Design and Development of Some Amperometric Biosensors Based on Enzymes, Antibodies and Plant Tissue

by

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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph.D is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my own work.

Signed: Eithne Dempsey *Date:* 10/9/93

Eithne Dempsey

For my Family and Boris

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CHAPTER 1

OVERVIEW OF BIOSENSORS AND THEIR APPLICATIONS

List of Symbols

Symbol	Meaning	Dimensions
A	electrode area	cm^2
C	bulk concentration of substrate	M
D	diffusion coefficient	$cm^2\ s^{-1}$
E	potential of an electrode vs. a reference	V
E_{act}	activation energy of a reaction	$KJ\ mol^{-1}$
$E_{1/2}$	measured half wave potential in voltammetry	V
ΔE	$\|E_a - E_c\|$ in cyclic voltammetry	V
d	thickness of enzyme Layer	cm
F	Faraday constant	C
i, I	current	A, μA
n	electrons per molecule oxidised or reduced	
R	gas constant	$J\ mol^{-1}\ K^{-1}$
R	reduced form of the standard system $O+ne^- \rightleftharpoons R$	
O	oxidised form of standard system $O+ne^- \rightleftharpoons R$	
T	absolute temperature	K
υ	viscosity	mPs^{-1} ($P = g\ cm^{-1}\ s^{-1}$)
ω	angular frequency of rotation 2π x rotation rate	sec^{-1}
K_m	Michaelis-Menton constant	M, mM

Design and Development of Some Amperometric Biosensors Based on Enzymes, Antibodies and Plant Tissue

Eithne Dempsey

ABSTRACT

Amperometric biosensors for various drugs and metabolites have been developed, based on immobilised enzymes in both aqueous and organic media. Chapter 1 serves as a general review of the field of biosensors, including both an overview of recognition methods together with the techniques involved in transduction. The remainder of the thesis is divided into four experimental chapters. The first of these describes the development of some amperometric enzyme electrodes for certain drugs and metabolites in aqueous solution. Various immobilisation methods, including use of polymers such as Nafion and Eastman AQ_{55D}, gelatine membranes, entrapment of the enzyme within electropolymerised films, together with direct adsorption, have been employed. Discriminative permselective films were found to show great effectiveness in excluding interfering compounds commonly found in serum. Application to the clinical and *in vivo* fields are discussed. Limits of detection down to the μM level were found. Fast wash times permitted application to flowing streams with frequencies of up to 200 samples per hour, with good precision. The second experimental chapter deals with the construction of biosensors for inhibiting compounds in the organic phase. A study of the kinetics of tyrosinase at the rotating disc electrode in non-aqueous media is also described. Reactions which are impossible in aqueous media due to kinetic or thermodynamic constraints become possible in non-aqueous solvents. The recent discovery that biocatalysts can function in extreme environments such as organic solvents has important implications for the implementation of biosensor technology in formerly inaccessible environments. It also extends the number of detectable analytes to include poorly water soluble organic species in the petrochemical, food and environmental areas. Chapter 4 deals with the electrochemistry of the anticancer drug 7-OH-coumarin and the development of an immunosensor for this compound. The final experimental chapter deals with the detection of some metal ions using a plant tissue modified carbon paste electrode, and includes speciation studies of copper.

CHAPTER 1

GENERAL INTRODUCTION AND REVIEW OF BIOSENSORS

1.1. INTRODUCTION

Since 1962, when Clark and Lyons [1] correlated the enzymatic oxidation of glucose with consumption of the oxygen coreactant, biosensors have received increasing interest and have become an integral part of both analytical chemistry and biochemistry. Biosensor design and technology is a developing area in the quest for innovative approaches to analysis. A biosensor may be thought of as comprising a bioactive substance that can specifically recognise a species of interest, in intimate contact with a suitable transduction device [2].

Biosensors have the potential to revolutionise analytical methodology by providing a powerful and often considerably less expensive alternative to older, well established laboratory techniques. Research efforts have become multi-disciplinary and have witnessed endeavours from both the scientific and commercial sectors. The impact of this area has been largely due to the advances made in bioelectrochemistry, microelectronics and micro-optic technology. It attracts scientists from far-ranging fields such as pharmacology, biochemistry, protein chemistry, electronics and physics.

Any analytical system that has minimal demand upon operator skills, and widens the horizons for practical exploitation, is an important advance. Achievement of such practical goals has motivated much of the current research into biosensors and explains why they have captured the attention of many scientists. They have the potential to form a powerful and inexpensive alternative to conventional analysers and should be capable of selectively determining analytes in complex environments, without the need for pretreatment or separation. Current problems in achieving these goals include a frequent calibration requirement, short operating lifetimes owing to instability of the biological component, narrow analyte range and the task of interfacing the sensor with the complex matrix, in which the analyte is present.

The numerous practical problems to be overcome stem partly from the stringent operational requirements placed upon what is an inherently metastable biolayer. There remains, however, a fundamental structural elegance to the bio-transducer combination which exploits the molecular resolving power of a biological reagent in a direct way.

Many reviews and books have been published in this area over recent years [3-8]. Biomolecular sensing may be defined as the detection of analytes of biological interest, using an enzyme or other receptor which can specifically

recognise a target molecule in a complex medium. In biomolecular sensing, two phenomena must occur:

- the selective molecular recognition of the target molecule; an important factor is the intrinsic specificity of the biological material involved in the recognition process, and problems of interference may occur;

- the occurrence of a physical or chemical signal consecutive to this recognition, subsequently converted by the transducer into a second signal, generally electrical, with a transduction mode which can be either electrochemical, thermal, optical or based on a mass vibration.

The major processes involved in any biosensor system, are shown below [9] (Figure 1.1.).

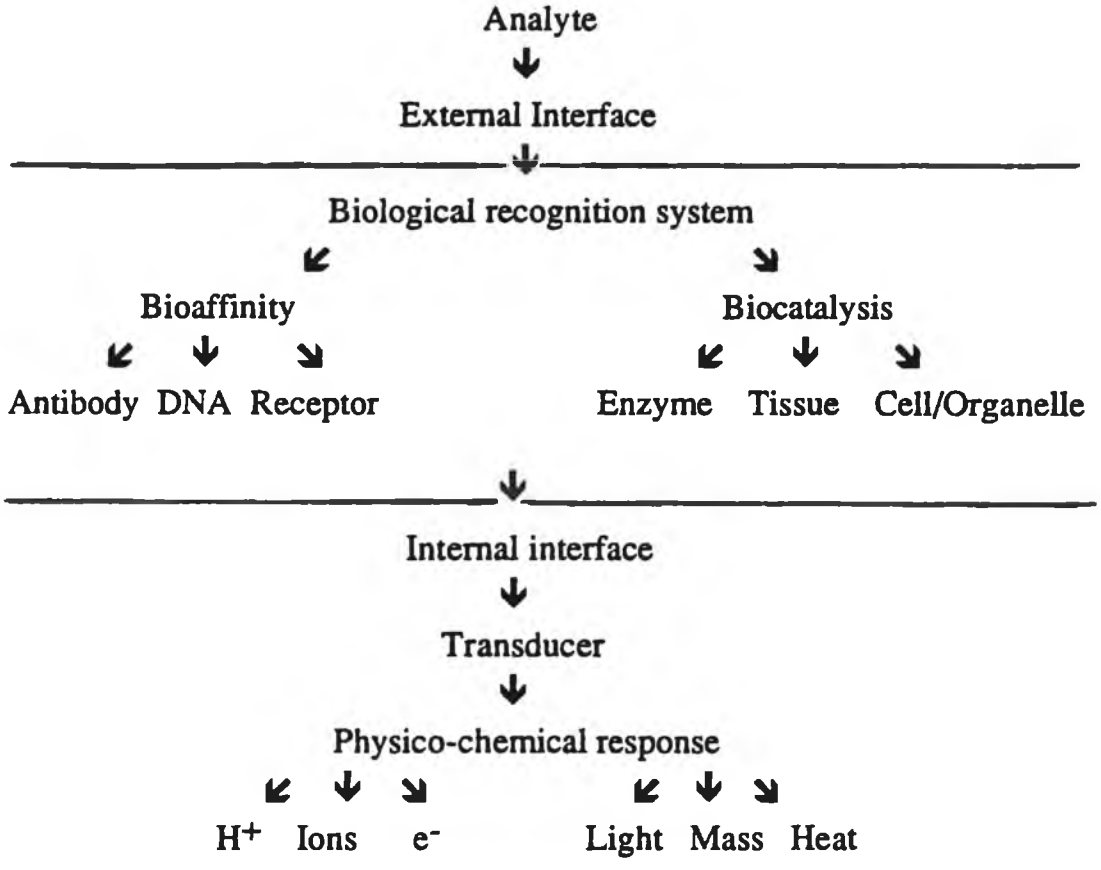

FIGURE 1.1. Scheme representing the processes taking place in a biosensor system; analyte recognition, signal transduction and readout.

The means of recognition and methods of transduction [10], will be discussed in the following sections.

1.2. MOLECULAR RECOGNITION

Molecular recognition, which is responsible for determining the selectivity of a biosensor, is mostly realised by biomolecules, such as enzymes and antibodies. It is based on interactions between molecules of complementary structure, binding to each other by coulombic forces, H-bonds, dipole-dipole or hydrophobic interactions. This recognition forms the basis of biological communication and organisation, and it is an integral part of the internal communication between the cells and organs of an organism.

A broad useful sub-division of biosensors, is between bioaffinity (e.g. antibody, binding protein or receptor) and biocatalytic (e.g. enzyme, microbial, plant/tissue) devices [11]. In the former, binding of an analyte leads to its passive capture with some subtle charge effect or conformational change in the bioreagent adduct. The bioaffinity receptor protein-ligand complex is stable enough to result in signal transduction. Here, unless a specifically labelled species is involved in the binding interaction, transduction of the binding event can prove difficult. Advanced biotechnology and monoclonal antibody production have provided strong support for bioaffinity biosensors, especially in the development of homogeneous immunosensors.

In the case of biocatalytic biosensors, the corresponding substrate is recognised and a product immediately generated by a specific reaction. A change in either the substrate or product is therefore detected. A variety of redox reactions, which involve electron transport in biological systems, are ensured by a set of redox enzymes and protein electron carriers and proceed at very high rates. Therefore, by combining electrode and enzymatic processes, the study of enzyme properties in electrochemical systems becomes a challenge, because of the high specificity and catalytic activity. Where initial binding is followed by analyte degradation, the re-coding of the analyte into some alternative reactive species allows for simpler and more classical modes of transduction. With respect to biocatalytic biosensors, the electron transfer type of enzyme sensor, in which the enzyme exchanges an electron directly with an electrode, has attracted much attention. This will be further discussed in section **1.3.**

Some of the complementary molecules used in biological recognition are outlined in Table 1.1.

Substance to be recognised	Complementary molecule	Recognition effect
Substrate, Inhibitor	Enzymes	Change of concentration or redox potential
Ion, molecule	Carrier protein	Change of concentration or redox potential
Drug, toxin, neurotransmitter	Receptor	Conformational change, membrane permeability change
DNA, RNA	DNA, RNA	Formation of H bridges
Carbohydrate	Lectin	Conformational change
Antigen, hapten	Antibody	Conformational/weight change

TABLE 1.1. Complementary molecules used in biological recognition processes.

The scheme shown in Figure 1.2. gives the concentration levels applying to the various recognition systems.

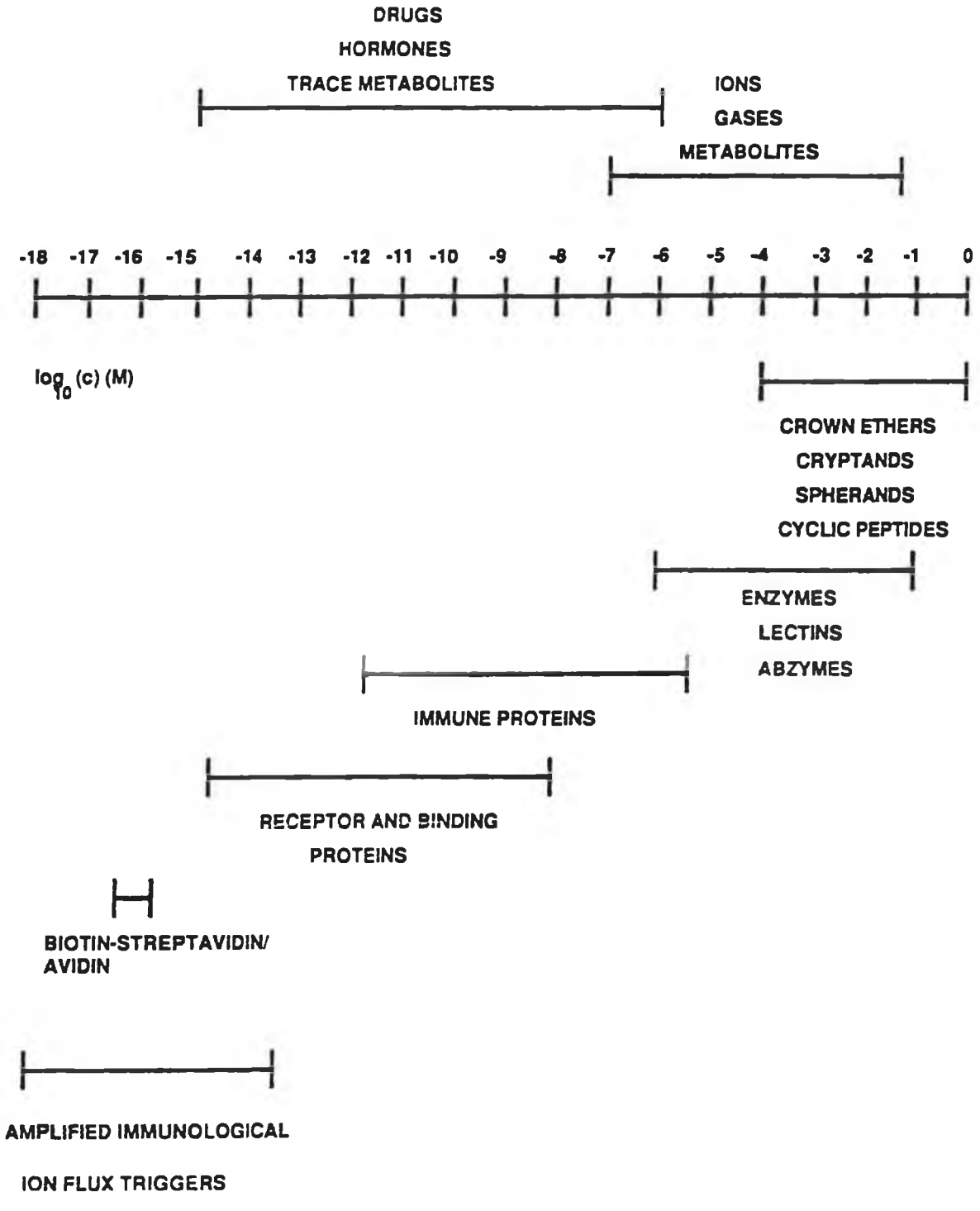

FIGURE 1.2. Levels of some recognition-response systems. Reproduced from [12].

1.2.1. *Enzymes as catalysts for recognition*

The use of an enzyme in association with an electrode was the first type of biosensor developed. Significant improvements have recently been proposed, by using new types of enzyme sensor associations, novel supports or immobilisation procedures, more appropriate detection techniques, or by purification of new enzymes. Genetic manipulation of enzymes may also enable production of 'tailor-made' biocatalysts with specific characteristics.

The first generation of biosensors were prepared by physical entrapment of soluble enzymes behind membranes or the fixation of enzyme membranes to a transducer. The second generation involved the application of bulk immobilisations of the biocompound onto the transducer. Currently the emphasis is on covalent binding of monolayers of oriented recognition molecules directly to the surface of semiconductor devices, resulting in fast response times.

Understanding the theoretical basis of enzyme function helps in explaining the response and performance of enzyme electrodes. Since enzymes are biological catalysts, the equilibrium constants of the catalysed reactions are not altered. Their use as analytical reagents is based on their selective effect on the reaction kinetics, a lowering of the activation energy and the formation of a lower energy barrier. The presence of the binding step determines the nature of the observed kinetics for enzyme catalysis and this can be illustrated by considering the simplest enzyme reaction scheme:

$$E + S \underset{k_{-1}}{\overset{k_1}{\Leftrightarrow}} ES \overset{k_2}{\Rightarrow} E + P \qquad (1.1.)$$

where k_1 and k_{-1} are the forward and backward rate constants for complex formation and k_2 is the rate constant of the rate limiting process stage for complex decomposition into product. After some data analysis the following equations may be derived:

$$v = (k_2[E]_o[S])/K_m + [S] \qquad (1.2.)$$

$$1/v = K_m/(k_2[E]_o[S]) \cdot 1/[S] + 1/(k_2[E]_o) \qquad (1.3.)$$

where υ is the rate of the enzymatic reaction as a function of the concentration of substrate [S] and $[E]_o$ represents the initial concentration of enzyme. The

Lineweaver-Burk plot [13] of reciprocal rate against the reciprocal substrate concentration is normally used in the determination of binding constants. The plot enables the rate of the enzyme catalysed step to be determined from the intercept, with the Michaelis constant, K_m, reflecting the association step from its slope. This constant expresses the [S] at which the rate of the reaction is half of its maximum value. K_m depends on the reaction conditions such as pH and temperature. The rate of the reaction is dependent on [S], and the initial rates of the reaction must always be used in the determination of K_m, as deviation from linearity occurs at high [S]. K_m can be determined, as in the case of amperometric enzyme electrodes, from a plot of the reciprocal of steady state current vs. 1/[S]. A linear Lineweaver-Burk plot indicates a catalytically - controlled reaction. As the [S] > K_m, a mass transfer/diffusion limited reaction is expected, also resulting in a linear plot, but only the effective K_m' may be calculated in this case, due to the diffusion limited process. [S]/K_m will depend on whether the system is diffusion or kinetically controlled.

There is no clear cut borderline between mass transport and catalytic control. Mass transport controlled rates will be observed if the rate of enzymatic catalysis is fast compared to the rate at which the substrate is transported to the catalytic layer. Conversely, catalysis limited rates will be observed if the opposite occurs. The detection of the product may show a mass transfer limitation regardless of catalysis/mass transport with respect to substrate. Under these conditions the overall conversion rate may be incorrectly labelled as mass transport limited, where in fact the product detection is limited or impaired. A non-linear Lineweaver-Burk or Eadie-Hofstee [13] plot is qualitatively indicative of a mass transport limited process.

It must be emphasised that the intrinsic kinetic parameters of immobilised enzymes are not necessarily the same as those of the free enzyme in solution, because of conformational changes, steric effects and diffusional constraints [14].

When the biocatalyst is present in a highly integrated state, i.e. buried in the cells of intact tissues, the catalysed reaction is determined, not only by the enzyme activity, but also by diffusion of the respective substrate through the cell wall and intracellular spaces.

The most common enzymes used in monoenzymatic systems are oxidases, the majority of these being flavoproteins which use natural electron acceptors such as oxygen and quinones in order to regenerate the reduced enzyme during the reaction. Enzymes not only recognise and convert their substrates, but they can also be reversibly inhibited by substances that modify

functional groups. With respect to these enzyme-based systems, the following advantages/disadvantages apply:

Enzyme based systems

- in kinetic equilibrium
- temperature dependant
- diffusion affects total signal

Advantages	Disadvantages
• highly selective	• high temperature coefficient
• sensitive	• irreversible thermal deactivation
• established methods	• pH sensitive
• ease of immobilisation	• sensitive to ionic strength
	• sensitive to inhibitors

The second group of enzymes normally employed - the dehydrogenases, require a separate cofactor, i.e. reduced nicotinamide adenine dinucleotide phosphate (NAD(P)H), for operation. In most cases this cofactor is bound simultaneously with the substrate in the active site of the enzyme, allowing transfer of a hydride ion from the substrate to $NAD(P)^+$. Reoxidation of the co-substrate at an appropriate electrode surface will lead to the generation of a current proportional to the concentration of substrate.

1.2.2. *Tissue biocatalysts as recognition agents*

Other types of biocatalysts, including bacterial cells and animal/plant tissues, have been shown to offer several advantages over isolated enzymes and have been reviewed recently [15]. Since flowers, leaves and fruit are structures related to plant growth, reproduction and nutrient storage, they have high concentrations of biocatalytic activity. Biosensors prepared from tissues open up several biosensing possibilities as multienzyme systems may work to an advantage. By changing the external experimental conditions, different substrates may be determined with the same biocatalytic material. Some examples of tissue biocatalysts are given below.

Mixed carbon paste electrodes have been shown to offer unique speed, sensitivity and miniaturisation advantages for biocatalysis. The use of closely spaced active biosurfaces and sensing microelectrode tips can provide useful information on both surface heterogeneity and the rate of biological processes. Scanning electron microscopy (SEM) has also been used to probe the bioactivity

of tissue - containing carbon surfaces [16]. The selective determination of dopamine at a mixed polyphenol oxidase containing banana carbon paste electrode was used to illustrate the new concept of a mixed plant tissue [17]. Sensor fabrication was accomplished simply by mixing the desired quantity of tissue into a conventionally prepared carbon paste electrode.

A photochemical cell using thylakoid membranes isolated from spinach leaves enabled a phytotoxicity biosensor to be constructed [18]. The photocurrent generated in the cell could be linearly correlated with the electron transport activity of the photosynthetic membranes. The approach was based on the fact that several pollutants or toxic compounds found in water can inhibit photosynthetic electron transport. The membranes therefore may be used for monitoring phytotoxicity.

The construction and dynamic response characteristics of a tissue porous electrode combination, composed of mushroom, potato and horseradish peroxidase tissues which are packed into reticulated vitreous carbon (RVC) has been described [19]. High sensitivity accrues from the high tissue loading and large surface area of the RVC. A bioelectrode for primary alcohols based on the incorporation of yeasts into the carbon paste matrix has also been reported based on the activity of alcohol dehydrogenase in yeasts [20].

1.2.3. *Whole cells as bioaffinity recognition agents*

Harnessing of the whole fully intact enzyme in its bioenvironment, i.e. in the whole cell or organelle, requires rather more in the way of biological manipulation and preparation. Hence rather less work has been reported along these lines. The advantage of an intact cellular system is that the enzyme is now retained in its natural immobilised state and is presupplied with any requisite cofactor/reagents. The drawback is that a cell retains many active enzymes, and response is sometimes not specific enough for analytical purposes. The advantages and disadvantages of such systems are summarised below:

Advantages of whole cell biosensors

- high degree of stability
- plethora of known species covering wide spectrum of metabolic activity
- capable of performing complex reaction sequences
- majority of species easy to grow, maintain and harvest

- amenable to genetic manipulation enabling production of 'tailor-made' biocatalysts with specific characteristics
- unlike enzymes, no requirement for costly extraction and purification prior to use as biocatalysts
- modest loading levels give high levels of catalytic activity
- possession of damage repair capabilities and replicative potential offers the prospect of biocatalyst recovery *in situ*

Disadvantages of whole cell biosensors

- low specificity
- slow response/recovery times
- fabrication and bulk production
- sterilisation

Microbial biosensors have been under development for environmental water monitoring. Tailoring of microbial species to have particular vulnerability to a toxin or toxin group would appear to be a requirement if meaningful specific data is to be obtained.

Heavy metal ions may be accumulated by micro-organisms such as algae [21,22] moss [23, 24] and lichens [25, 26]. The biopolymers in the cell walls of these plants are responsible for metal ion adsorption, and the binding site diversity gives the algae a broad applicability in electroanalysis and electrocatalysis.

A chitin-containing carbon paste electrode was found to give highly sensitive voltammetric waves for Fe(III) - EDTA chelates. Various conditions for the determination of trace iron were studied [27]. A clay modified electrode was characterised and optimised for analytical use in ion exchange voltammetry using $Ru(NH_3)_6^{+3}$ as a test ion [28]. The porosity of the clay film can be controlled via manipulation of the bathing electrolyte. The interlayer spacing for a well-orientated film has also been shown to control the overall porosity and hence conductivity of a clay film localised at an electrode surface. The strong affinity of natural ionic polysaccharides for certain metal ions has been exploited in the design of a new class of voltammetric sensing devices e.g. carbon paste electrodes containing pectic and alginic acids for the nonelectrolytic collection and subsequent voltammetric determination of copper and lead [29].

The oxidoreductases in bacterial-membranes may also be used as catalysts [30]. In this case *Gluconobacter suboxidans* was employed because the

properties of the membrane-bound oxidoreductases of this bacterium have been extensively studied. The results indicated that alcohol dehydrogenase activity in the cytoplasmic membranes of the bacterium had catalytic activity to oxidise ethanol when ferricyanide was used as an electron acceptor.

Cells of *Pseudomonas capacia* were immobilised in calcium alginate gel and their metabolic responses to a range of derivatised aromatic compounds studied, by placing the immobilised cells in close proximity to an oxygen electrode [31]. Cells of *Z. Mobilis* and invertase were coimmobilised in a gel membrane for specific determination of sucrose. The whole cell enzyme electrode was constructed by fixing the membrane on a pH electrode. L-ascorbic acid has been determined by concentration - step amperometry using a thin layer of carbon felt impregnated with cucumber juice as an enzyme solution of ascorbate oxidase. The dilute fruit juice was added on top of the carbon felt and the decrease in peak current caused by the enzymatic reaction measured [32].

Certain fish cells contain specialised cells with pigment granulas, which can be aggregated, by a transmitter substance, noradrenaline, released by the sympathetic nerve endings in the skin. It has been demonstrated that these fish scales may be used to monitor catecholamine levels in human blood plasma [33].

1.2.4. *Antibodies as bioaffinity recognition agents*

The chemical binding between an antibody [Ab] and an antigen [Ag] can be very strong indeed, and the binding process is frequently very rapid. The association reaction being characterised by the second order rate constant, k_a, and the dissociation reaction by k_d. The corresponding association ν_a and dissociation rates ν_d are given as :

$$\nu_a = k_a[Ab][Ag] \qquad (1.4.)$$

$$\nu_d = k_d[Ab\text{-}Ag] \qquad (1.5.)$$

At equilibrium the concentrations of reactants, [Ab] and [Ag], are such that the rates of association and dissociation are equal, giving an expression for the equilibrium constant, K_a for association :

$$K_a = [Ab\text{-}Ag] / [Ab][Ag] = k_a/k_d \qquad (1.6.)$$

The high specificity of antibody:antigen binding reactions has prompted considerable effort to use these immunochemical reactions to design immunosensors that allow the concentration of a biomolecule to be measured directly. Antibody properties for biosensor applications have been reviewed [34], together with future trends and future aspects of the research and development of immunosensors [35]. The selective reaction between an analyte and the corresponding antibody ensures the specificity of the immunosensor in biological fluid analysis. The specificities of immunosensors are determined in part by the affinity constants of the antibody:antigen interaction [36]. While a high affinity constant will give rise to high specificity, too high an affinity constant will result in an irreversible immunosensor, unless protein denaturing conditions are used. As most immunoassays are used to monitor low antigen concentrations over a narrow concentration range, assays based on amperometric detection are more sensitive than potentiometric devices [37].

An enzyme - coupled immunoasssay based on flow injection analysis and amperometric determination of NADH is feasible and practical, using phenyltoin as model analyte [38]. It is desirable if the rate constant of the enzyme used as an immunochemical label is as large as possible and it is also helpful if V_{max} is small. Both conditions help to ensure that the rate of product formation proceeds linearly for the greatest period of time, minimising the effect of variations in the time between addition of substrate and the initial signal measurement. In addition, an antibody incorporated into a semipermeable microchamber held at the tip of an optical fibre has enabled reagentless determination of phenyltoin [39].

A novel potentiometric enzyme immunoassay technique has been described, utilising polystyrene beads in conjunction with a gas sensing membrane electrode [40]. A biosensor using polymerised protein films containing the antibody and appropriate stabilisers to coat an interdigitated electrode transducer has also been reported [41]. Changes in the electrical field of the coated electrodes were related to specific interaction between the immobilised biomolecule and the analyte to be measured. An enzyme immunosensor was also constructed for the detection of human chorionic gonadotropin, an important hormone in the diagnosis of pregnancy [42]. In addition, a sequential injection immunoassay method has been described which utilises immunomagnetic beads to investigate short - time antibody binding [43]. Ghindilis *et al.* [44] have described an electrochemical immunosensor based on a new detection principle. Laccase was used as a label, which catalyses the reduction of oxygen. The formation of the complex between laccase-antibody and antigen on the electrode

surface resulted in a considerable shift of the electrode potential. The rate of increase in potential was proportional to the concentration of the insulin antigen.

