Ford Motor Company

AERONUTRONIC DIVISION

Newport Beach, California

Telephone: Area Code 714
675-1234, Ext. 1841

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STUDY OF BASIC BIO-ELECTROCHEMISTRY

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Prepared by: R. C. Bean
Y. H. Inami
M. H. Boyer

Electrode Reaction Mechanisms

The normal aerobic reaction products of the amino acid - D amino acid oxidase (DAO) system include the pyruvic acid derivative of the amino acid, hydrogen peroxide and ammonia. In addition, there are present in the reaction mixture the substrate-enzyme complex and two forms of the reduced enzyme. It is important to determine the degree to which each of these species participate in an electrode reaction.

Previous reports have discussed the fact that addition of ammonia to the anode compartment of the electrochemical cell produced no effect on the cell current, and it was concluded from this observation that ammonia is electrochemically inert under conditions which exist in this cell. During the past month, study of the possible contribution of H_2O_2 to the electrode reaction was carried out by determining the effect of catalase upon cell current. This study included first, determination of the current attributable to direct action of catalase, as that obtained when the anode compartment contained only catalase and buffer. It was found to be nil. Next, it was shown that cell current supported by an anolyte solution containing H_2O_2 decayed rapidly upon the addition of catalase. Finally, it was shown that addition of 0.0005M H_2O_2 to the DAO-D-tryptophane (D-T) system in the electrochemical cell gave about a 5% increase in cell current. When catalase was present, however, no change occurred upon addition of H_2O_2. It was further found that addition of catalase to the DAO-DT system caused an increase in the magnitude and stability rather than a decrease in cell current. These observations lead to the conclusion that oxidation of H_2O_2 does not contribute significantly to the electrode reaction in a bio cell based upon the DAO-DT system.

A further series of experiments was carried out to examine the possible electrochemical contribution of indole pyruvic acid (IPA) which is the major product of oxidation of tryptophane in the presence of DAO. For this purpose, a semi-quantitative method of determining the formation of IPA in the cell anode compartment was developed. The method involved spectrophotometric determination at the wavelength 306 mμ (see following section). The results (Table I) showed a definite correlation between IPA concentration and cell current.

Table I

RELATION OF CURRENT IN ELECTROCHEMICAL CELL TO
INDOLE PYRUVATE FORMED FROM D-TRYPTOPHAN BY ENZYMIC ACTION

IPA ($M \times 10^3$)	Atmosphere	Current Densities (uA/cm^2 @ 200 mv)
0.10	nitrogen	7.0
0.1	nitrogen	10.0
0.25	air	18
0.72	air	41
1.16	air	45
1.7	air	45

NOTE: Current densities were generally higher under nitrogen atmosphere for a short time after closing circuit but decayed rapidly to indicated values.

Determination of Indole Pyruvic Acid

A semi-quantitative method has been developed for determining the amount of indole-3-pyruvic acid (IPA) formed during the oxidation of D-tryptophane (D-T) by D-amino acid oxidase (DAO) in the electrochemical cell . This method is based upon a second absorption peak possessed by IPA at 306 mμ. . Preliminary experiments have shown that under proper conditions, this peak can be used to detect the formation of small quantities of IPA in the presence of other constituents of the cell electrolyte.

The most important interferring background absorption derives from D-tryptophane. It was found that at the concentration of this constituent (4 mg/l or 0.02M) normally used in the cell, the solutions were optically black in the region of interest. It was therefore necessary to work with somewhat reduced concentrations, either by dilution of the sample just before spectrophotometric analysis, or by use of more dilute D-T solutions (0.005M) in the electrochemical cell. The latter procedure was found to be more satisfactory and has been adopted.

Determination of IPA during operation of the cell involves the periodic removal of electrolyte samples and reading their optical density at 306 mμ against a reference solution of 0.00? M D-T in buffer. No interference is obtained from the buffer or the D-amino acid oxidase. The concentration of IPA is then known by

direct calibration with synthetic IPA (K and K Chemical Co.). Calibration curves are linear, with no indication of departure from Beer's law. The method is presently suitable for detection of IPA concentration as low as 0.0002M in 0.005M tryptophane. It should be possible to reduce this limit by further improvement in technique.

Enzyme Attachment to Electrodes

Studies have been initiated on the electrochemical characteristics of electrodes having films containing high concentration of enzymes confined at the electrode surface. Two techniques were used. In one, the electrode was enclosed in a cellulose membrane sack. Enzyme concentration inside the sack was then varied from very high to the same as in bulk electrolyte. In the other techniques the electrode was coated with an agar film incorporating the desired concentration of enzyme.

Data obtained to date show generally poorer performance by the coated electrodes as compared to electrodes in a free solution. However, since in the latter case, performance is strongly dependent upon stirring the interpretation of these observations is seriously impaired by the difficulty in establishing comparable diffusional and convective transport in the vicinity of the electrode for the two situations. No very attractive way out of this difficulty has become apparent as of this writing. However, study of the problem is continuing.

Chronopotentiometric Studies

Further work was carried out to improve the constancy of the $i\tau^{\frac{1}{2}}$ values in the ferrocyanide system. With the use of a modified electrode the reproducibility of the $i\tau^{\frac{1}{2}}$ values over a wide range of currents compared favorably with those reported by Reilley and co-workers.

The electrode consisted of a smooth platinum disc (approximately ¼ inch in diameter) which was sealed to an end of a soft glass tubing. Electrical contact was made by means of a nickel wire welded to the platinum disc. This electrode was inserted in a larger diameter glass tubing which served as a mantle. The mantle extended approximately one inch below the surface of the solution in the cell.

