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BIOSENSOR AND BIOELECTROCATALYSIS STUDIES OF ENZYMES

IMMOBILIZED ON GRAPHITE ELECTRODE MATERIALS

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CONTENTS

PAGE

Abstract

Acknowledgements

Index

List of Figures

List of Tables

General Abbreviations

Text 1 - 221

References 222 - 240

ABSTRACT

The immobilization of glucose oxidase and lipoamide dehydrogenase on graphite electrodes was studied for their application in mediated bioelectrochemical systems. These are techniques where the electrons involved in an enzymatic redox reaction are either supplied by, or given to an electrode from the enzyme. In the biosensor mode, where a current is measured due to product oxidation, the immobilized enzyme may be used to monitor its substrate, for example a biosensor for glucose based on the enzyme glucose oxidase. In the bioelectrosynthesis mode, when a cathodic potential is applied to the electrode, electrons may be transferred to the enzyme via a mediator thereby driving a thermodynamically difficult reaction, such as the reduction of the coenzyme NAD^+ .

A glucose sensor based on glucose oxidase immobilized onto graphite foil electrodes which had been doped with the mediator dimethylferrocene was found to produce both a linear and rapid response to changes in glucose concentration in solution. The kinetics of this particular system were studied in depth in order to explain its behaviour. Results showed that the porous nature of the electrode material resulted in immobilization of the enzyme throughout a number of layers. Consequently, diffusion of glucose into the electrode became the rate limiting process at low glucose concentrations, which resulted in linear calibration curves. This was characterized by a substrate modulus, ϕ , which was found to be

sufficiently large to predict diffusional limitations. From this the effective rate of glucose diffusion within the electrode was estimated, and found to be up to 5 times smaller than that in free solution. The rapid response of the sensor, on the other hand, was due to the absence of any mass transfer limitations external to the electrode surface.

When lipoamide dehydrogenase was employed in the same system it was found that complications arose due to coupling of NADH oxidation with the adsorbed mediator. By employing a less anodic potential of +150 mV vs. Ag/AgCl at NADH concentrations below 1 mM the detection of enzymatic oxidation of the coenzyme was achieved.

Enzymatic reduction of NAD^+ was achieved using lipoamide dehydrogenase immobilized onto graphite felt electrodes. This reaction was mediated by methyl viologen, with the enzyme-modified electrode used to reduce the mediator at a potential of -0.7 V (vs. SCE). In this fashion the reduced form of methyl viologen was recycled, thereby driving the reduction of NAD^+ . By coupling this system to lactate dehydrogenase it was possible to effect the reduction of pyruvate.

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CHAPTER 1 GENERAL INTRODUCTION

1.1.	Biotechnology	1
1.2.	Applications of Immobilized Enzymes	1
1.3.	Kinetics of Immobilized Enzymes	4
1.4.	Bioelectrocatalysis	13
1.5.	Biosensors	15
1.5.1.	The Transducer	17
1.5.2.	The Biological Component	21
1.5.3.	Immobilized Enzyme Electrodes	24
1.5.4.	Amperometric Enzyme Electrodes for Glucose	27

CHAPTER 2 GENERAL MATERIALS AND METHODS

2.1.	Reagents	33
2.2.	Fabrication of Graphite Foil-based Enzyme Electrodes	33
2.3.	Measurement of Current from Graphite Foil-based Enzyme Electrodes	34
2.4.	Electrochemical Experiments	37
2.5.	Protein Assays	40

CHAPTER 3 ENZYME IMMOBILIZATION ON CARBON ELECTRODE MATERIALS

INTRODUCTION

3.1.	Methods of Enzyme Immobilization	41
3.2.	Adsorption Techniques	41
3.3.	Physical Entrapment Methods	44

3.4.	Crosslinking Methods	45
3.5.	Covalent Coupling Methods	48
MATERIALS AND METHODS		
3.6.	Electrode Materials	51
3.7.	Oxidation of Electrode Materials	51
3.8.	Carbodiimide Activation	53
3.9.	Thionyl Chloride Activation	53
3.10.	Reduction of Surface Carboxylic Acid Groups Using Lithium Aluminium Hydride	54
3.11.	Silanization of Support Materials	55
3.11.1.	Organic Solvent Method	55
3.11.2.	Aqueous Method	55
3.12.	Glutaraldehyde Activation	56
3.13.	Enzyme Immobilization	56
3.14.	Washing Procedures	56
3.15.	Assay of Bound Enzyme Activity	57
3.15.1.	Oxygen Electrode	57
3.15.2.	Electrochemical Flow Cell	57
3.15.3.	Spectrophotometric Flow System	59
3.15.4.	Graphite Foil Electrodes	61
RESULTS AND DISCUSSION		
3.16.	Choice of Immobilization Methods	62
3.17.	Outline of Research	63
3.18.	Assay of Bound Enzyme Activity on Different Carbon Materials	63
3.19.	Oxygen Electrode Work	65

3.19.1.	Activity of Glucose Oxidase Immobilized on RVG 1000 Discs by Adsorption	65
3.19.2.	Carbodiimide Activation	66
3.19.3.	Glutaraldehyde Activation	66
3.19.4.	Silanization Techniques	70
3.20.	Determination of Kinetic Parameters and Bound Enzyme	71
3.21.	Assay of Immobilized Glucose Oxidase using an Electrochemical Flow Cell	79
3.22.	Immobilization of Lipoamide Dehydrogenase on RVG 1000	82
3.23.	Immobilization of Glucose Oxidase on Graphite Foil Electrodes	86

CHAPTER 4 KINETICS OF A GLUCOSE ENZYME ELECTRODE

INTRODUCTION

4.1.	Immobilized Enzyme Kinetics in Relation to Enzyme Electrodes	92
4.2.	The Glucose Oxidase-Dimethylferrocene Enzyme Electrode	96
4.3.	Outline of Model	96

MATERIALS AND METHODS

4.4.	Immobilization of Glucose Oxidase and Dimethylferrocene onto Graphite Foil Electrodes	102
4.5.	Measurement of Steady-State Currents	103

4.6.	Electrochemical Experiments	104
------	-----------------------------	-----

RESULTS AND DISCUSSION

4.7.	Determination of Apparent Kinetic Parameters	105
	K'_m and i_{max}	
4.8.	Evaluation of the Stability of the Enzyme Electrode	112
4.9.	Dependence of Enzyme Electrode Kinetics on the Concentration of Oxidized, Adsorbed Mediator	118
4.10.	Comparison of the Enzyme Electrode with Membrane Retained Enzyme	126
4.11.	Response Behaviour of the Enzyme Electrode	130
4.12.	Edge Effects of Graphite Foil Electrodes	137
4.13.	Effect of Enzyme Loading, Temperature and Electrode Diameter on Kinetic Parameters	141
4.14.	Temperature Effects	145
4.15.	Diffusional Inhibition of the Enzyme Electrode	151

CHAPTER 5 ELECTROCHEMICAL STUDIES OF IMMOBILIZED

LIPOAMIDE DEHYDROGENASE

INTRODUCTION

5.1.	NAD(H)-Linked Dehydrogenases	162
5.2.	Lipoamide Dehydrogenases	165
5.3.	Regeneration of Nicotinamide Coenzymes	167

MATERIALS AND METHODS

5.4.	Immobilization of Lipoamide Dehydrogenase	170
5.5.	Measurement of Activity of Graphite Foil Electrodes	171
5.6.	Measurement of Activity of RVG Electrodes	171

RESULTS AND DISCUSSION

5.7.	Development of an NADH-Sensing Enzyme Electrode	172
5.7.1.	Response Behaviour of Enzyme Electrodes	173
5.7.2.	Electrochemical Studies of the Oxidation of NADH at Graphite Foil Electrodes	177
5.7.3.	Detection of Enzyme Catalysed Currents	185
5.7.4.	Coupling of the Electrode to NAD-linked Dehydrogenases for Detection of Substrates	188
5.8.	Immobilized LipDH as an NADH Regenerating System	190
5.8.1.	Investigation of Suitable Mediators using Cyclic Voltammetry	191
5.8.2.	Bulk Reduction of NADH using Immobilized LipDH	200
5.8.3.	Coupling of Immobilized LipDH to Soluble Lactate Dehydrogenase	208

CHAPTER 6 GENERAL CONCLUSIONS AND FUTURE WORK

6.1.	Immobilization on Electrodes	213
6.2.	Kinetics of Immobilized Enzymes	215
6.3.	Kinetic Studies of a Glucose Biosensor	216

6.4.	NADH Oxidation at Modified Graphite Foil Electrodes	217
6.5.	NADH Recycling using Immobilized LipDH	219
6.6.	Future Work	219

LIST OF FIGURES	PAGE
1.1. Kinetic Parameters Associated with Immobilized Enzymes	6
1.2. Relationship between the Observed Rate of an Enzymatic Reaction and the Maximum Rates of Enzyme Catalysis and Mass Transport	8
1.3. Effect of External Diffusion on Immobilized Enzyme Kinetics	11
1.4. Effect of Internal Diffusion on Immobilized Enzyme Kinetics	12
1.5. A Typical Current-Voltage Curve for an Electroactive Species	18
1.6. Illustration of Two Different Types of Immunosensor	23
1.7. Bi-Enzyme Electrode Showing Different Modes of Operation	28
1.8. Different Types of Amperometric Enzyme Electrodes	29
2.1. Diagram of an Experimental Enzyme Electrode	35
2.2. Microcomputer Based System for the Analysis of Enzyme Electrodes	36
2.3. Two Compartment Electrochemical Cell	38
2.4. Three Compartment Electrochemical Cell	39
3.1. General Techniques of Enzyme Immobilization	42
3.2. Mechanism of the Reaction of Glutaraldehyde with Proteins	47
3.3. Immobilization of Enzymes using Silane Reagents	50
3.4. Electrochemical Flow Cell used to Monitor Glucose Oxidase Bound onto Different Graphite Electrode Materials	58
3.5. Spectrophotometric Flow System used to Monitor Lipoamide Dehydrogenase Bound onto RVG 1000	60

3.6.	Graph of Oxygen Consumption against Glucose Concentration for Glucose Oxidase Bound onto RVG 1000 Discs	69
3.7.	Graph of Oxygen Consumption against Glucose Concentration for Glucose Oxidase Bound onto Silanized RVG 1000 Discs	74
3.8.	Lineweaver-Burk Plot for Glucose Oxidase Bound onto Silanized RVG 1000 Discs	76
3.9.	Eadie-Hofstee Plot for Glucose Oxidase Bound onto Silanized RVG 1000 Discs	77
3.10.	Response Behaviour of the Electrochemical Flow System under Different Loading Conditions	80
3.11.	Typical Response Behaviour of the Spectrophotometric Flow System	85
3.12.	Hanes Plots for Glucose Oxidase Immobilized on Graphite Foil Electrodes	89
4.1.	Steady-State Currents from Graphite Foil Enzyme Electrodes after Progressive Removal of Surface Layers	97
4.2.	Model for the Enzyme Electrode	99
4.3.	Computer Printout of a Typical Calibration Experiment	106
4.4.	Calibration Curve Derived from Figure 4.3.	107
4.5.	Lineweaver-Burk Plot of the Data from Figure 4.4.	108
4.6.	Eadie-Hofstee Plot of the Data from Figure 4.4.	109
4.7.	Hanes Plot of the Data from Figure 4.4.	110
4.8.	Current Output of an Enzyme Electrode over a 24 Hour Period	113
4.9.	Computer Printout of a Typical Preconditioning Experiment	115
4.10.	Successive Calibration Curves for a Set of Enzyme Electrodes	116

4.11.	Computer Printout of a Set of Enzyme Electrodes Operated Overnight	117
4.12.	Calibration Curves of Enzyme Electrodes at Different Mediator Loadings	119
4.13.	Calibration Curves of Enzyme Electrodes at Different Applied Potentials	121
4.14.	Differential Pulse Voltammogram of a Graphite Foil Electrode Doped with Dimethylferrocene	123
4.15.	Linear Sweep Voltammograms of an Enzyme Electrode in Buffer and 100 mM Glucose	125
4.16.	Calibration Curves of a Standard Enzyme Electrode Compared with Membrane-Retained Glucose Oxidase	128
4.17.	Eadie-Hofstee Plot of the Data from Figure 4.16.	129
4.18.	Calibration Curves of Enzyme Electrodes at Different Enzyme Loadings	132
4.19	Transient Response Curves of Enzyme Electrodes at Low and High Glucose Concentrations	133
4.20.	Response Curve of a Membrane-Retained Glucose Oxidase Electrode	134
4.21.	Calibration Curves of a Standard Enzyme Electrode and One Covered by Dialysis Membrane	136
4.22.	Properties of Graphite Foil in Different Physical Planes	138
4.23.	Calibration Curves of Standard Enzyme Electrodes and Electrodes with Sealed Edges	139
4.24.	Eadie-Hofstee Plot of the Data from Figure 4.23.	140
4.25.	Arrhenius Plots for Enzyme Electrodes at Different Bulk Glucose Concentrations	147
4.26.	Transient Response Curves of Enzyme Electrodes at 6.6 mM Glucose between 1 - 45°C	149
4.27.	Transient Response Curves of Enzyme Electrodes at 74.1 mM Glucose between 1 - 45°C	150

4.28.	Dimensionless Calibration Curve for the Q Set of Enzyme Electrodes	153
4.29.	Dimensionless Eadie-Hofstee Plots for Different Sets of Enzyme Electrodes	154
5.1.	Structure of Nicotinamide Adenine Dinucleotide	164
5.2.	Schematic of the Mechanism of Action of Lipoamide Dehydrogenase and the Different Redox States of the Enzyme	166
5.3.	Computer Printout of Steady-State Currents from NADH-Sensing Enzyme Electrodes	175
5.4.	Calibration Curves for Bare and Enzyme Electrodes	176
5.5.	Hanes Plots of the Data from Figure 5.4.	178
5.6.	Linear Sweep Voltammogram of 10 mM NADH at a Graphite Foil Electrode	180
5.7.	Linear Sweep Voltammograms of a Graphite Foil Electrode Doped with Dimethylferrocene in Buffer and 10 mM NADH	181
5.8.	Differential Pulse Voltammograms of a Graphite Foil Electrode in Buffer, and Doped with Dimethylferrocene in Buffer and NADH Solution	183
5.9.	Linear Sweep Voltammograms of 10 mM NADH at a Graphite Foil Electrode with Time	184
5.10.	Calibration Curves for Mediator-Modified and Enzyme Electrodes at an Applied Potential of +150 mV vs. Ag/AgCl	186
5.11.	Hanes Plot Derived from Figure 5.10.	187
5.12.	Calibration Curve for an Enzyme Electrode with Alcohol Dehydrogenase Retained Behind a Dialysis Membrane	189
5.13.	Cyclic Voltammogram of 10 mM NAD ⁺ at a Pyrolytic Graphite Electrode	192
5.14.	Cyclic Voltammogram of Chlorophenol Red at a Glassy Carbon Electrode	193

5.15.	Cyclic Voltammogram of Anthraquinone-2-Sulphonic Acid at a Glassy Carbon Electrode	194
5.16.	Cyclic Voltammogram of Anthraflavic Acid at a Glassy Carbon Electrode	195
5.17.	Cyclic Voltammograms of Methyl Viologen at a Glassy Carbon Electrode in 10 mM NADH in the Presence and Absence of Lipoamide Dehydrogenase	197
5.18.	Cyclic Voltammograms of Benzyl Viologen at a Glassy Carbon Electrode in 10 mM NADH in the Presence and Absence of Lipoamide Dehydrogenase	198
5.19.	Schematic Showing the Reactions Involved in the Bulk Reduction of NAD^+ by Immobilized Lipoamide Dehydrogenase	201
5.20.	Graph Showing the Production of NADH with Time for Free and Immobilized Enzyme	205
5.21.	Current-Time Curves for Free and Immobilized Enzyme	206
5.22.	Variation of A_{340} and Current with Time for Immobilized Enzyme	207
5.23.	Variation of A_{340} with Time for Electrode IM5/1	209
5.24.	Variation of A_{340} with Time for Electrode IM5/2	211

LIST OF TABLES	PAGE
1.1. General Types of Biosensors	16
1.2. Immobilized Enzyme Electrodes	25
3.1. Properties of Different Carbon Materials	52
3.2. Glucose Oxidase Bound on Different Graphite Electrode Materials	64
3.3. Activity of Glucose Oxidase Adsorbed on RVG 1000 Discs	67
3.4. Activity of Glucose Oxidase Bound to Carbodiimide Activated RVG 1000	68
3.5. Activity of Glucose Oxidase Bound to Glutaraldehyde Activated RVG 1000	70
3.6. Activity of Glucose Oxidase Bound to Silanized RVG 1000	72
3.7. Kinetic Parameters for Glucose Oxidase Immobilized on RVG 1000 Discs	78
3.8. Kinetic Parameters and Immobilization Conditions for Yeast Lipoamide Dehydrogenase Bound onto RVG 1000 Discs	83
3.9. Conditions Employed for the Immobilization of Glucose Oxidase on Graphite Foil Electrodes	88
3.10. Kinetic Parameters for Glucose Oxidase / Graphite Foil Electrodes	88
4.1. Kinetic Parameters for Batch Q Electrodes	111
4.2. Kinetic Parameters Determined at Different Enzyme Loadings : Small Diameter / 1 ^o C	143
4.3. Kinetic Parameters Determined at Different Enzyme Loadings : Large Diameter / 1 ^o C	143
4.4. Kinetic Parameters Determined at Different Enzyme Loadings : Small Diameter / 25 ^o C	144
4.5. Kinetic Parameters Determined at Different Enzyme Loadings : Large Diameter / 25 ^o C	144

4.6.	Kinetic Parameters for Different Enzyme Electrodes Derived from Secondary Plots	156
4.7.	Kinetic Parameters Derived from Enzyme Electrode Model	156
5.1.	International Classification of Enzymes	163
5.2.	Lipoamide Dehydrogenase Bound onto RVG 1000 Electrodes	202
5.3.	Rates of NAD^+ Reduction for Lipoamide Dehydrogenase Bound onto RVG 1000 Electrodes	203

ABBREVIATIONS

Ag/AgCl	silver-silver chloride (reference electrode)
CV	cyclic voltammetry (or voltammogram)
DMFc	1,1'-dimethylferrocene
DPV	differential pulse voltammetry (or voltammogram)
FAD	flavin adenine dinucleotide
Fe(cp) ₂	1,1'-dimethylferrocene
GOD	glucose oxidase
ISE	ion-selective electrode
ISFET	ion-selective field effect transistor
LiAlH ₄	lithium aluminium hydride
LipDH	lipoamide dehydrogenase
LSV	linear sweep voltammetry (or voltammogram)
MV	methyl viologen
NAD ⁺	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)
NHE	normal hydrogen electrode
SCE	saturated calomel electrode (reference electrode)

NOTATION

A	area
A ₃₄₀	absorption at 340 nm

C_E	concentration of (active) immobilized enzyme
d	characteristic length of diffusion barrier
D_{eff}	effective diffusion coefficient
D_s	substrate diffusion coefficient
E	potential (volt) (electrochemical)
E^0	standard redox potential
E^*	activation energy
$E, [E]$	free enzyme concentration (kinetic)
$[E]_t$	total enzyme concentration
$ES, [ES]$	concentration of enzyme-substrate complex
F	Faraday's constant
h_d	mass transfer coefficient for dimethylferrocene within electrode
$h_s (h_p)$	mass transfer coefficient for substrate (product)
i	current
i_{ss}	steady-state current (of an enzyme electrode)
i_{max}	maximum current (of an enzyme electrode)
I	dimensionless current (i_{ss} / i_{max})
j_s	substrate flux into electrode
k	rate constant
k_e	rate constant for dimethylferrocene oxidation at electrode surface
K_m	Michaelis-Menten constant (for free enzyme)
K'_m	apparent Michaelis-Menten constant for immobilized enzyme

k_s	second-order rate constant for oxidation of reduced enzyme by ferricinium
n	number of electrons in redox reaction (electrochemical)
$P, [P]$	product concentration
r	radius (of electrode)
R	gas constant
$S, [S]$	substrate concentration
ΔS	concentration gradient of glucose (between interior and exterior of the electrode)
T	temperature
v_t	total enzyme activity of an enzyme electrode
V	overall or observed rate of an immobilized enzyme
V_{diff}	rate of mass transfer
V_{kin}	kinetic rate of an immobilized enzyme
V_{max}	maximum turnover rate of a free enzyme
V'_{max}	maximum velocity of an immobilized enzyme per unit volume of catalyst
β	dimensionless substrate concentration ($[S]/K'_m$)
ϵ	effectiveness factor for diffusion limited regime
η	effectiveness factor for an immobilized enzyme
κ	first-order rate constant for diffusion limited regime
μ	substrate modulus for external diffusional limitations

U

substrate modulus for enzyme electrode (as defined
by Mell & Maloy, 1975)

ϕ

substrate modulus for internal diffusional
limitations

CHAPTER 1

INTRODUCTION

1.1 Biotechnology

Biotechnology is concerned with the industrial exploitation of biological materials, and is an area of research that has expanded rapidly over the past few years. The use of microorganisms in the production of fermented foods, such as wine, cheese, beer, soy sauces and vinegar is centuries old, but it is only with the understanding of biochemical and cellular processes that has taken place this century both that microorganisms and cell-free enzymes have begun to be widely used in industry and medicine. Developments in many areas of research, including biochemical engineering, analytical chemistry, biocatalyst immobilization, recombinant DNA techniques and immunology have led to an expansion in both research and applications of biological catalysts, bringing about changes in the traditional industries and the development of new ones. Biotechnology is beginning to play a major role in the fermentation, waste treatment, agricultural and food industries and in many areas of medicine and health care (Higgins et al., 1985).

1.2. Applications of Immobilized Enzymes

Enzymes are nature's biological catalysts. Practically every

chemical reaction of importance that occurs within a living organism is catalysed by one or more enzymes. The characteristics of enzymes which are important for industrial use are their high specificity and their ability to function under mild conditions. The specificity of different enzymes may vary from hydrolytic enzymes such as esterases and proteases able to act on a wide range of substrates having similar functional groups to enzymes that act on only a couple of compounds with any degree of efficiency (for example urease and glucose oxidase). Enzyme catalytic rates also vary considerably, from 1-3 units mg^{-1} (1 unit = 1 μmole of substrate converted per minute under defined conditions) for enzymes such as L-amino acid oxidase, formate dehydrogenase and horse liver alcohol dehydrogenase, to 20,000 units mg^{-1} for catalase and 50,000 units mg^{-1} for pig pancreatic lipase. The specific activity of an enzyme, as well as its stability, will be an important factor in deciding whether an enzymatic method for carrying out an industrial process is economically viable.

Disadvantages of enzymes, such as their sensitivity to large fluctuations in environmental conditions and their cost, have been partly ameliorated by improvements in the large-scale purification of enzymes commercially, and by the development of immobilization techniques, whereby the catalyst is bound to a water-insoluble support. Immobilization allows reuse of the expensive catalyst and facilitates recovery. It also allows enzymes to be employed in continuous processes, and in many cases imparts improved

stability towards external conditions such as temperature and pH.

Industrial exploitation of immobilized enzymes began in the 1970's with the use of immobilized aminoacylase to produce L-amino acids such as alanine, phenylalanine and methionine from racemic mixtures of acyl-D,L-amino acids (Tosa et al., 1967 ; Chibata & Tosa, 1976) and immobilized glucose isomerase to produce high fructose corn syrups (Weetall, 1973 ; Hemmingsen, 1979).

Among the many applications that immobilized enzymes have found in industry can be cited the use of immobilized penicillin amidase to produce 6-aminopenicillanic acid (Giacobbe et al., 1978 ; Park et al., 1982), which is used as the precursor of semi-synthetic penicillins, the use of β -galactosidases to hydrolyse lactose from milk and from whey (Weetall et al., 1974 ; Pitcher, 1978), and the application of immobilization techniques to the use of carbohydrases such as amylases, invertases and glucoamylases in the starch industry (Marconi & Morisi, 1979).

Another area of research which is receiving increasing attention is the application of immobilized enzymes in organic synthesis. This work is concerned not only with the use of enzymes in non-aqueous environments but also with investigating novel substrates that are not found in vivo. Examples of the application of enzyme catalysis in the production of asymmetric and chiral compounds have been reported (Jones & Beck, 1976 ; Simon et al., 1985 ; Whitesides & Wong, 1985). The exploitation of enzymes immobilized in polyacrylamide-co-N-acryloxysuccinamide (PAN) gels

has been reported by Whitesides and co-workers (Dicosimo et al., 1981 ; Whitesides & Wong, 1983), and has recently been reviewed by Mosbach (1985).

As distinct from utilizing the abilities of enzymes to convert one compound to a more valuable product, much use has been made of the innate specificity of enzymes as part of analytical devices. Here again the advantage of immobilized enzymes over homogeneous catalysts has been a powerful impetus to the development of immobilized enzyme systems to replace standard enzyme assays. The development of such biosensors for the analytical determination of both inorganic and biological compounds is discussed in more detail in section 1.5., and has been extensively covered by Turner et al. (1987).

1.3. Kinetics of Immobilized Enzymes

An understanding of the effect of immobilization on enzyme kinetics is important in industry, and is also of interest to biochemists because immobilized enzymes may be used as models for the behaviour of enzymes in vivo (Engasser & Horvath, 1976). In free solution enzyme kinetics have traditionally been characterized by parameters such as the Michaelis constant (K_m), the maximum turnover rate (V_{max}) and inhibition constants. Upon immobilization the values of these parameters are often changed, which may be due to the following:

(i) The conformation of the bound enzyme may differ from that of the free enzyme.

(ii) The microscopic environment surrounding the immobilized enzyme can be different from the medium in which the measurements are made.

(iii) Diffusion of substrates and products may play a significant role, even in the simplest case of an enzyme immobilized on a flat, non-porous surface.

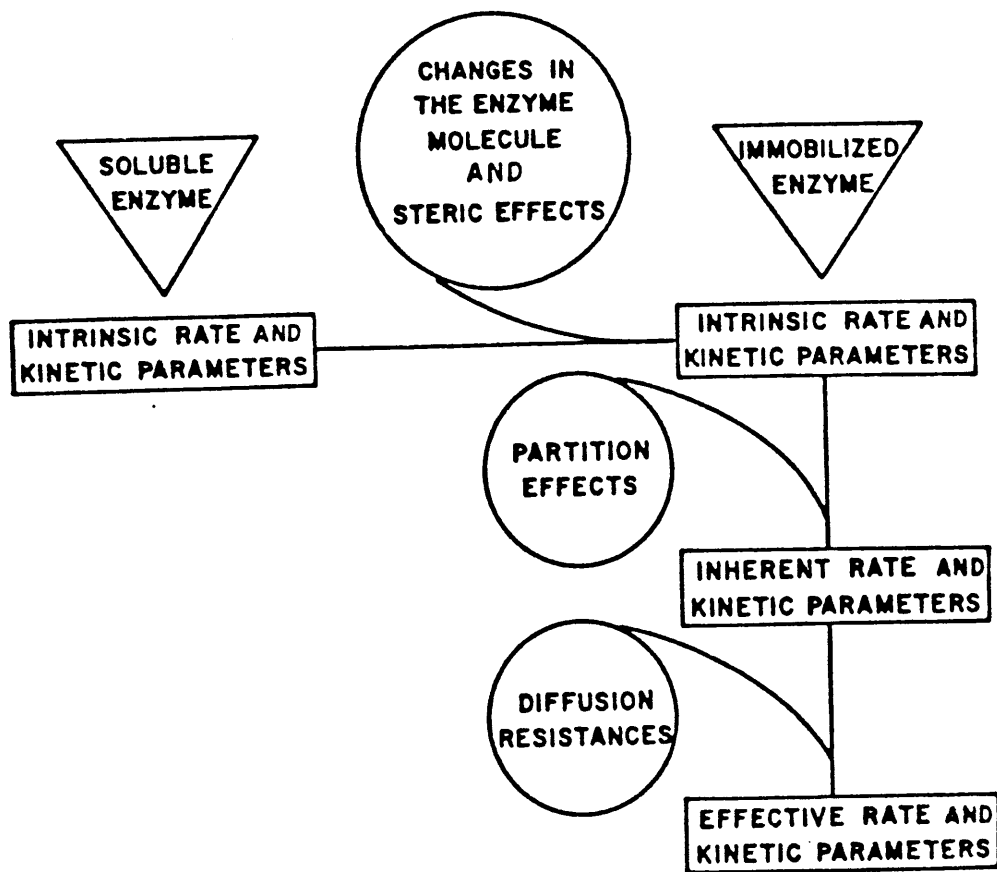
The way in which these processes may affect the observed kinetic behaviour of an immobilized enzyme can be conveniently summarized as shown in figure 1.1.

The intrinsic kinetics of the immobilized enzyme may differ from those observed for the enzyme in free solution due to conformational changes or to the nature of the support material.

The effective kinetic behaviour of the immobilized enzyme is that observed experimentally, and may differ from the intrinsic characteristics due to diffusion externally to the catalyst layer, or internally if the enzyme is retained within a porous matrix. Further complications to the observed kinetics may arise if there are any partition effects due to hydrophobic interactions, or if there are any electrostatic interactions between the support material and charged substrates and/or products.

The effects of diffusional limitations and electrostatic partitioning on the kinetics of immobilized enzyme reactions, both for single substrate enzyme kinetics and in the cases of substrate

FIGURE 1.1.



Kinetic parameters associated with immobilized enzymes
(adapted from Engasser & Horvath, 1976).

and product inhibition, have been reviewed by Katchalski et al. (1971), Laidler & Sundaram (1971), Wingard (1972) and Engasser & Horvath (1976).

The influence of diffusional limitations on the observed velocity of immobilized enzyme reactions will be considered further, since these are particularly relevant to the glucose biosensor discussed in chapter 4.

The overall (or observed) rate of a heterogeneous enzyme system, V , in the presence of diffusional resistances is a function of two limiting rates : the rate of the kinetic (enzyme) reaction , V_{kin} , and the rate of mass transfer, V_{diff} .

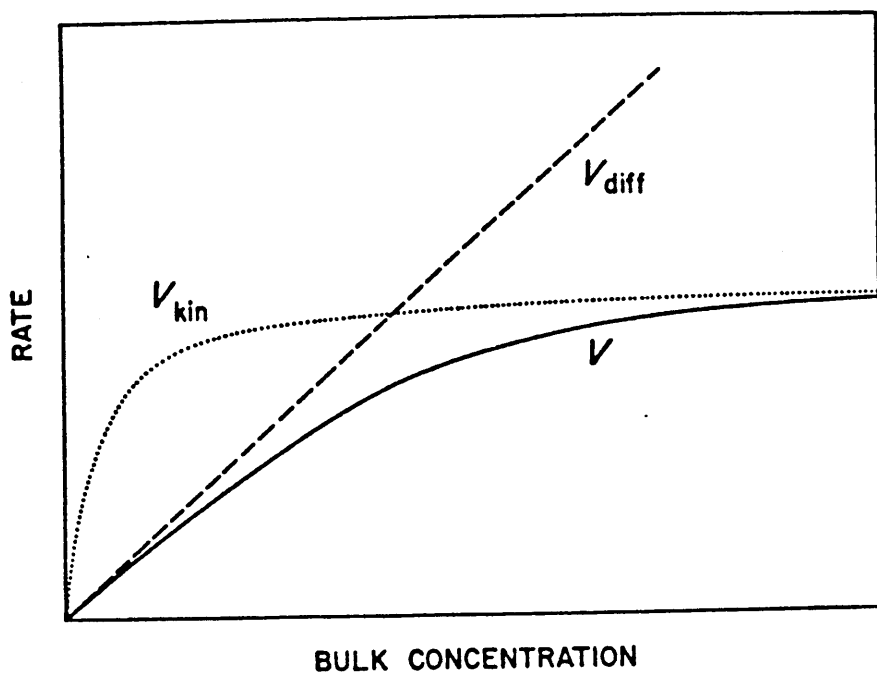
$$V_{kin} = \frac{V_{max} [S]}{K'_m + [S]} \quad [1]$$

$$V_{diff} = h_s [S] \quad [2]$$

Here V_{max} is the maximum rate of the enzymic reaction, $[S]$ is the substrate concentration, K'_m is the apparent Michaelis constant of the immobilized enzyme and h_s the mass transport coefficient for the substrate. The relationship between these rates is shown in figure 1.2.

The extent to which V is controlled by the rate of mass transfer can be expressed in terms of an effectiveness factor, η (Engasser & Horvath, 1973 ; Hamilton et al., 1974) as given in equation [3] :

FIGURE 1.2.



Relationship between the observed rate of an enzymatic reaction (V) and the maximum rates of enzyme catalysis (V_{kin}) and mass transport (V_{diff}).

(Adapted from Engasser & Horvath, 1974).

$$V = \frac{\eta V_{\max} [S]}{K'_m + [S]} \quad [3]$$

From equations [1] and [3] η may be defined as (Engasser & Horvath, 1976) :

$$V = \eta V_{\text{kin}} \quad [4]$$

Thus $1/\eta$ is a measure of the diffusional inhibition of the enzyme reaction, and kinetic control is obtained as η approaches unity. The value of η is a complex function of the kinetic parameters of the enzyme reaction, the substrate transport coefficient and the substrate concentration.

$$\eta = f [K'_m V_{\max} h_s [S]] \quad [5]$$

Effectiveness factors have been used to characterize the effects of diffusion on enzymes immobilized on spherical particles (Shuler et al., 1972 ; Engasser & Horvath, 1973 ; Kobayashi & Laidler, 1973) and entrapped in membranes (Moo-Young and Kobayashi, 1972).

The kinetic and transport parameters in equation [5] may be combined into one term, called the substrate modulus. Engasser & Horvath (1976) have defined a substrate modulus, μ , for external diffusion :

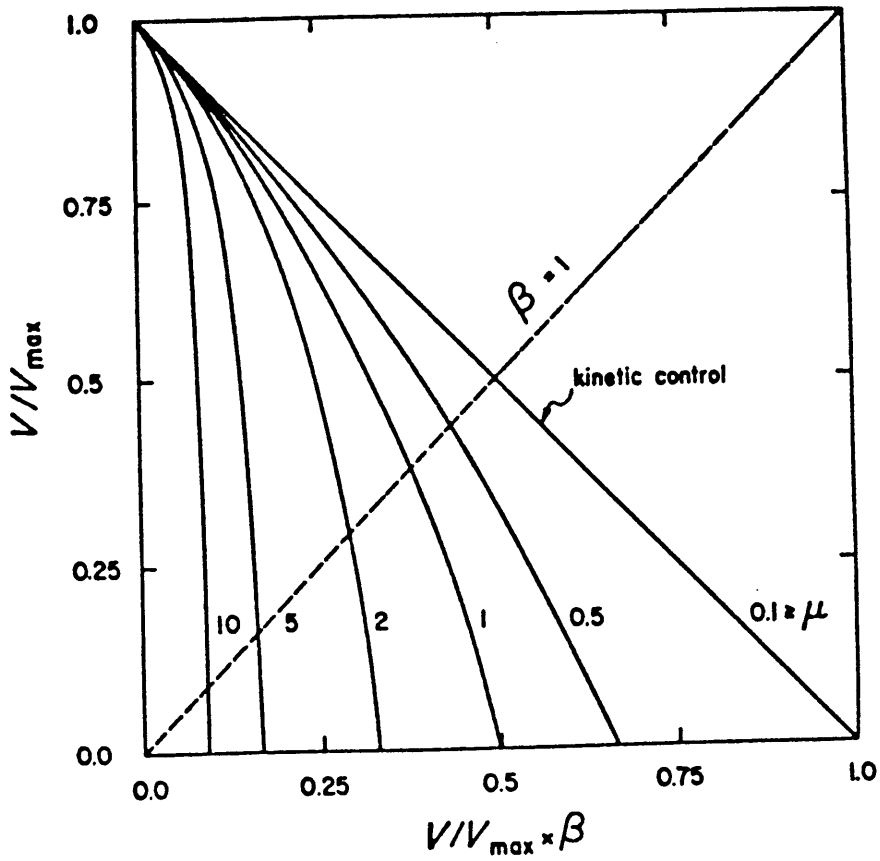
$$\mu = \frac{d V_{\max}}{D_s + K'_m} \quad [6]$$

where D_s is the substrate diffusion coefficient, d is the characteristic length of the diffusion barrier and $D_s / d = h_s$. In the case of internal diffusion a substrate modulus ϕ may be defined as (Engasser & Horvath, 1973 ; Hamilton et al., 1974) :

$$\phi = d \left[\frac{V'_{\max}}{D_s K'_m} \right]^{1/2} \quad [7]$$

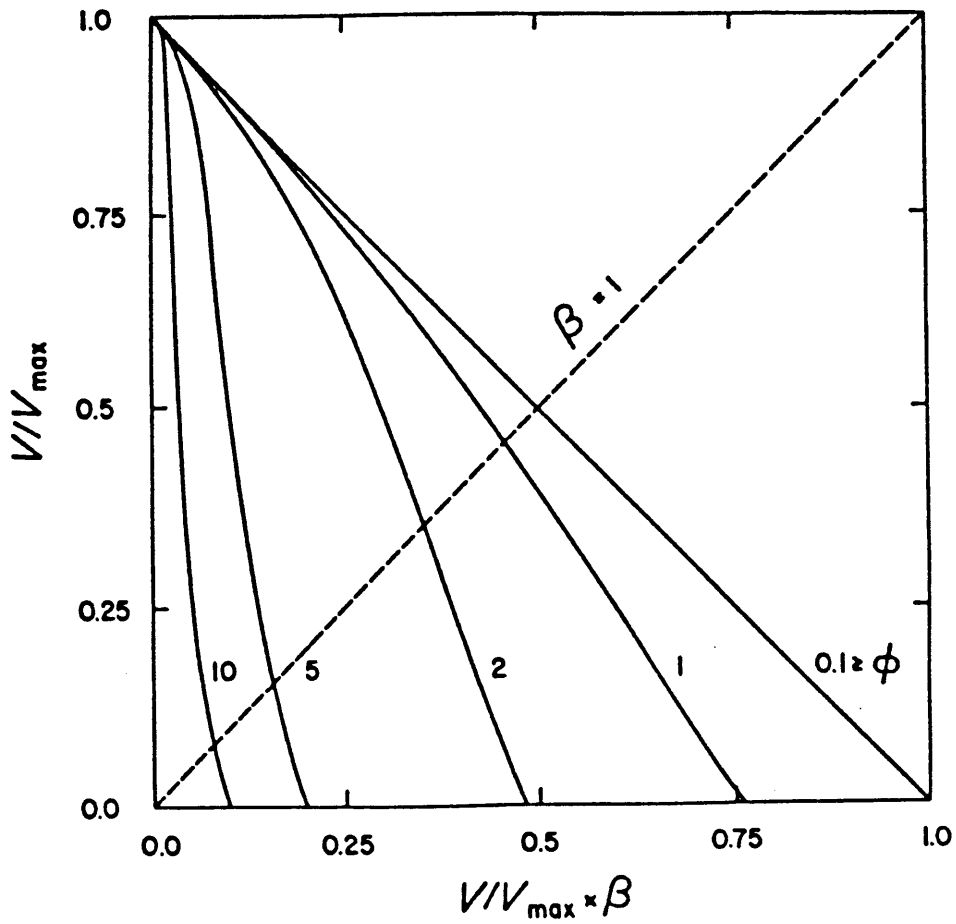
Here $V'_{\max}$ is the maximum velocity of the enzyme reaction per unit volume of the immobilized catalyst. Since the substrate moduli are independent of the substrate concentration they provide a more convenient measure of the extent of diffusional impairment of the enzyme reaction than the effectiveness factor η . In cases where V is no longer controlled by V_{kin} , Michaelis-Menten kinetics are not observed, and the characteristic plots used to study homogeneous enzyme reactions (Lineweaver-Burk, Eadie-Hofstee and Hanes plots) are no longer completely linear. Deviations from linearity are more pronounced on the Eadie-Hofstee variation of the Michaelis-Menten equation (Engasser & Horvath, 1973) and this is shown in figures 1.3. and 1.4. using dimensionless co-ordinates. Here β is the dimensionless substrate concentration as given in equation [8], and the rate (V) is normalized

FIGURE 1.3.



Effect of external diffusional limitations on enzyme kinetics as illustrated by dimensionless Eadie-Hofstee type plots at different values of the substrate modulus μ . The observed rate of reaction (V) is normalized with respect to the maximum reaction rate and the bulk substrate concentration to the apparent Michaelis constant (adapted from Engasser & Horvath, 1976).

FIGURE 1.4.



Effect of internal diffusional limitations on enzyme kinetics as illustrated by dimensionless Eadie-Hofstee type plots at different values of the substrate modulus ϕ . The observed rate of reaction (V) is normalized with respect to the maximum reaction rate and the bulk substrate concentration to the apparent Michaelis constant (adapted from Engasser & Horvath, 1976).

with respect to $V_{\max}$.

$$\beta = [S] / K_m \quad [8]$$

The effect of external diffusional limitations, expressed by the substrate modulus μ is shown in figure 1.3., and the effect of internal diffusion as expressed by ϕ is shown in figure 1.4. It is found that for internal diffusion the Eadie-Hofstee plot exhibits a sigmoidal character at high values of ϕ . Thus the shape of the Eadie-Hofstee plot can be used as a diagnostic tool for determining both the extent of diffusional inhibition present and the nature of the diffusional process concerned.

1.4. Bioelectrocatalysis

There are a considerable number of enzyme-catalysed reactions in vivo which involve electron transfer. The general class of enzymes catalysing these reactions is the oxidoreductases. In addition there are a number of redox proteins which act as electron carriers in vivo. The main groups of redox proteins and enzymes involved in these electron transfer reactions are the cytochromes, flavoproteins, ferredoxins, the copper "blue" proteins and the quinoproteins.

Bioelectrocatalysis is the study of the interaction of these electron transfer proteins with electrodes, where reducing equivalents are either supplied to, or given up by, the electrode.

Electrochemical techniques have been used to study the direct electron transfer between protein redox centres and electrodes (Eddowes & Hill, 1981). Direct electron transfer at chemically modified electrodes with cytochrome c has been reported (Eddowes et al., 1980 ; Lewis & Wrighton, 1981 ; Elliot & Martin, 1982), with spinach ferredoxin (Landrum et al., 1977) and with flavo-proteins such as glucose oxidase and xanthine oxidase (Ianniello et al., 1982 ; Cenas et al., 1983). Direct electron transfer at organic metal electrodes made from complexes of N-methylphenazinium (NMP^+) or N-methylacridinium (NMA^+) and tetracyano-p-quinodimethane (TCNQ^-) has been reported for cytochrome b_2 , glucose oxidase and horse radish peroxidase (Kulys et al., 1980 ; Kulys & Samalius, 1983). Other donors such as tetrathiofulvalene (TTF) have been used with the acceptor TCNQ^- for interaction with glucose oxidase (Albery et al., 1985). In other cases small molecule redox mediators have been employed to carry charge between the protein and the electrode (Weibel & Dodge, 1975 ; Prince et al., 1981 ; Cass et al., 1984).

There have been three main configurations in which bioelectrocatalysis has been exploited. The first case are the enzyme electrodes, where the electrochemical reaction is used to monitor the enzyme-catalysed reaction. These have been developed as analytical devices, and will be discussed further in section 1.5. Another method of utilizing the electrochemical reaction is to drive thermodynamically unfavourable reactions, such as reductions requiring NADH (Dicosimo et al., 1981 ; Simon et al., 1985).

Thirdly, enzymes may be used in a fuel cell to generate electrical energy directly from the free energy of the chemical reaction (Lahoda et al., 1975 ; Ahn et al., 1976 ; Plotkin et al., 1981). Considerable work has also been devoted to the development of bio-fuel cells based on microorganisms (Bennetto et al., 1983 ; Roller et al., 1984 ; Delaney et al., 1984), and reviews of both enzyme and microbial fuel cell technology have been published (Wingard et al., 1982 ; Aston & Turner, 1984 ; Bennetto, 1984). Another approach has been the use of bioelectrochemical fuel cells incorporating free enzymes (Laane et al., 1984) and whole cells (Simon et al., 1985) to synthesize chemicals of commercial value.

1.5. Biosensors

The general concept of a biosensor is a device coupling a biological component such as an enzyme or whole cell, to a transducer which measures a physicochemical change accompanying the action of the immobilized biocatalyst. The use of a biological component allows the exploitation of the specificity of biological recognition, e.g. enzyme / substrate, antibody / antigen or receptor / hormone. Table 1.1. lists both the different biological species and types of transducer which have been proposed for incorporation into biosensors.

TABLE 1.1.
GENERAL TYPES OF BIOSENSORS

BIOLOGICAL COMPONENT	DETERMINANT	TRANSDUCER	BIOSENSOR
ENZYME	SUBSTRATES ANALOGUES COFACTORS INHIBITORS	ELECTRODES (AMPEROMETRIC) ISE (POTENTIOMETRIC)	ENZYME ELECTRODES
		THERMISTOR	ENZYME THERMISTOR
		ISFET	ENFET
		OPTIC FIBRE	OPTRODE
ANTIBODY	ANTIGEN HAPTEN COMPLEMENT	ELECTRODES	IMMUNOSENSOR
MICROBIAL CELLS	INORGANIC SUBSTRATES - GASES & IONS ORGANIC BIOMOLECULES	ELECTRODES ISE	MICROBIAL SENSOR
ORGANELLE	SUBSTRATES	ELECTRODES	ORGANELLE SENSOR

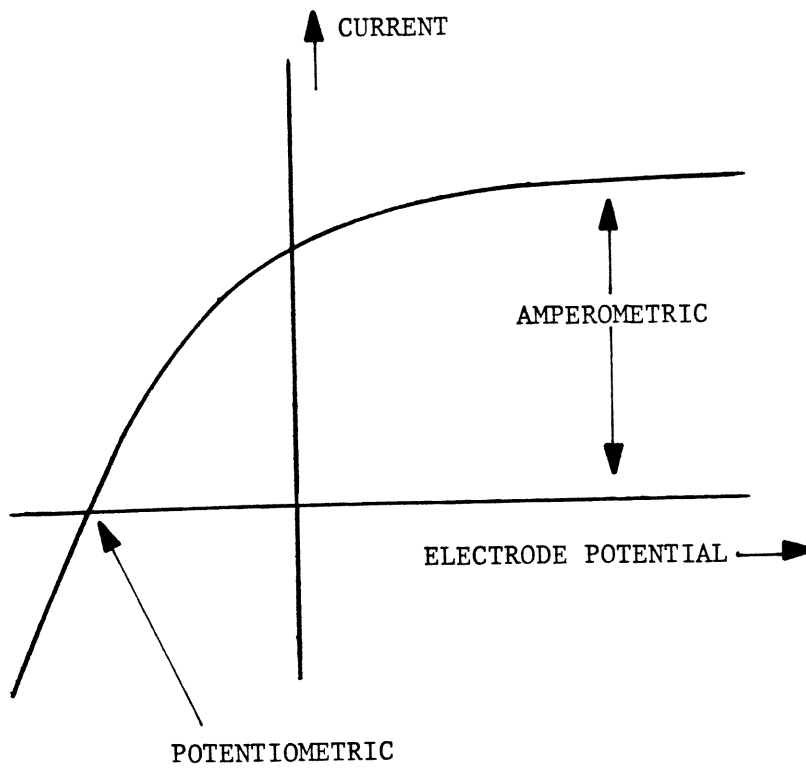
1.5.1. The Transducer

The function of the transducer is to convert the physical (e.g. heat, light) or chemical (e.g. charged species, gases) change occurring at the interface between the transducer and the layer of immobilized biocatalyst into an electrical signal, which may then be accurately related to changes in the concentration of the target compound in the analyte solution.

Electrochemical sensors have been the most widely investigated type of transducer. There are two main classes of electrochemical sensor, potentiometric and amperometric. In potentiometric devices the potential developed due to the analyte is measured under conditions of essentially zero current flow. The potential generated is proportional to the logarithm of the analyte concentration. Examples of conventional potentiometric sensors are the ion-selective electrodes (ISEs) for H^+ , NH_4^+ , Na^+ , K^+ and CO_2 , and also the semi-conductor based ion-selective field effect transistors (ISFETs). In amperometric sensors a potential is maintained between the working and reference electrodes, allowing a current to flow due to the presence of redox species. In this type of sensor the current measured is directly proportional to the concentration of the redox species present. The Clark-type oxygen electrode is a well-known example of an amperometric device. Figure 1.5. shows the points on the current-voltage curve of an electroactive species where potentiometric and amperometric measurement are made.

Potentiometric measurement is non-destructive since virtually

FIGURE 1.5.



A typical current voltage curve for an electroactive species. Potentiometric measurement is made at the point of zero current, and amperometric measurement in the region of transport limited current.

no electron transfer takes place between the electrode and the solution. Thus the measurement is made under conditions of thermodynamic equilibrium, and consequently becomes independent of mass transfer to the electrode surface. The opposite case is true for amperometric sensors, where continuous consumption of the electroactive species takes place at the electrode / solution interface. In this case mass transfer to the electrode surface must be well controlled or this process may become rate-limiting. The disadvantage of potentiometric devices lies in the requirement for an exact control of the potential applied to the working electrode. At the point on the current-voltage curve at which potentiometric measurement is made a change in the potential of 60 mV can alter the current by a factor of 10 (Albery et al., 1985). Because amperometric measurement is made at a potential more positive than the standard redox potential of the electroactive species, fine control of the potential is not required. Both types of devices, however, are able to offer a similar degree of sensitivity to about 10^{-6} mole dm^{-3} . Enzyme based electrochemical sensors have been reviewed by Aizawa (1983) and Aston & Turner (1984), and the use of electrochemical sensors in clinical analysis has been reviewed by Czaban (1985).

Alternative transducers which have been studied include thermistors (Weaver et al., 1976 ; Danielsson et al., 1981 ; Scheller et al., 1985), field effect transistors (Danielsson et al., 1979 ; Caras & Janata, 1980) optical fibre based biosensors (Freeman & Seitz, 1978 ; Goldfinch & Lowe, 1984 ; Arnold, 1985 ;

Place et al., 1985) and devices based on piezoelectric crystals (Olivera & Silver, 1980 ; Guilbault, 1983).

Biosensors employing thermistors are based on the principle that many types of biological recognition reactions, such as enzyme / substrate and antibody / antigen are exothermic. The resulting change in enthalpy may consequently be measured as temperature changes using suitably sensitive thermistors. In general the temperature difference is measured between a bare thermistor and one biologically modified. Analytical applications of enzyme thermistors have been reviewed by Danielsson et al. (1981) and Mosbach et al. (1983).

Retention of an active enzyme layer over the gate of an ISFET has led to the development of enzyme transistors or ENFETs. Here the enzymatic reaction may be monitored using a suitable FET, for example hydrogen- or ammonia-sensitive ISFETs. These devices have been reviewed by Danielsson et al. (1983), Lowe (1984) and Owen (1985).

Numerous configurations of optical fibre sensors have been reported (see reviews by Seitz (1984) and Peterson & Vurek (1984), although relatively few have incorporated enzymes or other biomolecules as part of the sensor. In optical fibre sensors changes in the optical properties of the analyte may be monitored via the optical fibre bundle. These changes may be due to spectral absorption by species present in the test medium (for example haemoglobin and oxyhaemoglobin), to fluorescent species such as NADH or to light scattering. Thus the whole of the optical fibre

bundle may be used for both the transmitted and reflected light, or two different sections of the optical fibre may be employed separately for these two operations. Many chemically sensitive optical fibres have been reported, e.g. for pH, pCO_2 , pO_2 and glucose (Peterson & Vurek, 1984) where a colorimetric or fluorometric reagent is sealed inside a suitable container at the end of an optical fibre bundle.

1.5.2. The Biological Component

Developments in the fields of protein purification and the immobilization of biomolecules have contributed to the considerable interest shown in recent years in the work on enzyme based biosensors. Biosensors coupling enzymes to all the types of transducer described in section 1.5.1. have been developed, the most widely reported type being " enzyme electrodes ", where redox enzymes are coupled to amperometric and potentiometric sensors. These biosensors are discussed in more detail in section 1.5.3. Biological sensors based on organelles (Karube et al., 1983), plant tissue slices (Kuriyama & Rechnitz, 1981) and microbial cells (Jensen & Rechnitz, 1978 ; Kobos et al., 1979 ; Wollenberger et al., 1980 ; Kobos & Pyon, 1981 ; Karube et al., 1982) have been developed. Biosensors based on microbial cells have been reviewed by Aizawa (1983), Karube (1983) and Karube & Suzuki (1984).

Monitoring of antigen / antibody reactions has been reported using suitably modified sensors (Olivera & Silver, 1980 ; Yamamoto

et al., 1981; Yamamoto & Hiroshi, 1981). Aizawa & Suzuki (1982) used catalase as a label to amplify the antibody / antigen interaction. This type of biosensor, often termed an immunosensor, can be employed in a competitive or a non-competitive (sandwich) method as shown in figure 1.6. In the first method of operation a known quantity of labelled antigen competes with antigen in the sample for the antibody bound to the surface of a suitable transducer. Thus the more antigen present in the sample the less labelled antigen that binds to the electrode, and thus the lower the measured signal. In the second method antigen in the sample is bound to antibody immobilized on the surface of the transducer. After rinsing labelled antibody is added. In this type of immunosensor the measured signal is proportional to the amount of antigen in the sample.

