With this technique, the problems of sample transfer, loss, recovery, contamination, and positioning of the microcell are eliminated. The bulk of the sample is readily recovered for use in additional spectrometric analyses. Since the cost of the microcell is negligible, it may be discarded rather than cleaned.

With the aid of the time averaging computer, a usable spectrum may be obtained from 100 transients on a 250-$\mu$g sample. Also, limited information may be obtained from 100-$\mu$g samples as shown in Figure 3.

## LITERATURE CITED

(1) Varian Instruments Application Report NMR-2.

# Optically Transparent Thin Layer Electrode for Anaerobic Measurements on Redox Enzymes

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A spectropotentiostatic method using an optically transparent thin layer electrode, OTTLE, for measuring $E^{\circ\prime}$ and $n$ values of redox enzymes was recently reported (1). The OTTLE was of the minigrid–microscope slide design with the bottom edge immersed in a small cup of solution (2). It was pointed out that measurements could be made on solution volumes of less than 1 ml with a cell design which eliminated the cup. This is an important consideration since many enzymes are available only in minute amounts. We describe here an OTTLE with which spectropotentiostatic measurements can be made on a total solution volume of down to 500 $\mu$l. An equally important feature of this cell is the ability to remove dissolved oxygen from solution by a vacuum degassing technique. Electrochemical measurements on the many redox enzymes which are oxidizable by molecular oxygen must be performed under such anaerobic conditions.

## EXPERIMENTAL

**Apparatus.** The optically transparent thin layer cell is shown in Figure 1. It is basically of the minigrid–microscope slide variety of OTTLE (2). The solution cup was eliminated and the auxiliary electrode was moved into the thin layer with the reference electrode probe located between the working and auxiliary minigrid electrodes. The cell was adapted to oxygen removal with vacuum/nitrogen cycling by using a previously reported vacuum degassing bulb and special Ag/AgCl reference electrode (3).

The front face of the cell consisted of clear lucite, $1 \times 1\frac{3}{4} \times \frac{1}{8}$ inches. Two blocks of $\frac{1}{4} \times \frac{1}{4} \times \frac{1}{4}$ inch lucite were attached to the front face with 1,2-dichloroethane. Holes were drilled through the blocks to accommodate valves (No. 1MM1, Hamilton Co., Reno, Nev.). The back of the cell was a microscope slide cut to the dimensions of the front. Three layers of 2-mil pressure-sensitive Fluorofilm DF-1200 Teflon tape (Dilectrix Corp., Farmingdale, N.Y.) cut in 2-mm strips, were placed around the edges of the glass for spacers. The working and auxiliary electrodes, $1 \times 3.5$ cm pieces of gold minigrid (500 wires per inch, 60% transmittance, Buckbee Mears Co., St. Paul, Minn.), were placed 5 mm apart and were sandwiched between the faces of the cell. The minigrids extended outside of the cell for electrical contact. The cell was sealed around the edges with epoxy. A silver/silver chloride, 1.0 M KCl reference electrode contacted the cell through valve V2 located between the minigrids. Placement of the reference probe between the auxiliary and working minigrids was critical for obtaining best potential control of the OTTLE minigrid electrode. A vacuum degassing bulb was attached to the other valve, V1; this bulb allowed the cell to be connected to a vacuum/nitrogen train. Details of the reference electrode, vacuum degassing bulb, and vacuum/nitrogen train have been described (3).

No problems were encountered in preparing vacuum-tight cells. Careful application of epoxy to completely cover the edges consistently resulted in good cells. Cell lifetime was usually limited by fraying of the fragile minigrid contacts rather than by the development of leaks.

A Princeton Applied Research Model 173 potentiostat in conjunction with a Model 175 Universal Programmer was used for applying potentials to the cell. Potentials applied for the spectropotentiostatic experiments were measured with a Digitec 261C digital voltmeter. Optical measurements were made with a Harrick Rapid Scan Spectrometer, Model RSS-B.

**Reagents.** Solutions were prepared in pH 7.00, 0.1 M phosphate buffer (Buffer Titrisol, EM Laboratories, Elmsford, N.Y.) and 0.1 M NaCl (Suprapur, EM Laboratories). Horse heart cytochrome $c$ (Type VI, 95–100% pure, Sigma Chemical Co., St. Louis, Mo.) and 2,6-dichlorophenolindophenol (99% pure, Fluka, Columbia Organic Chemicals, Columbia, S.C.) were used without further purification.

