THE AMPEROMETRIC ~~DETECTION~~ STUDY OF ENZYMES AND THEIR SUBSTRATES

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ABSTRACT

This thesis describes an investigation into the use of amperometric techniques for the detection of enzyme catalysed reactions.

Enzymes catalyse a vast number of biological reactions and the detection of their activity can lead to the development of analyses for their substrates.The specificity with which the enzyme recognises its substrate is unique and cannot be easily mimicked by chemical catalysis.Enzymatic analyses can therefore be highly discriminating, they are also often the best methods for biological analyses as they can be carried out under mild conditions.

The isolation of enzymes for use *in vitro* can also enable substrate analogues, which the molecule would not normally encounter, to be detected using that enzyme.

Traditional methods of detecting enzyme activity rely on expensive equipment and skilled operation.Current trends in analysis, especially in the medical field, are towards methods which can be used away from laboratories and devices capable of such analyses are called biosensors.Work carried out in this thesis uses the electrochemical technique of amperometry for the detection of enzyme catalysed reactions The simple instrumentation and rapidity associated with this technique facilitates the development of biosensors from the analyses described.

The thesis examines three different systems.The first is a specific sensor for paracetamol (acetaminophen), the second

a more widely applicable assay for dehydrogenases.This analysis is based on the amperometric detection of NADH using the mediated electrochemistry of the enzyme diaphorase.The same system has also been used to study the kinetics of the overall reaction which are compared with a kinetic analysis carried out by conventional methods.

The final assay is for the detection of catalase, this extremely active enzyme is also conjugated to antibody molecules such that an enzyme linked immunosorbent assay (ELISA) with amperometric detection can be developed.

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Chapter 2 Electrochemical theory and methods

Chapter 3 Materials and methods

INTRODUCTION

1.1The necessity for analysis

The ability to detect and quantify species is a fundamental requirement of the interaction between an organism and its environment.As the ability to control that environment evolves so the need to monitor change becomes more important, both to follow parameters which indicate a need for control and to assess the changes brought about by that control.

The techniques by which various parameters in the environment are monitored form the discipline of analytical chemistry.Analytical chemistry is necessarily broad since its methods find application in all branches of science and technology.

1.2 The techniques used in analysis

The method employed to measure a particular atom or molecule depends both upon the properties of the analyte and the type of information required.In general selectivity, sensitivity and precision are required from any analysis which must be capable of reproducibly discriminating the interesting analyte from other substances .

All analytical techniques provide an estimation of some parameter (either an intrinsic property of the analyte or one which changes in response to the presence of the analyte) which alters in proportion to the concentration of the species of interest.

Classical techniques of analytical chemistry either measure physical parameters or follow chemical reactions

which the analyte undergoes and it is possible to classify the techniques on the basis of the parameters to which they respond.Those most widely used are listed in table 1.1.(1)

1.3 The analysis of species in biological systems

The work described in this thesis is concerned with the analysis of molecules of biological importance.Such molecules are components of living systems and are either produced by organisms or exert some effect on them.

Until recently traditional physical and chemical techniques have been the only methods available for the analysis of biological systems in which they have several disadvantages.

The environments in which biologically interesting analytes are found are invariably complex and one of the problems of analysis of such molecules is the interference caused by other substances.The problem of interference is often accentuated by the unique specificity of many biological systems.The analyte of interest may be physically identical to other molecules in the same environment, for example optical isomers of the amino acids show completely different biological activities.

The methods of many classical analyses are also destructive,in biological analyses an *in vivo* measurement may greatly increase the utility of the result and such methods are useless for this type of experiment.

The way in which these problems can be solved is by using the biological processes themselves in analysis.The

Table1.1 The classification of analytical techniques by the parameter to which they respond

TECHNIQUE	PARAMETER
Gravimetry	Weight
Volumetry	Volume
Spectroscopy	Emission/absorbtion of electromagnetic radiation
Electrochemistry	Electrical properties in solution
Radiochemistry	Emission of nuclear radiation
Mass spectrometry	Abundance of molecular fragments
Chromatography	Differential migration

molecules studied are interesting because they affect a biological system. Discovering ways of measuring the effects of the analyte on that system can lead to direct methods for the detection of the analyte.The recognition of an analyte by a biological system can be much more sensitive than many chemical or physical processes and biological recognition can usually be non destructive.If a biological system can function in vitro it will often recognise molecules which it would not normally encounter as well as its natural substrates.

1.3.2 Transduction of biological effects into measurable signals

The analysis of a substance by its effect on a system requires that the effect can be transduced into a measurable signal.Analytical methods are thus made up of a recognition and a transduction component.Biological processes are able to recognise their effector molecules and are used in this thesis as the recognition component of analyses.The aim of the work carried out is to develop biosensors for the analysis of various substances and the properties of such devices are described in section 1.4.

Transduction of the recognition event can be by various means and is to an extent dictated by the type of device required, transduction mechanisms are again discussed below (see section 1.6).

1.4 Biosensors; concepts, development and use

A biosensor is simply a device that utilises a

biological recognition system in the detection of an analyte.In this sense many traditional methods of biological analysis are strictly biosensors (see section 1.6).More commonly however the definition of a biosensor includes both the means by which the analyte is recognised and the technology by which recognition is achieved. Present trends in analysis, especially in the medical field, are towards methods which require neither skilled operation nor complex technology and that can thus be excecuted away from a well equiped laboratory.The properties of an ideal biosensor are listed in table 1.2.In practice a compromise must usually be reached, achieving the most important properties at the expense of those which are not vital to a particular analysis.

The processes of recognition which can be exploited are outlined below.The work described in this thesis investigates the *in vitro* exploitation of biological recognition and methods by which these can be transduced into a measurable signal.

1.5 Biological recognition systems which can be exploited in analysis

There are four biological recognition systems which can be exploited in analysis and they are as follows;

1.5.1.Receptor-ligand interaction

Receptor-ligand interactions include all biological recognition systems which do not belong to any of the other three categories described below.

Table 1.2 The properties of an ideal biosensor

Sensitive
Selective
Accurate
Rapid analyses
Easy to use
Not dependent on complex equipment
Inexpensive
Preferably disposable

Table 1.3 Methods used for the transduction of recognition in a biosensor

Luminometry
Optoelectronics
Microcalorimetry
Potentiometry
Amperometry

The specific recognition of a compound (such as a hormone or drug) often occurs *via* receptors on the surface of the cells which that compound affects.Recognition can then elicit a wide range of effects which constitute the function of the compound.Receptor ligand interactions are ubiquitous and their effects are diverse (2).Specialised ligand receptors are cell bound, difficult to extract and often non functional *in vitro* (3).Receptors show high sensitivity (for example only forty molecules of pheromone are detectable in some insect systems (2)) thus they are usually present in very low concentrations which again hinders extraction for *in vitro* analysis.

As recognition systems receptor- ligand interactions possess the vital features of specificity and sensitivity required in analysis.They are however difficult to transduce into a measurable signal (see section 1.5.2.2)and this, along with difficulties in extraction, have meant that they have not yet been widely used in analysis.

1.5.2 Antibody-antigen interactions

A specific example of a receptor- ligand interaction which has been exploited by analysts is the binding of antibodies to antigens.

Antibodies constitute the humoral component of the mammalian immune response, they are all immunoglobulin proteins and can be separated into five different classes on the basis of their structure (4).The antibody molecule consists of two regions, one of variable amino acid sequence (the Fab region) and one of constant amino acid sequence (the

Fc region).Antibodies are produced in response to invasion of the body by foreign molecules and the composition and structure of the Fab region differs depending on the structure of the invasive molecule.

1.5.2.1 The humoral immune response

Invasion of an organism by a foreign molecule (an antigen) induces the proliferation and differentiation of two types of cells, the B and the T lymphocytes.B lymphocytes differentiate into antibody producing cells and these antibodies are capable of binding specifically to the invading antigen.The surface structure of a complex antigen will consist of several different regions and any one antibody molecule will specifically recognise only one of these regions which are called epitopes. Different antibodies not only recognise different epitopes on the same antigen but also show a variety of binding affinities to the epitope.

The humoral immune response therefore produces a heterogeneous mixture of antibodies.Recent developments have made possible the production of high concentrations of monoclonal antibodies, which bind to the same epitope with the same affinity (5), which can be used to develop highly selective immunoassays (12).

The humoral immune response elicits several effects in vivo.Firstly the antigen is immobilised by the binding of several antibodies to different regions of its surface.Immobilisation often impairs the antigens activity, for example viral antigens attatched to antibodies are unable

to bind to target cells(6).Secondly the frequency of ingestion of foreign molecules by phagocytic cells increases ten fold when those molecules are coated with antibodies (7).Finally a variety of killing mechanisms are initiated by the formation of an immune complex, these include contact lysis by monocytes and complement fixation leading to antigen destruction.Vasodilation and smooth muscle contraction are also induced which serve to isolate the affected area (8).

Since an antibody binds specifically to only one antigenic epitope the system is ideal for recognition of the antigen in its analysis.The possibility of exploitation is aided by the fact that antibodies are produced in high concentrations in response to antigen challenge thus isolation for use <u>in vitro</u> is possible.Such an analysis can be developed for any molecule against which antibodies can be raised.

The immune complex (figure 1.1) is held by electrostatic and hydrophobic interactions,van der Waals forces and hydrogen bonding which are all reversible non-covalent interactions between antibody and antigen.In practice the equilibrium shown in figure 1.1 lies so far towards immune complex formation (with dissociation constants between 10^{-6} and 10^{-12} M (9)) that binding is essentially irreversible.

Figure 1.1

$$Ab + Ag \underset{k-1}{\overset{k1}{\rightleftharpoons}} AbAg$$

Dissociation constant Kd = [Ab][Ag]/[AbAg]

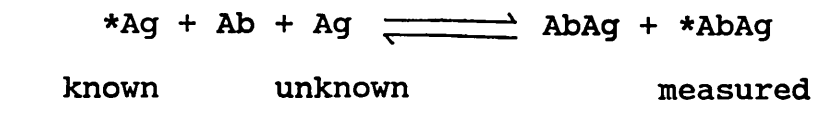
The natural effects of immune complex formation described above require the presence of molecules and cells other than just the antibody and antigen as do the effects of receptor ligand interactions (see section 1.5.1).These binding events thus have no overt effects in isolation and the *in vitro* transduction of binding requires the labelling of one or other of the components.

1.5.2.2 Immunoassays

The uniquely selective binding of antibody and antigen can be exploited in the analysis of any molecule against which antibodies can be raised.A label is essential for the visualisation of an immunoassay the principle of which is outlined in figure 1.2.Most immunoassays employ a radioisotope as label (10) but others are available including enzymes, which will be discussed further below.Immunoassays are classified by the type of label which is employed.Radioimmunoassay (RIA) and the more recently developed enzyme linked immunosorbent assay (ELISA) are both widely used in biology and medicine.

The bound and free molecules must be separable such that the concentration of unlabelled antigen can be estimated from the proportion of label in the bound fraction.Many variations of the immunoassay principle are now available which involve immobilisation of either antibody or antigen as well as alternative labelling strategies(11).

Figure1.2 The princple of a homogeneous immunoassay



* Denotes label

Most immunoassays employ polyclonal antibodies which recognise epitopes on the surface of the entire antigen and bind strongly to the molecule, monoclonal antibodies offer unique specificity but often bind weakly to the entire antigen and have not yet been widely used in immunoassay (12).

1.5.3 Complementary nucleotide interaction

The highly specific recognition of complementary nucleotide sequences is currently used in DNA fingerprinting for the identification of some inherited diseases (13)

Analysis is restricted to the detection of a particular nucleotide sequence and has only become useful recently since the alterations of DNA sequences which lead to certain diseases have been identified.

Again the technique requires a label, at present radiolabelling is used to follow the binding of a specific oligonucleotide probe to its complementary sequence in a preparation of DNA.

The rapid production of synthetic oligonucleotide probes is now technically possible but remains expensive and DNA

fingerprinting is not yet widely available.

1.5.4 Enzyme-substrate interactions

Enzymes are protein molecules which act as biological catalysts specifically enhancing the rate of reaction of both protein and non protein substrates.

The feature of an enzyme-substate interaction which is useful in analysis is that the enzyme is capable of specifically recognising the substrate and catalysing its conversion into a product molecule.If the conversion of substrate to product can be detected the use of labels is not necessary and analysis becomes much simpler.

The overall scheme of an enzyme substrate interaction is shown in figure 1.3.The rate of binding is governed by the law of mass action,in the same way as immune complex formation, and subsequent reaction steps are governed by the kinetic characteristics of the enzyme.

Figure 1.3 The enzyme-substrate reaction

$$E + S \rightleftharpoons ES \rightleftharpoons EP \rightleftharpoons E + P$$

1.5.5 Analysis using enzyme catalysed reactions

Enzymatic analysis encompasses not only the measurement of the substrates of the reactions but also study of the catalytic activity of the enzyme molecule.Often catalytic activity is affected by molecules other than the substrate

and these can also be measured by observation of the enzyme catalysed reaction.Enzymes are abundant in nature and catalyse a vast number of different reactions.Many enzymes are produced at high concentrations naturally and advances in microbiology have made possible the production of high yields of those that are less abundant.The production of high concentrations of enzyme makes their purification relatively simple.

Where labels are used to detect a reaction the label can be chosen to suit the desired detection method.

With enzyme substrate interactions the result of recognition is intrinsic to the recognition event and can be detected by a variety of techniques.

The work described in this thesis describes the development of detection methods for enzyme substrate interactions which can be applied to the production of biosensors (see section 1.4).Methods of measuring the effects of enzyme substrate interactions are described in the next section.

1.6 Measurement of enzyme substrate interactions

Enzyme catalysed reactions have been used for analysis since 1845 when G.Osann detected peroxide using peroxidase (14).By the 1880's enzymes were widely used ,for example in the analysis of foodstuffs (15).In these early analyses methods for the measurement of the reaction (usually by detection of the product) were limited and usually specific

to one reaction.The situation changed in 1935 when Warburg discovered that several different enzymes utilised the same molecules in their catalytic cycle (16a,16b).These molecules are the pyridine nucleotides which act as mediators of hydrogen transfer in several enzyme catalysed reactions.The nucleotide is usually associated with the enzyme protein and is inter converted between its oxidised and reduced form during the catalytic cycle (see figure 1.4).

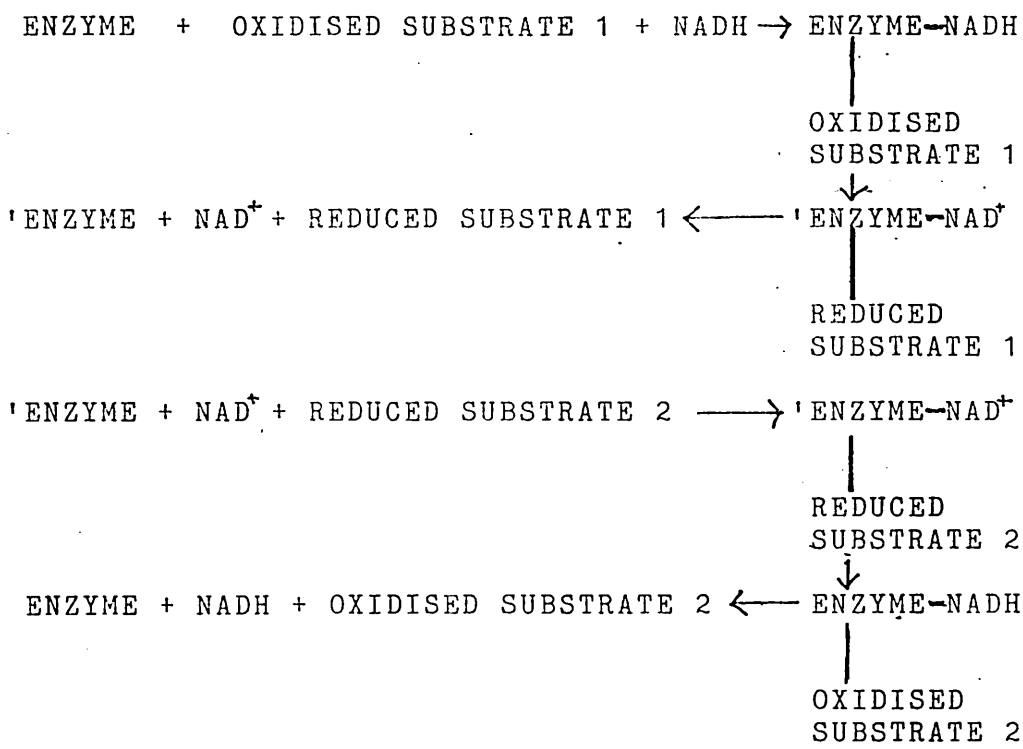
Warburg dicovered that the conversion of the nucleotide between its oxidised and reduced forms altered the wavelength of light which was absorbed by the nucleotide and that these reactions could be followed using absorption photometry (see section 1.6.1).The conversion of nucleotide can be exploited in the analysis of one of the enzyme's substrates provided that the second substrate is not supplied and the altered nucleotide can not be converted back into its original form.

A large number of pyridine nucleotide dependent enzymes were subsequently isolated, along with other enzymes whose substrates or products can be detected optically, greatly increasing the range of molecules which could be studied.The availability of reliable optical instruments after the second world war (17,19) and their later refinements have made absorption photometry the standard technique used in enzymatic analysis.

1.6.1 The technique of absorption photometry

The technique of absorption photometry is based on the proportionality between the concentration of a species and the amount of light which it absorbs.

Figure 1.4 The catalytic cycle of pyridine nucleotide dependant enzymes



The electrons in an atom or molecule are seen to exist in discrete energy levels with quantised energy differences between them.When light is shone at the atom or molecule energy is absorbed from the light which is sufficent to promote the transition of electrons into higher energy levels.

Light is electromagnetic radiation which can be seen as being propagated as a wave but absorbed and emitted as discrete particles (quanta) (18).The electromagnetic spectrum (shown in figure 1.5) can be divided into regions of different wavelength ranges determined by the way in which that light can be detected and by its energy.The properties of electromagnetic radiation are governed by the equations in below.

$$E = h\nu$$

where E = energy (joules)

h = Plancks constant

ν = frequency of propogation of the wave (hertz)

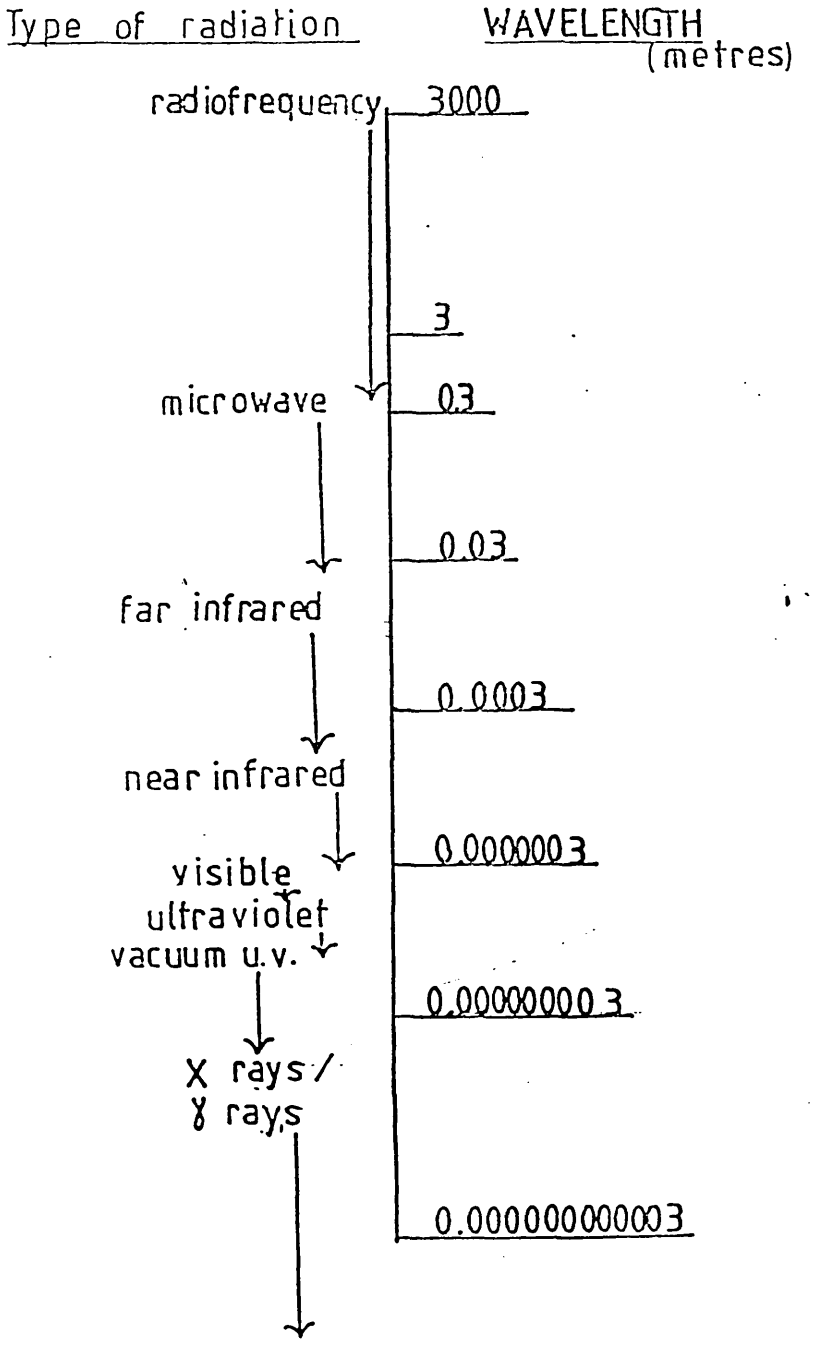
and

$$\nu = c/\lambda$$

where λ = wavelength (metres) and c the velocity (metres sec^{-1})

Light of a particular wavelength thus possesses a particular energy , if this matches the gap between energy levels in a particular atom or molecule it will absorb light of that wavelength.Species thus show a specific pattern of absorbance over a range of wavelengths which is termed their

Figure 1.5 The electromagnetic spectrum



absorption spectrum.

In order to quantify absorbance the amount of light transmitted through a solution of the interesting molecule must be measured.Quantitation can then be either by transmittance or absorbance where ;

Transmittance T = I/Io

Where I =Intensity of transmitted light

Io = Intensity of incident light

and absorbance A = log (1/T)

Transmittance decreases exponentially with concentration whereas absorbance is proportional to concentration , this proportionality is defined by Beer's law(19);

$A = \varepsilon cl$

where ε = molar extinction coefficent of absorbing species (M^{-1} cm^{-1})

c = concentration of absorbing species (M)

l = distance over which light is absorbed (cm)

Absorption photometry, commonly known as spectrophotometry, can thus be used to measure the concentration of any molecule, provided that it absorbs light strongly.Ultraviolet and visible wavelengths can be used in spectrophotometry and some molecules of interest show little or no absorbance in these regions.If a reaction is to be followed spectrophotometrically there must be a large differential absorbance between its substrates and products at a particular wavelength, and although the technique is

widely available it is not always applicable.

The properties of non-skilled operation not reliant on expensive laboratory equipment demanded of a biosensor are not met by spectrophotometry thus alternatives are sought.The transduction systems which have been used in the production of biosensors are listed in table 1.3 and their exploitation in biosensor devices is discussed more fully below.

1.6.2 Other optical transduction methods

Recent developments in optoelectronics and fibre optics have made possible the development of optical transducers that are smaller, simpler and less expensive than spectrophotometers.Some alternatives require instrumentation of similar sophistication to the spectrophotometer but enable analyses to be made with a specificity or sensitivity which cannot be achieved spectrophotometrically.The detection of bacterial luminescence for example has allowed the development of an extremely sensitive assay for pyridine nucleotide dependent enzymes (20).Surface plasmon resonance allows the direct detection of the formation of macromolecular complexes such as that formed between antibody and antigen (21).Both of these techniques require equipment that is as complex as a spectrophotometer but is not as widely available.

Technologically simpler optoelectronic devices, using light emitting diodes and light sensitive dyes, have been developed for the measurement of pH and serum albumin (22).

A device which is currently under intensive study is the

optical fibre.Incorporation of the recognition component into a fibre optic may lead to the production of a device which is sufficiently small and robust to be used in vivo.(23)

1.6.4 Microcalorimetry

Any chemical reaction involves a heat change and the measurement of this change (microcalorimetry) is applicable to the transduction of any biological recognition process (24).Microcalorimetry has not however been widely used because it requires the discrimination of small heat changes above large backgrounds which has proved to be a complex practical problem(25).

1.6.5 Electrochemical detection

Electroanalytical chemistry is concerned with the reactions which occur between a molecule and an electrode and has so far been the most widely used detection system in the development of biosensor devices.The methods of electroanalytical chemistry became widely available with the advent of operational amplifiers which greatly simplified the circuitry used in potential and current control (26).

Electroanalytical techniques can be broadly categorised as either amperometric, in which potential is controlled and current measured, and potentiometric ,in which potential is measured.The distinction between the two methods is discussed in more detail in chapter 2.

1.6.5.1 Potentiometric detection systems

One of the commonest potentiometric devices is the ion selective electrode, a variety of which have been produced

for the measurement of several different ions (27).These electrodes can now be used to detect an enzyme catalysed reaction which either uses or yields ions and such devices have been developed as biosensors (28).The most common examples of such enzyme electrodes are sensors for the substrates of decarboxylases and deaminases (28).These devices are based on the detection of carbon dioxide or ammonia by the base ion selective electrode and are not prone to interference.It is also technically possible to miniaturise potentiometric electrodes (29) which may become useful in the production of in vivo devices.

Potentiometric detection is restricted to the measurement of analytes which carry charge, the relationship between concentration and response is a logarithmic one.

Work described in this thesis is devoted to the study of the detection of enzyme-substrate reactions by electrochemical methods and concentrates on amperometric rather than potentiometric techniques .

1.6.5.2 Amperometric detection systems

The control of the potential difference between the analyte and the electrode dictates the current flow between the two.Current is also affected by the concentration of the analyte at the electrode surface, thus amperometry can be used to distinguish and measure an analyte.The control of reactions at the electrode surface will be discussed in more detail in the next chapter.

1.6.5.3 Amperometric techniques

The ability to apply different types of potential control in amperometry gives rise to a variety of different techniques for analysis.

For many years amperometry was used to detect trace amounts of analytes which can be oxidised or reduced at an electrode (30). The method used was polarography at a dropping mercury electrode which is a cumbersome technique and is now being replaced by analyses using a wide range of different electrode materials.The use of different electrodes has also increased the range of molecules which can be studied(31).

Specific amperometric analysis of enzyme substrates is possible if their products are detectable at an electrode surface.Many enzyme catalysed reactions involve the transfer of electrons in a way which is analagous to an electrode reaction, where such reactions occur it also is possible to examine the kinetics of the enzyme catalysed process.

Attempts have been made to obtain direct electron transfer between enzymes and electrodes.Direct transfer does not normally occur due to steric hindrance since the active site ,at which electrons are transferred to and from substrate molecules, is buried in the three dimensional structure of the enzyme.Electron transfer can thus only be acheived by modifying the electrode, such that surface groups can act as bridges of electron transfer, or by the use of soluble low molecular weight mediators which are able to shuttle electrons.

It is the use of mediators of electron transfer which is exploited in the amperometric observation of enzyme catalysed reactions in this thesis.

Many mediators have been described (32)but the most suitable for the study of a variety of enzyme catalysed reactions have been ferrocene and its derivatives (see figure1.6).Such a use of mediated electron transfer between enzyme and electrode has already led to the development of a practically applicable amperometric enzyme electrode (33).

1.7 Aim of the thesis

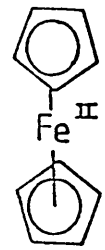
The studies described in this thesis have been undertaken to demonstrate the practical uses of amperometry in the observation of enzyme catalysed reactions.The applications of three separate recognition events, catalysed by different enzymes, in the development of biosensors has been investigated.

The first example is a sensor for paracetamol in which para aminophenol, formed by the enzyme catalysed hydrolysis of the drug is detected amperometrically.The second system uses mediated electron transfer from diaphorase to assay for the enzyme's substrate as well as to study the kinetics of the enzyme catalysed reaction.

Finally, in an analogous system catalase is assayed amperometrically, this assay is designed to detect the enzyme where it is used as a label in ELISA.

Three distinct assay systems are therefore investigated futhur details of which are given in the introduction to each chapter.

Figure 1.6 The ferrocene molecule



1.8 Development of biosensor devices

The requirement that a biosensor be an integrated device can be met by the immobilisation of the enzyme component of the sensor.This is facilitated by the use of a solid electrode as the base sensor since it can provide the insoluble support for the enzyme.Many methods for the immobilisation of enzymes to various supports have been described (63).Immobilisation often leads to increased stability of the enzyme as well as improving the efficency of tranduction in the biosensor.

Initial studies in this thesis have not included rigorous investigation of enzyme immobilisation since the aim has been to assess the suitability of particular enzyme/electrode combinations to specific analyses.

ELECTROCHEMICAL THEORY AND METHODS

2.1 Introduction

In this chapter the basic principles of electrochemistry and the electrochemical techniques used in this thesis are described.Such methods are often subjected to a rigorous mathematical treatment but here a qualitative approach is used to illustrate the important features of the techniques used.

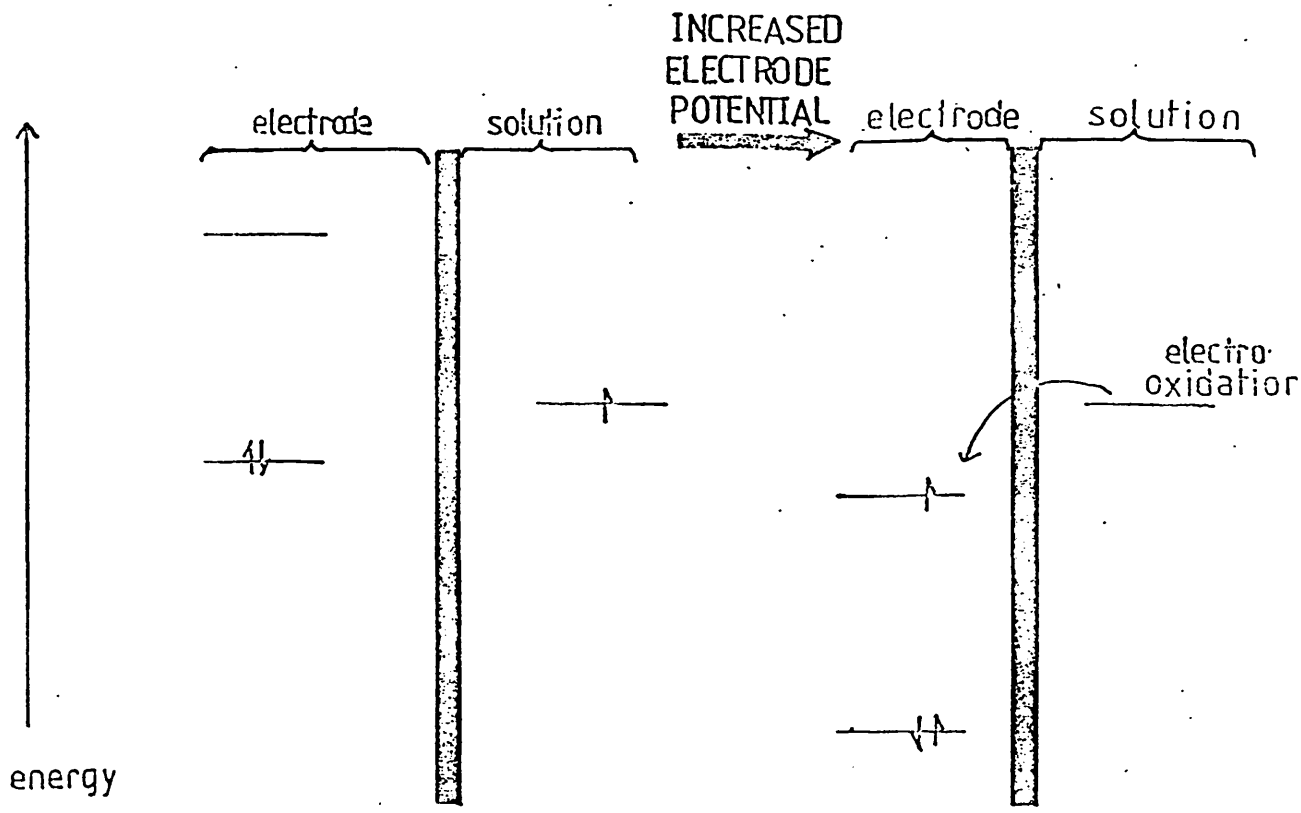
2.2 The fundamentals of an electrochemical reaction

An electrochemcal reaction occurs by the passage of charge across a phase boundary.The process is thus heterogeneous and the boundary is typically between a solid electrode and a solution of an electroactive species.Electrodes are usually metals or semiconductors and charge is transferred through them by the movement of electrons.Electroactive species are most commonly in ionic solutions but can also be in fused salts or ionically conducting solids.

The prerequisite for the passage of charge between electrode and solution is that a potential difference exists between them.Electrical potential of either phase is determined by the energy possessed by the electrons in that phase (see figure2.1).

If no potential difference exists between phases the energy of electrons in both is identical and no charge transfer will occur.If however there are unoccupied energy levels in one phase at a lower energy than those in the other phase (by virtue of a potential difference between the phases)

Figure2.1 The dependence of charge transfer on the energy states of the electrode and the species in solution



Horizontal lines denote energy levels and arrows denote electrons. An increase in electrode potential gives a corresponding decrease in energy of electrons and the highest unfilled levels are at a lower energy. When the energy gap is sufficiently wide electron transfer will occur from the solution species (see text).

electrons will transfer across the boundary in order to exist in a more favourable energy state.

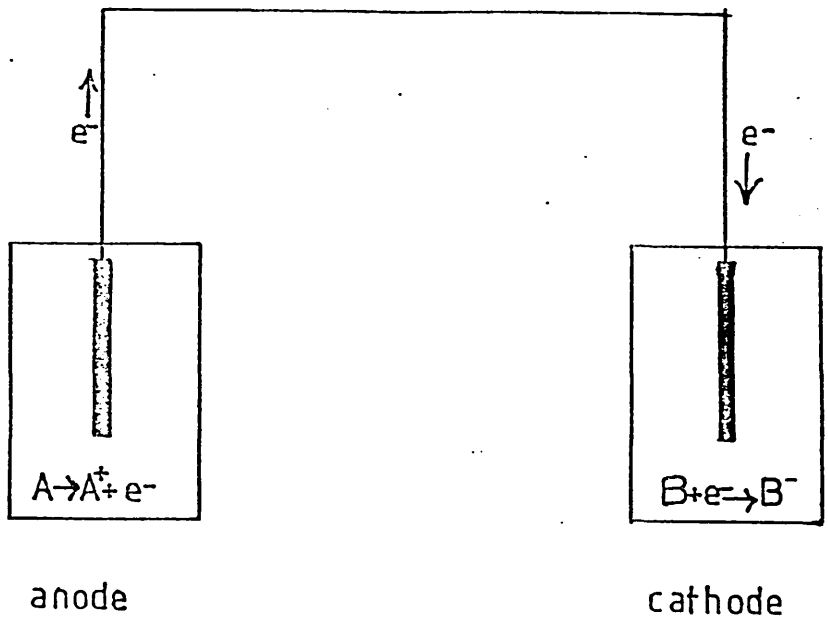
Electrodes at which charge transfer will occur readily contain many loosely associated electrons and accessible energy levels.The bonding in metals gives them a structure which has these properties and thus they, as well as molecules with a metallic structure, make good electrodes.

Charge transfer can be controlled by controlling the potential of the electrode and this is exploited in electroanalytical techniques, as will be described in more detail later in this chapter.

An isolated electrode/electrolyte phase boundary exists at an equilibrium controlled by the relative energy of the electrons in the two phases.An electrochemical reaction will occur if the equilibrium is shifted by the alteration of the potential of either phase.The potential of the electrode can be altered, to initiate an electrochemical reaction, if the electrode/ analyte system is incorporated into an electrochemical cell.

The electrochemical cell consists of two half cells, each an electrode/ solution interface, as shown in figure 2.2.The half cells are connected by an electrical conductor such that charge can transfer between the cells.If the potential difference at interface A is such that electrons pass to the electrode charge will pass from cell A to cell B and a reaction will be initiated at interface B where electrons pass into the solution.The reactions thus depend upon the

Figure 2.2 An electrochemical cell



potential at each interface and the tendency for charge transfer to occur at either interface is controlled by the difference in potential at each interface.

The tendency for charge transfer is defined by the half cell potential (or formal potential $E^{o'}$) of any interface when it is connected to a normal hydrogen electrode (NHE,see figure 2.3) in an electrochemical cell.The chemical composition of a half cell determines its electronic nature thus the passage of charge to or from the cell will alter its chemical composition and its potential.The normal hydrogen electrode is made in such a way that the passage of large amounts of charge either to or from it has very little effect on its chemical composition so that it retains a constant potential.Such a cell is termed non-polarisable and provides a reference potential against which the potential of other half cells can be measured.Such an arrangement is used for all electroanalytical techniques as the reactions occuring at a single half cell interface cannot be studied.

In practice the normal hydrogen electrode is difficult to use so alternative non-polarisable electrodes are employed.In work described here the saturated calomel electrode (SCE) is used which has a potential of +0.242 V versus the normal hydrogen electrode (38)

An electroanalytical technique involves either the measurement of the potential at an electrode/solution interface or the alteration of this potential, using an external energy supply, and the observation of the charge transfer initiated.Electroanalysis therefore falls into two

categories, depending on whether potential is measured or controlled and these are termed potentiometry and amperometry respectively .Work described in this thesis has concentrated on the use of amperometry for analysis and principles and practice of this technique are described in more detail below.

2.3 The principles of amperometry

Charge transfer at the interface results in either oxidation or reduction of the electroactive species as electrons pass to or from the electrode respectively.The charge transferred is defined by;

$$Q=nFAN \qquad 2.1$$

where;

Q = charge (Coulombs)

n = number of electrons tranferred per molecule of electroactive species

A = surface area of electrode (m^2)

N = number of moles of electroactive species at the electrode surface

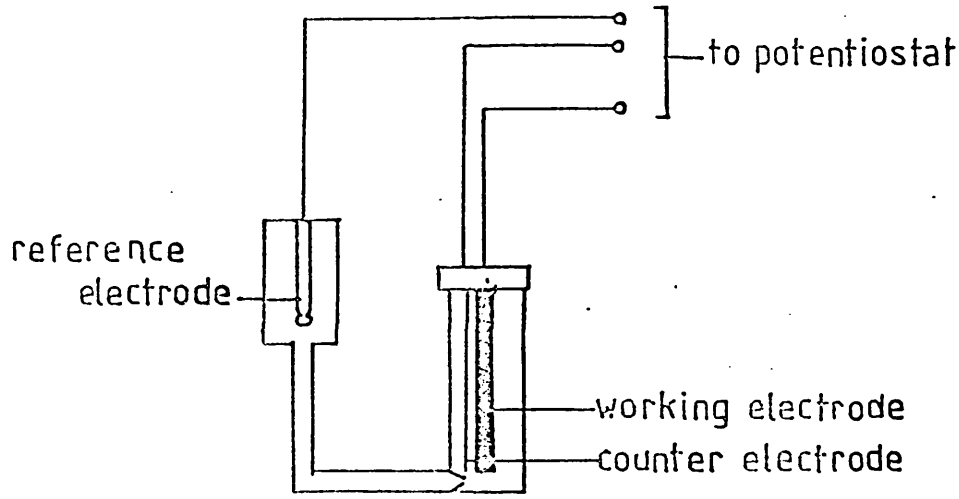
F = Faraday's constant = charge carried by one mole of electrons (coulombs/ mole)

In amperometry the rate of electron transfer (the rate of change of charge at the electrode surface) is measured ; this parameter is the current in amperes where;

$$\text{current} = i = dQ/dt = nFA\, dN/dt \qquad 2.2$$

The experimental arrangement of electrodes used in most electroanalytical techniques is shown in figure 2.4.The counter electrode acts as an electron sink such that

Figure 2.4 The standard three electrode arrangement used in an amperometric experiment



electrons do not pass to or from the reference electrode.

The charge transfer initiated by controlling the potential of the working electrode with respect to the reference depends upon the thermodynamic and kinetic properties of the electrode and the electroactive species.The occurence of charge transfer can thus be diagnostic for the presence of a particular electroactive species and the magnitude of the current obtained is proportional to its concentration.The nature of the potential control and the way in which the resulting current is measured can both be varied giving rise to a variety of different amperometric techniques, those which have been used in this thesis are decribed below.

2.4 Amperometric techniques

2.4.1Steady state voltammetry and chronoamperometry

The technique of steady state voltammetry is the simplest amperometric technique both in terms of the potential control and the interpretation of the current response.It is however not the first experiment to be performed on a new system since it requires a knowledge of the characteristics of the system under study.This information is gained using other methods which are described later in this chapter.

In steady state voltammetry the potential applied to the working electrode is sufficient to cause either the complete oxidation or complete reduction of the electroactive species.This results in the depletion of the electroactive species at the electrode surface.The current obtained is as

defined by equation 2.2 and determined by the rate at which electroactive species reaches the electrode.This rate is in turn controlled by the rate of diffusion of the species from the bulk solution which is dependent on the concentration gradient and thus the concentration in the bulk (see figure 2.5).

The amperometric methods can be applied analogously to both oxidation and reduction of electroactive species, in this chapter oxidation is discussed since this is the process occurring in the majority of the analyses used.

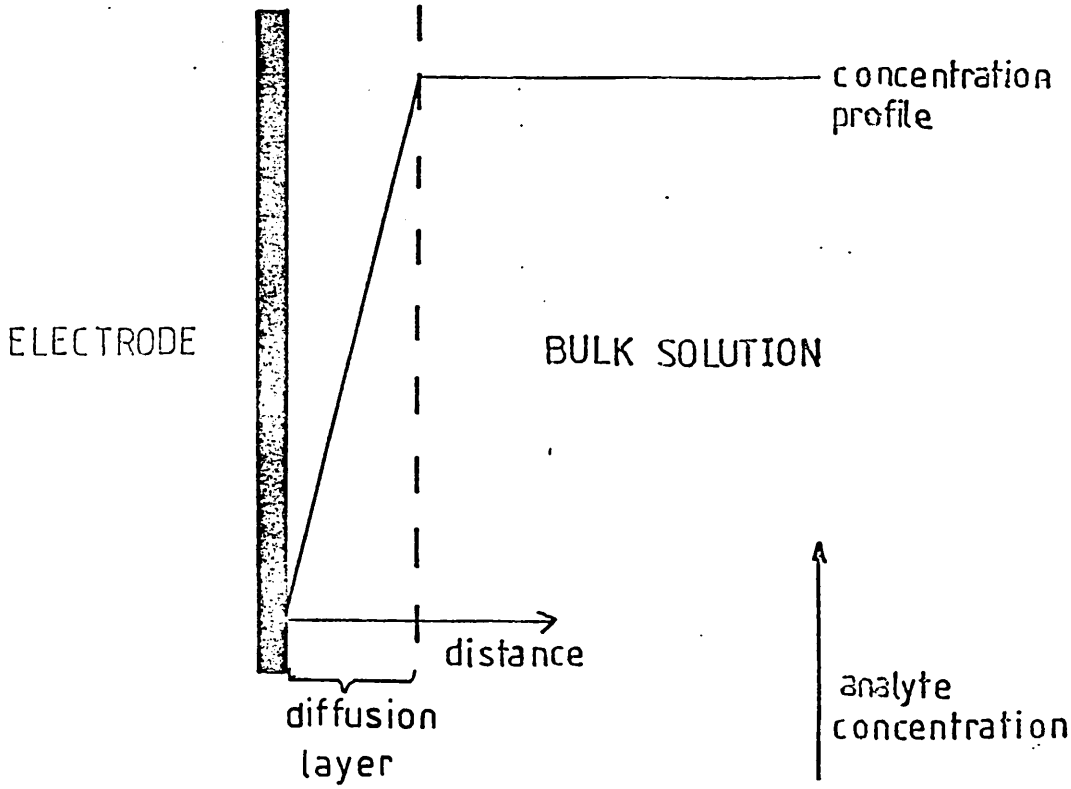
In steady state voltammetry the applied potential is maintained for the duration of the experiment and the solution of the electroactive species may be stirred to reduce the thickness of the diffusion layer.Where a stirred solution is not used the current decays as the thickness of the diffusion layer increases (see figure 2.5) and must be sampled at a fixed time after poising the electrode potential in order to calibrate the analyte concentration.

2.4.2 Chronoamperometry

In chronoamperometry a fixed potential , either oxidising or reducing, is again applied to a solution of the electroactive species.In this case, instead of recording the current at a fixed time after applying the potential, the current decay over a period of time is followed.

The way in which the current decays with time after poising the potential in a non-stirred solution of analyte is

Figure 2.5 The concentration profile of electroactive species in a steady state electrolysis



defined by the Cottrell equation;

$$i = nFAD^{1/2}C\pi^{-1/2}t^{-1/2} \quad 2.3$$

where t = time after poising the potential of the electrode (seconds)

D = diffusion coefficent of electroactive species (m^2sec^{-1})

and C = concentration of the electroactive species in bulk solution (Molar)

Chronoamperometry allows more information than simply the analyte concentration to be gained.Traditionally chronoamperometry has been used to determine the diffusion coefficients of various electroactive species but it can also yield information on the kinetics of homogeneous chemical reactions which are coupled to the electrochemical reaction. Coupled reactions and their investigation by chronoamperometry are discussed more fully in section 2.5.

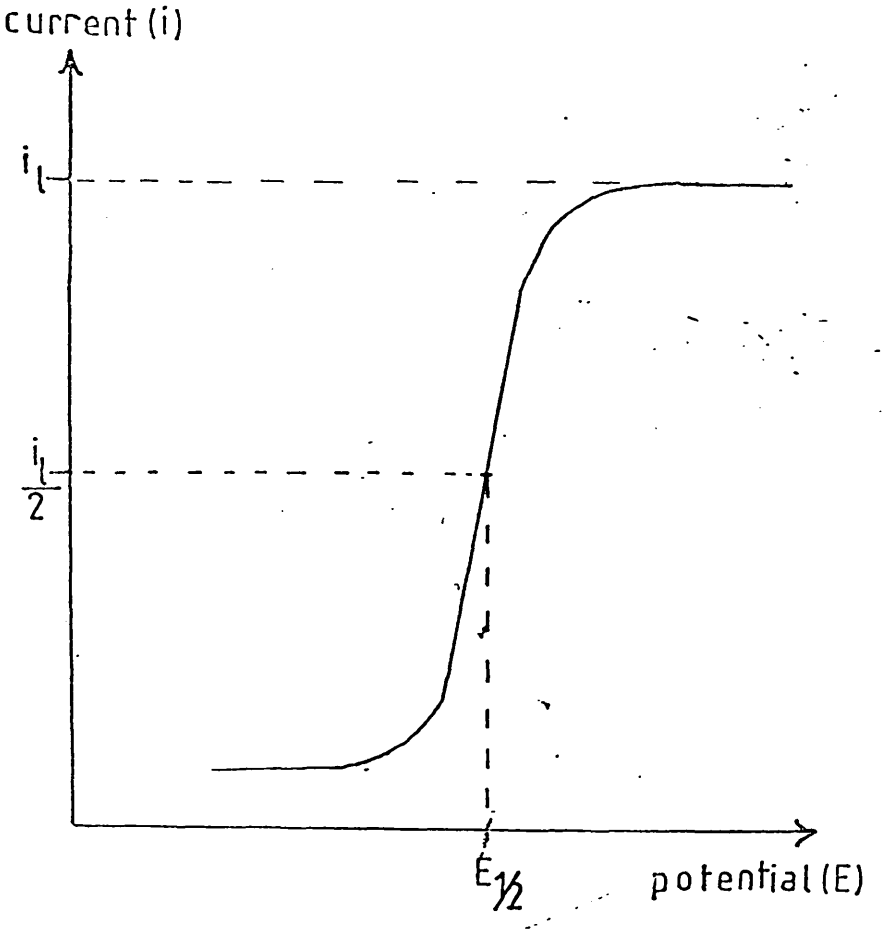
Steady state voltammetry and chronoamperometry give information about analytes when these are either fully oxidised or fully reduced at the electrode.The potential and electrode surface conditions necessary for complete electrolysis are dependent on the thermodynamic and kinetic properties of the electrode and the analyte relative to each other as explained above.Characterisation of the electrochemical reaction occurring is therefore necessary before electroanalysis is undertaken.The techniques of linear potential sweep voltammetry and cyclic voltammetry provide such a characterisation and are described below.

2.4.3 Linear potential sweep voltammetry

This technique involves the alteration of electrode potential in a stirred analyte solution at a fixed rate and measurement of the current as potential is swept.The potential is swept either anodic (towards more positive potentials) or cathodic (towards more negative potentials) of the starting potential to study oxidation or reduction of the electroactive species respectively.A linear potential sweep voltammogram is shown in figure 2.6.

If the electrooxidation of a species R is to be studied the potential is swept anodically from a potential at which the energy difference between the analyte and the electrode is insufficient to promote charge transfer.As the potential of the electrode is increased the energy of its electrons decreases and the energy gap between analyte and electrode becomes larger.Provided that charge transfer is kinetically favourable a potential will be reached where the energy gap is sufficient for electrons to pass from analyte to electrode resulting in analyte oxidation.As the potential difference increases the energy gap widens. The solution is stirred to replenish the electroactive species at the electrode surface. As the rate of charge transfer increases so does the current.A point is finally reached where the rate of diffusion of electroactive species to the electrode is equal to the rate at which charge transfer can occur across the interface and the current is at its maximum.

Figure 2.6 The output from linear potential sweep voltammetry



If electron transfer is not hindered the limiting current will be reached when the rate of replenishment of the electroactive species becomes limiting.The concentration of the electroactive species at the electrode is zero and the electrode reaction is said to be reversible.Under these conditions the limiting current, i_l, is defined by the Randels Sevick equation;

$$i_l = (2.69 \times 10^5) n^{3/2} A D^{1/2} \dot{V}^{1/2} C \qquad 2.4$$

where $\dot{V}$ = potential sweep rate (mV sec^{-1})

and C = concentration of electroactive species in bulk solution (Molar)

Dependance of the limiting current on the square root of the potential sweep rate indicates that the electrochemical reaction is reversible, that is that electron transfer across the interface is rapid.