A flow-through cell for the real time capacitance monitoring of immunochemical interactions has also been reported [45]. The feasibility of direct immunosensors based on capacitance measurements on silicon-silica heterostructures was shown for an immunosystem system based on G type antibody. Capacitance measurements succeed only if the successive bimolecular layers grafted onto the heterostructures are electrically insulating, and is therefore strongly dependant on the coupling procedure used for antibody fixation on the silanised silica layer [46]. By optimising a differential method and using a Langmuir-Blodgett technique to prepare an antigen bound electrode, Karube *et al.* have developed a promising sensitive potentiometric immunosensor for IgG [47]. IgG was immobilised on a wafer surface both covalently and through adsorption. The results of the incorporation of the antibody to human serum albumin into an electrochemically generated polypyrrole film on an electrode surface have been described by John *et al.* [48], with the electrochemical characterisation of the polypyrrole - antibody films after exposure to antigen.

Liposome-mediated immunoassay is a relatively recently developed technique allowing amplification of a signal to be achieved. A liposome is a tiny artificial spherical assembly of concentric phospholipid bilayers. It may be filled with up to 10^4 marker molecules (i.e. an ion that may be detected potentiometrically, or an enzyme marker). Marker species may be trapped within the aqueous spaces surrounded by bilayer lipid membranes. The analyte may be inserted into the membrane, and when antibody binds in the presence of complement, disruption of the bilayer occurs, resulting in the marker being released. The classical complement pathway is an antigen-antibody specific reaction that occurs when an antigen-sensitised liposome immunospecifically binds with a corresponding antibody. In the presence of the complement, usually a serum protein, the marker is released and detected, and is an indication that the immunoreaction has taken place. The use of liposomes as detectable reagents in solid phase immunoassays has been explored in flow injection immunoanalysis systems. This is based on the competitive binding of univalent analyte and multivalent liposomes to immobilised antibodies [49].

There are other routes founded on naturally occurring biological amplifiers, for example the cascade mechanism of blood clotting and glycogen mobilisation can achieve high gains by sequentially activating a series of catalysts leading to massive quantities of product from a small triggering

response. These natural resources may be trapped to a greater extent and incorporated in electrochemically-based assays [50].

1.2.5. *Receptors as bioaffinity recognition agents*

Much of the research published in the area of biosensors has been restricted to the selectivity afforded by the enzyme-substrate and immunochemical reactions and have largely ignored the combined selectivity - transducer physics offered by natural chemical sensing technology, such as that employed in olfaction and neurotransmitter electrophysiology. Therefore a more ambitious approach to bioaffinity biosensing is to use intact chemoreceptor organs present in some animal species.

The use of natural chemoreceptive mechanisms involves either modelling natural cell membranes function, or employing intact animal and plant tissues to act as biocatalytic materials for the synthesis of products which can be further analysed by potentiometric and amperometric methods. There has been much interest in the biochemical and physiological attributes of natural chemical sensing of the neural and olfactory system. The process employed in natural chemoreception rarely makes use of equilibrium or steady state conditions. Rather, signals are generated by transient phenomena which exist for brief periods. This implies that the analytical signal is derived from the perturbed physical signal which exists after the initial steady state is altered by receptor-stimulant interaction.

The isolated antennule of the Hawaiian crab has been used to measure stimulants such as trimethylene oxide [51]. Two species of Hawaiian crustaceans were shown to be effective for the construction of these antennular 'receptrodes'. The resulting chemoreceptor-based biosensors respond to stimulant compounds with short response times. Two severe problems in the chemoreceptor-transducer interface relate to reversibility and calibration issues. Leech and Rechnitz [52] have recently reviewed neuronal biosensors. They presented the progress of research in the development of intact chemoreceptor-based were used to detect the action potential firing upon binding of the stimulant to the receptor allowing quantitation of the analyte [53].

Chemoreceptors are large macromolecules which extend through and across the cellular membrane. Studies of the acetylcholine receptor reveal that it is a protein with a tubular tertiary structure. A channel extends through the length of the molecule and therefore through the membrane. When a compound binds at the binding site a conformational change occurs which allows the channel to

open. It is the high degree of specificity provided by the receptor binding site which allows the chemoreceptor to function as a highly specific molecular recognition element. The chemoreceptive neurons function as transducers converting the chemically added information received at the receptor binding site into electrical impulses.

It is possible to create a biosensor based on the concentration-frequency relationship if one could 'intercept' the action potential during its propagation. [51]. For instance a nicotinic acetylcholine receptor adsorbed directly onto a quartz optical fibre was used to bind to the toxin α-bungarotoxin with a remarkable retention of activity [54].

A report in Time Magazine on April 15, 1991 gave an account of how researchers discovered the first known genes of smell and opened up a whole new field of olfactory science [55, 56]. The use of sensor arrays for gas/odour or solute/taste detection is an exciting current development area in microelectronics as evidenced by recent literature [57-59]. The human olfactory system employs about 50 million olfactory receptors in a massively parallel neural architecture and it appears that four principle structural elements are deduced that define an odour or a gas: the size and shape of the molecule, and the nature and position of its functional groups. A successful artificial nose will need to be capable of acquiring this information [59]. Pearce *et al.* [60] have reported on an array of up to 12 conducting polymers, each of which had an electrical resistance that has partial sensitivity to the headspace of beer. The instrument, or electronic nose, is capable of discriminating between various commercial beers and more significantly between standard and artificially tainted beers.

Two other areas of sensor development involve sensors capable of stereospecific and molecular recognition. The integration of monolayer and molecular recognition was the basis of a recent paper by Rubenstein *et al.* [61]. The membrane reported consists of receptor sites that are experimentally designed to impart the desired selectivity within the monolayer matrix [62].

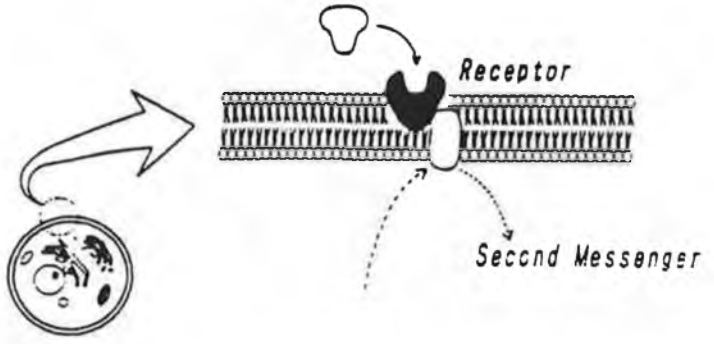

FIGURE 1.3.
Schematic illustration of molecular recognition by cellular receptors. Reproduced from [11].

1.3. IMMOBILISATION METHODS

One of the main factors affecting the performance of a biosensor is the biomolecule immobilisation procedure itself. Many different approaches to enzyme immobilisation have been used in the design of enzyme electrodes. Close juxtaposition of the molecular recognition molecule and the transducer surface is a key requirement for effective sensor operation. Each method has its own advantages and disadvantages and several factors should be considered before choosing an immobilisation technique. These include:

- applicability for a variety of surfaces
- immobilised biocatalyst should exhibit maximum activity
- immobilised biocatalyst should operate over a wide pH range
- immobilised biocatalyst should be stable
- the ability to co-immobilise more than one biocomponent
- little or no leakage of the biocomponent from the surface.

1.3.1. *Physical Methods*

1.3.1.1. *Adsorption*

Simple physical adsorption of the biocomponent on a transducer surface, is a popular technique. Advantages include the fact that it requires no reagents, with minimum activation and clean-up. It suffers, however, from greater susceptibility to changes in pH, temperature, ionic strength and substrate concentration. The lifetime of the biocomponent is short when compared with other methods of immobilisation. Physical adsorption at the external surface of a transducer is due to physicochemical bonds created by hydrophobic interactions and Van der Waals forces. Adsorption is a convenient, though not necessarily stable, means of immobilisation. A glucose sensor has for instance, been prepared by adsorption of the mediator Meldola Blue as well as glucose dehydrogenase on the surface of carbon [63]. The entrapment of an enzyme, either within a gel or through microencapsulation, is of general application and should not affect the enzyme activity [64]. Physical adsorption and gel/polymer entrapment are often supplemented by use of cross-linking agents to prevent leaching of the biocomponent into the bulk solution. The reaction characteristics and stability of a membrane-bound enzyme reconstituted in bilayers of liposomes has been reported [65].

1.3.1.2. *Membranes and Polymer Films*

The entrapment of biomolecules behind or within polymer films for immobilisation gives several advantages namely:

- inherent chemical and physical stability
- ability to support catalyst species
- rapid charge propagation among catalyst sites
- porosity towards substrate and electrolyte.
- size exclusion (for protection and selectivity)
- ion-exchange
- preconcentration

Membranes may be employed as a means of immobilisation. It is necessary that they have good adhesion to the transducer surface and should be thin, hydrophilic and porous. Cellulose acetate, polyvinylalcohol (PVA) and polyurethane are examples of commonly used enzyme membranes. Entrapment within polymer matrices, such as polyacrylamide, agarose and poly(N-methylpyrrole) have also been reported with successful results [66].

Physical entrapment in an inert matrix e.g. gelatine, is often employed due to the ease of preparation over many chemical procedures. The resulting enzymatic gel may be cast over an electrode, and maintained by a dialysis membrane. Gelatine membranes may be easily resolubilised by gently shaking in buffer at 37-40°C. A higher percentage of initial activity has been found after such resolubilisation of the membranes, compared to that of e.g. glutaraldehyde. This high residual activity, in gelatine indicates the protective effect of the 'native' environment created by the matrix [6].

Enzymes can be conveniently immobilised on a modified nylon mesh, produced by successive treatments of nylon with dimethylsulphate, lysine spacer and finally glutaraldehyde coupling agents. The resultant enzyme membrane, when held tautly over a platinum anode disc provides a high performance, long life glucose electrode, which can be housed in a cell adapted for flow injection analysis. Advantages of such a means of immobilisation include mechanical stability, which is sustained in the enzyme immobilised matrix and several enzymes may be simultaneously immobilised [6,8].

Some examples of such immobilisation procedures include an immobilised enzyme electrode for continuous creatinine determination in blood serum. The enzymes creatinine amidohydrolase, and sarcosine oxidase were immobilised

onto the surface of a polypropylene membrane of a Clark type electrode within a gelatine layer [67]. Villarta *et al.* [68] have coimmobilised aspartate aminotransferase and glutamate oxidase on a preactivated polymeric membrane for determination of their respective substrates. Other examples include the use of the purified enzyme L-glutamate oxidase from *Streptomyces platenis* which was immobilised onto a cellulose triacetate membrane held at an oxygen electrode [69] and xanthine oxidase immobilised via glutaraldehyde activation on a polycarbonate membrane mounted on the tip of an hydrogen peroxide electrode [70].

1.3.2. *Chemical Methods*

Covalent attachment to a transducer surface provides a more stable immobilised biocomponent and is more widely applicable than physical methods of immobilisation. The attachment often requires three steps: activation of the support, enzyme coupling and removal of loosely bound enzyme.
Chemical immobilisation of enzymes may create micro-environments that can :

- either attract/repel substrate, product or inhibitor by introducing a charge/hydrophobic group onto the support
- change the pH optima of enzymes by introducing positively/negatively charged groups into the support matrix.

1.3.2.1. *Crosslinking with Glutaraldehyde*

Chemical methods of immobilisation are based on the formation of covalent bonds between enzyme molecules and a functionalised compound, e.g. glutaraldehyde. A commonly used method of immobilisation involves covalent bonding of the enzyme with glutaraldehyde, usually in the presence of bovine serum albumin (BSA). The BSA provides additional coupling sites without blocking the electrode surface, thus acting like an intermediate spacer arm. The glutaraldehyde reacts with lysine amino groups in the enzyme, and by mutually cross-linking the enzyme with another protein (BSA), higher enzyme activity and greater stability can be obtained. It is a very stable method of immobilisation but quite severe, sometimes causing a loss of enzyme activity. A prolonged lifetime for a graphite electrode modified with glucose dehydrogenase was achieved by cross-linking the enzyme with glutaraldehyde [71].

1.3.3. *Langmuir-Blodgett films*

The Langmuir-Blodgett (LB) film technique, developed by Irving Langmuir and Catherine Blodgett in the 1930s, has the major advantage of allowing monomolecular layers to be built onto a wafer surface or solid substrate. Consequently, enzymes or antibodies with properties specific to the analyte to be detected, can be deposited directly on the transducer. A highly ordered molecular orientation is obtained and the microenvironment can be tailored to closely resemble the natural one encountered for instance inside biomembranes. If the molecules are not highly amphiphilic or are unable to interact effectively with supporting layers it is often difficult to deposit a film at the subphase.

LB films are created in troughs generally filled with highly purified water (subphase). The amphiphilic compound selected is dissolved in a volatile solvent, and then deposited onto the subphase. When the solvent is evaporated only the amphiphilic molecules in question remain on the surface. Two moving barriers allow the condensation of amphiphilic molecules in a two dimensional ordered film by compression. This film may be removed to the substrate by immersing the latter in the solution.

An interesting extension of artificial lipid membranes for electrochemical sensing is the construction of biometric ion-channel sensors. These devices are based on LB deposition of multilayers of acidic lipid membranes on glassy carbon electrodes. The most promising approach in biosensors would be via the change of the transmembrane conductance of an artificial bilayer lipid membrane due to specific interaction of the analyte with a biomolecule embedded in the membrane. Bi-lipid membranes may be deposited on hydrogel layers for amperometic and conductometric devices or on very thin silicon dioxide for the gate dielectric of potentiometric devices.

The high selectivity and affinity of antibody-antigen binding reactions have prompted considerable efforts to use these immunochemical reactions to prepare bioactive layers. The LB technique provides a simple way of obtaining well engineered molecular layers with controllable characteristics [72].

Even if the availability of natural hormone receptors and ion channels due to genetic organic methods, for the contribution of sensors were within the range of possibility, they could not be used for this purpose. These proteins need intact membranes. In fact the reconstitution of the nicotinic acetylcholine receptor in artificial bilayer membranes has been achieved and the electrical conductivity of this membrane modulated by means of the effector [73]. The main requisite for a general adaptation of this possibility of building a sensor would be to produce

monolayer/bilayer membranes on transducers and to change them with receptors in an oriented way. A possibility capable of realising this concept is the LB technique, which enables artificial mono/bilayer membranes to be produced and transferred [74].

1.3.4. *Effects of Immobilisation on Properties of Enzymes*

Changes in the chemical/physical properties of an enzyme upon immobilisation may be conveniently viewed as being due to either the nature of the support or to some alteration of the enzyme itself. The characteristics of the enzyme may be altered if the active site undergoes some conformational changes as a result of chemical/physical interactions between enzyme and support. Steric hindrances may occur, preventing free diffusion of substrate to all molecules of the enzyme or by forming electrostatic interaction with molecules of substrate or product. The pH optimum can change by as much as two pH units when the enzyme is immobilised, largely owing to the effect of the new microenvironment. The pH optimum of many enzymes moves to a more alkaline value if the support is anionic and to more acidic if cationic, due to changes in the degree of ionisation of amino acid residues at the active site. Saturation of an enzyme attached to an electrode will occur at a higher concentration of substrate than freely soluble enzymes, due to the Nernst layer [75]. Some of the factors involved here include:

- the diffusion layer surrounding the water-insoluble particle
- steric repulsion of substrate
- molecular size of polymer
- flexibility of polymer backbone
- degree of hydrophobicity
- electrostatic interactions

1.3.4.1. *Kinetic effects*

When the enzyme is immobilised, the measured reaction rate does not only depend on the substrate concentration, K_m, V_{max} and diffusion, but also on so called "immobilisation effects". A non-uniform distribution of substrate and product between the enzyme and the surrounding solution affects the measured kinetic constant. In a multilayer system at the surface of the sensor the substrate and product molecules are transferred by diffusion. Slow mass transfer occurs to

and within the concentration of reaction partners in the bulk solutions. Apparent kinetics are observed when internal/external diffusion affects the overall rate. Inherent kinetics prevail when only partitioning (not mass transfer) effects are present. The effects involved include:

- partitioning effects; the equilibrium substrate concentration within the support may be different from that in the bulk solution
- microenvironmental effects on the intrinsic catalytic parameters of the enzyme
- diffusional/mass transport effects; diffusional resistances to the translocation of substrate/product to or from the site of enzymatic reaction. Therefore, rate of diffusion of substrate is slower than the rate of its transformation by the enzyme.

The K_m may be affected by diffusion factors. Around each particle/membrane is an unstirred layer of solution, known as the Nernst diffusion layer. The substrate contained in such a layer will be quickly utilised by an enzyme immobilised within the membrane such that the subsequent reaction velocity will depend on the rate at which substrate from the bulk solution can diffuse through the unstirred layer and reach the enzyme. The steady state substrate concentration in the vicinity of the enzyme is less than that of external concentration of substrate. Thickness of the diffusion layer depends on the rate at which the bulk solution is stirred, such that it may be possible to increase the reaction velocity by more vigorous stirring. Ease of diffusion of product from enzyme is important, particularly if the reaction catalysed is subject to product inhibition. The rate of diffusion of substrate to enzyme will rise to a limiting value as the concentration of substrate in the bulk solution increases. If this limiting value is reached before an immobilised enzyme is completely saturated with substrate, then V_{max} is less in free solution. This phenomenon will be even more pronounced if the substrate is to diffuse through an additional layer before reaching the enzyme microenvironment.

1.4. TRANSDUCTION

The purpose of the transducer is to convert the biochemical signal into an electronic signal that can be suitably processed with data output allowed. Table 1.2. outlines the advantages and disadvantages of the various transduction techniques used in biosensors. The following sections will describe each method in detail.

TRANSDUCER	ADVANTAGES	DISADVANTAGES
ELECTROCHEMICAL Potentiometric	Wide response range, selective.	Responses to change in concentration are logarithmic.
Amperometric	Linear response, sensitive, inexpensive	Interferences and protein fouling
OPTICAL Fiber optics	Small, rapid response, no reference necessary, suitable for arrays.	Special instrumentation and indicators needed.
FIELD EFFECT TRANSISTORS	Very small size, suitable for automated systems.	Unstable, leakage problems, biocompatibility problems.
THERMISTORS	Simple, linear response	Non-selective, subject to drift.
PIEZOELECTRIC CRYSTALS	Operate over wide temperature range, sensitive	Work best in gas phase, slow recovery.

TABLE 1.2. Advantages and disadvantages of transduction methods.

1.4.1. *Electrochemical Transduction*

1.4.1.1. *Amperometric detection*

In the case of amperometric sensors, a voltage is applied between the working and reference electrode which forces non-spontaneous electron transfer reactions to occur. Amperometric techniques involve dynamic electrode processes in which current is allowed to flow. In amperometry, the response is a large amplitude faradaic current, resulting from the heterogeneous electron transfer of solution species undergoing oxidation/reduction reactions. Steady state is a condition at which the rates of diffusion of the electroactive surface are equal. In amperometry, electrochemical processes are generally complex and may be considered to be a succession of electron transfers and chemical events. The overall sensor current is thus dependant on many factors, including charge transfer, adsorption, chemical kinetics, diffusion, convection and substrate mass transfer. The pathway of a general electrode reaction is shown in Fig. 1.4.

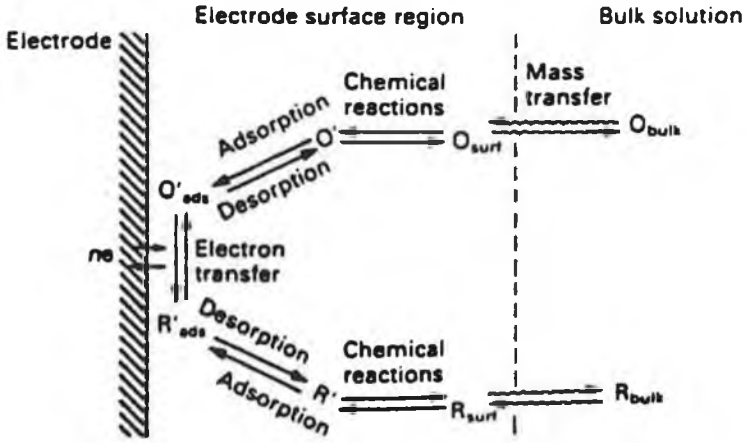

FIGURE 1.4.
Pathway of a general electrode reaction. Reproduced from [76].

The Clark electrode [1] was historically the first transducer associated with soluble glucose oxidase, for glucose monitoring. The sensor consisted of a platinum or gold disk cathode polarised at a potential of *ca.* -0.7 V vs. the Ag/AgCl anode, having a ring shape. This was covered with a film of polyethylene or polyvinylchloride, with a high permeability towards oxygen. This arrangement allows oxygen reduction at the cathode to proceed. The measured cathodic current resulting from this electrochemical reduction is proportional to the oxygen level in solution. Thus the depletion of oxygen at the biosensor tip due to the oxidase reaction can be easily correlated with the substrate concentration. A linear relationship between the current variation and the analyte concentration can be established in a definite range.

The various parameters affecting the steady state behaviour of immobilised enzyme electrodes include :

- diffusion of substrate to the electrode surface
- substrate diffusion within the biocatalytic layer
- conversion of substrate to product
- various product transport diffusion processes from the electrode surface to the bulk solution.

The combination of the selectivity offered by enzyme catalysis with the simplicity and low cost of an amperometric detector is an attractive approach to the production of chemical sensors for a range of biomedical and biotechnological applications. Amperometric sensors are usually based on oxidoreductase enzymes which commonly use oxygen as coreactant, and NAD^+ / $NADP^+$ as the electron acceptor, which recycles the enzyme after the substrate reaction. Transduction of the signal due to the enzymatic reaction in a biosensor can operate in one of three ways:

- the electrode simply detects either a consumed natural co-reactant, oxygen, or a product, hydrogen peroxide, produced during the biocatalysis; this current is proportional to the analyte concentration in a definite range
- more recently systems involving mediators have been described in the literature; these redox species function by replacing oxygen in oxidase-based reactions and require a lower operating potential with respect to hydrogen peroxide detection, they thus decrease the occurrence of electrochemical interferences at this potential

- an enzyme reacting directly and rapidly at the electrode itself constitutes a challenge for the design of amperometric biosensors; to achieve a feasible direct electron transfer the electrode must be used as a 'cofactor'. A promising approach to this problem is to employ more appropriate sensors, with conducting polymers deposited on the electrode surface.

The necessary cofactor has to be added to the system in the case of the dehydrogenase enzymes. Several approaches have attempted to overcome this limitation, e.g. development of coenzymes with enlarged molecular size which can be retained within the enzyme electrode yielding an 'artificial' prosthetic group [77].

1.4.1.1.1. *Types of electrodes used for amperometric sensing*

Glassy carbon electrodes are commonly employed as amperometric detectors. Electrochemical conditioning of glassy carbon electrodes results in the formation of electroactive surface compounds which enhance the electrode response via unspecified redox mediation processes. The mechanical and physicochemical techniques for activation of solid voltammetric electrodes including glassy carbon, i.e. polishing, chemical and electrochemical treatment and modification of the electrode surface by heat or electromagnetic radiation have been reviewed recently by Stulik *et al.* [78]. A study has also been made of the optimum conditions for electrochemical activation of carbon electrodes in the analysis of dopamine [79]. Glassy carbon amperometric flow detectors were shown to exhibit a substantial improvement in their stability following a simple preanodisation procedure [80]. Improvements in the performance of amperometric biosensors for alcohols was also achieved by subjecting the glassy carbon transducer to electrochemical pretreatment prior to the immobilisation of alcohol dehydrogenase [81]. The enzymatically generated NADH can thus be detected at a substantially lower potential compared to the untreated surface.

The *carbon paste electrode*, invented nearly 30 years ago by Adams, is well known for its low background current [82]. Carbon in the form of graphite is an inexpensive and versatile material and is particularly suitable for the fabrication of electrodes [83]. Carbon paste electrodes and composite electrodes modified with 20% silica gel within the electrode material have been found to exhibit many properties typical of carbon fibre microelectrodes in voltammetric measurements [84]. Amperometric biosensors based on mixed carbon paste redox enzymes have been the subject of considerable interest. Mixed carbon paste

bioelectrodes have been doped with biological entities (enzymes, micro-organisms, algae and tissues) which retain their activity while incorporated in the carbon paste matrix. The surface of the carbon paste bioelectrodes may be easily renewed, thus opening new possibilities for the determination of metabolites in real biological samples. Some applications of biologically modified carbon paste electrodes are described below.

Surface passivation during oxidation of NADH at a fluorocarbon - polymer carbon paste electrode (KEL-F (poly(chlorotrifluoroethylene) wax) was found to be 25 % less than that of the glassy carbon electrode or the carbon paste electrode. The enzymatic determination of ethanol was used as a sample to demonstrate the improvement in response and stability of the KEL-F Wax carbon paste electrode [85]. The modification of carbon paste electrodes by the incorporation of the enzyme glucose oxidase has also been described. The resulting probes can be operated as amperometric glucose sensors in the presence or absence of a mediator mixed into the paste as demonstrated by Amine *et al.* [86]. The simultaneous incorporation of ferrocene derivatives and glucose oxidase into the carbon paste matrix resulted in an effective microelectrode for sensing glucose. The incorporation of stearic acid into the enzyme - containing paste was found to greatly decrease the interference due to ascorbate [87].

Xanthine oxidase and alkaline phosphase have been adsorbed on a carbon paste electrode and physically entrapped behind a semipermeable membrane, to allow for development of sensors for xanthine and alkaline phosphatase [88]. The preparation of metal-dispersed carbon paste electrodes has been reported, based on the mixing of an organic binder with metalised graphite. These electrodes exhibit the ability to catalyse slow electrode reactions of certain compounds. The electrodes combine the efficient electrocatalytic activity of metal microparticles with the attractive properties of carbon paste matrices. Co-immobilisation of an oxidase enzyme allows coupling of a biocatalytic reaction with the electrocatalytic determination of the liberated peroxide [89].

The fabrication and performance of *graphite epoxy* modified electrodes, which exhibit desired characteristics for real life applications, have been reported by Wang *et al.* [90]. Unmodified epoxy-bonded graphite electrodes possess desirable electrochemical, mechanical and chemical properties and have been used for *in vivo* voltammetry and microanalysis. Modification of the graphite epoxy is easily accomplished by mixing the desired quantity of the modifier with the two individual graphite resin and accelerator components. Alcohol dehydrogenase and NAD^+ have been incorporated within rigid graphite epoxy matrices to yield renewable polishable alcohol sensors. The enzyme and cofactor

retain their bioactivity upon confinement in the graphite epoxy, with the oxidation current of the NADH formed as the response [91]. A study by Leech *et al.* [92] to illustrate the applicability of polishable ruthenium dioxides modified graphite epoxy electrodes to the determination of glucose has been reported. An amperometric enzyme electrode for bilirubin based on the incorporation of bilirubin oxidase and horseradish peroxidase within a graphite epoxy electrode has also been reported. The resulting bienzyme electrodes are renewable (by polishing) and offer low potential determination of bilirubin [93].

Reticulated vitreous carbon (RVC) has proved useful due to its excellent flow through properties and large surface area [94]. Its compact geometry provides a large non-microporous working area within a small external volume and allows efficient mass transfer due to the high void volume of the RVC. The higher surface area allows for more binding sites for enzyme immobilisation as well as making the RVC surface more reproducible. A platinised RVC electrode immobilised with glucose oxidase and electropolymerised with 1,2-diaminobenzene has been used by Sasso *et al.* [95] in the construction of a sensor for the determination of glucose in human serum. Fig 1.5. shows such a sensor based on RVC. Covalent immobilisation of lactate dehydrogenase on vitreous carbon was found to provide a surface endowed with both electrochemical and enzymatic activities [96]. In this report, a study was had of the effective masking of the electrode surface by attached proteins, which is difficult using electrodes with small working areas, due to the small amount of attached protein.