**Procedure.** The following procedure was used for oxygen removal from the enzyme solution and for filling the OTTLE. Close adherence to this procedure was necessary for optimum results. The thin layer cell was first evacuated by attaching it to the vacuum/nitrogen train by means of the degassing bulb and applying a vacuum. The stopcock on the bulb and the two valves were opened, and three cycles of vacuum-nitrogen were applied, leaving the cell under nitrogen pressure. The valves and stopcock were closed and the cell was removed from the train. A solution of previously degassed 1.0 M KCl was added by syringe to the reference tube, and the Ag/AgCl wire was reinserted. The cell was again attached to the train, the stopcock was opened, and two cycles of nitrogen–vacuum were applied to the bulb. The valve V1 to the cell body was opened and the vacuum–nitrogen cycle was repeated three more times, leaving the cell under nitrogen pressure. The reference valve V2 was then opened and the vacuum carefully applied until the KCl solution rose to the top of the reference tube, at
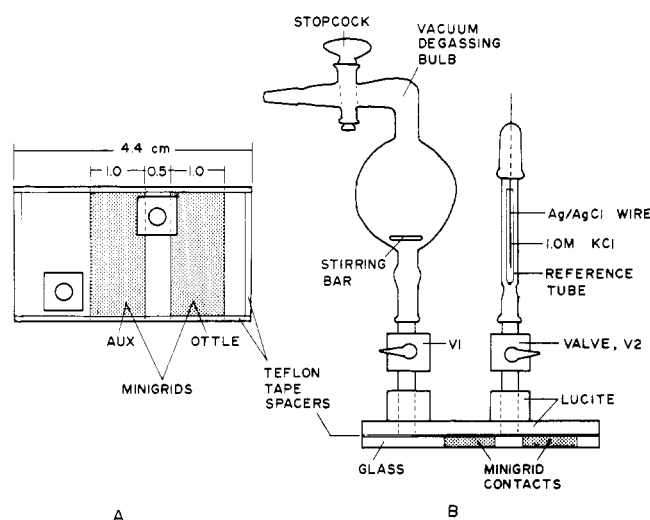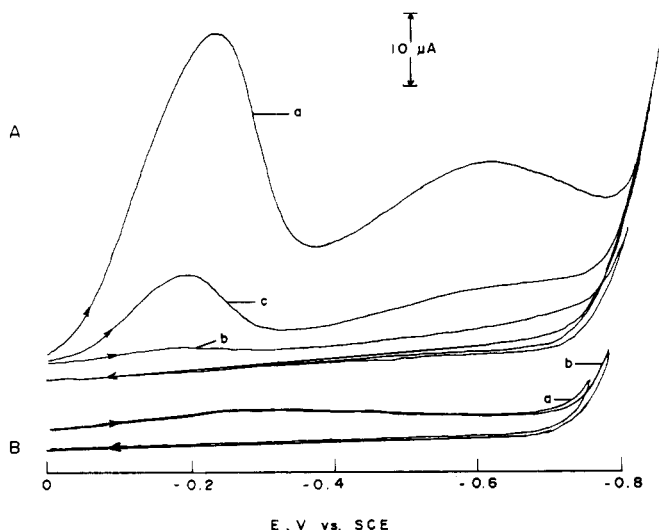


**Figure 1.** Optically transparent thin layer cell

(A) Top view, (B) Side view

**Figure 2.** Thin layer cyclic voltammograms of 0.1 M NaCl, 0.1 M phosphate buffer, pH 7.00. Scan rate 5 mV sec$^{-1}$

(A) OTTLE with solution cup. (a) Oxygen saturated solution. (b) Immediately after $O_2$ removal by reduction to $H_2O$. (c) 60 min after $O_2$ removal. (B) OTTLE in Figure 1. (a) Immediately after $O_2$ removal by vacuum/nitrogen cycling. (b) 60 min after $O_2$ removal

which time nitrogen was applied. This step was repeated three times to deoxygenate the KCl solution, leaving the reference under nitrogen pressure. The reference valve V2 was closed and vacuum applied for 5 min, followed by an application of nitrogen. The valve V1 and stopcock were closed and the cell removed from the train. Enzyme solution was then added by syringe to the vacuum degassing bulb. A lower limit of 500 $\mu$l of solution has proved to be sufficient for filling the cell. The cell was reconnected to the train, the stopcock opened, and three more cycles of vacuum–nitrogen were applied to deoxygenate the enzyme solution. Vacuum was then applied as the valve V1 to the cell body was opened. The vacuum and nitrogen were cycled until there were no bubbles in the thin layer cell. The stopcock was closed and the cell disconnected from the train while under nitrogen pressure. The reference valve V2 was opened, and the cap on the reference electrode was barely opened so that solution was forced into the reference electrode until the tip of the reference tube was immersed in solution. The valve V1 to the cell body was closed and the vacuum degassing bulb removed from the cell.

