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# Electrogenerated Chemiluminescent Determination of Oxalate

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The reaction between electrogenerated Ru(bpy)$_3^{3+}$ (bpy = 2,2'-bipyridine) and oxalate produces luminescence (i.e., electrogenerated chemiluminescence, ECL) in aqueous solution. The effect of oxalate concentration on the intensity of ECL emission was studied. ECL intensity was linearly related to concentration of oxalate over the region 10^{-6} to 10^{-4} M. This region encompasses the concentrations found in normal human blood and urine. Initial tests with synthetic urine samples suggest that this method is sufficiently selective for the determination of oxalate in urine.

High oxalate concentrations in the blood or urine accompany a number of maladies including renal failure, vitamin deficiencies, intestinal diseases, and hyperoxaluria (1). It has also been implicated in the formation of kidney stones; in this case the precipitation of calcium oxalate within the kidney occurs and this can cause renal tissue damage. The concentration of oxalic acid in the urine appears to be a key factor in the formation of kidney stones, so that selective and precise methods for determination of oxalate are important. Furthermore, since treatment sometimes involves a low oxalate diet, determination of the oxalate content of foodstuffs is also important. A number of methods for determination of oxalate, including volumetric, colorimetric, fluorimetric, enzymatic, and radioisotope methods, have been described (2); more recently liquid chromatographic (3) and cyclic voltammetric (4) methods have been suggested. Most of these methods require a preliminary separation of oxalic acid from the biological matrix (2), which tends to make them complicated and time-consuming. There is, therefore, a need for better methodologies for determination of oxalate, especially in biological samples. We report here preliminary results of a chemiluminescent method based on the rather specific electron transfer reaction of oxalate with Ru(bpy)$_3^{3+}$ (bpy = 2,2'-bipyridine).

The electrogenerated chemiluminescence (ECL) of oxalate with Ru(bpy)$_3^{3+}$ in acetonitrile solutions was first described by Chang et al. (5). More recent reports from this laboratory (6, 7) provided further details about the reaction conditions and mechanism and demonstrated that the reaction could be carried out successfully in purely aqueous solutions. ECL is produced as follows (6, 7). First Ru(bpy)$_3^{2+}$ is oxidized at a platinum or carbon electrode.
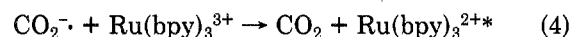
$$Ru(bpy)_3^{2+} \rightarrow Ru(bpy)_3^{3+} + e^- \qquad (1)$$
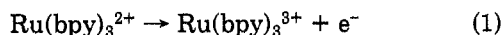
The following reactions then occur in the diffusion layer near the electrode surface.

$$Ru(bpy)_3^{3+} + C_2O_4^{2-} \rightarrow Ru(bpy)_3^{2+} + C_2O_4^-\cdot \qquad (2)$$

$$C_2O_4^-\cdot \rightarrow CO_2 + CO_2^-\cdot \qquad (3)$$
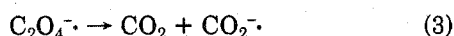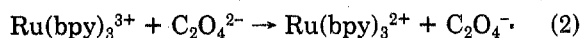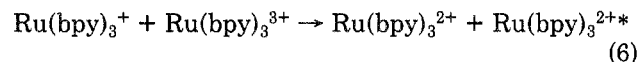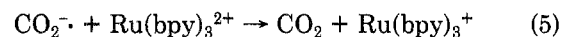
The intermediate radical anion, $CO_2^-\cdot$, is a strong reducing agent and produces the excited state, Ru(bpy)$_3^{2+*}$, in an

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electron transfer reaction with the 3+ species.

$$CO_2^-\cdot + Ru(bpy)_3^{3+} \rightarrow CO_2 + Ru(bpy)_3^{2+*} \qquad (4)$$

Alternatively, Ru(bpy)$_3^{2+*}$ may be produced by the sequence

$$CO_2^-\cdot + Ru(bpy)_3^{2+} \rightarrow CO_2 + Ru(bpy)_3^+ \qquad (5)$$

$$Ru(bpy)_3^+ + Ru(bpy)_3^{3+} \rightarrow Ru(bpy)_3^{2+} + Ru(bpy)_3^{2+*} \qquad (6)$$

The key factors in production of the excited state by this reaction sequence are the large free energy change associated with the rapid electron transfer reactions (4) and (6) and the production of a strong *reductant*, $CO_2^-\cdot$, by *oxidation* of oxalate. These unique features contribute to the specificity of the reaction. Intense orange emission is produced when the excited state decays to the ground state

$$Ru(bpy)_3^{2+*} \rightarrow Ru(bpy)_3^{2+} + h\nu$$
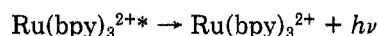
Since previous studies of this ECL system suggested that the emission intensity is proportional to the concentration of oxalate (6, 8), we investigated the possibility of developing an analytical method for oxalate based on this system.