Other enzymes such as glucose oxidase, peroxidase, glucose-6-phosphate dehydrogenase and alkaline phosphatase can be employed as a label, and the enzyme label may be monitored spectrophotometrically, thermometrically (Mosbach et al., 1983) or amperometrically (Aizawa et al., 1980 ; Cardosi et al., 1986).

Piezoelectric crystals have also been investigated for direct measurement of antibody / antigen interactions , changes in the oscillation frequency of the crystal detailing the concentration of antibody present in the sample solution (Shons et al., 1972 ; Roederer & Bastiaans, 1983).

FIGURE 1.6.

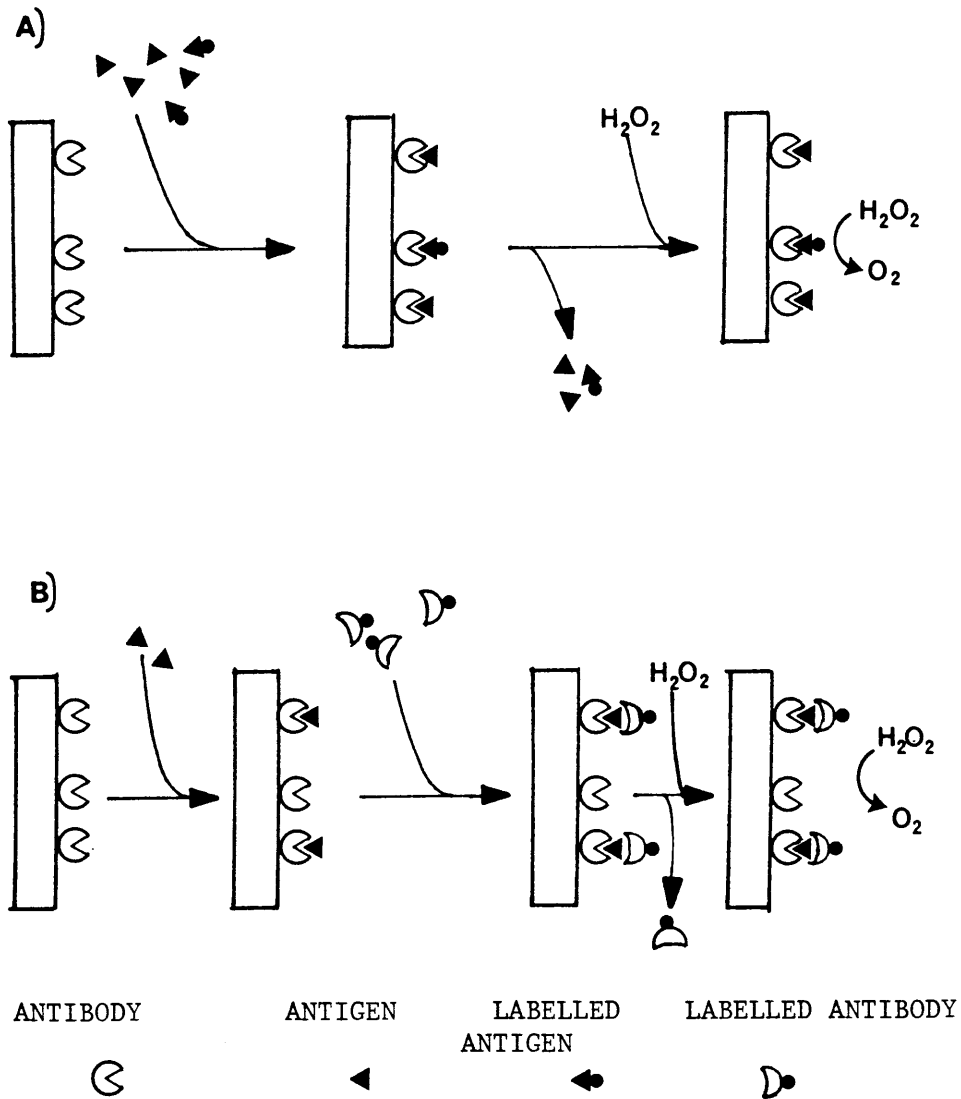


Diagram of an immunosensor based on A) competitive (single antibody) and B) non-competitive (sandwich) methods.

(Adapted from Aizawa & Suzuki, 1982)

1.5.3. Immobilized Enzyme Electrodes

The concept of an "enzyme electrode" was formulated by Clark & Lyons in 1962, where a layer of enzyme is retained over an electrochemical sensor, allowing measurement of the compound of interest via the enzyme-catalysed reaction. The first practical enzyme electrode based on a layer of glucose oxidase immobilized over a Clark oxygen electrode was described by Updike & Hicks in 1967. A similar system whereby urease was immobilized inside polyacrylamide gel over a pH electrode was used by Guilbault & Montalvo in 1969. Many enzyme electrodes have since been reported, and some typical examples are given in table 1.2.

The techniques used to immobilize enzymes on or in close proximity to the electrode surface have varied widely from simple adsorption, crosslinking with glutaraldehyde to covalent attachment. Recent trends in this area have been towards chemical modification of the electrode surface for the direct attachment of enzymes, and chemically modified electrode methodology has been reviewed by Pace (1981) and Murray (1984).

Further developments in the complexity of enzyme electrodes which may widen the range of detectable substrates have been reported using multi-enzyme probes. These have been reviewed by Renneberg et al. (1986). In these types of enzyme electrodes the two enzymes can be arranged to work antagonistically, competing for the same substrate, or synergistically enhancing the measured signal. An example of this is shown in figure 1.7. where bi-enzyme

TABLE 1.2.
IMMOBILIZED ENZYME ELECTRODES

SUBSTRATE	ENZYME	SENSOR	IMMOBILIZATION	REFERENCE
Acetyl- choline	Acetylcholinesterase	pH	Gelatin	[4]
Adenosine	Adenosine deaminase	NH ₃	Glutaraldehyde	[1]
Alcohol	Alcohol oxidase	O ₂	Glutaraldehyde	[15]
Alcohol	Alcohol oxidase	H ₂ O ₂	Adsorbed on paper	[2]
Amino acids	Amino acid oxidase	O ₂	Glutaraldehyde	[12]
Amino acids	Amino acid oxidase	H ₂ O ₂	Covalent coupling	[7]
Biotin	Catalase-labelled avidin	O ₂	Binding of labelled avidin to modified membrane	[8]
Catechol phosphate	Alkaline phosphatase	Direct product oxidation	Covalent coupling	[3]
Glucose	Glucose oxidase	O ₂	Glutaraldehyde	[5]
Glucose	Glucose oxidase	Benzoquinone- mediated	Membrane-retained	[16]
Glucose	Glucose oxidase	H ₂ O ₂	Covalent coupling	[7]
Glutamate	Glutamate decarboxylase	CO ₂	Glutaraldehyde	[14]

TABLE 1.2. CONTINUED

SUBSTRATE	ENZYME	SENSOR	IMMOBILIZATION	REFERENCE
Lactate	Lactate oxidase	O ₂	Adsorbed on membrane	[11]
Lysine	Lysine decarboxylase	CO ₂	Glutaraldehyde	[9]
Penicillin	Penicillinase	pH	Covalent coupling	[13]
Pyruvate	Pyruvate oxidase	O ₂	Adsorbed on acetylcellulose	[10]
Urea	Urease	NH ₄ ⁺	Polyacrylamide	[6]

REFERENCES :

- [1] Bradley & Rechnitz, 1985 ; [2] Clark, 1972 ; [3] Cleland & Enfors, 1983 ; [4] Durland et al., 1978 ; [5] Gough et al., 1985 ; [6] Guilbault & Montalvo, 1969 ; [7] Ianniello & Yacynych, 1981 ; [8] Ikariyama et al., 1985 ; [9] Kuriyama & Rechnitz, 1981 ; [10] Mizutani et al., 1980 ; [11] Mizutani et al., 1983 ; [12] Nanjo & Guilbault, 1974 ; [13] Papariello et al., 1973 ; [14] Razumas et al., 1983 ; [15] Verduyn et al., 1983 ; [16] Williams et al., 1970

layers are formed containing glucose oxidase (GOD) and NAD-linked glucose dehydrogenase (GDH). This system is monitored via the GOD reaction (see section 1.5.4.) and can be used to detect NAD^+ when both enzymes are acting antagonistically. In this mode of operation both enzymes compete for the common substrate (glucose) and the output of the GOD reaction is lowered as the level of NAD^+ in the analyte increases. Alternatively the same system may be used as an amplification technique to detect low levels of glucose if NADH is included in the analyte. In this situation the GDH catalyses the opposite reaction to the GOD and thereby recycles glucose within the bi-enzyme layer.

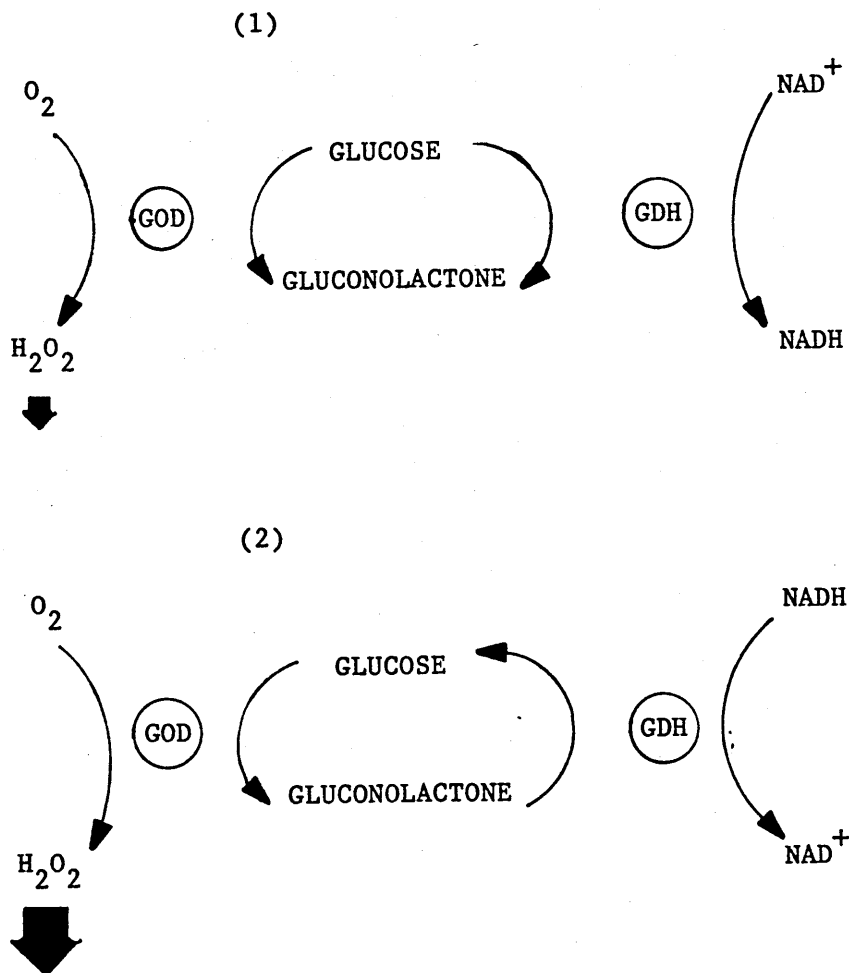
1.5.4. Amperometric Enzyme Electrodes for Glucose

In amperometry a current is measured due to the oxidation or reduction of a redox-active species. There are three major routes available for the coupling of a sensing electrode with an enzyme layer as shown in figure 1.8. :

- a) One of the substrates or products of the enzymic reaction undergoes electron-transfer at the electrode.
- b) Electrons from substrate oxidation are passed to the electrode via a small redox molecule, or mediator.
- c) The enzyme gives up its electrons from substrate oxidation directly to the electrode.

In the case of substrate reduction, the flow of electrons in

FIGURE 1.7.



Schematic illustrating the behaviour of coupled enzyme electrodes for glucose oxidase (GOD) and glucose dehydrogenase (GDH). The signal obtained from the GOD reaction is reduced in the presence of NAD^+ (1) where the two enzymes act antagonistically, and multiplied in the presence of NADH (2) where the enzymes act co-operatively with recycling of glucose within the bi-enzyme layer.

(Adapted from Renneberg et al., 1986)

FIGURE 1.8.

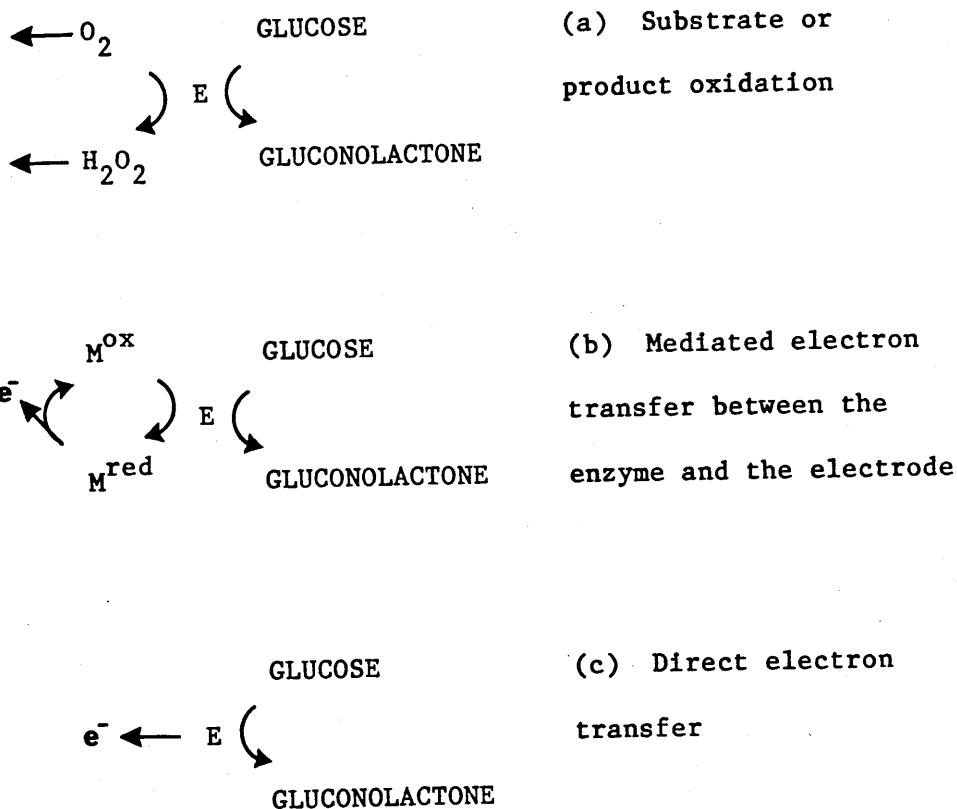
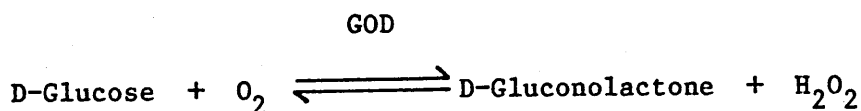


Diagram showing some of the different possible configurations of constructing an amperometric enzyme electrode for glucose. The enzyme (E), mediator (M) or both may be soluble or immobilized.

cases b) and c) is reversed. All three types of sensor configuration have been employed in the construction of amperometric biosensors for glucose based on the enzyme glucose oxidase (GOD, E.C. 1.1.3.4.). The reaction catalysed by this enzyme is :



Glucose oxidase from Aspergillus niger is a flavoprotein of 160,000 molecular weight containing two bound FAD groups (one per subunit). Biosensors based on this enzyme have been the most widely studied type of enzyme electrode, due both to the relative stability of the enzyme and the importance of monitoring glucose in medicine.

Glucose sensors based on the measurement of changes in either oxygen tension (Updike & Hicks, 1967 ; Enfors, 1981) or hydrogen peroxide concentration (Clark, 1972 ; Kamin & Wilson, 1980 ; Castner & Wingard, 1984 ; Gough et al., 1985) have been reported. These are the indirect type of biosensor shown in figure 1.8.a). The main disadvantage of this configuration is the dependence on a stable oxygen tension. Another problem is that at the potential required to oxidize hydrogen peroxide (around 0.7 V vs. SCE) other components of the analyte may also be oxidized (e.g. ascorbate in blood samples).

Alternative redox compounds to oxygen can also oxidize the enzyme (Schlapfer et al., 1974 ; Aleksandrovskii et al., 1981),

and biosensors based on benzoquinone (Williams et al., 1970), potassium ferricyanide (Schlapfer et al., 1974) and dimethylferrocene (Cass et al., 1984) have been constructed. The particular advantages of dimethylferrocene as a mediator include the fact that it is not autooxidizable (i.e. the reduced form is not readily oxidized by molecular oxygen), it has a relatively low toxicity and solubility in aqueous solution, and its low redox potential overcomes the problems outlined above for glucose enzyme electrodes based on hydrogen peroxide or oxygen measurement (Cass et al., 1984).

Direct electron transfer from glucose oxidase to graphite electrodes has been reported by Ianniello et al. (1982), although the currents obtained were very low. Alberly et al. (1985) have claimed that unmediated electron transfer takes place between glucose oxidase and an electrode made from the complex of two organic salts (for example $\text{NMP}^+ \text{TCNQ}^-$). The question of whether direct electron transfer occurs in this system or whether the enzyme is reoxidized by one of the components of the electrode material in solution, has been a matter of some debate. More recent evidence suggests that whilst direct electron transfer to the solid electrode does not take place (from the dependence of the current on the enzyme concentration) homogeneous reoxidation of the enzyme appears to be equally unlikely, and that some limited dissociation of the redox components of the electrode at the electrode surface is responsible. This direct enzyme electrode has advantages over the other types of glucose sensor

shown in figure 1.8., especially as far as the simplicity of the design is concerned. The rate at which the enzyme is oxidized by the electrode presents a problem in that the currents obtained are very low, due both to the slow rate of electron transfer and the size of the enzyme limiting the number of molecules that can be oxidized at the electrode surface at any given moment (Albery et al., 1985). Another major problem is the necessity to exclude oxygen from the vicinity of the probe, since even low oxygen levels may lower the rate of direct enzymatic oxidation (reoxidation of the enzyme by molecular oxygen being the faster process).

The use of mediator-modified electrodes such as the dimethylferrocene modified carbon electrodes seems to be the best practical compromise at the present time. This approach combines the advantages of direct electrochemistry with high catalytic rates and low interference characteristics.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1. Reagents

Unless otherwise stated all chemicals used were obtained either from BDH Chemicals Ltd (Poole, Dorset, England) or Sigma Chemical Company Ltd. (Poole, Dorset, England), and were of the highest purity available.

Graphite felts (RVG 1000 & RVG 4000) and carbon felt (RVC 1000) were purchased from Le Carbone (UK) Ltd (Portslade, Sussex, UK). Reticulated vitreous graphites R60S & R80S were obtained from Fluorocarbon Ltd (Anaheim, California, USA).

2.2. Fabrication of Graphite Foil-based Enzyme Electrodes

Discs were cut out of a roll of plain graphite foil (1 x 0.015 inches, Union Carbide, Ohio, USA) using either a no. 4 or no. 5 cork borer. Each disc was glued to the cut-off end of a pasteur pipette using araldite. Setting of the resin was achieved in an oven at 60°C for 20-30 minutes.

A piece of tinned copper wire (0.6mm, RS Components Ltd., Corby, Northants, England) was joined to the inside face of the graphite foil disc with silver-loaded epoxy resin (RS Components) and set in an oven at 60°C for 20 minutes.

Insulation of the wire-graphite foil contact was accomplished using a mixture of epon resin grade 815 and triethylenetetramine (1.8g of resin to 0.23g of amine, both obtained from Polysciences, Inc., Warrington, P.A., USA). The resin was allowed to set overnight at room temperature. Before use the resistance of each electrode was measured with a multimeter, the resistance being typically less than 5 ohms (electrodes showing high resistances were discarded). A diagram of one of these experimental electrodes is shown in figure 2.1.

2.3. Measurement of Current from Graphite Foil-based Enzyme Electrodes

Steady-state currents from graphite foil electrodes were measured using a microcomputer system as shown in figure 2.2. A BBC B microcomputer (Acorn Computers, Cambridge, UK) was used to generate the desired potential and to measure the resulting currents via an Artek Biosensor Interface (Artek, Lavendon, Bucks, UK).

The enzyme electrodes were immersed in 0.1 M phosphate buffer pH 7.0 containing 0.15 M potassium chloride inside a jacketed cell along with a silver / silver chloride (Ag / AgCl) reference electrode, which also acted as the auxillary electrode. This reference electrode was made by placing a piece of silver foil in a 0.5 M KCl solution and applying a potential of +400 mV vs. SCE and a resistance load of 5×10^3 ohms. A counter electrode of

FIGURE 2.1.

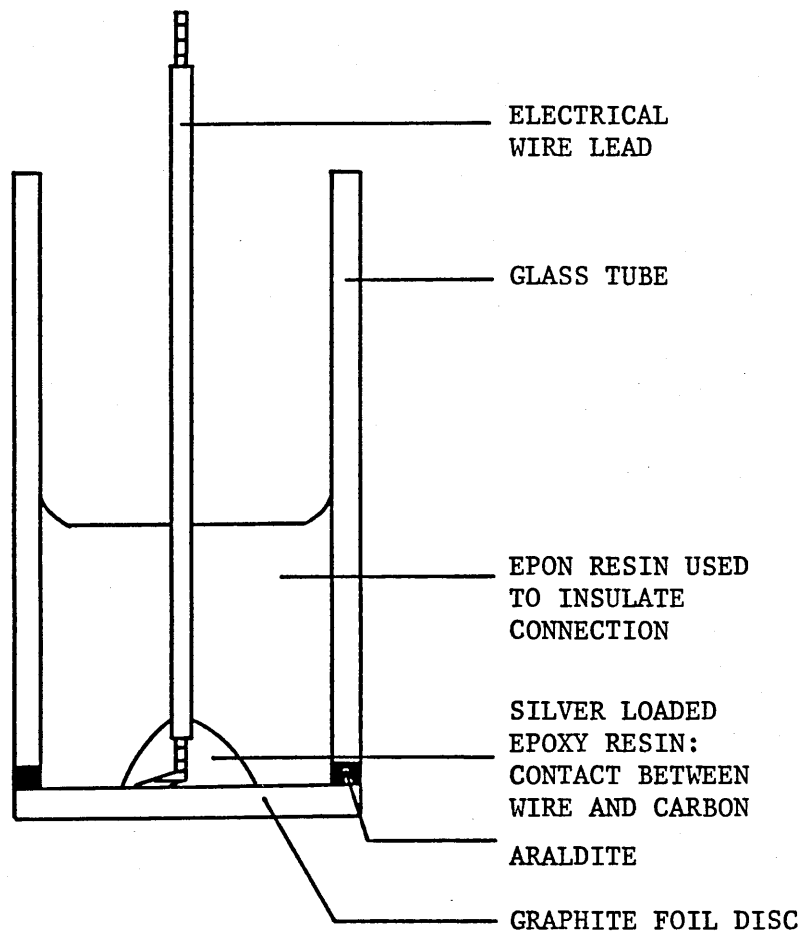
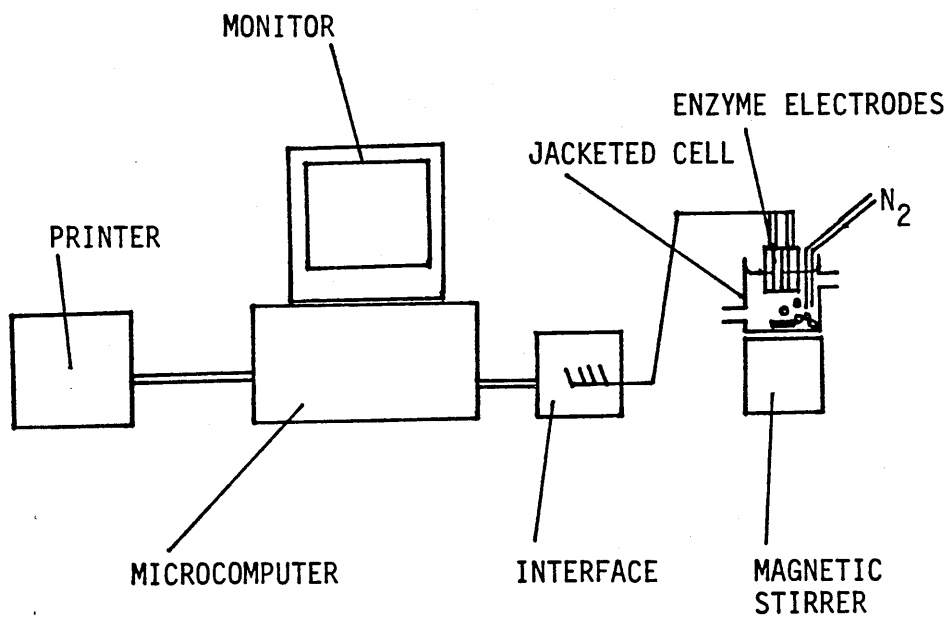


Diagram of an experimental electrode employed in the construction of biosensors for glucose and NADH.

FIGURE 2.2.

Microcomputer based experimental system used for the
measurement of currents from enzyme electrodes.

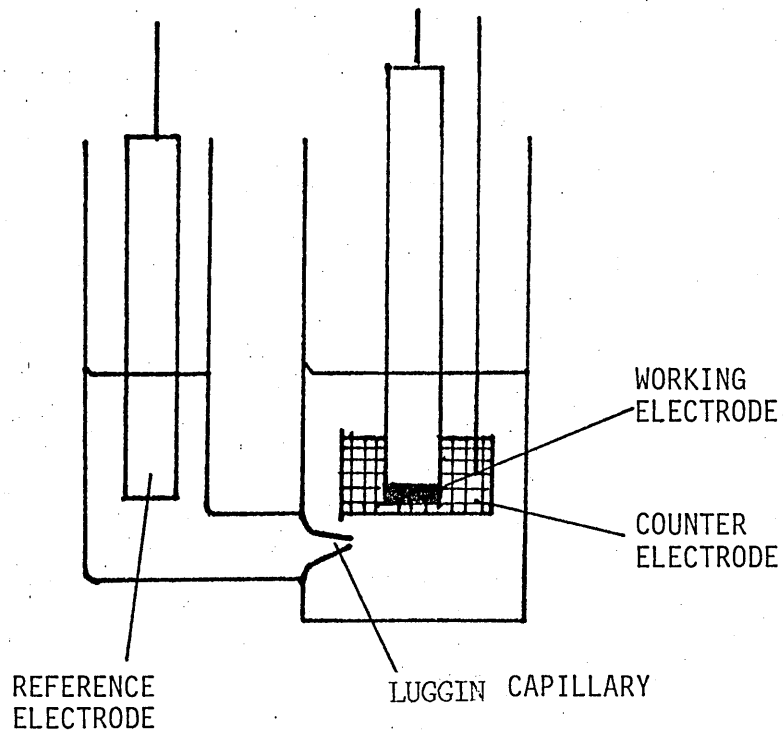
platinum gauze was used to complete the circuit. Temperature control was maintained by the use of a circulating water bath (Grant Instruments (Cambridge) Ltd., Cambridge, England). Degassing during the experiments was achieved using white spot nitrogen, which was first water-saturated by passage through a Drechsel flask containing distilled water to prevent excessive evaporation of liquid during the experiment. Stirring of the reaction vessel was achieved with a magnetic stirrer and stirring bar.

2.4. Electrochemical Experiments

Different electrochemical techniques, such as cyclic voltammetry (CV), differential pulse voltammetry (DPV) and linear sweep voltammetry (LSV) were used in a number of experiments. These were performed using the BAS 100 Electrochemical Analyzer (Bioanalytical Systems Inc., West Lafayette, Indiana, USA) with a two-compartment electrochemical cell of 2 ml working volume. The reference compartment was connected to the working compartment via a luggin capillary. A saturated calomel reference electrode (SCE) and a platinum gauze auxiliary electrode were used. This electrochemical cell is shown in figure 2.3.

Cyclic voltammetry of graphite felt (RVG 1000) electrodes was performed using an Oxford Instruments potentiostat and a JJ PL1000 xy/t chart recorder (JJ Lloyd Instruments Ltd., Warsash, Southampton, UK). In these experiments a three-compartment cell

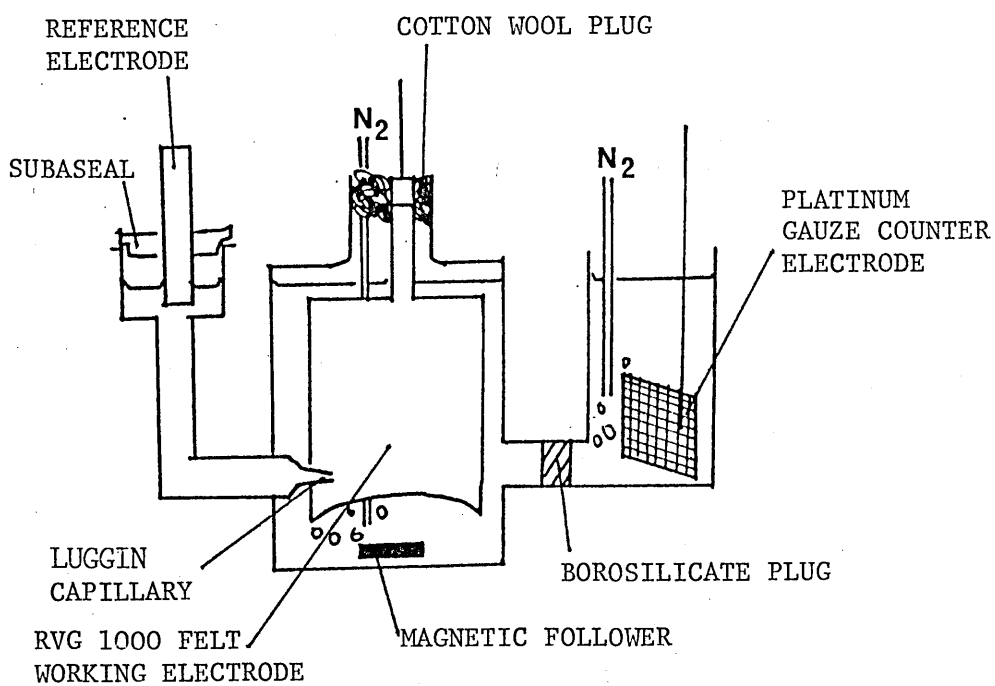
FIGURE 2.3.



Two compartment electrochemical cell used for
controlled potential experiments with the BAS 100

Electrochemical Analyzer.

FIGURE 2.4.



Three compartment electrochemical cell used for the methyl viologen mediated bulk reduction of NAD^+ using lipoamide dehydrogenase bound on graphite felt.

of 20 ml working volume was used, the reference compartment being connected to the working side via a luggin capillary, and the auxillary compartment being connected to the working compartment via a section of porous borosilicate. This type of cell is shown in figure 2.4.

2.5. Protein Assays

Estimation of the protein bound to different electrode materials was carried out using Bradford's reagent, prepared according to Bradford (1976). The protein remaining after the immobilization step was measured by comparison with a standard curve, and the difference between this value and the amount of protein originally used was taken to be the amount of enzyme immobilized.

CHAPTER 3

ENZYME IMMOBILIZATION ON CARBON ELECTRODE MATERIALS

INTRODUCTION

3.1. Methods of Enzyme Immobilization

The numerous methods by which enzymes may be insolubilized fall into four general types:

1. Retention by intermolecular forces such as adsorption on inert supports or by ionic interactions.
2. Physical entrapment of the enzyme within gels, membranes or hollow fibres.
3. Crosslinking with bi- or multi-functional reagents.
4. Covalent coupling to inert supports through amino acid side chains.

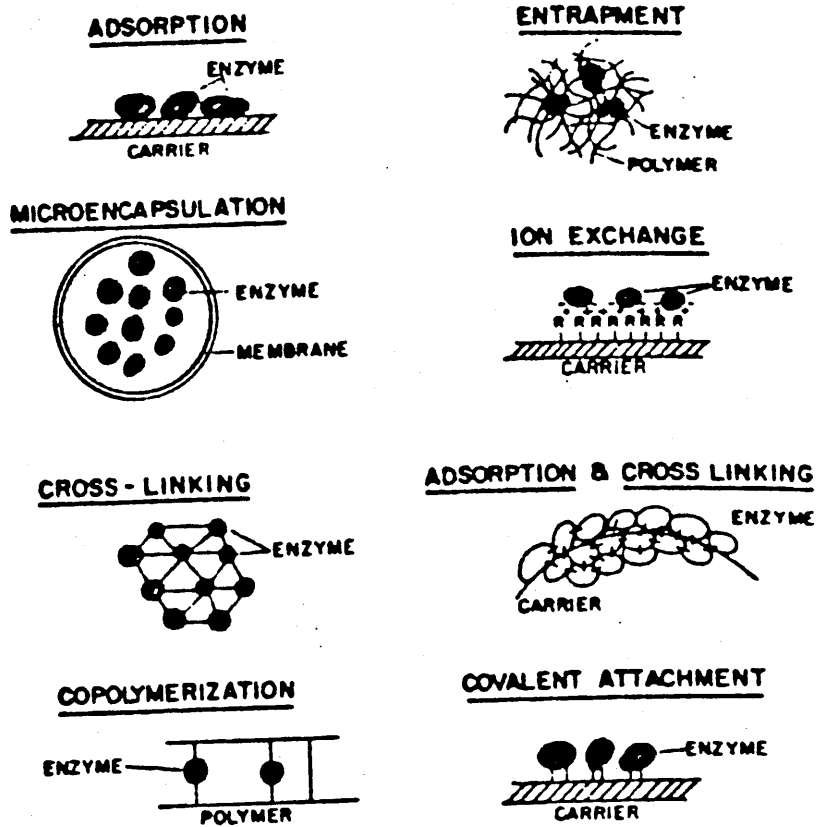
These different approaches to enzyme immobilization are shown graphically in figure 3.1. Techniques of enzyme immobilization have been reviewed amongst others by Mosbach (1976) and Barker (1980).

3.2. Adsorption Techniques.

Historically the oldest method of preparing immobilized proteins, adsorption methods are applicable to a variety of support

FIGURE 3.1

General Techniques of Enzyme Immobilization



materials. One major advantage of this type of immobilization procedure is its technical simplicity.

Carbohydrate polymers, such as DEAE-cellulose (Tosa et al., 1967), Sepharose 4B (Cowan & Daniel, 1982) and paper (Clark, 1972) have all been used to immobilize enzymes. Although the forces involved in physical adsorption of proteins are generally weak intermolecular forces, the strength of the adsorption and stability of the resulting conjugate may vary enormously. Wisdom et al. (1984) reported the adsorption of lipase onto different types of diatomaceous earths. The protein remained bound when used to catalyse triglyceride interesterification in organic media, but was relatively easily removed in aqueous solutions. By contrast almost irreversible adsorption of enzymes such as xanthine oxidase, lactate dehydrogenase, alkaline phosphatase and urease was found to occur with N-alkyl derivatives of Sepharose (Hofstee & Ortillio, 1973; Visser & Strating, 1975).

Enzymes may also be adsorbed very effectively onto inorganic supports. For example Kovalenko et al. (1981) have reported the adsorption of lactate dehydrogenase onto different forms of alumina, with 20 - 40% of the original activity retained by the adsorbed enzyme after 12 months storage at room temperature, and Koga et al. (1984) adsorbed alkaline phosphatase onto activated alumina.

3.3. Physical Entrapment Methods.

In these methods the enzyme is occluded within a structure that retains the enzyme, but allows access to substrates in the surrounding medium. This may be achieved by polymerization of the medium surrounding the enzyme. The most common method of this type has been the use of polyacrylamide gels (Updike & Hicks, 1967 ; Mosbach, 1970 ; Chen & Starzmann, 1982 ; Azari & Wiseman, 1982). Various derivatives of acrylic acid have been used, as well as different methods of polymerization.

Tikhonova et al. (1982) employed electrochemical initiation of the copolymerization of acrylamide and N,N'-methylene (bis) acrylamide to immobilize glucose oxidase. Kumakura et al. (1984) used gamma radiation to initiate copolymerization of various acrylate monomers such as 2-hydroxyethyl methacrylate. Other types of gel have also been employed, for example gelatin (Park et al., 1982) and poly (maleinic anhydride) to immobilize acetylcholinesterase (Bhattacharya et al., 1981).

Retention of enzymes by other physical methods includes the entrapment of enzymes within membranes and microcapsules. For example, Goldman et al. (1971) used collodion membranes, and Ortmanis et al. (1984) immobilized urease in cellulose acetate membranes. Davidson et al. (1974) constructed a urease-collagen membrane to treat waste water samples.

Another type of entrapment is the use of microcapsules. This involves retaining the enzyme within aqueous droplets in an

organic medium. For example, dispersion of an aqueous solution of enzyme containing 1,6-diaminohexane into an organic solution of hexanedioic acid dichloride results in polymerization on the water-solvent interface, forming a thin polyamide membrane around aqueous droplets of enzyme solution (Chang, 1964, 1984).

Similarly droplets of enzyme solution may be trapped within synthetic fibres (Marconi & Morisi, 1979) and liposomes (Gregoriadis & Buckland, 1973 ; Gregoriadis, 1978).

3.4. Crosslinking Methods.

In general crosslinking of proteins has been used in conjunction with other methods, such as adsorption or gel entrapment, as a means of further stabilizing and retaining the enzyme. The most common agent used has been glutaraldehyde, although other compounds such as 1,5-difluoro-2,4-dinitrobenzene, xylylene diisocyanate and dimethyl adipimide (Wold, 1972) and bisdiazobenzidine-2,2'-disulphonic acid (Goldman *et al.*, 1968) have been used. Other bifunctional reagents have been reviewed by Fasold *et al.* (1971).

Glutaraldehyde has been used for insolubilizing proteins not only for creating active immobilized enzyme preparations, but also for microscopy and X-ray crystallography. The chemical nature of the reaction, however, remained unclear for a long time. Richard & Knowles (1968) suggested the existence of unsaturated oligomers in commercial solutions, which might react with the

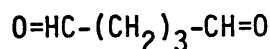
amine groups of amino acid side chains via a Michael-type condensation reaction. Monsan et al. (1975), using a variety of analytical techniques, were able to demonstrate the presence of oligomers of glutaraldehyde in commercial solutions. The stability of these oligomers, however, was found to depend on pH, easily reverting back to the monomer form at acidic pH values and precipitating out as polyglutaraldehyde at alkaline pH's. Using the model compound crotonaldehyde they further showed that the reaction takes place between these oligomers of glutaraldehyde and amine groups on the protein with the formation of imino bonds which are stabilized by resonance with adjacent ethylenic bonds. This proposed mechanism for the reaction of glutaraldehyde with proteins is shown in figure 3.2.

Glutaraldehyde has been used to crosslink a wide range of proteins onto many different types of support materials. Some of the many examples to be found in the literature have been the use of glutaraldehyde to crosslink proteins adsorbed on collodion membranes for kinetic studies (Goldman et al., 1968, 1971), to crosslink alcohol dehydrogenase in an alcohol biosensor (Verduyn et al., 1983) and to crosslink together lactate dehydrogenase and alcohol dehydrogenase on agarose beads, with their active sites juxtaposed via a bis-NAD analogue (Mansson et al., 1983).

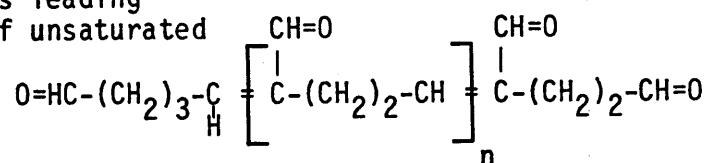
A number of bifunctional reagents have been investigated for the purpose of coupling enzymes to antigens and antibodies, for example p,p'-difluoro-m,m'-dinitrophenyl sulphone (FNPS), toluene diisocyanate and N,N'-o-phenylenedimaleimide. These have been

FIGURE 3.2.
Mechanism of the reaction of glutaraldehyde with proteins

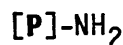
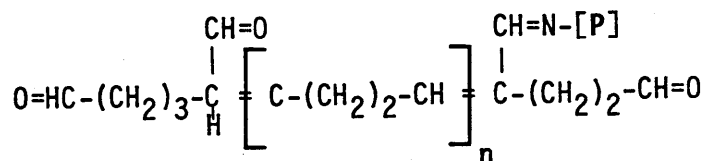
Glutaraldehyde exists in the monomer form or as unstable hydrates at acidic pH values.



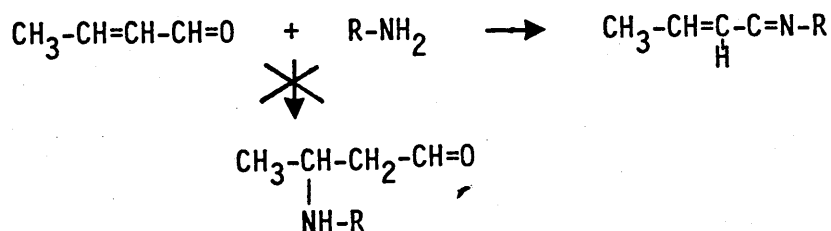
At neutral & alkaline pH's condensation occurs leading to the formation of unsaturated oligomers



Reaction with amino groups of proteins or support materials takes place via the formation of imino bonds.



In the case of the model compound crotonaldehyde reaction with amines proceeds via the aldehyde group and not through the ethylenic bond via a Michael-type condensation reaction.



reviewed by Avrameas et al. (1978). Kitigawa et al. (1981) studied 10 different heterofunctional reagents based on N-(m-maleimidobenzoyloxy)-succinamide, which are able to couple to thiol groups via the maleimide residue, and to amino groups through the N-hydroxysuccinamide ester. Techniques for the labelling of antibodies with reagents of this type have been reviewed by Ishikawa et al. (1983).

3.5. Covalent Coupling Methods

These types of immobilization methods, where covalent bonds are formed between the protein and the support material, have been the most widely used and studied. Many of the amino acid side chains are potential sites for coupling, the only prerequisite being that they are non-essential for the enzyme's catalytic activity. The most common side chains used are the amino groups of lysine, but the carboxylic acid groups of glutamic and aspartic acids, the thiol of cysteine and the phenolic group of tyrosine have also been employed.

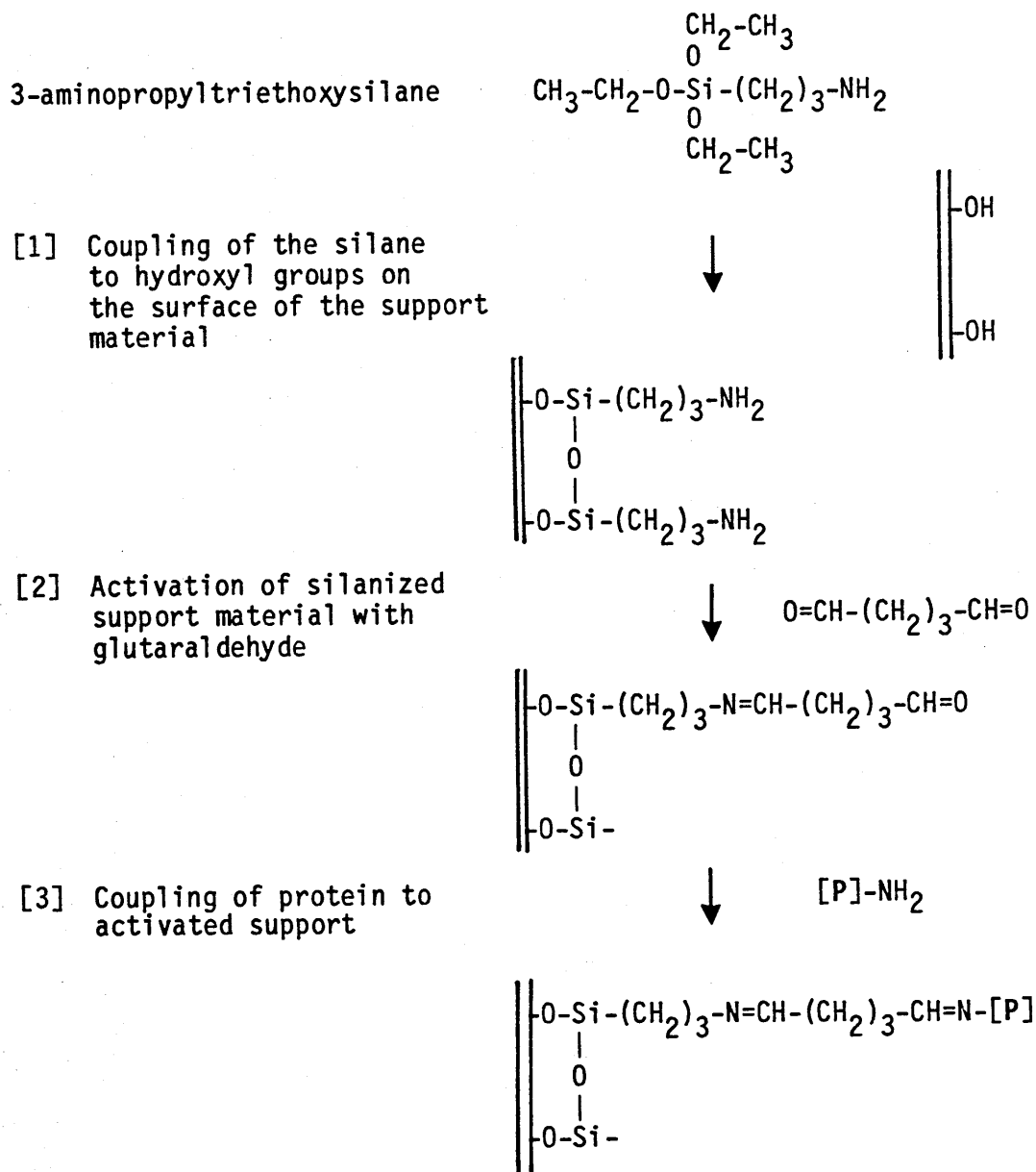
The coupling agent used to immobilize a protein depends to a large extent on the nature of the support material used. Coupling to support materials with carboxylic acid functions, such as CM-cellulose, silanized porous glass and different forms of carbon may be achieved by activation of the pendant carboxylic acid groups using N,N'-disubstituted carbodiimides (Bourdillon et al., 1979 ; Cho & Bailey, 1978, 1979 ; Janolino & Swaisgood, 1982 ; Osborn et

al., 1982). Other reagents, such as Woodward's Reagent K (N-ethyl-5-phenylisoxazolium 3'-sulphonate) have also been used (Woodward et al., 1966 ; Patel et al., 1967).

Polymers with pendant hydroxyl groups, such as celluloses and other polysaccharides may be activated using cyanogen bromide (Mosbach, 1970 ; Axen & Ernback, 1971 ; Robinson et al., 1973), cyanuric chloride (Kay & Lilley, 1970 ; Ianniello & Yacynych, 1981), titanium (IV) chloride activation (Gray et al., 1982 ; Alberti & Klibanov, 1982) and by using the acid-azide (Curtius) reaction (Lilley & Hornby, 1966). Alumina activated by titanium (IV) chloride has been used to immobilize sulphatase (Pelsey & Klibanov, 1983).

Another common method of covalently immobilizing enzymes is the use of silane reagents (figure 3.3). These techniques have also proved successfull with inorganic materials such as controlled pore glass (Weibel & Bright, 1971 ; Weetall, 1973 ; Weetall et al., 1974) and nickel oxide screens (Weetall & Hersh, 1970).

FIGURE 3.3.
Immobilization of enzymes using silane reagents



MATERIALS AND METHODS

3.6. Electrode Materials

The following high surface area carbon materials were investigated as possible supports for immobilization of glucose oxidase : reticulated vitreous carbon (R80S and R60S), carbon felt (RVC 1000) and graphitic felt (RVG 1000 and RVG 4000). Graphite foil was used as the electrode material and enzyme support for the work on the dimethylferrocene-mediated glucose biosensor. Some of the physical properties of these materials are given in table 3.1.

3.7. Oxidation of Electrode Materials

Graphite felts (RVG 1000, RVG 4000, RVC 1000) and reticulated vitreous graphite (R80S and R60S) were surface-oxidized using an equal volume ratio of concentrated sulphuric and nitric acids. Oxidation was carried out over a 24 hour period at room temperature. After removal from the acid bath, the pieces of electrode material were washed first under running tap water for 15 minutes, then by boiling in distilled water for 1 hour and finally rinsed with distilled water. The pieces of electrode material were dried in an oven at 160°C.

Oxidation of graphite foil for use in the construction of glucose oxidase based sensors was carried out in an oven at 160°C

TABLE 3.1.
PROPERTIES OF DIFFERENT CARBON MATERIALS

MATERIAL	CARBON CONTENT %	a) OXIDATION RESISTANCE %	RESISTANCE Ω cm	TENSILE STRENGTH kg/cm ²
GRAPHITE FELT				
RVG 1000	99	1	b) 1.7	c) 1
RVG 4000	99	1	b) 1.9	c) 1
CARBON FELT				
RVC 1000	94-97	4-12	b) 0.2	c) 4
RETICULATED VITREOUS GRAPHITE	N/A	N/A	0.47-0.69	3-12

a) Weight loss after 48 hr at 350°C in air

b) Of a 1 cm² square

c) Of a 5 cm strip

for at least 24 hours. This material is already oxidized to a large extent due to its method of manufacture.

3.8. Carbodiimide Activation

Oxidized pieces of electrode material were placed in a solution of water-soluble 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide p-methyltoluenesulphonate (20mg/ml) in 0.1M acetate buffer pH 4.5 for 90 minutes at room temperature. The derivatized materials were placed for 1 minute in 0.2M carbonate buffer pH 9.6, and then into enzyme solution (see section 3.11). This method was adapted from that reported by Cass et al. (1984).

In some experiments a higher concentration of carbodiimide (0.1M) was used. An alternative buffer (0.2M citrate buffer pH 5.0) was employed for the activation step with lipoamide dehydrogenase.

3.9. Thionyl Chloride Activation

Activation of acid-oxidized materials using thionyl chloride (SOCl_2) was carried out using a 10% (v/v) solution in chloroform. This approach was a modified version of the method reported by Watkins et al. (1975) where activation of graphite electrodes was accomplished using a solution of thionyl chloride in benzene. Owing to the carcinogenic nature of benzene an alternative solvent was employed, i.e. chloroform. The chloroform was dried before

use with molecular sieve type 4A (BDH Chemicals Ltd., Poole, Dorset, England) activated at 250°C. The reaction was conducted under reflux conditions for 4 hours with a drying tube attached (containing MgCl_2). After reflux the pieces of electrode material were washed three times with chloroform then placed inside a desicator which was flushed with nitrogen, then left overnight to dry.

3.10. Reduction of Surface Carboxylic Acid Groups using Lithium Aluminium Hydride

Pieces of electrode material were placed in ethoxyethane, which had been dried using molecular sieve type 4A (activated overnight at 250°C) and an excess of lithium aluminium hydride (LiAlH_4) added. The mixture was then refluxed for 2 hours. After allowing the solution to cool a small volume of 0.1 M acetic acid was added to remove any unreacted AlH_4^- ions. This method was essentially that reported by Ianniello & Yacynych (1981).

The pieces of electrode material were washed with three volumes of ether, standing in the final wash for 10 minutes. The material was then washed with 1M nitric acid to remove precipitated AlCl_3 , and finally with distilled water. The graphite was then dried in an oven at 60°C.

3.11. Silanization of Support Materials

3.11.1. Organic Solvent Method

Silanization, using 3-aminopropyltriethoxysilane, was carried out on both oxidized and LiAlH_4 -reduced materials with a 5% (v/v) solution in toluene (HPLC grade) as described by Weetall (1976). The derivitization was accomplished by reflux for 4 hours. The electrode materials were then washed twice with toluene and twice with acetone. Drying was carried out in an oven overnight at 60°C .

3.11.2. Aqueous Method

Silanization of LiAlH_4 -reduced graphite foil in aqueous medium was carried out using a 10% solution of 3-aminopropyltriethoxysilane acidified to pH 3.7 using concentrated hydrochloric acid as described by Weetall (1976). The silanization was carried out at 75°C for 4 hours. Excess silane reagent was evaporated off by placing the derivatized electrodes in an oven at 100°C for 45 minutes. Aqueous silanization was used with the graphite foil electrodes because of the adverse effect of refluxing in toluene on the araldite employed to retain the graphite disc onto the glass support (section 2.2.).

3.12. Glutaraldehyde Activation

Activation of a variety of modified support materials with glutaraldehyde was carried out using a 2.5% (v/v) solution in 0.1M phosphate buffer pH 7 at room temperature, typically for 90 minutes. This procedure was adopted after surveying a number of reported methods, and was based on the most common conditions employed.

