ENZYME ELECTRODES FOR FLOW INJECTION ANALYSIS

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Researches on amperometric enzyme electrodes with membranes of enzymes immobilised on the nylon mesh or directly on to platinum are discussed in terms of their applications for flow injection analysis.

KEYWORDS: Enzyme electrodes; amperometric electrodes; immobilisation of enzymes; flow injection analysis

1. INTRODUCTION

An enzyme electrode is essentially an electrode over which is placed a membrane of an immobilized enzyme. The electrode is used to monitor the consumption of a reactant or the appearance of a product of an enzyme-catalyzed reaction. For example, in the glucose oxidase catalyzed oxidation of glucose,

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$$\text{Glucose} + O_2 \xrightarrow[\text{oxidase}]{\text{Glucose}} \text{Gluconic acid} + H_2O_2 \qquad (1)$$

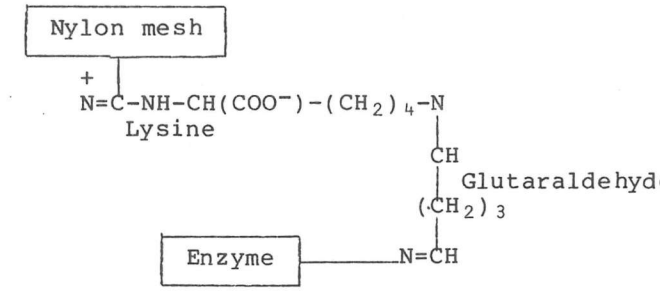
either oxygen or hydrogen peroxide is monitored.

Previously, enzyme electrodes could be summarized[1] as having a lifetime of two to three weeks, with a range of 0.1 to 10 mM and response times of 1 to 5 min. Improved methods of immobilization have led to a lifetime of two to three weeks, with a range of 0.1 to 10 mM and response times of 1 to 5 min. Improved methods of immobilization have led to longer lifetimes, while use of enzyme electrodes under the dynamic approach of flow injection analysis (FIA) ensures fast sample throughput.

There is considerable incentive for exploiting the facility in view of the selectivity of enzymes, while the high expense of enzymes can be minimised by the immobilizations which permit repeated use when dealing with large numbers of analyses. This paper discusses work in the author's laboratories which has led to promoting the use of enzyme electrodes in FIA without the need for stopped-flow reactors. The system has been extended to multi-enzyme electrodes, direct immobilization of enzyme on the metal electrode surface, and assessment of the significance of spacer molecules in the chemical immobilization of enzymes. Also discussed are some matters concerning standards for calibrating enzyme electrodes and the possible use of enzyme electrode for determining pollutants and related materials from their inhibitory effects on enzymes.

2. AMPEROMETRIC NYLON MESH ENZYME ELECTRODES

A convenient way of modelling enzyme electrodes for flow injection analysis (FIA) was the chemical immobilization[2,3] of glucose oxidase on nylon mesh with lysine spacer and glutaraldehyde following treatment with dimethylsulphate and sodium hydroxide[3,4].



The resulting thin enzyme membrane was stretched over a platinum electrode and held in place with an 'O' ring. The resulting enzyme electrode was set up in a microcell in a flow-injection analysis arrangement[2] (Fig.1) where glucose was monitored from the hydrogen peroxide produced by reaction (1).

