BBA 46082

A MICRO-METHOD FOR THE STUDY OF OXIDATIVE PHOSPHORYLATION

DAVID HOLTZMAN AND CYRIL L. MOORE

The Saul Korey Department of Neurology and the Department of Biochemistry, Albert Einstein College of Medicine of Yeshiva University, Bronx, N.Y. (U.S.A.)

(Received October 6th, 1970)

SUMMARY

1. A method is described for polarographic measurement of oxidative phosphorylation in mitochondria isolated from very small quantities of rat brain and liver. Measurements of O_2 consumption are made with an O_2 cathode and a standard polarization circuit using a reaction chamber with a volume of 40 μl.

2. The characteristics of this system are described. They are similar to the characteristics of polarographic systems requiring much larger quantities of mitochondria.

3. Oxidative phosphorylation was measured in mitochondria isolated from as little as 100 mg of tissue. Respiratory control ratios and ratios of phosphate acceptor to O_2 consumption (ADP/O) obtained are comparable to those measured in much larger volumes of mitochondria as reported in the literature.

INTRODUCTION

The study of oxidative phosphorylation and energy metabolism has advanced greatly in recent years. The use of polarography and the O_2 cathode allows the reproducible measurement of O_2 consumption and analysis of its dependence on the presence of ADP and various substrates in isolated mitochondria[1-7]. Improved methods of isolating mitochondria and studies on requirements for tight coupling of these preparations from brain[8-10] and muscle[11] have been described recently.

In our laboratory we have attempted to perfect a micro-system for studying oxidative phosphorylation in mitochondria isolated from very small amounts of tissue. The micro-system would allow us to extend our studies of oxidative phosphorylation to small quantities of mitochondria isolated from biopsy material or from tissue of laboratory animals.

In this paper we describe a system with which we have been able to study oxidative phosphorylation in mitochondria isolated from as little as 100 mg of brain tissue. This system includes the use of a reaction chamber with a volume of 40 μl. Respiratory control ratios[4] (defined as the ratio of the rates of substrate oxidation in the presence of ADP and in its absence) and the ADP/O values obtained with rat liver and brain mitochondria using this system are compared with results using conventional systems.

METHODS

The reaction chamber used in these experiments is diagrammed in Fig. 1. It was hand-lathed from lucite. The volume of the chamber with the stirring bar in place is 40 μl. The cap of the chamber is finished to a press fit. The reference electrode surface is flush with the bottom surface of the chamber cap; the O_2 electrode extends approx. 0.2–0.5 mm into the chamber.

The magnetic stirring bar, 2.5 mm in length and 1 mm in diameter, is constructed by heat-sealing a fine metal rod in the end of a Pasteur pipette. Mixing is considered adequate if the delay in response to an addition of ADP (state 4–3 transition) is less than 2 sec, and the rate must be constant throughout. Too rapid a rotation of the stirring bar produces an instability in the recording trace.

Additions are made by means of microsyringes introduced through the third hole set in the chamber top. The tip of the syringe is placed within the chamber but