3.13 Enzyme Immobilization

Glucose oxidase and lipoamide dehydrogenase were immobilized onto the derivatized support materials in buffered solutions. For support materials activated using carbodiimide an acidic pH was used (0.1M acetate buffer pH 4.5 for glucose oxidase, 0.2M citrate buffer pH 5.0 for lipoamide dehydrogenase), whereas for the other activation methods a neutral pH was employed (0.1M phosphate buffer pH 7). The concentration of the enzyme in the immobilization step was generally in the range 1-10mg/ml, although a wider range of enzyme concentrations was used for the graphite foil electrodes.

3.14. Washing Procedures

After enzyme immobilization the support materials with bound enzyme were washed with a sequence of buffers. This procedure

employed a low pH / high ionic strength buffer (0.5M citrate buffer pH 4.5) and a neutral pH / high ionic strength buffer (0.1 M phosphate buffer pH 7 containing 0.5M KCl or 0.5M phosphate buffer pH 7) in addition to the buffer used during the immobilization step.

Following this washing sequence the pieces of support material were thoroughly washed with distilled water and stored at 4°C in 0.1M phosphate buffer pH 7.

3.15. Assay of Bound Enzyme Activity

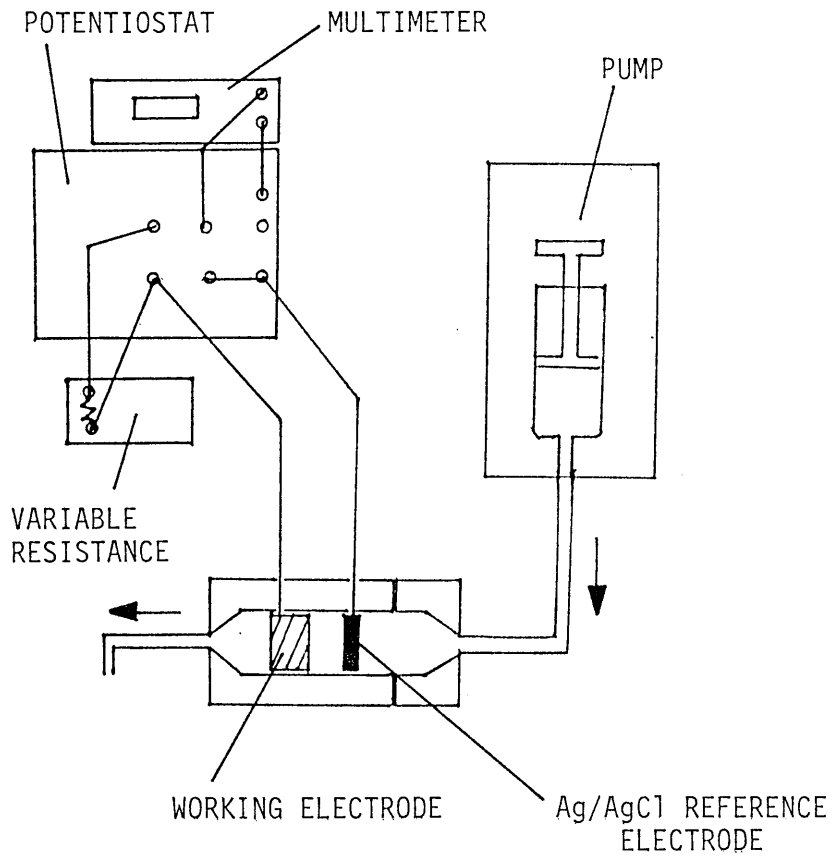
3.15.1 Oxygen Electrode

Assays using a Clark-type oxygen electrode (Rank Brothers, Bottisham, Cambridge, England) were performed for glucose oxidase immobilized onto discs of RVG 1000. Each disc was assayed separately. The jacketed oxygen electrode was maintained at 30°C using a thermostatted water bath.

3.15.2. Electrochemical Flow Cell

A simply designed flow cell was fabricated out of perspex for use with electrode materials too bulky to assay in the oxygen electrode. The experimental arrangement is shown in figure 3.4. A Sage Instruments pump (Sage Instruments Inc. (Orion Research Inc.) Cambridge, Massachusetts, USA) was used to feed solution

FIGURE 3.4.



Electrochemical flow cell used to monitor glucose oxidase bound onto different graphite electrode materials.

through the flow cell, and a Ministat potentiostat (H.B. Thompson & Associates, Newcastle-Upon-Tyne, UK) was employed to generate a potential between the working electrode and the Ag/AgCl reference/auxillary electrode. The experiments were performed at room temperature using 50 mM potassium phosphate buffer pH 5.75.

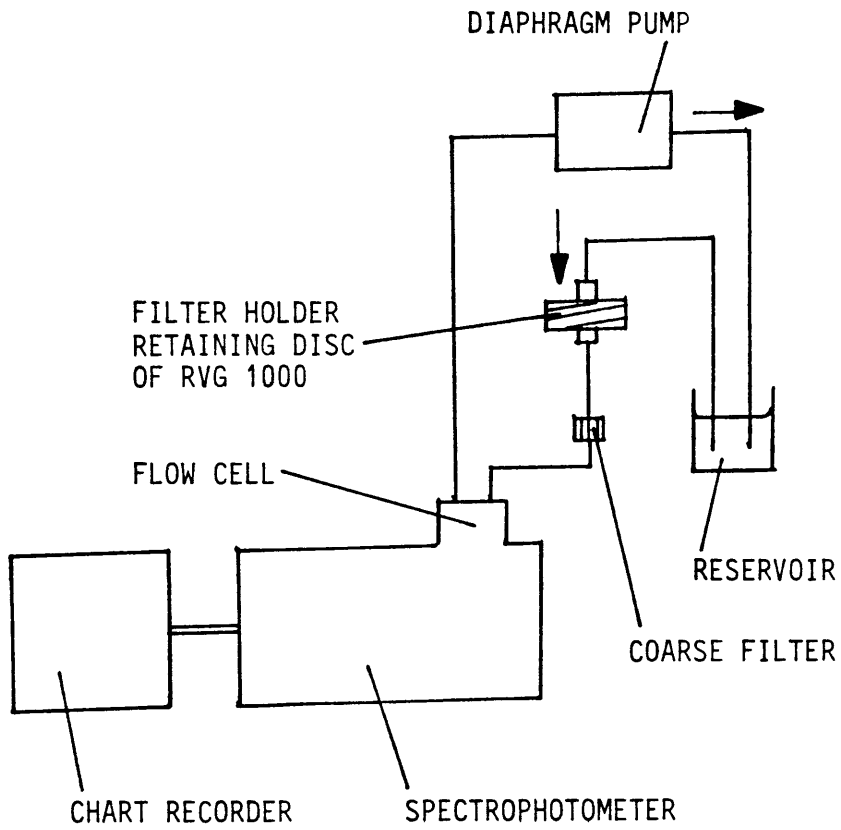
3.15.3. Spectrophotometric Flow System

Discs of RVG 1000 with bound lipoamide dehydrogenase were assayed for lipoic acid activity using the flow system shown in figure 3.5. The disc was retained inside a Sartorius filter holder (Sartorius GmbH, Gottingen, West Germany), and solution was circulated through the system by a KNF Neuberger diaphragm pump (KNF Neuberger UK Ltd, Wantage, Oxon, UK). This pump was able to achieve a flow rate of 30 ml/min through the system, higher than obtained with previously used pumps, and was therefore able to decrease the time required for equilibration of the system after addition of substrates to the reservoir.

The activity of the bound enzyme was followed at 340 nm. After allowing the system to equilibrate lipoic acid (typically 100 μ l of a 0.1 M solution in ethanol) was added to the reservoir. When a stable reading was achieved NADH solution (typically 100 μ l of a 0.2 M solution) was added and the change in A_{340} measured. The time required to obtain a steady rate after addition of NADH solution to the reservoir was about 1 minute.

Changes in A_{340} due to NADH oxidation were monitored using a

FIGURE 3.5.



Spectrophotometric flow system used to monitor the activity of yeast lipoamide dehydrogenase bound onto graphite felt discs.

Cecil spectrophotometer (Cecil Instruments Ltd., Cambridge, UK) fitted with a flow cell. The jacketed reservoir for the system was kept agitated with a magnetic stirring bar and stirrer, and temperature control was maintained with a Thermomix pump / heater (F.T. Scientific Instruments Ltd., Bredon, Tewkesbury, UK) and remote water bath.

3.15.4. Graphite Foil Electrodes

The activity of modified graphite foil enzyme electrodes was measured using the BBC microcomputer system described in section 2.3. The activity of the silanized electrodes was measured in a 3 electrode system using a Ministat potentiostat with a SCE reference electrode and a platinum gauze counter electrode.

RESULTS AND DISCUSSION

3.16. Choice of Immobilization Methods

As discussed in section 3.2. a wealth of immobilization techniques have been used to insolubilize enzymes onto organic and inorganic supports. Entrapment and encapsulation methods were not considered suitable for these experiments. Due to the shape and surface roughness of the graphite materials being investigated the application of a membrane would have been difficult to achieve. On the other hand entrapment of the enzyme inside a polymer matrix within the porous materials used would have led to severe diffusional inhibition of the immobilized enzyme (Mell & Maloy, 1975 ; Castner & Wingard, 1984), which would have made comparison of the different immobilization methods difficult. This reasoning left the following methods under consideration :

- (1) Adsorption
- (2) Adsorption followed by crosslinking
- (3) Covalent attachment
- (4) Covalent attachment followed by crosslinking

Glutaraldehyde was chosen as the crosslinking agent, and the covalent coupling methods examined were water-soluble carbodiimide, silanization and thionyl chloride activation.

3.17. Outline of Research

The aim of this work on immobilization methods was to evaluate the best way of immobilizing lipoamide dehydrogenase onto a high surface area electrode material for use in the methyl viologen mediated reduction of NAD^+ . This latter work is described in Chapter 5. Initially glucose oxidase was used as a model due to its higher activity, lower cost and better stability. Subsequently lipoamide dehydrogenase was examined in a spectrophotometric flow system, before being employed as the catalytic electrode to bulk reduce NAD^+ (Chapter 5).

Different methods were also used to immobilize glucose oxidase onto porous graphite foil for application as a biosensor for glucose.

3.18 Assay of Bound Enzyme Activity on Different Carbon Materials

Glucose oxidase was immobilized onto the high surface area electrode materials listed in section 3.4. and the protein bound measured using Bradford's reagent (section 2.5.). The results are shown in table 3.2.

The activity of the derivatized materials was assayed using both a standard polarographic oxygen electrode and an electrochemical flow cell (figure 3.4.) designed to assay sections of these materials acting as working electrodes.

TABLE 3.2.
GLUCOSE OXIDASE BOUND ON DIFFERENT
GRAPHITE ELECTRODE MATERIALS

GRAPHITE	IMMOBILIZATION METHOD	PROTEIN BOUND
RVG 1000	CARBODIIMIDE	0.45 mg / disc (a)
	THIONYL CHLORIDE	0.53 mg / disc (a)
RVG 4000	CARBODIIMIDE	1.5 mg (b)
R60S	CARBODIIMIDE	1.3 mg (c)

(a) 1.2 cm diameter

(b) 1.2 cm diameter, 2.5 cm in thickness

(c) 1.2 cm diameter, 2.7 cm in length

3.19 Oxygen Electrode Work

In the oxygen electrode, discs of the graphite and carbon felts and cylindrical sections of the reticulated vitreous graphites (R60S and R80S) were assayed by following the oxygen consumption of the immobilized enzyme in the presence of glucose. Assays were performed on a single disc at a time and at a single glucose concentration. It was found that only the thin felts RVG 1000 and RVC 1000 could be assayed effectively in the oxygen electrode chamber as the thicker materials hindered the action of the stirring bar and consequently gave inconsistent results. The discs of RVG 1000 and RVC 1000 on the other hand were observed to spin freely inside the oxygen electrode chamber above the stirring bar and reproducible results were obtained. In order to compare different immobilization methods it was decided to concentrate on the RVG 1000 material because of its higher purity and increased state of oxidation with respect to the carbon felt (table 3.1.).

The immobilization methods used in these studies were those listed in section 3.16., with carbodiimide activation and silanization chosen as examples of covalent coupling methods.

3.19.1. Activity of Glucose Oxidase Immobilized on RVG 1000 Discs by Adsorption

Glucose oxidase was adsorbed onto untreated and acid oxidized discs, and assayed for activity using the oxygen electrode.

Consecutive assays were performed at 20 mM glucose and 30°C using both phosphate buffer pH 7 and phosphate / citrate buffer pH 5.75. The results for an average of five or more assays is given in table 3.3. Higher enzyme activity was observed when the enzyme was adsorbed onto the oxidized discs, but no difference was noted between the two pH values employed. Although a large variation was observed between successive assays this did not appear to be due to inactivation of the enzyme. The activity of these discs was observed to be lower than those where the enzyme was bound using either glutaraldehyde crosslinking or covalent attachment.

3.19.2. Carbodiimide Activation

Untreated and acid oxidized discs were activated using water-soluble carbodiimide as described in section 3.6. Glucose oxidase was then immobilized onto these discs from a 1 mg/ml solution. The results for oxygen electrode assays are presented in table 3.4. and shown graphically in figure 3.6. The activity of the unoxidized discs were observed to be higher than where acid oxidation was employed.

3.19.3. Glutaraldehyde Activation

Acid oxidized discs of RVG 1000 were activated with glutaraldehyde as described in section 3.10. The results from oxygen

TABLE 3.3.
ACTIVITY OF GLUCOSE OXIDASE ADSORBED
ON RVG 1000 DISCS

UNTREATED

pH	Rate (nmol O ₂ min ⁻¹)	s _n	n
5.75	12.83	2.34	5
7.0	12.85	1.36	4

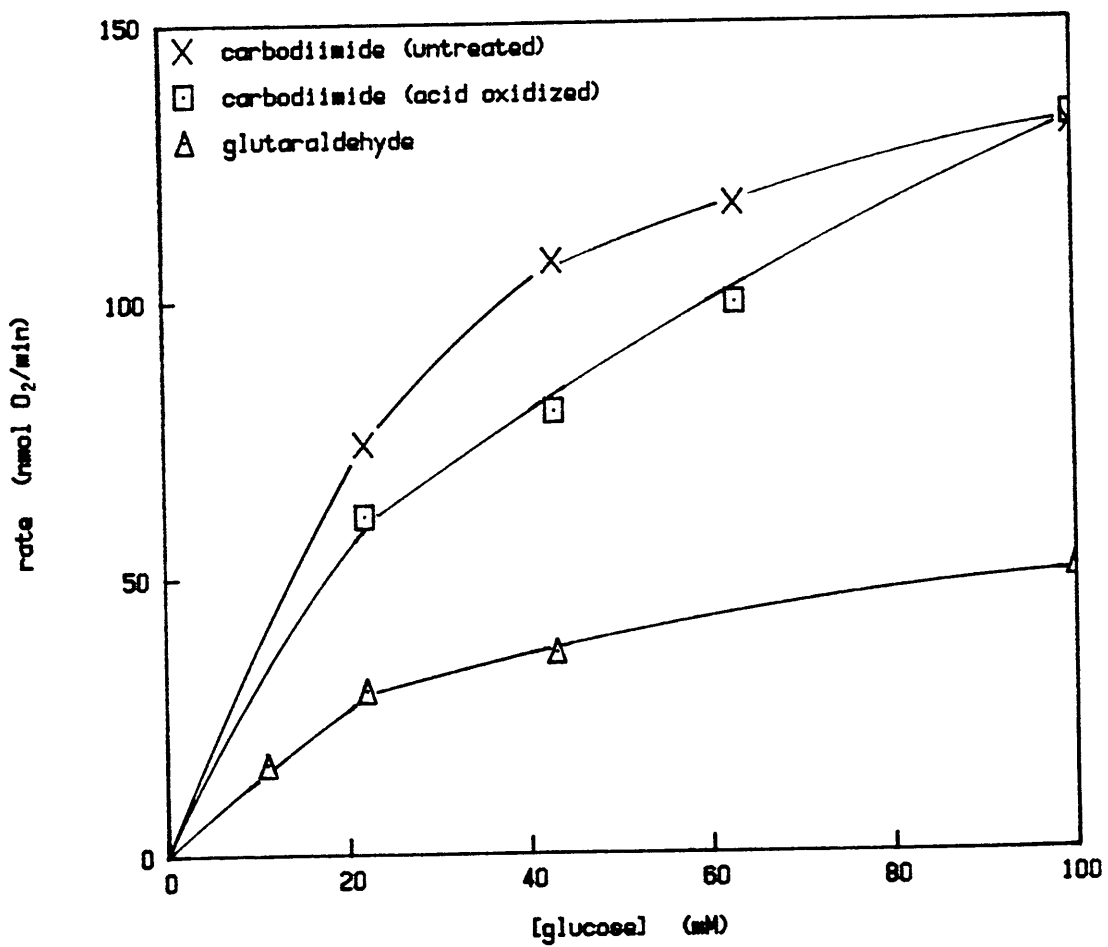
ACID OXIDIZED

pH	Rate (nmol O ₂ min ⁻¹)	s _n	n
5.75	15.43	2.46	5
7.0	15.61	4.77	5

TABLE 3.4.
ACTIVITY OF GLUCOSE OXIDASE BOUND TO CARBODIIMIDE
ACTIVATED RVG 1000

<u>UNTREATED</u>			
[GLUCOSE]	Rate	s_n	n
(mM)	(nmol O ₂ min ⁻¹)		
22	74.10	7.54	9
43	106.98	8.44	8
63	117.24	8.02	6
100	131.16	10.00	6
<u>ACID OXIDIZED</u>			
[GLUCOSE]	Rate	s_n	n
(mM)	(nmol O ₂ min ⁻¹)		
22	61.07	7.84	6
43	79.88	4.11	5
63	99.26	5.98	7
100	132.89	8.86	4

FIGURE 3.6.



Graph of oxygen consumption against glucose concentration for glucose oxidase bound onto RVG 1000 discs using different immobilization methods.

electrode assays are given in table 3.5. and shown graphically in figure 3.6. The activity of these discs was observed to be lower than those activated by carbodiimide but about twice as active as those discs where simple adsorption was used (section 3.19.1.).

**TABLE 3.5. ACTIVITY OF GLUCOSE OXIDASE BOUND TO GLUTARALDEHYDE
ACTIVATED RVG 1000**

[GLUCOSE] (mM)	Rate (nmol O ₂ min ⁻¹)	s _n	n
11	16.32	2.29	5
22	29.55	3.11	5
43	36.36	2.07	6
100	51.34	3.75	5

3.19.4. Silanization Techniques

Glucose oxidase was immobilized onto the following silanized discs :

- [1] Silane-treated
- [2] Silane-treated followed by glutaraldehyde activation

- [3] LiAlH_4 -reduced then silanized
- [4] LiAlH_4 -reduced, silane-treated then activated using glutaraldehyde

The results from oxygen electrode assays are presented in table 3.6., and rate curves are shown in figure 3.7. The most active preparations were obtained using method [2], which demonstrated more activity than method [4] where LiAlH_4 reduction was employed prior to silanization. This observation is paralleled by treatments [1] and [3] where the LiAlH_4 -reduced discs also demonstrated lower activity.

3.20. Determination of Kinetic Parameters and Bound Enzyme

The amount of protein bound to the discs was measured as described in section 2.5. The results for the discs derivatized by glutaraldehyde activation and by silanization are presented in table 3.7. The apparent kinetic parameters K'_m and V_{max} were determined using Lineweaver-Burk plots for discs derivatized by carbodiimide, glutaraldehyde and the various silanization methods, and the results are also given in table 3.7.

With the exception of the unoxidized discs activated using water-soluble carbodiimide, and set [4] of the silanized discs the values obtained for K'_m were within the narrow range of 36 - 40 mM glucose. The data presented in table 3.7. were derived from

TABLE 3.6

ACTIVITY OF GLUCOSE OXIDASE BOUND TO SILANIZED RVG 1000

[1] SILANIZED

[GLUCOSE] (mM)	Rate (nmol O ₂ min ⁻¹)	s _n	n
11	35.40	3.33	3
22	58.69	6.19	5
32	73.91	1.84	3
43	82.51	5.03	3
100	120.05	-	2

[2] SILANIZED / GLUTARALDEHYDE

[GLUCOSE] (mM)	Rate (nmol O ₂ min ⁻¹)	s _n	n
11	80.65	5.23	8
22	127.24	2.20	11
32	153.23	10.11	8
43	188.55	18.95	6
100	274.96	15.51	5

TABLE 3.6. CONTINUED

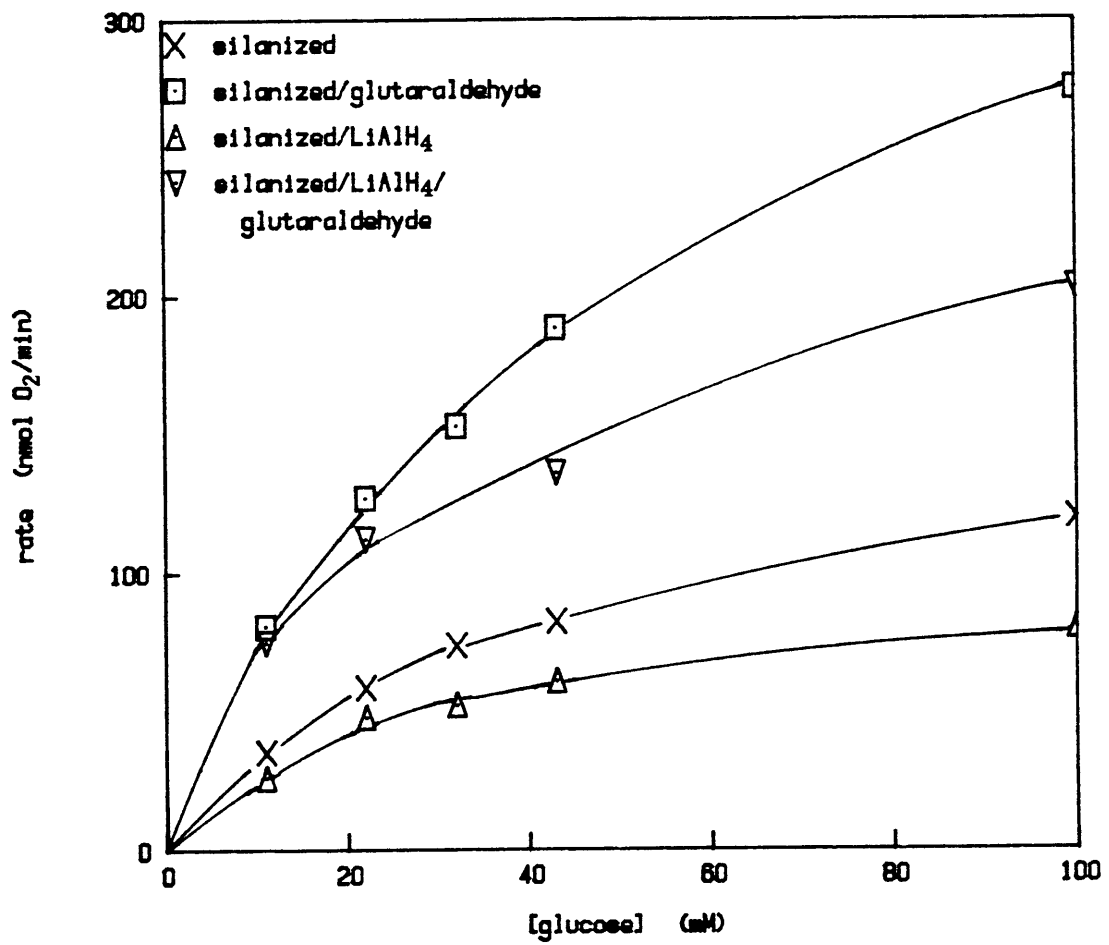
[3] LiAlH₄ / SILANIZED

[GLUCOSE] (mM)	Rate (nmol O ₂ min ⁻¹)	s _n	n
11	26.00	2.41	4
22	47.94	4.97	5
32	52.33	3.63	3
43	60.93	6.37	4
100	80.07	4.97	3

[4] LiAlH₄ / SILANIZED / GLUTARALDEHYDE

[GLUCOSE] (mM)	Rate (nmol O ₂ min ⁻¹)	s _n	n
11	74.89	8.25	7
22	112.43	9.95	10
43	136.30	12.91	7
100	203.06	19.24	7

FIGURE 3.7.



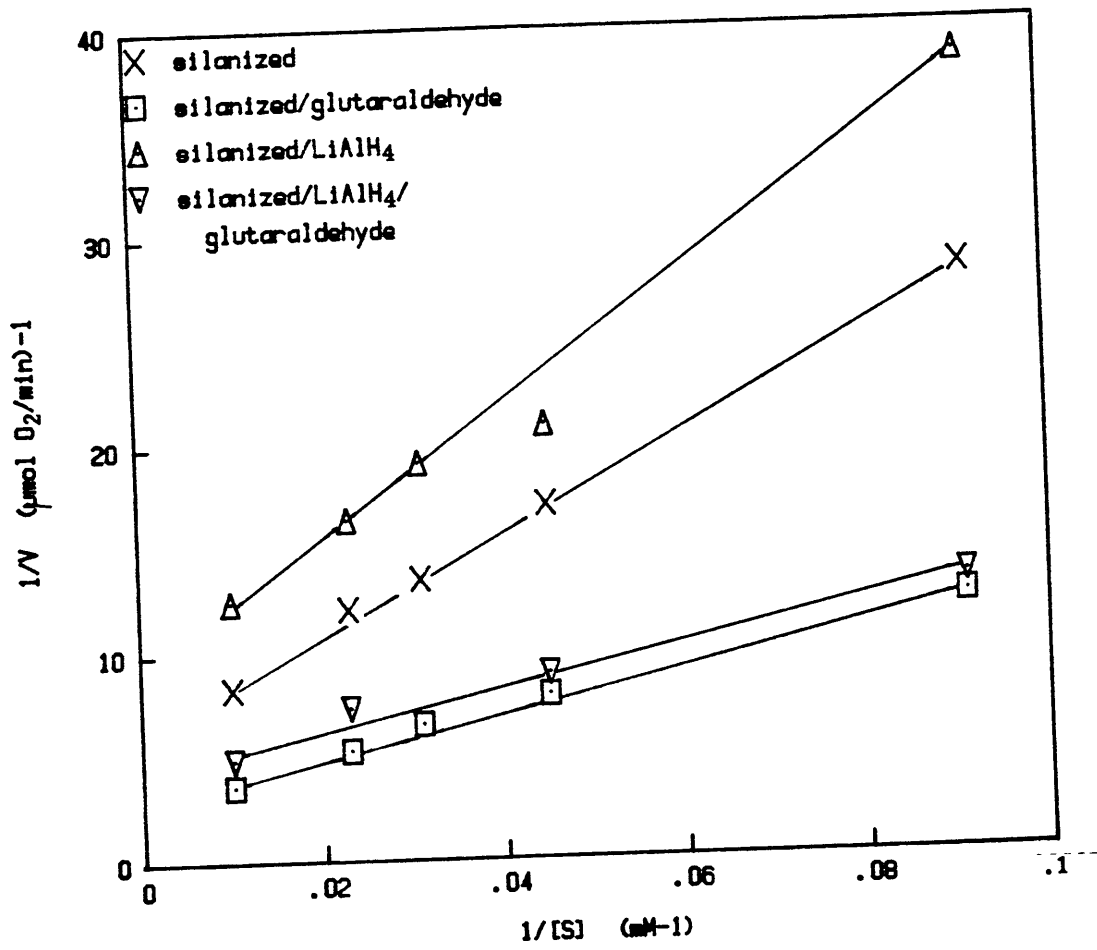
Graph of oxygen consumption against glucose concentration for glucose oxidase bound onto silanized RVG 1000 discs.

Lineweaver-Burk plots which are shown in figure 3.8. for the silanized discs. As demonstrated in chapter 4 for the glucose oxidase / graphite foil electrode (see section 4.7.) these kinetic plots tend to be linear even in the presence of diffusional limitations when the observed rate of reaction is not kinetically controlled. Kinetic plots using the Eadie-Hofstee variation for the silanized electrodes are shown in figure 3.9. There appears from these plots to be a difference between those electrodes which were treated with glutaraldehyde (silanizations [1] & [3]), and the other two sets of discs where this treatment was not employed (silanizations [2] & [4]), the former giving linear plots and the latter non-linear plots. This behaviour was not shown by discs treated with glutaraldehyde only, however, which gave linear Eadie-Hofstee plots.

It should be noted that the results obtained from the protein assays (table 3.7.) indicated that more enzyme was retained when glutaraldehyde was omitted, and that reduction using LiAlH_4 resulted in more enzyme being bound. These results were the opposite of the activity assays, which would suggest that in these cases a lower proportion of the enzyme bound was active.

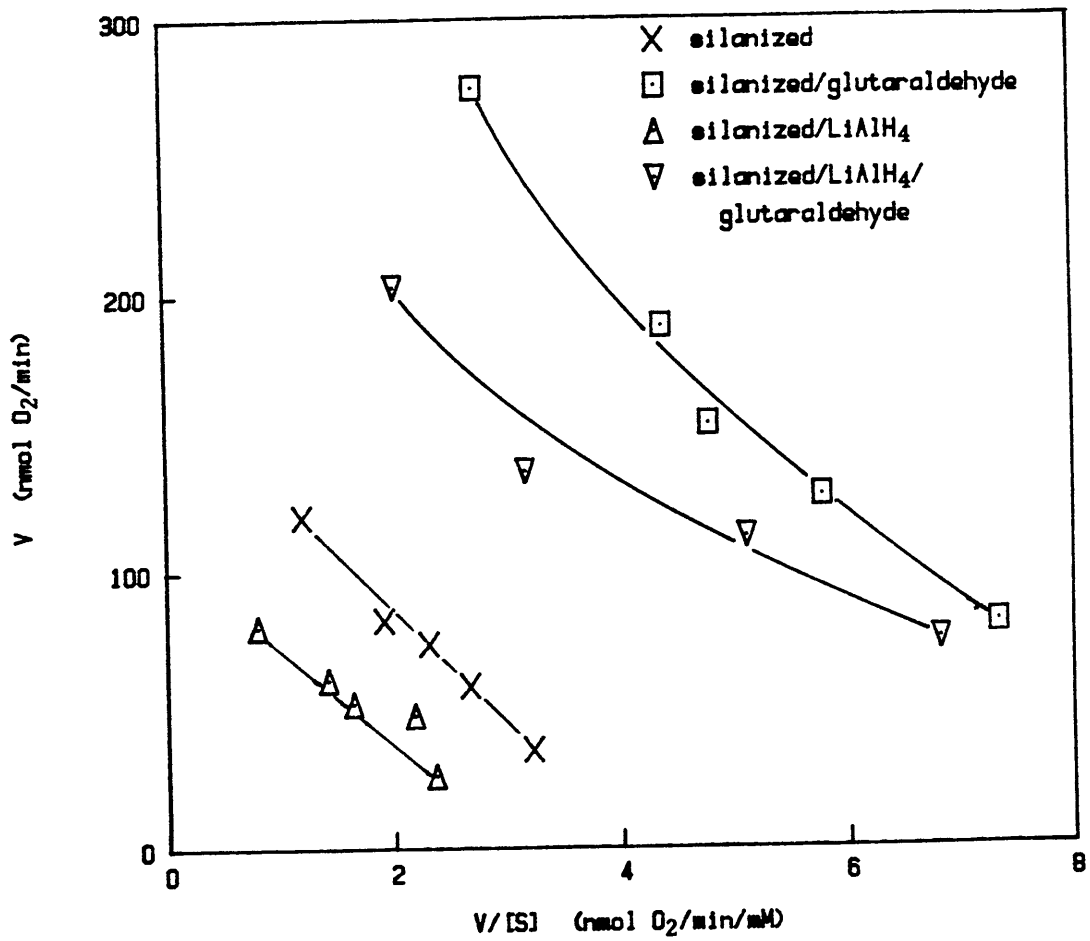
The similarity of the effective K'_m values obtained for all the silanized discs, and the fact that these values were higher than that reported for the enzyme in solution (6.8 mM given by Kamin & Wilson, 1980), indicated that diffusion of the substrates glucose and oxygen to the immobilized enzyme was partially rate-limiting for all the discs.

FIGURE 3.8.



Lineweaver-Burk plots of the data from figure 3.7. for glucose oxidase bound onto silanized RVG 1000 discs.

FIGURE 3.9.



Eadie-Hofstee plots of the data from figure 3.7. for glucose oxidase bound onto silanized RVG 1000 discs.

TABLE 3.7.
KINETIC PARAMETERS FOR GLUCOSE OXIDASE IMMOBILIZED
ON RVG 1000 DISCS

IMMOBILIZATION METHOD	PROTEIN BOUND (mg/disc)	$V_{\max}$ ($\mu\text{mole O}_2/\text{min}$)	K'_m (mM)
CARBODIIMIDE/UNOXIDIZED	-	0.172	28.2
CARBODIIMIDE/OXIDIZED	-	0.165	38.6
GLUTARALDEHYDE	0.66	0.071	36.2
SILANIZED	0.80	0.165	40.3
SILANIZED/GLUTARALDEHYDE	0.64	0.350	37.2
LiAlH_4 /SILANIZED	0.96	0.114	36.4
LiAlH_4 /SILANIZED/ GLUTARALDEHYDE	0.90	0.226	22.4

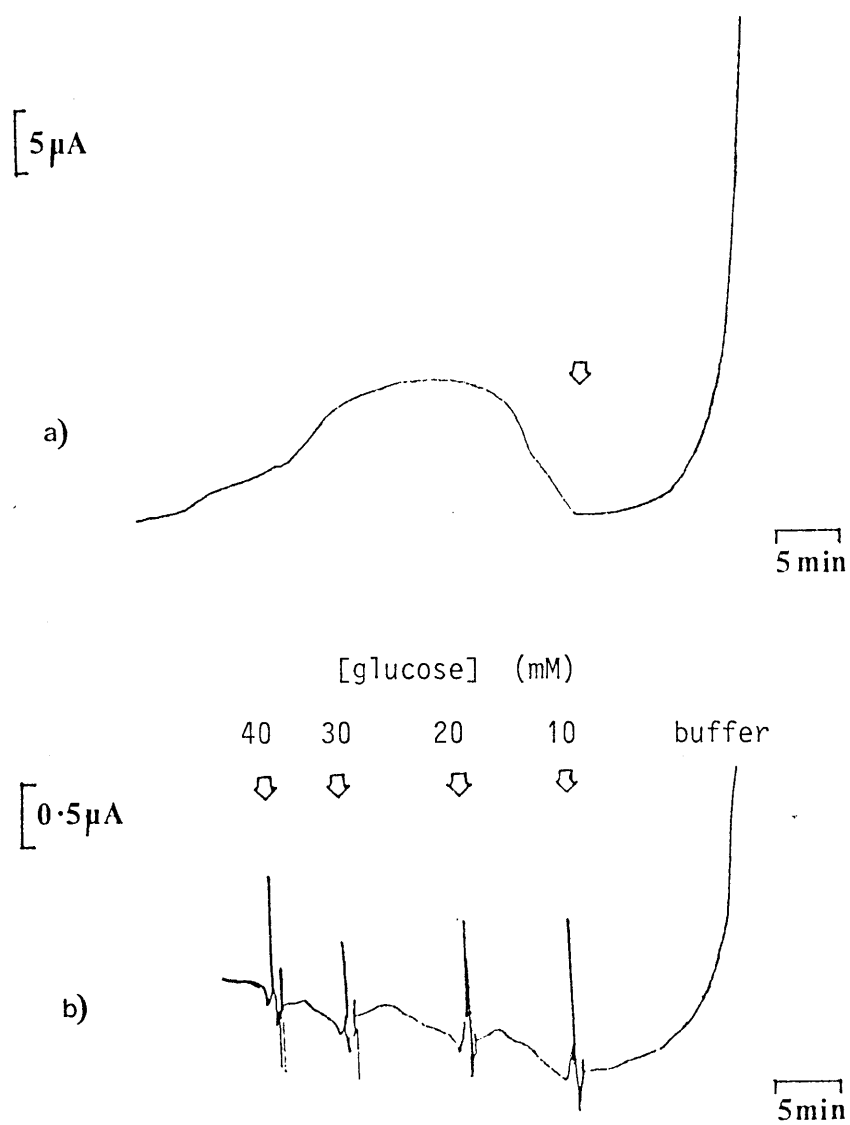
3.21. Assay of Immobilized Glucose Oxidase using an Electrochemical Flow Cell

The measurement of bound enzyme activity using the electrochemical flow cell shown in figure 3.4. was achieved with all the different electrode materials listed in section 3.6. The pump employed in these experiments did not pulse appreciably and a wide range of flow rates were obtainable. An operating flow rate of 1 ml/min was found to be satisfactory, which was increased to 10 ml/min during washing of the flow cell with buffer between assays.

Initially injection of glucose into the system was accomplished by means of a 3-way valve. Additions of 0.5 mmoles of glucose were made (0.5 ml of a 1M solution). The working electrode was held at +770 mV (against the Ag / AgCl reference) to measure the production of hydrogen peroxide by the enzyme-catalysed reaction. The typical type of response obtained from these experiments is shown in figure 3.10a. A maximum current of 18 μ A was obtained from a section of modified R60S, and 40 μ A from a disc of RVG 100. Although successive experiments were not performed for the RVG 1000 disc, the maximum current decreased to 11 μ A after three assays with the section of R60S. This apparent loss in activity was not characterized, but may have been due to loss of enzyme from the electrode (especially during the washing procedure) and / or due to poisoning of the enzyme by hydrogen peroxide.

In a second set of experiments a change of syringe was

FIGURE 3.10.



Response behaviour of the electrochemical flow system achieved by a) injection of 0.5 ml of a 1 M solution of glucose (arrow) and b) changing the syringe in the pump (arrows).

employed to expose the electrode to glucose solution (i.e. from one containing buffer only to one containing buffered glucose solution). In these experiments the current did not stabilize, but decreased after reaching a maximum value. However, successive changes of syringe containing increasing concentrations of glucose could be made which resulted in increases in the measured current (figure 3.10b.). Since the absence of a steady-state current could have been due to poisoning by hydrogen peroxide, the use of TMPD as an alternative oxidant was examined. In these experiments deoxygenated buffers containing 100 μ M TMPD were used, and the potential of the working electrode was maintained at +200 mV against the Ag/AgCl reference. Maximum currents of approximately 25 μ A were obtained at 100 mM glucose with RVG 1000 discs activated using both carbodiimide and thionyl chloride. Again steady-state currents were not obtained, and decreased currents were observed on repetitive assays. A further problem in these experiments was auto-oxidation of the mediator, even when freshly prepared solutions were employed. This auto-oxidation of the mediator, due to oxygen entering the system presumably through the silicone tubing, could not be overcome by degassing with nitrogen during the experiments because of the structure of the flow cell. Although higher concentrations of TMPD, up to 10 mM, were also investigated no discernable increase in stability of the enzyme-modified electrodes was noted.

Experiments using the electrochemical flow cell demonstrated that glucose oxidase could be successfully immobilized onto all

the carbon materials used. However, direct comparisons between different electrode supports and different immobilization methods were difficult to make because of the problems outlined above. Furthermore, silanization of the support materials, which was found to be the best immobilization method with the RVG 1000 discs assayed in the oxygen electrode, could not be used because of the insulating effect which resulted from this treatment. This effect was discovered when both silanized RVG 1000 and RVC 1000 were examined using cyclic voltammetry in potassium ferricyanide solution. With these silanized felts no anodic or cathodic waves were observed, indicating that silanization had insulated both materials. Similar results were obtained with silanized graphite foil (see section 3.23.).

3.22. Immobilization of Lipoamide Dehydrogenase on RVG 1000

Following on from the work with glucose oxidase a series of experiments were performed whereby lipoamide dehydrogenase from Torula yeast was immobilized onto modified RVG 1000 discs and assayed in the spectrophotometric flow system described in section 3.13.3. using lipoic acid as the substrate.

The two immobilization methods used for this work were the carbodiimide and the glutaraldehyde activation methods, and the immobilization conditions employed are listed in table 3.8. Lineweaver-Burk plots of the data from initial rate against lipoic acid concentration were used to determine the apparent kinetic

TABLE 3.8.
KINETIC PARAMETERS AND IMMOBILIZATION CONDITIONS FOR YEAST
LIPOAMIDE DEHYDROGENASE BOUND ONTO RVG 1000 DISCS

EXPERIMENT NO.	(a)		(b)	
	ACTIVATION	ENZYME CONC. (mg/ml)	$V_{\max}$ ($\mu\text{mol/min}$)	K'_m (mM)
[1]	C	0.1	0.052	2.55
[2]	C	1.0	0.040	1.84
[3]	C	5.0	0.033	1.50
[4]	G	0.1	0.030	1.90
[5]	G	1.0	0.059	1.71
[6]	G	5.0	0.065	1.37
SOLUBLE ENZYME :				
FLOW SYSTEM			9.98 (c)	4.11
CUVETTE			24.60 (c)	2.75

(a) C = Carbodiimide G = Glutaraldehyde

(b) Lipoic acid as substrate ; [NADH] = 0.2 mM

(c) $\mu\text{mol min}^{-1} \text{ mg}^{-1}$ enzyme

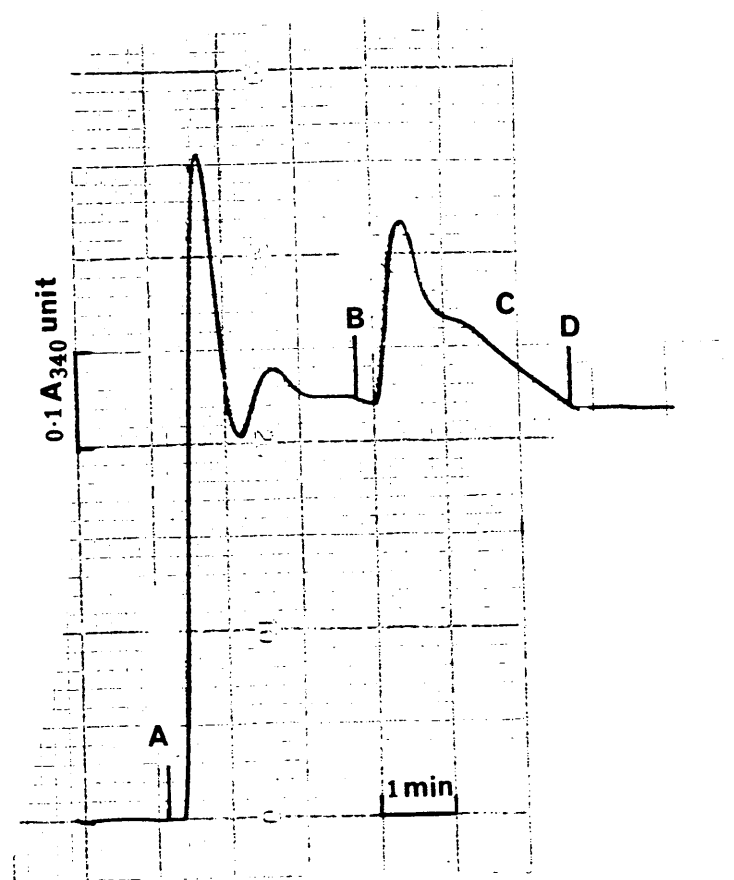
parameters, K'_m and V_{max} , of the immobilized enzyme. These values are also given in table 3.8., along with the data for the soluble enzyme determined both in the flow cell and in cuvette assays for comparison. It was noticeable that the specific activity of the enzyme determined in the flow system was about half that determined using a standard cuvette assay. This result was not surprising since the equilibration time necessary for complete mixing of the solution in the flow system (about one minute) made measurement of the maximum initial rate impossible. This can be seen in figure 3.11. which shows a typical recording of the adsorption at 340 nm against time during one of these experiments. Thus the rates measured with the immobilized enzyme were not an accurate measurement of the activity of the bound enzyme.

The results obtained with the immobilized enzyme, however, showed little variation between the immobilization methods, with experiment (6) being the most active. This conformity of the derived values for V_{max} suggested that the rates observed were not due to the bound enzyme but were due to mass transport limitations. The theoretical maximum rate of substrate conversion can be roughly calculated using Fick's law of diffusion, where the flux of mass transport (j_s) is given by the concentration gradient (ds/dx) multiplied by the diffusion coefficient (D_s) :

$$j_s = ds/dx \times D_s$$

In this case the limiting substrate concentration is that of

FIGURE 3.11.



Typical response of the spectrophotometric flow system showing the variation in A_{340} with time following :
 (a) addition of 0.1 ml of 20 mM NADH solution
 (b) addition of 0.1 ml of a 0.2 M solution of lipoic acid in ethanol. The rate measured by the immobilized enzyme is indicated at point (c). At (d) the pump was stopped, showing that the rate measured at (c) was not due to free enzyme.

NADH (0.2 mM), the diffusion coefficient being assumed to be about $6 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ and the diffusion path length 0.1 cm (the thickness of the graphite felt disc). This calculation gives a value for the flux of NADH of $1.2 \times 10^{-11} \text{ mole cm}^{-2} \text{ s}^{-1}$, or $7.2 \times 10^{-4} \text{ } \mu\text{mole cm}^{-2} \text{ min}^{-1}$. Taking the real area of these graphite felt discs as approximately 300 times the apparent geometric area (1.13 cm^2) gives a flux of $0.244 \text{ } \mu\text{mole min}^{-1}$ to the surface of the disc.

Since the flux of NADH reaching the whole graphite felt disc can be assumed to have been larger than this approximate value, the low rates observed with these modified discs would appear to have been due to only a small amount of enzyme activity being retained on the disc rather than because of mass transport limitations.

3.23. Immobilization of Glucose Oxidase on Graphite Foil

Electrodes

Some of the best methods used to immobilize glucose oxidase and lipoamide dehydrogenase onto graphite felt were employed in the study of the immobilization of glucose oxidase onto graphite foil electrodes doped with dimethylferrocene. The techniques used were the following :

- (1) Aqueous silanization
- (2) Carbodiimide activation
- (3) Carbodiimide activation followed by glutaraldehyde crosslinking

(4) Adsorption

(5) Adsorption followed by glutaraldehyde crosslinking

With the exception of the electrodes modified by silanization all the electrodes used in these experiments were assayed for activity using the microcomputer based system described in section 2.3. Preconditioning of these electrodes was employed (see section 4.5.). The silanized electrodes were assayed using a potentiostat in a three electrode system.

Aqueous silanization was used because of the adverse effect that refluxing in toluene had on the araldite used to retain the electrode material onto its glass tube support. These electrodes appeared to be severely diffusion inhibited, the response taking 15 to 20 minutes to reach a steady-state value. For this reason calibration curves were not obtained with these electrodes. As noted in section 3.21. silanization of graphite foil appeared to insulate this material, which these results confirmed, although it would appear that this insulating effect was not 100 % effective. Nevertheless silanization was not found to be a suitable immobilization method for use with the graphite foil enzyme electrode.

The conditions employed for the remaining modified electrodes are given in table 3.9. The steady-state currents obtained for each set of electrodes were averaged, and the data replotted by the Hanes method (figure 3.12.). The data at high glucose concentrations was used to obtain values for the apparent kinetic parameters K'_m and i_{max} (see section 4.7.), and these results are given in table 3.10.

TABLE 3.9.
CONDITIONS EMPLOYED FOR THE IMMOBILIZATION OF GLUCOSE OXIDASE
ON GRAPHITE FOIL ELECTRODES

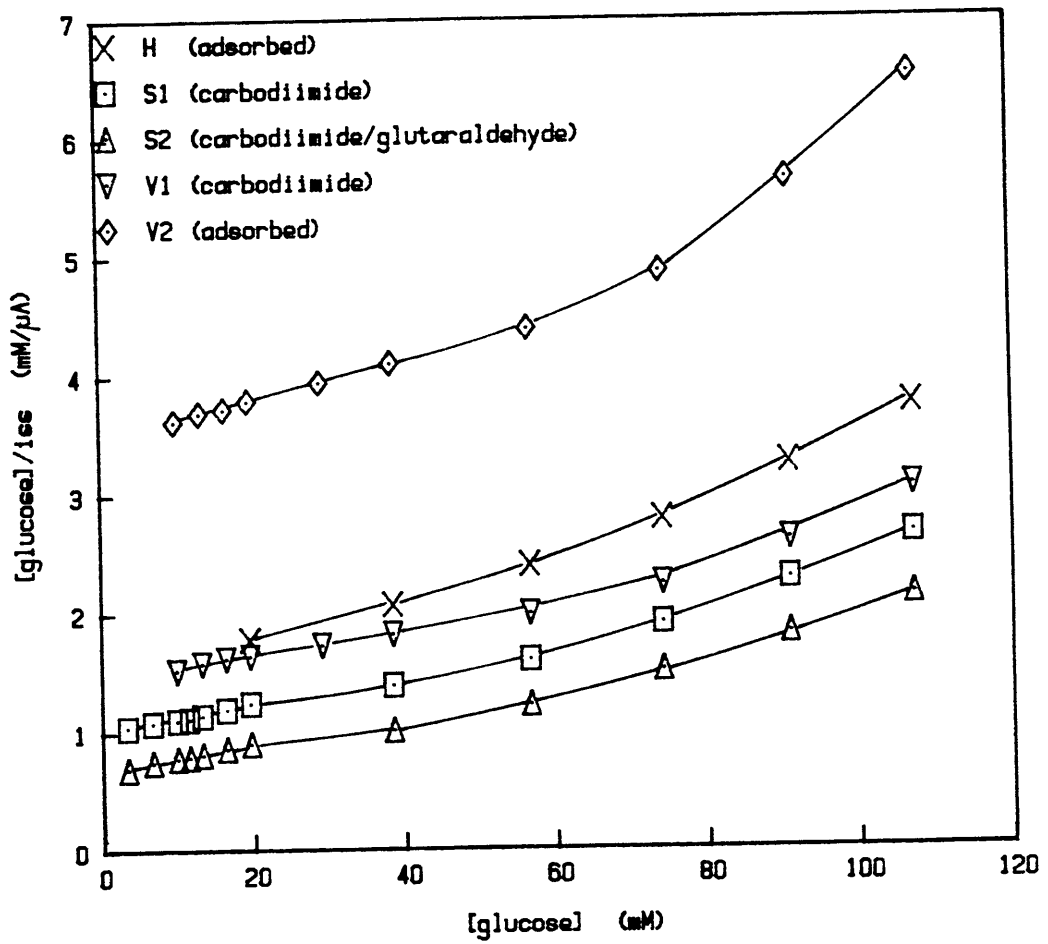
SET	IMMOBILIZATION	LOADING ^{a)} (mg ml ⁻¹)	ELECTRODE SIZE
H	ADSORBED	10	LARGE
V1	GLUTARALDEHYE	10	SMALL
V2	ADSORBED	10	SMALL
S1	CARBODIIMIDE	1	LARGE
S2	CARBODIIMIDE / GLUTARALDEHYDE	1	LARGE

a) Concentration of enzyme in the immobilization step

TABLE 3.10.
KINETIC PARAMETERS FOR GLUCOSE OXIDASE / GRAPHITE FOIL ELECTRODES

SET	K'_m (mM)	i_{max} (μA)	n
H	13.75	34.36	6
V1	20.16	40.32	4
V2	22.37	19.88	3
S1	9.47	44.05	5
S2	4.71	52.36	4

FIGURE 3.12.



Hanes plots for glucose oxidase immobilized on graphite foil electrodes by different methods.

In the case of the V set of electrodes the value of $i_{\max}$ obtained with adsorbed enzyme (V2) was approximately half that derived for the glutaraldehyde treated electrodes (V1). Similarly the value of $i_{\max}$ of set H (adsorbed) was about half that obtained for large diameter electrodes treated with glutaraldehyde (set R table 4.5.).

The results obtained with set S showed that electrodes which were exposed to glutaraldehyde (S2) demonstrated a higher activity than those treated with carbodiimide alone (S1). The values for $i_{\max}$ in both cases were lower than electrodes which had been treated with glutaraldehyde alone (set R, table 4.5.). Given the variation in activity within each set of electrodes (i.e. between electrodes treated in identical fashion) there was necessarily a large degree of overlap between electrodes treated by different methods.

The values obtained for K'_m given in table 3.10. vary between 4.71- 22.37 mM, which is a lower degree of variation than reported in the literature for glucose oxidase based amperometric sensors. Kamin & Wilson (1980) reported K'_m values of 3.1 - 19.1 mM, Boudillon et al. (1980) obtained values of 12-58 mM and Castner & Wingard (1984) reported K'_m values in the range 5-42 mM. The electrode materials used in these studies were either solid graphite electrodes or platinum electrodes. Thus the value of 4.7 mM obtained with the enzyme electrodes treated with carbodiimide / glutaraldehyde, although lower than the K_m for the enzyme in free solution, is not without precedent. It does, however, indicate

the difficulty of applying the term "Michaelis constant" to data derived from rates of immobilized enzyme activity rather than homogeneous systems.