At any one potential, before the limiting current, there will be a certain proportion of oxidised and reduced electroactive species at the electrode surface, this proportion is defined by the Nernst equation;

$$E = E^{o'} + RT/nF \ln ([OX]/[RED]) \qquad 2.5$$

where;

E = applied potential (volts)

$E^{o'}$ =Formal redox potential (volts vs NHE)

R = universal gas constant (J $mol^{-1}K^{-1}$)

T = absolute temperature (K)

F = faraday constant = charge carried by one

mole of electrons (coulombs mol^{-1})

[OX] = concentration of oxidised species at the electrode surface (moles l^{-1})

[RED] = concentration of reduced species at the electrode surface (moles l^{-1})

In practice this equation becomes;

$$E=E_{1/2} + RT/nF \ln([OX]/[RED]) \quad 2.6$$

where $E_{1/2}$= the potential at half the limiting current

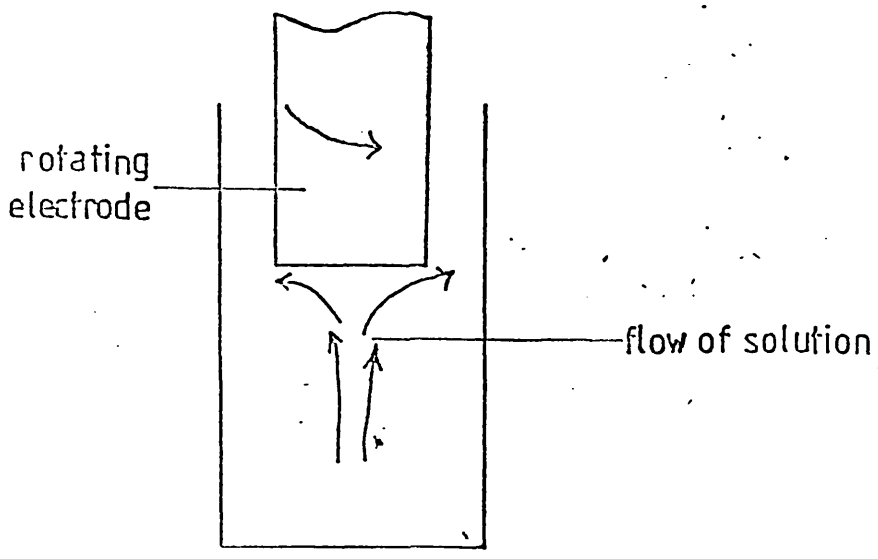
$E_{1/2}$ will approach the formal redox potential of the analyte if a true thermodynamic equilibrium is reached at the electrode surface.The $E_{1/2}$ value is thus a practical way of defining the oxidation or reduction potential of the electroactive species and can be determined by linear potential sweep voltammetry.The rate of potential sweep must be slow enough to enable oxidised and reduced forms to reach an equilibrium at the electrode surface.

In a steady state oxidation for analysis the potential applied is above that at which the limiting current is obtained such that complete electrolysis occurs.

2.4.4 Rotating electrode voltammetry

This technique is similar to linear potential sweep voltammetry except that the electrode is rotated rather than stirring the solution.The motion of the electrode initiates motion of the analyte solution as shown in figure 2.7.The advantage of this technique is that the flow of solution to the electrode surface is well defined and the kinetics of

Figure 2.7 The movement of solution induced by a rotating electrode



the electrode reaction can be studied (34).In studies described here the technique is used in exactly the same way as linear potential sweep voltammetry. In this case therefore the motion of the electrode stirs the solution.

2.4.5 Cyclic voltammetry

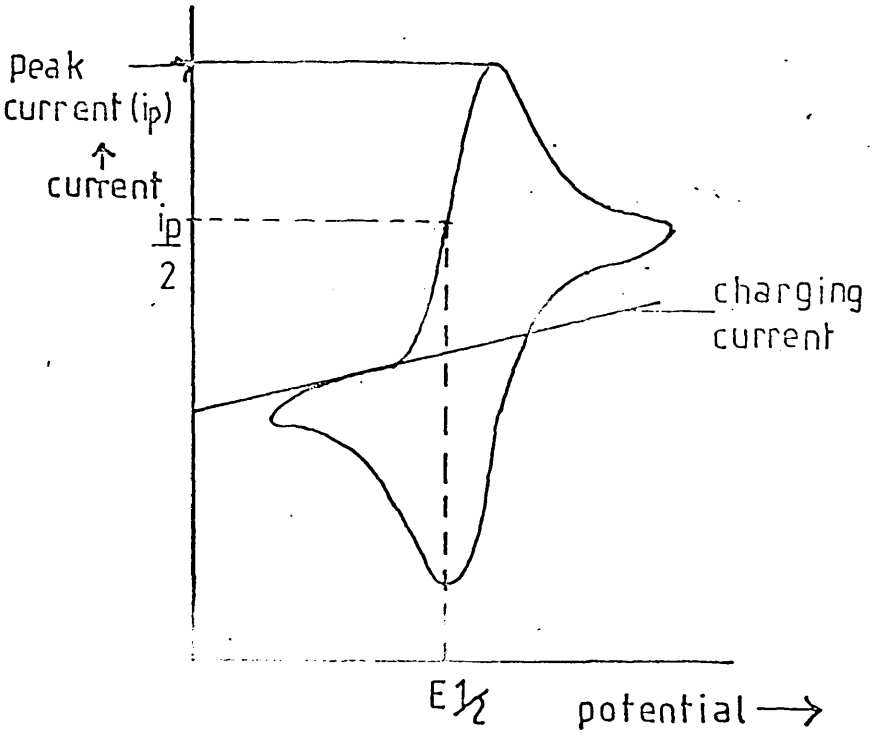
The potential input in cyclic voltammetry is the same as in linear potential sweep voltammetry but after the limiting current has been reached the direction of potential sweep is reversed and the potential swept back to that at which it started.The cyclic voltammetry experiment is performed in unstirred solution and gives a current vs potential curve of the type shown in figure 2.8.

The cyclic voltammogram thus shows two peaks, for the oxidation and subsequent reduction of electroactive species.Where electron transfer is rapid the electrochemical reaction is reversible and both peaks in the cyclic voltammogram will be the same size and will be separated by 59/n millivolts (35).The peaks are offset because the reduced electroactive species diffuses away from the electrode surface during the experiment and cannot be immediately re reduced once the potential sweep is reversed.

The peak current is again defined by the Randels Sevick equation and the criteria of peak height, peak separation and the proportionality of current with $\nu^{1/2}$ define the reversibility of the electrochemical reaction.

The techniques of cyclic voltammetry and chronoamperometry can be used for the analysis of reactions of non electroactive species provided that those species can

Figure 2.8. The output from cyclic voltammetry

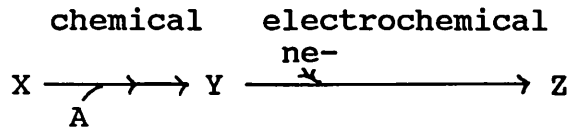


undergo a homogeneous chemical reaction which is coupled to the electrochemical process.

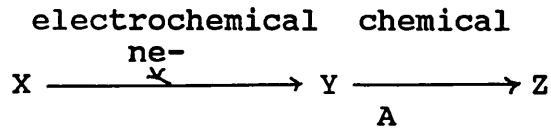
2.5 The effect of homogeneous reactions on electrochemical processes

Molecules placed in a solution of electroactive species which can react with either form of that species alter its electrochemistry and can thus be detected using electrochemical methods.Homogeneous reactions of this type fall into three broad categories as follows ;

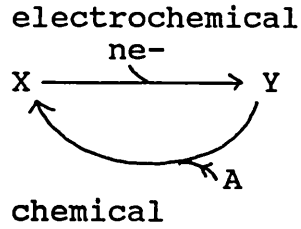
1. Preceding chemical reaction



2. Succeeding chemical reaction



3. Cyclic chemical reaction



Some of the studies described in this thesis use cyclic coupled reactions of flavoenzymes with electroactive species for the assay of the enzyme and its substrates.The enzyme is thus acting as the chemical reactant A as shown in category 3 above.

The perturbation of the normal electrochemical response

of the electroactive species in the presence of the chemical reactant allows the assay of the concentration of the chemical reactant and study of the kinetics of the reaction.

The deviations of a normal cyclic voltammogram and chronoamperogram in the presence of a coupled chemical reaction are shown in figure2.9.

In the cyclic voltammogram the reduced mediator is regenerated by the chemical reaction thus its concentration and the magnitude of the oxidation current increase.The current in a reversible cyclic voltammogram is proportional to $v^{1/2}$ as stated above and deviation from this (as shown in figure 2.10) is diagnostic of the presence of a regenerative chemical reaction.As sweep rate increases the time available for the chemical reaction to occur reduces as does the amount af reduced species regenerated and thus the current.

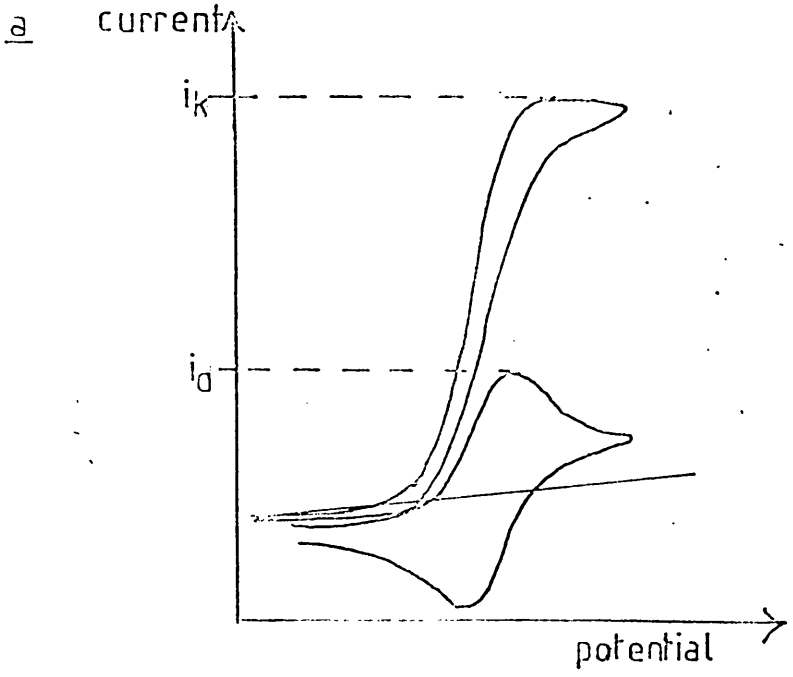
The current increase is dependent on the rate at which the reduced species is regenerated and can be used to calculate the rate constant for the reaction.

Values of peak current at various sweep rates are determined from cyclic voltammograms of the electroactive species with and without the chemical reactant.The current in the absence of a coupled reaction is termed i_d and the current in the presence of a coupled reaction is termed i_k.

The effects of homogeneous chemical reactions, with different velocities, on reversible electrochemical reactions have been mathematically modelled by Nicholson and Shain (36).

Figure 2.9 The effect of a homogeneous cyclic chemical reaction on a. The output from cyclic voltammetry

b. The output from chronoamperometry



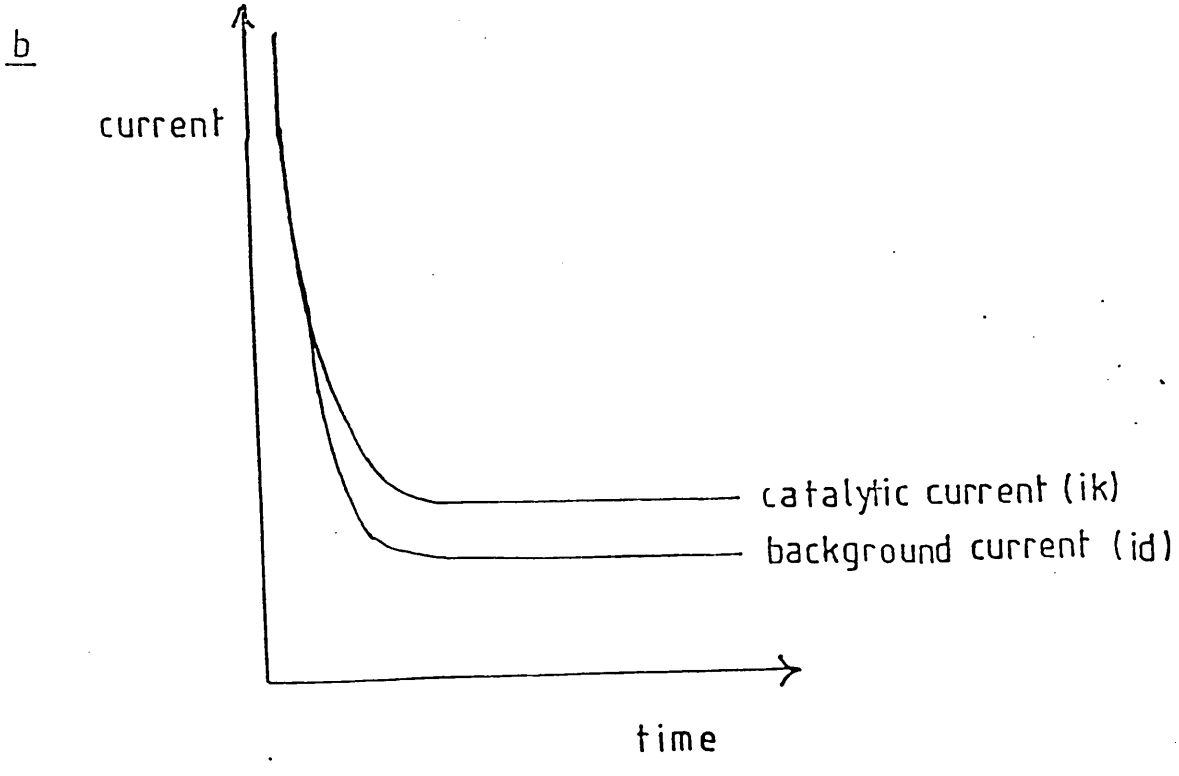
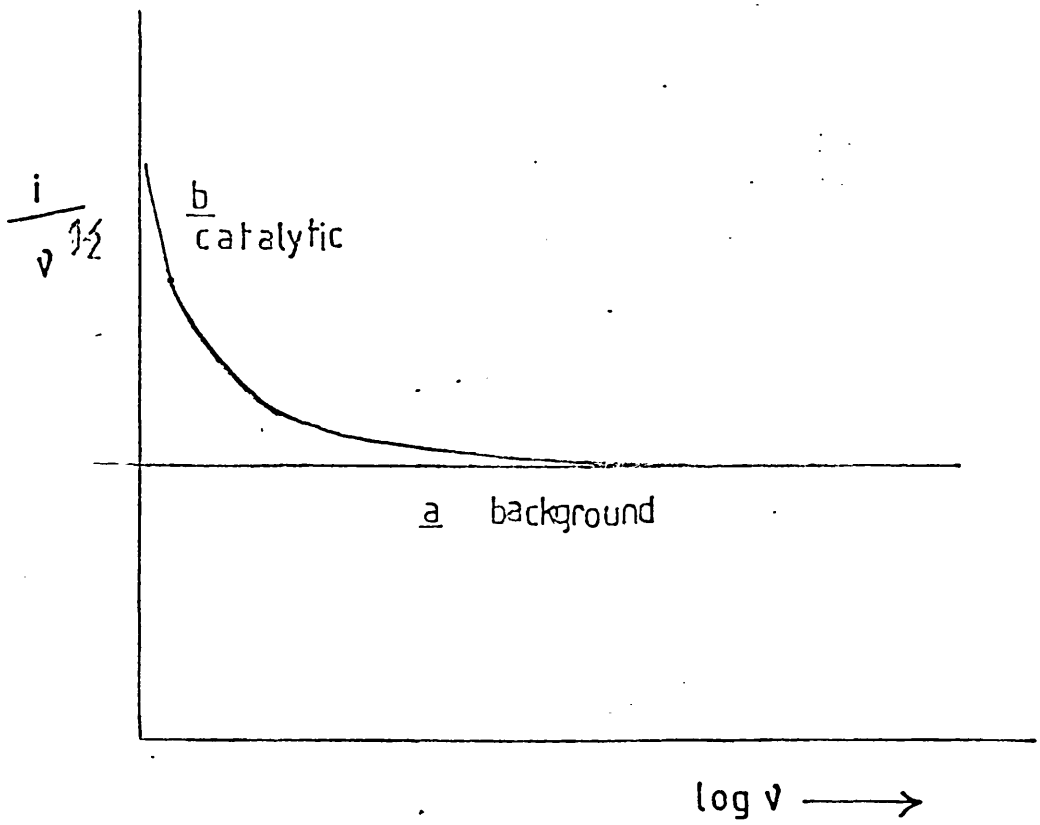


Figure 2.10 Diagnostic plot for a homogeneous cyclic reaction coupled to a reversible electrochemical reaction

a.reversible electrochemical reaction

b.reversible electrochemical reaction in the presence of a cyclic coupled homogeneous reaction.



From this analysis a working curve of i_k/i_d vs$\sqrt{k_f/a}$ has been produced, where

k_f = first order rate constant for the controlling reaction and

a = nFv/RT

Experimentally determined values of i_k/i_d at various sweep rates can therefore yield values of k_f/a from this working curve.The only variable in this term is v which can be removed by plotting k_f/a vs 1/v.This gives a line with slope =kfRT/nF from which kf can be determined.

In chronoamperometry the current is increased over the whole time scale measured as the apparent number of electrons transferred in the electrode reaction increases because more reduced species is available at the electrode surface.(see figure 2.5).

The apparent number of electrons transferred depends on the rate of the chemical reaction and can again be used to calculate the rate constant for the reaction.

In the presence of a coupled reaction the Cottrell equation becomes

$ik = n_{app}FAD^{1/2}C\,\pi^{-1/2}t^{-1/2}$

and $i_k/i_d = n_{app}/n$ which can be experimentally determined at various times after application of the potential.

A working curve of n_{app}/n versus log k_ft has been plotted from a mathematical analysis produced by Alberts and Shain (37).From this a k_f value can be extracted.

The techniques of amperometry described have been used, for reasons described elsewhere ,in the analysis of both simple electrode reactions and homogenous coupled reactions.

MATERIALS AND METHODS

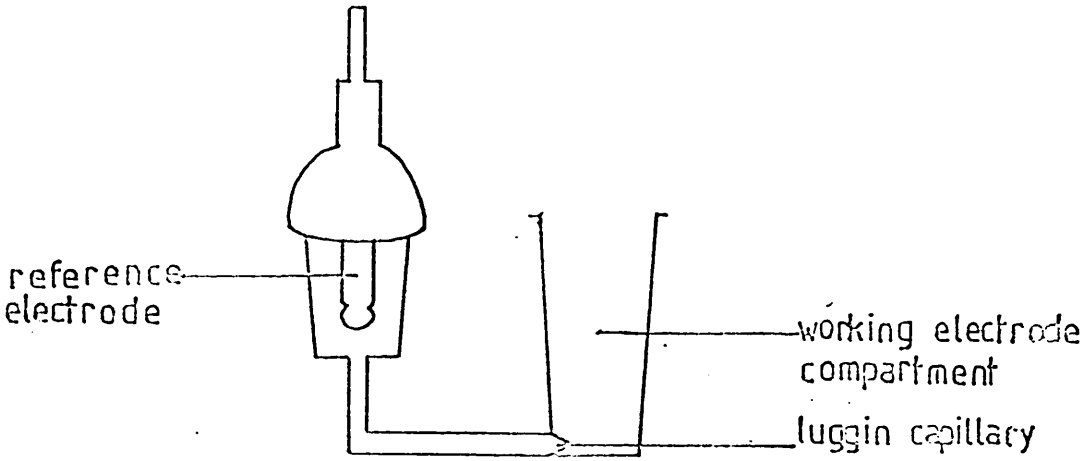
3.1 Electrochemical methods

3.1.1 Electrodes and electrochemical cells

Electrochemical experiments (apart from rotating disc voltammetry, see section 3.1.5.4)were carried out in a 1ml volume glass working cell connected to a separate reference compartment via a luggin capillary .This provides electrical contact between working and reference electrodes.Cells were manufactured in the biochemistry department at Imperial College.

Working electrodes were 0.7 mm diameter discs attached to a brass connecting rod using silver loaded epoxy resin.The brass rod was coated in poly (tetra-fluoroethylene) (PTFE) and held in the working compartment of the electrochemical cell using a Quickfit thermometer adaptor .A platinum counter electrode was held in the arm of the thermometer adaptor and the height of the electrodes adjusted such that both were close to the luggin capillary.Figures 3.1a and 3.1b show the electrochemical cell and the electrode configuration.Working electrodes were made from various metals or carbon and were all manufactured in the Chemistry Department.The reference potential was provided by a saturated calomel electrode (from Radiometer, Copenhagen) and was held in the cell by a Suba seal collar.This electrode has a potential of +242 mV vs a normal hydrogen electrode (38). Stirring of electrolyte solutions was achieved using a micro stir bar (PTFE coated), placed in the working cell conpartment, and a Stuart magnetic stirrer.

Figure 3.1a The cell used for electrochemical experiments



b. The working and counter electrodes

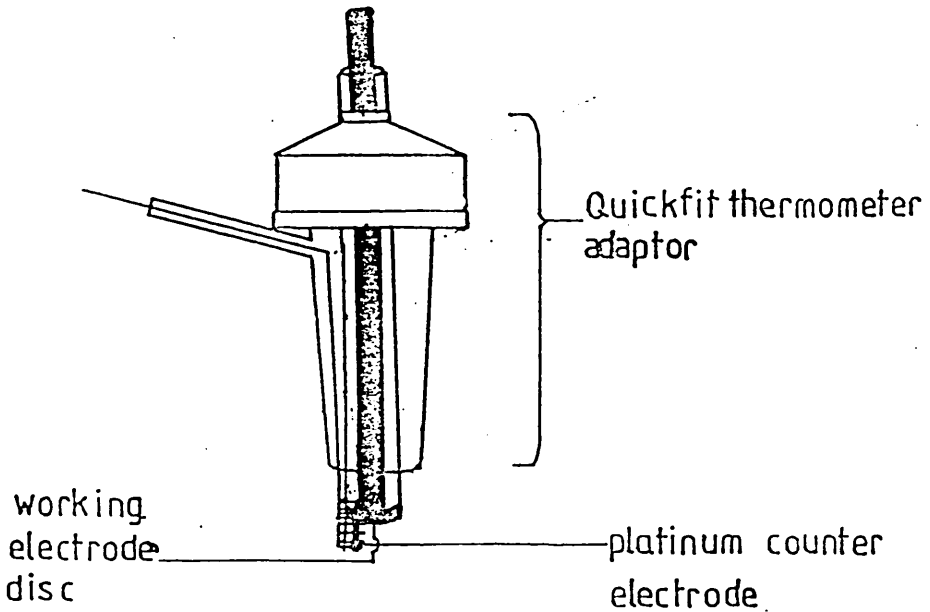
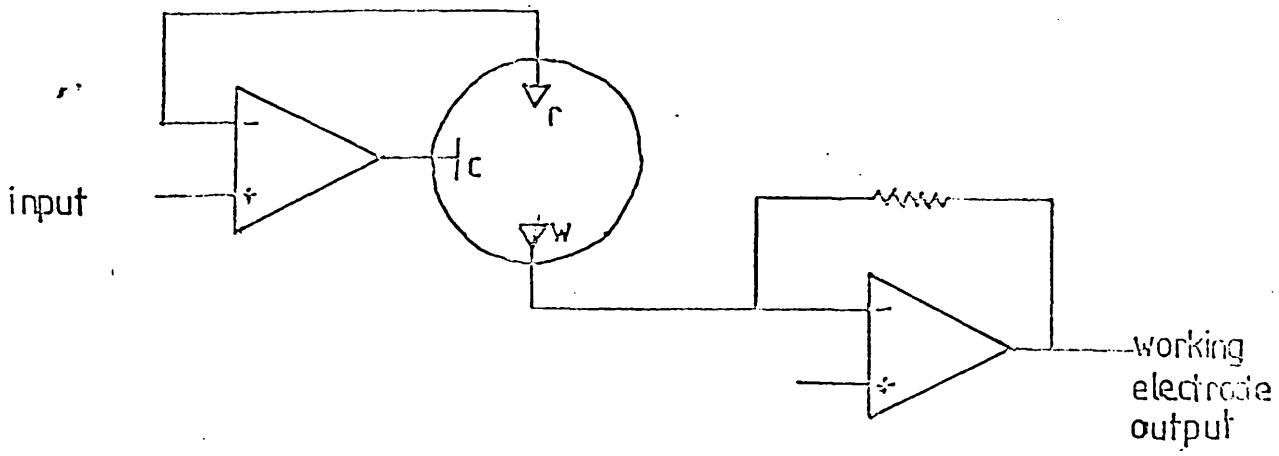


Figure 3.2 Standard potentiostat circuit for controlling a three electrode arrangement



r – connection for reference electrode
c – " " counter "
w – " " working "

3.1.2 Instrumentation

The potential of the working electrode was controlled by a standard potentiostat circuit which is shown in figure 3.2. This circuit enables the application of a steady potential whilst drawing negligable current from the cell ,this prevents reference electrode polarisation.The potentiostat used was either a model constructed in the chemistry department or a similar instrument obtained from Ursar Scientific Instruments.Currents were measured through a unity gain differential amplifier and recorded on a BBC Servogor XY chart recorder.Cyclic voltammetric studies employed the same potentiostats in conjunction with a triangular wave generator.

3.1.3 Enzymes and reagents

Enzyme sources and suppliers were as follows ;

1 Aryl acylamidase ex Pseudomonas from Dr. A.Atkinson of PHLS Centre for Applied Microbiology and Research, Salisbury Wiltshire.

2 Aryl acylamidase ex Rhodococcus from Genetics International (UK) inc.

3 Diaphorase ex Clostridium kluyverii and

4 Alcohol dehydrogenase ex Candida from Genzyme UK limited.

5 Alkaline phosphatase ex calf liver from Biozyme USA.

6 Catalase ex beef liver from Boehringer Mannheim limited.

3.1.3.2 Other reagents

Hydroxymethyl ferrocene was supplied by Strem Chemical Company, ferrocene monocarboxylic acid, ferrocene

carboxaldehyde, potassium ferricyanide and potassium ferrocyanide were from Sigma chemical company and all other ferrocene derivatives were made by the organic chemistry laboratory at Genetics international (UK)inc.Other reagents, where not explicitely stated were analytical grade and water, equivalent to triple distilled , from a Milli Q reagent water system supplied by Millipore , was used throughout.

3.1.4.Experimental conditions

All electrochemical experiments were performed at room temperature (20^{o}C) in the presence of 0.1 Molar buffer (as specified below) which also acted as supporting electrolyte.The working electrodes were polished in a slurry of 0.3 um diameter alumina and then sonicated in an ultrasonic bath, for 30 seconds, before each assay.

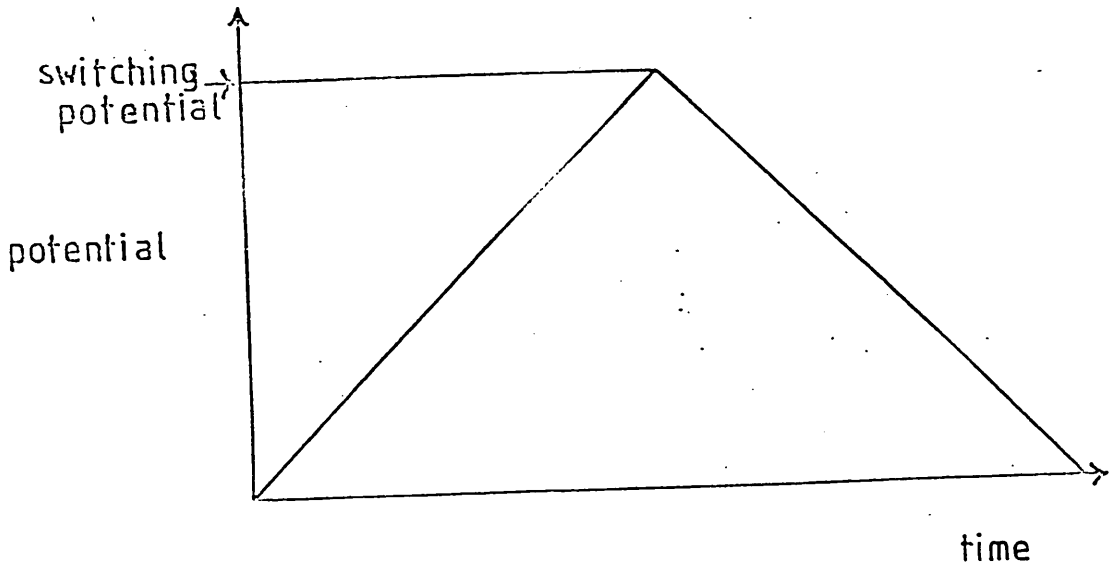
3.1.5 Electrochemical techniques

3.1.5.1 Cyclic voltammetry

The potential input in cyclic voltammetry was an anodic ramp followed by a cathodic ramp as illustrated in figure 3.3. The potential difference measured is that at the working electrode with respect to the reference electrode and the experiment is carried out in unstirred solution.

The switching potential (see figure 3.3) depends upon the redox potential of the electroactive species .In an experiment where the reaction of interest is an electrooxidation the switching potential must be at least 90/n mV positive of the peak oxidation potential of the analyte (where n is the number of electrons transferred in

Figure 3.3 The potential input for cyclic voltammetry



the electrode reaction) to ensure its complete depletion at the electrode surface (39).Similarly where reduction is studied the switching potential is at least 90/n mV negative of the peak reduction potential.

The potential was swept between these limits at different rates depending on the experiment and current was recorded with respect to the applied potential.

3.1.5.2 Chronoamperometry

Chronoamperograms were recorded at a fixed potential anodic or cathodic of the peak oxidation or reduction potential respectively (see section 3.1.5.4).The experiments were carried out in unstrirred solution and the current output was recorded with respect to time after application of the electrode potential.

3.1.5.3 Steady state voltammetry

Steady state experiments were similar to chronoamperometry but current was recorded at only one time after applying the potential and either stirred or unstirred solutions were used.Stirring in the electrochemical cell was with a micro stir bar made from PTFE and the rate was controlled using a Stuart magnetic stirrer.

3.1.5.4 Rotating disc voltammetry

3.1.5.4.1 Potential control

Potential input for a rotating disc voltammogram is as in cyclic voltammetry except that potential is swept in one direction only.

3.1.4.5.1 Electrodes and electrochemical cells

Working electrodes were 1cm diameter discs with brass connectors (as above) coated in PTFE and made from the same materials as the working electrodes described above .Electrodes were held in a motor driven rotor unit, supplied by Ursar Scientific, which was used to control the speed of electrode rotation.

The 10 ml volume cells were glass cylinders with side arms to house reference and counter electrodes, the rotating disc system is illustrated in figure 3.4.

3.1.6Electrochemical assays

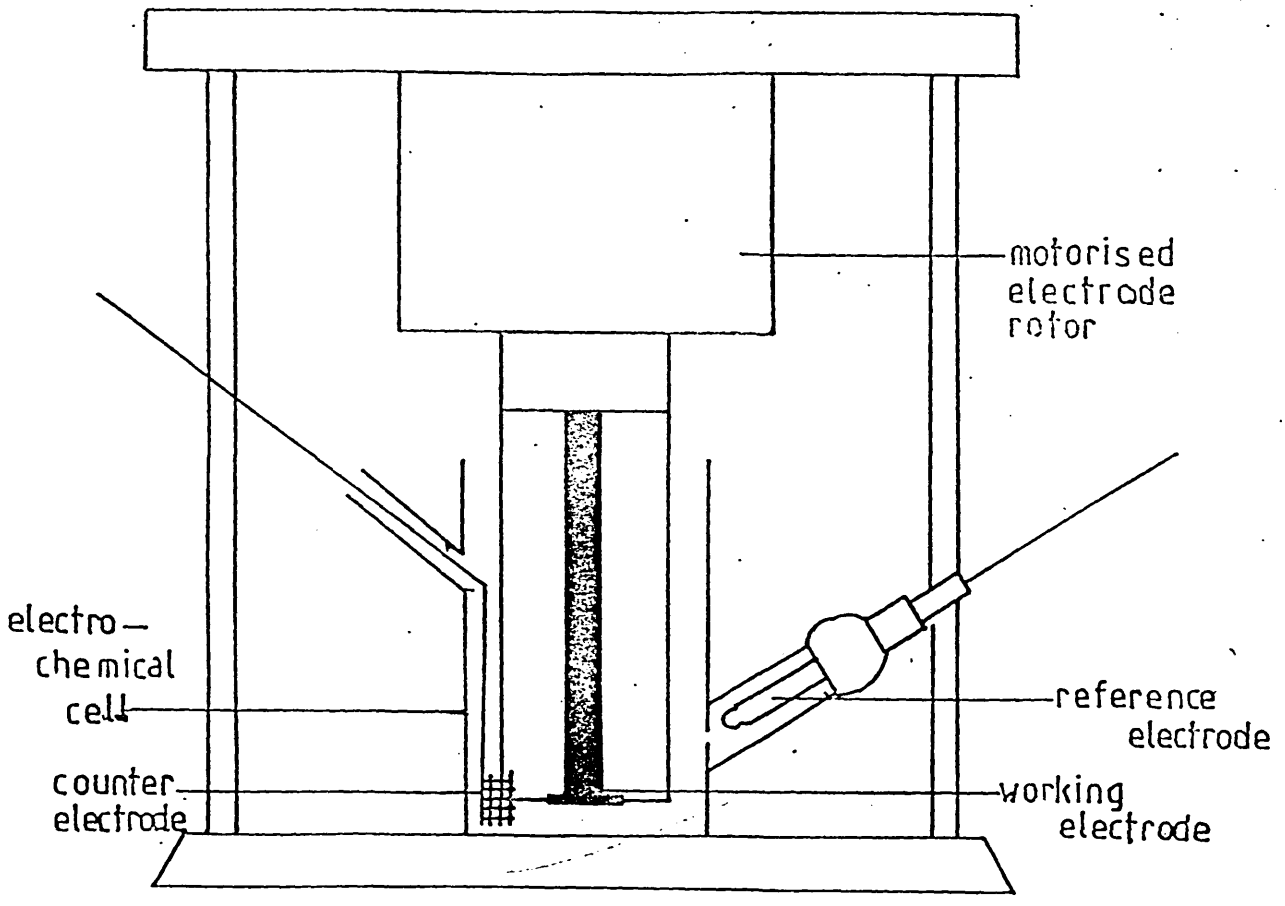
3.1.6.1 Assays for paracetamol and para aminophenol (PAP)

Steady state and cyclic voltammetric PAP and paracetamol assays were conducted at 20^{o}C in either 0.1M potassium phosphate buffer (pH 7)human whole blood (pH 7.2),human serum(pH 7.2) or 42 mg ml^{-1} bovine serum albumin (pH 7).Bovine serum albumin was supplied by Sigma and was used in place of human serum albumin because of its ready availability.

Blood samples drawn from a healthy volunteer were either treated with heparin and used immediately or placed in non-heparinised tubes, allowed to clot and centrifuged at low speed (to remove cells and debris).These serum samples could be stored frozen for later use.

Aliquots of PAP or paracetamol (0.01ml) were added to 1ml of liquid, in the electrochemical cell, to produce analyte concentrations between 0 and 500 ug ml^{-1}.

Figure 3.4 Cell and electrode arrangement for rotating disc voltammetry



Steady state assays for PAP and paracetamol in the presence of aryl acylamidase were carried out at a potential of +400 mV vs SCE and those for paracetamol alone were at +700 mV vs SCE.

Cyclic voltammograms were recorded between 0 and +700 mV vs SCE and rotating disc voltammograms between 0 and +1000 mV vs SCE.Glassy carbon working electrodes were used in all experiments.

3.1.6.2 Assays for diaphorase and NADH using ferrocene derivatives

Electrochemical characterization of the ferrocene derivatives and kinetic analyses of the enzyme ferrocene reaction were carried out using cyclic voltammetry.

3.1.6.2.1 Coupled experiments to study the kinetics of the diaphorase ferrocene interaction

An aliquot of the ferrocene derivative (1ml) at a concentration between 0.5mM and 2.0 mM and NADH at a concentration of 10mM in Tris (Tris(hydroxymethyl)aminomethane) /HCl buffer, pH 8.5, were placed in the cell.Cyclic voltammograms between different potential limits (depending on the ferrocene derivative) at sweep rates from 5 to 100 mV sec^{-1} were recorded.Diaphorase was added to give active enzyme concentrations between 6.72 $x10^{-8}$ M and 1.52$x10^{-6}$ M and cyclic voltammograms were again recorded.

3.1.6.2.2 NADH assays in the coupled system

Contents of the electrochemical cell were as for the kinetic

analysis except that NADH concentration was varied between 0 and 10mM and excess diaphorase(0.2 mM protein concentration) was added.

The working electrode was glassy carbon and was held at a potential of+400 mV vs SCE, the steady state current 15 seconds after poising the electrode potential was plotted against NADH concentration.

3.1.6.2.3 Ethanol assay using the coupled system

The assay was again a steady state experiment at a potential of +400 mV vs SCE and the current recorded 15 seconds after the potential was applied to a glassy carbon disc.

The contents of the electrochemical cell were as follows:

0.37ml of 5mM hydroxymethyl ferrocene,

0.3ml of 100mM NAD+ (from Boeringer Mannheim)

0.27ml of 90uM alcohol dehydrogenase and

0.06ml of 6.9mM diaphorase.

Various concentrations of ethanol (from James Burroughs Limited) were added and the current at each ethanol concentration recorded.

3.1.6.3 Experiments on other electroactive species

3.1.6.3.1 Ferricyanide and ferrocyanide

Cyclic voltammetry of 10 mM ferricyanide was carried out in 0.1 M phosphate buffer, pH7.0, in the presence of 0.1 M potassium chloride.The working electrode was pyrolytic graphite and the potential was swept between 0 and +700 mV vs SCE.

Steady state assays for ferrocyanide were in the same

supporting electrolyte at a potential of +400 mV vs SCE and the current obtained for ferrocyanide concentrations between 0.19mM and 1.53mM was recorded.

3.1.6.3.2 Oxygen

Rotating disc voltammograms were recorded at a glassy carbon electrode in 0.1M phosphate buffer, pH 7.0, between 0 and -1000 mV vs SCE.The buffer was either air saturated (containing approximately 240 uM oxygen),oxygen saturated or nitrogen saturated (containing no oxygen).The buffer in the electrochemical cell (10ml) was sparged with either O_2 or N_2 for 20 minutes before recording the voltammogram.

In all experiments the electrode was rotated at 16 sec^{-1} and the potential was swept at 5 mV sec^{-1}.

3.1.6.3.3 Methyl viologen

Cyclic and rotating disc voltammograms of 23.3 mM methyl viologen (from Sigma Chemical Company) were recorded in oxygen free and oxygen saturated buffer as described above.

3.1.6.4 Enzyme amplified assays with electrochemical detection

3.1.6.4.1 $NAD^{\pm}$ assay with detection of accumulated hydroxymethyl ferrocene

A mixture of 1mM hydroxymethyl ferricinium (synthesised as described in section 3.6), 8mM ethanol and either 2.5 or 5 Units each of diaphorase and ADH,in tris/HCl buffer, pH 8.5,(0.9ml)was added to a series of eppendorf microfuge tubes.NAD^+ (0.01ml) at various concentrations was added and the mixture incubated at room temperature for three

minutes.The reaction was stopped by the addition of 10 ul of stopping solution which contained mercury nitrate ,potassium orthophosphate (10%) and potassium chloride (1M).

The assay solution was transferred to the working compartment of an electrochemical cell.The current measured 15 seconds after poising a gold working electrode at +400 mV vs SCE in the solution was recorded and plotted against the NAD^+ concentration used.

3.1.6.4.2 Assays for pyridine nucleotides by the detection of hydroxymethyl ferrocene during reaction

A mixture of 1mM dimethyl ferrocene ethanolamine(DMFE), 130mM ethanol, 10 Units of diaphorase and 100 Units of alcohol dehydrogenase (1ml) was placed in the working compartment of the electrochemical cell .A 0.01ml aliquot of NAD^+ or NADH at various concentrations was added and the potential of the glassy carbon working electrode was poised at +400 mV vs SCE.Again the current 15 seconds after poising the potential was measured and plotted against the concentration of the pyridine nucleotide.

3.1.6.4.3 Alkaline phosphatase assay by measurement of accumulated ferrocyanide

Aliquots of alkaline phosphatase, 0.01ml at various concentrations, were added to 0.1ml of 50mM $NADP^+$ (grade 2 from Sigma chemical company) in 0.1M carbonate buffer pH 9.The solution was incubated at room temperature for 5 minutes before 0.01ml of 10 % orthophosphate was added to terminate the reaction.

A mixture of 8mM ethanol ,1mM potassium ferricyanide,3.6U of diaphorase and 3.6 U of ADH in tris HCl, pH 8.5, (0.9ml)was added and the mixture incubated for a further 3 minutes at room temperature.The reaction was stopped by addition of 0.01ml of stopping solution (see section 3.1.6.4.1)and current obtained at a gold electrode poised at +400 mV vs SCE was measured.

3.2 Spectrophotometric methods

3.2.1 Enzyme assays

3.2.1.1 Instrumentation

Absorbance measurements were taken using either a Perkin Elmer Lambda 3 spectrophotometer with a Perkin Elmer R100 chart recorder or a Varian DMS 200 spectrophotometer and an Epson LX86 printer.

3.2.1.2Enzymes and reagents

Enzyme sources and suppliers were as listed in section 3.1.3.1.

All other reagents ,where not specified, were analytical grade and made up in water from a Milli Q reagent water system.

3.2.2Spectrophotometric assay methods

3.2.2.1 Aryl acylamidase assay

Aryl acyl amidase was assayed by measuring the amount of PAP produced in a given time by a given amount of enzyme.PAP was calibrated in a colourimetric assay taken from Atkinson et al (40)

3.2.2.1.1 PAP calibration

1% aqueous orthocresol (1ml), 0.1 ml of ammoniacal

copper sulphate (made by mixing 25 ml of 0.2% w/v anhydrous copper sulphate solution with 0.4 ml of 0.880 ammonia) and 1.4 ml of water were placed in a disposable cuvette.Aliquots of a known PAP concentration (0.5ml) were added, the solution was mixed thoroughly and allowed to stand at room temperature for 5 minutes.The absorbance at 615 nm was then measured and plotted against PAP concentration.

3.2.2.1.2 Enzyme assay

2mM paracetamol (0.2ml) was incubated with 0.2 ml of various concentrations of aryl acylamidase and 0.6 ml of 0.1M phosphate buffer, pH 7.0, at room temperature for two minutes.An aliquot(0.5ml) of the reaction mixture was then placed in the same cresol/copper mixture described above.The solutions were mixed, allowed to stand for 5 minutes and the absorbance at 615nm measured.PAP concentration was interpolated from the calibration curve.

Addition of colour developing reagents is sufficent to terminate the enzyme catalysed reaction thus the concentration of PAP measured is that produced in a 2 minute incubation of 2mM paracetamol and aryl acylamidase, from this the enzyme activity in international units can be calculated as described in appendix 1.

3.2.2.2 Diaphorase

3.2.2.2.1 Activity in lyophilised powder

Diaphorase was assayed by the method described in the Worthington enzyme handbook (41).

Zinc chloride (0.12M) in 5mM HCl(0.1ml) ,0.1 ml of 6mM

NADH, 0.1 ml of 2,6,dichlorophenol indophenol (DCPIP) and 2.6 ml of 0.1 M tris/HCl buffer, pH 8.5, were placed in a disposable cuvette .0.1 ml of various diaphorase concentrations were added, the solution was thoroughly mixed and the rate of change of absorbance at 600nm was followed.DCPIP is reduced during the diaphorase catalysed reaction and its absorbance at 600nm decreases.From the initial rate of utilisation of oxidised DCPIP the activity of diaphorase can be calculated as described in appendix 2.

3.2.2.2.2 Concentration of active diaphorase in the lyophilised powder

For calculation of rate constants in kinetic studies it is important to know the precise concentration of active enzyme.The lyophilised preparartion exhibits a particular activity but this will be due to a proportion of the protein in the sample since some denaturation is inevitable.In order to estimate the active enzyme concentration in the powder a flavin bleaching assay was perfomed.

In the course of these studies two separate flavin assays were carried out on different batches of enzyme.

Either 18 or 13.35 mg ml^{-1} diaphorase in tris/HCl buffer,pH8.5,(0.9ml)was placed in a disposable cuvette.Either buffer or 100 mM NADH (0.1 ml) was added, the solutions were thoroughly mixed and their absorbance at 445nm measured.

The absorbance of diaphorase in the presence of NADH will be lower because the reaction of substrate with enzyme bleaches the absorbance of the flavin moiety .Provided that NADH is in sufficent excess all the active diaphorase

molecules will bind substrate.As no oxidant is provided substrate turnover cannot occur and the amount of flavin bleaching can be measured.Since each diaphorase molecule contains one flavin mononucleotide (FMN) the concentration of diaphorase able to be reduced by NADH can be calculated (see appendix 3).

3.2.2.3 Alcohol dehydrogenase assay

A solution of 15mM NAD^+ (1.5ml), 0.1ml of 96% (w/v) ethanol and 1.3 ml of sodium pyrophosphate buffer pH8.8 were placed in a disposable cuvette.An aliquot of ADH (0.1ml of 2ug ml^{-1}) was added, the solution was thoroughly mixed and the increase in absorbance at 340 nm was followed.The optical density increase is due to the increase in NADH concentration as it is formed from NAD and from the rate of NADH production ADH activity can be calculated (see appendix 4).

3.2.2.4 Alkaline phosphatase assay

Alkaline phosphatase was assayed by the method described in the Biozyme technical bulletin supplied with the enzyme (42).

A solution of 5mM para nitrophenyl phosphate (PNPP) (2.4 ml) and 0.5 ml of 50mM magnesium chloride in glycine buffer, pH 9.6, were placed in a disposable cuvette.Aliquots of alkaline phosphatase (0.1ml of approximately 0.8 U/ml) were added, the solution was thoroughly mixed and the increase in optical density at 400nm was recorded.Alkaline phosphatase catalyses the hydrolysis of the phosphate ester bond from PNPP to yield para-nitophenol which has an intense yellow

colour at alkaline pH.From the rate of cleavage of the phosphate ester bond the activity of the enzyme can be calculated as described in appendix 5.

3.2.2.5 Catalase assay

Hydrogen peroxide (0.1ml of an 8.8M solutio supplied by BDH chemicals) was added to 50 ml of phosphate buffer pH 7.The concentration of this solution was adjusted, by addition of either buffer or peroxide, until it showed an optical density between 0.55 and 0.52.Optical density readings were taken in a quartz cuvette at 240 nm.

An aliquot of catalase stock suspension (0.01ml) was added to 50 ml of buffer and 0.1ml of this added to 2.9 ml of the diluted peroxide solution in a quartz cuvette.The solution was thoroughly mixed and the time taken for A_{240} to drop from 0.45 to 0.4 was measured.The drop in A_{240} is due to utilisation of peroxide during enzyme catalysis and its rate is used to calculate catalase activity as described in appendix 6.

3.2.2.6 Kinetic experiments with diaphorase

3.2.2.6.1 Altering DCPIP concentration

Tris/HCl buffer, pH8.5,(2.7ml), 0.1ml of 0.1 mM NADH and 0.1 ml of DCPIP (between 23.2 and 127.2 uM) were placed in a disposable cuvette.Diaphorase (0.1ml of $1.4x10^{-7}$M) was added to the cuvette, the solution was mixed and the change of absorbance at 600 nm was followed.The rate of the diaphorase catalysed reaction was calculated as molar DCPIP reduced per minute using Beers law (see section 1.6.1) where the

extinction coefficent for DCPIP is 22000$M^{-1}cm^{-1}$(43).

3.2.2.6.2 Altering NADH concentration

The assay was as above except that DCPIP was held constant at 127.2 uM and NADH concentration was varied between 33.3 and 333.3uM.

3.2.2.7 The rate of a non-amplified $NAD^{\pm}$ assay

The ferricyanide/ethanol mixture used in the electrochemical amplified assays (0.1ml as specified in section 3.1.6.4.3) was placed in a quartz cuvette with 0.03ml of 5 mM NAD^{+}.3.6 units of ADH were added, the solution mixed and the rate of change of optical density at 340nm followed.

The rate of NADH production was calculated using the molar extinction coefficent of 6230 M^{-1}at 340nm.The rate of the reaction was measured only over the first 105 seconds of the experiment where substrate was not severely depleted.

3.2.2.8. Enzyme amplified assays

3.2.2.8.1 Measurement of the rate of an enzyme amplified $NAD^{\pm}$ assay

A solution of ferricyanide/ethanol and 5mM NAD^{+} (0.1ml) was placed in a quartz cuvette.3.6 units each of ADH and diaphorase were added, the solution was mixed and the rate of decrease in absorbance at 420 nm was followed.The optical density decreased at a constant rate due to the reduction of ferricyanide, the reaction rate was calculated as molar ferricyanide reduced per second using its extinction coefficent at 420 nm which is 1100 $M^{-1}cm^{-1}$ (44)

3.2.2.8.2 Alkaline phosphatase assay with enzyme amplification system from IQ Bio

Alkaline phosphatase was assayed using an AMPAK diagnostic kit supplied by IQ Bio limited, Cambridge.

AMPAK amplifier and substrate reagents were reconstituted 10 minutes before use.Aliquots of various alkaline phosphatase concentrations (0.01ml of between 29.7

nM and 2.97 uM) were added to 0.1ml of substrate in a disposable cuvette.The solutions were incubated at room temperature for 5 minutes after which 0.2 ml of amplifier was added.After a furthur 3 minutes incubation the reaction was terminated by the addition of 0.05 ml of stopping solution.

Tris/ HCl buffer, pH 8.5,(0.6ml) was added to each cuvette, after mixing the absorbance at 495 nm was measured.

The rate of INT formazan production was calculated from Beer's law using the an extinction coefficent of 19000 $M^{-1}cm^{-1}$ (45).

3.2.2.8.3 Alkaline phosphatase assay using ferricyanide as the indicator in the enzyme cycled assay

The assay was carried out in two stages as for the electrochemically detected assay (see section 3.1.6.4.3).In this case $NADP^+$ concentration was 5mM.

The amount of ferricyanide reduced was followed by the change in A_{420} over three minutes.The change of ferricyanide concentration was then measured as above (see section 3.2.2.8.1).

3.2.2.8.4 NADH assays with enzyme amplification and spectrophotometric detection

3.2.2.8.4.1 Detection of accumulated INT formazan

INT violet (0.1ml of 2.4 mM) , 25 units of diaphorase, 300 units of ADH , 0.1 ml of 1.5 M ethanol and 2.5 ml of tris/HCl buffer, pH8.5, were placed in a series of disposable cuvettes.0.1 ml aliquots of NADH from 0.33 to 3.3 uM were

added and the rate of change of absorbance at 495 nm was followed for 1 minute.The increase in INT formazan concentration over this time period was calculated as in section 3.2.2.8.2.

