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DEVELOPMENT OF AN AMPEROMETRIC BIOSENSOR FOR THE DETECTION OF ALCOHOL

A THESIS PRESENTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTERS IN SCIENCE IN BIOCHEMISTRY AT MASSEY UNIVERSITY

RUTH LARGE

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ABSTRACT

The aim of the following work was to design a biosensor for the detection of ethanol. A biosensor is an analytical device in which a biological sensing element is connected to or integrated with a physical transducing element. Amperometric enzyme biosensors utilise one or more enzymes to convert a substance which cannot be measured electrochemically to one which can be. In the case of an alcohol biosensor one of two enzymes (alcohol dehydrogenase and alcohol oxidase) can be used to convert electrochemically stable alcohol to either hydrogen peroxide or NADH which can be oxidised.

In the design of an alcohol biosensor there are three major variables to consider, these are; enzyme type, electrode material, and immobilisation technique. The goal was to select optimum conditions for the formulation of the desired sensor. In the present work the electrode materials used were platinum, carbon (foil and paste) and the conducting organic salt N-methyl phenazinium.Tetracyanoquinodimethane (NMP.TCNQ). The immobilisation techniques used were; adsorption, cross-linking to a protein matrix and covalent binding.

Of the biosensors produced from a selected combination or these variables each was tested by one or more of the following; cyclic voltammetry, enzyme assay, and amperometry. The most promising approach appears to be that of conjugating enzyme to haemin and allowing the conjugate to bind irreversibly to platinum via the haemin group. An electrode made with the organic salt NMP.TCNQ looked promising also but because the salt is readily oxidised it is unstable and therefore not an ideal electrode material.

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CONTENTS

LIST OF ABBREVIATIONS

ADH	Alcohol dehydrogenase
AO	Alcohol oxidase
BSA	Bovine serum albumin
CMC	1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluene sulphonate
DMF	Dimethyl formamide
DCC	Dicyclohexylcarbodiimide.
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC).
GA	Glutaraldehyde
GO	Glucose oxidase
HS	Hydroxysuccinimide
NAD(P)	ß-nicotinimide adenine dinucleotide (3′ phosphate)
NAD(P)H	ß-nicotinimide adenine dinucleotide (3′ phosphate) reduced form
NMP.TCNQ	N-methyl phenazinium. tetracyanoquinodimethane
PMS	Phenazine methosulphonate
TBApTS	tetrabutyl ammonium p toluene sulphonate.

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3.24 Platinum/haem-ADH electrode, response to ethanol

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Biosensor applications

Much of the science of analytical chemistry is concerned with the measurement of ions and fluids (Walt 1992). The ideal analytical instrument measures an analyte specifically and sensitively whilst avoiding the need to process the sample. An instrument which has these qualities and is also cheap, portable and robust would be the epitome of analytical devices. The recent development of electrochemical sensors for analytical applications has made a major contribution towards this goal (Schulz 1991, Roe 1992).

Electrochemical sensors are devices whose output reflects their chemical environment. Sensors in this category are as diverse as high-temperature oxide sensors, fuel cells, ion-selective electrodes and, more recently, enzyme electrodes and CHEMFETS (chemically-sensitive field-effect transistors). The final output of such sensors is either a voltage (potentiometric sensors) or an electrical current (amperometric sensors) (Hall 1988). Clark in the mid-1950s may be credited with the creation of the first electrochemical sensor: an electrode to measure dissolved oxygen in blood, utilizing a plastic membrane permeable to gases.

Biosensors are a group of electrochemical sensors defined as devices in which a biological sensing element (biointerface) is connected to or integrated with a physical transducer (Scheller et al). This addition capitalises on the high specificity of biological systems to add selectivity to the response of the physical sensor to a particular analyte. In this way, the sensing element, biointerface and the method for coupling the two may be optimised for any particular analytical problem (Byfield and Abuknesha 1991).

The potential commercial and scientific applications of biosensors are many and diverse, encompassing clinical medicine (assay of blood or urine concentrations), industry (monitoring of fermentation processes) (Luong et al 1991) and environmental monitoring for public health and safety purposes. The major advantage of biosensors is the ability to provide "on-line", real-time data, eliminating the need to take samples to the analytical laboratory. They are usually simple, rapid and dependable devices which do not require pre-processing of the sample.

1.2 Biosensor Classification

Biosensors consist of three different elements; a sensing element, a biological element and a transducing element to couple the two. The sensor portion of a biosensor may measure light emission or absorbtion, fluorescence, temperature change, optical change, electrical voltage or electrical current. The latter two sensing elements may be defined as electrochemical sensing elements.

The biological element may be either:

(a) a direct bioaffinity component utilising a binding event to detect substances (for example antigen and antibody interactions), or

(b) enzymatic or metabolic systems where recognition of the substrate by an immobilised receiver (enzyme) is followed by rapid chemical conversion of the substrate to a corresponding product which is detectable by the sensor.

Biosensors may also be categorised according to the method of immobilisation of the biological element on the sensor:

(a) First generation, involving membrane-entrapped or membrane-bound biological components

(b) Second generation, adding covalent coupling of the co-substrate

(c) Third generation, involving immobilisation of the biological component directly on an electronic element, such as the open gate of a field-effect transistor (FET).

These generations have evolved from the need to have the biological element in close proximity to the sensing element.(Guilbault 1988, Vadgama 1990).

1.3 Amperometric sensors

This subset of sensors is based on the exchange of electrons between an electrode poised at a fixed potential and an electroactive compound or ion in its environment. This "working" electrode may be either a cathode or an anode, depending on the expected direction of electron transfer, i.e. whether the electroactive material is to be oxidised or reduced.

Selection of an appropriate potential at which to poise the working electrode is commonly aided by the technique of cyclic voltammetry, in which the applied voltage is swept up and down a selected range and the redox current measured. A plot of current versus applied potential is a cyclic voltammogram and shows a characteristic increase in current at the potential where oxidation or reduction of an electroactive species occurs. In biosensor applications, the potential used is normally high enough to reduce the concentration of the electroactive species at the electrode surface to zero, so the current flowing in the circuit is limited by the rate of diffusion of this material to the

electrode. Factors affecting this rate include temperature, degree of solution mixing and the thickness of the biological membrane as well as the concentration of the electroactive species in bulk solution (Higgens 1988, Kemp 1985).

Amperometric sensors commonly use an interface containing an enzyme or enzymes to effect the conversion of an analyte which is not itself electroactive to one which can be measured amperometrically. In this case, sensor response also depends on the rate of conversion of the analyte to the electroactive species, i.e. the enzyme activity (Allen et al 1990).

The classical apparatus for applying a predetermined potential difference across two electrodes is a potentiometer. As shown in figure 1.1 (page 5), a potential (E) is applied between the counter and working electrodes and the current flowing through the working electrode is monitored. In theory any point in this system could be grounded but it is common practice to ground the current meter return to minimise stray noise pickup.

This "two-electrode" system is adequate when currents are small relative to the sizes of the electrodes. However if the solution resistance increases, the current at constant applied potential will decrease. This problem is overcome by replacing the counter electrode with two others: a reference electrode which monitors the potential of the solution near the working electrode and drives a potentiostat circuit whose output is connected to an auxiliary electrode which completes the circuit (figure 1.1).

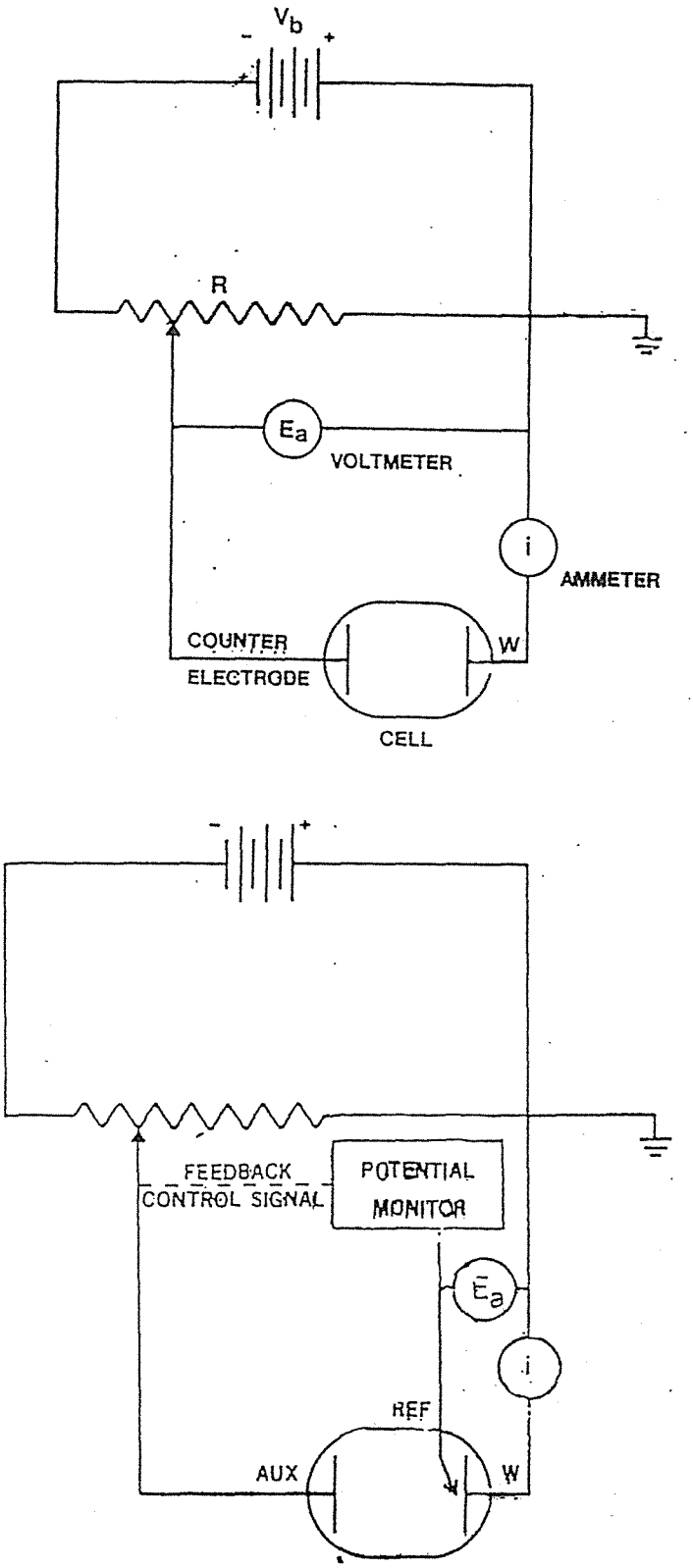


Figure 1.1 Electronic circuit for applying a potential difference to a working electrode. Upper: Two electrode system. Lower: Three electrode system.

This circuit allows the potential applied to the auxiliary electrode to vary so that the voltage across the working electrode/solution interface is maintained constant. In this way, all changes in current flowing in the working electrode circuit are due to changes in the transfer of electrons between the electroactive material and the electrode. With small electrodes, measurement of currents in the nanoampere range is found to be necessary.

1.4 Enzyme immobilisation

A sensitive and highly selective method for the determination of a given substrate utilises an immobilised enzyme in conjunction with either the two-electrode, or three-electrode system above, providing that (Kulys 1987):

(a) the compound to be measured is (or can be converted to) a substrate for the enzyme, and

(b) one product of the reaction is electroactive and can be oxidised or reduced at an appropriate "poised potential" on the working electrode.

The first practical electrode of this kind was reported by Updike and Hicks in 1967 who used glucose oxidase immobilised in a gel on a Clark-type amperometric oxygen electrode to measure glucose concentrations in biological fluids.

The most common materials in use for the design of working electrodes are platinum, gold and carbon. Of these carbon has the advantage of being cheap and readily available, while platinum has the advantage of a lower background current but is expensive.

Carbon as an electrode material is also very versatile ranging from carbon paste to spectroscopic graphite

(Dryhurst and McAllister 1984). Spectroscopic graphite is available in rods and is used in arc or spark emission spectroscopy. Carbon paste electrodes consist of an electrical contact at the bottom of an insulating well: the well is then filled with a paste of graphite in a suitable mulling liquid. Pyrolytic graphite is crystalline in structure and is anisotropic, rods can be cut from this material and the surface polished. Pyrolytic graphite has a variety of oxides and other functional groups on the surface making it a useful electrode for derivitisation. Carbon foil electrodes are made from compressed graphite and may be modified with mediators, adsorb enzyme or modified to covalently Glassy carbon electrodes are isotropic, impermeable to gases, electrically conductive and highly resistant to chemical attack, glassy carbon also has functional groups on its surface (Wring and Hart 1992).

An important aspect of sensor development is finding reaction conditions that effectively immobilise a sufficient concentration of the enzyme in an active, accessible and stable form to provide adequate sensor performance (Gibson and Woodward 1991). There are four main approaches to enzyme immobilisation; physical adsorption to a solid surface, entrapment in a polymeric gel or within microcapsules, crosslinking by means of bifunctional reagents often in combination with the aforementioned, and covalent binding to a reactive insoluble support.(Beh et al 1989,Sternberg et al 1988,Abdulla et al 1989, Weethall 1976, Lee et al 1989).

1.4.1 Adsorption

Substances such as alumina, charcoal, clay, cellulose, silica gel,glass, hydroxy-apatite and collagen are known to adsorb enzyme non-specifically. Ion-exchangers such as DEAE cellulose, CM cellulose can also be added to the list (Beh et al). The advantage of adsorption is the avoidance of complex reagents for electrode modification, and a minimum

of preparation and clean-up steps. Adsorption also tends to be less disruptive of the enzyme protein than chemical methods. However binding is more susceptible to environmental changes in pH, temperature and ionic strength.

1.4.2 Entrapment

Preparing a polymeric gel in a solution containing an enzyme results in entrapment of the enzyme. Polyacrylamide, starch, nylon and silastic gels can be employed for this purpose. However the relatively large thickness of gel layers presents a diffusional barrier to the analyte and gives a slow response to the sensor. Moreover, there is a continual loss of enzyme activity by surface leaching from the gel. Crosslinking with glutaraldehyde may overcome the latter (Guilbault and Neto 1985). Enzyme may also be entrapped in thin layers of polypyrrole and polyanaline.

1.4.3 Crosslinking

Bifunctional agents, such as glutaraldehyde, that induce non-specific intermolecular crosslinking between molecules of the enzyme protein can also bind enzymes to appropriate solid supports. Crosslinking of the enzyme itself yields an insoluble product but is expensive and inefficient since much activity is often lost. Diffusional limitations may result from this method of deposition and the lack of rigidity or mechanical strength is a disadvantage.

1.4.4 Covalent binding

This may be accomplished through functional groups in the enzyme protein which are not essential for its catalytic activity. Use is often made of nucleophilic functional groups present in amino acid side chains of proteins for coupling. It is important when utilising this technique to choose a support matrix which also has available functional

groups which can be activated by an appropriate reagent. Covalent bonding has the great advantage that the enzyme is unlikely to be released from the support matrix during use (Wingard 1985, Axen et al 1967).

1.5 Oxidase based electrodes

All enzymes catalysing oxido-reductions are termed oxidoreductases, which includes dehydrogenases and oxidases. An oxidase functions via a multiple redox electron carrier system using where oxygen acts as the terminal acceptor. All oxidases feature a flavin group and the enzyme itself exists in both oxidised and reduced forms depending on the oxidation state of the flavin group (Tatsuma et al 1991). Oxidases commonly produce the electroactive species hydrogen peroxide; these include xanthine oxidase, cholesterol oxidase, galactose oxidase, lactate oxidase, pyruvate oxidase, glucose oxidase, and alcohol oxidase. For an electrode-based system an oxidase enzyme can be immobilised at the electrode surface where exposure to substrate will lead to production of hydrogen peroxide which can then be measured amperometrically.

It would be ideal to couple the transfer of electrons from a redox centre couple inherent in the enzyme directly to the surface of the electrode rather than detecting the production of hydrogen peroxide. Unfortunately redox couples in enzymes such as glucose oxidase and alcohol oxidase do not undergo redox reactions with an electrode at sufficiently fast rates to make practical devices. However, addition of an external electron mediator can speed up this process. A mediator, in this context, is a low molecular weight redox couple which can transfer electrons from the active site of the enzyme to the surface of the electrode, thereby establishing contact between the two.(Cardosi and Turner 1991)

In order to be a suitable mediator a substance must:

(a) be chemically stable in the reduced and oxidised state, as it must be able to cycle constantly between the two states.

(b) be able to be reduced/oxidised within the pH range of the biosensor (usually between pH 5-9).

(c) be a specific electron acceptor for the enzyme and not participate in any side reactions.

(d) exhibit fast reaction kinetics.

Use of mediators to speed the rate of electron transfer offer distinct advantages in terms of improved sensitivity and the ability to use lower polarising voltages, which increases sensor specificity. However soluble mediators have the disadvantage of having to be added to the sample; a more practical approach would be to have the mediator firmly anchored to the surface of the electrode along with the enzyme.

1.6 Dehydrogenase based electrodes

The dehydrogenases are a group of oxidoreductase enzymes that are dependent on the nicotinamide cofactors NAD^+ or $NADP^+$ as electron acceptors. Dehydrogenases catalyse the oxidation of specific substrates with the concomitant production of the reduced cofactor NAD(P)H. The reduction of $NAD(P)^+$ to NAD(P)H can be utilised in biosensor systems by amperometric reoxidation of the reduced form at the working electrode (Tatsuma et al 1991).

At clean metal electrodes oxidation of NAD(P)H only occurs at large over-potentials and proceeds through radical

intermediates which can lead to interference and/or electrode fouling. The use of mediators to receive electrons from NAD(P)H and pass them on at a lower potential would again be desirable. Examples of such species are polymers containing orthoquinone and redox dyes adsorbed to graphite. Conducting organic salts can also be used, for example N-methyl phenazinium tetracyanoquinodimethanide (NMP.TCNQ), can be used in conjunction with alcohol dehydrogenase. Being electrically conducting this organic salt can be formed into pellets and used to make the working electrode (Bartlett 1990).

The need to add soluble nucleotide coenzymes to the system is another disadvantage to the use of dehydrogenases in biosensors. Attempts to immobilise the nucleotide on the sensor have generally resulted in large decreases in activity and loss of sensitivity.

1.7 An alcohol biosensor

Interest in a device to measure alcohol concentration is two-fold. Alcohol is a common analyte in the brewing industry and the clinical field requiring a portable measuring device. Monitoring of alcoholic fermentation has obvious applications in the brewing industry and medico-legal applications of an alcohol sensor for breath/blood levels would find wide use by law-enforcement and medical agencies. There is also a local interest by Massey University's Alcohol Research group, who require a device to continuously monitor ethanol concentration in metabolic experiments without taking samples.

It is possible to construct a microbial sensor for alcohol using alcohol-utilising micro-organisms and this was the basis for an electrode designed to be used in the measurement of fermentation processes, the electrode is robust but is not very selective or stable. A simple,

sensitive and accurate alcohol electrode has been obtained using a Clark-type oxygen electrode on which alcohol oxidase and catalase are immobilised (Verduyn et al 1983). The electrode is essentially an oxygen sensor which measures the decrease in oxygen tension in a layer of immobilized protein due to the action of alcohol oxidase after an addition of alcohol. Electrode responses were small and the electrode is not especially stable. There is an industrial analyzer for alcohol which employs alcohol oxidase sandwiched between a polycarbonate and a cellulose acetate membrane.

Alcohol oxidase systems are much the same as those used for glucose oxidase; alcohol response may be monitored by direct anodic detection of peroxide or with the use of a mediator. There are many publications on glucose sensors utilising glucose oxidase and some of the methods that have been reported have been repeated experimentally with alcohol oxidase as well as glucose oxidase in this thesis. Enzyme electrodes of alcohol dehydrogenase have been prepared using the conducting organic salt NMP.TCNQ (Zhao and Buck 1990), this electrode proved highly successful, giving responses to ethanol at a poised voltage of 0 V. Electrodes utilising membrane entrapped alcohol dehydrogenase with the mediator phenazine methosulpate have been reported . The use of phenazine methosulphate to lower the potential at which oxidation of NADH occurs is a continuing theme in the use of alcohol dehydrogenase as the basis for an alcohol biosensor as NADH oxidation occurs only at high potentials. Amperometric biosensors employing alcohol dehydrogenase (Suzuki et al 1975, Gestrelius et al 1975) have not been successfully applied to ethanol analysis in "real " samples.

1.7.1 Available enzymes

To achieve the aim of a working enzyme-based amperometric sensor two enzymes can be utilised: alcohol oxidase and alcohol dehydrogenase.

Alcohol oxidase catalyses the reaction;

alcohol + O_2 ----------> aldehyde + H_2O_2

Alcohol Dehydrogenase catalyses the reaction;

alcohol + NAD^+ ----------> aldedehyde + NADH + H^+

Glucose oxidase for reasons of economy, and availability, was used as a reference enzyme. Glucose oxidase was used to test techniques which could be adapted to alcohol oxidase because of the similarities between the enzymes.

Electrochemical sensors utilising these enzymes must be designed to detect hydrogen peroxide in the case of the oxidases and NADH in the case of alcohol dehydrogenase. The oxidase enzymes are perhaps the more promising enzymes to work with as they require no addition of cofactor. NAD must be added to the substrate or co-immobilised with the enzyme in the case of alcohol dehydrogenase.

Alcohol dehydrogenase is available at a high degree of purity, but has a low turnover number which limits the sensitivity in biosensor applications. On the other hand, the activity of commercially available alcohol oxidase is relatively low and it is less substrate specific: methanol is oxidised at higher rates than ethanol.

