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Calibration and Use of a Clark-Type Oxygen Electrode from 5 to 45°C[1]

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A calibration procedure for a Clark-type oxygen electrode over a wide range of temperatures is described. The autoxidation of duroquinol (2,3,5,6-tetramethyl-1,4-benzenediol) was used to verify the electrode's ability to accurately sense the total amount of dissolved O_2 in an aqueous buffer. Electrode response time was measured by using oxygenated ethanol to deliver a rapid increase in O_2 concentration to the reaction medium. An oxygen-producing system (spinach thylakoids) was utilized to test the range of O_2-evolution rates able to be sensed. It was concluded that a Clark-type oxygen electrode has the absolute sensitivity, rapidity, and range necessary to accurately track rates of O_2 production or consumption from 5 to 45°C.

KEY WORDS: enzyme kinetics; oxygenases; polarography; photosynthesis; photorespiration; plant systems.

The use of a Clark-type oxygen electrode (1) at temperatures lower than 25°C is mentioned only rarely in the literature (2,3) and is not recommended by Yellow Springs Instruments in their electrode instruction manual (4). A protocol for measuring the temperature dependency of photosynthetic oxygen evolution was needed in our lab; therefore, confirmation of the electrode's sensitivity from 5 to 45°C was necessary. Electrode "sensitivity" was defined as the ability to (a) accurately determine the total amount of dissolved oxygen over a range of temperatures, (b) respond rapidly to changes in dissolved oxygen concentration, and (c) accurately follow rates of oxygen production or consumption by leaf discs as photosynthesis and respiration take place.

MATERIALS AND METHODS

Experimental setup. Equipment consisted of a Clark-type O_2 electrode covered with a Teflon membrane (labeled "Standard" by YSI) and a Model 53 oxygen monitor (both from Yellow Springs Instrument Co., Yellow Springs, Ohio), a Zenith strip chart recorder, a Forma Scientific constant-temperature, water-circulating bath, a stirring plate, and a lamp. The 1.72-ml glass reaction vessel had a side port for exposure of the reaction mixture to the electrode and a ground-glass stopper with a capillary-size hole through the middle, and contained a magnetic stir bar. The reaction vessel was housed in a glass jacket of approximately 40 ml volume which contained the circulating coolant.

Electrode calibration. Duroquinol (DQH_2)[3], which autoxidizes rapidly at high pH and consumes stoichiometric amounts of oxygen, was obtained from ICN Radiochemicals (Irvine, Calif.). An approximately 12 mM DQH_2 solution was made up gravimetrically in 100% deoxygenated (bubbled with N_2) ethanol, acidified with 5 μl of 2 N HCl/10 ml ethanol, and stored under N_2, in the dark and on ice. Ten microliters of the

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[3] Abbreviations used: DQH_2 or duroquinol, 2,3,5,6 tetramethyl-1,4-benzenediol; DQ or duroquinone, 2,3,5,6 tetramethyl-2,5-cyclohexadiene-1,4-dione.

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ethanolic DQH_2 stock was diluted into 3 ml of 50 mM Hepes buffer (pH 6.0) in a 1-cm quartz cuvette and scanned from 240 to 315 nm in an Aminco DW2 spectrophotometer (Fig. 1, Scan III). The dilution was then fully reduced by the addition of a small amount of solid $NaBH_4$ and again scanned (Fig. 1, Scan I). A second dilution was made up, scanned (Fig. 1, Scan III), forced to full oxidation by the addition of 15 μl of 5 N NaOH, and scanned again (Fig. 1, Scan II). DQH_2 concentration in the stock solution equaled

$$[DQH_2] = \left[\frac{(3\text{ ml})}{(.01\text{ ml})}\right]\left[\frac{A_{265}^{ox}}{(21.2/\text{mM/cm})(1\text{ cm})}\right] \times \left[1 - \frac{(A_{265}^{stock} - A_{265}^{red})}{(A_{265}^{ox} - A_{265}^{red})}\right].$$

Three such stock DQH_2 solutions were made up, quantified spectrophotometrically, and used for the Clark electrode calibration.

A photosynthetic reaction buffer (60 mM Tricine–NaOH buffer, pH 7.6, 70 mM Na_2HCO_3, and 1 mM salicylhydroxamic acid) was adjusted to pH 9.0 with NaOH and equilibrated with atmospheric O_2 levels by bubbling with laboratory line air. After equilibration, the chart pen was set at the paper margins to represent 0 and 100% of the O_2 available for reduction. A 5- or 10-μl aliquot of the DQH_2 stock was added to the reaction vessel, resulting in O_2 consumption and a concomitant pen deflection (a similar addition of distilled H_2O caused only a small, transient blip on the recorder). The procedure was repeated three times for each of the three DQH_2 stocks at each of the nine temperatures.