3.2.2.8.4.2 Detection by the rate of utilisation of oxidised DCPIP

The assay mixtures were as in section 3.2.2.8.4.1 but INT was replaced by oxidised DCPIP (2.4 mM) and the decrease in absorbance at 600 nm was followed.The rate of DCPIP reduction was followed as in section 3.2.2.6.1.

3.3 Synthesis of catalase- IgG conjugates

Catalase and IgG were crosslinked using the one step glutaraldehyde method described by Avrameas (see reference 46).

A 5 mg ml^{-1} solution of bovine IgG (0.5ml), from Sigma Chemical Company and 0.5 ml of 76 mg/ml catalase were mixed and dialysed overnight against 1l of 0.1M phosphate buffer, pH 6.8, at room temperature.

Aqueous glutaraldehyde (0.05ml of 1%,grade 1 from Sigma) was added to the protein mixture and incubated at room temperature for 2 hours.

Glycine (4ml 0f 50mM)was added to destroy any unreacted glutaraldehyde and the solution was extensively dialysed against 1l of phosphate buffer (at least 24 hours).

Ammonium sulphate solution was added to a final concentration of 50% and allowed to equilibrate at room temperature for 1 hour.The final solution was centrifuged at maximum speed in a Beckman bench top apparatus for 15 minutes after which the supernatant was discarded.The precpitate was

washed in 50 % ammonium sulphate, the supernatant removed by centrifugation and the precipitate resuspended in phosphate buffered saline (10mM sodium phosphate containing 150mM sodium chloride, pH7.2).

An equal volume of glycerol was added to the final conjugation products which were stored at -20^{o}C

3.4 Immunoassay for catalase-IgG conjugates

The presence of conjugates can be detected by immunoassay where microtitre plates coated with anti bovine IgG will show bound catalase activity if the bovine IgG in the sample is conjugated to the enzyme.

3.4.1 Binding of conjugates to the micro titre plates

A 1/200 dilution of anti bovine IgG from Sigma (0.1ml) was placed in the well of 9 columns of a Nunc microtitre plate (8 rows by 12 columns) and incubated for 1 hour at room temperature. The plate was then extensively washed in PBS containing detergent (0.05% Tween 12) to remove non-specifically bound proteins.

Various dilutions of 0.1% bovine serum albumin (0.1ml) (Sigma grade 1)in PBS were added to all the wells to block unreacted protein binding sites, the plate was incubated for 1 hour and washed in PBS /tween.

Table 3·1

	blank	conjugate blank	conjugate blank	conjugate dilution 1/10	1/100	1/1000	1/10	1/100	1/1000	1/10	1/100	1/1000
1												
2												
3												
4												
5												
6												
7												
8												

The final conjugate mixture (0.1ml of various dilutions), produced as described in section 3.3 was added to the last 11 columns of the plate,the contents of each well are listed in table 3.1.The plate was again incubated for 1 hour and unbound conjugate removed by extensive washing with PBS tween.

Conjugate binding was then assessed by assaying each well of the plate for catalase activity.

3.4.2 Catalase assay on the microtitre plate

Hydrogen peroxide (0.1ml 0f 1mM) was added to each well of the plate and incubated for between 10 and 26 minutes.After incubation 5mM 2,2'azinobis(3 ethyl benzthiazoline sulphonic acid) (ABTS) and 2uM horseradish peroxidase (both from Sigma) were added and the optical density at 410 nm was measured using a Titertek Uniskan microtitre plate reader.

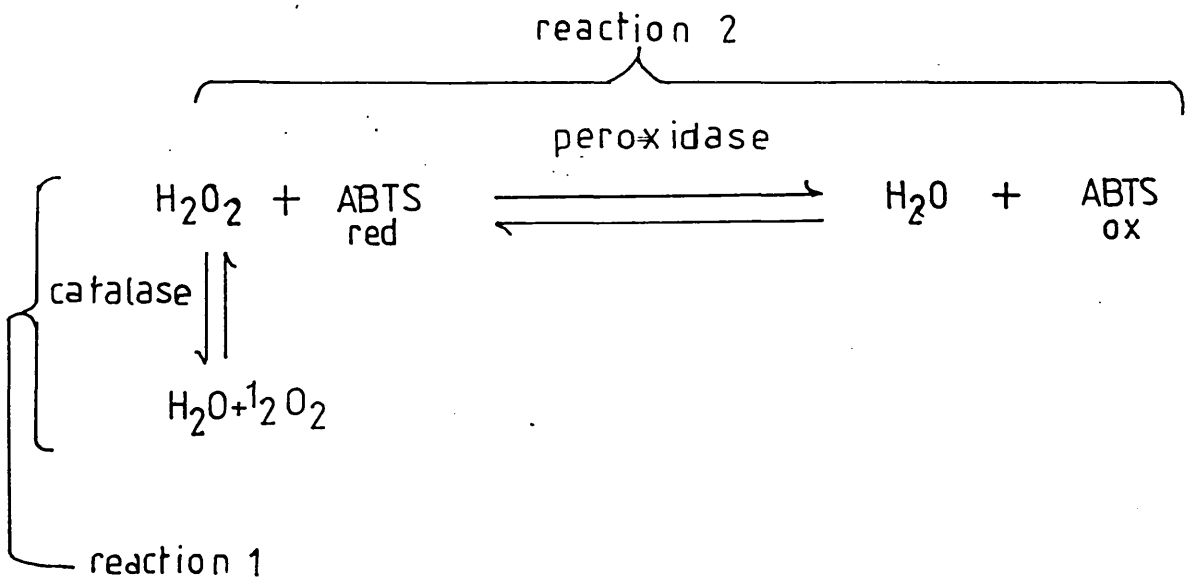
The indicator reaction (see figure 3.5)gives a measure of the peroxide concentration in each well.Oxidised ABTS absorbs light at 410 nm thus there will be a greater absorbance in the wells containing the least catalase.

3.5 SDS polyacrylamide gel electrophoresis of protein conjugates

3.5.1 Gel casting and running

Acrylamide/bis acrylamide (15%) resolving gels containing SDS(0.7cm thick) were cast in a Pharmacia gel casting tank.Stacking gels (5% acrylamide-bis acrylamide)forming six loading wells were cast above the

Figure 3.5 The indicator reaction used to assay for catalase on a microtitre plate



The reaction is carried out in two stages;

1. Incubation of conjugate with peroxide.
2. Indicator reaction.

Where catalase is present peroxide is used up and the concentration of oxidised ABTS is reduced.

resolving gel.

Proteins at 0.44 mg ml^{-1} were mixed with an equal volume of loading buffer, containing SDS, 2 mercaptoethanol and bromophenol blue.Samples of these mixtures (0.025ml) were loaded into each separate well of the gel stack , figure 3.6

shows the order of protein loading.

The gels were run, using Pharmacia gel tank and power pack,overnight at 14 milliamps constant current.

The gels were removed from their cassettes and the proteins fixed by soaking the gel in 10% glutaraldehyde (Sigma grade 2) for 30 minutes with mild agitation.

3.5.2 Gel staining

Protein bands were visualised by the silver staining method (47).

After fixing the gel was rinsed in 500 ml of distilled water and soaked on distilled water for 2 hours.

The gel was then transferred to freshly prepared ammoniacal silver nitrate solution (1.4 ml of 0.880 ammonia, 21 ml of 0.36 % sodium hydroxide and 4 ml of 19.4% silver nitrate) for no more than 15 minutes.

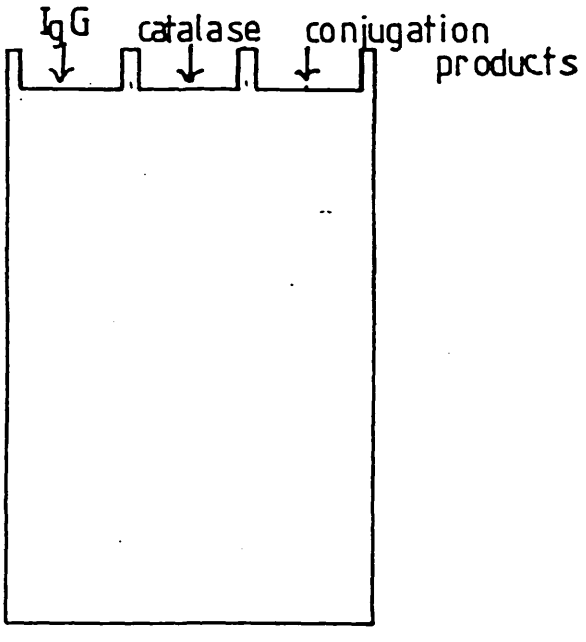
The gel was washed for two minutes in a new container of distilled water then transferred to a solution of 0.005% citric acid and 0.019 % formaldehyde.At this stage protein bands begin to appear and once they were sufficently clear the gel was removed and thoroughly washed in distilled water (at least 1 hour).

If necessary destaining can be acheived by the addition of a few crystals of ferricyanide after which the gel must be rigorously washed.

3.6 Synthesis of hydroxymethyl ferricinium

Hydroxymethyl ferrocene (215mg) was added to 5 ml of concentrated sulphuric acid and incubated for 2 hours at room temperature.The solution was made up to 40 ml with tris/HCl

Figure 3.6 The order of protein samples on the SDS polyacrylamide gel



buffer,pH 8.5, and filtered .The filtrate was adjusted to pH 8.5 by the addition of 5 M sodium hydroxide and the hydroxymethyl ferricinium concentration adjusted to 1mM (measured spectrophotometrically) by addition of buffer.

DEVELOPMENT OF AN AMPEROMETRIC ASSAY FOR PARACETAMOL IN HUMAN WHOLE BLOOD

4.1 Introduction

Paracetamol (N-acetyl para-aminophenol) is a commonly available antipyretic and analgesic drug.It is safe and effective at therapeutic doses and is therefore used in a wide variety of formulations.Unfortunately easy access to the drug has lead to an increase in its use in attempted suicides.

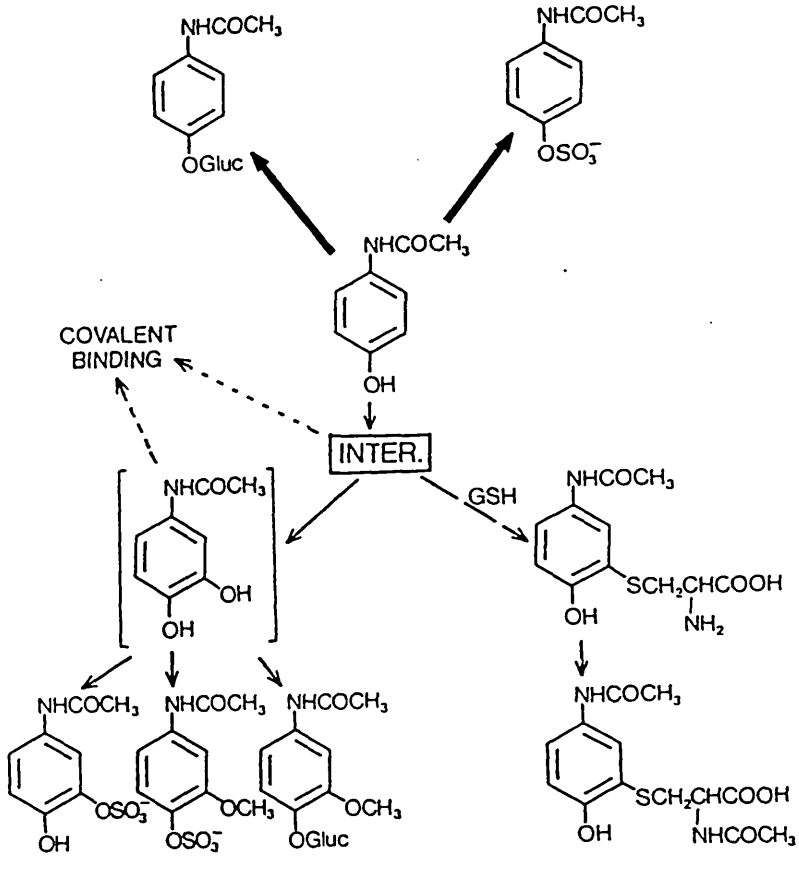
Ingestion of a paracetamol overdose gives rise to nausea and acute abdominal pain within 2 - 3 hours (48).After a lag of up to several days toxic products of paracetamol metabolism can lead to serious tissue damage and death.

Paracetamol metabolism occurs in the liver and it is thus the cells of this organ which are affected by toxic drug metabolites.Metabolism occurs by two distinct routes depending upon the concentration (49) (see figure 4.1).

In exerting its analgesic effect paracetamol is not used up and is transported to the liver before excretion.In low therapeutic doses paracetamol is conjugated to either glucose or sulphate and the conjugates excreted.Higher concentrations are oxidised *via* a cytochrome P450 dependent pathway to a reactive intermediate (as yet not fully characterised).The intermediate reacts with glutathione and the resulting cysteine and mercapturate conjugates are excreted.

The rate of depletion of hepatic glutathione is related to the amount of paracetamol ingested.When overdose occurs hepatic glutathione is completely used up and the

Figure 4.1 The metabolism of paracetamol in the liver
Taken from reference (49)



intermediate reacts instead with cellular proteins binding irreversibly and causing cell death (50).

Initial treatment of overdose is by gastric lavage or emesis to remove the drug from the stomach(48).Once paracetamol has reached the blood an antidote must be administered if the toxic effects are to be alleviated.Treatment with cysteamine inhibits the oxidation route (51) but has unpleasant side effects and can be dangerous if administered too long after overdose (52).The favoured antidote at present is N-acetyl cysteine which is a glutathione precursor and replenishes the hepatic pool enabling the safe excretion of the excess reactive intermediate.

If an antidote is not administered until cell death (the only overt symptom of paracetamol poisoning) is evident, the damage cannot be reversed.

The onset of liver damage depends upon both the dose ingested and the rate at which it is metabolised by the patient.Diagnosis of poisoning is by measurement of the half life of the drug in plasma which takes both of these factors into account (53) .A rapid and accurate estimation of blood paracetamol concentration is therefore required at two separate times after ingestion of the drug.

Currently, sensitive methods for measuring paracetamol (such as high pressure liquid chromatogrphy and gas liquid chromatography (54a,54b,54c))are lengthy and technically demanding.A rapid estimation procedure involving enzyme

catalysed hydrolysis of the drug followed by the colourimetric determination of the product ,para aminophenol, is however available in the form of a test kit.Although the method is relatively simple, skilled operations and expensive laboratory equipment are still required.Also, being a colour determination assay, it cannot be performed on whole blood and so red cells must be removed before analysis.

The experiments described in this chapter demonstrate the use of amperometry coupled to enzyme catalysed hydrolysis for the rapid estimation of paracetamol in human whole blood.

Previous attempts to estimate paracetamol amperometrically have been reported (55a,55b).In one case differential pulse voltammetry at a graphite electrode was used to estimate pararcetamol in plasma .The assay is rapid and sensitive but requires complex non-standard labaoratory equipment and lengthy calculation to extract the paracetamol concentration from the experimental results.

A second electrochemical assay involves the oxidation of paracetamol at a solid electrode using linear sweep voltammetry in buffered solution.This method has proved useful for the estimation of the stability of various dosage forms of the drug in aqueous solution .Oxidation of paracetamol at a solid electrode yields N- acetyl- para quinoneimine (56) and electrooxidation has been shown to be irreversible (see figure 4.4).Interestingly the intermediate formed electrochemically may also be the reactive intermediate formed by oxidation in the liver (57).Direct oxidation can be used to measure paracetamol in aqueous

solution but the extreme potential required causes high background currents in complex biological media, this will be discussed in more detail later in this chapter.

The paracetamol assay demonstrated in this chapter combines an amperometric assay for para aminophenol with enzyme catalysed paracetamol hydrolysis.Para aminophenol is oxidised at a potential 300 mV cathodic of paracetamol at the carbon electrodes used in the assay.

In any amperometric experiment the application of extreme potentials is undesirable because more non- specific interference is likely as is solvent decomposition.

Analysis at a lower potential should thus reduce problems of background current and electrode fouling in whole blood analyses.Incorporation of an enzyme catalysed reaction also gives the assay a specificity which cannot be obtained with an electrode reaction alone.

The overall reaction scheme used for paracetamol assay is that shown in figure 4.2.The enzyme catalysing the hydrolysis reaction is the same as that used in the currently available test kit (66).

4.2 Results and discussion

4.2.1 The electrochemistry of paracetamol and para aminophenol

The electrochemistry of aromatic amines has been extensively studied (57).This class of molecules shows specific electrode reactions which can be used in their analysis.

Figure 4.2 Scheme used in the amperometric paracetamol assay

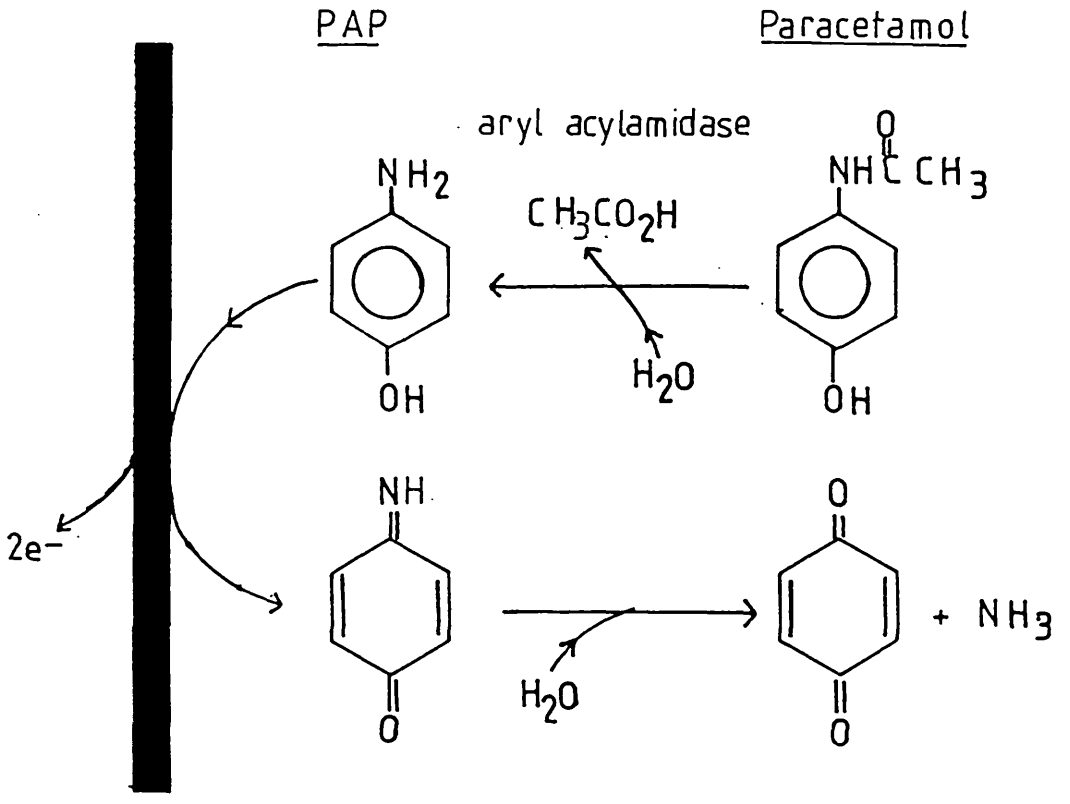


Figure 4.3 shows a rotating disc voltammogram of paracetamol on glassy carbon and gives an $E_{1/2}$ of +440 mV vs SCE, in agreement with previously determined values (56).A detailed study of the electrochemical reaction by Miner et al(56) has led them to propose the mechanism shown in figure 4.4. Cyclic voltammograms, see figure 4.5, show irreversible electrochemistry and are consistent with their findings.

Table 4.1 and figure 4.6 show currents obtained from the oxidation of paracetamol above the potential of limiting current (+700mV vs SCE).This direct assay for paracetamol shows low sensitivity and accuracy .The calibration was also poorly reproducible unless oxidation products, which coat the electrode surface , are rigorously removed by alumina polishing between each measurement.

Figure 4.7 shows a CV of human whole blood and reveals a large background current at the potential of paracetamol oxidation.If a direct paracetamol assay were carried out in whole blood this background would decrease the accuracy and sensitivity of the assay yet further.Blood composition also varies between patients thus standardisation of results would be impossible.

4.3.3 Para-aminophenol oxidation

Figure 4.8 shows a voltammogram of para-aminophenol(PAP) at glassy carbon giving an $E_{1/2}$ of 240 mV vs SCE and a plateau potential of +400 mV.Negligable background currents are seen in whole blood at this potential (see figure 4.7).Figure 4.9 shows a cyclic voltammogram of PAP which is again consistent with an irreversible oxidation mechanism as

Figure 4.3 Rotating disc voltammogram of paracetamol at a glassy carbon electrode

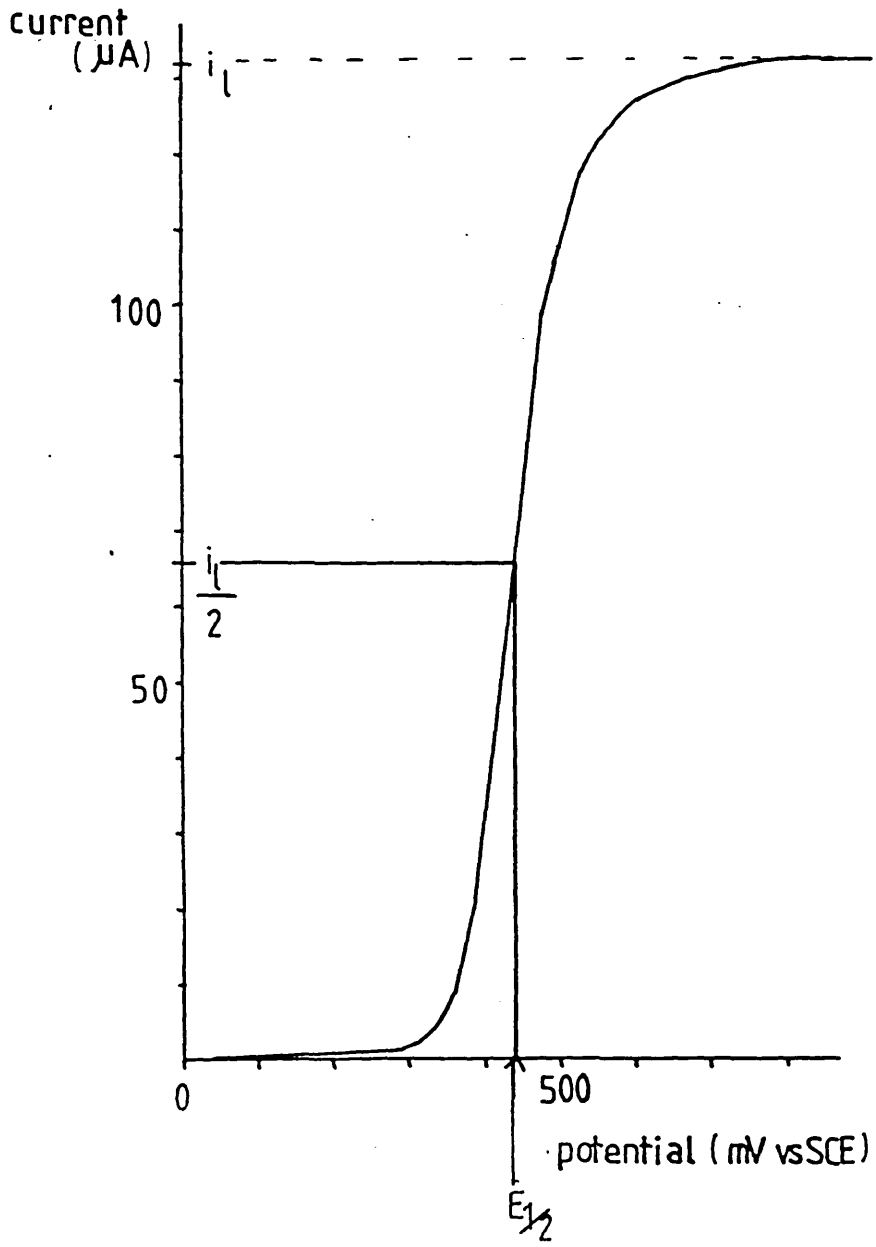


Figure 4.4 The electrooxidation of paracetamol

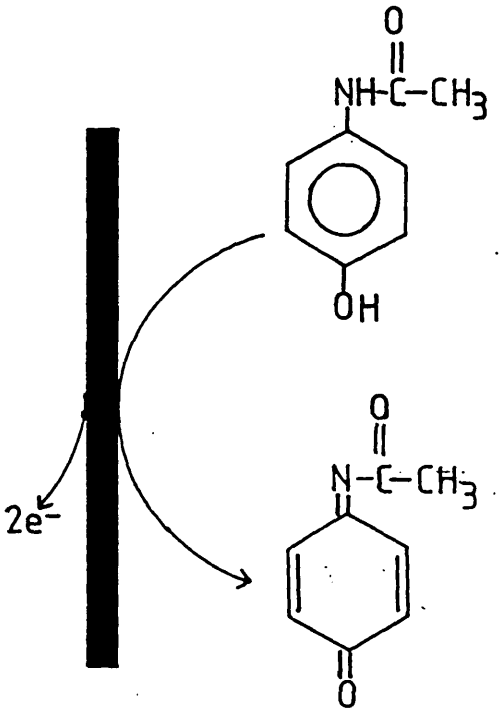


Figure 4.5 Cyclic voltammogram of paracetamol

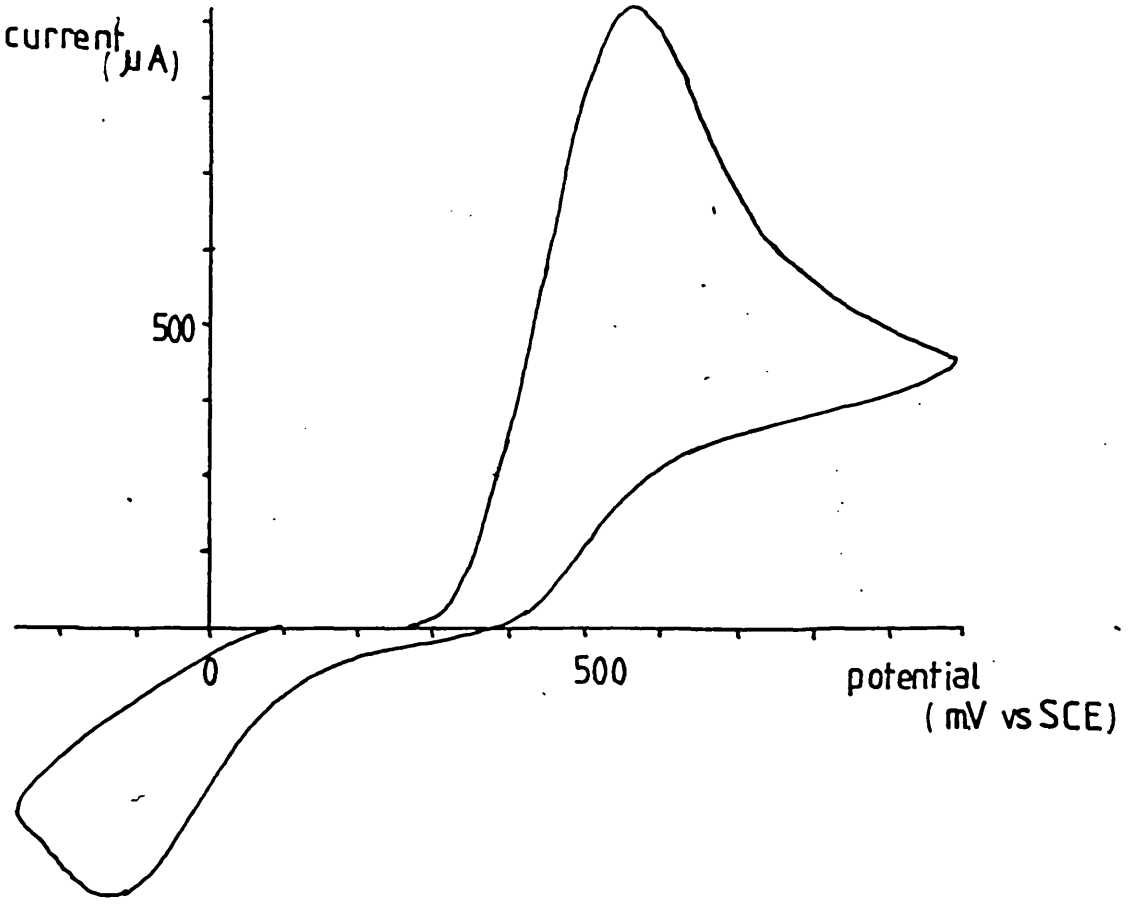


Table 4.1 Paracetamol calibration at +700mV vs SCE

Paracetamol concentration (/M x10^5)	Current μA
6.6	1.5
12.9	2
19.2	3
25.4	6
31.7	8
37.4	8
43.2	9.5
48.9	11
54.5	14
59.5	14
65.4	15.5

Figure 4.6 Amperometric paracetamol calibration at +700 mV vs SCE

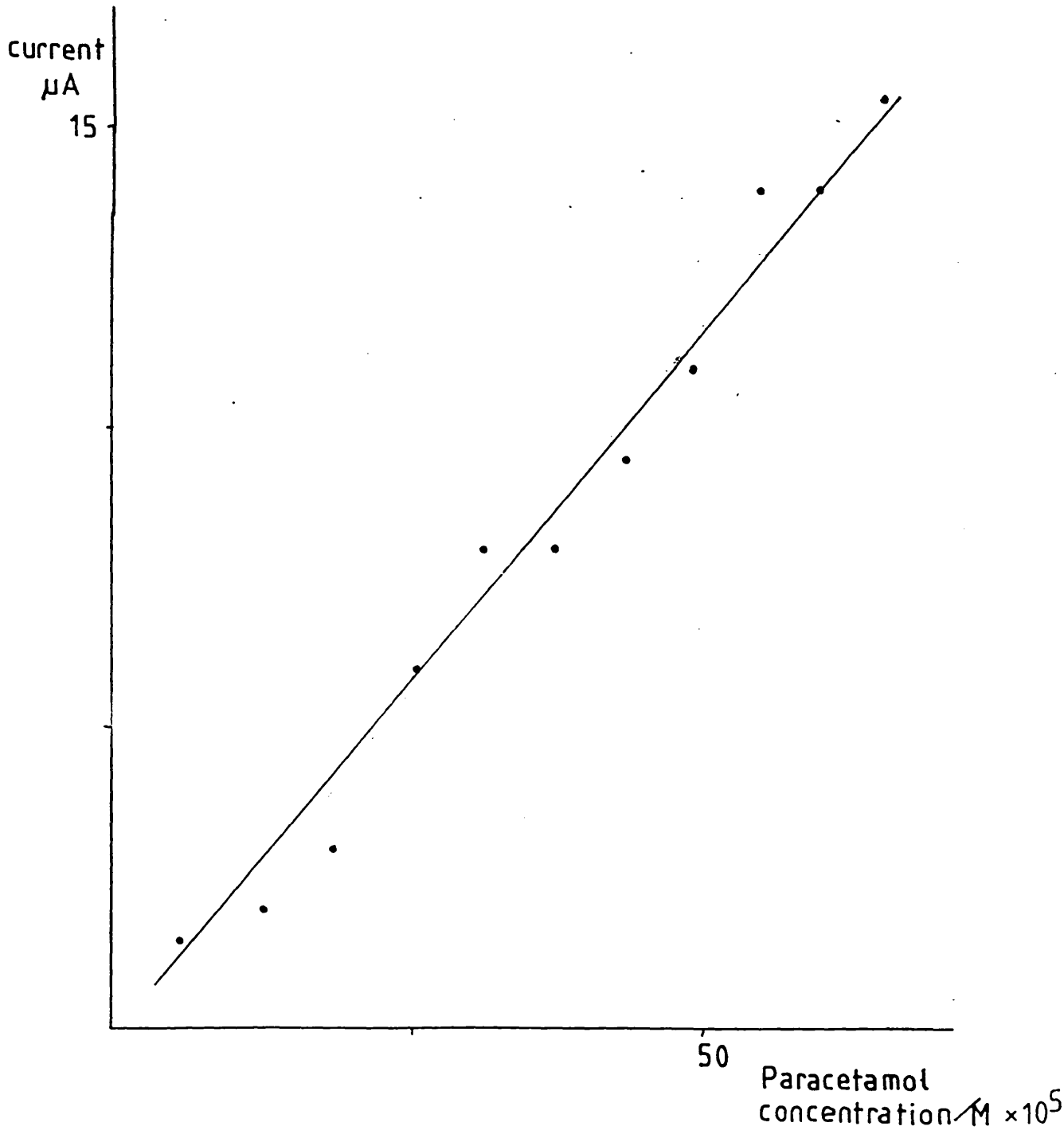


Figure 4.7 Cyclic voltammogram of human whole blood

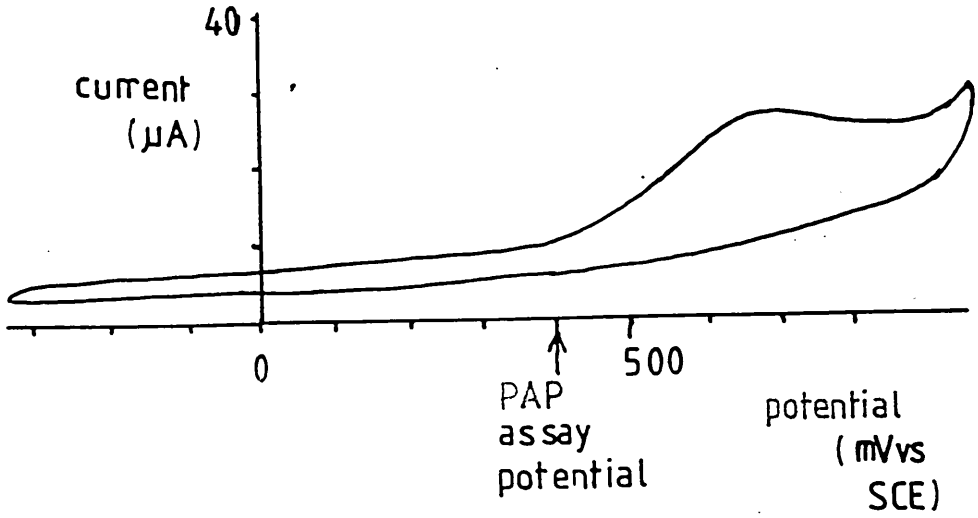


Figure 4.8 Rotating disc voltammogram of PAP

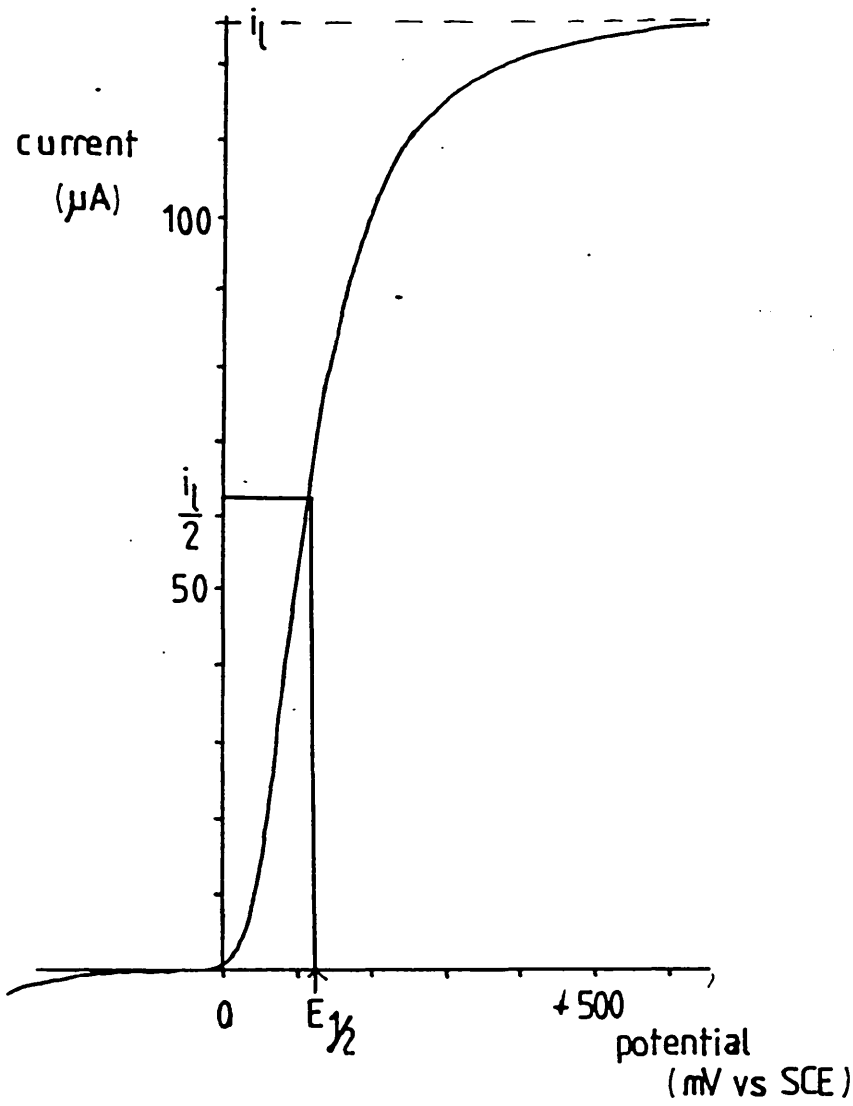
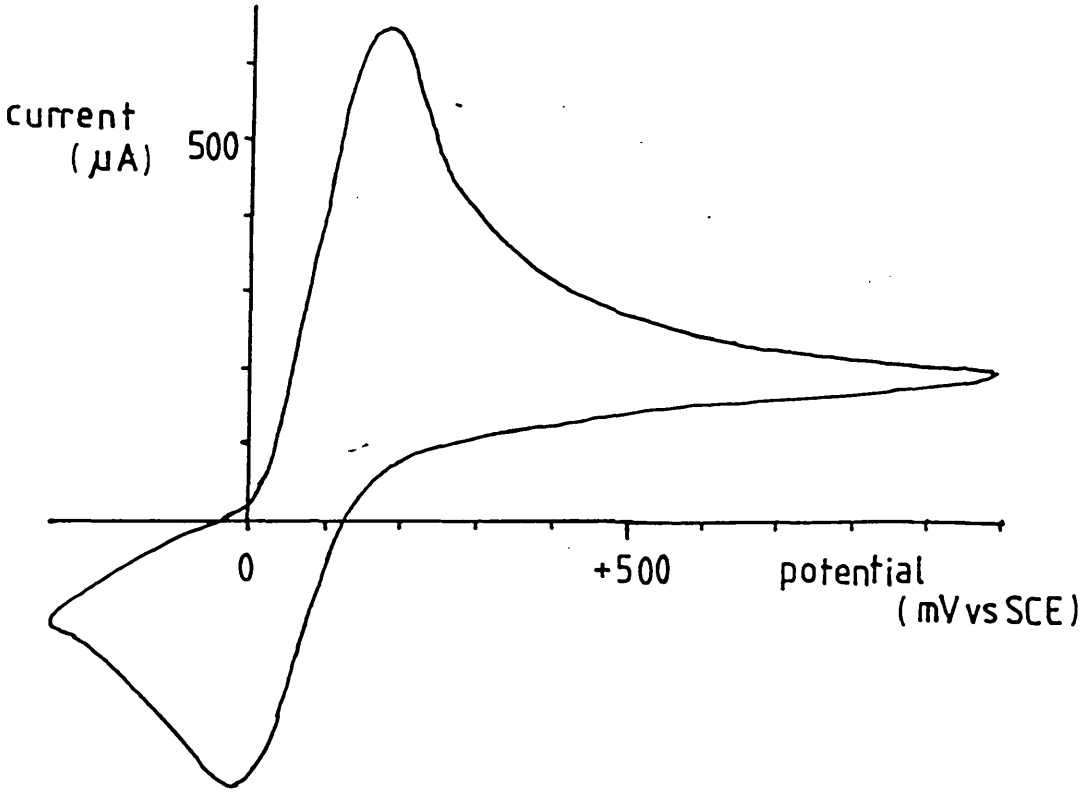


Figure 4.9 Cyclic voltammogram of PAP



shown in figure 4.10.

A steady state calibration for PAP at + 400 mV vs SCE in aqueous solution (see table 4.2 and figure 4.11) shows good accuracy and linearity in aqueous buffer.

PAP is the product of paracetamol hydrolysis and is formed slowly in aqueous solutions of the drug.Hydrolysis can be speeded up by a bacterial enzyme, aryl acylamidase which has been isolated from Rhodococcus and Pseudomonas species.

Enzyme catalysed hydrolysis (shown in figure 4.12) occurs optimally at 20 ^{o}C and pH 7 and no cofactors are required.These are close to the conditions in which the paracetamol assay will be performed ,that is room temperature and pH 7.2 which is that of whole blood.

4.2.4 Paracetamol assay

Figure 4.13 shows voltammograms of paracetamol in the absence and presence of aryl acylamidase .These show that the addition of 15 units of enzyme catalses the complete conversion of the paracetamol (at a concentration of 3.3 x10^{-4}M in the test solution) to PAP in 30 seconds.

A calibration curve for paracetamol in the presence of 15 units of aryl acylamidase at a potential of +400 mV vs SCE is shown in table 4.3 and figure 4.14.Comparison with figure 4.11 shows that higher currents are obtained when measuring paracetamol than for the same concentration of PAP.This is due to rapid air oxidation of the PAP which reduces the concentration in solution.Where paracetamol is estimated PAP is freshly produced in solution during enzyme catalysed

Table 4.2 PAP calibration at +400 mV vs SCE

PAP concentration ($/Mx10^5$)	Current μA
9.2	9
19.4	5.5
29	9.5
35.4	11.5
44.2	13.5
52.1	14.5
60.2	18
68.2	20
76	24.5
82.9	25.5
91.2	26
98.5	28
105.8	29.5

Figure 4.12 Aryl acylamidase catalysed reaction

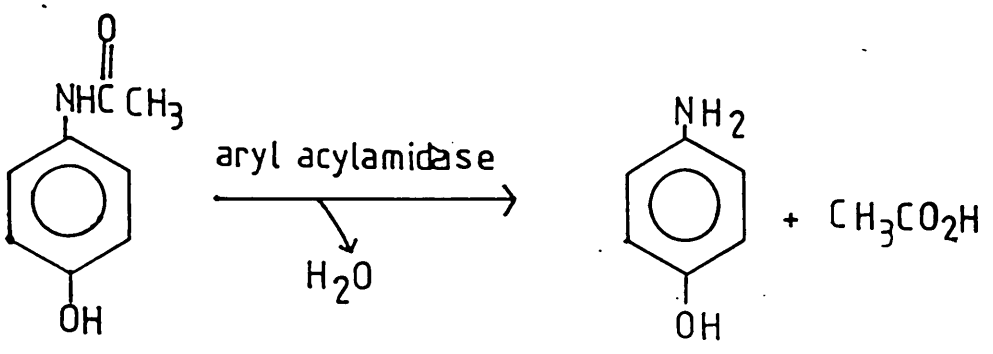


Figure 4.13 Stationary electrode polarograms

a. of 3.3×10^{-4}M paracetamol

b. as a, plus 15 Units of aryl acylamidase

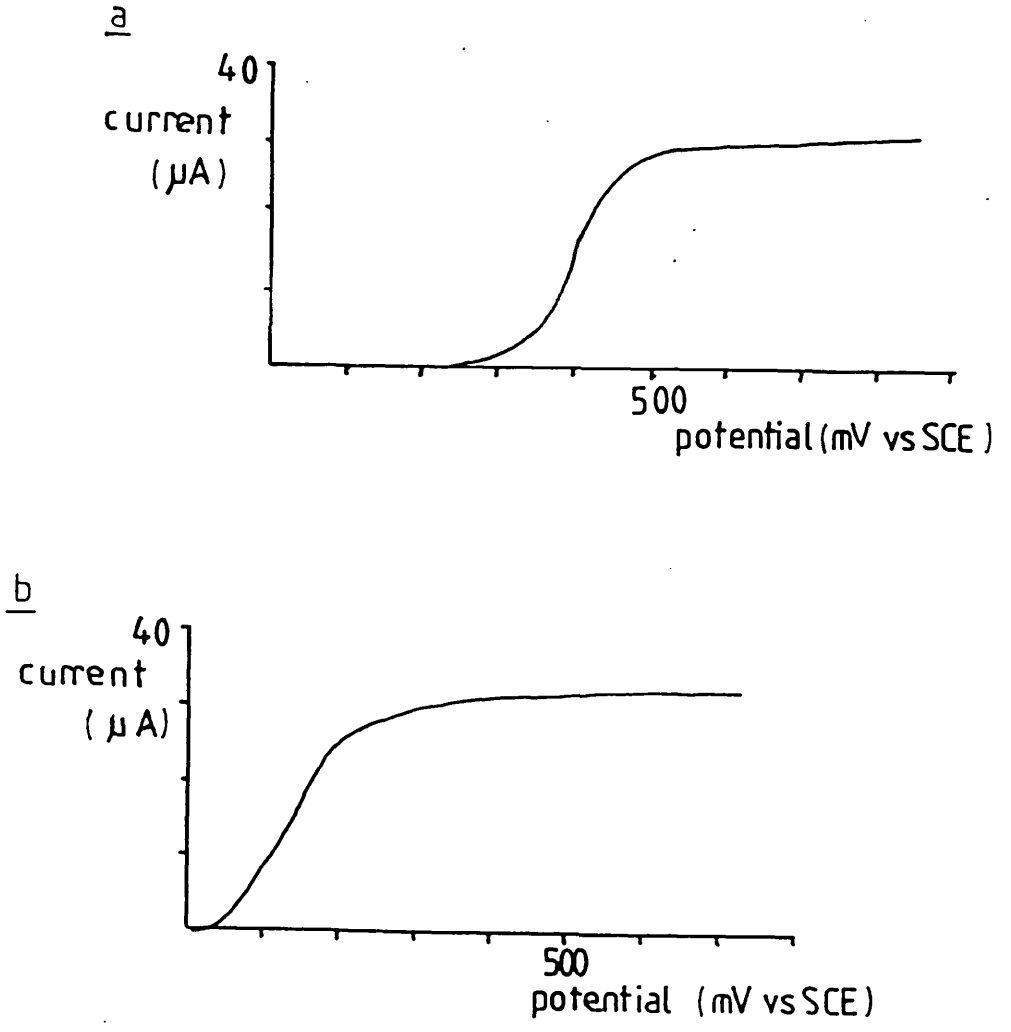
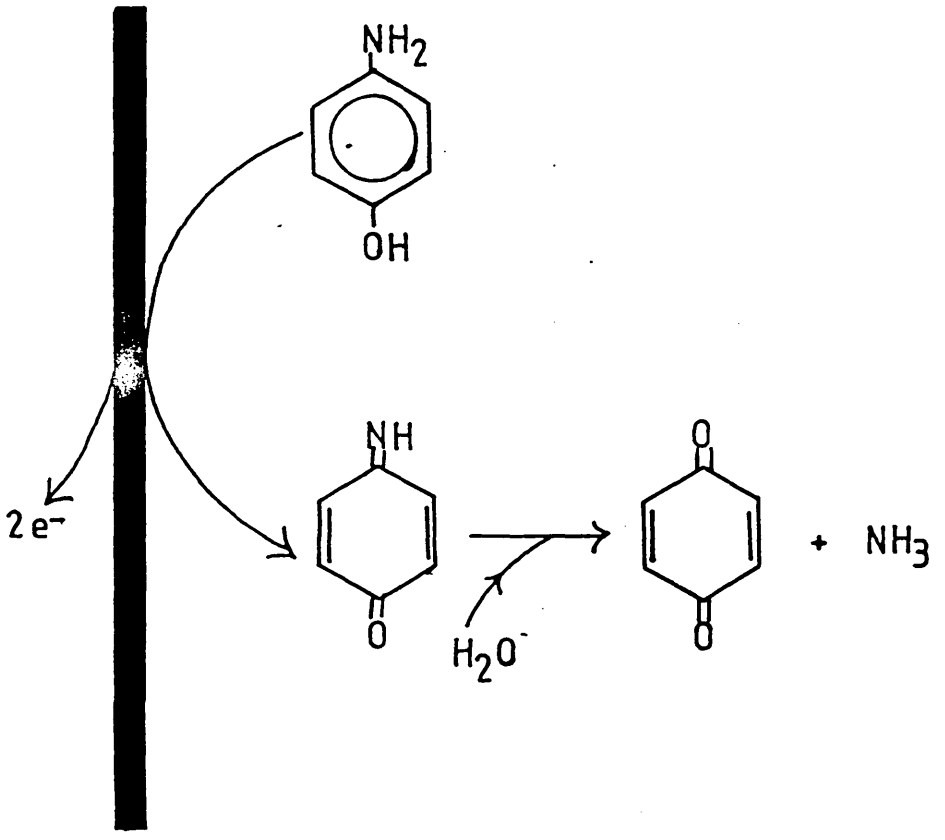


Table 4.3 Paracetamol calibration at +400 mV vs SCE in the presence of 15 units of aryl acylamidase

Paracetamol concentration ($/Mx10^{4}$)	Current (μA)
3.6	10
7.2	20
10.6	27
[illegible]	[illegible]
17.4	41
20.7	47
22.7	52
26.9	54
30	61
33	64

Figure 4.10 Electrooxidation of PAP



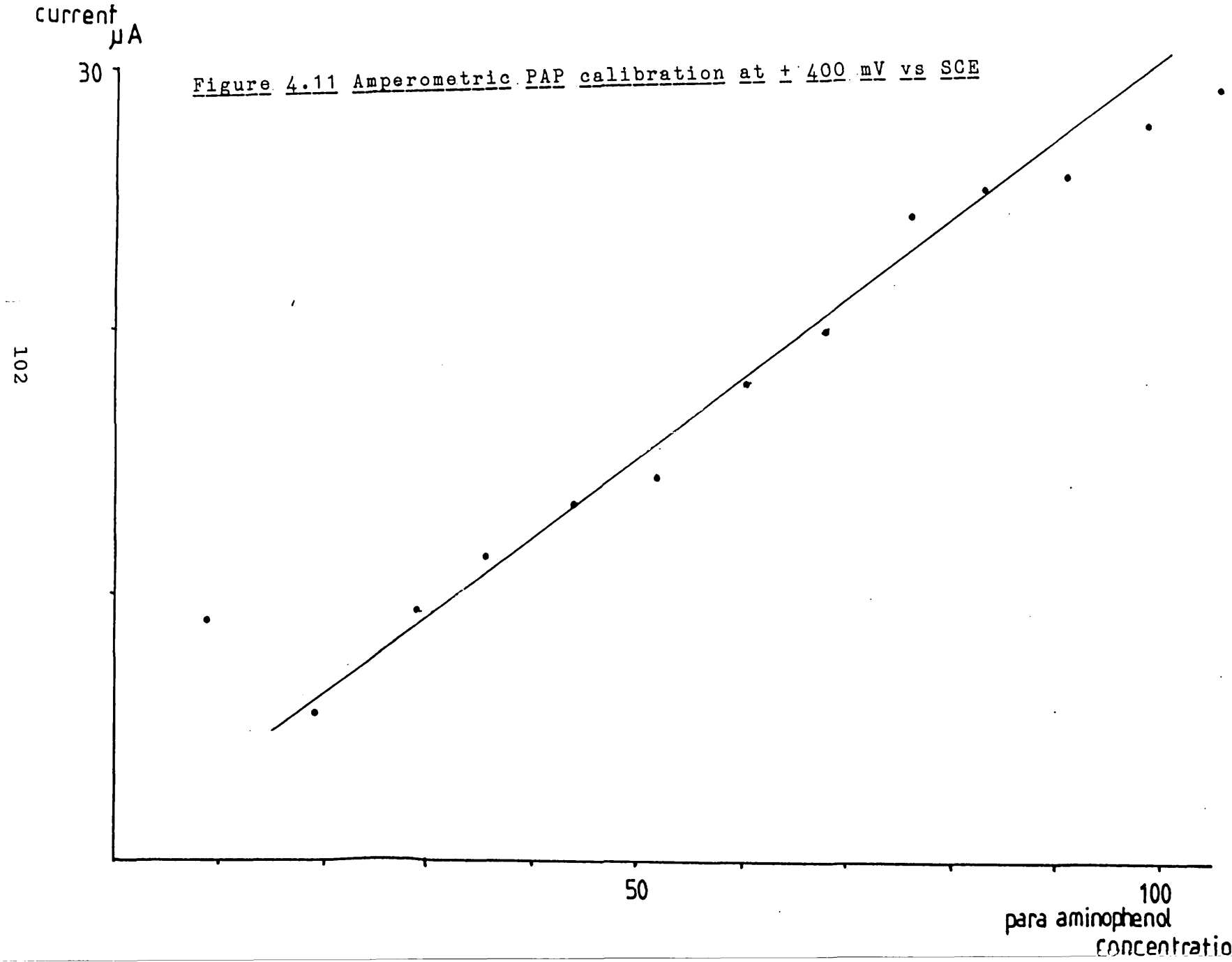
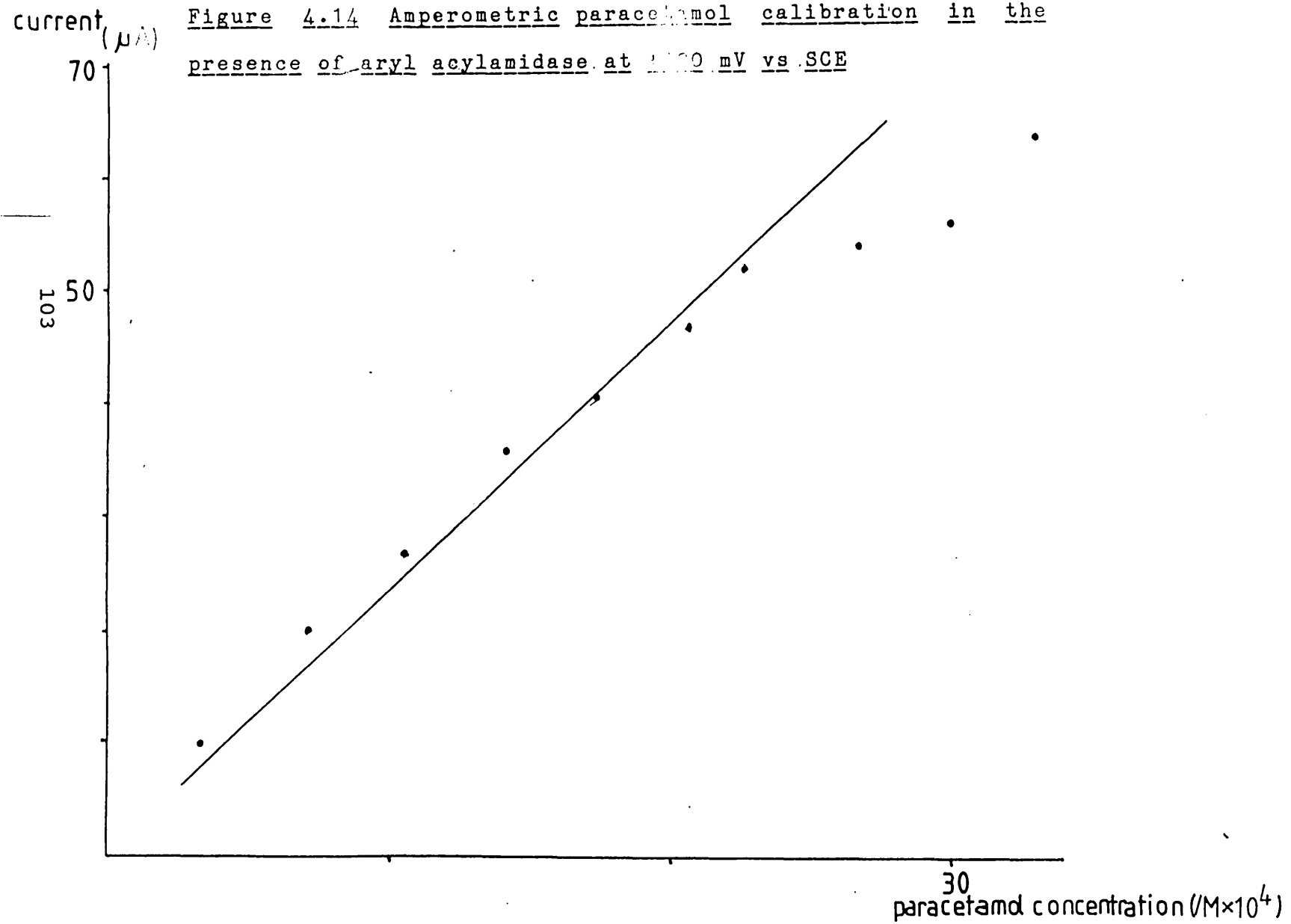


Figure 4.11 Amperometric PAP calibration at + 400 mV vs SCE

Figure 4.14 Amperometric paracetamol calibration in the presence of aryl acylamidase at [illegible] mV vs SCE



hydrolysis.