Utilising these two enzymes experiments were carried out to determine which combination of electrode material and immobilisation technique will render a good working electrode. Platinum, carbon and organic metals are good conductors and can be used in conjunction with either adsorption, entrapment, crosslinking, or covalent binding to construct a working electrode.

1.7.2 Aim of the present work

Thus the aim of this project was to determine the optimum combination of;

(a) enzyme

(b) electrode material, and

(c) immobilisation method

which would yield an alcohol biosensor of adequate sensitivity and stability.

CHAPTER 2: METHODS AND EQUIPMENT

2.1 Preparation of the organic metal N-methyl phenazinium tetracyanoquinodimethane (NMP.TCNQ)

From Bartlett (Cass 1990). Tetracyanoquinodimethane (TCNQ) (2.0 g) and lithium iodide (4.0 g) were refluxed together in 200 ml of spectroscopic grade acetonitrile for 2 hours and the solution allowed to cool overnight with stirring. The purple solid was filtered off over vacuum and the solid washed with spectroscopic grade acetonitrile until the washings were green, then washed with diethyl ether until the washings were colourless. The product was dried at room temperature under vacuum to constant weight (overnight). This gave 2.0 g of lithium tetracyanoquinodimethane (LiTCNQ), 100% yield.

LiTCNQ (1.03 g) was dissolved in refluxing absolute ethanol (100 ml) and added to phenazine methosulphonate (PMS) (1.5 g) dissolved in 50 ml of hot absolute ethanol. The solution was allowed to cool overnight with stirring and the black crystalline product filtered off over vacuum. The product was washed with a small quantity (approximately 5 ml) of diethyl ether and left to dry at room temperature overnight. This yielded 1.7 g of crude product. The crude product was dissolved in the minimum quantity (approximately 200 ml) of hot spectroscopic grade acetonitrile The hot solution was filtered through a fluted filter paper into a hot flask and the filtrate allowed to cool slowly overnight. The purified product was filtered off over vacuum as black crystals, washed with a small quantity of diethyl ether and allowed to dry at room temperature under vacuum to constant weight (overnight) to give approximately 1.5 g of purified material (75% yield overall).

2.2 Preparation of carbon foil electrodes

2.2.1. "End-on" electrodes

Discs of 0.5 cm diameter were punched from carbon foil, conducting wire was bonded to the carbon discs with silver loaded epoxy and left to set overnight. Glass tubing of 0.4 cm diameter cut into lengths of 5 cm. One end of the glass tubing was ground to a flat surface and the other end fire polished.

Araldite epoxy cement was used to seal the carbon foil disc to the ground end of the glass tubing and allowed to set overnight. To give strain relief the connection was bonded to the fire polished end of the glass tubing with araldite.

2.2.2. "Embedded" electrodes

Discs of 0.25 cm diameter were punched from carbon foil. Glass tubing of 0.26 cm diameter was cut into lengths of 4 cm. One end was then ground to a flat surface and the other fire polished. Araldite epoxy was then coated on the inside surface of the ground end of the glass tubing, the carbon disc pushed into the tubing and the araldite left to set overnight. Silver loaded epoxy was coated onto the tinned end of a conducting wire, pushed into contact with the carbon foil and left to set overnight. To give strain relief the conducting wire was bonded to the top of the glass tubing with Araldite.

2.3 Preparation of reference electrodes

A 20 cm length of glass tubing, 2.5 mm diameter, was heated in the centre and drawn out to form two electrode bodies. The tip of each was cut back to 0.5 mm diameter, a fibre of blue asbestos inserted in the tip and the tip melted in a gas flame. The tip was then polished on 600 grit carbon paper and the tube filled with Orion 90-00-01 reference electrode solution.

A 10 cm length of 0.6 mm diameter silver wire was polished

and coated with silver chloride by making it the anode of an electrolytic cell with a platinum cathode in 0.1 M HCl and applying 1.5 V for 15 minutes. This wire was then inserted into the tube to form a reference electrode.

The impedence of each electrode was roughly checked by connecting an ammeter across the electrode and an Orion 09.01 silver/silver chloride electrode in 1 M KCl. A good electrode had an impedence of 50-100 kOhms in this system: electrodes of lower resistance tended to leak too rapidly and those of higher resistance were polished back at the tip to decrease the resistance into the above range.

2.4 Conducting paste electrode

A teflon rod (9 cm long, 13 mm diameter) was drilled longitudinally with two 3 mm bores. A reference electrode (section 2.3) was inserted through one bore with the tip just exposed and through the other was inserted a brass rod with a platinum disc soldered to its end. This end was located 2 mm before the end of the teflon to form a well into which paste could be pressed.

2.5 Preparation of platinum electrodes

2.5.1. Wire electrodes

One end of a 10cm length of glass tubing (0.25 cm diameter) was rotated in a flame to reduce its bore to about 5 mm. 1 cm of platinum wire (0.25 mm diameter) was inserted through the orifice and the end of the glass tubing remelted to seal around the wire. Solder was introduced down the tube, melted in the flame, a connecting wire pushed into this molten solder and the whole allowed to cool.

2.5.2. Embedded electrodes

A 1 cm platinum wire was spot-welded onto one side of a 4 mm diameter disc punched from 0.5 mm platinum foil and a connecting lead of shielded cable soldered to the platinum

wire. The assembly was glued with epoxy cement into a glass tube (6 mm diam, 7 cm long) so that the disc was flush with one end and the connecting wire protruded from the other. The tube was then filled with polyurethane embedding resin for strain relief, and the assembly allowed to set. The glass/epoxy was then ground back until the platinum disk was exposed and the surface polished with alumina using a Bioanalytical Systems electrode polishing kit.

2.6 Auxiliary electrodes

Platinum auxiliary electrodes were used in all of the electrode systems. Platinum wire auxiliary electrodes were used in conjunction with platinum, carbon and polypyrrole working electrodes. A platinum mesh auxiliary electrode was used in conjunction with the organic metal electrode.

2.7 Instrumentation

2.7.1 Potentiostat

The potentiostat used for cyclic voltammetry was constructed by Dr. Greenway from a circuit published in (X), using operational amplifiers. The circuit uses the difference between an applied external voltage (Va) and the voltage at the reference electrode (Vr) located near to the working electrode to modulate the voltage applied to the auxiliary electrode. The phase of this modulation is such that the above difference is minimised: in practise Vr was always within 1 mV of Va, allowing for a sign difference.

2.7.2 Reference Voltage

This was supplied by a Keithley 230 Programmable Voltage Source which could be programmed to supply either a triangular wave form (for cyclic voltammetry) or a constant voltage (for amperommetry).

2.7.3 Current Monitor

This was a Keithley 485 Autoranging Picoammeter and was connected between the working electrode and ground.

2.7.4 Computer Interfacing

Both the Keithley voltage source and picoammeter had the capacity to (a) receive commands and (b) transmit data via a IEEE 488 (GPIB) bus to a x computer. This computer was an IBM 286 clone fitted with an Advantech IEEE 48 interface card, 2 megabytes memory and an 50 byte x disk for software and data-file storage.

2.7.5 Software

Programming of the instruments on the IEEE 488 bus, data collection and data manipulation was carried out by a package called ASYSTANT.GPIB (Asyst Conformation). Two programmes in ASYSTANT were written for the present work:

1. CYCLICVA- a set of routines to program the voltage source for a sawtooth output with user-determined limits and rates of change, trigger the picoammeter to take periodic readings (usually 1Hz) and store voltage and current readings to disk.
2. LOGAMPS- a simpler program to record current data at a constant applied voltage.

Asystant also allows the user to manipulate data files with a variety of mathematical and statistical freedoms and also to plot data on VDU screen or printer. The data-based illustration in this thesis were produced in this way.

2.7.6 Cyclic Voltammetry

In voltammetry, a potential is applied between two electrodes, and the current is measured. The applied potential can be varied, for example, to give a linearly increasing value versus time. The resulting current-potential data becomes the equivalent of an absorbance-wavelength spectrum, with the amplitude, shape, and relative

positions of the current peaks important variables. In cyclic voltammetry, the applied potential is varied at a fixed rate (20 mV/second) in one direction and then switched in sign and varied at the same rate but in the opposite detection. A schematic diagram of the experimental setup for cyclic voltammetry is shown in Figure 2.1. Current flows from electrodes X-to-Y, with reference electrode Z present for the measurement of the applied potential. More negative potentials correspond to a stronger reducing capability and vice versa for oxidation. Transfer of electrons produces an increase in current. Current peaks because the electroactive species in solution in the vicinity of the electrode surface is reduced. The direction of the scan is reversed and the reduced species now undergoes oxidation. When a substance with a reversible electron transfer step is present (for example the mediator, ferrocene) the cyclic voltammogram will show two peaks; one ascending (commonly called cathodic) and the other descending (commonly called anodic). In the case of the presence of an electron sink (for example the addition of an enzyme and its substrate) a large cathodic peak (or catalytic current) will replace the peaks of the previous cycle (Wingard 1985).

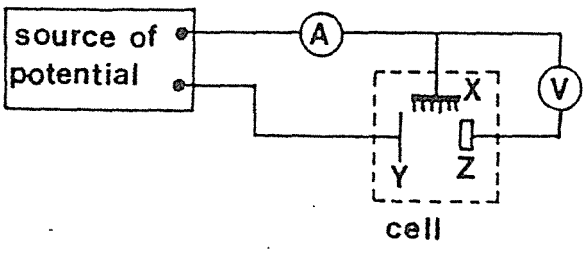


Figure 2.1 Schematic diagram for cyclic voltammetry, X is the working electrode, Y is an auxiliary electrode and Z is a reference electrode against which the applied potentials are measured. A represents the measured current and V the applied potential. (Wingard 1985).

2.8 Available enzymes

Alcohol oxidase (Sigma A0763-from Candida sp.) had an activity of 0.4 units per mg. Alcohol Dehydrogenase (Sigma A7011-from bakers yeast) had an activity of 290 units per mg. Glucose Oxidase (Sigma G2133 Aspergillus sp.) with activity of 6 units per mg solid.

2.9 Glucose oxidase assay on electrodes

REAGENTS:
Glucose oxidase buffer (0.1 M phosphate, pH 6.0). Dipotassium hydrogen phosphate (17.4 g) was dissolved in about 800ml MilliQ water, the pH adjusted to 6.0 with 1 M H_2SO_4 and made up to 1 l.
Substrate/chromagen reagent.

207 mg	phenol	(11 mM)
31.2 mg	4-aminoantipyrene	(0.77 mM)
14.4 g	glucose (anhydrous)	(0.4 M)
2.0 mg	peroxidase	(1.5 kU/ml)

These were dissolved in glucose oxidase buffer and made up to 200 ml with buffer.
Standard glucose oxidase. A stock of 20 mg ml^{-1} glucose oxidase of known activity was made up. A 1:100 dilution in buffer was made for assay. 50 ul of this dilution contains 10 ug enzyme (6 x 10^{-2} Sigma units).

PROCEDURE:
3.0 ml of substrate/chromogen reagent was pipetted into tubes of appropriate size for the electrodes and preincubated in a water bath at 35 C. For standards, 50 µl of diluted glucose oxidase was pipetted into a reagent tube at 35 C at zero time, mixed well and left at 35 C. Absorbance at 512 nm was read after exactly 5.0 minutes. Electrodes were dropped into a reagent tube at zero time, swirled or mixed continuously for 5.0 minutes at 35 C, the electrode removed and the absorbance at 512 nm measured. A

blank of reagent only was read at 512 nm was subtracted from the other readings.

CALCULATION:

If: U = units/mg of reference enzyme
S = ug of standard enzyme taken for assay
As = increase in absorbance of standard in 5 mins.
Ax = increase in absorbance of unknown in 5 mins.
Vs = ml of reagent used in standard
Vx = ml of reagent used for unknown

Then: Electrode-bound units of glucose oxidase =

$$\frac{S \times U \times Ax \times Vx}{1000 \times As \times Vs} = \frac{6 \times 10^{-2} \times Ax}{As}$$

2.10 Alcohol oxidase assay on electrodes

REAGENTS:

Alcohol oxidase buffer (0.15 M, pH 7.5). Dipotassium hydrogen phosphate (20.4 g) was dissolved in about 800ml MilliQ water, the pH adjusted to 7.5 with 1 M H_2SO_4 and made up to 1 l.

Substrate/chromogen reagent.

207 mg	phenol	(11 mM)
31.2 mg	4-aminoantipyrene	(0.77 mM)
2.24 ml	methanol	(0.4 M)
2.0 mg	peroxidase	(1.5 kU/ml)

These were dissolved in alcohol oxidase buffer and made up to 200 ml with buffer.

Standard alcohol oxidase. A stock of 3 mg ml^{-1} alcohol oxidase (0.4 units/mg) was made up.

50 µl contains 0.15 mg (6×10^{-2} Sigma units)

PROCEDURE:
Exactly as for glucose oxidase section 2.8

CALCULATION:
If: U = units/mg of reference enzyme
S = ug of standard enzyme taken for assay
As = increase in absorbance of standard in 5 mins.
Ax = increase in absorbance of unknown in 5 mins.
Vs = ml of reagent used in standard
Vx = ml of reagent used for unknown
Then: Electrode-bound units of glucose oxidase =

$$\frac{S \times U \times Ax \times Vx}{1000 \times As \times Vs} = \frac{6 \times 10^{-2} \times Ax}{As}$$

Note: one unit will oxidize 1.0 µmole of methanol to formaldehyde per min. at pH 7.5 at 25 C.

2.11 Alcohol dehydrogenase assay on electrodes

REAGENTS:
Alcohol dehydrogenase buffer (1 M Tris(hydroxymethyl)aminomethane), pH 7.5).
Standard NAD+ (30 mM), NAD^+ (0.2 g) was dissolved in 10 ml of alcohol dehydrogenase buffer. Standard Ethanol (1 M). 61.61 ml 95% ethanol in 1 l of alcohol dehydrogenase buffer.
Substrate.

1.5	ml	Buffer
0.3	ml	NAD+
0.3	ml	Ethanol
0.895	ml	Water

Standard alcohol dehydrogenase. A stock of 10 mg ml^{-1} alcohol dehydrogenase of known activity was made up. A 1:100 dilution with buffer was made. 145 Sigma Units.

PROCEDURE:
Into 16x100 mm tubes (or others to hold electrodes) 3.0 ml of substrate was pipetted and preincubated at 37 C. For standards, 5 ul of diluted alcohol dehydrogenase was pipetted into a reagent tube at 37 C at zero time, mixed well and absorbance read continually at 340 nm or at 30 sec., 60 sec. etc. for 5 minutes. Electrodes were dropped into a reagent tube at zero time, mixed well and absorbance recorded at 340 nm at 30 sec. intervals for 5 minutes. For both standards and electrodes the rate of absorbance per minute was calculated.

CALCULATIONS:
If: c = Concentration of alcohol dehydrogenase
dA = Change in absorbance per minute
E = Extinction coefficient of NADH (6220)
Then:

$$c = \frac{dA}{El}$$

Note; one unit of alcohol dehydrogenase will convert 1 μ mole of ethanol to NADH per min at pH 8.8 at 25 C.
1 unit will convert 0.73 μmole of ethanol to NADH per min at pH 7.5 at 37 C.

2.12 Amino group assay on electrodes

HEPES (HE) buffer; 0.15 M HEPES, 0.079 M NaOH, 1 Mm EDTA, pH 7.6.

Solvent/HEPES (SHE) buffer; 3 volumes HE buffer + 2 volumes N,N,dimethylformamide.

Electrodes were rinsed with SHE buffer and placed in 4 ml of SHE buffer. 3(-2pyridylitho)propionic acid N-hydroxy succinamide ester (SPDP) (10 mg) was dissolved in MilliQ and

0.2 ml of this solution was added to the electrode and was shaken for 1 hour at room temperature. The electrode was washed with SHE buffer and then several volumes of HE buffer to remove all excess SPDP. 5 ml of 0.2 M DTT/HE was added to electrode and rinsed for 45 min. This solution was made up to 25 ml in HE and the absorbance read at 343 nm.

2.13 Preparation of polypyrrole films on platinum electrodes

2.13.1 Deposition from acetonitrile

(Wynne and Stuart) Electrodes were suspended in 0.15 M tetrabutyl ammonium p toluene sulphonate (TBApTS) and 0.29 M pyrrole in acetonitrile. The electrodes were then cycled against a Ag/AgCl reference electrode between 1 V and 2 V for 40 minutes (scan rate of 20 mV/second).

2.13.2 Aqueous deposition

Electrodes were suspended in 0.2 M p toluene sulphonate (pH 6 with NaCl) and 0.2 M pyrrole. The polypyrrole was then deposited slowly while stirring and cycling over 1 V (scan rate of 20 mV/second), an even electrical field was important to avoid "dendritic" growth.

Miscellaneous chemicals

Haemin was obtained from Sigma, catalogue number H2250 (bovine), and was dried under high pressure for 4-5 hours before use.

Dicyclohexylcarbodiimide (DCC) was obtained from Mark-Schuchardt (R: 36/37/38, S: 7/8-23-24/25-37/39-45).

Hydroxysuccinimide (HS) was obtained from United States Biochemical Corporation (catalogue number 17470)

Dimethylformamide was dried over calcium hydride, distilled over reduced pressure and then filtered.

All of the above were stored containing a molecular sieve (4A beads with no indicator) in a vacuum desiccator over silica gel.

CHAPTER 3: RESULTS

Each electrode that was made was evaluated in one or more of the following ways;

1. Cyclic voltammetry, with and without added substrate.
2. Amperommetry, at various substrate concentrations.
3. Enzyme activity assay.
4. Stability to storage/washing.

Voltages are expressed relative to Ag/AgCl unless otherwise stated and the three electrode system was used in all cases.

3.1 Enzyme adsorption

3.1.1. On Carbon Foil

Glucose oxidase (GO) is strongly adsorbed to carbon foil electrodes placed in a 12.5 mg ml^{-1} solution of the enzyme (Mosbach 1988). An end-on carbon foil (Section 2.2.1) electrode was placed in a 12.5 mg ml^{-1} solution of glucose oxidase for 60 minutes and after successive washings cyclic voltammograms were performed in buffer with and without glucose (100 mM). Under these conditions no catalytic current was evident, and it was assumed that GO had not been adsorbed onto the electrode. Enzyme assays were then performed (Section 2.9) to verify this assumption, 46.0 x 10^{-2} units were found to be bound to the electrode. This indicates that GO was adsorbed onto the electrode and that there must be another reason for the lack of response to glucose.

A clean carbon foil electrode was used to investigate the reason for the lack of response to glucose. The electrode was poised at +0.7 V, chosen as this is the potential at which hydrogen peroxide is reduced at platinum electrodes, and allowed to reach a stable current output in 100 mM phosphate buffer, pH 7.5. The electrode was then placed in buffer containing 100 μM hydrogen peroxide but no

amperometric response was detected. 5ul of undiluted 100-volume hydrogen peroxide was added to solution, a small response was recorded at this high concentration. As this concentration of peroxide was more than could be produced enzymatically it is justifiable to conclude that enzyme-produced hydrogen peroxide is not oxidised at carbon foil electrodes at the potentials used in this experiment.

A mediator acting as an electron shuttle between the enzyme and the electrode would bypass the production of hydrogen peroxide and permit the use of carbon foil at low potentials (Cardosi and Turner 1991). Electrochemical investigations using cyclic voltammetry have shown ferrocene (bis(n^5-cyclopentadienyl)iron, $FeCp_2$) to be an excellent mediator for oxidase enzymes (Cass et al 1984). The mediator replaces dioxygen as a cofactor for GO and many other enzymes. Thus ferrocene acts as an electron acceptor and the reduced form is then re-oxidised at a suitable electrode as in the following scheme;

$$\text{glucose} + GO_{ox} \text{ -------->} GO_{red} + \text{gluconolactone}$$

$$GO_{red} + 2FeCp_2R^{+} \text{ ----------> } GO_{ox} + 2FeCp_2R + 2H^{+}$$

$$2FeCp_2R \text{ ----------> } 2FeCp_2R^{+} + 2e^{-}$$

The less soluble derivative 1,1 dimethylferrocene is a more useful mediator as ferrocene itself is significantly soluble in the reduced state and is lost into solution during use of the sensor.

Cyclicvoltammograms were performed on a clean carbon foil (end-on) electrode,revealing high basal currents which are characteristic of this group of electrodes, they are also very variable in nature, between individual electrodes and between successive cycles. The same electrode was then modified with 1,1 dimethylferrocene by solvent evaporation;

15 µl of 100 mM 1,1 dimethyl ferrocene in toluene was pipetted onto the carbon foil disc of the electrode and allowed to air dry. The peaks seen in the cyclicvoltammogram of the ferrocene modified electrode (Figure 3.1) are also very characteristic (Hill and Sanghera 1990) and exhibit reversible one-electron transfer.

Glucose oxidase was allowed to adsorb onto the modified electrode as described previously. Cyclic voltammograms of the modified electrodes were run with and without glucose (100 mM). The modified electrode with glucose oxidase adsorbed was poised at the potential indicated by the catalytic current evident on the cyclic voltammogram (+0.2 V) and an amperometric response curve to glucose in 100 mM phosphate buffer, pH 7.5 generated (Table 3.1).

Glucose concentration mM	Response above basal. µAmps
10	0.047
20	0.257
50	0.787

Table 3.1 Carbon foil/dimethyl ferrocene/GO adsorbed. Response to glucose.

This data indicated a graded though non-linear response to glucose concentrations which was readily repeatable. Enzyme assays were performed and 8.49×10^{-2} units were found to be bound to the electrode.