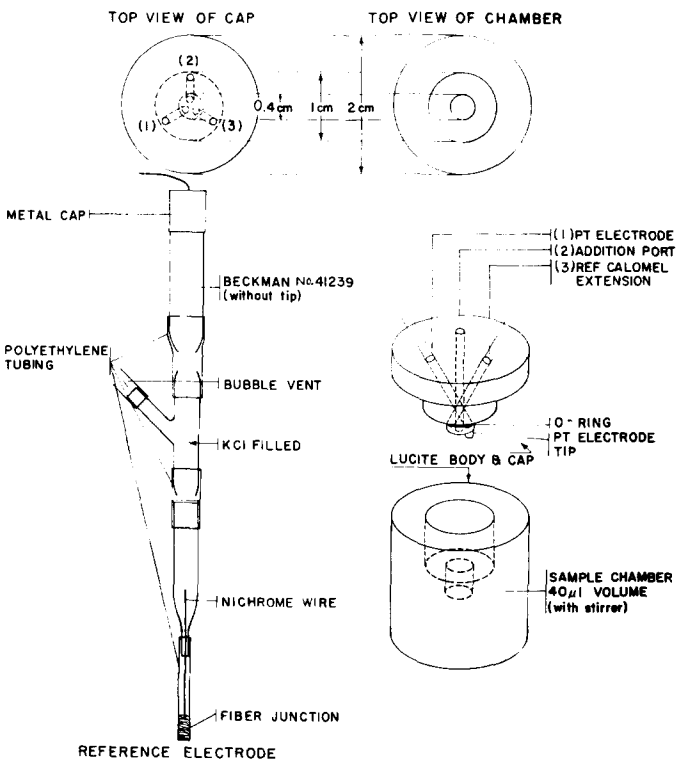

Fig. 1. Diagram of the Moore reaction chamber and reference electrode extension. The reference electrode extension includes a glass Y-joint for release of air bubbles and connections of polyethylene tubing. It is filled with 0.5 M KCl. The narrow polyethylene tubing, containing the fiber junction and nichrome wire in contact with the KCl, fits very tightly in its port (No. 3) in the chamber cap. These two pieces are kept together immersed in KCl solution between experiments. The end of the O_2 electrode, enlarged by heat-sealing the end of the platinum wire during construction, is carefully ground and coated with cellulose acetate so that the end can be inserted tightly in its port[1]. Stirring is accomplished by use of a stirring bar (*cf.* METHODS) and a Tri R magnetic stirring motor, which minimizes heat transfer to the chamber.

above the stirring rod, to avoid disturbing the smoothly rotating stirrer. Large transient changes in baseline sometimes occur when adding inhibitors dissolved in alcohol, probably because of the dissolved O_2 and the proximity of the syringe tip to the electrode surfaces. The addition port is just large enough to accommodate a size-18 hypodermic needle, through which the chamber is washed with alcohol and water. The chamber is emptied by suction aspiration through this port.

The O_2 cathode is constructed as described by HAGIHARA[5] and ESTABROOK[7]. The electrode consists of a thin platinum wire (28 gauge) heat-sealed in the end of a soft-glass tube (outer diameter 2 mm). The tip of the wire is exposed by careful grinding with fine sand paper. Before each experiment, the electrode tip is cleaned and coated with a thin layer of cellulose acetate dissolved in acetone to reduce changes in the reactivity of the electrode in the presence of mitochondrial solutions, as was shown by HAGIHARA[5]. If the coating is too thick, the reaction time of the electrode is prolonged excessively.

The polarizing voltage of 0.6 V is supplied by a 1.5-V flashlight battery through a conventional polarizing circuit[5-7]. The reference anode is a fiber junction extension of the Beckman calomel electrode No. 41239 (Fig. 1). A Varian recorder with pen writer is used. Full-scale pen deflection is 1 sec.

The response of this system to different concentrations of O_2 in solution was measured directly. The system was set up as for an experiment with the O_2 electrode pre-coated with cellulose acetate. The chamber was then filled with a solution of mitochondria in the reaction medium. Mixtures of O_2 and N_2, with O_2 concentrations

