Chapter II.8 A Respirometer for Monitoring Homogenate and Mitochondrial Respiration

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1 Introduction

Over the past 20 years, the polarographic oxygen sensor (POS) has become an increasingly important instrument for studying respiration. Investigations in this field often rely upon information about the oxygen requirements of tissues, cell homogenates and cell organelle suspensions (e.g., mitochondria). Not until the introduction of the POS was it possible to obtain detailed information about oxidative cell metabolism.

Widely used alternatives to the POS are the manometric measurement of the oxygen absorbed [6, 16] and the volumetric method [7]. Although both methods are extremely well suited for registering the oxygen consumption of tissues, homogenates and cell organelle suspensions, the addition of substances during the investigation is relatively difficult. Furthermore, these methods generally do not provide a good resolution of small respiratory fluctuations.

The POS is becoming increasingly attractive for oxidative cell respiration studies. Although mostly employed by physiologists and biochemists [5, 11, 12], experience shows that the POS is ideal for investigating the pharmacological and toxic actions on oxidative cell metabolism of pharmaceutically active entities and should be included in the basic equipment of every toxicological and pharmacological laboratory. Unfortunately, its advantages are at present not widely enough appreciated, although pharmaceutical research offers sufficient problems with respect to increasing therapeutic safety.

2 Principle of the Measuring Apparatus

In principle a typical measuring system consists of a reaction vessel in which the POS is inserted. The vessel can be sealed, and a fine opening on the upper end allows the

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Polarographic Oxygen Sensors (ed. by Gnaiger/Forstner)

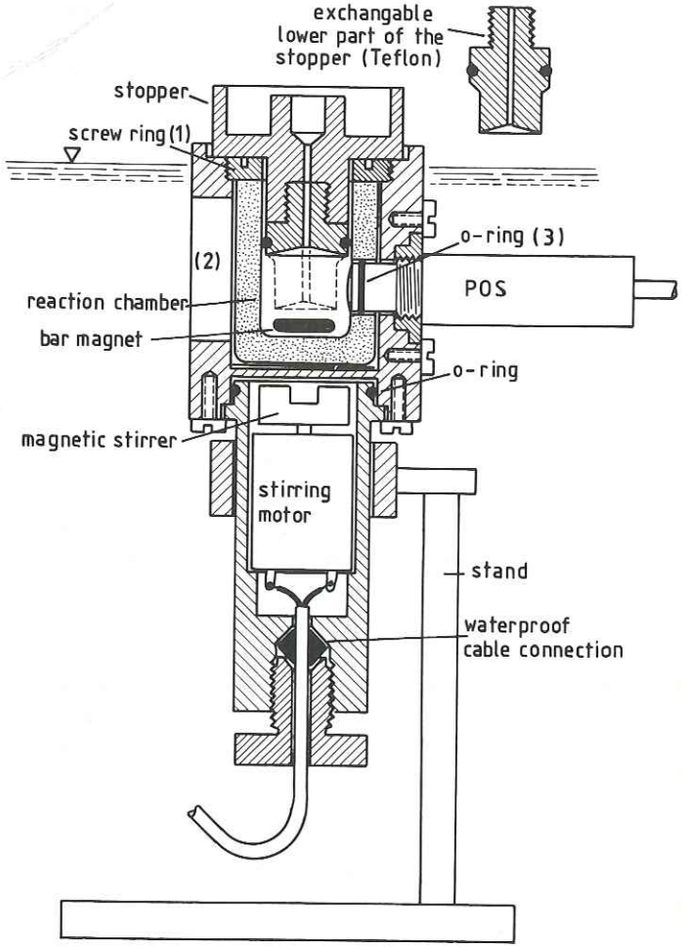

Fig. 1. Example of an oxygen-measuring cell constructed of glass with built-in magnetic stirrer. The glass chamber is built into a block of Plexiglas and fixed with the screw-ring (*1*). On three sides there are large openings (*2*), enabling the measuring cell to be surrounded with water circulated from a thermostatically controlled water bath. The POS is held horizontally in the Plexiglas block and is sealed with an O-ring where it enters the glass chamber (*3*). A further O-ring seals the Teflon stopper, the lower surface of which is slightly hollowed out in the form of a cone. This construction ensures the total expulsion of trapped air through the capillary during closure and offers a simple means of closing the chamber without the retention of air bubbles. The magnetic stirrer is mounted onto the Plexiglas block and sealed against the entry of water. The entire system is fixed in a thermostatically controlled water bath. Changing the size of the lower part of the stopper permits a variation of the reaction chamber volume

introduction of homogenate, cell suspensions, substrates, cofactors or pharmaceuticals. The medium under investigation is stirred using a magnetic bar driven by an externally applied rotating magnet. To ensure constant temperature, the reaction chamber can be fitted with a double wall with thermostated water circulating between the inner and

outer walls. Alternatively, the entire vessel, including a waterproof motor, is suspended in a thermostated water bath (Fig. 1).