The procedure for the spectropotentiostatic experiments has been described (1). At the end of each experiment, the potential of the Ag/AgCl reference electrode was measured vs. an SCE. The reference electrode was removed from the valve and the end immersed in 1.0 M KCl which also contained the SCE. The potential difference between the two electrodes was then measured.

## RESULTS AND DISCUSSION

**Oxygen Removal.** The OTTLE cell described here enables removal of dissolved oxygen by vacuum-nitrogen cycling, a technique which has proved effective in the study of autoxidizable enzymes such as cytochrome c oxidase and ferredoxins (4). The ability to remove oxygen by this procedure was compared with the previously reported method of removal by electrochemical reduction to water (1). The amount of oxygen present was measured by cyclic voltammetry.

Figure 2A shows cyclic voltammograms obtained with the conventional OTTLE in which the end of the cell is immersed in a cup of solution (1). Curve (a) is the initial scan on air saturated phosphate buffer at pH 7.0. The two reduction peaks correspond to reduction of $O_2$ to $H_2O_2$ and $H_2O_2$ to $H_2O$. Curve (b) was recorded immediately after electrochemical removal of $O_2$ by reduction to $H_2O$ at an applied potential of −0.70 V vs. SCE. The removal of dissolved $O_2$ in the minigrid region of the thin layer cell is indicated by disappearance of the two reduction peaks.
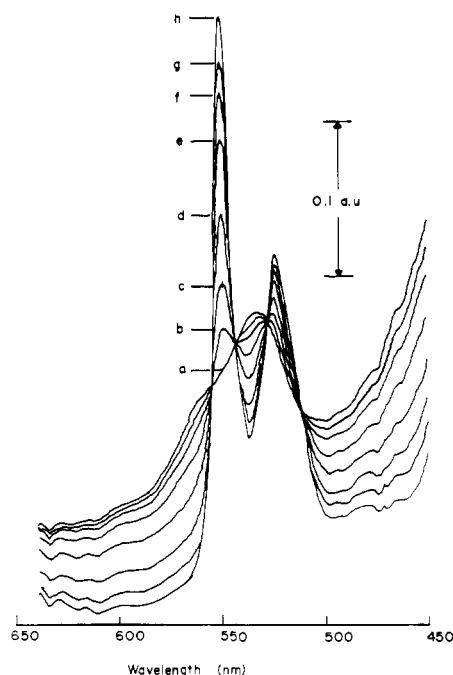


**Figure 3.** Thin layer spectra of 0.51 mM cytochrome c, 0.10 mM 2,6-dichlorophenolindophenol, 0.1 M NaCl, 0.1 M phosphate buffer, pH 7.00. Cell thickness 0.038 cm. $E_{applied}$: (a) 218, (b) 68.0, (c) 48.1, (d) 28.1, (e) 8.0, (f) −12.0, (g) −31.9, (h) −582 mV vs. SCE

Curve (c) was recorded after allowing the OTTLE to sit at open circuit for 60 min. Substantial diffusion of oxygen into the thin layer cell is evidenced by reappearance of the two reduction peaks. This oxygen diffuses into the cell from the solution above and below the minigrid where oxygen was not removed by the reduction to water procedure. Thus, any extended spectropotentiostatic experiment encounters the possibility of interference by oxygen.

Figure 2B shows the results which were obtained with the OTTLE described here. Curve (a) was recorded on a solution of pH 7.0 phosphate buffer immediately after oxygen removal by vacuum/nitrogen cycling. As evidenced by the absence of reduction peaks, oxygen was removed to an undetectable level. No oxygen could be detected 60 min later when curve (b) was recorded. It is apparent that dissolved oxygen can be effectively removed to a level which is undetectable by cyclic voltammetry [less than ca. $10^{-5}$ M] and maintained at that level for a sufficiently long period of time to pose no problem in most experiments.