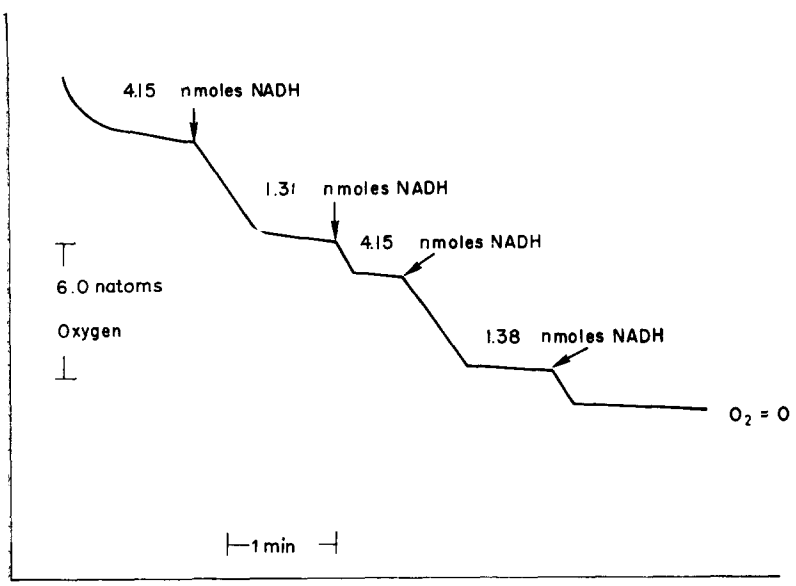

Fig. 2. Calibration of the O_2 electrode. NADH, in a spectrophotometrically determined concentration of 2.765 mM, is added in volumes of 0.5 and 1.5 μl to a solution of old, substrate-depleted rat liver mitochondria. The limiting quantity of NADH is stoichiometrically related to the O_2 consumed (see text and ESTABROOK[7]). A direct calibration is obtained by determining the change in current occurring upon complete oxidation of the added NADH. In this example, the concentration of O_2 in the 40-μl reaction chamber, before addition of the mitochondrial solution, is calculated to be 259 μmoles/l. All solutions are at room temperature (23°).

from 0 to 100%, were then bubbled vigorously into the chamber, and the reading taken when a steady value was reached. The response of the system was linearly related to the concentration of O_2 in each gas mixture.

The recorder scale is calibrated for O_2 concentration by measuring the response when a spectrophotometrically determined amount of NADH is introduced into the chamber containing substrate-depleted, repeatedly frozen and thawed liver mitochondria. The method, as described by ESTABROOK[7], calls for the use of beef heart submitochondrial particles. The amount of O_2 consumed is stoichiometrically related to the amount of NADH added.

Fig. 2 shows the results of a calibration with NADH. The response to a given amount of NADH (which is stable for several hours) is the same with the system

TABLE I

ADP/O AND RESPIRATORY CONTROL RATIOS FOR SUCCINATE AND THE NAD^+-LINKED SUBSTRATES (GLUTAMATE AND MALATE)

Rat brain mitochondria were from the regions shown. All experiments were conducted as shown in Fig. 3 and described in the text. Calculations were performed as described in the text. The quantity of mitochondrial protein used is given for those samples from which there was an adequate amount of mitochondria left over to measure the protein concentration. Each value represents a single determination.

Substrate	*Age of animal (days)*	*Region*	*Tissue weight (g)*	*Mitochondrial protein (μg)*	*ADP/O*	*Respiratory control ratio*
Glutamate and malate	40	Cerebral cortex	1.05	77	3.0	5.4
	60		1.15	9.6	2.8	6.3
	67		1.20	159	2.8	2.1
	40	Diencephalon	0.21	34	2.5	∞
	60		0.16	35	2.8	16
	40	Colliculi	0.18	70	2.7	∞
	60		0.18	23	3.0	∞
	67		0.23	165	3.0	2.0
	40	Cerebellum	0.29	36	2.2	∞
	60		0.33	16	2.1	22
	67		0.36	—	3.0	5.0
	40	Pons and medulla	0.26	94	2.5	5.6
	60		0.27	34	3.0	3.3
	67		0.31	—	3.0	1.5
Succinate	40	Cerebral cortex	1.05	77	2.0	∞
	60		1.15	9.6	1.3	2.6
	67		1.20	127	1.5	1.5
	40	Diencephalon	0.21	34	1.9	∞
	60		0.16	35	1.3	9.0
	67		0.20	102	1.5	3.8
	40	Colliculi	0.18	70	2.1	∞
	60		0.18	23	2.0	3.5
	67		0.23	165	1.8	1.6
	40	Cerebellum	0.29	36	1.7	15
	60		0.33	16	1.4	3.5
	67		0.36	—	1.4	1.8
	40	Pons and medulla	0.26	94	2.0	5.4
	60		0.27	34	1.5	2.6
	67		0.31	—	1.2	2.0

air-saturated and near the zero-end of the scale. However, responses with intact mitochondria are non-linear within the last 5 % of the scale (state 4–3 transitions are also imprecise within this part of the scale).

Calibration with NADH consistently gives full-scale values for O_2 concentration equivalent to 248–259 μmoles O_2 per l as compared to the accepted value of 255–265 μmoles/l (ref. 7) for air-saturated water at 23–25°.

