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Electrochemical Standardization

of the Dehydrogenase Assay Used in the

Estimation of Respiration Rates

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ABSTRACT

A primary coulometric titration is proposed as a standardization technique for the succinate dehydrogenase assay used in the estimation of respiration rates in marine plankton. The coulometric method, which is shown to be simple, accurate, and reproducible, eliminates the difficulties of using a chemical reducing agent. An alternative method using polarography is also discussed.

Introduction. Plankton respiration has been estimated from determinations of the activity of succinate dehydrogenase (Curl and Sandberg 1961, Pearre 1964, Packard 1967). This method is based on the reduction of 2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazolium chloride (INT) by succinate dehydrogenase extracted from the plankton. The usefulness of this method has been limited by the unreliability of the standardization. A standard curve based on the reduction of INT by ascorbic acid is prepared. However, auto-oxidation makes ascorbic acid a poor primary standard, and standard curves based on its reaction with INT [eq. (1)] are neither reproducible nor linear. Curl (personal communication) has proposed the use of cysteine as a substitute for ascorbic acid, but with its use the standardization takes at least eight hours. Since any reducing agent simply acts to supply electrons, we are proposing the use of an electrode that supplies the electrons directly. A linear relationship should exist between the number of coulombs passed through the cell circuit

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and the amount of formazin (reduced INT) produced, provided the INT is reduced stoichiometrically [eq. (2)]:

\[
\text{INT} + 2e^- + 2 \text{H}^+ = \text{INT-formazin} + \text{HCl}.
\] (2)

Electrolysis at constant current provides a simple and practical method for reducing INT. Only the fundamental quantities of current and time are involved, both of which are easier to measure than a chemical titrant (Willard et al. 1965). By performing a constant coulometric titration, the molar concentration of formazin \(C\) can be calculated from the cell current in amperes \(I\) by using the equation

\[
C = \frac{It}{nFV_s}.
\] (3)

where \(t\) is the time (in seconds) during which the current passes through the cell, \(F\) is one Faraday of electricity, \(n\) is the number of electrons gained per molecule of the reduced species, and \(V_s\) is the sample volume in liters. During this process, INT is reduced to a formazin, which is insoluble in water. Following the reduction, the formazin is extracted with an organic solvent and its absorbance is determined in a spectrophotometer. Thus, the standardization of INT is accomplished by timing the passage of a fixed current through the sample cell and by plotting the absorbance of the resulting formazin solution against the number of coulombs.

Usually, constant-current coulometric titrations are used to determine the total amount of a species in question. Current is passed through a cell until an independently measured end point is reached. Often another species is added to generate electrically an ion that in turn reacts with the one in question. For our purposes, a simple constant-current electrolysis would be sufficient, provided the reduction proceeds at 100% current efficiency. Initial polarographic studies have shown that the INT can be reduced electrochemically. The INT apparently undergoes a 2-electron reduction at potentials close to those at which oxygen is reduced and much lower than those at which hydrogen
is evolved; there appears to be no side reduction of the substrate. These preliminary observations suggested the potential usefulness of the method.

**Reagents.** The phosphate buffer (pH 7.7), the 0.04 M sodium succinate, the 2% INT solutions, and the tetrachloroethylene-acetone (1:1.5, v/v) mixture were prepared according to Curl and Sandberg (1961). A reagent mixture was prepared from these stock solutions with 1 ml of distilled water, 1 ml of INT, 2 ml of buffer, and 1 ml of sodium succinate solution.

**Polarography.** In the polarographic studies we employed a small H-cell equipped with a 2-sec to 5-sec capillary dropping mercury electrode, a mercury-pool auxiliary electrode, and a saturated calomel reference electrode. The three-electrode cell was powered by a universal polarographic analyzer designed by Enke and Baxter (1964) and assembled in our laboratory.

