METHODS OF BIOCHEMICAL ANALYSIS

Edited by DAVID GLICK
Stanford University Medical School
Palo Alto, California

VOLUME 17

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PREFACE

Annual review volumes dealing with many different fields of science have proved their value repeatedly and are now widely used and well established. These reviews have been concerned primarily with the results of the developing fields, rather than with the techniques and methods employed, and they have served to keep the ever-expanding scene within the view of the investigator, the applier, the teacher, and the student.

It is particularly important that review services of this nature should now be extended to cover methods and techniques, because it is becoming increasingly difficult to keep abreast of the manifold experimental innovations and improvements which constitute the limiting factor in many cases for the growth of the experimental sciences. Concepts and vision of creative scientists far outrun that which can actually be attained in present practice. Therefore an emphasis on methodology and instrumentation is a fundamental need in order for material achievement to keep in sight of the advance of useful ideas.

The current volume is another in this series which is designed to try to meet the need in the field of biochemical analysis. The topics to be included are chemical, physical, microbiological, and if necessary, animal assays, as well as basic techniques and instrumentation for the determination of enzymes, vitamins, hormones, lipids, carbohydrates, proteins and their products, minerals, antimetabolites, etc.

Certain chapters will deal with well-established methods or techniques which have undergone sufficient improvement to merit recapitulation, reappraisal, and new recommendations. Other chapters will be concerned with essentially new approaches which bear promise of great usefulness. Relatively few subjects can be included in any single volume, but as they accumulate these volumes should comprise a self-modernizing encyclopedia of methods of biochemical analysis. By judicious selection of topics it is planned that most subjects of current importance will receive treatment in these volumes.

The general plan followed in the organization of the individual chapters is a discussion of the background and previous work, a critical evaluation of the various approaches, and a presentation of the procedural details of the method or methods recommended by the author. The presentation of the experimental details is to be given in a manner that will furnish the laboratory worker with the complete information required to carry out the analyses.

Within this comprehensive scheme the reader may note that the treatments vary widely with respect to taste, style, and point of view. It is the Editor's policy to encourage individual expression in these presentations because it is stifling to originality and justifiably annoying to many authors to submerge themselves in a standard mold. Scientific writing need not be as dull and uniform as it too often is. In certain technical details, a consistent pattern is followed for the sake of convenience, as in the form used for reference citations and indexing.

The success of the treatment of any topic will depend primarily on the experience, critical ability, and capacity to communicate of the author. Those invited to prepare the respective chapters are scientists who either have originated the methods they discuss or have had intimate personal experience with them.

It is the wish of the Advisory Board and the Editor to make this series of volumes as useful as possible and to this end suggestions will always be welcome.

DAVID GLICK

CONTENTS

Oxygen Electrode Measurements in Biochemical Analysis*

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I. INTRODUCTION

The oxygen electrode depends on the electrolysis of dissolved O_2 at a weakly negative cathode. Diffusion lags can be reduced to a minimum by the immersion of the sensor directly in a medium which is constantly stirred, either by vibration of the electrode or the presence of a small, magnetic, stirring bar in the medium. Where stirring or movement of the electrode is not practical, it has been possible to design miniature electrodes which can "look at" the O_2 content in a very localized area (1). Under proper conditions, the calibrated O_2 electrode can give rapid, reliable information on the O_2 uptake or evolution from a wide variety of biological systems.

In 1897, Danneel (2), working in Nernst's laboratory, studied the electrolysis of O_2 at a Pt cathode. It was later observed that O_2 reduction at a Pt cathode gave a current–voltage curve with a plateau current proportional to the O_2 concentration. After many years of development, Heyrovsky and Shikata (3) designed the first combined polarizer and recorder for use with the dropping Hg electrode. This 1925 instrument was called the "polarograph," a term that has come into general use for all types of measurements involving electroreducible or electrooxidazable substances in solution. Davis (4) gives an excellent historical review of the development of the "oxygen cathode."

The earliest application of the Pt cathode for the study of biochemical systems may be that of Blinks and Skow (5), who in 1938 reported on the O_2 exchange of leaves and cell suspensions. About the same time, Petering and Daniels (6) adapted the dropping Hg electrode for the measurement of O_2 consumption by green algae, yeast, and blood cells. The dropping Hg electrode never became popular for biochemical measurements, but the Pt electrode underwent a rapid development and it was applied to the study of many biochemical systems (7–10).

Most investigators refer to the "polarographic method for oxygen determination" although the instrument originally designed as a *polarograph* is seldom used. More recently the terms *oxygen electrode* or *oxygen cathode* have been used interchangeably with the *Clark electrode*, which is a combined design (see Section II-3 for details) of the cathode and anode used for the measurement of oxygen tension or content by electroreduction of O_2.

Manometric methods gained widespread popularity because of the relative simplicity and reliability of the techniques involved. Until the development of the Clark type of electrode, manometry was the method of choice for the study of oxygen uptake or evolution from tissues, ho-

mogenates, and isolated enzyme systems. There are serious limitations in manometric techniques posed by the difficulty in following rapid changes in the gas phase, the relatively long time necessary for both equilibration and measurement, and the inability to measure differences in oxygen content and utilization in different parts of a biological system. The volumetric data obtained indicate only the average exchange of a sample over the period measured, whereas the O_2 electrode provides continuous information. Most biochemical systems function in intra- or extracellular fluid without the presence of the gas phase required in manometric methods. Oxygen-electrode systems operate well in solutions with variable composition as to substrate, gas content, and pH providing more natural conditions for *in vitro* measurements of metabolizing systems. They have also been applied for measurements *in vivo* (11,12). Although it is beyond the scope of this article to cover all applications of the O_2 electrode, a good idea of its utility for special problems can be gained from recent reviews and symposia (4,13–15).

II. TYPES OF ELECTRODES

1. The Open Microelectrode

This usually consists of a Pt or Au wire sealed in glass with either a pointed (for insertion into cells or tissues) or a flush tip (Figs. 1*A*–1*C*) with a calomel (KCl) or Ag–AgCl reference electrode. Davis (4) discusses the physical behavior of such electrodes in stationary media, indicating that the response time decreases from 400 msec for a 10-μ diameter wire to 4 msec for a 1-μ wire. Lübbers (16) indicates that the current available with minute electrodes is very small and requires expensive stable instrumentation for its measurement. Bare metal electrodes have an additional difficulty in that they are rapidly and require special techniques for maintenance of their sensitivity, which decreases by several per cent per hour.

Since the current available from an open electrode is greatly increased in a moving medium, a number of rotating and vibrating electrodes have been designed (4,17,18). The movement replaces the layers of solution near the electrode (which rapidly becomes depleted in O_2 content) and keeps the electrode more nearly in equilibrium with the material in solution. The movement also serves to keep the biological sample in more uniform suspension, resulting in a better measurement of the O_2 utilization or evolution into the solution. It is necessary to control the rotation or vibration of the electrode within small limits in order to obtain

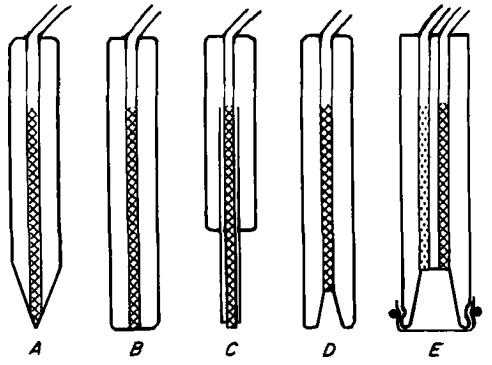

Fig. 1. Major types of O_2 electrodes. The cross-hatched area indicates a thin Pt or Au wire usually encased in glass or epoxy resin. The dotted area represents an Ag anode. (*A*) Bare needle type for insertion into tissues. (*B*) Flush form used for surface or vibrating electrodes. (*C*) Bare exposed type used for vibrating or rotating electrode. (*D*) Conical recessed form used for discontinuous or continuous measurement in unstirred medium or tissue. This form of Pt electrode is relatively free from artifacts of diffusion or movement. (*E*) Clark-type electrode with a Pt cathode and Ag anode sealed into a conical well covered with an O_2-permeable membrane.

reproducible results. Moving electrodes give much larger currents than stationary electrodes because there is no wait for a steady-state current to be established and diffusion transients are minimized. Rotating or vibrating bare electrodes permit very rapid O_2 measurements in solution during enzymatic reactions, although not as rapid as spectroscopic methods. Chappell (19) found that 1m*M* concentrations of $K_3Fe(CN)_6$, $K_4Fe(CN)_6$, HCN, ascorbate, indophenol, and several other organic substances interfered with O_2 measurements with the vibrating platinum electrode. He also stressed the need to design the reaction chamber so that O_2 diffusion into the medium from the air was minimized during the measuring sequence. The interference phenomena, described by Chappell, were not found when the Clark-type, membrane-covered electrode was used with the same solutions.

