (the oxidation–reduction potentials with respect to the standard hydrogen electrode) were calculated from the data of Tables I–IV. The data were averaged and the range of the values is specified. The free energy relationships were calculated for two phosphorylation sites. $\Delta E = E_h([NAD^+]/[NADH]) - E_h$ ([cytochrome c^{3+}]/[cytochrome c^{2+}]) where $E_h = E_m + (2.3RT/nF)\log([ox]/[red])$. $\Delta G_{ox\text{-}red} = -nF\Delta E$; $\Delta G_{ATP} = \Delta G'_0 + 1.36\log([ADP][P_i]/[ATP])$; $\Delta\Delta G = \Delta G_{ox\text{-}red} - \Delta G_{ATP}$.

perfused liver. It is assumed in these calculations that the transfer of 2 mol of electrons across two phosphorylation sites is accompanied by the synthesis of 2 mol of ATP. From the comparison of the values for ΔG of oxidation-reduction and ΔG for ATP synthesis, it is seen that the differences between the two ($\Delta\Delta G$) range from 0 to 0.20 kcal in suspension of liver cells to 0.8 to 1.5 kcal in perfused liver and ascites tumor cells.

The experimental results can also be expressed in terms of the mass action ratio for eq 1 and compared with the equilibrium constant calculated from the $E_{m7.0}$ values of the NAD couple and cytochrome c, and the $\Delta G_0'$ for the hydrolysis of ATP. The latter value (K_{eq} theoretical) is 4.4 $\times 10^7$ M^{-2} and the calculated mass actions are: 1.2×10^7 M^{-2} in ascites tumor cells, 3.9×10^7 M^{-2} in isolated liver cells, and 5.5×10^6 M^{-2} in perfused livers. The ratio of the two values [K_{eq} (theoretical)/K (measured)] can be calculated to be 1.0–1.4 for liver cell suspensions, about 3.7 for ascites tumor cells, and about 10 for perfused liver.

Discussion

If equilibrium between the mitochondrial oxidation–reduction reactions and the cytoplasmic [ATP]/[ADP][P$_i$] ratio exists, then the free energy change for the coupled reactions ($\Delta\Delta G$) is zero. The experimentally determined value for the $\Delta\Delta G$ across two phosphorylation sites is 0–0.2 kcal in isolated liver cells, 1.2–1.5 kcal in perfused rat liver, and 0.8 kcal in ascites tumor cells. The question arises whether a $\Delta\Delta G$ of about 1.3 kcal/2 sites is significantly different from zero. This value corresponds either to a 3.2-fold increase in [ATP]/[ADP][P$_i$] ratio or to errors in the measured redox states of the NAD couple and cytochrome c which combine to give a value of 30 mV in the ΔE of the oxidation–reduction reactions. In the absence of relevant experimental observation, the calculations are based on the assumption that [ATP], [ADP], and [P$_i$] are all free in solution. It is, however, quite likely that considerable amounts of these reactants are bound to cellular proteins and other cell constituents. The possible errors in the measurements of the redox state of the NAD couple include the uncertainty of its E_m value (for the discussion of various values, see Clark, 1960). The errors in the measurements of cytochrome c arise from difficulties in establishing 100% oxidized and reduced levels and in making corrections for catalase interference. The observed $\Delta\Delta G$ values of up to 1.3 kcal can therefore be explained by experimental short-comings and cannot be regarded as being different from zero.

The demonstration that the first two sites of oxidative phosphorylation are at near-equilibrium with the cellular [ATP]/[ADP][P$_i$] ratio in suspensions of liver cells and ascites tumor cells as well as perfused liver suggests that near-equilibrium exists for all three sites. The concept of near-equilibrium in the reaction

$$\text{NADH} + 2\text{cytochrome } a_3^{3+} + 3\text{ADP} + 3\text{P}_i \rightleftharpoons 3\text{ATP} + \text{NAD}^+ + 2\text{cytochrome } a_3^{2+} \quad (4)$$