TABLE II

ADP/O ratios and respiratory control ratios of mitochondria from rat liver obtained with NAD^+-linked substrate (glutamate and malate) and with succinate. All experiments were conducted as shown in Fig. 3 and described in the text. Calculations were performed as described in the text. ADP/O is in nmoles ADP added per natom O_2 consumed. Each value represents a single determination. ( ) * indicates second addition of ADP.

Substrate	*Age (days)*	*ADP/O*	*Respiratory control ratio*
Glutamate and malate	12	3.0	40
	21	3.0	9.5 (2.3) *
	25	2.8	3.1
	26	3.0	3.3
Succinate	12	1.5	21
	21	1.6 (1.7) *	5.2 (3.4) *
	25	1.3	2
	26	1.5	3.3

Mitochondria are added in volumes of 1–10 μl, depending upon the concentration. Reactants are added in volumes of 0.1 μl to a maximum of 2 μl. Final concentrations of mitochondrial protein and reactants are given in Tables I and II and in Fig. 3.

For calibration and experimental runs, the recorder is set at 100 % on the O_2 scale before adding mitochondria. This is necessary, since the added volumes of mitochondria represent 2.5–25 % of the chamber volume, and displace from the chamber a significant amount of the air-saturated reaction medium. The extent of the change in current, when the mitochondrial solution is added, is proportional to the volume and decreased O_2 content of the mitochondrial solutions.

ADP/O ratios are calculated by the method of CHANCE AND WILLIAMS[2]. Concentrations of ADP are determined as described by ADAM[12]. Change in the phosphate concentration was not measured directly, but was assumed to be equivalent to the amount of added phosphate acceptor[2,5,7]. Respiratory control ratios are calculated as defined by CHANCE[4].

Sprague–Dawley albino rats of both sexes are used for all experiments. The brains are immersed in cold isolation medium while freed of blood and pia and dissected into regions, as described by HOLTZMAN AND MOORE[13]. Brain mitochondria are isolated essentially as described by MOORE AND JOBSIS[10]. The volumes are scaled downwards for small samples of brain (500 mg wet weight). For every 100 mg of brain, 10 μg bovine serum albumin, 2 μg bacillus proteinase, and 10 μg $KHCO_3$ are added. The tissue is homogenized in a 2-ml glass homogenizer fitted with a teflon

pestle and allowed to stand for 2 min at 0° before centrifugation. The centrifugation procedure is the same as described before[10] with the exception that the Sorvall swinging bucket rotor is used for developing the modified ficoll gradient of 8, 10 and 13 % ficoll in 225 mM mannitol, 75 mM sucrose and 5 mM Tris–HCl (pH 7.4). Mitochondria sedimenting at the bottom of the gradient are collected and washed in 10 vol. of 225 mM mannitol, 75 mM sucrose and 5 mM Tris–HCl (pH 7.4). Mitochondria are isolated from liver by a modification of the method of SCHNEIDER AND HOGEBOOM[14]. Mitochondrial protein concentrations are determined on separate aliquots by the method of LOWRY *et al.*[15].