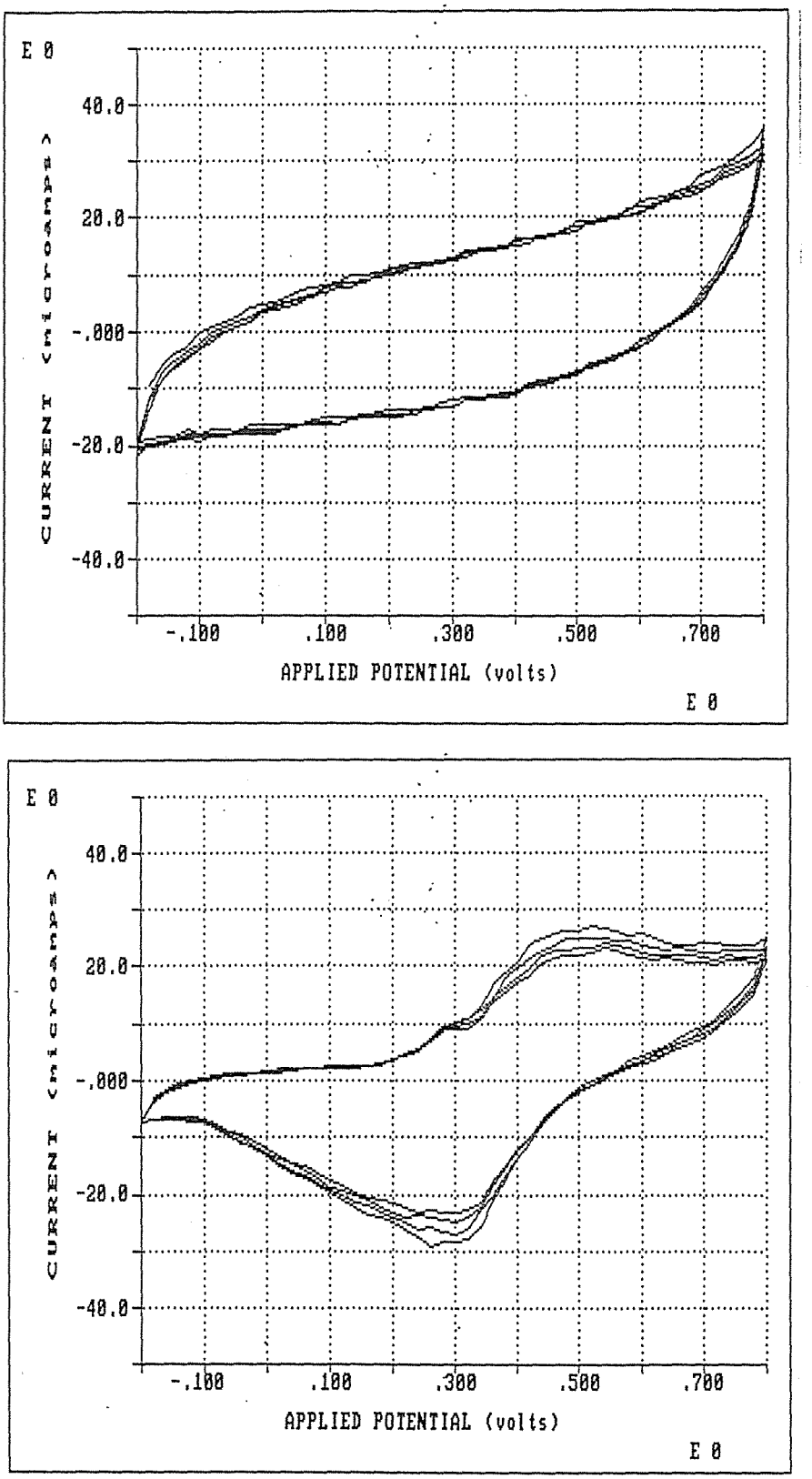


Figure 3.1 Carbon foil electrodes, cyclicvoltammogram. Upper: clean electrode. Lower: electrode modified with 1,1 dimethylferrocene.

Following the limited success of this experiment alcohol oxidase (AO) was allowed to adsorb onto a fresh carbon foil electrode modified with dimethyl ferrocene in the previous manner. Cyclic voltammograms indicated a change in catalytic current at +0.2 V, this voltage was applied and amperometric responses to ethanol in 100 mM phosphate buffer (pH 7.5) recorded (table 3.2). Enzyme assays were performed (Section 2.10); 1.00 units were bound to the electrode. The electrode had enzyme activity and exhibited a linear response with regards to ethanol (Figure 3.2). This experiment was readily repeatable but the magnitude of the response varied.

Ethanol concentration mM	Response above basal. µAmps
0.1	1.125
1.0	2.25

Table 3.2 Carbon foil/dimethylferrocene/AO adsorbed. Response to ethanol.

Direct adsorption of enzyme is simple, inexpensive and repeatable, but a major concern about this technique is whether the enzyme is bound irreversibly or will slowly leach out of the carbon foil. Electrodes made in this manner do not give consistent results and one reason for this may be the leaching of enzyme activity out of the electrodes. If the enzyme does leach out a decrease in sensitivity will be expected. Consequently GO immobilised on carbon using the technique above, was subjected to washing in phosphate buffer. Enzyme assays do indicate loss of enzyme following successive washings (Table 3.3).

Electrode	Electrode bound units
fresh electrode	15.82×10^{-1}
after further washing	2.88×10^{-1}
electrode store for one week	7.39×10^{-1}
after further washing	2.69×10^{-1}

Table 3.3 Electrode bound units of GO adsorbed to carbon foil electrodes.

It is evident from these results that GO does leach out of the carbon, however there is still enzyme present thus this cannot be the only source of electrode variability.

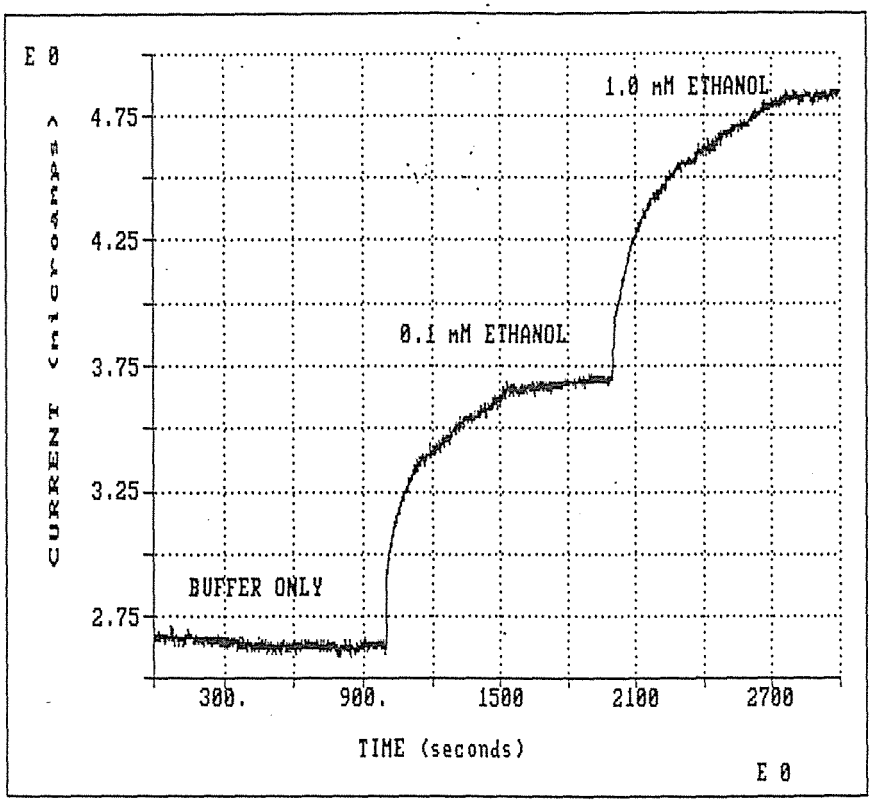


Figure 3.2 Carbon foil/dimethyl ferrocene/alcohol oxidase (adsorbed) electrode, response to ethanol. Amperometry trace.

3.1.2. N-methyl phenazinium.tetracyanoquinodimethane (NMP.TCNQ)

NMP.TCNQ is one of a family of conducting organic salts. In general conducting organic salts are made by the combination of an electron donor and an accepter. These species are typically planar molecules with delocalised pi-electron densities both above and below the molecular plane. To be conducting it is necessary that the donors and acceptors fall into different stacks (Figure 3.3). It is also necessary that the donor or acceptor form a new aromatic sextet by the loss or gain of an electron. There must also be partial charge transfer between the stacks (Bartlett 1990).

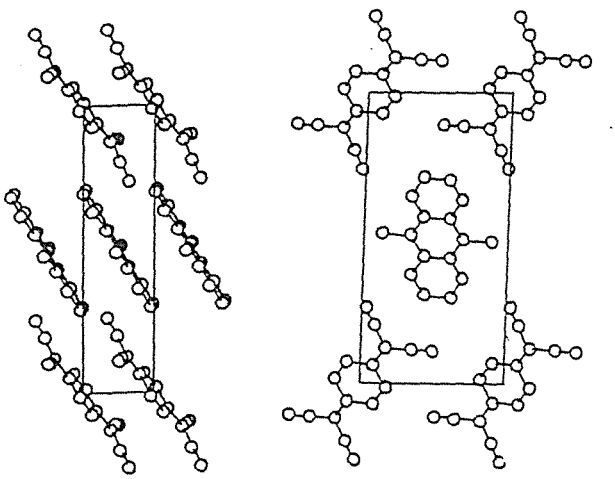


Figure 3.3 The crystal structure of NMP.TCNQ showing the separate stacks of NMP and TCNQ molecules (Bartlett).

NMP.TCNQ acts as a mediator for dehydrogenases, lowering the potential at which oxidation of NADH will occur (Cardosi and Turner). NMP.TCNQ was prepared (refer section 2.1) and mixed to form a paste with alcohol dehydrogenase (ADH) and NAD^+ in the proportions as follows:

5.3% alcohol dehydrogenase (0.57 mg)

10.6% NAD (1.14 mg)

84.1% NMP.TCNQ (8.99 mg)

to make 10.7 mg total (Zhao and Buck). A solution of 0.527 mg of low molecular weight poly(vinylchloride) in 70 µl tetrahydrafuran was allowed to evaporate until it had a consistency of "runny" honey. This PVC solution was then added to the above mix and stirred to give a consistency of putty. The paste was smeared into the cavity of a paste electrode and cyclicvoltammograms performed between 0.0V and +0.5 V with and without ethanol (100 mM) in tris buffer (100 mM) containing NAD^+ (300 mM) pH 7.5. The cyclicvoltammograms show very high currents (up to 100 µAmps) and no clear difference with and without ethanol. The choice to poise the electrode at 0.0 V was made as this is the potential at which Zhao and Buck (1991) and Sim (1991) poised their electrodes to obtain amperometric responses. The electrode did (Table 3.4) respond to ethanol but did not yield a reproducible response dependant on ethanol concentration (Figure 3.4).

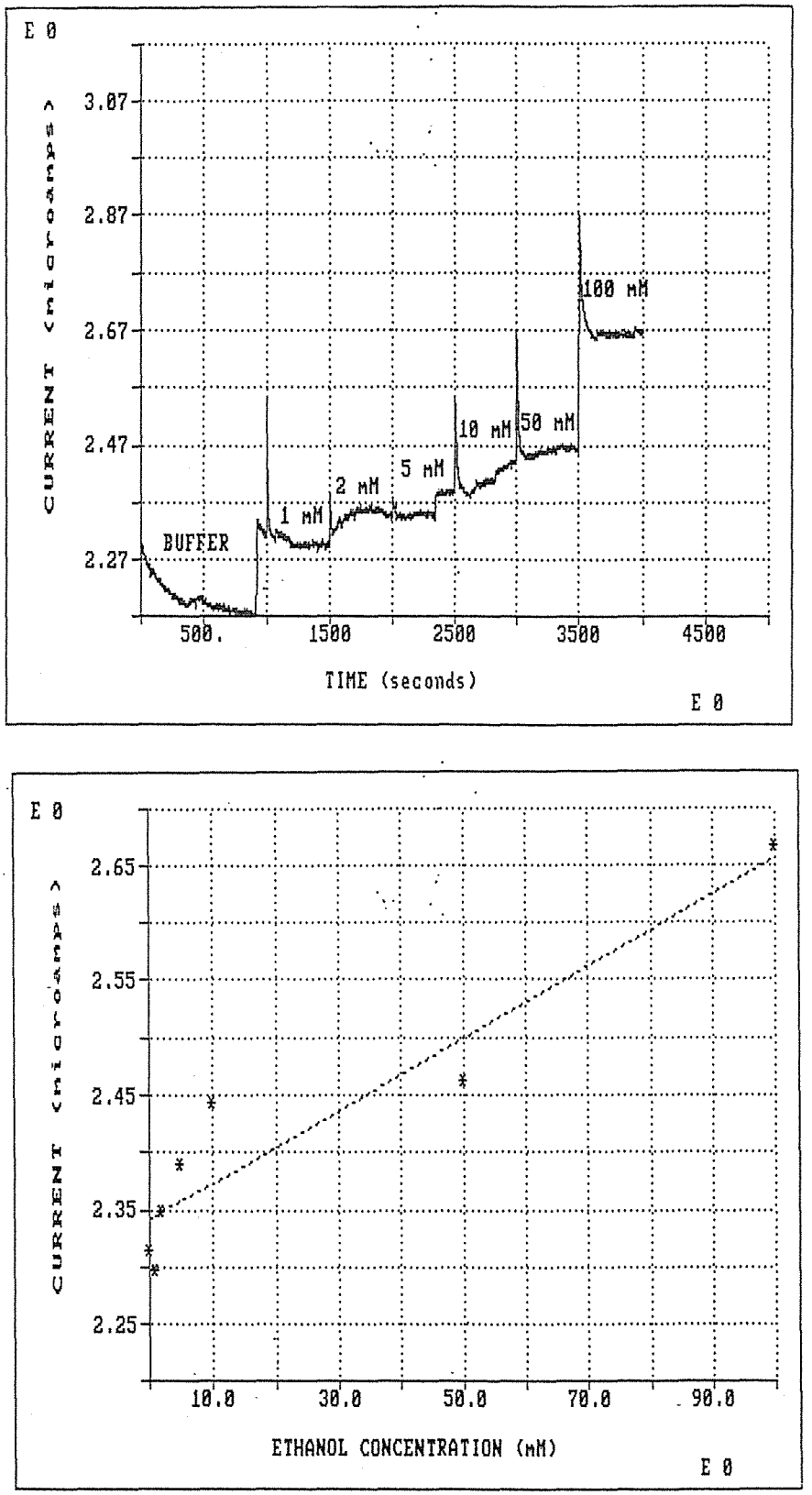


Figure 3.4 NMP.TCNQ/ADH electrode, response to ethanol. Upper: Amperometry trace. Lower: Response curve.

Concentration of ethanol mM	Response above basal. µAmps
1	-0.018
2	0.034
5	0.076
10	0.129
50	0.147
100	0.353

Table 3.4 NMP.TCNQ/ADH electrode. Response to ethanol.

In subsequent trials of electrodes made in the above manner no response was obtained. This is assumed to be due to the oxidation of the electrode material to give insoluble TCNQ. It is known that NMP.TCNQ is readily oxidised irreversibly (for example, at electrode potentials above +0.5 V versus standard calomel electrode) to yield insoluble TCNQ.

$$NMP.TCNQ \longrightarrow NMP^{+} + TCNQ^{ox} + e^{-}$$

NMP.TCNQ is also reduced irreversibly at potentials below -0.05 V. Although the electrodes were not exposed to potentials outside of these limits, colour changes in the buffer solution were observed during use. Blue-green streaks were evident stemming from the cavity of the electrode, probably due to soluble NMP^{+}.

Consequently it was assumed that the organic salt preparation was not adequate as the batch of salt used in the first case was different from that used in the subsequent experiments. At this point the decision was made to discontinue experiments with NMP.TCNQ due to the difficulty in preparation of the salt and the ease with which it could be oxidised or reduced. The response to

alcohol was not sufficient to warrant the continuation of these experiments and other avenues of endeavour were more promising.

3.1.3. Carbon paste electrodes

Gorton et.al.(1991) immobilised glucose dehydrogenase in carbon paste together with a mediator and the cofactor NAD^+ to produce a glucose biosensor which was both stable and sensitive. Kulys et al have also reported a successful pyruvate electrode made from carbon paste using pyruvate oxidase and methylene green. Because of the success of these two electrodes a similar technique was attempted with alcohol dehydrogenase.

Various redox mediators can shuttle the electrons from NADH to an electrode at a substantially reduced overpotential (for example NMP in section 3.1.2). A member of organic compounds containing quinoid structures reversibly oxidise NADH in aqueous solutions. Stability of these compounds can be increased when the basic p-phenylenediimine structure is incorporated into a phenoxazine or a phenothiazine. Such structures strongly adsorb on graphite electrodes to form chemically modified electrodes. The close coupling between the modifier and the graphite results in very fast charge-transfer rates between the modifier and the electrode. Meldola's Blue is one mediator in this class and was used by Gorton et. al. in their glucose dehydrogenase system. An attempt was made to extend its use to alcohol dehydrogenase.

Carbon paste electrodes were made by mixing 50 mg of carbon paste with 2.5 mg Meldola's Blue, 4 mg ADH and 25 mg NAD. The carbon paste mix was then smeared into a cavity electrode. In cyclic voltammetry, this electrode was cycled between -0.2 V and +0.8 V. A difference between cycles with and without ethanol is evident but at the potential where the greatest catalytic current is apparent (+0.5 V) the Meldola's Blue appeared to be unstable, leaching a blue

product into solution. At lower potentials the difference in the cyclic voltammograms is not as evident and although the Meldola's Blue remained stable there was no response to ethanol.

3.1.4 Polypyrrole

Polypyrrole is an insoluble conducting polymer formed by the anodic oxidation of pyrrole. Deposition from acetonitrile solution (section 2.12.1) gives a more coherent coating of polymer, but electrolysis of aqueous solution is more convenient since some soluble enzymes have been reported to co-deposit with the polypyrrole (Rishpon and Gottesfeld 1991,Kajiya et al 1991,and Tatsuma et al 1992, Schuhmann and Kittsteiner-Eberle 1991, Shuhmann et al 1991).

Using the aqueous deposition technique with 2 $mgml^{-1}$ of ADH present in solution a platinum electrode was cycled from +0.4 to +1.4 V (scan rate was 20 mV/second) for 40 minutes. Polypyrrole was deposited in places but was very patchy over the majority of the electrode. A gelatinous coat covered the areas where the polypyrrole was not deposited. Deposition was continued for a further 50 minutes with no further increase in the amount of polypyrrole deposited. Thus ADH appeared to inhibit the deposition of polypyrrole.

Deposition was attempted in the presence of a decreased concentration of ADH (0.5 $mgml^{-1}$) both aerobically and after bubbling with nitrogen gas. No polypyrrole was deposited in either case and protein flocculation was observed in anaerobic solutions indicating protein denaturation.

Yabuki et. al. incorporated ADH in polypyrrole films with phenazine methosulphonate (PMS) in a deoxygenated system. This approach yielded very stable electrodes and thus an attempt was made to repeat this work. 0.2 $mgml^{-1}$ of ADH, 30 mM NAD and 10 mM PMS were dissolved in deoxygenated 1 M pyrrole solution containing 100 mM KCl. A platinum electrode

was then placed in this solution and +0.8 V applied. A polypyrrole layer formed within 5 minutes and was very smooth in appearance. Cyclic voltammograms of this electrode in buffer (600 mM Tris, 400 mM lysine) pH 7.5 and in 100 mM ethanol were not different, indicating no catalytic current. Large peaks on the voltammogram may be due to the cycling between oxidised and reduced states of the polypyrrole itself.

On repeating this technique a smooth polypyrrole coating was again formed and the electrode prepared in this manner was poised at +0.3 V. After reaching a stable current in buffer (600 mM Tris, 400 mM Lysine, pH 7.5) containing 400 mM NAD^+, the electrode was placed in 100 mM ethanol in buffer. Any response to ethanol was limited to the nanoampere range.

The advantage of using this co-immobilisation technique is that a membrane forms quickly and is very durable. It is possible however that the NAD, being a small molecule does leach out of the polypyrrole film. Nevertheless it is probably useful in stabilising the enzyme during polymerisation by occupying its binding site on the protein. Because of more promising avenues of research this technique was not investigated further.

3.2 Protein coated enzyme electrodes

Enzymes may be incorporated into a protein matrix (for example gelatin or bovine albumin), the matrix allowed to set and the protein and enzyme bound together by a bifunctional reagent such as glutaraldehyde.

3.2.1. Platinum electrodes

Early biosensors often immobilised enzymes onto platinum by crosslinking the enzyme and gelatin or bovine albumin using glutaraldehyde (GA) (Clark and Lyons 1963, Alva 1991, Mullen et al 1986, Guasinham et al 1990), see Figure 3.5.