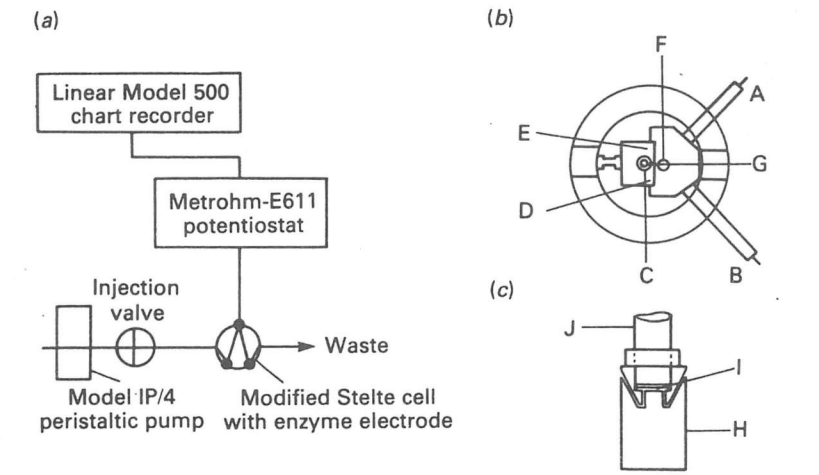


Fig.1 Flow injection analysis apparatus (a) with detector system based on a three-electrode Stelte cell (b) modified by means of a Perspex block H as depicted in (c) by a section from D to E in (b), in order to reduce dead volume. Key: A, reference electrode; B, auxiliary electrode; C, enzyme electrode chamber; D, sample inlet; E, sample outlet; F, reference and auxiliary electrode chamber; G, V-notch on back of Perspex block, H; I, etched channel; and J, enzyme electrode (from Ref.2).

This system, following optimization for flow rate, applied potential (+600 mV versus the silver/silver chloride electrode was chosen) and enzymatic reaction conditions gave good flow injection analysis peaks with short response times (<45 s) and wash times (≈45 s) (Fig.2a). The electrode could be calibrated between 0.01 and 3 mM glucose (Fig.2b) and possessed good capacity (24 h with continuous flow of 2.5 mM glucose) and lifetimes of more than 4 months with storage at 4°C.

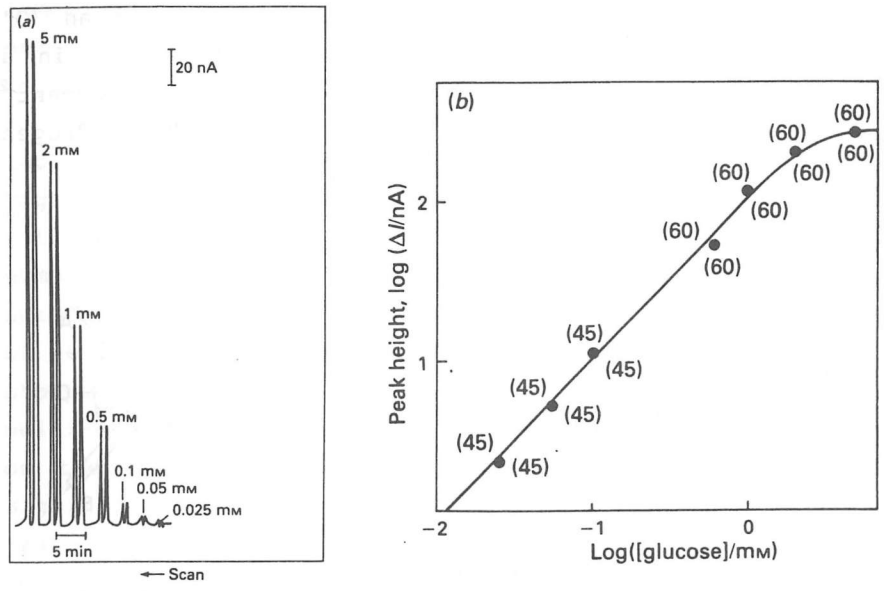


Fig.2 Illustration of recorder output (a) and calibration graph (b) for glucose obtained with a nylon membrane immobilized glucose oxidase enzyme electrode (from Ref.2).

The glucose oxidase electrode, set up in the modified Stelte cell for FIA proved effective for analysing glucose in various foodstuffs, such as molasses, ice cream and syrups, and the data agreed well with those obtained by soluble enzyme kit and Yellow Springs Instrument glucose analyzer methods[2] (Table 1).