Electrode response time. The temperature dependency of electrode response time was calculated by adding a known amount of O_2 to the reaction vessel. One hundred percent ethanol was equilibrated with atmospheric levels of oxygen by bubbling with lab line air and then added in 25-μl aliquots [47 nmol O_2 based on solubilities given in Ref. (5)] to a partially deoxygenated buffer in the reaction vessel.

Maximum rate of electrode response. To verify that the electrode can accurately track rates of O_2 depletion from or supply to the reaction mixture over a range of rates of physiological significance, an oxygen-evolving system (spinach thylakoids) was titrated into the reaction vessel. One hundred grams of deveined spinach leaves was homogenized

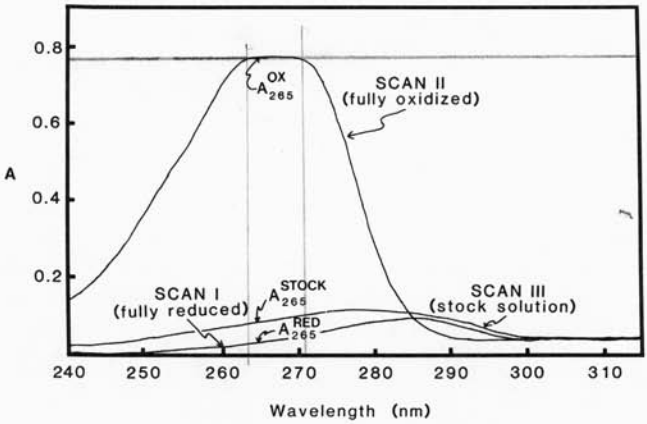

FIG. 1. Spectral scans of DQH_2 (Scan I), DQ (Scan II), and a diluted stock solution containing both reduced and oxidized duroquinol forms (Scan III) in aqueous solution.

with a Polytron mixer in a buffer of 0.4 M sucrose, 20 mM Tricine (pH 8.0), 15 mM NaCl, and 2 mg/ml bovine serum albumin, filtered through cheesecloth, and centrifuged at 1000*g* for 1 min. The resulting supernatant was decanted and centrifuged at 5000*g* for 10 min. The pellet was washed by resuspension in homogenization buffer and centrifugation again for 10 min at 5000*g*. The washed thylakoids were resuspended in a small amount of homogenization buffer and used for the titrations. Chlorophyll was determined according to Arnon (6).

The reaction medium was a 35 mM Tricine buffer (pH 7.4) containing 30 mM NaCl, 10 mM CH_3NH_2, and 2.5 mM $K_3Fe(CN)_6$. Partially deoxygenated buffer was used to prevent a supersaturation of oxygen during the experiment. Illumination was provided by 1500 $\mu E/m^2/s$ of red light measured at the surface of the coolant jacket.

RESULTS AND DISCUSSION

Electrode calibration. Calibration of the Clark electrode using known amounts of duroquinol (and therefore known amounts of oxygen depletion) agreed well with values calculated from the known solubility of oxygen at various temperatures (Fig. 2). The slight experimental overestimation at the cooler temperatures may be the result of a nonenzymatic decomposition of the H_2O_2 (Eq. [2]) produced during O_2 reduction by DQH_2 (Eq. [1]).

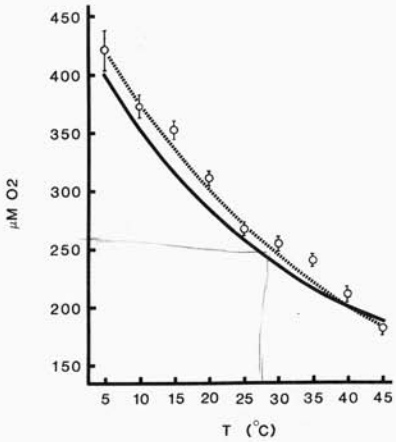

FIG. 2. Experimentally (---) and theoretically derived (—) values for the total amount of dissolved O_2 in aqueous solution after equilibration with air from 5 to 45°C. For experimental curve, mean ± SE, $n = 9$.

$$DQH_2 + O_2 \xrightarrow{\text{pH } 9.0} DQ + H_2O_2 \qquad [1]$$

$$H_2O_2 + H_2O_2 \rightarrow 2H_2O + O_2 \qquad [2]$$

The effect of combining equations 1 and 2 will be to underestimate the amount of oxygen consumed by the DQH_2 and therefore overestimate the amount of oxygen available for reduction. The electrode responds more rapidly at 45 than at 5°C (see Table 1 and the accompanying discussion), thus allowing less time for H_2O_2 decomposition and offering an explanation for the better agreement between experimental and calculated values obtained at the higher than at the lower temperatures (Fig. 2). When DQH_2 oxidation was followed spectrophotometrically, the total increase in absorbance (i.e., full oxidation) at 265 nm occurred in less than 1 s after a three-unit pH increase; therefore, a direct oxidation of DQH_2 by H_2O_2 as a source of the discrepancies seen in Fig. 2 seems unlikely. No autoxidation of the stock ethanolic DQH_2 solution was detectable spectrophotometrically after 24 h of storage under N_2 (in the dark, on ice and in the presence of 1.0 mM HCl). Overall, it was concluded that a Clark electrode can accurately measure the amount of dissolved O_2 in aqueous solution from 5 to 45°C.