CHAPTER 4

KINETICS OF A GLUCOSE ENZYME ELECTRODE

INTRODUCTION

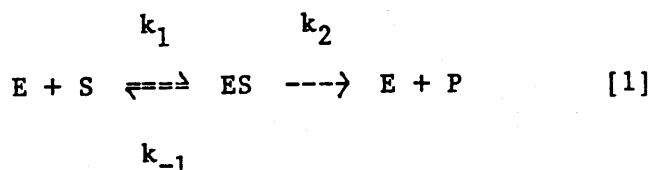
4.1. Immobilized Enzyme Kinetics in Relation to Enzyme Electrodes

The definition of an enzyme electrode (section 1.5.) is a layer of immobilized enzyme(s) combined with an electrochemical sensor. In the case of amperometric enzyme electrodes, where consumption of one of the species involved in the reaction takes place, any analysis of the kinetic behaviour of the immobilized enzyme has to accommodate the rate of this electrochemical reaction, as well as other possible effects of the immobilization (as outlined in section 1.3.).

The kinetics of amperometric enzyme electrodes based on glucose oxidase (GOD) have been studied in stirred solutions (Mell & Maloy, 1975), using a rotating disc electrode (Bourdillon et al., 1979), with a rotating ring-disc system (Shu & Wilson, 1976 ; Kamin & Wilson, 1980 ; Ianniello & Yacynych, 1981 ; Castner & Wingard, 1984) and employing a wall-jet rotating ring-disc enzyme electrode (Albery et al., 1985).

In the majority of these studies the rate of the enzyme reaction has been monitored via the electrochemical oxidation of hydrogen peroxide, one of the products of the enzyme reaction.

The enzyme reaction has been taken to follow Michaelis-Menten kinetics with glucose as sole substrate, the concentration of oxygen being assumed to be constant :



$$\text{and} \quad K_m = \frac{k_{-1} + k_2}{k_1}$$

Where E is glucose oxidase, S is glucose, P is gluconolactone and k a relevant rate constant. In general, studies of enzyme electrodes have been conducted under steady-state conditions, i.e. the system is in equilibrium with the various kinetic processes taking place (enzyme catalysis, substrate & product diffusion, electrochemical reactions) at the same rate. In order to satisfy steady-state conditions the concentration of substrate in the bulk solution is assumed to be invariable over the time course of the current measurement. Under these conditions the steady-state current (i_{ss}) is an exact measurement of the rate of the enzyme reaction.

Under conditions of catalysis (kinetic) control the variation of i_{ss} with substrate concentration can be described by a form of the Michaelis-Menten equation (Mell & Maloy, 1975 ; Shu & Wilson, 1976).

$$i_{ss} = \frac{i_{max} [S]}{K'_m + [S]} \quad [2]$$

where K'_m is the Michaelis constant for the immobilized enzyme and i_{max} is the maximum steady-state current, defined as :

$$i_{max} = n F A V'_{max} \quad [3]$$

Here n is the number of electrons involved in the electrochemical reaction, F is Faraday's constant, A is the electrode area and V'_{max} is the maximum rate of the enzyme reaction per unit volume of immobilized catalyst.

Variations of equation [2] have been used in the Lineweaver-Burk form (Kamin & Wilson, 1980) and the Eadie-Hofstee form (Kamin & Wilson, 1980 ; Castner & Wingard, 1984). Enzyme electrodes where the steady-state current is controlled by both the kinetic reaction and substrate diffusion have been described. Shu & Wilson (1976), using GOD immobilized onto carbon paste electrodes, found that the steady-state current was under catalysis control at a rotation speed of 900 r.p.m. In a further study Kamin & Wilson (1980) demonstrated catalysis controlled currents at a rotation speed of 1600 r.p.m., whilst at low rotation speeds (100 & 400 r.p.m.) non-linear Lineweaver-Burk and Eadie-Hofstee plots were attributed to external diffusional limitations.

Internal diffusional resistances were reported by Mell &

Maloy (1975) with GOD immobilized inside a polyacrylamide membrane gel. Castner & Wingard (1984) obtained both linear and non-linear Eadie-Hofstee plots for GOD immobilized by different methods onto platinum mesh electrodes at a rotation speed of 600 r.p.m.

To describe the relative effects of increasing diffusional limitations on the steady-state current, Mell & Maloy (1975) introduced a parameter U :

$$U = \frac{d^2 k_3 C_E}{D_s K_m} \quad [4]$$

where C_E is the concentration of active enzyme (or more accurately active sites), d is the characteristic length of the diffusion barrier and D_s is the substrate diffusion coefficient. This parameter, U , is similar to the internal substrate modulus ϕ defined in section 1.3. With $d k_3 C_E = V_{\max}$ then $\phi = U^{1/2}$. Using membrane-entrapped enzyme values of $U = 25$ for a thin membrane and $U = 50$ for a double-sized membrane were obtained.

Although the electrochemical reaction is generally not rate-limiting, Albery et al. (1985) have reported an enzyme electrode where the steady-state current is kinetically controlled at high glucose concentrations, but controlled by the electrochemical step (in this case the re-oxidation of the enzyme at an organic metal electrode) at low glucose concentrations.

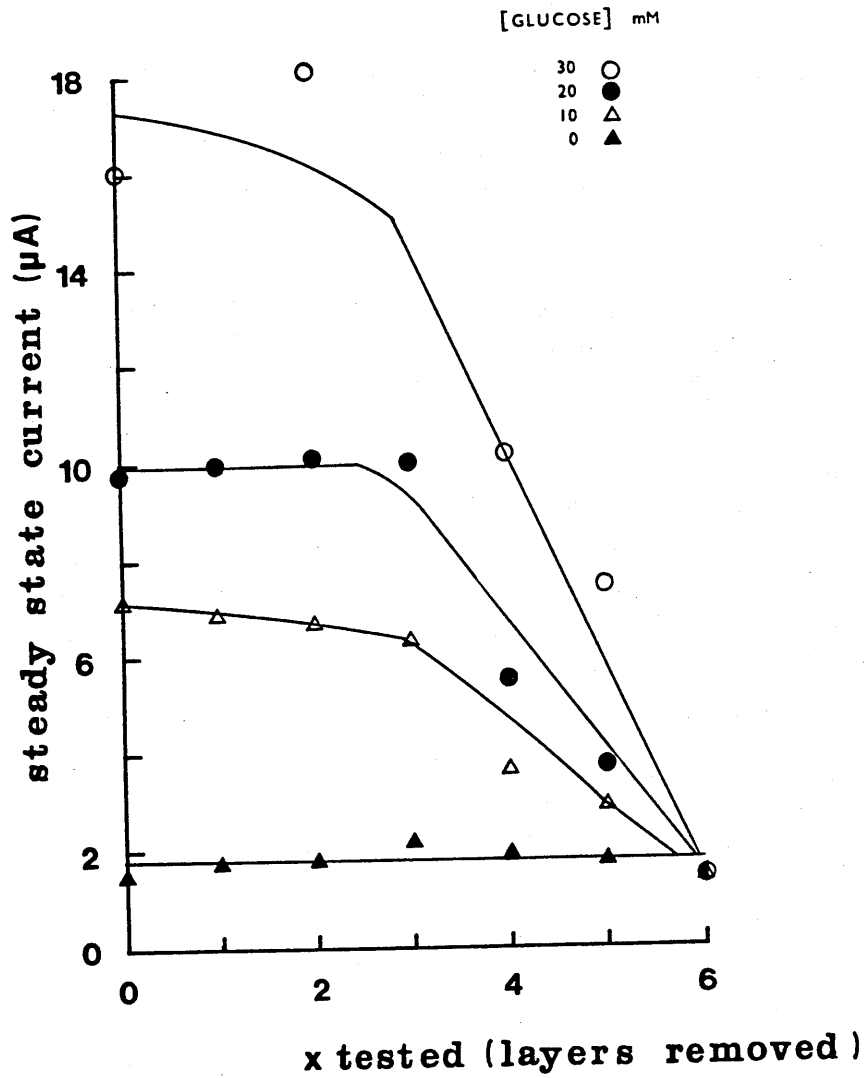
4.2. The Glucose Oxidase-Dimethylferrocene Enzyme Electrode

Ferrocene (dicyclopentadienyl iron) and its derivatives such as 1,1'-dimethylferrocene, decamethylferrocene, ferrocene monocarboxylic acid and ferrocene dicarboxylic acid are good oxidants for GOD (Cass et al., 1984), and the development of a glucose enzyme electrode based on dimethylferrocene (DMFc) adsorbed onto graphite foil electrodes was also reported in this paper. Initial studies on the kinetic behaviour of this enzyme electrode showed that the enzyme was not confined to the surface of the electrode, but in fact both the mediator and the enzyme were retained within the relatively porous electrode material (Schneider et al., 1984). This is shown in figure 4.1. where successive layers of graphite were removed from the surface of the electrode. This work suggested that the enzyme electrode was a more complicated system than initially thought, and that internal diffusional limitations could be significant in explaining its extensive linear range and non-linear kinetic plots.

4.3. Outline of Model

The salient features of the model proposed for the graphite foil based GOD / DMFc enzyme electrode are shown in figure 4.2. It should be noted that two molecules of DMFc are reduced by the enzyme for each molecule of glucose oxidized. The enzyme electrode can be considered as an open system, where substrate and product

FIGURE 4.1.



Steady-state currents at different glucose concentrations after progressive removal of surface layers from a graphite foil enzyme electrode.

can be freely exchanged with the surrounding fluid (Engasser & Horvath, 1973).

The reaction steps involved in this system are :

- (a) Mass transport of glucose from the bulk solution (electrode surface) to the enzyme's microenvironment inside the electrode.
- (b) Enzymatic oxidation of glucose
- (c) Re-oxidation of the enzyme by dimethylferricinium ions.
- (d) Re-oxidation of the dimethylferricinium ion at the electrode.
- (e) Transport of the mediator within the electrode

The concentration of glucose at the surface of the electrode can taken to be the same as that of the bulk solution under efficient mixing conditions. Thus the flux of glucose from the surface of the electrode to the vicinity of the enzyme is :

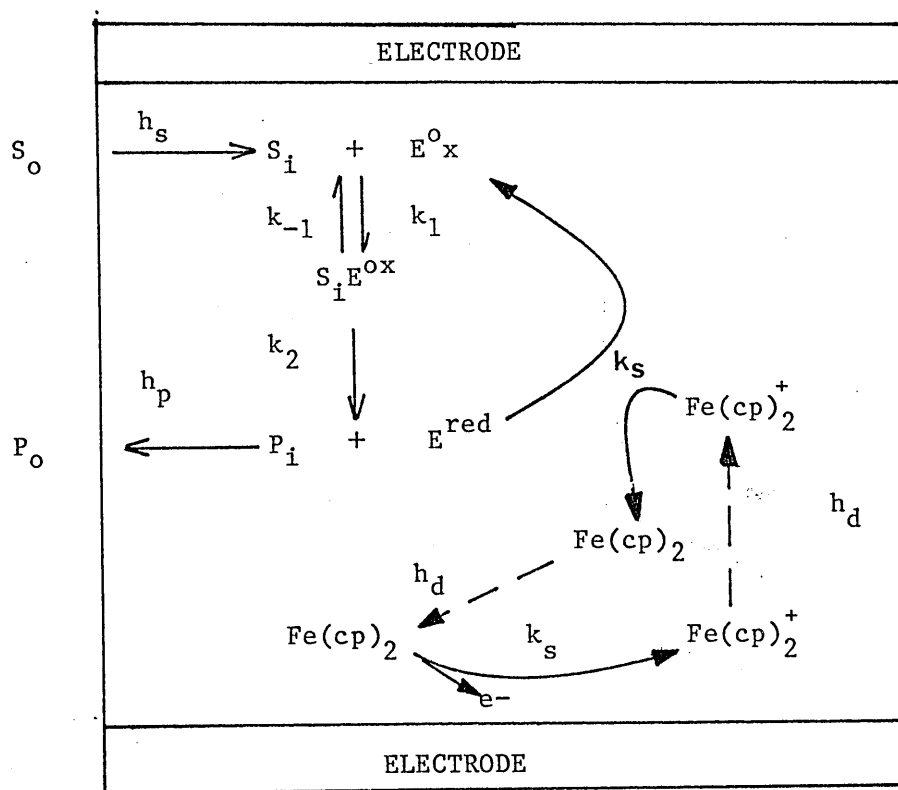
$$j_s = - D_{\text{eff}} \frac{d [S]_0}{d x} \quad [5]$$

where j_s is the flux of glucose through a unit area of the electrode and D_{eff} is the effective diffusivity of glucose inside the electrode. Assuming the concentration of dimethylferrocene remains constant during the reaction, and remains unchanged when the glucose concentration is varied, the reaction may be considered in terms of glucose as the only substrate and gluconolactone as the only product of the enzymic reaction.

Under steady-state conditions, and in the absence of diffusional limitations, the flux of substrate into the electrode, the

FIGURE 4.2.

MODEL FOR THE ENZYME ELECTRODE



S_o, S_i, P_o, P_i : concentration of substrate (glucose) and product (gluconolactone) inside & outside electrode respectively

h_s, h_p : mass transport coefficients for substrate and product

k_1, k_{-1}, k_2 : rate constants for the enzymatic reaction

E : enzyme (glucose oxidase) $Fe(cp)_2$: dimethylferrocene

k_s : second order rate constant for the oxidation of reduced enzyme by ferricinium

k_e : rate constant for the oxidation of the ferrocene at the electrode

h_d : mass transport coefficient for mediator within the electrode

rate of formation of the ES complex (equation [1]), the rate of product formation and the flux of product removal from the electrode are all equal. The material balance for glucose is :

$$j_s = k_1 [S] [E] + k_{-1} [ES] \quad [6]$$

For the ES complex :

$$k_1 [S] [E] = k_{-1} [ES] + k_2 [ES] \quad [7]$$

Conservation of the total amount of enzyme , $[E]_t$, requires that :

$$[E]_t = [E] + [ES] \quad [8]$$

Combination of equations [6], [7] and [8] leads to the following expression for the net flux of substrate (glucose) into the electrode :

$$j_s = \frac{k_2 [E]_t [S]}{([k_2 + k_{-1}] / k_1) + [S]} \quad [9]$$

The right-hand side of equation [9], which gives the net consumption of glucose in the system, is that of the Michaelis-Menten equation. Thus in an open system the intrinsic kinetics of the immobilized enzyme can be rigorously expressed by the

Michaelis-Menten equation (Engasser & Horvath, 1973).

Transport of glucose through the electrode material will occur by simple diffusion according to Fick's Law :

$$j_s = - D_{\text{eff}} \Delta S \quad [10]$$

where ΔS is the concentration gradient of glucose between the surface of the electrode and the interior.

The rate of reoxidation of the reduced enzyme by DMFc has been reported as $0.77 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ (Cass et al., 1984) and is therefore unlikely to be rate limiting. The ratio of oxidized to reduced DMFc present within the electrode material will depend on the potential applied according to the Nernst equation :

$$E = E^{\circ} - \ln \left[\frac{\text{Fe}(\text{Cp})_2^+}{\text{Fe}(\text{Cp})_2} \right] \frac{nF}{RT} \quad [11]$$

where E is the applied potential, $\text{Fe}(\text{Cp})_2$ is DMFc, and E° is the standard redox potential.

Reoxidation of reduced DMFc involves both the transport of the mediator from the enzyme to the active electrode surface and the subsequent electrochemical step. It is assumed for the purpose of generating a provisional model for the enzyme electrode that the rate of mass transfer of the mediator within the electrode is of the same order of magnitude as for glucose. Since the effective concentration of DMFc within the electrode can be assumed to be

saturating (see section 4.9.) then mass transfer of the mediator does not become a rate-limiting step. The oxidation of the mediator can be assumed to take place at a rate normally associated with electrochemical processes, and that this rate will be significantly faster than both the kinetic rate and the rate of passive diffusion of glucose within the electrode material.

Taking into account the assumptions made above the processes which may be rate limiting in the glucose biosensor can therefore be reduced to (a) diffusion of glucose within the electrode, and (b) the enzymatic oxidation of glucose. The slower of these two rates will control the rate at which glucose is oxidized, DMFc is reduced and hence the steady-state current measured.

MATERIALS AND METHODS

4.4. Immobilization of Glucose Oxidase and Dimethylferrocene onto Graphite Foil Electrodes

The surface of the electrodes (constructed as described in section 2.2.) were polished using soft tissue paper to remove any loose carbon. The electrodes were secured in a vertical position and 10 μ l of a solution of DMFc in toluene (20 mg / ml) placed on the surface of each electrode. The electrodes were then left for 45-60 minutes to allow the toluene to evaporate off.

Glutaraldehyde activation was carried out by placing the electrodes in a 2.5 % solution at pH 7 at room temperature for 90

minutes. Excess glutaraldehyde was removed by a brief washing with distilled water. The electrodes were then placed immediately into a solution of GOD. For those experiments where a high enzyme concentration was used (10 - 100 mg / ml) the electrodes were allowed to stand in the enzyme solution for two hours at room temperature. When a low enzyme concentration was employed (0.1 - 1 mg / ml) the immobilization was carried out overnight at 4°C. After removal of the electrodes from the enzyme solution weakly adsorbed enzyme was removed using the washing procedure described in section 3.12.

4.5. Measurement of Steady-State Currents

The variation of steady-state currents with glucose concentration and temperature were performed using the experimental apparatus described in section 2.3.

For both preconditioning and calibration experiments the following method was used. Typically 2 - 4 enzyme electrodes and the silver / silver chloride (Ag / AgCl) reference / auxillary electrode were placed in 15 ml of 0.1 M phosphate buffer pH 7.0 containing 0.15 M KCl in a jacketed cell maintained at the desired temperature, the buffer being stirred vigorously with a magnetic stirring bar. The solution was deoxygenated using white spot nitrogen for at least 10 minutes prior to applying a potential, and subsequently throughout the experiment. The charging current was allowed to decay and stabilize after which point addition(s)

of glucose were made. For the calibration experiments, which lasted for 60 - 90 minutes, additions of glucose were made usually over the range 3 - 107 mM glucose.

Temperature profiles were carried out by increasing the thermostat control on the water bath after a steady-state current had been reached at the previous temperature setting. The temperature inside the reaction vessel was continuously monitored using a thermometer. Measurement of steady-state currents at 1°C were achieved by partial filling of the water bath with ice and setting the thermostat control to 0°C.

4.6. Electrochemical Experiments

Cyclic voltammetry, linear sweep voltammetry, differential pulse voltammetry and bulk electrolysis were performed using the BAS Electrochemical Analyzer as described in section 2.4. Bulk electrolysis was used to measure in finer detail the current and charge passed for the enzyme electrode as occurred during the amperometric experiments.

RESULTS AND DISCUSSION

4.7. Determination of Apparent Kinetic Parameters K'_m and i_{max}

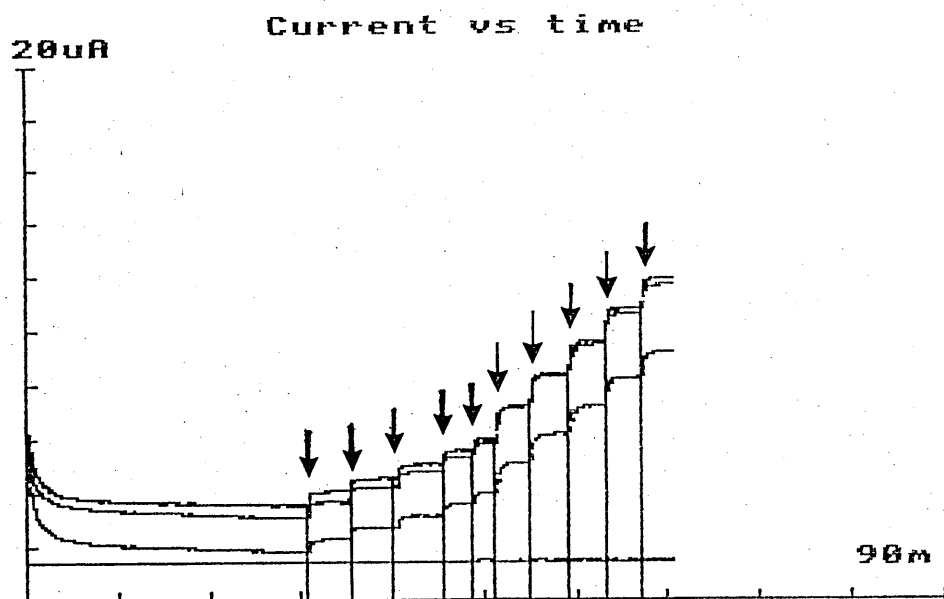
The results of a typical calibration experiment, as described in section 4.5., are shown in figure 4.3. When the background currents are subtracted a calibration curve of steady-state current, i_{ss} , against glucose concentration, $[S]$, can be plotted, as shown in figure 4.4.

The data from figure 4.4. was then replotted using the classical enzyme kinetic plots as shown in figures 4.5., 4.6. and 4.7. Figure 4.5. shows a Lineweaver-Burk type plot of $1/i_{ss}$ against $1/[S]$, figure 4.6 an Eadie-Hofstee type plot of i_{ss} against $i_{ss}/[S]$ and figure 4.7 a Hanes type plot of $[S]$ against $[S]/i_{ss}$.

Engasser & Horvath (1973 ; 1976) found that the Eadie-Hofstee formulation was the most affected by diffusional effects, and this was confirmed in the case of the graphite foil based glucose sensor. Whilst the Eadie-Hofstee plot was found to be almost sigmoidal in character, the Lineweaver-Burk plot proved to be less sensitive to diffusional effects giving a linear plot with a correlation coefficient of 0.9999 for the data shown in figure 4.5.

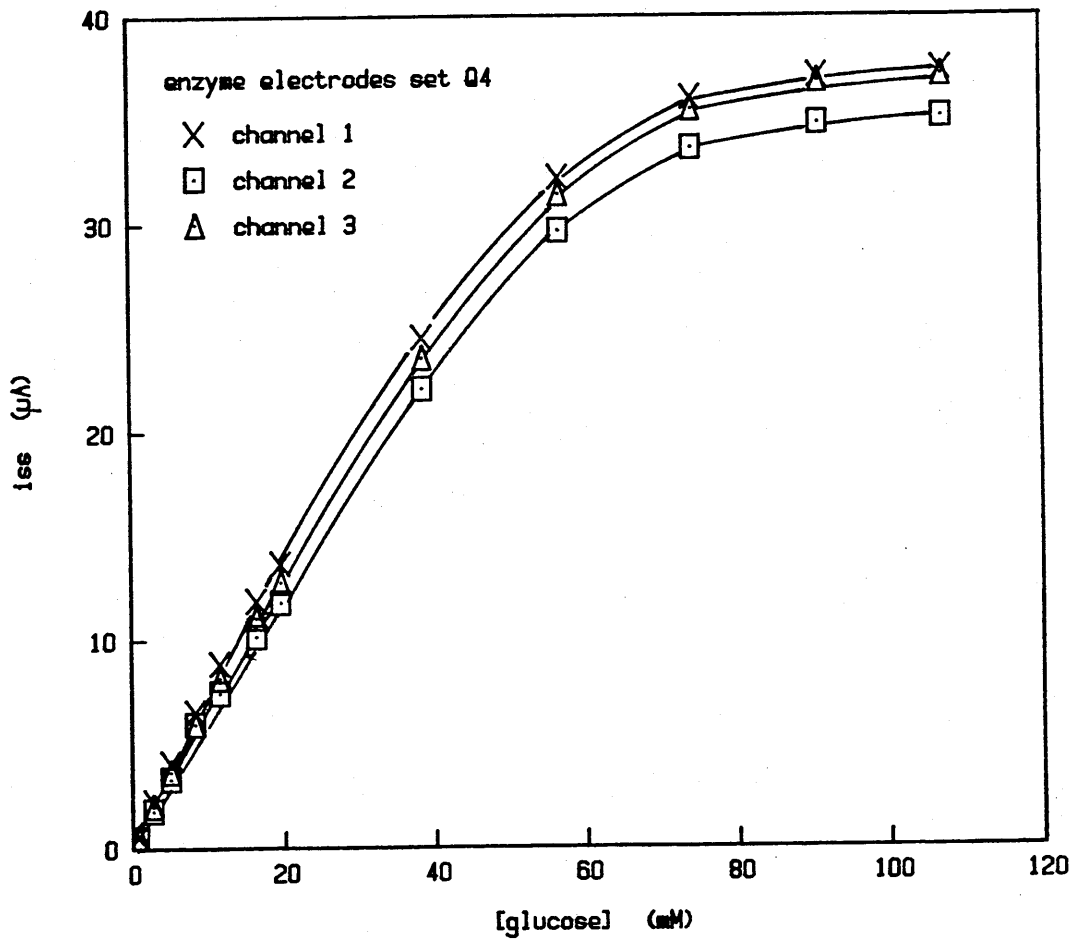
Determination of the kinetic parameters K'_m and i_{max} is difficult in the presence of diffusional limitations, since it is necessary to work outside the diffusion-limited regime, which is predicted only at very high substrate concentrations. The Hanes type plot was found to be the most suitable plot for this, tending

FIGURE 4.3.



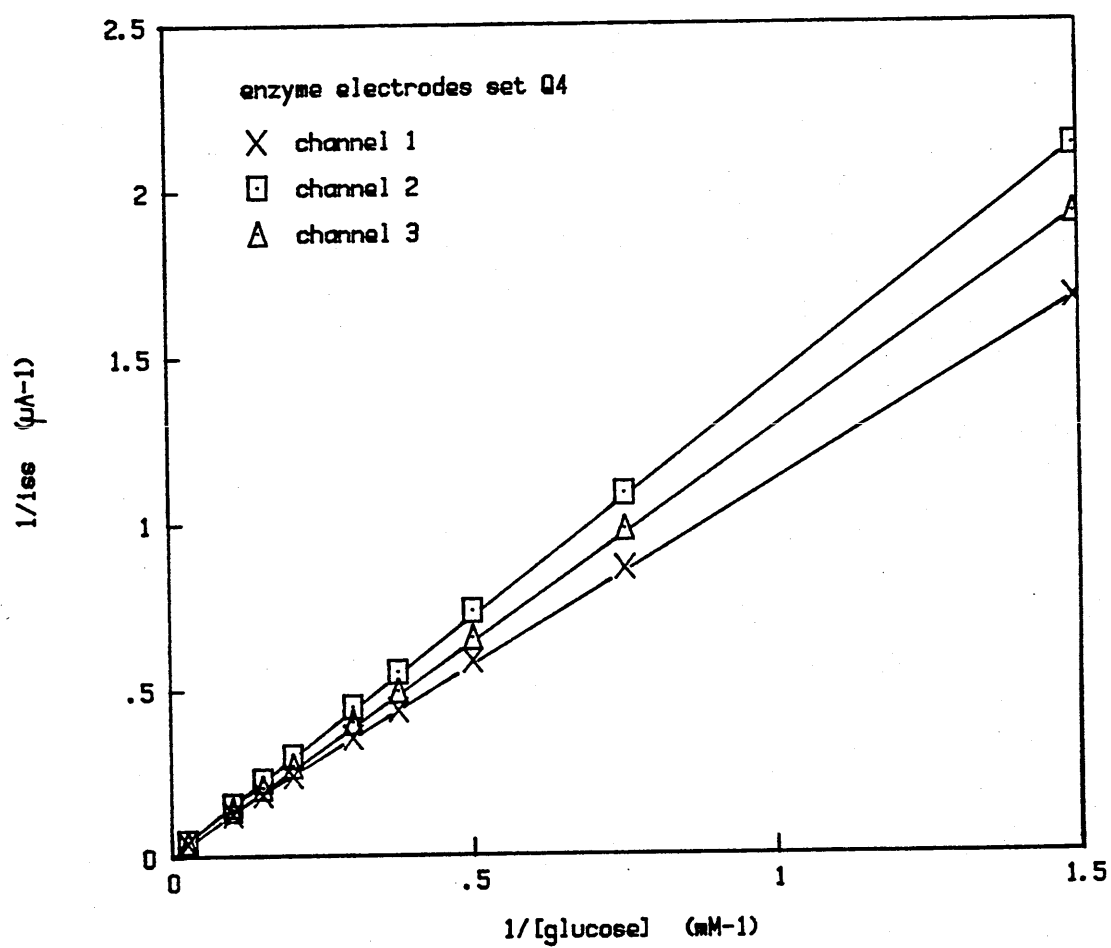
Computer printout of a typical calibration experiment of current against time for a set of ~~three~~ enzyme electrodes (set Q4). The experiment was carried out in 0.1 M phosphate buffer pH 7 containing 0.15 M KCl at room temperature, an applied voltage of +235 mV vs. Ag/AgCl and under nitrogen sparging. Arrows indicate additions of a 1 M glucose solution to give final concentrations between 0.67 - 107.1 mM.

FIGURE 4.4.



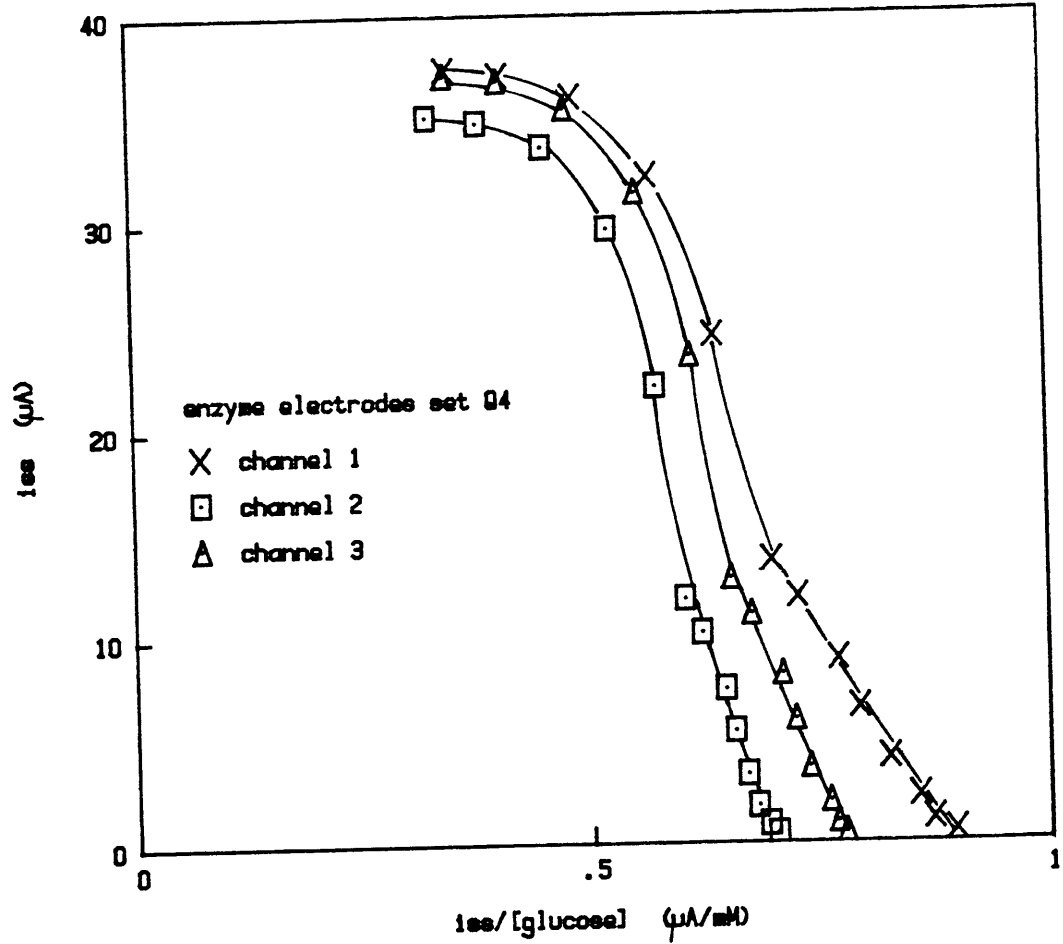
Calibration curves of steady-state current (corrected for background current) against glucose concentration derived from the data shown in figure 4.3.

FIGURE 4.5.



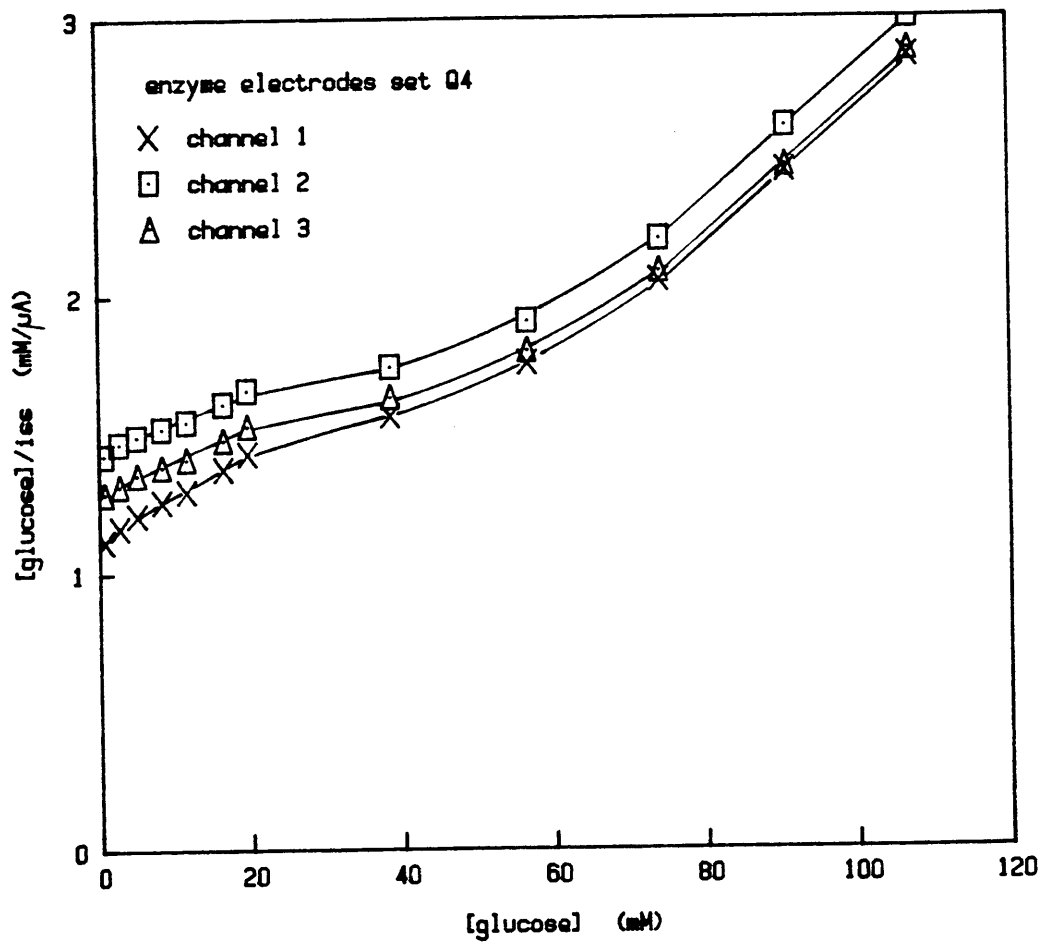
Lineweaver-Burk plots of $1/i_{ss}$ against $1/[S]$ derived from the calibration curves shown in figure 4.4.

FIGURE 4.6.



Eadie-Hofstee plots of i_{ss} against $i_{ss}/[S]$ derived from the calibration curves shown in figure 4.4.

FIGURE 4.7.



Hanes plots of $[S]/i_{ss}$ against $[S]$ derived
from the calibration curves shown in figure 4.4.

towards linearity at both extremes of substrate concentration.

Linear regression analysis was used for the data from a set of eight electrodes, including those presented in figures 4.4. - 4.7., for both Lineweaver-Burk and Hanes plots. In the case of the Hanes plots only the data at saturating glucose concentrations was used. The kinetic parameters determined from these two plots are given in table 4.1.

Significant differences in the values of K'_m and i_{max} can be observed between those derived at high substrate concentrations using Hanes plots, and those obtained from Lineweaver-Burk plots over the whole range of glucose concentrations.

TABLE 4.1.

Kinetic Parameters for Batch Q Electrodes

PLOT	K'_m	i_{max}
Lineweaver-Burk	63.19	47.39
Hanes	11.31	37.87

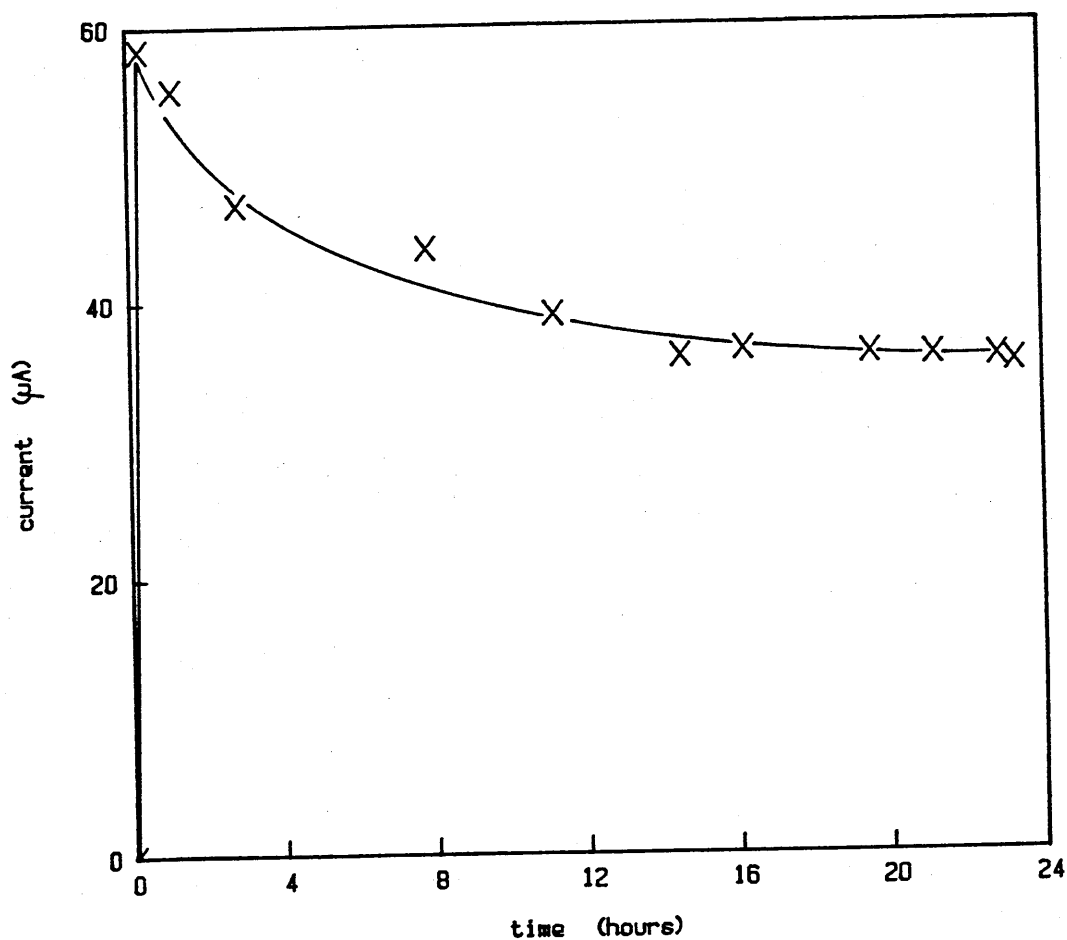
4.8. Evaluation of the Stability of the Enzyme Electrode

Two important criteria which needed to be evaluated for the model proposed in section 4.3. were : firstly, the assumption of steady-state behaviour with regard to the bulk substrate concentration, and secondly the stability of the enzyme electrode over the time period of the calibration experiment.

An experiment was performed where an enzyme electrode was operated for 24 hours at 38.5 mM glucose in 15 ml of buffer (GOD was immobilized onto this electrode from a 100 mg/ ml solution). Both the current and charge passed were measured continuously using the BAS 100 Electrochemical Analyzer. The total charge passed after 24 hours of continuous operation was 3.56 coulombs, which corresponded to 3.07 % oxidation of the glucose present (assuming 100 % efficiency according to the Nernst equation). Although the rate of change in the glucose concentration of the bulk solution will depend on the activity and the number of electrodes present, as well as the concentration of glucose and the volume of solution used, this result suggested that no appreciable change in the concentration of glucose in the bulk solution would be expected during the course of a typical calibration experiment (see section 4.5. for details).

The current measured during this experiment dropped to 60 % of its original value after 24 hours (figure 4.8.). A loss in current of 9.95 % was observed after the first 90 minutes, with subsequent decreases in current being 22 % after 5 hours and 33 %

FIGURE 4.8.



Current output of an enzyme electrode over a 24 hour period. The experiment was carried out at room temperature under nitrogen degassing, at a glucose concentration of 38.5 mM and an applied potential of +180 mV vs. SCE.

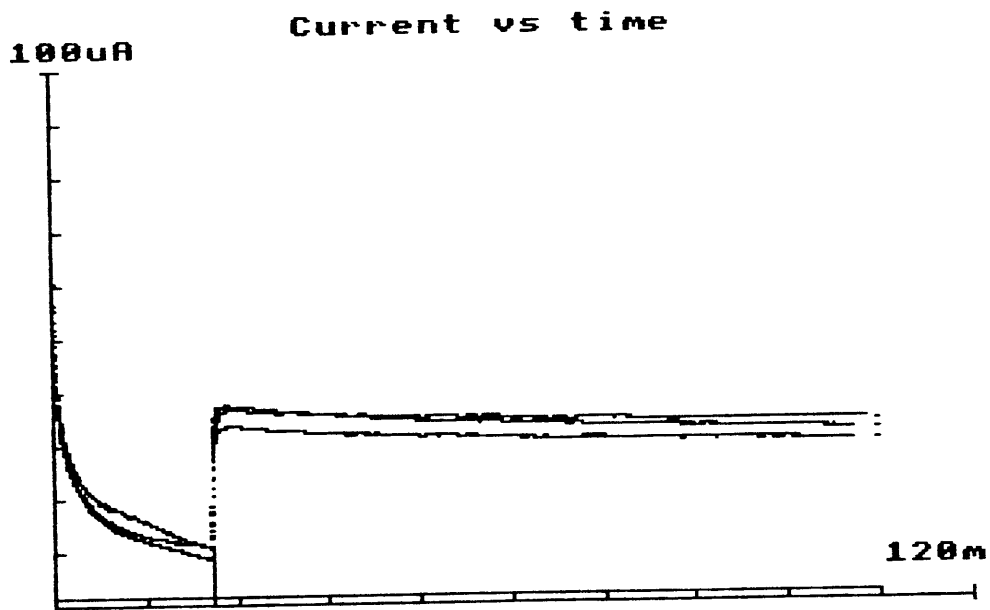
after 10 hours. The decay over the final 10 hours was about 2 %.

These results suggested that the stability of the enzyme electrode during the calibration experiment could be improved by preconditioning the electrodes for 90 minutes to remove this initial 10 % loss in activity. The preconditioning experiment for the electrodes described in section 4.7. is shown in figure 4.9. The decrease in current at the end of the experiment was on average 10.1 % of the maximum value recorded. A glucose concentration of 38.5 mM was employed, this concentration giving approximately 68 % of the saturating steady-state current (taken from a sample of 24 electrodes).

A loss of 5 % of the activity of the electrodes during the course of an experiment would have been an acceptable value for the purpose of obtaining a valid calibration curve. In fact a 3.3 % loss in activity was observed during the course of three consecutive calibration experiments (after preconditioning), as shown in figure 4.10.

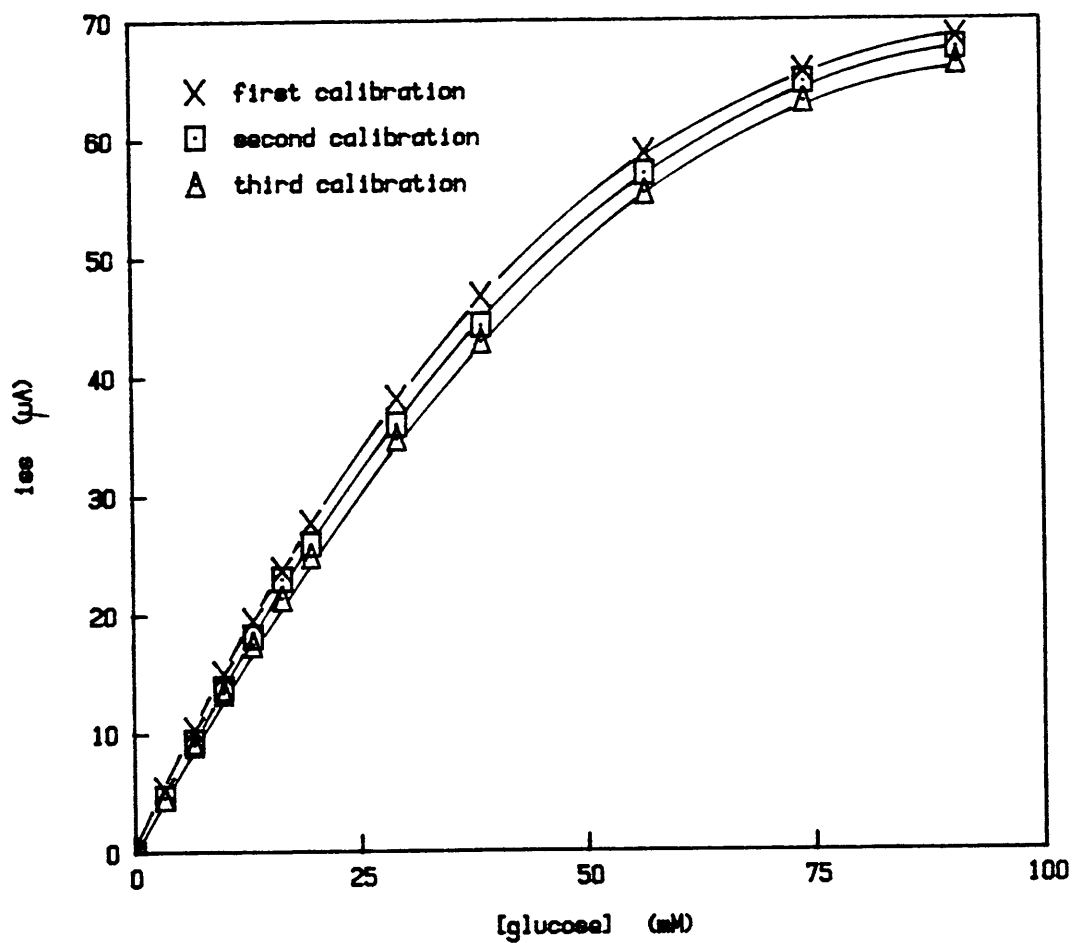
A similar set of results was obtained by running three electrodes overnight at 38.5 mM glucose after both preconditioning and calibration experiments. An average decrease in current of 7.98 % was observed after the first 3.3 hours (figure 4.11.). These results (figures 4.10. and 4.11.) showed that the expected loss in activity of the enzyme electrode during the course of a calibration experiment (after preconditioning) would be less than 3 %.

FIGURE 4.9.



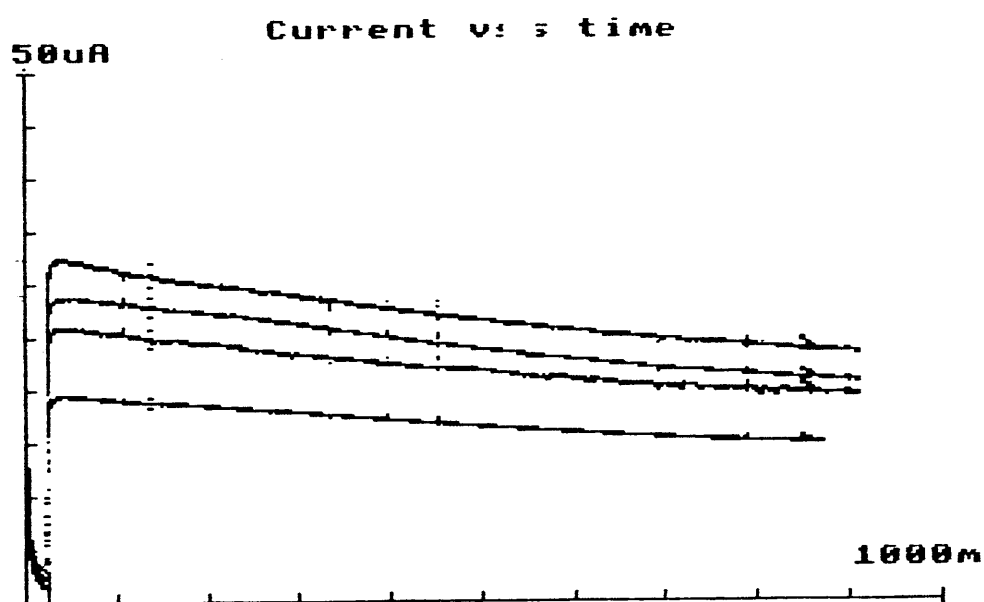
Computer printout of the preconditioning experiment for set Q4 enzyme electrodes. The experiment was carried out in 0.1 M phosphate buffer pH 7 containing 0.15 M KCl and 38.5 mM glucose, at room temperature, an applied voltage of +235 mV vs. Ag/AgCl and under nitrogen sparging. This experiment was performed prior to the calibration experiment shown in figure 4.3.

FIGURE 4.10.



Successive calibration curves carried out for a set of three enzyme electrodes (averaged). The experiments were carried out in 0.1 M phosphate buffer pH 7 containing 0.15 M KCl at room temperature, an applied voltage of +235 mV vs. Ag/AgCl and under nitrogen sparging.

FIGURE 4.11.



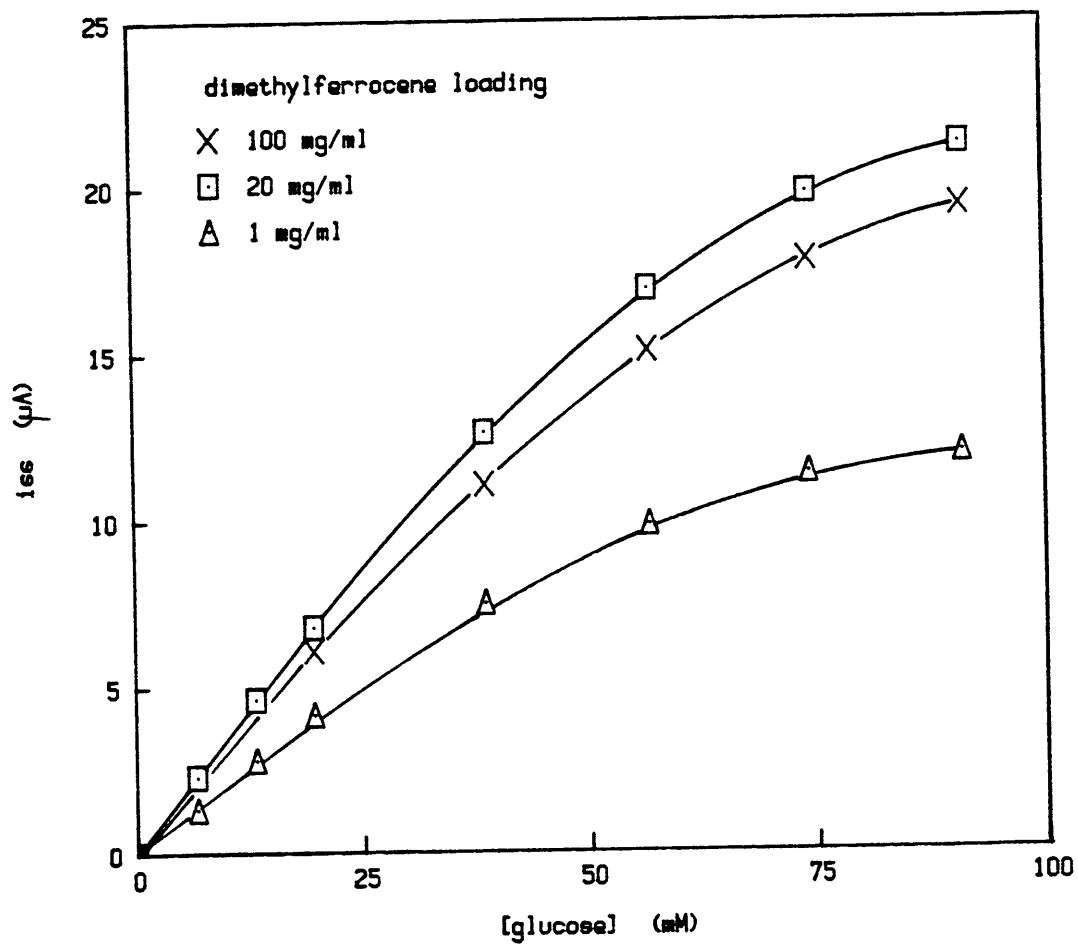
Computer printout of an overnight experiment carried out with T4 enzyme electrodes (glucose oxidase immobilized at 0.1 mg/ml concentration). The experiment was carried out in 0.1 M phosphate buffer pH 7 containing 0.15 M KCl and 38.5 mM glucose, at room temperature, an applied voltage of +235 mV vs. Ag/AgCl and nitrogen sparging.