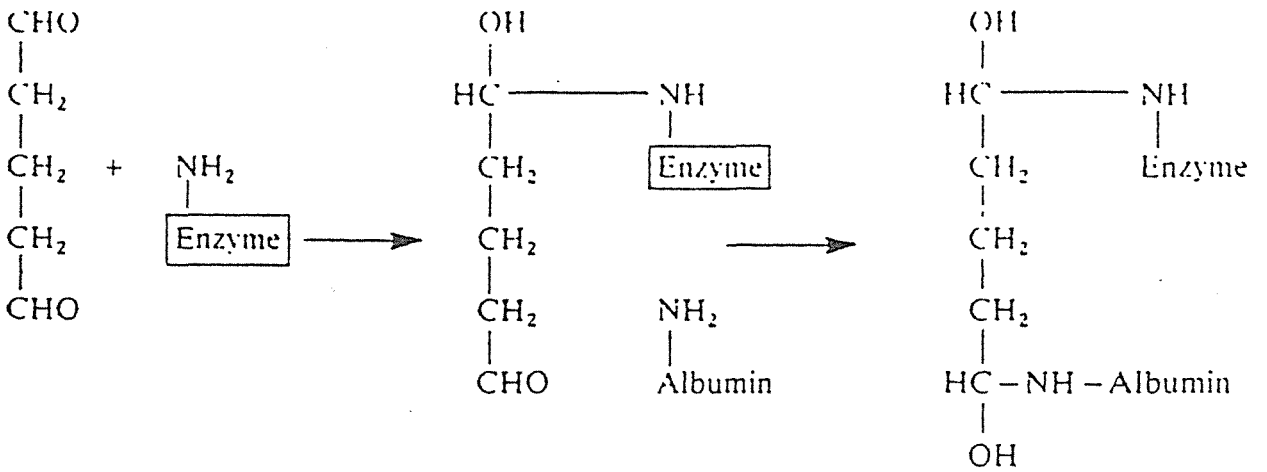
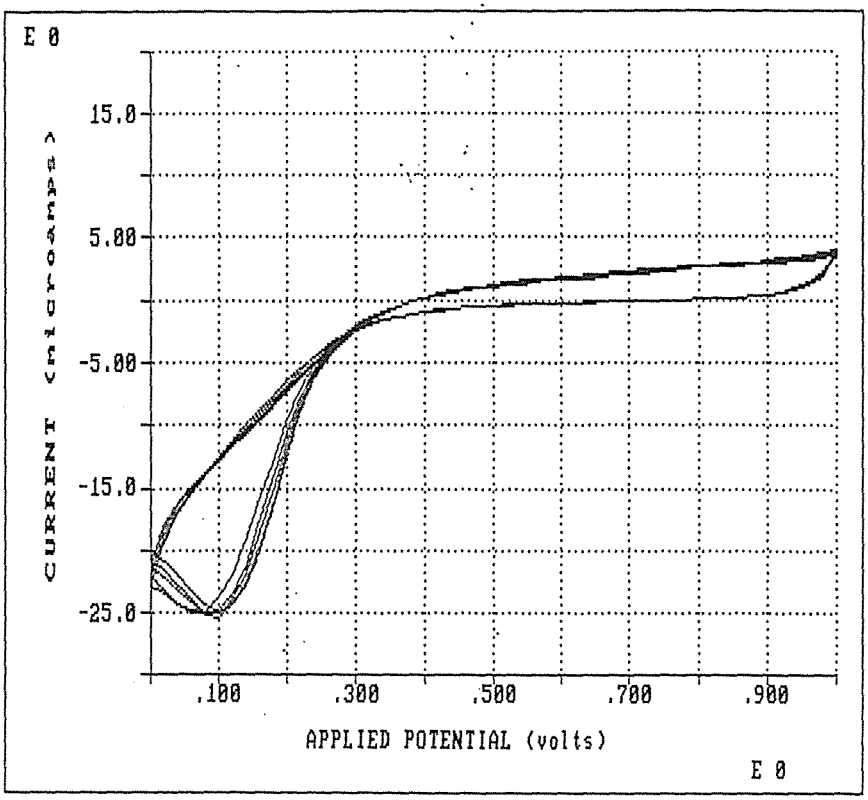


Figure 3.5 Reactions involved in the preparation of glutaraldehyde-attached enzyme.

20 $mgml^{-1}$ of GO was dissolved in 5% gelatin in pH 7 buffer at 35 C, a platinum wire was dipped into this mix, allowed to set and immersed into 1% glutaraldehyde for 1 minute. These mild crosslinking conditions had earlier been found to be optimum for fixing the maximum activity of urease to a metal electrode. Electrodes prepared in this manner were cycled with and without glucose (Figure 3.6). A difference at +0.5 V on the cyclicvoltammogram lead to the electrode being poised at a voltage of +0.5 V and responses to varying glucose concentrations measured amperometrically (Table 3.5).



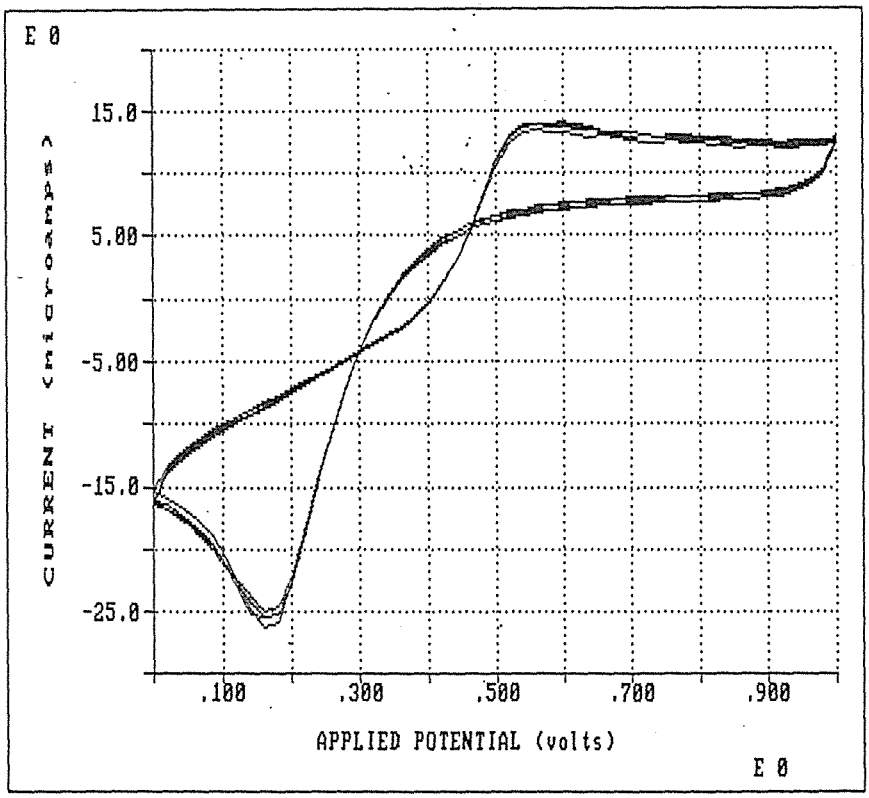


Figure 3.6 Platinum/GO immobilised by GA to gelatin. Cyclic Voltammogram in: upper; 0.1M phosphate buffer pH 7.5, and lower; 200 mM glucose.

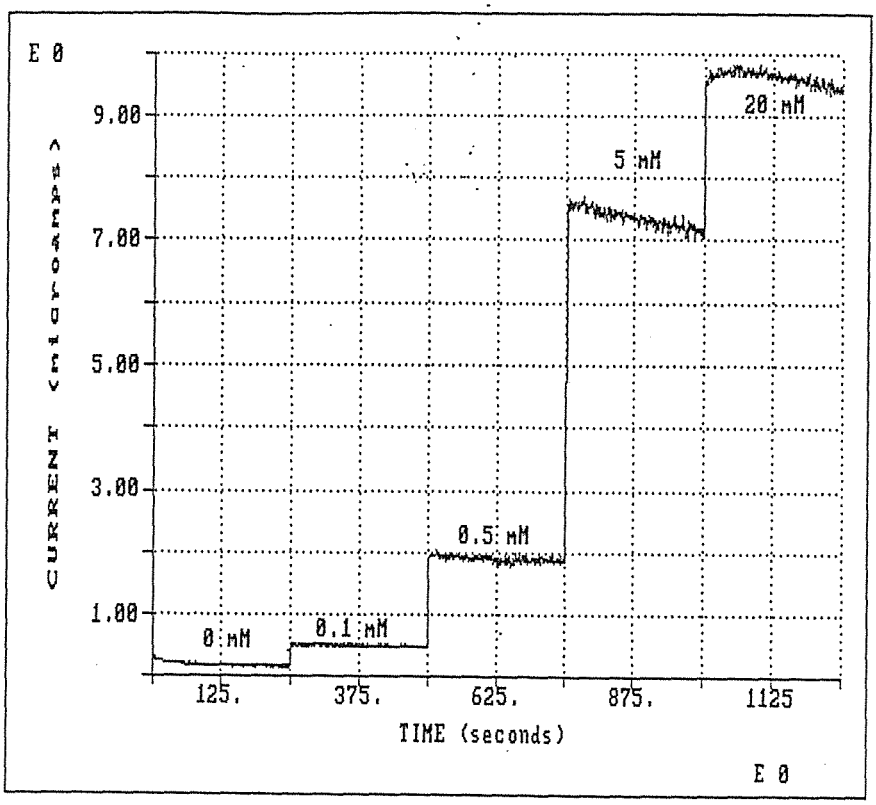
Glucose concentration mM	Response above basal. µAmps
0.1	0.3222
0.5	1.7181
5.0	6.9827
20.0	9.2527

Table 3.5 Platinum/GO crosslinked to gelatin by GA. Response to glucose.

A graded response curve was generated, although it was not linear (Figure 3.7) and enzyme assay indicated 14.29 x 10^{-2} enzyme bound units on the electrode.

Following this successful approach with GO, ADH as well as AO were treated similarly. As shown in Figure 3.8 the cyclic voltammogram of a clean platinum electrode shows a low background current characteristic of an ideal electrode material. A cyclic voltammogram was performed to determine the potential appropriate to oxidise NADH (Figure 3.8), and found to be +0.8 V. AO and ADH were separately incorporated into gelatin and crosslinked with glutaraldehyde as above but no amperometric response to ethanol was obtained. This being the case, an inhibition study of ADH activity by glutaraldehyde was carried out.

0.1 $mgml^{-1}$ ADH was mixed with an equal volume of buffer, 0.25% GA and 0.5% GA. 5 µl aliquots were then taken and assayed for activity at 1 min, 5 min, and 10 min intervals. Equal volumes of 1% GA and ADH were also mixed, 5µl aliquots taken and assayed at 0.5 min, 1 min, 2 min and 5 min. The sigma units of ADH were calculated for each aliquot and the percentage inhibition of enzyme activity calculated. (Table 3.6).



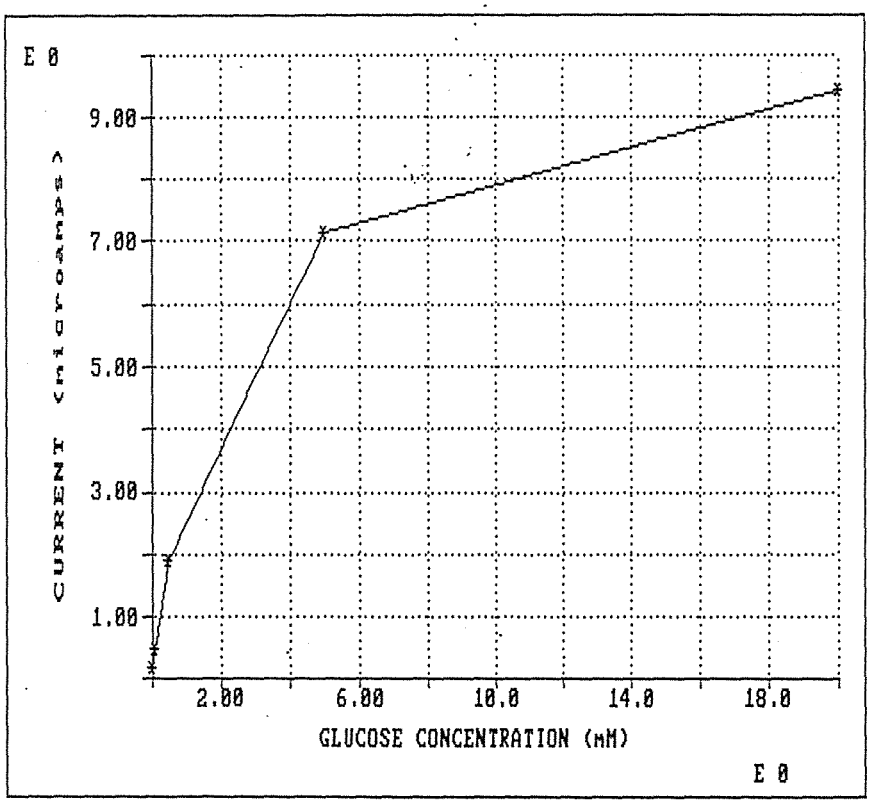


Figure 3.7 Platinum/GO immobilised by GA to gelatin, response to glucose. Upper: Amperometry trace. Lower: Response curve.

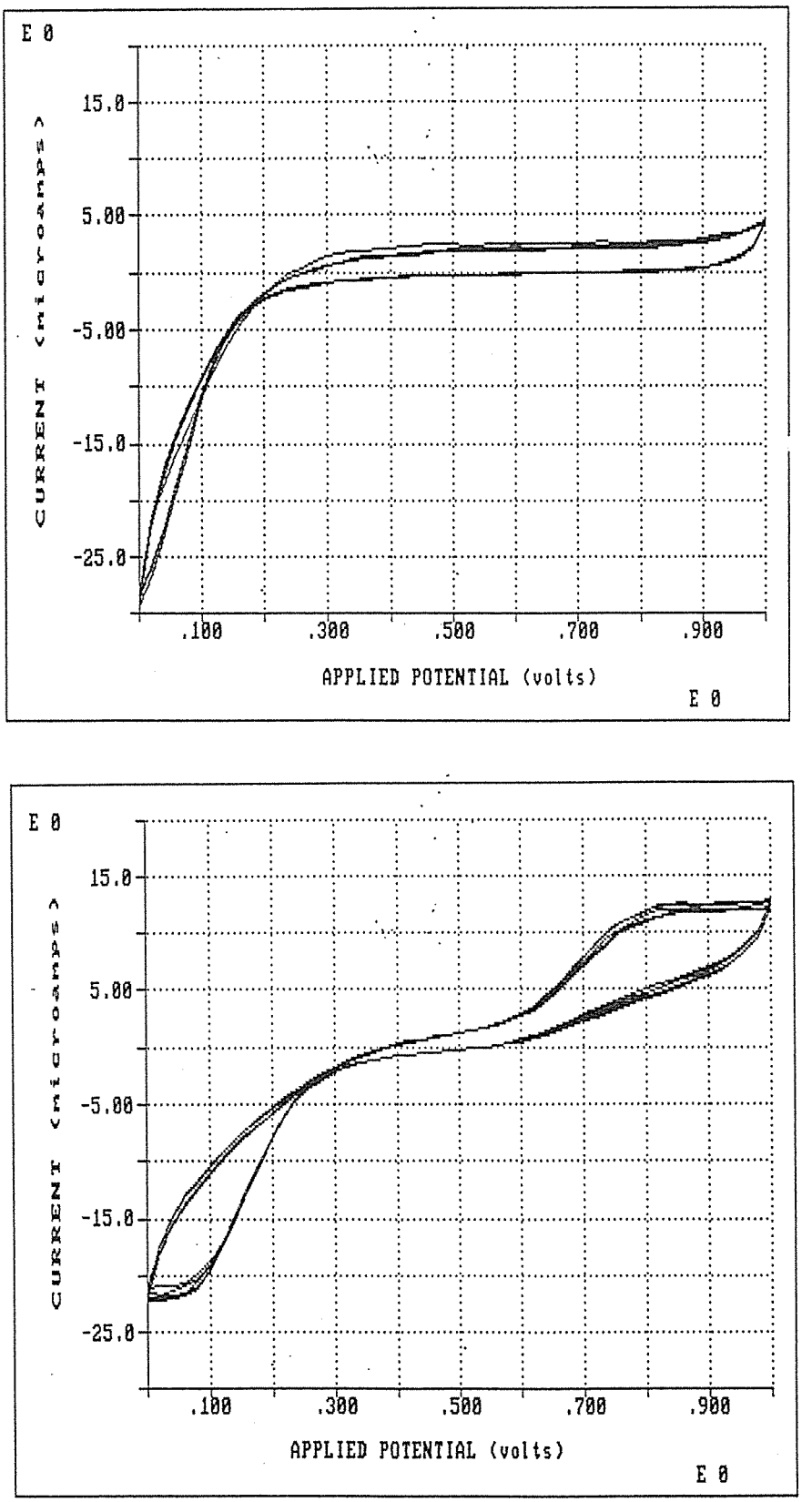


Figure 3.8 Clean platinum electrode. Upper: 100 mM phosphate buffer, pH 7.5. Lower: 300 mM NADH in buffer.

%GA	Exposure time to GA	Sigma Units	% Inhibition
1%	30sec	1.76	45.13
	1min	1.32	58.85
	2min	1.18	63.42
	5min	0	100
0.5%	1min	2.21	42.36
	5min	1.10	77.10
	10min	0	100
0.25%	1min	2.21	24.6
	5min	1.77	31.41
	10min	0.35	75.85
	15min	0.04	98.90

Table 3.6 Glutaraldehyde inhibition of ADH

It was therefore concluded that ADH is inhibited by GA, so an attempt was made to improve immobilisation conditions. Gelatin and BSA were compared as protein matrices for GA crosslinking. Furthermore premixed protein and GA was compared with dipping of protein-coated electrodes into GA. In the first treatment, 100 µl of 10 $mgml^{-1}$ ADH, 100 µl of BSA/gelatin and 100 µl of 0.5% GA were mixed. A platinum wire electrode was then dipped into this mix, allowed to dry and washed exhaustively. In the second treatment, 100 µl of 10 $mgml^{-1}$ ADH was mixed with 100µl of BSA/gelatin a platinum wire electrode dipped into this, allowed to dry and then immersed in 0.5% GA for 1min. The electrode was then allowed to dry and washed exhaustively.

Enzyme assays were performed on each separate electrode. Assays were then performed of the solution the electrodes had been stored in during the previous assay period (Table 3.7).

Electrode	Sigma Units	Sigma Units in supernatant (after electrode is removed)
BSA then GA dipped	1.06	0
BSA then GA dipped (2nd assay)	0.21	0
Gelatin then GA dipped	21.61	0.88
Gelatin then GA dipped (2nd assay)	0.37	0
Gelatin then GA dipped (3rd assay)	0.20	0
BSA + GA premixed	0.04	0
gelatin + GA premixed	0.55	0
gelatin + GA premixed (2nd assay)	0.55	0

Table 3.7 Electrode bound units of ADH on platinum electrodes premixed/dipped in gelatin/BSA and crosslinked with GA.

A table was prepared of the optimum conditions for ADH immobilisation on platinum wire using protein crosslinking with GA, (table 3.8). Similar data for AO was obtained and

shown in Table 3.9.

Electrode	Sigma Units ADH on electrode	Sigma Units ADH in supernatant (after electrode is removed)	Sigma Units ADH after 3 hours
2.5 min in 0.5% GA	7.07	2.21	0.91
1 min in 1% GA	5.95	0.09	0.19
30 sec in 2% GA	6.86	0.055	0.14

Table 3.8 Optimum conditions for crosslinking ADH with gelatin via GA.

Electrode	Electrode bound units of AO
1% GA, 30 sec	2.9×10^{-2}
1% GA, 1 min	0.8×10^{-2}
1% GA, 2 min	0
1% GA, 5 min	0
2% GA, 1 min	0.9×10^{-2}
2% GA, 30 sec	5.5×10^{-2}

Table 3.9 Optimum conditions for binding AO to gelatin via GA.

It can thus concluded from this study that both AO and ADH are inhibited by GA. Unfortunately, the conditions at which the enzymes can be immobilised with retention of high enzyme

activity result in enzyme leaching into solution and loss of activity at the electrode after successive washings.

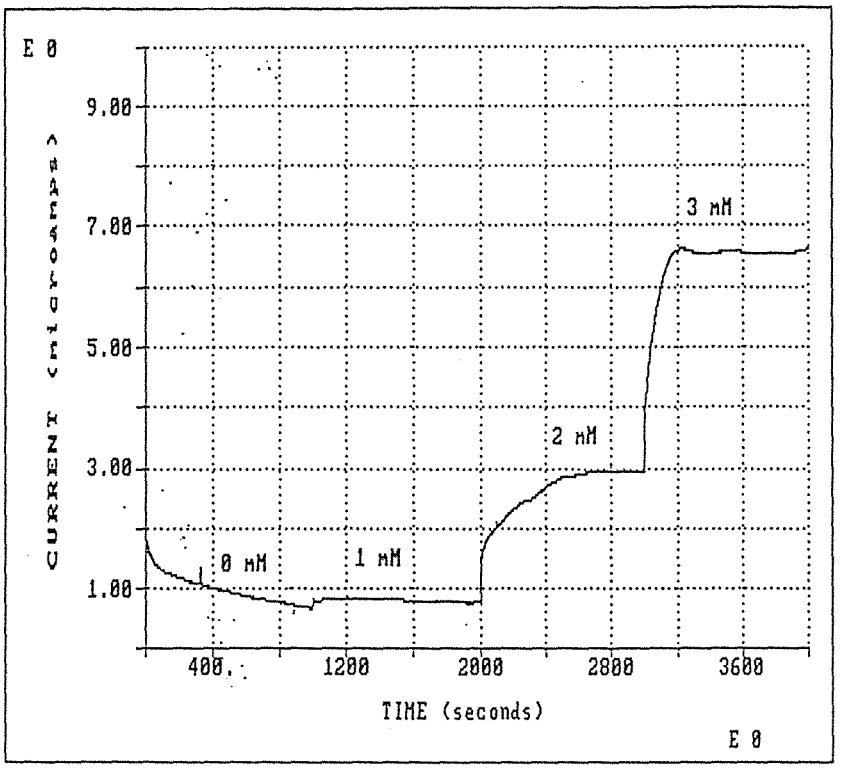
3.2.2. Carbon electrodes

Gelatin was used to immobilise GO onto carbon. Carbon foil electrodes (end-on) were modified with 1,1 dimethyl ferrocene (as in section 3.2.1) and then dipped into a mix of 12.5 $mgml^{-1}$ GO in 5% gelatin and then crosslinked with 2% GA for 30 seconds. Cyclic voltammograms with and without glucose showed a difference at +0.25 V.
The electrode was then poised at +0.25 V and the response measured amperometrically, (Table 3.10).