2. The Recessed Microelectrode

Davies and Brink (20) explored the possibility of using a recessed cathode for the determination of the absolute O_2 tension of solutions. By allowing the electrode, with the current off, to come into equilibrium with the medium and then turning the current on and measuring, it was possible to obtain accurate measurements of the absolute O_2 content

with a calibrated electrode. Solutions equilibrated with known concentrations of O_2 are necessary for the calibration of these electrodes. In practice, the initial current produced at different O_2 tensions is plotted and compared with the current produced in unknown solutions. Although this type of electrode is not suitable for continuous measurement, it has been used very effectively for the determination of O_2 content in a variety of solutions, notably blood and plasma. Recessed electrodes properly used show excellent current linearity with oxygen tension and give results comparable to the more rapidly responding, flush-type open electrodes (Fig. 1*B*). They are practically free of movement artifact because the solution in the recess is protected from convection. This is further enhanced in some designs by covering the orifice with a very thin membrane which does not interfere with oxygen diffusion.

An improvement in the design of recessed electrodes resulted in the conical recessed electrode. This has a funnel-shaped recess (Fig. 1*D*) and the advantage of being able to be continuously operated. The diffusion field is confined to the small recess which has a large-diameter orifice and a very small area near the electrode. Since the exposed electrode area is small compared to the orifice area, the oxygen consumption of the electrode is less than for a uniform diameter recess. There is a very small concentration gradient at the orifice as compared to that at the electrode surface. An electrode with a 13-μ Pt cathode and an 80-μ orifice has been shown to have a 90% response time of less than 2 min. This is one order slower than the flush-type electrode response, but does permit continuous rather than intermittent operation.

Some attempts were made to obtain faster electrodes, free from movement artifact, by covering the flush Pt metal surface with a very thin layer of porous, sintered glass or a microporous membrane (4,21). The principle of this type of electrode is the same as that of the conical recessed electrode because of the low effective diffusion coefficient of O_2 in the sintered glass or microporous membrane. Response time for this type of electrode may be very short (i.e., 90% complete in 30 msec), but because of technical difficulties it has not gained in popularity.

3. The Clark Electrode

The most successful electrodes for biochemical work have proved to be the bare platinum electrode (either vibrating or rotating) for very fast responses (Figs. 1*A*–1*C*) and the Clark-type, membrane-covered, Pt cathode–Ag anode electrode for following slower reactions (Fig. 1*E*). If ultrafast reactions involving O_2 must be followed, other methods such as

the recording spectrophotometer (22) can overcome inherent limitations of the response time of the electrode systems.

The chief advantages of the membrane-covered electrode are the isolation of the electrode current, the reduction in aging, and the decreased movement artifact. There is, however, a reduction in the speed of response as compared to the uncovered cathode. Electrodes have been covered with a wide variety of plastic materials ranging from collodion to cellophane, polyethylene, nylon, and Teflon. The combination of both Pt cathode and reference cathode under the same membrane, initiated by Clark in 1956 (23), was a major advance in design. In combined membrane-covered electrodes, the current flow is completely separated from the experimental medium by a thin, nonconducting membrane which is readily permeable to oxygen. A recent modification of this design (Fig. 2), in which the Pt or Au cathode and Ag anode are both within an epoxy casing (with flush metal surfaces exposed under a tightly stretched membrane) has proved to be very stable. The electrode is usually covered with 25-μ (1 mil) thick TEP Teflon stretched over a minimal amount of electrolyte, usually 50% saturated KCl. If it is desirable to work at temperatures below 25°C, very thin cellophane, Teflon, or polyethylene films have been successfully used down to 0°C. Response times for this type of electrode depend on the area of the cathode and the O_2 solubility in the membrane material. Macrocathodes give larger currents which require less amplification, but have slow response times (2–3 min). Semimicro and microcathodes (less than 50-μ diameter) give small currents, requiring amplification for recording or measurement, but have rapid response times. For example, a Clark electrode with a 600-μ diameter Pt cathode and an Ag anode covered with a 25-μ thick Teflon membrane and operated at 37°C has a 90% response time of 10 sec (Fig. 2.) Experimental electrodes have been covered with membranes of saran, nylon, cellophane, polyvinyl chloride, Mylar, natural rubber, and silicone rubber yielding different response times depending on the solubility of O_2 in the membrane material. The absolute solubility of O_2 in most of these materials has not as yet been worked out, but Michaels (25) determined the equation for the solubility of O_2 in polyethylene. Several Clark electrode designs have combined small thermistors (Fig. 3) in the unit with circuitry to correct for temperature differentials between the electrode and the solution where it is impractical to regulate the temperature of the experimental medium within small limits. Some specialized designs and their application to biochemical measurements are described in Sections IV and VI.

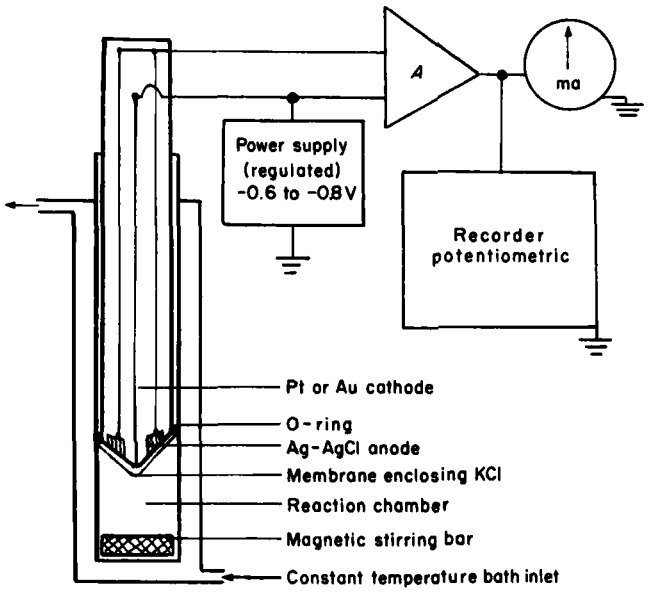

Fig. 2. Schematic drawing of a Clark oxygen electrode system as currently used for biochemical measurements [Lessler et al. (24)].

III. CONSTRUCTION AND THEORY

1. Fabrication

Both micro- and macroelectrodes have been made by sealing the Pt cathode and Ag anode in glass or epoxy. A wide variety of O_2-permeable membrane materials are available. The thickness of the membrane, solubility of O_2 in the material, and its ability to withstand some abrasion in use affect the response of the electrode. Early electrodes were made by sealing the Pt and Ag wire in glass but Ag does not seal well although Pt makes a tight bond with No. 6080 soda-lime glass. A wide variety of commercially fabricated electrodes are now available, but for special purposes many investigators construct their own. An example of a microelectrode (designed for measurement of oxygen utilization of the cornea) with a 25-μ diameter Pt cathode combined with a thermistor is shown in Figure 3. In fabrication, a great deal of attention must be given to the seal between the glass or epoxy and the metal or the electrode soon becomes defective. This is usually evidenced by failure of the electrode current to return to zero in O_2-free solution. Appar-

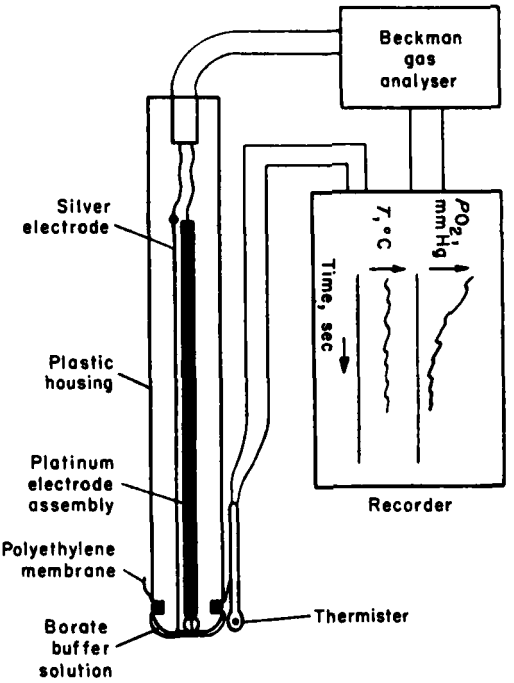

Fig. 3. Schematic drawing of a Clark-type microelectrode combined with a microthermistor for simultaneous recording of P_{O_2} and temperature. The electrode has a 5-mm overall diameter, a 25-μ diameter Pt cathode, an Ag anode, and is covered with a 12-μ polyethylene membrane. It is used for direct measurement of corneal O_2 utilization [Hill and Fatt (26)].

ently, a small amount of solution works its way along the glass or epoxy seal into a minute crack in the electrode. This provides a second electrical path to the reference electrode which interferes with the current measurement at the electrode tip. Only regular checking and calibration of the electrode can turn up this type of difficulty.