provides a basis for obtaining a quantitative expression for the control of the respiration of isolated mitochondria and of mitochondria in intact cells (Owen and Wilson, 1974). Rearrangement of the expression for the equilibrium constant for eq 4 gives

$$[a_3^{2+}] = K^{1/2}[a_3^{3+}]\left(\frac{[\text{NADH}]}{[\text{NAD}^+]}\right)^{1/2}\left(\frac{[\text{ADP}][\text{P}_i]}{[\text{ATP}]}\right)^{3/2} \quad (5)$$

which can be combined with the kinetic expression for the oxidation of reduced cytochrome a_3 by molecular oxygen to give a quantitative expression for the respiratory rate. Qualitatively the respiratory rate is a function of three variables: the mitochondrial [NAD$^+$]/[NADH], the cytoplasmic [ATP]/[ADP][P$_i$], and the oxygen concentration. The rate expression reported by several laboratories (Warburg and Kubowitz, 1929; Gibson and Greenwood, 1963; Chance, 1966)

$$v = k_1[a_3^{2+}][O_2] \quad (6)$$

was used by Owen and Wilson (1974) to derive an expression for the respiratory behavior of mitochondria. However, more recent data (Lindsay and Wilson, 1974) indicate that carbon monoxide (and by inference O_2) reacts not with reduced cytochrome a_3 alone but with a reduced cytochrome

a_3-reduced "invisible copper" complex. Therefore, eq 6, which assumes that the interaction of oxygen with cytochrome oxidase is a second-order reaction in which reduced cytochrome a_3 and oxygen are the only reactants, is oversimplified. The final rate equation used for a quantitative expression for respiratory control will have to incorporate both the role of the "invisible copper" and the behavior of any intermediates formed in the 4-electron reduction of oxygen to water.

It was first observed (Warburg and Kubowitz, 1929) that the respiratory rate of *Micrococcus candicans* cell suspensions remained almost independent of oxygen concentration to quite low values, suggesting a small k_m for oxygen. This observation was later extended to liver cell suspensions by Longmuir (1957). From these data it is reasonable to question whether mitochondrial function in the cell is dependent on the oxygen concentration at values greater than a few micromolar. The near-equilibrium model for the respiratory chain predicts that the cellular respiratory rate is dependent on three variables: (1) the mitochondrial [NAD^+]/[NADH], (2) the cytoplasmic [ATP]/[ADP][P_i], and (3) the oxygen concentration. In the experiments with cells noted above, the deceptively small apparent k_m for O_2 could result from variables 1 and 2 undergoing compensatory changes as the oxygen concentration decreased. Thus because the cellular ATP utilization *per se* is independent of O_2 concentration, a continuously decreasing [ATP]/[ADP][P_i] and/or decreasing mitochondrial [NAD^+]/[NADH] can maintain the respiratory rate at a constant value despite decreasing O_2 concentration.

The near-equilibrium model for the respiratory chain suggests that the cellular metabolism is dependent on the oxygen concentration to values much greater than would be expected for an enzyme system with a k_m of near 1 μM as measured by Longmuir (1957). Experimental verification will require technically difficult simultaneous measurements of the mitochondrial [NAD^+]/[NADH] and the cytoplasmic [ATP]/[ADP][P_i] in cell suspensions maintained at defined O_2 concentrations.

In suspensions of isolated mitochondria, when excess ADP is added the maximal respiratory rate ("state 3") is maintained until an [ATP]/[ADP][P_i] ratio of greater than 5×10^3 M^{-1} is reached (Wilson *et al.*, 1974). The respiratory rate then decreases with increasing [ATP]/[ADP][P_i] (control region) until a limiting value of each is attained ("state 4"). In cells, however, the respiration is controlled at [ATP]/[ADP][P_i] ratios of 3×10^2–3×10^3 M^{-1}. The reason for this difference lies principally in the mitochondrial [NAD^+]/[NADH] ratio which is 100-fold smaller in cells than in suspensions of isolated mitochondria. Substitution of these values into eq 5 shows that the same reduction of cytochrome a_3, and thus the same respiratory rate, is attained with a tenfold smaller [ATP]/[ADP][P_i] ratio in cells than in suspensions of isolated mitochondria.

