The design and construction of an oxygen micro-electrode for monitoring 20–100 μ l volumes

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Full instructions are given for the construction of a micro-respirometer. It is based on a membrane-covered platinum electrode of 10 μ m diameter that consumes little oxygen. The reaction chamber, made entirely of glass, can be varied in volume between 20 and 100 μ l by adjusting the sealer assembly. Although specifically designed to study the properties of small samples of mitochondria from tiny organisms, it can be used equally well either for oxygen dissolved in aqueous solutions or dry gas mixtures containing oxygen. Measurements can be made with about one-hundredth the amount of material required for commercially available macro-respirometers, and when connected via an impedance-matching device to a pen recorder it gives a stable output of 100–200 mV in air.

The respiratory properties of mitochondria are universally studied by using a respirometer with a membrane-covered platinum electrode as first described by Clark (1956). Commercially available respirometers of this type have a relatively large volume and require several millilitres of a mitochondrial suspension for their successful operation. This is rarely a limitation when studying mammalian tissues, but it renders impossible measurements on the respiration of the small numbers of mitochondria that can be isolated from tiny organisms. In our laboratory we wished to study the properties of mitochondria isolated from the muscle and reproductive organs of the sheep parasitic nematode *Haemonchus contortus*. Since each nematode weighs only about a milligram, the quantity of mitochondria that can be prepared at any one time under physiological conditions is very limited.

The size of respirometers using the normal Clark electrode is dictated by the oxygen consumption of the electrode itself, for only when this is a negligible proportion of the tissue oxygen consumption are experimental results accurate. The electrode described in the present paper employs Woolaston wire in its construction so that the surface area of platinum on the exposed end of the electrode is small and its oxygen consumption is correspondingly low. Silver (1963) used this method successfully in the construction of a flow cell for

measuring oxygen in the blood vessels of small animals. Atkinson & Smith (1973) described a respirometer with a fixed volume of 0.25 ml designed to measure the uptake of oxygen by a single nematode over a long period, but it would be quite unsuitable for mitochondrial studies. The low permeability of the polypropylene membrane would make it slow to respond to changes in oxygen partial pressure and there is no facility for the rapid addition and mixing of substrates and inhibitors. Two other respirometers have been described with volumes of 0.3–0.5 ml (Tucker, 1967; Hughes *et al.*, 1982). Both were designed for measuring oxygen in blood, and consist of small chambers constructed around commercially available oxygen macro-electrodes. Neither would be suitable for studying the properties of small mitochondrial samples. The respirometer described in the present paper not only has a rapid response but measures successfully the oxygen consumption of as little as 20 μ l of a mitochondrial suspension. It would be a useful tool in most biochemical laboratories, and has wider biological applications since it operates equally well whether the electrode chamber is dry or contains an aqueous medium.

Materials and equipment

There are four main sections to the respirometer (Fig. 1), the reaction chamber (A), the Pt-electrode assembly (B), the stirrer and sealer assembly (C)

Abbreviation used: PTFE, polytetrafluoroethylene.

Fig. 1. *Vertical section through the respirometer*

Key: A, reaction chamber; B, Pt-electrode assembly; C, stirrer and sealer assembly; D-H, water jacket and lower part of Ag/AgCl-electrode assembly; J, tube for supporting Pt-electrode assembly; K, upper part of Ag/AgCl-electrode assembly. Two of the Perspex items illustrated at K are made up and pushed into the inclined tubes screwed into E. Into one of them is inserted the Ag/AgCl electrode, and a small flexible tube that is pushed to the bottom of the KCl reservoir as shown. The other end of this is attached to a filter-pump to facilitate draining off the saturated KCl solution when necessary. The rippled shading shows the location of the heating water and the saturated KCl solution between the Pt and Ag electrodes.

and the water jacket and Ag/AgCl-electrode assembly (D-H).

Reaction chamber

This is made by joining a 3.5cm-long piece of thick-walled borosilicate glass tubing of 10mm outer diam. and 3mm inner diam. to a 3cm long piece of tubing of 8mm outer diam. and 6mm inner diam. Just before the junction the thick-walled tubing is blown out until its outer diameter is about 12mm. The thick-walled end of the composite tube is ground flat and an annular groove ground into it to take a BS/USA size 009 'O' ring. This is accomplished by rotating in a drilling machine a brass tube preformed to the correct profile, pressing it against the end of the glass tube and lubricating with oil and silicon carbide powder (The Carborundum Co., Manchester, U.K.). The ground surface of the annular groove is smoothed by heating in a flame, and the end surface of the tube is re-ground. Careful annealing is essential after each heating stage. Perspex [poly(methyl methacrylate)] cannot be used to construct a reaction chamber of this size because it absorbs and releases significant amounts of oxygen (Silver, 1963).