Concentration of glucose mM	Response above basal. µAmps
1	0.1
2	2.253
3	5.93

Table 3.10 Carbon foil/dimethyl ferrocene/GO crosslinked with gelatin by GA, response to glucose.

There definitely is a response albeit non-linear (Figure 3.9). Since a crosslinked layer is relatively thick, it may be that only the molecules of enzyme near the electrode have access to the mediator so that only a thin layer of enzyme is able to transfer electrons at this low potential.



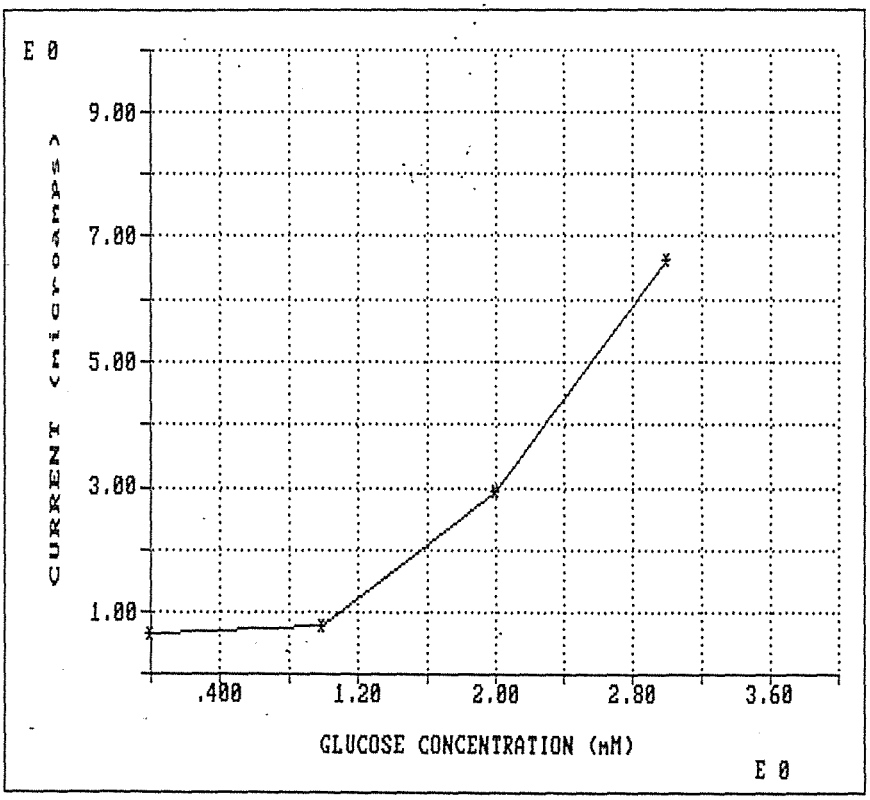


Figure 3.9 Carbon foil/dimethylferrocene/GO crosslinked with gelatin by GA, response to glucose. Upper: Amperometry trace. Lower: Response curve

3.3 Covalently bound enzymes

3.3.1 Amino-Silanisation of "anodised" platinum

Platinum may be oxidised electrolytically to form functional groups on the surface, these may be exposed to aminopropyltriethoxysilane (APTS) and enzyme attached via the amino groups using GA (Wingard and Woodward).

Platinum electrodes (embedded) were placed in 100 ml of 0.25 M H_2SO_4 and cycled voltammetrically against a Ag/AgCl reference electrode between +1.86 V and -0.5 V at -0.05 mV/sec, the electrode was then poised at +1.86 V for 300 seconds. The electrode was then exposed to a solution of 0.5 ml τ-aminopropyltriethoxysilane (τ-APTS) in 4.5 ml toluene 14 for 1 hr at 0 C. Electrodes were soaked in 10% GA in GO buffer for 1 hr at 0 C. The electrodes were then soaked in 0.5 ml of 20 $mgml^{-1}$ ADH in 0.017 M NAD in 1 M Tris buffer at pH 7.5.

The electrodes were assayed for enzyme activity (Section 2.11) and found to have 26.70 units bound on the electrode.

Next day cyclic voltammograms were recorded for each electrode. The electrodes were cycled between -0.2 V and +0.8V but no change was seen between cycles with and without ethanol. The electrodes were then poised at +0.8 V, chosen as this is the potential which has been determined to oxidise NADH on platinum electrodes (Section 3.2.1), and the response to ethanol measured amperometrically. There was no response to ethanol and further enzyme assay indicated that there was no enzyme activity on the electrode. The silanisation procedure is reported to require stringent anaerobic conditions (personal communication, Dr Alan Hart). As the equipment for such work was not available it was assumed that this was the major reason for the lack of results in this experiment.

3.3.2 Carbodiimide

3.3.2.1 Carbon

GO is known to be immobilised onto carbon electrodes via carbodiimide linkage to functional carboxyl groups on the carbon (Cass et al 1984). An embedded carbon foil electrode (Section 2.) was modified with 1,1 dimethylferrocene (as in section 3.1.1) and the electrode tip immersed in 1 ml of 150 mM 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluene sulphonate (CMC) in 100 mM acetate buffer at pH 4.5 for 80 min at room temperature. After washing in MilliQ water the electrode was placed in a stirred solution of 0.1 M carbonate buffer at pH 9.5 containing 12.5 $mgml^{-1}$ GO for 90 min. After use the electrode was stored at 4 C in 50 mM phosphate buffer pH 7.5.

Before use the electrode was poised at +0.2 V overnight in 0.7 mM glucose in 100 mM phosphate buffer, pH 7.5. Cyclic voltammograms were performed between -0.2 V and +0.8 V (refer Figure 3.10), and are typical of ferrocene modified electrodes (Section 3.1). The ferrocene peaks are expected to completely disappear with addition of substrate and instead a large catalytic current should flow at oxidising potentials; this current is due to the regeneration of ferrocene (Hill and Sanghera 1990).The addition of 100 mM glucose in this case does decrease the ferrocene peaks but a catalytic current is not seen, possibly due to the large basal current, generated by this particular electrode, "swamping" the expected response. Enzyme assays of GO electrodes indicated 10.15 x 10^{-2} electrode bound units.

Thus there is enzyme activity on electrodes and a response to GO can be measured amperometrically using these electrodes, at +0.2 V (table 3.11).

Glucose concentration mM	Response from basal µAmps
10	0.08
20	0.3
50	0.8

Table 3.11 Carbon/dimethylferrocene/GO immobilised with CMC. Response to glucose.

These results were readily repeatable with fresh electrodes but the magnitude of the response differs between electrodes, and the responses are linear (figure 3.11).

Electrodes of this kind were prepared with AO in exactly the same manner, using 20 $mgml^{-1}$ AO instead of GO. Cyclic voltammograms were performed, indicating a catalytic current at +0.2 V. The electrodes were then poised at +0.2 V and the responses recorded (Table 3.12).

Concentration of EtOH mM	Response above basal. µAmps
0.5	0.01240
1	0.0149
2	0.0275
5	0.0933

Table 3.12 Carbon foil/dimethylferrocene/AO immobilised with CMC, response to ethanol.

Enzyme assays were performed and 2.96×10^{-2} units of AO were found to be on the electrode. Thus AO was immobilised by this method and produces a relatively linear amperometric response to ethanol (Figure 3.12).

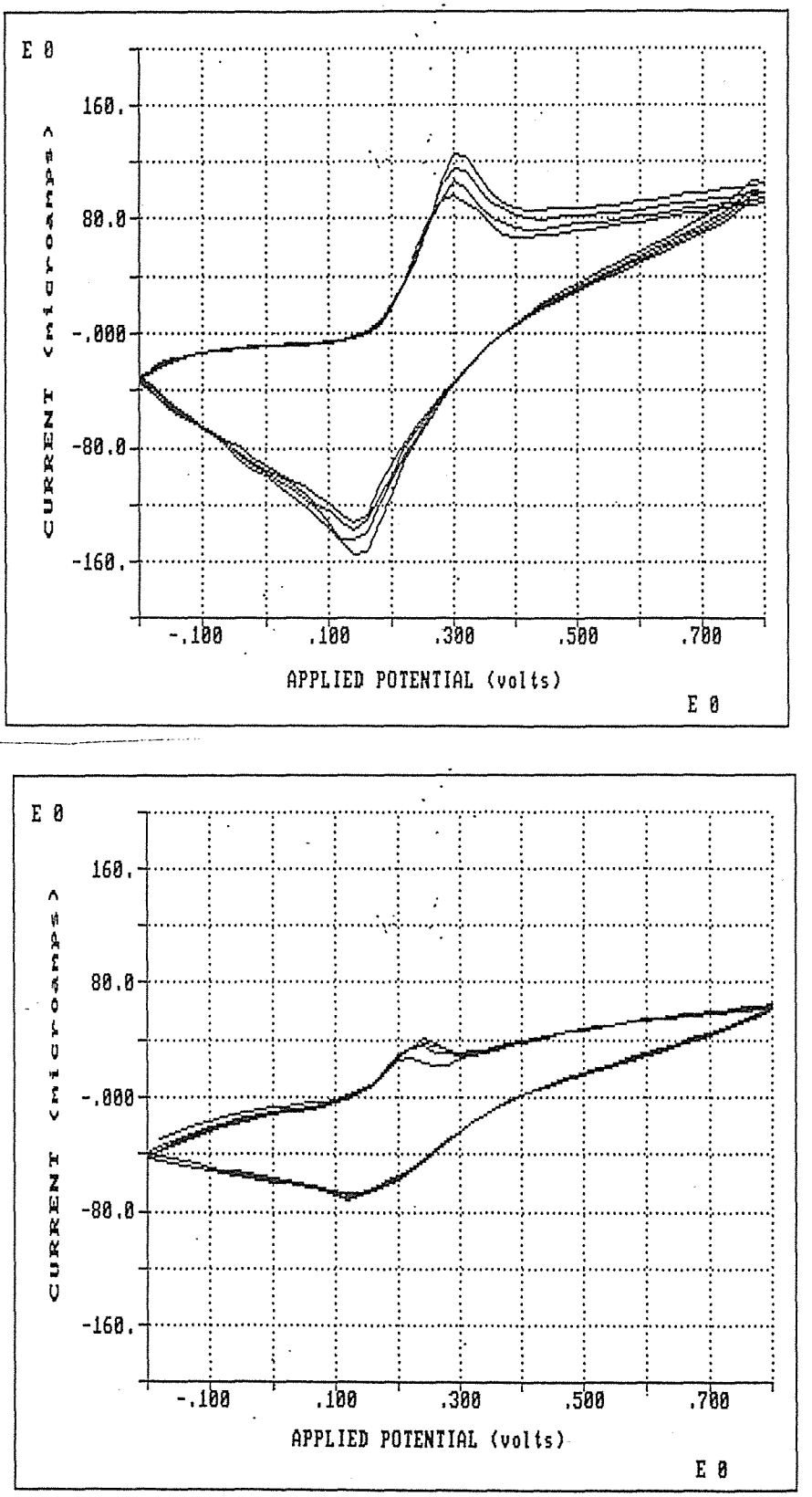


Figure 3.10 Carbon foil/dimethylferrocene/GO immobilised with CMC, cyclicvoltammograms. Upper: 100 mM phosphate buffer, pH 7.5. Lower 100 mM glucose in buffer.

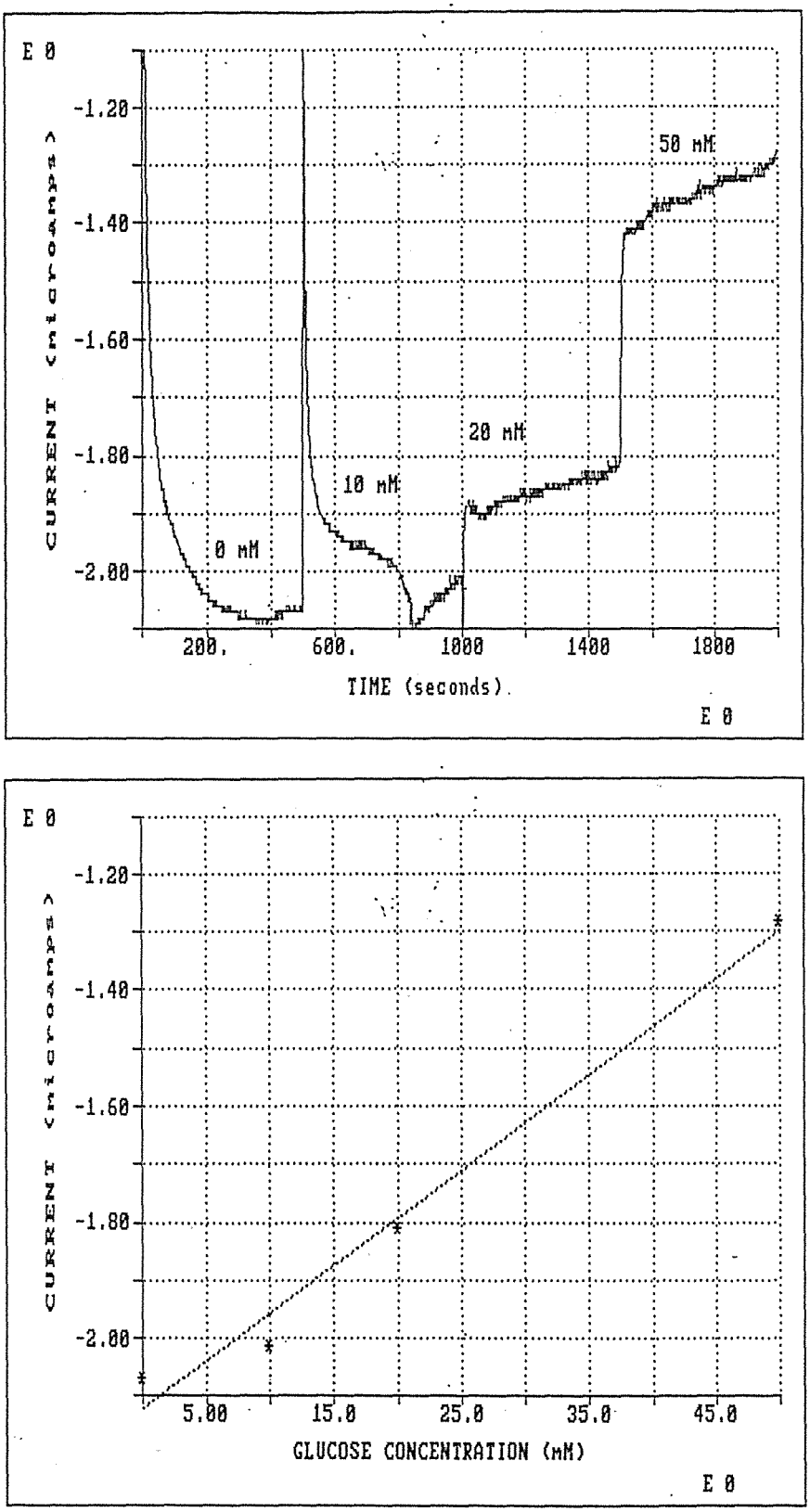


Figure 3.11 Carbon foil/dimethylferrocene/GO immobilised with CMC, response to glucose. Upper: Amperometry trace. Lower: Response curve.

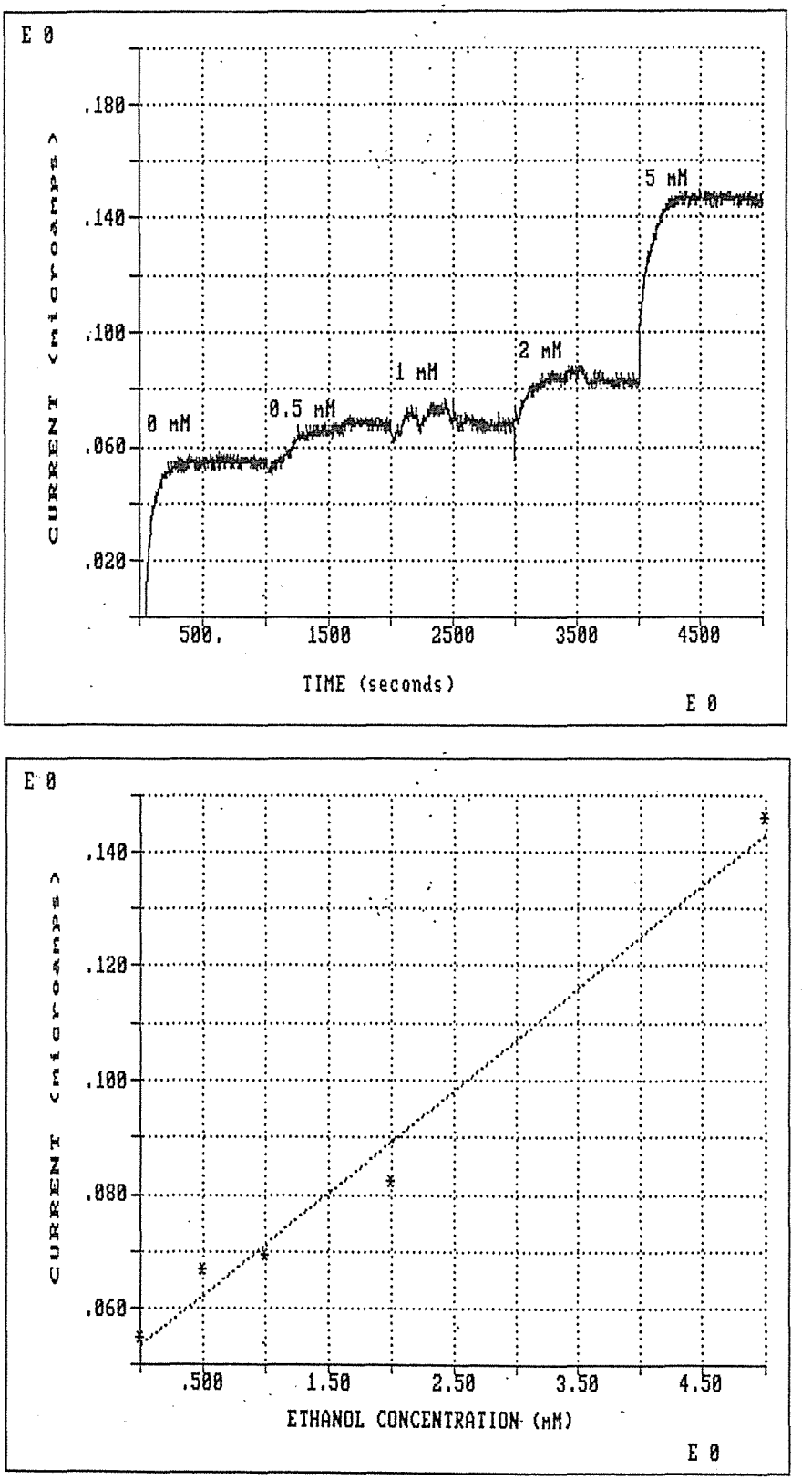


Figure 3.12 Carbon foil/dimethylferrocene/AO immobilised with CMC, response to ethanol. Upper: Amperometry trace. Lower: Response curve.

3.3.2.2 Aminopropyl glass

Porous glass particles with bound amino propyl groups are commercially available (Sigma G-5019) and were used to formulate a method for immobilisation of enzymes to the free amino groups. The amino content of the glass is given as 71 μmoles NH_2 g^{-1}.

A method developed for coupling with water soluble carbodiimides in affinity chromatography was adapted to use for amino propyl glass beads. After deaerating, 100 mg of the glass was stirred in 1 mM succinic anhydride (10 ml), and the pH maintained at 6.0 with 5.0 M NaOH. The pH was monitored until it was stable for at least 30 min without further addition of NaOH. The glass was then allowed to incubate for 5 hours in this solution at 4 C. The carboxylated material was added to 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (40 mg) dissolved in water (10 ml) and adjusted to pH 4.0. 16.3 mg of ADH was added and stirred at room temperature overnight. The reaction was terminated by washing with water and 40% aqueous dimethylformamide.

Enzyme assay indicated that 270.73 units of ADH was bound to 100 mg of the carboxylated glass. Given that ADH can be bound to aminopropyl glass in this manner, the next step was to utilise electrode materials which can be derivatised to yield amine groups. Using the technique set out above the derivatised electrodes can covalently bind to the amino groups of enzyme proteins.

3.3.2.3 Covalent binding to polypyrrole films

Polypyrrole is readily nitrated and reduced to yield amino groups which can then be made use of in the technique as set out above for aminopropyl glass.

Polypyrrole was deposited onto platinum wire electrodes from acetonitrile as given in Section 2.12.1. The electrode was

rinsed with acetonitrile and then placed in a solution of cupric nitrate trihydrate (700 mg) in acetic anhydride (20 ml). Nitration was stopped after 5 minutes by transferring the electrode to another beaker and rinsing with acetonitrile. The nitro groups were then reduced in acetonitrile/TBAPTS by cycling between +0.5m V and -2.5 V. The electrode was then assayed for amino groups, 7.27×10^{-2} μmoles amino groups per electrode.

