CHAPTER 5

Electron Transport and Redox Titration

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1. INTRODUCTION

Photosynthesis is essentially a light-driven oxidation-reduction reaction involving coupled synthesis of ATP. Even a whole-plant physiologist measuring leaf CO_2 uptake can therefore be said to be engaged in measurement of electron transport by indirect means. This chapter is restricted to more direct, continuous measurement of electron transport through pathways located primarily in the chloroplast thylakoid membrane system, and with redox titration of individual components of such pathways. Measurement of electron transport in intact chloroplasts and in leaf discs is also discussed. Assay of some individual electron carriers is covered in Chapter 4, while ATP synthesis coupled to electron transport is described in Chapter 6.

1.1 **Electron Transfer**

When one or more electrons are transferred from a species A to a species B, A becomes oxidised and B becomes reduced.

$$A_{red} + B_{ox} \xrightarrow{ne^-} A_{ox} + B_{red}$$

In such an oxidation-reduction ('redox') reaction A may be described as the electron donor or reductant and B as the electron acceptor or oxidant: A *reduces* B and B *oxidises* A. The whole transfer may be viewed as the sum of two half-reactions, oxidation of the donor, A

$$A_{red} \rightarrow A_{ox} + ne^-$$

and reduction of the acceptor, B

$$B_{ox} + ne^- \rightarrow B_{red}$$

Any particular half-reaction, or couple, has a specific mid-point redox potential which depends on the identity of the chemical species involved. The direction of electron transfer as any two half-reactions approach equilibrium can be predicted from their individual mid-point redox potentials — the donor will be the species with the half-reaction of lower potential. The mid-point redox potential at pH 7.0 (i.e. the standard mid-point redox potential) is written E_{m7}. These terms are more rigorously defined in Section 3.1. Measurement of E_{m7} for any electron carrier is covered in Section 3.

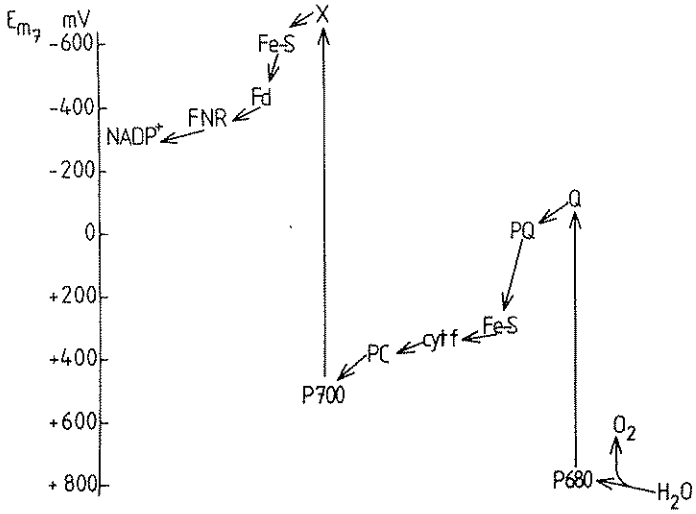


Figure 1. The 'Z-scheme' for non-cyclic electron transport in photosynthesis.

1.2 The Electron Transport Chain of Plant Photosynthesis

The Z-scheme for photosynthetic electron transport (*Figure 1*) is obtained simply by plotting the sequence of electron carriers on a vertical scale of E_{m7}. Two abrupt drops in E_{m7} correspond to the two photochemical reactions of plant-type photosynthesis, and the Z-scheme model when first proposed (1) provided an explanation for a number of lines of evidence suggesting that two such light reactions are linked in series. In fact this model applies only to the electron transport chains of green plants, algae and cyanobacteria; the purple and green photosynthetic bacteria have single photosystems driving cyclic or non-cyclic electron transport. Although this volume is concerned largely with green-plant photosynthesis, bacterial photosynthesis has played an essential role in the development of this subject (2) and comparisons of plants and bacteria are continuing to be highly informative for students and research workers alike (3).

In the Z-scheme model the two photochemical reactions are driven by distinct pigment systems, and are termed photosystem I (PSI, the first to be discovered) and photosystem II (PSII). PSI drives electron transport from plastocyanin (or a *c*-type cytochrome in certain algae) at $E_{m7} = +390$ mV to ferredoxin at $E_{m7} = -420$ mV. PSII drives electron transport from water at $E_{m7} = +820$ mV to plastoquinone at $E_{m7} = 0$ mV. Electron transport from plastoquinone occurs 'downhill' through a cytochrome complex which therefore links PSI and PSII. Electron transport from ferredoxin to NADP$^+$ at $E_{m7} = -320$ mV occurs 'downhill' through a flavoprotein, ferredoxin-NADP reductase. Certain electron carriers associated with each photosystem have even lower E_{m7} values than plastoquinone and ferredoxin. These participate in the primary photochemical reactions and are probably phaeophytin together with certain plastoquinone species (PSII) and chlorophyll together with additional iron-sulphur species (PSI).

The whole electron transport chain therefore requires a minimum of two quanta of radiation (one for each photosystem) in order to drive each electron uphill through a total redox span of 1140 mV. The end products are oxygen, NADPH and ATP. It is also clear that PSI can pass electrons to plastoquinone and hence to itself, thereby driving a cyclic electron transport pathway the end-product of which is ATP alone.

With this minimum of background information on the electron transport chain it should be possible for the reader to make sense of the techniques of measurement to be described. Adoption of a practical approach will not, of course, remove the need to begin by asking the right sort of question.

Familiarity with the field of investigation through reviews (4) and primary research articles is presumably a minimal requirement for the design of informative experiments.

2. MEASUREMENT OF ELECTRON TRANSPORT

2.1 **Principle of Measurement: the Hill Reaction**

Non-cyclic electron transport from water to any acceptor through all or part of the chain depicted in *Figure 1* can be described in a single equation:

$$H_2O + A \xrightarrow{2e^-} AH_2 + \tfrac{1}{2}O_2$$

This is one formulation of the Hill reaction, which is the light-dependent evolution of oxygen by isolated chloroplasts. It requires reduction of any suitable added electron acceptor, or 'Hill oxidant', represented by A in the equation.

Measurement of non-cyclic electron transport can therefore take the form of measurement of the oxygen produced, or of reduction of the electron acceptor. Both these principles of measurement are widely used in photosynthesis research, though oxygen evolution is obviously impossible for related reactions where an artificial electron donor replaces water. Measurement of reduction of the acceptor is also preferred where the stoichiometry of net oxygen evolution is in doubt (due perhaps to competing oxygen uptake). With these two exceptions, measurement of oxygen evolution is generally the most convenient and widely used principle of measurement.

2.2 **Comparison of Techniques**

As indicated above, measurement of oxygen evolution or uptake is frequently the method of choice for determining electron transport rates. Before considering the oxygen electrode in detail (Section 2.3), we briefly review the other techniques which are available for measuring electron transport.

2.2.1 *Manometry*

Before the 1960s, most measurements of electron transport involved manometric techniques of one kind or another, and high sensitivity and accuracy could be obtained by skilled practitioners of the art. On the whole the oxygen electrode (Section 2.3) has replaced the Warburg manometer as the standard method of measuring photosynthetic electron transport, and such special applications of manometry that remain are likely to be displaced by the increasing range and versatility of polarographic sensors. Anyone determined to use manometry is referred to the comprehensive treatise of Umbreit *et al.* (5).

2.2.2 *Spectrometry*

Optical absorbance, fluorescence and electron paramagnetic resonance spectroscopy are all used extensively in photosynthesis research and their major applications are described in Chapter 4. Infra-red gas analysis (IRGA) is a favoured method of measuring carbon dioxide uptake, having replaced the manometric techniques originally employed in this area, though many routine IRGA measurements are now likely to be replaced by the simpler and less expensive leaf-disc oxygen electrode (Section 2.8) or by more direct polarographic measurement of CO_2. Mass spectrometry using [^{18}O]- and [^{16}O]oxygen was important historically in providing decisive evidence for photolysis of water as the source of evolved oxygen, and continues to be invaluable where it is necessary to discriminate between oxygen uptake and oxygen evolution in the same experimental system (6).

A more recently developed technique for measuring photosynthetic electron transport is photoacoustic spectrometry, where modulated light provides pulses of oxygen evolution which may be detected as pressure changes with a microphone. The acoustic signal is amplified by a phase-sensitive detector which is locked to the frequency of the modulated light. Photoacoustic measurements of complex transients (e.g. state 1 − state 2 transitions; see Chapter 4) are now possible, and the technique provides a promising, non-invasive way of measuring photosynthetic yield in intact systems (e.g. leaves) directly rather than by having to infer it from (equally non-invasive) fluorescence yield (7). A photoacoustic sensor is available from Applied Photophysics, London (see Appendix to chapter).

It is likely that ion-selective electrodes will become increasingly used in measurement of photosynthetic electron transport. As indirect measurements, both carbon dioxide and nitrate or nitrite electrodes have been used in algae, bacteria and intact chloroplasts and protoplasts. A sulphide electrode has been used for electron transport measurement in green bacteria. The chief advantage of polarographic techniques is that they provide continuous measurement: they are also comparatively cheap and easy to use. It is likely that the new technology of biosensors will produce even more selective and useful polarographic techniques in due course. Meanwhile, the most useful polarographic technique, at least for plant-type photosynthesis, is the oxygen electrode (Section 2.3).

2.2.3 *Absorption Changes of an Added Electron Acceptor on its Reduction in the Hill Reaction*

A suitably buffered chloroplast suspension prepared by the techniques described in Chapter 2 will reduce any one of a range of electron acceptors (Hill oxidants) in the light. A low-cost way of demonstrating the electron transport activity of isolated chloroplasts is to illuminate the chloroplast suspension for a period of time (say 10 min) and to measure an optical absorbance change caused by reduction of the electron acceptor. Suitable controls are obtained by leaving an identical sample in the dark or by illuminating another sample in the presence of the electron transport inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) at a final concentration of at least 10 μM.

One widely used electron acceptor is the dye 2,6-dichlorophenolindophenol (DCPIP or DCIP). The oxidised form of DCPIP has a broad red absorbance band centred at 590 nm and appears blue. On reduction the red absorbance band disappears and the solution becomes colourless — this can be demonstrated by adding the reductant ascorbic

Table I. Properties of Three Electron Acceptors Whose Reduction can be Measured by Optical Absorbance.

Acceptor	E_{m7} *(mV)*	λ *max (nm)*	*Millimolar extinction coefficient* (10^{-3} M^{-1} cm^{-1})	*Molecular weight*	*Soluble in*
DCPIP	+217	590 (oxidised form)	16	290 (disodium salt)	Ethanol
Potassium ferricyanide[a]	+430	420 (oxidised form)	1.04	329	Water
$NADP^+$	−320	340 (reduced form)	6.22	743	Water (most stable at neutral pH)

[a] See ref. 22.

acid to a blue DCPIP solution. Oxidised DCPIP has a high enough extinction coefficient for it to be feasible to measure electron transport as a decrease in red absorbance (at around 590 nm) in a complete reaction mixture with chloroplasts still present. Thus 3 ml of chloroplast (thylakoid) suspension containing 0.1 mM DCPIP in a standard 10 mm cuvette will have an absorbance of about 1.5 at 590 nm relative to a reference cuvette containing no DCPIP. A chloroplast concentration equivalent to 30 μg of chlorophyll will give an absorbance decrease in the region of 0.1 min^{-1} as the DCPIP becomes reduced at reasonable light intensity (e.g. a desk lamp at 50 cm, preferably behind a bottle of water to act as a heat filter). DCPIP, like DCMU, is only sparingly soluble in water, and a stock solution of 0.1 M is best made up in ethanol, taking care to keep the final ethanol concentration in the reaction mixture below 1% (it would be 0.1% in the above example).

Other electron acceptors that can be used in this way are ferricyanide and $NADP^+$. $NADP^+$ is of course the physiological Hill oxidant, and for thylakoid preparations the soluble electron carrier ferredoxin at 5 μM must be added to replace that lost by dilution during the chloroplast isolation. With pea thylakoids it has been found that it is necessary to add ferredoxin-$NADP^+$ reductase as well. A suitable reaction mixture can be devised from the extinction coefficients given in *Table 1*. All three acceptors of *Table 1* are effectively PSI acceptors in intact thylakoids. Ferricyanide and DCPIP will also act as PSII acceptors in sub-chloroplast particles: a kinetic barrier prevents their reduction in intact thylakoids. Mild trypsin digestion (Section 2.5.2) removes this barrier along with the DCMU-sensitivity of their photoreduction. For ferricyanide (absorbing at 420 nm) and NADP (absorbing at 340 nm) it is not usually practical using simple spectrophotometers to measure reduction in the presence of chloroplasts with their strong light-scattering as well as chlorophyll absorbance band at these wavelengths, so it is necessary to spin down the chloroplasts in a bench centrifuge and measure the absorbance change of the supernatant. Ferricyanide and $NADP^+$ reduction can however be measured directly using dual-wavelength spectrophotometry (see Chapter 4).

