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The Effect of Energization on the Apparent Michaelis–Menten Constant for Oxygen in Mitochondrial Respiration

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Lineweaver–Burk plots of $1/v$ against $1/[O_2]$ for rat liver mitochondrial respiration with succinate or ascorbate+*NNN'N'*-tetramethyl-*p*-phenylenediamine as substrates are non-linear. In state 3u (uncoupled by trifluoromethoxycarbonyl cyanide phenylhydrazone) such plots tend to be concave upward, whereas in state 4 (energized) the plots were concave downward. The apparent K_m for oxygen is larger in state 4 than in state 3u, despite the higher turnover in the latter system. It is postulated that at least one reversible reaction occurs between cytochrome *c* and cytochrome *c* oxidase, whose rate is increased on energization (reversed electron transfer); a model including such a reaction is proposed which accounts semiquantitatively for the observations.

In early studies of oxygen consumption by biological systems a hyperbolic relation was believed to exist between oxygen partial pressure (pO_2) and respiration rate (v_R) (Tang, 1933). However, Winzler (1941) showed that at higher pO_2 values the relation ceased to be hyperbolic. Bänder & Kiese (1955) found that in mitochondria, yeast and bacteria the respiration rate followed eqn. (1):

$$v_R = \frac{V_{max.}\,(pO_2)^{1.4}}{K+(pO_2)^{1.4}} \qquad (1)$$

Longmuir (1957) confirmed this, but noted that eqn. (1) was only valid from 10 to 90% of the maximal respiration rate. Schindler (1964) emphasized that the apparent K_m for oxygen is not a fixed parameter for any system, but is linearly related to the maximal rate of respiration, according to eqn. (2), where n is a constant with a value of about 0.65 (Chance, 1965), TN is the turnover number (catalytic-centre activity) of the oxidase (electrons/s per mol of cytochromes $a+a_3$), and k_1 is the velocity constant ($M^{-1}\cdot s^{-1}$) for the reaction of cytochrome a_3 with oxygen:

$$K_m = \frac{n\mathrm{TN}}{4k_1} \qquad (2)$$

Moreover in intact mitochondria the apparent K_m for oxygen was also dependent on the energy state (Bienfait & Jacobs, 1971; Degn & Wohlrab, 1971; Schindler, 1964). In the presence of a high ATP/ADP ratio (state 4) K_m was much higher than in the presence of uncoupler (state 3u).

This increase in K_m for oxygen as mitochondria become energized might be expected to reflect either an increase in turnover (TN) or a decrease in k_1 (eqn. 2), but TN at a high ATP/ADP ratio (state 4) is considerably lower than TN at a low ATP/ADP ratio (state 3) or in the presence of uncoupler (state 3u); and flow-flash observations show that k_1 (the rate of reaction of cytochrome a_3 with O_2) is not diminished by ATP to the extent that would be needed to account for the observed difference in K_m (Chance & Erecinska, 1971). We are thus presented with a problem.

The behaviour of reduced cytochrome a_3 towards O_2 is also relevant to the question raised by Slater *et al.* (1973) as to the location of the third site of phosphorylation. If energy is conserved in the reaction with O_2, then the K_m observations may provide information about the energization process.

By using a new technique (Degn & Wohlrab, 1971) we have reinvestigated the oxygen kinetics of the mitochondrion, and developed a simple theory to account for some of the energy-state effects. This theory represents an extension of the previous models of Higgins (1959) and Schindler (1964).

Experimental

In principle the technique is that described by Degn & Wohlrab (1971). The steady-state respiration rate (v_R) is equal to the rate of oxygen transfer (v_T) from a gas phase of oxygen partial pressure T_G to a liquid phase of the oxygen partial pressure T_L according to eqn. (3):

$$v_R = v_T = k(T_G - T_L) \qquad (3)$$

where k, measured when respiring material was omitted, is the oxygen-transfer constant of the system. The respirograph system described previously (Degn & Wohlrab, 1971) has been extended in several ways. T_G and T_L are now both measured with

Clark electrodes; a new electronic gas-gradient mixer has been developed (Lundsgaard & Degn, 1973) and an on-line computer system has been adapted to the respirograph, so that calculation and double-reciprocal plotting of v_R against T_L are performed automatically during the experiment from the recorded T_G and T_L values.