4.2.5 Estimation of paracetamol in whole blood

Calibration curves for paracetamol and PAP in whole blood are shown in table 4.4 and figure 4.15.

The currents obtained here are lower than in buffer. Due to the greater viscocity of the medium, the steady state current obtained is dependent on diffusion of PAP to the electrode surface (see section 2.4.1) and this is slower in the more viscous solutions.Comparing figures 4.15c and 4.15d a divergence of the two curves occurs as the paracetamol concentration increases which cannot be explained solely by a difference in viscocity, the reason for this divergence is discussed below.

4.2.5.1 Explanation of the divergence between whole blood and buffer calibrations

Human whole blood is a complex mixture of molecules a large proportion of which are proteins.One of the most abundant blood proteins is albumin (59) which plays an important role in the transport of fatty acids during lipid biosynthesis (60).Serum albumin can also bind other hydrophobic molecules and would be expected to bind paracetamol.Such binding will shield the drug from enzyme catalysed hydrolysis in the assay described.The binding of molecules to serum albumin is a passive process controlled by mass action.As the concentration of paracetamol increases the proportion bound to serum albumin and protected from hydrolysis will increase, thus the proportion converted into

Table 4.4 Calibration of PAP and paracetamol in buffer and whole blood at +400 mV vs SCE

PAP conc. (/M $\times 10^4$)	Current in buffer (μA)	Current in blood (μA)	Paracetamol conc. (/M$\times 10^4$)	Current in buffer (μA)	Current in blood (μA)
5.1	3	3	3.6	10	12
10	8	6	7.2	20	20
14.8	11	10	10.6	27	26
19.6	15	12	14.1	36	31
24.2	18	14	17.4	41	37
28.8	20	17	20.7	47	43
33.2	22	19	22.7	52	46
37.6	24	23	26.9	54	52
41.2	27	25	30	61	57
46	27	26	33	64	62

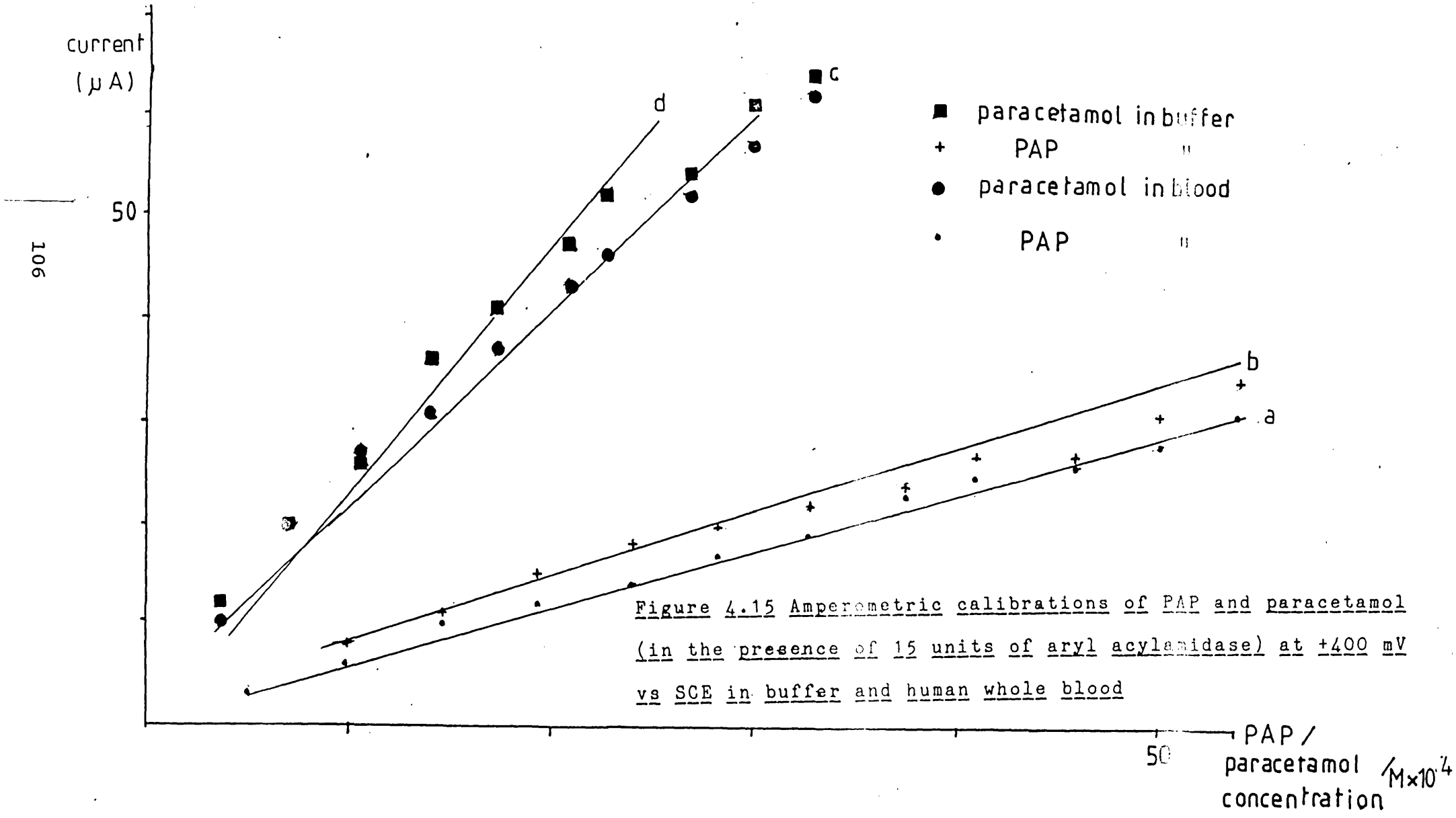


Figure 4.15 Amperometric calibrations of PAP and paracetamol (in the presence of 15 units of aryl acylamidase) at +400 mV vs SCE in buffer and human whole blood

PAP decreases as will the current obtained.

The same effect can be demonstrated in a solution containing serum albumin alone, as shown in table 4.5 and figure 4.16.The divergence is much larger than that in whole blood but the serum albumin concentration is at the high end of the range found in blood (61) and is thus likely to be higher than that in the blood sample used.PAP is more hydrophilic than paracetamol and would not be expected to bind as strongly to serum albumin hence no divergence of the calibration curves is seen.

Where an overdose of paracetamol is taken binding of the drug to serum albumin will prevent its metabolism in the liver and the amperometric estimation, of free rather than bound paracetamol, gives an estimation of the amount which is able to initiate liver damage.

4.2.6 Background currents in whole blood

Under the assay conditions used whole blood gives a very low background current (see figure 4.17a).For comparison figure 4.17b shows the background current from whole blood in the same experiment but at a potential of +700 mV vs SCE.The current here is almost half that measured at low paracetamol concentrations and will vary between samples as the composition of the patients blood varies.The presence of this large and variable signal confirms the necessity for a low measuring potential in the paracetamol assay (see section 4.1).

Table 4.5 Paracetamol calibration at +400 mV vs SCE in buffer and 42mg/ml bovine serum albumen

Paracetamol concentration	Current in buffer	Current in 42mg/ml BSA
(/Mx10^4)	μA	μA
32.7	8	9
62.7	27	18
96.4	49	26
127	69	32
157	86	37
187	103	46
216	123	52
244	129	58

Figure 4.16 Amperometric paracetamol calibration in buffer and 42mg/ml BSA (at +400 mV vs SCE in the presence of 15 units of aryl acylamidase

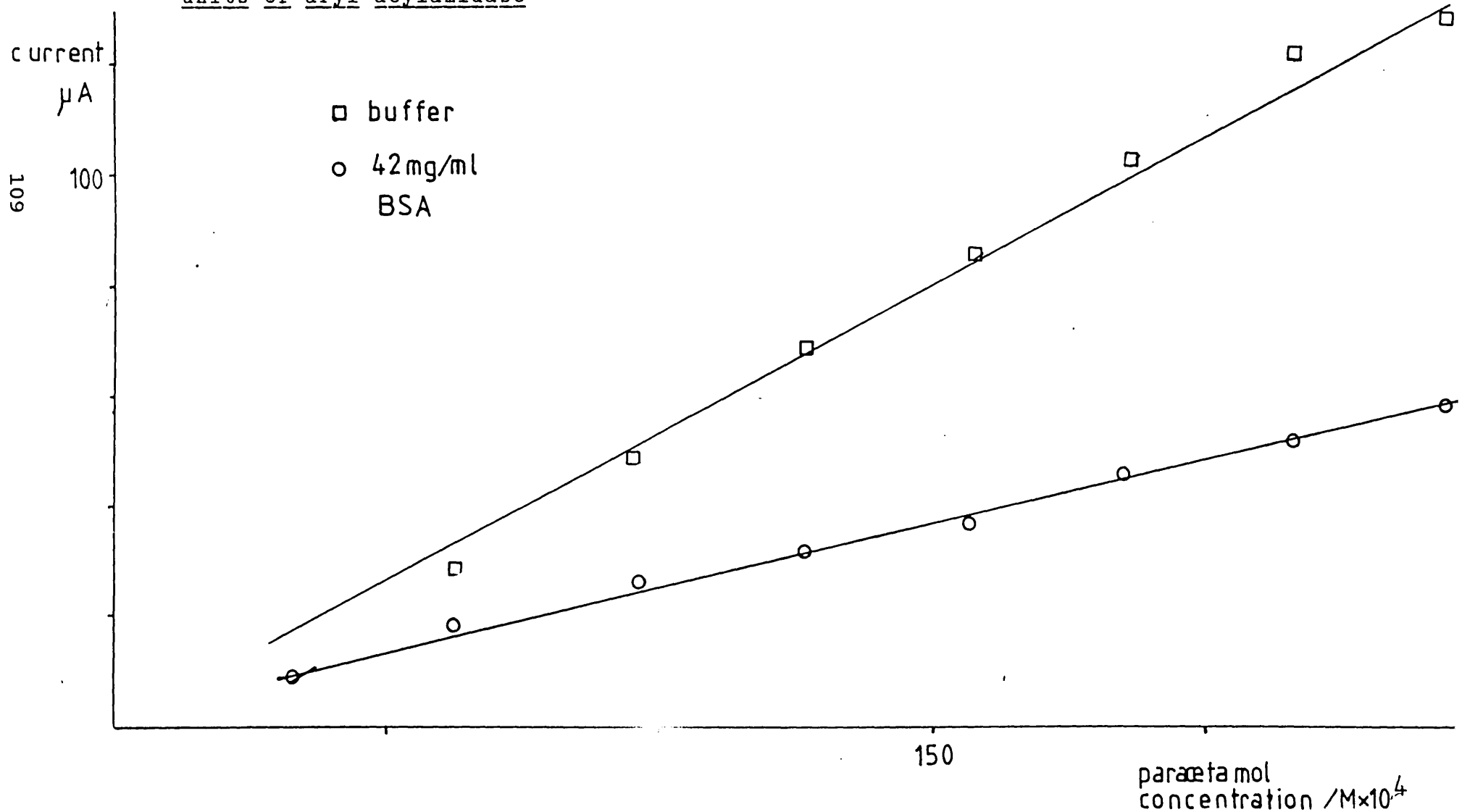


Figure 4.17a Steady state current for buffer and paracetamol in human whole blood in the presence of 15 units of aryl acylamidase

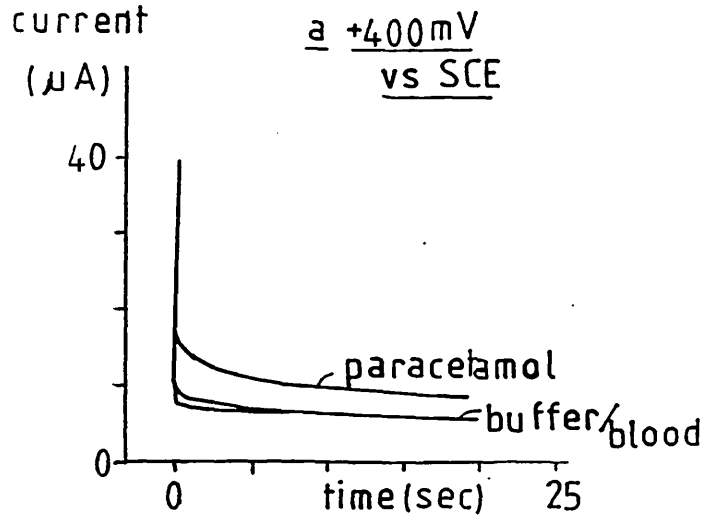
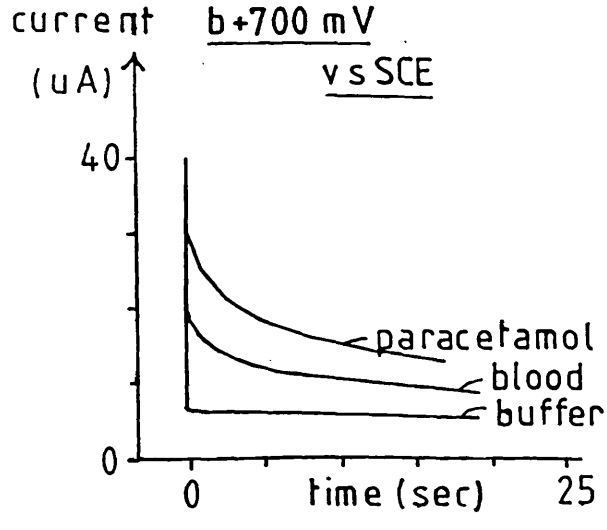


Figure 4.17b Steady state currents for buffer and paracetamol in human whole blood at + 700 mV vs SCE



4.2.7 The clinical estimation of paracetamol levels

4.2.7.1 Clinically important concentrations

The severity of a paracetamol overdose is estimated by both the concentration of the drug (in plasma or whole blood) and the rate at which it is metabolised.Previously determined concentrations at given times after drug ingestion have been seen in most cases to have the outcomes detailed in table 4.6.The assay must therefore be capable of accurate estimations between 50 ug ml^{-1} and 300 ug ml^{-1}.Linear paracetamol calibrations have been obtained up to 500 ug ml^{-1} using the assay described here.

The calibration range available electrochemically is higher than that in the currently used colourimetric assay (63) which requires dilution of the blood samples in more severe overdose cases (above 200 ug/ml) to bring the concentration into the linear calibration range.

4.2.7.2 Recovery experiments

To assess the efficacy of the assay in a clinical situation blood samples were spiked with paracetamol and the measured concentration interpolated from a paracetamol calibration performed in either buffer or whole blood was compared with the actual concentration.The calibration curves used are shown in figure 4.18 and the results of the recovery experiments are shown in table 4.7 and figure 4.19.

The concentrations measured relative to a calibration in buffer are lower because only free paracetamol is measured,

Table 4.6 The clinical consequences of different plasma paracetamol concentration at variuos times after injestion

Time after injestion (hours)	Plasma paracetamol concentration (µg/ml)	Clinical effect
4	> 300	Probable hepatic necrosis
4	120-300	Possible necrosis
4	< 120	Necrosis unlikely
12	> 120	Probable necrosis
12	50-120	Possible necrosis
12	< 50	Necrosis unlikely

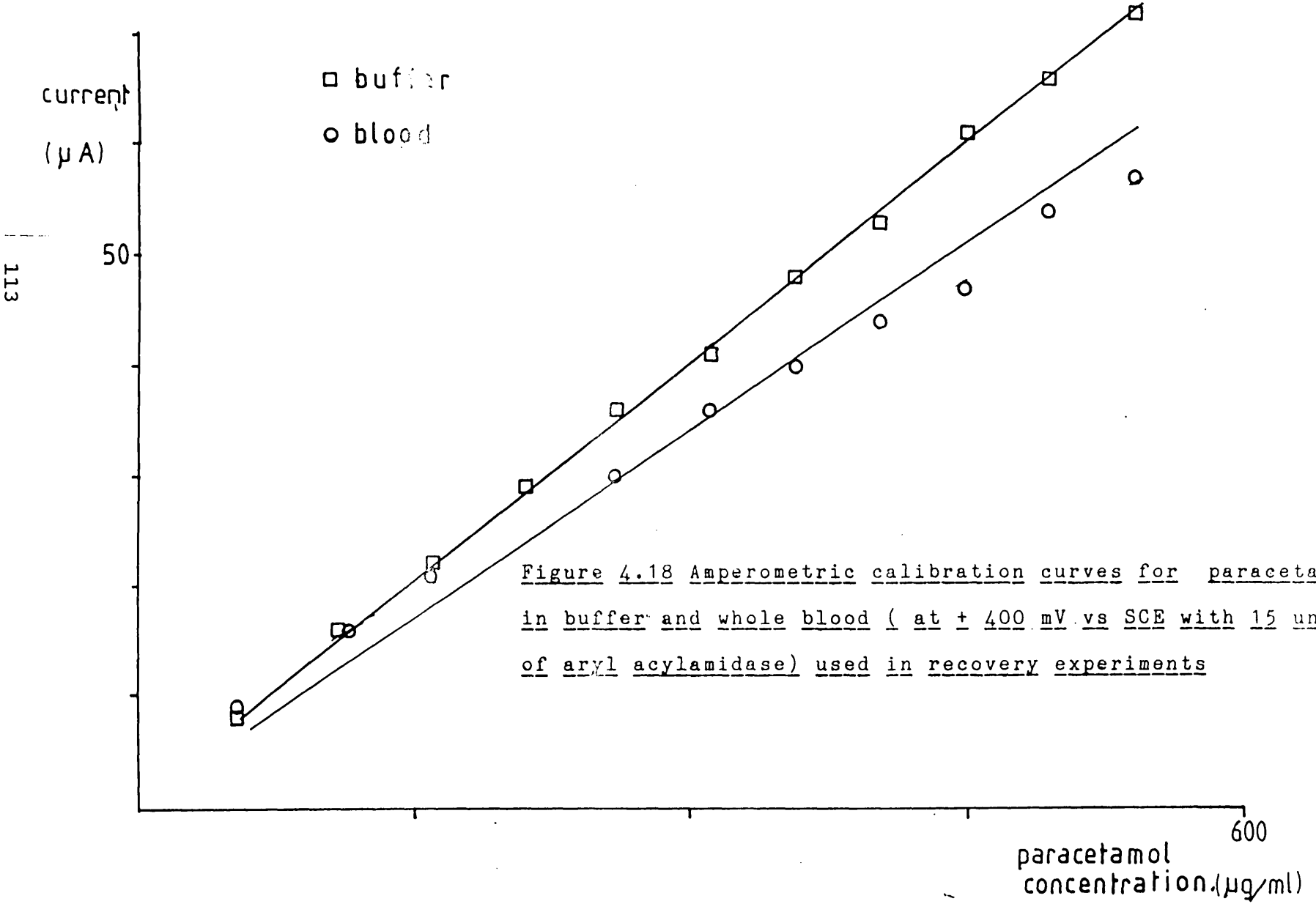
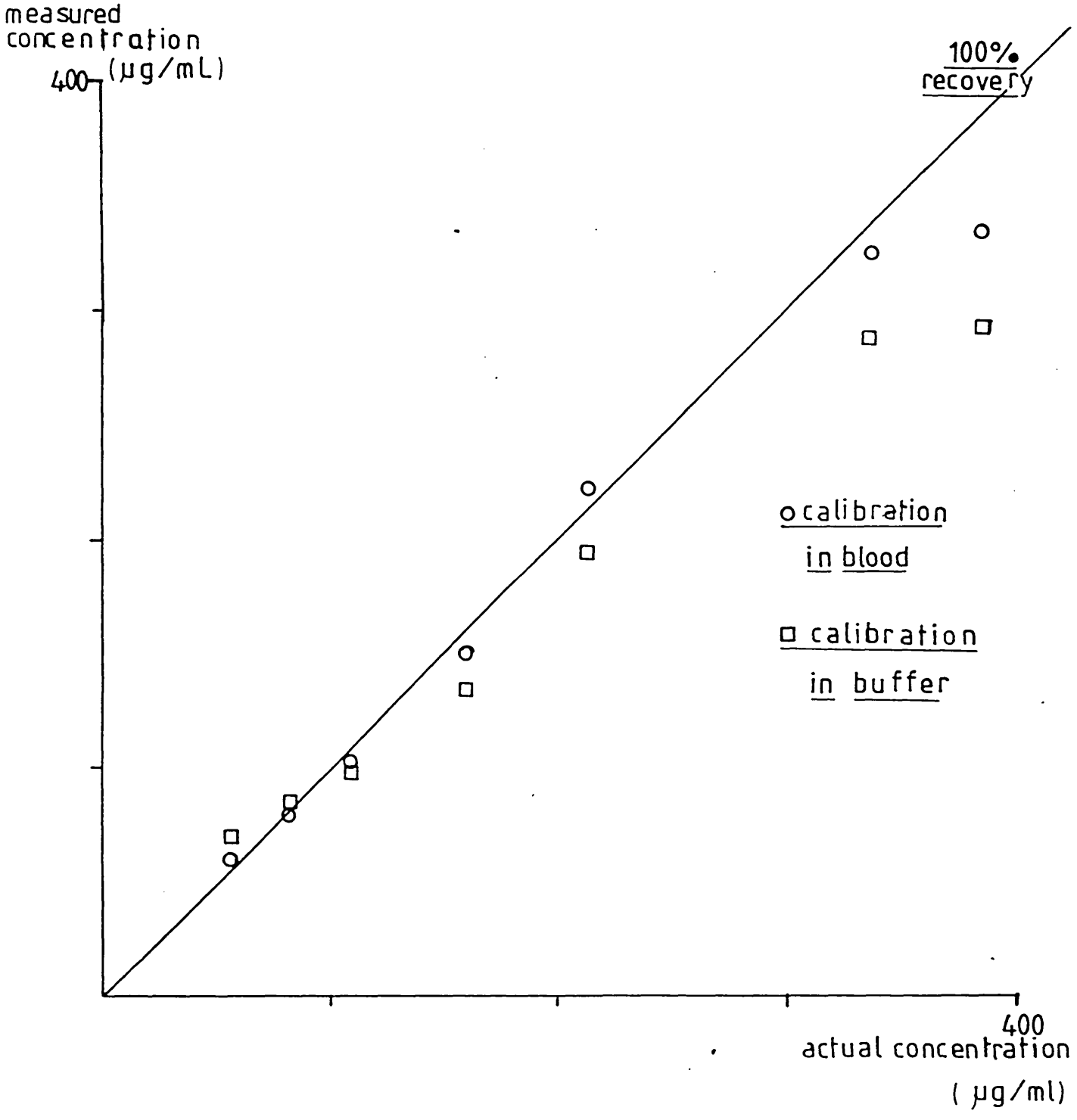


Figure 4.18 Amperometric calibration curves for paracetamol in buffer and whole blood (at + 400 mV vs SCE with 15 units of aryl acylamidase) used in recovery experiments

Table 4.7 Actual and measured concentrations of paracetamol in recovery experiments

Actual concentration (µg/ml)	Concentration from blood calibration (µg/ml)	Concentration from buffer calibration (µg/ml)
55	60	70
82	80	85
109	103	98
161	150	135
213	223	195
337	325	288
385	335	292

Figure 4.19 Paracetamol recovery experiments



as described above.Where paracetamol was calibrated against standards estimated in whole blood the points are all close to an ideal graph which has a slope of 45 ^{o}.

The recovery experiments show that the assay has a sufficiently high reproducibility to be used for clinical estimations. That is, the enzyme catalysed and electrode reactions give consistent results when calibrating a series of concentrations as well as when measuring concentrations at random. The method can be used with either a buffer or blood calibration to yield total or unbound paracetamol concentrations respectively.Since blood serum albumin concentration will vary between patients greater accuracy will be obtained in an assay using a buffer calibration and where only the potentially harmful drug will be estimated (see section 4.2.5.1).

4.3 Concluding remarks

The amperometric method for the estimation of paracetamol has been shown to provide a rapid and accurate estimate of drug concentrations within and above the range of clinically important concentrations.It is vital that all the paracetamol is hydrolysed before the electrode reaction occurs and thus excess aryl acylamidase must be present.

Development and production of a workable biosensor device from the system demonstrated requires that the enzyme component be immobilised at the electrode surface giving an integrated device.Several methods of enzyme immobilisation are available and that chosen depends upon the properties of the particular enzyme used (64).Preliminary experiments,

using cellulose acetate membranes to trap the aryl acylamidase at the electrode, yielded a reasonably stable enzyme membrane .Leaching of the enzyme from the membrane along with difficulties in controlling the thickness of the cellulose acetate layer made this method unsuitable for use in a biosensor and further studies are required before an integrated device can be produced.

The paracetamol assay described employs the simplest form of amperometric analysis using an enzyme catalysed reaction.This type of analysis is limited to those substrates which can be converted ,by an enzyme catalysed reaction, to products which show distinct electrochemistry.The following chapter describes the investigation of enzyme activity using amperometry which has broader applications in various assay systems.The requirement for the direct coupling of an enzyme catalysed reaction to an electrode reaction is that electron transfer is possible between the active site of the enzyme and the electrode.This is achieved either by modification of the electrode or by the use of low molecular weight mediators of electron transfer.The type of analysis to be demonstrated is applicable to a wide range of enzyme catalysed reactions and thus provides a general route for the development of a variety of sensors.

AMPEROMETRIC STUDIES ON DIAPHORASE USING COUPLED ELECTROCHEMISTRY

5.1 INTRODUCTION

The class of enzymes known as 'diaphorases' are redox enzymes which catalyse the oxidation of reduced nicotinamide adenine dinucleotide (NADH) and its phosphorylated derivative (NADPH) (see figure 5.1) using a variety of dye molecules as electron acceptor (see figure 5.1).

Diaphorase activity was first isolated from pig heart tissue by Straub in 1939 (65) at which time the enzyme's physiological role was unknown.Subsequently the same activity has been isolated from many sources including various mammalian tissues, higher plants and bacteria.In the case of the diaphorases isolated from mammalian tissues and aerobic bacteria their physiological role is now known to be that of a lipoamide dehydrogenase.The enzyme exists as part of the pyruvate dehydrogenase and keto- acid dehydrogenase complexes where it catalyses the oxidation of lipoic acid (6,8 thiooctic acid) (66).These multi enzyme complexes catalyse the oxidative decarboxylation of pyruvate (to acetyl coenzyme A) and ketoglutarate (to succinyl coenzyme A as part of the tricarboxylic acid cycle see figure 5.2), and are thus vital components of aerobic metabolism.

The diaphorase used in the studies reported here was first isolated in 1969 by Kaplan et al (67) and is extracted from Clostridium kluyverii.This particular diaphorase differs in several respects from the lipoamide dehydrogenases isolated previously (see table 5.1). Some of these

Figure: 5.1 a.Nicotinamide adenine dinucleotide

b.Nicotinamide adenine dinucleotide phosphate

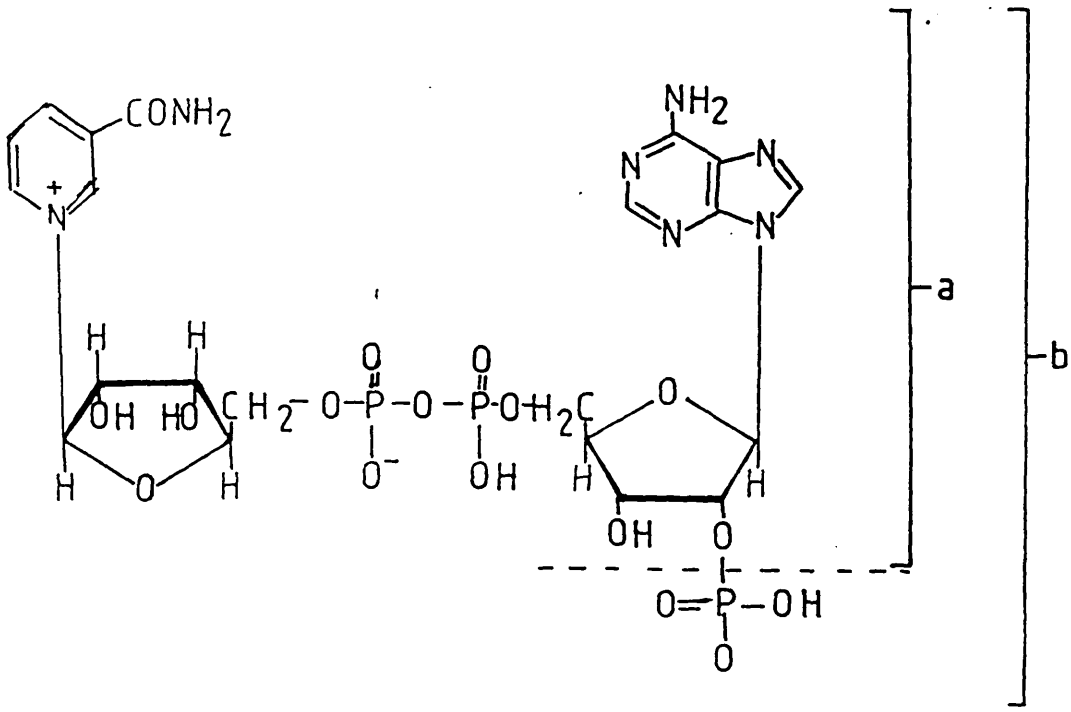


Figure 5.2 The tricarboxylic acid cycle showing the role of lipoamide dehydrogenase in the keto-acid dehydrogenase complex

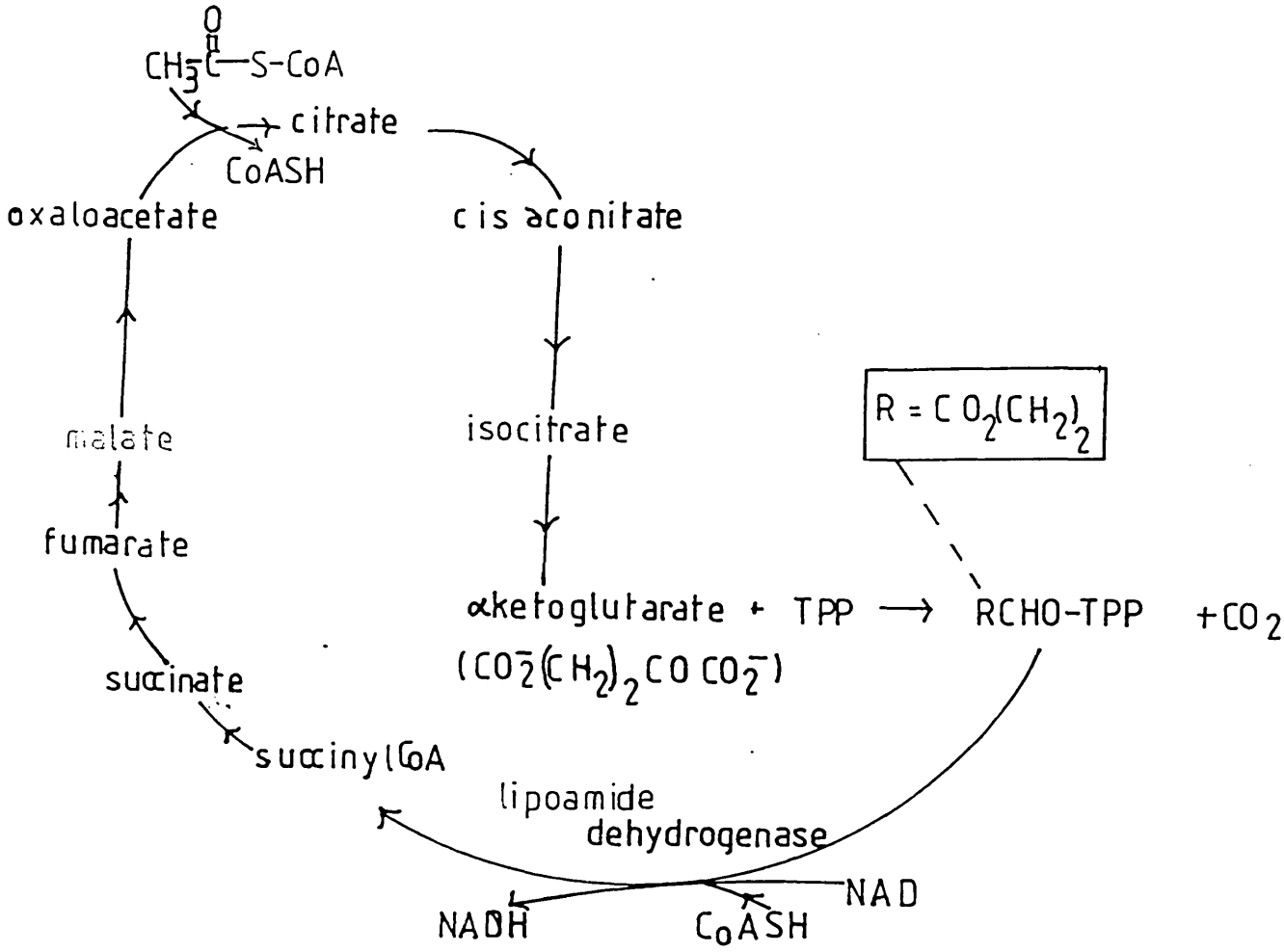


Table 5.1 Differences between CLOSTRIDIUM KLUYVERII diaphorase and previously isolated lipoamide dehydrogenases

Diaphorase	Lipoamide dehydrogenase
Acts on both NADPH and NADH	Acts on either NADPH or NADH
Cannot reduce O_2 or cytochrome c	Can reduce O_2 and cytochrome c
No disulphide at active site	Disulphide at active site
24,000 molecular weight	Most higher than 24,000
Does not catalyse hydrogen exchange between pyridine nucleotides	Can catalyse hydrogen exchange between pyridine nucleotides

differences (for instance inability to reduce oxygen, to be expected as C.kluyverii is an anaerobe) may indicate that the enzymes' roles are different. The role of diapherase in C.kluyverii is as yet uncharacterised (67).

All diaphorases have flavin moieties in a close but non-covalent association with the protein (66).Sulphydryl groups also appear to be important for activity in all diaphorases and they constitute part of the active site in lipoamide dehydrogenases (66).The enzyme bound flavin is involved in the electron transfer processes which occur during catalysis and is vital to the electrochemical study of the enzyme.

5.1.1 The study of diaphorase electrochemistry using mediated electron transfer

In principle, since redox enzymes catalyse reactions involving electron transfer it should be possible to observe their action using an electroanalytical technique.In practice unless a specifically modified electrode is used (69)steric hindrance prevents the transfer of electrons to or from the electrode during catalysis (see section 2.5).To overcome this problem small molecules which are able to shuttle electrons between the electrode and the enzyme's active site are used as electron transfer mediators.The way in which mediated electrochemistry is used to observe the kinetics of an enzyme catalysed reaction has been described in section 2.5.The type of system described in section 2.5 provides a technique both for studying the kinetics of the enzyme catalysed reaction and for assaying for the enzyme's

substrates.

When using mediated electrochemistry as an analytical technique it is important that the mediator reacts rapidly at the electrode, as evidenced by reversible electrochemistry (see section 2.4.5).If this is the case then electron transfer between mediator and electrode will not limit the rate of the overall reaction.The reactions occuring during mediated enzyme electrochemistry are shown in figure 5.3.

The scheme shown in figure 5.3 is of the type which is set up where the mediated electrochemistry of diaphorase is studied.The mediator is thus reduced by the enzyme (in the presence of the reducing substrate, NADH) and re-oxidised at the electrode.

Various mediator molecules have been used with different electrochemical techniques (32), the most useful for the amperometric study of flavoenzymes are the ferrocene derivatives.The stucture of ferrocene is shown in figure 5.4, rapid electron transfer occurs to and from the molecule and various substitutions of the cyclopentadienyl rings provide a family of molecules with different physical and electrochemical properties.Ferrocenes have been found to act as mediators for several flavoenzymes, for which they are substrates, as electron transfer can occur between the flavin moiety and the ferrocene molecule

Both diaphorase and lipoamide dehydrogenase show a low degree of substrate specificity for the oxidant and a wide variety of dyes can be reduced, the most commonly used being

Figure 5.3 Mediated electron transfer between enzyme and electrode

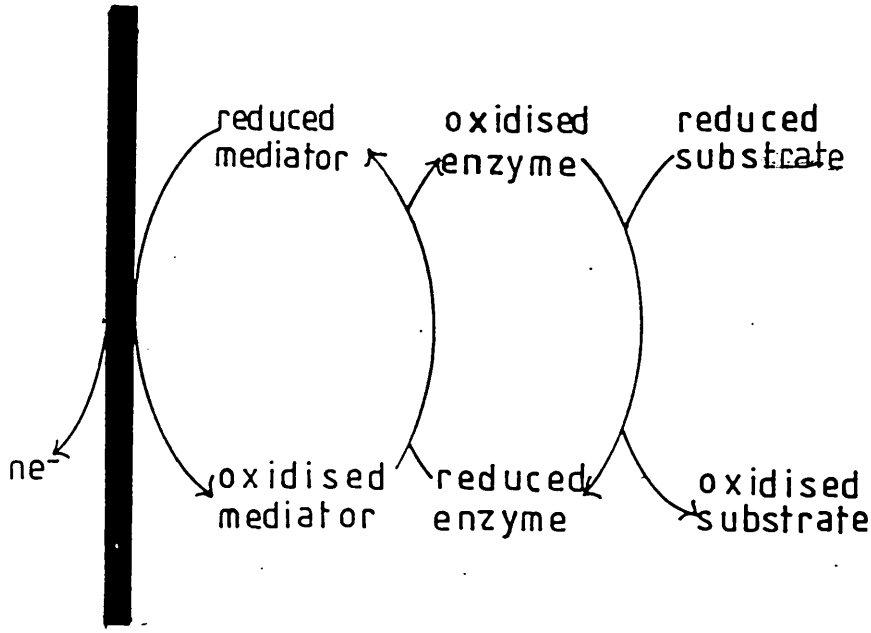
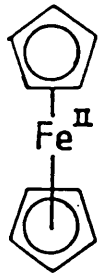


Figure 5.4 The ferrocene molecule



methylene blue and 2,6 dichlorophenolindophenol (DCPIP).Since both enzymes also contain a flavin which is active in electron transfer the dye molecule can be replaced by ferricinium (oxidised ferrocene) facilitating amperometric studies by the type of scheme described above.

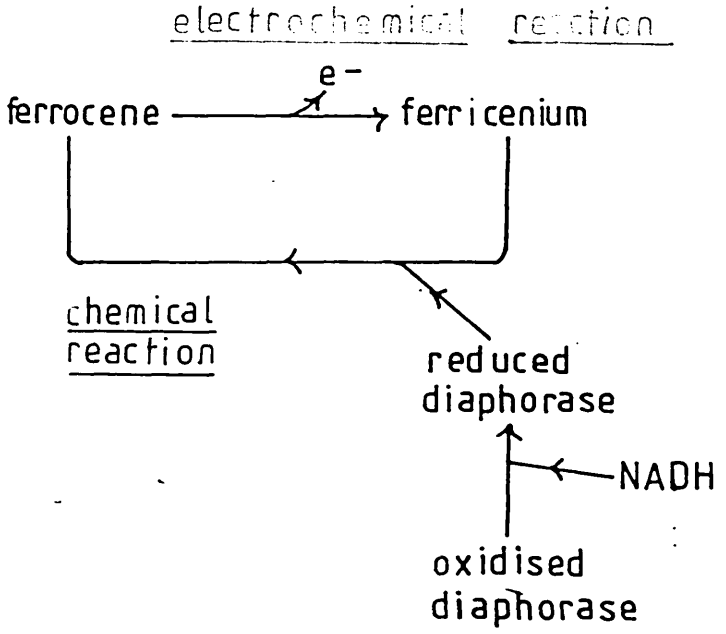
5.1.2 Amperometric assay systems for diaphorase

In the amperometric assay diaphorase, reduced by NADH in solution, transfers electrons to ferricinium which is produced by the oxidation of ferrocene at the surface of a solid electrode.The reaction scheme is shown in figure 5.5.

Different assay configurations enable either enzyme kinetics or substrate concentrations to be studied.Where NADH is maintained in excess diaphorase is assumed always to be in its reduced form and is thus treated as A in the analysis of cyclic coupled chemical reactions (see section 2.5) .At the peak potential of ferrocene oxidation the ferricinium concentration at the electrode surface is at its maximum and is determined by the concentration of ferrocene in bulk solution.Where the electrode reaction is fully reversible (see section 2.4.5) the ferricinium concentration at the electrode will be the same as that of ferrocene in bulk solution.

The assay configuration shown in figure 5.5 can also be used to assay for NADH by simply altering the substrate concentration over the desired range and measuring the current at or above the peak mediator oxidation potential.Substrate assays can be carried out at fixed

Figure 5.5 Mediated electron transfer from reduced diaphorase



potential and it is normally necessary to increase the enzyme concentration to produce detectable currents.

An assay for the nicotinamide coenzymes has a potentially vast application.There are over 250 nicotinamide dependent enzymes known at present (70), if one of these is included in the assay the scheme shown in figure 5.6 is established and the concentration of substrate X can be measured amperometrically.