TABLE 1 Glucose analysis by enzyme electrode and soluble enzyme test kit (data from Ref.2)

Sample	Sample Pretreatment	Test Kit /g%	Enzyme Electrode /g%
Strawberry ice cream	Carrez solutions I & II	4.4	4.5
Vanilla ice cream		4.7	4.6
Glucose syrup	Dissolve in phosphate buffer (0.1M, pH 7) gentle warming at 35°C	14.8[a]	13.9
Glucose powder	Dissolve in buffer with gentle warming at 35°C	88.0	88.7

a Yellow Springs Instrument Glucose Analyzer

3. AMPEROMETRIC BI-ENZYME ELECTRODES USED WITH A REDOX MEDIATOR

3.1 Glucose Analysis

At the high electrode potentials employed in the above systems (+600 mV versus the silver/silver chloride electrode), other electroactive species, such as ascorbic acid and uric acid, frequently present in samples, will also be oxidized. In order to overcome the resulting interference, the glucose oxidase electrode system may be in the redox mediated mode[5]. Thus, the use of hexacyanoferrate(II) mediator permits a low applied potential (-100 mV versus the silver/silver chloride electrode), enabling the determination of glucose in blood serum without difficult sample pretreatment[5]. Peroxidase is needed to catalyse the hexacyanoferrate(II) reaction with hydrogen peroxide:

$$H_2O_2 + 2Fe(CN)_6^{4-} + 2H^+ \xrightarrow{\text{Peroxidase}} 2H_2O + 3Fe(CN)_6^{3-} \quad (3)$$

The generated hexacyanoferrate(III) is returned to the hexacyanoferrate(III) state at a platinum electrode at low applied potential, that is, -100 mV. The peroxidase was co-immobilized with glucose oxidase on nylon mesh[5], when the optimum glucose oxidase:peroxidase rate was 2:1.

The blood serum samples (3.8 mM to 19.2 mM in glucose) analysed were diluted 10-fold in phosphate buffer (100 mM at pH 7.0) containing potassium hexacyanoferrate(II) (2.5 mM). Samples (500 mm^3) were used for FIA using the glucose oxidase/peroxidase electrode, and the results showed good correlation[5] (0.997) with glucose analyses made by a colorimetric soluble enzyme kit method according to

$$[\text{Glucose}]_{\text{Electrode}} = 1.00[\text{Glucose}]_{\text{Soluble enzyme test kit}} - 0.219 \quad (4)$$

3.2 Hypoxanthine Analysis

The above studies on glucose were extended to xanthine-hypoxanthine analysis[5]. This demanded a lower applied potential because of the electroactivity of xanthine and hypoxanthine at the 600 to 700 mV required for direct hydrogen peroxide monitoring[5]. Also, the uric acid product of the enzymic xanthine oxidation interferes at such high applied potentials. Therefore, the bienzyme electrode approach was used[5]. The enzymes (2:1 xanthine oxidase:peroxidase) were attached to the nylon by dipping the activated nylon mesh in a mixture of the enzymes for 2 h at room temperature and then overnight at 4°C. The electrode was assembled by stretching the bienzyme membrane over a platinum electrode[2].

A typical recorder output for the electrode calibration with respect of xanthine, the primary substrate, is shown in Fig.3. The calibration extends over the range 2 to 100 µM according to (the correlation coefficient is 0.999).

$$\text{Log(current/A)} = 0.93 \log([\text{Xanthine}]/\text{M}) - 2.08 \quad (5)$$

A similar calibration profile holds for hypoxanthine[5].