With fibre-optic actinic illumination it is now quite simple to make continuous measurement of reduction of many electron acceptors, provided care is taken to use an appropriate combination of optical filters to define the actinic beam and to shield the detecting system of the spectrophotometer. Thus for DCPIP reduction a blue actinic beam can be used in conjunction with a red filter covering the entrance to the photomultiplier housing. The absorption band of DCPIP is also sufficiently broad for a long-pass red filter (e.g. a 663 nm cut-off filter which has 50% transmission at 663 nm and transmits more at longer wavelengths; Appendix II) to be used for the actinic beam with the photomultiplier protected by a broad-band blue filter such as a Corning 4-96.

The tendency of chloroplasts and thylakoids to sediment from the suspension during measurement can be overcome by means of an electronic stirring head in the base of the cuvette holder with a magnetic follower ('flea') in the cuvette. An electronic stirrer which fits under a standard 1 cm cuvette is manufactured by Rank Brothers, Bottisham, Cambridge. Use of an electronic stirrer and magnetic 'flea' inevitably generates noise in the absorbance signal, but if the flea is kept below the optical light-path this disadvantage is more than offset by the elimination of drift caused by sedimentation and by the opportunity for thorough mixing of reagents added during the course of the reaction.

2.3 **The Liquid-phase Oxygen Electrode**

Various types of oxygen electrode have been used in photosynthesis research for over 40 years. A rapidly-responding oxygen electrode used with a modulated light source is most useful for measuring rapid transients (8). For steady-state measurements, however, a combined oxygen electrode and reaction vessel is now widely used in photosynthesis research. Commercially produced models are available from Hansatech, King's Lynn, and Rank Brothers, Bottisham, and 'when all else fails, read the manufacturer's instructions' is as sound a principle here as elsewhere. The reader is also referred to the article of Delieu and Walker (9) on whose design the Hansatech electrode is based. The Rank electrode is derived from a design of Chappell, but the same general principles apply since both models are variants of the Clark oxygen electrode, using a platinum cathode and a silver anode connected by a KCl bridge.

2.3.1 *Principles and Construction*

A block diagram of a basic oxygen electrode set-up is given in *Figure 2*. The oxygen electrode chamber is kept at constant temperature by circulating water from a thermostatically-controlled water bath through a transparent water jacket which surrounds the chamber. Electron transport in isolated thylakoids is generally most stable at 15°C, though intact chloroplasts work better at 20 or 25°C. A slide projector is a good, cheap light source but a bottle of water or other heat filter should be placed between the projector and the electrode unit in order to minimise heating effects. Heating should be avoided for two reasons: firstly oxygen solubility is temperature-dependent, and secondly there is the obvious need to keep the reaction mixture at constant temperature. The oxygen electrode disc contains the anode and cathode which are maintained at a constant potential difference by a control box (usually battery powered). The control box also has an output to a chart recorder. A simple potentiometric strip-chart recorder (Y/t recorder) will then record oxygen concentration as a function of time.

Figure 3 shows assembled Hansatech and Rank oxygen electrodes; *Figure 4* shows the electrode discs. In both cases the electrical current passing between the anode and cathode varies linearly with the oxygen concentration in the vicinity of the cathode, where oxygen is reduced electrochemically.

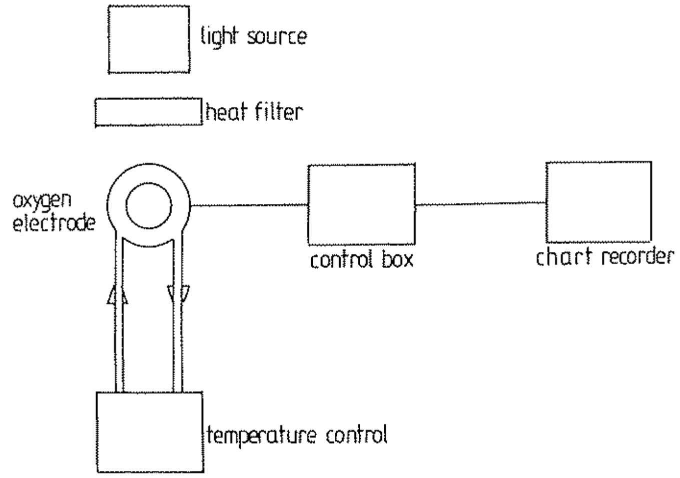


Figure 2. General layout of an oxygen electrode system.

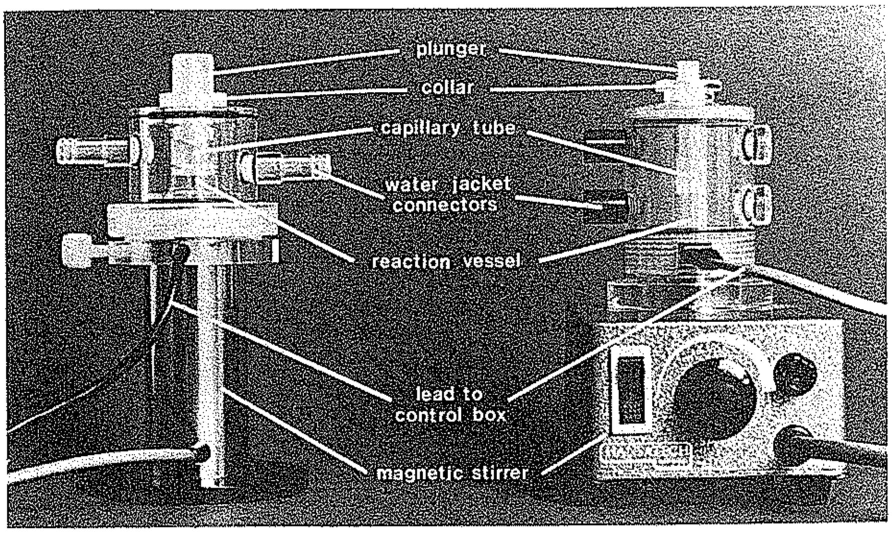


Figure 3. Rank **(left)** and Hansatech **(right)** oxygen electrodes, assembled and mounted on magnetic stirrers.

At the cathode:

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

At the anode:

$$4\ Ag \rightarrow 4\ Ag^+ + 4e^-$$
$$4\ Ag^+ + [4\ Cl^-] \rightarrow 4\ AgCl$$

The KCl solution in the vicinity of the platinum cathode is separated from the reaction mixture by a thin membrane (usually of Teflon) which is freely permeable to oxygen.

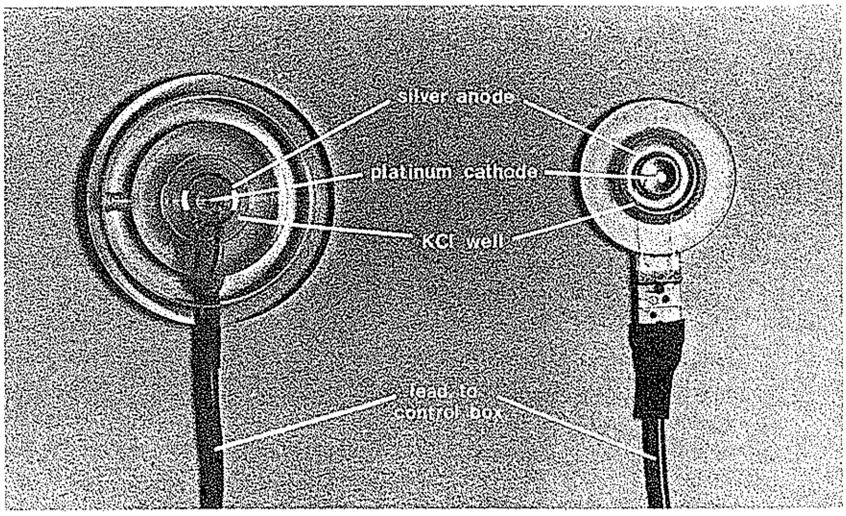


Figure 4. Rank **(left)** and Hansatech **(right)** oxygen electrode discs.

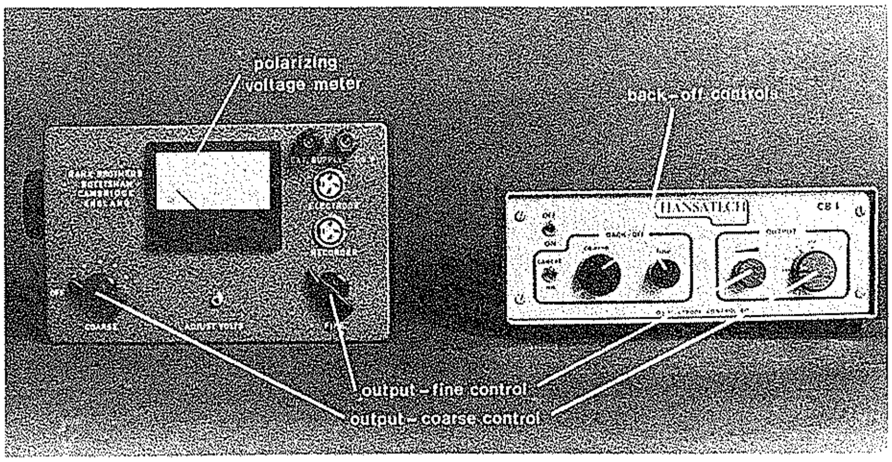


Figure 5. Rank **(left)** and Hansatech **(right)** oxygen electrode control boxes.

The current flowing is therefore proportional to the oxygen concentration in the reaction mixture, with the response time of the apparatus (a few seconds) being limited by diffusion of oxygen from the stirred reaction vessel to the cathode.

The response time decreases with increased cathode surface area [the rapidly responding 'Joliot' electrode used for flash-yield measurements (8) has a large surface area in direct contact with the sample suspension] but increased surface area also increases consumption of oxygen by the cathode itself. For continuous measurements a small cathode is preferred so that electrochemical consumption of oxygen is negligible compared with photosynthetic rate.

Control boxes (*Figure 5*) normally have three basic controls:

(i) A control for the polarising voltage applied between the anode and cathode. This should be set for 650 – 700 mV. The polarising voltage is pre-set on some control boxes.
(ii) A gain or 'output' control (or combination of coarse and fine controls) for adjusting the scale of the output to the chart recorder.
(iii) A zero-suppression or 'back-off' control for adjusting the zero position on the chart recorder without affecting the scale.

It is important that the control box is compatible with the chart recorder. Most control boxes require a fairly sensitive recorder, ideally with a minimum input of 1 mV, whereas one recent control box (Hansatech CB1) will actually give an output of about 1 V. Multiple recorder inputs selected by a switch (say 1, 5, 10, 20, 50, 100 mV, etc.) are useful for changing the expansion factor by a known amount: for example, switching from 10 mV to 1 mV input to give a 10-fold scale expansion.

2.3.2 *Setting Up*

The following instructions for setting up the oxygen electrode are based on those written for well-tried class practical schedules.

(i) Take the electrode disc and cover the silver cathode completely with a few drops of saturated KCl solution. The well in which the anode sits should contain a continuous layer of KCl solution, but should not be over-filled to the extent that KCl solution spills over the rest of the disc.
(ii) Place a further drop of KCl solution on the platinum cathode. Place a small (~2 cm) square of cigarette paper or lens tissue on the cathode, and a similar sized square of Teflon membrane on top of that. The membrane should not protrude over the edge of the well. The tissue acts as a 'spacer' to maintain a constant layer of KCl solution between cathode and membrane.
(iii) Push a rubber O-ring over the raised projection (containing the cathode) in order to fasten the membrane in position. Avoid trapping air bubbles under the membrane.
(iv) Fasten the disc, now complete with membrane, into the base of the reaction vessel by screwing up the collar. Take care to avoid rotating the disc relative to the reaction vessel as this can tear the membrane. The collar needs to be quite firmly hand-tight so that the rubber ring forms an effective gasket between the reaction vessel and the well in the electrode disc (undertightening is a common fault for beginners).

The Rank oxygen electrode has a single rubber ring which serves as a gasket as well as to hold the membrane in place. The Hansatech oxygen electrode has separate rings for each of these functions, as well as a third ring of larger diameter to prevent leakage of the KCl solution onto the outside of the electrode unit.

(v) Pipette a few millilitres of distilled water into the reaction vessel — if this is left empty for long periods the KCl solution will dry up and the membrane will have to be replaced.

(vi) Connect the electrode to the control box, and the control box to the chart recorder. Place the electrode over a magnetic stirrer and put a magnetic follower or 'flea' in the reaction vessel. Do *not* place the cap or plunger in position yet, the water in the reaction vessel should remain open to the air for equilibration.

(vii) Switch on the magnetic stirrer and set it to give smooth rotation of the flea. Switch on the chart recorder and the water supply to the water jacket. Adjust the zero position on the chart recorder so that the pen records zero deflection with the input selector on zero. Then select an appropriate input sensitivity (usually 10 mV; 1 V on the Hansatech CB1).

(viii) Switch on the control box, and adjust the polarising voltage, if necessary, so that the meter (if present) reads 650–700 mV. Turn the coarse output control clockwise (a few steps if stepped) until the pen on the chart recorder reaches 60–80% of full-scale deflection. The pen will drift slightly and the rate of drift will decrease with time. It should have stopped completely after 10–15 min at the most.