Misra and Fridovich previously pointed out (7) that such factors as ionic strength, partial pressure of oxygen, and temperature can make calibration of a Clark electrode via the known solubility of oxygen [as in Ref. (8)] a method with the potential for imprecision. Other techniques [as in Ref. (7)] also have their disadvantages. The advantage of using DQH_2 is that the strong absorbance from 260 to 270 nm allows for a very accurate spectrophotometric determination of con-

TABLE 1

RESPONSE TIMES (IN SECONDS) FOR RECOGNITION OF 50% ($t_{1/2}$) AND 100% (t) OF AN O_2 ADDITION (VIA ETHANOL) AT VARIOUS TEMPERATURES

	Temperature (°C)				
	5	15	25	35	45
$t_{1/2}$	7.4 ± 0.2	5.2 ± 0.4	5.0 ± 0.3	1 ± 0.1	<1
t	28 ± 3	16 ± 3	15 ± 1	9 ± 0.4	4 ± 0.4

Note. Mean ± SE, n = 9.

centration. Due to oxidation and hydration during storage, and the uncertainties associated with weighing out small quantities of solids, concentration determinations based on gravimetric measurements are subject to considerable error. The three "12 mM" stock solutions used in this study actually had duroquinol concentrations of 10.8, 11.4, and 11.2 mM.

Electrode response time. At all nine temperatures, an addition of O_2 to the reaction vessel was immediately recognized by the electrode but 100% recognition showed a temperature-dependent time lag (Table 1).

It was concluded that the rapidity of the initial response to added O_2 was sufficient at all temperatures to record any actual changes effected by the leaf tissue. The reason for the temperature-dependent time lag was not further investigated but may result from a decrease in O_2 permeability that the Teflon electrode membrane demonstrates with decreasing temperatures (4). This possibility is supported by the observation that at 5°C a membrane stretched very tightly across the electrode made for a more rapid response than one stretched not so tightly (and therefore thicker). No differences in the steady-state rate of oxygen sensing were seen. However, at 45°C a tight membrane resulted in a much "noisier" electrode than when the membrane was left unstretched.

Maximum rate of electrode response. Figure 3 demonstrates the ability of a Clark electrode to respond in a linear fashion to increasing levels of O_2 production at four temperatures. Of particular importance is that the electrode response remains linear well beyond the range of photosynthetic measurements typically recorded at all temperatures (dashed portions of each line on Fig. 3). The electrode can therefore give an accurate reading of the changing oxygen status in the reaction buffer. A similar titration with spinach thylakoids

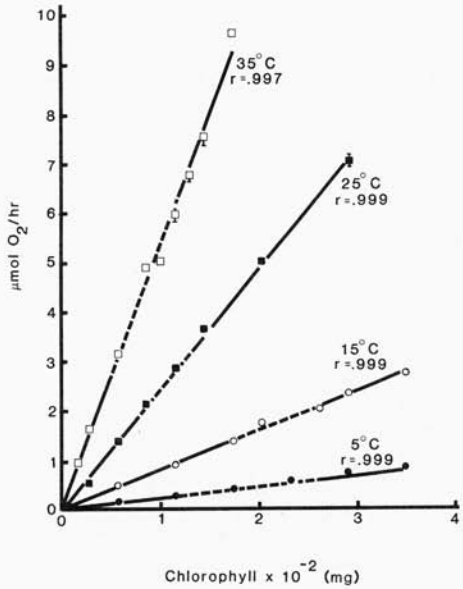

FIG. 3. Titration of maximum electrode response rate at four temperatures. The dashed portion of each line represents values on the ordinate corresponding to the range of photosynthetic measurements typically seen in leaf discs. Each datum point is the mean ± SE of three replicates.

was attempted at 45°C but water oxidation was completely inhibited.

Although developed in our lab to measure photosynthesis of leaf discs over a wide temperature range, the temperature dependency of any oxygen-consuming or -producing process can be followed with the Clark electrode. Such systems as tissue discs or slices, plant protoplasts, cells in liquid suspension culture, isolated organelles, suborganellar particles, and enzymes can be easily investigated at a variety of temperatures.

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