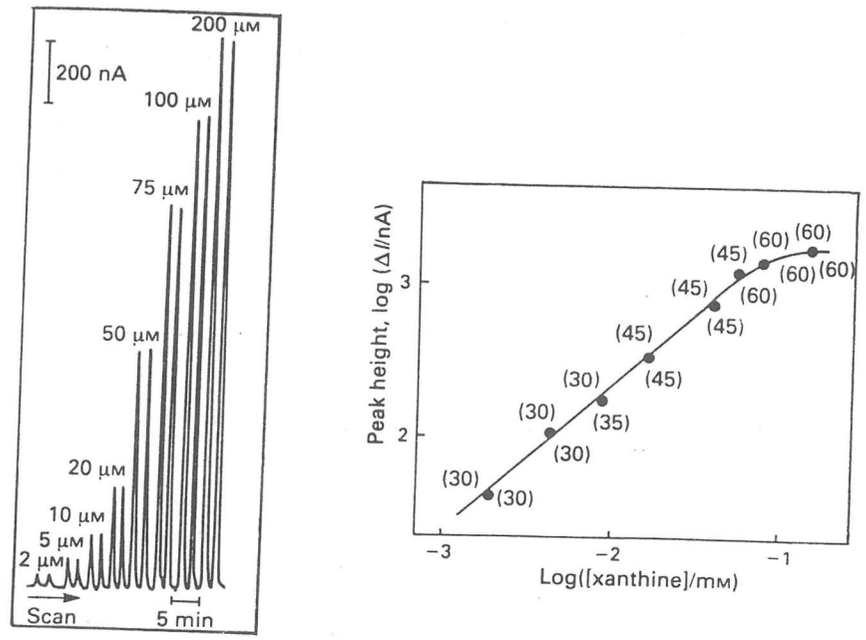


Fig.3 Chart recorder output and calibration plot for hexacyanoferrate(II) mediated xanthine calibration of the xanthine oxidase-peroxidase bi-enzyme electrode (from Ref.5).

An application of the xanthine oxidase electrode is for monitoring fish meat quality, as reflected by increases in hypoxanthine levels during storage[6]. The above electrode was tested on four different kinds of fish meat[5] and the results compared (r = 0.998) with those of a reference spectrophotometric method of the Analytical Methods Committee[7] (Table 2) for the combined data relating to fresh fish and stored fish:

$$[\text{Hypoxanthine}]_{\text{Electrode}} = 1.01[\text{hypoxanthine}]_{\text{AMC}} + 4.0 \times 10^{-3} \quad (6)$$

TABLE 2 Analysis of hypoxanthine in fish meat, using a bienzyme electrode (BE) of xanthine oxidase and peroxidase and the AMC spectrophotometric approach (data from Ref.5)

Fish meat type	Hypoxanthine/μmol g^{-1}			
	Fresh fish		After 20 storage at 20°C	
	BE	AMC	BE	AMC
Rainbow trout	0.32	0.32	0.42	0.39
Herring	0.74	0.88	1.87	1.84
Hake	0.59	0.63	2.19	2.40
Plaice	0.95	0.91	1.89	1.76

4. A TRI-ENZYME ELECTRODE APPROACH FOR SUCROSE ANALYSIS

The use of enzyme electrodes for the analysis of sucrose generally involve the co-immobilization of enzymes in one way or another[8]. The simplest is the random co-immobilization on a single membrane as in the co-immobilization of invertase, mutarotase and glucose oxidase on nylon mesh placed over a platinum electrode used in the amperometric mode in a modified three-electrode Stelte cell to give an effective arrangement for the flow injection analysis of sucrose[8].

The nylon mesh was activated in the usual way with dimethyl sulphate, reacted with lysine spacer and glutaraldehyde, and then treated with the triple enzyme solution for 2 hours at room temperature followed by overnight contact at 4°C. The resultant sucrose-sensing membrane was then firmly fitted over the platinum indicating electrode of the Stelte cell with an 'O' ring[8]. Varying proportions of invertase, mutarotase and glucose oxidase were assessed[8]. These were based on enzyme units (Table 3), which were diluted in phosphate buffer (0.1 mM, pH 7 at 25°C) to give a total protein concentration of about 3 mg cm^{-3}.