4.9. Dependence of Enzyme Electrode Kinetics on the Concentration of Oxidized, Adsorbed Mediator

It was assumed for the model proposed in section 4.3. that the concentration of DMFc within the electrode was saturating, and that the kinetics of the processes associated with the reoxidation of enzyme-reduced mediator were not rate limiting. The effect of changes in the concentration of oxidized DMFc on the observed kinetic behaviour of the enzyme electrode was investigated by varying both the concentration of the mediator doped onto the electrode and the applied potential.

A rough estimate of the concentration of DMFc within the electrode can be made. Taking the diameter of the graphite foil electrode as 1.2 cm and its thickness as 0.1 cm, the volume of the electrode is approximately 0.11 cm^3 . Since 10 μl of a 20 mg / ml solution was used, then the concentration of mediator in an equivalent volume of solution can be calculated as 9.4 mM. This value is the lowest theoretical limit, since the graphite itself occupies a significant proportion of the electrode volume. Nevertheless, this calculation suggested that the DMFc loading employed as standard (10 μl of a 20 mg/ml solution) would be saturating providing that no significant loss of DMFc occurs during the calibration experiment. This was shown when enzyme electrodes were doped with 10 μl of a 100 mg / ml solution of DMFc. As shown in figure 4.12. these electrodes demonstrated slightly lower currents than electrodes doped with the standard 20 mg / ml solution. This loss in

FIGURE 4.12.



Calibration curves of enzyme electrodes employing different concentrations of dimethylferrocene applied to the electrode surface. The experiments were carried out in 0.1 M phosphate buffer pH 7 containing 0.15 M KCl at room temperature, an applied voltage of +235 mV vs. Ag/AgCl and under nitrogen sparging.

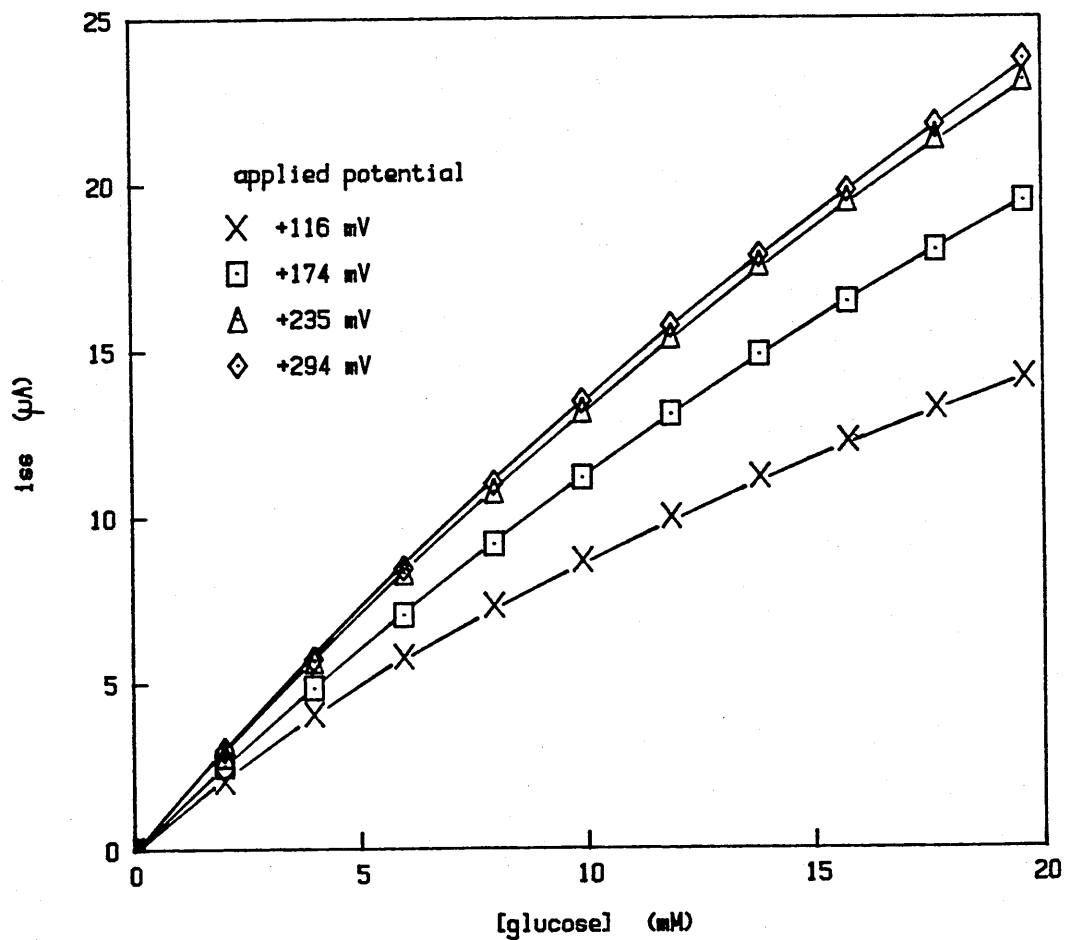
activity may have been due to poisoning of the enzyme by excess DMFc. It was noted that after allowing the solvent to evaporate, crystallized DMFc was present on the surface of electrodes which had been doped with 100 mg / ml solution. This suggested that using this concentration of mediator the capacity of the electrode to fully adsorb the mediator was exceeded.

A third set of electrodes were doped with 10 μ l of a 1 mg / ml solution of DMFc. In this case the steady-state current was considerably lower than that produced by the standard electrodes (figure 4.12.), indicating that in this case the concentration of oxidized DMFc " seen " by the bound enzyme was not saturating, and was therefore rate limiting.

Brooks et al. (1986) have shown that under continuous operation DMFc is rapidly lost from the electrode. This did not appear to present a problem in these experiments, partly due to the short time scale required to complete a calibration experiment and to the preconditioning employed. Non-dependance of the measured currents on the DMFc loading due to mediator loss was shown graphically in figure 4.10.

Similar behaviour was observed when the applied potential was lowered. As shown in figure 4.13. lowering the potential from +235 mV to +116 mV (vs. Ag/AgCl) reduced the measured steady-state currents. However, a further increase in potential to +294 mV had little effect, the steady-state currents at this potential being less than 3 % greater than at +235 mV. This difference in measured currents was less than the variation between each pair of

FIGURE 4.13.



Calibration curves for a set of three enzyme electrodes (averaged) at different applied potentials. The experiments were carried out in 0.1 M phosphate buffer pH 7 containing 0.15 M KCl at room temperature and under nitrogen sparging. Potentials are given with respect to the Ag/AgCl reference used, and were measured directly using a multimeter.

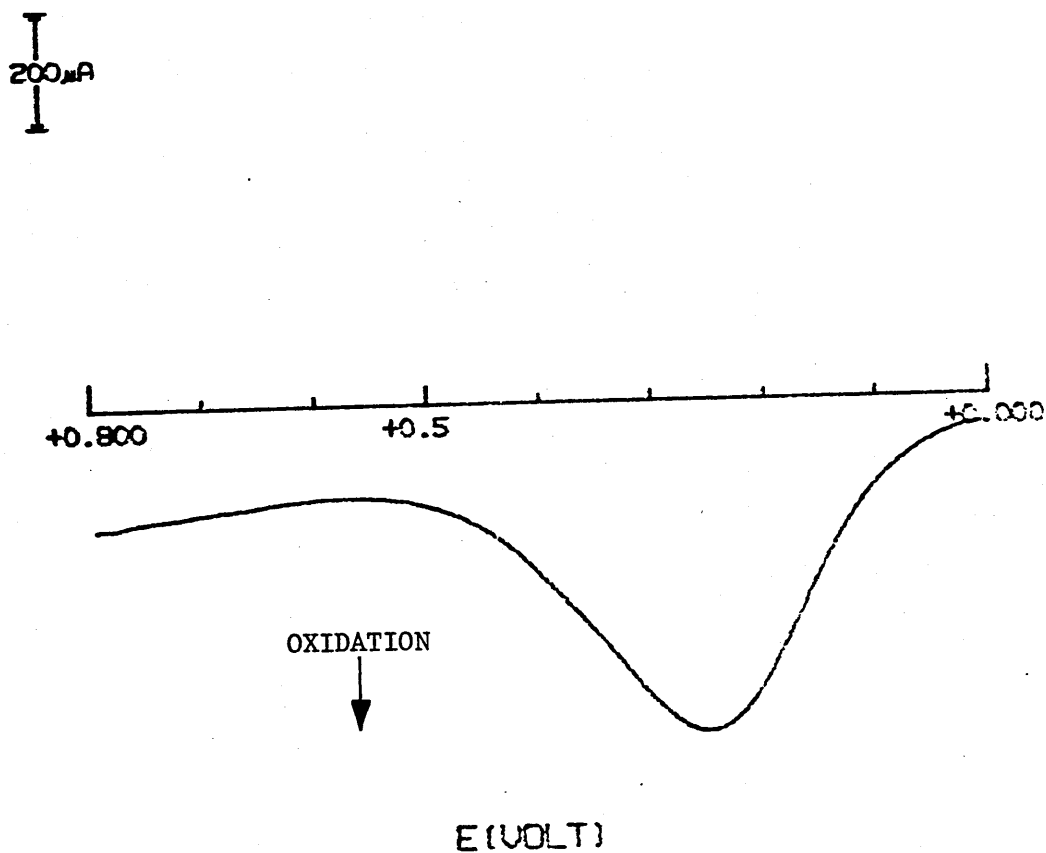
electrodes.

The half-wave potential of the adsorbed mediator, as determined by differential pulse voltammetry, was +248 mV vs. SCE (figure 4.14.). Thus the standard potential used for the calibration experiments was about 27 mV more positive than the potential at which half of the adsorbed mediator is in the oxidized state. Using the Nernst equation (section 4.3. equation [11]) it can be calculated that at +235 mV vs. Ag/AgCl the ratio of oxidized to reduced DMFc is approximately 2 : 1. The measured half-wave potential of adsorbed DMFc was found to be considerably more positive than that reported elsewhere (+100 mV vs. SCE; Cass *et al.*, 1984).

The results presented in figures 4.12. & 4.13. indicated that under the conditions used in these experiments (10 μ l of a 20 mg/ml solution, applied potential of +235 mV vs. Ag/AgCl) the concentration of oxidized mediator "seen" by the immobilized enzyme was not rate limiting. Concerning the processes involved in the oxidation of enzyme-reduced mediator it was not possible to determine directly whether these events influenced the observed kinetics of the enzyme electrode. As discussed in section 4.3., and from the results shown in figure 4.12. it was considered that diffusion of reduced mediator within the electrode was not a rate-limiting step.

Cass *et al.* (1984) showed that the heterogeneous electron transfer reaction (oxidation of ferrocene monocarboxylic acid at the electrode) was fast compared to the reaction between ferric in-

FIGURE 4.14.



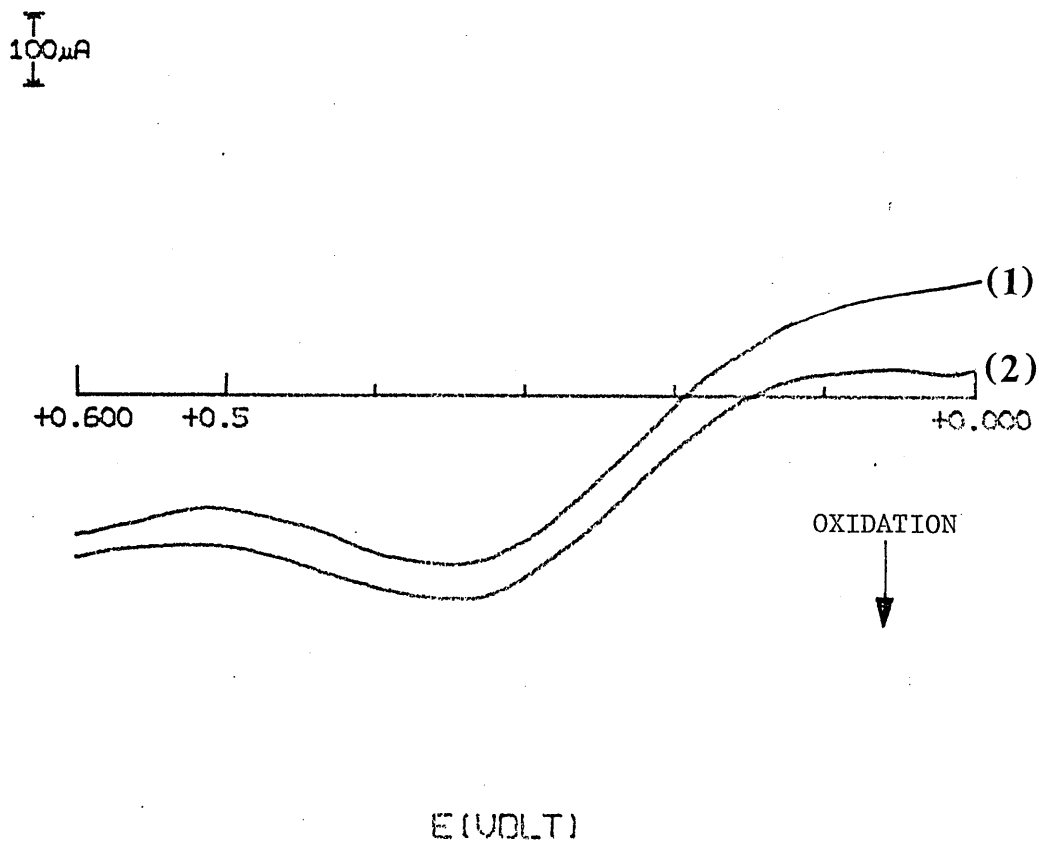
Differential pulse voltammogram of a graphite foil electrode doped with dimethylferrocene. Conditions employed were :
scan rate = 4 mV s^{-1} ; pulse amplitude = 50 mV ; sample width = 17 msec ; pulse width = 50 msec ; pulse period = 1000 msec

ium ions and GOD. Thus although the electrochemical kinetics of the adsorbed mediator did not appear to be a classically reversible system, by reference to this work (Cass et al., 1984) this process was also taken as being non-rate-limiting.

It was of interest to see whether catalytic oxidation waves could be obtained using the immobilized GOD / DMFc system. Cass et al. (1984) showed that in solution, cyclic voltammetry of ferrocene monocarboxylic acid was substantially altered in the presence of both the enzyme and substrate (glucose). This effect can be seen in figure 5.17. for the soluble lipoamide dehydrogenase / NAD^+ / methyl viologen system.

In the case of the GOD-based enzyme electrode linear sweep voltammetry was employed, principally because background sweeps could be subtracted with this technique using the BAS 100. Figure 4.15. shows linear sweeps of an enzyme electrode in buffer and in the presence of 100 mM glucose (performed under anaerobic conditions). In both cases background sweeps using an unmodified electrode were subtracted. Although a characteristic catalytic wave for a coupled system was not observed there was a definite increase in current at oxidizing potentials when glucose was present in the medium. This result is open to more than one interpretation. One conclusion would be that the amount of active immobilized enzyme present was insufficient to keep the electrode surface continually supplied with reduced mediator. This could have been partially due to the extensive electrode area available. Alternatively, depletion of glucose within the porous electrode material may have

FIGURE 4.15.



Linear sweep voltammograms of an enzyme electrode : (1) in buffer only, and (2) in buffer containing 100 mM glucose. Background sweeps of a bare graphite foil electrode were subtracted.

been such that the concentration of glucose became rate limiting. When figures 4.14. & 4.15. are compared it can be seen that the half-wave potentials were substantially different. This was a common occurrence when electrochemical techniques were employed with DMFc-doped graphite foil electrodes. The reasons for these variations are unclear, but they may reflect the variability of the electrode material itself.

4.10. Comparison of the Enzyme Electrode with Membrane Retained Enzyme

The results presented in section 4.7. demonstrated that the steady-state response of the immobilized enzyme electrode was not dictated by enzyme kinetics alone. It was of interest to elucidate whether the behaviour of the immobilized enzyme electrode could be accounted for by the presence of a diffusional barrier external to the electrode surface. One of the simplest methods of inducing an external diffusion barrier to an enzyme system is the use of a membrane. When an enzyme is immobilized inside a membrane, on the other hand, diffusion of reactants can be characterized by an internal diffusional process (see section 1.3.).

For direct comparison it was necessary to use a membrane that did not induce any other complicating factors into the system, e.g. charge distribution, and additionally to use the DMFc-doped graphite foil electrode as the base sensor. It was therefore decided to retain a solution of GOD (5ul of a 100 mg/ml solution)

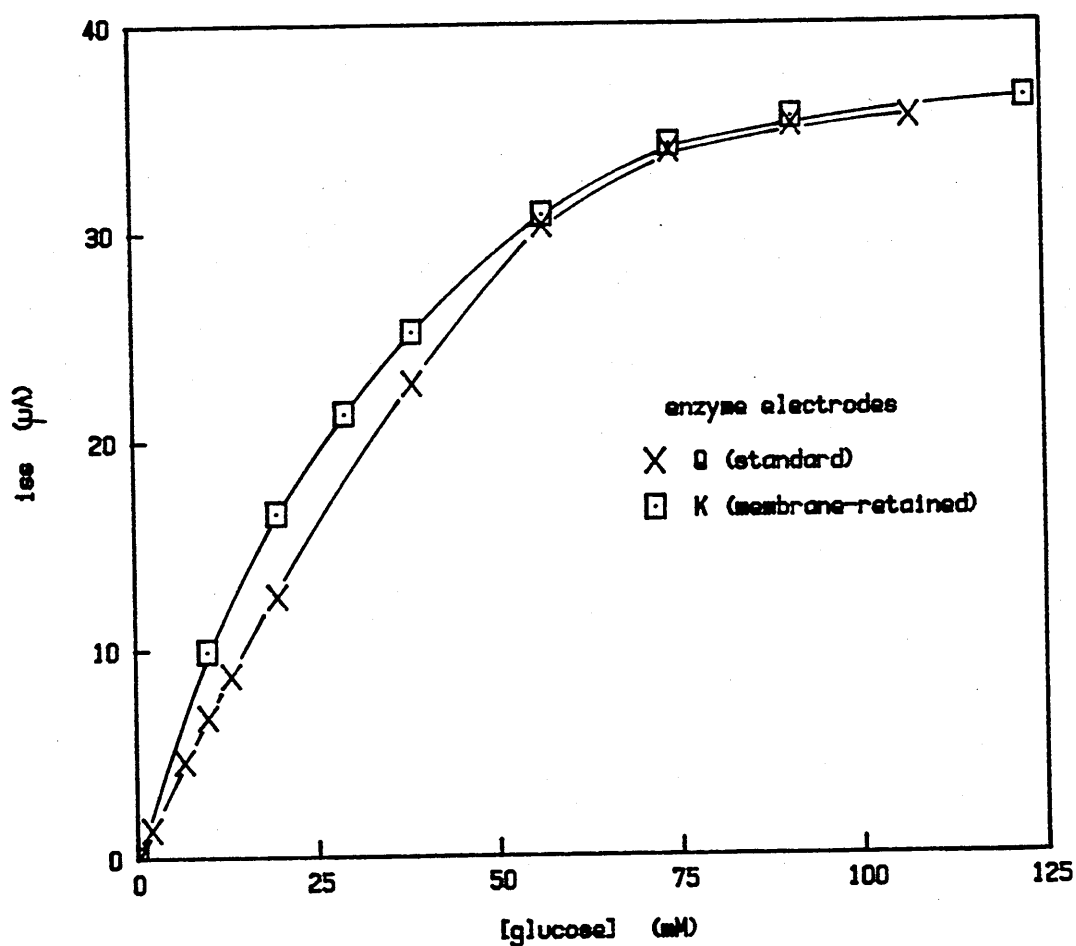
behind a piece of dialysis membrane, which was then held in place over the electrode with a ring of silicone tubing.

A calibration curve for one of these electrodes is shown in figure 4.16. along with data for an immobilized enzyme electrode. This particular enzyme electrode was chosen for comparison because it produced very similar steady-state currents at saturating glucose concentrations. Whereas the currents obtained for the two electrodes are almost identical at glucose concentrations greater than 50mM, below this value the shape of the i_{ss} -[S] curves differ sharply, the immobilized system giving a more linear response. The use of Eadie-Hofstee type plots, however, shows up these two facets of the response curve more graphically.

As can be seen in figure 4.17. the two curves coincide quite well at saturating glucose concentrations (which would be expected from figure 4.16.). At lower glucose concentrations, however, they demonstrate completely different behaviour, the membrane-retained enzyme (electrode K3) being characterized by a linear region, indicating a first-order regime. Similar types of Eadie-Hofstee plots of i_{ss} against $i_{ss}/[S]$ have been obtained by previous workers, who attributed this behaviour to external diffusional limitations (Castner & Wingard, 1984).

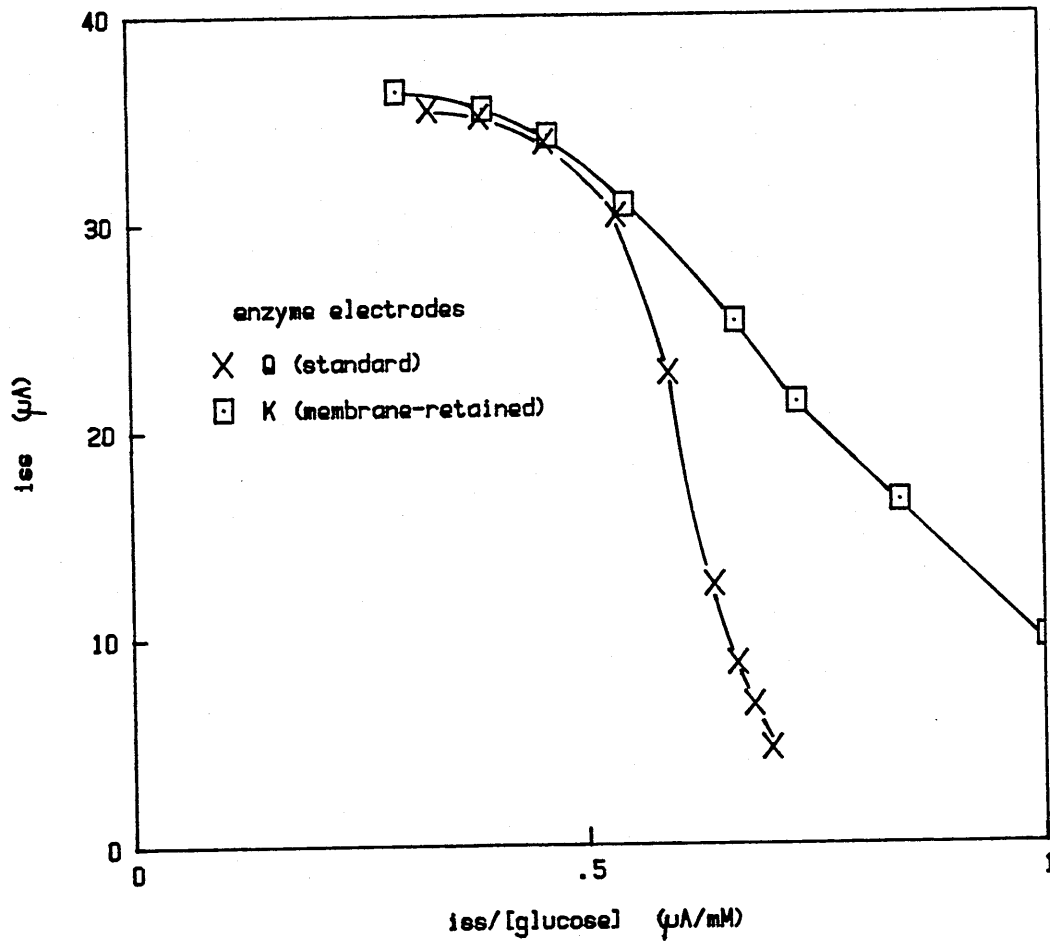
The comparison of these two types of enzyme electrode suggested that the behaviour of the electrode where the enzyme was constrained within the electrode material could not be accounted for simply by a diffusion barrier external to the electrode surface.

FIGURE 4.16.



Calibration curves for an enzyme electrode made by retaining glucose oxidase behind a dialysis membrane over a graphite foil electrode doped with dimethylferrocene (K) compared with a standard enzyme electrode (Q). The experiments were carried out under standard conditions.

FIGURE 4.17.



Eadie-Hofstee plots of the data from figure 4.16.

for the enzyme electrode based on membrane-retained enzyme (K) and a standard enzyme electrode (Q).

4.11. Response Behaviour of the Enzyme Electrode

Two important characteristics of an amperometric enzyme electrode are the linearity of the steady-state current to substrate concentration, and the time required for the response of the system to reach equilibrium. Racine & Mindt (1971) used cellophane membranes of different permeabilities to retain cytochrome b_2 . With membranes of lower permeability the linear range of the sensor to lactate concentration was increased, but with concomitant increases in the response times required to obtain steady-state currents. A linear range of 20 mM lactate was achieved with a membrane of low permeability ($0.84 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$), the response time being 7 minutes.

Using GOD immobilized inside polyacrylamide membranes Mell & Maloy (1975) obtained linear responses in excess of the K_m of the enzyme (6.8 mM). With a membrane of 0.5 mm thickness a linear range of approximately 20 mM glucose was observed with a response time of 212 seconds. They concluded that an increase in enzyme loading would also increase the concentration range over which a linear response was observed due to greater depletion of substrate within the immobilized enzyme layer.

The data presented in figure 4.4. shows a good linear response of the enzyme electrode to about 40 mM glucose. A closer examination of enzyme electrodes with both different enzyme loadings and electrode diameters revealed that the linear range was more

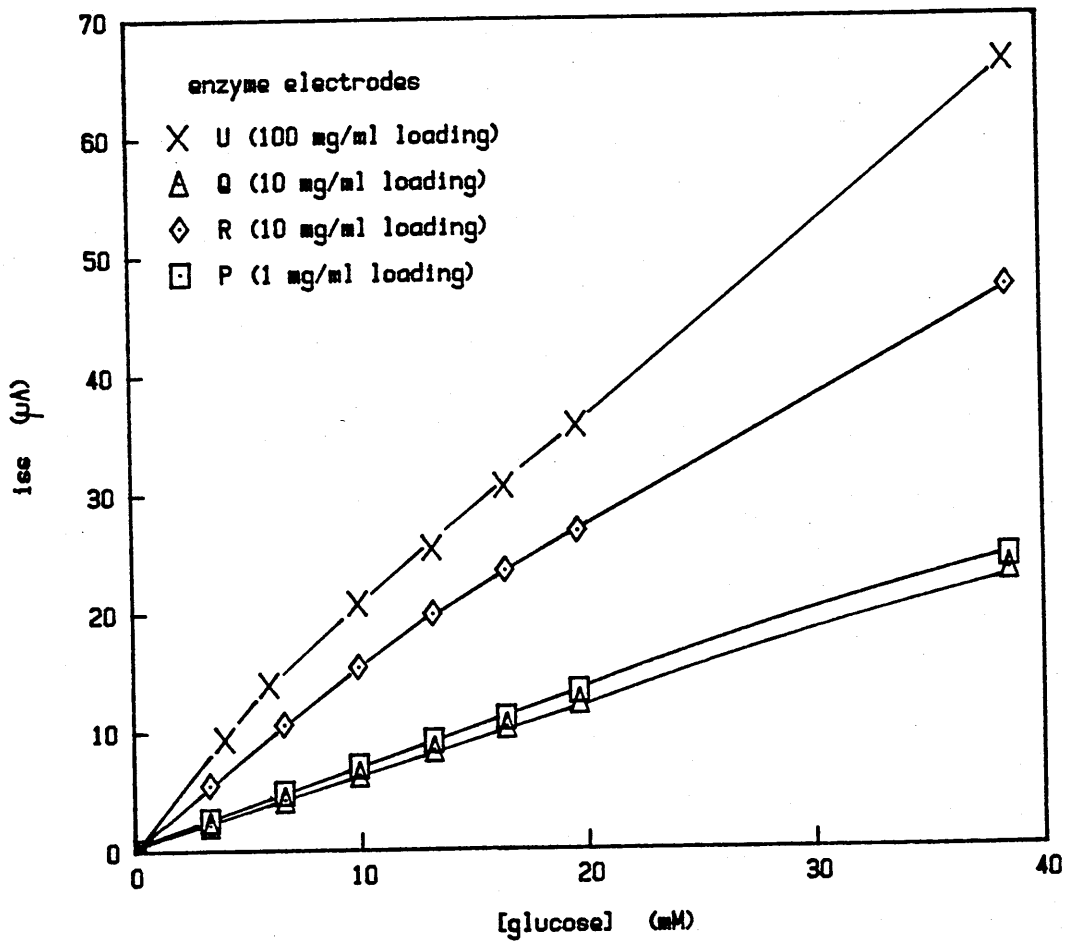
affected by the size of the electrode than by its activity. This can be clearly seen in figure 4.18. over the range 2 - 40 mM glucose. Small diameter enzyme electrodes showed a good linearity to approximately 40 mM glucose, although there was a slight degree of curvature after 20 mM glucose, whereas the larger diameter electrodes demonstrated smaller linear ranges. These differences may have been due partly to the increased currents obtained with the larger electrodes, since normalization of the data shown in figure 4.18. reduced these differences in linearity observed between the different batches of electrodes.

The linear response of the enzyme electrodes was also affected by the potential used, as can be seen from figure 4.13., where lower poised potentials gave decreased linear ranges.

The approach to steady-state in the measured current did not appear to be affected by these parameters, no significant differences being observed for different enzyme loadings, potential or DMFc content. Typical steady-state responses to low and high glucose concentrations are shown in figure 4.19. Steady-state currents were reached after approximately 60 seconds, although small increases (generally less than $0.2\mu\text{A}$) were observed during the following 2 - 3 minutes.

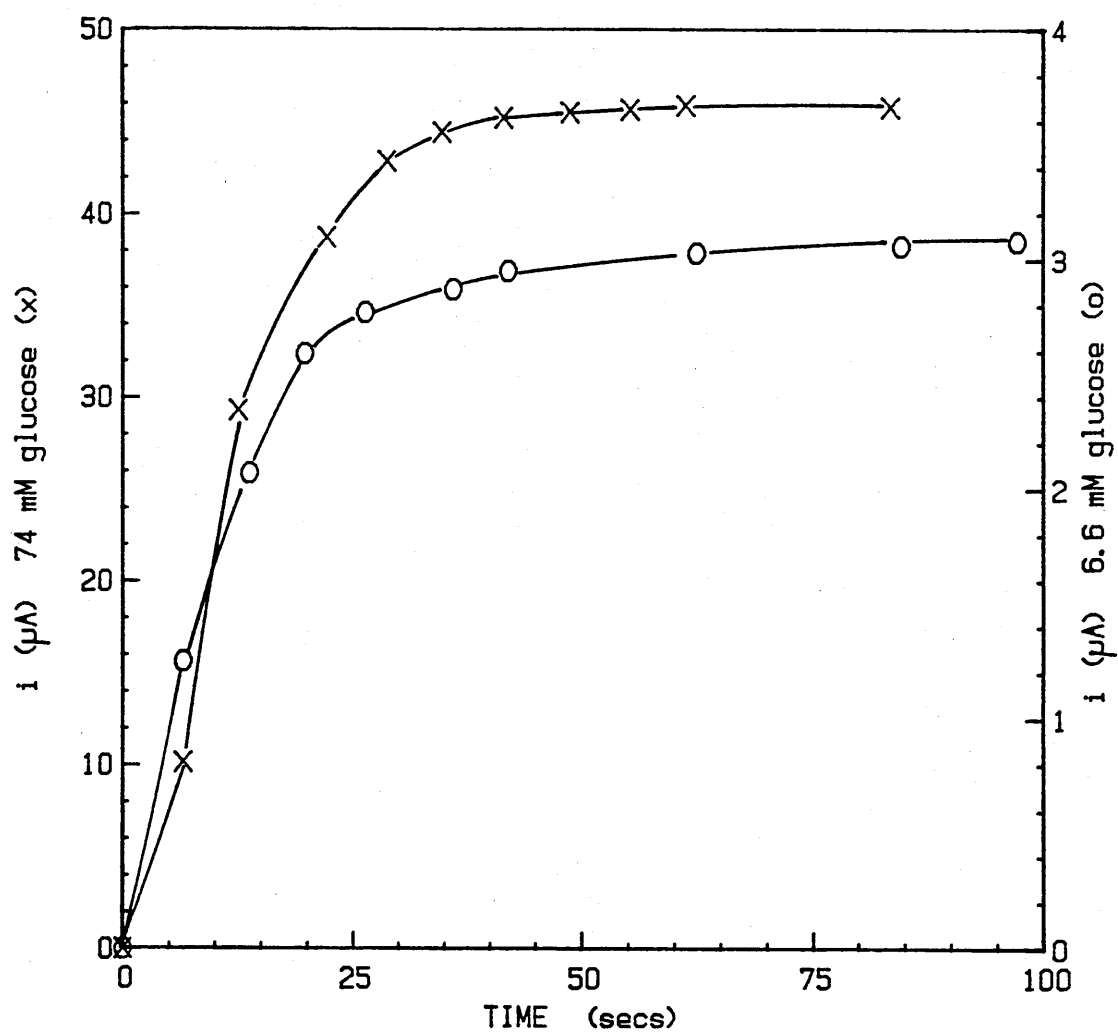
The quick response of the enzyme electrode (figure 4.19.) was found to be in marked contrast to the dialysis membrane system described in section 4.10. The approach to steady-state for this type of enzyme electrode is shown in figure 4.20. A stable current was not reached after more than 6 minutes following the addit-

FIGURE 4.18.



Calibration curves of both large diameter (U and R) and small diameter (Q and P) enzyme electrodes (averaged) based on different enzyme loadings. The experiments were performed under standard conditions and at room temperature. (Further details of these enzyme electrodes are given in tables 4.4. and 4.5.).

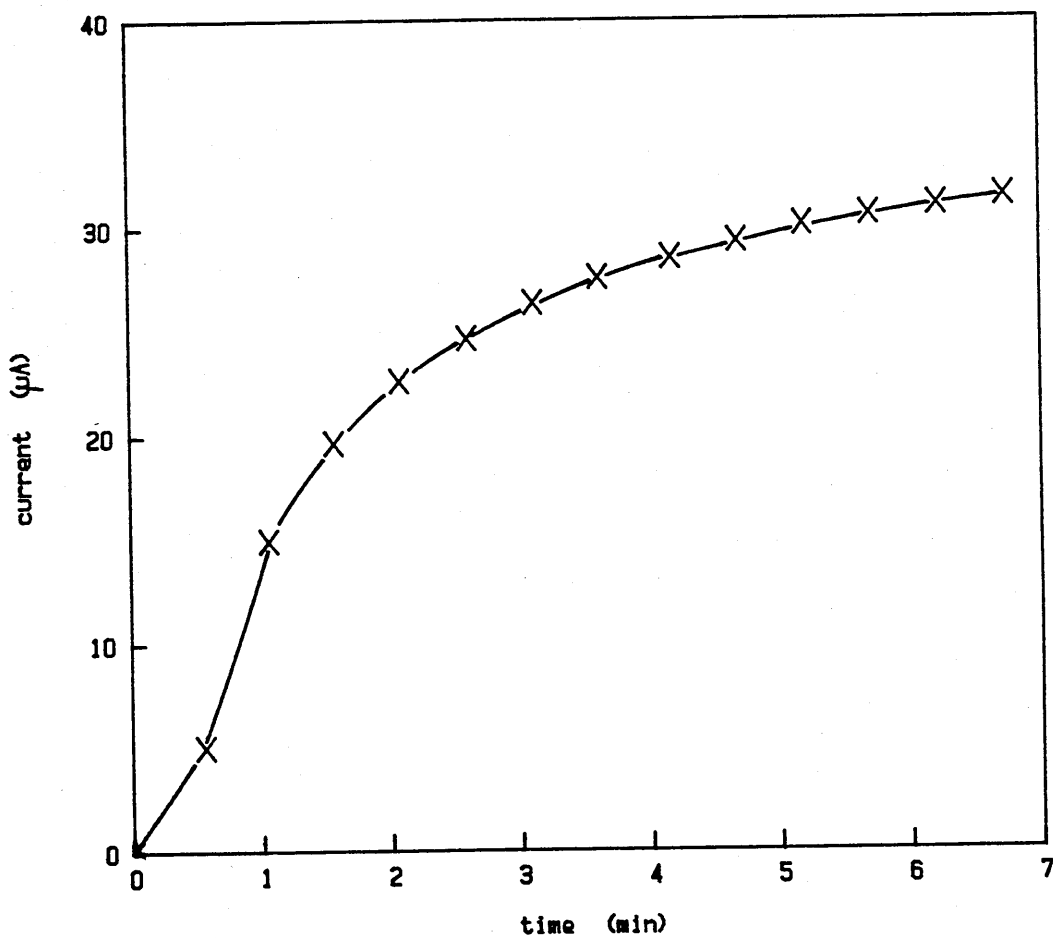
FIGURE 4.19.



Transient response curves of enzyme electrodes

(averaged) at low & high glucose concentrations.

FIGURE 4.20.



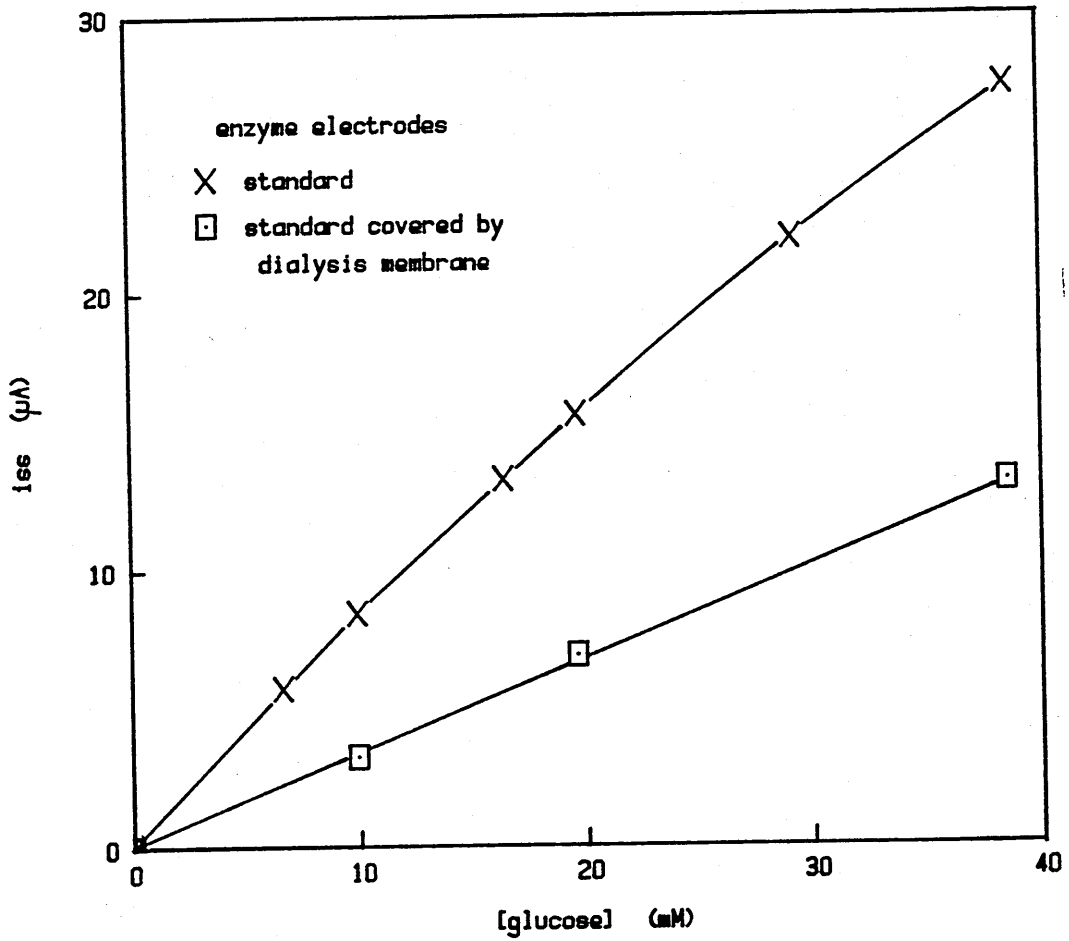
Response curve of current against time for an enzyme electrode based on membrane-retained glucose oxidase. The experiment was carried out under standard conditions.

ion of glucose to the system. When a dialysis membrane was placed over the " internal " immobilized system the current-time response was increased, giving a curve similar to that shown in figure 4.20. Calibration curves of an enzyme electrode operated conventionally and covered with dialysis membrane are presented in figure 4.21. This latter enzyme electrode demonstrated a more linear response up to 40 mM glucose than the standard sensor, although the steady-state currents were considerably lower.

These results demonstrated the effect of external diffusional limitations on the enzyme electrode, where both the linear range of the sensor and the response time were increased. The lower steady-state currents obtained when the enzyme electrode was covered by a membrane would appear to have been due to the reduced flux of glucose in this system reaching the immobilized enzyme layer.

An interesting observation was the fact that in both types of enzyme electrodes, with glucose oxidase retained either within the electrode or at the surface by dialysis membrane, saturation occurred at almost identical glucose concentrations. This suggested that the local concentration of glucose at the microenvironment of the immobilized enzyme was similar in both cases, especially at saturating glucose levels. For those enzyme electrodes where the enzyme was bound within the porous electrode material the approach to steady-state was found to be considerably more rapid, indicating that in this system at least some of the immobilized enzyme was freely accessible to glucose in the bulk solution.

FIGURE 4.21.



Calibration curves of a standard enzyme electrode and one covered by dialysis membrane at non-saturating glucose concentrations. The experiments were performed under standard conditions.

4.12. Edge Effects of Graphite Foil Electrodes

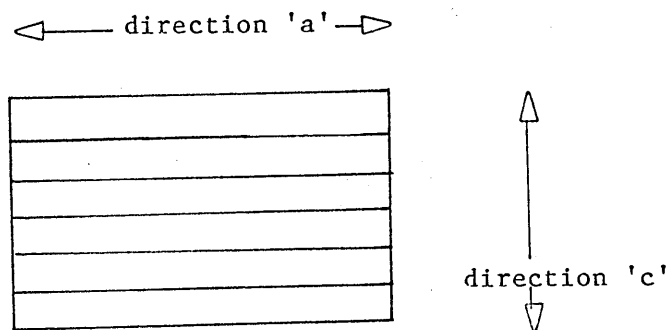
The results presented in section 4.10 showed that the behaviour of the immobilized enzyme electrode resembled more closely a system affected by internal, rather than external, diffusional resistances. This analysis was backed by the data presented in figure 4.1. , which demonstrated that the enzyme activity was not confined to the surface layer of the electrode, but was distributed throughout a number of layers. Due to the nature of graphite foil, which is an amorphous type of graphite with different properties in different directions (figure 4.22.), the most probable route for access to enzyme and substrate was considered to be through edges of the material. Figure 4.22. lists some of the properties of graphite foil in both physical planes.

To assess the validity of this assumption that the edges of the electrode played the important role in enzyme immobilization and substrate access, the edges of half the electrodes in a set were sealed off using araldite. This procedure was carried out before adsorption of the mediator, the subsequent glutaraldehyde activation and enzyme immobilization steps. Calibration curves for two of these electrodes, one having sealed edges, are shown in figure 4.23. This graph clearly demonstrates that blocking the edges of the electrode has had an adverse effect on the measured steady-state currents resulting from a decrease in the amount of active enzyme immobilized.

Linear regression analysis was used on the data from Hanes

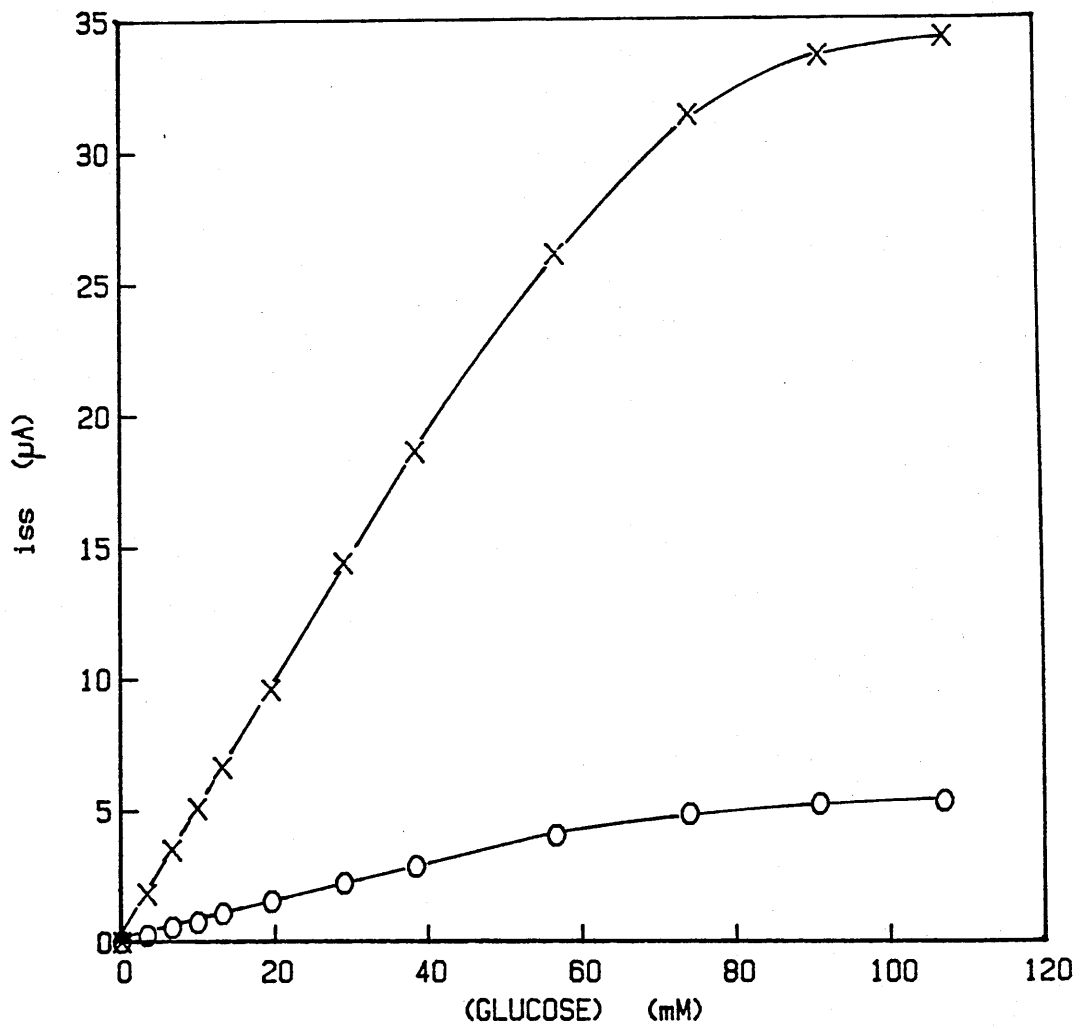
FIGURE 4.22.

PROPERTIES OF GRAPHITE FOIL IN DIFFERENT PHYSICAL PLANES



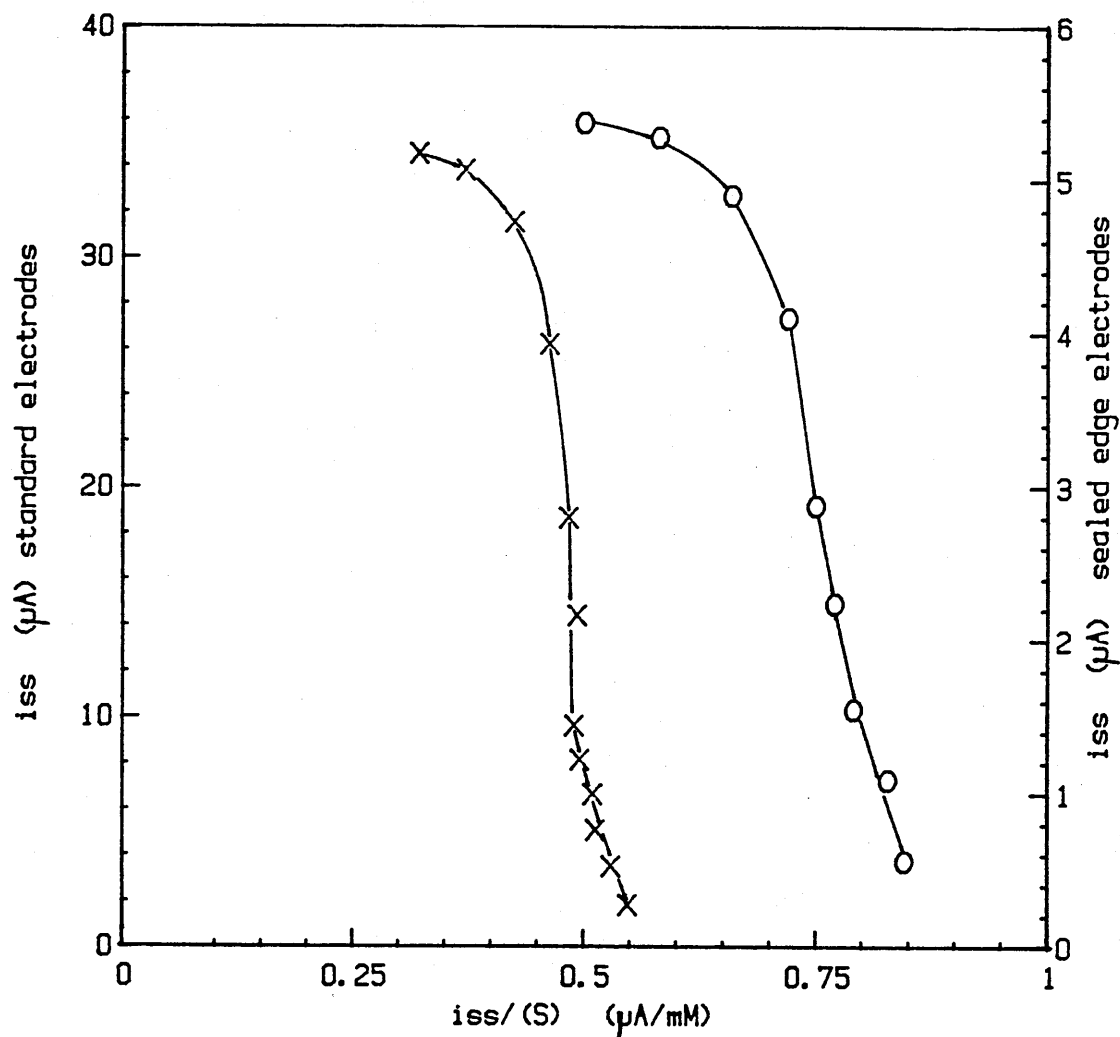
PROPERTY	UNITS	DIRECTION 'a'	DIRECTION 'c'
Ash content	%		0.1
Tensile strength	M N m ⁻²	6.5	-
Expansion coefficient between 20 and 1000°C	10 ⁻⁶ °C ⁻¹	nil	25-28
Thermal conductivity at ambient temperature	W cm ⁻¹ °C ⁻¹	160	4
Resistivity	Ω cm	0.001	0.05

FIGURE 4.23.



Calibration curves for standard enzyme electrodes
(X) and electrodes with sealed edges (O).

FIGURE 4.24.



Eadie-Hofstee plot of the data from figure 4.23. for standard enzyme electrodes (X) and electrodes with sealed edges (O). The X-axis data for the sealed edge electrodes was multiplied by a factor of 10 to provide a direct comparison with the standard electrodes.

plots at saturating glucose concentrations for six electrodes of each type. Values for $i_{\max}$ of 42.47 μA for the unmodified electrodes and 6.06 μA for the "blocked" electrodes were obtained. Thus when the edges of the electrode were sealed off only 14 % of the enzyme activity was immobilized compared to the control electrodes.

The retention of some enzyme activity by the "blocked" electrodes could have been accounted for by the ability of carbon materials to strongly adsorb proteins. However, the shape of Eadie-Hofstee type plots of the data from figure 4.23. for both sets of electrodes were very similar, as shown in figure 4.24. This would suggest that in fact the sealing of the edges of the electrodes with Araldite was not 100 % effective, which allowed for some enzyme immobilization to take place.

4.13 Effect of Enzyme Loading, Temperature and Electrode Diameter on Kinetic Parameters

When the overall behaviour of an immobilized enzyme system is controlled by both diffusional resistances and enzyme kinetics, a change in the absolute value of one of these processes may radically alter the observed kinetics. This was demonstrated in section 4.9. by the use of a membrane, imposing a greater degree of diffusional inhibition on the enzyme reaction. Similarly an increase in the enzyme activity of the system, whilst raising the experimentally derived values for the maximum steady-state current, may

also provoke a change in the balance between the kinetic and diffusional processes, leading to a change in the calculated values for K'_m .