With the performance of the apparatus and the electrode satisfactorily established the chronopotentiometric technique was applied to the D-tryptophan-D-amino acid oxidase system because this system was found to exhibit the greatest

bio-electrochemical activity in previous polarization studies. Anodic chronopotentiometric studies were carried out with "oxidized", "reduced" and "clean" electrodes in unstirred and stirred solutions. When measurements were made in unstirred solution the electrode was withdrawn approximately ½ inch from the end of the glass mantle; in stirred solution the electrode was extended slightly beyond the end of the mantel. The solution was de-aerated prior to an experimental run and blanketed during the run with prepurified nitrogen. The temperature of the cell was maintained at 38°C.

"Oxidized" electrode refers to an electrode which was brought chronopotentiometrically to the potential of oxygen evolution. "Reduced" electrode refers to an oxidized electrode that was reduced chronopotentiometrically to the potential of hydrogen evolution. "Clean" electrode refers to an electrode which was treated in the following way: anodized and cathodized in concentrated nitric acid, cathodized in H_2SO_4 (pH≅0), rinsed with distilled water, wiped dry, placed in the sample solution and reduced chronopotentiometrically to a potential of hydrogen evolution. The electrode was subjected to one or the other of these pretreatment procedures prior to the taking of each potentiogram.

In an unstirred solution of 5×10^{-3}M tryptophan in sulfuric acid (pH≅0), the potential-time curve indicated that oxidation of tryptophan does occur at the "clean" electrode; nevertheless, the chronopotentiogram was not sufficiently defined for quantitative determination of the transition time. Chronopotentiometric determinations in stirred solution gave well defined potential-time curves on the "clean" electrode. The $i\tau^{\frac{1}{2}}$ values were substantially constant (standard deviation of ±5%) for currents ranging from 100 to 500 μa. This constancy of the $i\tau^{\frac{1}{2}}$ value seems to indicate that the oxidation reaction is diffusional controlled in the stirred solution and that stirring merely increases the thickness of the diffusion layer. Since no significant oxidation was observed on electrodes successively subjected to the "reduced" treatment it is deduced that adsorbed oxidation products inhibit the oxidation of tryptophan.

Because it has been postulated in the previous reports that indole-3-pyruvic acid may be the electrochemically active specie in the tryptophan oxidation, the oxidation behavior of this compound (5×10^{-3}M) was examined chronopotentiometrically in 0.1M sodium pyrophosphate buffer solution of pH 8.3, using a "clean" electrode. Although the chronopotentiograms obtained to date were not defined well enough for

quantatative determination of the transition time, some pertinent features were revealed. In an unstirred solution the potential-time curve showed an oxidation wave at 0.6V vs SCE. However, in a stirred solution, theoxidation wave occurred at 0.1V vs SCE. The wave obtained at the lower potential is likely to be due to the oxidation of an impurity which is present in concentration too small to be detected chronopotentiometrically in the unstirred solution.

Anodic chronopotentiograms of stirred and unstirred solution of $5x10^{-3}$M tryptophan in 0.1M Na_4BO_7 buffer solution were essentially identical. Anson and Schultz reported on the oxidation of oxalic acid at the platinum electrode that the transition time for the oxalic acid oxidation was independent of stirring in buffered solution above pH 3. They were able to account for this behavior on the basis of an oxalic acid adsorption mechanism and on the fact that extensive oxidation of platinum occurs at pH-values above 3. It is plausible that an adsorption mechanism is also involved in the oxidation of tryptophan. Addition of 1 ml of D-amino acid oxidase to 25 ml of $5x10^{-3}$M tryptophan solution produced no significant difference in thepotential-time curves from those obtained in the absence of D-amino acid oxidase. Furthermore, the addition of 1 ml (1 mg/ml) of indole-3-pyruvic acid solution to the 25 ml of solution mentioned above produced no detectable change in the potential-time curve.

In the following series of experiments, the influence of enzyme or of tryptophan on the oxidation of $5x10^{-3}$M ferrocyanide in 0.1MKNO_3 was investigated. The addition of 2 ml. of enzyme to 25 ml of ferrocyanide solution decreased the transition timer for the oxidation of ferrocyanide at the "clean" electrode (12.5 seconds as compared to 14.0 seconds). Similar effects were observed on a "reduced" electrode, provided the potential of the electrode was kept below 0.5V vs SCE in the oxidation cycle of the previous run. In the case of tryptophan, a $5x10^{-3}$M tryptophan in ferrocyanide solution had no effect on the oxidation of ferrocyanide ion at the "clean" electrode. However, an electrode which was brought to a potential of 1.2V vs SCE and subsequently reduced would not oxidize ferrocyanide ion. As long as the potential of the electrode was kept below 0.9V, this electrode on subsequent reduction would oxidize ferrocyanide ions. From these results it is tentatively concluded that the oxidation product of tryptophan is adsorbed on the electrode surface and inhibits the oxidation of ferrocyanide.

Plan for Next Period

1. Work will be continued on enzyme and organism attachment to the electrode with emphasis on effects related to alteration of diffusional and convective transport in the vicinity of the electrode.

2. The urea-urease system will be investigated, both in free solutions and with attachment to the electrode.

3. Identification of IPA oxidation products in the electrochemical cell reactions will be attempted.

4. Further work will be done on establishing the stoichiometry of the current production and the substrate utilization.

5. Chronopotentiometric studies will be continued along present line. Emphasis will be placed on attempts to obtain quantitative data.

6. The usefulness of a wax-impregnated carbon electrode will be evaluated for these studies, since the platinum electrode is known to be oxidized extensively in solutions above pH 3.