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# MEASUREMENT OF STEADY-STATE VALUES OF RESPIRATION RATE AND OXIDATION LEVELS OF RESPIRATORY PIGMENTS AT LOW OXYGEN TENSIONS. A NEW TECHNIQUE

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SUMMARY

1. An apparatus was developed for the simultaneous measurement of steady-state values of respiration rate and oxidation level of respiratory pigments at low oxygen tensions. An open reaction system is utilized. The liquid sample is in contact with a gas mixture whose oxygen tension can be increased linearly with time at a rate so slow that the system is always practically at a steady state.

2. Assuming Michaelis–Menten kinetics in the respiration, theoretical curves for oxygen tension in the liquid and oxidation level of the terminal oxidase during a linear increase of the oxygen tension in the gas were calculated.

3. Measurements were performed on rat liver mitochondria. Steady-state curves for oxygen tension in the liquid and oxidation level of the terminal oxidase, cytochrome $a_3$, obtained with coupled mitochondria resembled the theoretical curves. For uncoupled mitochondria the cytochrome $a_3$ curve was sigmoidal, deviating strongly from the theoretical curve.

4. The apparent $K_m$ for oxygen uptake of coupled mitochondria in the presence of pyruvate and malate, in the absence of phosphate was found to be 0.5 $\mu$M. In the case of uncoupled mitochondria the oxygen tension in the liquid could not be measured with sufficient accuracy to allow comparison with Michaelis-Menten kinetics. The apparent $K_m$ for oxygen uptake was less than 0.05 $\mu$M.

## INTRODUCTION

Although respiration has been a predominant topic for experimental studies throughout the history of biochemistry, remarkably little is known about the dependency of this process on the concentration of its primary reactant, oxygen. This lack of knowledge is due to the difficulties in measuring oxygen in the relevant low concentration range. The affinity of most respiring systems for oxygen is very high, and,

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Abbreviations: TES, *N*-tris (hydroxymethyl) methyl-2-aminoethane sulphonic acid; TMPD *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

consequently, the respiration rate and degree of oxidation of the respiratory pigments show dependencies on the oxygen tension only at very low values of the latter. The only practical technique for measuring low oxygen tensions in suspensions of cells or subcellular particles is polarography[1]. This technique is adequate for measurements on samples with apparent $K_m$ values for oxygen uptake down to about 0.1 $\mu$M. In all but one[2] of the very few published studies[1–5] of the dependency of respiration rate on oxygen tension the experiments have been performed in a closed system where the oxygen tension was falling to zero due to the respiration. In such a system the polarographic oxygen trace falls linearly with time until a very low value is reached where the rate of fall begins to decrease. The final nonlinear part of the oxygen trace is recorded in an expanded scale, and respiration rate *versus* oxygen tension curves can be calculated, the respiration rate at any time being determined as the slope of the oxygen tension curve at that time. It is a disadvantage of this technique that the oxygen tension passes through the nonlinear range in such a short time that it is not justified to assume that the results are steady-state rates of respiration as functions of oxygen tension. Likewise, simultaneous measurements of light absorption due to respiratory pigments is of limited value because the resulting curves are transients rather than steady-state curves.

In the work to be reported here we have developed an apparatus which allows simultaneous measurements of respiration rate and degree of oxidation of respiratory pigments under steady-state conditions at low oxygen tensions. The apparatus which we propose to call a respirograph consists of an optical cuvette fitted with a vibrating platinum electrode for polarographic oxygen determination. The surface of the liquid sample in the cuvette is in contact with a gas mixture consisting of $N_2$ and $O_2$. The gases are mixed by a linear oxygen gradient apparatus producing a mixture whose $O_2$ content increases linearly with time. The cuvette is situated in a dual wavelength spectrophotometer for measurement of the degree of oxidation of the respiratory pigments. A schematic representation of the respirograph is given in Fig. 1.

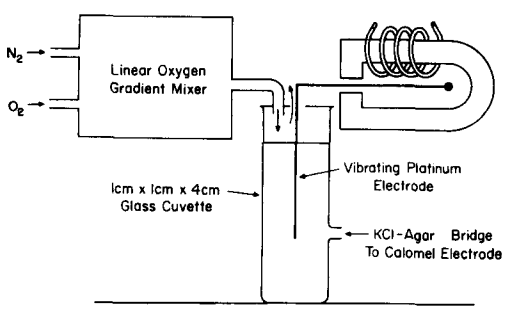



Fig. 1. Schematic diagram of respirograph.