Pt-electrode assembly

First a piece of blue-lead-glass tubing is obtained about 6cm long of 3mm outer diam. and 1mm inner diam. Lead glass is essential for fusing to platinum without incurring expansion cracks. A length of 10 μ m Pt wire with a silver covering to a total diameter of 125 μ m (Woolaston wire; Johnson, Matthey and Co., Royston, Herts., U.K.) is twisted on to a piece of solid Pt wire also of 125 μ m thickness to make good electrical contact, then threaded along the lead-glass tube so that the twisted junction is near the middle. The glass tube is laid on the bench, the solid Pt wire is clamped and the Woolaston wire trailed over the edge of the bench and tensioned with a small weight of about 100mg. Conc. HNO₃/water (1:1, v/v) is introduced into the glass tube until all the silver of the Woolaston wire within the tube has dissolved, then the acid is washed away with distilled water. The tubing and solid Pt wire are raised and clamped vertically, with care being taken not to swing the weighted end of the Woolaston wire sufficiently to break the 10 μ m portion within the tube. The lead-glass tube is then heated to fuse it on to the prepared Woolaston wire and part of the 125 μ m Pt wire to clamp the junction. The excess Woolaston wire is then cut off at the end of the lead glass.

The outer part of the electrode assembly is made from a piece of borosilicate glass tubing with an inner diameter of dimensions to make a seal on the outer edge of a BS/USA size 004 'O' ring. The end

is thickened and fashioned to the dimensions shown in Fig. 1. Before the lead glass containing the Pt wire is mounted inside this tube, a length of copper braiding about 15cm long, taken from a coaxial cable, is soldered to the free end of the Pt wire. Two BS/USA size 004 'O' rings were fitted inside the borosilicate-glass outer tubing to the positions shown in Fig. 1, and the blue-lead-glass tube pushed through them until 2mm of it stands proud. Araldite epoxy resin adhesive (Ciba-Geigy, Duxford, Cambs., U.K.) is introduced and the tube warmed until the adhesive flows down to the upper 'O' ring. Sufficient is introduced to form a domed surface at the top of the tube. Araldite is also introduced into the other end of the tube to lock the copper braiding in place. When the adhesive has set the top surface and the end of the lead glass containing the Pt wire is ground down to a slightly convex surface with the use of 320 carborundum silicon carbide powder. It will be found that the glass grinds down more rapidly than the Araldite, and a needle file is required to adjust the latter to the correct shape.

Stirrer and sealer assembly

The sealer is made from a $\frac{1}{4}$ in (6.4mm) rod of PTFE about 9cm long. The rod is held in a lathe chuck and drilled down the centre to a depth of 4.3cm with a 2mm drill. It is then drilled a further 2.7cm with a 0.7mm drill mounted on the end of a 1.5mm-diam. brass rod. This extended drill is left in the PTFE rod as a stiffener to facilitate further turning operations. The rod is reversed in the lathe chuck and the other end supported in a revolving centre in the tail-stock. The first 7cm nearest the headstock is then turned down to 2BA thread clearance size, and the distal 3.3cm of this section is further turned to be a push fit in the reaction chamber (approx. 3mm diam.). The larger section is then threaded 2BA. The rod is removed from the lathe and the end cut off flush with the supporting extended drill, which is then removed, leaving a central hole down which the stirrer can be introduced. Finally, a longitudinal groove is cut down the outside of the entire length of the rod, just sufficiently deep to allow the insertion of an 11.5cm Hamilton-syringe needle into the reaction chamber. To enable the height of the sealer assembly to be adjusted, a 2.5cm-diam. disc cut from $\frac{1}{4}$ in (3.2mm) polythene sheet is drilled through the centre, tapped 2BA and screwed on to the PTFE body. The stirrer is made from a 13cm length of 23S.W.G. (0.61mm) Pt wire hammered out at the end almost to the diameter of the reaction vessel and twisted so that, when turning, it forces the liquid in the vessel downwards. It is driven by a laboratory stirrer fitted with a pin chuck.