2. Electrode Behavior

The main condition for measuring oxygen tension is that the oxygen must be transported to the electrode by diffusion. This depends on the gradient of oxygen tension between the electrode surface and the sample. The P_{O_2} at the surface of a properly functioning electrode is zero because the reduction current depends only on the oxygen tension of the sample. A constant diffusion zone can be established by a membrane in front of

the electrode. If no additional oxygen diffuses into the sample, the O_2 reduction current measures exactly how much oxygen is transported through the membrane by diffusion, hence the oxygen tension of the solution.

When a Pt cathode is maintained at 0.4–0.8 V negative with respect to a suitable reference electrode in an oxygen-containing solution, oxygen undergoes electrolytic reduction at the cathodal surface. The velocity of reduction is limited chiefly by the maximum rate of diffusion of O_2 to the electrode surface. At these low potentials, the current produced by the Pt–Ag–AgCl cell has been found to be directly proportional to the O_2 tension (P_{O_2}) of the solution.

Many types of reference electrodes may be used, but for most biochemical applications the calomel (KCl) or the Ag–AgCl electrodes are the most popular. In the Clark-type electrode where the electrolyte volume is small, concentrated solutions such as saturated KCl or 50% saturated KCl have been used to prevent significant changes in chloride concentration during use. Hill and Fatt (27) recommend an electrolyte of 0.1*M* KCl in a pH 9.5 borate buffer for use with microelectrodes, while Carey and Teal (28) found that 1*M* KOH gave a good electrode response.

More complete knowledge of the behavior of an electrode can be gained by plotting the current–voltage curves obtained in solutions equilibrated with known concentrations of O_2 (Fig. 4). Such curves show that the plateau current recorded when the cathode is maintained at -0.4 to -0.8 V, is directly dependent on the O_2 concentration. Thus, the steady-state current observed is linearly dependent on the O_2 concentration as shown in Figure 5. This curve was obtained with a 200-μ diameter Pt cathode, but similar linear curves have been observed with smaller and larger cathodes (29). Measurements of this type can be done in unstirred solutions, but ideally should be made in a uniformly stirred solution which allows for rapid equilibration and production of a steady-state current from the electrode in a relatively short period of time.

At lower potentials, the current produced is limited by both the energy barriers of the reduction process and the availability of O_2 molecules as determined by their concentration gradient at the electrode surface. The plateau found between potentials of about -0.4 and -0.8 V indicates that O_2 is gaining access to the electrode surface at the maximum possible rate for the given diffusion conditions. In this plateau region, the electrolysis current is affected to only a minor degree by electrode potential, and maximally by P_{O_2}. At voltages above -0.8 V the electrode current rapidly rises because another reaction (the direct reduction

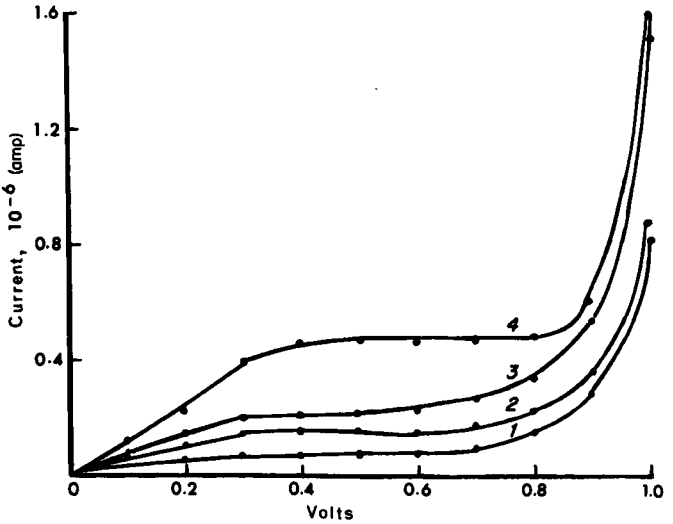

Fig. 4. Current–voltage curves of a Clark-type microelectrode indicating the nature of the plateau at different O_2 concentrations. (*1*) = 2.5% O_2, (*2*) = 5.0% O_2, (*3*) = 7.0% O_2, (*4*) = 95.0% O_2. Measurements were made with a 15-μ diameter Pt cathode, covered with a 25-μ Teflon membrane and a 12-μ cellophane membrane [Redrawn from Gleichmann and Lübbers (29)].

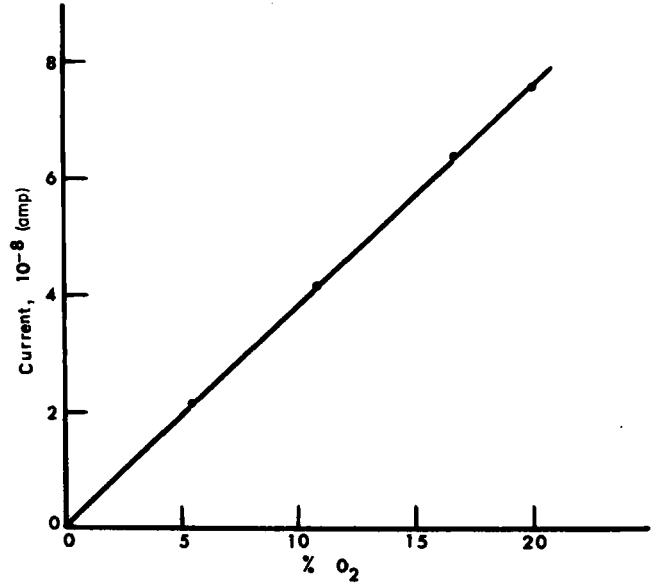

Fig. 5. Electrode current as a function of oxygen concentration. Typical curve of steady-state current versus per cent O_2 with which the solution is equilibrated.

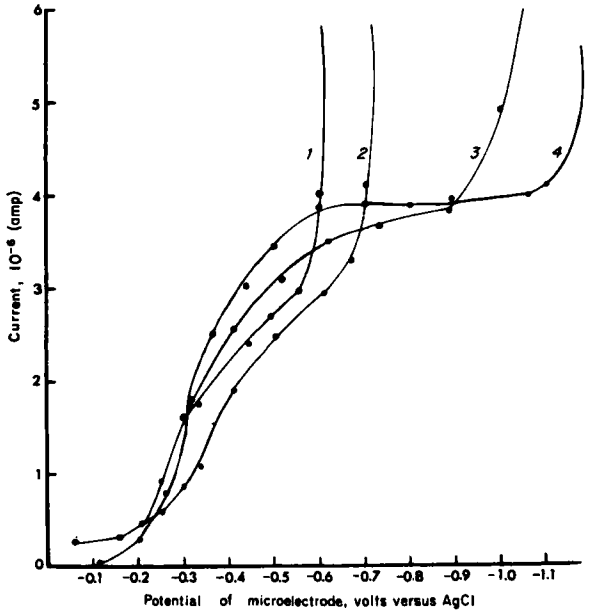

Fig. 6. Current–voltage curves for open-type oxygen electrode in unstirred, aerated solutions at different pH's. (*1*) = Phthalate buffer at pH 3.0, (*2*) = acetate buffer at pH 4.7, (*3*) = borate buffer at pH 9.0, (*4*) = 0.01*M* NaOH in 0.1*M* KCl at pH 12.0 [redrawn from Kolthoff and Lingane (30)].

of H^+ ions) occurs, interfering with measurement of O_2 reduction. Other factors which influence the equilibrium current are whether or not the solution at the electrode is flowing or stationary and the effect of pH on the behavior of the electrode (Fig. 6).

Most systems designed for biochemical measurements involve uniformly stirred solutions, thus reducing the stirring artifact. It should be stressed that a uniform stirring rate must be maintained both for calibration and measurement. The Clark-type electrode is particularly sensitive to stirring artifacts, but is relatively insensitive to wide variations in pH during measurement, except where very high or very low pH may damage the membrane covering the electrode.

3. Electrode Reactions

Much of our knowledge of the electrode reaction is indirect, based on current–voltage curves in stirred or unstirred solution, without actual analysis of the reaction products (Figs. 4 and 5). The equilibrium potential of Pt in solutions with dissolved O_2 is very sensitive to traces of

H_2O_2 and the electrode behavior in H_2O_2 solutions is very sensitive to traces of O_2. The reduction process at the Pt cathode has been postulated to involve either two or four electrons, depending on the investigator and his method of measurement. Davis (4) gives an excellent review of the physical theory behind the reaction of oxygen and the cathode.