## THEORY OF THE RESPIROGRAPH

The experimental system described above is an open system because oxygen can diffuse across the boundary between the gas and the liquid phases. When the liquid contains a respiring sample the net transport of oxygen will be in the direction from the gas to the liquid. Provided both the gas and the liquid phases are homogeneous,

the rate of transport of oxygen is linearly dependent on the difference in oxygen tension between the gas and the liquid. This relationship is expressed by the equation.

$$v_t = K(T_G - T_L) \tag{1}$$

where $v_t$ is the rate of transport of oxygen from the gas to the liquid, $T_G$ and $T_L$ are the oxygen tensions in the gas and the liquid, and $K$ is a constant, usually called the oxygen transfer constant. This constant depends on the surface area, the volume of the liquid and the temperature. If the gas mixture is allowed to flow rapidly through the volume above the liquid it is practically homogeneous and its composition is not significantly affected by the exchange of oxygen with the liquid phase. In order to obtain the required homogeneity in the liquid phase stirring is necessary. In the present case this is done efficiently by the vibrating platinum electrode. Great care must be taken that the stirring does not create bubbles because these will affect the surface area of the solution and change the oxygen transfer constant.

The measurement of respiration rate by the respirograph depends on the relationship given by Eqn. 1. At a steady state, *i.e.* when the oxygen tension in the liquid is constant, the rate of oxygen consumption by the respiring sample and the rate of transport of oxygen into the liquid are equal. If the oxygen tension in the gas and the oxygen transfer constant are known, and the oxygen tension in the liquid is measured polarographically, the rate of transport of oxygen (= respiration rate) can be calculated from Eqn. 1.

Ideally the measurements should be performed by setting the oxygen in the gas at a constant value and observing the oxygen in the liquid until constant. By resetting the oxygen in the gas and waiting for the new steady state to occur, a point by point determination of the steady-state respiration rate as a function of the oxygen in the liquid could be made. However, the measurements can be done in a much less tedious way by the help of the linear oxygen gradient mixer. If the oxygen tension in the gas is allowed to increase continuously at a very slow rate the system will always be practically at a steady state. As the oxygen in the gas runs through a slow linear sweep, steady-state values of oxygen tension in the liquid and degree of oxidation of respiratory pigments can be recorded continuously.

Because the respiration of biological samples usually approximates the Michaelis-Menten model of an enzymatic reaction it is of interest to calculate the theoretical curve for the oxygen tension in the liquid as a function of the oxygen tension in the gas when the sample is assumed to utilize oxygen according to Michaelis–Menten kinetics. Assume that the respiration rate $v_r$ depends on $T_L$ according to the equation

$$v_r = \frac{v_{max} T_L}{K_m + T_L} \tag{2}$$

where $v_{max}$ is the maximal respiration rate and $K_m$ is the Michaelis constant. At steady states $v_t = v_r$, and Eqns. 1 and 2 yield

$$T_G = T_L \left( \frac{v_{max}}{K(K_m + T_L)} + 1 \right) \tag{3}$$

Eqn. 3 is represented by the curve in Fig. 2. The constants used in the calculation of the curve were $K = K_m = 1$ and $v_{max} = 100$. It is observed that the oxygen tension in the liquid approaches asymptotically a straight line with the slope 1 as the maximal

respiration rate of the sample is approached. The asymptote intersects the $X$-axis at the point $v_{max}/K$. It is also shown in Fig. 2 how $K_m$ is evaluated by a geometrical construction in the $T_L$ *versus* $T_G$ curve. A line with the slope 1 is drawn through the point $v_{max}/2K$ on the $X$-axis. The ordinate of the intersection between this line and the curve is $K_m$.

We shall now calculate a theoretical curve for the degree of oxidation of the terminal oxidase in the respiring sample as a function of the oxygen tension in the gas. Again we assume that the respiration follows Michaelis–Menten kinetics. The reaction

$$O_2 + a \rightarrow O_2^- + a^+ \tag{4}$$

between $O_2$ and the reduced form, $a$, of a terminal oxidase apparently is practically irreversible[6]. Therefore the reaction rate is