Commercially available reaction chambers are seldom ideally suited for a particular experiment. Self-construction of the reaction vessel is therefore often necessary [13, 14] according to the following design criteria:

1. The volume of the reaction chamber has to be adapted for the specific requirements (usually about 1–10 cm^3).
2. The POS forms an integral part of the reaction chamber and thus need not be removed for cleaning purposes or during a change of the experimental medium. The position of the sensor should prevent attachment of air bubbles. These precautions protect the membrane from damage and minimize the time required for cleaning the chamber and changing the buffer.
3. The size of the electrode should be appropriate for the chamber volume. The high oxygen consumption of large electrodes (Chap. I.1) comprises a substantial source of error in small reaction chambers.
4. During sealing of the chamber the total exclusion of air bubbles must be ensured.
5. The medium must be well stirred to prevent the formation of a zone of reduced oxygen content directly on the membrane.
6. The entire reaction chamber, including the sensor tip, must be kept at constant temperature.
7. It must be possible to introduce substrate or test substances into the reaction chamber at any time during the measurement, without the infusion of foreign oxygen.
8. The material used for constructing the reaction chamber must possess good heat-exchange properties to permit measurements at defined temperatures.
9. Provision must be made for the insertion of a thermistor into the chamber during the experiment. It is sometimes advantageous to equip the chamber with a built-in thermistor.
10. Disassembly of the oxygen sensor must be easy, permitting simple replacement of the membrane.
11. The material chosen for the construction of the chamber must be easy to clean. It must not permit dissolution of oxygen or interfering substances.

3 Preparation of Buffer Solutions with a Defined Oxygen Partial Pressure

The measurement of oxygen consumption and the calibration of the sensor [15] should be conducted using thermostated solutions either saturated with oxygen or with a gas mixture at a defined partial pressure of oxygen. The apparatus shown in Fig. 2 proved useful for the preparation of such media.

Air, or a gas mixture, is blown continuously through a heat exchanger immersed in the water bath, then through a sterile filter (25 mm in diameter, pore size 0.45 μm) and through an aeration stone into the water contained in bottle B. The air or gas mixture thus becomes saturated with water vapor, and passes into bottle A which contains

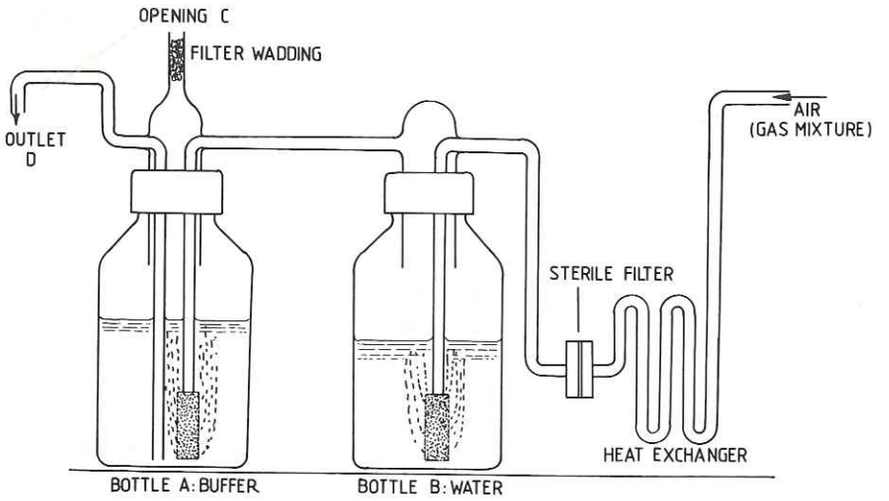

Fig. 2. Apparatus for preparing solutions of defined oxygen partial pressure. Bottles *A* and *B* contain the buffer (test medium) to be equilibrated, and water, respectively. Air (gas mixture) is thermostated in a heat exchanger, sterile filtered, saturated with water vapor in bottle *B* and finally flows through the buffer in bottle *A*. The entire apparatus stands in a temperature-controlled water bath

the buffer solution (test medium). For calculating the p_{O_2} at equilibrium see Appendix A and B. The use of a gas mixture saturated with water vapor prevents the vaporization of water in bottle A which would lead to an increased buffer concentration. The sterile filter protects the buffer from contamination by microorganisms. The excess air (gas mixture) escapes through the exit C. Buffer solution can be removed by closing the opening C with a finger: the increased pressure causes the expulsion of buffer through tube D.