The amino-derivatised polypyrrole electrodes were suspended in 1 mM succinic anhydride and the pH maintained at 6.0 with 5.0 M NaOH. The pH was monitored until it was stable for at least 30 min without further addition of NaOH. The electrodes were then allowed to incubate for 5 hours in this solution at 4 C.

The carboxylated electrode was added to 40mg 1 ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) dissolved in 10 ml MilliQ and adjusted to pH 4.0, then washed excessively in MilliQ water.

16.3 mg ADH was added and stirred at room temperature overnight. Next day the reaction was terminated by washing in water and 40% aqueous DMF. The electrodes were then assayed for enzyme activity;
Units bound on electrode= 79.7
There is therefore activity on the electrode but reassay next day indicates loss of all of the activity and no amperometric response.

3.3.3 Binding of enzymes to cellophane membranes

Cellophane membranes can be derivatised to form functional groups on their surfaces, these can then be used to covalently bind enzyme to the membrane which can then be wrapped around an electrodes giving a versatile approach to enzyme bound biosensors.

3.3.3.1 Benzoquinone

A 2cm square of dialysis membrane was cut and washed with 0.1 M phosphate buffer at pH 8 with 20% (v/v) ethanol. 15 ml of EtOH containing 0.25 g benzoqinone was added to the membrane while agitating and left agitating for 2 hours at room temperature. The membrane was then washed with a water/EtOH mixture (80/20,v/v) then washed 3 times with 1 M NaCl and 3 times with MilliQ water (Beh et al 1989).

The activated membrane was then mixed with 100 ml of 0.1 M bicarbonate buffer at pH8 containing 1 $mgml^{-1}$ ADH and agitated overnight at 4 C. After washing with bicarbonate buffer the membrane was washed with 100 mM acetate buffer at pH 4 and twice with 1 M NaCl. After treating with 1 volume of 100 M buffered ethanolamine at pH 8.5 for 3 hours at 4 C the excess was washed off and the membrane remained a brown colour. The enzyme activity of the membrane was then measured and found to be insignificant.

3.3.3.2 Cyanuric chloride

A 2cm square of dialysis membrane was cut and suspended in 100 ml of pure acetone. The membrane was suspended in the acetone for 45 minutes agitating slowly. 15 ml of acetone containing 0.5 g cyanuric chloride, and 15 ml of deionized water was added and the membrane stirred for 5 min. After washing in 50% acetone in 100 mM phosphate buffer at pH 6.7 followed by washing in 500 mM borate buffer at pH 8.7 the membrane was mixed with 1 ml of borate buffer containing 1 $mgml^{-1}$ of ADH. The membrane was then left agitating overnight at 4 C and next day washed with borate buffer. Following treatment of the membrane with 1 volume of 100 mM buffered ethanolamine at pH 8.5 for 3 hours at 4 C the ethanolamine was rinsed off and the membrane assayed for enzyme activity; no activity was found.

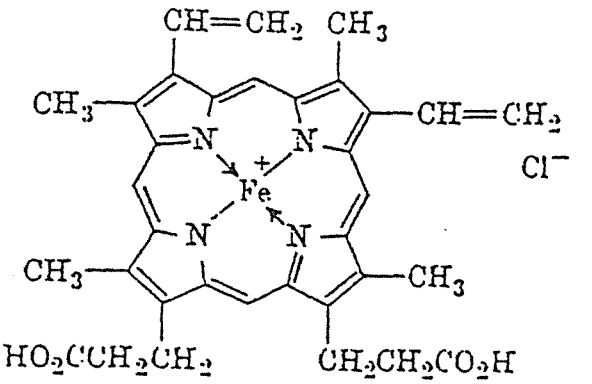
3.3.5 Haemin

Substances containing conjugated double bonds (olefinic), for example haemin, are known to bind irreversibly to platinum by sharing pi electrons with the d-orbitals of the platinum surface (Lane and Hubbard 1973). Utilising this knowledge GO was conjugated to haemin which contains conjugated double bonds with the hope that the conjugate would then bind to platinum giving the electrode GO activity. Haemin has two available carboxyl groups which can be activated via the hydroxy succinimde/carbodiimide method illustrated in Figure 3.13. The method for conjugation was derived from techniques utilised by Al-Bassam et. al. (1979), Rajkowski and Cittanova (1981),and Sauer et. al. (1986, and 1989).

3.3.5.1 Glucose oxidase

6.82 mg of haemin was dissolved in 22.18 µl of 50 $mgml^{-1}$ dicyclohexylcarbodiimide (DCC) in DMF, 12.38 µl of $50mgml^{-1}$ hydroxysuccinimide (HS) in DMF and 15.44 µl DMF. This haemin was then left to be activated for 1 hour. The activated haemin solution was then added to 1.95 ml of stirred 25 $mgml^{-1}$ GO in phosphate buffer pH 7.0. The GO-haemin solution was then left stirring overnight at 4 C.

Next day 0.25 ml of the GO-haemin solution was added to the top of a Sephadex G-25 column 1 cm x 19 cm and washed in with phosphate buffer pH 7.0. 3 ml fractions were collected and a brown band was visible moving down the column. The brown band eluted out at 7 ml, and a further dark brown band remained at the top of the column.



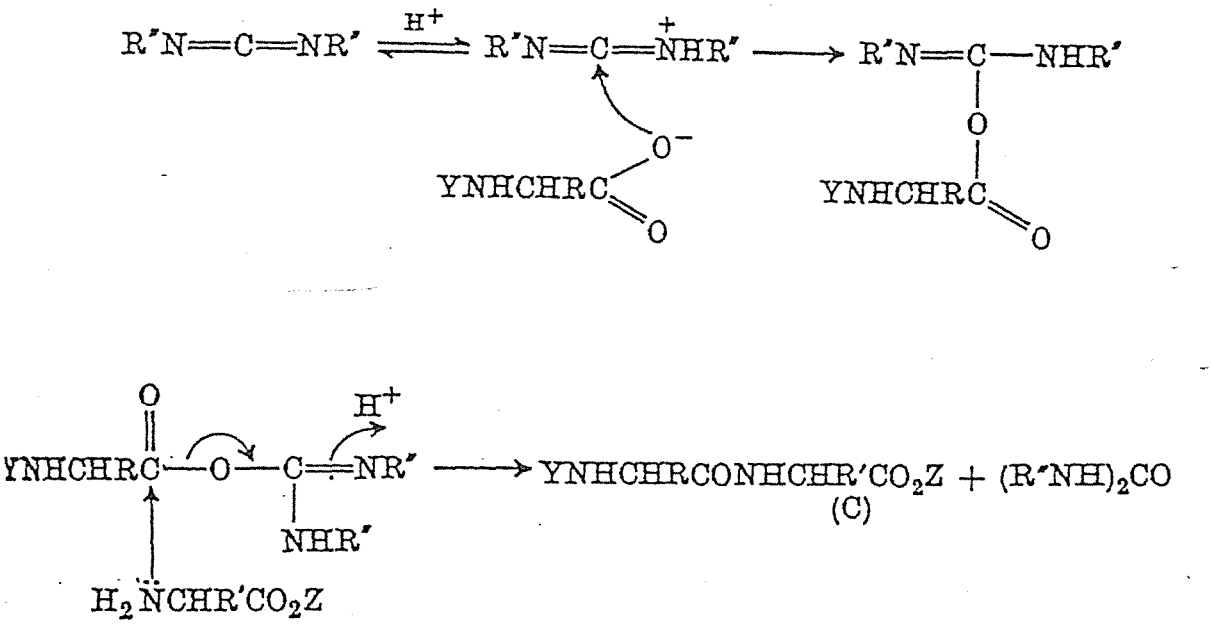


Figure 3.13 Upper: Structure of haemin. Lower: Method of conjugation of haemin to protein (Tedder).

The brown colour of the fraction collected indicated that haemin was present in the fraction, haemin is a small molecule and to have eluted off the column in the void volume it must have conjugated on to a bigger molecule, ie. GO. The brown fraction collected, postulated to be haemin-GO conjugate was assayed for enzyme activity as well as an absorbtion spectra recorded between 250 nm and 500 nm. Absorbance spectra of 1 mg ml^{-1} GO and haemin were recorded for comparison. 300 µl of the eluate of the band was added to 2.7 ml buffer and the spectrum recorded. As shown in Figure 3.14, peaks corresponding to both haemin and GO were present, indicating that conjugation has taken place. Enzyme assays indicated that 3.61 enzyme units were present in the eluted fraction. This data proved that haemin-enzyme conjugates had been made.

A platinum embedded electrode was immersed in the eluted haemin-GO conjugate at 4 C overnight. Next day the electrode was cycled between -0.2 V and +0.8 V, the same shaped cycle is seen as that of a comparative cycle of unconjugated haemin on platinum (figure 3.15), indicating that the haemin is bound to the electrode. Furthermore a difference is obvious in the cyclic voltammograms between with and without glucose (Figure 3.16). The electrode was poised at +0.5 V in 100 mM phosphate buffer pH 7.5 and responses to glucose recorded (Table 3.13).

Concentration of glucose mM	Response above basal. µAmps
1	0.287
5	1.518
20	3.909

Table 3.13 Platinum/haemin-GO conjugate, response to glucose.

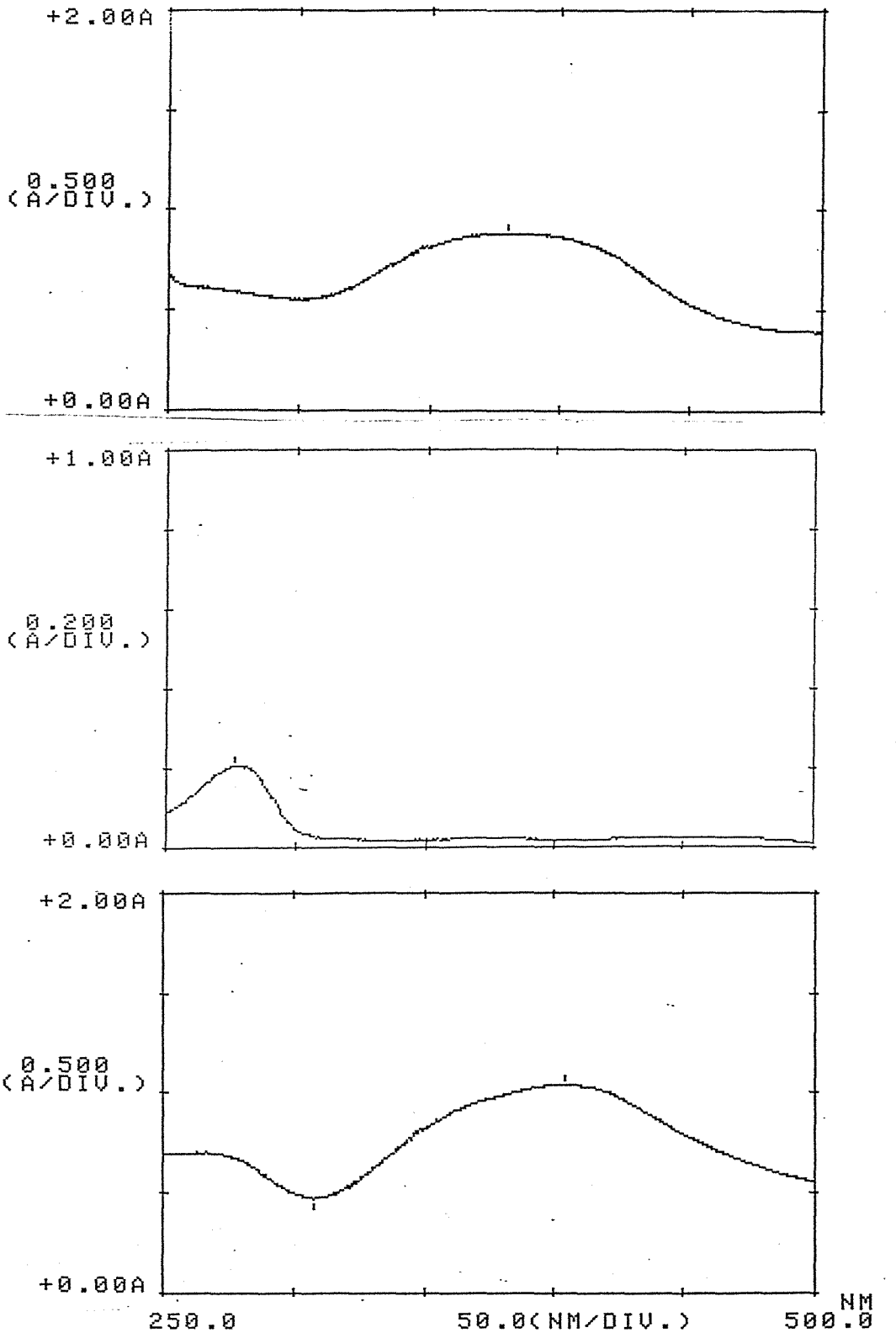


Figure 3.14 Absorbance spectra of haemin (upper), GO (middle) and haemin-GO conjugate (lower).

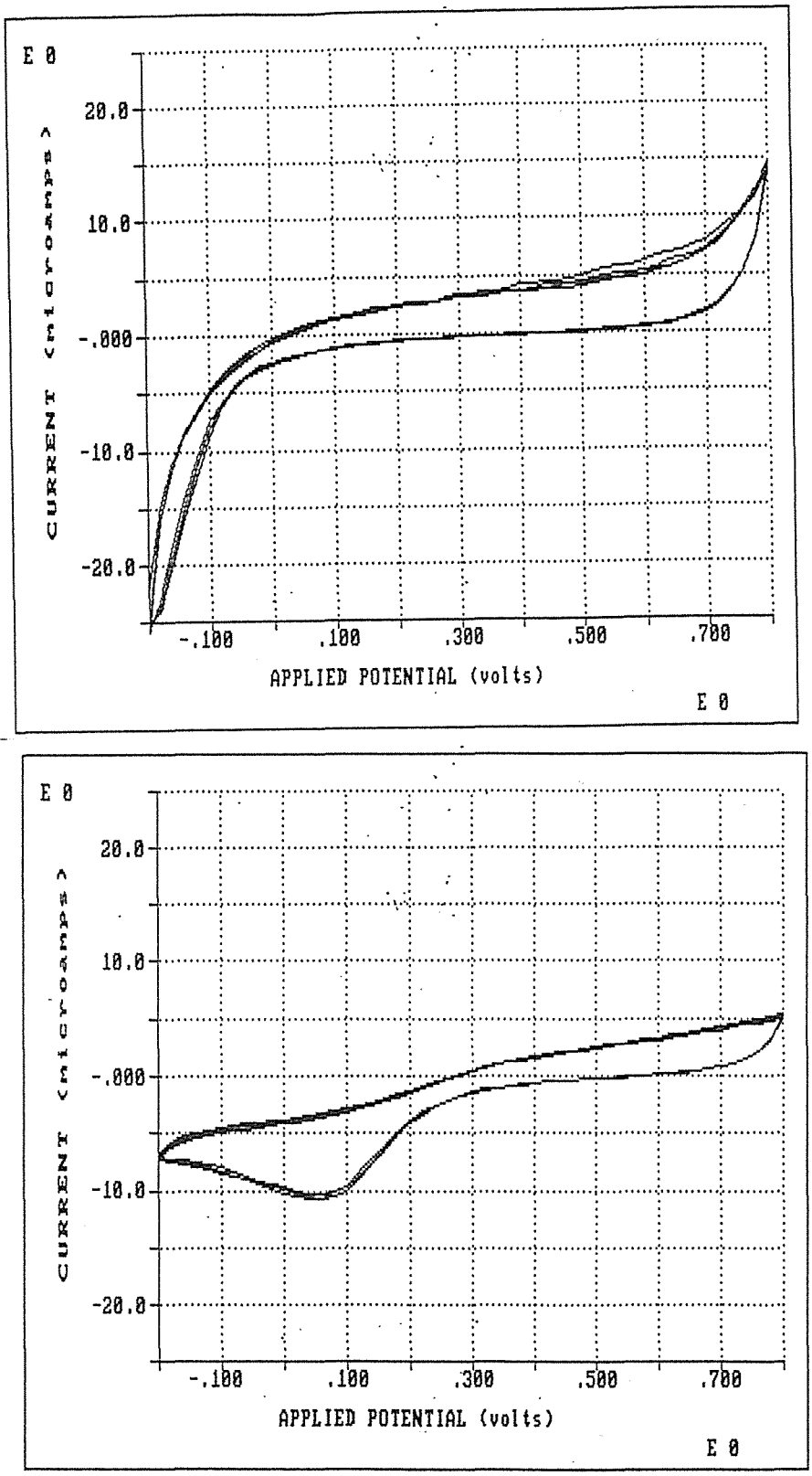


Figure 3.15 Upper: Clean platinum electrode, cyclic voltammogram in buffer. Lower: Platinum electrode with haemin bound, cyclic voltammogram in buffer.

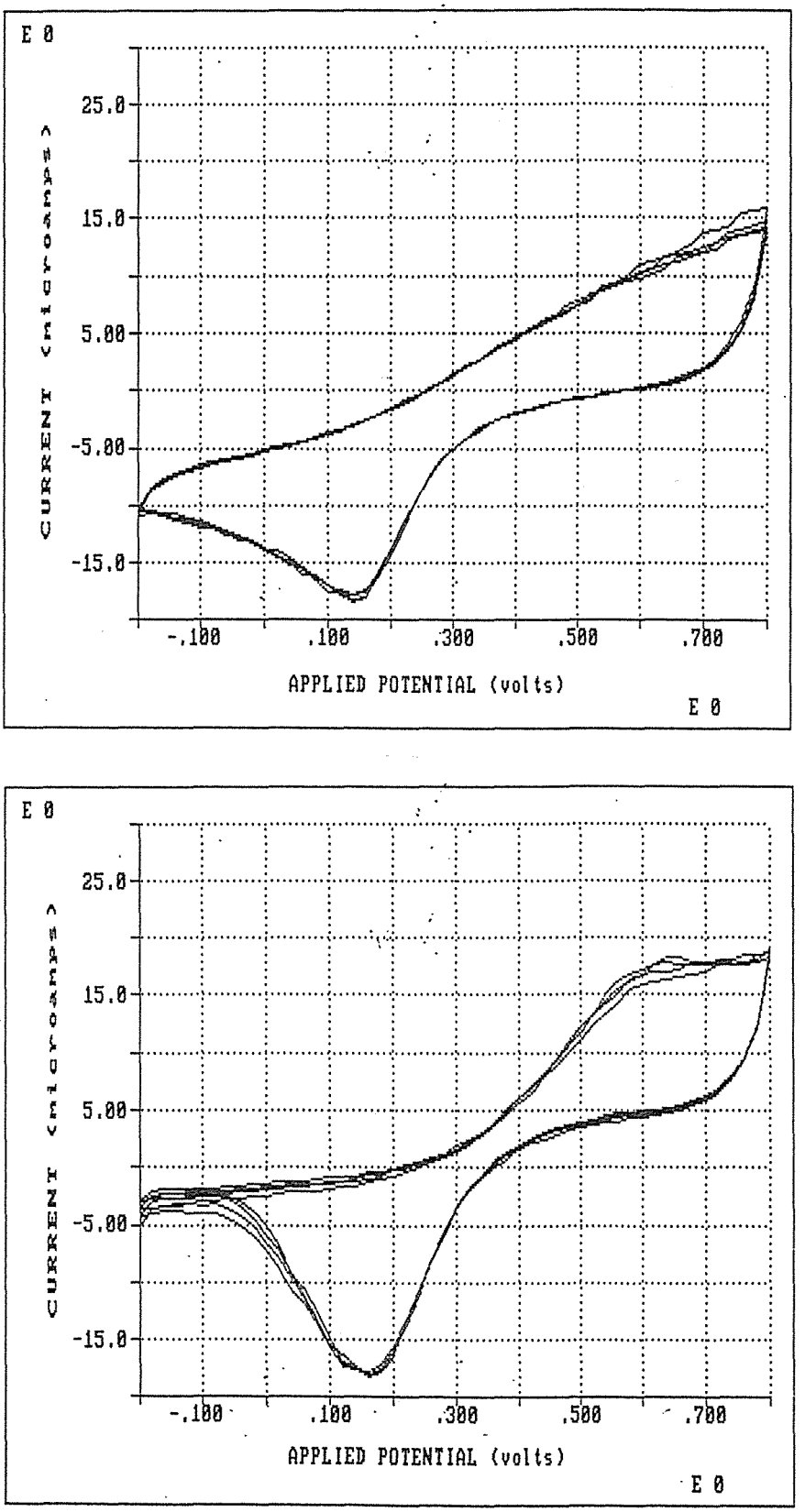


Figure 3.16 Platinum/Haemin-GO conjugate, cyclic voltammogram. Upper: Buffer. Lower: 100 mM glucose in buffer.