(ix) When the drift has stopped, use a combination of the coarse and fine output controls to bring the pen to read 100%, that is, full-scale deflection. The distilled water in the reaction vessel will now have a known concentration of dissolved oxygen, since it will have equilibrated with the air. The reading of 100% on the chart recorder now corresponds to the concentration of oxygen in air-saturated distilled water at the defined temperature and at normal atmospheric pressure. *Table 2* gives oxygen concentrations at various temperatures. In order for the calibration of the oxygen electrode to remain constant (at 100% deflection for this oxygen concentration) the output controls should not be changed at all during the experiment.

(x) Check the response of the oxygen electrode as follows. Set the chart moving at about 30 mm min^{-1}, drop the pen, and add a few milligrams of solid sodium dithionite (just enough to cover the end of a microspatula) to the water in the reaction vessel. This quickly reduces all the dissolved oxygen. An alternative to adding dithionite is to bubble the water with nitrogen gas from a cylinder. In either case the pen should make an immediate excursion, ideally reaching the zero position on the chart after a minute or so. In practice a reading of less than 10% is acceptable. If the pen does not get this far, add a little more dithionite. If it still does not comply, consult the fault-finding section (Section 2.3.4). Alter-

Table 2. The Oxygen Concentration of Air-saturated Water [from Delieu and Walker (9)].

Temperature (°C)	*O_2 concentration (mM)*
0	0.442
5	0.386
10	0.341
15	0.305
20	0.276
25	0.253
30	0.230
35	0.219

natively, a quick and convenient way of checking that the oxygen electrode is responding without changing the contents of the reaction vessel is to switch off the magnetic stirrer. Consumption of oxygen by the cathode then produces a slow but steady pen excursion which is completely reversed when the stirrer is switched on again.

(xi) Stop the chart and lift the pen. Thoroughly rinse out the dithionite solution and replace it with water. The pen should return to 100%. A vacuum line is the simplest way of removing the liquid contents of the reaction vessel. The end of the line should be a piece of tubing soft enough not to damage the membrane and of internal diameter smaller than the diameter of the magnetic flea.

(xii) If oxygen *evolution* is to be measured in the experiment, the pen will have to be backed-off using the back-off or zero-suppression control on the control box or the zero control on the chart recorder. It is usually advisable to back-off to no less than 10%, to allow for unexpected oxygen uptake. Backing-off may be carried out as necessary during the course of an experiment since it merely alters the position of the oxygen scale on the chart without altering the scale itself. Provided the output controls are not moved, the calibration will be unaffected.

(xiii) Decide on the experimental system, load the reaction vessel with sample and reaction mixture, then secure the cap or plunger in the reaction vessel (the screw collar flush with the top of the water jacket) in such a way that the surface of the reaction mixture rises a few millimetres into the cap's capillary tube; there should be no 'dead' volume (i.e. air bubbles) in the reaction vessel. Additions during the course of a single measurement can be made through the capillary tube by means of a microlitre syringe. For a microlitre syringe with a 50 mm needle the barrel of the syringe can normally be pushed against the top of the plunger without fear of the tip of the needle puncturing the membrane. This procedure ensures that the solution from the syringe enters the bulk of the reaction mixture and quickly becomes stirred by the rotating flea. A common mistake for beginners is to make additions only into the capillary tube of the plunger, presumably from fear of puncturing the membrane.

(xiv) Leave the control box, chart recorder, stirrer and circulating water supply on at all stages during an experiment, even over coffee or lunch-breaks. If it is necessary to leave the electrode for a while between runs, it is a good idea to leave it with water in the reaction vessel, to make sure that the initial baseline has been maintained. Only the chart drive need be switched off, and the pen raised. At the end of a sequence of experiments, the fastidious will appreciate the value of leaving water in the reaction vessel, giving a final opportunity to check the baseline and back-off position. It also helps subsequent setting-up if all controls (including the chart recorder input) are returned to the zero position and the control box switched off.

2.3.3 *Calibration*

Accurate absolute calibration can be carried out by measurement of the release of oxygen from hydrogen peroxide by catalase, though great care has to be taken to standardise the hydrogen peroxide. This method is described by Delieu and Walker (9). In practice the values given in *Table 2* have been found to be highly reliable, and the concentration of dissolved oxygen in air-saturated reaction mixtures is rarely significantly different from those given in *Table 2* for water. However, it is as well to check the calibration of the oxygen electrode at least at the initial stages of an experimental programme, and obviously where absolute values of oxygen yield are crucial to the interpretation of results.

A useful method of calibration under the conditions prevailing in an experiment with isolated thylakoids is to add limiting amounts of freshly made up and standardised potassium ferricyanide solution. A freshly made up solution of 20 mM, when diluted 20 times (e.g. add 150 μl to water in a cuvette to make up to 3.0 ml) will give an absorbance at 420 nm of 1.04 (*Table 1*). 10 μl of 20 mM stock solution (i.e. 200 nmol) added to a suspension of thylakoids in the oxygen electrode will give an increment of light-dependent oxygen evolution equivalent to 50 nmol O_2, since $O_2 = 4\ Fe(CN)_6{}^{3-}$. This will, for example, correspond to 16.4% of full-scale deflection for a 1 ml reaction mixture at 15°C or 18.1% at 20°C if the oxygen concentrations in *Table 2* are correct. A possible source of error here would be the presence of endogenous or added reductant (such as ascorbate), and it is therefore best to make four or five consecutive additions of ferricyanide, noting the total increment of oxygen evolution in each case. After endogenous reductant has been titrated out, equal volumes of ferricyanide should give equal increments of oxygen if the response of the oxygen electrode is linear. If the standard pen deflection corresponding to each oxygen increment on addition of 10 μl of ferricyanide is y% then the total amount of oxygen in the solution at 100% pen deflection will be

$$\frac{4.81 \times A_{420}}{y}\ \mu\text{mol } O_2$$

where A_{420} is the measured absorbance of the 20-fold diluted stock ferricyanide solution.

2.3.4 *Maintenance and Fault Finding*

Provided electrical connections are all sound and the membrane correctly installed on the electrode disc, oxygen electrodes are surprisingly reliable and even student-proof. Routine maintenance consists simply of cleaning the electrode disc and the base of the reaction chamber free of KCl crystals every time the membrane is changed. Periodically the AgCl deposit, which visibly tarnishes the anode, should be cleaned off with a mildly abrasive metal polish or wadding. The polish should then itself be washed off with detergent. A build-up of AgCl will adversely affect response time. The electrode disc itself should be stored clean and dry (and in the dark — fluorescent striplights discolour the plastic) since KCl deposits can in time cause electrical tracking or short-circuiting. This will lead to a residual signal even in the absence of oxygen. The membrane should be replaced and the disc cleaned of KCl at least once a week if the electrode is in daily use. Replacing the membrane requires a little skill and, particularly for those with long fingernails, a membrane applicator may be a worthwhile investment. The symptom of membrane damage is periodic spikes in the trace which become more frequent and greater in amplitude with time.

Certain reagents such as 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) are apparently adsorbed onto the membrane with the result that their effects carry over to subsequent traces. This carry-over effect can be prevented by rinsing the reaction vessel (but not the water jacket!) with ethanol. Periodic oscillations with a frequency of around 1 min may be symptomatic of discharged batteries, but are more likely to reflect temperature changes due to a coarse thermostat setting in the supply to the water jacket. Chattering or oscillations of frequency less than 1 sec may be stirrer noise — this can be checked by varying the stirrer speed. The axis of rotation of an undersized flea may itself rotate, giving much slower oscillations of the trace. In each case the remedy is to replace the flea or to re-position the electrode over the stirrer. Another common stirrer fault is for the speed of rotation of the magnet to increase as the stirrer warms up until the original stirrer setting becomes too fast for the flea to keep up with the magnet.

Any other malfunction of the electrode or recorder can be isolated fairly quickly by checking the response of the electrode [Section 2.3.2 (x), (xi), — just switching the stirrer off will usually do] before and after replacing a suspect component or connector. The success of systematic replacement of components (leads, control box, recorder, electrode disc) depends on there being a very high probability that the replacement is itself in working order. The situation where many components of different kinds have a history of inadvertent damage but have not been repaired or replaced is to be avoided at all costs. Class practicals using such equipment can quickly degenerate into chaos, and frantic replacement of components by others with which the existing electrical connectors are incompatible adds another interesting dimension. In the research laboratory it is probably best to buy your own apparatus and keep it entirely separate from teaching set-ups, for much the same reason than an ex-driving school car is unlikely to provide trouble-free transport.

2.3.5 *Light Sources*

A standard slide projector is the cheapest satisfactory light source. Fibre-optic light sources have some advantages: the lamp may be remote from the oxygen electrode, and the light pipe itself acts as a heat filter. A stabilised power supply is a good idea if a constant light output is essential, though the response of the oxygen electrode itself is slow enough to damp out mains ripple completely.

The wavelength of light used should be defined according to the needs of the experiment, though it is generally sensible to use a broad red filter even for routine work, since low wavelength components of white light can drive purely photochemical oxygen uptake at significant rates, particularly where zwitterionic buffers (e.g. Hepes, MES) are used in the presence of reducing agents and flavins. Ferricyanide and phenazine methosulphate (PMS, the co-factor of cyclic electron flow) are also photosensitive and should be used only with red light unless blue light is required for some other reason (such as excitation of fluorescence).

Light-emitting diodes (LED) of sufficient intensity for photosynthesis have recently become available, and offer the advantages of a stable and well-defined output together with a fast rise time obviating the need for a mechanical shutter.

2.3.6 *Units*

The commonest units for expression of electron transport rates are μmol O_2 (mg chl)$^{-1}$

h^{-1}, typical uncoupled rates at saturating light intensity being several hundred μmol O_2 (mg chl)$^{-1}$ h^{-1}.

It is usually convenient to measure rates initially as pen movement (% of full-scale deflection) per minute, the value being calculated from a linear region of the trace or from a construction line drawn through it. 'Raw' data can be tabulated as '% min^{-1}' and a single conversion factor used for conversion to μmol O_2 (mg chl)$^{-1}$ h^{-1}. This factor is

$$0.6 \times \frac{c.v}{chl}$$

where c is O_2 concentration (mM) at air saturation at that temperature, v is volume of reaction mixture (ml) and chl is the amount of chlorophyll present (mg). For 50 μg of chlorophyll in 1 ml at 20°C the factor is 3.312.

2.4 **Whole-chain Electron Transport**

2.4.1 *Reaction Medium*

A suitable thylakoid reaction medium for most electron transport measurements (including electron transport partial reactions) contains sorbitol (0.1 M), $MgCl_2$ (5 mM), NaCl (5 mM) and Hepes buffer (50 mM) to maintain pH 7.6 (*Table 3*). A stock solution containing twice these stated concentrations can be added to give exactly half the required final volume of reaction mixture; the remainder of the final volume is then made up of the following: water, chloroplast suspension equivalent to the desired total chlorophyll (final concentration usually 50 μg ml^{-1}) and any other reagents that must be added for the particular experiment.

Table 3. Media Recipes.

Thylakoid reaction medium

	Final concentration
Sorbitol	0.1 M
$MgCl_2$	5 mM
NaCl	5 mM
Hepes (free acid)	50 mM

Adjust to pH 7.6 with dilute KOH

Medium for oxygen uptake; PSI and PSII (Mehler reaction)

Sorbitol	0.1 M
$MgCl_2$	5 mM
NaCl	5 mM
Hepes (free acid)	50 mM
Methyl viologen	0.1 mM
Sodium azide	5 mM

Adjust to pH 7.6 with dilute KOH

Medium for oxygen uptake; PSI

Sorbitol	0.1 M
$MgCl_2$	5 mM
NaCl	5 mM
Hepes (free acid)	50 mM
DCMU	10 μM
DCPIP	0.1 mM
Ascorbate	5 mM

Adjust to pH 7.6 with dilute KOH

Intact chloroplast medium

Sorbitol	0.33 M
EDTA	2 mM
$MgCl_2$	1 mM
$MnCl_2$	1 mM
Ascorbate	2 mM
Hepes (free acid)	50 mM

Adjust to pH 7.6 with dilute KOH

2.4.2 *Oxygen Evolution*

The physiological electron transport chain can usually be reconstituted by adding ferredoxin (5 μM) and NADP$^+$ (2 mM) to the reaction medium. This system gives net oxygen evolution in the light, but a variable proportion of electron flow to oxygen also occurs, making the system unsuitable for absolute measurement of electron transport unless some correction factor for oxygen reduction is introduced. Oxygen evolution is linear until all the NADP$^+$ becomes reduced, whereupon the oxygen uptake reaction can be observed.

Potassium ferricyanide (5 mM) is a useful electron acceptor that gives light-dependent oxygen evolution dependent on both PSI and PSII acting in series. There is no evidence for competing oxygen uptake. Prolonged incubation of thylakoids with ferricyanide in the dark can impair electron transport and photophosphorylation, and it is therefore preferable to add ferricyanide in a small volume with a microlitre syringe soon after the light has been switched on.