Changes in the values of K'_m and i_{max} were also investigated in relation to increased enzyme loading, with regard to two different diameter graphite foil electrodes and two different temperatures. The two electrode sizes are also discussed in section 4.15. in relation to the determination of a value for the substrate modulus of the enzyme electrode, and a more detailed study of the effect of temperature on the system is given in section 4.14.

Values for K'_m and i_{max} , derived as described in section 4.7., for small and large diameter electrodes at 1°C and 25°C are given in tables 4.2. - 4.5. The increase in i_{max} with higher enzyme loadings was found to be more pronounced at 1°C than at 25°C, with the exception of set U, although the values of i_{max} were considerably higher at 25°C than at 1°C. The larger diameter electrodes gave higher maximum steady-state currents than the smaller electrodes as would be expected.

The changes in K'_m values for these different variables was more interesting. The first major difference which was observed was the higher values of K'_m obtained at 1°C. With the exception of set U all the enzyme electrodes examined at this temperature gave K'_m values of 30 - 36 mM. At 25°C the range of derived values was 7 -14 mM, much closer to the K_m of the enzyme in free solution (9.2 mM given by Weetall & Herish, 1970 ; 6.8 mM given by Kamin & Wilson, 1980). The values of K_m for the soluble enzyme quoted

TABLE 4.2

KINETIC PARAMETERS DETERMINED AT DIFFERENT ENZYME LOADINGS

SMALL DIAMETER ELECTRODES : 1°C

SET	LOADING (mg/ml)	n	K' _m	i _{max}
P	1	5	33.35	10.77
N	100	3	30.93	14.34

TABLE 4.3.

KINETIC PARAMETERS DETERMINED AT DIFFERENT ENZYME LOADINGS

LARGE DIAMETER ELECTRODES : 1°C

SET	LOADING (mg/ml)	n	K' _m	i _{max}
G	0.1	6	34.36	11.04
E	1	5	36.27	14.60
F	10	5	35.48	20.49
U	100	3	24.26	36.85

TABLE 4.4

KINETIC PARAMETERS DETERMINED AT DIFFERENT ENZYME LOADINGS

SMALL DIAMETER ELECTRODES : 25°C

SET	LOADING (mg/ml)	n	K'm	i _{max}
P	1	5	9.45	44.44
Q	10	8	11.31	37.87
N	100	3	14.33	49.95

TABLE 4.5.

KINETIC PARAMETERS DETERMINED AT DIFFERENT ENZYME LOADINGS

LARGE DIAMETER ELECTRODES : 25°C

SET	LOADING (mg/ml)	n	K'm	i _{max}
G	0.1	5	11.98	63.98
R	10	8	12.86	72.45
U	100	3	13.50	124.00

above were also obtained at 25°C, but a change in this constant with temperature would not be expected to occur since it simply a ratio of the rate constants for the various steps of the enzyme reaction (section 4.1., equation [1]), which would all be equally affected by temperature changes. The set of electrodes used for edge effect experiments also gave K'_m values of approximately 30 mM (section 4.12.), although the reasons for this remain unclear, since these were the only set of electrodes to give K'_m values higher than about 15 mM at 25°C. The higher values obtained for the electrodes at 1°C would appear to have been due to increased diffusional resistances within the electrode relative to the activity of the enzyme, leading to greater substrate depletion, which was supported by the effect of temperature on the response behaviour of the electrodes as described in section 4.14. This fact was shown most graphically by the more linear response of the U set of electrodes at 1°C compared to the calibration curves carried out at 25°C.

At 25°C there was a general increase in the values of K'_m derived from Hanes plots at saturating glucose concentrations as the enzyme loading increased, as predicted by Mell & Maloy (1975).

4.14. Temperature Effects

The rate of chemical reactions, including enzymatically catalysed ones, approximately doubles with an increase in temperature of 10°C. This is due to the increase in the rate constants of the

reaction with temperature. The activation energy of the reaction can be obtained using the Arrhenius equation :

$$\ln(k) = - \ln(A) - \frac{E^*}{RT}$$

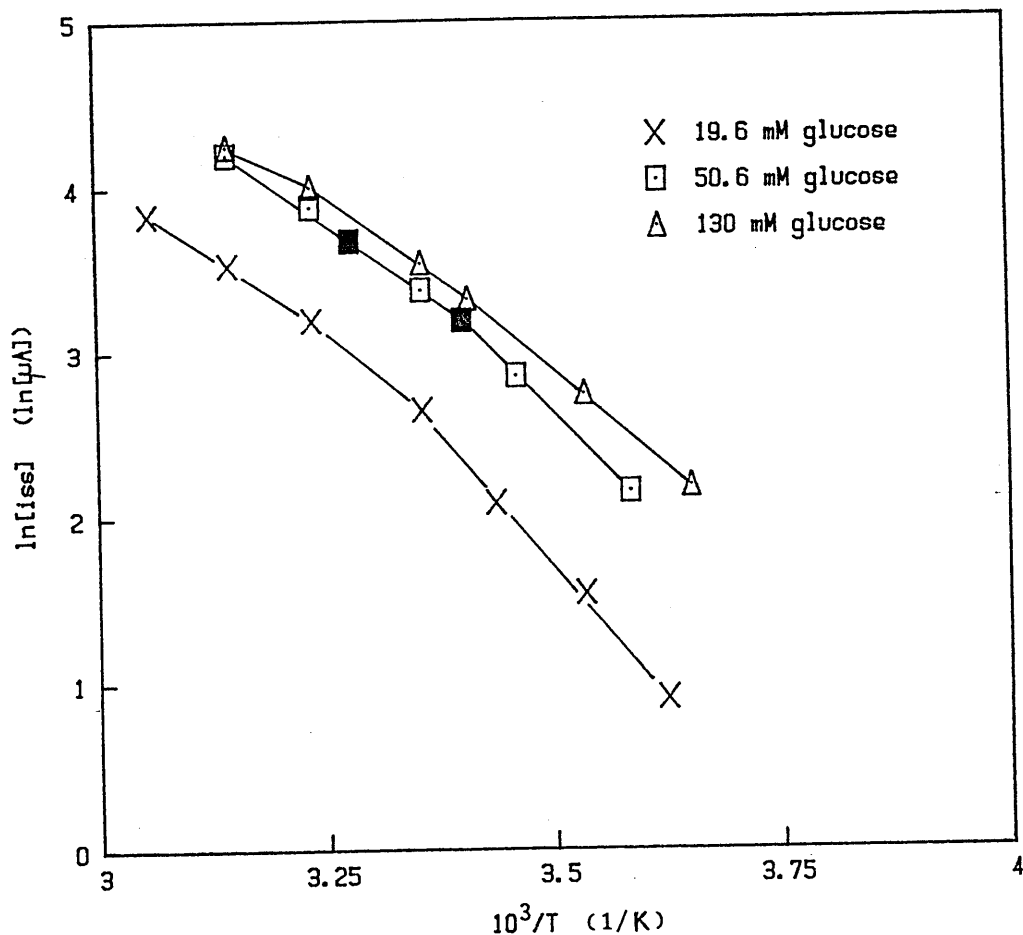
where E^* is the activation energy of the reaction, A is a constant and k is the rate constant for the reaction.

By plotting the reciprocal temperature ($1/T$, with T measured in Kelvin) against the logarithm of the rate constant, linear plots can be obtained with the slope equal to $-E^*/R$.

For immobilized enzyme systems the presence of diffusional resistances may result in a change in the shape of the Arrhenius plot (Engasser & Horvath, 1976). To investigate this possibility temperature-current profiles were conducted for a number of different enzyme electrodes at different glucose concentrations as shown in figure 4.25. It was found that small deviations from linearity were obtained at both saturating (130 mM) and non-saturating (19.6 & 50 mM) glucose concentrations. The first dog-leg observed in these plots was found to be similar in all three cases, and occurred at about 20 - 25°C.

Further deviations from linearity also occurred as the temperature increased, and these were probably due to enzyme inactivation or mediator loss (the melting point of DMFc being about 37°C). It should be noted that no hysteresis effect was observed when the temperature was increased and subsequently decreased, as

FIGURE 4.25.



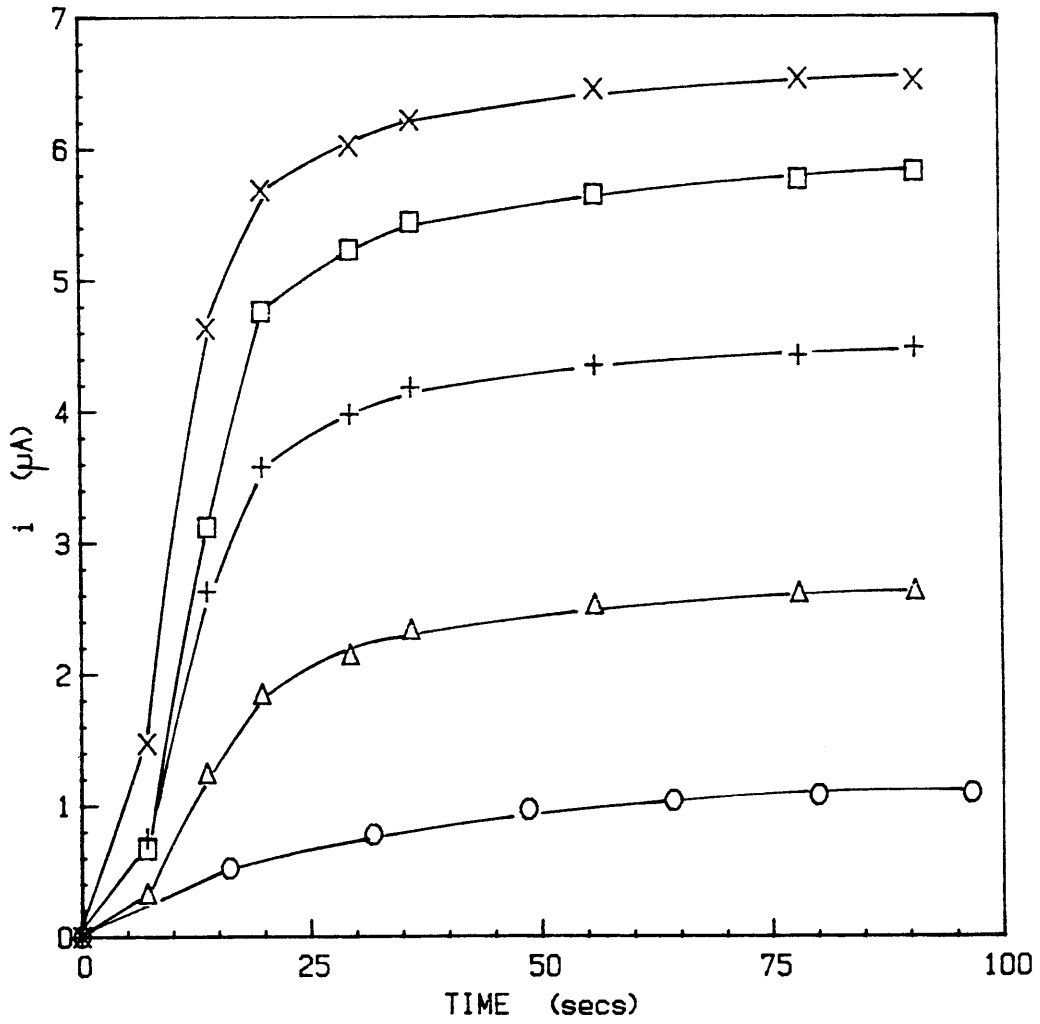
Arrhenius plots showing the effect of temperature on the steady-state currents from enzyme electrodes performed at different glucose concentrations. The experiments were carried out in 0.1 M phosphate buffer pH 7 containing 0.15 M KCl at an applied voltage of +235 mV vs. Ag/AgCl and under nitrogen sparging. The filled in points for the data at 50.6 mM glucose refer to steady-state currents measured on subsequent lowering of the temperature.

shown by the filled in points for the Arrhenius plot at 50 mM glucose in figure 4.25.

From the slopes of the Arrhenius plots shown in figure 4.25. rough values for the activation energy, E^* , may be obtained. The slopes were measured below 25°C, and gave values for E^* of 38.94 kJ mole⁻¹ at saturating glucose concentrations. The value of E^* appeared to increase as the glucose concentration became non-saturating, being 44.74 kJ mole⁻¹ at 50 mM glucose and 54.21 kJ mole⁻¹ at 19.6 mM glucose. This latter value compared well with the value obtained by Cass et al. (1984) of 50 kJ mole⁻¹ at 10 mM glucose. When values of $i_{\max}$ derived from Hanes plots were used instead of steady-state currents measured at saturating glucose levels, a lower activation energy of 32.68 kJ mole⁻¹ was obtained.

Because of these small deviations from linearity observed on the Arrhenius plots at both low and high temperatures it was of interest to examine whether temperature had any effect on the response behaviour of the enzyme electrode. The transient responses of two enzyme electrodes (averaged) with increasing temperature are shown in figures 4.26. and 4.27. at 6.6 mM and 74 mM glucose respectively. All the curves were observed to be similar, with limiting steady-state currents being achieved after approximately 60 seconds. However, a lag in the initial response was observed at the lower temperatures used, with this phenomenon being the most pronounced at 74 mM glucose and 1°C. These results were consistent with previously obtained data, such as the more linear calibration curves found with the U set of electrodes at 1°C relative to 25°C,

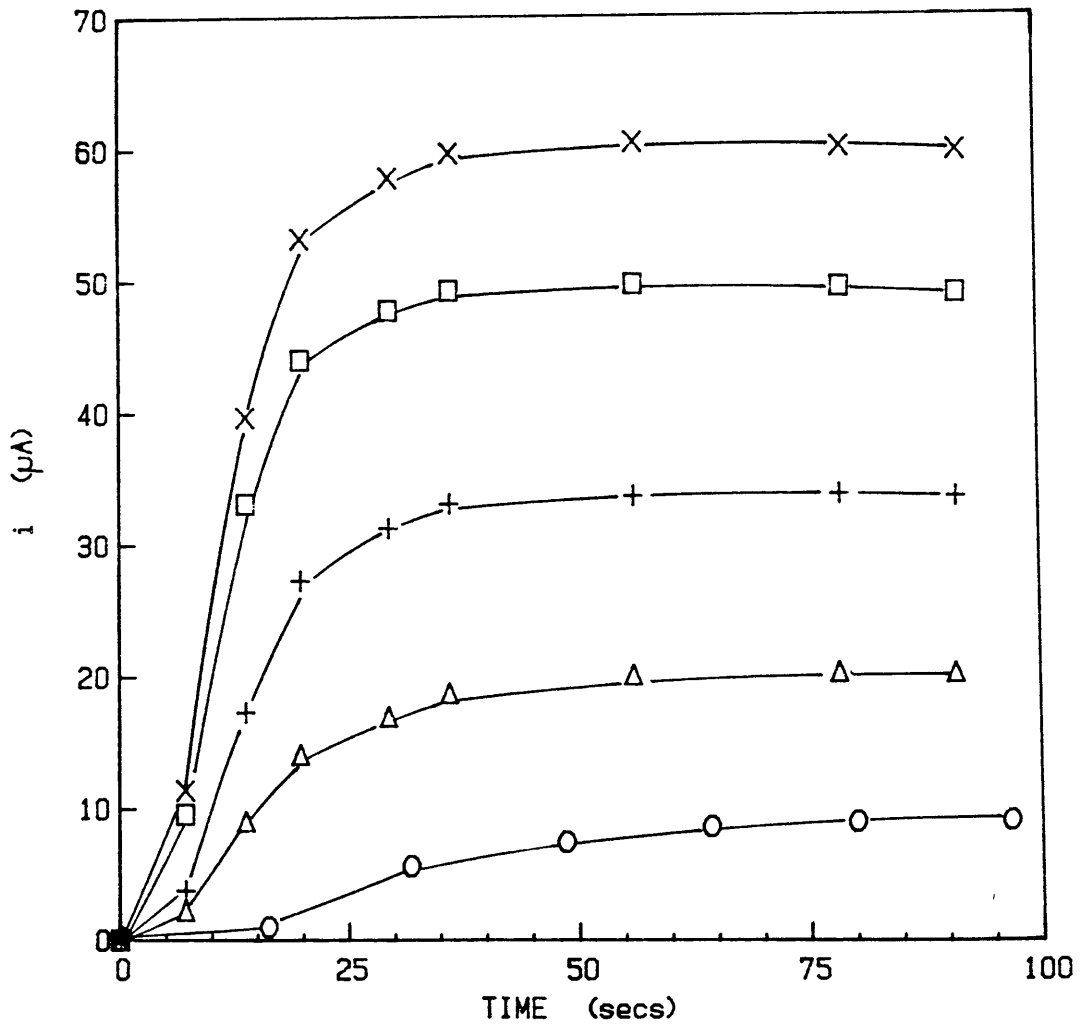
FIGURE 4.26.



Transient response curves of enzyme electrodes at
a low glucose concentration (6.6 mM) over the
temperature range 1 - 45°C.

○ 1°C	△ 15.5°C	+ 25°C
□ 36.5°C	× 45°C	

FIGURE 4.27.



Transient response curves of enzyme electrodes at a high glucose concentration (74.1 mM) over the temperature range 1 - 45°C.

○ 1°C	△ 15.5°C	+ 25°C
□ 36.5°C	X 45°C	

which demonstrated that the electrodes were more diffusion limited at 1°C than at 25°C.

4.15. Diffusional Inhibition of the Enzyme Electrode

The results presented in this chapter have shown that the glucose oxidase / dimethylferrocene graphite foil enzyme electrode exhibited a complicated relationship between the measured steady-state currents and the bulk glucose concentration, which was best explained by internal diffusional limitation within the porous electrode.

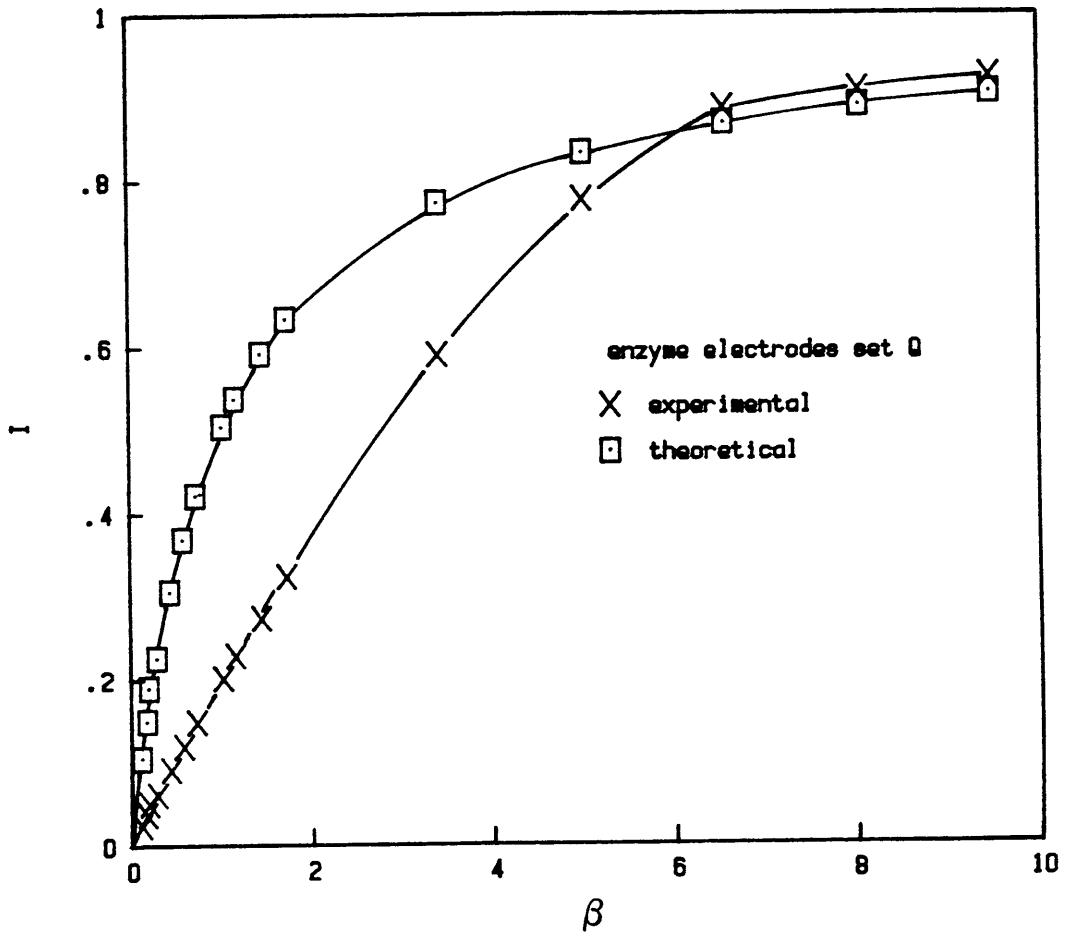
The non-linear plots shown in figures 4.6. & 4.7. demonstrated that only at saturating glucose concentrations using the Hanes plot could a reasonable estimate of the kinetics of the immobilized enzyme be made. At non-saturating glucose concentrations diffusion of glucose within the porous electrode material became a rate limiting factor. At glucose concentrations $< K'_m$ the measured currents appeared to be under diffusion control since this corresponded to the linear part of the calibration curve. At intermediate glucose concentrations a situation was observed where the measured currents were not clearly controlled by either diffusion or by enzyme kinetics.

This behaviour can be shown the most clearly by using dimensionless plots which allow both a comparison of enzyme electrodes exhibiting different amounts of enzyme activity, and also with theoretical plots of an immobilized enzyme layer confronted with

increasing degrees of diffusional inhibition. Here a dimensionless substrate concentration, β can be defined as $[S]/K'_m$, and a dimensionless current, I as i_{ss}/i_{max} . Figure 4.28. shows a dimensionless rate curve for the Q set of electrodes using the kinetic parameters K'_m and i_{max} determined from Hanes plots at saturating glucose concentrations. Alongside the experimental data is shown a theoretical curve derived using the same values of the two kinetic parameters but with values of i_{ss} calculated from the variation of the Michaelis-Menten equation given in section 4.1. equation [2]. It can be seen that at non-saturating glucose concentrations the parameter I is lower for the experimental data than the kinetic curve, indicating that the reaction is not kinetically controlled. This type of behaviour was observed by Mell & Maloy (1975) where $I/2$ was plotted against β for GOD immobilized inside polyacrylamide membranes. With thicker membranes the reaction became subject to greater diffusional limitations, which was characterized by increased values of the substrate modulus (section 1.3.) resulting in curves similar to those shown in figure 4.28.

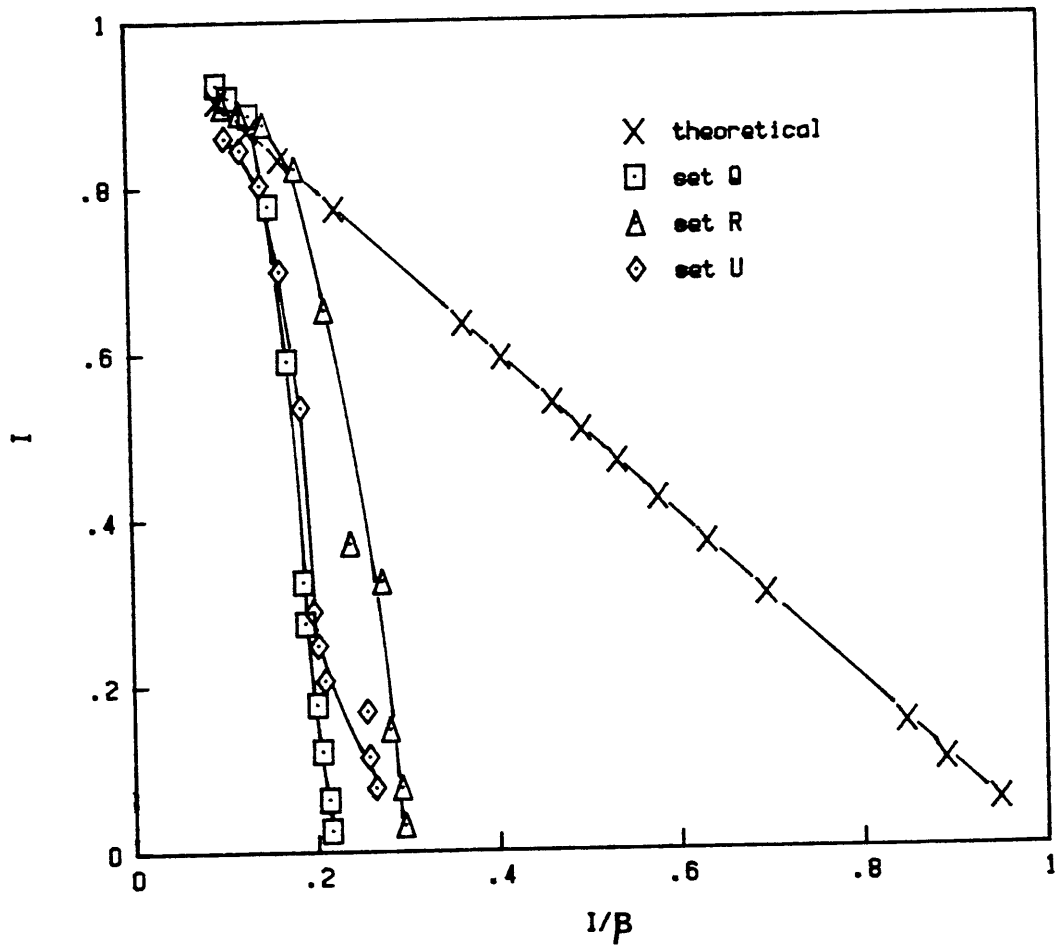
Engasser & Horvath (1974, 1976) used the substrate modulus ϕ to characterize the degree of internal diffusional limitation on an immobilized enzyme, and this was shown graphically using a dimensionless Eadie-Hofstee plot (figure 1.4.). Similar plots were made for three sets of enzyme electrodes (Q, R & U) and the results are shown in figure 4.29. with a theoretical plot (based on the Michaelis-Menten equation) added for comparison. For all three sets of enzyme electrodes deviation from the straight line

FIGURE 4.28.



Dimensionless calibration curves of the normalized current, I (i_{ss}/i_{max}) against β ($[S]/K'_m$) for the Q set of enzyme electrodes. For comparison a theoretical curve based on the Michaelis-Menten equation and the values of K'_m and i_{max} given in table 4.4. is shown.

FIGURE 4.29.



Dimensionless Eadie-Hofstee plots of the normalized current, I (i_{ss}/i_{max}) against I/β (where $\beta = [S]/K'_m$) for the Q, R and U sets of enzyme electrodes. For comparison a theoretical curve based on the Michaelis-Menten equation is also shown.

predicted for kinetic control is observed, and by comparison with figure 1.4. a rough value for the substrate modulus of $\phi = 5$ for the Q set of enzyme electrodes can be estimated, the larger diameter electrodes showing less diffusional inhibition.

From figure 4.28. it can be seen that at low values of β a linear relationship is observed between substrate concentration and response, indicating that the measured steady-state current is under diffusional control. Thus a first-order constant K may be defined where

$$K = \frac{K'_m}{\epsilon(\phi)} \quad [a]$$

and ϵ , which is a function of ϕ , is the effectiveness factor for this first-order regime. The three kinetic plots shown in figures 4.5. - 4.7. were used to derive values for K and ϵ according to the method proposed by Engasser & Horvath (1974). It was found that although the values of K obtained from the three different plots were almost identical, consistent values of ϵ could not be obtained. Equation [a] given above was therefore used to determine values of ϵ . An approximation given by Engasser & Horvath (1974) that when $\phi \geq 5$ for membrane entrapped enzyme $\epsilon \cong 1/\phi$ was employed to derive values of ϕ . Table 4.6. lists the values of K obtained from the kinetic plots and the values of ϵ and ϕ which were derived as outlined above.

The values of ϕ given in table 4.6. corresponded with the

TABLE 4.6.

KINETIC PARAMETERS FOR DIFFERENT ENZYME ELECTRODES DERIVED
FROM SECONDARY KINETIC PLOTS

ELECTRODE SET	SIZE	$i_{\max} (\mu\text{A})$	$K_m (\text{mM})$	κ	ϕ
Q	Small	37.87	11.31	50.95	4.50
R	Large	72.45	12.86	40.14	3.17
U	Large	124.00	13.50	50.05	3.71
U (1°C)		36.85	24.26	62.72	2.58 (a)4.65

(a) Taking $K_m = 13.50 \text{ mM}$

TABLE 4.7.

KINETIC PARAMETERS DERIVED FROM ENZYME ELECTRODE MODEL

ELECTRODE	$V_{\max}$ mole $\text{cm}^{-3} \text{ s}^{-1}$	D_{eff} $\text{cm}^2 \text{ s}^{-1}$	ACTIVE ENZYME BOUND μg
Q	2.67×10^{-7}	1.43×10^{-6}	0.12
R	3.91×10^{-7}	4.84×10^{-6}	0.23
U	6.69×10^{-7}	5.76×10^{-6}	0.39
U (1°C)	1.99×10^{-7}	1.97×10^{-6} $1.09 \times 10^{-6} \text{ a)}$	0.11

a) Taking $K_m = 13.50 \text{ mM}$ and $\phi = 4.65$

graphical representation given in figure 4.29., the substrate modulus for the Q set of enzyme electrodes being the largest. As discussed in section 1.3. larger values of ϕ are indicative of increased diffusional limitations within the immobilized enzyme layer. According to Mell & Maloy (1975) higher levels of immobilized enzyme activity would lead to increased dependence of the measured response on diffusion, and therefore result in larger values for ϕ . Whilst this relationship was observed between the U and R sets of enzyme electrodes, it did not appear to be the case with the Q set of electrodes, which may have been due to the different size of the latter.

As discussed briefly in section 4.14. calibration curves were also performed with the U set of electrodes at 1°C. The value of ϕ obtained from this data was calculated using the K'_m derived at both 1°C and 25°C. It should be noted that the value of ϵ obtained from equation [a], and hence ϕ , is dependent on the K'_m .

Having obtained approximate values of the substrate modulus ϕ for the graphite foil enzyme electrodes it should be possible to estimate the effective diffusional coefficient for glucose within the electrode material, D_{eff} , using the following equation (Engasser & Horvath, 1976) :

$$\phi = d (V'_{max} / D_{eff} \times K'_m)^{1/2} \quad [b]$$

where V'_{max} is the maximum velocity of the immobilized enzyme per unit of porous catalyst and d is the characteristic length of

the system. By definition (Castner & Wingard, 1984)

$$i_{\max} = n F A d k_3 C_E \quad [c]$$

where A is the electrode area and $k_3 C_E = V'_{\max}$ (mole s⁻¹ cm⁻³). In order to estimate the value of D_{eff} for the different enzyme electrodes certain assumptions were found to be necessary. These were firstly in the calculation of $V'_{\max}$ it was necessary to find an approximate value for the volume of electrode occupied by the bound enzyme. The volume of the small diameter electrode (0.35 cm radius) was calculated as 0.0147 cm³. Given that the density of the graphite foil is about 1 g/cm³, whereas the density of graphite varies from 1.8 - 2.1 g / cm³, it can be roughly calculated that graphite foil is approximately 50 % space. However, it is unlikely that the immobilized enzyme fully occupied all the space available. Consequently the value for the volume of the enzyme layers (i.e. A x d) was taken 1/10 of the electrode volume, i.e. 1.47×10^{-3} cm³. In the case of the larger diameter electrodes (0.4 cm radius) this value was calculated as 1.92×10^{-3} cm³. Thus for the Q set of enzyme electrodes :

$$V'_{\max} = \frac{i_{\max}}{n F A d} \quad [d]$$

$$V'_{\max} = \frac{37.87 \times 10^{-6}}{1 \times 96485 \times 1.47 \times 10^{-3}}$$

$$V'_{\max} = 2.67 \times 10^{-7} \text{ mole s}^{-1} \text{ cm}^{-3}$$

It should be noted that changes in the approximated value for the volume of electrode occupied by the enzyme, which will affect the calculated values of $V'_{\max}$, do not alter the calculation of the total amount of enzyme activity immobilized on the electrode. This is because the values of $i_{\max}$ were derived for each whole electrode, and do not therefore depend on the electrode area.

The second assumption made in this set of calculations was in the estimation of a value for d . Since as previously shown the measured steady-state currents are diffusion controlled at glucose concentrations $< K'_m$ it can be predicted that not all of the bound enzyme is in contact with glucose diffusing into the electrode from the bulk solution. This would be the situation facing for example enzyme immobilized in the centre of the graphite foil disc. For this reason the characteristic length of the system was taken as being one tenth that of the radius of the electrode, i.e. $d = r/10$. Thus for the Q set of electrodes $d = 0.035 \text{ cm}$, $\phi = 4.5$ and $V'_{\max} = 2.67 \times 10^{-7} \text{ mole s}^{-1} \text{ cm}^{-3}$. These values can be inserted into equation [b] to give :

$$\phi = d (V'_{\max} / D_{\text{eff}} \times K'_m)^{1/2} \quad [b]$$

$$D_{\text{eff}} = d^2 \times V'_{\max} / \phi^2 \times K'_m \quad [e]$$

$$= 1.43 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$$

This calculated value for the diffusion coefficient of glucose within the porous electrode material is about 5 times smaller than that for glucose in free solution ($6.7 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$). Similar calculations were carried out for the R & U sets of electrodes and the results are given in table 4.7. It can be seen that the calculated value for D_{eff} in the case of the U set of enzyme electrodes was about 3 - 4 fold smaller at 1°C than at 25°C .

The dependence of the calculated values of D_{eff} on both V'_{max} and d is quite apparent from equation [e], with D_{eff} being proportional to $d^2 V'_{\text{max}}$. The amount of enzyme activity, expressed as μg of soluble enzyme activity, immobilized on the different enzyme electrodes can be calculated either from $V'_{\text{max}} \times A d$ or from $v_t = i_{\text{max}}/nF$ (where v_t is total enzyme activity of an electrode). Thus for the Q set of electrodes the total enzyme activity was calculated as $3.92 \times 10^{-10} \text{ mole s}^{-1}$, or $0.024 \mu\text{mol min}^{-1}$ (units).

Taking the specific activity of GOD in solution as 2×10^5 units / g gives a value of $0.12 \mu\text{g}$ of soluble enzyme activity immobilized. Table 4.7. lists the calculated values for the amounts of soluble activity immobilized on the different sets of electrodes. Mell & Maloy (1975) found that only 1 % of the enzyme immobilized in polyacrylamide gels was active, and Castner & Wingard (1984) showed that according to electrochemical measurements between 0.5 - 13 % of the enzyme immobilized onto platinum electrodes was active. Assuming that about 1 % of the bound enzyme

was active, then the total amount of enzyme bound onto the graphite foil electrodes varied from 11 - 39 μg . These values would explain why Bradford assays were unable to detect any change in the concentration of GOD in the immobilization solutions before and after the immobilization step, since these amounts of enzyme in the combined washings would have been below the limits of detection of this technique (Bradford, 1976).

CHAPTER 5

ELECTROCHEMICAL STUDIES OF IMMOBILIZED LIPOAMIDE DEHYDROGENASE

INTRODUCTION

5.1 NAD(H)-Linked Dehydrogenases

There are currently over 2,000 known enzymes which catalyse a wide range of chemical reactions. These have been placed into six general categories by the International Union of Biochemistry (table 5.1.). The first group of enzymes in this list are those which catalyse oxidation-reduction reactions, such as the flavoproteins, ferredoxins and quinoproteins. One of the largest groups of redox enzymes are those dehydrogenases which require one of the nicotinamide coenzymes, NAD(H) or NADP(H) for catalytic activity. The structure of these coenzymes are shown in figure 5.1., which depicts the portion of the molecule which is responsible for electron transfer.

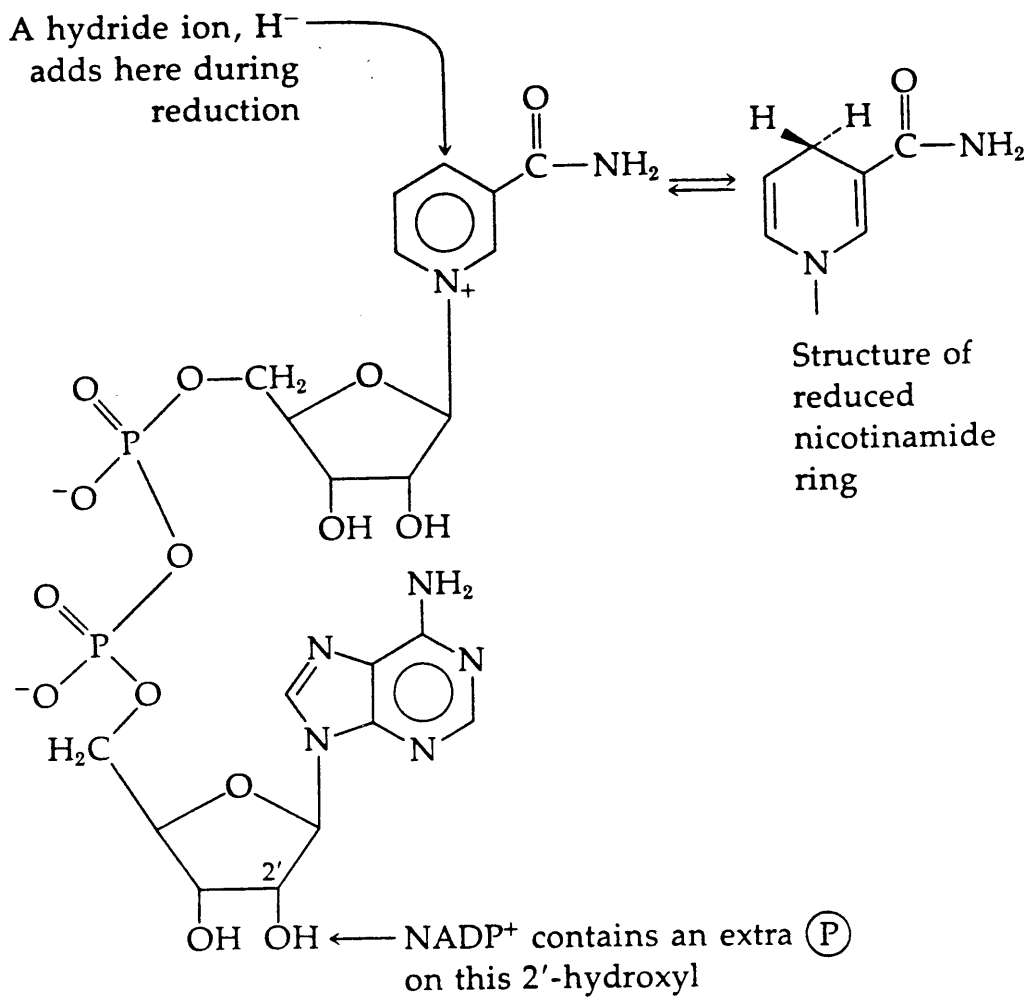
Oxidation or reduction of NAD(P)(H) is a two-electron process with electrons being transferred to (or from) the 4 position on the pyridine ring as a hydride ion (H^-). The second atom of hydrogen (as H^+) is derived from water (i.e. from H_3O^+). The nicotinamide coenzymes are only bound to dehydrogenases during the electron transfer reaction, and play a role in vivo as a pool of reducing equivalents which may be channeled to provide energy, for

TABLE 5.1.

INTERNATIONAL CLASSIFICATION OF ENZYMES

1. OXIDO-REDUCTASES : oxidation-reduction reactions
 - 1.1. Acting on CH-OH
 - 1.2. Acting on C=O
 - 1.3. Acting on C=CH-
 - 1.4. Acting on CH-NH₂
 - 1.5. Acting on CH-NH-
 - 1.6. Acting on NADH ; NADPH
2. TRANSFERASES : transfer of functional groups
3. HYDROLASES : hydrolysis reactions
4. LYASES : addition to double bonds
5. ISOMERASES : isomerization reactions
6. LIGASES : formation of bonds with ATP cleavage

FIGURE 5.1.



Structure of nicotinamide adenine dinucleotide (NAD)
 showing the oxidized and reduced forms of the coenzyme.

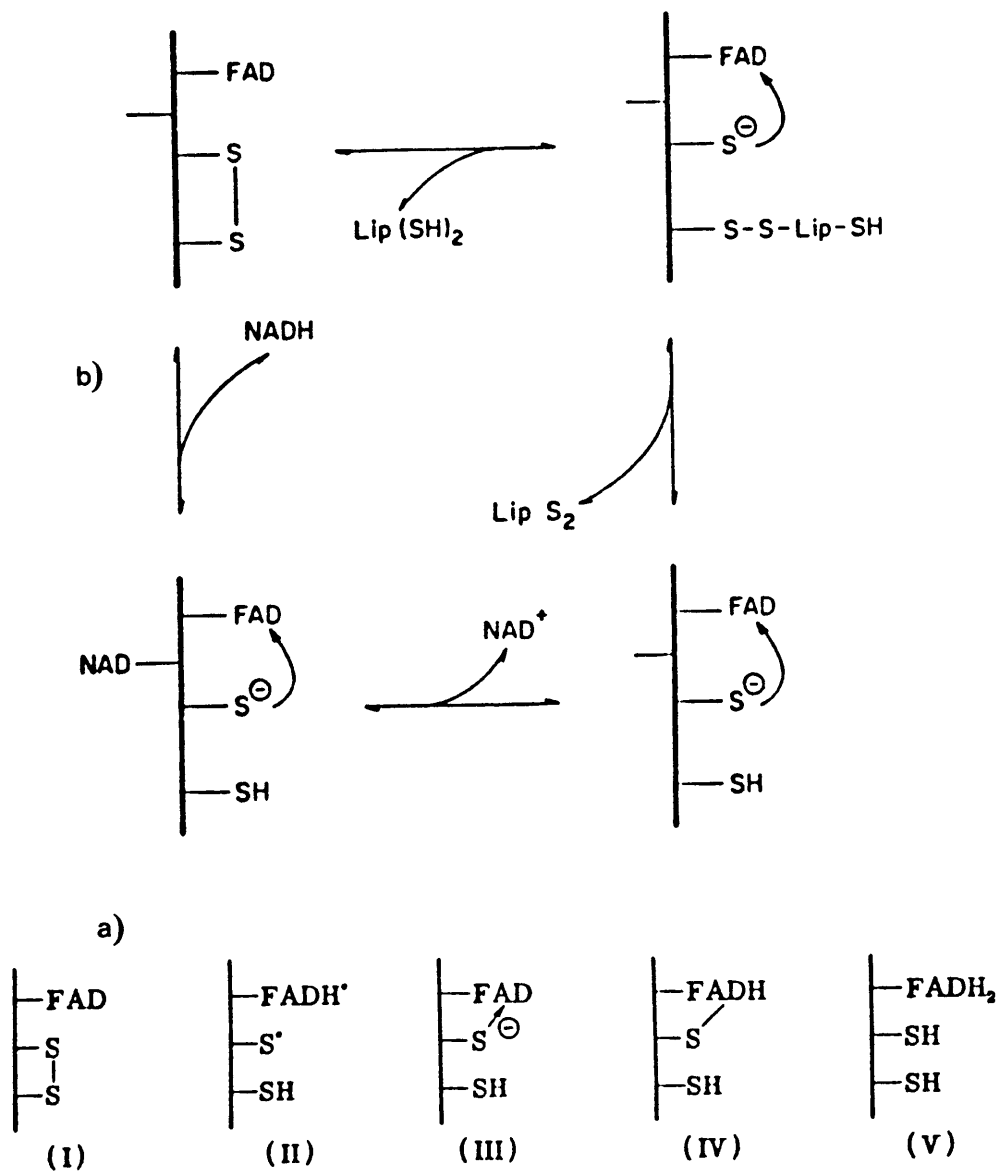
example in the electron transport chains of mitochondria, or in anabolic metabolism (synthesis of new biological material).

5.2. Lipoamide Dehydrogenases

The oxidation of pyruvate, succinate and α -ketoglutarate is catalysed by multi-enzyme complexes in many organisms. One of the component enzymes of these complexes is lipoamide (lipoyl) dehydrogenase (LipDH), also referred to as diaphorase. The natural substrates of LipDH are enzyme-bound dihydrolipoic acid and NAD^+ . The substrate specificity of these enzymes is quite broad, with both derivatives of lipoic acid and suitable pyridine analogues of NAD^+ acting as substrates. The enzyme can also use electroactive compounds as oxidants such as potassium ferricyanide, methylene blue, dichlorophenolindophenol (DCPIP) and vitamin K_3 . The properties of LipDH from different sources has been reviewed by Massey (1963) and Williams (1976).

LipDH from porcine heart has a molecular weight of about 110,000 daltons, and is comprised of two subunits, each containing one FAD molecule and one active disulphide group. During catalysis the enzyme cycles between the oxidized state (figure 5.2.I) and a spectrophotometrically characteristic reduced state in which the two electrons transferred to each subunit of the enzyme are shared between the FAD and the disulphide. This half-reduced form of the enzyme EH_2 is catalytically active, whereas the fully reduced form of the enzyme EH_4 is inactive

FIGURE 5.2.



Schematic showing a) the different oxidation states of lipoamide dehydrogenase : (I) represents the fully oxidized state E ; (II) - (IV) the half-reduced state EH_2 ; (V) the fully reduced form EH_4 ; and b) the mechanism of action with lipoamide (Lip(SH)₂) as substrate.

(figure 5.2.V). The exact chemical nature of EH_2 is not known, and cannot be adequately described by any single nomenclature. Structures which have been given to EH_2 are shown in figure 5.2.II - IV ; structure II represents a biradical, III a charge transfer complex in which the thiol is a donor and FAD the acceptor, and in structure IV a covalent bond is postulated to exist between the FAD and one of the sulphur atoms of the cysteine group (Williams, 1976).

5.3. Regeneration of Nicotinamide Coenzymes

At present the most industrially important class of enzymes are the hydrolases, for example carbohydrases (amylases, amyloglucosidases, cellulases), aminoacylases, amidases (e.g. penicillin amidase) and proteases. This widespread use of hydrolytic enzymes is partly due to the simplicity of their catalytic action. Many of the classes of enzymes listed in table 5.1. require additional inorganic or organic cofactors to be present for catalytic activity. Thus the industrial use of NAD-linked oxidoreductases has been limited by the coenzyme requirement of these enzymes. Use of stoichiometric quantities of the coenzyme is prohibited on a cost basis, and research into industrial applications of these enzymes has been directed in part towards developing recycling methods for regenerating the required form of the coenzyme. With efficient regeneration only catalytic amounts of the coenzyme are necessary (Jones & Beck, 1976).

There are three general methods of recycling NAD(H) ; chemical, enzymatic and electrochemical. Various compounds are capable of coupling to NAD(H) such as sodium dithionite, flavin mononucleotide (FMN) and its derivatives, ferricyanide, various quinones (Cenas et al., 1984 ; Carlson & Miller, 1985), phenazine methosulphate (PMS) and DCPIP (Jones & Beck, 1976). The advantage of this type of recycling method is the low cost of the chemical catalysts. Disadvantages of this method are separation of these low molecular weight materials from the system, undesirable side reactions and especially the low efficiency of this method. Chemical methods of NAD(H) recycling have been reviewed by Jones & Beck (1976).

Numerous enzymatic techniques have been investigated for recycling of NAD(H). The simplest method is the single substrate technique, where the form of the coenzyme required for the desired reaction is regenerated by the same enzyme via a secondary substrate. One application of this method has been the synthesis of deuterated compounds, which would be difficult to achieve using conventional techniques. In this scheme lactaldehyde is reduced to deuterated propane-1,2-diol by alcohol dehydrogenase employing 1-[²H]-ethanol as the auxillary substrate (Jones & Beck, 1976). More commonly used is the coupled enzyme method where the required form of the cofactor for the main reaction is regenerated via a second enzyme. Coupled enzyme methods have been reported using immobilized enzymes, and also employing immobilized coenzyme, and have been reviewed by Jones & Beck (1976), Wang & King (1979)

and Simon et al. (1985).

Electrochemical methods for the direct oxidation of NADH or reduction of NAD^+ have been reported. The theoretical advantage of electrochemical regeneration is the simplicity of the technique. Oxidation of NADH proceeds at both graphite and platinum electrodes at an overvoltage depending on the electrode material. One of the major problems encountered, especially with graphite electrodes, is fouling of the electrode by adsorption of NAD^+ (Cenas et al., 1983b) which becomes more pronounced as the NADH concentration increases. Enzymatically active NAD^+ has been electrochemically produced from NADH using both bare and chemically modified electrodes. For example Aizawa et al. (1975) employed a bare platinum wire electrode at a potential of +0.7 V vs. SCE, and Huck et al. (1984) have reported the catalytic oxidation of NADH on graphite electrodes modified by the dye 3- β -naphthoyl-Nile blue. The oxidation of NADH on modified electrodes has been reviewed recently by Gorton (1986).

Electrochemical reduction of NAD^+ is complicated by dimerization of the 1 electron reduced form of the coenzyme. This has been overcome by employing NAD^+ immobilized onto alginic acid (Aizawa et al., 1976a). A similar method has been reported for the reduction of immobilized NADP^+ (Aizawa et al., 1976b). Efficient reduction of the bound coenzyme was obtained, although the rates of reduction of the NAD-alginate complex reported were low.

Another approach to the problem of NADH regeneration has been

to combine electrochemical and enzymatic methods. Thus NADH may be regenerated by an NAD^+ reductase, for example lipoamide dehydrogenase, and coupled to a suitable mediator such as methyl viologen. The reduced form of methyl viologen can subsequently be replenished electrochemically. This type of recycling method has been reported by Ito & Kuwana (1971) for the enzyme ferredoxin-NADP-reductase, by DiCosimo et al. (1981) using immobilized lipoamide dehydrogenase, and has been reviewed recently by Simon et al. (1985). This approach to the problem of NADH recycling combines both the specificity of enzyme catalysis (ensuring that the NADH produced is in the biologically active form) with the efficiency of electrochemical techniques.

MATERIALS AND METHODS

5.4. Immobilization of Lipoamide Dehydrogenase

Glutaraldehyde activation was used to immobilize lipoamide dehydrogenase (LipDH) from porcine heart (supplied by BCL, The Boehringer Corporation Ltd. Lewes, England) onto both graphite foil and graphite felt electrodes.

Immobilization onto graphite foil electrodes was carried out as described for glucose oxidase (section 4.4.), the concentration of enzyme used being 1-2 mg / ml, and the reaction being allowed to continue overnight at 4°C.

LipDH was bound onto 4 cm² squares of graphite felt using

different concentrations of glutaraldehyde, the enzyme immobilization step being carried out as described above for the graphite foil electrodes.

The graphite felt electrodes were cut out in squares with a thin strip of material left on one side as an electrical contact.

5.5 Measurement of Activity of Graphite Foil Electrodes

Steady-state currents from LipDH modified electrodes were measured using the BBC microcomputer-based system (section 2.3) as described for the glucose oxidase electrodes in section 4.5. These experiments were carried out without nitrogen sparging in 0.1 M phosphate buffer pH 7 containing 0.15 M KCl. A preconditioning regime was also used for these electrodes as described for the glucose oxidase electrodes (section 4.5.) at 1.4 mM NADH.

5.6. Measurement of Activity of RVG Electrodes

The methyl viologen mediated reduction of NAD^+ by LipDH bound to graphite felt electrodes was conducted in a three compartment electrochemical cell (figure 2.4.). A platinum gauze counter electrode and a SCE reference electrode were used.

All three cell compartments were filled with 0.5 M phosphate buffer pH 7 containing 0.5 M KCl and sparged with white spot nitrogen. Both the working and auxillary compartments were degassed throughout the experiment. A potential of -0.7 V vs. SCE

was applied to the modified graphite felt electrode using a Ministat potentiostat (H.B. Thompson & Associates, Newcastle Upon Tyne, UK) and bulk reduction of methyl viologen continued until a stable background current was obtained. At this point an addition of NAD^+ into the system was made and the increase in current measured using a single pen Gould chart recorder (Gould Bryans Instruments Ltd., Mitcham, Surrey, UK). Throughout the experiment 100 μl aliquots of the reaction mixture were taken, diluted immediately with 400 μl of buffer and mixed using a rotamix (Hook & Tucker Instruments Ltd., Croyden, UK) to oxidize all the viologen present. The absorption at 340 nm was then measured. The current due to methyl viologen reduction was monitored continuously with a multimeter and a record made using the Gould chart recorder.

RESULTS & DISCUSSION

5.7. Development of an NADH-Sensing Enzyme Electrode

The glucose sensor based on the co-immobilization of GOD and DMFc onto graphite foil electrodes (Chapter 4) demonstrated many useful properties including a quick response time and a linear relationship between substrate concentration and steady-state response over a wide range of glucose concentrations. These results prompted research into whether an effective biosensor for NADH could be constructed by replacing GOD with LipDH. It had been

concurrently shown that DMFc could act as an effective oxidant for the soluble enzyme (Davis, 1985).