The design, fabrication and evaluation of chemically modified *screen printed carbon electrodes* has been described by Wring *et al.* [97, 98]. The technology can be usefully applied to the mass production of inexpensive, reproducible and sensitive disposable electrochemical sensors for the detection of trace levels of compounds in biological fluids. A method for determination of acetaminophen in whole blood was described using aryl acylamidase, incorporated on a disposable screen-printed dry strip [99]. Disposable single-use sensors have also been reported by Green *et al.* [100] and medical diagnostic advantages discussed.

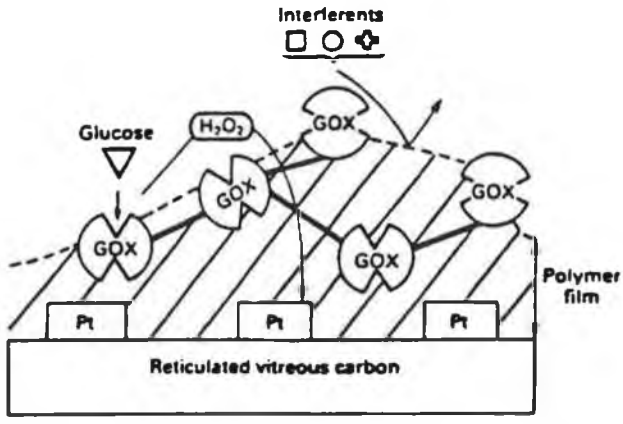

FIGURE 1.5. Schematic diagram of possible enzyme/electrochemically polymerised 1,2-diaminobenzene layer. Glucose unlike hydrogen peroxide does not penetrate the polymer and only exposed enzyme sites are active. Reproduced from [9].

Platinum has been used widely as an electrode material based on hydrogen peroxide and oxygen consumption modes because of its inherently superior electrocatalytic response. Platinum is, however, susceptible to electrode poisoning due to oxide formation and the adsorption of impurities. A Nafion film dispersed with platinum particles formed on a glassy carbon electrode combines the electrocatalytic activity of platinum with the background stability of glassy carbon. It has served as a selective and sensitive electrode surface for an amperometric glucose enzyme electrode [101]. Mass transport conditions for amperometric determinations at macroelectrodes has to be carefully controlled. With the advent of *microelectrode* theory and technology, the need for strict mass transport control can be minimised. The small surface area of carbon fibres permit increased current densities with decreased surface functionalities and a decrease in the interior order of these fibres.

Some features of microelectrodes [102] include:

- radii the same order of magnitude as the distance an analyte molecule can diffuse on the time scale of a voltammetric experiment
- higher faradaic current / charging current ration than macroelectrodes
- insensitivity to changes in convective conditions in solution
- negligible alteration of the bulk medium which is critical particularly for *in vivo* applications
- small capacitances and low total currents observed with such electrodes allow for their use in highly resistive solutions, and at extremely high voltammetric scan rates.

Disk-shaped microvoltammetric electrodes have been fabricated with radii of less than 7 μm. Gold and platinum wires, as well as carbon fibres, have been used in rigid insulating materials to construct electrodes that have sufficient physical strength such that the electrodes can be polished and resurfaced as is done with electrodes of conventional size [103]. Their most striking feature is that they exhibit sigmoidal shaped voltammograms at low scan rates under conditions of diffusional mass transport.

Dopamine has been detected voltammetrically in the cytoplasm of a single biological cell, as shown by Lau *et al.* [104]. An enzyme microelectrode for the detection of catechol by immobilising horseradish peroxidase on a carbon fiber electrode via a covalently bound biotin/avidin/biotin complex was reported [105]. Some other examples of microbiosensors include such a sensor for urea based on immobilised urease on the tip of a 10 μm diameter fibre [106], together with a micro-enzyme sensor using creatine amidinohydrolase and sarcosine oxidase for measuring creatine [107]. The sensor consisted of platinum and silver wires fixed in glass tubing with epoxy resin adhesive and both enzymes immobilised on their tip by a cross-linking method. The water soluble polymer poly(n-vinyl pyrrolidone) has been immobilised on a microelectrode surfaces by gamma radiation and the utility of such films as electrode coatings for the determination of catechol compounds in the presence of the electroactive interferent ascorbic acid demonstrated [108]. A one-step fabrication of a miniaturised glucose sensor based on the electrochemical codeposition of rhodium microparticles and glucose oxidase onto a carbon fibre electrode yields

a powerful electrocatalytic surface towards the anodic detection of the liberated hydrogen peroxide [109].

A novel microelectrode flow-through cell has been reported recently [110-112]. The electrode is simple and flexible, easy to prepare and change, and is stable in organic solvents. The preparation of a reagentless glucose oxidase based biosensor for application in such a flow system has been described by Rohde *et al.* [113]. The enzyme was first codeposited with a redox polymer and glutaraldehyde on the surface of a platinum electrode and subsequently covered with an electropolymerised layer of pyrrole. The electron transfer from the reduced $FADH_2$ group in the core of the enzyme to the electrode surface is facilitated via the redox polymer/polypyrrole system.

An electrochemical microband thin layer flow cell that incorporated a thin glassy carbon electrode was fabricated and tested and exhibited properties of a microelectrode with excellent electroactivity and signal to noise characteristics. The small surface area of the nanoband allows for rapid stabilisation of chromatographic background, following the application of the potential [114].

1.4.1.1.2. *Immobilisation Strategies for Amperometric Biosensors*

1.4.1.1.2.1. *Polymer films*

Perhaps the most widely used anionic polymeric material employed for the immobilisation of biocatalysts on electrode surfaces, has been the perfluoro-sulphonate material, Nafion. Such polymeric films may be deposited by dip coating with the 5 % ionomer solution. Nafion films tend to adsorb proteins and other cationic species readily and these may interfere with the sensor response. Consequently, they are more effectively employed when coated with an external polymer layer such as polyurethane or cellulose acetate, which is more inert in this respect. By virtue of its sulphonate side chain Nafion is anionic, but has no cross-linking of exchange sites and the fluorocarbon backbone is hydrophobic [115].

The perfluorosulphonic acid polymer Nafion leads to highly reproducible electrode preparation and performance, when used as a dialysis membrane material on a GOD platinum electrode for the determination of glucose in whole blood [116]. A stable ferrocene mediated glucose biosensor was developed using an anionic ion exchange polymer blend [117]. A blend of poly[(4-vinylpyridine)-co-styrene] and polystyrene with quarternised amine groups solubilised in methanol was used for the immobilisation of glucose oxidase. The

ferrocenecarboxylic acid was incorporated into the film and coating achieved using Nafion, in order to prevent leaching of the mediator.

A polyester sulphonated material from Eastman Kodak (AQ_{55D}) has also been introduced as an electrode modifier and a covering membrane. Studies have illustrated the attractive characteristics of these ionomers, including the ion exchange affinity towards large hydrophobic counterions, exclusion of co-ionic species, prevention of fouling in the presence of proteins, strong adherence to the electrode, simple coating procedures and commercial availability in a dissolved low cost form. This Eastman AQ polymer undergoes minimal swelling, is insoluble and maintains structural integrity in acetonitrile. The Eastman AQ_{55D} polymer is applied to the electrode in a similar manner to Nafion.

Coating microvoltammetric electrodes with a poly(ester-sulphonic acid) [118-120] film has been found to offer substantial improvements in their selectivity towards cationic neurotransmitters. A blend of Eastman AQ_{29D} and AQ_{55D} polymers dispersed in water has been used for immobilisation of enzymes at platinum surfaces, which is then covered by a thin layer of Nafion to avoid dissolution in water [121].

Some of the reasons for using polymers such as Eastman AQ or Nafion include the fact that immobilisation of the enzyme may be achieved during the casting procedure of the polymer/enzyme solution at the surface of the electrode. In addition negative charges of the polymer/enzyme film can act as a barrier for the negatively charged biological interferents found in biological fluids. Polymer/enzyme films offer the possibility to incorporate by ion exchange, positively charged electron mediators, which are used as a shuttle for electron transport from the redox site of the enzyme to the surface of the electrode.

Some additional examples of the use of polymers for immobilisation in amperometric sensors include an amperometric sensor for cholesterol based on a bilayer film coating first with cobalt tetrakis(o-aminophenyl) porphyrin polymer, followed by the enzyme film [122]. Enzyme sensors have also been prepared by entrapment in photocrosslinkable polyvinyl alcohol - SbQ Gels [123]. The resulting membrane exhibits a good mechanical resistance.

1.4.1.1.2.2. *Electrochemical polymerisation*

This is a very convenient technique for the immobilisation of biocomponents on electrode surfaces [124]. Common polymer films prepared by electropolymerisation include polypyrrole, polythiophene, polyaniline. These

coating polymers may have attached redox centres to facilitate the electron transfer properties of the electrode material.

Electropolymerised films are generally formed by the oxidation of a monomer. These are insoluble, conducting or insulating films that coat the electrode surface. They are self-regulatory films, with uniform thickness that completely cover the electrodes surface, regardless of size/shape. The film maintains its uniformity because it only grows thick enough to become an insulator. Polymerisation continues until the surface is completely covered, which is signalled by the current decreasing to a minimum, because the monomer can not penetrate the film.

Chemical, electrochemical, photo-induced or plasma-induced polymer procedures have been described. The conducting polymer polypyrrole may be grown potentiostatically/galvanostatically on platinum, gold, glassy carbon and graphite and semiconductor surfaces. The morphology and hence the chemical and physical properties of these polymer films is controlled by the polymerisation conditions, especially the oxidation potential applied, the counter ion incorporated during the polymerisation conditions, temperature, reaction time and solvent composition. Functionalities on, for example, polypyrrole may be obtained by polymerisation of the functionalised monomer or by substitution reactions of the polymer coating on the electrode surface. An overview of the analytical applications of conducting polymers has been given by Ivaska [124].

The electrocatalytic properties of polypyrrole in amperometric electrodes have been described [125]. With polypyrrole it is possible to directly incorporate a range of biologically active proteins. It has been demonstrated that direct incorporation of oligonucleotides is possible and have shown that these can form the basis of unique biosensors [126]. Iwakura *et al.* described a method in which GOD and the mediator ferrocene carboxylic acid are polymerised simultaneously into a polypyrrole film by electropolymerisation of pyrrole [127]. In the resulting enzyme-loaded films the behaviour is determined by the balance of the diffusion of reactants and products within the film and the kinetics of the immobilised enzyme.

Foulds and Lowe [128] reported on the immobilisation of GOD in ferrocene-modified pyrrole polymers. They used a redox copolymer [(ferrocenyl)-amidopropyl] pyrrole, and entrapped GOD electrochemically into the polymer film to create a reagentless glucose sensor. They concluded that the simplicity of the anodic polymerisation of polypyyrole may be combined with the well behaved electrochemistry of mediators such as ferrocene, in order to trap

enzymes into tailor-made polypyrrole matrices bearing redox functionalities that will accept electrons from the reduced form of the enzyme.

The electropolymerisation of pyrrole and N-methylpyrrole from buffered aqueous solutions can be used to immobilise glucose oxidase and other enzymes at electrode surfaces. These processes have been modelled and the predictions compared to experimental results [129]. Enzyme-based bilayer conducting polymer electrodes consisting of polymetallophthalocyanines and polypyrrole-GOD thin films have also been reported [130].

An amperometric biosensor for hypoxanthine was constructed by Nguyen *et al.* by forming a layer of crossslinked xanthine oxidase on a platinum electrode, followed by electropolymerisation of a submolecular film of rescorcinol and para-aminobenzene [131]. The adsorption of glucose oxidase at a platinum electrode followed by immobilisation in an electrochemically polymerised phenol film was found to be a reproducible method for the fabrication of enzyme microelectrodes to glucose [132]. Another polymer employed as an enzyme immobilisation matrix is polythiophene [133, 134].

Polymer films deposited on thin film noble metal electrodes on silicon chips, utilising plasma-polymerisation have been shown to be suited for immobilisation of GOD [135]. A non-aqueous photopolymer system with dispersed enzyme has been used to make photolithographically patterned enzyme membranes for amperometric glucose electrodes based on thin-film platinum electrodes [136].

The utility of colloidal gold as a biocompatible enzyme immobilisation matrix suitable for the fabrication of enzyme electrodes has also been described. Gold sols with adsorbed enzymes have been electrodeposited onto conducting matrices to make enzyme electrodes, which retain enzymatic activity, and give an electrochemical response to substrate in the presence of mediator [137].

The special constructional needs of microelectrodes has prompted a range of 'fine-tuned' enzyme deposition methods. Thus enzymes have been covalently attached to platinised platinum wire electrodes with the electrode platinisation in enzyme solution allowing incorporation of the enzyme within the platinum matrix [138].

A strategy has been presented for linking horseradish peroxidase covalently to the surface of a carbon fibre micro-electrode via a hydrophilic tether using biotin/avidin/biotin as a coupling technique. Since the enzyme is linked through a tether that is covalently attached to the surface, this strategy allows much more control over the extent of coverage and the localisation of the

enzyme on the surface than does adsorption, without creating a diffusional barrier [139].

1.4.1.1.3. *Tailoring Surfaces and Permselectivity in Amperometric Devices*

An interferent is a species that is electroactive at the operating potential of the amperometric sensor. The specificity of the biochemical transducer is compromised by the partial selectivity of the electrode. Interferents such as uric acid, acetaminophen and ascorbate which are present in human serum and other complex matrices, limit the practical use of biosensors for direct measurement.

There are several methods for deliberately tailoring the chemical sensor to suit a particular application.

- acceleration of electron transfer reactions
- prevention of fouling
- introduction of permselective of membrane barrier effects for interfering electroactive or surface active species
- selective binding
- preconcentration of an analyte in the modified layer
- biocatalysis
- electrochemical detection of electroinactive species.

Modification of the electrode with a permselective coating thus brings into the measurement a separation step performed *in situ* at the electrode surface. Surface modification represents a means by which the problem of co-existing surface active materials or electroactive species which result in a degraded response, may be alleviated. Coverage of the sensing surface with an appropriate permselective film can greatly enhance the sensor selectivity and stability.

A poly(vinyl chloride) membrane system has been described for use as a high selectivity barrier in amperometric sensors and biosensors [140]. Comparative results for a cellulose acetate layer, commonly employed in amperometric sensors and biosensors for clinical monitoring, demonstrates the superior selectivity of ascorbate.

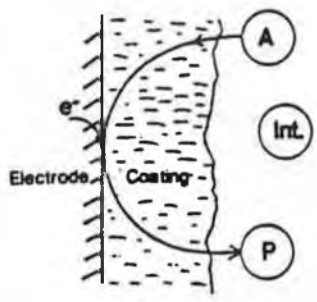

FIGURE 1.6.
A permselective coating for amperometric sensing (A, analyte; P, product; Int, interferent). Reproduced from [141].

Various approaches have been employed to alleviate the protein fouling problem. Size-selective cellulose acetate films have been particularly attractive for this task [142]. Wang *et al.* have coupled electrocatalytic and permselectivity by modifying glassy carbon electrodes with cobalt phthalocyanine/cellulose acetate [143]. A degraded and irreproducible response characterises the deactivation process from adsorption of proteins onto the electrode surface. An approach based on tissue-modified electrodes for the prevention of biofouling has also been described [144]. The rich biocatalytic activity of tissue-containing surfaces was exploited for enzymatic digestion of interfering proteins, e.g. papaya containing proteases and zucchini electrodes containing ascorbate oxidase [145]. Such *in situ* destruction of potential interferences should simplify sample cleanup procedures and eliminate the need for protective membranes or reaction schemes. A tissue bioreactor for eliminating interferences in flow analysis has also been developed [146] and the utility of tissue bioreactors for the elimination of potential interferences in FIA described.

An elegant means of rejection of interferents is the application of enzymatic anti-interference system containing enzymes, these layers positioned in front of the sensor catalyse the conversion of the interfering compounds to inert products [147]. In biospecific electrodes the elimination enzymes are directly integrated in the sensor in a membrane immobilised form and separated from the indicator enzyme layer by a semipermeable membrane. The eliminator enzyme membrane has to be diffusion controlled so as to assure complete conversion of the interferent penetrating into the membrane. To eliminate endogenous glucose an anti-interference layer containing co-immobilised GOD and catalase may be used [148].

Electropolymerised films have been employed to prevent fouling [149]. Insulating films with permselectivities that allow the passage of water but prevent interferences from reaching the electrode surface have been employed. The effectiveness of various electropolymerised films have been evaluated. An electropolymerised film of 1,3 diaminobenzene/rescorcinol was found to give the best protection and stability [150]. The permselectivity of films prepared by electrochemical oxidation of phenol and amino-aromatic compounds have also been investigated [151]. Wang *et al.* [152] reported on the use of different electropolymerisation conditions for controlling the size-exclusion selectivity of polyaniline, polypyyrole and polyphenol films. They demonstrated the potential of the permselective polypyrrole and polyaniline films for selective amperometric detection to selectively detect acetaminophen/catechol in the presence of NADH/Uric acid.

Composite electrode coatings based on a mixture of phosphatidylcholine and cholesterol offer remarkable mechanical stability under vigorous hydrodynamic conditions. Improved stability was attained without compromising the attractive permselective response of lipid electrodes for amperometric detection of hydrophobic substances in flowing streams [153]. In biological taste reception the lipids in the biological membrane are suggested to play an important role. A taste sensor was developed [154] which consists of a multichannel electrode with transducers composed of lipid membranes immobilised with a polymer i.e. eight kinds of lipid analoges mixed with PVC and plasticiser. The sensor responds to five basic taste substances in five different ways.

TRANSPORT MECHANISM	COATING	REFERENCE
Size exclusion	Cellulose acetate, polyanaline	[155,156]
Charge exclusion	Nafion, PVP, AQ	[157,158]
Polarity	Phospholipid	[159]
Mixed control	Cellulose acetate- Nafion	[160]

1.4.1.1.4. *Mediation and electron transfer for enzyme electrodes.*

In general, the active site of redox enzymes is deeply buried within the protein molecule, making direct electron transfer difficult. To overcome this problem, redox mediators, with a redox potential adapted to that of the enzyme in question, have been used to transport electrons from the enzyme to the electrode by means of a shuttle mechanism. This results in a decrease in the overpotential to the formal potential of the mediator (at which the residual current is low and where there are few electrochemical interferences from other oxidising/reducing reactions e.g. ascorbic acid). Mediators are generally organometallic compounds with a redox couple exhibiting fast electron transfer reactions [162].

A practical mediator needs to be :

- electrolytically active at an electrode of a given potential
- of low molecular weight
- easily attached to the surface of the electrode
- reversible
- fast reacting
- regenerated at low potential
- pH independent
- stable in both oxidised and reduced forms
- unreactive with oxygen
- non-toxic.

1.4.1.1.4.1. *First generation mediators*

These include water soluble electron acceptors, e.g. ferricyanide, quinones, organic dyes [163,164] and are generally used with soluble enzymes. Hu and Turner [165] have described an amperometric enzyme electrode for the analysis of glucose utilising adsorbed benzoquinone on a carbon electrode to mediate electron transfer from glucose oxidase. In addition, an enzyme electrode for nucleosides was constructed by immobilising nucleoside oxidase on a carbon paste electrode containing p-benzoquinone [166]. The cathodic current for the reduction of benzoquinone at the glassy carbon electrode was increased in the presence of nucleoside oxidase and inosine. This was attributed to the regeneration of benzoquinone from hydroquinone by the laccase-like reaction of

the enzyme. Glucose oxidase and glucose dehydrogenase immobilised microelectrodes were fabricated and their characteristics evaluated using 1,4-benzoquinone and ferricyanide as electron mediators [167].

An amperometric glucose sensor was described based on carbon paste chemically modified with glucose dehydrogenase, NAD^+ and Meldola Blue [168]. The kinetics of a glucose oxidase electrode with a soluble mediator (1-methoxyphenazinemethosulphate) has also been reported [169]. Other electron acceptors for GOD include hexacyanoferrate [170], tetrathiafulvalene [171] methyl viologen [172] and phenazine methosulphate [173].

1.4.1.1.4.2. *Second generation mediators*

Most second generation mediators are hydrophobic in nature, e.g. ferrocene (bis η^5-cyclopentadienyl) iron and its derivatives, and may be retained on carbon electrodes by simple adsorption. They have well characterised electrochemistry and operate at a redox potential that is generally low. Ferrocene shows a well defined good one electron quasi-reversible electrochemistry and a large catalytic current is observed at oxidising potentials when glucose oxidase is added. Many substituted ferrocenes are available with different overall charges and a wide range of solubilities in various solvents. In their oxidised ferricinium ion forms, they will act as electron acceptors to a number of flavoproteins. For ferrocene-mediated glucose electrodes the electron transfer between the electrode surface and the enzyme is performed by a 'pseudo' shuttle mechanism supported in one part by the diffusion of the sufficiently soluble ferricinium cation from the electrode to the active site of the reduced enzyme, where it is reduced, and in the second part, an electron transfer back to the electrode by the virtually water-insoluble reduced mediator or by electron hopping between mediator molecules.

Direct current and cyclic voltammetry was used to investigate the suitability of a ferrocene derivative as a mediator with galactose, glycolate and L-amino acid oxidase [174]. Some amperometric enzyme based sensors based on ferrocene [175-178] and dimethylferrocene [179,180] as mediator have been reported. The modification of carbon paste electrodes by incorporation of glucose oxidase has also been described with 1,1 dimethylferrocene mixed into the paste. The ferricinium ion competes favourable with the natural mediator oxygen to oxidise the reduced glucose oxidase. The reoxidation of the reduced ferricinium

can be monitored at the electrode at low potentials to minimise interferences [86]. An enzymatic amperometric sensor for hydrogen peroxide has been reported [181] using ferrocene as mediator immobilised in a graphite-paraffin oil paste. Jonsson *et al.* improved the stability of the electrode by favouring a stronger adsorption of the mediator to graphite rather than entrapping it. This improved adsorption was achieved by use of an anthracene-ferrocene ester, which served to increase the number of aromatic nuclei in the mediator molecule [182].

The charge transfer process for the determination of glucose at a ferrocene-modified electrode is shown in Fig. 1.7.

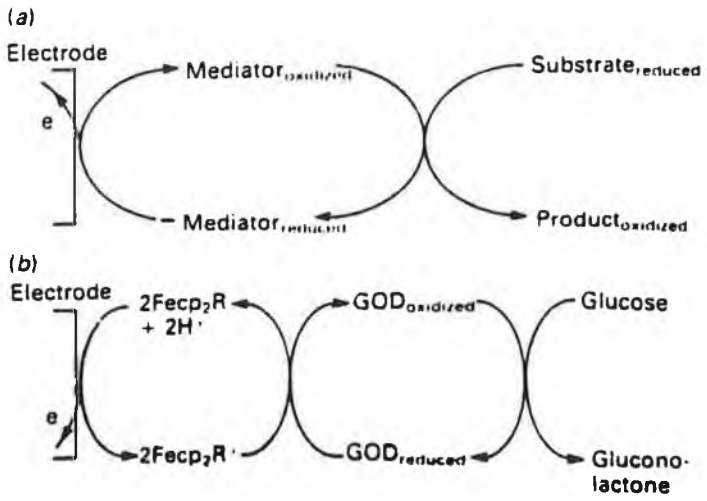

FIGURE 1.7.

(a) Schematic diagram of a mediated charge transfer process.

(b) Charge transfer process for the determination of glucose at a ferrocene-modified electrode.

Reproduced from [178].

1.4.1.1.4.3. *Third generation mediators*

These are based on the principle of direct electron transfer from the biological receptor to a modified/unmodified electrode [183]. Even with optimum orientation of the proteins, an electron transfer can only be explained by a charge transfer/tunnelling process. In the past decade, an impressive number of promotor-modified metal electrodes have been used to study the direct electrochemistry of redox proteins, both positively or negatively charged. Thiol or disulphide-containing organic molecules are particularly good gold modifiers because they chemisorb through a strong gold-sulphur bond. They provide certain selectivity in achieving electron transfer between electrodes and proteins.

An obvious interest is to tackle the catalytic redox chemistry of enzymes via fast interfacial charge transfer without the need for mediators. The goal is to obtain a well-defined voltammetric response that can be identified as a clean manifestation of the active site catalytic chemistry, without the need for an overpotential that exceeds the requirement in the physiological system. The direct electron transfer between various electrode materials and immobilised peroxidases have been reported and the electrocatalytic reduction of the hydrogen peroxide achieved. By co-immobilising an oxidase with the peroxidase, amperometric biosensors can be made within a potential range essentially free from interfering electrochemical reactions [184]. A peroxidase model electrode was devised for reagentless sensing of hydrogen peroxide. A small model molecule, which mimics the vicinity of the reaction centre of a redox enzyme, can communicate electrochemically with an electrode [185]. Horseradish peroxidase was found to strongly adsorb on the surface of spectroscopic graphite with retained catalytic activity. Efficient electron transfer from the graphite to the oxidised form of adsorbed graphite was used as a sensor for hydrogen peroxide [186]. Jonsson and Gorton [187] described a glucose sensor made of a carbonaceous electrode covered with a catalytic layer of palladium and gold sputtered onto the graphite to allow hydrogen peroxide detection at 300 mV. Benetto *et al.* immobilised GOD by direct cross linking on a platinised carbon electrode (not requiring a mediator) [188].

The two $FADH_2$ redox centres of reduced glucose oxidase are prevented from transferring electrons to metal electrodes by an insulating glycoprotein shell. If redox enzymes could undergo direct charge transfer to the electrode for biological reason, then all redox enzymes would have to come to the same potential. Ferrocenyl derivatives, ruthenium complexes or polymers incorporating osmium complexes have been bound to glucose oxidase rendering it effective for

electron transfer to an unmodified electrode. Heller incorporated into the enzyme 'electron relays' including ferrocene derivatives which effectively 'short out' the interior and exterior of the enzyme [189,190]. A polycationic osmium redox polymer has been used to form an electrostatic complex with the polyanionic enzyme, wherein the electron transfer distance is reduced. The complex is 'stretched' by internal electrostatic repulsion. Low ionic strength allows formation of the complex and electron transfer occurs [191] (see Fig 1.8.).

Electrical communication between the FAD redox centres of glucose oxidase and a conventional electrode was achieved using a two electron transfer relay system based on ferrocene-modified poly(ethylene oxide) [192]. The results indicate that the mediating ability of the polymers is dependant on the density of bound ferrocene moieties along the polymer chain. Glucose microelectrodes have been formed with glucose oxidase immobilised in poly(vinyl pyridine $Os(bipy)_2Cl$) derivative-based redox hydrogels, on bevelled carbon fibre electrodes. The high current density and lower oxygen sensitivity point to the efficient collection of electrons through their diffusion in the redox hydrogel to the electrode surface [193]. Electrical communication between the FAD redox centres of glucose oxidase and a conventional carbon paste electrode has also been achieved by using electron transfer relay systems based on polysiloxanes [194]. Ye *et al.* [195] have prepared glucose electrodes by 'wiring' quinoprotein glucose dehydrogenase to glassy carbon with an osmium complex containing a redox-conducting epoxy network, allowing direct electron transfer from enzyme to the surface.

Modification of glucose oxidase by the coupling of ferrocenecarboxylic acid allows for mediator modified enzymes which undergo direct oxidation at the electrode [196]. It has been shown that a positive charge on the mediator is particularly important when it is reacting with an enzyme known to have positive charges around its active site. The covalent attachment of mediators to redox enzymes is interesting in that it offers the prospects of reagentless biosensors, and secondly such modified enzymes might well be suited to *in vivo* applications.