Water jacket and Ag/AgCl-electrode assembly

The top plate D (Fig. 1) is made from $\frac{1}{2}$ in (12.7 mm)-thick Perspex sheet turned to 3 in (76 mm) diameter. Parts E and F are made from 3 in (76 mm) Perspex rod and G from 2 in (51 mm) Perspex rod drilled and turned to the correct shape (Fig. 1). Part H is a length of 3 in (76 mm) Perspex tube of $\frac{1}{8}$ in (3.2 mm) wall thickness. The KCl reservoirs are also made from Perspex rod, each arm being about 12 cm long to avoid congestion around the reaction vessel. The Ag/AgCl electrode is made from a length of 19S.W.G. (1 mm) Ag wire. To coat the wire with AgCl it is dipped into 1 M-HCl and connected in series with a 1.5 k Ω resistor to the positive terminal of a 1.5 V battery. With a Pt wire as cathode, a current is passed overnight. The operation is carried out in the dark.

Setting up the apparatus

All 'O' rings are greased with Apiezon L grease. Parts G and H are pushed on to base-plate F, then part E is pushed on to parts G and H. The assembly is kept in place by a brass plate located under part F with two vertical bars screwed into part E as shown in Fig. 1. Only one of the vertical bars is shown, although in the apparatus the two are diametrically opposite one another. They are fitted in a position that avoids fouling the tubes projecting from the main body of the apparatus, and this cannot be shown in a plane section. The KCl-reservoir parts are screwed into place and the apparatus is mounted on a Palmer stand to facilitate raising and lowering.

The PTFE membrane is stretched and held tight over the Pt electrode by two BS/USA size 012 'O' rings (Fig. 1). Fitting the membrane on to the electrode is facilitated by the construction of a simple tool. This is made from about 8 cm of PTFE rod turned down to 12.5 mm, and one end is hollowed out to a depth of 4.5 mm to form a tubular section that can be slipped over the end of the electrode. The rod is tapered towards the other end to facilitate the introduction of the 'O' rings. A 4 cm $\times$ 4 cm square of PTFE membrane, cut from a 12 μ m-thick sheet, is placed on the tubular end of the fitting tool and the electrode is pressed into it. The electrode is raised and saturated KCl solution introduced into the hollow thus formed in the membrane. The electrode is lowered again and the two 'O' rings are pushed up the tool from the other end, slipped off it and on to the membrane. The membrane is stretched tight by pulling at the edges, and the electrode stem is now pushed through the 'O' rings in the centre of block F and pulled down as far as it will go. The reaction vessel is pushed upwards through the lower face of the top plate D as far as it will go and the plate is screwed

into position on block E. The electrode is now pushed upwards until it butts on to the 'O' ring mounted at the lower end of the reaction vessel, it being ensured at the same time that the blue-glass part of the electrode is in the centre of the reaction vessel. The electrode is held in place by Perspex tube J attached to base-plate F with coil springs. The reservoirs are filled with saturated aqueous KCl solution, and the Ag/AgCl electrode is positioned in one of them. The stirrer and sealer assembly are positioned in the reaction vessel and adjusted until the vessel has the required volume. It is essential to keep the saturated KCl solution in the electrode chamber electrically insulated from the heating water. For this reason double 'O'-ring seals and occluded air spaces are used so that, when part G is married to both parts E and F, compressed-air chambers separate the two liquids. A potential is applied to the electrodes by using a 1.5 V cell and a variable resistor as shown in Fig. 2. The current flowing is monitored as the potential difference across a 1 M Ω resistor in the circuit. In order to monitor this voltage with the use of a pen recorder it is necessary to connect an impedance-matching device between the 1 M Ω resistor and the recorder. The stabilized 5 V supply was initially powered by two 9 V batteries, but later by a simple mains unit.

Application

The current flowing between the electrodes was directly proportional to the oxygen partial pressure in the respirometer chamber. This was checked by blowing nitrogen on the membrane followed by gas mixtures containing 10% oxygen, 50% oxygen, air and finally oxygen. A plot of oxygen partial pressure against current gave a straight line with a regression coefficient of 0.99954. The current consumption was 0.55 nA/mmHg oxygen partial pressure. Fig. 3 shows how the current consumption of the cell varies with the voltage applied to the electrodes when in equilibrium with air-saturated water at 37°C. It was found that the speed of response to changes in oxygen partial pressure was greater the higher the voltage applied to the electrodes, so the respirometer was normally run at the end of the plateau in Fig. 3, i.e. with 1 V applied. At this voltage and in equilibrium with dry air blowing on the membrane, when the air was changed to nitrogen the time for 95% electrical response to the change was 11 s and for complete equilibrium with nitrogen was 20 s. Similar values were obtained for the speed of electrical response when the cell contained aqueous solutions. This was measured by equilibrating it with stirred air-saturated water at 37°C and adding a few micro-litres of Na₂S₂O₄ solution. Fig. 4 shows pen-

Fig. 2. Voltage supply to electrodes and output circuit

A suitable voltage is applied to the electrodes and the current drawn produces a potential difference across the 1 MΩ resistor. This is applied via an impedance-matching device to the input terminals of a pen recorder. A 5 V regulated supply was first powered by two 9V batteries, but these were later replaced by a simple mains unit.