5.1.3 Enzyme amplified assays

The presence of a reagent cycle in the assay scheme shown in figure 5.6 (where NAD^+ is regenerated) allows the development of sensitive assays for the cycled component. The rate at which cycling occurs is proportional to the reagent concentration and controlled by the rates of the two enzyme catalysed reactions.An assay of this type for NAD^+ or NADH has been developed where the rate of cycling is measured spectrophotometrically.The assay scheme was developed by Self using the coupled action of diaphorase and alcohol dehydrogenase to cycle NAD^+ between its oxidised and reduced forms (71).The reaction scheme is shown in figure 5.7.Conditions must be controlled such that the NAD^+ or NADH concentration limits the rate of the enzyme catalysed cycle.Accumulation of INT formazan for different periods of time allows alteration of the sensitivity of the assay.The spectrophotometric assay has been successfully exploited in the measurement of phosphatase enzymes by the addition of a further step in the reaction in which the phosphatase cleaves $NADP^+$ producing NAD^+ the concentration of which is then

Figure 5.6 A dehydrogenase assay using the mediated electrochemistry of diaphorase

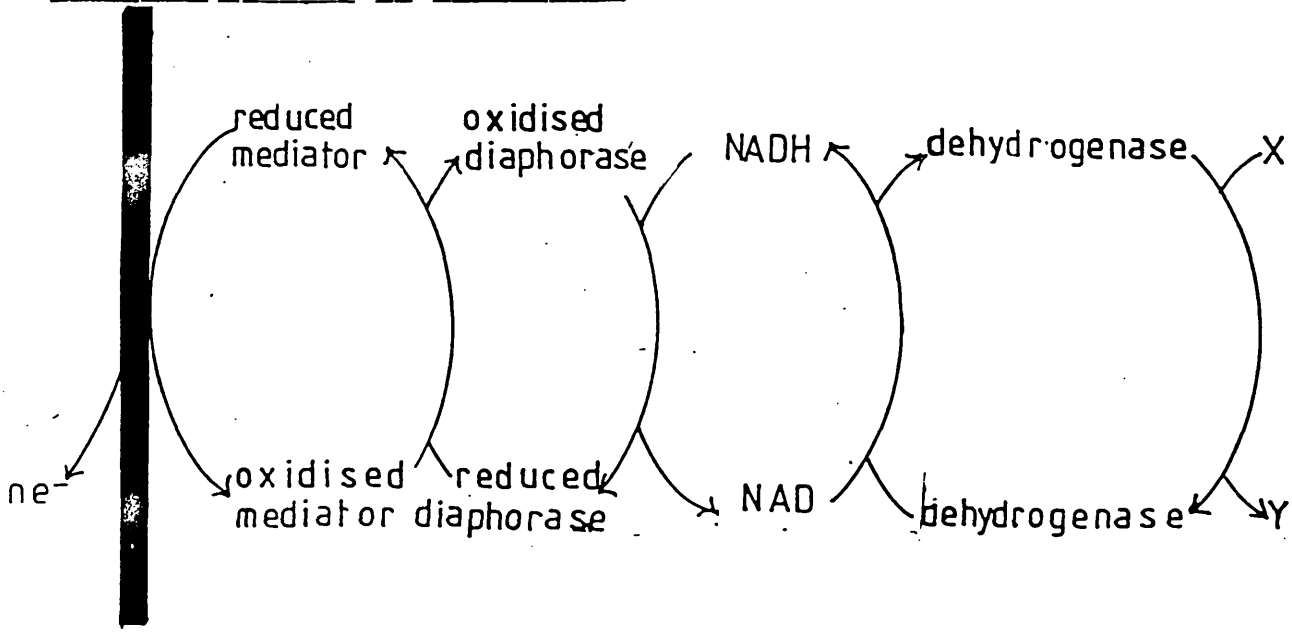
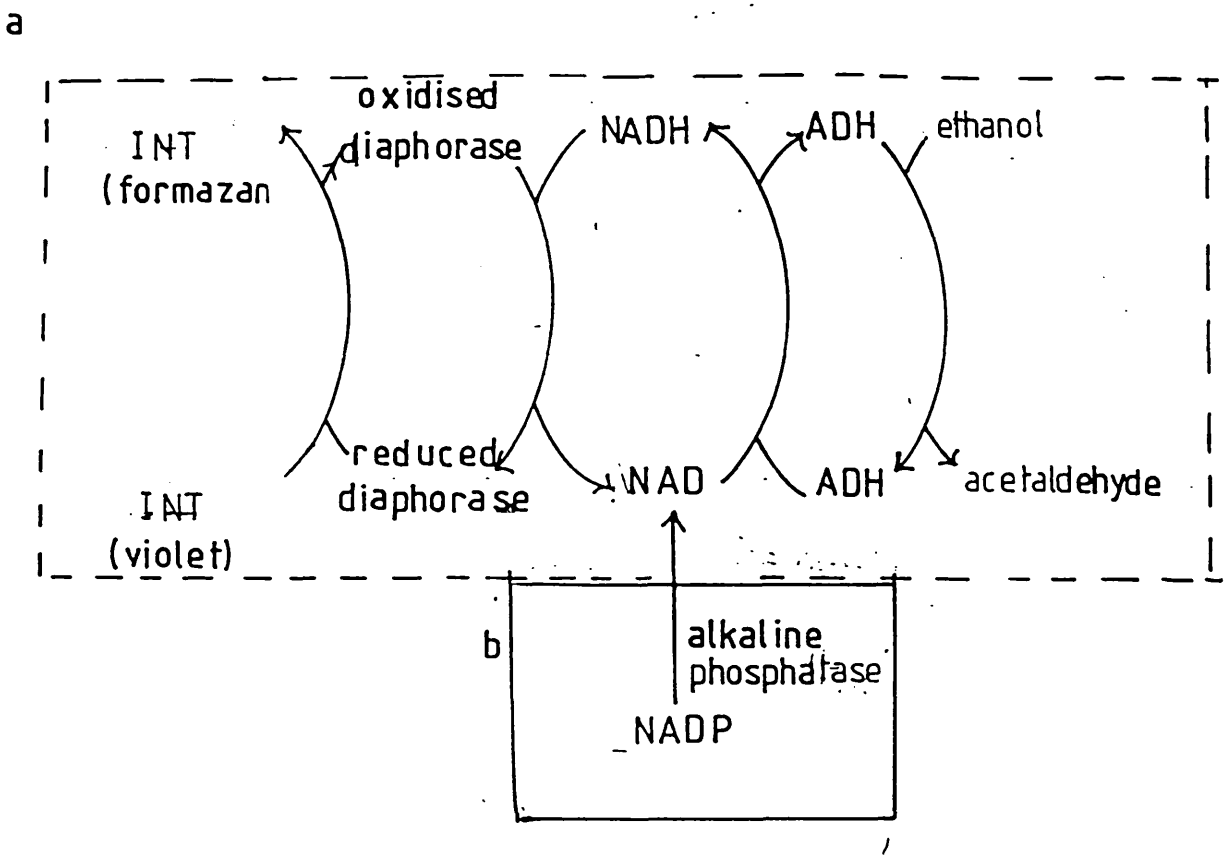


Figure 5.7 Enzyme amplified assays.

a. for NAD or NADH

b. for NADP or alkaline phosphatase



assayed by the amplified scheme (figure 5.7b) (71).

Studies described in this and the following chapter demonstrate the amperometric detection of diaphorase activity and, with the established enzyme amplified cycle, show how it can be used to develop a sensitive electrochemical assay for alkaline phosphatase.

Phosphatase enzymes are ubiquitous and often important indicators of disease states in man (72) and alkaline phosphatase is widely used as a label in ELISA.The determination of phosphatases is therefore useful in the areas of both medicine and science.

5.2 Results and discussion

5.2.1 The electrochemical characteristics of the ferrocene derivatives

A number of ferrocene derivatives were studied in order to find the one most suitable for mediating diaphorase electrochemistry.Initial examination of mediator electrochemistry was by cyclic voltammetry, a sample cyclic voltammogram (CV) is shown in figure 5.8 and the electrochemical characteristics derived from the CV's of several ferrocene derivatives are given in table 5.2.

In using coupled electrochemistry the mediator must react rapidly at the electrode and the CV of an electroactive species indicates whether this is the case.The CV of a reversible (rapid) electrochemical reaction has anodic and cathodic peaks of equal magnitude (i_{pa}/i_{pc} = 1) and a peak separation of close to 59 mV (73).

From table 5.2. close to reversible characteristics were obtained from hydroxymethyl ferrocene (HMF) and dimethyl ferrocene ethanolamine (DMFE).Both of these derivatives are reasonably soluble in aqueous solution and are thus suitable for solution studies.

The oxidation potential of the mediator is also an important consideration .As in all amperometric studies application of extreme potentials at the working electrode is undesirable (see section 4.1).In studies involving diaphorase the application of a low oxidation potential is even more important because NADH itself is oxidised at approximately +700 mV versus SCE at carbon electrode surfaces (74).Direct

Figure 5.8 Cyclic voltammogram

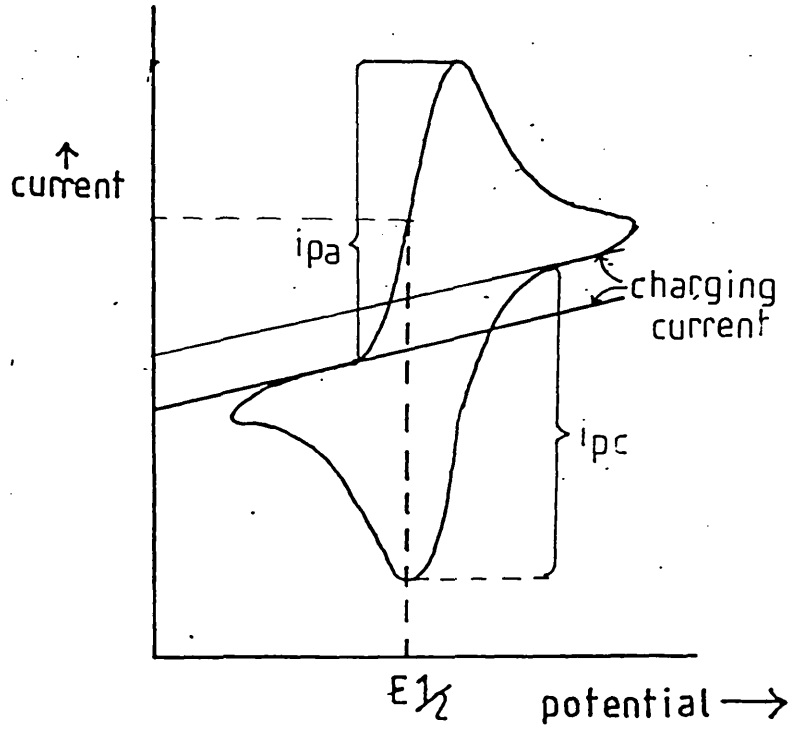


Table 5.2 Electrochemical characteristics of the ferrocene derivatives

Derivative	$E_{1/2}$ (mV vsSCE)	ip_a/ip_c	Peak separation (mV)
HMF	+170	1	70
DMFE	+115	1	100
Ferrocene carboxylic acid	+250	1	75
Ferrocene carboxaldehyde	+400	2.25	110
DCPIP	+10	1.3	80

oxidation of NADH is not used analyticaly because oxidation products severely foul the electrode surface and NADH quantitation is unreliable (75).If mediated amperometric studies on NADH containing solutions are carried out near its oxidation potential these products will poison the electrode and NADH oxidation current may interfere with the measurement of catalytic currents.The effect of a coupled enzyme reaction is observed by an increase in current at the peak oxidation potential of the mediator (see section 2.5) .In the diaphorase system the mediator oxidation potential must therefore be well below + 700 mV vs SCE so that the NADH oxidation neither interferes with the observed current nor results in fouling of the electrode.Both HMF and DMFE fulfil this criterion and were used in all subsequent diaphorase studies.

The electrochemistry of a non-ferrocene mediator, DCPIP, was also examined and its properties are listed in table 5.2.Despite its poor electrochemistry amperometric studies were carried out using this mediator as it is also used as the electron acceptor in the spectrophotometric assay and a direct comparison between the two techniques was sought.

5.2.2 Reduction of electrochemically generated ferricinium derivatives by reduced diaphorase - kinetic studies using cyclic voltammetry and chronoamperometry

5.2.2.1 Cyclic voltammetry

The theory behind the use of cyclic voltammetry to study the kinetics of a homogeneous chemical reaction coupled

to a heterogeneous electrode reaction is given in section 2.5.

The effect of the homogeneous reaction of reduced diaphorase with hydroxymethyl ferricinium (HMF^+) on the CV of HMF is shown in figure 5.9 In this experiment NADH is in sufficient excess that the diaphorase is assumed to be fully reduced, regeneration of ferrocene at the electrode surface then occurs according to the scheme described by Nicholson and Shain (36) as shown in figure 5.5.

5.2.2.2 Chronoamperometry

Excess substrate was again used in chronoamperometric studies and the same reaction occurs (figure 5.5).The effect of the coupled reaction on the HMF chronoamperogram is shown in figure 5.10, the theory from which kinetic information is derived from altered chronoamperograms is given in section 2.5.

In both chronoamperometry and cyclic voltammetry working curves, calculated from mathematical models (36 and 37) allow the extraction of values of a kinetic rate constant, k_f, from values of catalytic currents determined experimentally.The value of k_f depends on the rate limiting reaction in the whole process and will be discussed more fully in section 5.2.4.The working curves for the two techniques are given in figures 5.11 and 5.12.In cyclic voltammetry the current obtained is sweep rate dependent (see section 2.4.5) thus i_k and i_d are determined over a range of sweep rates in order that this dependence can be eliminated.

Figure 6.1 Cyclic voltammograms of hydroxymethyl ferrocene

a. 0.5 mM HMF + 10 mM NADH

b. 0.5 mM HMF + 10mM NADH + 1.75×10^{-5} M diaphorase

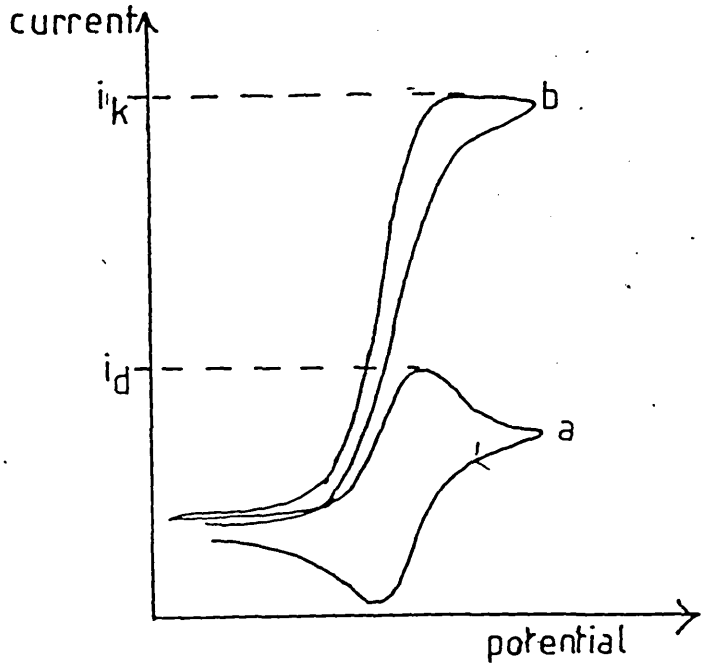


Figure 5.10 Chronoamperograms of hydroxy methyl ferrocene

a. with 10 mM NADH

b.with 10mM NADH and 8.4×10^{-8} M diaphorase

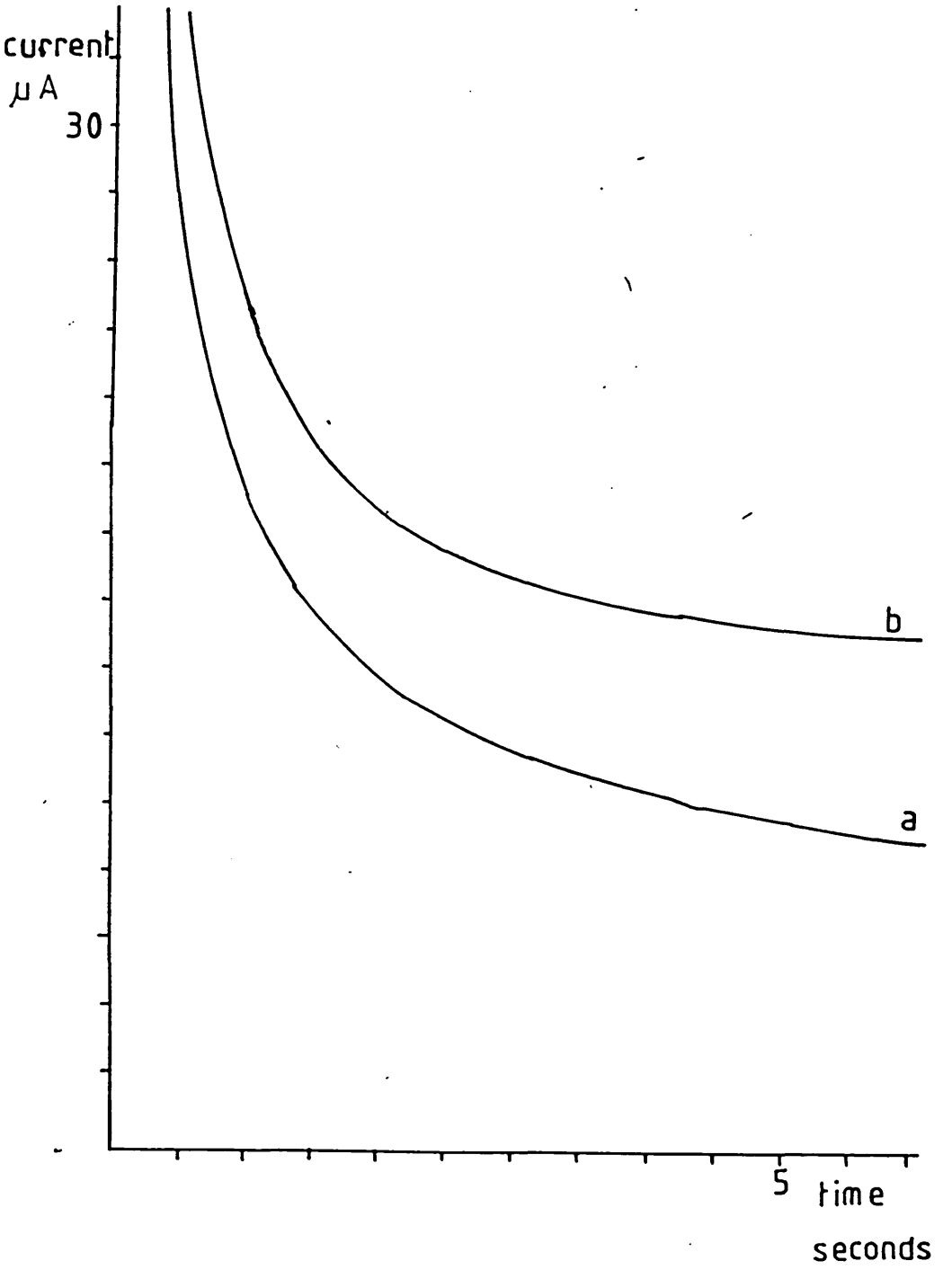


Figure 5.11 Working curve for coupled reactions studied by cyclic voltammetry

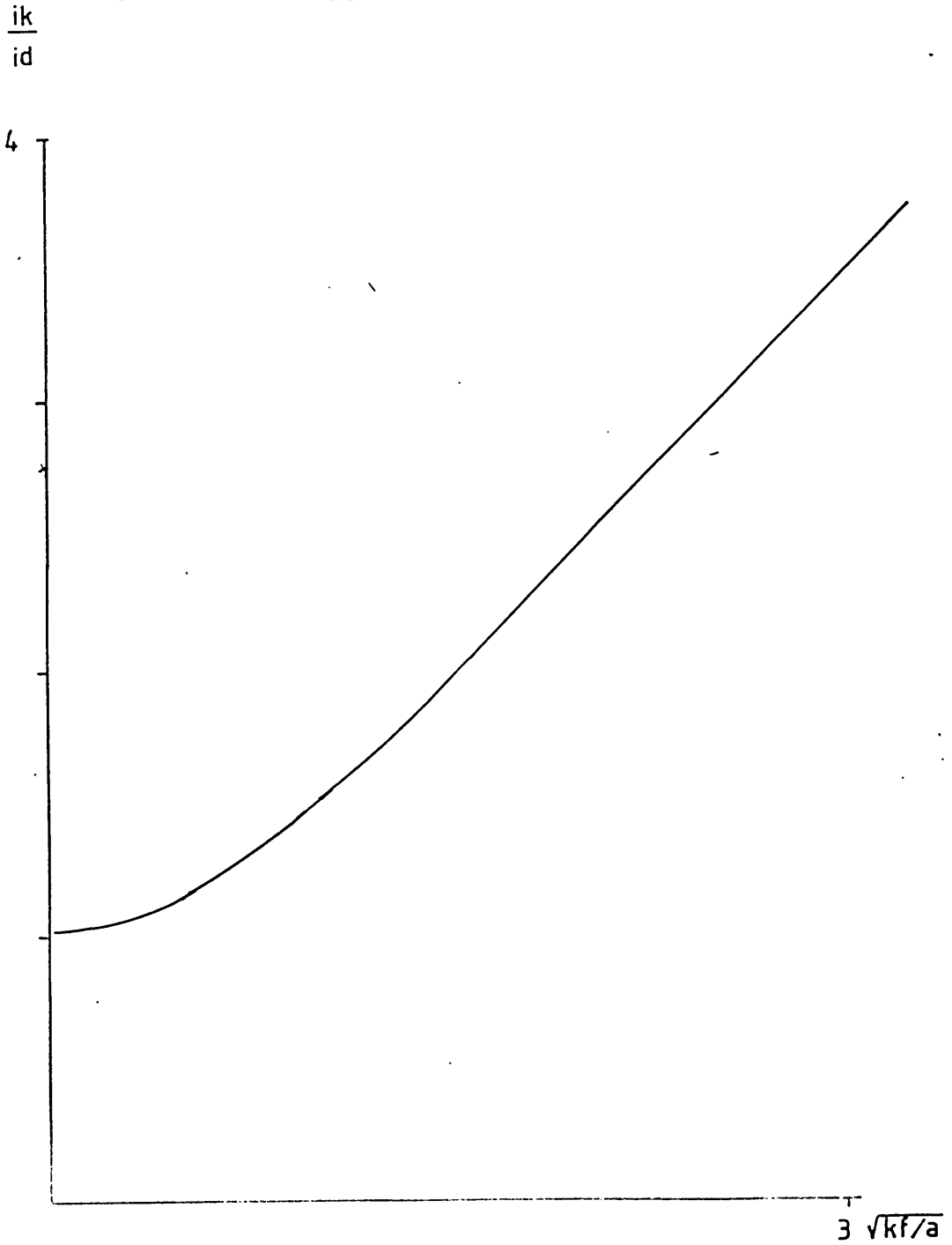
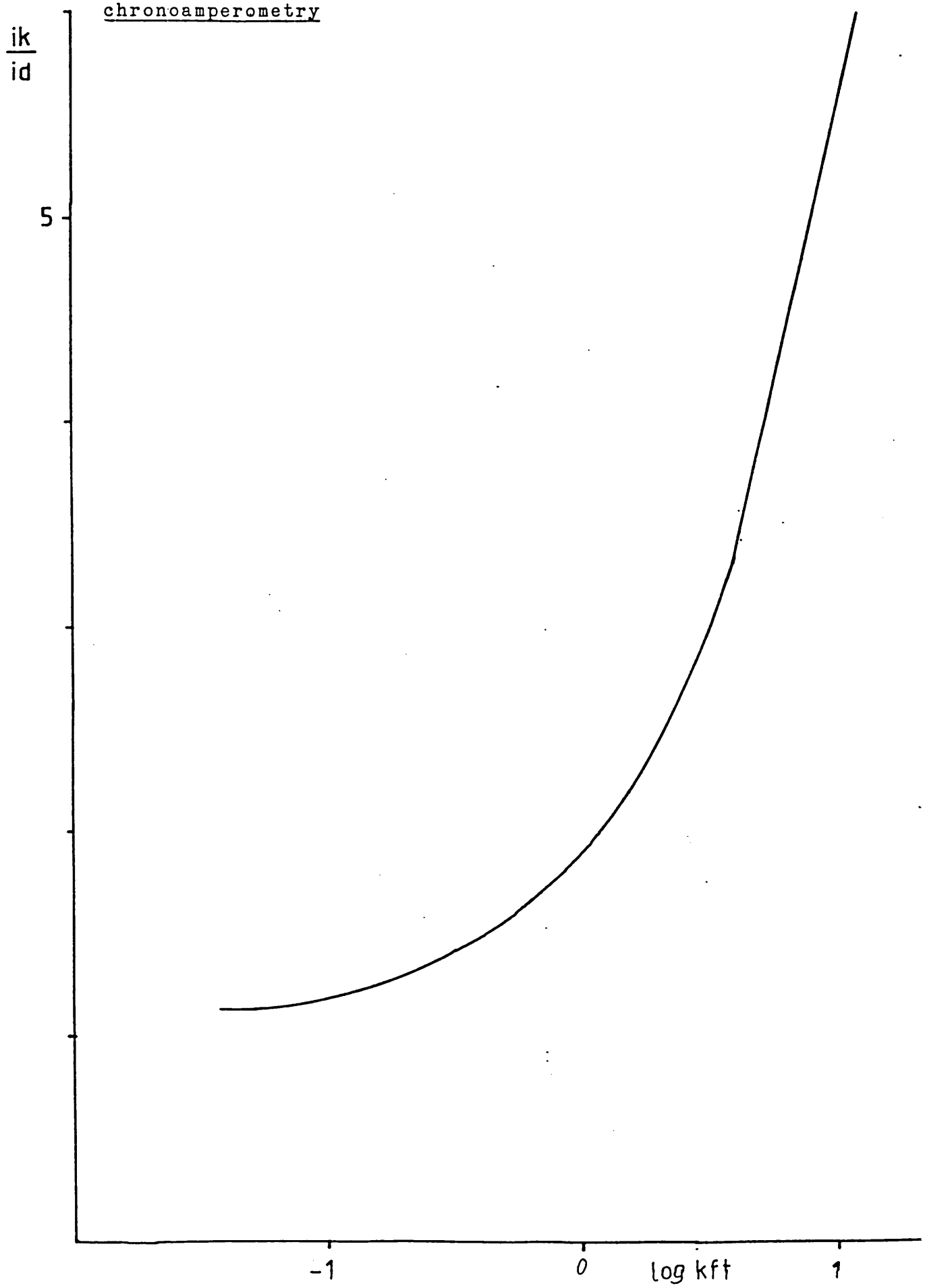


Figure 5.12 Working curve for coupled reactions studied by chronoamperometry



CV's of HMF over a range of sweep rates (v) are shown in figure 5.13.

Catalytic currents at various sweep rates were determined for HMF, DMFE and DCPIP ,alone and in the presence of three different concentrations of diaphorase at a constant NADH concentration.Results from these experiments are given in tables 5.3 to 5.11 and plots of $i/(v^{1/2})$ against log v are shown in figures 5.14 to 5.16.

The values of k_f and k_s for the different mediators in the presence of various diaphorase concentrations are given in table 5.16.

Chronoamperometry was carried out under the same conditions as the cyclic voltammetry experiments described and the values of k_f determined are given in tables 5.12 to 5.15.The values of k_f show poor reproducibility (except where DMFE is the mediator) and were not considered sufficeintly reliable to be considered in the kinetic analysis of the system described below.

The rate constants obtained reflect the rate at which the mediator is regenerated at the electrode surface (see section 2.5).In this set of experiments a high NADH concentration is used and the reaction occuring at the electrode surface is that shown in figure 5.5.

The concentration of oxidised mediator is determined by its bulk concentration as described in section 2.3.Maintaining excess NADH ensures that oxidised diaphorase is rapidlly reduced such that the reduced diaphorase

Figure 5.13. Cyclic voltammograms of HMF at, a. 5mV sec^{-1}, b. 10mV sec^{-1}, c. 20mV sec^{-1}, d. 50mV sec^{-1}, e. 100mV sec^{-1}.

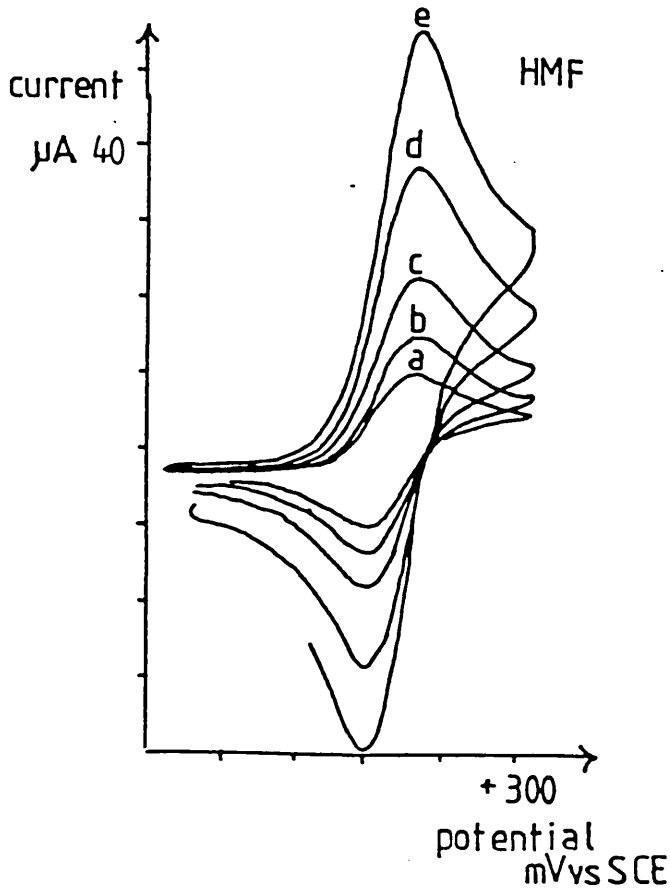


Table 5.3 Cyclic voltammetry of hydroxymethyl ferrocene in the presence of 4.2×10^{-8} M reduced diaphorase

v (mv sec-1)	logv	$v^{1/2}$	id µA	ik µA	$id/v^{1/2}$	$ik/v^{1/2}$	ik/id
5	0.7	2.24	3.7	18	1.65	8.04	4.73
10	1	3.16	5	17.5	1.58	5.54	3.26
20	1.3	4.47	7.2	17.4	1.61	3.89	2.29
50	1.7	7.07	11.5	17.7	1.62	2.5	1.47
100	2	10	16.5	18.8	1.65	1.88	1.11

v V/sec	1/v sec/V	ik/id	$\sqrt{kf/a}$	kf/a
0.005	200	4.73	2.74	7.51
0.01	100	3.26	1.86	3.5
0.02	50	2.29	1.26	1.59
0.05	20	1.47	0.64	0.41
0.1	10	1.11	0.2	0.04

Table 5.4 Cyclic voltammetry of hydroxymethyl ferrocene in the presence of 1.05×10^{-7} M reduced diaphorase

v (mv sec-1)	logv	$v^{1/2}$	id µA	ik µA	$id/v^{1/2}$	$ik/v^{1/2}$	ik/id
5	0.7	2.24	6.2	26	2.77	11.6	7.48
10	1	3.16	7	25	2.2	7.9	5.1
20	1.3	4.47	8.7	23.6	1.95	5.3	3.42
50	1.7	7.07	12	21.4	1.7	3.03	1.95
100	2	1	16.1	16.8	1.6	1.68	1.1

v V/sec	1/v sec/V	ik/id	$\sqrt{kf/a}$	kf/a
0.005	200	7.48	4.4	19.4
0.01	100	5.1	3	9
0.02	50	3.42	1.9	3.61
0.05	20	1.95	1.04	1.1
0.1	10	1.1	0.2	0.04

Table 5.5 Cyclic voltammetry of hydroxymethyl ferrocene in the presence of 2.1×10^{-7} M reduced diaphorase

v (mv sec-1)	logv	$v^{1/2}$	id µA	ik µA	$id/v^{1/2}$	$ik/v^{1/2}$	ik/id
5	0.7	2.24	5	30.8	2.23	13.75	11.1
10	1	3.16	5.8	30.4	1.84	9.6	7.74
20	1.3	4.47	7.2	29.6	1.6	6.62	5.34
50	1.7	7.07	9.9	28.4	1.4	4.02	3.24
100	2	10	13.5	26.4	1.35	2.6	2.1

v V/sec	1/v sec/V	ik/id	$\sqrt{kf/a}$	kf/a
0.005	200	11.1	6.6	43.6
0.01	100	7.74	4.6	21.2
0.02	50	5.34	3.2	10.2
0.05	20	3.24	1.84	3.4
0.1	10	2.1	1.12	1.25

Table 5.6 Cyclic voltammetry of dimethyl ferrocene ethanolamine in the presence of 4.2×10^{-8} M reduced diaphorase

v (mv sec-1)	logv	$v^{1/2}$	id µA	ik µA	$id/v^{1/2}$	$ik/v^{1/2}$	ik/id
5	0.7	2.24	4.7	15.8	2.1	7.05	4.41
10	1	3.16	5.9	15.5	1.9	4.91	3.07
20	1.3	4.47	8.9	16.5	2	3.69	2.48
50	1.7	7.07	11.9	16.9	1.68	2.39	1.49
100	2	10	16.2	18.4	1.62	1.84	1.15

v V/sec	1/v sec/V	ik/id	$\sqrt{kf/a}$	kf/a
0.005	200	4.41	2.56	6.55
0.01	100	3.07	1.76	3.1
0.02	50	2.48	1.36	1.85
0.05	20	1.49	0.64	0.41
0.1	10	1.15	0.2	0.04

Table 5.7 Cyclic voltammetry of dimethyl ferrocene ethanolamine in the presence of 1.05×10^{-7} M reduced diaphorase

v (mv sec-1)	logv	$v^{1/2}$	id µA	ik µA	$id/v^{1/2}$	$ik/v^{1/2}$	ik/id
5	0.7	2.24	4.9	21.5	2.2	9.6	6.86
10	1	3.16	5.8	20.7	1.84	6.55	4.68
20	1.3	4.47	7.2	19.3	1.61	4.32	3.09
50	1.7	7.07	10.1	17.5	1.43	2.48	1.77
100	2	10	18.5	18.8	1.55	1.88	1.34

v V/sec	1/v sec/V	ik/id	$\sqrt{kf/a}$	kf/a
0.005	200	6.86	4	16
0.01	100	4.68	2.7	7.29
0.02	50	3.09	1.76	3.1
0.05	20	1.77	0.9	0.81
0.1	10	1.34	0.54	0.29

Table 5.8 Cyclic voltammetry of dimethyl ferrocene ethanolamine in the presence of 2.1×10^{-7} M reduced diaphorase

v (mv sec-1)	logv	$v^{1/2}$	id µA	ik µA	$\frac{id}{v^{1/2}}$	$ik/v^{1/2}$	ik/id
5	0.7	2.24	4.6	26.7	2.1	11.92	8.16
10	1	3.16	5.6	26.6	1.77	8.42	5.77
20	1.3	4.47	7	25	1.57	5.59	3.83
50	1.7	7.07	10.6	22.8	1.5	3.22	2.21
100	2	10	14.7	22	1.47	2.2	1.51

v V/sec	1/v sec/V	ik/id	$\sqrt{kf/a}$	kf/a
0.005	200	8.16	4.9	24.01
0.01	100	5.77	2.4	11.56
0.02	50	3.83	2.2	4.84
0.05	20	2.21	1.12	1.25
0.1	10	1.51	0.7	0.49

Table 5.9 Cyclic voltammetry of DCPIP in the presence of 5×10^{-7} M reduced diaphorase

v (mv sec-1)	logv	$v^{1/2}$	id µA	ik µA	$id/v^{1/2}$	$ik/v^{1/2}$	ik/id
5	0.7	2.24	2.8	8.5	1.25	3.8	2.9
10	1	3.16	4	9.5	1.27	3	2.3
20	1.3	4.47	5.8	9.6	1.3	2.15	1.7
50	1.7	7.07	9.6	11.5	1.36	1.63	1.3
100	2	10	14.4	14.6	1.4	1.5	1.2

v V/sec	1/v sec/V	ik/id	$\sqrt{kf/a}$	kf/a
0.005	200	2.9	2.42	5.9
0.01	100	2.3	1.86	3.6
0.02	50	1.7	1.26	1.6
0.05	20	1.3	0.72	0.52
0.1	10	1.2	0.58	0.34

Table 5.10 Cyclic voltammetry of DCPIP in the presence of 1.02×10^{-6} M reduced diaphorase

v (mv sec-1)	logv	$v^{1/2}$	id µA	ik µA	$id/v^{1/2}$	$ik/v^{1/2}$	ik/id
5	0.7	2.24	2.8	11.2	1.25	5	3.8
10	1	3.16	4	12	1.27	3.8	2.9
20	1.3	4.47	5.8	13	1.3	2.9	2.2
50	1.7	7.07	9.6	14.8	1.36	2.1	1.6
100	2	10	14.4	19.2	1.4	1.9	1.5

v V/sec	1/v sec/V	ik/id	$\sqrt{kf/a}$	kf/a
0.005	200	3.8	3.28	10.76
0.01	100	2.9	2.44	6
0.02	50	2.2	1.76	3.1
0.05	20	1.6	1.14	1.3
0.1	10	1.5	1	1

Table 5.11 Cyclic voltammetry of DCPIP in the presence of 1.53×10^{-6} M reduced diaphorase

v (mv sec-1)	logv	$v^{1/2}$	id µA	ik µA	$id/v^{1/2}$	$ik/v^{1/2}$	ik/id
5	0.7	2.24	2.8	13.1	1.25	5.8	4.5
10	1	3.16	4	13.8	1.27	4.4	3.4
20	1.3	4.47	5.8	14.6	1.3	3.3	2.5
50	1.7	7.07	9.6	16	1.36	2.3	1.7
100	2	10	14.4	17.8	1.4	1.8	1.4

v V/sec	1/v sec/V	ik/id	$\sqrt{kf/a}$	kf/a
0.005	200	4.5	3.64	13.2
0.01	100	3.4	2.9	8.4
0.02	50	2.5	2.04	4.2
0.05	20	1.7	1.26	1.6
0.1	10	1.4	0.88	0.77

Figure 5.14. Diagnostic plots for coupled reactions studied by cyclic voltammetry a.0.5 mM HMF + 10mM NADH b. as a. + 4.2 x 10^{-8} M diaphorase c. as a. + 1.05 x 10^{-7}M diaphorase d. as a. + 2.1x 10^{-7}M diaphorase.

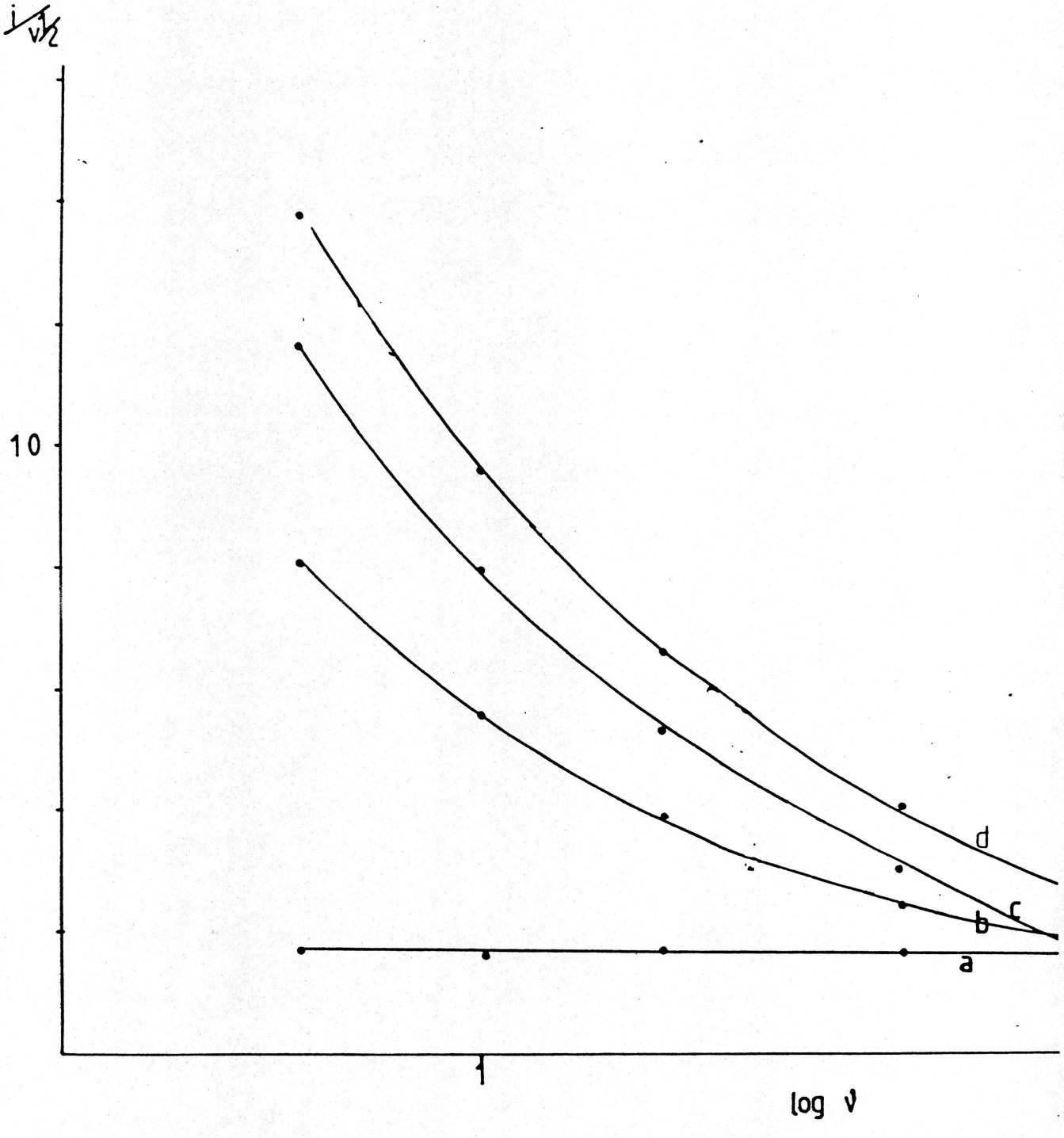


Figure 5.15. Diagnostic plots for coupled reactions studied by cyclic voltammetry a. 0.5mM DMFE + 10mM NADH b. as a. +4.2 x 10^{-8} M diaphorase c. as a. + 1.05 x 10^{-7}M diaphorase d. as a. + 2.1 x10^{-7}M diaphorase.

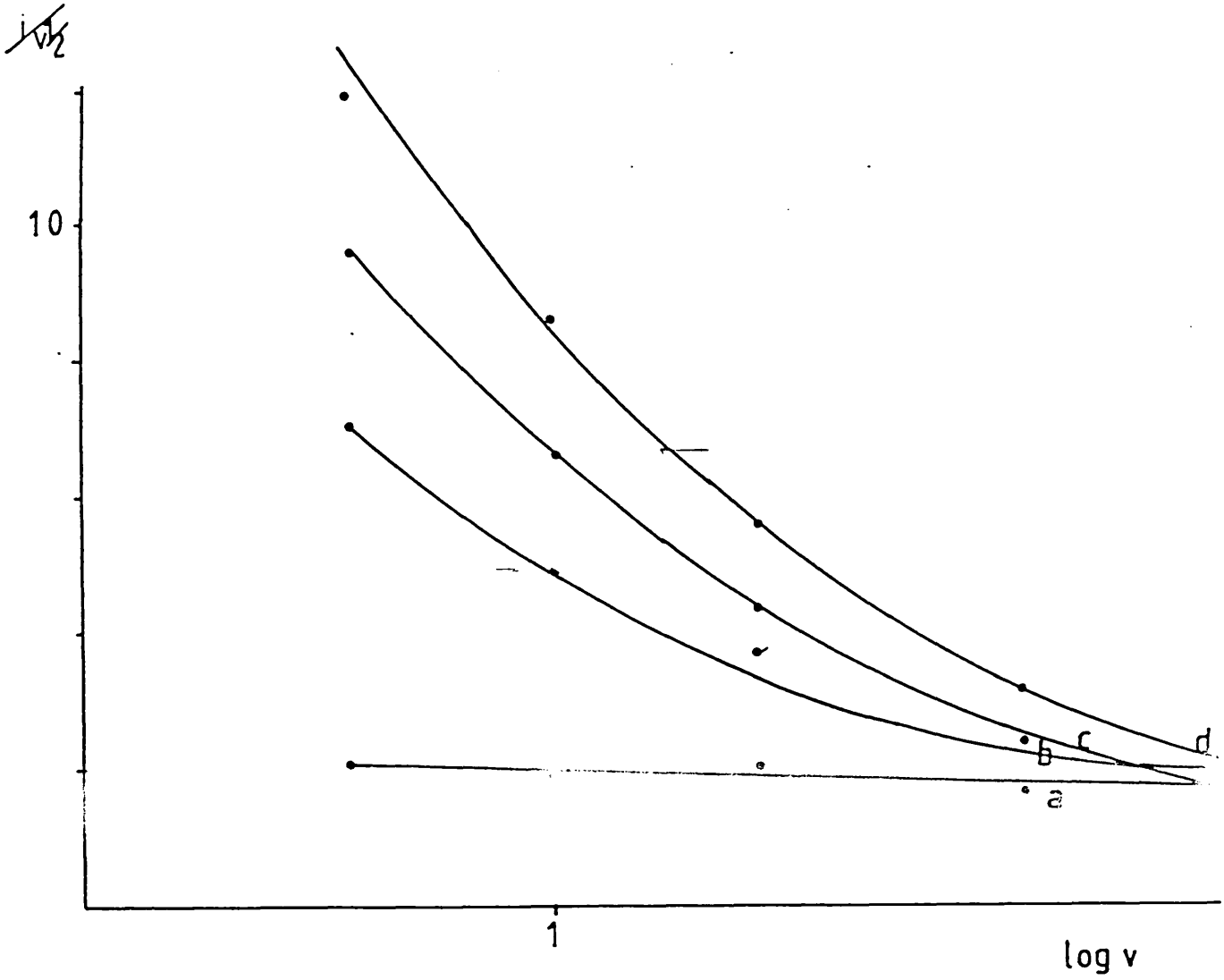


Figure 5.16. Diagnostic plots for coupled reactions studied by cyclic voltammetry a. 1mM DCPIP + 10mM NADH b. as a. + 5×10^{-7} M diaphorase c. as a. + 1.02×10^{-6} M diaphorase d. as a. + 1.53×10^{-6} M diaphorase.

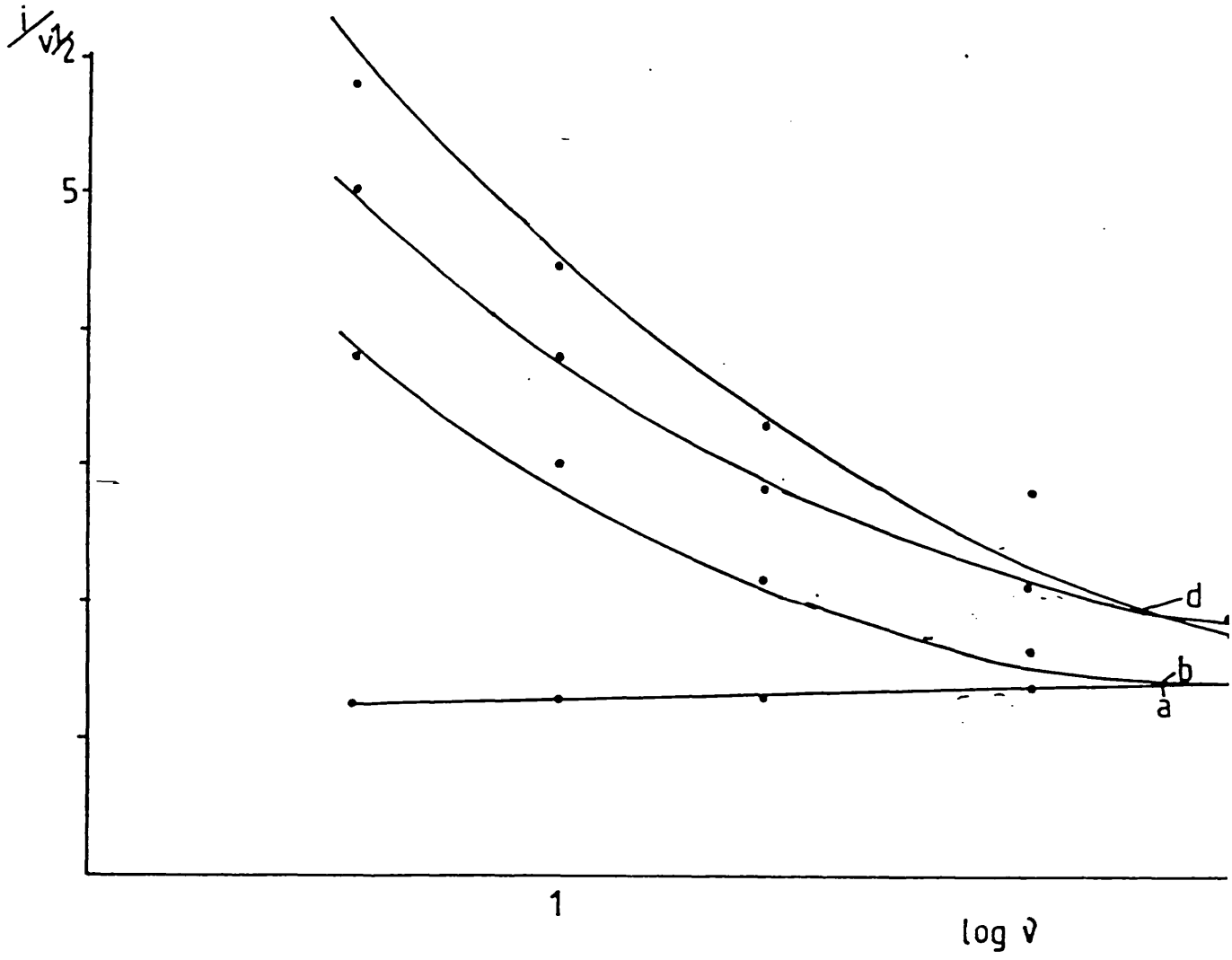


Table 5.12. Chronoamperometry of HMF in the presence of 8.4×10^{-8} M diaphorase and 10mM NADH.

t (secs)	$t^{-1/2}$	id μA	ik μA	ik/id	log kft	kf (sec-1)
0.5	1.41	26.4	42.2	1.6	-0.28	1.05
1	1	18.6	34	1.8	-0.06	0.87
1.5	0.82	15.3	31.4	2.1	0.14	0.92
2	0.71	13.5	30.1	2.23	0.21	0.81
2.5	0.63	12.3	29.2	2.37	0.32	0.84
3	0.56	11.4	28.8	2.53	0.34	0.73
3.5	0.53	10.8	28.4	2.63	0.4	0.72
4	0.5	10.2	28	2.75	0.42	0.66

Table 5.13. Chronoamperometry of DMFE in the presence of 6.73

t (secs)	$t^{-1/2}$	id uA	ik uA	ik/id	log kft	kf (sec-1)
0.5	1.41	46.8	47	1	0	0
1	1	27	32.4	1.2	-0.96	0.22
1.5	0.82	21.6	28.9	1.34	-0.62	0.24
2	0.71	18.4	27.2	1.48	-0.41	0.19
2.5	0.63	16.4	16.4	1.61	-0.2	0.22
3	0.56	15	25.8	1.84	-0.24	0.3
3.5	0.53	14	25.4	1.92	0.04	0.3

Table 5.14 Chronoamperometry of DCPIP in the presence of 6.72 $x10^{-8}$M diaphorase and 10mM NADH

t (secs)	$t^{-1/2}$	id uA	ik uA	ik/id	log kft	kf (sec-1)
0.5	1.41	4.8	12.1	2.52	0.33	4.3
1	1	3.6	8	2.22	0.2	1.6
1.5	0.82	3.2	6.5	2.03	0.1	0.84
2	0.71	2.9	5.6	1.93	0.02	0.52
2.5	0.63	2.7	5	1.85	-0.01	0.39
3	0.56	2.6	4.6	1.77	-0.08	0.28
3.5	0.53	2.4	4.3	1.79	-0.08	0.24
4	0.5	2,4	4	1.67	-0.19	0.16

Table 5.15 Mean kf's for different mediators from chronoamperometry

Mediator	Diaphorase concentration (M)	Mean kf (sec^{-1})
HMF	8.4×10^{-8}	0.865
DMFE	6.72×10^{-8}	0.245
DCPIP	" "	1.04

Figure 5,18 The reaction between enzyme and substrate proposed by Michaelis and Menten

$$E + S \rightleftharpoons ES \rightleftharpoons EP \rightleftharpoons E + P$$

concentration is assumed to be determined by the concentration of free diaphorase added to the cell.

Equation 5.1 defines the rate of the reaction shown in figure 5.5.

$$v = k_s [E][M] \qquad 5.1$$

and $v/[M] = k_s [E]$

[M] for any single experiment is a constant and the parameter v/[M] is equal to the electrochemically determined k_f.

Determination of the value of k_f at various enzyme concentrations allows the determination of the second order rate constant for the overall reaction (see table 5.16).

As described in section 1.6 the majority of enzymatic analyses, both for substrate determination and kinetic study, have been conducted spectrophotometrically.In attempting to use amperometry as an alternative technique it was necessary to carry out conventional spectrophotometric studies on diaphorase for comparison.