It has been observed that minute amounts of H_2O_2 appear in solution when O_2 is being reduced along with an elevation in pH in the medium adjacent to the electrode. The alkaline shift is due to the production of OH^- ions with no change in the H^+ ion concentration at the electrode. Kolthoff and Lingane (30) suggest that two separate reductions occur, each involving the same number of electrons per original O_2 molecule. It appears that a product of the first reduction is a reactant of the second reduction. The appearance of small amounts of H_2O_2 probably takes place during the first reduction and it is then almost completely reduced in the second step of the reduction process. These observations strongly suggest the following sequence of reactions at the O_2 cathode:

$$O_2 + 2H_2O + 2e^- \rightarrow H_2O_2 + 2OH^-$$

$$H_2O_2 + 2e^- \rightarrow 2OH^-$$

Davis (4) points out that the sequence is insensitive to pH since H^+ ions are not utilized except to form water. This reaction would also tend to produce small amounts of H_2O_2, if the second stage of reduction did not proceed at maximum velocity. This sequence of reactions also accounts for the observed increase in alkalinity at the cathode during O_2 electroreduction. Kolthoff and Jordan (31) found that traces of O_2 enhanced the electroreduction of H_2O_2. They also found that when O_2 was carefully excluded, H_2O_2 in concentrations of $10^{-3}M$ or less was reduced only at elevated potentials, and the current produced was smaller than the expected values for H_2O_2 diffusion currents. The catalytic effect of O_2 on the electrolysis of H_2O_2 may explain the absence of a second wave in the current–voltage curve for O_2 reduction at a Pt electrode because peroxide is reduced at less negative potentials in the presence of O_2.

Connelly et al. (32) indicate that the major factors determining O_2 concentration near the electrode are: (*1*) the geometry of the system, (*2*) the O_2 content of the solution, (*3*) the diffusion coefficient of O_2 in the membrane, (*4*) the rate of O_2 consumption by the sample, and (*5*) the rate of O_2 consumption by the electrode. These authors also present a mathematical analysis of the boundary conditions when oxygen electrodes are applied to or inserted into tissues. Silver (33) recently reanalyzed the parameters involved in the measurement of O_2 tension in

tissues. The situation in a stirred or unstirred solution is simpler than in a tissue where O_2 is utilized and replaced at variable rates. Careful analysis of the O_2 tension profile in solution has led Fatt (34) to propose the following equation to describe the boundary conditions.

When $x = 0$ at the electrode surface and is positive moving from that surface to the layer above, then

$$dP/dx = 0$$

and

$$d^2P/dx^2 - (Q/DK) = 0$$

when $x = L$, $P = P_a$ and where P = O_2 tension, mm Hg; Q = rate of O_2 consumption, ml O_2/(ml layer)(sec); D = O_2 diffusion coefficient, cm^2/sec; K = O_2 solubility, ml O_2/(ml layer)(mm Hg); x = distance variable, cm; L = thickness of layer, cm; and, P_a = O_2 tension at open surface layer, mm Hg.

IV. CALIBRATION AND EVALUATION

1. Residual and Zero Current

Residual current is the small current observed with oxygen cathodes even in the complete absence of O_2. Alben (35) studied the residual current of a YSI Clark electrode* with a Pt cathode and an Ag anode wet with saturated KCl under a thin polyethylene membrane. Using vacuum-boiled distilled water through which completely deoxygenated N_2 was bubbled, he found electrode currents of 1×10^{-10} A at 9°C. This corresponds to 0.0052% O_2, or a sea-level P_{O_2} of 0.04 mm Hg. These data indicate that significant measurements of P_{O_2} can be made with this type of electrode down to about 0.1 mm Hg.

A simpler method for testing the residual current of oxygen electrodes in the laboratory is to use a dilute solution of Na_2SO_3. In neutral or mildly basic solution, 0.02M Na_2SO_3 gives residual currents corresponding to a P_{O_2} of water through which N_2 is being bubbled (commercially purified N_2 usually has less than 0.01% O_2). The *residual current* should be distinguished from the *zero current* described below.

Zero current is the electronic zero (or dark current) observed after warmup of the electrode–amplifier–recorder system and when there is no potential difference between cathode and anode. This zero current is characteristic of the equipment used. The amplifier–recorder combi-

* Yellow Springs Instrument Company, Yellow Springs, Ohio.

nation should be designed so that the zero (or dark) current can be nulled. For accurate calibration and measurement, it is necessary to adjust the potentiometer scale so that it read zero either with no potential difference between cathode and anode (zero current) or when the electrode is in an essentially anerobic solution (residual current).

2. O_2 Tension and Content

The preliminary step in calibrating an electrode for the determination of O_2 tension is the nulling of the zero current mentioned above. The electrode should then be immersed in distilled H_2O through which N_2 is being bubbled. After equilibration of the electrode with the deoxygenated H_2O (4–5 min), transfer it to distilled H_2O through which filtered air is being bubbled, or for a simpler procedure, briefly wave the electrode in the air. This will give a reading of the current produced by 20.9% O_2 at the ambient barometric pressure. It is also useful to measure the electrode current at other O_2 concentrations by equilibrating the aqueous phase with analyzed, commercially available, gas mixtures under carefully controlled conditions of temperature and barometric pressure. The latter method is commonly used to calibrate an electrode for the determination of unknown O_2 concentrations by use of a calibration curve (Fig. 5).

The oxygen electrode gives readings of current which can be directly converted to *oxygen tension* (P_{O_2}) in millimeters of mercury. Using a calibration curve can provide information on the per cent of O_2 in solution, but this may or may not indicate the *O_2 content* of the solution if significant amounts of O_2 are bound by substances in the solution. By mixing blood, plasma, or other solutions with specifically prepared reagents to free bound O_2, the true O_2 content of such solutions can be determined [see Clark and Lyons (36)]. Relatively simple calculations enable the investigator to calculate the absolute amount of O_2 (i.e., μ-atoms) utilized in a reaction (see Section V).

In the absence of analyzed gas mixtures, there is a convenient and rapid means for accurate determination of the oxygen content of a reaction medium, using submitochondrial electron transport particles (e.g., heart muscle ETP) with limiting concentrations of DPNH (19). The high affinity of the heart muscle ETP for DPNH permits a stoichiometric titration of oxygen content. Using spectrophotometrically standardized DPNH, we can add limiting concentrations of DPNH and determine the change in current occurring on complete oxidation of the DPNH. In this manner a direct calibration can be obtained. In addition, ex-

periments of this type permit the estimation of back-diffusion of oxygen into the reaction medium. If sufficient DPNH to utilize 50% of the oxygen in the reaction vessel is added to heart muscle ETP suspended in a suitable buffer, the current drift observed after complete oxidation of the DPNH can be measured and used to establish the rate of back-diffusion of oxygen into the reaction vessel.

3. Difficulties and Their Correction

The most commonly used criterion for the evaluation of new oxygen electrodes is the shape of the current–voltage curve. If the electrode gives a long plateau, it is probably a good electrode (Fig. 4). This means that a diffusion-limited current has been reached at a low potential and that oxygen diffusion to the electrode is restricted relative to the intrinsic reaction rate. Occasionally, the entire electrode assembly is accidentally immersed and water gets into the insulated connection of the wires in the electrode or into the plug. This will disturb the current voltage curve, but it indicates a wet and not a bad electrode. Simply drying the electrode overnight in a 50 – 60°C oven will usually correct this difficulty. In any case, drying the electrode thoroughly should be tried before discarding it.

The most commonly encountered difficulty with the oxygen electrode is its tendency to age. This is indicated by a falling off in electrode current even though a constant oxygen tension is maintained in the medium. In relatively simple salt solutions or distilled water, this tendency is slight, but in phosphate-buffered solutions containing divalent ions, aging may occur at an appreciable rate. Aging occurs in both naked and membrane-covered electrodes and is usually due to the presence of substances which become attached to the metallic surfaces. This condition requires careful cleaning of the electrode surfaces and replacement of the membrane in membrane-covered electrodes. The Ag anode of the Clark-type electrode becomes oxidized and should occasionally be cleaned with 50% NH_4OH which is immediately washed away with distilled water. It is good practice to have an illuminated magnifier handy for visual checking of the electrode surfaces, for the presence of bubbles in the solution under the membrane, and for the presence of folds or holes in the covering plastic film. Some techniques for interrupting and reversing the electrode current which have been developed aid in avoiding both diffusion effects and electrode aging (37).

Periodic recalibration of the electrode is necessary in complex solutions. This must be done more often in some solutions than in others

(for example, in solutions with a high protein content such as blood or serum). It is important to routinely check the electrode calibration and the barometric pressure to insure duplication of absolute measurements. This is not as important in relative measurements, but calibration and checking of the electrode response can improve agreement between repeated experiments.