2.4.3 *Oxygen Uptake*

Another method of measuring whole-chain electron transport utilises the autoxidation reaction of a terminal electron acceptor. Using methyl viologen (MV^{2+}) as an example, reduction of methyl viologen by the chain

$$H_2O + 2\ MV^{2+} \rightarrow 2\ MV^+ + \tfrac{1}{2}O_2 + 2H^+$$

gives oxygen *evolution* in the usual stoichiometry of the Hill reaction ($O_2/2e^- = \tfrac{1}{2}$), but oxygen is simultaneously consumed at twice this stoichiometry by a combination of the autoxidation reaction

$$2\ MV^+ + 2O_2 \rightarrow 2\ MV^{2+} + 2O_2^-$$

with dismutation of superoxide

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

The sum of these three equations (electron transport, autoxidation, dismutation of superoxide) is a special case of the Hill reaction, termed the Mehler reaction

$$H_2O + \tfrac{1}{2}O_2 \xrightarrow{(MV)} H_2O_2$$

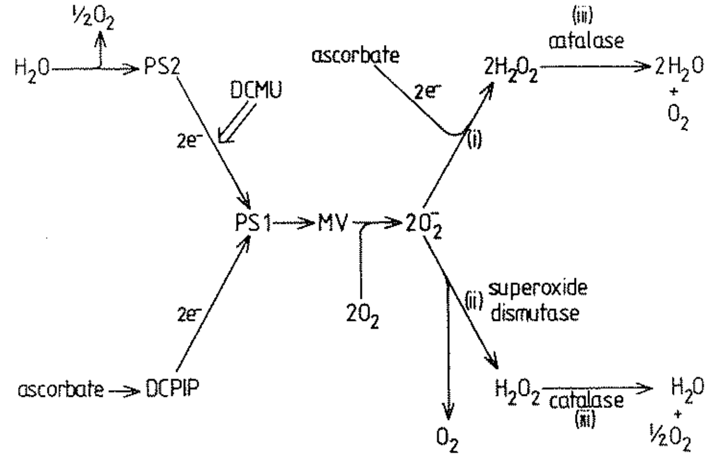
which gives a stoichiometry of oxygen *uptake* of

$$O_2/2e^- = \tfrac{1}{2}$$

Oxygen is therefore consumed at exactly the same overall rate as that at which it would be evolved in a straightforward Hill reaction. Possible departures from $O_2/2e^- = \tfrac{1}{2}$ are shown in *Table 4*. In order to ensure that this stoichiometry holds, it is necessary to avoid the presence of reducing agents such as ascorbate or Mn^{2+} ions, which will interfere with the dismutation of superoxide, or else to add the enzyme superoxide dismutase to at least 10^3 units ml^{-1}. It is also necessary to inhibit the endogenous catalase activity of the chloroplast preparation (e.g. with sodium azide) to suppress further release of oxygen from H_2O_2, or else to add excess catalase (4×10^3 units) with ethanol (5 mM) whereupon H_2O_2 is consumed by peroxidation of ethanol to

Table 4. Stoichiometries of Oxygen Uptake when Methyl Viologen (MV) is used as an Electron Acceptor.

Enzyme additions		*Reactions involved*	$O_2/2e^-$
Water as electron donor; PSI and PSII			
Without ascorbate	none SOD	(ii)	½
	catalase SOD + catalase	(ii) + (iii)	0
With ascorbate	none	(i)	1½
	SOD	(ii)	½
	catalase	(i) + (iii)	½
	SOD + catalase	(ii) + (iii)	0
Ascorbate as electron donor; PSI only			
	none	(i)	2
	SOD	(ii)	1
	catalase	(i) + (iii)	1
	SOD + catalase	(ii) + (iii)	½



acetaldehyde.

The simplest assay medium for measurement of whole-chain electron transport by the Mehler reaction therefore contains methyl viologen (0.1 mM) and sodium azide (5 mM) in addition to the components given in Section 2.4.1 (see *Table 3*). In a standard oxygen electrode, consumption of oxygen will proceed at a constant rate until 15–20% of the initial oxygen concentration remains, whereupon the apparent rate declines. A number of bipyridylium compounds will mediate photosynthetic oxygen uptake in this way, all at concentrations of 0.1 mM. These include methyl viologen itself ('paraquat'), benzyl viologen, diquat and triquat. The reaction can also be mediated by anthraquinone (0.1 mM), flavin mononucleotide (0.1 mM), adrenochrome (0.1 mM) and by ferredoxin (25 μM) (*Table 5*).

2.4.4 *Photosynthetic Control*

Electron transport is coupled to ATP synthesis (Chapter 6) and it is often useful to be able to assess the degree of coupling in a thylakoid preparation by observing the effect of ATP synthesis on the rate of electron transport. Photosynthetic control can be demonstrated using any whole-chain electron transport system, and some electron transport partial reactions.

The simplest technique is to carry out an initial electron transport measurement (a 'state 2' rate) with K_2HPO_4 (5 mM) also present in the reaction medium and then to add (e.g. after 1 min of linear electron transport) a limiting amount (e.g. 0.2 μmol) of ADP. The effect of ADP on the oxygen electrode trace of well-coupled thylakoids is shown in *Figure 6*. The rate of electron transport increases (to the 'state 3' rate) as thylakoid $\Delta\bar{\mu}_{H^+}$ is dissipated by ATP synthesis; it decreases again (to the 'state 4' rate) once all the ADP has been used up. State 4 is normally slower than state 2 since the ATP synthesised during the cycle serves to block the main channel of proton leakage through the coupling ATPase. The total extra increment of oxygen evolved or consumed, given by B in *Figure 6*, is calculated in μatom or natom equivalents. On the assumption that state 3 is completely a phosphorylating electron transport rate, the number of ATP molecules synthesised per pair of electrons transferred through the chain (the P/2e$^-$ ratio) will be equal to the measured ADP/O ratio, provided $[O] = 2e^-$ (that is, $O_2/2e^- = ½$).

Routine measurement of the ADP/O ratio of thylakoids by these means usually gives a value of 1.33 or thereabouts, though the 'true' P/2e$^-$ ratio is still in dispute. Thus addition of 0.2 μmol (e.g. 10 μl of 20 mM) ADP (A) will normally give an oxygen increment (B) of 150 natom equivalents (75 nmol) of oxygen, corresponding to 24.6% full-scale deflection for a 1 ml reaction volume at 15°C. Where oxygen uptake is measured, it is important not to confuse the transition from state 3 to state 4 with the decline in response of the oxygen electrode at low oxygen concentrations. Addition of NH_4Cl (to 5 mM) after a steady state 4 has been reached should demonstrate a high, linear, uncoupled rate.

A criterion of the integrity of the thylakoid preparation is the photosynthetic control ratio, that is, the ratio of the state 3 rate to the state 4 rate. This is a function of the capacity of the membrane to support a $\Delta\bar{\mu}_{H^+}$ that will exert feedback control on electron transport. The photosynthetic control ratio has a value of three or more in well-coupled thylakoids.

2.5 **Electron Transport Partial Reactions**

2.5.1 *Photosystem I*

For PSI operating alone it is necessary to measure oxygen uptake or reduction of the terminal acceptor since oxygen evolution does not occur. Obviously it is also necessary to add an artificial electron donor in place of water. It is desirable to eliminate any PSII activity that may complicate the results. Thus a simple assay medium for PSI electron transport contains (in addition to components given in Section 2.4.1) DCMU (10 μM) as PSII inhibitor and DCPIP (0.1 mM) with ascorbate (5 mM) as an electron

Table 5. Some Electron Donors and Acceptors, Inhibitors, Mediators etc. for Electron Transport and Redox Measurements.

Reagent	*Function*	*Molecular weight*	*Soluble in*	*Suitable stock concentration*	*Effective final concentration*
ADP (adenosine 5′-diphosphate)		427.2^a	Water	20 mM	0.2 mM
Anthraquinone	Mediator		Ethanol	Saturated solution	10 μl saturated solution
Ascorbate (sodium salt)	Reductant - donor	198 (anhydrous)	Water	0.5 M	5 mM
ATP (adenosine 5′-triphosphate)		507.2^a	Water	200 mM	0.2 mM
Benzyl viologen (1,1-dibenzyl-4,4′-bipyridylium dichloride)	Autoxidising acceptor or mediator	409.4	Water	10 mM	0.1 mM
Catalase	H_2O_2 dismutation	—	Water	Crystall. susp.	2500 units ml^{-1}
Cyanide (sodium salt)	Catalase and SOD inhibitor	65	Water	0.5 M (small volume)	5 mM
DBMIB (2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone)	Inhibitor	164.2	Ethanol	1 mM	1–10 μM
DCMU [Diuron; 3-(3,4-dichlorophenyl)-1,1-dimethylurea]	Inhibitor	233.1	>10% Ethanol	10 mM in ethanol diluted to 1 mM in 10% ethanol	20 μM
DCPIP (2,6-dichlorophenol indophenol)	Electron donor (PSI) or acceptor (PSII)	290.1 (Na salt) 268.1 (free acid)	Ethanol	10 mM	0.1 mM
Dithionite	Reductant-donor	210.2 (Na salt) 174.1 (anhydrous)	Water	1 M (in 1 M Tris pH 9.0)	10 mM
DPC (diphenyl carbazide)	Electron donor to PSII	242.3	Ethanol	50 mM	0.5 mM
Duroquinone (tetramethyl-*p*-benzoquinone) ('TMQ')	Electron donor as quinol	164.2	Ethanol	50 mM	0.5 mM
EDTA (ethylene diamine tetra-acetic acid)	Chelating agent	292.25 (free acid)	Water		
FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone)	Uncoupler	254	Ethanol	1 mM	10 μM
Ferredoxin	Electron carrier; autoxidisable acceptor	—	—	—	5–20 μM
Ferricyanide (K salt)	Electron acceptor; oxidising agent	329.3	Water	0.2 M	2 mM
Ferrocyanide	Donor	329.3	Water	50 mM	0.5 mM
FMN (flavin mononucleotide)	Autoxidising acceptor	456	Water	10 mM	50 μM

Table 5. continued.

Reagent	*Function*	*Molecular weight*	*Soluble in*	*Suitable stock concentration*	*Effective final concentration*
Hepes (N-2-hydroxyethylpiperazine-*N*′-2-ethane sulphonic acid)	Buffer	238.3 (free acid)	Water		
Hydroxylamine	Donor	33	Water	50 mM	0.5 mM
Methyl viologen (1,1′-dimethyl-4,4′-bipyridylium dichloride)	Autoxidising electron acceptor; mediator	257.2^a	Water	5 mM	50 μM
MOPS [3-(N-morpholino) propanesulphonic acid)]	Buffer	209.3 (anhydrous)	Water		
NADP (nicotinamide adenine dinucleotide phosphate)	Acceptor	765.4^a	Water	100 mM	1 mM
NADPH$_2$ (reduced form)		833.4^a			
Oxaloacetate	Acceptor		Water	100 mM	1 mM
PD (*p*-phenylene diamine)	Acceptor/donor	108.1	Ethanol	10 mM	0.1 mM
3-Phosphoglycerate (disodium salt)	Acceptor	230^a	Water		
PMS (N-methyl phenazonium methosulphate)	Cyclic co-factor	306	Water	10 mM	0.1 mM
Semi-carbazide	PSII donor		Ethanol	50 mM	0.5 mM
SOD (superoxide dismutase)	O_2^- dismutation	—	—	—	—
Sodium azide	Catalase inhibitor	65.02	Water	1 M	5 mM
Sodium borohydride	Reducing agent	37.8	Add solid		
Sodium pyrophosphate	Inhibitor of Pi translocator	446.1 (decahydrate)	Water	500 mM	5 mM
TMPD (tetramethyl-*p*-phenylene diamine)	Mediator	164.3	Water	10 mM	0.1 mM
Tris (Tris hydroxymethyl methylamine)	Buffer	121.14	Water	As required	

[a] These molecular weights are for anhydrous reagents. The amount of H_2O combined varies, refer to suppliers' data sheet for exact molecular weights.

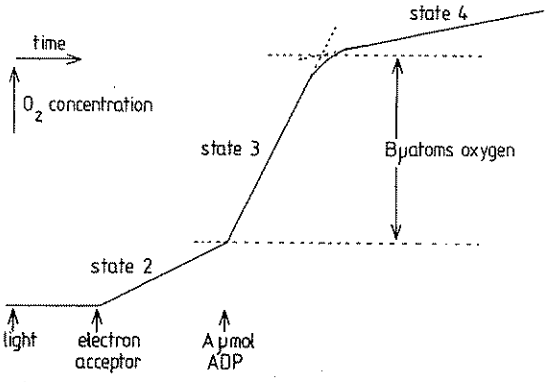


Figure 6. Oxygen electrode trace obtained with isolated thylakoids in an ideal photosynthetic control cycle. ADP/O ratio = A/B. Photosynthetic control ratio = state 3/state 4. K_2HPO_4 (at 5 mM) is present at the start and ADP (A μmol) (e.g. A = 0.2 μmol) is added where shown. NH_4Cl (to give 5 mM) is added during state 4.