Fig. 3. Relation between voltage applied to electrodes and the current drawn when the respirometer is equilibrated with air-saturated water at 37°C
For details see the text.

recorder tracings of the respirometer in use and compares it with tracings from a normal-sized commercially available respirometer (Rank Brothers, Cambridge, U.K.). Both tracings show the consumption of oxygen during the oxidation of successive additions of NADH in the presence of phenazine methosulphate (Sigma Chemical Co., Poole, Dorset, U.K.) and catalase in accordance with the method of Robinson & Cooper (1970).

The volume of the micro-respirometer reaction chamber was 60 μl, and 1 μl additions of 40 mM-NADH were made, but the Rank respirometer contained 4.5 ml, and 10 μl additions of 40 mM-NADH were made. When the respiratory properties of mitochondria from rat liver were studied the micro-respirometer gave pen-recorder tracings of the same standard as those from the Rank respirometer, but with one-hundredth of the amount of material.

Discussion

In the design of the micro-respirometer the Ag and Pt electrodes are separated by a considerable distance and they require a large reservoir of saturated KCl solution to interconnect them. This arrangement has a number of advantages. First, it confers electrical stability on the apparatus. The KCl solution is earthed through a 1 kΩ resistor and, together with the water jacket, which is fed from an earthed water bath, it provides a screened cover around the respirometer cell. Secondly, the large reservoir of KCl solution eliminates the possibility of the electrode membrane drying out, and membranes remain in perfect working order for many months. They do, however, take a little while to stabilize after being fitted, and for about the first 24 h the current drawn by the apparatus rises continuously before attaining a steady value. This

Fig. 4. Calibration of micro-respirometer and Rank electrode for comparison

The consumption of oxygen during the oxidation of successive additions of $1\ \mu\text{l}$ of 40 mM-NADH in the described micro-respirometer (a) and $10\ \mu\text{l}$ of 40 mM-NADH in a Rank respirometer (b) was measured in accordance with the method of Robinson & Cooper (1970). The volume of the micro-respirometer was $60\ \mu\text{l}$, and the Rank respirometer contained 4.5 ml. Both were operated at 37°C .

is most probably due to the gradual wetting of the membrane by the KCl solution.

Another design feature helps to make the electrode very sensitive to changes in oxygen partial pressure. The ground glass and ground Araldite on the top face of the Pt electrode allow rapid diffusion of KCl solution under the PTFE membrane and eliminate the necessity of fitting a lens-tissue layer between the membrane and the

electrode. Above the membrane, however, the lightly greased 'O' ring pressing on it and also on the fire-polished bottom of the reaction chamber provides a good barrier against the leakage of KCl solution between the membrane and the reaction chamber.

As well as being used extensively in our laboratory for mitochondrial studies, the respirometer has been used dry to measure the oxygen consumption of single specimens of the small flour beetle *Tribolium confusum*. A beetle can be persuaded to remain still in the reaction chamber if it is introduced resting on a small piece of tissue paper. For measurements under dry conditions like this, the stirrer is removed and the PTFE sealer assembly is replaced by a similar one without the longitudinal groove used to pass the Hamilton-syringe needle into the reaction chamber. It is still necessary to retain the hole down the centre of the shaft, otherwise the air in the chamber becomes compressed as the shaft is pushed home. When the chamber has been adjusted to the required volume, it is sealed with a lightly greased stiff wire the same diameter as the stirrer shaft. The outer surface of the PTFE sealer assembly is also greased because air diffuses rapidly into the chamber if the seal is not completely air-tight. Grease is not necessary when the reaction chamber contains water, since measurements show that air does not diffuse at an appreciable rate through the water trapped between the PTFE sealer, the Pt stirrer and the walls of the reaction chamber. However, it is important to ensure that no air bubbles are trapped in these spaces, and for this reason the entire reaction-chamber vessel should be filled with the aqueous medium and the stirrer and sealer assembly should be clean and dry before it is lowered into position. The aqueous medium then flows up inside the assembly around the Pt stirrer, forming an effective air barrier.

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