Bottles and tubes should be sterilized before use.

4 Introduction of Substances During Measurements

The stopper of the reaction chamber is equipped with a capillary through which substances such as substrate, inhibitors, ADP etc. can be introduced. To prevent oxygen from entering the chamber, the capillary should have a length of at least 15–20 mm. Microliter syringes (e.g., Hamilton) are best suited for introducing substances; tuberculin syringes suffice for larger quantities up to 1 cm^3.

It is recommended that large volumes be brought to the experimental temperature before being injected. Ideal for this purpose are commercially available syringes with a temperature jacket. By the eddition of small volumes (relative to that of the reaction vessel), the temperature of the solutions introduced can be ignored in most cases.

5 Cleaning the Reaction Chamber

After each measurement, the reaction chamber is emptied with a water pump and thoroughly cleaned with water. An intensive treatment with suitable cleaning agents is, however, sometimes necessary as the measuring system can become contaminated with interfering substances.

5.1 Contamination with Water-Insoluble Chemicals

When water-insoluble substances (e.g., dissolved in ethanol) are added during the measuring process, traces of them remain on the chamber walls after emptying. For this reason, after being cleaned with water, the reaction vessel must be rinsed with solvent. Water-soluble solvents are finally removed by rinsing with water. The use of hydrophobic solvents necessitates cleaning with suitable detergents. Water is always employed for final rinsing.

Of particular importance is the thorough cleaning of awkward areas, for example those around the sensor. Possible incompatibility with the chamber material must always be considered in the choice of cleaning solvents.

5.2 Contamination by the Diffusion of Substances into Plastics

A large number of substances diffuse rapidly into many polymeric materials. A plastic chamber (e.g., of Plexiglas) may be incompatible with the substances to be used during the investigation (mostly inhibitors or pharmaceuticals). It should be noted that diffusion can lead to changes in the initial concentrations [1–4, 8–10]. With clomethiazole in plastic chambers we observed more than 20% material loss. Furthermore, substances which have diffused into the chamber material cannot be removed totally and traces will leak into the test medium during subsequent investigations. Glass reaction vessels offer the only alternative in such cases.

A further problem is the possibility of diffused oxygen in the plastic material of the reaction chamber. This oxygen diffusion may lead to false results particularly in very small chambers.

5.3 Contamination of the POS Electrolyte

Although polythene, polycarbonate, and Teflon are thought to be impermeable except for gases, it is possible for various substances to pass through the sensor membrane into the electrolyte solution [1, 8, 9]. This can lead to changes in the electrode characteristics and contamination of the samples subsequently examined, if the absorbed substances diffuse back through the membrane into the reaction chamber.

An effective solution is not possible here. One should, however, be aware of the situation and its implications. When necessary, the electrolyte should be renewed and

exposure to contamination reduced to a minimum. Sometimes, improvement can be achieved by using thicker sensor membranes, even if this causes a decline in the sensitivity and a longer response time of the POS.

5.4 Contamination Due to Strong Basic Solutions and Detergents

Basic solutions and detergents are difficult to remove. Cleaning is considerably easier if the chamber is treated for a short time with 0.1 mol dm^{-3} hydrochloric acid and then thoroughly rinsed with water.

5.5 Contamination of the Reaction Chamber with Microorganisms

The chief source of error in the oxygen reaction chamber is due to contamination with algae and bacteria. Plastic vessels are problematic, as microorganisms grow particularly well on this material (Chap. II.9).

Membranes of POS which have been exposed to contaminated solutions for some hours or days are covered with bacteria. Such membranes must be replaced. The chamber must be cleaned with water after each measurement. Ideally, it should be rinsed hourly with 50% methanol or isopropanol.

During larger pauses between measurements (e.g., overnight or at weekends), the reaction vessel should be filled with 30% methanol. Before use, the chamber must be carefully washed with water. Instead of methanol, sodium azide or 0.06% phenylmercuricborate solution can be used.

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