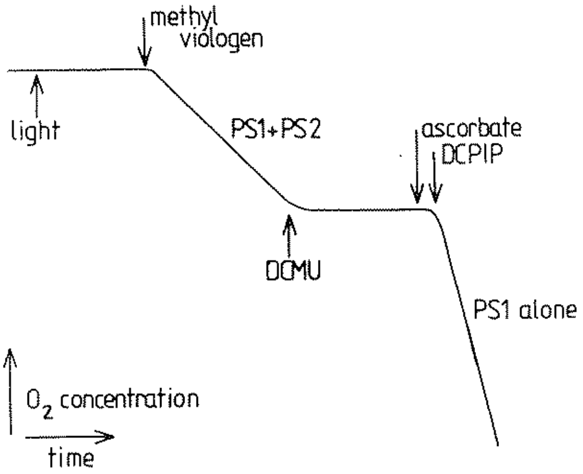


Figure 7. Oxygen electrode trace obtained during measurement of electron transport through PSI and PSII together (following addition of methyl viologen) and of PSI alone (following addition of ascorbate and DCPIP to DCMU-inhibited thylakoids). Final concentrations: methyl viologen, 0.1 mM; DCMU, 10 μM; DCPIP, 0.1 mM; ascorbate, 5 mM. Sodium azide (5 mM) is present throughout.

donor couple (DCPIP effectively mediating electron transport from ascorbate to P700), see *Table 3*.

Electron transport may then be measured as $NADP^+$ reduction at 340 nm (Section 2.2.3) or as oxygen uptake in an oxygen electrode with methyl viologen (0.1 mM) and sodium azide (5 mM) (Section 2.4.3). The oxygen electrode in fact provides a simple way of screening thylakoids for blocks in electron transport (due to chemical inhibition or mutation, for example) associated with PSI or PSII (*Figure 7*). A block in PSII will give a decreased rate of initial oxygen uptake (PSI + PSII) but not of oxygen uptake due to PSI alone. A block in PSI will decrease both rates.

A number of acceptors can be used in place of methyl viologen (Section 2.4.3) and donating mediators in place of DCPIP (Section 2.5.3).

Although the ascorbate → DCPIP → PSI → MV → O_2 pathway provides a satisfactory *relative* measure of PSI electron transport, a number of additional factors must be controlled if it is used as an *absolute* measure. In the simplest system, free of catalase or superoxide dismutase, the $O_2/2e^-$ ratio has a value of 2 (10). This value is obtained because ascorbate reduces the superoxide intermediate of oxygen reduction to peroxide, as well as functioning as an alternative donor to water. Thus the component reactions are:

(i) electron transport (two electrons):

$$\mathrm{AscH_2 + 2MV^{2+} \xrightarrow[PSI]{DCPIP} Asc + 2MV^+ + 2H^+}$$

(ii) autoxidation of methyl viologen:

$$\mathrm{2MV^+ + 2O_2 \rightarrow 2MV^{2+} + 2O_2^-}$$

(iii) reduction of superoxide to peroxide:

$$2O_2^- + AscH_2 + 2H^+ \rightarrow 2H_2O_2 + Asc$$

The sum of these three reactions is:

$$2\ AscH_2 + 2O_2 \xrightarrow[PSI]{DCPIP,\ MV} 2Asc + 2H_2O_2$$

and hence the $O_2/2e^-$ ratio has a value of 2. In the presence of superoxide dismutase the dismutation reaction:

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

will compete with reduction of superoxide by ascorbate, giving a changed overall stoichiometry of $O_2/2e^- = 1$. Thylakoids can normally be washed free of competing superoxide dismutase activity and, if in doubt, the rate of oxygen uptake can be titrated with added superoxide dismutase: the rate should be exactly halved by saturating superoxide dismutase if the enzyme was absent initially. Alternatively, potassium cyanide (10 mM) can be added to inhibit superoxide dismutase.

Similar considerations apply to contaminating catalase, though this can be inhibited with sodium azide (5 mM). At these concentrations, cyanide (10 mM) has been found to inhibit both catalase and superoxide dismutase, while azide (5 mM) inhibits only catalase (10). These effects on oxygen uptake stoichiometry are illustrated by the trace shown in *Figure 8*. *Table 4* shows $O_2/2e^-$ ratios prevailing for all oxygen-consuming electron transport measurements of the kind outlined here. The simplest protocol is probably to use well-washed thylakoids with 5 mM sodium azide and to assume $O_2/2e^- = ½$ for PSI and PSII together and $O_2/2e^- = 2$ for PSI alone.

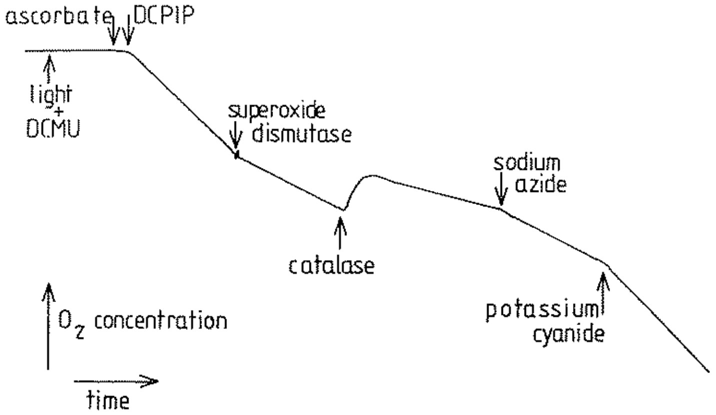


Figure 8. Oxygen electrode trace obtained during measurement of PSI electron transport as in *Figure 7*. Superoxide dismutase (800 units) halves the observed rate of oxygen uptake, and this rate in turn is halved by addition of catalase (4×10^3 units). Sodium azide (to 10 mM) inhibits catalase and potassium cyanide (to 10 mM) inhibits both catalase and superoxide dismutase. The rate of electron transport is constant throughout the experiment; only the stoichiometry of oxygen uptake is changed. See ref. 10 for a more complete description.

2.5.2 *Photosystem II*

In intact thylakoids the lipophilic electron acceptors diaminodurene (DAD) and *p*-phenylenediamine (PD) are useful for measurement of electron flow through PSII alone. They should be freshly made up in buffered medium containing excess ferricyanide at three times the stock concentration of DAD or PD. A suitable final concentration of the acceptor couple is then DAD or PD (0.4 mM) with ferricyanide (1.2 mM). PSI must be inhibited, for example by DBMIB (1 μM), or by pre-incubation for 60 min with KCN (30 mM). Mediators of PSII oxygen uptake have been reported (11), including DBMIB at higher concentrations (20 μM) than those used for electron transport inhibition (1 μM).

In the absence of lipophilic electron acceptors such as DAD or PD, DCPIP and ferricyanide will function as PSII electron acceptors only if the kinetic barrier to their reduction is removed. This is likely to be the case in sub-chloroplast particles. In intact thylakoids, mild trypsin digestion (80 μg ml^{-1} for 10 min at neutral pH in the dark) reveals a DCMU-insensitive electron flow to ferricyanide that may be measured as oxygen evolution. This effect presumably results from removal of the lipophilic shield represented by the Q_B (Chapter 1) or herbicide-binding protein, though the list of likely side-effects of trypsin digestion is long and somewhat limits the usefulness of this technique. Similar reservations apply to the use of silicomolybdate, which also gives DCMU-insensitive PSII electron transport and oxygen evolution. Silicomolybdate can be made up as a stock solution of 25 mM in 50% dimethylsulphoxide (DMSO), and a final concentration of 1 μM will give limited PSII electron transport and coupled photophosphorylation.

2.5.3 *Further Donors, Acceptors and Inhibitors*

More specific localisation of electron transport partial reactions is possible, and comprehensive accounts of these are given in the reviews of Trebst (12,13) and Izawa (14).

(i) *PSI donors other than DCPIP.* One problem that arises from the use of DCPIP/ascorbate as a PSI electron donor (Section 2.5.1) is that very little of the intermediary electron transport chain is involved, as shown by the insensitivity of this reaction to DBMIB, an inhibitor of plastoquinone oxidation. It is often useful when working with thylakoids to use donors to specific points of the chain prior to P700. Duroquinol, for example, functions as a donor to plastoquinone and gives a DBMIB-sensitive PSI electron transfer reaction. Duroquinone is kept as a stock solution (50 mM in ethanol). Prior to use, mix 2 mg of sodium borohydride with 0.5 ml of the stock solution, and leave the mixture on ice until the yellow duroquinone goes colourless on its reduction to duroquinol. Add 5 μl of concentrated HCl to remove excess borohydride. Addition of the duroquinol to a final concentration of 0.5 mM gives DBMIB-sensitive PSI electron transport in intact thylakoids. The difference between the sites of donation by duroquinol and DCPIP is indicated by the oxygen electrode trace in *Figure 9*. The oxygen uptake stoichiometry of the duroquinol–PSI reaction has not been investigated, though it is likely that the same considerations hold as for DCPIP/ascorbate (Section 2.5.1), and hence that $O_2/2e^- = 2$. The electron transport sequence duroquinol → PQ → PSI → MV → O_2 is also coupled to photophosphorylation, presumably because the native

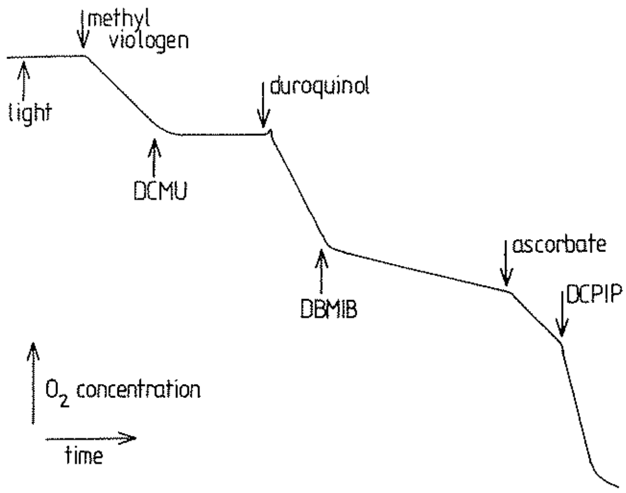


Figure 9. Oxygen electrode trace illustrating further dissection of the non-cyclic chain (cf. *Figure 7*). Duroquinol (to 0.5 mM) donates to plastoquinone and therefore bypasses inhibition by DCMU (5 μM) but not by DBMIB (20 μM). DBMIB is an inhibitor of plastoquinone oxidation. Ascorbate (5 mM) with DCPIP (0.1 mM) bypasses inhibition by DBMIB. From ref. 24.

proton-translocating cycle is retained. DCPIP/ascorbate also gives some coupled photophosphorylation, though photosynthetic control is difficult to observe.

Other electron donors to PSI can be used with ascorbate in place of DCPIP. Tetramethyl-*p*-phenylenediamine (TMPD) gives fast electron transport that is not coupled to ATP synthesis and which is DBMIB-insensitive since it bypasses the plastoquinone proton shuttle. DAD/ascorbate gives coupled electron transport also insensitive to DBMIB — in this case it is likely that DAD catalyses an artificial proton shuttle, replacing plastoquinone.

(ii) *Artificial PSII donors.* In general, PSII donors replace water only where water-oxidation has been inactivated by some means, for example by heating (6 min at 55°C), by Tris treatment (0.8 M Tris, pH 8, for 15 min) or by hydroxylamine treatment (5 mM hydroxylamine at pH 7 for 15 min).

Membranes prepared in one of these ways will then oxidise a variety of artificial donors, including ascorbate, hydroquinone, semicarbazide, ferrocyanide, hydrogen peroxide and PD. The electron transport is DCMU-sensitive. 1,5-Diphenylcarbazide (at 0.5 mM) is probably now the most widely used electron donor to PSII. Since oxygen evolution is absent in these systems, DCPIP reduction measured at 600 nm (Section 2.2.3) is probably the best technique for following the course of the reaction, though PSI acceptors (e.g. $NADP^+$ with ferredoxin) can also be used.

2.6 Measurements with Intact Chloroplasts

2.6.1 *Reaction Conditions*

An intact chloroplast preparation offers a more 'physiological' system for electron transport measurement, but it suffers the drawback of the impermeability of the chloroplast envelope to many added donors, acceptors and inhibitors. Nevertheless, it is often desirable to monitor the complete photosynthetic electron transport chain in this way. Conditions for isolation and storage of intact chloroplasts are given in Chapter 2. A reliable isotonic reaction medium for intact chloroplast studies (*Table 3*) contains sorbitol (0.33 M), EDTA (2 mM), $MgCl_2$ (1 mM), $MnCl_2$ (1 mM), ascorbate (2 mM) and Hepes (50 mM) at pH 7.6. The function of EDTA in the presence of Mg^{2+} and Mn^{2+} is unclear, but this particular medium is nevertheless widely used.