![Figure 1](image-url)

Figure 1. Polarogram of $5.82 \times 10^{-4} M$ INT solution (curve A) and reaction mixture with no INT present (curve B).
To show that INT can be reduced electrochemically, an INT solution was subjected to polarographic analysis. The polarogram (Fig. 1) shows that INT is no more difficult to reduce than dissolved oxygen and that the reduction occurs in steps. Visual observation of the appearance of red colloids indicated that formazin is produced at potentials of less than $-0.75\, \text{v}$ versus saturated calomel electrode (SCE). Evidently the half-wave potential $[E_{1/2(1)}]$ for the first electron addition is at $-0.1\, \text{v}$ and the potential $[E_{1/2(2)}]$ for the second addition is at $-0.4\, \text{v}$; $E_{1/2(2)}$ for dissolved oxygen is about $-0.1\, \text{v}$ versus SCE. The reductions at voltages that are more negative than $-0.75\, \text{v}$ may be further additions of electrons at the nitrogen sites on the tetrazolium ring. The low half-wave potentials probably explain the instability of the reagent mixture. The diffusion current, $I_d$ (Fig. 1), should increase linearly with concentration if the polarographic behavior of INT is quantitative. In Table I, the ratios of $I_d/c$ for four different INT solutions are nearly constant, indicating that polarography alone could provide useful estimates of INT concentrations.

### Table I. Results of polarographic analysis. Diffusion current shown in microamperes.

<table>
<thead>
<tr>
<th>INT Conc. (c)</th>
<th>Diffusion current ($I_d$)</th>
<th>$I_d/c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$5.82 \times 10^{-4}, \text{M}$</td>
<td>2.60</td>
<td>$0.45 \times 10^{-4}$</td>
</tr>
<tr>
<td>$5.06 \times 10^{-4}, \text{M}$</td>
<td>2.10</td>
<td>$0.41 \times 10^{-4}$</td>
</tr>
<tr>
<td>$3.16 \times 10^{-4}, \text{M}$</td>
<td>1.40</td>
<td>$0.44 \times 10^{-4}$</td>
</tr>
<tr>
<td>$2.03 \times 10^{-4}, \text{M}$</td>
<td>0.85</td>
<td>$0.42 \times 10^{-4}$</td>
</tr>
<tr>
<td>$0.00 \times 10^{-4}, \text{M}$</td>
<td>0.00</td>
<td>$0.00 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

Coulometry. The constant-current source is shown in Fig. 3. The 45-v potential drop through the high-load resistance provides the constant current. The electrolysis cell (Fig. 2) is a 1.9-cm ID glass vessel with a capillary stopcock drain. The vessel has an anode half-cell and an N$_2$ inlet protruding through the rubber stopper. Contact with the mercury-pool cathode is made through a platinum wire sealed in the bottom of a vessel. The anode vessel is a 0.7-cm ID glass tube with a pressed-asbestos frit at the bottom end. This arrangement allows electrical continuity between the electrodes but retards diffusion of electrolysis products from the anode chamber into the substrate. A 2.4-cm$^2$ platinum foil is fitted into the tube for the anode.

**Procedure.**

(i) Fill the electrolysis cell to level A (Fig. 2) with clean mercury. Introduce 5 ml of the reaction mixture into the cell and attach the anode and bubbler. A flow of nitrogen sufficient to overturn the mercury surface and slowly mix the substrate is satisfactory.

(ii) Start and time the electrolysis. For times of less than 1 min, activate the timer with the starting switch; otherwise, manual activation is satisfactory. The INT is reduced on the mercury surface and red colloids of the formazin
appear. Following electrolysis, rinse the stopper, anode, and bubbler with the organic-solvent mixture.

(iii) Drain mercury from the cell. Then remove the organic and aqueous solutions and combine with them the solvent-mixture washings of the cell. Withdraw the aqueous layer with a pipette and clean the organic layer by centrifugation if necessary.

(iv) Adjust the volume of the formazin solution to 8.0 ml. If the absorbance is judged to be above 0.5, or if more than 0.05 coulomb has been passed through the cell, reduce the color intensity by appropriate dilution.
(v) Determine the absorbance of the formazin solutions at 490 mµ against a pure solvent blank in a Beckman DU spectrophotometer (Curl and Sandberg 1961). If extra dilution is necessary, normalize the absorbance values to the 8.0-ml volume.

Results. In Table II and Figs. 4, 5 is shown the relationship between the number of coulombs passing through the electrolysis cell and the formazin produced in the cell. Solutions A and B were prepared identically; the slight difference in slope apparently results from aging and demonstrates the neces-
The results for solution B show a linear increase in formazin production throughout the titration. After the end point at 810 millicoulombs (the intersection of
the dotted lines) is reached, a further passage of electricity causes no increase in formazin. The concentration of the initial INT stock solution can be calculated from this end point. This calculation for solution B (Fig. 5) yielded an INT concentration of 0.183%, indicating that only 92% of the original 2 g of INT dissolved or survived aging. The concentrations of INT reported in Table I were calculated on the assumption that 92% of the initial INT was present.