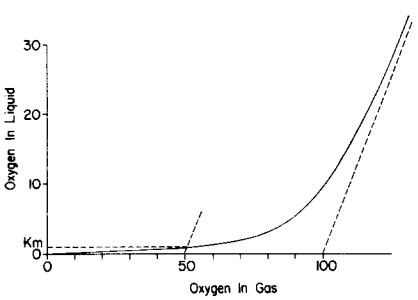
$$v_r = kT_L a \tag{5}$$

where $k$ is the rate constant. Under steady-state conditions $v_t = v_r$ and $T_L$ can be eliminated from Eqns. 1, 2, and 5. Hereby the following relationship between the oxygen tension in the gas and the concentration of the reduced form of the terminal oxidase is obtained

$$T_G = \left(\frac{v_{max}}{ka} - K_m\right)\left(\frac{ka}{K} + 1\right) \tag{6}$$

Using the same values for the constants as shown above and in addition $k = 1$ a theoretical curve for $a$ as a function of $T_L$ was calculated. From this and the conservation equation for cytochrome, $a + a^+ = 100$, the degree of oxidation of the terminal oxidase on a per cent scale was calculated as a function of the oxygen tension in the gas. The resulting curve is shown in Fig. 3. Coincidentally the respiration rate as a function of the oxygen tension in the gas is described by exact the same curve.

Intuitively it would be expected that, at an increasing oxygen gradient, the



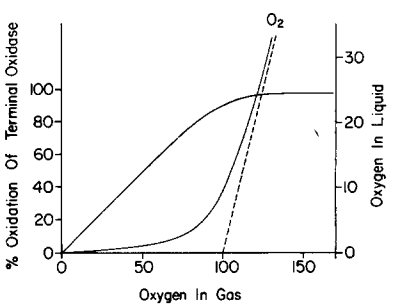




Fig. 2. Plot of oxygen tension in the liquid ($T_L$) *versus* oxygen tension in the gas phase ($T_G$) in the presence of a system respiring according to Michaelis–Menten kinetics. The dotted line to the right with the slope 1 is the asymptote of the $T_L$ *versus* $T_G$ curve. It intersects the abscissa axis at the point $v_{max}/K$. The dotted line to the left with the slope 1 is drawn through the point $v_{max}/2K$ on the abscissa axis. Its intersection with the $T_L$ *versus* $T_G$ curve indicates $K_m$.

Fig. 3. Theoretical curves showing the steady-state degree of oxidation of the terminal oxidase (decreasing slope) and the oxygen tension in the liquid (increasing slope) as functions of the oxygen tension in the gas. The curves were calculated with the assumption that the terminal oxidase–oxygen reaction is essentially irreversible, and the system is respiring according to Michaelis–Menten kinetics.

cytochrome next to the terminal oxidase in the respiratory chain sequence will reach its maximal steady-state oxidation later than the terminal oxidase. Generally any pigment in the respiratory chain must reach its maximal steady-state oxidation later than the one which precedes it in the chain sequence and before the one which follows it in the sequence. Accordingly the steady-state data obtained by the respirograph may provide a means of establishing the sequence or other organization of the pigments in the absence of inhibitors.

Examples are known of single enzymes or cellular species whose oxygen uptake deviate strongly from Michaelis–Menten kinetics. In such cases there may be two or more steady states of oxygen tension in the liquid corresponding to the same oxygen tension in the gas, or the steady state may be more or less unstable, and damped or undamped oscillations of the oxygen tension in the liquid take place. Theoretical and experimental results concerning such abnormal phenomena in the system open to oxygen have been presented in earlier papers[7–10].

## THE APPARATUS

The apparatus used in the experiments is a slightly modified form of the one used in the earlier published experiments on peroxidase kinetics[7,9]. The major innovation is the linear oxygen gradient mixer. For this purpose a Matheson mixing flow meter was used. This instrument has two inlets which are controlled by needle valves, and it has a common outlet. The nitrogen flow through one inlet was set at a constant rate, and the oxygen flow through the other inlet was variable, the shaft of the needle valve being connected through a reduction gear to a synchronous motor. At this inlet the normal needle was replaced by a specially made needle with a 3° cone, and the orifice for the needle was made correspondingly small. The needle shaft was turning at a rate of 0.05 rev./min or less, depending on the oxygen gradient desired.

The use of a motorized needle valve in the gas mixer is far from a good solution because the oxygen gradient is sigmoid rather than linear. However, we found that when a bottle of a suitable volume, serving as a capacity, is inserted between the gas mixer and the gas outlet in the cuvette, and the needle valve is initially set at a certain slightly open position, an oxygen gradient can be produced which has a fairly long linear section beginning from time zero. The capacity cannot be used to straighten out the gradient when the oxygen is decreasing with time. For this reason only increasing gradients were used in the present work. We are presently investigating other principles for producing the oxygen gradient which avoid this difficulty.