In other respects an intact chloroplast preparation can be used in the liquid-phase oxygen electrode in much the same way as a suspension of thylakoids. It is usually desirable to increase the chlorophyll concentration to 100 μg ml^{-} and to use a higher temperature of 20 or 25°C. Catalase at about 5×10^3 units is often added to the chloroplast suspension to prevent inhibitory effects of any hydrogen peroxide produced by oxygen reduction.

2.6.2 *CO_2-dependent Oxygen Evolution*

Saturating CO_2 concentrations can be produced by inclusion of sodium bicarbonate (10 mM). In the presence of K_2HPO_4 (0.5 mM), high quality intact spinach chloroplasts will then show physiological rates of oxygen evolution, after an initial lag phase, of 200 μmol O_2 (mg chl)$^{-1}$ h^{-1} or even more. With isolated pea chloroplasts (ideally from 10-day-old pea seedlings) it is necessary to replace the K_2HPO_4 with sodium pyrophosphate (5 mM) and ATP (1 mM), apparently to discourage an adenylate translocator on the chloroplast envelope which will otherwise inhibit photosynthesis by depleting the chloroplast of ATP.

The electron transport measured in such a system is obviously sensitive to DCMU and, less obviously, to uncouplers [e.g. carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) at 1 μM or NH_4Cl at 40 mM]. The action of uncouplers is indirect: inhibition of ATP synthesis inhibits recycling of $NADP^+$ by the coupled phosphoglycerate kinase and triose phosphate dehydrogenase reactions, and effectively removes the terminal electron acceptor. The Calvin cycle is generally thought to require ATP and NADPH in the ratio 3:2, and it is a matter of debate whether the non-cyclic electron transport chain alone provides ATP with a sufficiently high stoichiometry for CO_2 fixation.

2.6.3 *Other Oxygen-evolving Reactions of Intact Chloroplasts*

It is also possible to replace $NaHCO_3$ with 3-phosphoglycerate (1 mM) or with oxaloacetate (1 mM). In both cases the full non-cyclic electron transport chain operates, but these reactions are of use because of their differing stoichiometric requirements for ATP. 3-Phosphoglycerate-dependent oxygen evolution results from regeneration of $NADP^+$ by an isolated section of the Calvin cycle, and requires ATP and NADPH in equimolar amounts. The reaction, like CO_2-dependent oxygen evolution, is sensitive to inhibition by uncouplers, but any auxiliary photophosphorylation (cyclic or pseudocyclic) is probably not required. Oxaloacetate reduction requires no ATP and oxaloacetate-dependent oxygen evolution is therefore stimulated by uncouplers.

2.7 Combining Electron Transport with Other Measurements

2.7.1 *Sampling*

Liquid-phase oxygen electrodes of the type described in Section 2.3 afford the opportunity of withdrawing samples of the reaction mixture at intervals for further analysis, thereby allowing direct comparison of the oxygen trace with a plotted time-course of some accompanying, related reaction. Examples of interesting reactions that can be followed are reduction of a terminal electron acceptor (e.g. $NADP^+$), ATP synthesis (using ^{32}P incorporation or the luciferin-luciferase assay, Chapter 6), protein phosphorylation [each sample being run on sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and the gel autoradiographed and bands counted], CO_2 fixation (incorporation of ^{14}C into acid-state products), and concentrations of metabolic intermediates (a suitable assay being carried out on each sample). Samples obviously may also be withdrawn for spectroscopic analysis, e.g. injection of the sample into a tube which is then immersed in liquid nitrogen for fluorescence or e.p.r. spectroscopy (Chapter 4).

Provided there is uniform illumination of the reaction vessel, it does not matter if the total volume of sample taken is an appreciable proportion of the initial volume of reaction mixture — the trace continues to record the rate of change of oxygen concentration, which is independent of volume. An event marker on the chart recorder is particularly useful where sampling techniques are used. Each sample should be taken with a microlitre syringe of the appropriate capacity, and with a needle length (50 mm for Rank and Hansatech oxygen electrodes) that will allow rapid removal of a sample from the centre of the reaction vessel without any danger of puncturing the membrane. For work with radioisotopes, it is convenient to keep one oxygen electrode unit specifically for this purpose.

2.7.2 *Continuous Measurement of Other Variables*

A modification introduced by Horton (15) of the Delieu and Walker oxygen electrode has been incorporated into a unit produced commercially by Hansatech. This consists of an oxygen electrode completely enclosed within an opaque case with five ports for illumination or detection of light. This oxygen electrode permits continuous optical or fluorescence measurement during the time-course of an electron transport reaction. Light can be provided by fibre-optics or by an LED array plugged directly into one of the ports. This type of oxygen electrode has been most extensively used for combined chlorophyll fluorescence measurements. Here photodiode detectors can be plugged directly into one or more ports or a photomultiplier can be mounted close to each port, or at the end of a light-pipe.

This oxygen electrode unit has a number of other potential applications, including measurement of thylakoid ΔpH by 9-aminoacridine fluorescence quenching (Chapter 6) or, indeed, any other fluorescence signal.

It is also possible to measure light scattering and optical absorbance (Chapter 4) using suitable light sources, detectors and filter combinations. Ambitious experiments using four or more channels in addition to the oxygen signal are possible. Oxygen electrode 'polygraphy' of this kind is most easily obtained using a multi-channel chart recorder, and this may be facilitated by the use of recorders (e.g. certain Rikadenki models) which can store one or more channels in order to present the signals onto the chart without the usual offset on the time-axis between channels. Alternatively, a suitably programmed microcomputer offers enormous advantages for the economical storage and subsequent manipulation of the data, though at the time of writing there is no commercially available package designed for this specific application.

2.8 The Gas-phase Oxygen Electrode

Photosynthetic electron transport can also be measured as oxygen evolution by leaf discs. A commercially available gas-phase electrode (Hansatech) based on the design of Delieu and Walker (16) works on the same principle as the liquid-phase oxygen electrode but the reaction vessel is replaced by a chamber which allows space (~5 ml) for a leaf disc (up to 25 mm diameter). Capillary matting is also provided to hold a small volume of bicarbonate solution as a source of CO_2. Calibration is achieved by altering the oxygen partial pressure by inserting or removing a known volume (e.g. 1 ml) of air. The most useful version of the gas-phase electrode is likely to be the cased version equipped with ports for simultaneous fluorescence measurement.

The major advantage of leaf discs is that they circumvent all the biochemical problems of obtaining functional particles such as chloroplasts in suspension, and offer an electron transport system functioning essentially the same as *in vivo*. They are likely therefore to be of most use where it is necessary to make simple comparisons of overall photosynthetic rate, perhaps between different species or stages of leaf development. The disadvantages are that the electron transport system that leaf discs contain is almost immune to conventional, invasive, biochemical analysis. Introduction of well-defined photosynthetic inhibitors that can penetrate leaf discs in the gas phase would do something to offset this disadvantage, though tortuous diffusion paths and slow response would still presumably make the liquid-phase electrode and isolated chloroplasts indispensable for serious biochemical analysis of electron transport and energy coupling in plants.

The trade-off between assurance of physiological conditions *in vivo* and simplicity and intelligibility of components isolated *in vitro* is a permanent dilemma in biology. Partial circumvention of this dilemma is the chief advantage of making non-invasive, biophysical measurements on intact tissue as well as on well-defined biochemical model systems. Chlorophyll fluorescence, for example, can report on fundamental events in photosynthesis even in leaves or leaf discs (Chapter 4), and simultaneous measurement of chlorophyll fluorescence yield and electron transport has much to commend it at all levels of complexity.

3. REDOX TITRATION

3.1 Oxidation-reduction Potentials

An appreciation of the energetics of the constituent reactions of photosynthetic electron flow is important in understanding the process of photosynthetic energy conservation as a whole. Because the pathway comprises a series of reduction-oxidation reactions (redox reactions) the thermodynamic relationships involved can be conveniently described in electrochemical terms.

The following is only a brief description of the thermodynamics of electron transfer systems; for a fuller account the reader is referred to references 17–19.

A generalised redox reaction:

$$A_{red} + B_{ox} \rightleftharpoons A_{ox} + B_{red}$$

can be considered as the sum of two 'half-cell' reactions of the type

$$A_{red} \rightarrow A_{ox} + ne^-$$

where A_{red} and A_{ox} together constitute a 'redox couple' and n indicates the number of electrons involved.

For such a half-cell, the relationship between the free energy available and the activities of the reactants is given by:

$$E = E_o + \frac{2.3\ RT}{nF} \log_{10} \frac{A_{ox}}{A_{red}} \qquad \text{Equation 1}$$

where E is the redox potential, E_o the standard redox potential, F the Faraday Constant, R the Universal Gas Constant, T the temperature in K, and A_{ox} and A_{red} the activities, or more usually the concentrations, of oxidised and reduced forms of the couple, respectively. This is directly analogous to the familiar Gibbs Free Energy relationship with $E_o = -nFG^o$, where G^o is the standard free energy. At room temperature (25 °C) the value of $\frac{2.3\ RT}{F}$ is 59 mV.

Redox potentials have no absolute value and are therefore expressed relative to a standard half-cell, usually the standard hydrogen electrode,

$$H_2 \rightleftharpoons 2H^+ + 2e^-$$

which is given a redox potential $E_o = 0$ mV.

The expressions used above apply to standard conditions at pH 0. It is more useful, with biological systems, to refer to standard conditions at pH 7; a standard redox potential defined at pH 7 is symbolised E_{m7} or sometimes E_o'. Similarly, when referring to standard conditions at pH 7, E becomes E'. The E_o' for the standard hydrogen electrode is −420 mV. The term E_h, frequently encountered in the biochemical literature, signifies that the redox potential is expressed relative to the standard hydrogen electrode.

Therefore the version of Equation 1 usually encountered in bioenergetics is:

$$E_h = E_{m7} + \frac{59}{n} \log_{10} \frac{A_{ox}}{A_{red}} \qquad \text{Equation 2}$$

E_{m7} is frequently referred to as the mid-point potential and is that redox potential where the concentrations of oxidised and reduced forms of the couple are equal. Thus for the generalised redox reaction above, with two participating half-cells

$$\Delta E_h = \Delta E_{m7} + \frac{59}{n} \log_{10} \frac{A_{ox} \cdot B_{red}}{A_{red} \cdot B_{ox}} \qquad \text{Equation 3}$$

where ΔE_h and ΔE_{m7} are the difference between the parameters, E_h and E_{m7}, for the two half-cells; n being the number of electrons involved and A_{ox}, A_{red}, B_{ox}, B_{red} the activities, or in most practical applications, the concentrations, of the reactants. For a more extensive discussion of this relationship see references 17 and 18.

In the situation where either the oxidised or the reduced form is preferentially bound by another chemical species, the apparent mid-point potential will shift depending on the concentration of the ligand present. The analysis of such interactions is discussed in (18). The involvement of protons in bioenergetics gives particular importance to the case where the ligand is a proton bound to the reduced form of the couple, i.e:

$$A_{ox} + e^- + H^+ \rightleftharpoons A_{red} \qquad \text{Equation 4}$$

The mid-point potential then depends on the pH in the following manner:

$$E_{mpH} = E_{m7} + 59\ (7\text{-pH}) \qquad \text{Equation 5}$$

where E_{mpH} is the mid-point potential at the given pH.

When the proton is bound to the oxidised form of the couple, the relationship is similar but the E_{mpH} increases rather than decreases with pH.

3.2 **What Redox Titrations Tell You**

Measurement of redox potential allows components of electron transport chains which have the same or similar absorbance spectra (or e.p.r. spectra) to be resolved. This is particularly useful when several species of the same component are present in a membrane. Once they have been resolved on the basis of redox potential it is often possible to show small differences in their absorbance spectra (19). In addition, equilibrium measurements of redox potential show the sequences of electron transport components in a membrane that are thermodynamically feasible, under given ambient conditions such as pH. However, for a true picture of the reaction pathways involved, the kinetics of oxidation and reduction of the components can be measured at different redox potentials. As photosynthetic electron flow is initiated by a photochemical reaction, it is technically a fairly simple matter to establish a given ambient redox potential, excite the sample with flash illumination and then observe electrons flowing through (at least) some of the components. In a simple linear sequence of optically detectable components, of course, a series of complementary transient reductions and oxidations would be seen at a potential optimal for electron flow. In fact, real life is more complex than this; nevertheless, considerable progress has been made over the last 15 years in understanding photosynthetic systems using this approach.

At this point, it is worth mentioning that the mid-point potential is not necessarily the working potential of the component. Little information is, as yet, available on the working poises of redox components in photosynthesis, and presumably they will change with varying conditions. The redox poise of any one component will depend on that of other redox components in the chain, and also on other membrane phenomena, such as the protonmotive force.

It has also proved possible, by redox poising, to titrate other reactions in bioenergetic membranes such as proton binding, electric field-indicating absorbance changes and the phosphorylation of light-harvesting pigment-protein complexes, and to correlate these with electron transport events.

3.3 Measuring Oxidised and Reduced Components

To measure the mid-point potential, E_{m7}, of a component it is necessary to be able to measure the ambient redox potential, E_h, and the degree of oxidation (or reduction) of the component of interest simultaneously. Equation 2 can then be used to calculate a value for E_{m7} under those conditions.