Cleaning and polishing the electrode surfaces should never be done with Fe-containing abrasives. Iron-containing materials deposit minute amounts of Fe at the cathodal surface which cause rapid aging of the electrode. A very fine mixture of silicon carbide suspended in light machine oil or in a rubber matrix is a good polishing agent. If the electrode surface becomes coated or dull, it can be cleaned with a toothbrush and toothpaste containing a fine abrasive. When the surface of an Ag anode becomes gray or spotted, it should be cleaned by rubbing with a cotton-tipped applicator wet with 50% NH_4OH. After either cleaning or polishing, the electrode should be carefully washed with distilled water and the surface examined with a 10× magnifier for the presence of residual particles. Routine examination of the electrode surface during the calibration period prior to an experiment is advised. We use a 10× illuminated magnifier for the electrode inspection and it has saved many hours of fruitless effort.

Reaction vessels may be constructed of glass or Plexiglas with or without a temperature-controlled jacket. If Plexiglas is used, it should be remembered that this material will hydrate and can become fairly permeable to O_2. Plexiglas is also difficult to clean without scratching and cannot stand sterilization. Glass becomes the material of choice for experiments that run several hours, where it is advisable to start the reaction with sterile solutions and reaction vessels. The reaction vessel may be sealed, but usually closure by a simple press-fit, which permits the addition of reagents, is adequate. The closed vessel should be tested for back diffusion of O_2 under experimental conditions. This is more critical for experiments of long duration than for reactions that go to completion in a few minutes.

It should be remembered that the electrode itself consumes O_2 at a constant rate from the solution. The rate is dependent on the size and design of the electrode and the P_{O_2} of the solution under study. An electrode error, based on the amount of O_2 consumed by the electrode, may be an important factor in systems that use O_2 slowly. For example, an electrode current of 1 μA in air-equilibrated solution (at 37°C, 760 mm Hg) represents an O_2 consumption of 0.3×10^{-6} g O_2/hr or approximately 0.24 μl/hr (see Table I). If the same solution had been equili-

brated with 100% O_2, the O_2 consumption of the electrode would have been 5 times as great or 1.2 μl/hr. The electrode error depends on both the amount of O_2 dissolved in the solution and the rate of O_2 consumption by the sample. For example, a sample in 4 ml of air-equilibrated Ringer's solution which utilizes 10 μl O_2/hr at 37°C will deplete the solution of 50% of its O_2 in 1 hr with an electrode error of 2.4%. The same sample equilibrated with 100% O_2, instead of air, would remove approximately 10% of the O_2 in solution in 1 hr with an electrode error of 12%. Note that the electrode error is greater at higher O_2 pressures. A significant improvement in both accuracy and the time required for measurement of 50% depletion occurs when the solution under study is equilibrated with air. Another way of decreasing the electrode error is to add more biologically active material so that the reaction requires a shorter period of time for measurement.

TABLE I

Conversion Factors (37°C, 760 mm Hg)

Conditions	O_2, μl
1.25×10^{-6} g O_2	1.0
1 μA Pt cathode 0.5 mm diameter	0.24
1 ml air-saturated distilled H_2O	5.6
1 ml 100% O_2-saturated distilled H_2O	28.0
1 ml air-saturated Krebs-Ringer	5.1
1 ml 100% O_2-saturated Krebs-Ringer	25.5

4. Rapidity of Response

There is an upper limit to the rapidity with which the O_2 electrode can follow the changes of O_2 tension in solution. This depends on many factors such as the size of the cathode, the solubility of O_2 in the membrane, and the time constant of the amplifier–recorder system used. It is well beyond the scope of this paper to discuss the details of response time, but it is important that the investigator has some idea of the response time of the biochemical system under investigation as compared to the electrode–amplifier–recorder response time. As the size of the cathode is reduced, the O_2 electrode gives a more rapid response, but the current produced becomes very small, making necessary increased sophistication (and cost) of the measuring system. Fatt (1) has confirmed this by designing microelectrodes with Pt cathodes of various sizes down

to 1μ in diameter. For example, a Clark electrode* with a 25-μ diameter cathode covered by a 12-μ cellophane–Teflon membrane "looks at" (draws O_2 from) a region only 5–10 times the diameter of the cathode and has a 90% response time of 3 sec. For any given diameter of O_2 cathode, the response time can be reduced by use of a thinner membrane or by changing to a membrane material which is more permeable to O_2. This is the reason that the bare Pt cathode has a faster response time than the membrane-covered electrode. The response time of earlier Clark-type electrodes was about 30 sec for a 90% response. Reduction in the size of the cathode and other improvements in design and membrane materials has led to the development of currently available electrodes with 95% response times of 3 sec or less.

V. CALCULATIONS

Oxygen in solution as monitored by an O_2 electrode is usually calculated as partial pressure of O_2 (P_{O_2} mm Hg) of the gas phase with which the solution is in equilibrium. The recorded P_{O_2}, however, does not always correspond to the same O_2 concentration in solution because the solubility of O_2 varies with temperature and to a lesser degree with the composition of the solution. The amount of O_2 in simple solutions can be calculated from its solubility coefficient which depends on the temperature and the electrolyte composition of the medium. The solubility coefficient (α) for distilled water and some salt solutions (based on the *International Critical Tables*, *3*, 271 (1928)), is available in most chemical and physical handbooks. A short table of O_2 solubility in Ringer s solution can be found in Umbreit et al. (38). An experimental determination of O_2 solubility in complex buffered solution by Chappell (19) provides more accurate values for calculation of the O_2 content of currently used biochemical solutions than can be obtained by use of the α values mentioned above (see Table II).

Although O_2-electrode measurements are initially recorded as P_{O_2} in mm Hg change per unit time, many situations arise where it is desirable to express these data as absolute values in volume of O_2 (STP) or in microgram atoms of O_2 (μatoms). If careful records are kept of the barometric pressure (we use a recording barometer) and the calibration is made with air (20.9% O_2) at the temperature of the reaction, it is possible to calculate the volumetric or gravimetric O_2 values rapidly and accurately.

* Beckman-Spinco No. 161-940, Beckman Instruments Inc. ,Palo Alto, California.

TABLE II
Solubility of O_2 in Buffered Medium

Temperature, °C	μg atoms O_2/ml[a]
15	0.575
20	0.510
25	0.474
30	0.445
35	0.410
40	0.380

[a] Experimentally determined solubility of O_2 in a solution of $NADH_2$ + P_i + isolated mitochondria in a buffered medium [Chappell (19)].

At 37°C, saturated with air at sea level (a P_{O_2} of 159 mm Hg), distilled water contains 5.6 μl O_2/ml and Krebs-Ringer solution approximately 5.1 μl O_2/ml (see Table I). If the distilled water and Krebs-Ringer were equilibrated with 100% O_2, their O_2 contents would be 28 and 25.5 μl O_2/ml, respectively. Since the O_2 electrode is a "percentage response" instrument, the values obtained during the course of an experiment can be used for direct calculation of the initial amount of O_2 in the medium and the volume of O_2 utilized by the sample. This procedure involves calibrating the meter or recorder to 100 divisions with a known volume of equilibrated solution, recording the percentage change with time due to the activity of the sample, and conversion of these data to the volume of O_2 utilized.

Many biochemists prefer to express the O_2 utilization in gravimetric (μatoms) rather than volumetric (μl) form. This is usually done by calibration of the instrument with either air or 100% O_2 and using the conversion factors of either Umbreit et al. (38) or Chappell (19)(Table II). If a reaction is carried out at 30°C in 4 ml of medium and there are 0.445 μatoms of O_2 per ml (see Table II), then for a full-scale deflection of 50 divisions the following calculation is used.

$$\frac{4 \text{ ml} \times 0.445 \text{ μatoms/ml}}{50 \text{ scale division}} = 0.0356 \text{ μatoms/division of recorder}$$

Further details of calculations for specific reactions are in Section VI-1-A.

VI. APPLICATION TO SPECIFIC REACTIONS

In theory any reaction which takes up or gives off oxygen or any reaction which can be coupled to such a process is amenable to measure-

ment by oxygen electrode procedures. Citation of all of the individual applications which have been reported is obviously beyond the scope of the present discussion. However, some of the more common reaction systems are summarized below. Polarographic procedures have been applied to the study of tissue oxidation at almost every stage of organization from slices and homogenates through purified enzymes. Since most of the tissue respiratory activity is recovered in the mitochondrial fraction, special emphasis will be placed on studies involving isolated mitochrondia.