TABLE 3 Sucrose trienzyme electrode response (/nA) to glucose (I_G) and sucrose (I_S) (1 mM each) at pH 6.5, flow rate 4.2 cm^3 min^{-1}, and 500 mm^3 sample volume (data from Ref.8)

Membrane type	A	B	C	D	E
IU ratio/200 INV:MUT:GO[a]	10:10:10	10:10:1	10:5:1	5:10:1	20:10:1
Enzyme/buffer for [Enzyme] of 3mg cm^{-3}/cm^3	7.5	1.9	1.85	1.25	3.25
I_G/nA	3613	1195	1373	1283	1328
I_S/nA	200	160	923	103	145
I_G:I_S ratio	18.1	7.5	1.5	12.5	9.2

[a] INV = Invertase; MUT = mutarotase; GO = glucose oxidase

Except for the expected response to glucose, sucrose response is not affected by fructose, arabinose, galactose, raffinose, maltose, lactose, sorbose[8]. Also, there is no interference from gluconic, citric, lactic and acetic acids, but ascorbic acid raises the readings.

The optimum enzyme composition corresponded to membrane C, that is, 2000:1000:200 IUs of invertase:-mutarotase:glucose oxidase, respectively. The corresponding electrode[8] exhibited a good calibration range (0.001 to 1 mM sucrose), short response times (10 s to 20 s), long lifetime (just 6% reduction in signals after >14 h continuous flow of 1 mM sucrose) and good storage stability for intermittent use (38 d when stored in 0.1M phosphate buffer of pH 7 at 4°C).

5. MODIFIED ELECTRODES WITH ENZYME COATINGS

Although immobilization of enzymes on nylon mesh gives effective enzyme electrodes, permitting a substrate analysis every 90 s or so, the membrane is still relatively thick and will hinder fast analysis. Therefore, glucose oxidase has been immobilized directly on to

silanized, anodized platinum wire[9] to give PtO[3-aminopropyltriethoxysilane)-glutaraldehyde-enzyme] The approach is similar to a procedure[10] of silanizing a 0.5 cm^2 platinum plate, followed by a coating of a mixture of glutaraldehyde, bovine albumin and enzyme on the silanized platinum, except that here[9] the agency of bovine albumin was not used.

The resulting electrode was assembled in a micro flow-through cell[9] and gave a fast and sensitive response. For continuous exposure to glucose, achieved by pumping glucose (10 and 2.5 mM) over the electrode at 3.5 cm^3 min^{-1}, the electrode functioned well[9] for 9 h. This is less than the 24 h observed for an electrode with glucose oxidase immobilized on nylon mesh[2] (loading 22 nmol cm^{-2} min^{-1}) and may relate to the smaller enzyme loading on the wire (5-10 nmol cm^{-2} min^{-1}), small surface area and/or weakness of the nature of the Pt-O bonding. The calibration (r = 0.999) was

$$\text{Log(current/A)} = 0.992\ \log([\text{glucose}]/\text{M}) - 3.94 \quad (7)$$

and lifetime during normal use was 10 days[9].

A considerably enhanced signal (by a factor of 6) is obtained by immobilizing the glucose oxidase on platinized platinum[11]. The virtue of this is well illustrated by the FIA chart (Fig.4), while Fig.5 emphasises the gain in signal by the platinized platinum and the enhanced calibration range (0.005 - 30 mM glucose) when compared with the electrodes obtained by immobilizations of platinized or thermally oxidised platinum (0.05 - 30 mM glucose).

A further gain of the platinized platinum (and also of the thermally oxidised platinum) approach was the lifetime of 15 h obtained when subjected to a continuous flow of 10 mM glucose[11] compared with just 9 h for anodised platinum[9].