For components which have oxidised and reduced forms with significantly different optical extinction coefficients at a convenient wavelength, usually in the visible or near i.r. regions of the spectrum, absorbance spectrophotometry is the method of choice (Chapter 4). A major problem encountered with spectrophotometry in photosynthetic systems is that the relatively massive absorbances of the chlorophyll and carotenoid pigments give a high background absorbance often at the wavelengths where the redox components absorb. In the case of purple non-sulphur bacteria, several mutant strains exist, with shifted carotenoid spectra, which leave the α-bands of the cytochromes visible.

Even when the sample is carefully balanced initially with a reference sample, problems can still arise, particularly if the sample is left in the instrument for some time. It is useful to have a spectrophotometer with the facility for shutting off the measuring beam between measurements. The length of time the sample spends at high or low extremes of potential should be minimised. Chlorophylls can become oxidised at high potentials, bleaching the sample.

Most spectrophotometers of adequate precision can be used for redox titrations, although minor modifications may be necessary to house the cuvette and to allow additions to be made to the solution during the titration. Scanning spectrophotometers have some advantages over double-beam instruments and can be used to titrate more than one component (for example *b*- and *c*-type cytochromes) in a single run. The addition of an on-line computer can eliminate tedious analysis of the results.

For components without convenient optical absorbance signals, the use of other measuring techniques is described in the literature. Electron paramagnetic resonance has been widely used to monitor quinones and iron-sulphur centres, including the components on the acceptor side of both photosystems. It is necessary to freeze the sample rapidly in order to see the e.p.r. signal and an apparatus is described in (19) for doing this and for freezing-in the redox state of the components. Redox potential is sensitive to temperature, but the methods available for freezing appear to monitor faithfully the room temperature equilibrium (see ref. 19 for a discussion of this point).

Other techniques used with redox titrations include chlorophyll fluorescence yield (to indicate the oxidation state of reaction centre components; see Chapter 4) and phosphorylation of light-harvesting pigment-protein complexes (monitoring the activation of a protein kinase).

It is possible to get functional information about the behaviour of redox components by following the kinetics of the changes which occur after a brief flash of light (preferably eliciting only a single turnover). In this type of experiment, the potential is poised as required and then electron transport started by flash excitation. The transient oxidation/reduction of components can be followed spectrophotometrically. The system is equilibrated with the electrode before and after, but clearly not during, the transient. A picture of the behaviour of each visible component can then be followed over the range of potentials at which the chain works, and the properties of components not visible spectrophotometrically can be inferred from the behaviour of the visible components. These invisible components may then be correlated with components seen using other techniques, e.g. e.p.r.

3.4 **Measuring Redox Potential**

Ambient redox potential is followed by an inert metal electrode immersed in the solution; platinum is generally used as the electrode. The potential is measured by comparison with a reference electrode using a high resistance voltmeter. The standard hydrogen electrode, to which potentials are referred, is, in practice, substituted by either a calomel or a silver/silver chloride reference electrode.

The calomel electrode consists of mercury together with a paste of mercury and mercurous chloride (calomel) in contact with a solution of potassium chloride saturated with mercurous chloride, the half-cell reaction is:

$$\tfrac{1}{2}Hg_2Cl_2 + e^- \rightleftharpoons Hg + Cl^-$$

and the mid-point potential is +244 mV at 25°C with saturated KCl.

The silver/silver chloride electrode consists of silver in contact with silver chloride in 1 M potassium chloride. It has a half-cell reaction:

$$AgCl + e^- \rightleftharpoons Ag + Cl^-$$

and a mid-point potential of +222 mV at 25°C.

When calculating values for E_h, the potential of the reference electrode must, of course, be taken into account.

3.5 **Experimental Assembly**

The apparatus for carrying out redox titrations of optically detectable components is described below. It consists of an enclosed cuvette, incorporating an electrode and a stirring device, through which nitrogen or argon is bubbled to maintain anaerobic conditions. Many standard pH meters can be used to record the potential, with digital meters being easy to read.

3.5.1 *Electrodes*

The measuring electrode is made of an inert metal, usually platinum, although gold electrodes can also be used. Commercially, platinum electrodes are available on their own or in combination with a silver/silver chloride reference electrode. For use in a cuvette, where space is a limiting factor, a micro-combination electrode is the most suitable size.

As an alternative to a commercial reference electrode, a calomel electrode outside the cuvette, but connected with the buffer via a salt bridge, can be used. This is less convenient, since the salt bridge has to be made up each time, but it occupies less space in the cuvette and can be used also with a top stirrer. The buffer from the cuvette is brought into electrical contact with the potassium chloride in the reference electrode via a three-way glass tap.

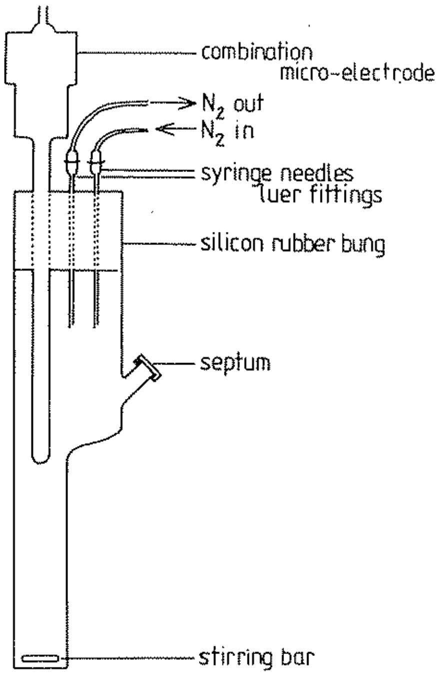


Figure 10. Cuvette for redox titrations (see ref. 19).

3.5.2 *Cuvette*

The cuvette can be made entirely of glass with the electrode and gas inlet/outlet supported in a rubber stopper as in *Figure 10*. The inlet port for making additions at the side should be covered with a septum, sufficiently soft to allow the repeated entry of a microsyringe — the septa used on intravenous canulae are ideal.

3.5.3 *Anaerobiosis*

It is essential to exclude oxygen rigorously and the measurements should be carried out under argon or nitrogen gas containing less than 1 p.p.m. O_2. All tubing should be made of an oxygen-impermeable material such as copper, Nylon 6 or Teflon. If necessary, Fieser's solution (20) can be used to remove residual oxygen from the gas. An apparatus of the 'Nilox Scrubber' type can be used — the details of this can be found in ref. 21, but they are also available commercially. After passage through Fieser's solution the gas should be cleaned in a dilute acid wash, a lead acetate wash and finally distilled water for pure gas. A recipe for Fieser's solution is given in *Table 6*. The gas inlet and outlet on the cuvette can conveniently be made from syringe needles and the attachments made by standard Luer fittings.

3.5.4 *Stirring*

Because of the depth of the column of liquid in a redox cuvette, adequate stirring can be a problem. The most efficient method is to stir from the top although this can be

Table 6. Fieser's Reagent[a].

1. Add 2 g of anthraquinone-2-sulphonic acid (sodium salt) and 15 g of sodium dithionite to a warm 20% aqueous potassium hydroxide solution.
2. Stir until they dissolve to give a blood red solution. Cool.
3. 100 ml will take 788 ml oxygen.
4. Requires a dilute acid wash, a lead acetate wash (for hydrogen sulphide) and a distilled water wash.

[a] See ref. 20.

difficult to accommodate with commercially available electrodes because of space considerations. Dutton (19) describes an apparatus where a magnetic flea is used but is held to the side of the cuvette. Modern magnetic stirrers can be fitted into the base of the cuvette housing and the solution stirred from the bottom.

3.5.5 *Mediators*

Proteins, whether membrane-bound or soluble, are not able to interact directly with a platinum electrode. It is necessary to add small molecules which are able to interact both with the electrode and with the redox centres in the proteins to bring the two into equilibrium, and to ensure that the redox potential experienced by the protein is the same as that measured by the electrode; these substances are known as mediators.

Mediators are themselves redox couples and function efficiently only near to their own mid-point potential. Therefore a range of mediators is needed to cover the range of potentials to be titrated. Mediators should be stable, and for spectrophotometric titrations should be uncoloured in the region of interest. Unfortunately mediators generally have at least one highly coloured form and it is important to check that there is no spectral interference with the titration. Titrations should be independent of the mediator concentration used, and to check that mediators do not interfere, the titration should give the same results if the mediator concentration is changed, say by a factor of 5 or 10. If membrane-bound components are to be titrated, the mediators should be hydrophobic enough to interact with them. These ideals are frequently difficult to meet and it is often necessary to compromise on the mediators actually used.

Most mediators are unstable to light or air (or both) over periods of days, and ideally should be made up fresh for each day's experiments. A list of useful mediators, together with their mid-point potentials, is given in *Table 7*.

Mediators are selected bearing in mind that they work most effectively near their mid-point potential, say 60 mV either side of the mid-point potential if $n = 1$. Mediators should be present to cover effectively the whole range of the titration. For example, for a titration from $E_h = +100$ mV to $E_h = -50$ mV a mixture of N-methyl phenazonium methosulphate (PMS), N-ethyl phenazonium ethosulphate (PES), duroquinone, pyocyanine and 5-hydroxl-1:4-naphthoquinone should give adequate mediation. Generally, there is no harm in including more mediators covering the correct range than the absolute minimum; moreover the mediation should be more effective. There are regions of potential where few good mediators are available at the moment and extra care is needed to ensure full equilibration between electrode and sample. Care should be taken to avoid using a mediator which absorbs strongly at the wavelengths of interest. For example, titrations of α-bands of cytochromes cannot be done with TMPD because

Table 7. Mediators.

Name	E_{m7}	*n*	*Typical concentration*	*Dissolve in*
Potassium ferricyanide[a]	+430 mV	1	7.5 μg ml^{-1}	Aqueous
Hydroquinone	+280 mV	2	2.5 μg ml^{-1}	Ethanol
TMPD (N,N,N′,N′-tetramethyl phenylene diamine)	+260 mV	1	3 μg ml^{-1}	Ethanol
DAD (2,3,5,6-tetramethyl phenylene diamine)	+220 mV	2	12 μg ml^{-1}	Ethanol
PMS (N-methyl phenazonium methosulphate)	+80 mV	2	7 μg ml^{-1}	Aqueous
PES (N-ethyl phenazonium ethosulphate)	+55 mV	2	7 μg ml^{-1}	Aqueous
5-Hydroxyl-1,4-naphthoquinone	+50 mV	2	3 μg ml^{-1}	Ethanol
Duroquinone	+10 mV	2	4 μg ml^{-1}	Ethanol
Pyocyanine (N-methyl-1-hydroxy phenazonium methosulphate)	−34 mV	2	1.6 μg ml^{-1}	Aqueous
Anthraquinone	−100 mV	2	1 in 500 dilution of saturated solution	Ethanol
2-Hydroxyl-1,4-naphthoquinone	−145 mV	2	3 μg ml^{-1}	Ethanol
Anthraquinone-2,6-disulphonate	−185 mV	2	7.5 μg ml^{-1}	Aqueous
Anthraquinone-2-sulphonate	−225 mV	2	7.5 μg ml^{-1}	Aqueous
Benzyl viologen (1,1′-dibenzyl-4,4′-bipyridylium dichloride)	−311 mV	1	1 μg ml^{-1}	Aqueous
Methyl viologen (1,1′-dimethyl-4,4′-bipyridylium dichloride)	−430 mV	1	1 μg ml^{-1}	Aqueous

[a] See ref. 22.

of such an overlap of absorbance. Some mediators have specific interactions with particular components which may be undesirable: for example, neutral red is reported to interact specifically with the low potential electron acceptor, Q_L, of PSII, and many quinones quench chlorophyll fluorescence. Published titrations will give an idea of the mediators to use for any particular component.

The concentrations of mediators generally used are in the range 10−50 μM. For redox poising experiments, where flash-induced kinetics are being followed, it may be necessary to use lower concentrations of mediator than for equilibrium titrations.

It is important that the interaction of the mediators with the redox chain is slow compared with the change being measured. Some mediators such as PMS can act to bypass electron transport components and themselves reduce other members of the chain; if the mediator is present in too high a concentration the titration will be distorted. In any case, the titration should always be checked by repeating with a different concentration of mediators present.

3.5.6 *Testing*

When a new redox apparatus is put into use it can be tested by titrating known components. One of the simplest is the ferri/ferrocyanide couple, which does not require the use of any additional instrumentation. Values for the mid-point potential of the ferri/

ferrocyanide couple are given in ref. 22. If a spectrophotometer is available a mediator can be titrated and followed optically, or more biologically, cytochrome *c* can be used; information on the mid-point potential for cytochrome *c* is given in ref. 23.

If difficulty is experienced in maintaining low potentials, this may indicate that oxygen levels are too high; either the gas contains too much oxygen, or there is a leak in the system. It is essential that stirring is maintained at an adequate rate; if additions of reductant or oxidant take a long time to alter the measured potential it may be that stirring is insufficiently vigorous.