5.7.1. Response Behaviour of Enzyme Electrodes

Enzyme electrodes using glutaraldehyde crosslinked LipDH were assayed for their ability to oxidize NADH under identical conditions employed for the GOD-based enzyme electrodes. Steady-state currents were obtained with these modified electrodes over the concentration range 0.1 - 36 mM NADH. The response behaviour characteristics were comparable to the GOD electrodes, steady-state currents being obtained 60 to 90 seconds after addition of NADH.

The absence of limiting currents at high NADH concentrations, however, suggested that the measured NADH oxidation was not due simply to enzyme turnover. The K_m for NADH of LipDH is of the order of 0.2 mM (Williams, 1976), and although it was expected to observe enzyme activity at NADH concentrations up to $10 \times K_m$, the absence of NADH saturation at $100 \times K_m$ suggested that the steady-state currents observed were not simply the result of enzymatic NADH oxidation (by comparison with the glucose enzyme electrode described in Chapter 4).

The effect of increased ionic strength on steady-state currents was investigated since it has been reported that the enzyme may be activated by higher ionic strength (Massey, 1963). It was found, however, that the opposite effect was observed for these enzyme electrodes, the current decreasing when higher KCl

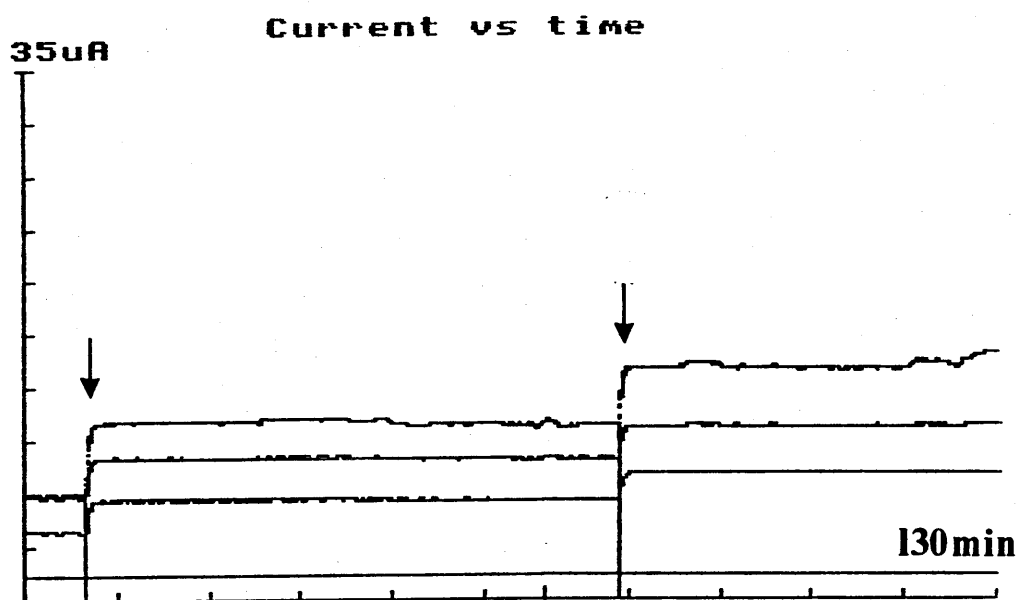
concentrations were used. This may have been due to the fact that increasing the chloride ion concentration lowers the potential of the Ag/AgCl reference electrode.

The steady-state currents from these electrodes were observed to be more stable than those produced by the glucose electrodes (see for example figure 4.7.). As shown in figure 5.3. no drop in current was observed over the course of a 3 hour experiment at two NADH concentrations. These levels of current, however, were not reproducible; the steady-state current measured at a fixed NADH concentration decreased consecutively over the course of 9 assays. This effect can be attributed to fouling of the electrode surface by NADH and its oxidation products. Previous workers have reported fouling of electrodes during bulk oxidation of NADH, which necessitated periodic cleaning of the electrode surface (Wallace & Coughlin, 1977).

The differences in behaviour between these electrodes and the GOD-based electrodes indicated that non-enzymatic oxidation of NADH could be taking place at the electrode surface, thereby masking the expected behaviour of any immobilized LipDH present.

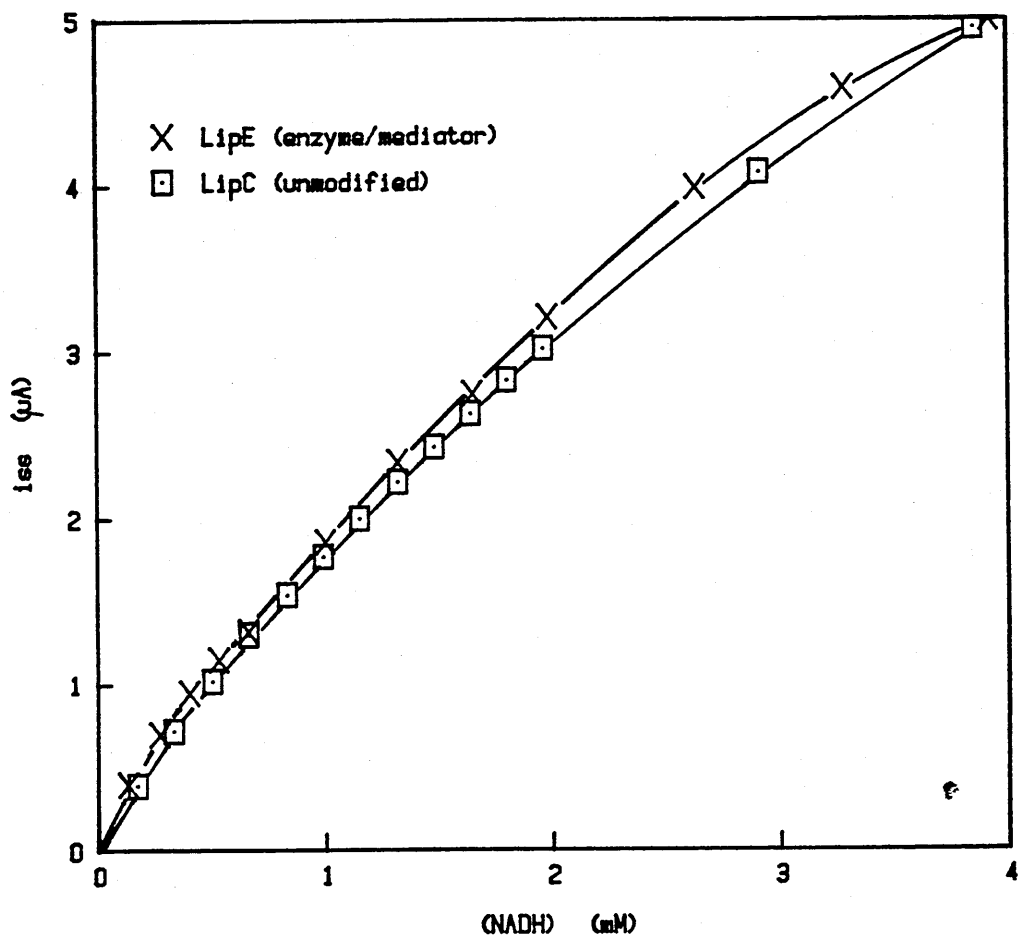
The calibration experiments were therefore repeated using plain graphite foil electrodes without either mediator or enzyme. The results of this set of electrodes, and the set of enzyme electrodes described above, are shown in figure 5.4. over the range 0.1 - 4 mM NADH. Each curve is an average of three enzyme (lipE) and four unmodified (lipC) electrodes. It was observed that the two curves were almost identical over the range 0.1 - 4 mM NADH,

FIGURE 5.3.



Computer printout of current vs. time for graphite foil electrodes modified with dimethylferrocene and lipoamide dehydrogenase. The experiment was carried out in 0.1 M phosphate buffer pH 7 containing 0.15 M KCl at room temperature and an applied voltage of +235 mV vs. Ag/AgCl. Arrows indicate the additions of NADH made to the cell.

FIGURE 5.4.



Calibration curves of steady-state current against NADH concentration for bare (unmodified) graphite foil electrodes (LipC), and graphite foil electrodes modified with lipoamide dehydrogenase and dimethylferrocene. The experiments were carried out in 0.1 M phosphate buffer pH 7 containing 0.15 M KCl at room temperature and an applied voltage of +235 mV vs. Ag/AgCl and under nitrogen sparging

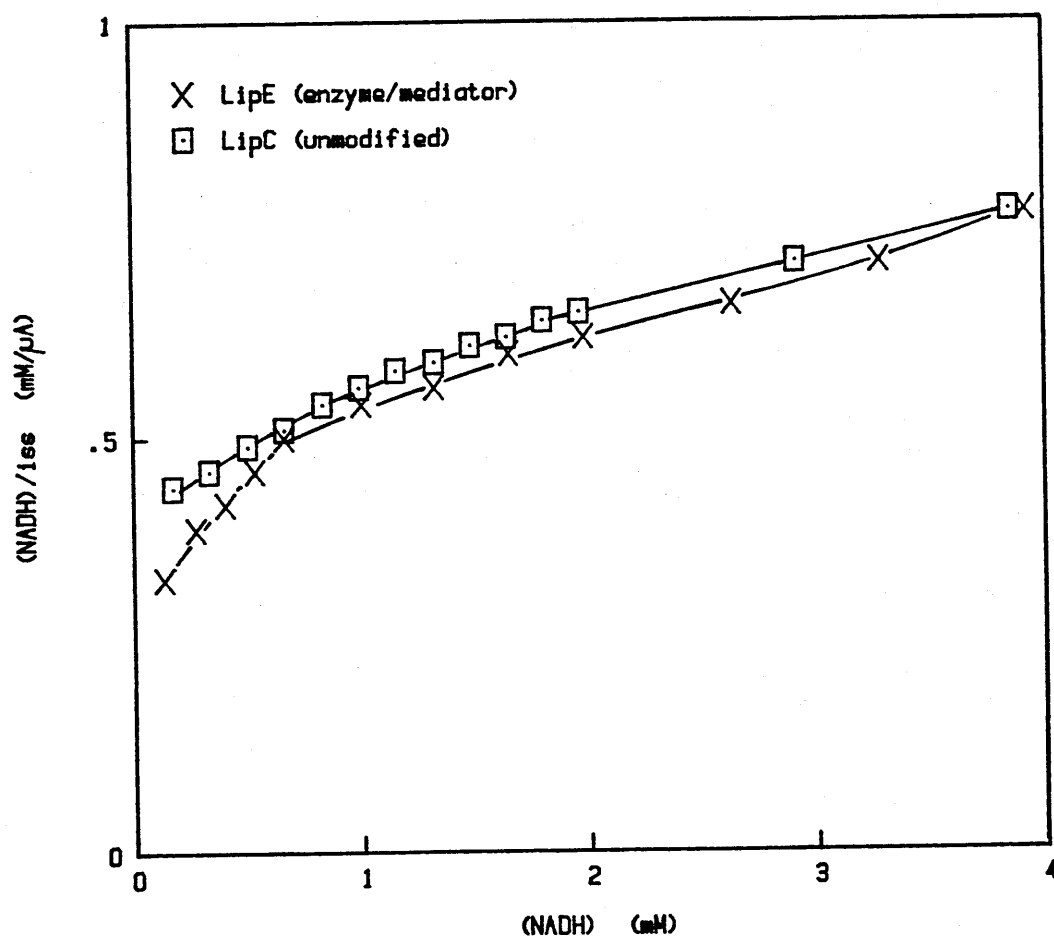
the enzyme-modified electrodes producing a steady-state current approximately 0.1 μA more than the bare electrodes, although differences of up to 1.5 μA were noted at higher NADH concentrations ($> 7 \text{ mM}$).

Hanes plots of the data (figure 5.5.) similarly showed little differences between the enzyme-modified and bare electrodes, although the plot for the enzyme-modified electrodes appeared to be more curved. Interpretation of these secondary plots is difficult, especially given the small differences between the current due to direct electrochemical oxidation of the NADH, and that attributable to enzymatic oxidation.

5.7.2. Electrochemical Studies of the Oxidation of NADH at Graphite Foil Electrodes

The potential employed in the calibration experiments of both bare and modified graphite foil electrodes (+235 mV vs. Ag / AgCl, +275 mV vs. SCE) represented an overvoltage of 500 mV compared to the standard redox potential of the $\text{NAD}^+ / \text{NADH}$ couple, but was lower than many reported values for the anodic peak potential for NADH oxidation on graphite electrodes. Braun et al. (1975) reported a peak potential of +0.67 V for NADH oxidation at glassy carbon electrodes, and Tse & Kuwana (1978) obtained a value of +0.45 V using radiofrequency treated pyrolytic graphite electrodes. Lower anodic peak potentials have been reported by Torstensson & Gorton (1981) using chemically modified spectro-

FIGURE 5.5.

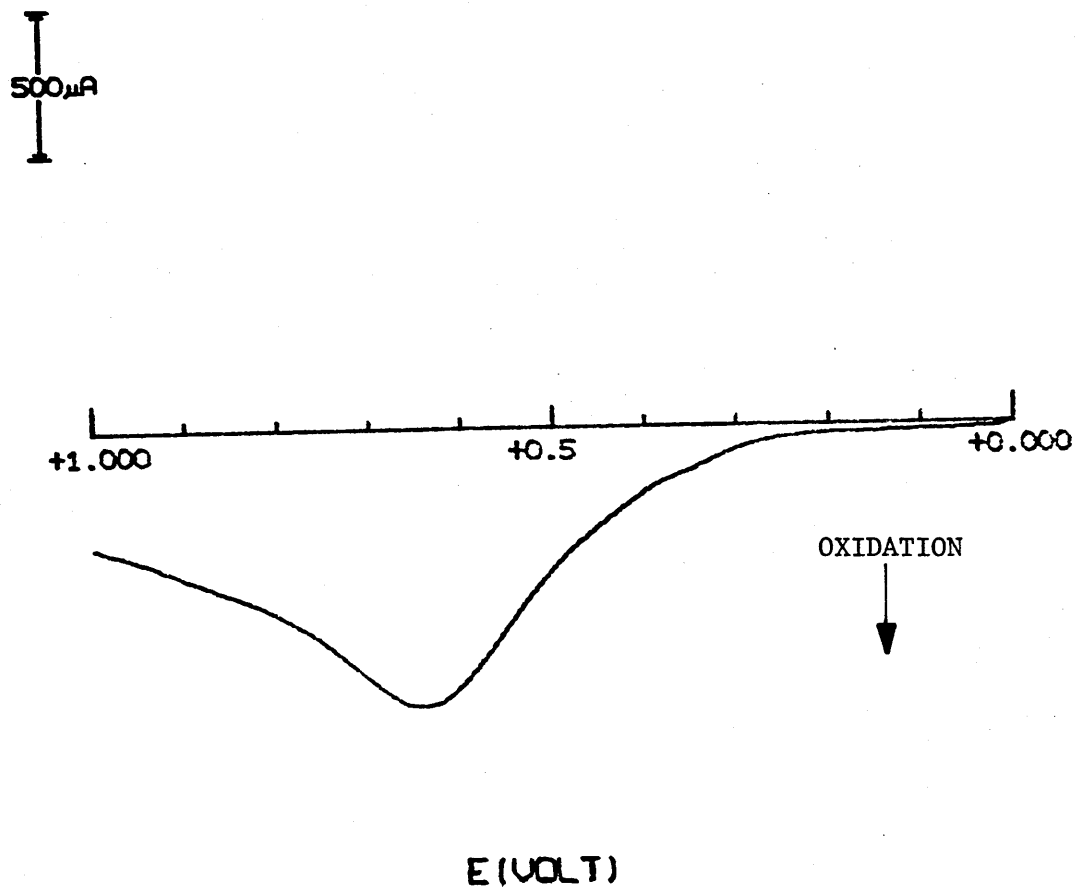


Hanes plots of $[NADH]/i_{ss}$ against $[NADH]$ derived from the data shown in figure 5.4. for bare (LipC) and enzyme (Lip E) graphite foil electrodes.

graphic graphite electrodes. Peak potentials of -50 mV and -100 mV were obtained respectively with phenazine methosulphate (PMS) and phenazine ethosulphate (PES) modified electrodes. More recently NADH oxidation has been shown to proceed effectively on electrodes made from organic salts such as NMP^+ and TCNQ^- at potentials of about +100 mV (Kulys, 1981 ; Albery *et al.*, 1985). All the above potentials are given with respect to the saturated calomel electrode (SCE). Gorton (1986) has recently reviewed the use of chemically modified electrodes for the oxidation of NADH to NAD^+ .

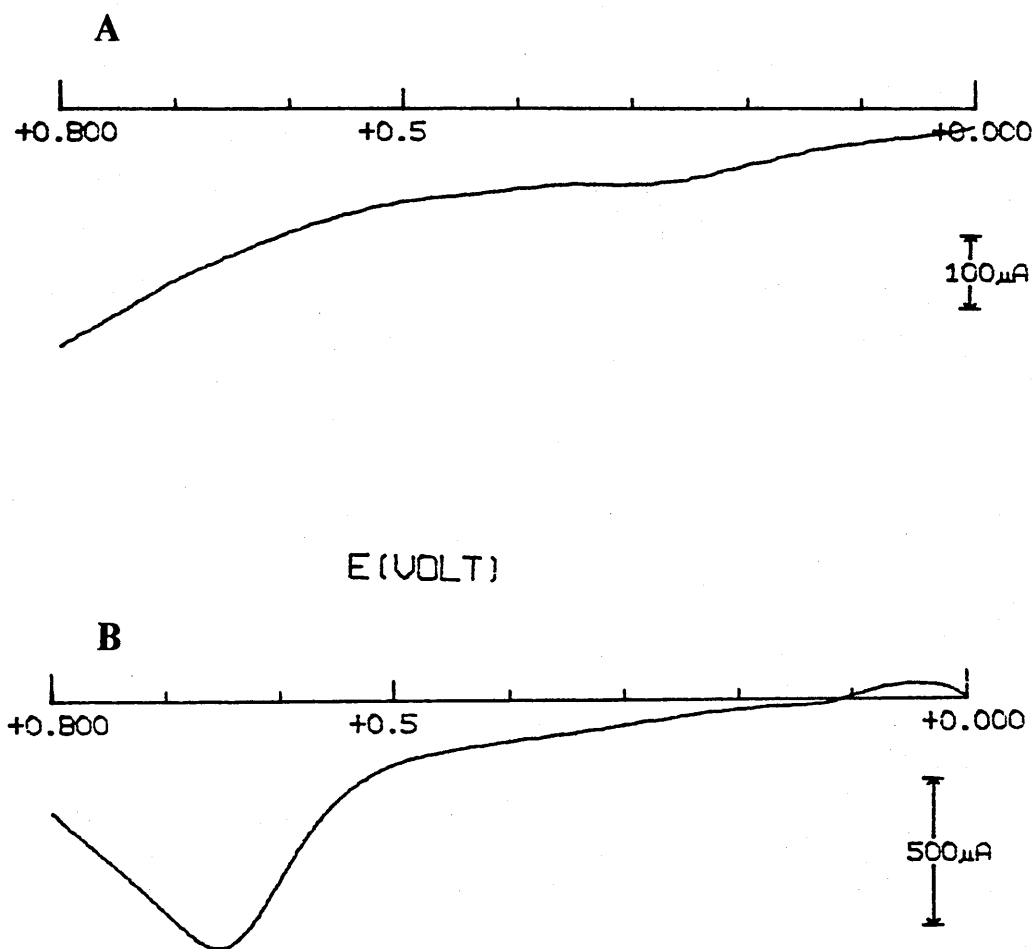
It was of interest therefore to examine the effect of DMFc on the oxidation of NADH on the graphite foil electrode. Initially linear sweep voltammetry (LSV) was used to investigate the oxidation of NADH on both graphite foil and glassy carbon electrodes. These experiments were performed using the same electrolyte as employed for the calibration experiments (0.1 M phosphate buffer pH 7 containing 0.15 M KCl) at different NADH concentrations. At 10 mM NADH graphite foil and glassy carbon electrodes gave half-wave potentials of +640 and +586 mV respectively. Figure 5.6. shows the voltammogram obtained with an unmodified graphite foil electrode. When LSV was used with DMFc-modified electrodes a small peak for the adsorbed mediator was observed (figure 5.7.a). In the presence of 10 mM NADH this modified electrode gave a similar voltammogram to the unmodified electrode (figure 5.7.b). This result suggested that NADH oxidation was not coupling to the adsorbed mediator. From figure 5.7.a it can be seen that a well-

FIGURE 5.6.



Linear sweep voltammogram of a 10 mM solution of NADH in 0.1 M phosphate buffer containing 0.5 M KCl at a graphite foil electrode at a sweep rate of 100 mV s^{-1} . Background correction was employed in obtaining this voltammogram.

FIGURE 5.7.



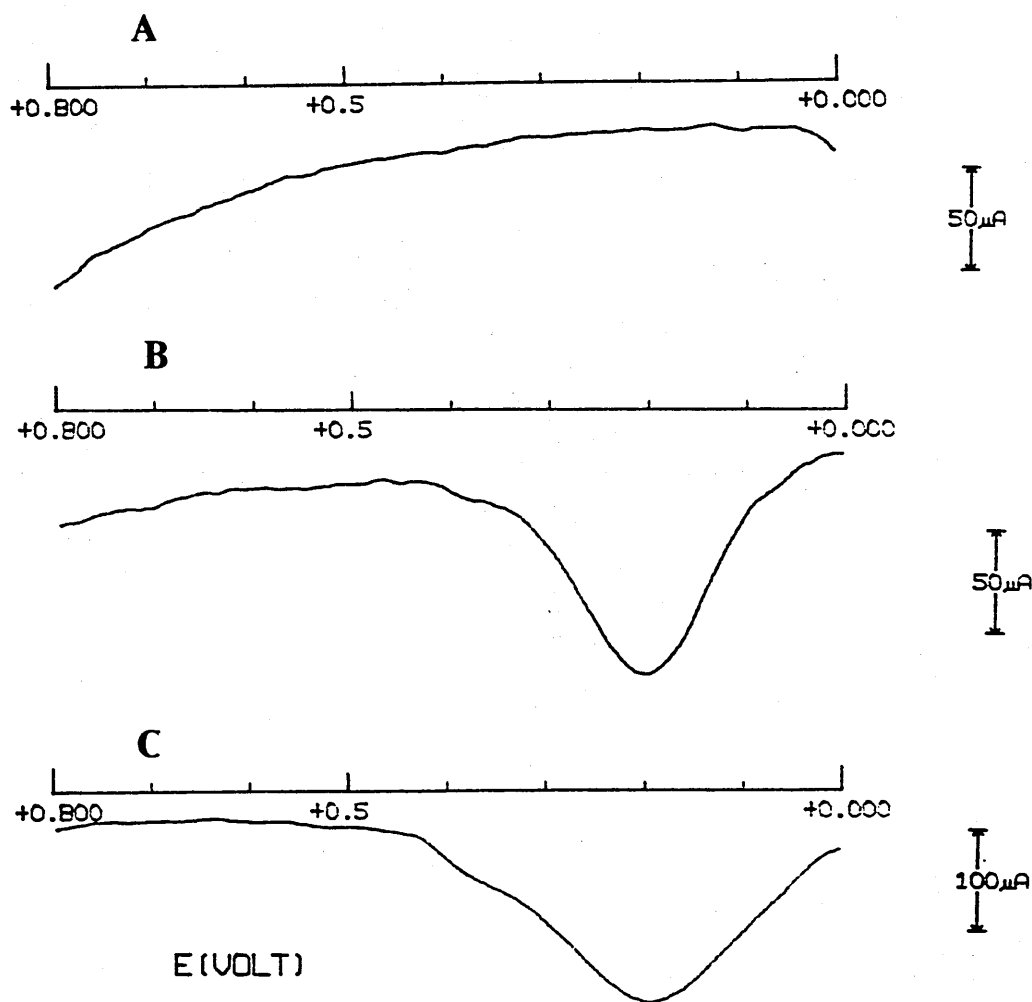
Linear sweep voltammograms of a graphite foil electrode modified with dimethylferrocene at 100 mV s^{-1} in :
(a) 0.1 M phosphate buffer containing 0.5 M KCl, and
(b) in the same solution containing 10 mM NADH. IR compensation was employed when obtaining these voltammograms.

defined peak for DMFc oxidation was not evident. This was partly due to large background currents observed with the graphite foil. By using differential pulse voltammetry (DPV) oxidation of the adsorbed mediator could be readily observed. In DPV small amplitude pulses (50 mV) are superimposed upon a staircase waveform (the potential is scanned in small steps), and the current is sampled just before and after each pulse. Since large differences between these two current samples are only observed in the region around the E^0 of a redox species, a peak output is obtained.

Differential pulse voltammograms of graphite foil electrodes before and after modification with DMFc are shown in figure 5.8.a and 5.8.b respectively. When the experiment with the DMFc-modified electrodes were repeated in the presence of 1 - 10 mM NADH no separate current peak for NADH oxidation was observed, as shown in figure 5.8.c (at 1 mM NADH), although an increase in the DMFc peak was noted. However, it was difficult to achieve reproducible voltammograms of NADH at unmodified electrodes, which makes it difficult to conclusively state that coupling between the adsorbed mediator and NADH was occurring. However, the results obtained with LSV indicated that if the adsorbed mediator is oxidizing NADH then it must be a relatively slow process.

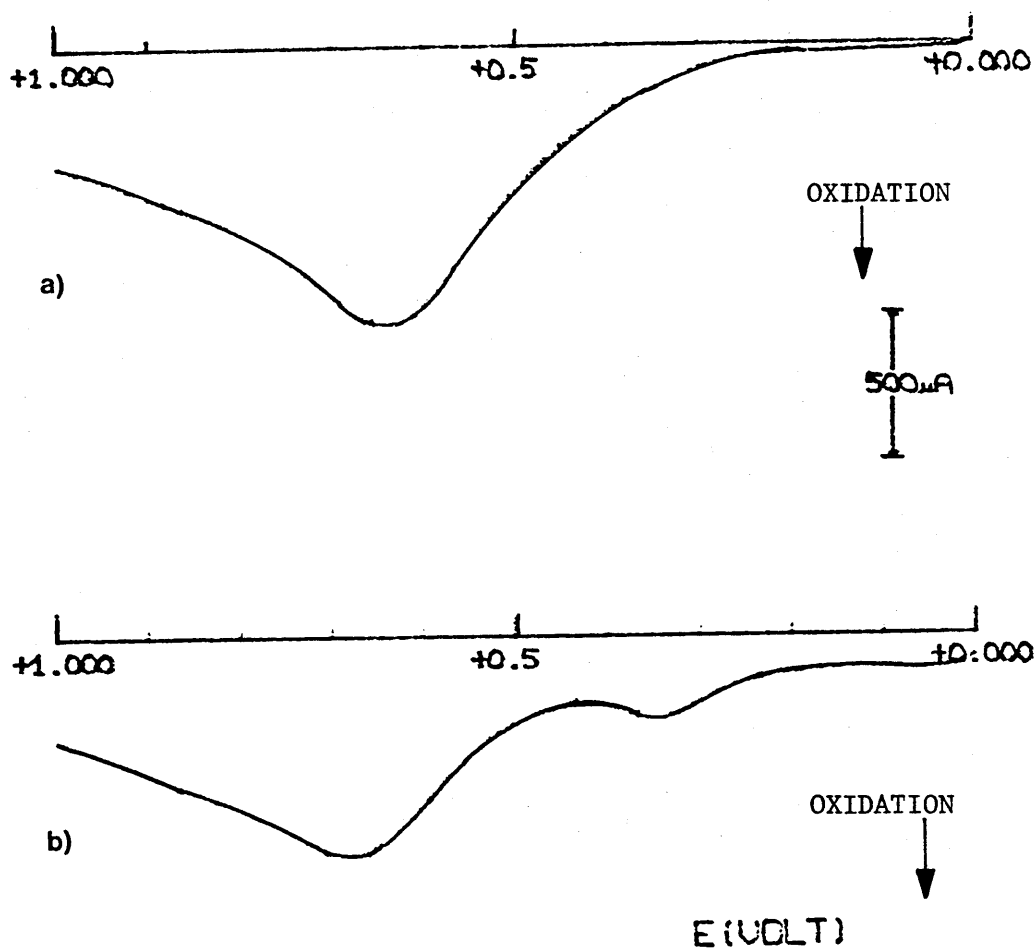
Although little current due to NADH oxidation was observed below +250 mV in figure 5.6., it was observed that on repeated scans a second peak developed at +340 mV (figure 5.9.). This phenomenon of a pre-peak occurring with NADH oxidation on graphite electrodes has been reported previously by Moiroux & Elving

FIGURE 5.8.



Differential pulse voltammograms of a graphite foil electrode in 0.1 M phosphate buffer pH 7.0 containing 0.5 M KCl :
 (a) in buffer only (b) doped with dimethylferrocene in buffer
 (c) doped with dimethylferrocene in buffer containing 1 mM NADH.
 The conditions employed were : scan rate = 4 mV s^{-1} ; pulse amplitude = 50 mV ; sample width = 17 msec ; pulse width = 50 msec ; pulse period = 1000 msec. IR compensation was used when obtaining these voltammograms.

FIGURE 5.9.



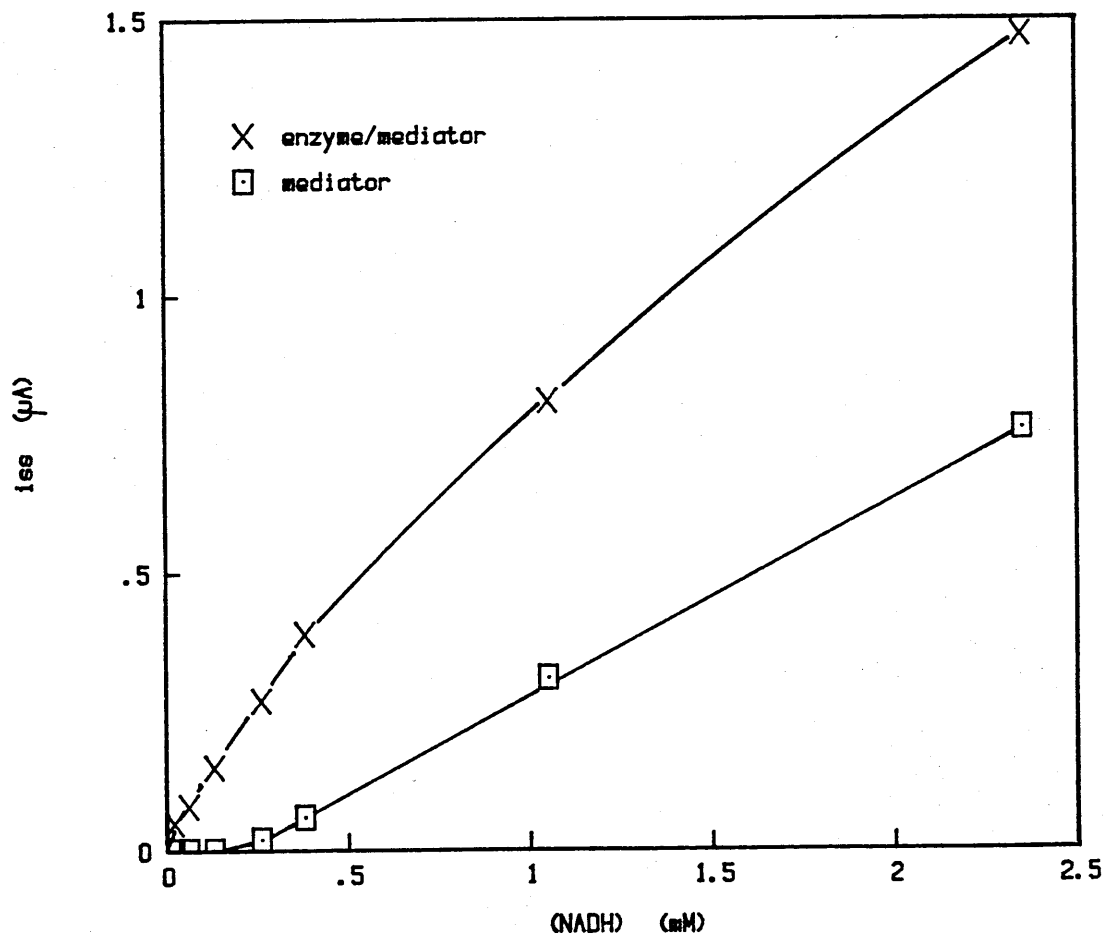
Linear sweep voltammograms of a 10 mM solution of NADH in 0.1 M phosphate buffer containing 0.5 M KCl at a graphite foil electrode at a sweep rate of 100 mV s^{-1} . a) first sweep b) after immersion of the electrode in the NADH solution for 1 hour. Background correction was employed in obtaining these voltammograms.

(1978), and was considered to be due to adsorption of NAD^+ . The currents obtained with the bare electrodes during the calibration experiments may therefore have been due to this phenomenon. Since this pre-peak was observed to increase with the contact time of the electrode in the NADH solution (figure 5.9.), the use of a pre-conditioning regime with the unmodified electrodes may have had a similar effect.

5.7.3. Detection of Enzyme-Catalysed Currents

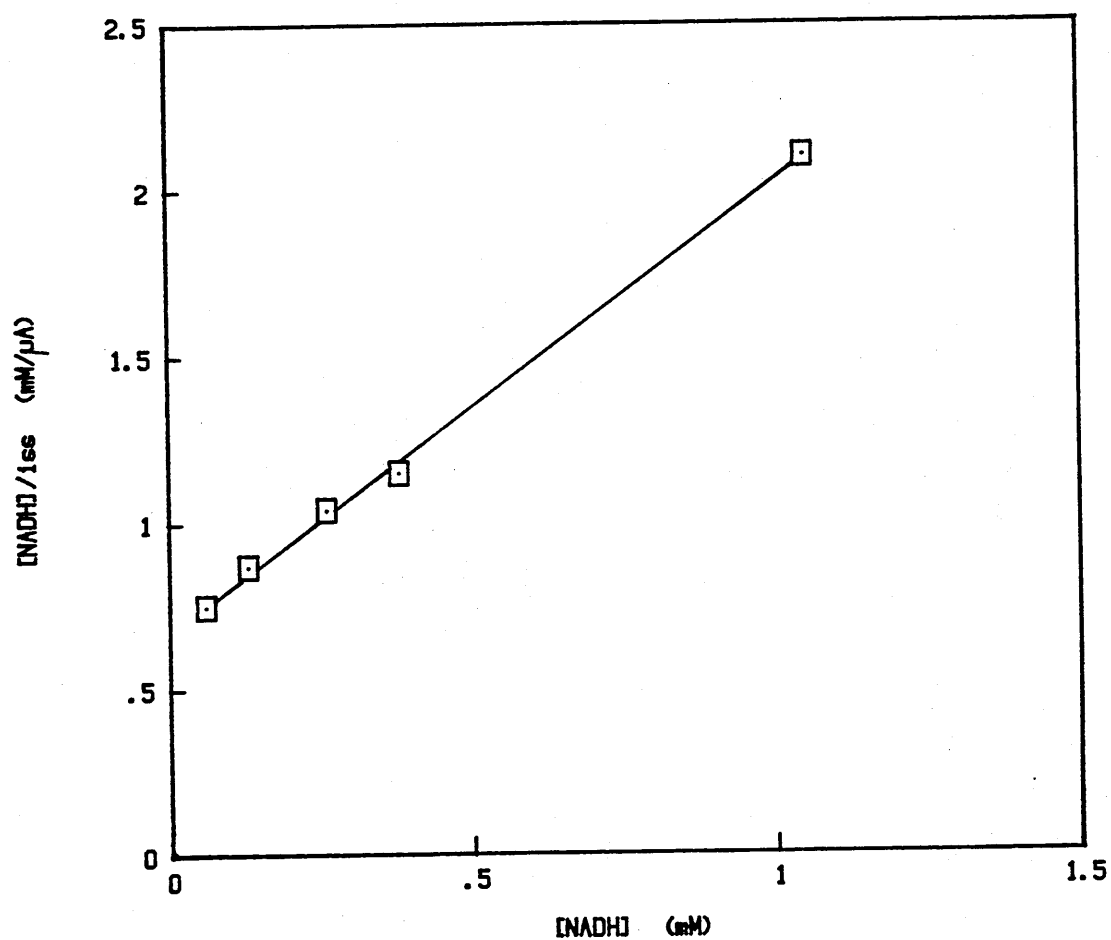
In order to determine whether any enzymatic activity was present on the enzyme modified electrodes an experiment was performed at a lower applied voltage, +150 mV vs. Ag / AgCl, and at NADH concentrations below 1 mM. Two enzyme electrodes were assayed along with two DMFc-modified electrodes as a control. The results (each pair of electrodes averaged) are shown in figure 5.10. No detectable current was measured with the mediator-modified electrodes below 0.25 mM NADH. The steady-state currents from the latter electrodes were subtracted from those of the enzyme electrodes, and a Hanes plot was constructed, as shown in figure 5.11. A straight line was obtained, giving values for $K'_m = 0.49$ mM and $i_{\max} = 0.72 \mu\text{A}$. The low value of $i_{\max}$ indicates that, as for the glucose oxidase electrodes, very little active enzyme was bound within the electrode material.

FIGURE 5.10.



Calibration curves of steady-state current against NADH concentration for mediator-modified and enzyme electrodes obtained under standard experimental conditions and an applied potential of +150 mV vs. Ag/AgCl.

FIGURE 5.11.



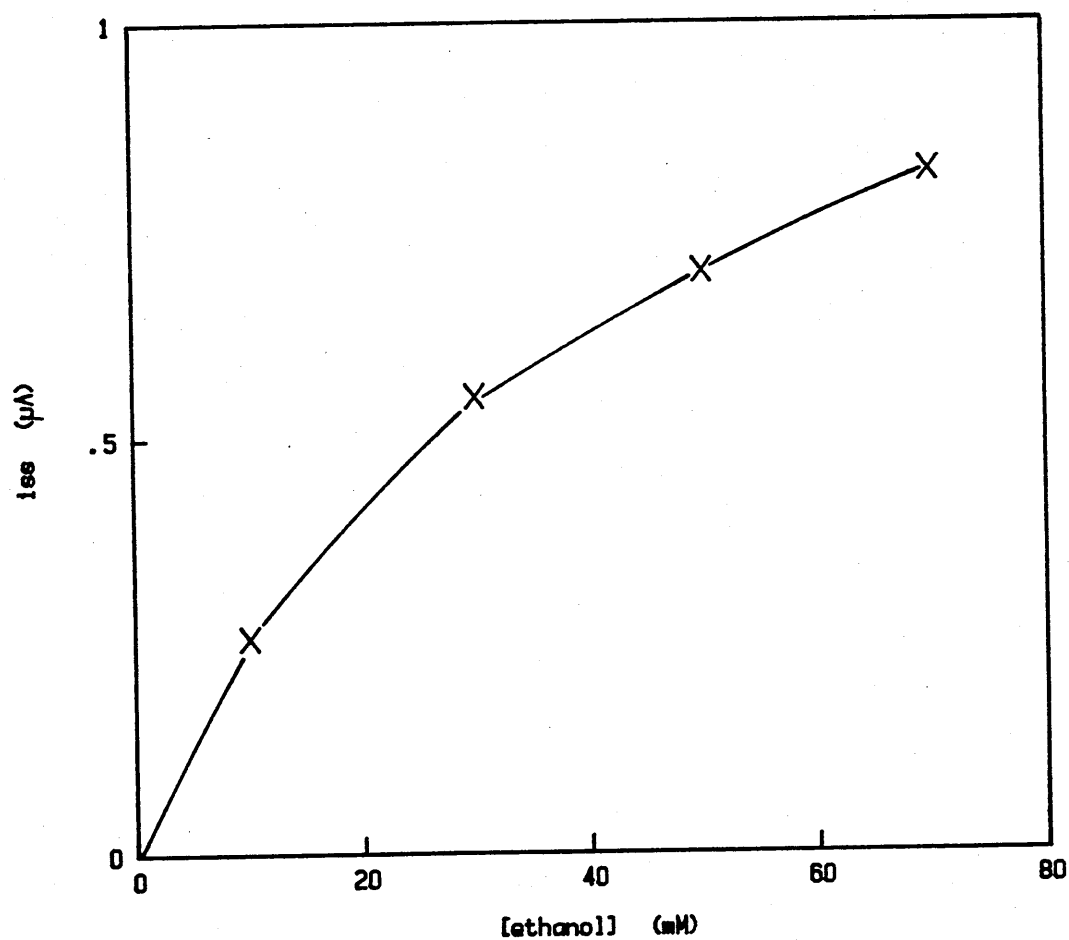
Hanes plot derived from the data shown in figure 5.10. obtained by subtraction of the steady-state currents produced by the mediator-modified electrodes from those measured with the enzyme electrodes.

5.7.4. Coupling of the Electrode to NAD-Linked Dehydrogenases for Detection of Substrates

Since the graphite foil electrode, whether modified or not, was capable of detecting NADH, it was of interest to demonstrate the principle that this sensor for NADH could be used to detect the substrates of NAD-linked dehydrogenases. In two sets of experiments the enzymes alcohol dehydrogenase (ADH) and glutamate dehydrogenase (GLDH) were retained behind dialysis membrane over the end of a graphite electrode modified both with DMFc and LipDH. The standard working potential of +235 mV vs. Ag / AgCl was employed. After allowing the background current to decay NAD⁺ was added to the system (to a final concentration of 3.85 mM), followed by additions of substrate (ethanol in the case of ADH, glutamic acid for the GLDH).

In both cases steady-state currents were observed upon addition of the appropriate substrate, although millimolar concentrations of both substrates were required to obtain a measurable response. Figure 5.12. shows the response of an ADH enzyme electrode against ethanol concentration. As can be seen from figure 5.12. relatively high concentrations of ethanol were necessary to obtain a response, which may have been due to the effects of the dialysis membrane. Coughlin et al. (1975) used an ethanol concentration of 0.4 M in their system, where ADH immobilized in a column was coupled to a flow through electrochemical cell for reoxidation of the NADH produced.

FIGURE 5.12.



Graph of steady-state current against ethanol concentration for a graphite foil electrode modified with lipoamide dehydrogenase and dimethylferrocene, and with alcohol dehydrogenase retained behind a dialysis membrane. The experiment was carried out under standard conditions.

The steady-state responses from enzyme electrodes based on both ADH and GLDH were found to be relatively short lived, lasting less than 10 minutes before starting to decrease.

5.8. Immobilized LipDH as an NADH Regenerating System

Whilst the direct oxidation of NADH at electrode surfaces to form biologically active NAD^+ has been reported (Coughlin et al., 1975 ; Wallace & Coughlin, 1977 ; Kulys, 1981 ; Huck et al., 1984 ; Albery et al., 1985) the efficient direct reduction of NAD^+ has proved more difficult to achieve without the formation of biologically inactive dimers. One approach to solving this problem has been to immobilize the coenzyme on polymers, where the physical separation of the coenzyme molecules prevents dimerization (Aizawa et al., 1976a). The alternative approach has been to couple enzymatic reduction of NAD^+ with electroregeneration of a suitably powerful reductant (Ito & Kuwana, 1971 ; DiCosimo et al., 1981), such as 1,1'-dimethyl-4,4'-bipyridylum dichloride (methyl viologen).

The aim of the work presented in this section was to demonstrate the feasibility of using an electrode surface as both the support for an NAD^+ reductase (LipDH) and as the working electrode for mediator regeneration, and to couple this system to a soluble NAD-linked dehydrogenase.

5.8.1. Investigation of Suitable Mediators using Cyclic Voltammetry

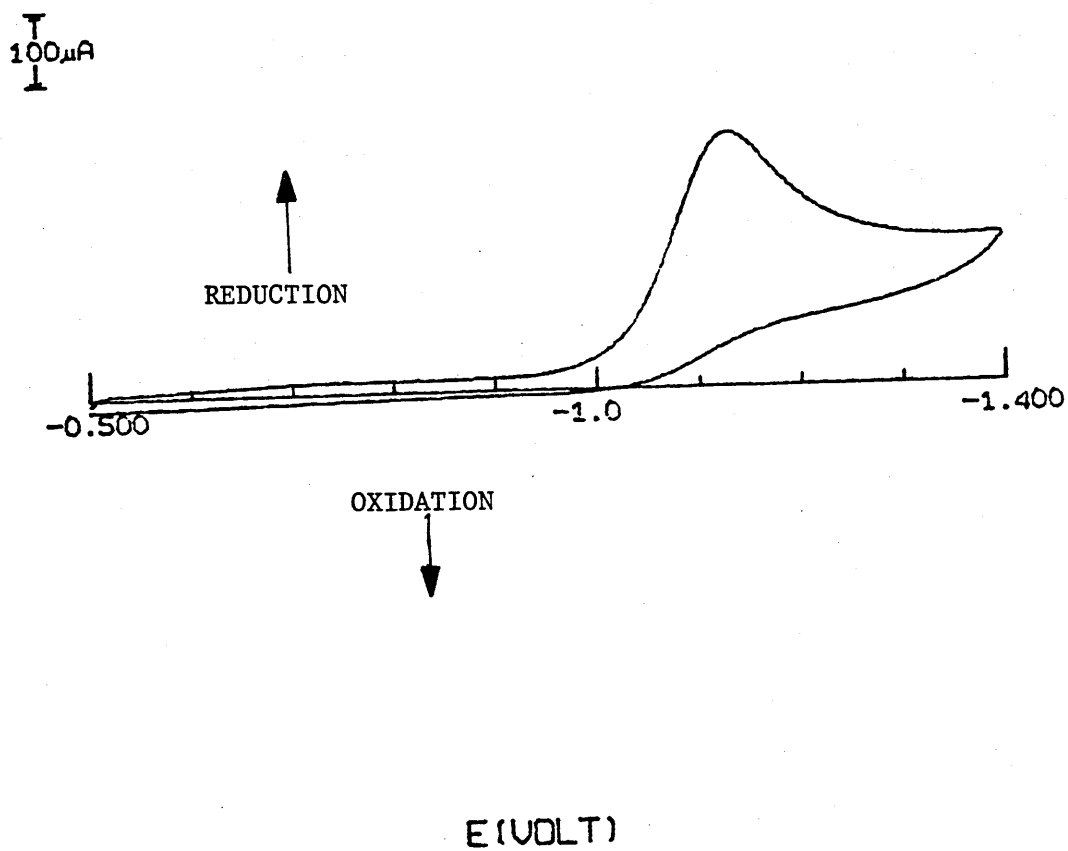
The standard redox potential of the $\text{NAD}^+ / \text{NADH}$ couple is -320 mV vs. the normal hydrogen electrode (NHE), or -560 mV vs. SCE. In order to drive the enzymatic reduction of NAD^+ it was necessary to employ a mediator with a more cathodic redox potential. The redox potentials of a number of possible redox compounds were investigated using cyclic voltammetry (CV) at a glassy carbon electrode.

As shown in figure 5.6. an overvoltage was required at the graphite foil electrode to oxidize NADH, and similar results were obtained when a glassy carbon electrode was used. Similarly an overvoltage was also required to achieve NAD^+ reduction at the glassy carbon electrode, as can be seen from figure 5.13. The cathodic peak potential measured was -1.1 V, an overvoltage of approximately 500 mV relative to the standard redox potential.

A series of dyes having redox potentials more cathodic than -560 mV were investigated. Irreversible electrochemistry was observed for all of the dyes tested, and a typical CV obtained is shown in figure 5.14. for chlorophenol red.

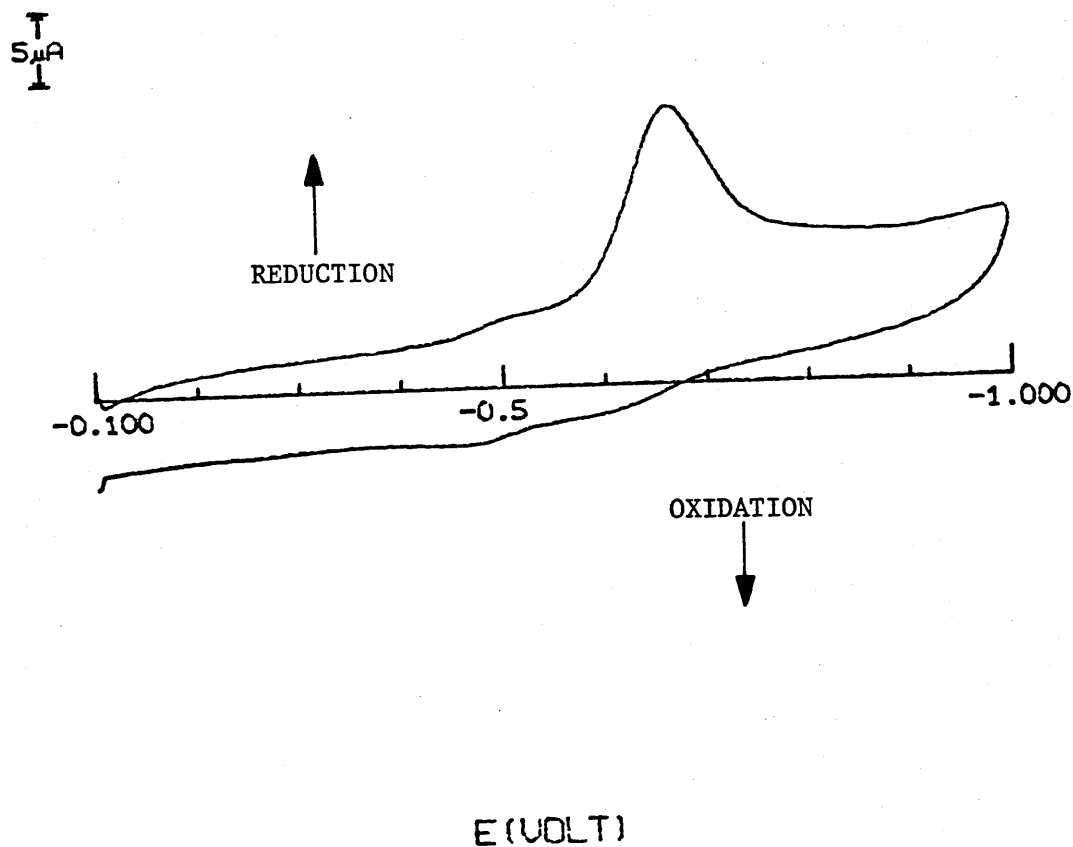
Some anthraquinone derivatives have been reported with cathodic redox potentials (Prince et al., 1981). A series of voltammograms of anthraquinone-2-sulphonic acid at different sweep rates are shown in figure 5.15. Although reversible electrochemistry of this compound was observed, it was nevertheless also

FIGURE 5.13.



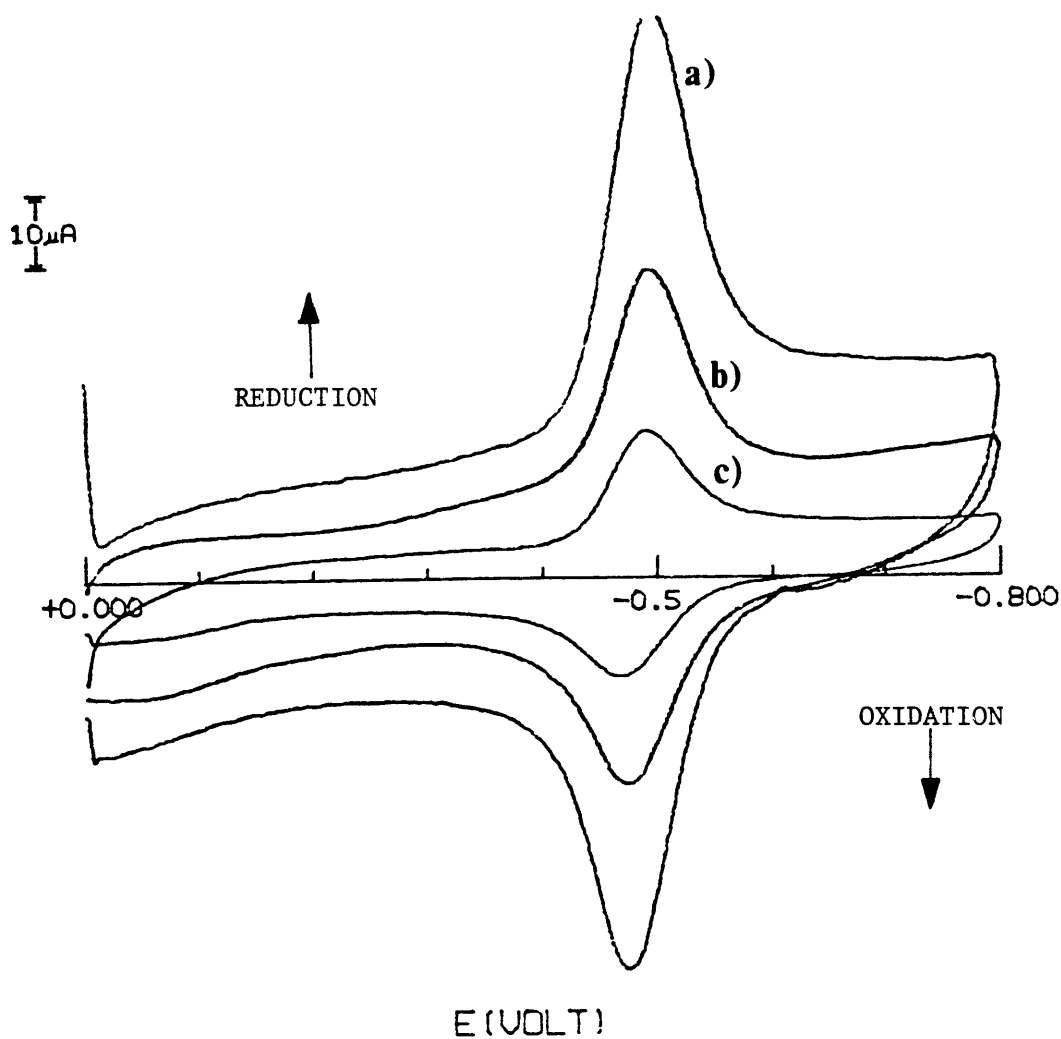
Cyclic voltammogram of a 10 mM solution of NAD^+ at a pyrolytic graphite electrode in 0.1 M phosphate buffer pH 7 containing 0.5 M KCl at a sweep rate of 100 mV s^{-1} .