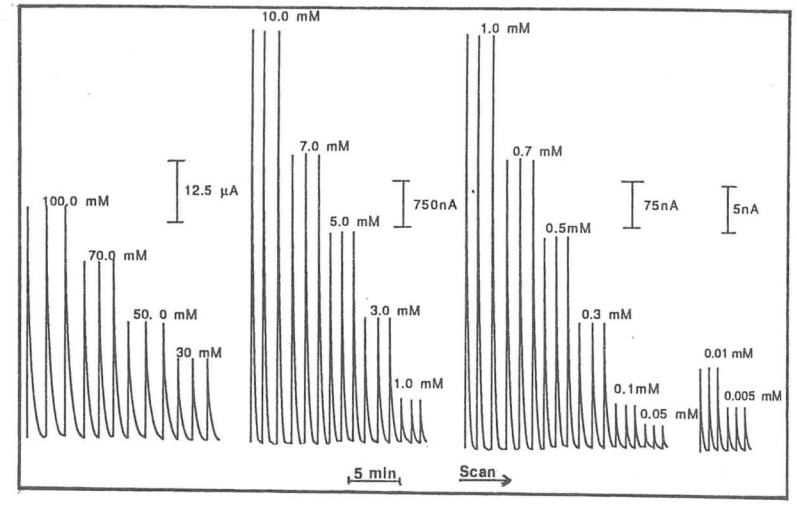


Fig.4 A FIA recorder output for glucose standards obtained during the calibration of a platinized platinum glucose oxidase electrode (from Ref.11)

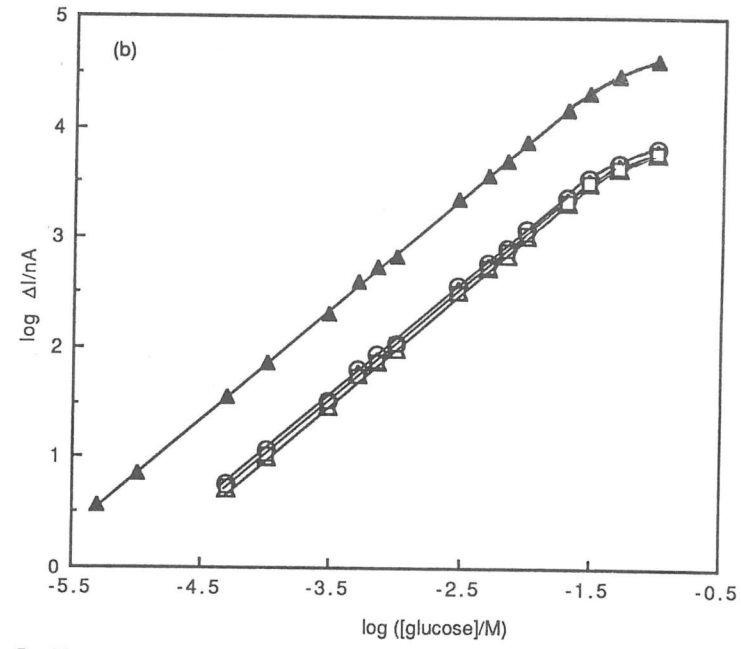


Fig.5 Glucose calibration plots for glucose oxidase electrodes based on anodized platinum with 20% silanization (Δ), anodized platinum with 10% silanization (□), thermally oxidised platinum (o), and platinized platinum (▲) (from Ref.11)

6. CONSIDERATIONS CONCERNING SPACER MOLECULES AND COUPLING AGENTS

Spacer molecules, such as the lysine mentioned above for the enzyme immobilisations on nylon net, have been advised[3] for their utility in effecting higher enzyme activity. Thus, although useful electrodes were obtained for anodised and platinised platinum without the use of spacers, it was claimed[3] that the apparent enzyme activity decreased by about half for no-spacer compared with an enzyme immobilised with a spacer. In a study of the relative utility of spacer molecules in the immobilisation of glucose oxidase on nylon net, it was found[12] that the current output of the enzyme electrode decreased with each coupling agent in the order lysine > arginine > asparagine > p-phenylene diamine > ornithine > m-phenylene diamine > glutamine > no spacer. Hence, the choice of lysine as a spacer molecule is well merited.

