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ELECTRODE MEASUREMENT OF OXYGEN TENSION WITH 1-ms TIME RESOLUTION

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SUMMARY

A continuous flow device utilizing a Clark oxygen electrode was constructed; this device had a dead time and resolution of 1 ms. Mixing was tested by observing the neutralization of acid with base, and at the maximal flow rate, the mixing was 94% complete within 1 ms and better than 98% complete within 2 ms after initial mixing. Observation of the oxygenation of hemoglobin gave data which agreed with previous data obtained by a stopped-flow optical experiment. The respiration of phosphorylating submitochondrial particles was measured utilizing this device. The burst of respiration in submitochondrial particles was triphasic, with a very rapid burst lasting some 60 ms, followed by a longer burst of respiration lasting more than 4 s.

INTRODUCTION

Oxygenation of suspensions or solutions provides a useful method of observing the kinetics of oxygen consumption.

Optical studies are capable of measuring the rates at which simple systems such as hemoglobin solutions interact with oxygen, but the consumption of oxygen by more complex systems, such as mitochondrial suspensions, requires the use of electrode measurement [1–3].

The continuous flow electrode devices used in previous studies had a minimal dead time of about 15 ms and a time resolution of 10 ms. However, it was necessary to run material through the apparatus at wastefully high rates in order to achieve the dead time of 15 ms, and the dead time commonly used was 30–40 ms. Early experiments on submitochondrial particles during these previous studies had shown no rapid phase of respiration at times from about 40 ms onward. Thus the device described here was constructed to investigate this problem with a higher time resolution.

EXPERIMENTAL

The continuous flow apparatus

The device was similar in principal to that described earlier [1], but some of the details of construction were different. The two solutions were forced through the

apparatus by means of two Yale O-ring Robb syringes, driven by a large capacity dual syringe pump (Harvard Apparatus Co., Model 2206). In order to obtain a sufficiently steady flow, it was necessary to select the drive screw from several that were provided by the Harvard Apparatus Co. The syringes were adapted to thick walled plastic tubing of the type previously described [1] by means of luer-lock adapters (Chromatronix). The solutions were then led to a block which contained both the mixer and the measuring block holding the electrode. The mixer and the measuring block were arranged in a nested configuration which minimized the distance between the point of mixing and the closest point at which the electrode could be placed. The measuring block was mounted to a fixed plate, while the mixer was mounted on a movable plate so that it could be pressed against the measuring block. In order to obtain reproducible results it was necessary to adjust this pressure to a definite value with a torque wrench. Fig. 1 shows a cross-section of the mixing and measuring blocks. Short delay times, in the range of 2–50 ms, were obtained by inserting plastic spacers of appropriate thickness between the mixer and the measuring block. O-rings and dental dam material were inserted at appropriate points to prevent leakage of this device. Longer time points were obtained by insertion of a Chromatronix six-position switching valve between the mixer and the measuring block. With this switch it was possible to run the drive syringe continuously and to switch quickly from one delay to another, without exposing the oxygen electrode to the much higher oxygen pressures of the atmosphere between the measurements. This switching device allowed the gathering of more points of greater reliability from a given amount of sample. The same type of oxygen analyzer, oxygen electrode, and teflon membrane were used as previously [2].

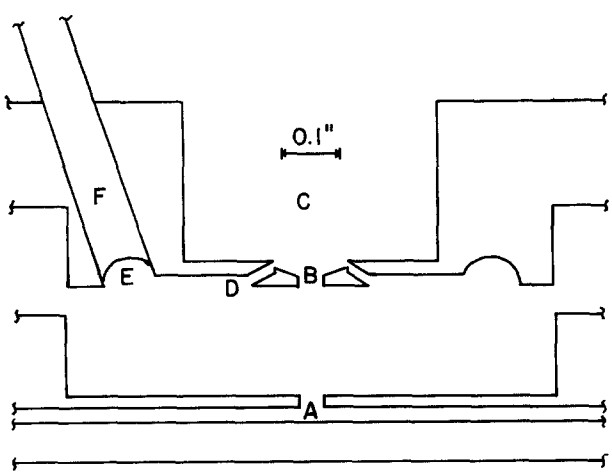

Fig. 1. Diagram of mixing and measuring blocks. This figure shows a cross-section through the middle of the blocks. Shown are the mixing point (A); the measuring chamber (B); the hole into which the electrode was fitted (C); the drain passage (D), of which there are six; the drainage ring (E); and the canula through which the solution was removed (F). The distance between A and B was varied by inserting delays of various lengths between the two blocks.