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Mechanism of Cooperative Oxygen Binding to Hemoglobin: Kinetic Aspects†

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ABSTRACT: The theory of oxygen binding to hemoglobin formulated by T. R. Chay and D. Brillhart ((1974), *Biochemistry,* to be published) has been used to study the kinetics of ligand binding to hemoglobin. We find that the theory is in good agreement with Gibson's recent stopped-flow experiment on human hemoglobin at pH 7. Our model predicts that a large amount of DPG comes off from non-stripped hemoglobin at about the fourth oxygenation stage and that oxygen goes to the β chain initially, but the β chain does not retain oxygen for a long period of time.

About 4 years ago, Gibson (1970) measured the kinetics of the oxygenation process of hemoglobin (Hb)[1] by stopped-flow methods. Interpretations of these data have been made with the kinetic versions of the Adair equation by using eight Adair-rate parameters (Gibson, 1970) and of the allosteric transition model of Monod *et al.* (1965) using five parameters (Hopfield *et al.,* 1971). The basic assumption behind these interpretations is that the α and β chains in Hb are equivalent. A recent experiment by Gibson (1973), however, indicates very significant differences between the α and β chains. In particular, Gibson has found experimentally that, in the presence of phosphates, the association and dissociation rate constants of oxygen to the β chain are larger than those of the α chain in such a way that in equilibrium, the ligand affinity of the α chain is larger than that of the β chain. This means that the kinetic equivalences of the Adair equation (Gibson, 1970) and of the allosteric transition model (Hopfield *et al.,* 1971) are quite unsuited to represent the kinetics of the oxygen-Hb reaction (Gibson, 1973). A modification of the Adair equation, which accounts for the nonequivalent α and β chains, has been proposed by Gibson and his coworkers (Olson and Gibson, 1972,1973; Tan *et al.,* 1973; Cole and Gibson, 1973). These workers showed that in general 32 kinetic parameters are needed to describe the reaction. Since there are too many parameters, the modified Adair equation is very impractical. A simple theory is needed to study the kinetic mechanism of the oxygen binding process of Hb. The model of Chay and Brillhart (1974) has been shown to be capable of fitting the equilibrium oxygenation curves. In this paper, we use this model to see whether or not the kinetic data can be explained by our model. We also use the model to study the time courses of ligand binding to Hb, of the DPG effect, and of the inhomogeneity of the α and β chains.

Kinetic Equation for the Model

Very recently, we have developed a model (Chay and Brillhart, 1974) of the oxygen binding process of Hb on the basis that (i) in the absence of phosphate, the binding of oxygen to Hb follows the sequential theory of Koshland *et al.* (1966); (ii) 1 mol of organic phosphate (OP) can combine with 1 mole of Hb in any oxygenation stage in a reversible manner (Tyuma *et al.,* 1973); and (iii) in the presence of OP, the oxygenation of Hb follows the scheme presented by Figure 1. In Figure 1, the upper portion of the scheme shows the reaction between oxygen and OP-Hb complex, and the lower portion of the scheme shows the reaction between oxygen and OP-free Hb (*i.e.,* stripped Hb).

The equilibrium parameters associated with the lower portion of the scheme (*i.e.,* OP-free Hb) are Q and Z, which are defined respectively as (Chay and Ho, 1973)

$$Q = K_L K_t (K_{AA}/K_{BB})^{3/2} \quad (1)$$

and

$$Z = K_{AB}/(K_{AA}K_{BB})^{1/2} \quad (2)$$

where Q is a measure of the relative stability between the conformation A (unliganded subunit conformation) and B (liganded subunit conformation), and Z is a measure for K_{AB}, the strength of the subunit interaction between A and B, relative to K_{AA} and K_{BB}, which are defined as the strengths of the interactions between AA and BB conformations, respectively. In eq 1, $K_L = (BL)/(B)(L)$ is the ligand

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[1] Abbreviations used are: Hb, hemoglobin; OP, organic phosphate; DPG, 2,3-diphosphoglycerate.