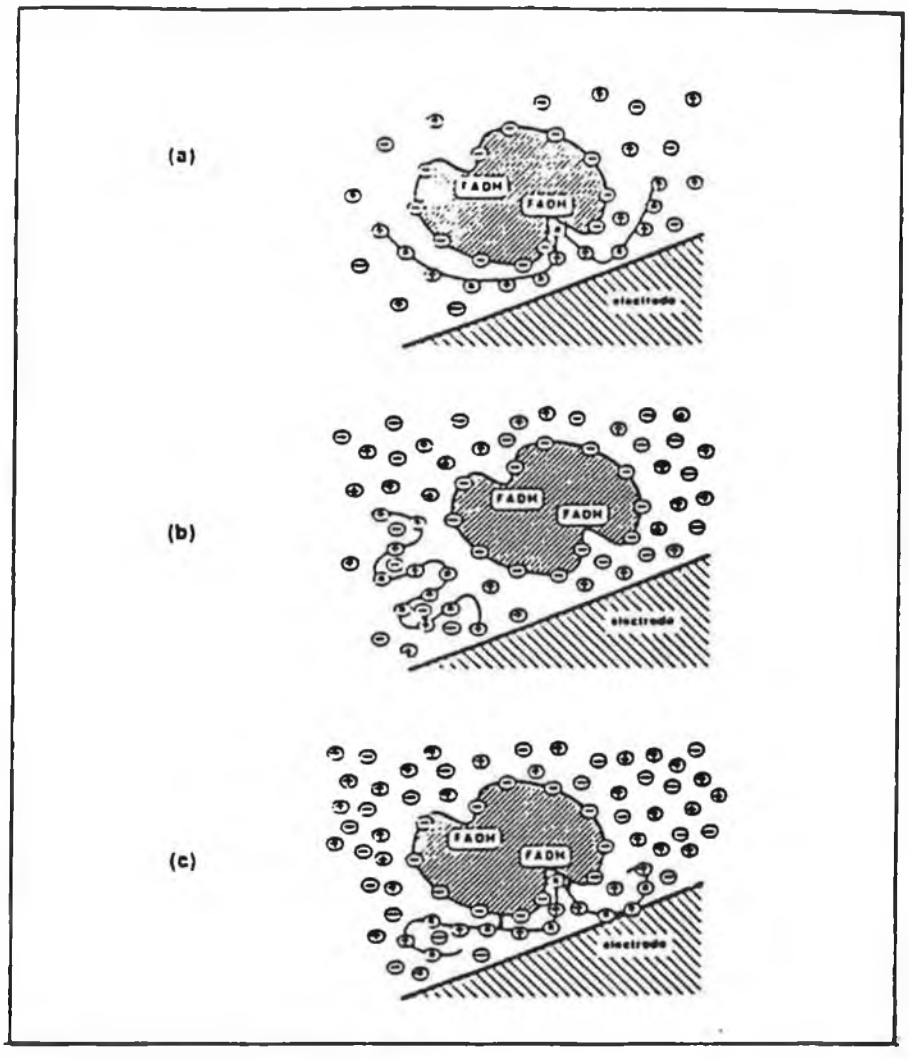

FIGURE 1.8.

(a) Electrostatic bonding of a polycationic redox polymer to a polyanionic enzyme at low ionic strength brings redox centres of the two within range for electron transfer. Electrons are transferred to the electrode via the polymer.

(b) Charge screening and coiling of the redox polymer at high ionic strength leads to dissociation of the electrostatic complex stopping electron transfer, and thereby electrooxidation of glucose

(c) After covalent bonding of the redox polymer to the enzyme, the complex does not dissociate at high ionic strength and electrooxidation of glucose persists.

Reproduced from [197].

1.4.1.1.5. *Enzymatic Amplification*

The use of multiple enzymes extends the applicability of enzyme electrodes to new analytes and enhances their sensitivity by substrate recycling. Enzyme amplification was extensively used by Scheller *et al.* [198] who developed substrate recycling schemes for many substances and achieved valuable enhancements in response. The operation is based on an enzyme catalysis regeneration of the substrate of a second enzyme. This has been achieved by coupling dehydrogenases with oxidases/transaminases. For example L-glutamate can be determined by recycling in a bienzyme system consisting of glutamate dehydrogenase and glutamate pyruvate dehydrogenase.

Substrate recycling was used for making a highly sensitive enzyme electrode for determination of ADP/ATP [198]. Glucose-6-phosphate dehydrogenase, pyruvate kinase and hexokinase were all immobilised on a graphite electrode. The recycling produces NADH which is detected by oxidation at the modified electrode. The cycle is driven by the conversion of phosphoenolpyruvate to pyruvate and by conversion of glucose to gluconic acid with simultaneous production of NADH.

The application of these recycling systems to real samples is restricted by their sensitivity to both substrates of the enzymatic cycle e.g. lactate and pyruvate. Therefore the determination of pyruvate in plasma by this method could require removal of the endogenous lactate first. Table 1.4. shows some of the analytes and enzymes involved in substrate recycling processes [199].

ANALYTE	ENZYMES	TRANSDUCER	REF.
Lactate	LOX and LDHase	Thermistor	200
Glucose	GOD and GDHase	O_2 electrode	201
Lactate/pyruvate	Cytochrome b_2 + LDHase	Pt electrode	201
Glutamate	Glutamate dehydrogenase and alanine aminotransferase	Modified carbon electrode	202
Ethanol	Alcohol oxidase and alcohol dehydrogenase	O_2 electrode	203

TABLE 1.4. Substrate recycling methods.

1.4.1.2. *Potentiometric and ISFET devices*

Potentiometric techniques involve static non-faradaic electrode processes with the measurement of a potential under thermodynamic equilibrium conditions where no current flows. Both electrolysis and charge transport over the electrode / solution junction occur at the indicator electrode. They operate on the principle of measuring a potential developed at an electrode interface, as a result of the accumulation of charge density brought about by some selective equilibrium processes. They are based on the Nernst equation relating electric potential and the logarithm of the activity of the known species in solution. The constant current gives a logarithmic response curve and allows a concentration of over a 100 fold to be probed.

Potentiometric enzyme electrodes are fabricated by immobilising or covalently binding an enzyme to an ion/gas selective electrode [62]. The biocatalyst may be immobilised on or in close proximity to the ion selective membrane. The consumption of a substrate or the formation of products by the enzymatic reaction changes the activity of an ion or a gas for which the membrane is selective. A large number of enzymes have been used but only a few of these electrodes are available, the majority being pH, and electrodes for ammonia originating from hydrolysis of urea, creatinine and other amides, and from oxidation or oxidative deamination of amines and amino acids.

In the area of potentiometric sensors, three devices, i.e. ion selective electrodes (ISE), coated wire electrodes (CWE) and field effect transistors (FET) have been the subject of ardent research. The same basic features applies to potentiometric sensors based on the FET. The trend to miniaturisation and to internal electronic amplification of the potentiometric measuring principle led to the development of ion selective FETs (ISFETs), which are derived from metal oxide semiconductor FETs by replacing the metal gate with an electrolyte junction and a reference electrode. The gate insulator is directly exposed to the analyte solution or is coated with an ion selective membrane. Because insulated gate FETs are basically charge-measuring devices, any strongly adsorbing species that changes the interfacial charge density of the gate should change the drain current. By analogy with the concept of ISEs, FETs can become ion selective after coating of their gate with corresponding membranes. Such a device may be used in combination with the selectivity of an enzymatic reaction catalysed by an enzyme immobilised on the surface of the gate insulator. Penicillinase was immobilised within a membrane gel on the gate of an ISFET and the device was selective for penicillin owing to local pH changes as a consequence of the

enzymatically catalysed reaction. Electrodes for glucose, urea and other substrates utilising pH ISFETS have been described by many researchers [204, 205]. Table 1.5. gives some examples of enzyme potentiometric devices [206].

Fabrication of reliable chemically sensitive field effect transistors still poses significant technical difficulties. Problems related to integrity of the solid state part of the device, integrity of the final sensor package, definition of the sensitive areas in multisensors and casting of the selective membranes have been addressed by Domansky *et al.* [207].

SUBSTRATE	ENZYME	ELECTRODE	REFERENCE
Adenosine	Adenosine deaminase	pNH_3	[208]
Creatinine	Creatinase	pNH_3	[209]
Glucose	GOD	pH-glass	[210]
Lactate	Lactate dehydrogenase	Redox membrane (PVC + ferrocene)	[211]
Penicillin	Penicillinase	Sb/Sb_2O_3	[212]
Uric acid	Uricase	pCO_2	[213]

TABLE 1.5. Potentiometric enzyme biosensors.

1.4.2. *Optical Transduction*

Over the past few years, considerable effort has been directed towards the development of biosensors based on optical transduction. Optical biosensors monitor changes in the light transmission due to the effect of the sample on evanescent waves or surface plasmons set up by absorption of light. In these cases the optical fibre acts like a transducer. Such biosensors have been reviewed recently [214-216]. The biocomponent may be either chemically bound or physically entrapped near the interface or in the bulk of the sensing layer or directly immobilised on the surface of the optical element and changes in pH or in oxygen consumption detected [217,218]. A chemical sensor can be obtained from a single optical fibre or from a bundle of these fibres by immobilisation of an 'indicator' on the tip, preferably by its binding to a polymer. The best known realisations are optrodes for penicillin, urea [219], and glucose [220-222].

One of the most promising and straightforward approach is to utilise a change in the intensity of absorbed or emitted light from an indicator dye that can

in turn interact with a biological reporter molecule. One such device for glucose, developed by Takai *et al.* [223], is based on a cellulose acetate membrane incorporating a benzidine derivative, where hydrogen peroxide generated by glucose oxidase can result in a detectable absorbance change.

Despite a few limitations, optical sensors have the potential to provide alternatives to other sensing systems due to the many advantages these sensors impart in analytical measurements.

The chemical transducer consists of immobilised chemical reagents, which are normally analyte specific. The reagent systems are often employed in the solid phase for convenient handling. The reagent phase is localised in the sensing region of the optical fibre either by direct deposition on the fibre or by encapsulation with a polymeric membrane. Immobilisation of chemical reagents may be carried out physically or chemically. The physical methods of immobilisation include gel entrapment, adsorption and electrostatic attraction, and use simple and economical procedures. Chemical immobilisation is based on the formation of a covalent bond between the reagent molecule and an activated or functionalised form of the polymeric solid support. This method is the most irreversible of the immobilisation techniques, but requires several steps in the synthesis of the immobilised reagent phase [224].

An optical label is advantageous for realising an immunosensor for homogeneous immunoassays. The core of an optical fibre may be modified by a layer of antibody, with labelled antigen, and free antigen to be determined in solution. For a competitive assay format, a fluorophore antigen can be used to compete with an unlabelled molecule (analyte) for binding to a limited amount of antibody on the waveguide [11]. After equilibration, coupling of surface fluorescent light from the label back into the waveguide then allows for estimation of the amount of surface captured label. This overall construction has been used recently for devices measuring hCG and rubella antibody [225,226].

The fluorescence of flavoproteins (glucose oxidase and lactate monooxygenase) changes with the interaction between enzyme and substrate which may be used for substrate determination. As the reduced prosthetic group is reoxidised by oxygen, the process is reversible, so in this case the enzyme is self-regenerating, as demonstrated by Trettnak *et al.* [227]. A novel fibre optic biosensor based on chemiluminescence and bioluminescence reactions for hydrogen peroxide and ATP has also been reported [228, 229].

It has been known that it is possible to recognise optical isomers on a molecular level. This is due to the preferred interaction of the receptor and the substrate with one of the two enantiomers due to several geometric factors and

differences in binding energy [230]. There is a substantial interest in quantitation and determination of optically active species. Most drugs and biomolecules are active in one enantiomeric form. If the receptor/carrier is enantioselective, a fairly specific recognition of enantiomers of biogenic amines e.g. drugs will be possible. Future possibilities for optical biosensors are extensive as has been discussed by Lungstrom and Gustafsson [231].

1.4.3. *Calorimetric Transduction*

Numerous attempts to find a universal transducer that matches any kind of reaction have been reported. Heat variation appears very attractive for transduction. Heat may become indicative for a given system when the latter is selectively converted, under the influence of a catalyst, especially an enzyme. The enthalpy changes during the enzymatic reaction include evolution/absorption of heat which may be considered as reaction product. Enzyme thermistors do not exactly fit with the definition of a biosensor, because they do not consist of a transducer surrounded by an immobilised enzyme, but represent a thermistor at the end of a small enzyme reactor in a flow system. A review on this subject, including aspects of instrumentation methods and applications, has appeared in the literature recently [232].

Enzymes are deposited at the tip of the thermistor and left to dry, with a second enzyme-free thermistor used as reference. The enzymatic reaction gives rise to temperature changes which induce a variation in the measuring thermistor resistance and an electrical signal is sent to an amplifier and then to a recorder.

Enzyme thermistors have so far been described for the detection of urea, penicillin and other amides, for glucose, sucrose, cholesterol, uric acid and lactate, and they have been used preferentially for medical analyses. The concentration of glucose in diluted whole blood has been measured by Hedberg *et al.* using a miniaturised thermal biosensor based on the enzyme thermistor principle [233].

A thermal assay probe (enzyme thermistor) was used to study the performance of enzyme analyses by immobilised enzymes in organic solvents and in mixtures of organic solvents and aqueous buffer [234]. This showed the use of a thermal assay probe or enzyme thermistor to measure the heat produced by immobilised enzyme in a continuous flow of organic solvents/water-solvent mixtures.

1.4.4. *Piezoelectric Transducers*

Piezoelectric biosensors usually employ quartz crystals, either as resonating crystals, or as bulk or surface wave devices. Their action depends on changes in mass upon binding of analyte, with the sensitivity of most systems being inversely proportional to the crystal thickness. When a mechanical stress is exerted on quartz in a particular direction, electrical changes are induced on the surface. Conversely, an applied electric field leads to a mechanical deformation of the crystal in particular. An AC voltage under resonance conditions will cause the crystal to oscillate. The general approach in exploiting the piezoelectric crystal involves the immobilisation of a material that exhibits high selectivity to the substance to be determined. This attachment of the analyte on the surface of the sensor brings about a change in the resonant frequency of the sensor. Bioaffinant surfaces have been achieved by adsorption, polymer binding or cross-linking of antibodies and enzymes. Advantages of such devices include the fact that electroactive products do not interfere while sensing and they give as output, not an analogue signal, but a frequency modulated digital signal which can be easily interfaced to a digital device like a microprocessor.

A system has been described in which acoustic impulses are launched across a very thin polymer film [235]. Bound mass causes small changes in the transit time which is monitored sequentially on the two sensing areas. A limitation of most of the quartz devices is the mass sensitivity, which, being inversely proportional to the crystal thickness, is inadequate for many purposes. This paper describes how by replacing the crystal with a thin film of piezoelectric polymer, the sensitivity may be greatly enhanced .

Usually twin systems are employed, having one crystal for monitoring of the analyte and the other for compensating the binding of unspecific molecules [236]. Coupling the selectivity provided by the biological coating with the inherent sensitivity of the piezoelectric devices enhances the development of new biosensors. The principle makes them ideally suited for sensing immunogens which would deposit on immunoglobins immobilised on the quartz crystal. The use of antibodies as immobilised affinants is the most common way to obtain piezoelectric biosensors [237]. A further enhancement of affinity and sensitivity can be achieved by means of a streptavidin-biotin affinity system and this seems even to open up the possibility of using piezoelectric affinity sensors in solution. [238, 239].

1.4.5. CHARACTERISATION OF BIOSENSORS

The time necessary to reach a plateau defines the sensor steady state **response time.** For a bioprobe, in most cases, dealing with monolayer systems, the response time is of the order of 1 - 3 minutes. A multienzyme system has a longer response time, because of the enzyme sequence. Response time is usually of the order of several minutes, maybe longer for bacterial electrodes, depending on the type of electrode, nature and thickness of the membrane, amount of immobilised biocatalyst, pH, membrane permeability, temperature and stirring rate. At present the maximum sample throughput of commercial enzyme-electrode-based analysers is about 100 samples per hour. Recently, further progress in increasing the measuring frequency of enzyme electrodes has been achieved. Wang reported that several thousand aqueous glucose samples have been processed per hour. In these experiments a composite graphite epoxy electrode containing the biological entity was integrated within the injector valve of a FIA device [240].

The operational stability of an enzyme-based system, defined as the residual conversion efficiency after operation for different periods, depends not only on the enzyme itself but also on the stability of the support, the immobilisation procedure and the kind of samples [241]. An increased rigidity of the structure of the enzyme may lead to increased stability preserving the native conformation of the protein and making unfolding less probable. Enzymes become inactivated during the course of their reaction. In addition to thermal denaturation, two kinds of operational inactivation can be distinguished

- autoinactivation is due to transient reactive intermediates generated during catalysis
- interaction of the enzyme with final reaction products may also lead to decrease of activity.

The storage stability of amperometric enzyme electrodes has been enhanced by a combination of a soluble positively charged polymer, diethyl-aminoethyl dextran and lactitol. Alcohol biosensors based on alcohol oxidase were found to have increased stability under conditions of thermal stress at 37 °C [242]. The useful lifetime of biosensors usually depends on the retention of the biological activity of the biocomponent of the sensor. This combination of additives stabilises the activity of the enzymes alcohol oxidase, peroxidase and L-glutamate oxidase, used in the fabrication of alcohol and L-glutamate biosensors. The enzyme

loading to a major extent determines the stability of an enzyme sensor. An enzyme reserve is built up by employing more enzyme activity in front of the probe than the minimum required to achieve complete analyte conversion.

A large excess of enzyme in the membrane keeps the effect of pH variations on the measuring process small. Therefore pH profiles in the linear measuring range and with diffusion control should be substantially less sharp than those of the respective enzyme in solution. Enzyme stabilisation by immobilisation is frequently reflected by an increase in the optimum temperature for substrate conversion. If kinetic and diffusional control are superimposed the higher activation energy results in a substantial acceleration of the enzyme reaction with increase in temperature.

Kinetics of inactivation can be described as first order and usually are more complicated, partly because of a multistep inactivation process or the presence of several enzymatic forms which inactivate at different rates.
The principles of stabilisation may be summarised [243] :

- localisation of enzyme molecules in the parts of the supports where they are sterically inaccessible for the action of microorganisms and proteases
- mutual spatial fixation of enzyme molecules to prevent intermolecular inactivation processes
- multipoint attachment of enzymes to the supports to prevent unfolding of the protein molecules
- attachment of enzymes to supports with surface groups possessing buffer properties to prevent pH inactivation
- use of properly chosen/water immiscible nonaqueous cosolvents to prevent inactivation of enzyme in water organic-media
- to prevent against the action of chemical inactivation, use of supports for immobilisation, catalysing the decomposition of these inactivators
- dehydration of the enzyme molecules and their fixation by a polymeric matrix
- increased rigidity of enzyme molecules by intramolecular cross-linking to prevent unfolding
- mutual fixation of cellular components to prevent solubilisation of membrane-bound enzymes and intracellular proteolysis
- reactivation of mutarotated enzyme through use of 'unfolding-refolding' procedures.

1.5. APPLICATIONS

As biosensors pass from the conceptual into the developmental stage, future work inevitably will be increasingly application-driven. Although many technical problems have yet to be overcome, biosensors offer a prospect of measuring biomolecules important in medicine, biotechnology, and other areas with convenience and speed, as well as at low cost.

Between 12 and 15 billion US $ per year are spent for analytical purposes worldwide [244]. In this sum, the analytical usage of enzymes in clinical chemistry, food and cosmetic industry and biotechnology for the routine measurement of *ca.* 80 substances, mainly low molecular weight metabolites, but also effectors, inhibitors and the activity of enzymes themselves, is included. Potential applications in industry include drug testing and manufacturing, as parts of artificial organs, food quality and environmental control. In biophysical research, biosensors could replace slower and costlier analytical methods. In hospitals, they could speed up the analysis of blood samples and biopsies, giving the results at the patient's bedside within a few minutes. Medicine and food provide the prime focus for new products, but many smaller companies are searching for niche markets and may furnish some fascinating new gadgets.

Driven by the promise of large sales in the environmental health care and food industry markets, North American and Japanese research organisations, both industrial and academic, are funding strong programmes on biosensors. Biosensors are enjoying a growing role in a number of important applications, e.g. in the detection of water, soil and air pollution. Japan has more than forty large companies in the field, but American firms are increasingly venturing into biosensors. At YSI Inc. Yellow Springs Ohio, a research team which have developed the first commercial biosensor, has introduced a new immobilised enzyme type of biosensor. It can measure the level of lactic acid present in the blood of athletes after exertion, or of glucose, in the case of a diabetic [245].

Ultimately, a biosensor has to be a device with a useful practical analytical end function, irrespective of whether this is targeted to applied biology/basic research. Possibilities for innovative configurations should provide the necessary sound basis for applications, that the applied scientist would consider relevant and the industrialist to be of commercial importance.

1.5.1. *Medical Applications*

Clinical diagnostics have dominated biosensor development to date and are likely to continue to be the focus for commercialisation successes for the next few years. These applications are driven by the need for cost effective patient care. The world's most successful biosensor is the pen-shaped blood glucose monitor. The printing technology that has been developed for the mass production of one-shot devices might find application in reusable or continuous use devices. The coupling of biocatalytically active material with physicochemical transducer devices in biosensors continues to be an expanding area of research. Up to now *ca.* 20 self-contained analysers based on enzyme electrodes for the determination of ca. 15 analytes have been brought on the market [246].

Implanted voltammetric sensors are hampered by contacting the biological fluid for extended periods of time, and it is hoped that new modification schemes will be developed for improving their stability. Neurotransmitters have a very important role in the brain since they are the key link in communication between neurons. *In vivo* voltammetry provides a unique way to monitor chemical changes. The electrode must be planted in the brain and monitored for at least 8 hours in most experiments, so opportunities are not available for electrode resurfacing [247]. An electrochemical microsystem using three electrodes has been described by Ponchon *et al.* Using pulse amperometry, these electrodes have been used *in vivo* to study the release of dopamine in the rat neostriatium [248]. Future work on *in vivo* electrochemistry will undoubtedly focus on the development of selective probes for organic drugs or additional electrolytes, the coupling of enzymatic and immunochemical reactions with *in vivo* electrodes, the design of new noninvasive and multispecies probes, and the search for a means of protecting such electrodes against matrix effects.

Characterisation of voltammetric signals with microelectrodes in the living brain is fraught with difficulties. In addition to being anatomically complicated, brain tissue presents the analytical electrochemist with a complex chemical environment that includes surfactants, electrode poisons, and a tissue matrix that both resists mass transport to the electrode surface and reacts physiologically to the presence of the probe. Identification of electrochemical signals recorded *in vivo* with carbon paste electrodes was discussed by O'Neill in the context of these problems [249].

While sensor function *in vitro* depends on diffusion, enzyme reaction and electrochemical response, in *in vivo* applications it is also influenced by

circulation, microconvection/diffusion in tissue, and endogenous metabolic function [250]. The sensor surface is in receipt of protein deposits, resulting in an unstable 'biolayer' that causes signal errors of clinical significance and curtails the lifetime of the sensor. Miniaturisation of biosensors is important for a variety of reasons, including *in vivo* neurological and physiological studies, improved biocompatibility and for use in multisensor arrays and probes. *In vivo* sensors are needed as biofeedback devices that provide information on a real-time basis e.g. for artificial organs, clinical monitoring devices etc. The requirements for implantable glucose sensors include:

- linearity in 0 - 20 mM range with 1 mM resolution
- specificity for glucose; not affected by changes in metabolite concentrations and ambient conditions.
- biocompatibility
- small - causes minimal tissue damage during insertion and there is better patient acceptability for a small device
- external calibration and < 10% drift in 24 hours.
- response time < 10 min
- prolonged lifetime at least several days, preferably weeks in use.

The ExacTech pocket blood glucose meter (see Figure 1.9.) is an amperometric device in the shape of a pen (MediSense Abingdon, UK and Cambridge U.S.A.), and is designed for the mediated determination of glucose in whole blood. It consists of a pen-shaped barrel, housing a custom-built single chip microprocessor, a sealed power source and operating button. The strips are elegantly designed enzyme electrodes screen printed onto a plastic substrate. They consist of two electrodes screen printed onto plastic strips, consisting of an Ag/AgCl reference electrode and carbon-based working electrode which contains the assay reagents. Electrochemical measurements are performed by placing a drop of the test sample on the electrode area.

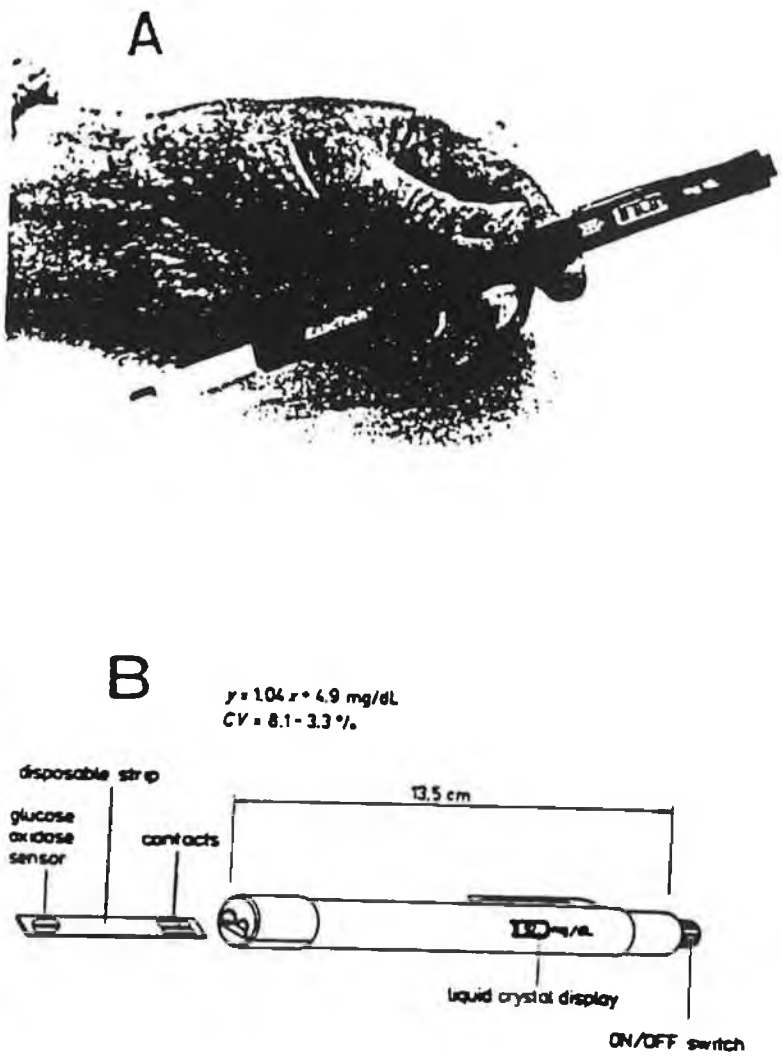

FIGURE 1.9.

(A) Pen-sized glucose analyser developed at Cranfield Institute of Technology (UK) for Genetics International.

(B) Exactech blood glucose meter.

Reproduced from [251].

An implantable glucose sensor has been recognised for many years as the critical component necessary for optimal control of blood glucose concentrations in diabetic patients. A sensor that would yield continuous readings of blood glucose levels such that a person with diabetes could take the appropriate corrective steps during the initial onset of hyper/hypoglycemia would be a valuable addition to diabetes treatment. In addition, incorporation of such a device into a closed loop system with a microprocessor and an insulin infusion pump could provide automatic regulation of the patients blood glucose.

An electrochemical glucose sensor has been integrated, together with a pH sensor, on a flexible polyimide substrate for *in vivo* applications based on the measurement of hydrogen peroxide produced by the membrane-entrapped enzyme GOD [252]. The enzymes GOD and catalase were immobilised into two layers of photolithographically patterned hydrogels. The intended use of this device is the short term monitoring of glucose and pH in intensive care units and for neurosurgical applications. The developed immobilisation technique can also be used to create integrated multi-sensor chips for clinical analysers.

A new prototype direct reading glucose electrode, with GOD and hydrogen peroxide was preliminary tested clinically during insulin-induced hypoglycemia in eight healthy subjects and hyperglycemia in five diabetic patients. In situations with fast metabolic perturbations e.g. surgery and deliveries repetitive and prompt bedside blood glucose determinations are important for correlating an insulin treatment thus avoiding hyper/hypoglycemia. The glucose electrode itself consisted of an amperometric hydrogen peroxide sensor with a glass rod and a central platinum wire covered with a four layer membrane [253].