Preparation of hemoglobin and particles

Submitochondrial particles were prepared in the presence of Mg^{2+} and Mn^{2+} according to the method of Hansen and Smith [4] as described by Beyer [5]. Phosphorylation by these particles was also checked [5] and was found to be adequate. Hemoglobin was prepared by the method of the Adairs' [6], and was stripped of organic phosphates by passage through a Bio-Gel column.

Experimental procedure

In all experiments, an anaerobic solution of hemoglobin or suspension of submitochondrial particles was mixed with a medium not containing hemoglobin or submitochondrial particles but containing a certain amount of oxygen. In reporting the experimental results, all concentrations are given at the values obtained after mixing of the two solutions. If some component of the final mix, such as hemoglobin or oxygen, was present in only one of the syringes before mixing, then that component was present before mixing at double the concentration cited. All of the experiments on submitochondrial particles were carried out in a medium which was 0.15 M in sucrose, 0.05 M in KCl, 5 mM in potassium succinate and about 50 μM in oxygen. Concentrations of submitochondrial particles are expressed in mg protein.

Experiments with conventional mixing were made utilizing the same electrode and metering system, except that the electrode was set up in the standard manner, using the membrane supplied by the manufacturer. The steady-state rate of oxygen consumption by the particle suspension in a stirred vessel was observed and recorded on a strip chart recorder.

Computer analysis of results

The differential equations for the kinetics of hemoglobin oxygenation were solved by means of a digital computer using Hamming's modified predictor-corrector method for the solution of general initial-value problems. The data from the experiments with submitochondrial particles were fitted by means of a least-squares procedure to the equation previously used for this purpose [3].

RESULTS

Test of mixing

In order to verify that the four-jet mixing device was adequate to obtain time resolution of 1 ms, the efficiency of its mixing was tested in the following way.

1 M NaOH, carbonate free, was mixed with 1 M HCl. The neutralization of acid by base is extremely rapid and quite exothermic, so that the extent of the neutralization reaction is a good measure of the amount of mixing which has occurred. A small thermistor was placed in the flow tube at various distances from the mixer and the temperature was measured. The percentage of the full temperature change which had been achieved was proportional to the percentage of mixing; the full temperature change was about 7 °C.

The results obtained are shown in Fig. 2. Better than 90% mixing was obtained under all the conditions observed for aqueous solutions and for solutions which were 10% glycerol. More viscous solutions showed somewhat poorer mixing performance. The suspensions of submitochondrial particles in 0.25 M sucrose had a viscosity of

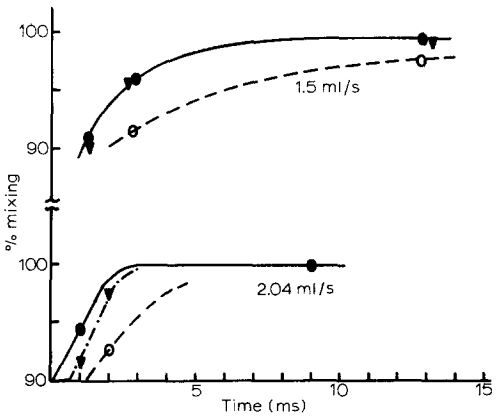

Fig. 2. Efficiency of mixing. This figure shows the efficiency of mixing versus time after mixing, at two different flow rates. Flow rates most commonly used in other experiments lay between the two flow rates on which these measurements were made. ●, the efficiency of mixing of aqueous solutions (viscosity 0.98 cP); ▲, efficiency of mixing of 10 % glycerol solutions (1.29 cP); ○, the efficiency of mixing of 20 % glycerol solutions (1.51 cP).

about 1.17 cP, so that the mixing should be quite adequate for measurements down to times of 1 ms.