The gases used were bubbled through water before mixing. Nevertheless the gas stream caused evaporation from the sample. This loss of water was found to be about 2 $\mu$l/min and was compensated by pumping water into the cuvette at that rate.

Moisture from the gas coming out of the cuvette can create electric conductivity on adjacent insulators. Even a very low conductivity of the insulators suspending the vibrating electrode can seriously disturb the high sensitivity polarographic measurement. For this reason the vibrator with the electrode suspension was completely encapsulated except for a hole permitting the free movement of the vibrator, and a stream of dry nitrogen was continuously flushed through the vibrator unit, escaping through the vibrator hole.

## MATERIALS AND METHODS

Rat liver mitochondria were prepared according to the method of SCHNEIDER[11]. The medium used in the experiments consisted of 0.22 M mannitol, 0.07 M sucrose, 200 $\mu$M $Na_2EDTA$, and 20 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES). The pH was adjusted to 7.4 with 1 N KOH. As an uncoupler was used 5-chloro-3-*tert*-butyl-2'-chloro-4'-nitrosalicylanilide (S-13). This substance was a gift from Monsanto Chemical Co. TES was obtained from Sigma. The concentration of mitochondria was determined on the basis of their cytochrome *c* oxidase content[12]. For the gas mixture was used air from the laboratory compressed air outlet and nitrogen from Matheson which contains maximally 0.03 % of oxygen. This low oxygen content is insignificant in the present experiments.

## RESULTS AND DISCUSSION

When 2.4 ml of the medium, originally saturated with atmospheric oxygen, was placed in the cuvette and the gas phase was pure nitrogen, the oxygen tension in the liquid was observed to decrease according to first order kinetics with a half time of 0.92 min. From the half time, $t_{\frac{1}{2}}$, the oxygen transfer constant, $K$, in Eqn. 1

$$K = \frac{\ln 2}{t_{\frac{1}{2}}} = 0.75 \text{ min}^{-1}$$

can be calculated. In all the following experiments a total volume of 2.4 ml was used, and the above value for $K$ is used in the calculation of the respiration rate.

In the experiments with mitochondria the medium and the substrate were first mixed in the cuvette, and oxygen was removed by a pure nitrogen gas phase. Mitochondria were then added, and the light absorption measurement with the dual wavelength spectrophotometer was initiated. When the light absorption trace was constant, usually after about 10 min, the linear oxygen gradient mixer was started. At the end of the linear section of the gradient the oxygen was turned off, and the gas phase was again pure nitrogen. The return of the oxygen and light absorption traces to the base lines was then observed. This procedure was followed in four consecutive experiments measuring at 447–460 nm (cytochromes $a_3$ and $a$), 550–540 nm (cytochrome $c$), 560–575 nm (cytochromes $b$ and $b_5$), and 605–620 nm (cytochromes $a$ and $a_3$) The resulting light absorption curves were replotted in a scale of per cent of maximal steady-state oxidation and combined in one graph, shown in Fig. 4. The light absorption measured at 447–460 nm is predominantly due to cytochrome $a_3$ which is the terminal oxidase. The light absorption at 605–625 nm is predominantly due to cytochrome $a$[13]. It is observed that the curves corresponding to the different cytochromes follow each other in accordance with the generally accepted sequence of the cytochromes in the respiratory chain[14]. The apparent $K_m$ for the oxygen uptake of the mitochondria is about 0.5 $\mu$M as evaluated from the oxygen trace in Fig. 4.

The light absorption traces returned rapidly to the baselines when the oxygen in the gas was turned off in all experiments except the one where cytochrome $b$ was measured (560–575 nm). In this case the trace first returned rapidly to about 1/3 of the maximal value. It then levelled off, and after some time the fall accelerated again and the trace fell off to the baseline. A similar sudden fall in the light absorption trace

was also observed to occur about 15 min after the mitochondria were introduced into the medium but before the oxygen sweep was started. If the oxygen sweep was started before this phenomenon occurred, the light absorption trace during the sweep looked like the one in Fig. 4. However, if the sweep was started after the sudden fall had occurred, the light absorption trace exhibited an initial sudden increase of the same magnitude as the preceding sudden fall, as shown in Fig. 5. This behaviour of the light absorption at 560–575 nm was made the subject of a more detailed study, which will be described in another paper[15]. Briefly, it was found that cytochrome $b_5$ is responsible for the sudden jumps in the light absorption.