The respirograph system consisted of a hexagonal cuvette, light-path 16mm, containing 5.2ml of sample, placed in a Perkin–Elmer 356 dual-wavelength spectrophotometer. Stirring was performed by means of a synchronous motor.

The computer system consisted of a NOVA 1200 minicomputer with 8K memory from Data General Corporation, Southboro, Mass., U.S.A., supplied with interface units for analogue in-and-out modes and for digital in-and-out modes, including a 'real-time' clock. All orders, including orders for communication with external units were given in 'BASIC' with 'CALL-statements'. The system was programmed in such a way, that T_G and T_L were monitored every 10s. By using eqn. (3), $1/T_L$ and $1/v_R$ were generated as the analogue output and recorded on an x,y-recorder.

The liquid phase of the cuvette was in contact with a continuously flowing gas phase of known composition given by the gas-gradient mixer. Each experiment was started with pure N_2 in the gas phase. When the absorbance as well as the zero current of the oxygen electrodes had stabilized, progressively increasing amounts of O_2 were added to the gas phase. The signal from the liquid oxygen electrode was amplified 100-fold. The method ensures that changes in T_L and absorbance are relatively slow and thus permits most of the 'noise' to be removed by damping.

Rat liver mitochondria were isolated by the method of Hogeboom (1955) as modified by Myers & Slater (1957). The protein was determined by the method of Gornall *et al.* (1949). The medium used in the experiments contained 40mM-KCl, 40mM-Tris–HCl, 8mM-$MgCl_2$ and 1.6mM-EDTA adjusted to pH7.8 with 1M-KOH. Hexokinase was obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Trifluoromethoxycarbonyl cyanide phenylhydrazone was from Dr. P. G. Heyter, Du Pont Co., Wilmington Del., U.S.A., and *NNN'N'*-tetramethyl-*p*-phenylenediamine-hydrochloride was from BDH Chemicals Ltd., Poole, Dorset, U.K.

Results

Fig. 1 shows recordings of T_G, T_L and $\Delta E_{445-455}$ during a typical experiment. Three experiments with the same quantity of mitochondria oxidizing succinate are illustrated: (*a*) respiration under state 4 conditions, (*b*) respiration under state 3 conditions, obtained with the hexokinase system (glucose plus hexokinase plus ADP) and (*c*) respiration under state 3u conditions obtained by addition of trifluoromethoxycarbonyl cyanide phenylhydrazone. The

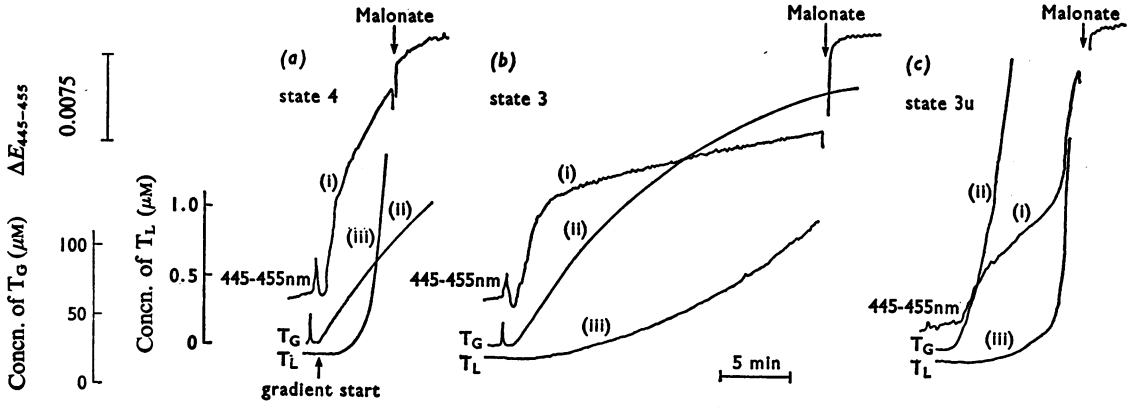

Fig. 1. *Steady-state measurements of T_G, T_L and $\Delta E_{445-455}$ as O_2 increases in the gas phase*