In a spectrophotometric assay the increase or decrease of product or substrate concentration with time gives a measure of reaction velocity.The rate determining steps controlling this velocity differ depending on the substrate concentration as will be described below.

5.2.3 Michaelis Menten enzyme kinetics

Several models of the mechanism of an enzyme substrate reaction have been proposed.These models enable the extraction of kinetic information from measurements of

Table 5.16 Second order rate constants determined by cyclic voltammetry

Mediator	Diaphorase concentration (M)	kf (sec^{-1})	ks ($sec^{-1}M^{-1}$)
HMF	$4.2x10^{-8}$	1.84	$4.2x10^{7}$
HMF	$1.05x10^{-7}$	3.8	$3.6x10^{7}$
HMF	$2.1x10^{-7}$	8.3	$4.0x10^{7}$
DMFE	$4.2x10^{-8}$	1.2	$2.9x10^{7}$
DMFE	$1.05x10^{-7}$	2.97	$2.83x10^{7}$
DMFE	$2.1x10^{-7}$	4.66	$2.22x10^{7}$
DCPIP	$5\ x10^{-7}$	1.22	$2.4x10^{6}$
DCPIP	$1.02x10^{-6}$	1.81	$1.77x10^{6}$
DCPIP	$1.53x10^{-6}$	3	$1.96x10^{6}$

Mediator	Mean ks ($sec^{-1} M^{-1}$)
HMF	$3.93x10^{7}$
DMFE	$2.65x10^{7}$
DCPIP	$2.04x10^{6}$

reaction velocity,the most widely used model is the Michaelis Menten kinetic scheme which will be outlined below.

Figure 5.18 shows the general mechanism of an enzyme substrate reaction.

The equation is a simplified one dealing with the conversion of one of the enzymes substrates to its corresponding product.In practice most enzymes have more than one substrate and if a kinetic analysis of the reaction is to be undertaken all substrates not being varied must be in excess (76).

Where one of the substrates is held in excess its concentration is assumed to be constant. The velocity of a two substrate reaction is defined by equation 5.2.

$v = k[A][B]$

where v = reaction velocity,

[A] and [B] are substrate concentrations,

and k the rate constant for the reaction.

When [A] is a constant

$v = k'[B]$

Where $k' = k[A]$

Provided that the concentration of A does not change significantly during the reaction this may be considered a first order process, and is termed pseudo-first order.

Michaelis and Menten derved equation 5.3 which defines the velocity of the enzyme substrate reaction at varying substrate concentrations.

$$v = Vm[S]/(Km + [S]) \qquad 5.3$$

where v =reaction velocity

Vm = maximum reaction velocity

[S] = substrate concentration

and Km = Michaelis constant for the enzyme substrate reaction

A graph of v against [S] defined by this equation is shown in figure 5.19.

The reaction reaches its maximum velocity when all the enzyme is bound to substrate. Under these conditions the reaction is first order, velocity being dependent upon enzyme concentration and independent of substrate concentration. A plot of the type shown in figure 5.19 allows the determination of Vmax and Km. These are characteristic of a particular enzyme substrate reaction under pseudo-first order conditions.

In reactions studied spectrophotometrically the initial reaction velocity is measured such that substrate depletion or product inhibition do not complicate reaction kinetics. Tables 5.17 and 5.18 and figures 5.20 and 5.21 show change of velocity with substrate concentration for the diaphorase catalysed reaction in which either DCPIP or NADH is the variable substrate

Figure 5.19 Reaction velocity against substrate concentration where Michaelis Menten kinetics occur

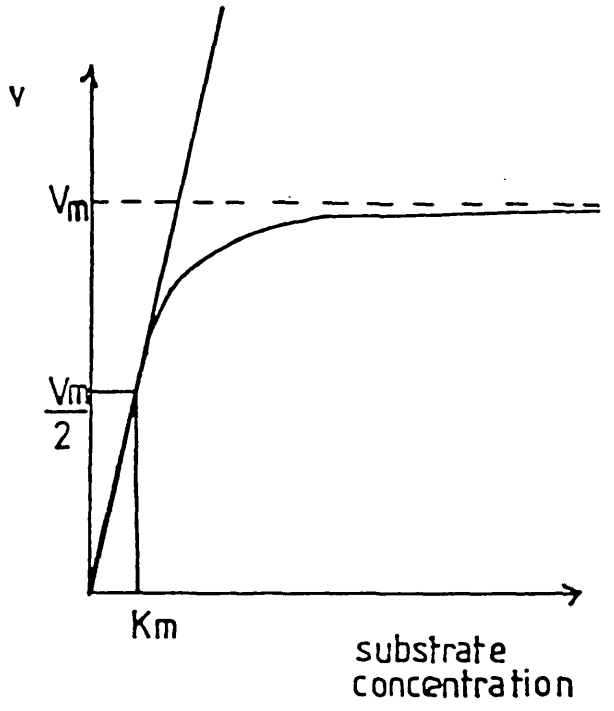


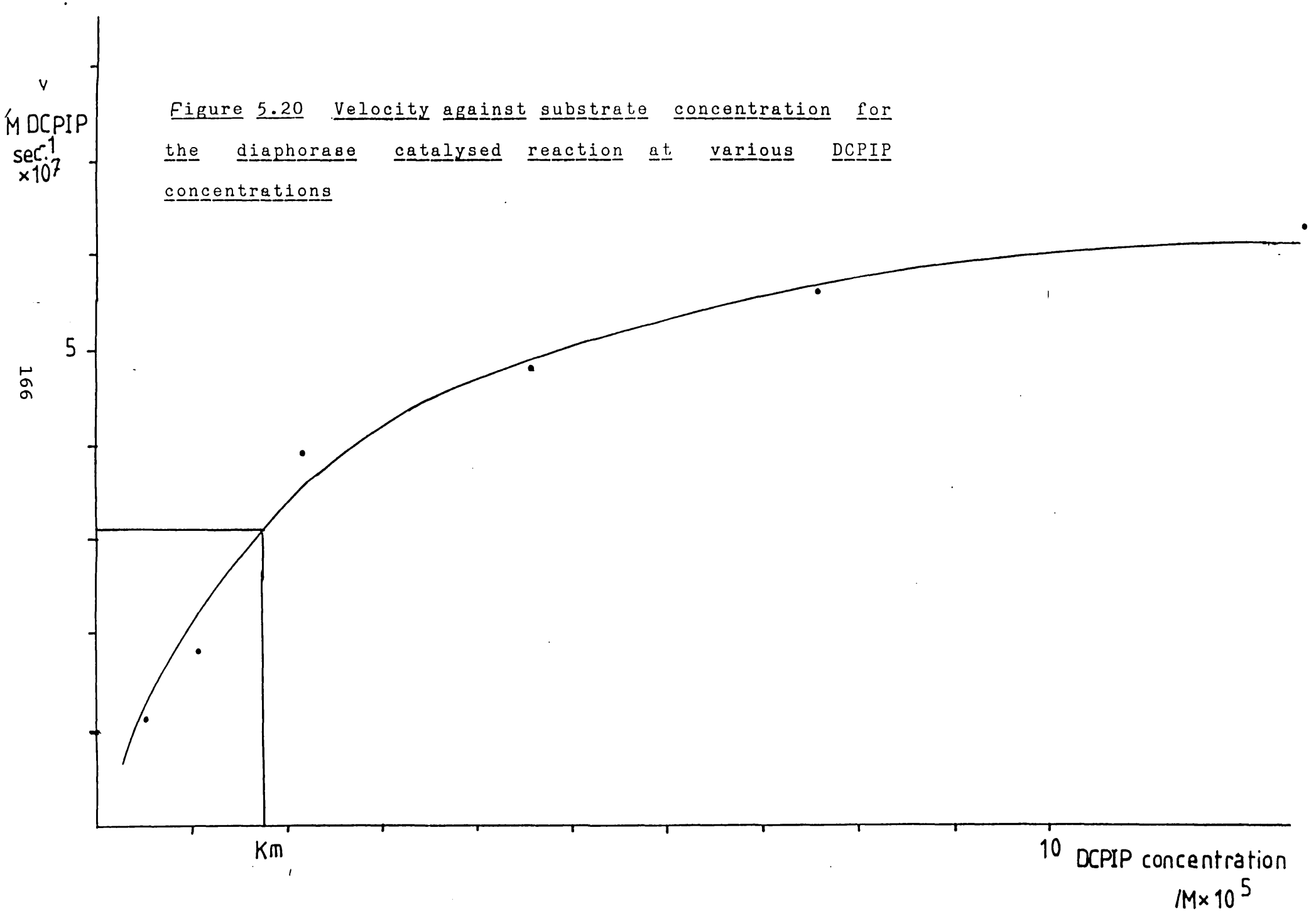
Table 5.17 Velocity of the diaphorase catalysed reaction at various DCPIP concentrations

DCPIP concentration (/M×10^5)	Velocity (/M DCPIP sec^{-1} x10^7)
0.51	1.23
1.14	1.82
2.32	3.9
4.53	4.8
7.56	5.6
12.72	7.3

Table 5.18 Velocity of diaphorase catalysed reaction at various NADH concentrations

NADH concentration (/M×10^6)	Velocity (/M DCPIP sec^{-1} x10^7)
33.3	6
66.7	10
100	12.2
133.3	11.5
166.7	12.5
200	11.8
300	11.7

Figure 5.20 Velocity against substrate concentration for the diaphorase catalysed reaction at various DCPIP concentrations



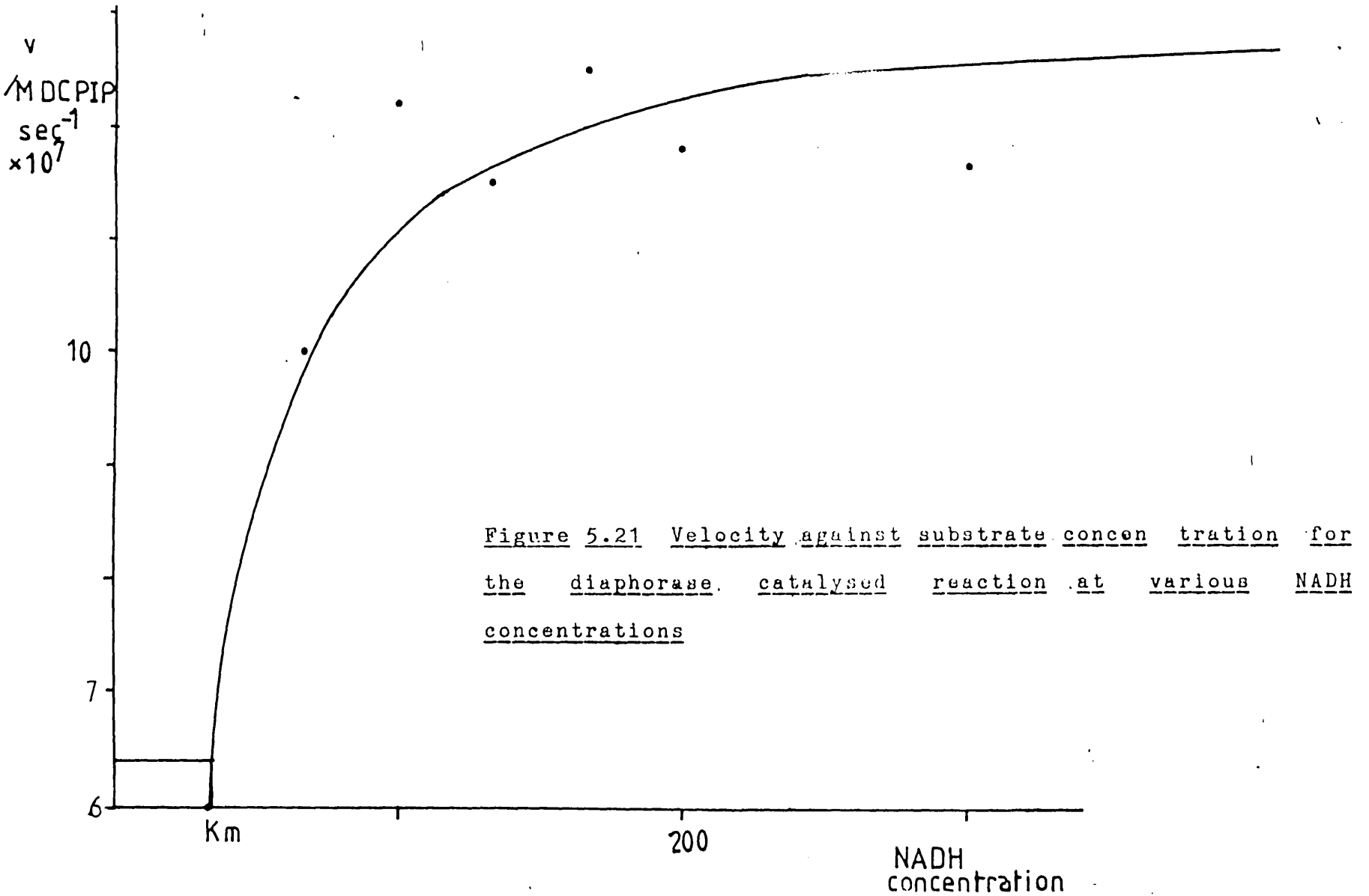


Figure 5.21 Velocity against substrate concen tration for the diaphorase catalysed reaction at various NADH concentrations

Where DCPIP concentration was varied the NADH concentration was held at 3.33 uM. Figure 5.20 shows that this NADH concentration is not sufficeint to saturate the enzyme. The reaction therefore not pseudo-first order and the rate constants determined below cannot be used in subsequent comparisons.

The parameters detemined from the model define the characteristics of the enzyme catalysed reaction and in this study these are later compared with rate constants derived by amperometry. This enables both the comparison of the two techniques and gives an understanding of the rate determining reaction steps in the amperometric scheme.

5.2.4 Comparison between amperometric and spectrophotometric assays,rate constants and reaction mechanisms.

In the spectrophotometric assay increasing the substrate concentration ([S])leads to saturation kinetics where the rate determining step changes as [S] is increased.

Initially, where [S] is much lower than Km, the rate of reaction is limited by the enzyme substrate interaction.Under these conditions the Michaelis Menten equation reduces to;

$$v = kcat/Km\ ([E][S]) \qquad 5.4$$

kcat/Km is an apparent second order rate constant for the encounter of enzyme and substrate to which the electrochemically determined rate constant k_s is analagous (see section 5.2.7).

Where [S] is much greater than Km the enzyme becomes saturated with substrate and the rate of reaction is limited

Table 5.19 Rate constants for diaphorase from spectraphotometric assay in which DCPIP concentration was altered

Vmax (M DCPIP sec^{-1})	6.2×10^{-7}
Km (M DCPIP)	1.75×10^{-5}
kcat (sec^{-1})	155

Table 5.20 Rate constants for diaphorase catalysed reaction from spectraphotometric assay where NADH concentration was altered

Vmax (M DCPIP sec^{-1})	12.75×10^{-7}
Km (M DCPIP)	34×10^{-6}
kcat (sec^{-1})	671

by the breakdown of the ES complex.This process is first order and equation 5.3 now reduces to

$$v = k_{cat}[E] \qquad 5.5$$

where kcat is the turnover number of the enzyme and expresses the maximum number of substrate molecules which can be converted to product per second per molecule of enzyme.At saturation v=Vm and a plot of v against [S] gives values of Vm and Km from which kcat and kcat/Km can be determined.

A spectrophotometric assay thus yields values of kcat and kcat/Km which refer to the steps which control the reaction velocity under first and second order conditions respectively. Tables 5.19 and 5.20 give values of the kinetic parameters determined spectrophotometrically. The values in table 5.20 were determined under pseudo-first order conditions and show good agreement with previously published data.

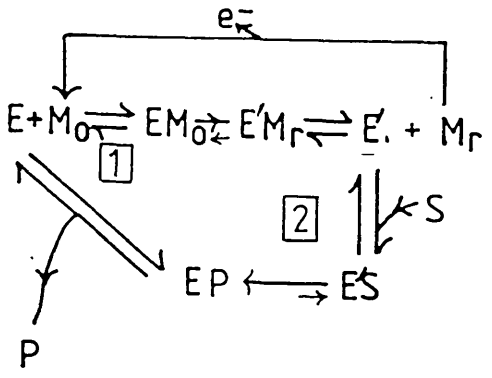
Several different graphical representations of the Michaelis Menten equation can be used to determine kinetic parameters from this type of data. The plot of v against [S] was used because it is the simplest representation of the data, although it may be innaccurate if high substrate concentrations are either inhibitory or stimulatory to the enzyme.

5.2.5 Kinetic analysis of the electrochemical reaction

In the electrochemical assay the reaction scheme is slightly different and is shown in figure 5.22.

The electrochemical analysis (see section 2.5) yields a rate constant kf which is dependent on the enzyme concentration therefore the rate determining step must be a

Figure 5.22 The reactions occuring during mediated electron transfer between enzyme and electrode



E = enzyme

M = mediator

S = substrate
P = product
} of the enzyme catalysed reaction

second order process.There are now two second order steps in the reaction (steps 1 and 2 in fig 5.22) compared with only one in the spectrophotometric assay (see fig 5.19), and either of these could be rate limiting.The way in which the rate limiting reaction can be assigned is described below.

Table 5.21 lists k_s values determined at three different DMFE concentrations and second order rate constants, determined both spectrophotometrically and electrochemically, are collected in table 5.22. The spectrophotometrically determined rate for the encounter of NADH with oxidised diaphorase is essentially the same as the electrochemically determined k_s values.k_s and kcat/Km are analogous and their value is determined by the rate detemining reaction.

It is not possible to measure kcat/Km for the reaction between reduced diaphorase and oxidised mediator since ferrocenes show low optical absorbance. If it were possible to determine this value, and if it were higher than both kcat/Km for the reaction between diaphorase and NADH, and k_s (determined electrochemically) then reaction 2 in figure 5.22 could be assigned as the rate determining step.

Observation of kinetic rate constants at a single concentration of mediator and NADH thus provides insufficeint information from which to determine the rate limiting reaction. However, electrochemically determined k_s values are also the same for different types and concentrations of mediator (see tables 5.21 and 5.22).Since the reaction rate is not influenced by the mediator the reaction of diaphorase with NADH must be the rate determining step.This will be

Table 5.21 Second order rate constants at various DMFE concentrations

DMFE concentration ($/M \times 10^3$)	ks ($M^{-1} sec^{-1}$)
0.25	2.29×10^7
0.5	2.83×10^7
1.0	2.7×10^7

Table 5.22 Second order rate constants for diaphorase determined spectrophotometrically or electrochemically

Technique	Mediator/ variable substrate	ks ($M^{-1} sec^{-1}$)
Spectrophotometry	DCPIP	8.86×10^6
"	NADH	1.09×10^7
Amperometry	HMF	3.93×10^7
"	DMFE	2.65×10^7
"	DCPIP	2.04×10^6

discussed further below.

5.2.6 NADH calibration using an amperometric assay

Having established that the reaction of NADH with diaphorase can give rise to an electrochemical response the reaction scheme can be used to assay for NADH (see fig 5.17).

The NADH assay is carried out at a fixed potential anodic of that at which maximum oxidation of the mediator occurs (in this case at +400 mV vs SCE).

Table 5.23 and figure 5.23 show the results of a steady state assay in which current flowing in response to NADH by the reduction of diaphorase then ferricinium is measured.

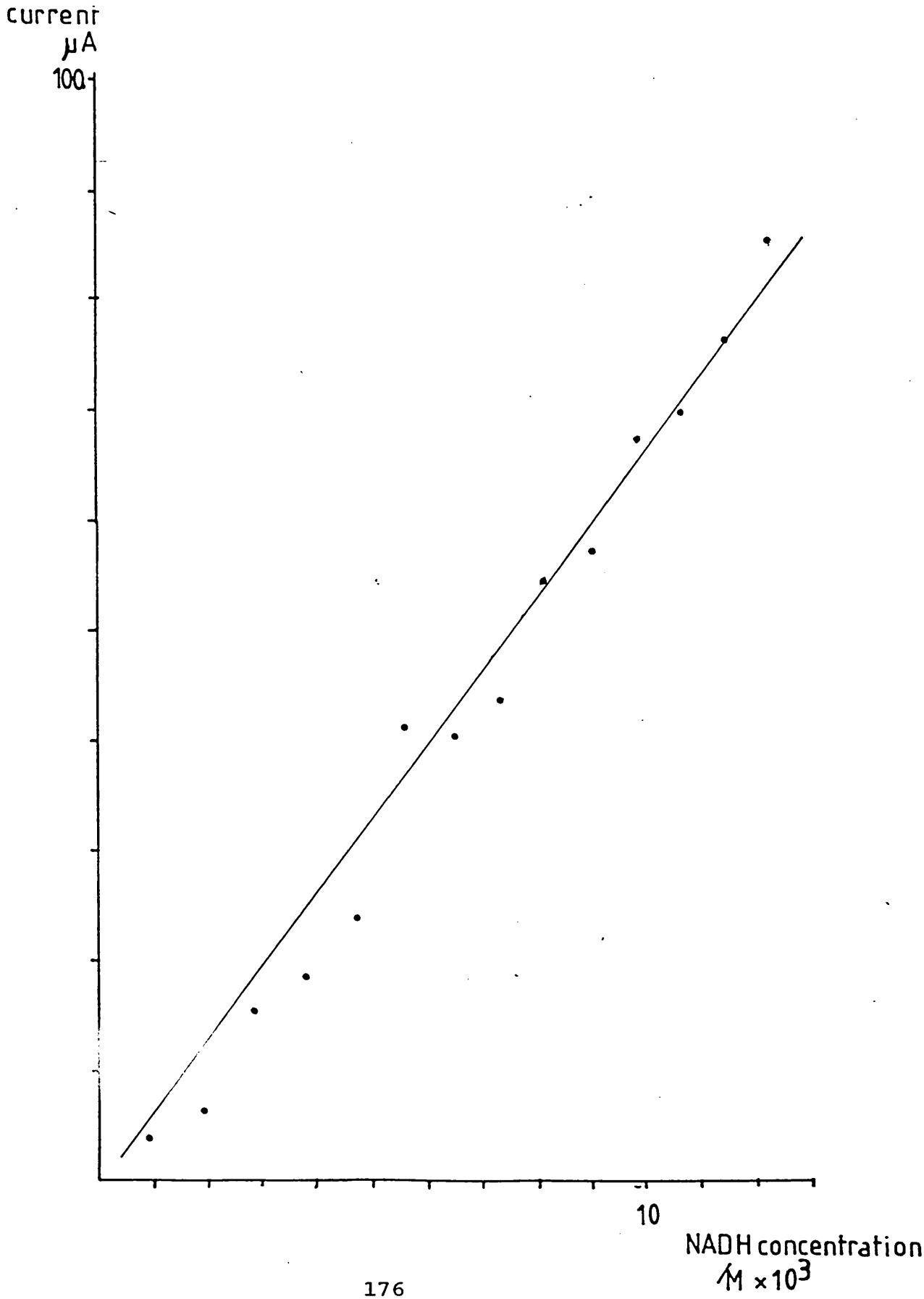
A straight line calibration is obtained up to 13 mM NADH confirming that at these concentrations the reaction of diaphorase with NADH is rate limiting.The assay is similar to a chronoamperometry experiment and k_s is thus proportional to NADH concentration over the range assayed (see section 2.5).

In the spectrophotometric assay NADH saturates the enzyme at a concentration of 250 uM.The electrochemical assay however gives a linear response to NADH concentrations up to at least 13mM.Since the diaphorase reaction is not being saturated Vm cannot have been reached and thus the NADH concentration to which the enzyme is exposed must be well below the Km.This occurs because oxidised diaphorase is only present at the electrode surface.Initially the NADH concentration at the electrode is the same as that in bulk solution.However as the electrochemical reaction proceeds NADH becomes depleted at the electrode surface and its

Table 5.23 Electrochemical NADH calibration

NADH concentration ($/Mx10^{3}$)	Current (uA)
0.99	3.6
1.96	6.2
2.91	15.4
3.85	18.3
4.76	23.6
5.66	41
6.54	40
7.41	43.5
8.23	54
9.09	57
9.9	67
10.7	69
11.5	76
12.28	85

Figure 5.23 Amperometric NADH assay



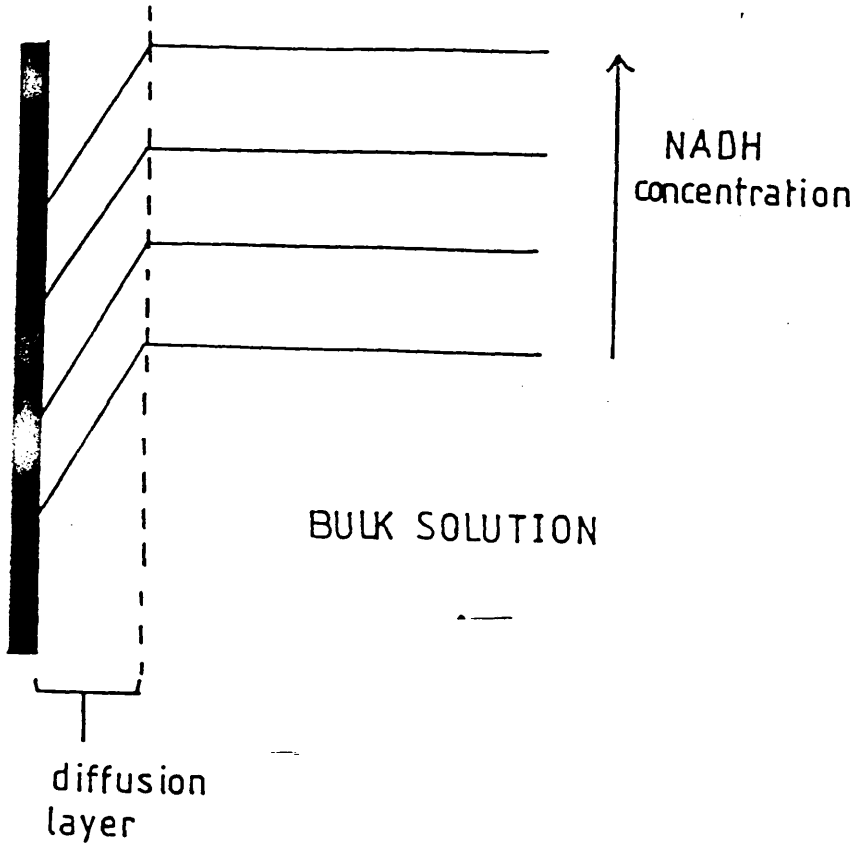
concentration there is determined by diffusion from the bulk solution (see figure 5.24).A higher bulk concentration thus sustains a higher electrode surface concentration and gives a larger current.

In the electrochemical assay the necessity for diffusion to the electrode increases the linear range of the assay but also decreases its sensitivity compared with the spectrophotometric assay.Higher diaphorase concentrations are also required in the electrochemical assay to give a measurable response.

5.2.7 Summary of the kinetic analysis of the electrochemical reaction scheme

Cyclic voltammetry experiments have allowed the direct determination of k_f, a first order rate constant, for the electrochemical reaction shown in fig 5.5.Since k_f is dependent on enzyme concentration [E] the second order rate constant k_s ,determined from k_f and [E] refers to either of the reaction steps which are first order for enzyme (steps 1 and 2 in reaction 5.22).Both of these are second order overall as they are each dependent on mediator and NADH.Spectrophotometrically determined second order constants (kcat/Km) for the reaction of oxidised diaphorase with NADH and reduced diaphorase with DCPIP are the same as the k_s's determined electrochemically.This agreement confirms that electrochemistry can be usefully applied to a kinetic analysis of the reaction but assignment of the rate limiting reaction is required if this constant is to be compared with

Figure 5.24 The concentration profile of NADH at the electrode surface in an amperometric assay



spectrophotometrically determined values.The fact that kcat/Km for reaction 1 in fig 5.22 cannot be determined spectrophotometrically means that this information alone cannot be used to distinguish which step is rate determining. Further experiments however have shown that, in the electrochemical experiment the reaction rate is dependent on NADH concentration but it is independent of both mediator type and concentration.Therefore reaction 2, the reduction of diaphorase by NADH, must be the rate limiting step under the conditions used.

In the diaphorase- NADH reaction assayed electrochemically saturation does not occur up to 13mM NADH.The response of a glucose sensing electrode based on the same principle as described here saturates at reducing substrate (glucose) concentrations of 35mM (69) (the Km of glucose oxidase for glucose is 4mM(77)).A similar kinetic analysis to that described was carried out in the presence of saturating glucose (100 mM)and k_s was found to be dependent on mediator type; this is consistent with reaction 1 being rate limiting.Oxidised mediator concentration at the electrode must be assumed to be that of reduced mediator in the bulk, where a fast electrode reaction occurs (see section 2.4.1).Under conditions where both reduced and oxidised substrate are saturating (if it is possible to supply such concentrations of each) in the electrode reaction k_f would approach kcat and the rate will be a maximum for that enzyme concentration.These conditions cannot be demonstrated in the diaphorase system because NADH interferes with the catalytic

current when present at high concentrations (see section 5.2.1)

5.2.8 The development of a biosensor from the NADH assay demonstrated

Practical enzyme electrodes for use as biosensors are usually constructed with the enzyme either attached to or trapped behind a membrane over the electrode surface.This arrangement provides an integrated device and the enzyme stability is often improved compared with that in solution.The membrane component also serves to increase the diffusional barrier to substrate further extending the linear range of the assay.

An initial kinetic analysis of the system as described must however be conducted in solution as immobilization may alter the properties of the enzyme.

The ferrocene mediated electrochemistry of diaphorase has been demonstrated, analysed kinetically and its use as an NADH assay described.Some applications of this NADH assay are investigated in the next section and in chapter 6.

5.2.9 Application of the ferrocene mediated electrochemistry of diaphorase to the measurement of ethanol

NADH produced either chemically or biochemically can be assayed amperometrically via diaphorase and ferrocene as described above.The addition of any dehydrogenase enzyme to the electrochemical cell provides the scheme shown in figure 5.6.This scheme provides an assay system for the dehydrogenase substrate.

Table 5.24 and figure 5.25 show the results of an ethanol assay in which NADH produced in the alcohol dehydrogenase (ADH) catalysed reacton of NAD and ethanol is measured amperometrically.

The calibration curve shows a high background current of 7.9 uA which corresponds to an NADH concentration of approximately 1.7 uM.The background may be due to an NADH impurity in the NAD^+ added .The NAD^+ concentration is 30 mM and the background NADH would thus represents a minor impurity. This is unlikely since background currents were subtracted from currents measured in the presence of ethanol. The non-zero intercept must be due to the addition of some background electroactive species but it is unclear what this contaminant might be. The explaination of the intercept is therefore not known.

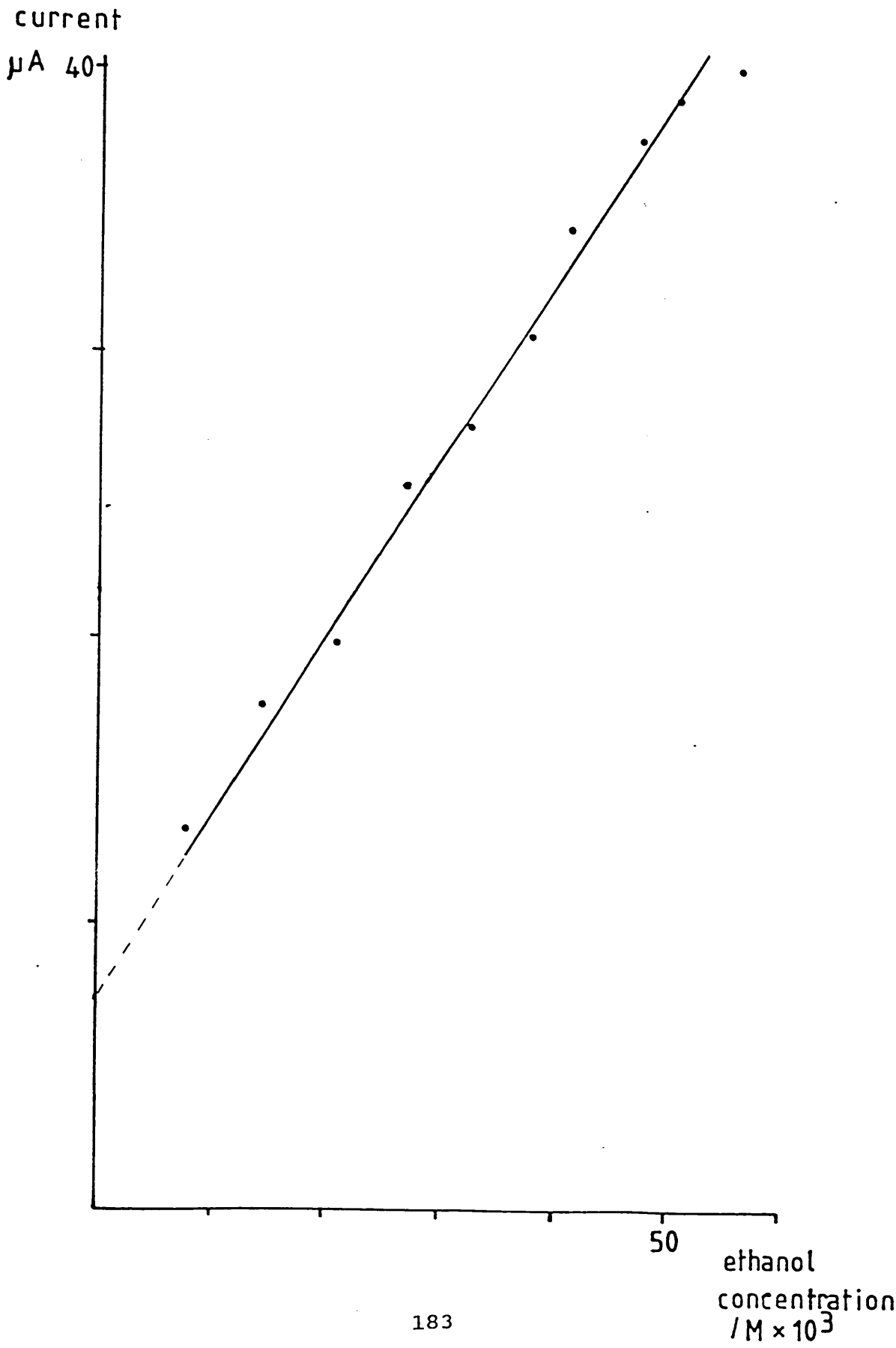
The calibration curve shows a similar current-concentration response to another amperometric ethanol sensor based on the detection of NADH at an electrode made from the conducting organic salt NMP+TCNQ-(79).

Several applications exist for an alcohol sensor in both medicine and industry (80) and this demonstrates only one of the dehydrogenases whose substrates can be detected with this assay system.

Table 5.24 Electrochemical ethanol calibration

Ethanol concentration $(/Mx10^{3})$	Current (uA)
7.6	13.2
14.6	17.6
21.1	19.8
27.2	25.4
32.9	27.4
38.2	30.6
43.2	34.4
47.9	37.6
52.3	39
56.5	40

Figure 5.25 .Amperometric ethanol calibration



APPLICATION OF THE AMPEROMETRIC NADH ASSAY TO THE DETECTION OF LOW CONCENTRATIONS OF PYRIDINE NUCLEOTIDES

6.1 Introduction

6.1.1 Enzyme amplified assays

The sensitivity of an enzyme assay can be drastically improved if an enzyme catalysed cycle is present.The principle of this type of assay is that two enzymes are used to catalyse the interconversion of a substrate between two forms.The enzymes will also each require the presence of one or more other substrates for catalysis as shown in figure 6.1.The rate at which the cycle turns is proportional to the concentration of the limiting substrate (in this case either NAD^+ or NADH), provided that neither enzyme is saturated with this substrate, and can be measured by following the accumulation of Q or the disappearance of X in figure 6.1

Enzyme cycling has produced several sensitive assays for the pyridine nucleotides (71), which are ideal analytes since many enzymes catalyse their interconversion between oxidised and reduced forms.

The amperometric scheme described in section 5.1.3 is analogous to that shown in figure 6.1 and in the presence of excess ethanol provides an amplified assay for either NAD^+ or NADH, a spectrophotometric version of this already exists (see section 5.1.3).

The work described in this chapter demonstrates the application of the amperometric NADH assay described in chapter 5 to the detection of low concentrations of pyridine

Figure 6.1 The scheme of an enzyme amplified assay

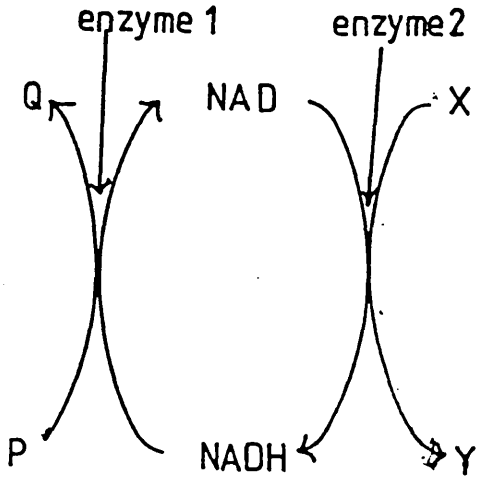


Figure 6.1 The scheme of an enzyme amplified assay

Scheme used in the AMPAK test kit is as above where;

P = INT violet

Q = INT formazan

X = Ethanol and

Y = Acetaldehyde

nucleotides and/or alkaline phosphatase by the use of an enzyme catalysed cycle.

6.1.2 Alkaline phosphatase detection in enzyme linked immunosorbent assay (ELISA)

The principle of ELISA has been described in section 1.5.2.2 .The main practical disadvantage of using enzyme labels instead of radiolabels in immunoassays is that they cannot be detected with the same high sensitivity(71).Enzyme labels are however more stable, safer and cheaper to detect and thus methods of improving ELISA sensitivity are sought.

Conventional enzyme assays, based on the detection of either substrate or product, (see section 1.6) are limited to the sensitivity with which these can be measured.The enzyme amplification scheme shown in figure 6.1 can enhance the sensitivity of pyridine nucleotide detection and if the enzyme label used in an ELISA measurement can catalyse the production of either NAD^+ or NADH the scheme can be applied to ELISA systems.Unfortunately the assay cannot detect dehydrogenase enzymes because alcohol dehydrogenase is present in excess.The production of the nucleotide must therefore be by a separate reaction which is irreversible under the conditions of the assay.

One of the commonest labels in ELISA is alkaline phosphatase(81) which is normally detected by a colourimetric assay in which it catalyses the hydrolysis of paranitrophenyl phosphate.The resulting paranitrophenol has a strong yellow colour at alkaline pH and can be measured by its absorbance at 410nm.Alkaline phosphatase shows broad substrate

specificity and can also catalyse the cleavage of the phosphate ester bond in $NADP^+$.The resulting NAD^+ can then be sensitively detected by the use of an enzyme amplified cycle.

6.2 Results and discussion

6.2.1 Spectrophotometric enzyme amplified assays

The assay developed by Self et al(71,72) (outlined in figure 6.1) is available in the form of a test kit for alkaline phosphatase.For comparison with amperometric assays alkaline phosphatase was calibrated using this kit.The results of the assay are given in table 6.1 and figure 6.2.

The reaction is performed in two steps; $NADP^+$ cleavage followed by NAD^+ estimation using enzyme amplification with detection of INT formazan.The $NADP^+$ cleavage step must be terminated before the amplifying reagents are added to obtain a linear calibration for alkaline phosphatase.

Alkaline phosphatase concentrations down to $1x\ 10^{-7}M$ can be easily detected in the assay. In the presence of excess ethanol and INT violet the assay sensitivity is dependent on the rate of the diaphorase and ADH catalysed reactions and the time for which the formazan is allowed to accumulate.

Since the reagents being used in this assay are a commercial preparation the enzyme concentrations are unknown,therefore further spectrophotometric assays were performed in the presence of known amounts of enzymes.The assays were for NADH and the results with either INT or DCPIP as indicator are given in tables 6.2 and 6.3 and figures 6.3 and 6.4.

Table6.1 Alkaline phosphatase calibration using AMPAK test kit

Alkaline phosphatase concentraton (/Mx10^{8})	Reaction velocity (/M INT sec^{-1} x10^{8})
2.97	8.5
5.94	14.95
8.91	23.8
11.9	32.3
14.9	38.2
17.8	50.5
20.8	63.3
29.7	92

Figure 6.2 Alkaline phosphatase using AMPAK test kit

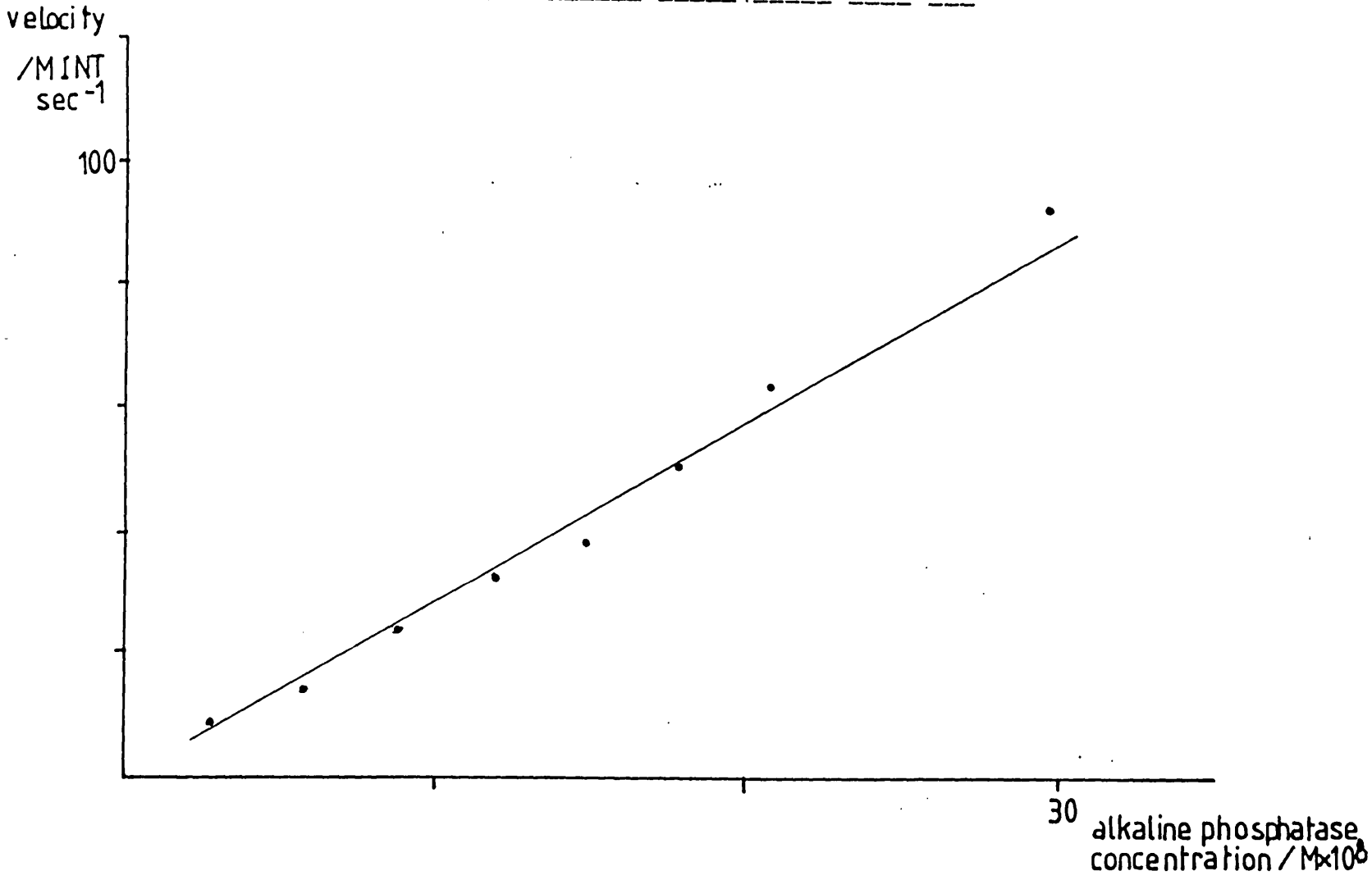


Table 6.2 NADH assay using spectraphotometric determination of INT fomazan

NADH concentration ($/Mx10^{6}$)	reaction velocity ($/M\ INT\ sec^{-1}\ x10^{8}$)
0.33	3.5
0.66	8.08
1	8.51
1.33	16
1.66	18.7
2	23.9
2.33	27.6
2.66	27.7
3	33.9
3.33	42.5

Figure 6.3 NADH calibration using spectrophotometric detection of INT formazan

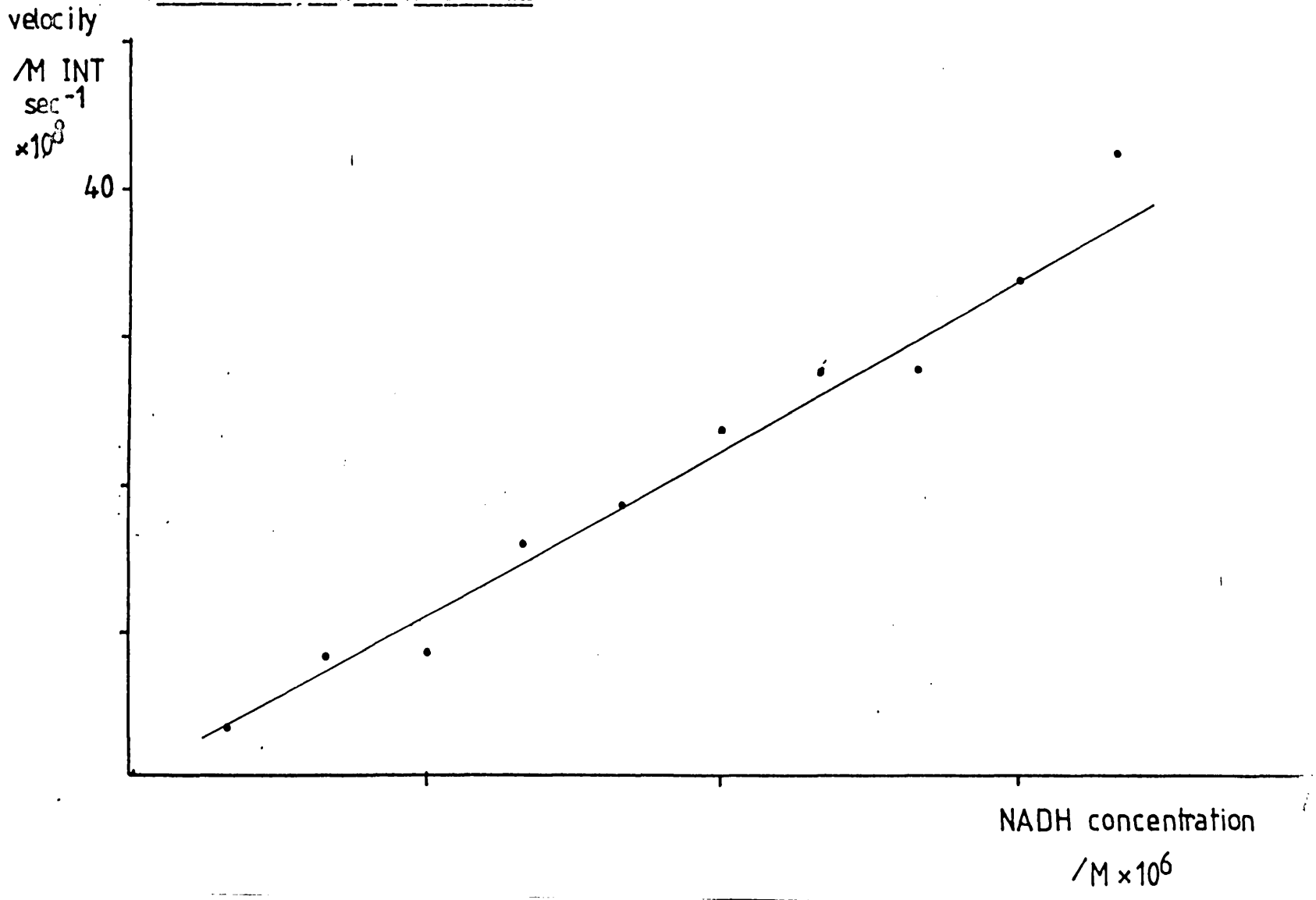


Table 6.3 NADH concentration using spectrophotometric detection of reduced DCPIP

NADH concentration ($/M \times 10^{6}$)	reaction velocity ($/M$ DCPIP $sec^{-1} \times 10^{8}$)
0.33	5.7
0.6	11.4
1	19
1.33	22.7
1.66	27.7
2	34.5
2.33	36.8
2.66	40.5
3	49.6

Assays with either of these indicators under the conditions used give linear calibration at the micromolar level, optimizaton of enzyme concentrations and incubation times would be expected to improve this sensitivity.

6.2.2 Electrochemical enzyme amplified assays

6.2.2.1 Amplified assay with direct detection of NADH through diaphorase and ferrocene

Tables 6.4 and 6.5 show the results of amplified assays for NAD^+ and NADH using diaphorase coupled DMFE electrochemistry as the detection system.These results are plotted in figures 6.6 and 6.7 along with the results of a non-amplified NADH assay(see tables 6.6and figure 6.5).All three assays were carried out using the same concentrations of diaphorase and DMFE.

6.2.2.2 Mechanism and amplification in the enzyme cycled system

From figures 6.5 and 6.7 a NADH concentration of $4x10^{-5}M$ yields a current of 3.45uA in the amplified assay.This is equivalent to that produced by a NADH concentration of $33.6x10^{-5}M$ in a non-amplified system and represents an 8.4 fold increase in effective NADH concentration.