Electroenzymatic glucose sensors have been designed for implantation into subcutaneous tissue (see Fig. 1.10.). Sensors implanted in the subcutaneous tissue of normal human subjects showed an excellent correlation between the concentration of glucose measured by the sensor and capillary finger sticks measured with a commercial analyser [254]. Wilson *et al.* reported another such sensor which is needle-shaped and flexible and must be implanted subcutaneously using a catheter which is then removed. The success in implantation depends on the reaction of the tissue surrounding the implant so as not to interfere with the proper functioning of the sensor [255].

Up to now *in vivo* applications of glucose sensors have been hampered by immunological reactions of the organism against the implanted material. Coverage of needle-type glucose sensors with silanised membranes increases the biocompatibility of the probe [256]. As judged by STM, deposition of protein on the membrane was less drastic in tissue than in the bloodstream. Another problem

with the development of implantable sensors is the need to calibrate the sensor *ex vivo*. This requires a high sensor stability.

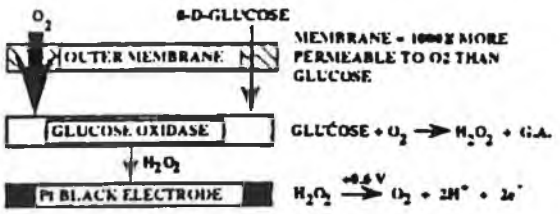

FIGURE 1.10.
Block schematic of a hydrogen peroxide-based electroenzymnatic glucose sensor with a differentially permeable outer membrane layer.
Reproduced from [257].

The importance of a rapid, specific micromethod for the measurement of lactic acid is apparent in the medical literature. An increase in spinal fluid lactate in meningitis was first noted in 1924. Spinal fluid lactate measurements are also valuable in clinical an experimental research involving glycolytic and oxidative metabolism. Such a sensor has been described by Clark *et al.* (see Fig. 1.11.) [258]. Lactic acid has been demonstrated to be the product of the activation of the glycolysis anaerobic pathway and it has been shown that its concentration increases dramatically when the exercise intensity reaches a fixed threshold. The detection of lactate is becoming increasing popular in the diagnosis of shock and myocardial infarction and in sports medicine.

Potentiometric devices may be used for direct and indirect determinations. In direct testing, the sensor is placed in contact with the whole blood plasma or serum sample. Direct measurement lends itself to a *stat* or emergency type of application. In indirect potentiometry, the sample in diluted prior to

measurement. For *stat* measurements of lactate or glucose in whole blood, amperometric sensors seem more attractive.

A method for rapid bedside estimation of plasma and whole blood lactic acid, has been described [259]. In the YSI 23L instrument (USA), Lactate oxidase is immobilised between a cellulose acetate membrane and a polycarbonate membrane [260], the latter serving to exclude high molecular weight interferences.

Direct measurement of undiluted samples is also required for implantable biosensors, *in situ* application in reactors, and non-invasive measurements. Very promising approaches are non-invasive measurements at the surface of the skin. In this manner, lactate has been quantified [261], and correlated to the fatigue of the patient.

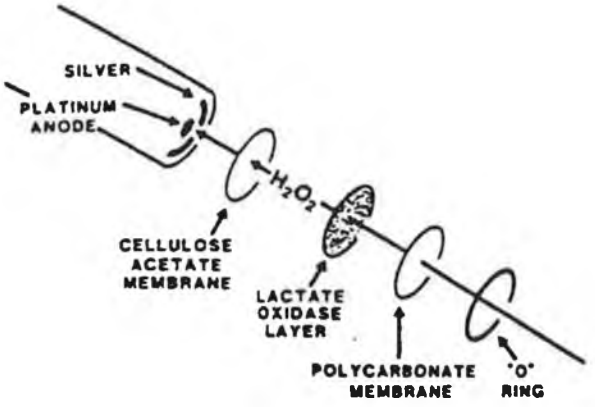

FIGURE 1.11.
Diagram of a lactate sensor. Reproduced from [258].

In addition, a very successful sensor for urea measurement in blood has been developed for Hitachi (Japan) [262]. Table 1.6. shows some examples of commercially available instruments for sensing in the biomedical field.

MANUFACTURER	INSTRUMENT	ANALYTE	TECHNOLOGY	STABILITY
AIC Anal. Inst. Corp. Japan	Glucoroder E	Glucose	Immobilised GOD, O_2 electrode	6000 assays 2 months
Baxter Travenol Lab. U.S.A.	Exactech	Glucose	GOD + ferrocene	One-shot test
Fuji Electric Co. Japan	Gluco 20 and amylase glucose analyser UA-300	Glucose Uric acid	Immobilised GOD, and uricase at H_2O_2 electrode	> 500 samples
Kyoto daiichi Kagaku, Japan	Glucose Auto and Stat GA-1120	Glucose	Immobilised GOD H_2O_2 electrode	1000 assays
Omron, Tateisi Electronics Co. Japan	HER-100	Lactate	Immobilised LOD, H_2O_2 electrode	ND
Seres, France	Enzymat	Glucose, Lactate, Lysine	Cross-linking of oxidase and gelatine, O_2 electrode	ND
Setric SGI, France	Microzym-L	L-lactate	Yeast lactate dehydrogenase and hexacyanoferrate	200 samples or two weeks

YSI (Yellow Springs Instrument Co.), U.S.A.	YSI 27	Glucose Sucrose Alcohol L-lactate	Kits with immobilised oxidase enzymes, H_2O_2 electrode.	10 days
	YSI 2000	Glucose and L-lactate	Immobilised oxidase enzymes H_2O_2 electrode.	3 weeks 2 weeks
	YSI 2300 G YSI 2300 L YSI 2300 GL	Glucose L-lactate Glucose and L-lactate	Glucose oxidase Lactate oxidase	ND

TABLE 1.6. Some commercially available instruments based on amperometric enzyme electrodes available for medical applications.
Reproduced from [263].

Miniaturisation will be critical for any *in vivo* health care applications. Microelectronics are linked to miniaturisation because of the small circuitry and electronics produced by photolithographic semiconductor technology. The goal of the union between biosensors and microelectronics is to form a sensing bioelectronic system.

1.5.2. *Food Industry*

Biosensors find application in the food area where the analysis of food samples during manufacture and processing prior to sale is carried out by the food industry for economic reasons and to ensure compliance with government legislation. A recent review article [264] assesses the status of electroanalysis in the food industry. Trends in electroanalysis, offer unique possibilities for assays of food and drink and for monitoring food production processes. Particular attention is given to the utility of new biocatalytic sensors, stripping techniques and electrochemical flow detectors in the food industry. Tests include the estimation of meat and fish freshness based on the analysis of monamines arising from decarboxylation of amino acids released during proteolysis and determination of hypoxanthine using a xanthine oxidase based enzyme electrode [265]. The determination of L-glutamate is important in fermentation control in the foodstuff industry, because many kinds of food contain glutamate as an essential flavour compound [266]. Enzyme sensors involving hydrogen peroxide sensing electrodes can be readily employed for the determination of sucrose in sugar beet juice and of lactose in milk. The application of biosensors of any kind in fermenters are connected with significant problems such as:

- direct stabilisation of biosensors is impossible
- discrete measurements are necessary
- in most cases the analyte concentration is greater than the linear range
- various interfering substances have to be expected
- the sensor stability is affected by mechanical and thermal stress

TOXINS	ENZYMES
Antibiotics in meat	Catalase
Bacterial toxins	Lipase
Mycotoxins	Peroxidase
FOOD COMPONENTS	**CHEMICAL PARAMETERS**
Fat/fatty acid composition	Oxygen
Rancidity	Hydrogen peroxide
Sugars	pH
Vitamins	Nitrates/nitrites
Fish, meat and fruit content	Sulphites

TABLE 1.7. shows some analytes for the food and beverage industries

1.5.3. *Environmental Monitoring*

The primary purpose of controlling environmental pollution is to protect human health and the ecological integrity of the biosphere on which humans and other organisms depend. Electroanalysis is widely used for environmental monitoring. A review by Kalvoda [267], summarises the most progressive electroanalytical methods and the development of novel sensors that are primary part of monitors and analysers for environmental purposes.

Biosensor examples include the biochemical oxygen demand (BOD) sensor for measuring biodegradable organic compounds in waste water. It consists of micro-organisms immobilised on an oxygen sensor [268]. An activated sludge-based biosensor for rapid IC_{50} estimation and on-line toxicity monitoring has also been described recently [269].

The basic action of organophosphorous pesticides is associated with their ability to inhibit acetylcholinesterase in the central and peripheral nervous system where the enzyme plays an important role in the transmission of nerve impulses [270]. In environmental control, the acetycholine esterase electrode for studying the toxic inhibitors of choline esterase, is very useful. Rawson *et al.* have also described a whole cell biosensor for monitoring pollutants such as herbicides [271]. An amperometric biosensor for the detection of the photosynthetic

inhibiting herbicide atrazine has been reported. The sensor, incorporating the enzyme tyrosinase, was sensitive to a wide range of di- and triphenols. The biosensor displayed a notable decrease in response in the presence of atrazine [272]. Table 1.8. gives some examples of sensors for pesticide analysis.

BIOCATALYST	SUBSTANCE	TRANSDUCER	RANGE/L.O.D
Monoclonal antibodies	2,4-dinitrophenol	Potentiometric electrode	10^{-6}-10^{-5}M
Polyclonal antiserum	Atrazine, propazine	Fluorimeter	0.01-10 µg/l 0.002-10 µg/l
Polyclonal antiserum	Parathione Malathione	Piezoelectric crystals	36 ppb
Butyrl-choline-esterase	Organophosphorous pesticides and carbamates	pH electrode	3-15 ppb
Butyrl-choline-esterase	Organophosphorous pesticides and carbamates	Potentiometric cell	0.1-20 ppm
Acetylcholine-esterase	Organophosphorous pesticides and carbamates	Photometer Amperometric electrode	Carbofuran 0.5-500 µg/l

TABLE 1.8. Some biosensors for determination of pesticides

Some environmental areas for applications of sensors are given below:

- ozone depletion
- acid rain
- industrial atmospheres
- fugitive emissions
- soil and groundwater analysis
- detection of personal contamination
- home and workplace

The operational requirements for environmental sensors are as follows:

- capable of recording at sufficiently frequent intervals
- respond to concentrations of pollutants which endanger human life
- robust/ease of maintenance
- easy to install/calibrate by semiskilled operator
- low maintenance times
- long shelf lives for replacement sensors

1.5.4. *Industrial Applications*

Sensors have numerous application in industry and in process control. In particular in the following areas :

- process monitoring
- fermentation, food, chemical, petrochemical and pharmaceutical industries
- quality control
- contamination detection
- water, air and effluent monitoring
- hazardous/toxic waste detection
- adulterants and food contaminants
- QA/QC of pharmaceuticals
- pyrogens
- stereo and optical isomers.

Potential uses of biosensors in biotechnology and pharmaceutical production are closely related to and partly overlap biomedical applications. However, present trends in biotechnology suggest that monitoring fermentation processes and antibody production by means of biosensors may especially be of crucial importance. Similarly the pharmaceutical industry has requirements for monitoring the purity and potency of pharmaceutical measurements at the level of therapeutic action. Other logical industrial applications in which biosensors could play a major role include monitoring worker safety, product testing, especially in the personal care and beauty product field, and monitoring raw materials or process intermediates. Few of these industrial opportunities will be realised within the next five years because the conditions of use are often too harsh for biological sensors.

1.5.5. *Markets for Biosensors*

There is a general agreement that biosensors have the capacity to offer easy to use, cost effective and rapid analyses in a wide variety of operating environments. In reality, however, only a few biosensor products have emerged, and even fewer have achieved mass market commercial success. Biosensor technologies often do not lend themselves to manufacture - a factor underestimated by many scientists. The necessary features are shown in Table 1.9.

FEATURE	BENEFIT
Targeted Specificity	Versatility
Electronic Processing	Ease of use and Versatility
Selective Measurement in Complex Samples	Decreased User Time Ease of Use (No Sample Preparation)
Fast Measurement	Increased Workload Decreased User Time
Continuous Measurement	Increased Accuracy Increased Efficiency
Small Size	Fit with Current Practise Versatility Economical
Associated Disposables	Increased Revenue

TABLE 1.9. Required features and benefits of biosensors in order to be eligible for marketing.

Additional performance criteria for biosensors are listed below:

- performance/price ratio >> existing
- should permit new measurements to be made
- rapid/real time measurements

- precision
- sensitivity
- facile use by lay personnel
- small size
- rugged
- inexpensive
- manuafacturable
- rapid response
- biocompatibility
- ready interfacing with computers

A biosensor can be successfully utilised in three different ways, by providing :

- superior alternatives to existing methods
- an existing measurement in a novel environment
- an entirely novel measurement

In practise, most successful biosensor applications have been in the first two areas, where the developmental costs can be lower than in the last category.

Compared with the *in vivo* market which is developing at a much slower rate, *in vitro* diagnostics presents the largest market opportunity for biosensors in medicine. Until now, application of new technologies has largely gone unregulated and little analysis of the cost versus benefits obtained has been carried out. It has been reported that we are entering a period when growth will slow and close scrutiny will be applied by regulatory agencies to the costs and efficacy of diagnostic products. These changes will provide challenges and opportunities for companies which are in tune with them and whose technologies provides affordable healthcare of high quality.

The area of *in vivo* diagnostics is extremely specialised and complex. The technical difficulties caused by allergenicity, biocompatibility, consequences of malfunction etc. are enormous and have yet to be overcome. Product development costs are therefore very high and there are considerable legal and ethical barriers to introducing biosensors *in vivo*. In summary, these constraints, as well as a lack of sufficient fundamental clinical knowledge, will severely hinder the development of *in vivo* biosensors.

	1990 U.S. $K	1995 U.S. $K	2000 U.S. $K
Clinical Diagnostics	20	85	250
Industrial Analysis	3	30	145
Agriculture/Veterinary	2	50	100
Defence	2	15	85
Medical Devices	5	25	50
Environmental	2	20	35
Robotics	1	5	10
Other	0	10	20

TABLE 1.10. World Biosensor Market

The following points exhibit the need for sensor research and development.

- sensors represent an alternative analytical strategy
- decentralisation of laboratory analyses
- real-time analysis
- automated manufacturing processes
- remote sensing in adverse environments
- replacement of existing bioassays
- technological spin-offs
- scientific spin-offs

1.6. CONCLUDING REMARKS

In this introductory chapter the main aspects of the biosensing process have been discussed. A recent review is given of the vast amount of literature available in this area. The field of biosensors encompasses diverse fields such as biochemistry, electronics, analytical chemistry, medicine, and biotechnology.

The remaining chapters will deal with the development and characterisation of some novel biosensing devices based on various enzymes, antibodies and plant tissues, incorporated within or immobilised on different electrode materials. Such sensors have wide applicability in the biomedical and biotechnological areas.

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CHAPTER 2

THE DEVELOPMENT OF SOME AMPEROMETRIC ENZYME ELECTRODES BASED ON VARIOUS IMMOBILISATION TECHNIQUES

2.1. INTRODUCTION

The development of some novel amperometric sensor systems, based on the principles in Chapter 1, are described in the following sections. Analytes such as the important drug theophylline, metabolites such as choline and lactate and the amino acid lysine, act as substrates to their specific enzymes. As discussed earlier, molecular recognition by enzymes has provided the majority of publications in this area, in particular the oxidase-based systems, which have been very well documented as catalysts for recognition. An important parameter affecting performance of the biosensor is the biocatalyst immobilisation on the transducer surface. Different means of immobilisation are employed in this section, including physical methods e.g., entrapment within polymeric coatings such as Nafion together with mild matrices such as gelatine, and chemical methods involving electropolymerised o-phenylenediamine films. Mediators are employed to provide electron transfer to the electrode surface. The theophylline oxidase, choline oxidase and lysine dehydrogenase based biosensors utilise ferricyanide as mediator with the enzyme lysine dehydrogenase requiring the additional presence of NAD^+ as cofactor. No mediator was employed in the case of the lactate sensor, where the direct detection of hydrogen peroxide was employed. Physical and electrochemical conditions were optimised in order to achieve the best compromise between stability, sensitivity, and speed. Possible areas of application are given and ideas for future research discussed.

2.2. DEVELOPMENT OF AN AMPEROMETRIC ENZYME ELECTRODE FOR THEOPHYLLINE

Theophylline (1,3-dimethylxanthine) is a widely used bronchodilator drug employed in the management of various asthmatic conditions. Theophylline is also a metabolite of caffeine and is used as an indicator of liver function. The wide use of theophylline preparations in medical practice creates the necessity for a simple, rapid and accurate method of analysis.

It is widely prescribed as a diuretic and powerful relaxant of involuntary muscles. Because of its narrow therapeutic index and the wide interpatient variability in its pharmacokinetics, serum theophylline concentrations are a useful guide to dosage titrations [1]. In addition, adequate control of asthma is generally achieved with 8-20 μg/ml theophylline, but in cases of toxicity, levels as high as

60 μg/ml can be encountered. Owing to this toxicity the use of the drug as a therapeutic agent necessitates close monitoring.

There are many physiological and environmental factors that significantly influence the plasma clearance of theophylline and may lead to an unpredictable relationship between maintenance dosages and its concentration in serum. Theophylline is eliminated primarily by demethylation and oxidation by the liver. Theophylline pharmacokinetics are susceptible to change by co-administration of theophylline with various compounds and by hepatic disease. The major metabolites found in urine are 3-methylxanthine, 1,3-dimethyluric acid and 1-methyluric acid.

Because of its therapeutic significance, theophylline has been the focus of intense bioanalytical efforts. There is a considerable market for sensors capable of monitoring therapeutic drugs present in blood at μM levels. Above the therapeutic range of 55-110 μM/L, theophylline rapidly becomes toxic, and so effective monitoring of its concentration is desirable. Many methods have thus been developed for measuring theophylline concentrations, including gas or liquid chromatography, ultraviolet spectroscopy, immunoassays and pulse voltammetry [2-6]. Some of these methods will be discussed in the following sections.

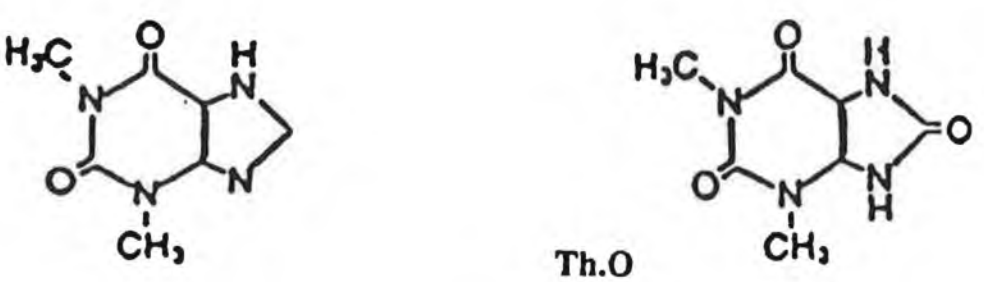
A method for determination of theophylline and paraxanthine in urine samples by liquid chromatography has been described recently [7]. The problem here was not the detection of theophylline but the interferences encountered by many exogenous and endogenous compounds. The presence of the pharmacologically active dietary methylxanthine and caffeine, as well as theobromine and paraxanthine, may influence patient compliance and may also complicate the evaluation and interpretation of theophylline therapeutic drug monitoring. The standard additions method provides excellent results in the determination of both theophylline and paraxanthine at therapeutic levels.

An alternative HPLC method for the determination of theophylline concentrations in plasma from both healthy rats and from rats with carbon tetrachloride induced cirrhosis has been reported [8]. No interferences were observed from theophylline's major metabolites or from endogenous components co-extracted from plasma. A rapid on site AccuLevel Theophylline Assay, based on an immunochromatographical method has been evaluated and compared to the reference method of HPLC [9]. The rapid result provided by this assay was necessary in order to provide more timely treatment for patients presented with acute decompensation of their asthma, and subsequently requiring a quantitative serum theophylline determination early in the course of treatment.

A flow injection immunoassay for theophylline using a Protein A immunoreactor has also been described [10] with p-aminophenyl phosphate as substrate and based on p-aminophenol for detection. This hydrolysis product p-aminophenol may be detected amperometrically at + 0.2 V [11]. A liposome immunosensor for theophylline has also been described which is composed of a Clark-type oxygen electrode and actively sensitised liposomes [12]. It monitors liposome lysis induced by specific anti-theophylline antibodies and complement which are monitored by the release of entrapped enzymes. When the entrapped enzyme horseradish peroxidase is released, the decrease in oxygen may be monitored using the oxygen electrode.

An amperometric enzyme electrode for the determination of theophylline in serum has been described recently by McNeill *et al.* [13]. The method is based on the catalysed oxidation of theophylline by the haem-containing enzyme theophylline oxidase. The development of such an enzyme electrode offers many prospects for clinical diagnosis, particularly where self-testing or *in vivo* monitoring are concerned, as well as for high-speed automated analysis. An indirect electrochemical assay for theophylline, based on its inhibiting action on alkaline phosphatase, has also been reported recently [14].

The research reported in this section involves the use of theophylline oxidase for direct amperometric biosensing of theophylline. The recent isolation of theophylline oxidase (Th.O. [15]) has opened the door for enzymatic methods for theophylline. This enzyme can oxidise theophylline in the presence of ferricytochrome C as electron acceptor according to:



Th.O

Theophylline + ferricytochrome C ⇨ 1,3 dimethyl uric acid + ferrocytochrome C **(2.1.)**

Gupta *et al.* [16] described an optical enzymatic approach for theophylline based on monitoring the ferrocytochrome C produced at 550 nm. The amperometric assay described in this work, couples the enzymatic reaction (of eqn. 2.1) with

the use of the water soluble ferricyanide mediator that reacts with the generated ferrocytochrome C:

$$Fe(CN)^{3-}_6 + \text{ferrocytochrome C} \Rightarrow Fe(CN)^{4-}_6 + \text{ferricytochrome C} \quad (2.2.)$$

The ferricyanide ion thus produced is then oxidised at +0.4 V (vs. Ag/AgCl) to yield a sensitive and selective detection of theophylline. Simultaneous regeneration of the cofactor is also obtained. These results regarding the optimisation and characterisation of the theophylline electrode are reported in the following sections.

2.2.1. EXPERIMENTAL

2.2.1.1. *Apparatus*

The 10 ml electrochemical cell (Model VC-2, Bioanalytical Systems (BAS)) containing the working electrode, Ag/AgCl reference electrode (Model RE-1, BAS), and platinum wire auxillary electrodes was connected to an EG&G PAR Model 264A polarographic analyser, the output of which was displayed on a strip-chart recorder (Model 4500 Microscribe, The Recorder Co.). The flow injection system consisted of a reservoir containing the carrier solution, a Rainin Model 5041 sample injection valve (20 µl loop), interconnecting tubing and a platinum thin-layer detector (Model TL-10A, BAS).

2.2.1.2. *Electrode Preparation*

Prior to coating, the platinum disk (Model 2013, BAS) was polished with a 0.05 µm alumina slurry and then was sonicated in water for 2 min. Modification of the platinum electrode was achieved by covering its surface with two 5 µl aliquots of the mixed polymer/enzyme/cofactor solution (1 % Nafion/8.3 U/ml Th.O./30 µM ferricytochrome C). The first layer was allowed to dry (using a heat gun) before the second aliquot was applied. The resulting film was subsequently covered with a thin layer of a Nafion solution (5 µl of 1 % Nafion (diluted in ethanol)).

2.2.1.3. *Reagents*

All solutions were prepared with deionised water. Experiments were conducted in 0.05 M phosphate buffer (pH 6.9). Theophylline oxidase (20 U/ml) and ferricytochrome C (70 μM) were obtained from GDS Diagnostics. Stock solutions of theophylline (Sigma) were dissolved in 0.4 M HCl and appropriate dilutions made in phosphate buffer. Potassium ferricyanide (Fisher), ascorbic acid, uric acid, acetaminophen, glucose, caffeine, L-α-phosphatidylcholine (Type XI-E from egg yolk), and cholesterol were all obtained from Sigma. The 5 % Nafion solution was obtained from DuPont.

2.2.1.4. *Procedure*

Batch and flow experiments were performed at room temperature by applying a potential of +0.4 V and allowing transient currents to decay. For batch measurements, the electrolyte solution (containing 1 mM potassium ferricyanide) was stirred at 400 rpm. Theophylline additions were made and current-time data were recorded. In flow injection analysis, the sample and carrier phosphate-buffer solutions contained 1 mM ferricyanide and a flow rate of 2.0 ml/min was employed throughout.

2.2.2. RESULTS AND DISCUSSION

2.2.2.1. *Amperometry and Chronoamperometry*

Figures 2.1. and 2.2. compare the amperometric and chronoamperometric responses of (A) the unmodified and (B) the Th.O. modified electrodes to successive additions of theophylline, each addition effecting a $5x10^{-5}M$ increase in concentration. In the absence of biocatalytic activity, the unmodified electrode was not responsive to the addition of theophylline. In contrast, the enzyme electrode responded rapidly to micromolar changes in the substrate concentration. A steady-state amperometric response was produced within 3 min. The low noise level allowed convenient quantitation of low theophylline concentrations and both techniques yield current signals proportional to the substrate concentration. With DC amperometry, linearity prevailed from up to $3.5x10^{-4}M$ (see inset Fig. 2.1.), with a sensitivity (slope of linear portion) of 0.807 μA/mM (correlation coefficient, r = 0.999). The maximum therapeutic level of theophylline, encountered in cases of toxicity, i.e. $3.3x10^{-4}M$ [14], thus falls within the linear

portion of the calibration curve. Coated electrodes, containing no enzyme (but only the cofactor within the Nafion film) yielded no responses to theophylline (not shown).

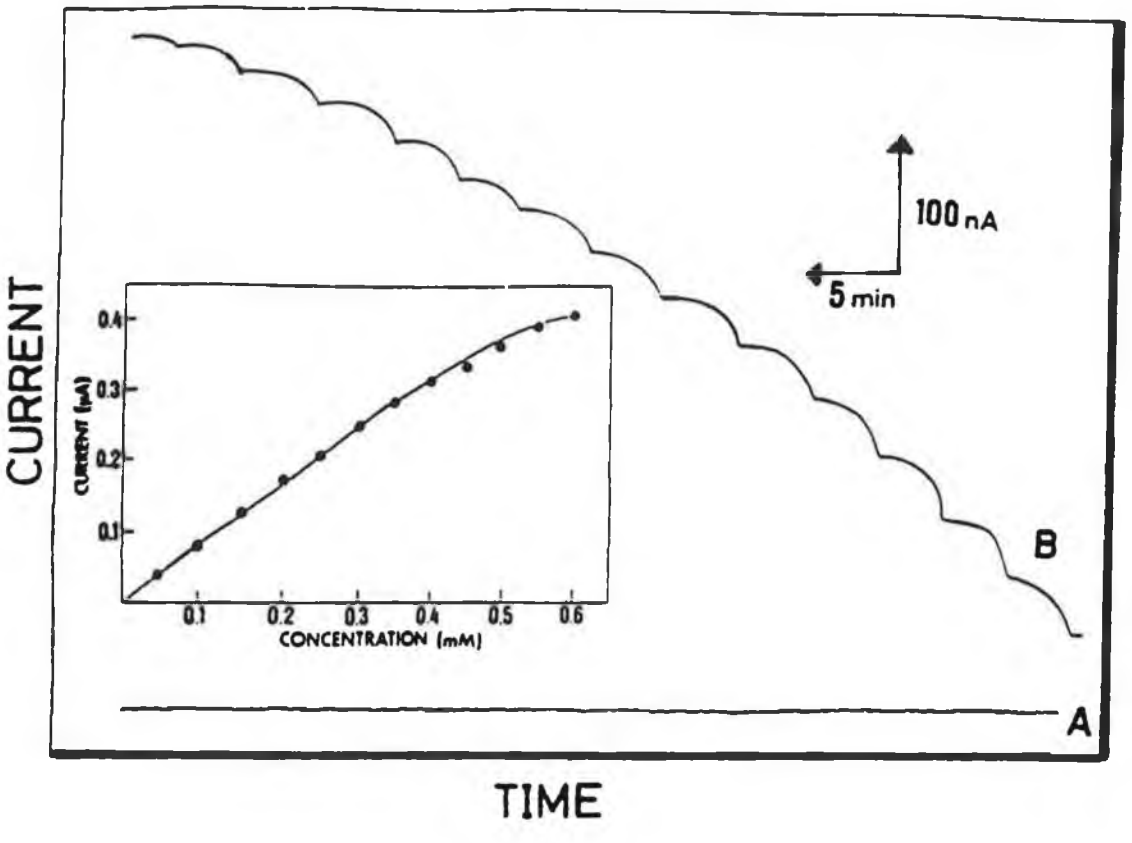


FIGURE 2.1.