1. Mitochondrial Oxidation

Mitochondria are organelles which in most tissues are specialized for the oxidative degradation of a variety of sustrates, and the production of ATP for utilization outside the mitochondrion. Oxidation reactions in coupled mitochondria involve the participation of (*a*) primary dehydrogenase enzymes such as those of the tricarboxylic acid cycle, (*b*) the electron transport system, and (*c*) the oxidative phosphorylation mechanism. The individual components of these systems and the reactions involved have been discussed in a number of recent reviews (39–43). In the simplest terms, a flow of components and reactions such as that diagramed in Figure 7 obtains. Substrates such as pyruvate, malate, or β-hydroxybutyrate enter the mitochondrion and are oxidized by the various primary dehydrogenases (i.e., the Krebs cycle, the fatty acid spiral, and ancillary enzymes) to CO_2 with the reducing equivalents trapped as either DPNH or in the reduced form of flavoproteins. The mitochondrial electron transport system then oxidizes the reduced pyridine nucleotide or flavin in a series of stepwise redox reactions with O_2 as the ultimate electron acceptor. During these oxidation–reduction reactions, energy is conserved in the form of some unknown compound or condition (see ref. 39) designated X$\backsim$I in Figure 7. X$\backsim$I is then utilized in the synthesis of ATP from ADP and P_i. Alternatively, X$\backsim$I can support ion transport and other energy-linked reactions (44,45), or can be discharged by uncouplers of oxidative phosphorylation.

The O_2 electrode has largely supplanted the traditional manometric procedures (46) for the study of these reactions. Polarographic methods provide a continuous record of the time course of the reaction and have a number of other advantages over manometric methods. Assays with the oxygen electrode permit convenient addition of a number of reagents during an experiment so that several meaningful rates can be evaluated from a single incubation (see Fig. 8). The reaction conditions can be

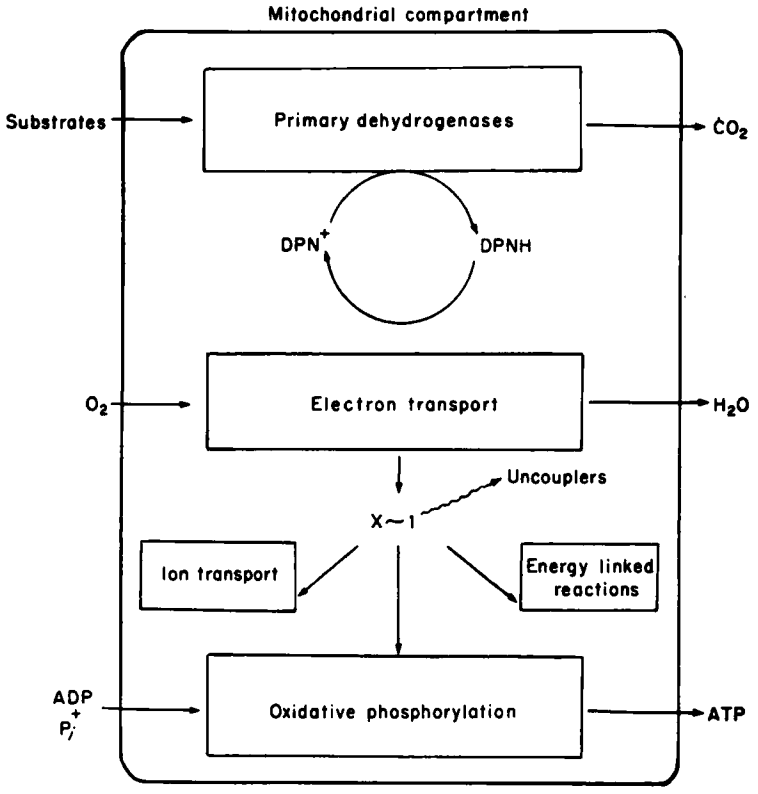

Fig. 7. Flow pattern of mitochondrial reactions and components.

adjusted so that a minimum time of incubation is required, and the secondary side reactions (which often complicate longer incubations) can be kept to a minimum. In addition, as will be discussed below, the oxygen electrode can be added conveniently to a cuvette or reaction chamber in which other reaction parameters are monitored simultaneously. The direct measurement of O_2 tension in the reaction medium eliminates the gas-exchange problems which were encountered when investigators tried to follow rapid oxidations by manometric methods (46–49). Disadvantages of polarographic procedures include the fact that only one reaction can be run with each electrode. Slower reactions are often more conveniently followed by simultaneous observation of a bank of several manometers. The time of the reaction in a polarographic chamber is limited by the amount of O_2 which can be dissolved in the incubation

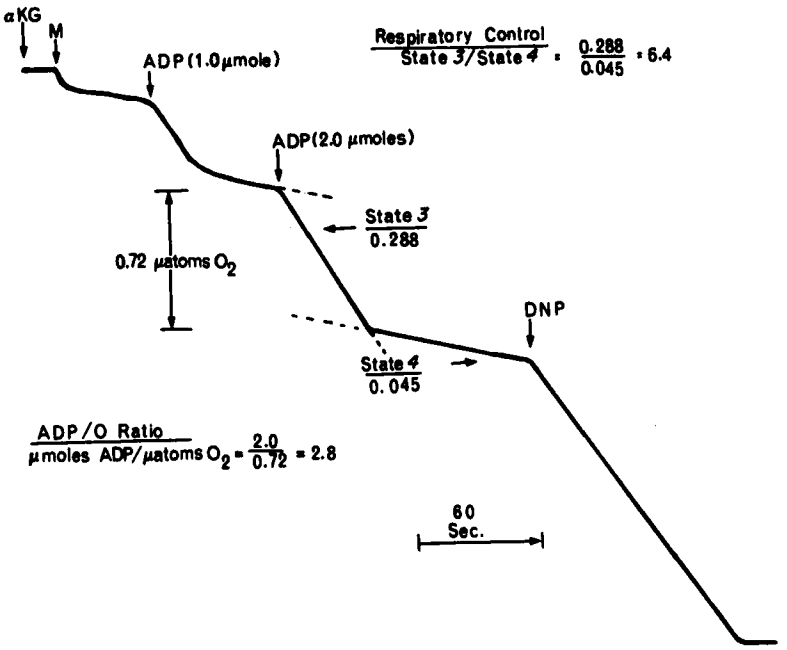

Fig. 8. Evaluation of ADP/O ratio and respiratory control ratio in a typical mitochondrial respiration experiment. Isolated beef heart mitochondria prepared by the Nagarse (Enzyme Development Corporation, 64 Wall Street, New York, N. Y.) procedure of Hatefi et al. (51) as modified by Brierley et al. (52); 3.75 mg of protein were added to 6 ml of an incubation medium consisting of sucrose (0.25*M*), $MgCl_2$ (10m*M*), Tris phosphate (5m*M*, pH 7.0) and Tris-α-ketoglutarate (5m*M*). The temperature was maintained at 25°C and the rate of oxygen uptake was monitored with a 48I #53 electrode. At the indicated points 2.0 μmoles of ADP were added using a syringe microburet. The concentration of dinitrophenol (DNP) was 0.1m*M*.

medium (Table I). This O_2 limitation is sometimes a source of difficulty when estimation of changes in P_i or substrate concentration is necessary for direct calculation of P/O ratios.

A. RESPIRATORY CONTROL AND P/O RATIOS

These procedures have been reviewed recently by Estabrook (50) and will be summarized only briefly here. A suspension of tightly coupled mitochondria added to a suitable medium shows a low rate of respiration in the absence of added ADP since the availability of the acceptor system is rate limiting (7). In the illustrated example (Fig. 8), addition of 2.0 μmoles of ADP increased the rate of respiration with α-ketoglutarate from 0.045 to 0.288 μatoms of O_2/min/mg of protein. The respiratory

control ratio is defined by Chance and Williams (7) as the ratio of the respiration in the presence of the acceptor system (state *3*) to the respiration which is observed after the added ADP has been converted to ATP (state *4*). In the illustration given in Figure 8, this value would be 0.288/0.045 or 6.4. To evaluate these rates, the slope of the trace of O_2 consumption is measured in chart divisions per unit of time. If the recorder has been adjusted to give full-scale deflection when an air- and temperature-equilibrated solution is compared with an anaerobic one, then the chart divisions per μatom of O_2 can be calculated from the volume of the suspension and the solubility of O_2 in the suspending medium. Since most mitochondrial oxidations involve the passage of two electrons down the electron transport chain to O_2, whereas the reduction of a molecule of O_2 to water is a 4-electron change, the usual convention is to express the rates and amounts of O_2 uptake in μatoms of O_2 (μmoles of $O_2 \times 2$).

Since the O_2 uptake during the rapid phase (state *3*) of the reaction is proportional to the amount of ADP added and the conversion of added ADP to ATP in this phase of the reaction is nearly quantitative, the efficiency of the oxidative phosphorylation reaction can be estimated (7). In the illustration (Fig. 8), addition of 2.0 μmoles of ADP resulted in an accelerated rate of respiration which consumed a total of 0.72 μatoms of O_2. The ADP/O ratio (which is equivalent to the P/O or $P/2e^-$ ratio) for this experiment is therefore equal to 2.0/0.72, or 2.8.