FIGURE 5.14.



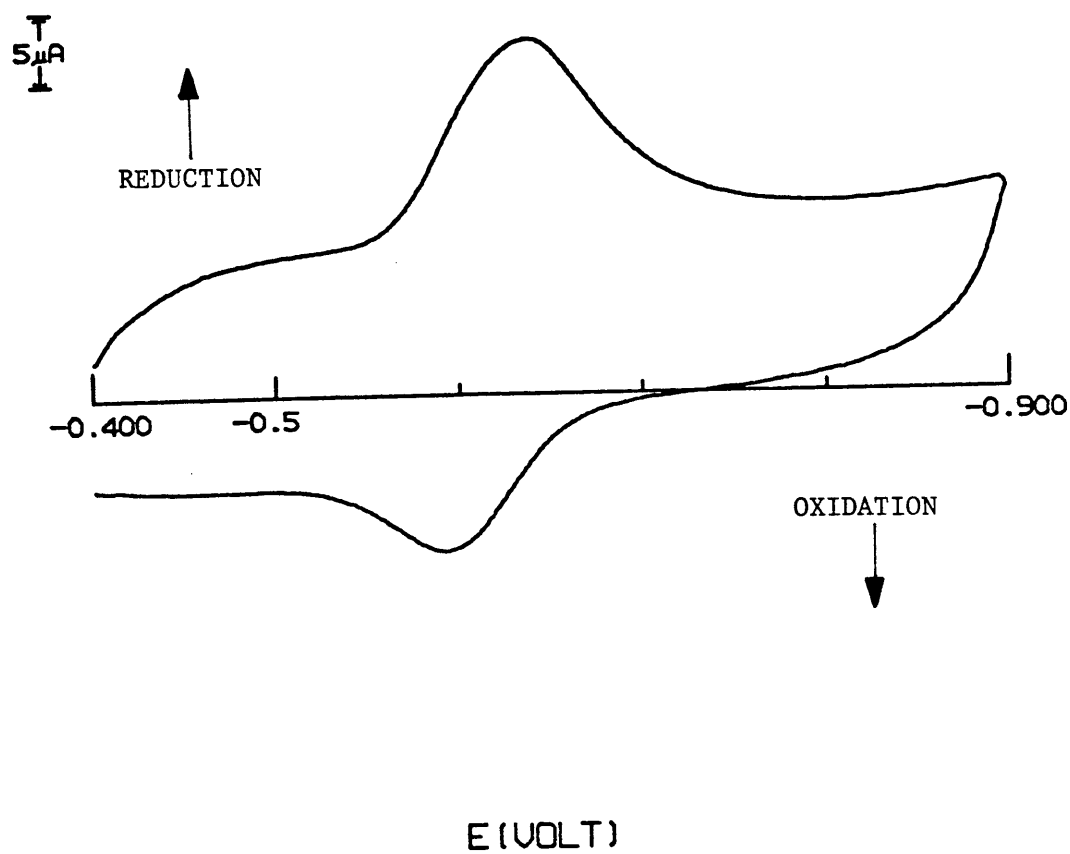
Cyclic voltammogram of a 1 mM solution of chlorophenol red at a glassy carbon electrode in 0.1 M phosphate buffer pH 7 containing 0.5 M KCl at a sweep rate of 20 mV s^{-1}

FIGURE 5.15.



Cyclic voltammogram of an aqueous solution of anthraquinone-2-sulphonic acid at a glassy carbon electrode in 0.1 M phosphate buffer pH 7 containing 0.5 M KCl at sweep rates of a) 100 mV s^{-1} b) 50 mV s^{-1} and c) 20 mV s^{-1}

FIGURE 5.16.



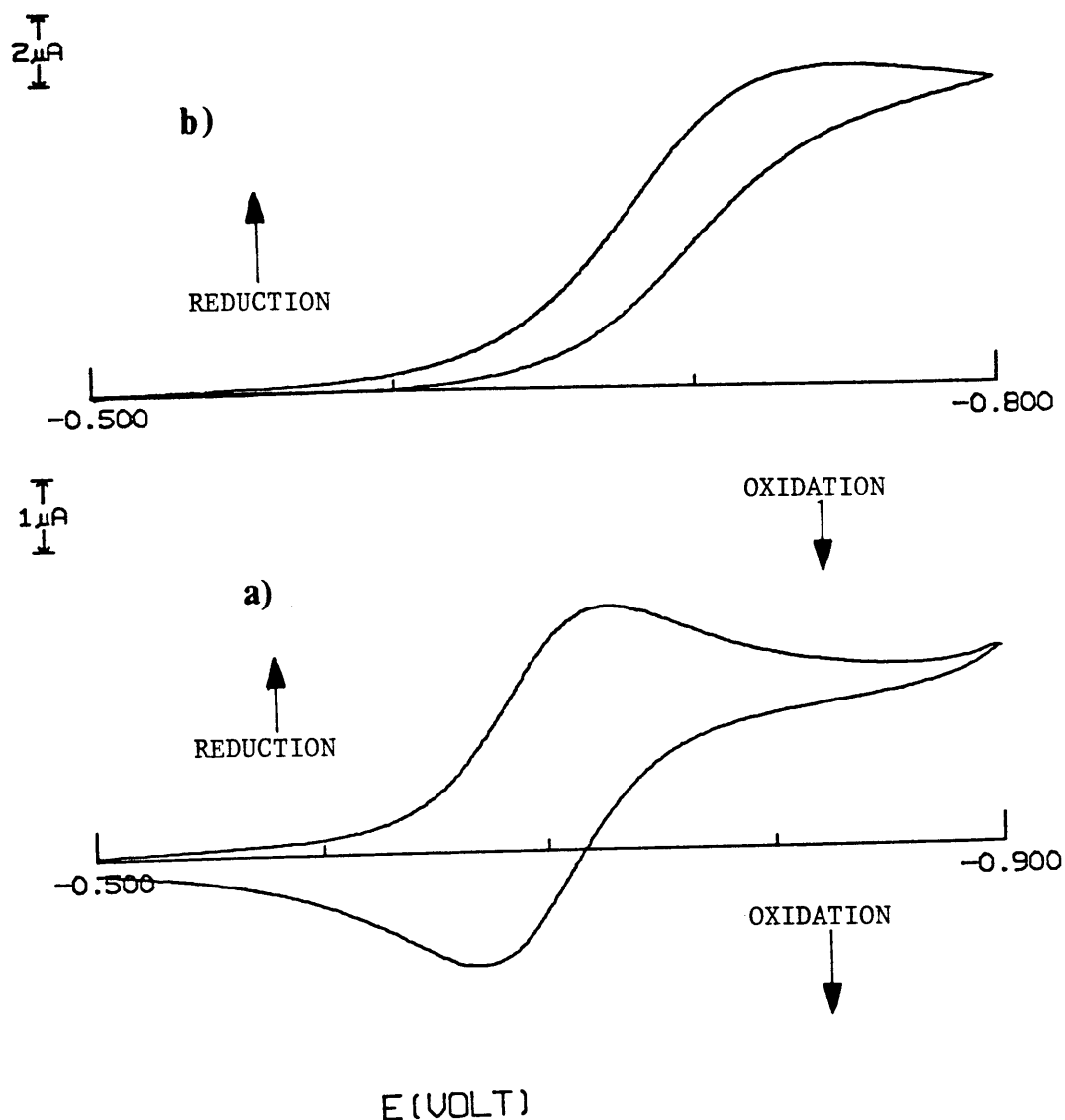
Cyclic voltammogram of an aqueous solution of anthraflavic acid at a glassy carbon electrode in 0.1 M phosphate buffer pH 7 containing 0.5 M KCl at a sweep rate of 100 mV s^{-1}

relatively insoluble and the mid-point potential of -480 mV was not sufficiently cathodic. The fact that both the oxidation and reduction mid-point potentials were nearly identical at increasing sweep rates suggests that the electrochemistry observed was due to adsorbed material (Murray, 1984).

With anthraflavic acid, on the other hand, both reversible electrochemistry and a sufficiently cathodic mid-point potential of -615 mV was noted (figure 5.16.). However, this compound was also found to be relatively insoluble in aqueous medium at neutral pH (both figures 5.15. & 5.16. were obtained using saturated solutions of the two anthraquinones).

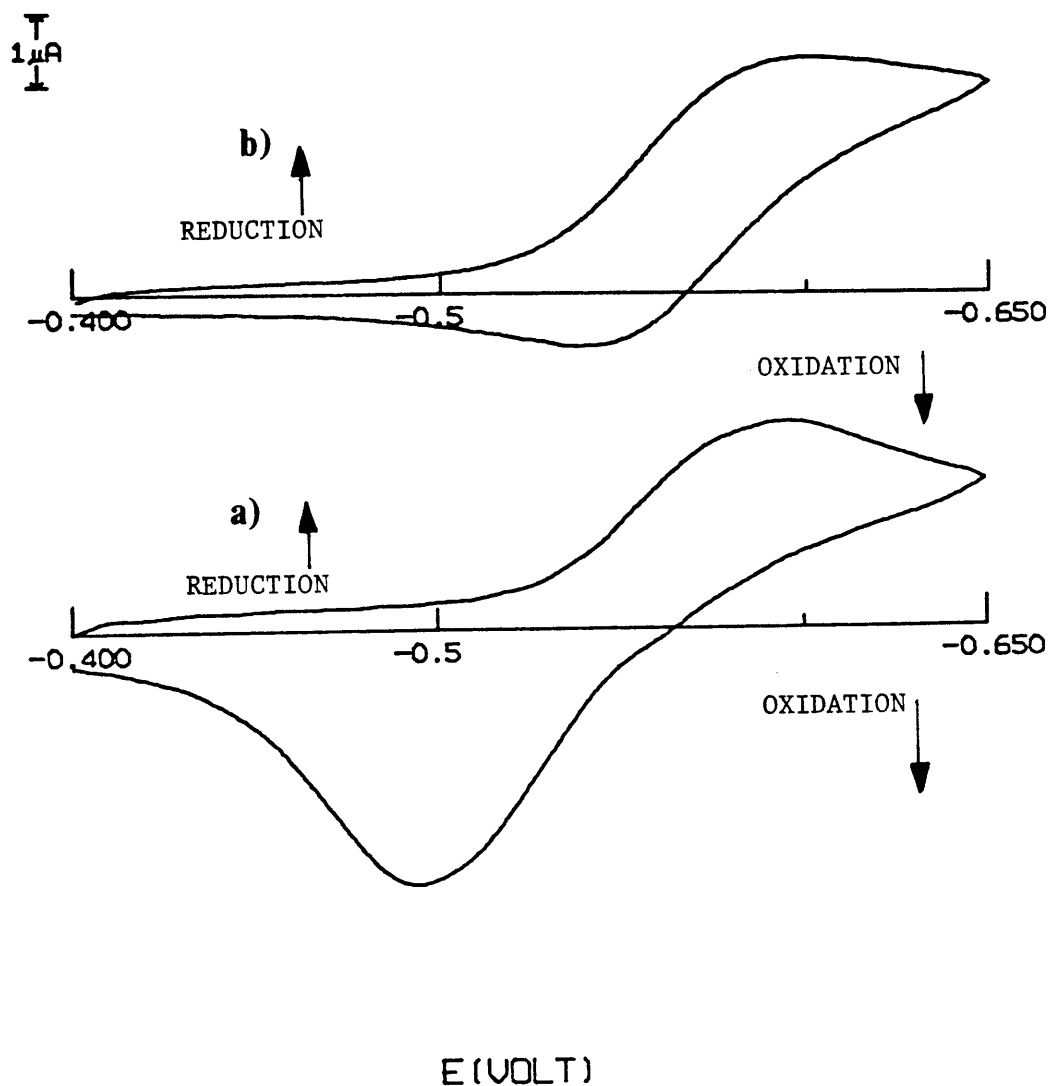
The ability of methyl viologen to act as a reductant for LipDH had been previously reported (DiCosimo et al., 1981), and it was of interest to examine this process using electrochemical methods. Based on the theory developed by Nicholson & Shain (1964) the technique of cyclic voltammetry has been proposed as a diagnostic tool for looking at coupled reactions between enzymes and redox active compounds (Cass et al., 1984 ; Davis, 1985). As shown in figure 5.17.a cyclic voltammetry of methyl viologen is unaffected by the presence of NAD^+ . On the addition of enzyme to the solution the CV shown in figure 5.17.b was obtained. In this case a large catalytic current flowed at cathodic potentials due to enzymatic reoxidation of the electrochemically reduced viologen. On the reverse sweep no oxidation wave was observed, again due to enzymatic consumption of all the reduced viologen formed. Following the method detailed by Davis (1985) a series of voltammograms

FIGURE 5.17.



Cyclic voltammograms of a) 1 mM solution of methyl viologen in 0.1 M phosphate buffer pH 7 containing 0.5 M KCl and 10 mM NAD^+ at a glassy carbon electrode employing a sweep rate of 1 mV s^{-1} b) as in a) but including 400 μg of lipoamide dehydrogenase

FIGURE 5.18.



Cyclic voltammograms of a) 1 mM solution of benzyl viologen in 0.1 M phosphate buffer pH 7 containing 0.5 M KCl and 10 mM NAD^+ at a glassy carbon electrode employing a sweep rate of 1 mV s^{-1} b) as in a) but including 400 μg of lipoamide dehydrogenase

were obtained at different sweep rates and different enzyme concentrations, and the second order rate constant for the reduction of LipDH by methyl viologen was then calculated. The value obtained was $0.26 \times 10^5 \text{ litre mole}^{-1} \text{ s}^{-1}$, which is almost identical to the value obtained for this constant with ferrocene monocarboxylic acid as the oxidant. This fact casts doubt on the validity of this method, as one would not expect identical rate constants for two mediators of such different chemical structure. It is known, for example, that the maximum turnover rates of NADH oxidation by LipDH varies when DCPIP and ferricyanide are used as electron acceptors (Massey, 1963). The catalytic current observed in figure 5.17.b does conclusively show, however, that methyl viologen is an efficient reductant for LipDH.

It was of interest to look at the same effect with benzyl viologen as the reductant. This viologen has a more anodic redox potential than methyl viologen (-598 mV compared to -689 mV vs. SCE), and would therefore be expected not to couple as well to the enzyme. A series of voltammograms were obtained using benzyl viologen under the same conditions employed with methyl viologen (10mM NAD^+ , $14 \mu\text{M LipDH}$), and the results at a scan rate of 1 mV s^{-1} are shown in figure 5.18. An enhancement of the cathodic peak was observed when enzyme was included in the solution and a decrease in the anodic peak was also noted. However these two processes were less marked than with methyl viologen as the reductant, demonstrating that benzyl viologen is not as good a reductant for the enzyme.

The results obtained with the different redox species confirmed that methyl viologen would be the best mediator to use for the studies involving immobilized LipDH to effect the bulk reduction of NAD^+ .

5.8.2. Bulk Reduction of NADH using Immobilized LipDH

The reactions involved in this system are shown schematically in figure 5.19. Table 5.2. lists the immobilization conditions used with the enzyme modified electrodes employed in the bulk reduction studies. Data from Bradford protein assays for these electrodes is also given in table 5.2. With the exception of the third set of electrodes (IM3) the amount of enzyme bound onto these electrodes was found to be consistent.

Bulk reduction of NAD^+ using these modified electrodes was carried out using saturating concentrations of both NAD^+ (2 mM) and methyl viologen (10 mM) in 0.5 M phosphate buffer pH 7.0 containing 0.5 M KCl.

These results are shown in table 5.3. for both the initial rates of NADH reduction (assayed spectrophotometrically) and the corresponding amount of active bound protein (taking 20 $\mu\text{mole min}^{-1} \text{mg}^{-1}$ as the specific activity of the enzyme in this reaction (Allen & Bowen, 1985).

The first enzyme modified electrode (IM1) was assayed in the small electrochemical cell shown in figure 2.3., which necessitated the removal of the lower third of the electrode material.

FIGURE 5.19.

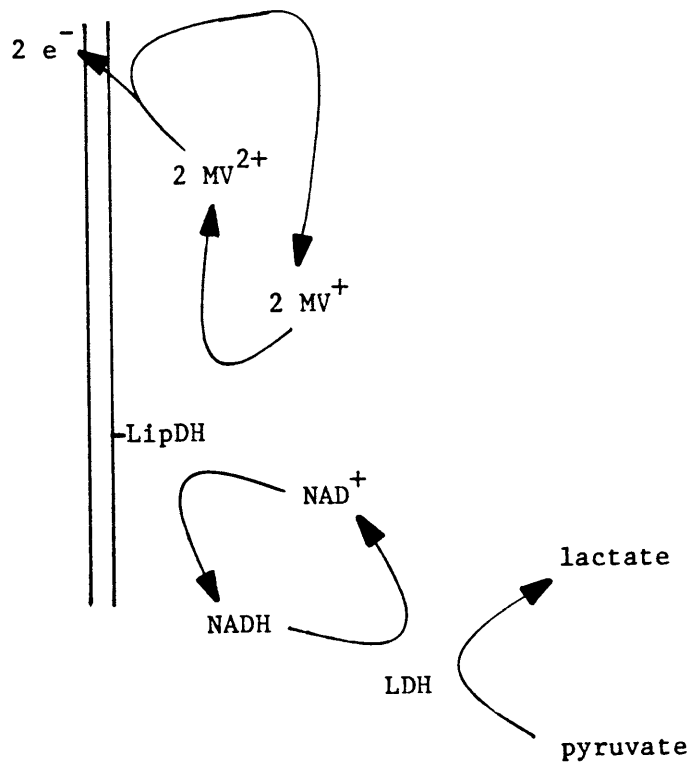


Diagram showing the reactions involved in the bulk reduction of NAD^+ by immobilized lipoamide dehydrogenase (lipDH), mediated by methyl viologen (MV) and coupled to soluble lactate dehydrogenase (LDH).

The other electrodes were assayed in the three compartment cell

TABLE 5.2.

LIPOAMIDE DEHYDROGENASE BOUND ONTO RVG 1000 ELECTRODES

IMMOBILIZATION	GLUTARALDEHYDE (%)	PROTEIN BOUND (mg/electrode)
IM1	2.5	0.93
IM2	2.5	0.98
IM3	1.0	0.48
IM4	0.5	1.10

shown in figure 2.4. The three sets of electrodes IM2 - IM4 demonstrated a general increase in bound activity as the concentration of glutaraldehyde in the activation step was increased up to the maximum employed of 2.5 %. Higher concentrations of glutaraldehyde were not used as previous experiments with the flow system (section 3.22.) showed that virtually no active enzyme was bound when a 5 % glutaraldehyde concentration was used. It should be noted that the lower initial rate obtained with electrode IM1 was due to the removal of the lower third of the material.

Electrode IM2/2 demonstrated the highest amount of active bound protein (approximately 5.9 %). All the electrodes showed

TABLE 5.3.

RATES OF NAD⁺ REDUCTION FOR LIPOAMIDE DEHYDROGENASE BOUND ONTO
RVG 1000 ELECTRODES

IMMOBILIZATION	RUN	a) INITIAL RATES		b) ACTIVE BOUND
		$A_{340} \text{ min}^{-1}$	$\mu\text{mole min}^{-1}$	ENZYME (μg)
IM1	1	0.295	0.318	15.9
	2	0.105	0.113	5.7
	3	0.060	0.065	3.2
IM2/1	1	0.238	0.766	38.3
	2	0.162	0.521	26.0
	3	0.031	0.100	5.0
IM2/2	1	0.360	1.158	57.9
	2	0.268	0.862	43.1
	3	0.042	0.133	6.7
IM3/1	1	0.179	0.576	28.8
	2	0.057	0.182	9.1
IM3/2	1	0.260	0.836	41.8
	2	0.030	0.096	4.8
IM4/1	1 c)	0.083	0.267	13.3
	2	0.031	0.100	5.0
IM4/2	1	0.077	0.248	12.4
	2	0.024	0.077	3.9

a) $\text{Rate (mole min}^{-1}\text{)} = A_{340} \text{ min}^{-1} \times \text{vol (litre)} / 6.22 \times 10^3$

b) Based on specific activity of $20 \mu\text{mole min}^{-1} \text{ mg}^{-1}$

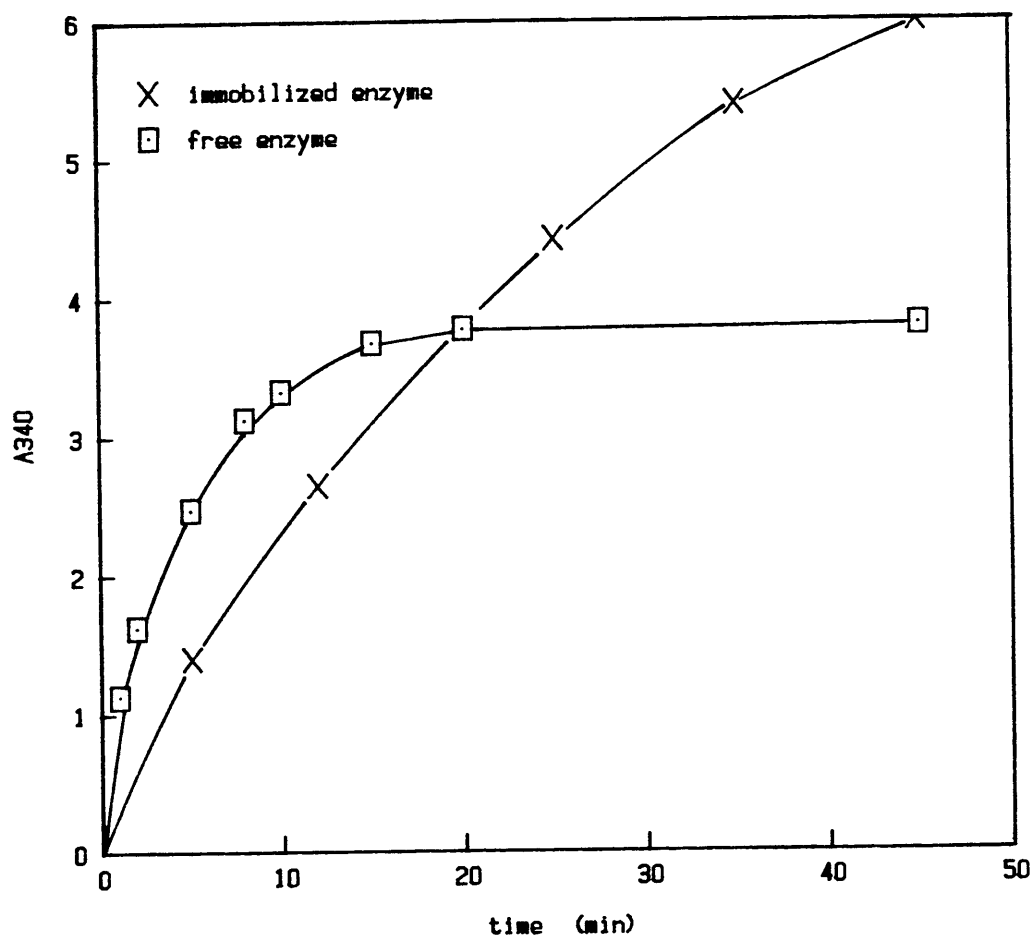
c) Gold foil working electrode

lower initial rates on subsequent assays. On average the second assay gave an initial rate of NAD^+ reduction 31 - 37 % of the first assay (for electrodes IM1, IM3/1, IM4/1 and IM4/2). In contrast the two electrodes IM2/1 and IM2/2 gave values of 68 % and 74 % respectively.

A comparison of the free and bound enzyme is shown in figure 5.20. Whilst no increase in NADH concentration was observed for the soluble enzyme after 20 minutes the bound enzyme demonstrated around 50 % of the initial activity. The immobilized LipDH showed continuing activity 2 hours after addition of NAD^+ to the system. The difference in behaviour of the free and bound enzyme was also reflected in the current generated during NAD^+ reduction. As shown in figure 5.21. an increase in current for the soluble enzyme was immediately followed by a gradual decrease. With the immobilized enzyme the maximum current remained stable for a much longer period. A closer monitoring of the current produced by the modified electrode using a multimeter showed that the current-time profile reflected that of the rate of NAD^+ reduction (figure 5.22), a slow down in the rate of NAD^+ reduction being accompanied by a decrease in the measured current.

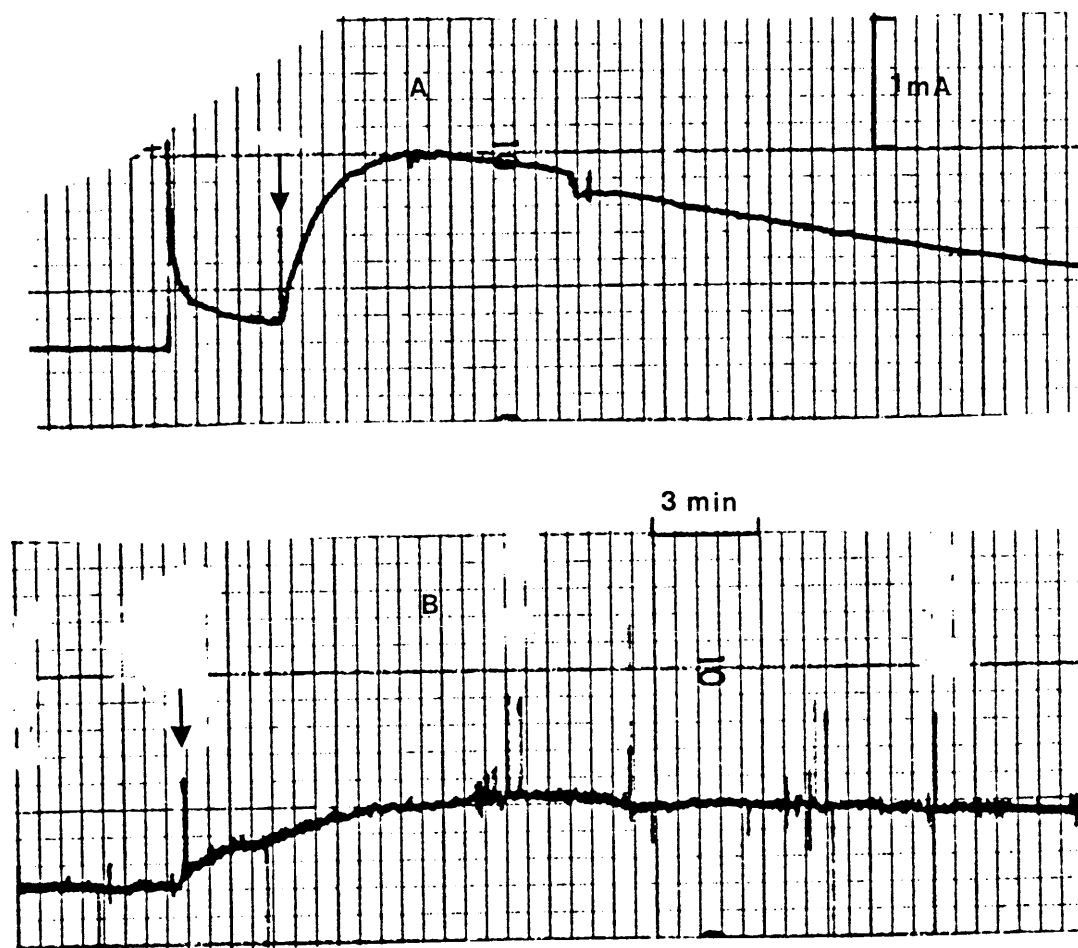
The final absorption at 340 nm obtained with the soluble enzyme was 7.3 (figure 5.20.). Taking the A_{340} of a 1 mM NADH solution as 6.0, this gives a value of 60 % reduction of the NAD^+ added. Values of 70 % after 90 minutes and 82 % after 60 minutes were obtained with electrodes IM2/2 and IM1 respectively. It should be noted that the actual amounts of NAD^+ reduced differed

FIGURE 5.20.



Graph showing the production of NADH with time (monitored at 340 nm) for the lipoamide dehydrogenase / methyl viologen system for free and immobilized enzyme. The experiments were carried out in 0.5 M phosphate buffer pH 7.0 containing 0.5 M KCl, at room temperature, an applied potential of -0.7 V and under nitrogen sparging.

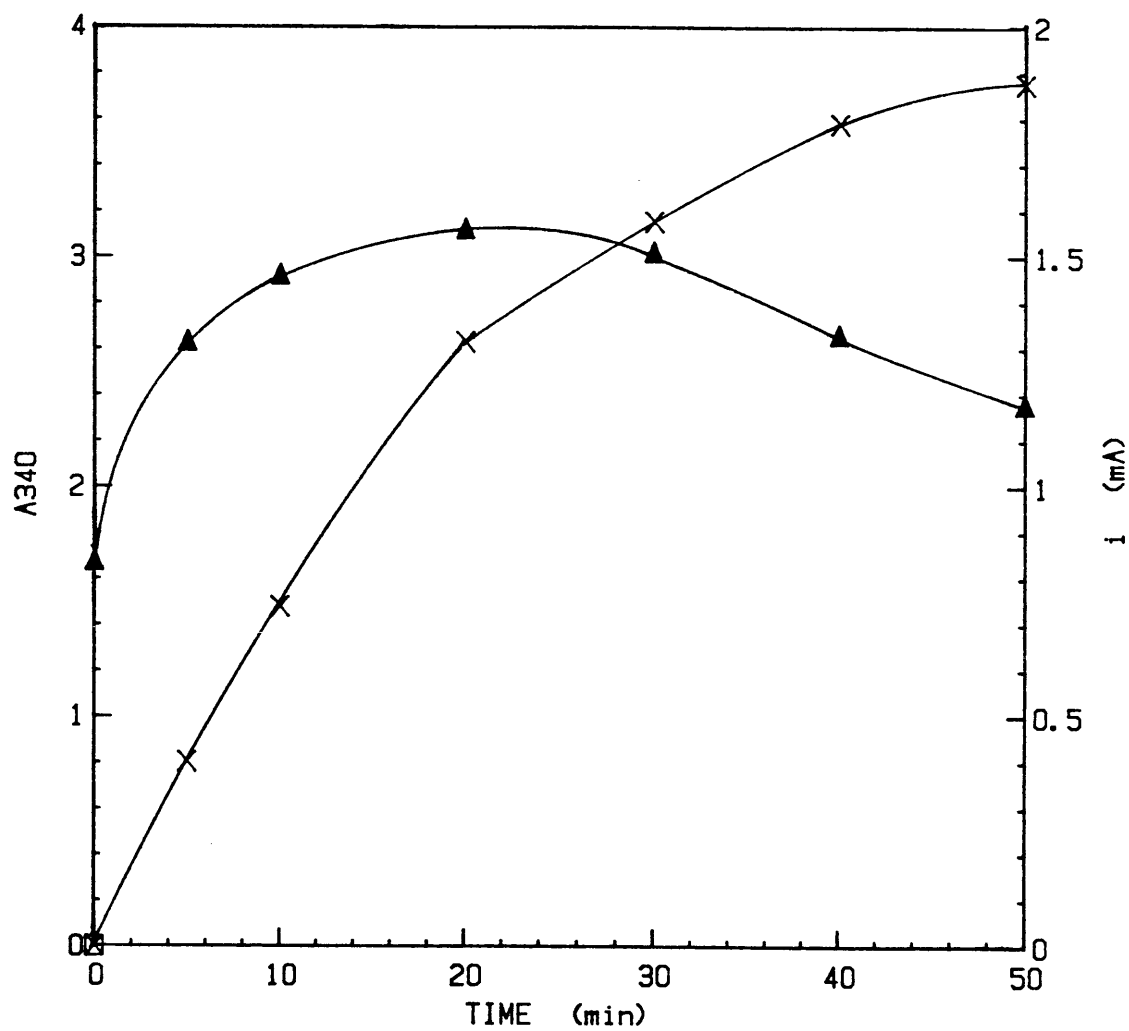
FIGURE 5.21.



Current-time curves for a) free and b) immobilized lipoamide dehydrogenase in the methyl viologen mediated bulk reduction of NAD^+ .

Arrows indicate the point of addition of NAD^+ to the cell.

FIGURE 5.22.



Variation of X A_{340} and ▲ current with time for immobilized lipoamide dehydrogenase in the bulk reduction of NAD^+ .

in these two experiments due to the volume of solution employed in each case (6.7 ml for IM1 and 20 ml for IM2/2). The remaining experiments were stopped before such high percentages of the NAD^+ present were reduced.

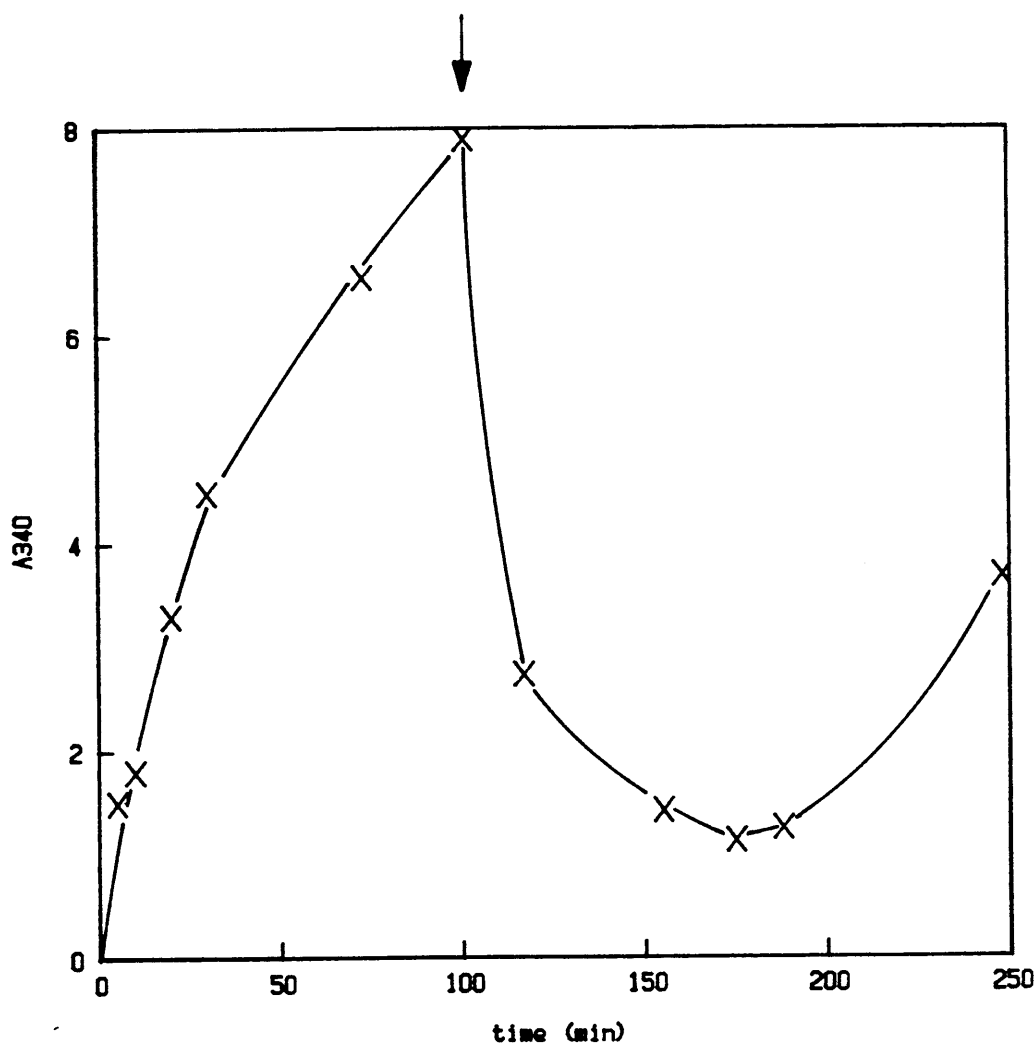
For the first experiment with electrode IM4/1 a gold foil working electrode was used to reduce the methyl viologen, the modified felt electrode acting only as the enzyme support. The initial rate obtained in this case was very close to that measured for electrode IM4/2. This result indicated that employing the enzyme modified felt as the working electrode did not affect the enzymatic reaction.

5.8.3. Coupling of Immobilized LipDH to Soluble Lactate Dehydrogenase

Coupling of the enzyme electrode to soluble lactate dehydrogenase was investigated using a further set of electrodes (IM5) employing the same experimental conditions as described for the bulk reduction studies. These electrodes were also activated using a 2.5 % solution of glutaraldehyde.

Figure 5.23. shows the profile of A_{340} against time for electrode IM5/1. After allowing for a build up of NADH within the cell, additions were made of pyruvate (40 μmole) and lactate dehydrogenase (30 units). After addition of the enzyme a rapid decrease in A_{340} was observed. The level of NADH eventually stabilized and then began to increase again. It was calculated

FIGURE 5.23.



Graph showing the variation in A_{340} with time for electrode IM5/1. The experiment was carried out in 0.5 M phosphate buffer pH 7.0 containing 0.5 M KCl, at room temperature, an applied potential of -0.7 V and under nitrogen sparging. The cell also contained 30 units of soluble lactate dehydrogenase. The arrow indicates addition of pyruvate to the cell.

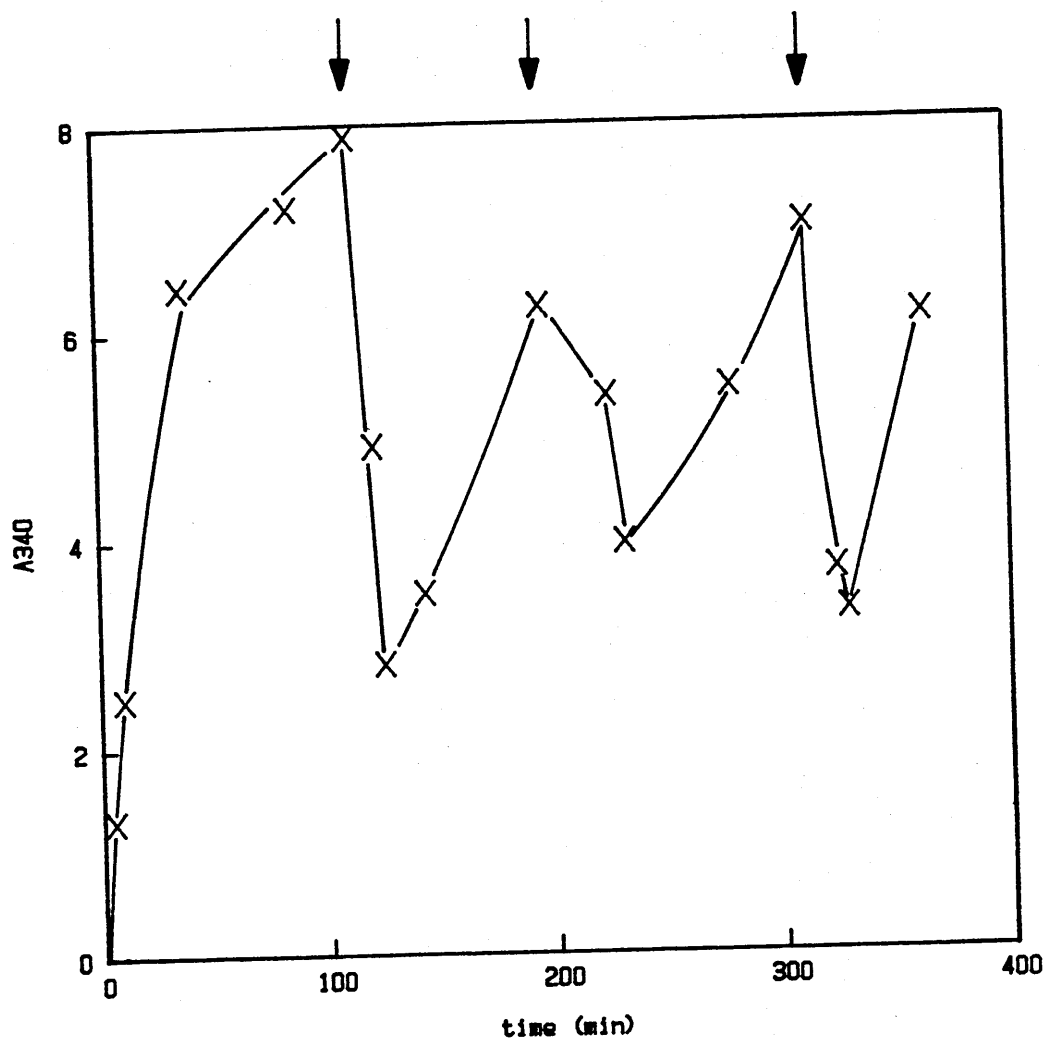
that a total of 32.9 μmoles of NADH were consumed during the experiment, which corresponds to about 82 % conversion of pyruvate to lactate. This calculation assumed a rate of NADH production after the addition of lactate dehydrogenase equivalent to the final rate measured at the end of the experiment. This figure of 82 % substrate conversion is therefore a conservative estimate.

The results of the experiment performed with electrode IM5/2 are shown in figure 5.24. In this experiment three additions of pyruvate were made. In contrast to the results shown in figure 5.23. the rapid decrease in A_{340} following additions of pyruvate were quickly followed by an increase in the level of NADH. The final rate observed at the end of the experiment, however, was higher than those measured between additions of substrate. This fact was taken into account when calculating the amounts of NADH consumed and produced during the experiment. Approximately 28.6 μmoles of NADH were consumed after the first addition of pyruvate (72 % conversion), 33.5 μmoles were consumed after the second addition of pyruvate (84 % conversion) and 13.2 μmoles of NADH were consumed after the third addition of pyruvate (33 % conversion). A total of 99.5 μmoles of NADH were produced during the course of the 6 hour experiment.

Similar initial rates of 0.836 and 0.842 $\mu\text{mole} / \text{min}$ were obtained for electrodes IM5/1 and IM5/2 respectively. The final rate measured for electrode IM5/2 was approximately 32 % of its initial value.

These results demonstrated that the enzyme-modified electrodes

FIGURE 5.24.



Graph showing the variation in A_{340} with time for electrode IM5/2. The experiment was carried out in 0.5 M phosphate buffer pH 7.0 containing 0.5 M KCl, at room temperature, an applied potential of -0.7 V and under nitrogen sparging. The cell also contained 30 units of soluble lactate dehydrogenase. The arrows indicate additions of pyruvate to the cell.

could be used continuously over a period of hours to reduce NAD^+ to NADH, even though experiments with electrodes IM1 - IM4 showed that loss of activity occurred on repeated use.

CHAPTER 6

GENERAL CONCLUSIONS AND FUTURE WORK

6.1. Immobilization on Electrodes

The two main types of graphite material employed in the work reported here, the graphite foil and the graphite felt, presented different properties with regard to enzyme immobilization, and hence the results of alternative methods showed differing degrees of success depending on which material was used. The graphite foil, in consequence of being a porous material, allowed successful immobilization of GOD using just simple adsorption. Although the use of water-soluble carbodiimide to covalently attach the enzyme, or glutaraldehyde to crosslink adsorbed enzyme, resulted in larger amounts of enzyme bound (which was shown by the increased steady-state currents obtained) as seen from table 3.12. these improvements were not large. Higher currents were more easily obtained in this system by increasing the enzyme loading (the enzyme concentration used in the immobilization step) and / or the electrode size (tables 4.3. - 4.5.). More recently an alternative immobilization method has been reported which gives significantly higher levels of bound enzyme activity on this type of graphite material (Brooks et al., 1986). The nature of the graphite itself, however, meant that sufficient enzyme could be retained

using adsorption to carry out kinetic studies.

In the case of the graphite felt, a high surface area material, higher levels of enzyme activity could be immobilized using covalent coupling techniques as opposed to adsorption with or without subsequent crosslinking (see table 3.7.). Thus whilst more active enzyme was retained on the felt when glutaraldehyde crosslinking was employed, covalent coupling techniques such as carbodiimide activation and especially silanization resulted in higher levels of bound activity. The optimum silanization technique, however, was not the one expected. Since silanization works by coupling to hydroxyl groups it was envisaged that reduction of the surface of the graphite felt using LiAlH_4 would lead to more enzyme being bound. As shown in table 3.7. LiAlH_4 reduction of the graphite felt lead to an increase in the amount of glucose oxidase immobilized, but with less activity being retained. The use of glutaraldehyde to crosslink the enzyme had the opposite effect of lowering the amount of enzyme bound but increasing the level of bound active enzyme. These effects may have been due to the degree of silane polymerization which occurred, which whilst increasing the amount of enzyme bound (with LiAlH_4 reduction) led to a greater degree of enzyme inactivation.

Whilst silanization appeared to be the best method examined for immobilization of glucose oxidase onto graphite felt, the insulating effect which resulted meant that this method could not be used when the modified felt was employed as an electrode. Consequently, given the low specific activity of this enzyme com-

pared to GOD the immobilization methods employed with LipDH were found to give rather low levels of bound enzyme activity.

Using adsorption followed by glutaraldehyde crosslinking resulted, in the best case, in only 5.9 % of the bound enzyme being active (based on the activity of the enzyme in solution). In this experiment (electrode IM2 tables 5.2. & 5.3.) the amount of enzyme immobilized was $61 \mu\text{g} / \text{cm}^2$ of felt (geometric surface area). With GOD the loadings obtained varied from $40 \mu\text{g} / \text{cm}^2$ for carbodiimide activation (table 3.2.) to $85 \mu\text{g} / \text{cm}^2$ for LiAlH_4 -reduced / silanized discs (table 3.7.). With this type of electrode material covalent coupling techniques were found to give a large improvement over adsorption methods.

6.2. Kinetics of Immobilized Enzymes

As explained in section 1.3. when an enzyme is immobilized onto a water-insoluble support the observed rates of substrate conversion may be affected by diffusional processes. In these cases the measured rates are no longer controlled by enzyme kinetics alone. In all the studies carried out the observed rate of reaction of the immobilized enzymes was affected to one degree or another by diffusion. When graphite foil was employed internal diffusion of glucose within the porous electrode became a rate-limiting process at low glucose levels. In the case of graphite felt the nature of the diffusional limitations was not completely elucidated, but the increased values of the apparent Michaelis

constant (K'_m) suggested that the rates of enzyme reaction were being affected by diffusion. With LipDH bound onto graphite felt discs and analysed in a spectrophotometric flow system the values of K'_m for the immobilized enzyme appeared to decrease relative to the K_m determined for the free enzyme (table 3.8.). Analysis of these results were complicated by the equilibration time of the system (figure 3.11.) which meant that the measured rates of NADH oxidation were not the initial rates. Thus determination of a " K'_m " in this case most likely had an indirect relation to the intrinsic kinetics of the bound enzyme.

6.3. Kinetic Studies of a Glucose Biosensor

Early studies on the kinetic behaviour of the amperometric glucose sensor based on GOD and DMFc immobilized onto graphite foil electrodes suggested that the enzyme was located within the porous electrode material. The linear calibration curves (which exceeded the K_m of the enzyme) and non-linear kinetic plots were tentatively attributed to internal diffusion of glucose within the electrode being a rate-limiting process (Schneider *et al.*, 1984). Subsequent experimental evidence has shown that this initial analysis was essentially correct, with the steady-state current (i_{ss}) being diffusion limited at low glucose levels ($< K'_m$), enzyme-controlled at saturating glucose concentrations ($> 50 \text{ mM}$), and dependent on both of these processes at intermediate concentrations. No evidence was shown for any external (to the electrode surface)

diffusional limitations. The effect of decreasing the temperature appeared to be an increase in the effect of diffusion on the measured currents, resulting in for example increased values obtained for K'_m at 1°C relative to 25°C.

The response of the enzyme electrode to glucose was found to be independent of the immobilization conditions, being only affected to a limited degree by lowering of the temperature. This response was in marked contrast to a membrane system where the response became markedly slower. This fact, coupled to the sigmoidal shape of the Eadie-Hofstee type plots, suggested a system where the outside layer of enzyme was freely open to the bulk solution, but enzyme immobilized deeper within the electrode was subjected to a large gradient of glucose concentration and consequently was not being reached. By making certain assumptions the diffusion coefficient for glucose within the electrode (D_{eff}) was calculated for three sets of enzyme electrodes, and was found to be lower in all cases than the value for glucose diffusion in free solution.

6.4. NADH Oxidation at Modified Graphite Foil Electrodes

Amperometric measurement of NADH was achieved using untreated, mediator-modified and enzyme / mediator-modified graphite foil electrodes. On further study, using linear sweep and differential pulse voltammetry, it was found that this oxidation process was not due simply to the ability of graphite foil to oxidize NADH at

lower potentials than other graphite electrodes. With the plain electrode an oxidation peak at a potential of +340 mV (vs. SCE) was found to occur upon contact of the electrode with NADH in solution, which was less than 100 mV more positive than the operating potential employed in the amperometric experiments (+275 mV vs. SCE). With reference to the work of Moiroux & Elving (1978) this phenomenon could have been due to NAD^+ adsorption. When the graphite foil electrodes were treated with DMFc, some coupling of the oxidation of NADH to the adsorbed mediator appeared to occur. It is known that other redox species such as ferricyanide also oxidize NADH at a slow rate. Further work would be necessary to confirm this effect, and to establish whether DMFc is an efficient oxidant for NADH. By employing a less anodic potential in the calibration experiments, and by concentrating on micromolar levels of NADH, it was possible to detect enzymatic oxidation of NADH.

With all the electrodes used non-linear calibration curves of steady-state current against NADH concentration were obtained. However, a linear relationship between these parameters was observed from enzymatic NADH oxidation over the limited range of 20 - 380 μM NADH.

Entrapment of alcohol and glutamate dehydrogenases behind dialysis membrane over the surface of a graphite foil electrode enabled the substrates of these enzymes (ethanol and glutamic acid respectively) to be detected.

6.5. NADH Recycling using Immobilized LipDH

The results presented in sections 5.8.2. & 5.8.3. showed that LipDH bound onto graphite felt electrodes was able to utilize methyl viologen, which had been electrochemically reduced on the support material, to reduce NAD^+ . It was also demonstrated that this system could be coupled to a soluble NADH-requiring dehydrogenase such as lactate dehydrogenase.

The immobilized enzyme did not appear to be particularly stable, showing a decrease in activity on repeated use. The immobilization procedure itself was optimized in relation to the glutaraldehyde step, but the best method only gave 14.5 ug of enzyme activity per cm^2 of graphite felt (based on the specific activity of LipDH in this reaction in solution).

By comparison to the behaviour of the enzyme in solution, which under a variety of conditions appeared to lose activity after less than 20 minutes (figure 5.20.) the immobilized enzyme showed continuing activity after more than 6 hours (see figure 5.24.). Whether this enhanced stability of the enzyme with regard to catalytic activity was due to diffusion or to conformational changes is not clear, and further work would be necessary to examine the mechanism of this system.

6.5. Future Work

The continuing interest in biosensors is shown by the many

different configurations reported in the literature for sensing the same substrate. These developments have been concerned not only with the immobilization method employed to trap the enzyme at the interface between solution and transducer, but also with regard to the sensing element itself. Thus the study of immobilizing enzymes directly onto electrode surfaces is by no means a closed book. With materials such as the graphite felt future studies need to be directed at the chemical nature of the surface of the graphite, i.e. as to the existence of phenolic, carboxylic acid and hydroxyl groups for example, their relative abundance, and how this surface can best be derivatized. The high surface area of the graphite felt, and its satisfactory behaviour as an electrode material make it suitable for future studies involving bioelectrochemical biotransformations.

The kinetics of immobilized enzymes is additionally an area where effort is required, since improvements in the behaviour of a biosensor (speed of response, range of detection etc.) depend on an understanding of the processes involved in the production of the electrical signal. From this work it appeared necessary to produce a system where diffusion limits the sensor response over the range of substrate concentration of interest, but does not in consequence cause a large increase in the response time of the system. Future work could therefore be directed towards discovering alternative electrodes which mimic the structure of the graphite foil (enzyme bound within thin layers of electrode material). An alternative method of measurement which could over-

come slow responses of an enzyme electrode would be the transient measurement of the rate of response rather than the steady-state signal.

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