Three experiments are shown. Each experiment was started with N_2 in the gas phase. The signal from the oxygen electrode in the liquid (T_L) was amplified 100 times. After some time T_L and absorbance were constant. This value is taken as the 'fully reduced'. The oxygen gradient was then started (as shown by the arrow). When the system was oxygenated, malonate or antimycin A was added to obtain the 'fully oxidized' value. State 4 respiration (*a*) was obtained by adding mitochondria to a final concentration of 1.3mg of protein/ml to a reaction mixture of 40mM-KCl, 40mM-Tris–HCl, 8mM-$MgCl_2$, 1.6mM-EDTA, 0.15μM-rotenone, 10mM-potassium phosphate and 10mM-potassium succinate, pH7.8. To obtain state 3 respiration (*b*) 1mM-ADP, 10mM-glucose and 10mg of hexokinase/ml were further added to this reaction mixture, and to obtain state 3u respiration (*c*) 0.8μM-trifluoromethoxycarbonyl cyanide phenylhydrazone was added. (i), $\Delta E_{445-455}$; (ii), concentration of T_G; (iii), concentration of T_L.

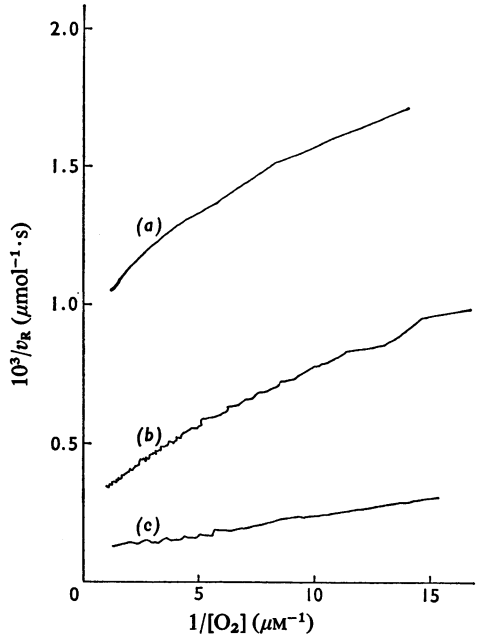

Fig. 2. *Reciprocal plots of succinate oxidation (v_R) against [O_2] in different energy states*

$1/v_R$ and $1/[O_2]$ were obtained by means of on-line computation as described. The three experiments are those described in Fig. 1; (*a*), state 4; (*b*), state 3; (*c*), state 3u.

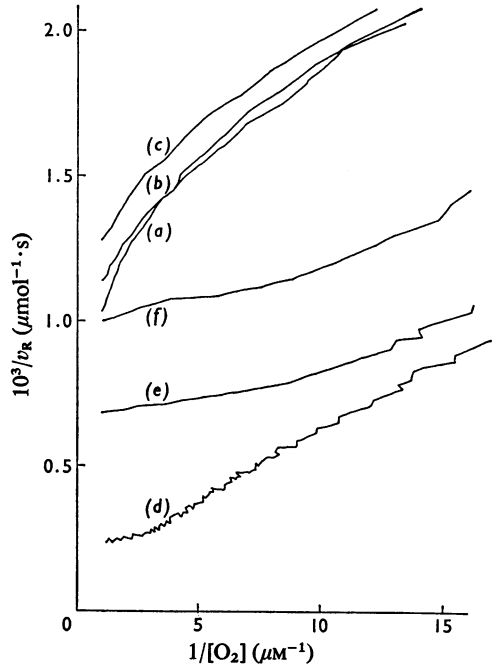

Fig. 3. *Reciprocal plots of succinate oxidation (v_R) against [O_2] in the presence of malonate*