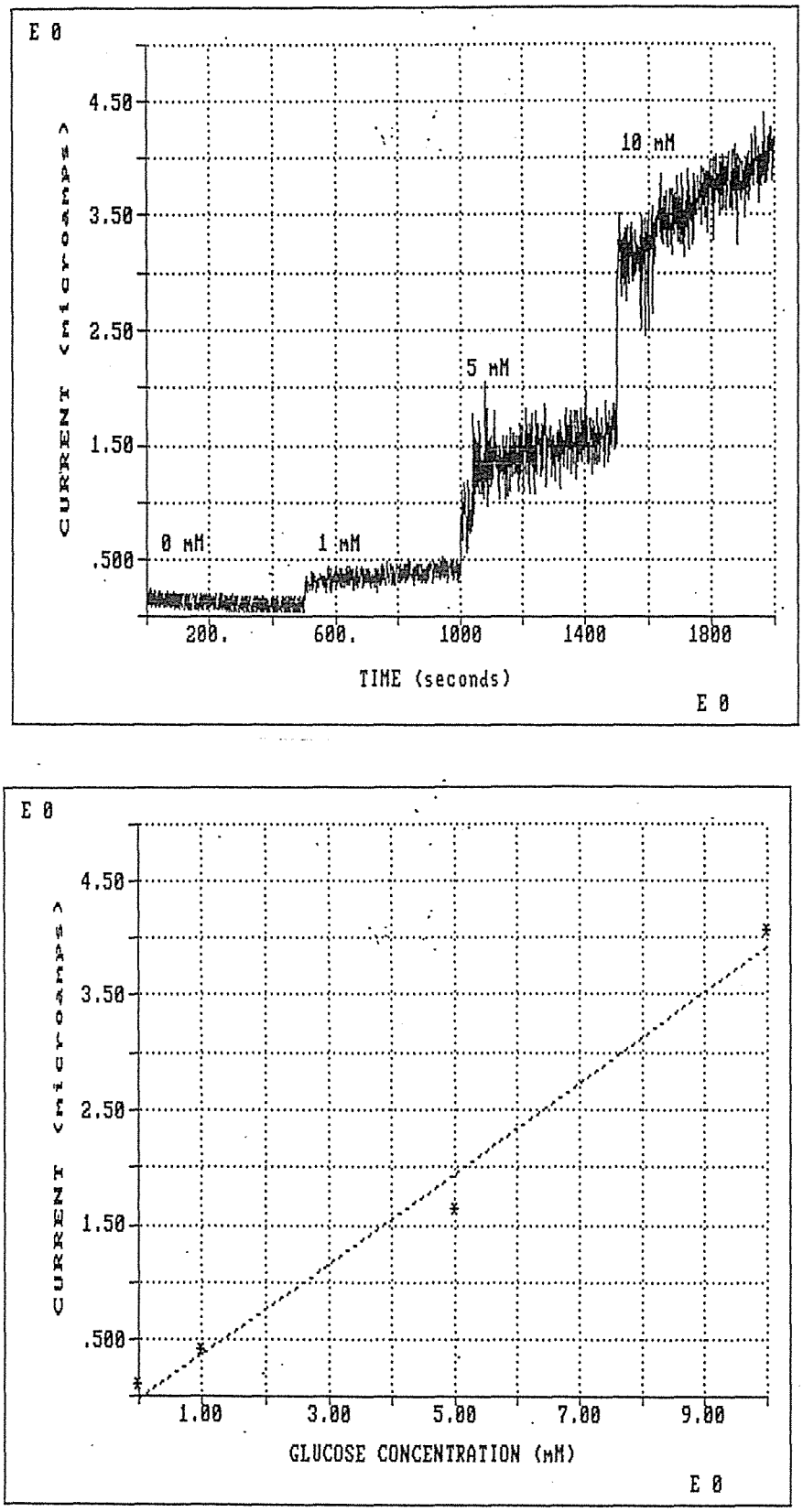


Figure 3.17 Platinum/Haemin-GO conjugate, response to glucose. Upper: Amperometry trace. Lower: Response curve.

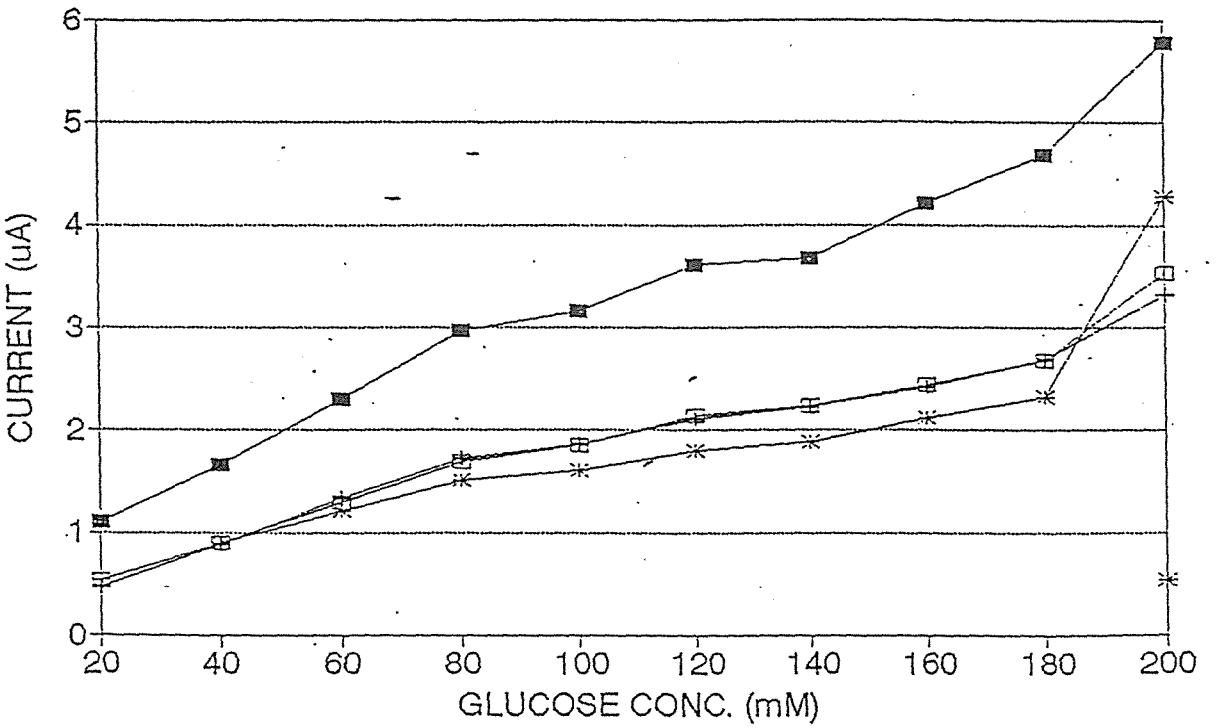


Figure 3.18 Platinum/Haemin-GO conjugate, flow injection analysis, response to glucose.

This method of immobilisation on platinum electrodes was readily repeatable and gave a linear amperometric response to glucose (Figure 3.17). The same conjugated GO preparation was used to produced glucose sensors for use in flow-injection analysis.

A platinum electrode was placed in the haemin-GO conjugate solution for 1.5 hours, washed and placed into a flow cell containing a reference electrode and an auxiliary electrode. Samples were automatically fed into the system every 90 seconds. A series of responses was generated the same day (Run 1) and another 3 series of responses were generated consecutively from an electrode left overnight in the same conjugate solution (Runs 2, 3 and 4), see Table 3.14.

Glucose concentration mM	Response above basal. Run 1. µAmps	Response above basal. Run 2. µAmps	Response above basal. Run 3. µAmps	Response above basal. Run 4. µAmps
20	1.1	0.47	0.53	0.53
40	1.66	0.88	0.90	0.90
60	2.3	1.33	1.21	1.29
80	2.97	1.72	1.51	1.69
100	3.16	1.86	1.60	1.85
120	3.61	2.11	1.79	2.15
140	3.68	2.22	1.88	2.23
160	4.21	2.42	2.12	2.44
180	4.68	2.69	2.33	2.67
200	5.79	3.32	4.28	3.54

Table 3.14 Platinum/Haemin-GO conjugate electrode in FIA system, response to glucose. Comparison between 4 electrodes.

The responses were linear up to 80 mM glucose (Figure 3.18). Following the success of this experiment the same technique was used to conjugate AO and ADH to haemin and then to bind the conjugate onto platinum electrodes.

3.3.5.2 Alcohol oxidase

2.11mg haemin was dissolved in 6.88 µl of 50 $mgml^{-1}$ DCC in DMF, 3.84 µl of 50 $mgml^{-1}$ HS in DMF, and 39.28 µl DMF. The haemin was allowed to be activated over 1 hour at room temperature and was then added in 20 µl aliquots to 1.5 ml 25 $mgml^{-1}$ AO in phosphate buffer at pH 7 and allowed to

conjugate overnight. 2.5 ml of haemin-AO conjugate was added to a G-25 sephadex column and a brown band was eluted in the void volume. The eluted band was assayed for enzyme activity and an absorbtion spectra was recorded. An absorption spectra of AO is also recorded and compared with the spectra obtained from the eluted band. Spectras coincide (Figure 3.19) and the eluted band has AO activity (0.35 units).

A platinum electrode was then left in solution overnight, next day a cyclicvoltammogram was recorded which showed a typical haemin cycle and a difference between with and without ethanol (100 mM) (figure 3.20). Responses were then recorded amperometrically at +0.3 V, there was a minimal response to ethanol but there was a response to 50 mM methanol as shown in Figure 3.21.

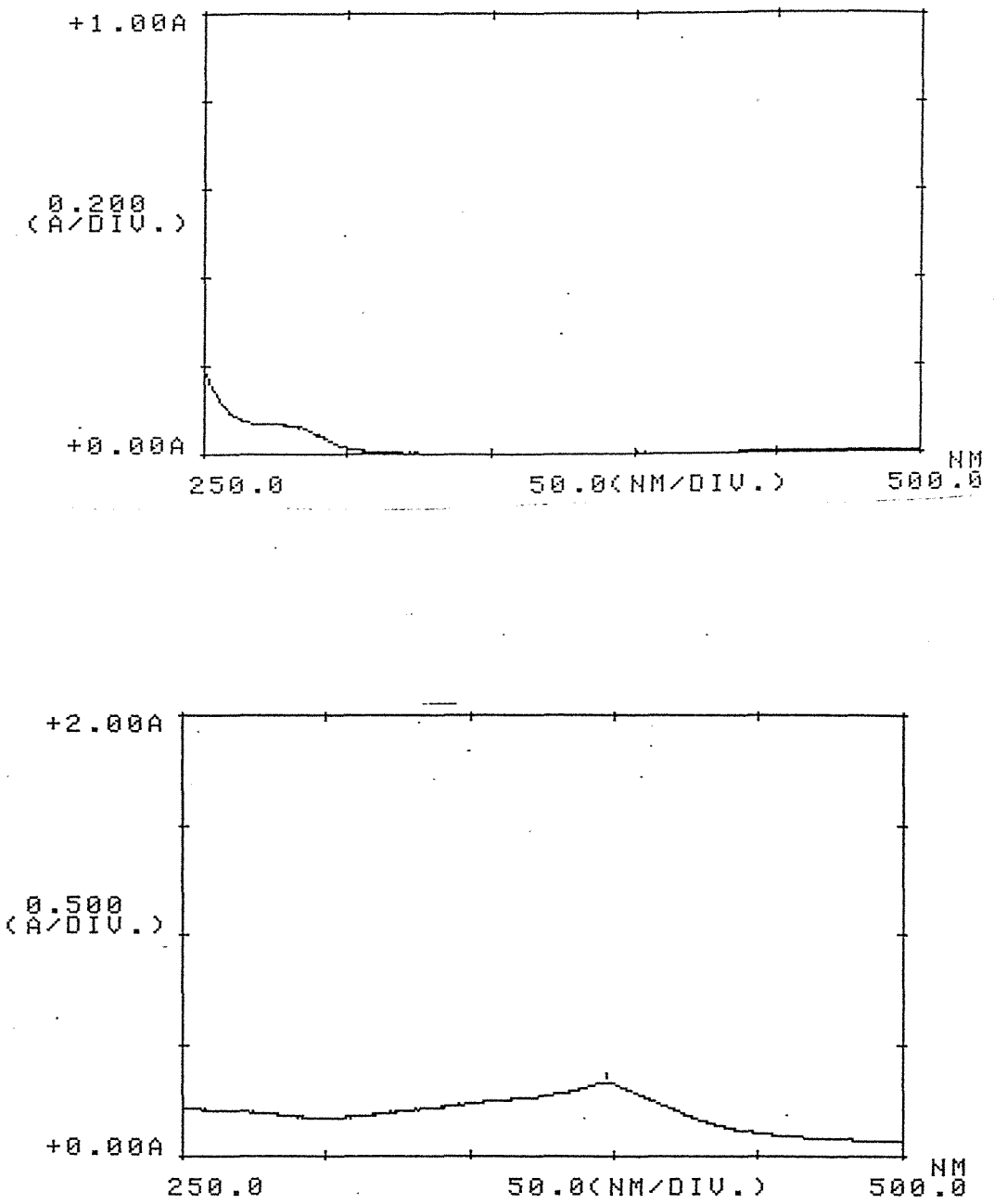


Figure 3.19 Absorbance spectra. Upper: AO. Lower: Haemin-AO.

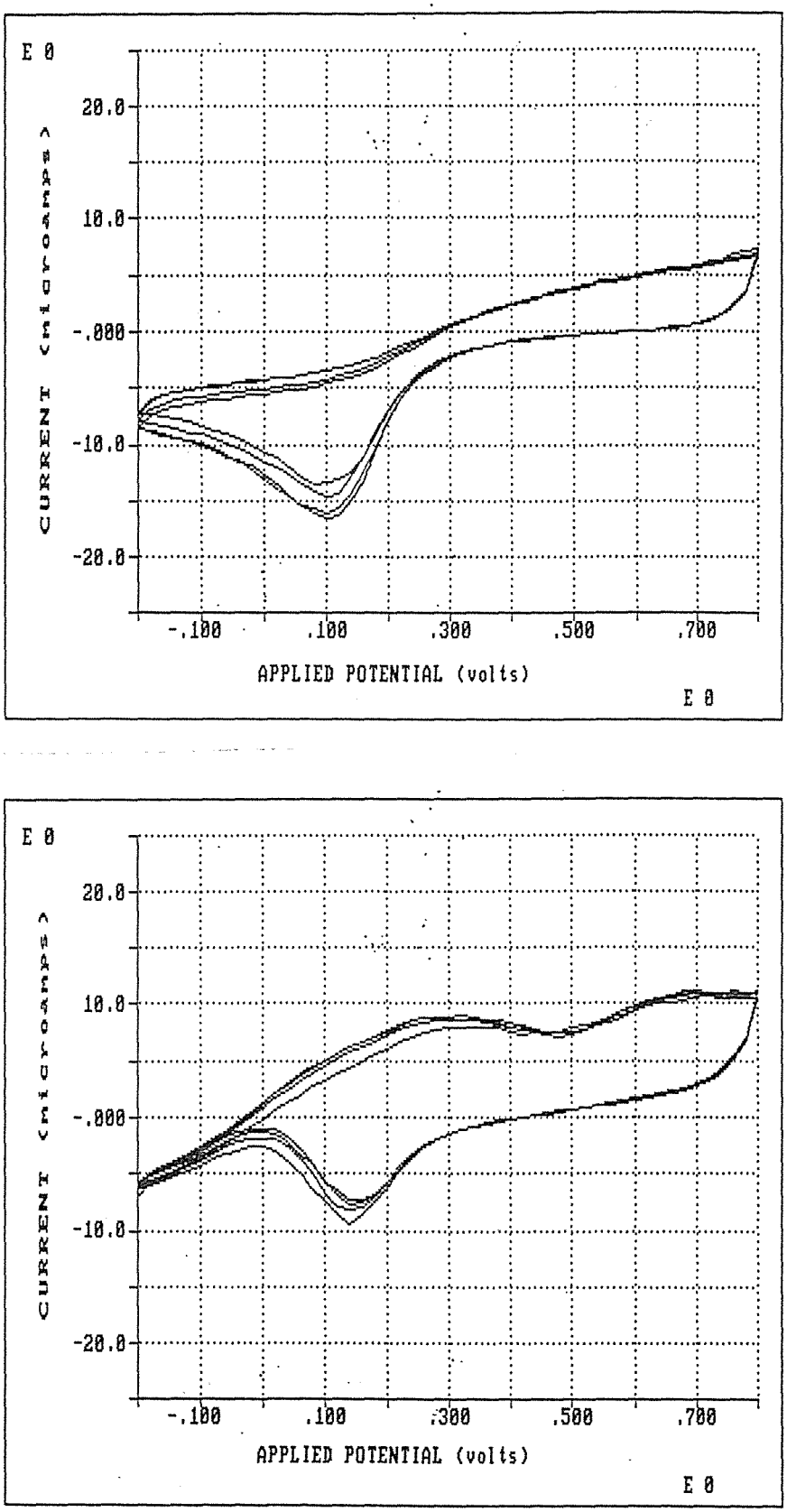


Figure 3.20 Pt/Haemin-AO conjugate, cyclicvoltammogram. Upper: Buffer. Lower: 100 mM ethanol.

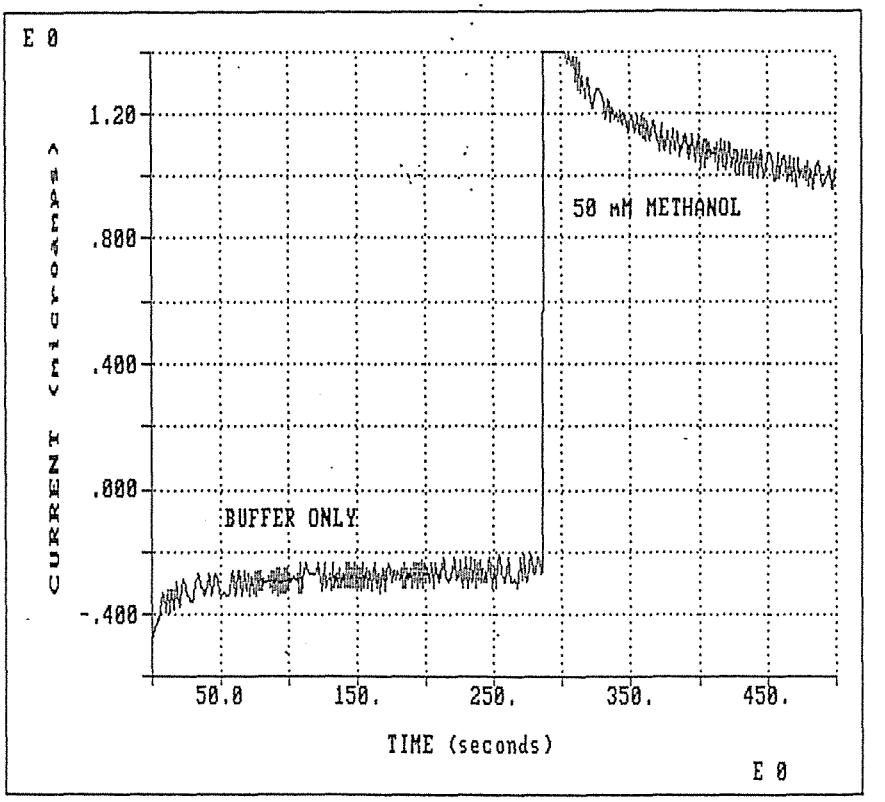


Figure 3.21 Pt/Haemin-AO conjugate,response to 50 mM methanol.

3.3.5.3 Alcohol dehydrogenase

8.56mg haemin was dissolved in 27.88 µl of 50 $mgml^{-1}$ DCC in DMF, 15.56 µl of 50 $mgml^{-1}$ HS in DMF, and 6.5 µl DMF. The haemin was allowed to be activated over 1 hour at room temperature and was then added in 20 µl aliquots to 1.95 ml of 25 $mgml^{-1}$ ADH in phosphate buffer at pH 7 and allowed to conjugate overnight. 0.2 ml of haemin-ADH conjugate was added to a G-25 Sephadex column and a brown band was eluted in the void volume. The eluted band was assayed for enzyme activity and an absorbtion spectra was recorded. An absorption spectra of ADH is also recorded and compared with the spectra obtained from the eluted band. Spectras coincide (Figure 3.22) and the eluted band has enzyme activity (123.0 units).

A platinum electrode was placed in the ADH-haemin conjugate at 4°C overnight. Next day the electrode was cycled but does not show haemin peaks even though enzyme assay showed ADH is present, figure 3.23. The haem group may act as a mediator shuttling electrons between the enzyme and the electrode in the absence of NAD^+. Poised potentials of haemin-ADH electrode are made, there was no response to ethanol but a response to methanol was recorded (Table 3.16) which is linear (figure 3.24).

Methanol concentration mM	Response above basal µAmps
10	0.9
20	1.9
30	4.5

Table 3.16 Platinum/haematin-ADH electrode, response to methanol.