EXPERIMENTAL SECTION

Electrochemical measurements were made with a PAR (Princeton, NJ) Model 175 programmer, Model 173 potentiostat, and Model 179 digital coulometer. Cyclic voltammograms were recorded on a Houston Instruments (Austin, TX) X-Y recorder. The three-electrode cell employed a Pt disk working electrode (area, 0.20 cm^2), a Pt flag auxiliary electrode, and a Ag wire quasi-reference electrode (9). The use of a quasi-reference electrode simplifies the cell arrangement and avoids possible contamination from conventional reference electrode salt bridges. The electrochemical cell had a flat glass window for observing ECL emission. A Hamamatsu R928 photomultiplier tube operated at 700 V was used to measure the emission intensity; the tube output was recorded (vs. time) on the X-Y recorder. The cell and tube were enclosed in a lightproof box during the ECL measurements.

Ru(bpy)$_3$(ClO$_4$)$_2$ was prepared from the dichloride salt (G. F. Smith Chemical Co.) (10). All other reagents were of reagent grade. Unless otherwise noted, solutions were prepared with triply distilled water.

RESULTS AND DISCUSSION

A cyclic voltammogram for 10^{-3} M Ru(bpy)$_3^{2+}$ in 0.1 M acetate buffer (pH 6.0) is shown in Figure 1. Because a quasi-reference electrode was used, voltammograms such as this one were obtained daily so that the potential region where Ru(bpy)$_3^{2+}$ oxidation proceeds at the diffusion controlled rate could be located (e.g., at potentials ≥0.95 V in Figure 1). To obtain ECL from solutions containing both oxalate and Ru(bpy)$_3^{2+}$, the potential was stepped from values at the foot of the wave (e.g., 0.40 V) to ca. 0.95 V. Typical recordings of the resulting emission intensity vs. time, for a solution of 10^{-3} M Ru(bpy)$_3^{2+}$, 10^{-4} M Na$_2$C$_2$O$_4$, and 0.1 M acetate buffer (pH 6.0), are shown in Figure 2. For simplicity, and to illustrate that the method is useful even with unsophisticated measurement techniques, the peak intensity obtained on an X-Y (or strip chart) recorder was employed. Use of the in-
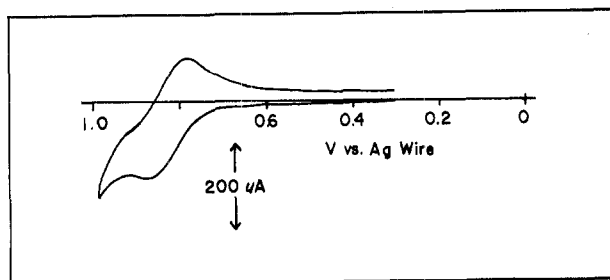
Figure 1. Cyclic voltammogram for solution 1 mM in Ru(bpy)$_3^{2+}$ and 0.1 M in pH 6.0 acetate buffer at 0.20 cm^2 Pt disk electrode. Sweep rate = 100 mV s^{-1}.
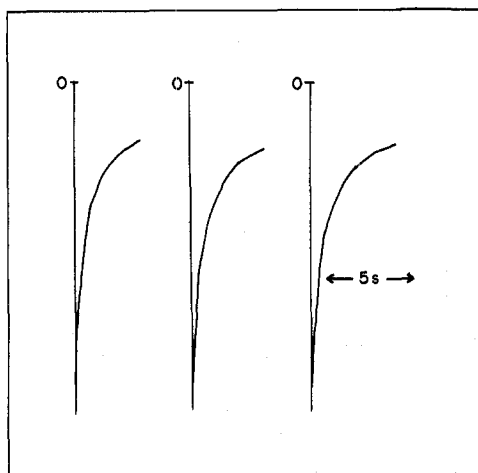

Figure 2. ECL emission intensity (in arbitrary units) vs. time for a solution 1 mM in Ru(bpy)$_3^{2+}$, 1 × 10^{-4} M in Na$_2$C$_2$O$_4$, and 0.1 M in pH 6.0 acetate buffer.