A popular coupling agent is glutaraldehyde, but p-benzoquinone also has prospects for, in a study of its performance compared with glutaraldehyde, its use as a coupling agent gave glucose oxidase nylon membrane enzyme electrodes of increased range, namely, 0.01 - 7 mM glucose compared to 0.01 - 2 mM glucose for glutaraldehyde[12],

7. SOME UNUSUAL INTERFERENCES OF ENZYME ELECTRODES: SUBSTRATE STANDARDS AND ENZYME INHIBITORS

There are occasions when it is difficult to obtain proper standards for testing and calibrating enzyme electrodes. This has been experienced in the FIA of cholesterol for a nylon mesh cholesterol oxidase electrode[13], principally because of the interference of the solubilising surfactant. Hence, it was recommended that aqueous cholesterol calibrating standards should be confined to ca. 1% V/V Triton X-100, when a respectable calibration range of 0.010 - 1.035 mM cholesterol could

be obtained. However, cholesterol oxidase electrodes are not as straightforward as others discussed above, since the quality of the commercially available enzyme, as determined by isoelectric focusing and steric exclusion chromatography is more heterogeneous than glucose oxidase, xanthine oxidase and peroxidase.

Of course, enzyme electrode performance can be affected by the presence of inhibitors with the substrate. For example, some heavy metal cations, principally copper(II) , mercury(II) and silver(I) of the 16 metals studied, can inhibit the response of a nylon mesh glucose oxidase electrode[14]. Fortunately, the enzyme electrode could be reactivated by simply washing with buffer stream or, more speedily, with EDTA in the buffer[14]. The inhibition and the ability to reactivate the enzyme electrode provides a basis for adapting the electrode for the FIA of enzyme inhibitors. Thus, such an arrangement has been modelled[14] for copper(II) determination from its inhibition of a glucose oxidase electrode according to the relation (r = 0.994)

$$I/A = -9.49 \times 10^{-7} \log([Cu]/M) + 4.84 \times 10^{-8} \quad (8)$$

for the 0.25 - 5 mM copper concentration range.

CONCLUSION

Clearly, attention to immobilization of enzymes yields good enzyme electrodes for direct analysis of substrated in flowing streams. These can be based on multi-enzyme membranes and are adaptable for a wide range of applications in the analysis of substrates with some prospect also for analysing enzyme inhibitors.

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THE ELECTROREDUCTION OF OXYGEN *

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Abstracts : *Oxygen electroreduction is a slow reaction and, therefore, to obtain electric currents high enough for practical applications, a catalyst is needed. For this purpose many materials have been scrutinized, and surface modification of some of them to achieve higher catalytic efficiencies have been attempted. A comprehensive reference is first made to the several classes of materials tested so far. Then the techniques that have been applied to the study of oxygen electroreduction are reviewed, with some emphasis being given to the analysis of the electrochemical data. The application of these techniques are finally illustrated with a detailed discussion of the electroreduction of oxygen on pure and modified surfaces of carbon and gold.*

1. *Introduction*

The electroreduction of oxygen is a reaction of critical importance to several areas of practical electrochemistry, namely:

- fuel cells:
  - * acid (mainly phosphoric acid);
  - * alkaline;
  - * high temperature (melts and solid electrolytes);
- metal-air batteries:
  - * zinc-air;
  - * iron-air;
  - * aluminium-air;
- sealed-storage batteries:
  - * Ni-Cd;
  - * Ni-Zn;
  - * Pb acid;
- chlor-alkali industry;
- metallic corrosion:
  - * iron and steel;
  - * copper;

(*) Based on a plenary lecture delivered at the 4th Meeting of the Portuguese Electrochemical Society, Estoril, March 1989.

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