Curves (*a*), (*b*) and (*c*): coupled mitochondria (state 4). Curves (*d*), (*e*) and (*f*): uncoupled mitochondria (state 3u). Mitochondria to a final concentration of 0.83 mg of protein/ml were added to a reaction mixture of 40 mM-KCl, 40 mM-Tris–HCl, 8 mM-$MgCl_2$, 1.6 mM-EDTA, 0.15 μM-rotenone, 2 mM-arsenite and 9.6 mM-succinate. (*a*) No further additions; (*b*) 0.40 mM-malonate; (*c*) 0.96 mM-malonate; (*d*) 0.8 μM-trifluoromethoxycarbonyl cyanide phenylhydrazone; (*e*) 0.8 μM-trifluoromethoxycarbonyl cyanide phenylhydrazone plus 0.48 mM-malonate; (*f*) 0.8 μM-trifluoromethoxycarbonyl cyanide phenylhydrazone plus 0.96 mM-malonate.

three corresponding double-reciprocal plots of respiration rate against oxygen concentration (as plotted by the on-line computer) are shown in Fig. 2. Fig. 1 shows that in states 3 and 4 the steady-state $\Delta E_{445-455}$ has reached about 50% oxidation before any appreciable oxygen appears in the liquid phase. The pO_2 value corresponding to 50% steady-state oxidation in the uncoupled mitochondria is higher. In Fig. 3 the effect of slowing the flux at the succinate dehydrogenase end of the chain is tested. Addition of malonate to a final concentration of 0.48 mM (Fig. 3, curve *b*) or 0.96 mM (Fig. 3, curve *c*) has little effect on the coupled respiration, indicating that respiration is limited by phosphorylation. Addition of the same amounts of malonate to uncoupled mitochondria (Fig. 3, curves *e* and *f*) produces a larger effect and an apparent change in the kinetics is observed. Fig. 4 shows the Lineweaver–Burk plots obtained when *NNN'N'*-tetramethyl-*p*-phenylenediamine+ascorbate is the reducing system used. Differences in oxygen kinetics are seen at three different concentrations of *NNN'N'*-tetramethyl-*p*-phenylenediamine, but the characteristic effect of state 4 conditions in changing the shape of the curves is seen here as in Fig. 2. The plots are non-linear under all conditions, and marked differences occur between the kinetics of coupled and uncoupled mitochondria. The Lineweaver–Burk plots with coupled mitochondria are concave downward in both states 3 and 4 with succinate or *NNN'N'*-tetramethyl-*p*-phenylenediamine+ascorbate as the substrate. The Lineweaver–Burk plots of uncoupled mitochondria are more complicated. When the maximal respiration rate is large, as when succinate respiration is uninhibited (cf. Fig. 3, curve *d*) or at a high *NNN'N'*-tetramethyl-*p*-phenylenediamine concentration (cf. Fig. 4, curve *d*), the plots appear to be concave upward at high pO_2 values, but straight or concave downward at lower pO_2 values. With decreasing maximal respiration rate the Lineweaver–Burk plots tend to linearity at high oxygen partial pressure (cf. Figs. 3 and 4, curves *e* and *f*).