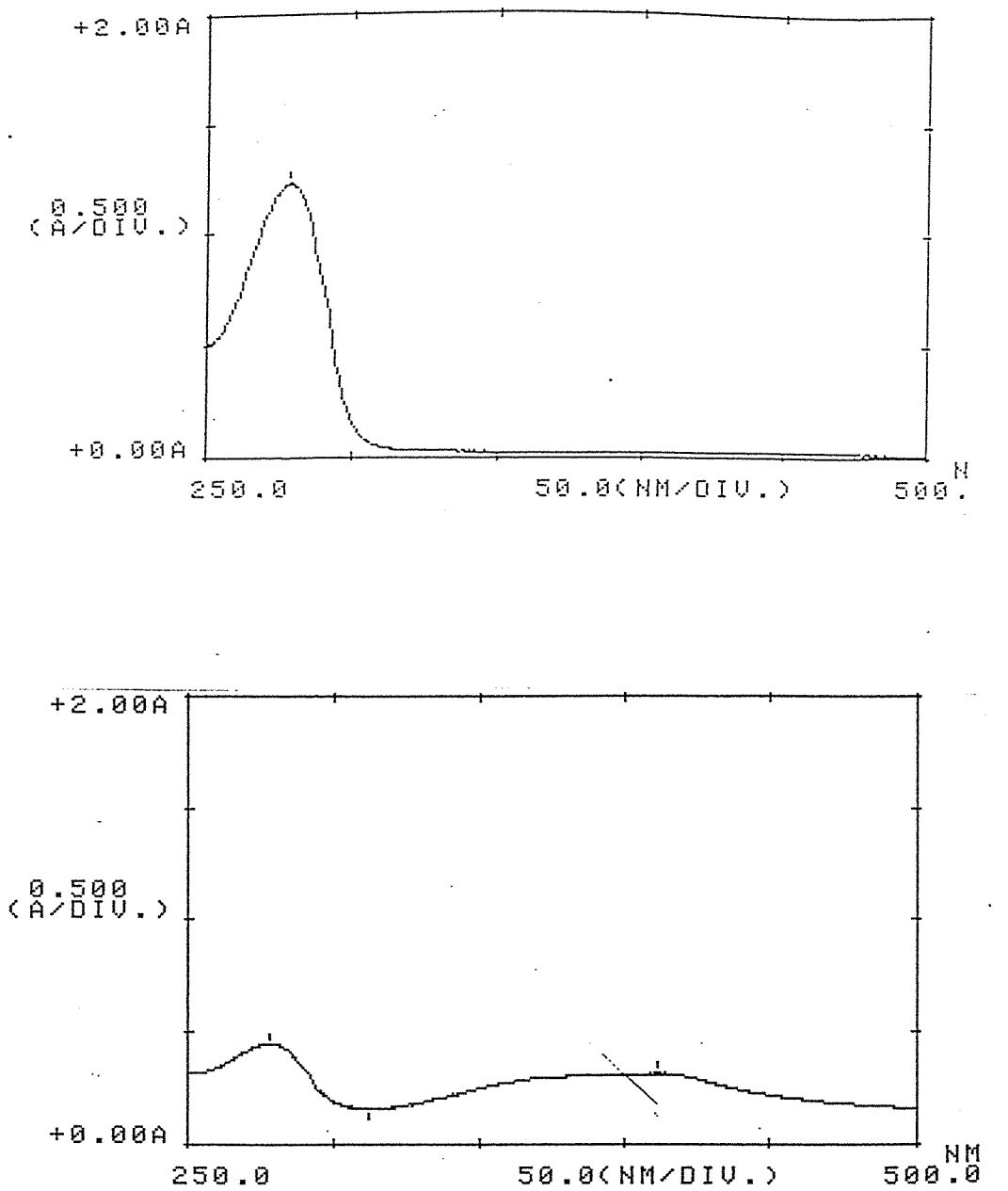


Figure 3.22 Absorbance spectra. Upper: ADH. Lower: Haemin-ADH.

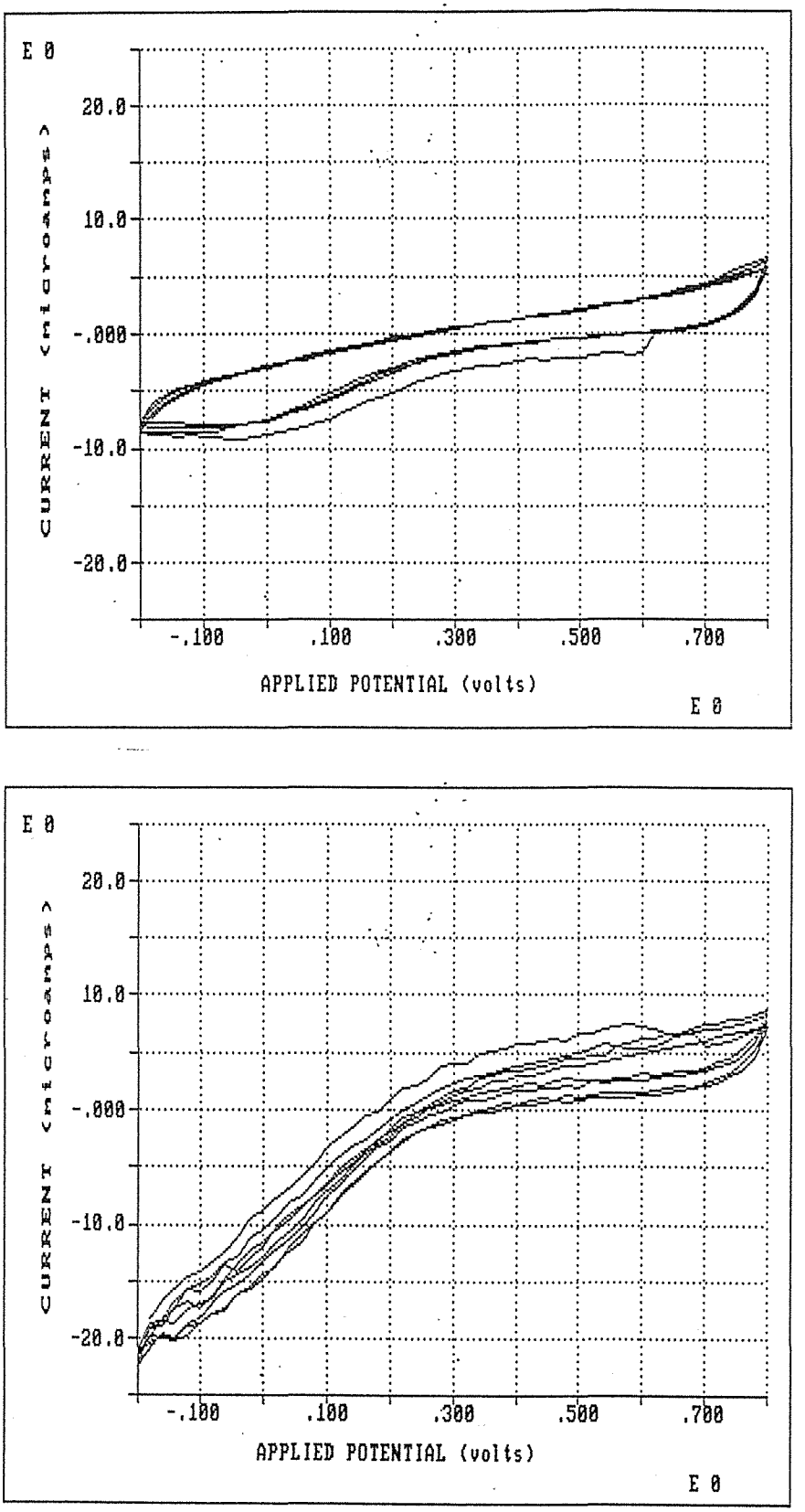


Figure 3.23 Platinum/Haemin-ADH electrode, cyclicvoltammograms. Upper: Buffer. Lower: 100 mM ethanol.

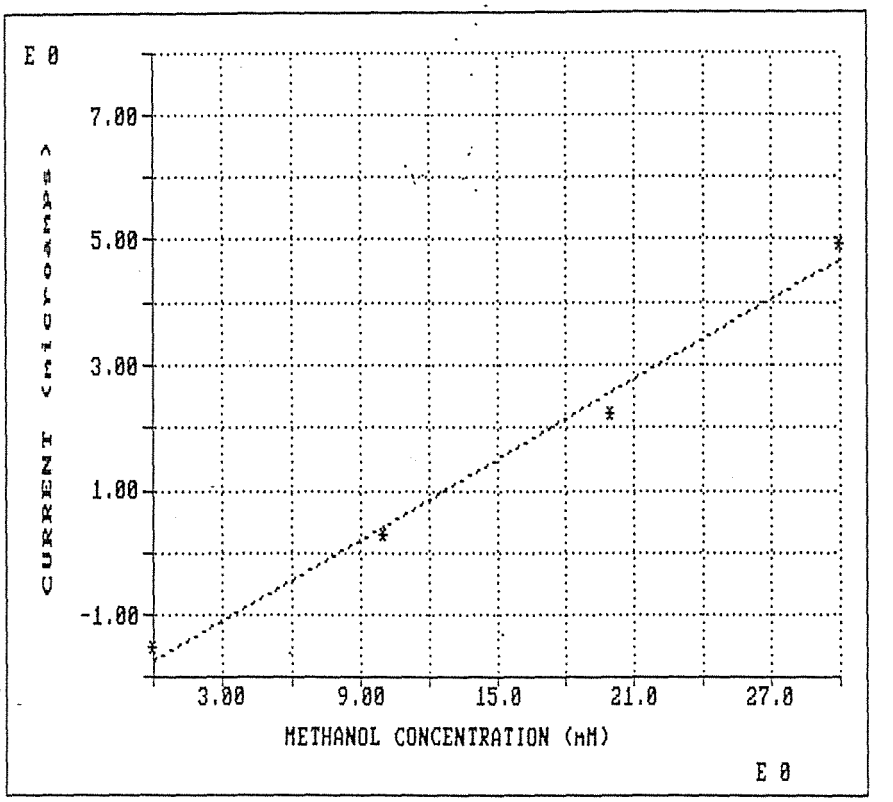


Figure 3.24 Platinum/Haem-ADH electrode, response to methanol. Response curve.

CHAPTER 4: DISCUSSION

In this thesis three main variables have been considered in the investigation of the optimisation of a design for an alcohol biosensor. These were; type of enzyme, electrode material, and method of immobilisation. Some electrode combinations required that consideration had also to be given to the electron mediator used. The main approach in this thesis work was to reproduce and then expand upon other approaches being reported in this field. In this manner it was hoped that techniques reported to be successful with other enzymes, in particular GO, would prove to be applicable also to either AO or ADH.

4.1 Enzyme type

Glucose oxidase was used because of its availability, high activity, low cost, and its use in other biosensor research internationally (Wilson and Turner 1991). GO was used to evaluate techniques which could then be extended to alcohol oxidase.

For the purpose of an alcohol biosensor alcohol oxidase appears to be a more favourable enzyme than alcohol dehydrogenase due to the low turnover number of the latter enzyme and it's requirement for the coenzyme NAD^+. An appropriate mediator can improve the value of ADH or an approach which binds the coenzyme to the enzyme itself (Laidler and Mazid 1987, Gestrelius et al 1975), thus removing the need to add NAD to the assay. AO does have its faults, mainly that it is subject to product inhibition and it is very unstable, being denatured easily.

Clark, in 1972, reported the use of alcohol oxidase in the determination of methanol and ethanol with an enzyme electrode. Clark's electrode lacked specificity, linearity and stability. In 1983 Verduyn et al recorded a method of constructing an alcohol electrode also using alcohol

oxidase. Verduyn used an oxygen electrode with AO immobilised with catalase on nylon net and while initial results of his electrode did look promising, a high voltage (+0.8 V) was applied to obtain them. Since this time, the majority of attempts to make an alcohol biosensor reported in the literature have been based upon ADH.

4.2 Electrode material

For this thesis four main electrode materials were used; carbon foil, carbon paste, NMP.TCNQ, and platinum.

Carbon foil (made from compressed graphite) is the cheapest substrate for use as an electrode material, however it is not robust, tending to shear at slight pressure (even in solution), has high background potentials, differing surface area between individual electrodes and tends to be hydrophobic. An attempt to produce carbon foil electrodes which were more stable to shearing forces than the original "end-on" mounted disks (section 2.2.1), was made by embedding carbon discs in epoxy cement so that only the surface of the foil was exposed. Another disadvantage of carbon is that hydrogen peroxide, a product of oxidases, is not easily oxidised at carbon electrodes, and an electron mediator must be used in conjunction with carbon electrodes (Cass).

Some groups (Mosbach) have reported success in adsorbing GO to carbon foil electrodes modified with 1,1 dimethyl ferrocene and this approach was attempted. GO adsorption was demonstrated by enzyme assay and indeed by amperommetry, however GO was washed out of the electrode and into solution. These results were shown to be valid also for AO and ADH. Enzymes may be crosslinked with GA once adsorbed to carbon and this may increase the stability of the electrode (Mosbach). Although this technique was tried in relation to AO and ADH, because of the high susceptibility to GA electrodes fabricated in this manner did not have any

activity. Pyrolytic graphite was investigated as a more hydrophillic carbon substrate but electrodes of this material had high background currents which rendered them useless for this purpose.

Carbon paste electrodes can be made and these are relatively cheap, enzymes can be incorporated into the paste, as can mediators. Gorton et. al. have reported an electrode made from carbon paste including Meldola's Blue and glucose dehydrogenase and this approach was used. Kulys et al (1992) have also reported a pyruvate carbon paste electrode, incorporating pyruvate oxidase and Methylene Green, which was successful. Electrodes were made in a similar way containing Meldola's Blue as a mediator for ADH, but the mediator proved to be soluble in the potential range where NADH was reduced and therefore this particular technique using Meledola's Blue gave no good results.

One way of utilising the economical attribute of carbon combined with the detection of hydrogen peroxide by platinum is to platinise carbon (Hajizadeh 1991). This technique is being explored by others in the Biosensor group at AgResearch but does not yield reproducible electrodes, the electrodes are not reusable and nor are they any more resistant to shearing force. Thus platinisation of carbon was rejected as the only real value appeared to lie in economy, the adsorptive quality of the electrodes are not retained and thus another method for enzyme immobilisation must still be sought.

NMP.TCNQ has been reported to be a good mediator for dehydrogenases and was used in this thesis as an electrode material as a paste incorporating ADH as well as NAD^+. Other groups have had a great deal of success using this approach (Sim 1990, Zhao and Buck 1991), having found that electrodes made in this manner can be poised at 0 V and give a substrate response. Zhao and Buck found their electrode to

be very reproducible and have reliable responses to ethanol from 0.1 to 10 mM which increased in 5 μA steps. There is no good reason why electrodes made in a similar manner to that of Zhao and Buck would not yield similar results, however several attempts were made with highly non-reproducible results. The organic salt is not easy to make and is readily oxidised to form insoluble TCNQ rendering it useless as a mediator. Thus although initial results using NMP.TCNQ did look promising it was discarded as an electrode material as other avenues of research looked more promising.

Platinum electrodes have a very low background current making them eminently desirable as biosensor working electrodes. They are easy to clean and robust. However although it is reusable platinum is expensive, it will not adsorb enzyme and nor does it have any functional groups on its surface to assist immobilisation of the enzyme, thus to take advantage of platinum's characteristics a method for immobilisation must be sought.

4.3 Method of immobilisation

The simplest immobilisation technique, after absorbtion, is crosslinking by applying a bifunctional agent (eg. GA) to a protein matrix containing the enzyme. Miyamoto et al (1991) immobilised ADH and NAD^+ in a triple-layer using BSA and GA on platinum. Without a mediator the potential applied to a platinum electrode must be around +0.75 V so this is not an ideal system and is only slightly better when using an oxidase enzyme. Currents generated by Miyamoto et al's electrode were in the nanoampere range. However previous successes of other authors (Cass) with GO gave encouragement to apply the technique to AO and ADH.

GA crosslinking was attempted with both platinum and carbon foil electrodes. Good glucose sensors were made using this method on platinum. GA inhibition studies showed that AO and ADH were inhibited by GA at low concentrations.Those

concentrations which were not inhibiting resulted in too weak crosslinking to retain the enzyme, which was leached out into solution. Results were obtained for an AO electrode crosslinked in gelatin on carbon but this electrode was unstable and erratic in its response to ethanol.

Carbon has, effectively, free carboxyl groups on its surface and these can be utilised for covalently binding enzyme (Mosbach). Water soluble carbodiimide methods were used to bind separately GO, AO and ADH to the electrode surfaces of modified (with dimethyl ferrocene) carbon foil electrodes. Enzyme assay and amperommetry indicated that enzyme was bound by this method but the approach, although repeatable, yielded electrodes differing markedly in response. Further work would be needed to identify the source of variability. One possibility is that a proportion of the enzyme activity is adsorbed to the carbon foil disc as well as being covalently bound and this may take time to wash out. The assumption in this approach is that there are indeed free carboxyl groups available, because this is difficult to test more time could be spent determining what the surface of the electrodes is like and whether there is a difference in surface between each individual electrode. The electrodes may be taken to high temperatures (700 ^{o}C) or chemically oxidised to give carboxyl groups.

A conducting polymer can be formed by the electro-oxidative polymerisation of polypyrrole. Polypyrrole films can thus be deposited on platinum electrodes and then be derivatised, or enzyme can be included at the polymerisation step and incorporated in the polypyrrole membrane (Buguslarsky et al).Foulds and Lowe (1988) modified polypyrrole with ferrocene and then immobilised GO in the polymer. Results from this experiment were promising and thus a similar technique was attempted with ADH. Polymer films would not form in the presence of ADH: however if phenazine methosulphonate (PMS) was present films did form but did not

have any activity. Presumably the conditions of electro-polymerisation were too harsh for the enzyme.

Polypyrrole coated electrodes were derivatised to form amino groups on the surface which were activated using EDC, ADH was then exposed to the activated polpyrrole membrane. Although amino groups were demonstrated on the electrode, no ADH was immobilised. It is presumed that this experiment failed at the carbodiimide coupling step. The desirability of polypyrrole lies in its ease of deposition and the fact that generally smooth, robust surfaces are formed within a short period of time. More time could be spent optimising the conditions for co-depositing enzyme with the polypyrrole.

Many biosensors have been formed in the past using polymer membranes wrapped around an electrode, for example the first biosensor made by Clark and Lyons in 1962 was based on the principle of a membrane covered oxygen electrode. The membrane can either physically entrap enzyme as in Clark and Lyons' electrode or the enzyme may be covalently bound to the membrane (Dong et al 1991, Wilson and Thevenot (Cass)). It was hoped that methods used for binding protein to affinity columns could be made use of to bind enzyme to cellulose membranes which could then be wrapped around an electrode system or on membranes pre-deposited on to a working electrode. Benzoquinone and cyanuric chloride methods were used to activate cellulose membranes which were then exposed to ADH. No positive result was obtained in either of these methods.

In an attempt to add functional groups to platinum, platinum electrodes were electrolytically oxidised and then amino-silylated, the resulting amino groups being then crosslinked to ADH. Wingard recorded this procedure in 1985 and because of platinum's ideal qualities as an electrode material, this technique looked promising. Although amino groups appeared

to be present there was no enzyme activity on the electrode. Personal communication (Dr. Alan Hart) stressed the importance of stringently anaerobic conditions during the electro-oxidation. It is possible that failure in this case was due to incomplete deoxygenation of the solution.

Substances with conjugated double bonds, of which haemin is a good example, are known to bind irreversibly to platinum. This knowledge was utilised to bind both ADH, AO and GO to platinum electrodes by first conjugating the enzymes to haemin. Although the technique of binding conjugated enzyme to platinum electrodes is not evident in the literature recipes for conjugating carboxyl groups to amino groups is readily available. Haemin was conjugated to the enzymes using an active ester method and the conjugate then exposed to a platinum electrode. All results were positive with enzyme assays indicating activity as well as a linear response to glucose in the case of GO electrodes and a response to alcohol in both the case of AO and ADH. In view of the fact that electrode responses were obtained at polarising voltages as low as +0.2 V, it is possible that haemin could be acting as a mediator in its own right: having an iron atom central to the porphyrin ring the haemin could conceivably fluctuate between oxidised and reduced forms. The particular advantage of a technique such as this lies in the use of platinum as the electrode material, thus all the advantages of platinum are combined with an easy and reliable technique of immobilisation.

CHAPTER 5: CONCLUSION

This thesis work has been primarily involved in finding those conditions which will yield a sensitive and accurate alcohol biosensor. Many methods of immobilisation on different electrode materials have been attempted, the majority following a successful report in the literature. Carbon foil electrodes, chosen because of economy and their adsorptive properties yielded some promising results, particularly with regards to covalently binding enzyme to carboxyl groups of the carbon. These electrodes did however differ markedly between runs and also between individual electrodes and would require more work before being used as possible candidates for an alcohol electrode. No success at all was forthcoming with carbon foil electrodes and likewise with immobilisation on cellulose membranes. Conducting polymers appear to be a possible line to follow, being robust and easy to prepare, however no success was had in this direction either. Crosslinking either ADH or AO in a protein matrix with GA can also be rejected due to the enzymes' susceptibility to denaturation by GA.

As platinum has such characteristics as being reusable, easy to clean, and capable of reducing hydrogen peroxide at convenient potentials a method of adding functional groups was attempted. No enzyme activity was present on electrodes made in this way, and this technique was also rejected. Another approach to utilising platinum was to conjugate enzyme to haemin and then allow the haemin to bind to platinum, via the association of d-orbitals of the platinum with the pi-electrons of the haemin. This approach was successful and an added bonus of this technique appeared to be the lowering of the potential that the electrode must be poised at to reduce hydrogen peroxide, possibly due to a mediator effect of the haemin.

In conclusion the method of choice of all the techniques studied in the preparation of this thesis would be the novel idea of haemin-enzyme conjugate binding to platinum. This technique appears ideal, a high enzyme activity is present on electrodes, the electrode material is platinum, and in the case of GO the responses are linear and recorded in μAmps range. However AO and ADH electrodes do not seem to have high sensitivity and activity does seem to be lost over time. Reducing the amount of dimethyl formamide in preparation may help but ideally some method of stabilisation of the enzymes must be sought. Thus this technique appears very promising but more work on the preparation of the conjugate, separating bound haemin from unbound haemin and retaining enzyme stability is warranted. Further study on the stability of the electrode and investigations into amplifying the electrode response would be a useful next step toward the goal to designing a biosensor for the detection of alcohol.

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