The reaction medium for mitochondrial oxidations varies considerably from laboratory to laboratory. Most investigators employ isotonic sucrose or saline containing a phosphate buffer. Most mitochondrial preparations require P_i as well as ADP to establish a state *3* rate although exceptions have been noted. The heavy beef heart mitochondrial preparation of Hatefi and Lester (53), for example, contains 30–50 μmoles of endogenous P_i/mg of protein and shows little requirement for added P_i in the initial deflections induced by the addition of ADP. Many investigators obtain better results with the addition of components such as Mg^{2+}, serum albumen, and EDTA to the reaction medium. Our own experience would indicate that the simplest possible suspending medium is often an advantage in interpreting results, and we would therefore recommend that the requirement for additional reagents be established experimentally for the preparation of mitochondria to be studied. The optimal concentration of substrate should also be established experimentally in the medium employed. Most mitochondrial oxidations involve a number of sequential enzymic reactions so that the overall reaction which occurs may be quite complex. Tarjan and Von

Korff (54), for example, have established that the respiratory control ratio of rabbit heart mitochondria is affected by the presence of endogenous substrates, by the nature of the endogenous substrates, by the concentration of ATP, and by the concentration of mitochondria in the reaction mixture. With beef heart mitochondria, levels of 1 mg protein/ml medium support a rate of respiration sufficiently rapid to make diffusion of O_2 into the measuring chamber a minor source of error. Higher concentrations of mitochondrial protein must be added when preparations with less vigorous respiratory activity are employed.

B. ION TRANSPORT EFFICIENCY

Chance (55) noted that addition of small amounts of Ca to suspensions of respiring mitochondria produced elevated rates of respiration and, as in the case of ADP addition, the duration of the elevated oxidation rate was proportional to the amount of Ca^{2+} ion added. Chappell and co-workers (56) noted a similar response to Mn^{2+} ion addition. Subsequent studies in a number of laboratories have attempted to relate the Ca^{2+} induced respiration increment to the efficiency of accumulation of Ca by the mitochondria [see Lehninger et al. (44) for a review]. These ratios have been found to vary extensively with the conditions of measurement and to depend on pH, the prior treatment of the mitochondria, and the type and concentration of cations in the suspending medium. The efficiency of other respiration-dependent mitochondrial ion transport reactions, such as the uptake of Mg^{2+} in the presence of parathyroid hormone (57) and the accumulation of K^+ induced by valinomycin (58), has been estimated by similar techniques.

C. MULTIPLE PARAMETER MEASUREMENTS

Respiration measurements with the oxygen electrode can be combined with the simultaneous determination of a number of other parameters by incorporating other electrodes and optical systems in the measuring chamber. A number of investigators (57–61) have found such multiple-parameter measuring systems to be extremely helpful in studies of mitochondrial ion transport and other reactions. Pressman (59) has discussed the use of specific ion electrodes in these reactions and has given the details for constructing a multiple-parameter measuring system. Various combinations, including measurement of pH with a glass electrode, measurement of concentrations of K^+, Na^+, NH_4^+, and Ca^{2+} using ion-specific electrodes, and measurement of light scattering, absorbance, and fluorescence have been used effectively in conjunction with O_2-electrode studies of respiration. Such measurements have been

applied to ascites cell and microbial suspensions as well as to isolated mitochondrial preparations and show great promise in helping to define events in these more complicated systems (62,63).

D. FIRST DERIVATIVE OF O_2 UPTAKE

Circuits have been designed (59,60) which permit the recording of the first derivative of the output from an O_2 electrode. This method of recording is often useful in detecting small changes in the rate of oxidation. For example, Pressman (59) cited an experiment in which marked oscillation of the K^+ concentration and light scattering traces occurs in suspension of mitochondria treated with valinomycin. A corresponding change in O_2 rate was not seen in traces of O_2 uptake but was readily apparent in the first derivative trace.

E. MITOCHONDRIAL FRAGMENTS AND COMPLEXES

Phosphorylating fragments of mitochondria can be prepared by sonic irradiation or mechanical fragmentation. These particles are usually loosely coupled and show little if any respiratory control when assayed by procedures similar to those just outlined for mitochondrial oxidations. Lee and Ernster (45) have pointed out that addition of oligomycin to such a preparation respiring with DPNH as substrate results in marked inhibition of respiration, and this inhibition can be relieved by the subsequent addition of dinitrophenol. This type of respiratory control is extremely sensitive to the concentration of Mg^{2+} present in the medium (45) and can be useful in estimating the coupling potential of various submitochondrial particle preparations.

Polarographic procedures have also been used successfully to evaluate the rate of oxidation of isolated enzyme complexes such as DPNH oxidase and cytochrome oxidase. Wharton and Griffiths (64) compared manometric, polarographic, and spectrophotometric assays for isolated cytochrome oxidase, and found that the three procedures were in agreement, provided phospholipid sols were added to the manometric assay to prevent enzyme aggregation, and that specific activities were extrapolated to infinite concentration of cytochrome *c*.

2. Other Oxidation Reactions

A number of nonmitochondrial oxidation reactions have been studied with polarographic procedures. The TPNH-dependent O_2 consumption stimulated by the microsomal *N*-demethylation of drugs such as aminopyrine has been studied by Ernster and Orrenius (65). A metal-

catalyzed lipid peroxidation which is activated by ADP has also been studied in suspensions of microsomes using a Clark electrode [Hochstein et al. (66)]. This reaction is inhibited by the oxidative demethylation reaction (67) and it has been suggested that the two processes compete for a common TPNH-oxidizing enzyme. The O_2 electrode also has been used in the study of oxygenases and hydroxylases from the cytosol (soluble fraction) of various tissues.

3. Oxygen Evolution Reactions

Several reports have appeared describing application of polarographic procedures to the measurement of O_2 evolution. For example, Shugarman and Appleman (68) have recently described the construction and use of a Lucite chamber and a Clark electrode for estimation of photosynthetic O_2 evolution in *Chorella*. Measurement of photosynthetic O_2 evolution, and dark-period respiration can be completed in less than 10 min using the modifications described. Earlier studies of quantum efficiency and the action spectra of various phases of photosynthesis by Haxo and Blinks (69), Blinks (70), Myers and French (71), and others have made use of O_2 electrodes. An experimental arrangement for such studies was designed by Myers and Graham (72).

Cockburn et al. (73) studied the O_2 evolution of isolated chloroplasts with CO_2 as the hydrogen acceptor. The O_2 electrode has also been used to determine catalase activity and the production of H_2O_2 in biochemical reactions (74). By titrating with H_2O_2, catalase activity can be accurately determined while H_2O_2 production can be assayed by the evolution of O_2 when known amounts of catalase are added to the reaction mixture.

VII. SUMMARY

The extensive use of O_2 electrodes for biochemical and other applications has led to the design of a number of excellent systems. There is little doubt that better electrodes are in the process of development, and that both reliability and response time will be improved. The construction of reaction vessels which can be used in spectrophotometers or fluorometers, and which can accept the Clark-type O_2 electrode along with pH and specific ion electrodes, will enable investigators to obtain continuous information on several parameters of a reaction while measuring O_2 utilization or evolution.

The most significant design improvements in O_2 electrodes for monitoring biochemical activities are:

a. Reduction in the diameter and mass of the cathode and the positioning of a shaped Ag anode in close proximity to the cathode.

b. Covering the cathode and anode with a thin, tightly stretched, O_2-permeable membrane to protect the electrode surfaces, and separate the electrical circuit from the experimental solution.

c. Reduction in the volume of electrolyte in a recessed electrode or under the membrane of a Clark-type electrode.

d. Improvements in the technique for obtaining uniformly stretched membranes and keeping them securely attached to the electrode.

e. Development of O_2 microelectrodes (cathode of 1–50 μ diameter) which are relatively insensitive to convection and stirring artifacts.

The construction of stable O_2 microelectrodes has led to the possibility of rapid O_2 measurements in very small volumes. This permits O_2 studies with very small amounts of active material, and can be used to follow the course of separations and purifications. In other areas, systems with the O_2 electrode, the CO_2 electrode, and the combined pH electrode have been successfully used for the determination of blood gas and acid–base parameters of small blood samples (9,15,16). The O_2 microelectrode has also been used for *in vivo* measurements of tissue respiration of cornea (12), brain and other tissues (16), as well as for O_2 measurement both on and in blood vessels. Oxygen electrodes have also been designed for the rapid assay of the O_2 content of streams and sewage, as well as for the determination of bacterial action on sewage.