In both amplified and non-amplified assays the current measured is due to the oxidation of ferrocene at the electrode surface.In the non-amplified assay the current increases with NADH concentration as this determines the rate at which ferrocene is regenerated in the diaphorase catalysed reaction (see section 5.2.6).In the amplified assay however

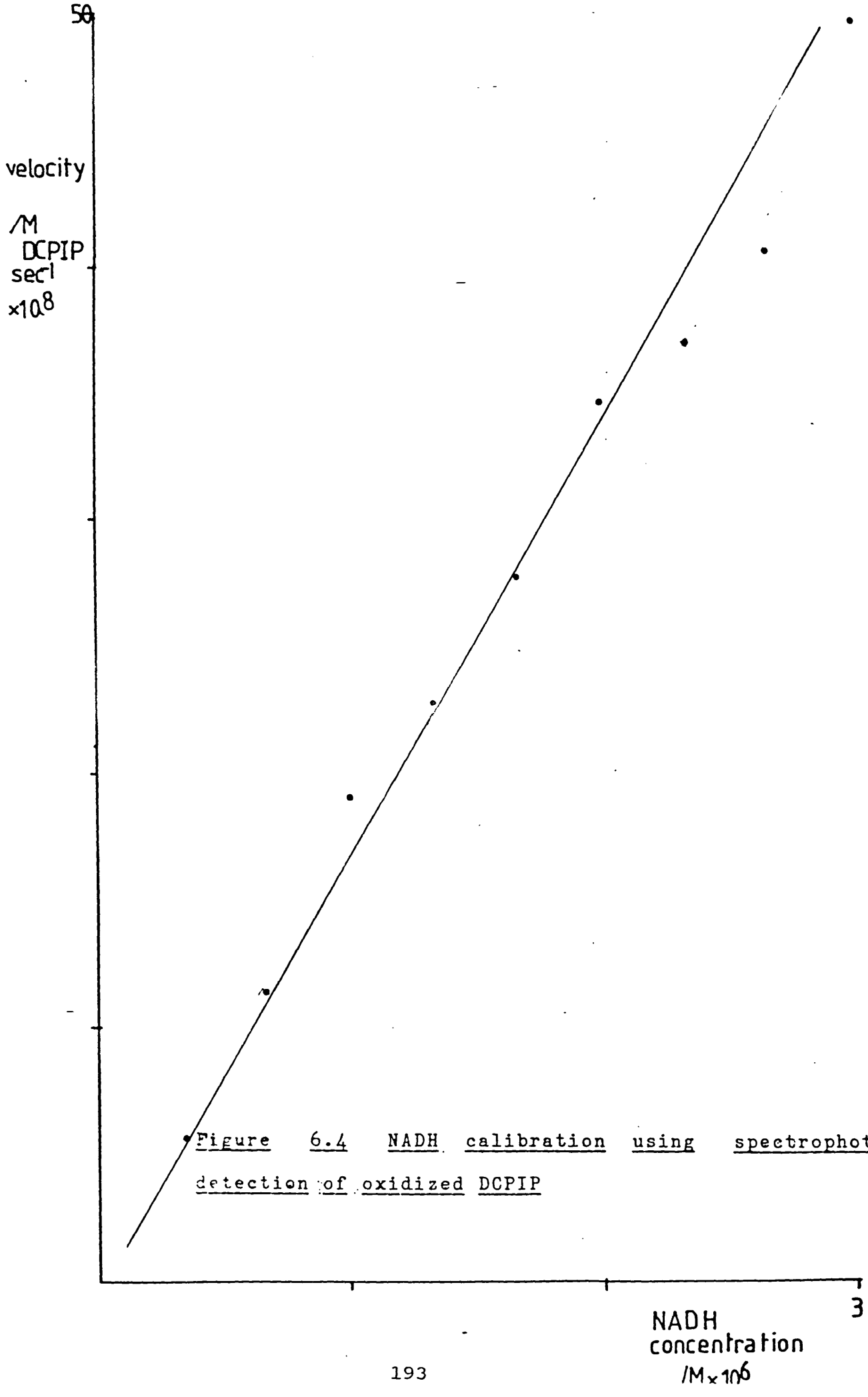


Figure 6.4 NADH calibration using spectrophotometric detection of oxidized DCPIP

Table 6.4 Amplified assay for NAD^+ with amperometric detection of DMFE

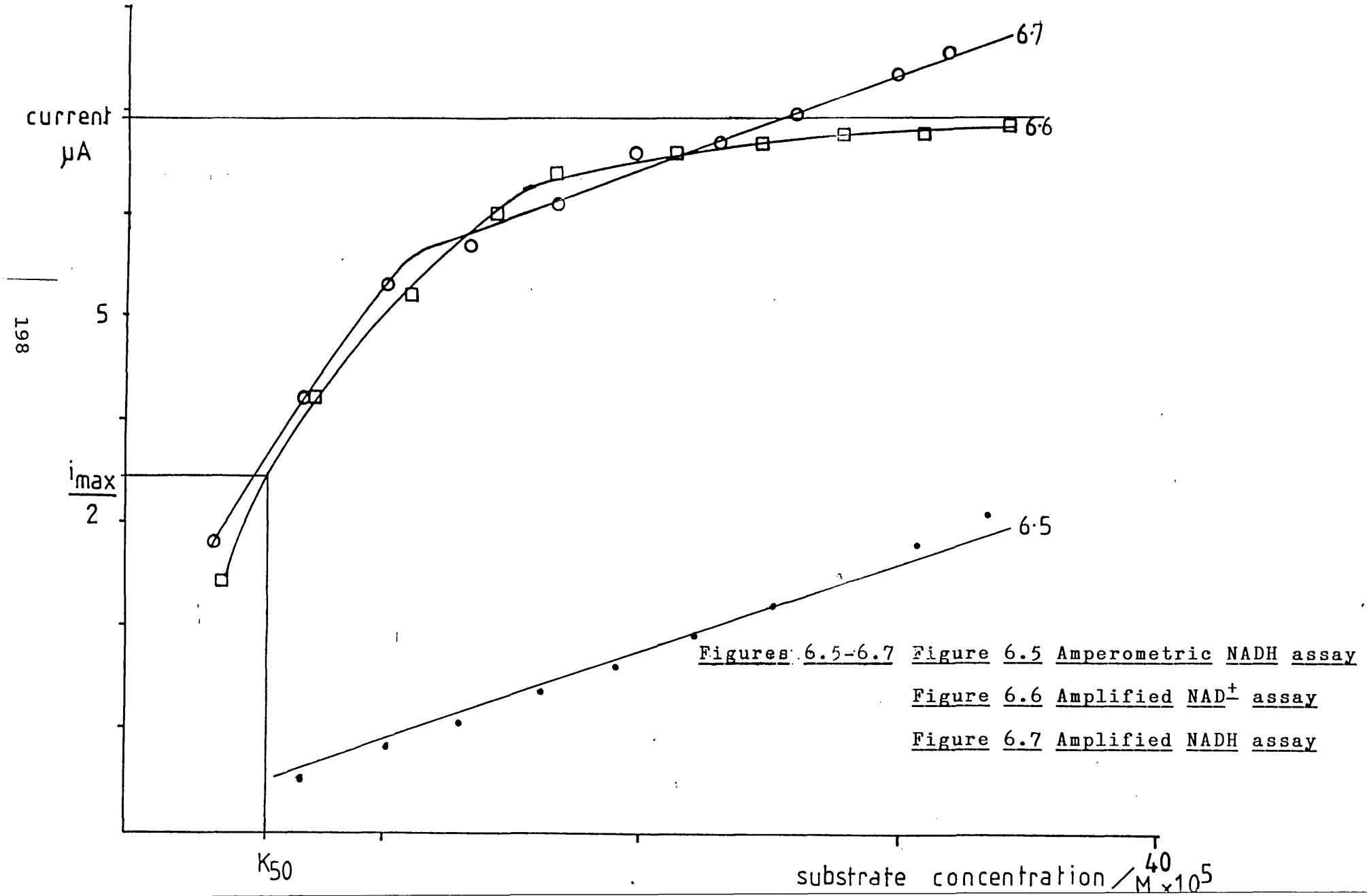
NAD concentration (/Mx10^5)	Current (μA)
3.8	2.4
7.4	4.2
11	5.2
14.4	6
16.7	6.4
21.3	6.6
24.6	6.7
27.8	6.8
30.9	6.8
34.1	6.9

Table 6.5 Amplified NADH assay with amperometric detection of DMFE

NADH concentration ($/Mx10^5$)	Current (μA)
3.4	2.8
6.9	4.2
10.2	5.3
13.4	5.7
16.7	6.1
19.8	6.6
23	6.7
26	7
28.8	7.4
31.8	7.6

Table 6.6 Non amplified amperometric NADH assay

NADH concentration ($/Mx10^5$)	Current (μA)
3.4	0.3
6.6	0.5
9.8	0.8
13	1.05
16.1	1.35
19.04	1.6
22.1	1.9
25.1	2.2
27.9	2.5
30.7	2.8
33.5	3.1



Figures 6.5-6.7 Figure 6.5 Amperometric NADH assay

Figure 6.6 Amplified $NAD^{\pm}$ assay

Figure 6.7 Amplified NADH assay

the rate of ferrocene regeneration is determined by the rate at which NADH is produced in the alcohol dehydrogenase catalysed reaction.The rate of this reaction is in turn controlled by the NAD^+ concentration added to the electrochemical cell and the rate at which it is produced in the diaphorase catalysed reaction.

In the non - amplified amperometric assay the NADH concentration required to saturate the diaphorase catalysed reaction is much higher than that required in a homogeneous spectrophotometric assay.This is due to the NADH concentration at the electrode surface being lower than that in bulk solution , as described in section 5.2.6.

The amperometric amplified assays however reach a maximum current at bulk substrate concentrations which do not saturate a non-amplified amperometric NADH assay (see figures 6.5-6.7).Initially, the concentration of either NAD^+ or NADH at the electrode surface will be the same as that added to bulk solution.Once cycling commences the concentrations of both nucleotides are determined by the rate of the enzyme catalysed cycle.

In figure 6.6 the amplified NAD^+ assay levels off completely whilst the current in the NADH assay continues to rise.The slope of the latter half of the NADH calibration curve is the same as that of a non-amplified NADH assay.This result suggests that at high NADH concentrations the reduced nucleotide can still be assayed through diaphorase and ferrocene and it must therefore be the alcohol dehydrogenase which has become saturated.Once the alcohol dehydrogenase

catalysed reaction has reached Vmax the enzyme catalysed cycle cannot turn any faster and the addition of further aliquots of NAD^+ gives no increase in current.

The value of substrate concentration at half the limiting current has been designated as K_{50} in figure 6.6.This parameter has a value of 5.6 x 10^{-5}M, determined by approximating imax to 6.9 uA as shown in figure 6.6.This value will be determined by the substrate concentration at which the rate limiting reaction saturates and thus on the Km of this reaction.In this case it is the Km of the alcohol dehydrogenase catalysed reaction which will determine K_{50} since this saturates first.

The Km values of diaphorase for NADH and alcohol dehydrogenase for NAD^+ are $2.7x10^{-5}$M and $7.4x10^{-5}$M respectively (67,78).The similarity of these values suggests that, as the alcohol dehydrogenase reaction saturates first, the NAD^+ concentration at the electrode surface is higher than the NADH concentration.This in turn suggests that the diaphorase catalysed reaction is faster than the ADH reaction thus the latter will be rate limiting.

Although this configuration of the amperometric amplified assay provides considerable current enhancement compared with a non-amplified assay, aswell as facilitating the determination of both oxidised and reduced forms of the nucleotide, the assay sensitivity cannot be altered for a constant enzyme concentration.In the IQ Bio amplified alkaline phosphatase assay and the spectrophotometric enzyme

amplified assays described above the sensitivity is dependent on the cycling time allowed and can therefore be altered according to requirements.This flexibility is lost in the direct amperometric assay, higher sensitivity can only be acheived by the addition of more enzyme.This approach will eventually lead to an upper limit of sensitivity as high protein concentrations will interfere with the electrode reaction (83)

The next section describes an amperometric assay in which reduced mediator is accumulated before measurement at the electrode in an attempt to combine flexible sensitivity with the ease of electrochemical detection.

6.2.2.3 Amplified assay with amperometric detection of accumulated mediator

In the direct amperometric assay described the substrate for diaphorase is produced by an electrode reaction.If accumulation of reduced mediator is to occur excess oxidised mediator must be present at the beginning of the assay (as for the spectrophotometric assay shown in figure 6.1 where P is provided in excess).In order to use an oxidised ferrocene (ferricinium) derivative as the oxidant in the diaphorase reaction, to provide an assay analogous to that described in section 6.2.2.1,this must first be synthesised as none is commercially available.

6.2.2.4 Ferricinium synthesis

Ferricinium derivatives are unstable compared with ferrocene.They can however be synthesised by oxidising ferrocene using concentrated acid and this method was used

here.

After oxidation the ferricinium concentration was measured at timed intervals to assess its stability.The concentration was estimated by absorbance at 615nm (ε =335 $M^{-1}cm^{-1}$) in buffered solution at pH 8.5 (the optimum for the cycling assay).The results of the stability study both before and after an ether extraction step are given in table 6.7 and figure 6.8.

The ferricinium concentration remained constant for over 24 hours after oxidation suggesting a reasonable degree of stability.After ether extraction however the ferricinium concentration dropped until a steady value was again reached.Ether extraction removes some of the less polar ferrocene from the reaction product mixture.The simultaneous drop in ferricinium concentration suggests that there is a dynamic equilibrium between the oxidised and reduced forms of the molecule.Removal of ferrocene thus causes the equilibrium to shift to compensate for the loss and reproduce the stable molecule.Thus a certain proportion of the reaction mixture will be ferricinium which is only stable in the presence of sufficent ferrocene.It has been reported that the stability of ferricinium increases with decreasing pH and that the shift in equilibrium occurs by disproportionation of the oxidised molecule to a mixture of ferrocene and ferric hydroxide, of which approximately 2/3rds is ferrocene(84).

The equilibrium mixture of ferrocene and ferricinium produced was used to provide the oxidant in the enzyme

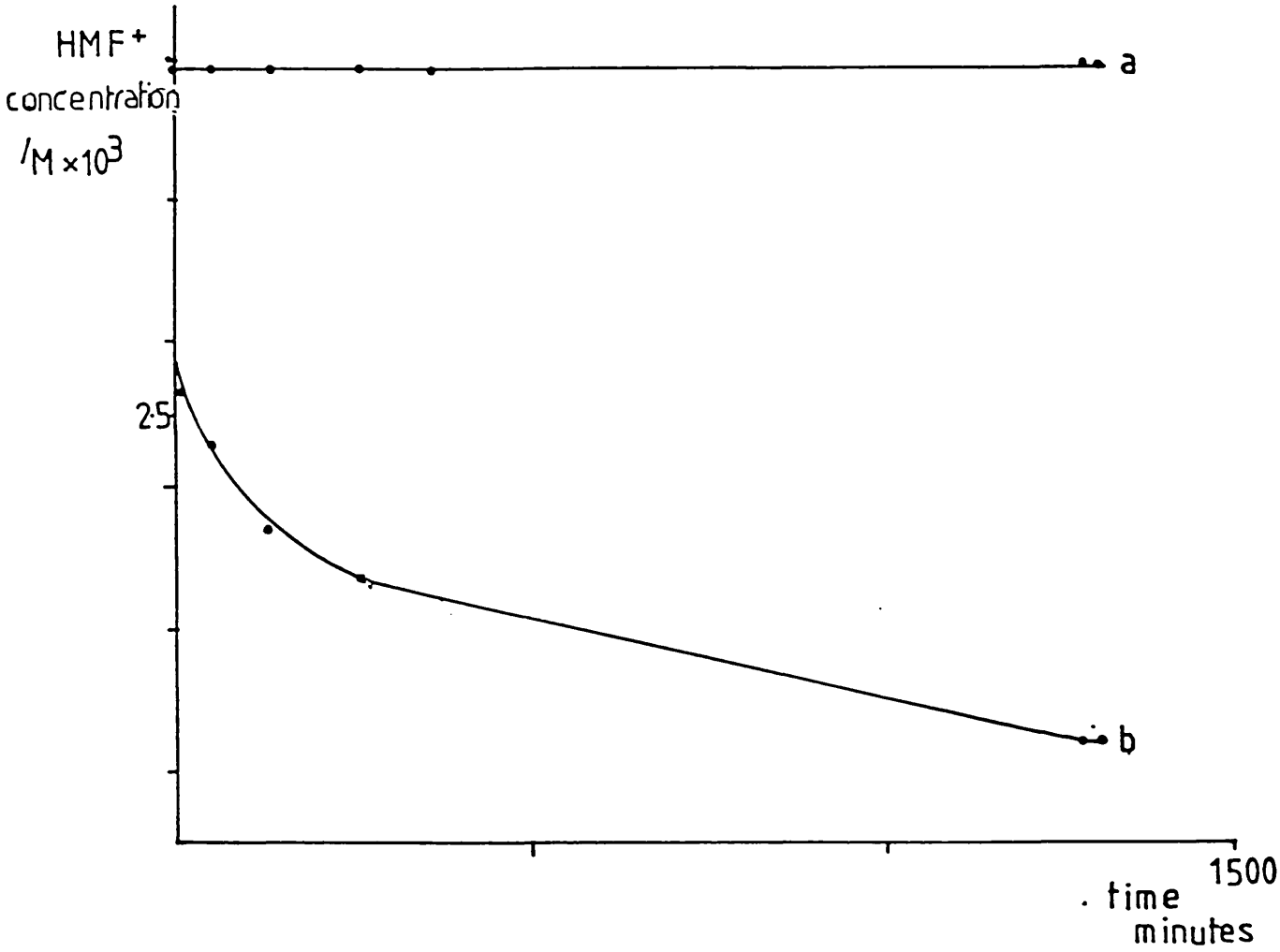
Table 6.7 Concentration of hydroxymethyl ferrocenium in ether extracted and non extracted preparations at various times after synthesis

Time (minutes)	Concentration no extraction ($/Mx10^3$)	Concentration after ether extraction ($/Mx10^3$)
0	2.99	2.54
50	"	2.46
130	"	2.34
260	"	2.27
360	"	2.24
1290	"	2.04
1310	"	2.04

Figure 6.8 Ferrocenium concentration at different times after synthesis

a. Before ether extraction

b. After ether extraction



amplified assay.

6.2.2.5 Enzyme amplified assay with electrochemical detection of reduced ferrocene

Table 6.8 and figure 6.9 show the results of an enzyme amplified assay with chemically synthesised ferricinium as oxidant in the presence of two different enzyme concentrations.The two calibration curves confirm that doubling the enzyme concentration increases the slope of the graph by a factor of two and thus increases the sensitivity of the assay.

The ferrocene present at the beginning of the assay (see section 6.2.2.5) gives a large background current and the calibration above this background is innaccurate.The innaccuracy will be due partly to errors involved in measuring current above such a high background and partly to the instability of ferricinium.In order to improve the accuracy of the system stable ferricinium is required in a solution without ferrocene.Ferricinium would be stabilised to some extent by lowering the assay pH but this would also decrease the activity of the cycling enzymes and the sensitivity of the assay.Alternatively it may be possible to synthesize a stable derivative of ferricinium by attachment of stabilizing moieties to the cyclopentadienyl ring.Drastic derivatization may also reduce the rate of reaction of the molecule with diaphorase which would be detrimental to the assay.In an attempt to overcome these problems potassium ferricyanide was used as an alternative electron transfer mediator for diaphorase and its use in an amplified assay is

Table 6.8 Amplified NAD^+ assay using detection of accumulated hydroxymethyl ferrocene

NAD^+ concentration ($/Mx10^6$)	Current with 2.5 units of each enzyme (μA)	Current with 5 units of each enzyme (μA)
0	11.4	10.15
10	13.1	13.9
20	13.8	16.1
30	15.5	19.2
50	17.3	19.5
60	18.5	22.4
80	19	24.2
100	20.3	26.6

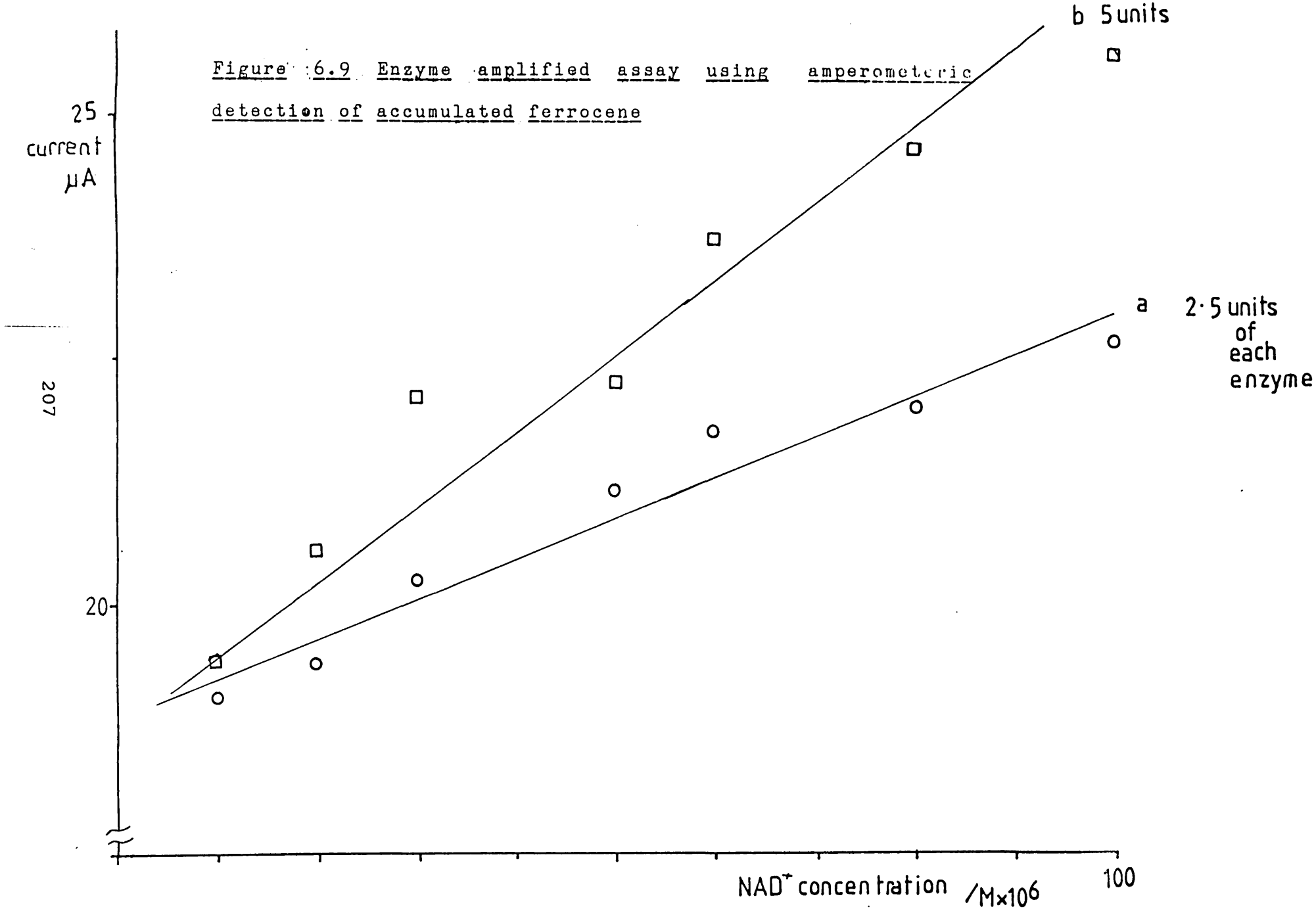


Figure 6.9 Enzyme amplified assay using amperometeric detection of accumulated ferrocene

described below.

6.2.2.6 Electrochemical enzyme amplified assay with ferricyanide as oxidant

Hexacyanoferrate contains a hexacoordinated iron atom whose oxidation state is altered between 3+ and 2+ by the addition or removal of electrons.The molecule is stable in aqueous solutions of both ferri and ferro forms and has been used in spectrophotometric assays as an electron acceptor for diaphorase (at 420 nm =1100 $M^{-1}cm^{-1}$)(44).

A cyclic voltammogram of 10 mM potassium ferricyanide is shown in figure 6.10.The molecule shows a reversible electrode reaction only in the presence of high concentrations of supporting electrolyte(85) and thus 0.1 M potassium chloride is added to the electrochemical cell.Table 6.9 and figure 6.11 show a calibration for ferrocyanide at a potential of +400 mV versus SCE showing the molecules electroactivity.Since ferricyanide also shows an optical absorption, the concentration of NAD^+ or NADH can be estimated by both electrochemical and spectrophotometric amplified assays where ferricyanide is the oxidant for diaphorase.

6.2.2.7 Enzyme amplified assay with spectrophotometric determination of ferricyanide

Table 6.10 and figure 6.12 show the results of an enzyme amplified assay for alkaline phosphatase where the final electron acceptor is ferricyanide the concentration of which is determined spectrophotometrically.

Figure 6.10 Cyclic voltammogram of potassium ferricyanide

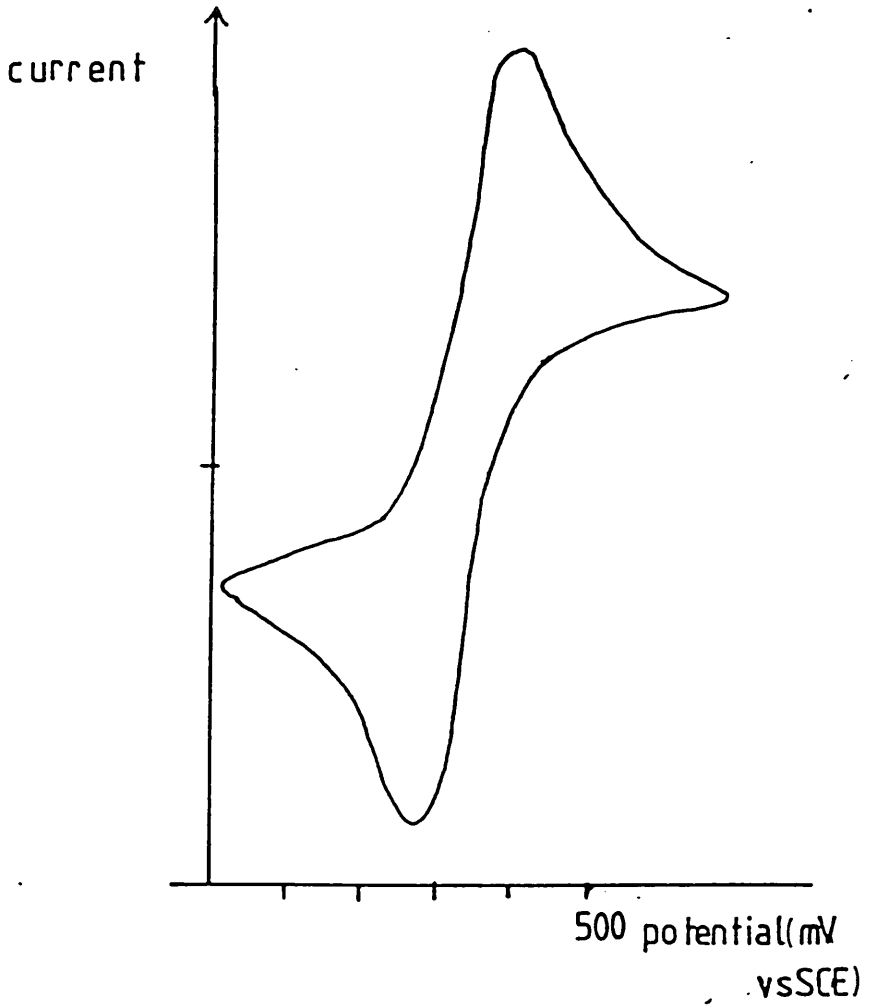


Table 6.9 Amperometric ferrocyanide assay at a gold

Ferrocyanide concentration ($/Mx10^{3}$)	Current (μA)
0.19	1.15
0.38	2.35
0.57	4.9
0.74	5.72
0.91	6.83
1.07	7.65
1.23	8.6
1.38	9.95
1.53	10.85

Figure 6.11 Amperometeric ferrocyanide assay at +400 mV vs SCE

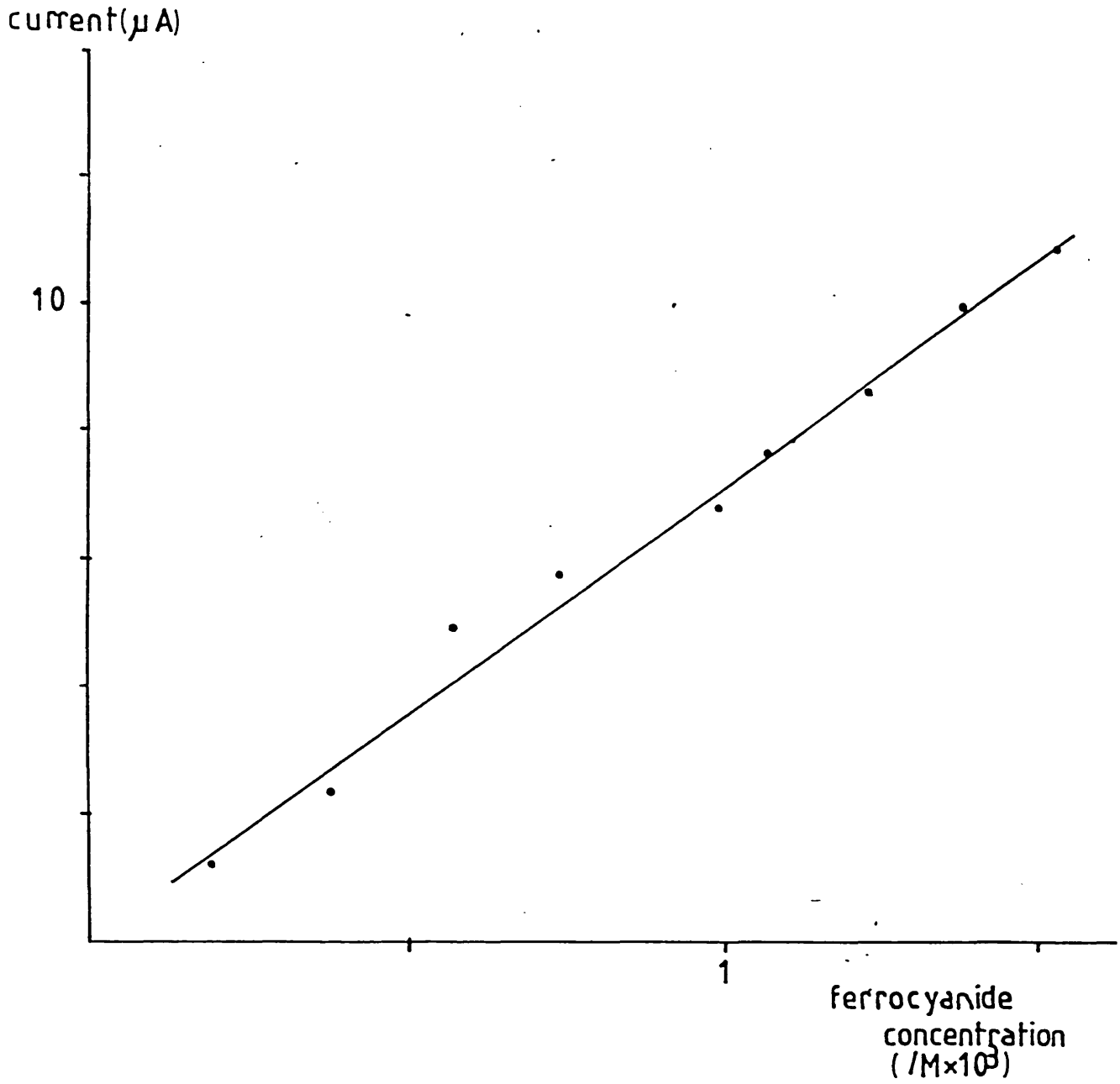
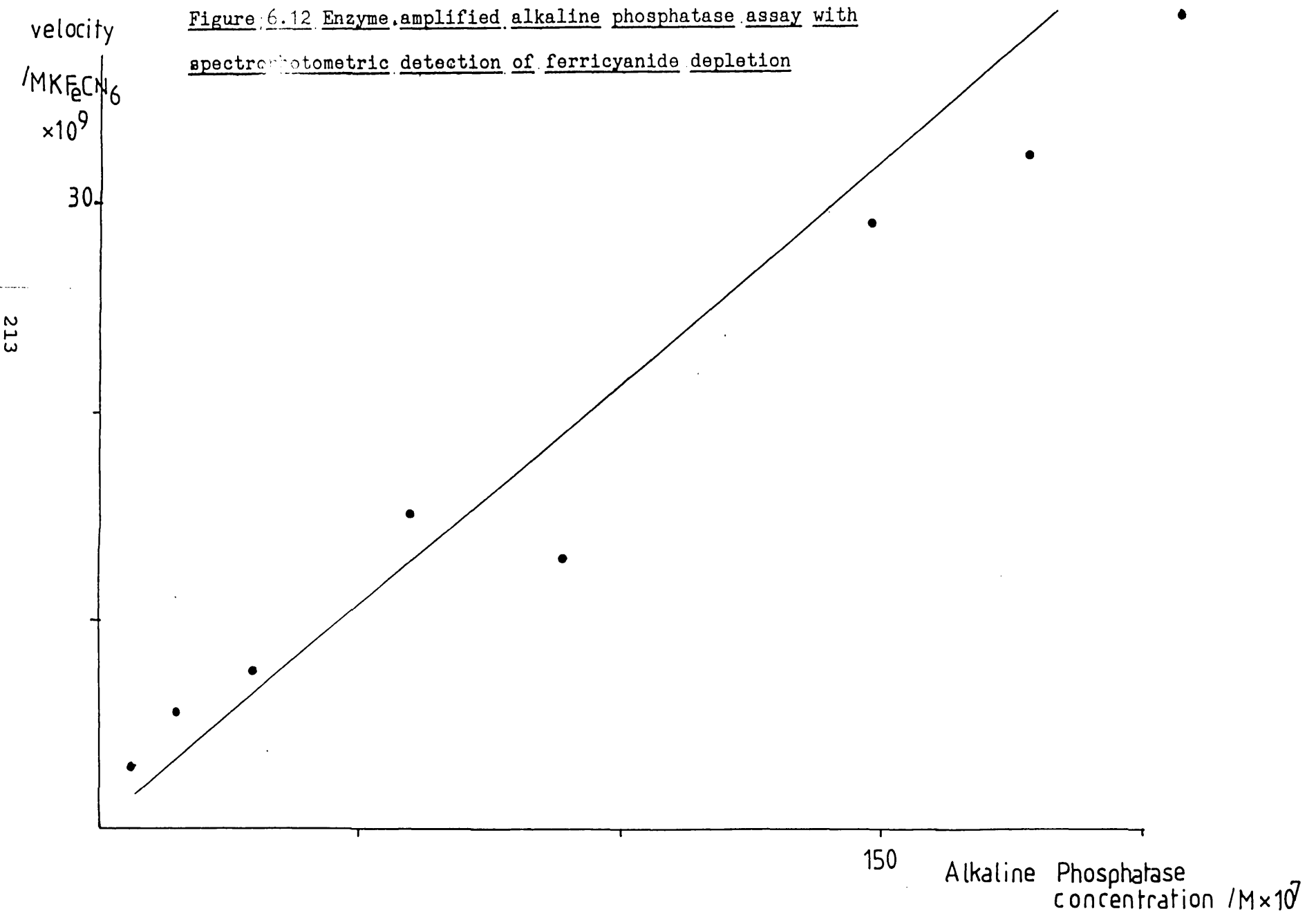


Table 6.10 Alkaline phosphatase assay with spectrophotometric detection of ferricyanide

Alkaline phosphatase concentration (/Mx10^7)	reaction velocity (/M ferricyanide sec^{-1} x10^9)
5.9	2.8
14.8	5.2
29.7	7.7
59.4	15
89.1	12.9
148.5	29
178.2	32
207.9	39
237.6	43

Figure 6.12 Enzyme amplified alkaline phosphatase assay with spectrophotometric detection of ferricyanide depletion



Inorganic phosphate was used to terminate the alkaline phosphatase catalysed reaction before adding cycling reagents and after incubation the cycling reaction was stopped by the addition of a mercury salt.At this stage potassium chloride was also added, which is important in the electrochemical estimation of ferricyanide (see section 6.2.2.5)and does not interfere with the optical density measurement.

The reduction in ferricyanide absorbance at 420nm is followed so the initial electron acceptor concentration is limited to that which obeys Beer's law (generally absorbances above 2.0 tend to deviate from a linear relationship with concentration, see section 1.6.1).This also limits the range of the assay since the electron acceptor eventually becomes depleted.The same problem exists in an assay using DCPIP as electron acceptor hence INTviolet is the preferred oxidant in a spectrophotometric assay.

The assay gives linear calibration of alkaline phosphatase concentration but higher rates of cycling than in assays using DCPIP or INT will be required to produce a detectable change in absorbance because ferricyanide has a lower extinction coefficent.

Although the spectrophotometric assay with ferricyanide is less sensitive than other systems it demonstrates that this molecule can act as terminal electron acceptor for diaphorase and further investigation,to determine the degree of amplification yielded by cycling, were carried out using the colorimetric ferricyanide system.

6.2.2.8 Amplification provided by enzyme cycling

Tables 6.11 and 6.12 and figures 6.13 and 6.14 show the results of non amplified and amplified assays for NAD^+ in which the final product of the enzyme catalysed reaction is measured spectrophotometrically.Reagent concentrations are the same in both assays but in the first no diaphorase is added and the absorbance of NADH, at 340 nm, is measured.In both cases the rate of change of absorbance is followed for a fixed NAD^+ concentration.

Without diaphorase NAD^+ becomes depleted and the initial reaction rate is taken for comparison with the amplified assay.The increase in rate of absorbance change is 4.6 fold (by comparison of data in tables 6.11 and 6.12) in the presence of 3.6 units of each enzyme.The amplification factor would be expected to increase further by increasing the enzyme concentration as described above.

6.2.2.9 Enzyme amplified assay for alkaline phosphatase with electrochemical detection of accumulated ferrocyanide

The amplified assay was carried out as for the colourimetric estimation described above but the ferrocyanide accumulated was measured by oxidation at a gold electrode at +400 mV versus SCE which is above the peak oxidation potential of the molecule (see figure 6.10) .Results of this assay are given in table 6.13 and figure 6.15

The calibration yields low currents above a high background.The residual current could be due to a slow turnover of the cycle by the high concentration of $NADP^+$ added

Table 6.11 Non amplified NAD^+ assay with spectrophometric detection of NADH

Time (sec^{-1})	NADH concentration ($/Mx10^{6}$)
5	1.29
25	14
45	21.5
65	32
85	42
105	50
125	53.5
145	54.3

Figure 6.13: Spectrophotometric assay of non amplified NAD^+ reduction

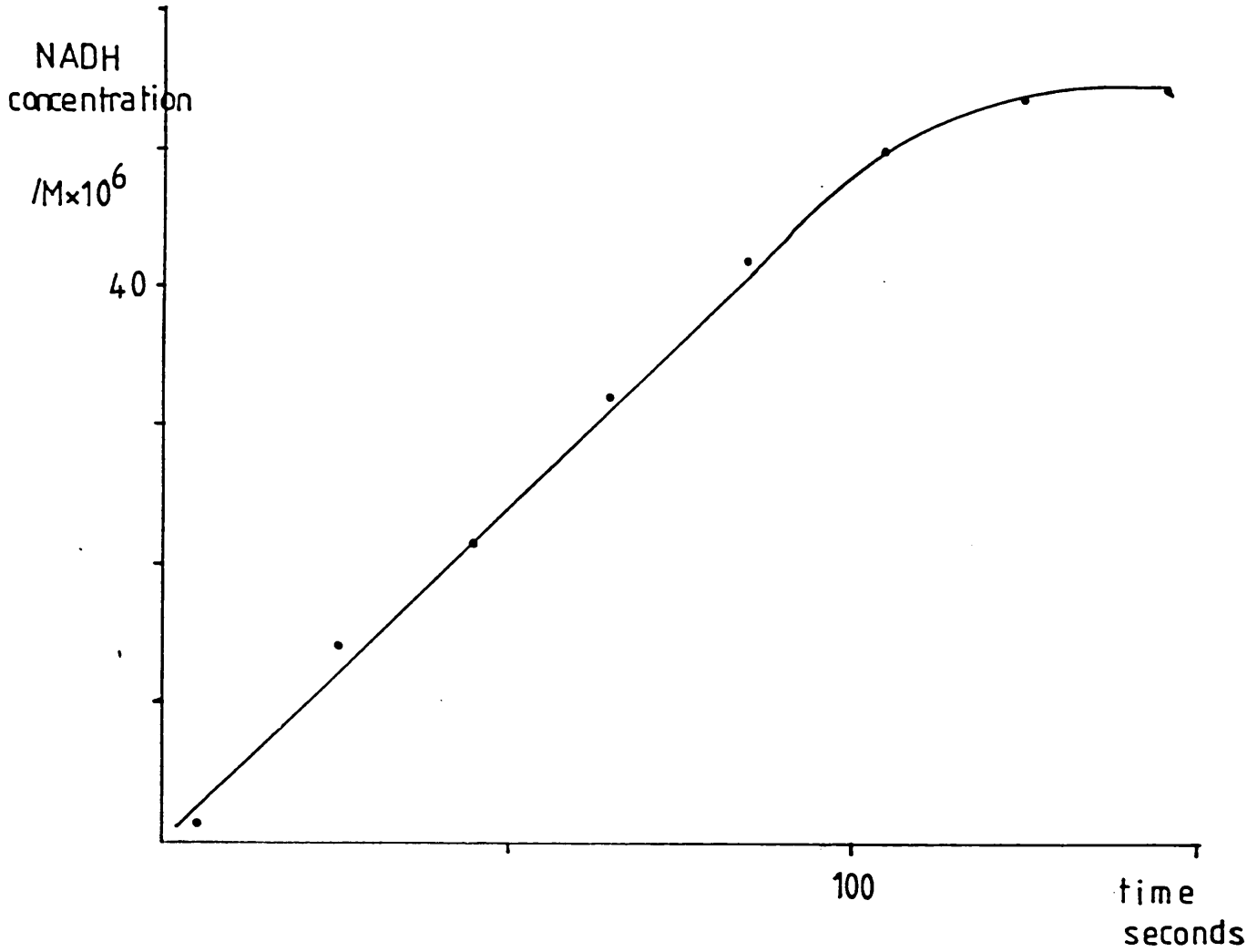


Table 6.12 Amplified NAD^+ assay with spectrophotometric detection of ferricyanide

Time (sec^{-1})	ferricyanide concentration ($/Mx10^{4}$)
5	10.07
25	9.6
45	9.15
65	8.78
85	8.35
105	7.85
125	7.32
145	7.12

Figure 6.14 Enzyme amplified assay for alkaline phosphatase with amperometric detection of ferrocyanide

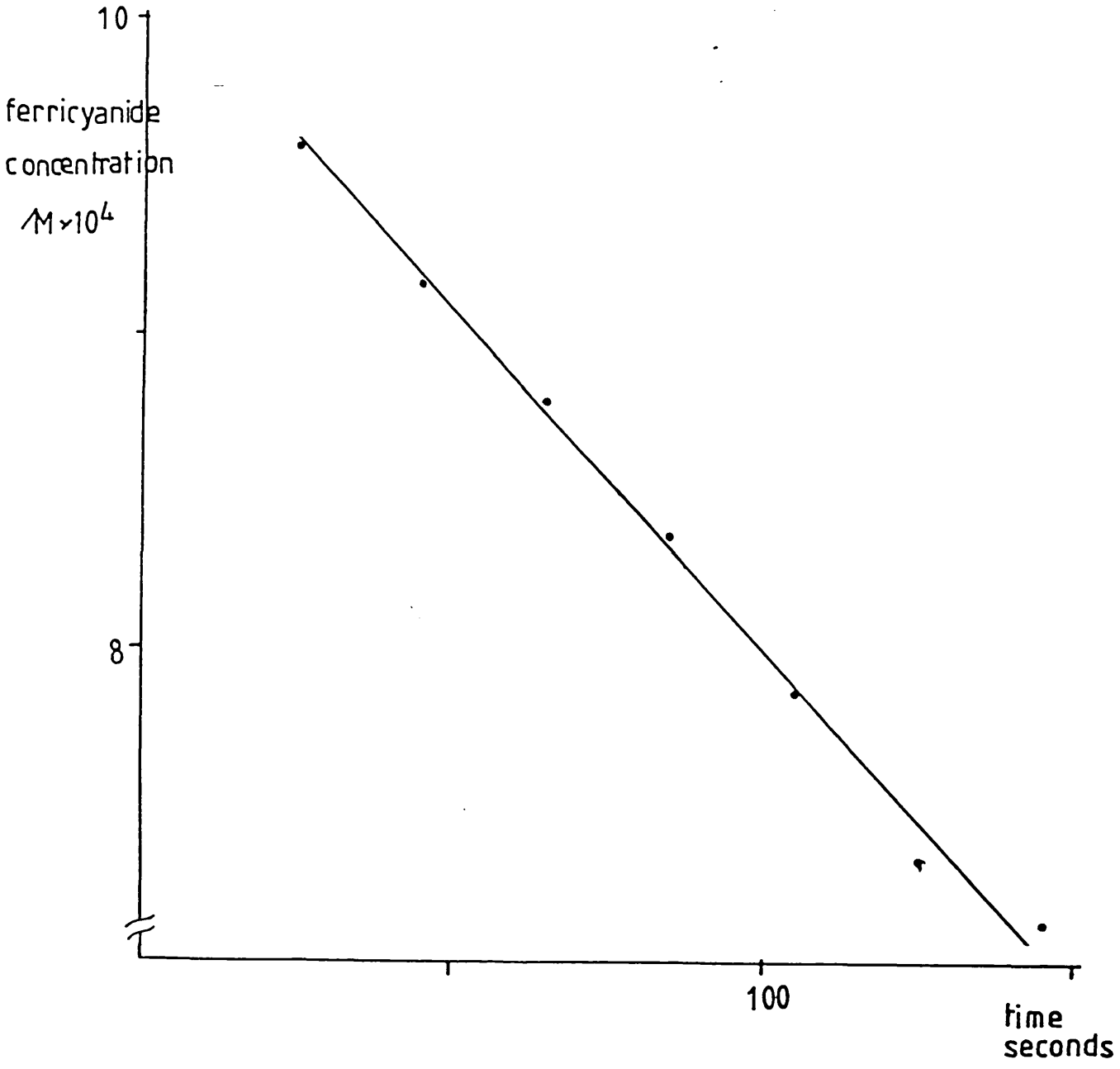
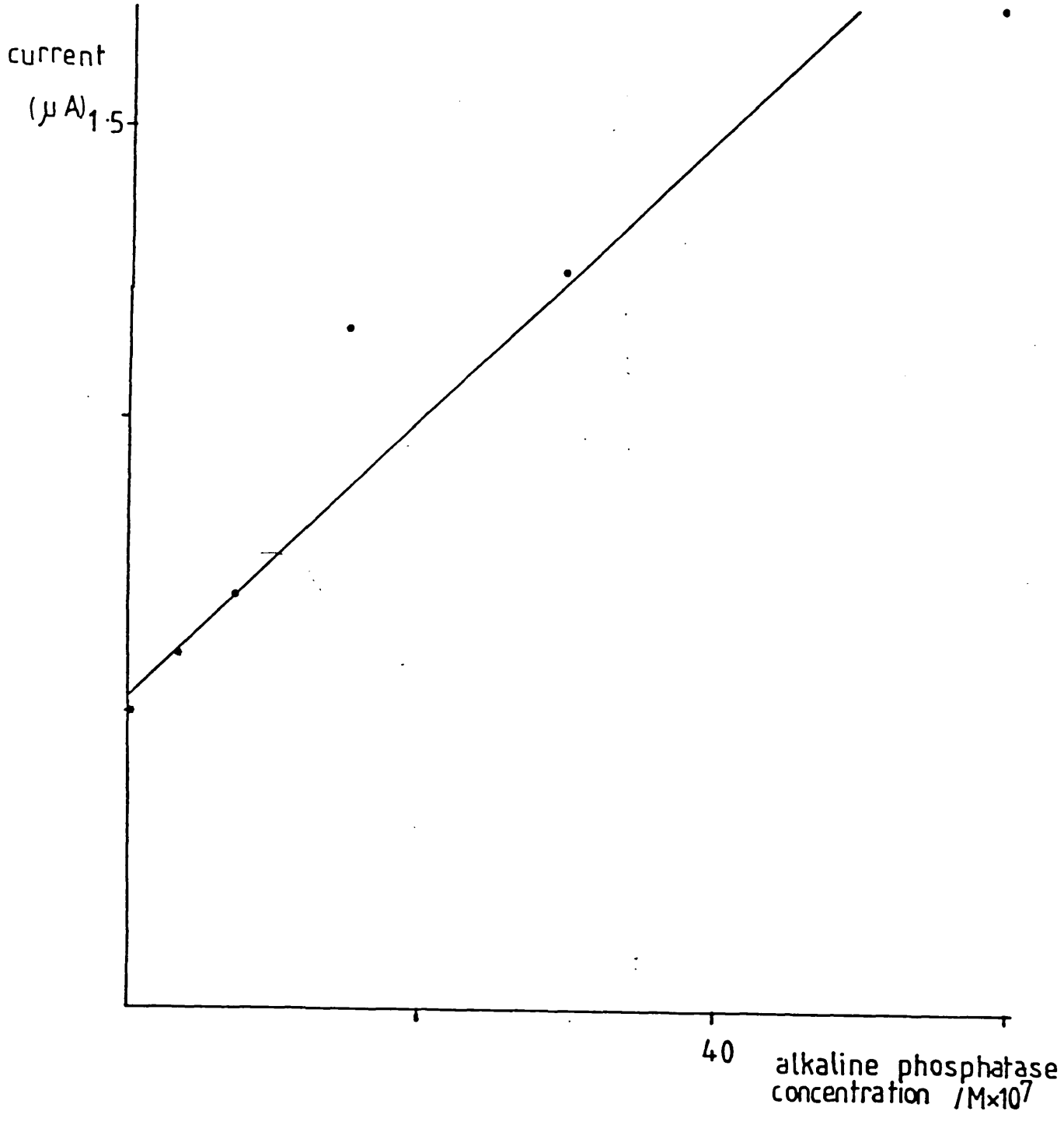


Table 6.13 Amplified alkaline phosphatase assay with amperometric detection of accumulated ferrocyanide

Alkaline phosphatase concentration (/Mx10^7)	Current (μA)
1.86	0.5
3.71	0.6
7.43	0.7
14.85	1.15
29.7	1.25
59.4	1.7

Figure 6.15 Enzyme amplified assay for alkaline phoshpatase with amperometric detection of ferrocyanide



as $NADP^+$ may act as a poor substrate for ADH .This turnover will also occur in the spectrophotometric assay where only a low background absorbance is detected.The success of the spectrophotometric assay indicates that it is in the amperometric estimation of ferrocyanide that the inaccuracies in calibration occur.Repeated attempts to assay ferrocyanide at a solid electrode from bulk solution have produced very variable results although overall a response to ferrocyanide concentration can be obtained.

The high background currents must therefore be due to the poor electrochemistry of ferrocyanide which is also indicated by the scatter on the calibration curve.