**Spectropotentiostatic Evaluation.** In order to determine whether the OTTLE cell could be reliably used for the study of redox enzymes, spectropotentiostatic experiments (1) were performed on the enzyme cytochrome c in the presence of the mediator-titrant 2,6-dichlorophenolindophenol. Thin layer spectra recorded for various values of applied potential are shown in Figure 3. Curve (a) corresponds to cytochrome c in its entirely oxidized form; curve (h) shows the entirely reduced form with sharp absorbance bands at 550 and 520 nm.

A Nernstian plot of $E_{applied}$ vs. log ([O]/[R]) where the ratio [O]/[R] was obtained from the absorbance behavior at 550 nm produced an $E^{\circ\prime}$ of 262.9 mV vs. NHE (std dev = ±1.3 mV, $N$ = 3) for cytochrome c from the potential-axis intercept. This agrees well with the value of 262 mV which was previously obtained by the spectropotentiostatic method in the conventional OTTLE (1). A slope for the Nernstian plot of 58.4 mV (std dev = ±1.6 mV, $N$ = 3) was obtained. This is slightly lower than the theoretical value of 59.1 mV which was reported previously (1).

**Reference Electrode.** Stability of the reference electrode potential is very important in the OTTLE spectropotentiostatic method for measuring $E^{\circ\prime}$. Any changes in the potential of the Ag/AgCl reference electrode would cause error in the $E^{\circ\prime}$ value. Initial measurements with the OTTLE gave irreproducible results which were traced to changes in potential of the Ag/AgCl reference. The potential shifted to a different value if some enzyme solution was inadvertently transferred into the reference tube containing the 1 M KCl during the last step in the procedure for filling the cell. The potential of the Ag/AgCl electrode was found to vary from −4.4 to +18.1 mV vs. SCE. Although the potential varied from experiment to experiment, the potential drift over a 24-h period was less than 1 mV, once solution was added to the reference tube. This suggested that the problem could be avoided by measuring the potential of the Ag/AgCl electrode vs. that of an SCE at the end of each experiment (see Procedure). Any potential shifts could then be corrected. When this procedure was adopted, good agreement between $E^{\circ\prime}$ values measured in separate experiments was obtained. The potentials reported in Figure 3 were corrected to the SCE in this manner.

## CONCLUSION

The cell described here is well suited for spectropotentiostatic measurements on redox enzymes. The total solution volume required is only 500 μl. Removal of dissolved oxygen by vacuum–nitrogen cycling enables experiments to be performed under essentially anaerobic conditions.

## LITERATURE CITED

(1) W. R. Heineman, B. J. Norris, and J. F. Goelz, *Anal. Chem.*, **47**, 79 (1975).
(2) R. W. Murray, W. R. Heineman, and G. W. O'Dom, *Anal. Chem.*, **39**, 1666 (1967).
(3) F. M. Hawkridge and T. Kuwana, *Anal. Chem.*, **45**, 1021 (1973).
(4) W. H. Orme-Johnson and H. Beinert, *Anal. Biochem.*, **32**, 425 (1969).

## CORRECTION

### Normal Pulse Polarographic Analysis Based on Mercury Anodization: Sulfide and Iodide

In the paper by John A. Turner, Roger H. Abel, and Robert A. Osteryoung, *Anal. Chem.*, **47**, 1343 (1975), Equation 1 as given in the paper is incorrect and should read

$$-i(t) = \frac{nFA\sqrt{D_R}}{\sqrt{\pi t}} \left( C^{\circ} - \frac{1}{f_R} \exp\left[ \frac{-nF}{RT}(E - E^{\circ}) \right] \right) \quad (1)$$

where $f_R$ is the activity coefficient of the diffusing anion and the other terms have their usual meaning. The simulation to obtain Figure 1 was carried out properly assuming $f_R = 1$. We are indebted to Richard Burnette of the Department of Chemistry of the University of Tennessee for calling this to our attention.

## CORRECTION

### Prediction of Continuum Intensity in Energy-Dispersive X-ray Microanalysis

In this article by C. E. Fiori, R. L. Myklebust, K. F. J. Heinrich, and Harvey Yakowitz, *Anal. Chem.*, **48**, 172 (1976), the symbol $b$ is missing in Equation 5. The equation should read

$$N(E) = f_E P_E \bar{Z} \left[ a\frac{E_o - E}{E} + b\frac{(E_o - E)^2}{E} \right] \quad (5)$$