Oxygenation of hemoglobin

In order to further verify the operation of this device, the kinetics of a known reaction were observed. The oxygenation of hemoglobin has been observed spectrophotometrically by Gibson [7] using a stopped-flow device. By utilization of special

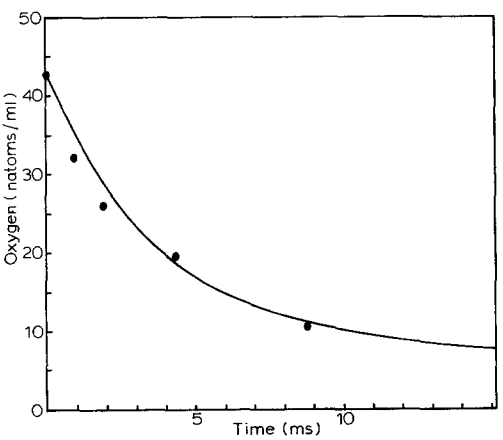

Fig. 3. Kinetics of oxygenation of hemoglobin. Concentration of free oxygen in solution is plotted versus time. The solid line represents the course of the reaction calculated from the constants previously reported for these conditions [7]. The measurements were at 20 °C on a hemoglobin solution which was 52 μM in heme. The solution was 50 mM in bis(2-hydroxyethyl) imino-tris(hydroxymethyl)methane buffer (Bis-Tris) at pH 7.0. The initial oxygen concentration was obtained by measuring the oxygen concentration in the oxygenated solution and dividing by two.

techniques, he was able to estimate the four rate constants involved. Our measurements were directly comparable with his, being made at the same pH, temperature, and at the same concentration of the same buffer. The disappearance of oxygen was monitored rather than the change in the heme spectrum. Fig. 3 shows the points obtained, as compared with a line generated from the four rate constants of Gibson. The agreement between the data reported here and the curve reported by Gibson [7] is well within experimental error. In fact, the agreement between the curve generated by solution of the differential equations and the experimental points reported by Gibson [7] is no better than the agreement between this curve and the points reported here. This demonstrates that very good agreement is obtained between the optical results and the electrode results even for very short times.

Results with submitochondrial particles

When respiration by submitochondrial particles was measured utilizing the device previously described [2], no burst of respiration was observed during the first few hundred ms, and the longer time rate observed was faster than that observed by conventional means. This seemed rather peculiar, since a well defined rapid burst of respiration was observed with intact mitochondria, and their rate of oxygen consumption at longer times agreed well with that observed by conventional means. The availability of a device with higher time resolution allowed a more critical evaluation of the kinetics of respiration by submitochondrial particles. Fig. 4 shows the presence of an initial, extremely rapid, phase of respiration. Since the half-time of the exponential part of the curve fitted to these points was only 22 ms, this component occurred too rapidly to be observed with the earlier device. In experiments done with the earlier device the shortest time points were usually in the range of 30–40 ms, since it was excessively wasteful of sample to run at the speed required to get times as short as 15 ms. This initial burst was followed by a phase in which oxygen was consumed at a

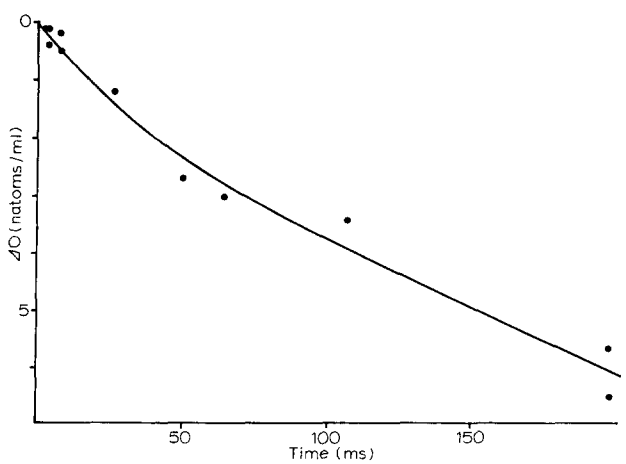

Fig. 4. Respiration by submitochondrial particles during the first 200 ms. The solid line represents the best fit by a curve containing both an exponential and a linear component [3]. The appearance of a short lived burst of respiration is apparent in this figure. The half-time for the exponential component of the curve was 22 ms. The concentration of protein was 2 mg/ml.