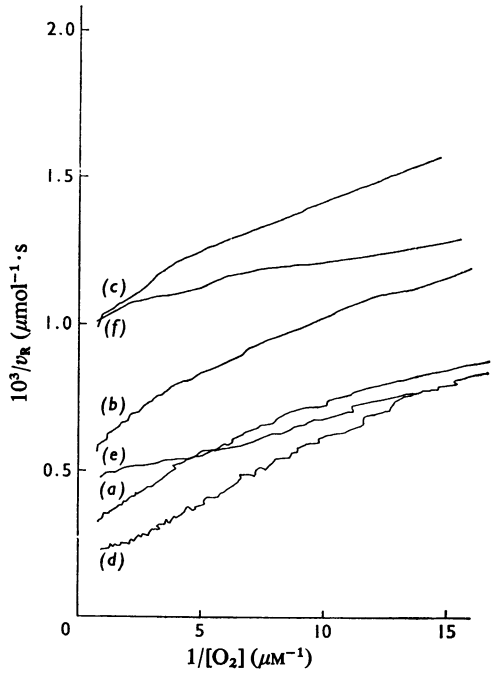

Fig. 4. *Reciprocal plots of NNN'N'-tetramethyl-p-phenylenediamine+ascorbate oxidation (v_R) against [O_2] at different NNN'N'-tetramethyl-p-phenylenediamine concentrations*

Mitochondria to a final concentration of 0.83mg of protein/ml were added to a reaction mixture of 40mM-KCl, 40mM-Tris–HCl, 8mM-$MgCl_2$, 1.6mM-EDTA, 0.38μg of antimycin A/ml, 10mM-ascorbate. (*a*) 116μM-*NNN'N'*-Tetramethyl-*p*-phenylenediamine; (*b*) 58μM-*NNN'N'*-tetramethyl-*p*-phenylenediamine; (*c*) 29μM-*NNN'N'*-tetramethyl-*p*-phenylenediamine; (*d*) 0.8μM-trifluoromethoxycarbonyl cyanide phenylhydrazone plus 116μM-*NNN'N'*-tetramethyl-*p*-phenylenediamine; (*e*) 0.8μM-trifluoromethoxycarbonyl cyanide phenylhydrazone plus 58μM-*NNN'N'*-tetramethyl-*p*-phenylenediamine; (*f*) 0.8μM-trifluoromethoxycarbonyl cyanide phenylhydrazone plus 29μM-*NNN'N'*-tetramethyl-*p*-phenylenediamine.

Discussion

This work confirms that in mitochondria the respiration rate does not follow simple Michaelis–Menten kinetics as a function of the oxygen partial pressure. The concave-downward Lineweaver–Burk plots of coupled mitochondria indicate an apparent reaction order of less than 1. This is in contrast with the findings of Bänder & Kiese (1955) and of Longmuir (1957). Only the concave-upward parts of the plots with uncoupled mitochondria are consistent with an apparent reaction order higher than 1.

The addition of uncoupler causes a decrease in the apparent K_m. This change would be equivalent to a change in the 'on' constant for oxygen (k_1) if the simple relation of eqn. (2), proposed by Schindler (1964) and Chance (1965), was accepted. However, the more complex picture of the oxygen kinetics which emerges from the Lineweaver–Burk plots suggests that this relation is not generally valid.

We propose the following relatively simple scheme for consideration (eqn. 4):

$$\left.\begin{array}{ll} \mathrm{AH}+\mathrm{cyt.}\,c^{3+} \xrightarrow{k_{+3}} \mathrm{A}+\mathrm{H}^+ +\mathrm{cyt.}\,c^{2+} & (a) \\ \mathrm{Cyt.}\,c^{2+}+\mathrm{cyt.}\,a_3{}^{3+} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \mathrm{cyt.}\,c^{3+}+\mathrm{cyt.}\,a_3{}^{2+} & (b) \\ \mathrm{Cyt.}\,a_3{}^{2+}+\mathrm{O_2} \xrightarrow{k_{+1}} \mathrm{cyt.}\,a_3{}^{3+}(+\mathrm{O_2}^-) & (c) \end{array}\right\} (4)$$