The above experiments were repeated with the addition of the uncoupler S-13. The resulting curves are shown in Fig. 6. The light absorption curves are profoundly different from those obtained with coupled mitochondria. It is remarkable that all curves except the one at 560–575 nm are nearly identical and strongly sigmoidal. The deviating behaviour of the 560–575-nm trace is probably caused by cytochrome $b_5$. Whereas the 447–460-nm curve in the experiment with coupled mitochondria (Fig. 4) has the shape predicted for a terminal oxidase (Fig. 3) the 447–460-nm curve, measured when uncoupler was added, is strongly sigmoidal in disagreement with the theoretical curve. This may indicate that one of the assumptions in the theory does not hold in the case of uncoupled mitochondria. Most likely to be violated is the con-

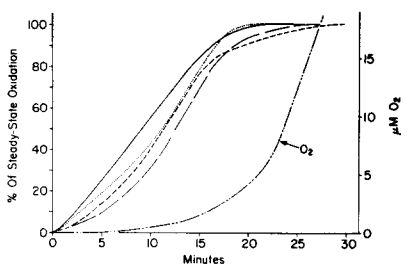



Fig. 4. Per cent of steady-state oxidation of mitochondrial cytochromes *versus* time after the start of the oxygen gradient and the corresponding oxygen tension in the liquid phase (——·——). Rat liver mitochondria were suspended in 0.22 M mannitol–0.07 M sucrose–200 μM $Na_2$ EDTA–20 mM TES medium at 0.18 μM cytochrome oxidase (6 mg protein per ml) in the presence of 10 mM sodium pyruvate and 5 mM sodium malate. The following wavelengths were monitored: 447–460 nm (———), 605-620 nm (·····), 550–540 nm (-------), and 560–575 nm(— —).

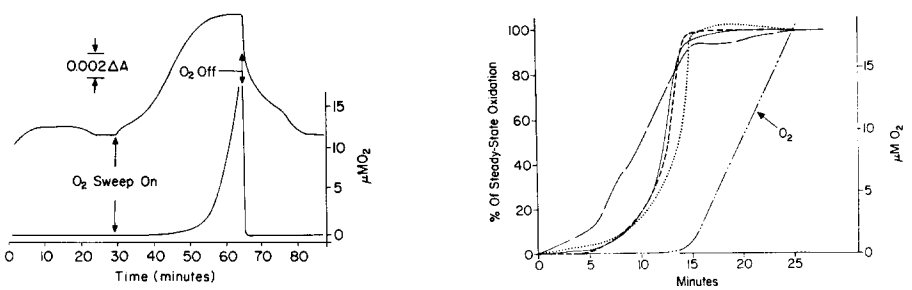



Fig. 5. Anomalous behaviour at 560–575 nm (upper trace) due to interference by mitochondrial cytochrome $b_5$. The lower curve segments show the oxygen tension in the liquid. At about 65 min the oxygen supply was turned off, and the resulting increase in absorbance was determined.

Fig. 6. Steady oxidation states of uncoupled rat liver mitochondria. Experimental conditions were the same as for Fig. 4. However, 0.6 mole of S-13 per mole of cytochrome oxidase was present.

dition that the respiration rate as a function of oxygen tension follows Michaelis–Menten kinetics. Unfortunately the sensitivity of the oxygen measurement was insufficient to record the lower part of the oxygen tension curve in this experiment, and the assumption could, therefore, not be checked. Because of the insufficient sensitivity of the measurement only an upper limit for the apparent $K_m$ for the oxygen uptake of uncoupled mitochondria can be given. This is 0.05 $\mu$M, *i.e.* it is at least 10 times lower than the apparent $K_m$ for the coupled mitochondria. This result is contrary to results obtained by CHANCE[16], who holds that $K_m$ increases linearly with increasing electron flux. The maximal respiration rate was increased about 50% after the addition of uncoupler in the present experiments.

Finally the same experiments as above were performed, using ascorbate–TMPD as the substrate instead of pyruvate–malate. Uncoupler was not added. The resulting curves are shown in Fig. 7. It is seen that in this case the 447–460-nm trace resembles the theoretical curve in Fig. 3. The light absorption traces at different wavelengths are not as well ordered as in the experiment where pyruvate–malate was used as the substrate. The oxygen trace approximates the theoretical curve which was calculated assuming Michaelis–Menten kinetics. The apparent $K_m$ is about 0.5 $\mu$M, which is the same as was found when pyruvate–malate was the substrate.