Acknowledgments

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References

1. I. Fatt, *J. Appl. Physiol., 19,* 326 (1964).
2. J. Danneel, *A. Elektrochem., 4,* 227 (1897).
3. J. Heyrovsky and M. Shikata, *Rec. Trav. Chim., 44,* 496 (1925).
4. P. W. Davis, "The Oxygen Cathode," in *Physical Techniques in Biological Research,* Vol. 4, W. H. Nastuk, Ed., Academic Press, New York, 1962, p. 137.
5. L. R. Blinks and R. K. Skow, *Proc. Natl. Acad. Sci. U.S., 24,* 420 (1938).
6. H. G. Petering and F. Daniels, *J. Am. Chem., Soc., 60,* 2796 (1938).
7. B. Chance and G. R. Williams, *J. Biol. Chem., 217,* 383 (1955).
8. M. Schwartz, *Biochim. Biophys. Acta, 131,* 548 (1967).
9. J. W. Severinghaus and A. F. Bradley, *J. Appl. Physiol., 13,* 515 (1958).
10. J. B. Stoner, *Brit. J. Exptl. Pathol., 44,* 384 (1963).
11. C. M. Connelly, *Federation Proc., 16,* 681 (1957).

12. R. M. Hill and I. Fatt, *Science, 142*, 1295 (1963).
13. D. Glick, *Quantitative Chemical Techniques of Histo- and Cytochemistry*, Vol. 1, Interscience, New York, 1961, p. 302.
14. J. P. Payne and D. W. Hill, *Oxygen Measurements in Blood and Tissues and Their Significance*, Little, Brown, Boston, 1966.
15. W. Feder, Ed., "Bioelectrodes," in *Ann. N.Y. Acad. Sci., 148*, Art. 1 (1968).
16. D. W. Lübbers, *Intern. Anesth. Clin., 4*, 103 (1966).
17. B. Hagihara, *Biochim. Biophys. Acta, 46*, 134 (1961).
18. B. Chance and G. R. Williams, *Nature, 175*, 1120 (1955).
19. J. B. Chappell, *Biochem. J., 90*, 225 (1964).
20. P. W. Davies and F. Brink, Jr., *Rev. Sci. Instr., 13*, 524 (1942).
21. I. Fatt, *J. Appl. Physiol., 19*, 550 (1964).
22. B. Chance and B. Hess, *Science, 129*, 700 (1959).
23. L. C. Clark, *Trans. Am. Soc. Artificial Internal Organs, 2*, 41 (1956).
24. M. A. Lessler, E. Molloy, and C. M. Schwab, *Federation Proc., 24*, 336 (1965).
25. A. Michaels, *J. Polymer Sci., 50*, 393 (1961).
26. R. M. Hill and I. Fatt., *J. Am. Optometric Assoc., 35*, 873 (1964).
27. R. M. Hill and I. Fatt, *Nature, 200*, 1011 (1963).
28. F. G. Carey and J. M. Teal, *J. Appl. Physiol., 20*, 1074 (1965).
29. U. Gleichmann and D. W. Lübbers, *Arch. Ges. Physiol., 271*, 431 (1960).
30. I. M. Kolthoff and J. J. Lingane, *Polarography*, Interscience, New York, 1952.
31. I. M. Kolthoff and J. Jordan, *J. Am. Chem. Soc., 74*, 4801 (1952).
32. C. M. Connelly, D. W. Bronk, and F. Brink, *Rev. Sci. Instr., 24*, 683 (1953).
33. I. A. Silver, *Intern. Anesth. Clin., 4*, 135 (1966).
34. I. Fatt, *Ann. N.Y. Acad. Sci., 148*, 81 (1968).
35. J. O. Alben, Ph.D. thesis, University of Oregon Medical School, Portland, Oregon, 1959.
36. L. C. Clark and C. Lyons, *Ann. N. Y. Acad. Sci., 102*, 29 (1962).
37. F. D. Carlsen, F. Brink, and D. W. Bronk, *Rev. Sci. Instr., 21*, 923 (1950).
38. W. W. Umbreit, R. H. Burris, and J. F. Stouffer, *Manometric Methods*, 4th ed., Burgess, Minneapolis, 1964.
39. A. L. Lehninger, *The Mitochondrion*, Benjamin, New York, 1964.
40. J. M. Tager, S. Papa, E. Quagliariello, and E. C. Slater, Eds., *Regulation of Metabolic Processes in Mitochondria*, Elsevier, Amsterdam, 1966.
41. M. E. Pullman and G. Schatz, *Ann. Rev. Biochem., 36*, 539 (1967).
42. R. W. Estabrook and M. E. Pullman, Eds., *Methods in Enzymology*, Vol. 10, Academic Press, New York, 1967.
43. E. Quagliariello, S. Papa, E. C. Stater, and J. M. Tager, Eds., *Mitochondrial Structure and Compartmentation*, Adriatica Editrice, Bari, Italy, 1967.
44. A. L. Lehninger, E. Carafoli, and C. S. Rossi, *Advan. Enzymol., 29*, 259 (1967).
45. C. P. Lee and L. Ernster, in *Regulation of Metabolic Processes in Mitochondria*, J. M. Tager, S. Papa, E. Quagliariello, and E. C. Slater, Eds., Elsevier, Amsterdam, 1966, p. 218.
46. E. C. Slater, in *Methods in Enzymology*, Vol. 10, R. W. Estabrook and M. E. Pullman, Eds., Academic Press, New York, 1967, p. 166.
47. G. Lenaz and R. E. Beyer, *J. Biol. Chem., 240*, 3653 (1965).
48. J. M. Halsam, *Biochim. Biophys. Acta, 105*, 184 (1965).
49. G. P. Brierley, *Biochem. Biophys. Res. Commun., 19*, 500 (1965).

50. R. W. Estabrook, in *Methods in Enzymology*, Vol. 10, R. W. Estabrook and M. E. Pullman, Eds., Academic Press, New York, 1967, p. 41.
51. Y. Hatefi, P. Jurtshuk, and A. G. Haavik, *Arch. Biochem. Biophys.*, *94*, 148 (1961).
52. G. P. Brierley, C. T. Settlemire, and V. A. Knight, *Arch. Biochem. Biophys.*, *126*, 276 (1968).
53. Y. Hatefi and R. L. Lester, *Biochim. Biophys. Acta*, *27*, 83 (1958).
54. E. M. Tarjan and R. W. Von Korff, *J. Biol. Chem.*, *242*, 318 (1967).
55. B. Chance, *Proc. Intern. Congr. Biochem.*, *3rd*, Brussels, *1955*, 1956, 300.
56. J. B. Chappell, M. Cohn, and G. D. Greville, in *Energy-Linked Functions of Mitochondria*, B. Chance, Ed., Academic Press, New York, 1963, p. 219.
57. H. Rasmussen and E. Ogata, *Biochemistry*, *5*, 733 (1966).
58. B. C. Pressman, *Proc. Natl. Acad. Sci. U.S.*, *53*, 1076 (1965).
59. B. C. Pressman, in *Methods in Enzymology*, Vol. 10, R. W. Estabrook and M. E. Pullman, Eds., Academic Press, New York, 1967, p. 714.
60. J. B. Chappell and A. R. Crofts, *Biochem. J.*, *95*, 393 (1965).
61. G. P. Brierley and C. T. Settlemire, *J. Biol. Chem.*, *242*, 4234 (1967).
62. C. E. Wenner, E. J. Harris, and B. C. Pressman, *J. Biol. Chem.*, *242*, 3454 (1967).
63. W. Hempfling, M. Hofer, E. J. Harris, and B. C. Pressman, *Biochim. Biophys. Acta*, 141, 391 (1967).
64. D. C. Wharton and D. E. Griffiths, *Arch. Biochem. Biophys.*, *96*, 103 (1962).
65. L. Ernster and S. Orrenius, *Federation Proc.*, *24*, 1190 (1965).
66. P. Hochstein, K. Nordenbrand, and L. Ernster, *Biochem. Biophys. Res. Commun.*, *14*, 323 (1964).
67. S. Orrenius, G. Dallner, and L. Ernster, *Biochem. Biophys. Res. Commun.*, *14*, 329 (1964).
68. P. M. Shugarman and D. Appleman, *Anal. Biochem.*, *18*, 193 (1967).
69. F. T. Haxo and L. R. Blinks, *J. Gen. Physiol.*, *33*, 389 (1950).
70. L. R. Blinks, *Proc. Natl. Acad. Sci. U.S.*, *46*, 327 (1960).
71. J. Myers and C. S. French, *J. Gen. Physiol.*, *43*, 723 (1960).
72. J. Myers and J. Graham, *J. Plant Physiol.*, *38*, 1 (1963).
73. W. Cockburn, C. W. Baldry, and D. A. Walker, *Biochim. Biophys. Acta*, *131*, 594 (1967).
74. M. Rorth and P. K. Jensen, *Biochim. Biophys. Acta*, *139*, 171 (1967).

Separation and Determination of Bile Pigments

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