For such a system, we have:

$$v = 4k_{+1}[\mathrm{O_2}][\mathrm{cyt.}\,a_3{}^{2+}] = k_{+3}[\mathrm{AH}][\mathrm{cyt.}\,c^{3+}]$$
$$= \frac{k_{+2}[\mathrm{cyt.}\,c^{2+}][\mathrm{cyt.}\,a_3{}^{3+}]}{[\mathrm{cyt.}\,a_3]_{\mathrm{total}}}$$
$$- \frac{k_{-2}[\mathrm{cyt.}\,c^{3+}][\mathrm{cyt.}\,a_3{}^{2+}]}{[\mathrm{cyt.}\,a_3]_{\mathrm{total}}} \quad (5)$$

where k_{+3} and k_{+1} are second-order constants ($M^{-1}\cdot s^{-1}$) and k_{+2} and k_{-2} first-order constants (s^{-1}), and it is assumed that $[\mathrm{cyt.}\,c]_{\mathrm{total}} \simeq [\mathrm{cyt.}\,a_3]_{\mathrm{total}} = $ 'e' in the mitochondria. Because eqn. (5) contains products of variables it is not amenable to a simple steady-state solution. For a given fractional reduction, c_s, of cytochrome c ($c_s = [\mathrm{cyt.}\,c^{2+}]/[\mathrm{cyt.}\,c]_{\mathrm{total}}$) we have eqn. (6):

$$\frac{c_s e}{v} = \frac{1}{k_{+2}} + \frac{k_{-2}}{4k_{+1}k_{+2}} \cdot \frac{1}{[\mathrm{O_2}]} + \frac{(k_{+2}-k_{-2})c_s}{4k_{+1}k_{+2}[\mathrm{O_2}]} \quad (6)$$

Expressions of this type are useful when the steady-state reduction of cyt.c is correlated with changes in v and [O_2]. From eqn. (5) we have:

$$v = k_{+3}[\mathrm{AH}](1-c_s)e \quad (7)$$

Substituting in eqn. (6) we obtain:

$$v + \frac{A_1 v}{[\mathrm{O_2}]} - \frac{A_2 v^2}{[\mathrm{O_2}]} = A_3 \quad (8)$$

where

$$A_1 = \frac{k_{+2}k_{+3}[\mathrm{AH}]e}{4k_{+1}(k_{+2}+k_{+3}[\mathrm{AH}])}$$

$$A_2 = \frac{k_{+2}-k_{-2}}{4k_{+1}(k_{+2}+k_{+3}[\mathrm{AH}])}$$

and

$$A_3 = \frac{k_{+2}k_{+3}[\mathrm{AH}]e}{k_{+2}+k_{+3}[\mathrm{AH}]}$$

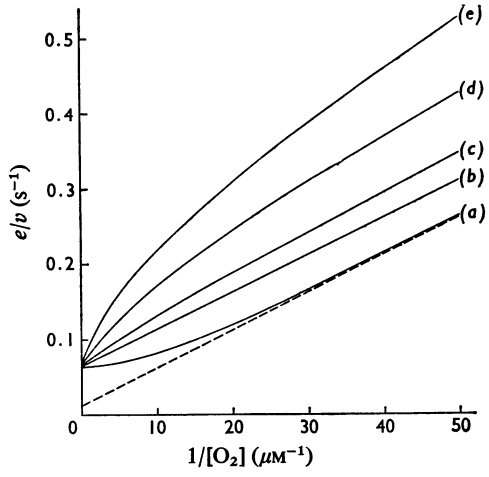

Fig. 5. *Theoretical plots of e/v against* $1/[O_2]$ *for various values of* k_{-2} *in eqn.* (4)

$$AH \xrightarrow{k+3} \text{cyt. } c \underset{k-2}{\overset{k+2}{\rightleftharpoons}} \text{cyt. } a_3 \xrightarrow{k+1} O_2$$

$k_{+3}[AH] = 20s^{-1}$; $k_{+2} = 80s^{-1}$; $4k_{+1} = 2\times10^8 M^{-1}\cdot s^{-1}$. (*a*) $k_{-2} = 0$; (*b*) $k_{-2} = k_{+2} = 80s^{-1}$; (*c*) $k_{-2} = 2k_{+2} = 160s^{-1}$; (*d*) $k_{-2} = 5k_{+2} = 400s^{-1}$; (*e*) $k_{-2} = 10k_{+2} = 800s^{-1}$. ----, Asymptote for (*a*), $k_{-2} = 0$, $[AH] = \infty$.