Typical current-time recording for successive $5x10^{-5}M$ increments of theophylline concentration obtained at the unmodified (A) and Th.O.-containing (B) electrodes. Applied potential, + 0.4 V; solution stirring, 400 rpm; electrolyte, 0.05 M phosphate buffer (pH 6.9), containing 1 mM $K_3Fe(CN)_6$. Also shown (inset) is the resulting calibration plot for the modified electrode.

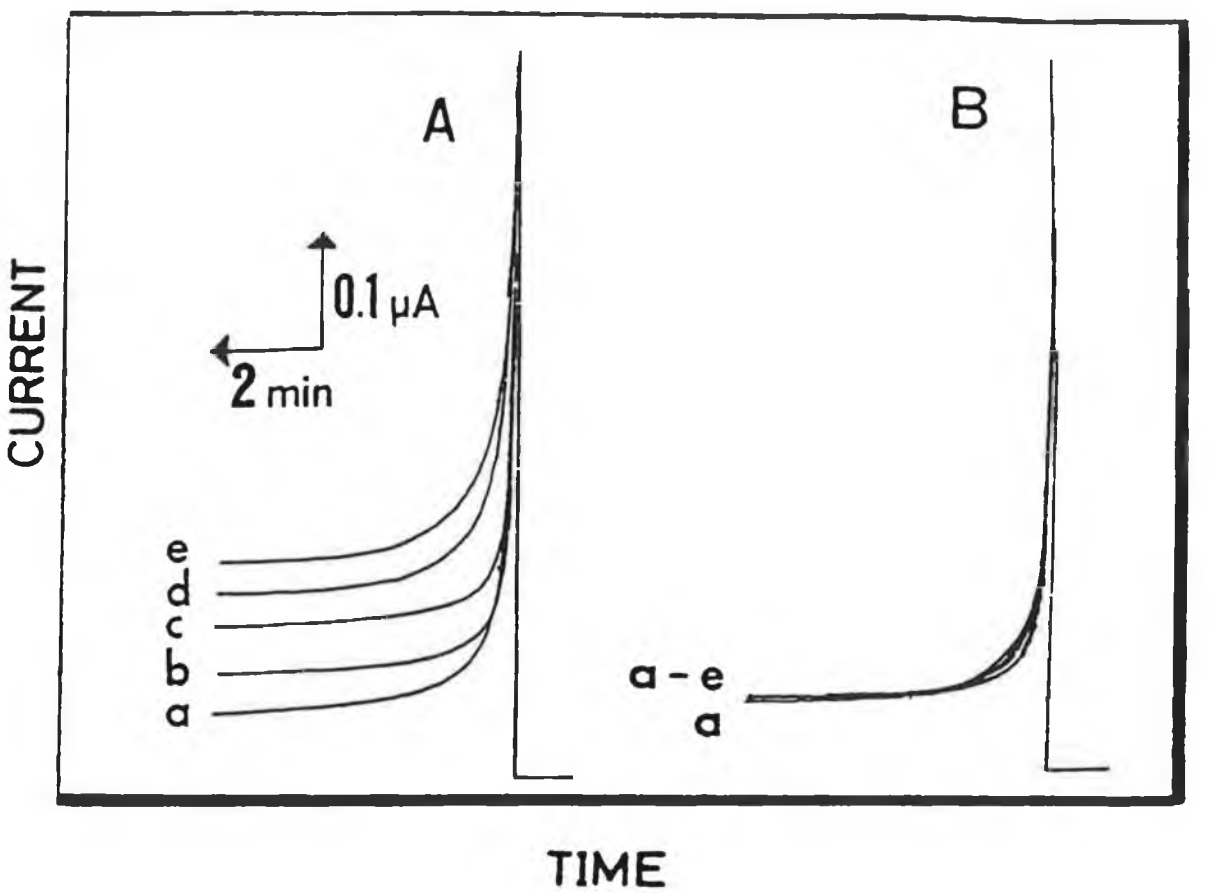


FIGURE 2.2.

Chronoamperometric response to $5x10^{-5}M$ increments in theophylline concentration (b-e), along with the blank response (a), as obtained at Th.O.-containing (A) and unmodified (B) electrodes. The potential was stepped to +0.5 V.

2.2.2.2. *Optimisation of Operating Conditions*

The sensitivity of the theophylline electrode was affected by various preparation and operational conditions. As expected, the response increased rapidly with an increase in the enzyme surface loading (between 25 to 60 mU (Fig. 2.3A); a levelling-off was observed for higher loadings. The solution pH also had a profound effect upon the sensitivity (Fig. 2.3B). The current increased with pH over the range 5.4-6.9, with a gradual decrease at higher values. Figure 2.4A shows the dependence of the theophylline response on the concentration of the redox-mediating ferricyanide ion. The response increased rapidly with the ferricyanide concentration up to $1x10^{-3}$M, after which it starts to level off. The small response observed in the absence of ferricyanide was attributed to the regeneration of the cofactor, as expected for monitoring the oxidation potential between 0.2 and 0.4 V (Fig. 2.4B). A gradual decrease in the response, observed at higher potential values, was attributed in part to electrochemical "consumption" of the substrate. While operating at the optimum potential for biosensing (+0.4 V), no direct oxidation of theophylline was observed (e.g. Fig. 2.1A), such a reaction can, however, occur at higher potential values.

The Th.O. electrode, operated under the above optimum conditions, permits quantitation of low concentrations, as desired for clinical theophylline monitoring. Successive standard additions of $5x10^{-6}$ M theophylline were used to estimate the detection limit (Figure 2.5.). The signal-to-noise characteristics (S/N =3) indicated a detection limit of $2x10^{-6}$M. Such a level is well below the normal therapeutic range for theophylline ($5.5x10^{-4}$M - $1.1x10^{-4}$M [14]).

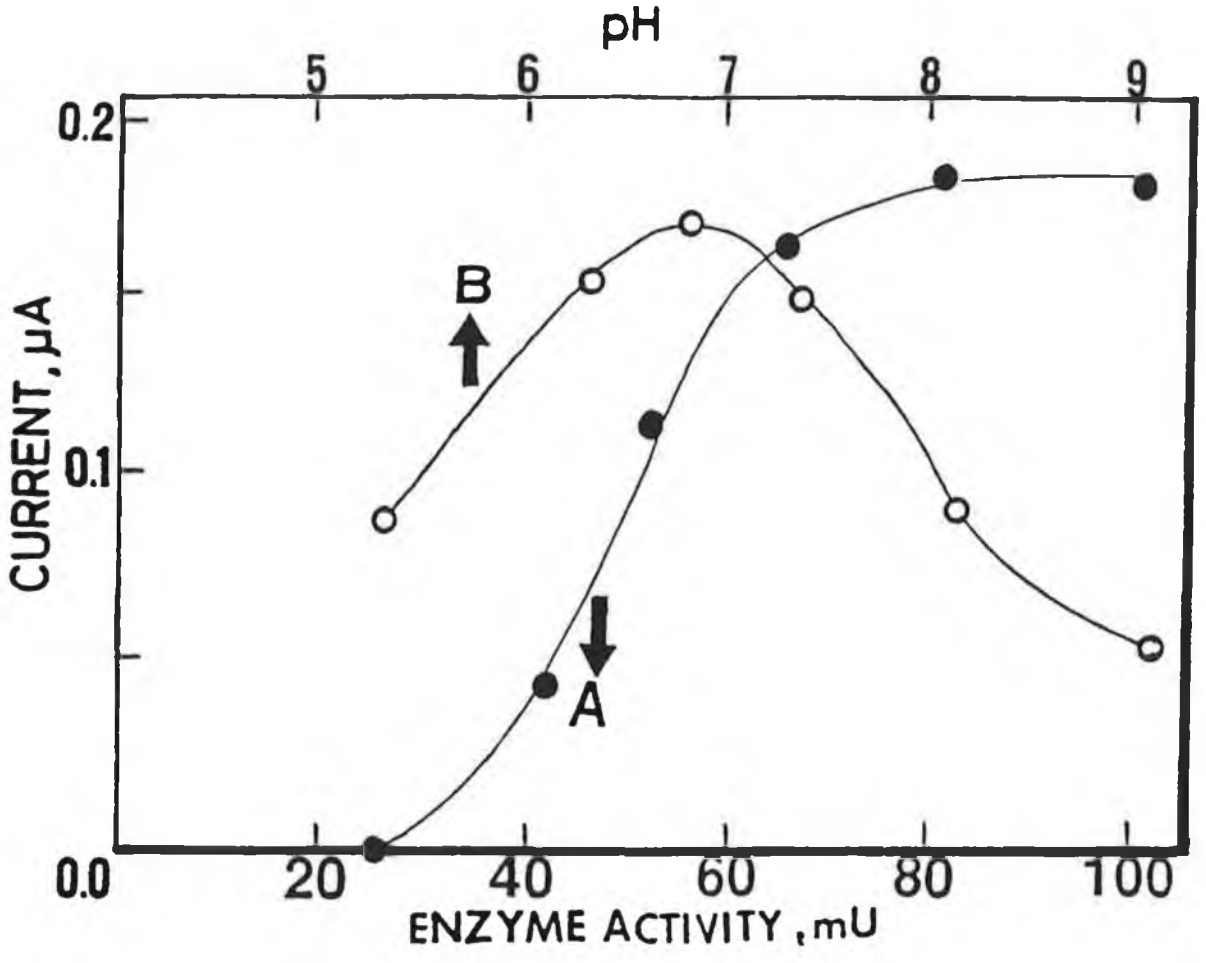


FIGURE 2.3.

Dependence of the current upon the enzyme loading (A) and solution pH (B). Response to $5x10^{-4}$M theophylline. Other conditions as in Figure 2.1.

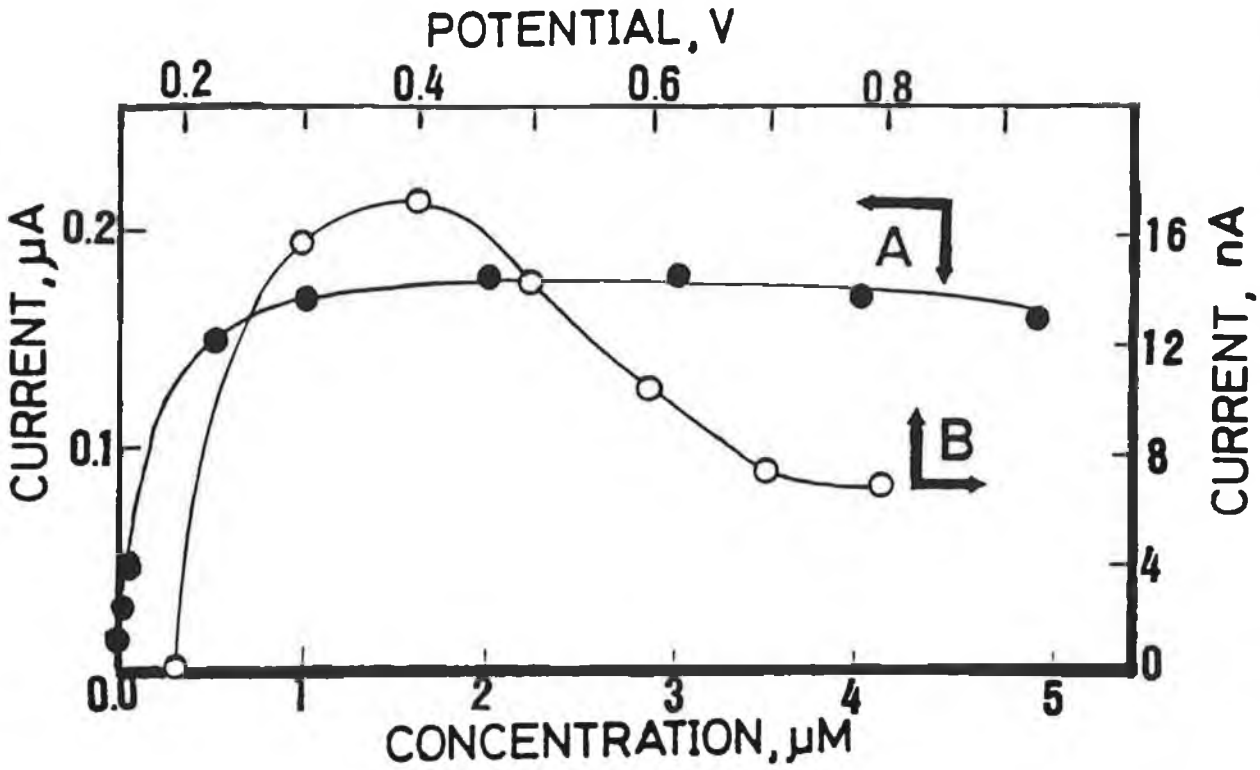


FIGURE 2.4.

Dependence of the current upon the $K_3Fe(CN)_6$ concentration (A) and applied potential (B). Response to $5x10^{-4}$ M (A) and $5x10^{-5}$M (B) theophylline. Other conditions, as in Figure 2.1.

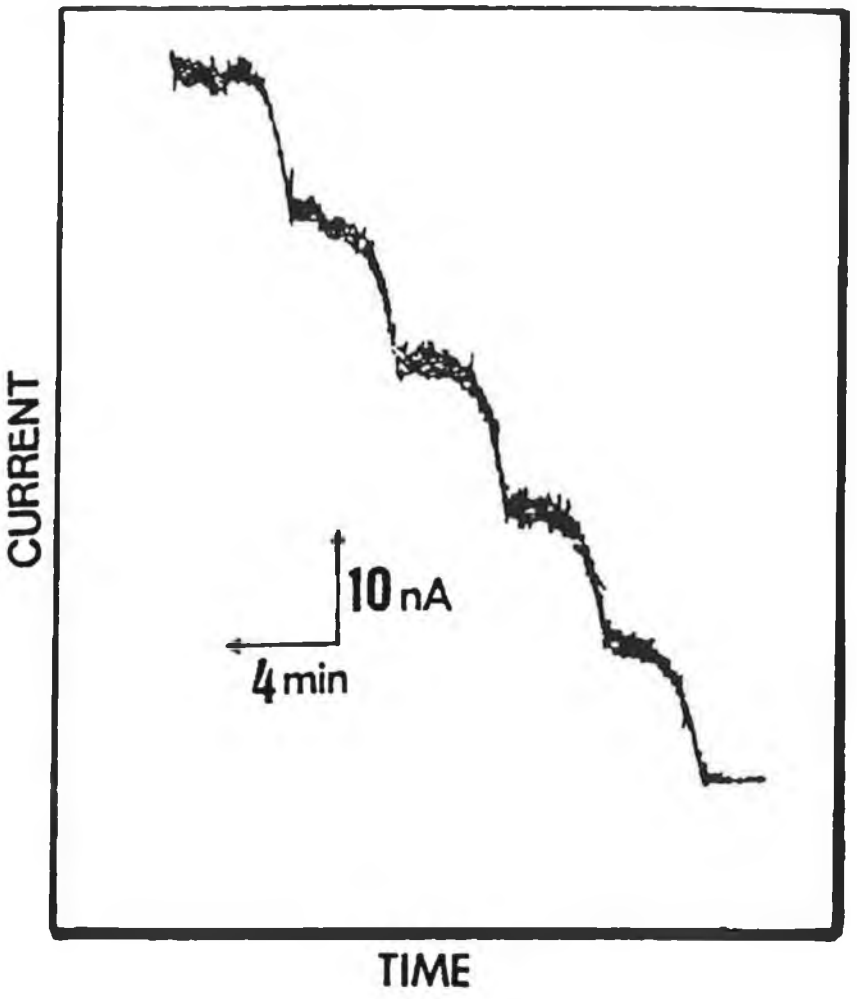


FIGURE 2.5.
Current-time recording for successive 5×10^{-6}M increments of theophylline. Other conditions as in Figure 2.1.

2.2.2.3. *Flow Injection Studies*

The fast response of the Th.O.-containing electrode makes it suitable for use in dynamic flow systems. Figure 2.6. compares flow-injection response peaks for a series of 25 repetitive injections of a $5x10^{-4}$M theophylline solution at the bare (A) and Th.O. (B) electrodes. As expected, no response was observed with the naked surface (A). In contrast, the coated electrode exhibited well-defined and sharp peaks (B). A reproducible response was observed over this prolonged series of injections (RSD = 3 %, mean = 1.9 nA, range = 18-20 nA), indicating the stability of the enzyme layer under vigorous hydrodynamic conditions. The fast response and wash times resulted in peak widths of 20 s and an injection rate of 180 samples/hour. The peaks shown in Fig. 2.6B were part of a long run of 90 successive injections for which a RSD of 4 % was obtained. The flow injection response was linear for samples containing up to $1x10^{-3}$M theophylline (Figure 2.7.). A curvature was observed at higher concentrations. The detection limit (of the flow injection operation) was $1x10^{-5}$M theophylline; the sensitivity (slope of the linear portion) corresponds to 32 nA/mM; correlation coefficient, 0.997.

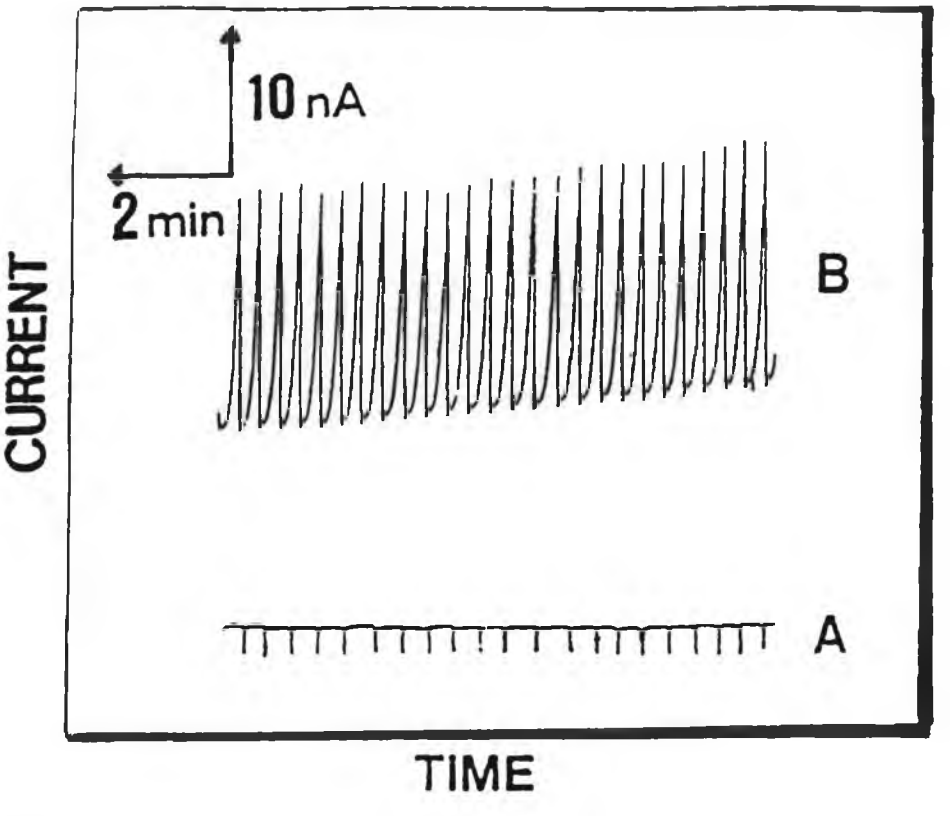


FIGURE 2.6.
Detection peaks for repetitive injections of $5x10^{-4}$M theophylline solution at the bare (A) and Th.O.-containing (B) electrodes. Flow rate, 2.0 ml/min. Applied potential, + 0.4 V. Electrolyte and carrier, 0.05 M phosphate buffer (pH 6.9) containing 1 mM ferricyanide.

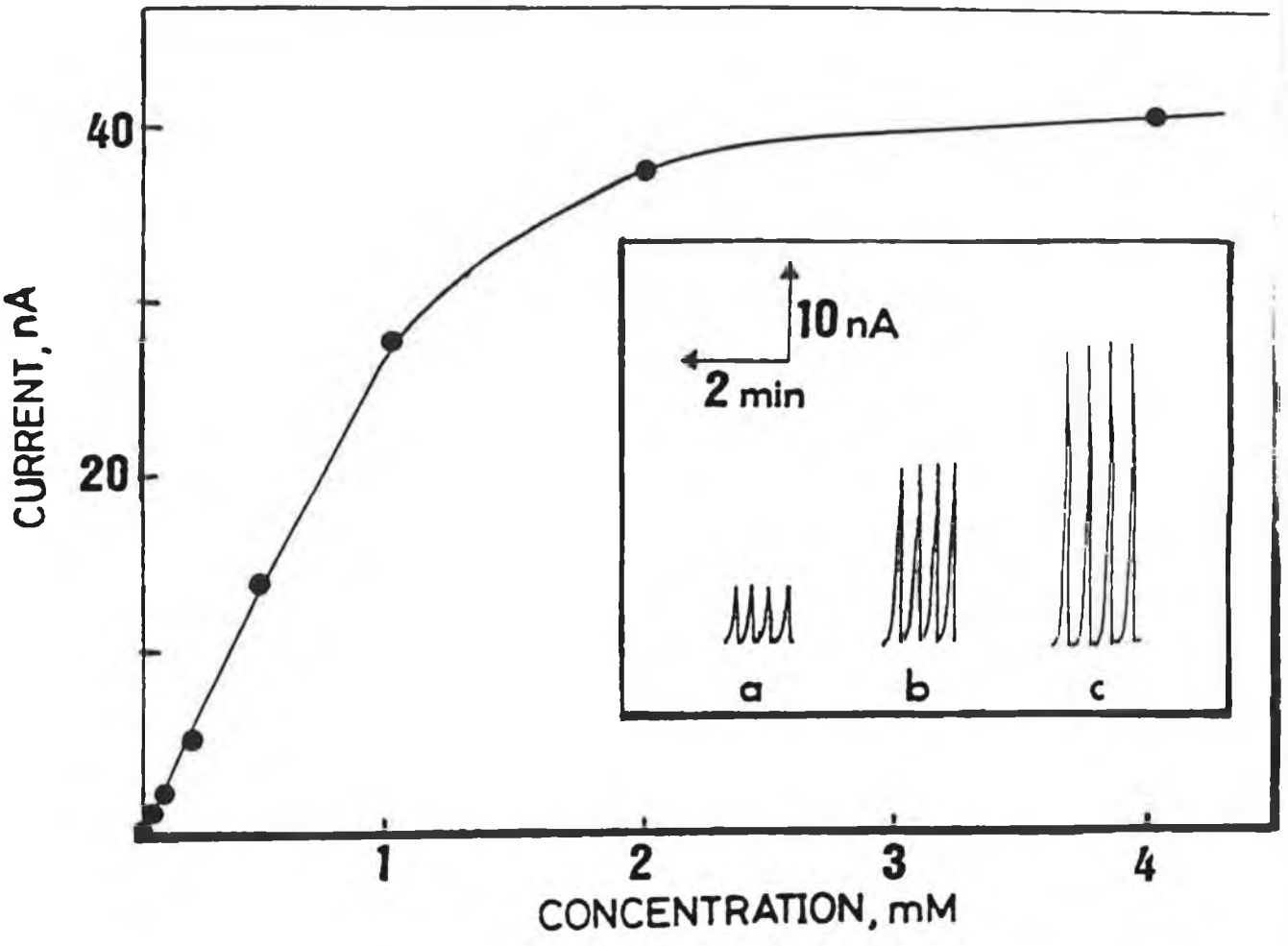


FIGURE 2.7.
Calibration plot for theophylline obtained in flow injection analysis. Conditions as in Fig. 2.6.

2.2.2.4. *Interference Studies*

Interference studies were conducted in the presence of several physiologically relevant compounds. While glucose, acetaminophen and caffeine exhibited a negligible effect (0-1 %) upon the response, a large interference was observed in the presence of uric and ascorbic acid (50 and 25 % respectively). This was overcome by placing by placing a lipid layer (20 µl of a solution containing 20 mg phosphatidylcholine/14 mg cholesterol per 1 ml chloroform) on top of the Nafion/Th.O film, which reduced the uric acid and ascorbic acid response to 0-1 %. Similar selectivity improvements have been reported for a lipid modified glucose sensor [17].

The Th.O. electrode had an analytically useful response for up to 35 days (with intermittent usage and storage at 4°C in phosphate buffer). A slowly decreasing sensitivity was however observed during this period (with *ca.* 15 and 30 % decreases within 10 and 20 days, respectively) (Fig. 2.8.). Because of the fast fabrication and low cost of the Th.O. electrode, the reasons for its slow deactivation were not elucidated.

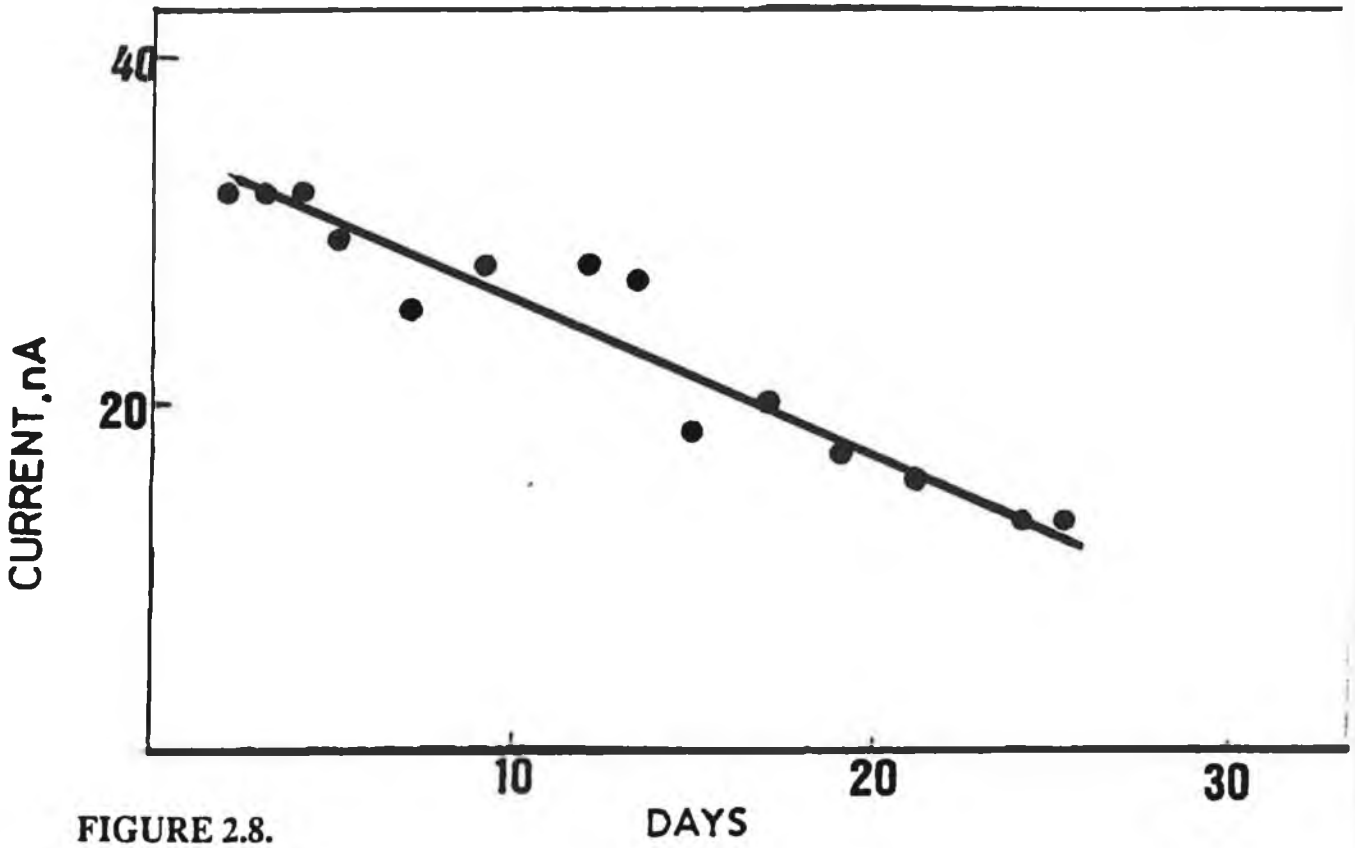


FIGURE 2.8.
Enzyme electrode stability over a period of 24 days with intermittent storage at 4 °C. Injection of 0.25 mM theophylline: sensitivity 200 nA full scale and other conditions as Fig. 2.1.