Discussion. In our early attempts to reduce INT electrochemically, a platinum disk was used as the cathode. The evolution of hydrogen gas from the platinum surface resulted in low current efficiency and erratic absorbancies. Also, the formazin tended to coat the electrode, increasing the cell resistance so that the cathode potential assumed a high negative value. The need for an electrode with a renewable surface and a higher hydrogen overvoltage was indicated. These requirements were met by using a mercury-pool cathode. The higher hydrogen overvoltage of mercury prevents hydrogen evolution, and the mercury surface is renewed by agitation.

The coulometric titration is a more desirable standard for INT solutions than is the ascorbic acid standard because it is linear, more reliable, and easier to use. The efficiency of the ascorbic acid reduction of INT can be determined by comparing the actual and theoretical formazin production. The actual formazin production in an 8 x 10^-4 M ascorbic acid solution was 0.055 absorbance unit (Fig. 4). If the reduction in this solution had been 100% efficient, the equivalent of 154.2 millieoulombs would have been used. Thus, the theoretical formazin production in normalized absorbance units is 2.27 (Fig. 5). This indicates that the ascorbic acid reduction of INT is only 4.7% efficient. If this efficiency were constant, ascorbic acid might be a usable standard, but the efficiency varies during handling, preparation, and storage.

A primary coulometric titration, as used in this study, differs from other coulometric titrations because the principal reduction occurs on the cathode surface. The application of primary coulometric titrations is rare because the

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Millieoulombs</th>
<th>Normal. formazin absorb.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>14.7</td>
<td>0.265</td>
</tr>
<tr>
<td>6</td>
<td>29.2</td>
<td>0.476</td>
</tr>
<tr>
<td>10</td>
<td>48.9</td>
<td>0.742</td>
</tr>
<tr>
<td>15</td>
<td>73.5</td>
<td>1.105</td>
</tr>
<tr>
<td>20</td>
<td>98.2</td>
<td>1.470</td>
</tr>
<tr>
<td>Solution B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>2.4</td>
<td>0.046</td>
</tr>
<tr>
<td>1</td>
<td>4.9</td>
<td>0.128</td>
</tr>
<tr>
<td>3</td>
<td>14.6</td>
<td>0.243</td>
</tr>
<tr>
<td>6</td>
<td>29.2</td>
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<tr>
<td>20</td>
<td>98.4</td>
<td>1.56</td>
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<tr>
<td>60</td>
<td>294.0</td>
<td>4.46</td>
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<tr>
<td>85</td>
<td>417.0</td>
<td>6.36</td>
</tr>
<tr>
<td>95</td>
<td>466.0</td>
<td>7.15</td>
</tr>
<tr>
<td>130</td>
<td>636.0</td>
<td>10.0</td>
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<tr>
<td>165</td>
<td>813.0</td>
<td>12.1</td>
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<tr>
<td>190</td>
<td>922.0</td>
<td>13.1</td>
</tr>
<tr>
<td>260</td>
<td>1265.0</td>
<td>12.5</td>
</tr>
<tr>
<td>281</td>
<td>1371.0</td>
<td>12.4</td>
</tr>
</tbody>
</table>
current efficiency falls below 100% as the titration proceeds. Since the reactant concentration decreases and since the flux of the product ions increases near the electrode surface, the electrode potential increases until the medium is electrolyzed; this results in a lower current efficiency. These difficulties usually are eliminated by generating electrically a reagent to react spontaneously with the substance to be determined. However, in our standardized case this method was not necessary because the formazin was insoluble and no product ions were ever present.

In preliminary polarographic analyses, the close correlation between the concentration of INT and the magnitude of the diffusion current demonstrated the possibility of using polarography to study INT reactions. In future work, enzymatic reactions might be followed directly in the polarographic cell by using the decrease in diffusion current at a fixed potential to indicate the amount of INT reduced by the enzyme, provided there were no adverse effects from the presence of mercury.

Because it is simple and absolute, we propose the coulometric titration of INT for standardization of the succinate dehydrogenase assay of Curl and Sandberg. Although the assay is still complicated by the extraction of formazin, this problem may be eliminated in the future by directly sensing succinate dehydrogenase activity (Kovacs and Jaki 1964) with an electrometric method.

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