Table I. Peak ECL Intensity as a Function of Concentration of Oxalate[a]

concn of oxalate, M	peak ECL intensity (arbitrary units)
0	28.3
1 × 10^{-6}	48.5
3 × 10^{-6}	79.0
1 × 10^{-5}	231
3 × 10^{-5}	609
1 × 10^{-4}	1955

[a] All solutions 10^{-3} M in Ru(bpy)$_3^{2+}$ and 0.1 M in acetate buffer (pH 6.0).

tegrated intensity of the emission transient (e.g., as employed by Maloy and co-workers (11) in the CL determination of glucose) would probably yield more precise data.

To determine the dependence of ECL intensity on concentration of oxalate, emission profiles were obtained from solutions 10^{-3} M in Ru(bpy)$_3^{2+}$ and 0.1 M in phosphate buffer (pH 6.0) containing various levels of oxalate. The results are summarized in Table I. A plot of peak intensity vs. concentration of oxalate for the data shown in Table I is linear (slope = 1.93 × 10^7; standard deviation of slope, σ_S = 8.5 × 10^4; intercept = 30; standard deviation of intercept, σ_I = 4; correlation coefficient = 0.99997). The line does not pass through the origin because ECL was observed for solutions to which no oxalate was added (see Table I). The origin of this background ECL, which arises during oxidation of Ru-(bpy)$_3^{2+}$ in oxalate-free solutions, is not known. The intensity of the background signal varied little with the components of the buffer system and was about the same for acetate at pH 6.0 (relative intensity 25) and phosphate at pH 6.0 (relative intensity 28) but was slightly less (relative intensity 10) for 0.2 M Na$_2$SO$_4$ at pH 6.0. Furthermore, the background in-

Table II. Peak ECL Intensity as a Function of Concentration of Oxalate in Diluted Synthetic Urine (See Text)

concn of oxalate, M	peak ECL intensity (arbitrary units)
0	36.5
1 × 10^{-5}	149
3 × 10^{-5}	468
6 × 10^{-5}	814
1 × 10^{-4}	1600

tensity was relatively insensitive to pH; 0.1 M phosphate buffers at pH 4.0, 5.0, and 6.0 produced relative intensities of 20, 26, and 28, respectively. This suggests that reaction of Ru(bpy)$_3^{3+}$ with OH$^-$, which is known to cause weak luminescence (12), is not the major source. This also suggests that the background ECL is not caused by reaction of Ru-(bpy)$_3^{3+}$ with oxalate because oxalate induced emission decreases sharply at low pH (6). The background ECL, however, depends on the water used to prepare the solutions and was about five times higher when water from a commercial purification system (which does not reflux the water over permanganate) was used. These data suggest that the background ECL probably comes from the oxidation by Ru(bpy)$_3^{3+}$ of some trace impurity.

Oxalate concentrations in normal urine range from about 1.6 × 10^{-4} to about 5.5 × 10^{-4} M (13); concentrations in normal blood range from about 1.7 × 10^{-5} to about 3.9 × 10^{-5} M (14). Thus, the ECL method has sufficient sensitivity to determine oxalate in both of these clinically interesting fluids. As a preliminary test of the selectivity of the ECL system, an oxalate calibration curve was obtained for a synthetic urine. The synthetic urine was an aqueous solution containing appropriate (15) inorganic electrolytes, lactic (0.5 × 10^{-3} M) uric (2 × 10^{-3} M), hippuric (8 × 10^{-3} M), and ascorbic (1 × 10^{-4} M) acids as well as urea (0.4 M), glucose (3 × 10^{-4} M), and human albumin. All constituents were added at concentrations found in normal urine (15).

The synthetic urine was diluted 10-fold with 0.1 M phosphate buffer at pH 6.0 and this solution was made 10^{-3} M in Ru(bpy)$_3^{2+}$. Known quantities of a standard Na$_2$C$_2$O$_4$ solution (also at pH 6.0) were added and ECL emission profiles were obtained. The data are summarized in Table II. A plot of peak intensity vs. concentration of oxalate for the data shown in Table II is linear (slope = 1.6 × 10^7, σ_S = 0.1 × 10^7, intercept = −34, σ_I = 75, correlation coefficient = 0.994). Since the synthetic urine was diluted 10-fold, the concentration region shown in Table II would correspond to the region 10^{-4} to 10^{-3} M oxalate in a urine sample; this region easily encompasses the range of concentrations found in normal urine. A background ECL signal was again observed (see Table II).