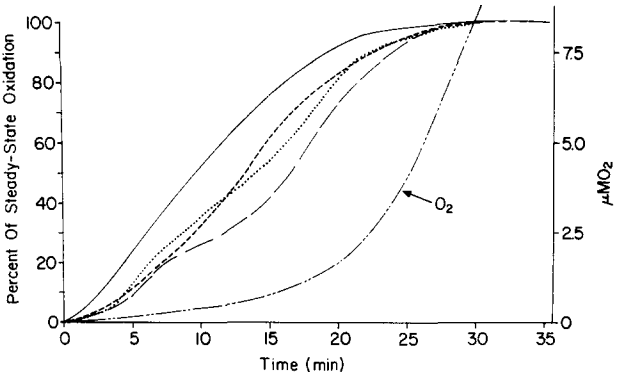



Fig. 7. Steady states of coupled rat liver mitochondria in the presence of sodium ascorbate and *N*, *N*, *N'*, *N'*-tetramethyl-*p*-phenylenediamine (TMPD). Same experimental conditions as for Fig. 4, however, the substrate was 20 mM sodium ascorbate with 200 $\mu$M TMPD. Rat liver mitochondria were at a concentration of 0.08 $\mu$M cytochrome oxidase (3 mg protein per ml).

In all the experiments reported above a fresh sample of mitochondria was taken for each oxygen sweep. However, some experiments were also done where the sweep was repeated on the same sample. This was done under the three different experimental conditions employed. In all cases it was found that the oxygen traces as well as the light absorption traces were repeatable on the same sample. This shows that although the duration of the experiments is quite long, deterioration of the mitochondria does not take place to any significant degree during the experiment.

## CONCLUSION

In the present work we have developed a new technique for the simultaneous measurement of steady-state values of respiration rate and oxidation level of respiratory pigments at low oxygen tensions. The purpose of such measurements is to

obtain data from which the rate constants of the reactions between the components of the respiratory system can be calculated. The advantage of measuring steady-state concentrations is that the theoretical analysis of the results is facilitated by the substitution of algebraic equations for differential equations[17]. Another advantage of the steady-state measurements is that they permit the determination of rate constants of fast reactions without the use of fast responding equipment.

The full interpretation of the data obtained by the respirograph requires extensive computer work, however, some results such as the apparent $K_m$, the turnover number and the rate constant, $k$, of the reactions between $O_2$ and cytochrome oxidase can be obtained manually. From the experiment with coupled mitochondria we find by the geometrical method shown in Fig. 1 the apparent $K_m = 0.5$ $\mu$M $O_2$. By the help of Eqn. 1 we find $v_{max} = 38$ $\mu$M $O_2 \cdot min^{-1}$ and hence the turnover number of cytochrome oxidase 14 heme $a^{-1} \cdot sec^{-1}$. From Eqn. 6 we find $k = 7 \cdot 10^6$ $sec^{-1} \cdot M^{-1}$. In Table I these results are compared with those obtained for rat liver mitochondria by SCHINDLER[6], who measured oxygen by means of luminescent bacteria. The cause of the discrepancies is not clear. We are not aware of any other work determining the same set of data.

The experiments performed in this work were designed to prove the feasibility of our technique for measuring steady-state concentrations of species involved in respiration. We think that the results are successful in that respect. We anticipate that a fairly complete set of steady-state curves for the components of the respiratory system can be obtained when the dual wavelength spectrophotometric measurements are supplemented with fluorescence measurements.

TABLE I

COMPARISON OF OUR DATA FOR CYTOCHROME OXIDASE WITH THOSE OBTAINED BY SCHINDLER[6] USING A DIFFERENT TECHNIQUE IN EXPERIMENTS ON RAT LIVER MITOCHONDRIA

| *Data* | SCHINDLER[6] | *Our results* |
|---|---|---|
| Apparent $K_m$ | 0.04 $\mu$M $O_2$ | 0.5 $\mu$M $O_2$ |
| Turnover number | 6.8 heme $a^{-1} \cdot sec^{-1}$ | 14 heme $a^{-1} \cdot sec^{-1}$ |
| $k$ | $2.6 \cdot 10^7$ $M^{-1} \cdot sec^{-1}$ | $7 \cdot 10^6$ $M^{-1} \cdot sec^{-1}$ |

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