6.3 Concluding remarks

Clearly the successful amperometric calibration of alkaline phosphatase by an enzyme amplified system is possible but the methods investigated here have failed to provide the accuracy or sensitivity of the spectrophotometric experiment.A recent report of a successful amperometric assay using ferricyanide is difficult to assess as the exact details of the electrochemical methods used are not specified (87).Ferricyanide electrochemistry may be improved by alteration of solution conditions , it has been reported that magnesium ions can act as bridges, between ferrocyanide and the electrode, across which electrons can transfer (88).Improved electron transfer rates have also been found at high concentrations (up to 1 M) of supporting electrolyte (88).The provision of such solution conditions would not only increase the complexity of the assay but could also effect

enzyme activity and the necessary reagents would probably need to be added after cycling.

Alternatively either electrode surface modifications or use of a different terminal electron acceptor could provide routes to an improved amperometric assay and furthur investigation of these routes is required.

THE AMPEROMETRIC DETECTION OF CATALASE FOR THE DEVELOPMENT OF AN ALTERNATIVE ELISA

7.1 Introduction

The search for ways of improving ELISA sensitivity such that it can provide a realistic alternative to RIA (see section 6.1.2)can be either by improving the sensitivity of detection of existing enzyme labels (as described in chapter 6) or developing sensitive assays using alternative labels.

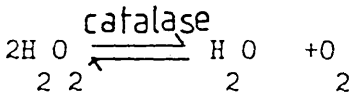
This chapter describes an example of the second approach where an amperometric assay for catalase is investigated.

Catalase is chosen as an enzyme label because it has one of the highest catalytic activities known (kcat =5 $x10^6$ s^{-1}) thus low enzyme concentrations are required to convert high concentrations of substrate to product.

7.1.1 Catalase detection methods

Catalase catalyses the conversion of hydrogen peroxide to oxygen and water as shown in figure 7.1.Present catalase assays are based on measurement of the disappearance of peroxide spectrophotometrically at 240nm (89).The Michaelis constant of catalase for hydrogen peroxide is 1.1 M and the enzyme is inhibited by peroxide at concentrations above 10mM (90).It is therefore impossible to assay for the enzyme in the presence of saturating substrate concentrations and the high reaction rate means that substrate depletion occurs very rapidly.Low initial peroxide concentration gives a low optical density and thus a reproducible spectrophotometric

Figure 7.1 Catalase-catalysed reaction



catalase assay is very difficult to achieve.

This chapter describes attempts to develop an accurate amperometric catalase assay and to label antibodies with the enzyme for use in ELISA.

7.1.2 Amperometric detection of catalase

The scheme of the amperometric catalase assay to be studied is outlined in figure 7.2.

The assay is analogous to the detection of diaphorase by the rate of regeneration of ferrocene; peroxide produced by reduction of oxygen at the electrode surface is broken down in the catalase catalysed reaction.The oxygen regenerated by this reaction is then re-reduced and an increased current is obtained.

No reagents need be added to the electrode buffer as oxygen is present at a concentration of 240 uM in normal aqueous solution (91).

The continual production of peroxide at the electrode should overcome the problem of depletion described above and lead to the production of a reproducible assay.

7.2 Results and discussion

7.2.1 Electrochemical experiments

7.2.1.1 The electrochemistry of oxygen at a carbon electrode

A rotating disc voltammogram of oxygen saturated buffer at a glassy carbon electrode is shown in figure 7.3.

The voltammogram shows a single oxygen reduction wave which disappears when the buffer is sparged with nitrogen before recording the voltammogram(see figure 7.4)

Figure 7.2 Amperometric catalase assay

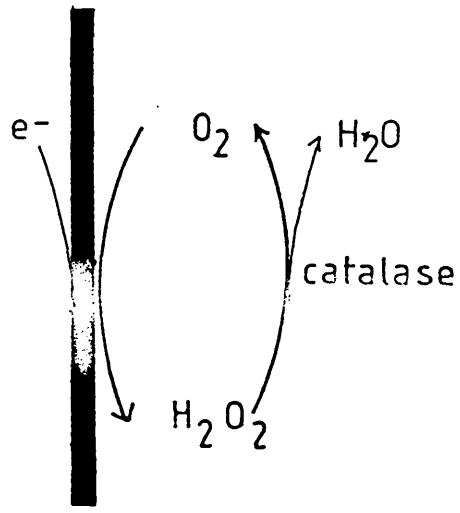
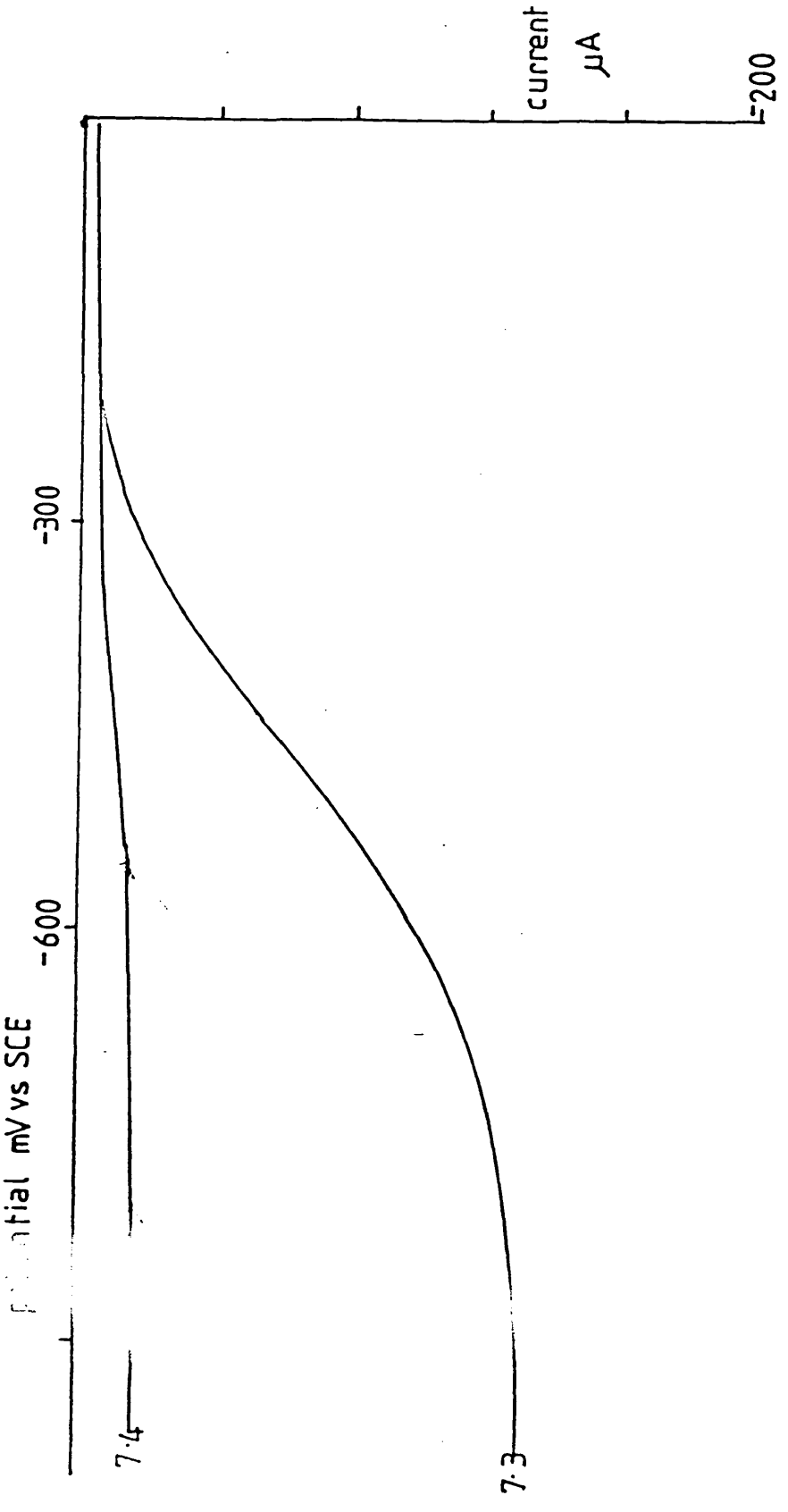


Figure 7.3 Rotating disc voltammogram of oxygen saturated buffer

Figure 7.4 Rotating disc voltammogram of oxygen free buffer



Oxygen reduction at various electrode surfaces has been intensively studied (97) .Initial investigations were at a dropping mercury electrode (DME) .Here the working electrode is a continual stream of mercury drops falling from the electrode housing.The drop rate is steady and the falling mercury stirs the solution close to the new electrode surface.The system therefore provides a clean electrode of defined proportions in a solution stirred at a constant rate (92).

A polarogram of oxygen saturated buffer taken at a dropping mercury electrode is shown in figure 7.5.Two distinct reduction waves are obtained .The first wave corresponds to the reduction of oxygen to hydrogen peroxide by the mechanism shown in figure 7.6 (94).The second wave is due to the reduction of the hydrogen peroxide to water which occurs at a more cathodic potential.The mechanism of this reduction is not yet understood (74).

The rate determining step at the potential of peroxide production has been shown to be the initial formation of the superoxide radical, $O_2^{\cdot}$ in figure 7.6 (95).Subsequent rapid protonation can be supressed if the experiment is carried out at alkaline pH in the presence of a surfactant.Under these conditions it is possible to estimate the concentration of added superoxide dismutase enzyme (SOD) by the production of a catalytic current, as SOD catalyses the regeneration of oxygen and peroxide from superoxide radicals.The estimation of catalase using this electrode has also been demonstrated at a reduced pH without surfactant, by the scheme shown in

Figure 7.5 Oxygen reduction at the dropping mercury electrode (taken from reference 103)

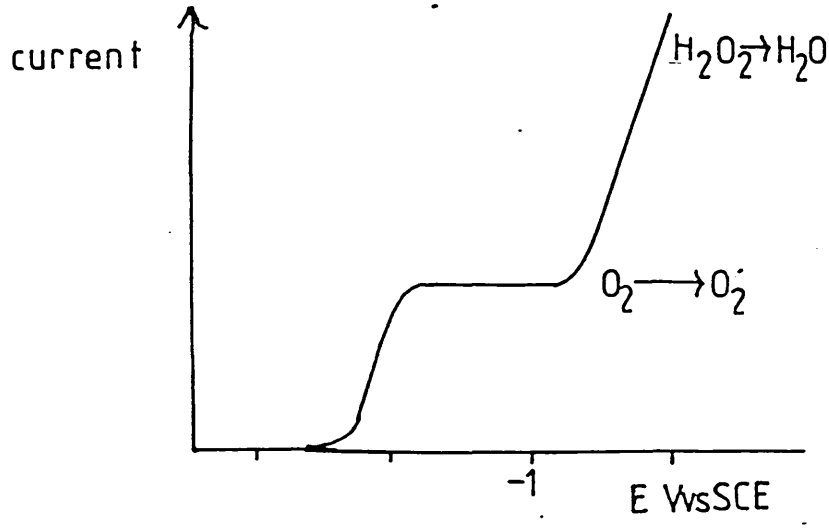


Figure 7.6 Mechanism of oxygen reduction at a dropping mercury electrode

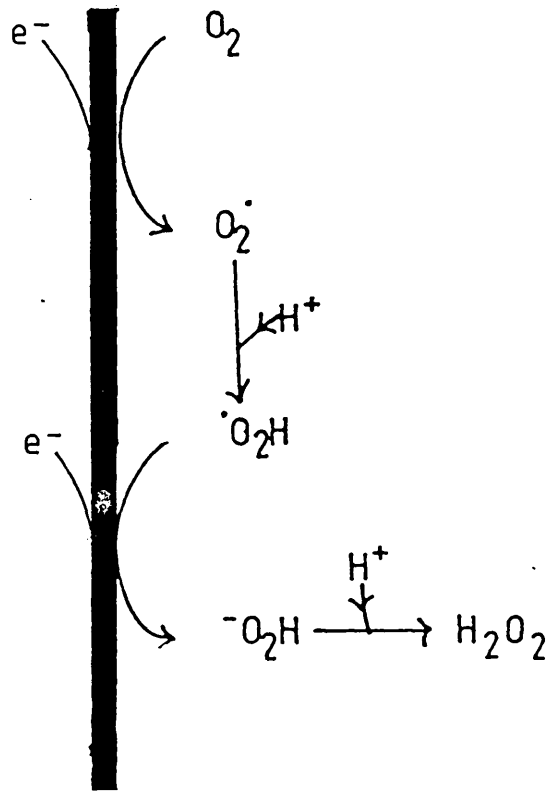


figure 7.2(96).

Polarography at the DME is a complex technique requiring sophisticated equipment and some of the reactions exhibited at the mercury surface can now be carried out using various solid electrodes.

The difference in oxygen reduction polarograms at DME and glassy carbon indicate however that the mechanism of the reaction is different in each case.The mechanism of oxygen reduction at carbon electrodes has not yet been characterised but the presence of only one reduction wave indicates that oxygen reduction to water may be occuring in a single four electron transfer reaction at one potential (94).

Addition of catalase has no effect on the voltammogram at carbon as would be expected if peroxide was not formed as an intermediate.

The complexity of the DME and the toxicity of mercury excludes its use in biochemical assays therefore an attempt was made to alter the mechanism of oxygen reduction at glassy carbon to form a stable hydrogen peroxide intermediate, to allow the assay proposed.

To alter the oxygen reduction mechanism methyl viologen was added to the solution in the electrochemical cell.Methyl viologen is reduced at a variety of electrodes by two single one electron reduction reactions (98).The first reduction produces the radical cation of methyl viologen ($MV^{+\cdot}$ see figure 7.7) which has been shown to react rapidlly with molecular oxygen.The presence of the radical cation should catalyse the reactions shown in figure 7.8.$MV^{+\cdot}$ is formed by

Figure 7.7 The electroreduction of methyl viologen

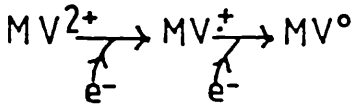
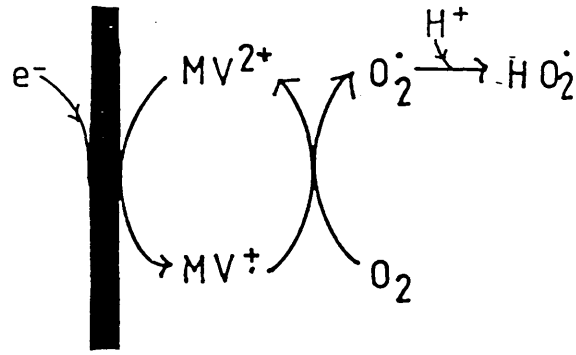
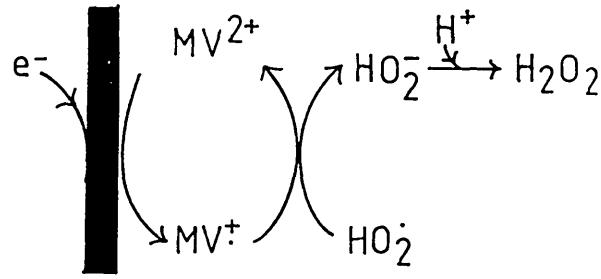


Figure 7.8 Catalysis of oxygen reduction by methyl viologen radicals

a



b



the reaction of MV^{2+} at the electrode.The radical cation then reacts with molecular oxygen to form superoxide which is rapidly protonated to yield the hydroperoxy radical (see figure 7.8a).This radical can then be further reduced by $MV^{+\cdot}$, at the same electrode potential, and a second protonation leads to the formation of hydrogen peroxide.

The rationale behind the use of methyl viologen radicals as a catalyst for the one electron reduction of oxygen was based on their fast rate of the reaction (99) and the fact that peroxide production has been demonstrated at electrodes modified with polymeric viologens(99).In these studies poly (cyanuroviologen) (PCV) is coated on to a graphite electrode in a monolayer .In the presence of oxygen the background viologen reduction peak was anodically shifted and increased in height.The limiting current was also dependent on the oxygen concentration indicating that the reaction shown in figure 7.8 was occurring.The shift in peak reduction potential was used to calculate the rate constant for oxygen reduction which was found to be $2x10^{9}$mole $cm^{-2}sec^{-1}$(99)

7.2.1.2 The electrochemistry of methyl viologen

Voltammograms of methyl viologen in the absence and presence of oxygen are shown in figure 7.9 .In the absence of oxygen the reversible one electron reduction of MV^{2+} occurs.The fact that this is a one electron reaction is confirmed by plotting log $i/(i_L-i)$ vs E as shown in table 7.1 and figure 7.10.The slope of this graph is 65 mV ,very close to the value of 59mV expected for a 1 electron transfer reaction (see appendix 7).

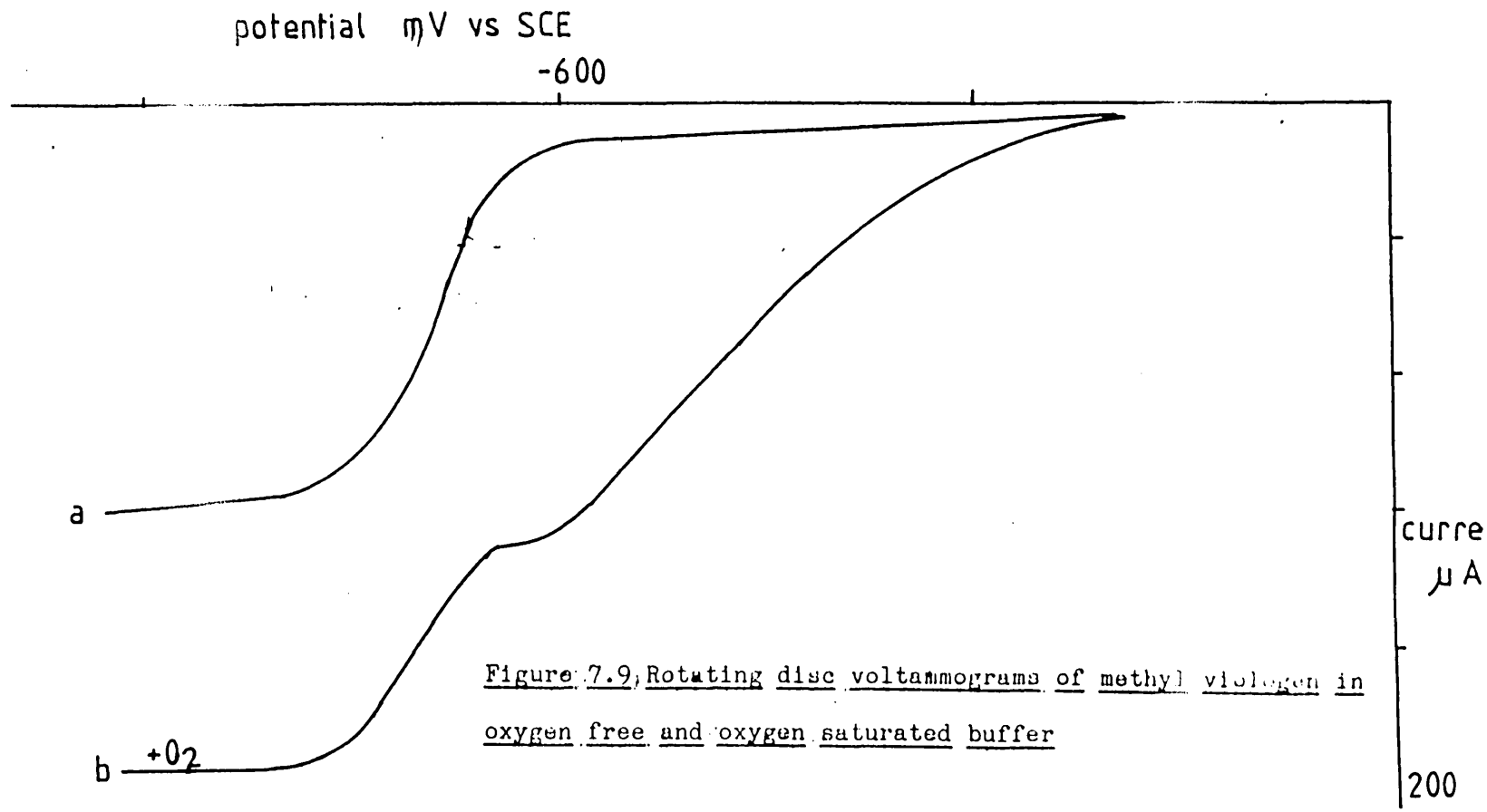
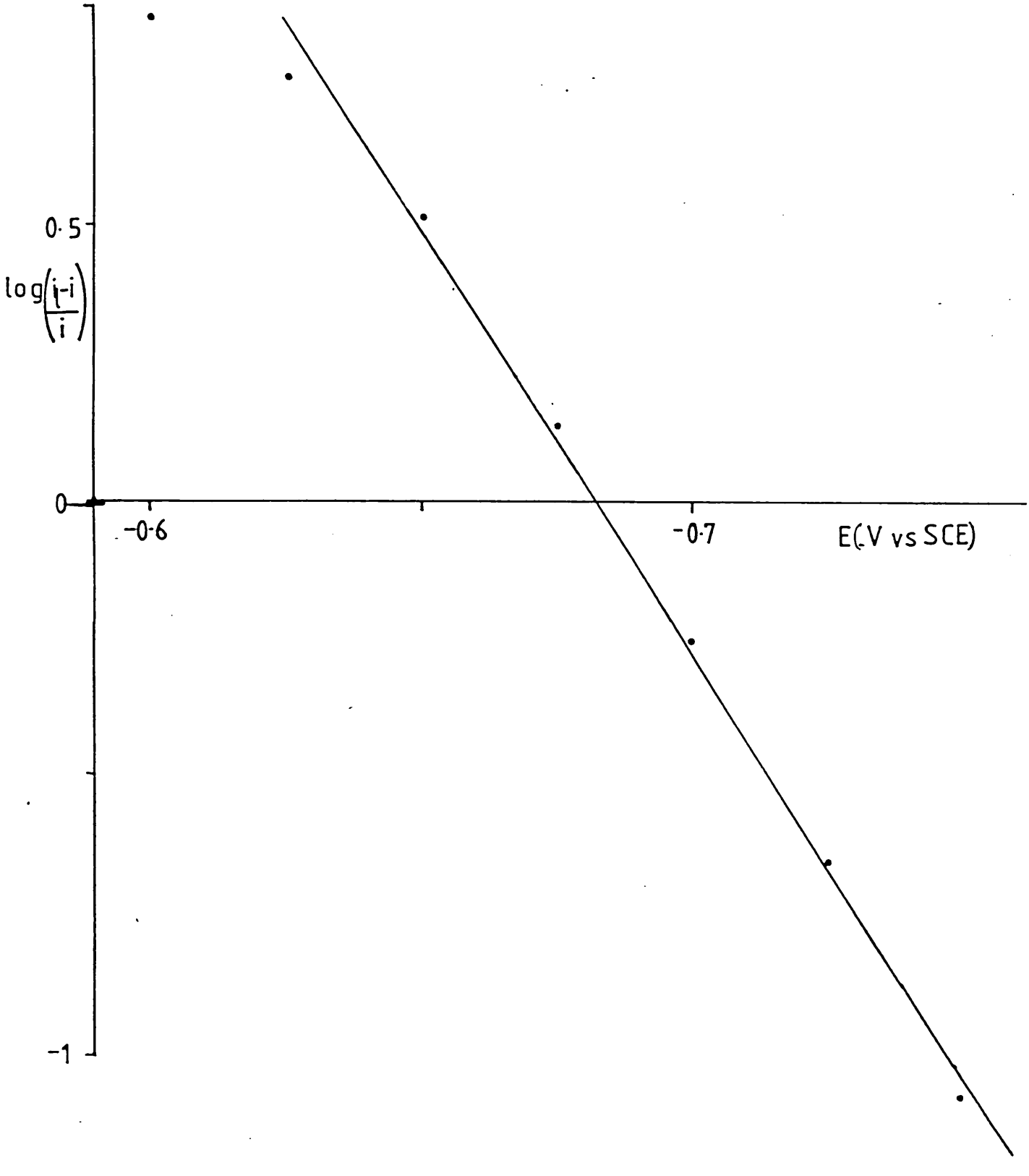


Figure 7.9, Rotating disc voltammograms of methyl viologen in oxygen free and oxygen saturated buffer

Table 7.1 Data to determine the number of electrons tranferred in the first reduction of MV^{2+}

Potential (mV vsSCE)	$\log(i_l - i)/i$
-0.6	0.87
-0.625	0.77
-0.65	0.51
-0.675	0.13
-0.7	-0.26
-0.725	-0.66
-0.75	-1.08

Figure 7.10 Plot to calculate the number of electrons transferred in the first reduction of methyl viologen



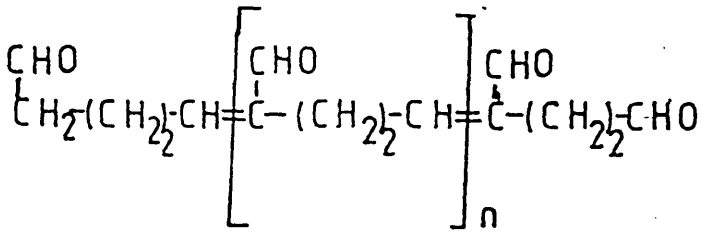
If catalytic regeneration were occuring in the presence of oxygen, as shown in figure 7.8,an anodic shift in the plateau potential should be observed (99).No such shift is seen in figure7.9 and the current obtained in the presence of oxygen is merely an addition of MV^{2+} and oxygen reduction currents.

The addition of catalase to the electrochemical cell containing MV^{2+} gives no increase in limiting current.

As oxygen reduction is known to be catalysed by $MV^{+\cdot}$the failure to produce a catalytic current when MV^{2+} is present in solution must be attributable to the difference between MV^{2+} in solution and that in a polymer layer.

Several explanations of the difference can be postulated.First, since the reaction between $MV^{+\cdot}$ and molecular oxygen is a second order process the concentration of each reactant will affect the reaction rate.The reactions with both monomer amd polymer were carried out in oxygen saturated buffer therefore the oxygen concentration in both cases should be the same.The concentration of viologen active sites on the polymer modified electrode was calculated as $1x10^{-10}$ moles cm^{-2}(99).By integration of the area under the reduction wave of a cyclic voltammogram (after correction for the charging current as shown in figure 7.11) the number of molecules of MV^{2+} being reduced to $MV^{+\cdot}$ was found to be $1x10^{-8}$moles cm^{-2}.The reduction of methyl viologen in solution therefore produces a higher electrode surface concentration of MV^{2+} than where a polymer modified electrode is used.Since the $MV^{+\cdot}$ concentration at a polymer

Figure 7.12 The glutaraldehyde molecule



modified electrode is sufficient to react with the oxygen in solution then this reaction should be even more likely to occur where methyl viologen is supplied in solution.

A second explanation could be that the direct electron transfer to oxygen is inhibited by the polymer matrix.If this is the case, and the direct four electron reduction of oxygen is the favourable electrode reaction at these potentials the latter will occur at the bare carbon electrode, whilst reduction will be forced to occur in two stages, as shown in figure 7.8,where a polymer layer is present.

Slow electron transfer has indeed been demonstrated at some polymer modified electrodes (100) but in the case of PCV, which is the most effective in the reduction of oxygen, the monolayer electrode does not impede direct charge transfer (99) .

Finally it may be that peroxide formed in the $MV^{+\cdot}$ catalysed reaction is rapidly reduced to water at the same potential and is therefore not present for long enough to react with catalase.This again seems unlikely because in this case catalytic regeneration of MV^{2+}would still be occurring and an anodic peak potential shift would be expected.

No obvious explanation exists for the failure of oxygen to regenerate methyl viologen when the monomer is added to the electrode solution.Whatever the reason further investigation into the production of an amperometric catalase assay should concentrate on the catalysis of peroxide production by poly(viologen) modified solid electrodes.

7.2.2 Conjugation of catalase and antibodies

7.2.2.1 Introduction

Preliminary experiments, in conjunction with the electrochemical studies described, were carried out to investigate whether it is possible to label antibodies with catalase by protein-protein conjugation.

One of the simplest methods available for protein conjugation is the linking of ε-amino groups of surface lysine residues using the bifunctional reagent, glutaraldehyde. (see figure 7.12).

The method requires no preactivation of the proteins provided that both have free NH_2 groups on their surface.

A one step glutaraldehyde conjugation reaction was carried out with bovine immunoglobulin G (IgG) and beef liver catalase.Conjugates in the product mixture were detected using the immunological screening technique described in section 3.4.

7.2.2.2 Conjugate assay results

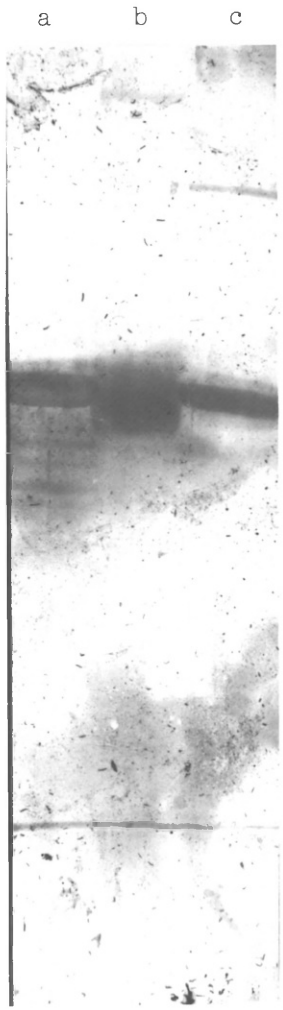
Conjugate detection depends on the binding of catalase to the microtitre plate via its attatched IgG molecule.Where large amounts of catalase are bound the final absorbance at 410 nm is low.The results of the immunoassay (see figure 7.13) show that the final reaction mixture contains catalase-IgG conjugates.

The conjugation products expected are catalase-catalase, IgG-IgG and catalase-IgG.Blocking of the microtitre plate with BSA ensures that no non specific binding occurs, this prevents the presence of catalase homoconjugates interfering

Figure 7.13 Results from the immunological screening of conjugation products

	blank	conjugate blank	conjugate blank	conjugate dilution 1/10	1/100	1/1000	1/10	1/100	1/1000	1/10	1/100	1/1000
1	H	H	H	1·7	1·9	H	1·7	1·75	1·8	1·9	H	H
2	H	H	H	0·91	1·7	H	1·2	1·2	1·4	1·7	1·81	1·7
3	H	H	H	0·85	H	H	1·3	0·89	1·26	1·5	1·75	1·86
4	H	H	H	0·75	1·35	H	0·87	0·93	1·2	1·5	1·7	1·9
5	H	H	H	0·71	1·14	H	0·77	0·76	0·92	1·21	1·5	1·9
6	H	H	H	0·61	1·31	H	0·67	0·9	0·97	1·4	1·6	H
7	H	H	H	0·54	1·13	H	0·6	0·71	0·93	1·0	1·52	1·9
8	H	H	H	0·5	1·11	H	0·62	0·59	0·81	0·92	1·4	1·8

Figure 7.14 SDS polyacrylamide gel of catalase, IgG and conjugation products



a.IgG

b.Catalase

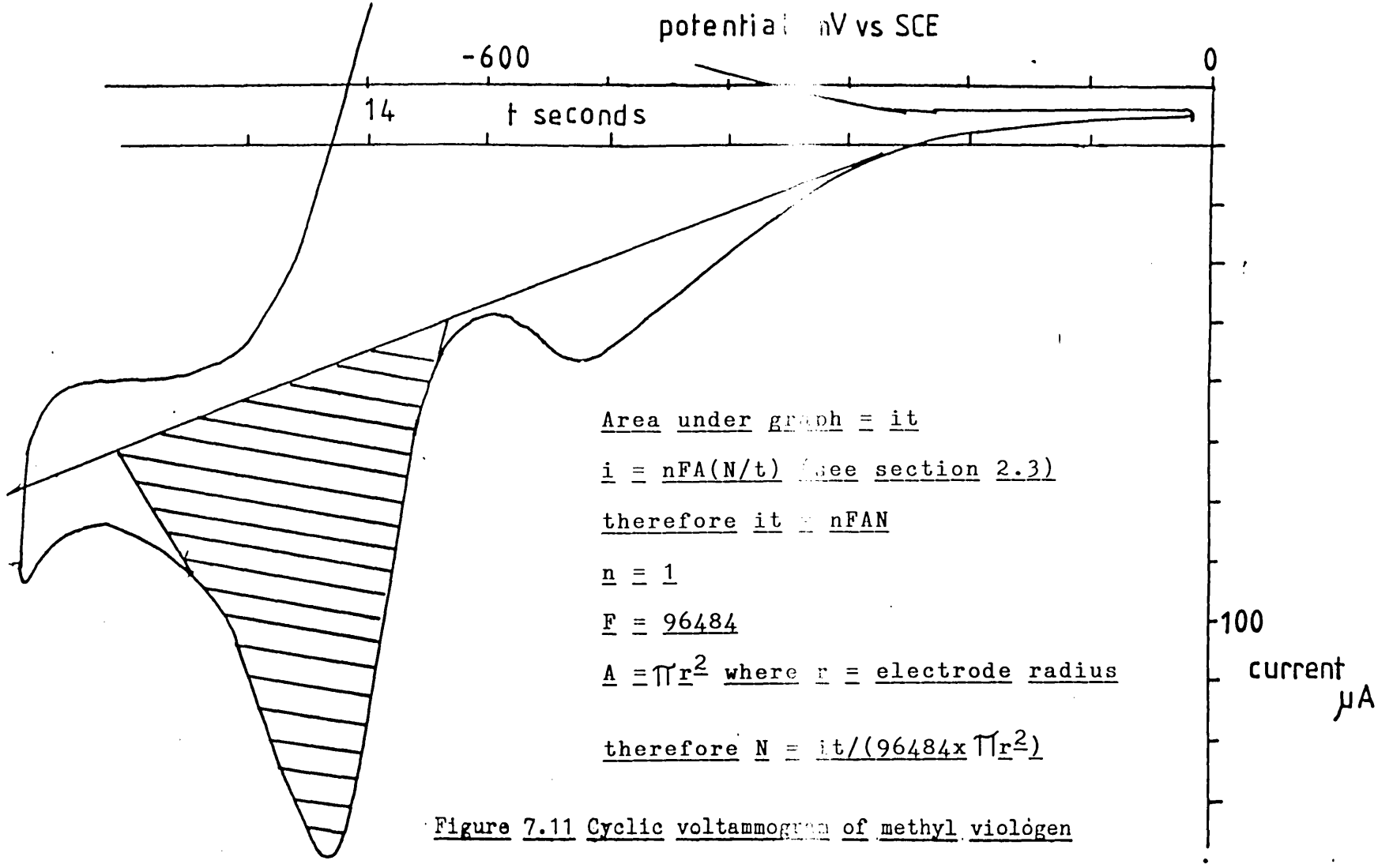
c.Conjugation products

in the indicator reaction.IgG homoconjugates can bind specifically to the anti-IgG but are not detected in the indicator reaction.Provided therefore that blocking and washing of the plate is rigorous any positive result in the indicator reaction can only be due to the presence of cayalase-IgG conjugates.

7.2.2.3 Discussion of the conjugation procedure

The conjugation method employed depends upon the reaction of exposed lysine groups with glutaraldehyde and is therefore unsuitable for proteins in which these groups are at a low concentration or are protected on the protein surface.Glutaraldehyde is rarely available in pure monomeric form and the long chain polymers, which are readily formed, are inefficient conjugation bridges.The one step method will yield a large concentration of homo conjugates (IgG-IgG or catalase -catalase) and one or other of these will be formed preferentially if either of the proteins shows favourable activity with the crosslinker.Glutaraldehyde is also a potent enzyme inhibitor and its concentration in the conjugation mixture must be carefully controlled.

A catalase assay on the conjugate mix showed that the enzyme activity was reduced 5.4 fold.Catalase is therefore initially added in excess of IgG to ensure that sufficent conjugated enzyme remains active.The crude conjugate mixture therefore contains large amounts of catalase which can be seen on an SDS polyacrylamide gel of the mixture (see figure 7.14).Although excess catalase is necessary for the successful conjugation of active enzyme it must be completely



Area under graph = it

i = nFA(N/t) (see section 2.3)

therefore it = nFAN

n = 1

F = 96484

A = πr^2 where r = electrode radius

therefore N = it/(96484x πr^2)

Figure 7.11 Cyclic voltammogram of methyl viologen

removed if the conjugate is to be assayed by its catalase activity.

Possible purification protocols include gel filtration, to separate the high molecular weight conjugate, or affinity purification by the binding of the IgG portion to a protein A column (there should be little or no unreacted IgG in the mixture).Preliminary experiments using purification on a Superose gel filtration column using a fast protein liquid chromatography system (from Pharmacia ,Upsala Sweden) yielded very low conjugate concentrations.It may be advantageous to attempt alternative conjugation methods to produce higher initial conjugate concentrations and ease purification, several alternative conjugation techniques are available (101).

7.3 Concluding remarks

Although the amperometric catalase assay attempted was unsuccessful futher investigation, refining the working electrode used, should allow the development of the proposed ayatem.Initial conjugation experiments indicate that it is relatively straightforward to couple catalase to antibodies for use in ELISA.

GENERAL DISCUSSION

The work described in this thesis has demonstrated that the measurement of enzyme catalysed reactions by amperometry can allow the sensitive detection of the enzymes' substrates and the observation of enzyme reaction kinetics.

High specificity is conferred in substrate concentration analysis by the use of an enzyme rather than a chemical catalyst.The use of enzyme catalysed reactions makes such analyses particularly suited to medical and biological assays since these are the systems in which the enzymes show their natural activity.In practice enzymes often react with other molecules which are structurally related to their substrate and this increases the range of molecules which can be analysed.

The assays described are based on the detection of the product of an enzyme catalysed reaction.In the paracetamol sensing system product concentration after complete hydrolysis of the drug is estimated amperometrically.Where diaphorase provides recognition, the amperometric detection of the product yields the enzyme substrate, thus the opposite redox reaction occurs at the electrode and in the enzyme catalysed scheme.This phenomenon can be used to investigate the reaction of any redox protein provided that electrons can be freely transferred between the enzyme and the electrode.

The observation of enzyme activity has allowed the kinetics of the enzyme catalysed reaction to be studied using an amperometric assay.The two possible rate limiting processes can be distinguished by the dependence of the

reaction rate on either mediator or substrate concentration.The electrochemical analysis yields rate constants which show excellent agreement with those determined by spectrophotometry.The occurrence of the enzyme catalysed reaction at the electrode surface rather than in solution introduces a diffusion step into the scheme which is not present where the enzyme is assayed spectrophotometrically and which prevents direct determination of the Michaelis Menten constant for the enzyme.The reaction saturates at an artificially high added substrate concentration because that added to the bulk solution is higher than that at the electrode surface where it becomes depleted by the enzyme catalysed reaction.This factor increases the linear range of the amperometric assay but also makes the measurement less sensitive to low substrate concentrations.

Indirect measurement of Km is theoretically possible by providing bulk concentrations of both mediator and substrate that cause saturation of the reaction rate.Under these conditions kf determined electrochemically would approach the kcat of the enzyme.From this and a value of k_s determined with limiting concentrations of one substrate the Km for the limiting substrate could be determined (since k_s= kcat/Km).This value was not determined for diaphorase because firstly high NADH concentrations interfere with the amperometric measurements(see section 5.2.1), and secondly the solubility limit of the mediator in aqueous solution

would probably preclude the addition of saturating concentrations.

The amperometric detection of paracetamol has allowed the development of a specific assay for the drug which can be performed on samples of whole blood.The assay is not only faster than the currently available colourimetric test kit, which requires both removal of red cells from whole blood and several incubation steps(102), but also provides an estimation of the unbound plasma paracetamol which can potentially cause liver damage.The method described could be readily converted into an integrated form by immobilisation of the enzyme at the electrode surface.The immobilisation of enzymes often increases their stability and several techniques for the attachment of proteins to various insoluble supports have been described (103).

The advantage of amperometry as the detection method is that relatively simple electronic circuitry can be used to produce a device which can be used outside a clinical laboratory.The ease and speed of the assay will allow rapid estimation of paracetamol half-life in whole blood in either a doctor's surgery or casualty ward and thus aid the effective treatment of overdose patients.

As well as establishing a kinetic analysis of the enzyme catalysed reaction the amperometric assay of diaphorase provides a sensor for the rate of NADH production which is currently followed in a wide range of dehydrogenase enzyme assays (104).

Experiments have demonstrated the successful assay of

ethanol concentration using the ferrocene mediated electrochemistry of diaphorase as detector.This assay could be treated as described for the paracetamol sensor (by immobilisation of both diaphorase and the dehydrogenase) to produce a biosensor for ethanol and the system can be applied to the detection of any pyridine nucleotide dependent dehydrogenase.

Just as the combined actions of the enzyme and the electrode reactions regenerate the mediator so the combination of ADH and diaphorase catalysed reactions regenerate NADH and the detection of this provides a sensitive assay for either NAD^+ or NADH.Such cyclic coupled enzyme reactions have been known for many years but have recently become important in enzyme linked immunosorbent assays (ELISA).

The sensitive detection of alkaline phosphatase using amperometric NADH detection, both direct and indirect, has been described.The assay which provides flexible sensitivity requires the provision of excess oxidised mediator and further investigations into either the stabilization of ferricinium or improvement of the amperometric detection of ferrocyanide, are necessary to produce an assay that is as sensitive as the currently available spectrophotometric method.

The final assay system described was aimed at producing an amperometric assay for catalase.The high catalytic activity of the enzyme makes it an obvious choice as an

immunoassay label and its conjugation to antibodies has been demonstrated.Colourimetric determination of the enzyme is difficult due to substrate inhibition and the fast rate of the reaction.The electrochemical assay described should provide a more accurate assay for the enzyme because the substrate will be continually produced at the electrode at a concentration which will not limit catalase activity.The attempt to produce the necessary peroxide for a coupled assay in a simple electrode reaction was unsuccessful but previous studies suggest that a polymer modified electrode will yield high enough concentrations of peroxide to enable the observation of catalytic currents.

The investigation of three separate biosensor systems has lead to the development of both a specific paracetamol assay and a general base sensor for the detection of dehydrogenases and their substrates.

Problems encountered in the systems investigated have been in the electrode reactions used to detect the products of the enzyme catalysed reactions.Generally biosensor devices developed to date have been based upon detection of the natural reaction of the enzyme and thus it is in the development of electrochemical detection systems that future advances in biosensor research and development will be made.

Approaches to the development of biosensors and their many applications have not been discussed in detail here.Several comprehensive reviews are however available (see references 106 and 107) which provide information on recent developments in this field.

Biosensor devices are presently passing from research into production but much further investigation of both specific and general devices is required before they become available for the wide range of analyses in which they would be useful.

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APPENDIX 1

Calculation of aryl acylamidase activity

From calibration curve A 1.1 the concentration of PAP is X ug ml^{-1}.

Molecular weight of PAP is 109 D therefore

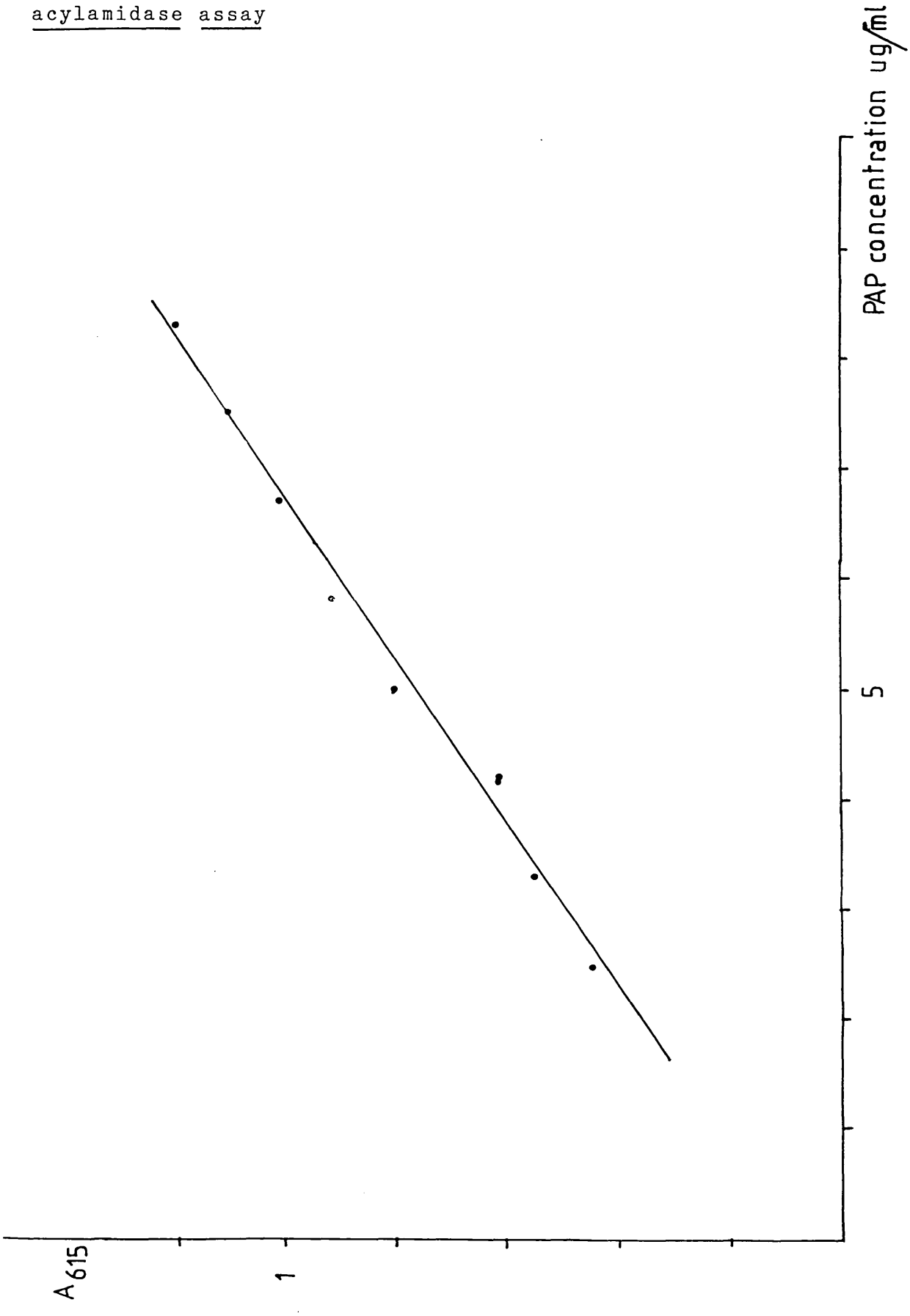
Xug ml^{-1} = X/1000x109 M in a 1 ml reaction volume therefore ammount of PAP produced = $X/10^6$ x109 moles, this is produced in two minutes.

0.2 ml of Ymg ml^{-1} aryl acylamidase was added to 1ml reaction mixture therefore enzyme concentration = Y/5 mg ml^{-1}.

One international unit of enzyme activity is defined as the amount of enzyme which catalyses the conversion of 1 u mole of substrate to 1 u mole of product per minute.

Number of units of enzyme activity is therefore X /10^6x109x2 and Units per mg of enzyme = ($X/10^6$x109x2)/(Y/5).

Figure A 1.1 PAP calibration for colourimetric aryl acylamidase assay



APPENDIX 2

Calculation of diaphorase activity

Rate of change of A_{600} = X min^{-1}.

ε_{600} = 22000 M^{-1} therefore

rate of change of product concentration =

X/22000 M min^{-1} = Xx3/22x10^6 moles min^{-1} =

Xx3/22 u moles min^{-1}.

0.1 ml of Y mg ml^{-1} diaphorase was added to a 3ml reaction volume therefore enzyme concentration = Y/30 mg/ml therefore total amount of enzyme = Y/10mg

Units (as defined in appendix 1) per mg = (Xx3/22)/(Y/30)

APPENDIX 3

Calculation of active diaphorase concentration

A_{445} without NADH = X

A_{445} with NADH = Y

change in absorbance due to flavin bleaching = Y-X

ε_{445} for FMN = 11300 M^{-1} therefore

Change in FMN concentration = Y-X/11300 M

since each diaphorase molecules has 1 molecule of associated flavin concentration of active diaphorase = Y-X/11300 M

APPENDIX 4

Calculation of alcohol dehydrogenase activity

Rate of change of A_{340} = $X min^{-1}$

ε_{340} for NADH = 62300 M^{-1} therefore

rate of change of NADH concentration = X/62300 M min^{-1}

rate of product production in a 3 ml reaction volume =

$Xx3/6.23x10^{6}$ moles min^{-1}

$=Xx3/6.23$ u moles min^{-1}

0.1 ml of Ymg ml^{-1} ADH was added to a 3 ml reaction volume therefore enzyme concentration = Y/30 mg/ml and total amount of enzyme = Y/10 mg ml^{-1}

Units per mg (see appendix 1) (Xx3/6.23)/(Y/10)

APPENDIX 5

Calculation of alkaline phosphatase activity

Rate of change of A_{400} = X min^{-1}

ε_{400} for PNP = 18300 M^{-1} therefore

rate of change of PNP concentration = X/18300 M min^{-1} =

Xx3/18.3 $x10^{6}$ moles min^{-1} =

Xx3/18.3 u moles min^{-1}

0.1 ml of Y mg ml^{-1} alkaline phosphatase was added to a 3 ml reaction volume therefore enzyme concentration = Y/30 mg/ml and enzyme amount =Y/10 mg.

Units per ml (see appendix 1) = (Xx3/18.3)/(Y/10)

APPENDIX 6

Calculation of catalase activity

A drop in A_{420} from 0.45 to 0.4 corresponds to the reaction of 3.45 u moles of H_2O_2 (105).

Time taken for this drop = t mins therefore total number of Units (see appendix 1) = 3.45/t.

10ul of Y mg/ml catalase was diluted 5000 fold therefore catalase stock concentration = Y/5000 mg ml^{-1}

0.1 ml of this was added to a 3 ml reaction volume therefore enzyme concentration = Y/15000 mg ml^{-1} and enzyme ammount = Y/5000 mg

Units per mg = (3.45/t)/(Y/5000)

APPENDIX 7

Graphical determination of the number of electrons transferred during an electrochemical reaction

The Nernst equation (equation 2.5) reduces to

$E = E_{1/2} = RT/nF \ln [OX]/[RED]$

under experimental conditions .

For an oxidation i_l represents [OX] and i_l-1 represents[RED]

Therefore a plot of E vs ln $i_l/(i_l-i)$ has a slope of 25.7/ n mV and a plot of log $i_l/(i_l-i)$ has a slope of 59.11/n mV.

For a reduction i_l represents [RED] and i_l-i represents [OX] thus a plot of E vs log $(i_l-i)/i_l$l has a slope of -59.11 mV.