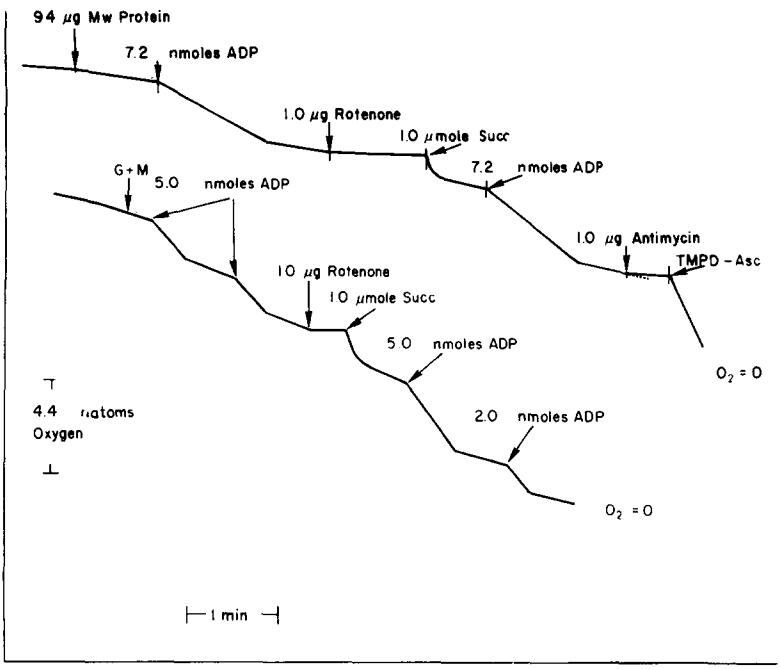

Fig. 3. Polarographic assay of oxidative phosphorylation of rat brain and liver mitochondria. Upper trace: mitochondria prepared from the pons and medulla of a 40-day-old rat. Tissue wet weight is 260 mg. The mitochondrial sample, containing 94 µg of protein, is added to the reaction medium containing bovine serum albumin and 0.5 µmole each of glutamate and malate. Additional reactants are added as indicated. With glutamate and malate as substrates, ADP/O = 2.5 and respiratory control ratio = 5.6. For succinate, ADP/O = 2.0 and respiratory control ratio = 5.4. Lower trace: mitochondria, isolated from the liver of a 21-day-old rat, in the reaction medium containing bovine serum albumin. With 0.5 µmole each of glutamate (G) and malate (M) as substrate, ADP/O = 3.0 and respiratory control ratio = 9.5 and 2.3. With succinate, ADP/O = 1.6 and 1.7 with respiratory control ratio = 5.2 and 3.4, respectively.

The reaction medium for brain mitochondria consists of: 225 mM mannitol, 75 mM sucrose, 10 mM Tris–phosphate (pH 7.4), 5 mM Tris–HCl (pH 7.4), and 0.2 mM EDTA (pH 7.4). The reaction medium for liver mitochondria consists of: 250 mM sucrose, 5 mM Tris–HCl (pH 7.4), 10 mM Tris–phosphate (pH 7.4), 5 mM KCl. The final pH of each solution is 7.4. Fresh bovine serum albumin is added to each medium in the reaction chamber to a final concentration of 1 mg/ml.

Mannitol, ADP, sucrose, EDTA, Trizma base, Antimycin A, and substrates were obtained from Sigma Chemical. Rotenone obtained from Sigma was recrystallized

3 times from alcoholic solutions. Bovine serum albumin obtained from Sigma was defatted before use. Tetramethyl-*p*-phenylenediamine was obtained from Eastman Kodak. All other chemicals were laboratory reagent grade.

RESULTS

Fig. 3 shows the results of two experiments carried out in the reaction chamber described above. The upper trace is from an experiment run with mitochondria isolated from the pons and medulla of a single 41-day-old rat (0.26 g of tissue). The sample of mitochondria contained 94 μg of protein. The lower trace is from a similar experiment run with a sample of mitochondria isolated from the liver of a 21-day-old animal.

The results of studies of oxidative phosphorylation on mitochondria isolated from regions of individual rat brains are given in Table I. As little as 9.6 μg of mitochondrial protein were used in some of these assays.

Respiratory control ratios in Table I cover a wide range, up to infinity (*i.e.* the slope of state 4 respiration = 0). Values for respiratory control in brain mitochondria are comparable to those reported by OZAWA *et al.*[8], and MOORE AND JOBSIS[10] for mitochondria from the cerebral cortex. No attempt was made to determine optimal concentrations of EDTA or to use Mg^{2+} as the latter authors did. Respiratory control ratios tended to be higher with glutamate and malate than with succinate as substrate, as was reported by OZAWA *et al.*[8] for tightly coupled brain mitochondria and LARDY[16] for mitochondria from other tissues.

The ADP/O ratios given in Table I are comparable to those reported for polarographic measurements of much larger samples of mitochondria from the cerebral cortex[8,10]. The ADP/O ratios were reproducible and independent of the large variations in the respiratory control ratios in any given experiment. It is to be noted that with this method, differences in the respiratory control of the brain are clearly and reproducibly determined.