This is formally similar to equations obtained by Schindler (1964) and Higgins (1959), with the difference that the reverse reaction of eqn. (4*b*), governed by the constant k_{-2}, was previously neglected.

From eqn. (8) we can obtain a dimensionless parameter '*r*' (Schindler, 1964) such that:

$$r = \frac{A_2A_3}{A_1} = \frac{k_{+2}-k_{-2}}{k_{+2}+k_{+3}[AH]} \qquad (9)$$

When $r = 0$, eqn. (8) reduces to the Michaelis equation. This can occur either when [AH] becomes very large or when $k_{+2} = k_{-2}$. When $r = 1$, eqn. (8) becomes the equation of a 'titration' ($n = 0.5$ in eqn. 2). When $1 > r > 0$, eqn. (8) will give a Lineweaver–Burk plot concave upward, as observed for uncoupled systems. When $r < 0$, that is, when $k_{-2} > k_{+2}$, eqn. (8) will give a Lineweaver–Burk plot concave downward, as observed for coupled (or state 4) mitochondria. Fig. 5, which should be compared with Figs. 2, 3 and 4, illustrates the theoretical curves for the system of eqn. (4) with $k_{+3}[AH]$ set at $20s^{-1}$, k_{+2} at $80s^{-1}$ and $4k_{+1}$ at $2\times10^8 M^{-1}\cdot s^{-1}$.

At the lowest O_2 concentrations, all the plots of $1/v$ against $1/[O_2]$ are linear with a slope proportional to $1/4k_{+1}$. The extrapolated intercept on the e/v axis is equal to $(k_{-2}+k_{+3}[AH])/k_{+3}[AH]k_{+2}$ (or $1/k_{+2}$ when $k_{-2} = 0$), but the real intercept is $(k_{+2}+k_{+3}[AH])/k_{+3}[AH]k_{+2}$. The K_m calculated from the intercept on the abscissa of a line tangential to the Lineweaver–Burk plot at a given O_2 concentration decreases with increasing $[O_2]$ when k_{-2} is less than k_{+2}, but increases with $[O_2]$ when k_{+2} is less than k_{-2}. The concentration of oxygen giving a half-maximal respiration rate (apparent K_m) is therefore considerably larger in state 4 than in state 3u.

This difference in apparent K_m for oxygen in state 4 can thus be explained semiquantitatively by reference to the increased back-reaction rates in the energized state. The experimental observations with ascorbate and *NNN'N'*-tetramethyl-*p*-phenylenediamine as substrates seem to be consistent with a k_{-2} of about twice k_{+2}. However, the tendency for the two curves (state 4 and state 3u) to approach one another as $[O_2]$ tends to zero (cf. Figs. 2–4) cannot be accounted for by means of the present model. It is nonetheless clear that it is not necessary to postulate any major effects of energization on k_{+1} at the present time. However, to decide the real value of k_{+1} and to determine whether it is at all energy-dependent will demand more detailed knowledge of the K_m for oxygen, and of its relation to the reaction mechanism.

Measurements of the steady-state oxidation of the cytochromes at low oxygen concentrations are required to provide such information.

From our results we draw the following provisional conclusions.

(*a*) Although the apparent K_m for oxygen (the concentration giving 50% of the maximum respiration rate) is dependent on the mitochondrial energy state, this does not imply that k_{+1} (the rate of reaction of oxygen and reduced cytochrome a_3) is energy-dependent.

(*b*) The respiratory control ratio for mitochondria (the ratio of respiration rates in low- and high-energy states) can be a variable, dependent on oxygen concentration.

(*c*) In both the system with ascorbate plus *NNN'N'*-tetramethyl-*p*-phenylenediamine as substrate, and the system with succinate as substrate, the forward reaction (governed by k_{+2} in eqn. 4*b*) is less sensitive to energy-state changes than is the back reaction (governed by k_{-2} in eqn. 4*b*). That is, the systems are subject to a rate control that is more thermodynamic (Slater *et al.*, 1973) than kinetic (Chance, 1965) in character.

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