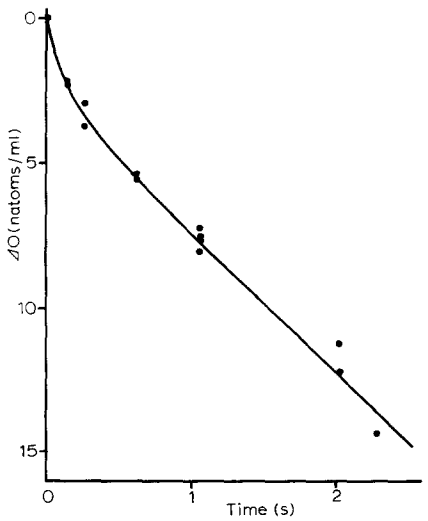

Fig. 5. Respiration by submitochondrial particles during the first 2.5 s. After the initial burst of respiration, a period ensued during which respiration was nearly linear with time. The protein concentration was 1 mg/ml. The temperature was 23.5 °C and the linear portion of the respiration showed a respiration rate of 292 natoms $0 \cdot mg^{-1} \cdot min^{-1}$.

TABLE I

COMPARISON OF RESPIRATION RATES OBSERVED BY CONTINUOUS FLOW AND CONVENTIONAL METHODS

All experiments were done in 0.15 M sucrose, 0.05 M KCl, 5 mM potassium succinate. Experiments with intact mitochondria were carried out as previously described [1]. Measurements on mitochondria treated with fluorescein mercuric acetate were made immediately after addition of this reagent. In the case of Triton X-100-treated mitochondria, the mitochondria (6 mg/ml) were aged 50 min at room temperature in the presence of 0.08 % Triton X-100; final concentrations of mitochondria and Triton X-100 were half as great. The experiment with submitochondrial particles alone was done at a protein concentration of 3 mg/ml, in the presence of 10 μM rotenone. The experiment with submitochondrial particles and carbonylcyanide metachlorophenylhydrazone (CCCP) was done at one-third the above concentrations of particles and rotenone, in order to observe accurately the accelerated oxygen consumption.

	Rate of oxygen consumption (natoms $\cdot mg^{-1} \cdot min^{-1}$)		
	Continuous flow	Conventional	Temperature (°C)
Mitochondria treated with fluorescein mercuric acetate	66	61	26
Frozen mitochondria	250	293	24
Mitochondria treated with Triton X-100	373	368	24
Submitochondrial particles	314	137	25
Submitochondrial particles, 0.33 μM CCCP	700	228	27

constant rate as shown in Fig. 5. Thus it appeared that the situation with regard to submitochondrial particles was similar to that which existed for mitochondria [2, 3] except that the burst of respiration was more rapid and of somewhat smaller magnitude in the case of submitochondrial particles. However, the discrepancy between the respiration rates observed by the continuous flow device and those seen by conventional methods showed that a second change in the rate of respiration occurred in submitochondrial particles at much longer times. This discrepancy appeared using the new apparatus also, even though the experiments were done in a different laboratory. As was previously discussed [2], after the initial burst of respiration the rate of respiration observed for intact mitochondria agreed very well with the rate observed by conventional means. The situation observed for submitochondrial particles was quite different. The rate of respiration observed in the time span between 10 ms and 2 or 3 s was still about 2.5 times greater than the rate observed by conventional methods. The results shown in Table I are examples chosen from among a large number of such experiments, all of which showed about the same magnitude of difference between the rates measured by the continuous flow method and by the conventional method.

DISCUSSION

It is clear from the successful application of this device that a continuous flow device with 1 ms time resolution can be constructed for use with any type of electrode which has a relatively small sensing area, regardless of the overall shape of the electrode. Devices previously described [8] required very thin electrodes which are of limited availability. In the case of a system such as that described here, the upper part of the block can be made to accomodate almost any shape of electrode.

The triphasic character of the respiration by submitochondrial particles was unexpected, but highly reproducible. Although it resulted from the comparison of two different kinds of data, the difference between the rates observed at times of 1–4 s, and those observed at longer times were seen on entirely separate sets of apparatus in two different laboratories, and thus must be considered reliable.

The value of this higher resolution design was demonstrated by the detection of the rapid phase of respiration of sub-mitochondrial particles, which had been missed by earlier designs. This apparatus should be useful for the measurement of rapid oxygen consumption in other complex systems for which optical methods are inadequate.

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