The results of studies of oxidative phosphorylation in mitochondria isolated from the livers of animals of different ages are given in Table II. As with brain mitochondria, a wide range of respiratory control ratios was obtained. The higher values are comparable to those reported as "optimal" with systems using far larger samples of liver mitochondria[4]. The ADP/O ratios for NAD^+-linked substrate are comparable to those reported in the literature[2,16,17]. The ADP/O values measured with succinate as the substrate are lower than those generally reported. The wide range of respiratory control ratios and the low ADP/O ratios with succinate may be due to the fact that the livers were in most cases removed several minutes after the removal of brains from the decapitated animals.

The independence of respiratory control ratios and ADP/O values in mitochondria from different tissues has been noted by others[4,18]. The large variation in respiratory control ratios, which we observed, is probably due to several factors. First, the respiratory control ratio is thought to reflect the intactness of the mitochondrial structure which will vary with the inevitable variation in the conditions and procedures involved in the isolation of mitochondria[4,8,18]. Secondly, the apparent state 4 respiration and, therefore, the respiratory control ratio are affected by the adequacy of the coating of the platinum electrode, as has been observed also by

HAGIHARA[5]. The presence of fresh bovine serum albumin in the reaction medium does improve the consistency of attaining high control ratios by removing free fatty acid and by increasing the protein concentration of the system.

The reproducibility of data, and their adequate comparison to those observed with 30–60 times larger systems lends confidence to the method. Use of this method allows us to study the functional behavior of mitochondria from small, chemically diverse regions of tissues such as the central nervous system. In addition, biopsy specimens of brain, muscle, or perhaps even mitochondria-rich liver, would provide a sufficient quantity of tissue to study the metabolic properties of isolated mitochondria or of tissue homogenates in human disease.

ACKNOWLEDGMENTS

We thank Misses Cecilia Kovac and Celia Prario for their expert and reliable technical assistance.

This research was supported by the National Institutes of Health Grant NS 08777 and NS 03356. D.H. is a National Institutes of Neurologic Diseases and Stroke Special Fellow (Grant No. 1 FII NS 02262). C.L.M. is a Health Research Council Career Scientist of the City of New York.

REFERENCES

1 P. W. DAVIES AND F. BRINK, JR., *Rev. Sci. Instr.*, 13 (1942) 524.
2 B. CHANCE AND G. R. WILLIAMS, *Nature*, 175 (1955) 1120.
3 L. C. CLARK, JR., *Trans. Am. Soc. Artificial Internal Organs*, 2 (1956) 41.
4 B. CHANCE, *Ciba Found. Symp. on the Regulation of Cell Metabolism*, 1958, Little, Brown, Boston, 1958, p. 91.
5 B. HAGIHARA, *Biochim. Biophys. Acta*, 46 (1961) 134.
6 P. W. DAVIES, in W. L. NASTUK, *Physical Techniques in Biological Research*, Vol. 4, Academic Press, New York, 1962, p. 137.
7 R. W. ESTABROOK, in R. W. ESTABROOK AND M. E. PULLMAN, *Methods in Enzymology*, Vol. 10, Academic Press, New York, 1967, p. 41.
8 K. OZAWA, K. SETA, H.TAKEDA, K. ANDO, H. HANDA AND C. ARAKI, *J. Biochem.*, 59 (1966) 501.
9 K. OZAWA, K. SETA and H. HANDA, *J. Biochem.*, 60 (1966) 268.
10 C. L. MOORE AND F. F. JOBSIS, *Arch. Biochem. Biophys.*, 138 (1970) 295.
11 C. L. MOORE AND P. M. STRASBERG, submitted for publication.
12 H. ADAM, in H. U. BERGMEYER, *Methods in Enzymatic Analysis*, Academic Press, New York, 1965, p. 573.
13 D. HOLTZMAN AND C. L. MOORE, in preparation.
14 W. C. SCHNEIDER AND G. H. HOGEBOOM, *J. Biol. Chem.*, 183 (1950) 123.
15 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
16 H. A. LARDY, *Proc. 3rd Intern. Congr. Biochem., Brussels, 1955*, Academic Press, New York, 1956, p. 287.
17 H. A. LARDY AND H. WELLMAN, *J. Biol. Chem.*, 195 (1952) 215.
18 A. L. LEHNINGER, *The Mitochondrion*, Benjamin, New York, 1964, pp. 132–136.