2.2.3. CONCLUSION

In summary, an amperometric biosensor for theophylline, based on the recently isolated enzyme theophylline oxidase has been described. The enzyme was entrapped together with a ferricytochrome C cofactor, within a polymeric (Nafion) coating. The anodic detection (at 0.4 V vs. Ag/AgCl) was facilitated by the addition of a redox-mediating ferricyanide ion. The influence of various experimental variables were described. The limit of detection was found to be $2x10^{-6}M$ theophylline, with linearity prevailing up to $3x10^{-4}M$. The fast response times allow for rapid flow injection measurements, with frequency of 180 samples per hour and a relative standard deviation of 3.0 %.

Because of the great significance of theophylline in the clinical arena, additional work is required to improve the performance (particularly the stability and selectivity) of the sensor and to thoroughly assess its suitability for assays of relevant biological fluids. The Nafion film alone did not serve to fully eliminate interfering compounds. It was therefore necessary to apply an overlaid lipid layer on top of the enzyme film, which served to eliminate ascorbate and uric acid, but was found to be unstable. A lowering of the detection potential is also required for application to biological samples. A recent paper by McNeill *et al.* [13] described a rapid determination of theophylline in serum at 0.1 V vs. Ag/AgCl, based on Th.O. using an organic conducting salt for construction of the enzyme electrode. Measurements in serum were carried out using a dual working electrode system. The electrodes were covered with a polycarbonate membrane, which excluded serum proteins from the electrode and prevented fouling effects caused by passive adsorption on the electrode.

Further improvements required are coimmobilisation of the ferricyanide mediator to obtain a reagentless device. When coupled with the fast and sensitive response, such improvements and studies should lead to reliable and rapid testing for theophylline based on disposable strips or automated flow analysis.

2.3. DEVELOPMENT OF AMPEROMETRIC SENSORS FOR CHOLINE ACETYLCHOLINE AND ARSENOCHOLINE

Acetylcholine was the first documented transmitter of motor neurons in both the spinal cord and in nerve skeletal junctions in vertebrates. Several experiments have demonstrated that acetylcholine may either accelerate or retard the firing rate of individual neurons [18]. Deficiency of choline in animals leads to the development of fatty liver and renal failure. Choline is used by cholinergic neurons to synthesise acetylcholine and has also been used to treat certain disorders of the Central Nervous System. The current keen interest in understanding the acetylcholine nerve function in the body has given great importance to development of analytical methods for its determination. This interest has resulted in the development of biosensors based on immobilised enzymes, with applications in different areas of medicine, food and environmental analysis.

Previous methods which permitted the quantitation of acetylcholine include radiolabelling [19,20] and gas chromatographic techniques [21]. These methods are time-consuming, involve complicated procedures and require expensive equipment. Several acetylcholine sensors have since been developed based on enzyme electrodes with potentiometric [22,23] or amperometric detection of oxygen [24,25] or hydrogen peroxide [26-28]. However, the consumption of oxygen may be strongly affected by fluctuations in the ambient oxygen concentration, while, in the case of hydrogen peroxide detection, the applied potential is very high and hence the method is susceptible to easily oxidisable interferents.

A kinetic fluorimetric method for the determination of choline and acetylcholine based on the oxidation of these analytes by Ce(IV) in a sulphuric acid medium yielding fluorescent Ce(III) has also been proposed [29].

The co-immobilisation of the enzymes acetylcholine esterase (ACE) and choline oxidase (COx) on an alkylamino-bonded silica surface using glutaraldehyde has been incorporated as the enzyme reactor in a HPLC system for detection of acetylcholine and choline [30]. The amperometric detection of acetylcholine and choline in a liquid chromatographic system with an immobilised enzyme reactor has also been reported [31]. The hydrogen peroxide produced enzymatically in the enzyme reactor, after separation of acetylcholine and choline by the reversed phase column, was monitored amperometrically.

An enzyme sensor has been described for determination of acetylcholine and choline in rat brain extracts [32]. Another enzyme-modified carbon paste

tetrathiafulvalene (TTF) electrode has been reported for the determination of acetylcholine [33]. TTF was shown to efficiently reoxidise the reduced flavin adenine dinucleotide centre of choline oxidase.

Some enzyme modified carbon fiber electrodes have been described for the determination of acetylcholine and choline. The mechanism is based on the detection of hydrogen peroxide which is the product of the sequential enzyme reactions of acetylcholine esterase and choline oxidase [34]. The enzymes are co-immobilised in polyvinyl alcohol containing styryl pyrydinium (photo-crosslinkable polymer). A major improvement in the selectivity of carbon fiber microelectrodes for acetylcholine and choline, by coating with Nafion, has been achieved [35]. The coating was found to be particularly impermeable to ascorbic acid and anionic biogenic amine metabolites. An ultramicro acetylcholine sensor based on modified carbon fiber electrodes, with entrapment of both enzymes within a polyvinyl alcohol - quarternised stilbazole matrix, has also been reported recently [36].

The esterase enzymes play an important role in biology because they are responsible for the hydrolysis of acetylcholine, and in their absence the original state of the post-synaptic membranes cannot be established. It is of interest therefore to study the inhibiting action exerted by some compounds on this class of enzymes. The behaviour of the esterase/pesticide systems, and the analytical possibilities arising from the inhibiting action exerted by pesticides on esterases have been examined by several authors. A biosensor consisting of a Clark oxygen electrode, modified with a dialysis membrane containing acetylcholine esterase and choline oxidase has been constructed and its application to the analysis of ecologically important matrices described [37].

Pesticides such as paraoxon and aldicarb have been determined with an amperometric hydrogen peroxide-based choline sensor [38]. An important toxicological index is defined as the amount of compound which causes a percentage of cholinesterase inhibition equivalent to that produced by a known amount of a pesticide (e.g. parathion) taken as a reference compound.

Biosensors for choline, choline esters and inhibitors of choline esterase have been developed by coupling immobilised choline oxidase and its esterase to a hydrogen peroxide electrode [39]. When a kinetically controlled bienzyme sensor with a low activity of cholinesterase is used, a diminished sensitivity is obtained for acetylcholine with an increased sensitivity for inhibitors such as NaF, butoxycarboxime, trichlorfon or dimethoate.

Other previously published methods for the determination of toxic substances, e.g. organophosphorous and carbamate pesticides, based on the

inhibitory effects of these compounds on acetylcholinesterase activity have made use of pH electrodes which indicate the generation of protons in the cholinesterase - catalysed reaction [40, 41]. Choline electrodes for pesticide analysis have also been previously described [42, 43]. A screen-printed sensor based on 7,7,8,8-tetra cyanoquinodimethane - modified graphite and immobilised butyrylcholinesterase, was found to be sensitive to non-competitive inhibition by organophosphates in the range 0.06 to 8 ppm [44]. Another recent report of an amperometric biosensor for pesticides was based on a cobalt - phthalocyanine modified carbon paste electrode with a cholinesterase enzyme membrane [45].

Preliminary experiments in our laboratory indicated that the signal to noise ratio could be improved by immobilising the enzymes at the surface of the electrode rather than by mixing them directly in the carbon paste electrode. A similar observation was found by Skladal *et al.* [46]. This work involved the development of a sensor based on the co-immobilisation of ACE and COx on a glassy carbon electrode, permitting the quantitative measurement of acetylcholine and choline. The soluble mediator potassium ferricyanide has been shown to efficiently reoxidise the reduced flavin adenine dinucleotide centres of choline oxidase. An important arsenic complex of choline, namely arsenocholine, found in fish tissue, could be determined using the choline oxidase modified glassy carbon electrode, thereby allowing the development of a novel biosensor for the quantitation of this compound.

2.3.1. EXPERIMENTAL

2.3.1.1. *Apparatus*

Amperometric measurements were performed using an EG & G PAR Model 174A polarographic analyser connected to a Philips Model PM 8251 recorder. Cyclic voltammetry was performed with an EG & G PAR Model 264A polarographic analyser and a JJ Instruments Model PL4 recorder. Experiments were carried out in a 10 ml electrochemical cell using a saturated calomel reference electrode, a platinum wire auxiliary electrode and a glassy carbon working electrode. A magnetic stirrer and bar provided the convective transport.

2.3.1.2. *Reagents*

All chemicals were of analytical grade, and deionised water, obtained by passing distilled water through a Milli-Q water purification system, was used to prepare all solutions. Choline oxidase, acetylcholinesterase and choline were all purchased from Sigma, while acetylcholine and Nafion were obtained from Aldrich. Arsenocholine was obtained as part of a study of arsenic speciation organised by the Bureau of Certified Reference Materials (BCR) of the European Communities.

2.3.1.3. *Electrode Preparation*

Prior to coating, the glassy carbon electrode was polished using an alumina slurry. Modification of the electrode was achieved by applying 5 µl of a choline oxidase solution containing 5 mg/ml enzyme (12 Units/mg), resulting in an activity of 0.382 Units/cm^2, for the choline electrode. In the case of the acetylcholine sensor, an additional 0.5 µl of acetylcholinesterase solution containing 1 mg/ml of enzyme (950 Units/mg) was applied to the surface (resulting in an activity of 0.475 Units ACE/cm^2) and allowed to dry using a heat gun. 5 µl of a Nafion solution (diluted 1 in 10 with deionised water) was then placed on the enzyme layer and subsequently dried until a stable film was obtained. When not in use, electrodes were stored at 4^{o}C in phosphate buffer pH 7.0.

2.3.2. RESULTS AND DISCUSSION

2.3.2.1. *Constant potential measurements*

Preliminary studies on the amperometric detection of H_2O_2 with the choline oxidase modified electrode were performed in phosphate buffer (0.1 M, pH 6.5). The background transient current was allowed to decay to a constant value before samples of a stock choline solution were injected into the electrolyte. The time required to reach 95 % of the amperometric steady state current was typically < 5 sec after addition of the choline sample. As was expected in the absence of biocatalytic activity, the unmodified electrode showed no response to choline. Despite the high operating potential (0.85 V) required for monitoring the hydrogen peroxide produced in the enzymatic reaction, favourable signal to noise ratio characteristics were observed.

However, when the electrolyte contained 1 mM of the hexacyanoiron(III) ion, the current density for choline addition was considerably greater, due to recycling of the mediator between the Fe(II) and Fe(III) states.

$$\text{Acetylcholine} \xrightarrow{\text{ACE}} \text{Choline} + \text{Acetate} \qquad (2.3.)$$

$$\text{Choline} + 2O_2 + H_2O \xrightarrow{\text{COx}} \text{Betaine} + H_2O_2 \qquad (2.4.)$$

$$Fe(CN)_6^{3-} \Rightarrow Fe(CN)_6^{4-} \qquad (2.5.)$$

Therefore in subsequent experiments, 1 mM potassium ferricyanide was used in solution and reoxidation of the hexacyanoiron(II) ion achieved by applying a potential of 0.25 V.

2.3.2.2. *Development of an amperometric electrode for choline*

The sensitivity of the choline electrode was affected by various preparation and operational parameters. At low surface loadings of the enzyme the response exhibited the greatest current but poorest linearity; hence a loading of 0.382 Units/cm^2 electrode surface was chosen as a compromise. The solution pH was found to have a profound effect on the sensitivity (Fig. 2.9.), with a decrease in response observed with increasing pH. The best S/N ratio was obtained with a solution of pH 6.5, which was subsequently used in further experiments. Since choline has a pK_a of 13.9, it will be positively charged at pH 6.5 and therefore will partition preferentially in the anionic polymeric Nafion layer. The dependence of the choline response on the concentration of the redox mediating hexacyanoiron(III) ion is shown in Fig. 2.10. The catalytic current increases rapidly with mediator concentration until 1 mM, after which it was not found to be the limiting factor.

A typical response trace of current density vs. time for choline under the optimum conditions outlined above, allows a limit of detection of 5 x 10^{-5} M. Calibration curves for 1 mM additions of choline, for two different enzyme loadings may be seen in Fig. 2.11. In the case of 0.30 Units choline oxidase per

cm^2 electrode surface, linearity extended between 1 and 10 mM choline with a correlation coefficient r = 0.999 and sensitivity of 16.5 nA/mM. For a loading of 0.382 Units/cm^2, linearity was observed between 1 and 13 mM (r = 0.999) with a sensitivity of 19.0 nA/mM.

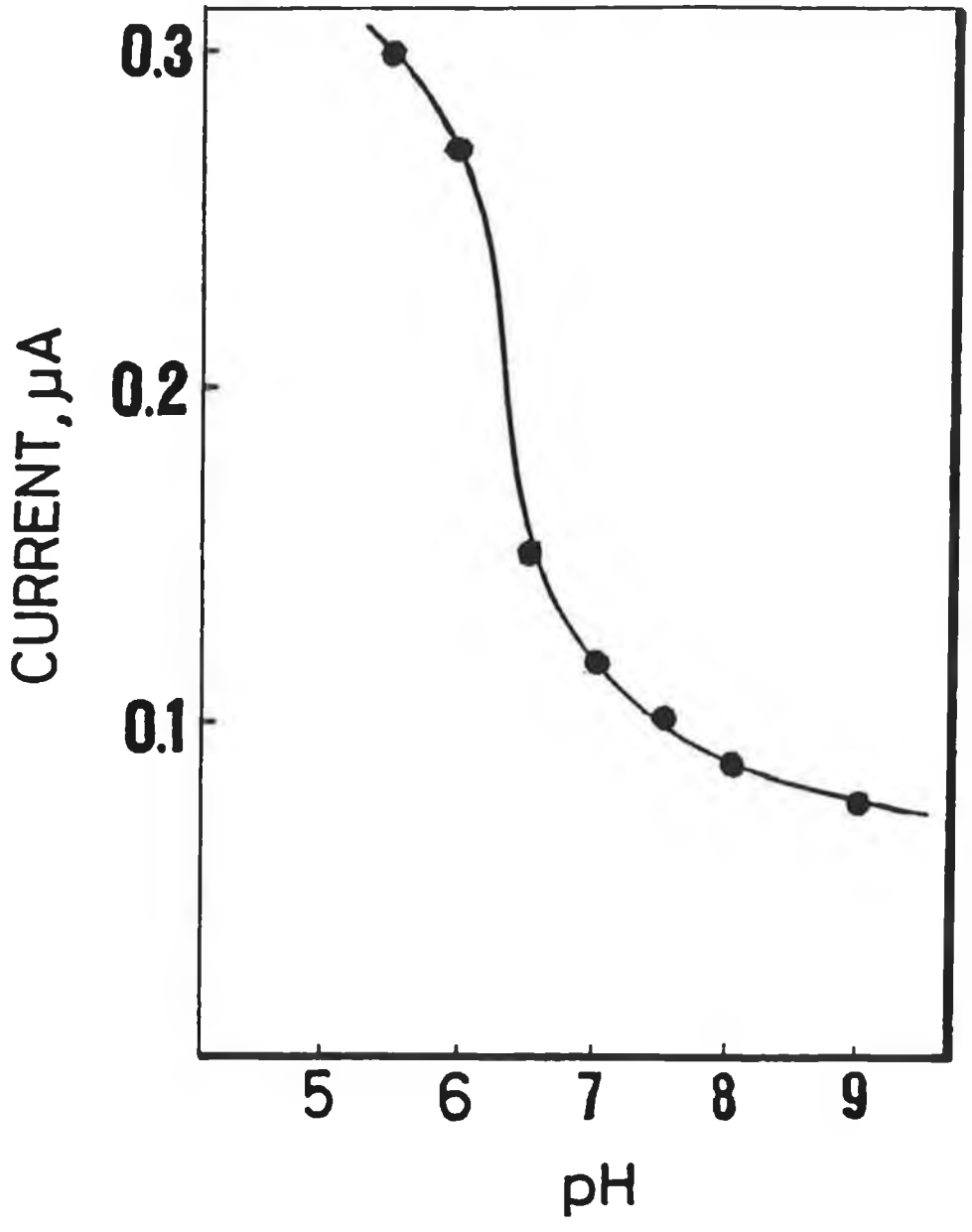


FIGURE 2.9.
Dependence of the choline response upon the solution pH. Electrolyte : phosphate buffer (0.1 M) containing 1 mM potassium ferricyanide; applied potential, 0.25 V, loading 0.382 Units COx/cm^2; solution stirring rate, 200 rpm; sensitivity, 2
µA full scale.

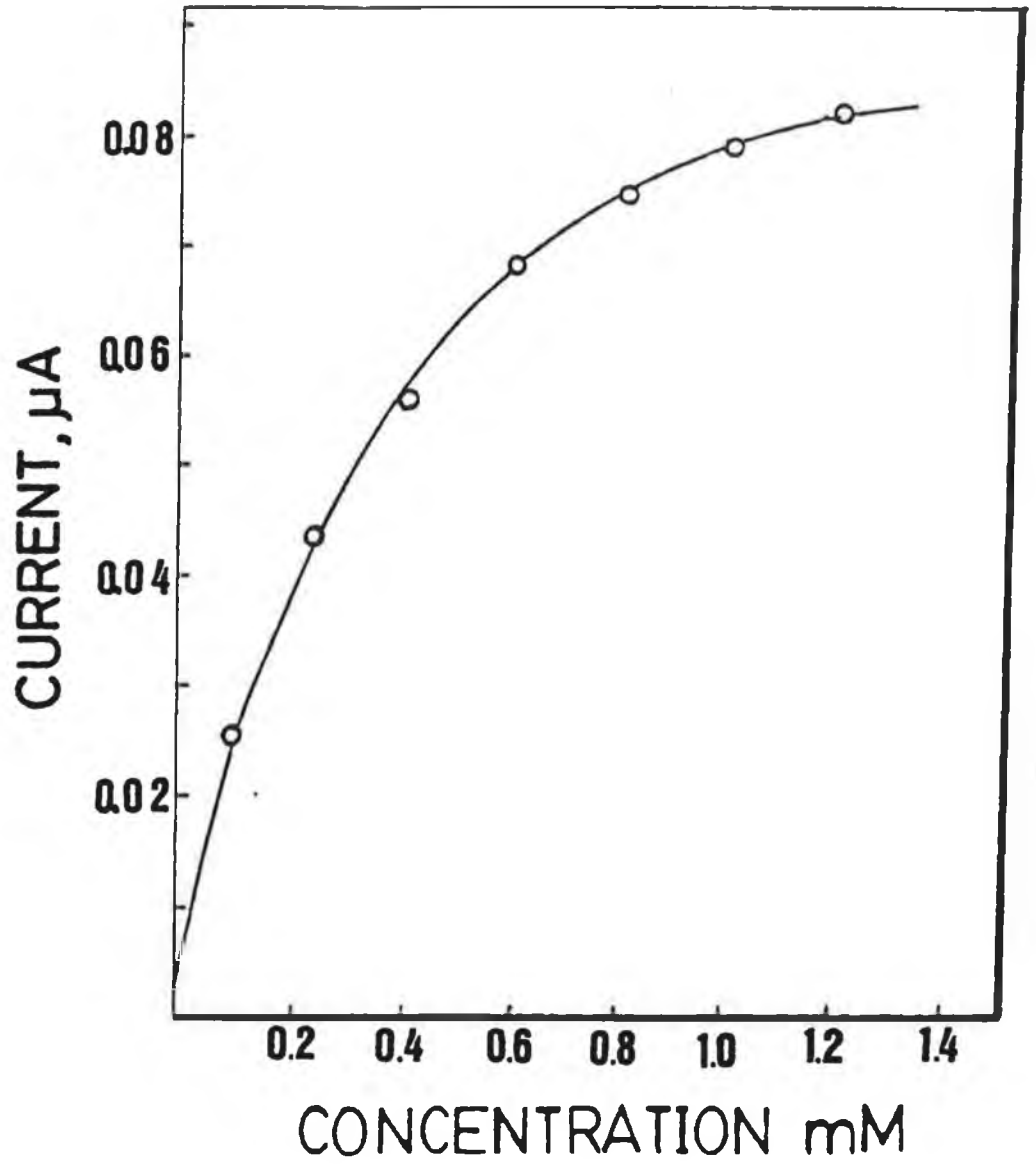


FIGURE 2.10.
Optimisation of mediator concentration in solution. Loading 0.077 Units/cm^2 COx. Injections of 2 mM choline. Conditions as Fig. 2.9.

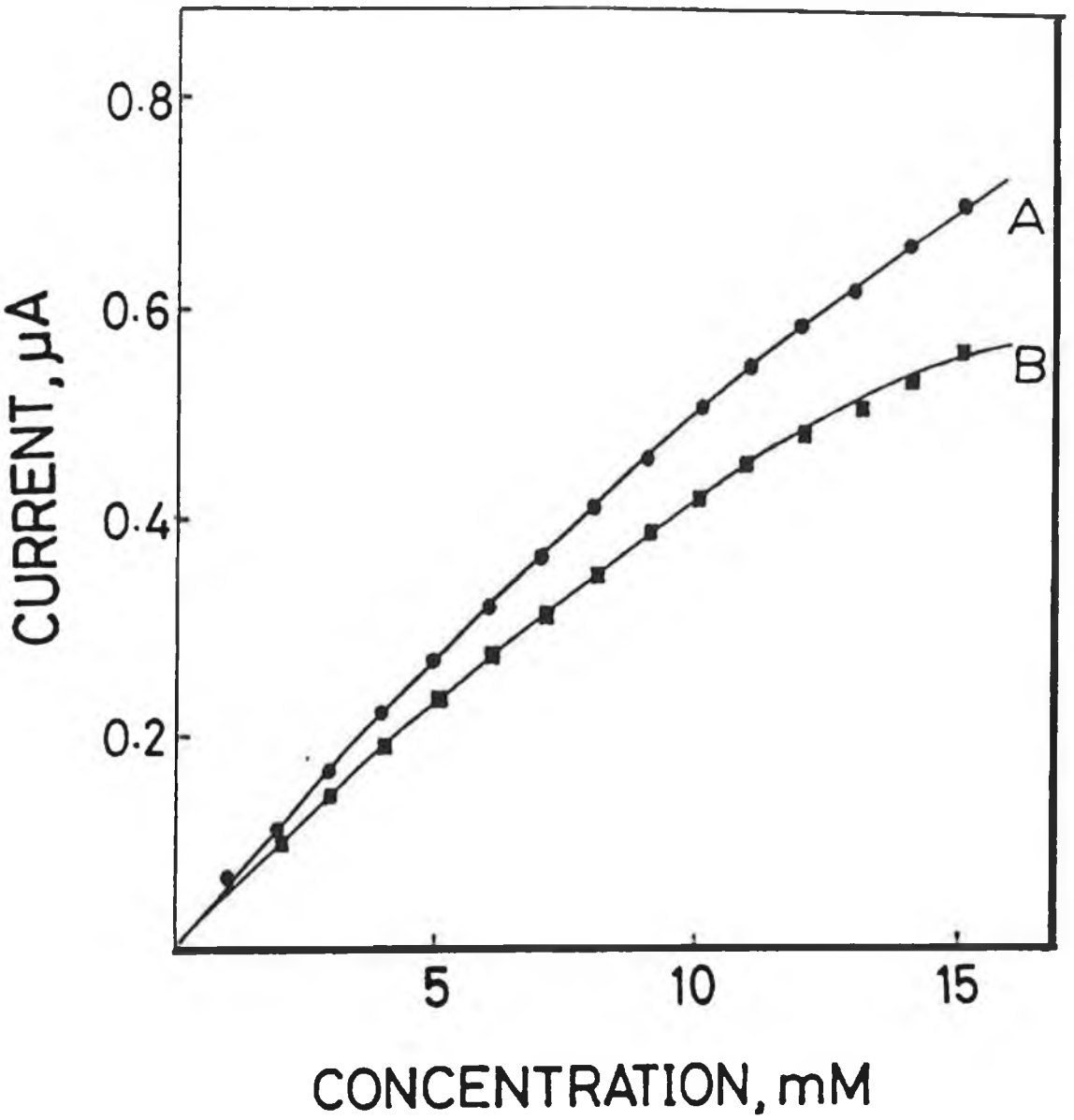


FIGURE 2.11.
Calibration curves for two surface loadings of COx (a) 0.382 Units/cm^2 and (b) 0.307 Units/cm^2. Injection of 0.5 mM choline. Sensitivity, 0.5 μA full scale. Other conditions as Fig. 2.9.

2.3.2.3. *Development of an arsenocholine sensor*

An application of this sensor was demonstrated in the determination of arsenocholine. The amperometric response to 0.04 mM (10 ppm) additions may be seen in Fig. 2.12. Linearity was found to extend from 0.04 - 0.06 mM with r = 0.998 and a sensitivity of 0.44 µA/mM. The greater sensitivity observed for arsenocholine may possibly be due to interaction between the metal and the enzyme active site.

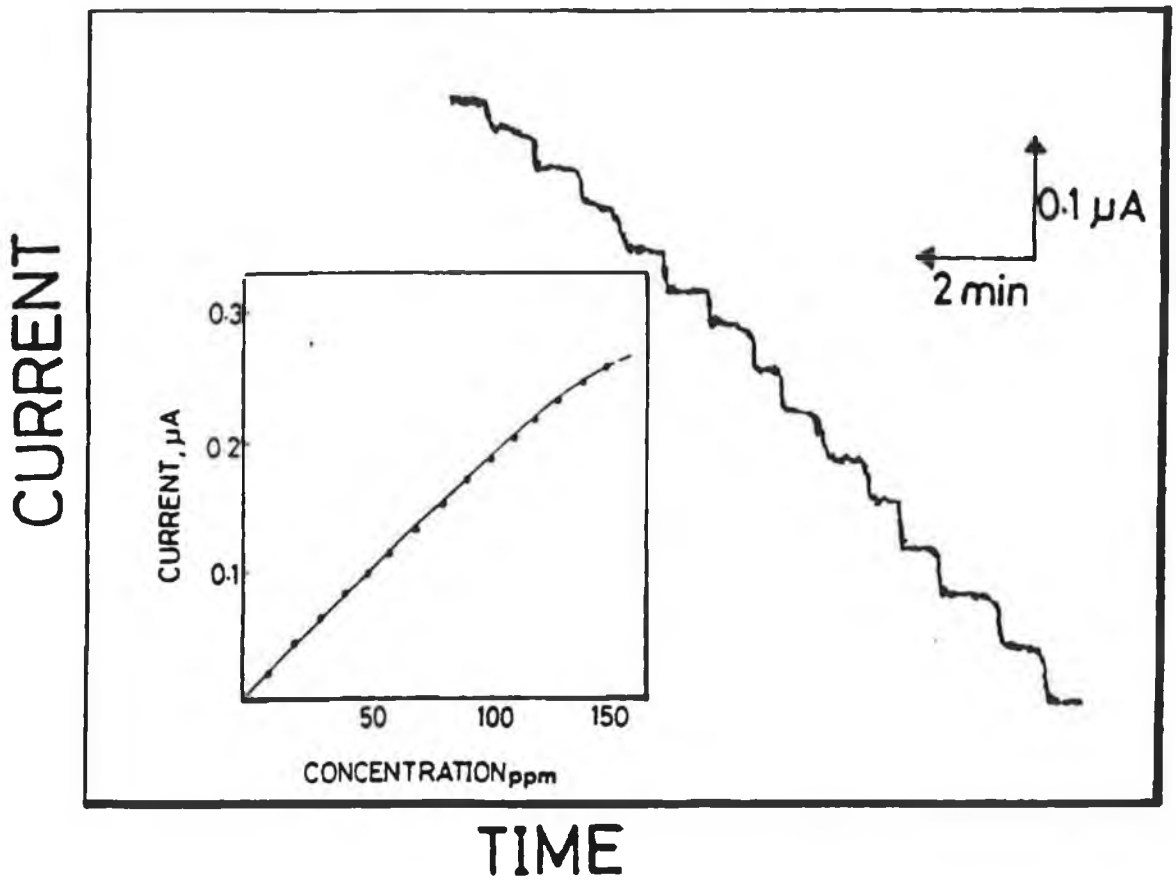


FIGURE 2.12.
Current - time response for successive 10 ppm injections of arsenocholine. Loading 0.307 Units/cm^2 COx.