3.6 **Performing Redox Titrations**

The precise details of the technique will depend on the type of measurements involved and on the instrumentation available. The description below is given as a guide and refers to a titration of a membrane preparation of the photosynthetic bacterium *Rhodopseudomonas capsulata* Str. AlAPho$^+$. This strain is defective in carotenoid synthesis and makes no carotenoids with significant absorbance at wavelengths where the α-bands of cytochromes absorb, permitting the redox state of *b*- and *c*-type cytochromes to be monitored easily. In this case a *c*-type cytochrome has been titrated by following the absorbance at 551 − 540 nm.

Because of light scattering problems and absorbance of pigments, it is often necessary to limit the concentration of the species to be titrated. It is also necessary, however, to have sufficient absorbance change between the totally oxidised and the totally reduced forms of each species present to be able to measure possibly as many as 15 or 20 different points. For most purposes the total reduced-minus-oxidised absorbance can conveniently be measured using potassium ferricyanide to oxidise the sample and sodium dithionite to reduce it, these reagents having potentials beyond the ranges of most components involved in photosynthetic electron flow.

(i) To estimate the concentration of sample required in the titration, suspend the membranes in the buffer to be used [e.g. 50 mM 3-(N-morpholino)propanesulphonic acid (MOPS), 50 mM KCl, pH 7.0] to about the right concentration — 150 μM bacteriochlorophyll (BChl) under the above conditions.

(ii) Oxidise the reference sample completely with a few crystals of potassium ferricyanide.

(iii) Add a little solid sodium dithionite to reduce the sample (solid dithionite is easily oxidised on exposure to air so the bottle must be kept sealed and the surface layer of dithionite avoided). Leave for a couple of minutes and record the spectrum.

(iv) Check that the sample is totally reduced by adding a little more dithionite and recording another spectrum. From the total reduced minus oxidised absorbance, the optimum concentration to use can easily be calculated, typically 100−200 μM BChl. An excess of dithionite may cause the membranes to precipitate. In the absence of mediators, some components, particularly membrane-bound components, are only slowly reduced by dithionite because of kinetic limitations.

Having established the optimum BChl concentration, the titration can now be performed.

(i) Dilute the sample in buffer that has been thoroughly bubbled with argon (or nitrogen) to remove the oxygen. Transfer to the redox cuvette and, passing argon over the solution continuously, stir for about 20 min. If a reference cuvette is required, enough sample should be diluted to be split between reference and sample cuvettes to ensure a good optical match between the two. Argon needs only to be bubbled through slowly once the apparatus is set up, one bubble every couple of seconds is enough to maintain anaerobicity. This can be monitored by bubbling the outlet gas from the cuvette through water. At this stage ensure there is no gas leak from the system.

(ii) Add the mediators through the septum. The choice of suitable mediators is discussed above. DAD and TMPD are slowly destroyed at high potential so should only be added when the potential is below +300 mV. Ferricyanide was the only mediator present in the titration illustrated in *Figure 12*.

(iii) If a reference cuvette is being used, it should be brought to the desired potential. Since that absorbance has to be constant over the whole course of the titration, which may be 2 h or so, the poise of the redox components has to remain steady for that length of time. The simplest approach is to oxidise them completely by addition of a crystal or two of ferricyanide.

(iv) Adjust the potential of the redox cuvette to the starting point for the titration. Make up a range of concentrations of sodium dithionite and potassium ferricyanide. As a starting solution, dissolve enough to cover the end of a microspatula in 1 ml or so of buffer.

(v) Add aliquots of 1 or 2 μl of dithionite through the septum to decrease the potential, and of ferricyanide to increase the potential. The amount that it is necessary to add to achieve the desired change in potential must be established by trial and error. Many samples, such as bacterial chromatophores, will contain endogenous reductants which act to bring the redox potential down during some parts of the titration, without the addition of external reductant. This can be exploited, if the rate of drift of the potential is compatible with full equilibration, as a convenient way to decrease the potential.

(vi) Allow the sample to remain at the starting potential for long enough for full equilibration. When the sample is equilibrated there should be no change in the absorbance while there is no change in the potential. The time required to reach equilibration will depend on how far the potential has been changed; at the initial point of the titration, which may mean a relatively large change of potential, at least 10 min should be allowed for the sample to equilibrate. If the potential is unstable and drifts away significantly from the desired potential it must be brought back and re-equilibrated. If the potential does not change on addition of dithionite or ferricyanide, check that the solution is being stirred efficiently; if it is, then try with a stronger solution of dithionite or ferricyanide.

Unbuffered dithionite solution is not stable at room temperature, in air, and must be made up in buffer and left with a stream of argon or nitrogen bubbling through. It is more stable in basic solution than in acidic solution. It is generally obvious when the dithionite solution has 'gone off' since it is no longer effective in bringing the potential down and begins to accumulate a dirty white precipitate. Potassium ferricyanide solution is stable for 1 day and should then be discarded.

(vii) Scan a spectrum over the desired range of wavelengths, which will include a wavelength to measure the change and an isosbestic wavelength as a reference (Chapter 4).

(viii) Change the potential by about 9–10 mV by addition of aliquots of dithionite or ferricyanide. Allow to equilibrate for about 4 min at the new potential (equilibration of membrane-bound components is generally more difficult than that of soluble components) and record another spectrum. Continue to the desired end point or until there is no longer any change. If titrating several components over a wide range of potential it is better to do more than one titration, over different parts of the range, overlapping to allow them to be matched up. The presence of endogenous reductants and differences in redox buffering through the mediators can lead to it being easier to poise the potential in some regions than in others.

(ix) At the end point of the titration, take the potential back up to the starting point and record the spectrum again to check that it has not altered. One or two additional points of the titration can also be checked, making sure that after each change of the potential the sample is left sufficiently long to equilibrate.

(x) Repeat the titration using a fresh sample but this time changing the potential in the opposite direction. There should be no hysteresis between the two titration curves; if there is, it indicates that the sample was not fully equilibrated. Try leaving it a longer time at the potential before taking a reading at each point and check that the correct mediators for that potential range are present in the correct concentrations.

3.7 Treatment of Data

(i) At each potential, record the difference in absorbance between two suitable wavelengths — a measuring wavelength and a wavelength which is isosbestic for the absorbance change of the component of interest. Plotting the value of the absorbance difference against ambient potential will give a graph of the type shown in *Figure 11*. *Figure 11a* represents a redox couple where one electron is involved ($n = 1$), *Figure 11b* a redox couple where two electrons are involved. However, this type of plot is less useful for equilibrium titrations than the one discussed below.

(ii) To get a straight line graph, from the relationship above,

$$E_h = E_m + \frac{59}{n} \log_{10} \frac{\text{oxidised}}{\text{reduced}}$$

where $2.3\frac{RT}{F} = 59$ mV at 25°C and n is the number of electrons involved in the reduction, plot $\log_{10} \frac{\text{oxidised}}{\text{reduced}}$ against E_h.

(iii) Calculate a value for the amount of oxidised component at each potential E_h, by taking the difference between the absorbance of the totally reduced component and the absorbance of the reduced component at E_h, for all potentials.

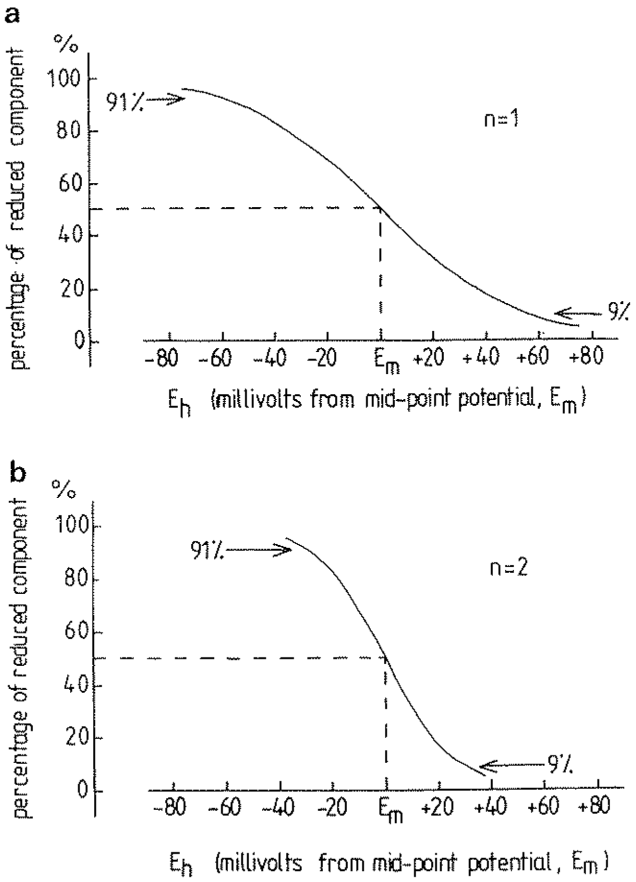


Figure 11. The dependence of the proportion of reduced species on redox potential for **(a)** a one-electron ($n = 1$) reaction and **(b)** a two-electron ($n = 2$) reaction.

(iv) Calculate the ratio of oxidised to reduced at each potential. Plot the logarithm of this ratio against ambient potential E_h, to get a graph of the sort shown in *Figure 12*.

If an $n = 1$ couple is titrated, a slope of 59 mV/decade change should be found, for an $n = 2$ couple the slope is 29.5 mV/decade change at 25°C. If a straight line is not obtained it may indicate a lack of equilibration between measurements (Section 3.6). The mid-point potential can then be read from the graph at the point where the ratio of oxidised to reduced species is equal to 1, i.e. where $\log_{10}$ (oxidised/reduced) is equal to 0. This corresponds to the point of maximum slope on the plot shown in *Figure 11*.

In the example given, the mid-point potential of the *c*-type cytochrome is +344 mV and the slope of the line indicates a one-electron reaction.

It may be that the titration involves more than one component with similar spectral

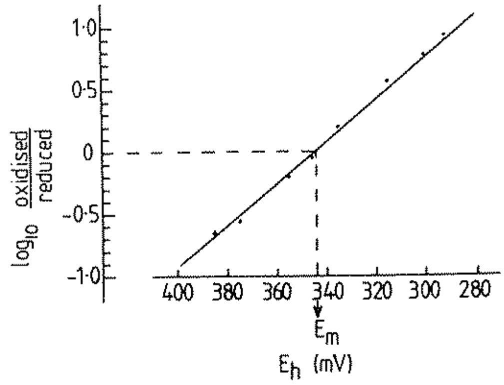


Figure 12. Redox titration of chromatophores of *Rhodopseudomonas capsulata* AlAPho$^+$, grown with N_2 as the sole nitrogen source, suspended to 150 μM BChl in 50 mM MOPS, 50 mM KCl, pH 7.0. 100 μM potassium ferricyanide was present as a mediator. The titration was conducted in the direction of decreasing potential. The measurements were made on a computer-linked single beam scanning spectrophotometer (N.G. Holmes and A.R.Crofts, unpublished observation).

characteristics, but with different mid-point potentials. As a rule of thumb, for an $n = 1$ plot, the change from 9% to 91% oxidised (or reduced) takes 118 mV (see *Figure 11*), similarly for an $n = 2$ plot, 9% to 91% takes 59 mV; if changes occur over a much wider span, then this suggests the titration of more than one component. It may be necessary in such multi-component titrations to try a number of plots, varying the parameters, and seeing which gives the optimal fit. This is most simply done by computer fitting of the data. In any case, if the components are close together then it becomes impossible to resolve them by eye and a computer must be used.

If the components are not too close together, and a clear plateau is visible between the two regions of changing absorbance with potential, they can be calculated as though they were two separate titrations.

4. ACKNOWLEDGEMENTS

We would like to thank the many colleagues who have contributed unwittingly to this chapter through comments and discussions over a number of years. We are indebted to Christine Sanders for artwork and for help with preparation of the manuscript, and thank Alan Logan for photography.

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APPENDIX

Suppliers

Cuvette	York Glassware Services, 9 The Crescent, Blossom Street, York Y02 2AW, UK
Redox electrode	Kent Industrial Measurements, EIL Analytical Instruments, Hanworth Lane, Chertsey, Surrey, UK Russell pH Ltd., Station Road, Auchtermuchty, Fife KY14 7DP, UK
Septum and tubing	Portex Ltd., Hythe, Kent CT21 6JL, UK
Oxygen electrodes	Hansatech Ltd., Paxman Road, Hardwick Industrial Estate, King's Lynn, Norfolk, UK Rank Bros., High Street, Bottisham, Cambridge CB5 9DA, UK
Electronic stirrers	Rank Bros., High Street, Bottisham, Cambridge CB5 9DA, UK
Fibre-optic light sources	CUEL, 12 Tulip Tree Avenue, Kenilworth, Warwickshire CV8 2BU, UK
LED light sources	Hansatech Ltd., Paxman Road, Hardwick Industrial Estate, King's Lynn, Norfolk, UK
Photoacoustic sensor	Applied Photophysics, 20 Albemarle Street, London W1X 3HA, UK