CONCLUSIONS

The results obtained indicate that the ECL system described is suitable for the determination of oxalate. While further study of the specificity of the ECL reaction is required, the rather severe constraints on components which produce sufficiently strong reducing intermediates on oxidation suggest that this reaction is quite specific. We might note that in a study of the CL reaction of hydralazine with Ru(bpy)$_3^{3+}$ in strongly acidic solutions (8), the authors questioned the selectivity of the method for biological fluids. However, that study was concerned with attempts at determinations at much lower levels (i.e., hydralazine concentrations at 5 × 10^{-7} to 3 × 10^{-5} M) and effects of important variables such as solution pH were not investigated. We might also note that while the study carried out here employed electrochemical oxidation of Ru(bpy)$_3^{2+}$ to produce emission, chemical oxidants (e.g., Ce^{4+}, PbO$_2$) have also been shown to produce chemilu-

minescence (7) and these should also be applicable to analytical methods. A report from this laboratory on an analogous luminescent reaction involving *reduction* of Ru(bpy)$_3^{2+}$ in the presence of peroxydisulfate has appeared recently (16). ECL and chemiluminescence reactions based on this reaction also appear possible and are under investigation.

Registry No. Ru(bpy)$_3^{3+}$, 18955-01-6; oxalic acid, 144-62-7.

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Enzyme Electrode and Thermistor Probes for Determination of Alcohols with Alcohol Oxidase

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Enzyme electrode and thermistor probes have been prepared by chemically binding alcohol oxidase from *Candida boidinii*. By use of the electrode probe (enzyme immobilized onto an O$_2$ electrode) from 1 to 100 mM methanol, ethanol, or butanol can be assayed with a precision of about 2.0–2.5%. With an enzyme thermistor probe (enzyme immobilized onto glass beads) in a flow system, as little as 0.2 mM, with linearity to 2.0 mM, of methanol, ethanol, and butanol can be assayed with a precision of about 1.5%. The thermistor probe system is quite stable for several hundred assays and the electrode probe, for about 100 assays.

Blood alcohol analysis has been the subject of considerable interest, particularly in legal cases where there is a need for simple, quick analysis.

Alcohols have been determined by gas chromatography (1–3) and spectrophotometry (4, 5). Some assays have used enzymes like alcohol dehydrogenase and a solution spectrophotometric procedure (6, 7). Guilbault and Lubrano (8) have attempted to build an enzyme electrode probe, amperometrically by detecting the hydrogen peroxide formed during the enzymatic reaction. Because of low activity, the enzyme could not be immobilized. Guilbault and Nanjo (9) described an enzyme electrode that could be used in concentrations as low as 1 mg %, but the electrode used an enzyme that was not commercially available.

In recent years bioanalytical devices have been developed, in which the "sensing" enzymes are placed in close proximity to the transducer—an electrode or a thermistor probe (10, 11). Apart from the obvious possibility of repeated use of the

immobilized biocatalyst, the advantages gained by such an arrangement include higher sensitivity, quicker response, stabilization of the enzyme, and the possibility of applying such devices in continuous flow operations. The calorimetric principle of analysis possesses unique universality, since most enzyme reactions are accompanied by considerable heat evolution in the range of 5–100 kJ/mol (10, 11). A number of applications to the analysis of biochemical systems using calorimetric measurements have been described (10–14).

In this paper we report on the use of a new alcohol oxidase enzyme, *Candida boidinii*, immobilized by chemical bonding, for the assay of methanol, ethanol, and butanol. Both an enzyme electrode probe and a continuous flow approach, using a column of bound enzyme and a thermistor probe, are described and compared. The use of this enzyme in a column with an O$_2$ electrode for assay of ethanol has been reported by Gulberg and Christian (15).

EXPERIMENTAL SECTION

Reagents. Catalase (hydrogen peroxide:hydrogen peroxide oxidoreductase E.C. 1.11.1.6, beef liver, Boehringer, a suspension in water of 1 300 000 U/mL).

Alcohol oxidase (alcohol:oxygen oxidoreductase, E.C. 1.1.3.13), from *Candida boidinii*, obtained from Boehringer Mannheim, had a specific activity of 6.5 U/mg of enzyme protein. The enzyme (50 units) was dialyzed against 0.1 M phosphate buffer, pH 7.0, overnight. The enzyme solution was then freeze-dried.

Methanol, ethanol, 1-propanol, and 1-butanol were all obtained from Merck and were C.P. analytical grade. Water was doubly distilled.

Immobilized Enzyme Reagents. The enzyme electrode probe was prepared by placing a layer of pig intestine (obtained from Universal Sensors) onto a Universal Sensors O$_2$ electrode (Universal Sensors, P.O. Box 736, New Orleans, LA 70148), and affixing with the rubber O-ring provided for the O$_2$ electrode. The intestine is wet with doubly distilled water, then 10 μL of a 5% albumin solution is added to the membrane. Into this 10 μL is then dissolved 2 mg of the freeze-dried alcohol oxidase, and

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