2.3.2.4. *Development of an acetylcholine sensor*

The acetylcholine sensor fabrication involved co-immobilisation of both choline oxidase and acetylcholinesterase, this layer being subsequently covered with the polymeric Nafion coating. The same electrolyte and conditions were employed as for the choline determination. Changes in the surface loading were not found to appreciably modify the amperometric response for either choline or acetylcholine. The amperometric response of this electrode to successive additions of both compounds may be seen in Fig. 2.13. with each addition effecting a 0.5 mM increase in concentration. A shorter linear range was obtained in the case of the acetylcholine response, extending only up to 5 mM with limit of detection (calculated as 3 times the standard deviation of the noise) of 4.3 x 10^{-5} M.

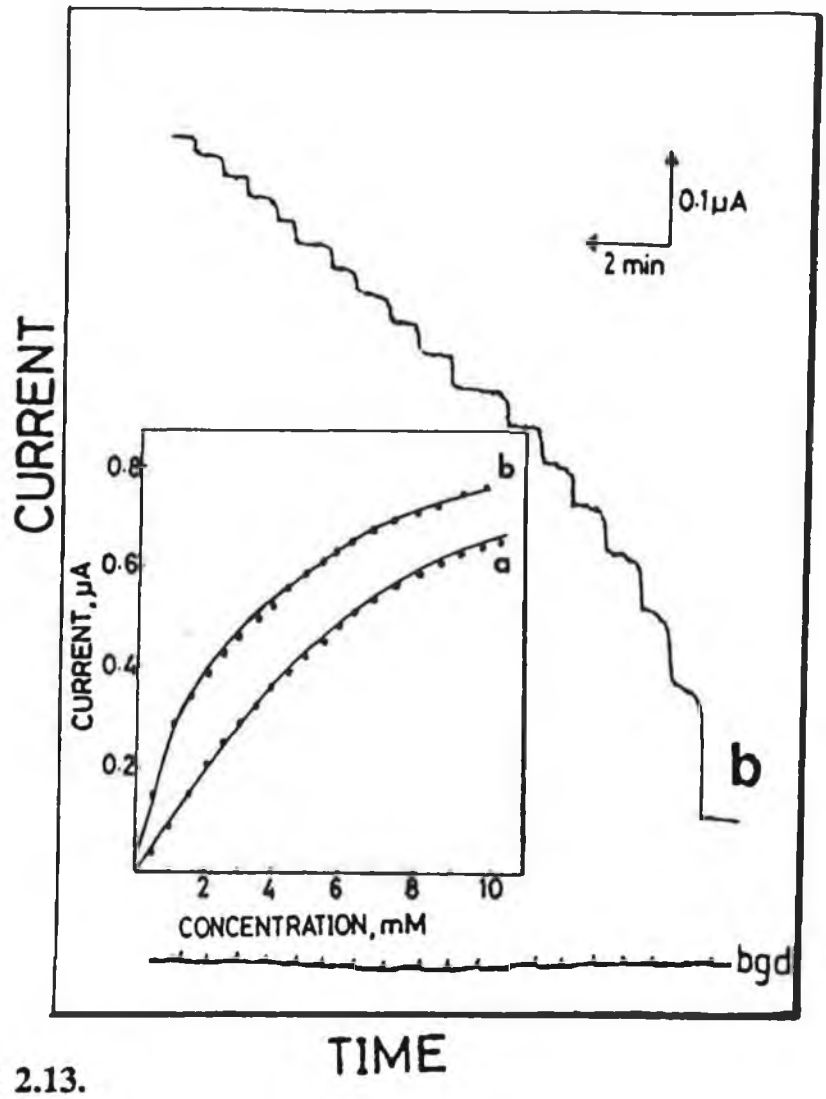


FIGURE 2.13.

Amperometric response for successive (a) 0.5 mM additions of choline (b) 0.5 mM acetylcholine. Loading 0.382 Units COx and 0.475 Units ACE per cm^2 electrode surface. Sensitivity 1 μA full scale.

2.3.2.5. *Cyclic Voltammetry*

Cyclic voltammetry of the hexacyanoiron(III) ion at a Nafion-coated glassy carbon electrode showed a completely reversible behaviour (Figure 2.14.). The current obtained here, however, was lower than that obtained at the bare glassy carbon, perhaps due to some electrostatic repulsion. When the glassy carbon electrode was modified with both choline oxidase and Nafion, the mediator also exhibited reversible behaviour, and the current was only slightly larger, perhaps due to the ionic interaction between the enzyme and the polymer, resulting in lower net charge within the film in contact with the solution. The effect of modification with acetylcholinesterase resulted in irreversible behaviour for the mediator. The non-linear relationship between i_p and $\nu^{1/2}$ indicates a slow mass transfer process, probably due to the additional complexity of this film when modified with this enzyme as opposed to choline oxidase. This phenomenon was even more pronounced when the electrode was modified with both enzymes. Fig. 2.15. shows the relationship between current and $\nu^{1/2}$ for the various modified electrodes. These curves show initial diffusion control followed by limitation due to electron transfer at high scan rates. The potential difference between E_a and E_c increased as the complexity of the film increased, as shown in Table 2.1.

2.3.2.6. *Apparent Michaelis - Menten Constants*

The linear response range of the sensors can be estimated from a Michaelis-Menten analysis of different calibration curves (Table 2.2.) by using the Lineweaver-Burk plot of $1/i_{ss}$ (μA) vs. 1/[substrate] (mM), the slope of which allows calculation of the apparent K_m value. This value provides a measure of the substrate/concentration over which the electrode response is linear, and characterises the enzyme electrode - not the enzyme itself. I_{max} represents the maximum current density measured under conditions of enzyme saturation. The additional diffusional restrictions imposed as a result of increased loading, and therefore complexity on the electrode surface, causes alteration of the basic catalytic properties and causes a shift in apparent K_m.

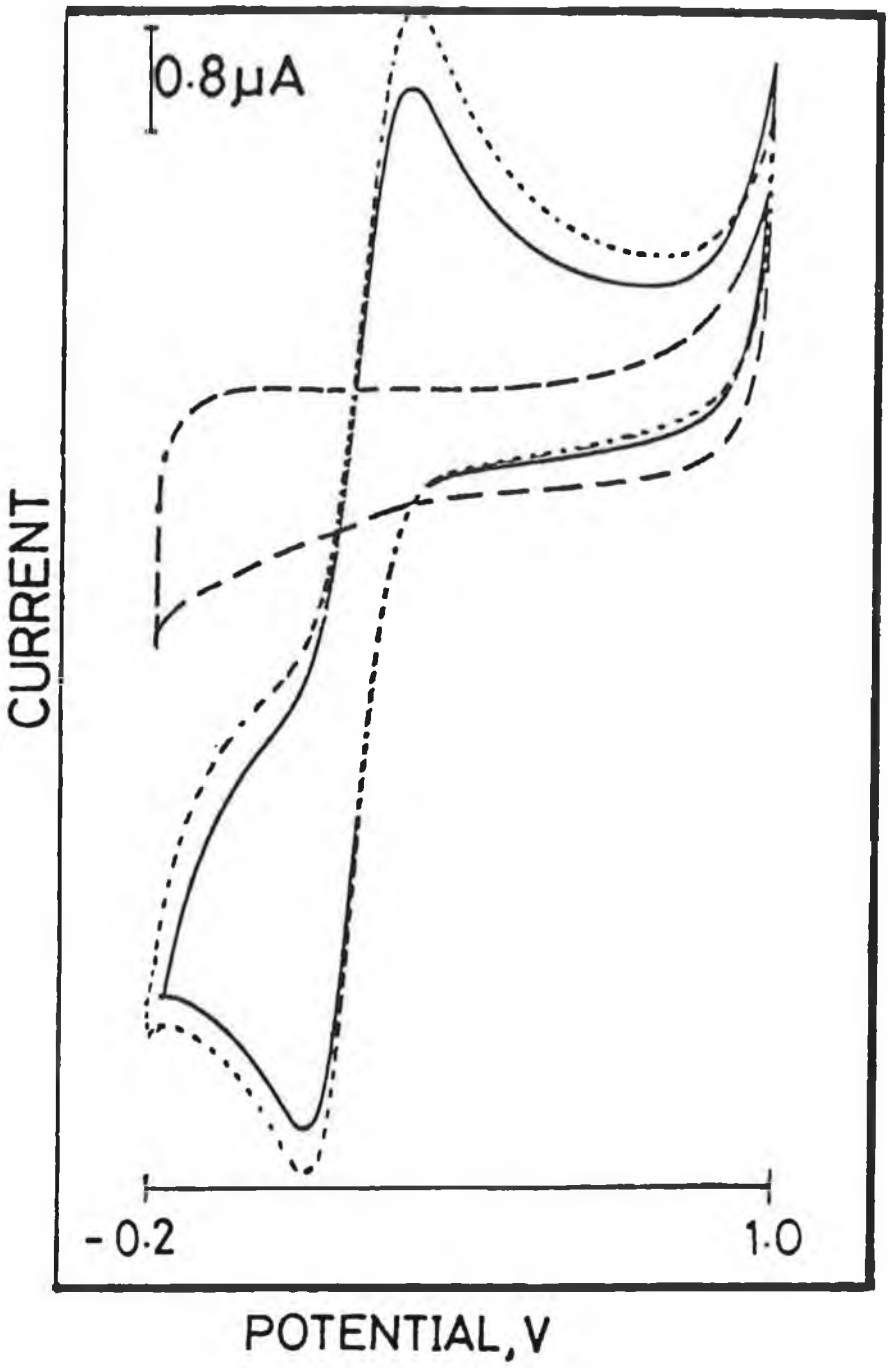


FIGURE 2.14.

Cyclic voltammogram for 1 mM potassium ferricyanide at a COx modified glassy carbon electrode (0.36 U/cm^2). Scan range -0.2 to 1.0 V, scan rate 50 mV/cm, 10 µA full scale. Full line represents the case after addition of 5 mM choline.

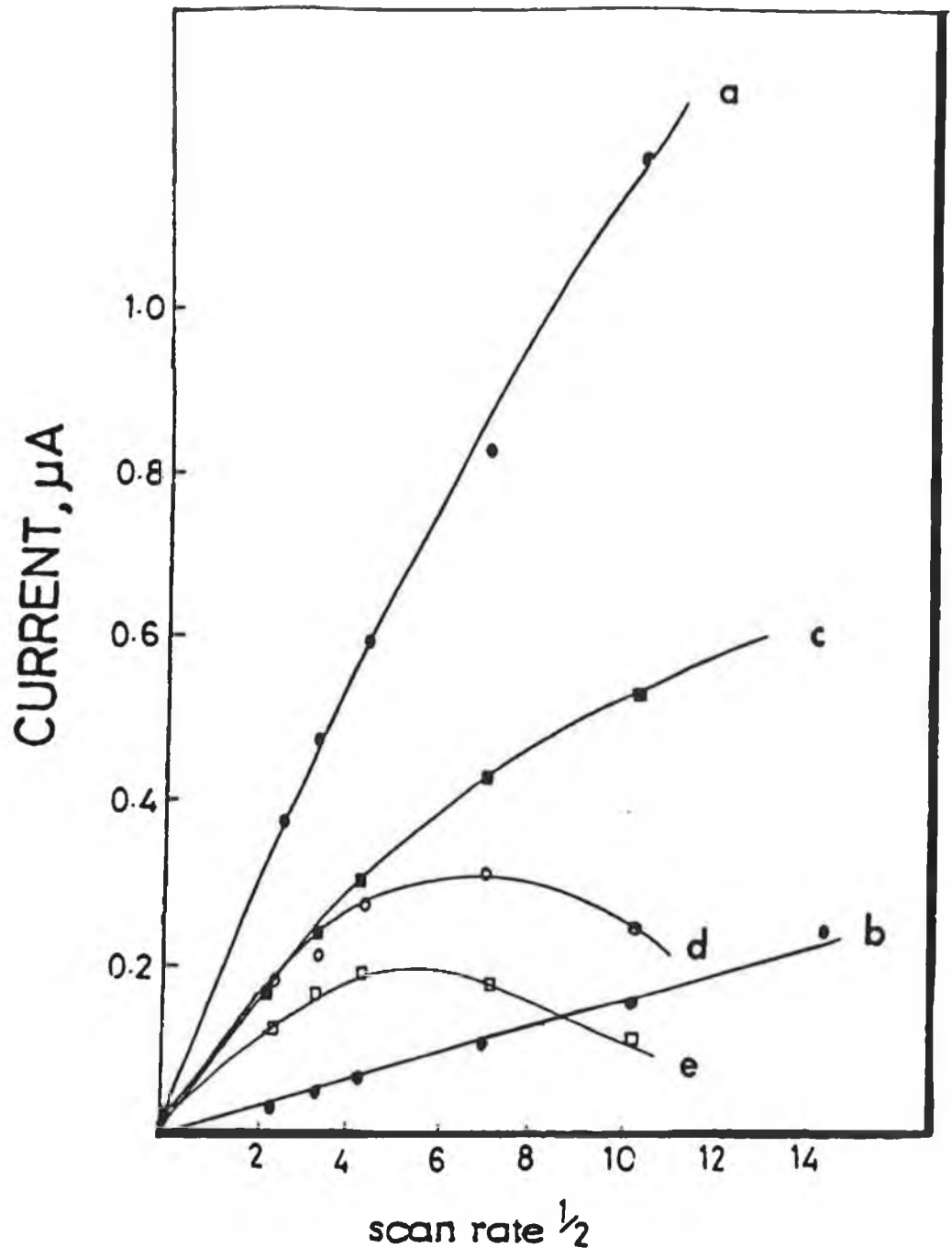


FIGURE 2.15.

Steady state current vs. scan rate$^{1/2}$ for (a) bare glassy carbon electrode, (b) Nafion modified electrode, (c) Nafion and COx modified electrode (d) Nafion and ACE modified electrode (e) COx and ACE (0.382 Units COx and 0.602 Units ACE/electrode) modified electrode.

ELECTRODE	Eox (V)	Ered (V)	E (mV)
(a) Bare	0.24	0.15	90
(b) Nafion coated	0.20	0.15	50
(c) COx + Nafion	0.24	0.14	100
(d) ACE + Nafion	0.34	0.05	290
(e) COx/ACE(i) + Nafion	0.31	0.02	290
COx/ACE(ii) + Nafion	0.44	-0.07	520

TABLE 2.1. Oxidation and reduction potentials for various modified electrodes. (a) to (d) as Table 2.1. with (e) (i) 0.382 Units COx and 0.602 Units ACE/electrode and (e) (ii) 0.382 Units COx and 0.475 Units ACE/electrode.

Analyte	Apparent K_m	I_{max}
(A)		
Choline 0.30 U	83.60 mM	5.00 µA
0.24 U	51.96 mM	2.50 µA
(B)		
Arsenocholine	1.35 mM	0.87 µA
Choline	67.05 mM	1.20 µA
(C)		
Acetylcholine	1.37 mM	1.14 µA
Choline	11.63 mM	1.33 µA

TABLE 2.2. Comparison of apparent Michaelis-Menten constants and I_{max} values for different analytes.
(A) Different loadings of COx.
(B) Arsenocholine and choline responses for the same electrode.
(C) Acetylcholine and choline responses under the same conditions.

2.3.3. CONCLUSION

Immobilisation of acetylcholine esterase (ACE) and choline oxidase (COx) within a polymeric coating on a glassy carbon electrode resulted in the development of a sensor for the important transmitter acetylcholine and its metabolite choline. Anodic detection at + 0.25 V vs. SCE was facilitated by the addition of the redox-mediating hexacyanoferrate (III) ion. The influence of various experimental parameters has been described. Linearity for acetylcholine and choline responses extended up to $5x10^{-3}$ M and $13x10^{-3}$ M with limits of detection of $4.3x10^{-5}$ M and $5x10^{-5}$ M respectively. The simple immobilisation procedure and the rapid reaction kinetics between the mediator and the enzyme, coupled to a fast heterogeneous rate constant, resulted in rapid response times and good precision. An important application of the biosensor was shown for the determination of arsenocholine, using the choline oxidase immobilised electrode, resulting in a response four times that obtained for choline, with linearity extending up to 150 ppm and a LOD of 0.01 mM. The high selectivity offered by this sensor was indicated by the lack of interference offered by easily oxidised compounds normally found in serum, because of both the charge exclusion properties of the Nafion polymeric coating and the low operating potential required to re-oxidise the mediator.

These results demonstrate therefore the feasibility of constructing an amperometric biosensor for acetylcholine that allows a very fast response time while operating at low applied potentials, where the oxidation of interferents, e.g. ascorbic acid in neurochemical applications and the reduction of oxygen, are minimised. The polymeric coating Nafion enhances the discriminative properties of the electrode surface, though a response was observed for ascorbate even at this low potential. The polymer layer alone therefore, was not sufficient to reject anionic interferences and problems may result in the direct analysis of biological fluids using this sensor. The high sensitivity of the bienzyme sensor makes it useful for future measurements in biological media with stability extending up to 3 weeks. An *in vivo* acetylcholine sensor would be a powerful tool for elucidating the site of acetylcholine action in the brain. In order to apply this sensor to real life applications, miniaturisation using carbon fiber microelectrodes, will be necessary, together with a lowering of the limit of detection in order to reliably measure actual neurotransmitter release. The sensor system could also be useful in environmental studies related to arsenic accumulation in fish.

2.4. DEVELOPMENT OF A LYSINE-DEHYDROGENASE BASED ELECTRODE FOR BIOSENSING OF L-LYSINE

The analysis of amino acids and their metabolites is of major importance in the biological and biomedical fields. Reliable analytical methods are essential for determinations in complex matrices such as blood and urine. The monitoring of amino acids in protein hydrolysates is another area of considerable interest. Electroanalytical techniques have been applied to investigations into amino acid levels in biological fluids; in this particular area, LCEC methods are showing great promise. In the area of biomedical analysis, LCEC has been used to measure simultaneously circulating amino acids and their metabolites; such determinations are of obvious importance in studies of metabolic disorders [47].

L-lysine is an essential amino acid of great biochemical, biotechnological and nutritional values. It is the first limiting amino acid in cereals and finds wide application as a supplement in animal feed and human dietary nutrition. Therefore, its level in foodstuffs or body fluids may indicate the nutritional quality or certain diseases respectively. Electrochemical biosensors for the determination of L-lysine, based on immobilised lysine decarboxylase [48], or lysine oxidase [49], coupled with the monitoring of the liberated carbon dioxide or consumed oxygen, respectively, have been reported. General chemical methods for amino acid determination have also been attempted e.g. direct detection with ninhydrin, but these methods have limited selectivity and fail because the samples are usually contaminated by other amino acids and ammonia. Colorimetric methods have been employed using amino acid decarboxylase [50], lysine aminotransferase [51], lysine oxidase [52] and yeast saccharophine [53]. These methods however, prove to be complicated and require long incubation times for colour development.

An optical biosensor for lysine has been developed based on the use of lysine decarboxylase and a cadaverine-sensitive membrane [54]. A plasticised PVC membrane containing a lipophilic tartrate as the amine carrier served as the optical cadaverine sensor. The transport of the cadaverine cation into the membrane is coupled to the transport of a proton (of an indicator dye) out of the membrane. The spectral change may be related to the cadavarine concentration. The whole sensor is highly specific for lysine, with nicotine being the only major interferent. This scheme is unlike others involving the measurement of oxygen consumption (in the case of amino acid oxidases) or carbon dioxide production (using decarboxylases), where the background levels of the respective gases have to be kept constant in order to specifically measure the concentration changes

caused by the enzymatic reaction.

The work reported in this section involves the development of a novel electrode for L-lysine based on the immobilisation of the recently isolated enzyme L-lysine dehydrogenase (EC 1.4.1.; LysDH) [55] from *Agrobacterium tumefaciens*. This enzyme catalyses the oxidative deamination of the amino group of L-lysine in the presence of NAD^+ to give L-α-aminoadipic-δ-semialdehyde, which is spontaneously converted into Δ^1piperideine-6-carboxylate. Biosensing applications of this promising enzyme have not yet been reported.

Immobilisation within gelatine was employed in this case. This immobilisation procedure has currently been receiving increased attention because of its potential use as a convenient matrix in chemical sensors. Also the outer dialysis membrane may resist the fouling phenomenon due to adsorption of species from solution. Therefore these permselective membrane films are very important for exclusion purposes. The preparation, characterisation and advantages of the amperometric LysDH electrode are described in the following sections.

2.4.1. EXPERIMENTAL

2.4.1.1. *Apparatus*

Batch and flow amperometric measurements were performed using an EG&G Princeton Applied Research Model 264A analyser and a 4500 - Microscribe strip chart recorder. Batch experiments utilised a 10 ml cell (Model VC-2 BAS). The platinum working electrode (1 mM diameter disc, BAS), Ag/AgCl reference electrode (Model RE-1, BAS) and platinum wire auxiliary electrode were joined to the cell by holes in its Teflon cover. The flow injection system consisted of the carrier reservoir, a plastic Model 7010 injection valve (20 µl sample loop), and interconnecting Teflon tubing. The flow detector (Prufgerate - Werk Medingen, Germany) was equipped with a platinum working electrode and reference electrode. Flow of the carrier solution was maintained by gravity.

2.4.1.2. *Reagents*

All solutions were prepared with doubly-distilled water. Electrochemical measurements were conducted at room temperature in a 0.05 M phosphate buffer solution (pH 9.5). Lysine dehydrogenase (50 U/ml) was received as a gift from

Kochi University, Nankoku-Shi, Japan. Potassium ferricyanide, D-fructose and 2-propanol were obtained from Fisher Scientific, whereas methanol, 1-propanol, n-butanol and gelatine were purchased from Baker. Sigma Chemical Co. provided the L-lysine, nicotinamide adenine dinucleotide, L-lactic acid, L-glutamic acid, galactose, glucose, sucrose, L-ornithine, L-proline, glycine, L-alanine, L-tryptophan and L-leucine. Allyl alcohol was obtained from Eastman Organic Chemicals while the dialysis membrane (12-14 k MW cut-off) was purchased from Spectrum Medical Industries (Los Angeles, CA).

2.4.1.3. *Electrode Preparation*

The platinum electrode was first polished with 0.05 μm alumina slurry after which it was sonicated for five minutes in deionised water. Immobilisation of the enzyme proceeded by mixing 20 μl of the 5 % gelatine solution (prepared by heating at 35°C for one hour [54]) with 40 μl enzyme. Five aliquots of this solution were spread on an even cellulose dialysis membrane to cover an area of 0.5 cm^2. The membrane was held at 4°C overnight prior to placement onto the surface of the platinum electrode to give 0.83 U/electrode (0.3 cm^2) and were held tightly in place using a rubber cap.

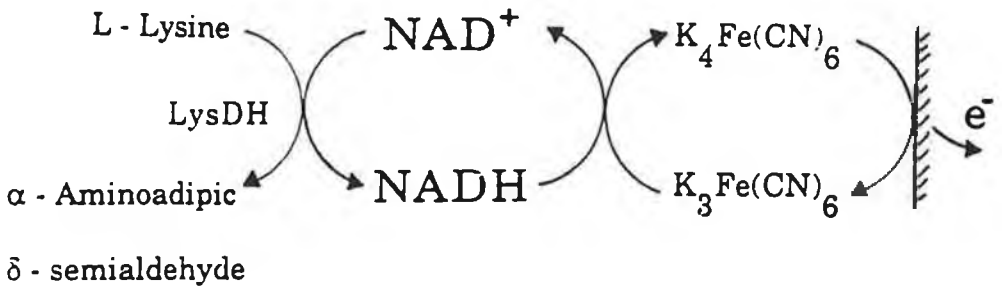
2.4.1.4. *Procedure*

For batch experiments the electrolyte solution containing 1 mM potassium ferricyanide and 0.5 mM NAD^+ was stirred at 400 rpm. The solution was spiked with the required amount of stock lysine solution and the current-time response recorded. In flow injection analysis the sample and carrier phosphate buffer solutions contained the same concentrations of both mediator and cofactor. A sample volume of 20 μl and a flow rate of 1 ml/min were used throughout. Both batch and flow experiments were performed by applying a potential of 0.4 V and allowing transient currents to decay prior to injection. The stability of the electrode was ascertained by monitoring the current response at twenty-four hourly intervals over a period of 25 days. When not in use the electrode was stored at 4°C in phosphate buffer (pH 7.4) in the presence of 1 mM lysine (as required for activation of the enzyme).

2.4.2. RESULTS AND DISCUSSION

2.4.2.1. *Batch Amperometric Experiments*

In the presence of NAD^+, LysDH catalyses the conversion of L-lysine to L-α-aminoadipic Δ-semialdehyde. The electrochemical biosensing was thus based on the amperometric determination of the liberated NADH. Such detection was facilitated by the presence of the redox mediating ferricyanide ion (Scheme 2.6.):



(2.6)

Figure 2.16. shows the response of the LysDH electrode to successive additions of L-lysine; each addition effecting a 5 μM increase in concentration. The enzyme electrode responded rapidly to the change in the substrate concentration, producing a steady-state response within 30 s. High sensitivity was indicated from the well-defined response for low (micromolar) concentrations of the substrate. The signal-to-noise (S/N) characteristics indicated a detection limit of 7×10^{-8}M. The data of Figure 2.16. yielded a linear calibration plot, with slope of 1.1 nA/μM (correlation coefficient, 0.999). Linearity prevailed up to 7×10^{-4} M, as was indicated from another standard addition experiment involving successive 1×10^{-4}M concentration increments. As expected, no response was observed for analogous measurements in the absence of biocatalytic activity within the gelatine membrane (Fig. 2.17.A).

Several hereditary disorders of lysine degradation, e.g. familial hyperlysinaemia result in elevated concentrations of L-lysine in the plasma at 6.6

x 10^{-4}M, compared to a normal value of 2.2 x 10^{-4}M, both of which fall within the linear portion of the calibration curve.

Activation of the enzyme has been reported to result from the association of its two dimeric subunits to form a tetramer [55], when incubated in the presence of the substrate at 35°C for 10 min. The activated enzyme resulted in higher binding affinity for the substrate and the value of I_{max} calculated was found to double, with hyperbolic as opposed to sigmoidal saturation curves being obtained for the activated enzyme electrode response. Figure 2.17. exhibits this effect showing a slight curve at the beginning of the calibration curve.

Numerous experimental parameters affecting the amperometric response were evaluated in order to optimise the analytical performance. Figure 2.18. shows the effect of the enzyme surface loading. The sensitivity increased linearly with the surface loading up to 800 mU per electrode; a levelling-off was observed for higher enzyme activity. The effects of the operational potential and solution pH are shown in Figure 2.19. (A and B respectively). As anticipated for the detection of the liberated ferrocyanide ion, a rapid increase of the current was observed upon increasing the potential between 0.1 and 0.4 V. A levelling off was observed between 0.4 and 0.8 V, with some decrease at a higher value. All subsequent work was performed at a potential of 0.4 V. There was no direct oxidation of NADH observed at 0.4 V, whereas this does occur at higher potentials. The current increased with the pH over the 7.0 - 9.6 range, with a rapid decrease at higher values. LysDH is known to possess its maximum reactivity for the oxidative deamination of L-lysine at pH 9.7 [55].

The concentrations of the NAD^+ cofactor and ferricyanide mediator have a profound effect on the response of the LysDH electrode (Fig 2.20.). As expected from the reaction stoichiometry (Scheme 2.6., NAD^+ reduction requiring two electrons and that of the mediator only one), the response for 1 x 10^{-4}M L-lysine increased rapidly with the concentration of these species up to 4 x 10^{-4}M, after which it starts to level off. No response was observed in the absence of either mediator or cofactor at 0.4 V vs. Ag/AgCl (not shown). Thus in the subsequent experiments 0.5 mM NAD^+ and 1 mM $K_3[Fe(CN)_6]$ were employed in order to provide a sufficient concentration of each so that no limitation of the analytical response may occur.

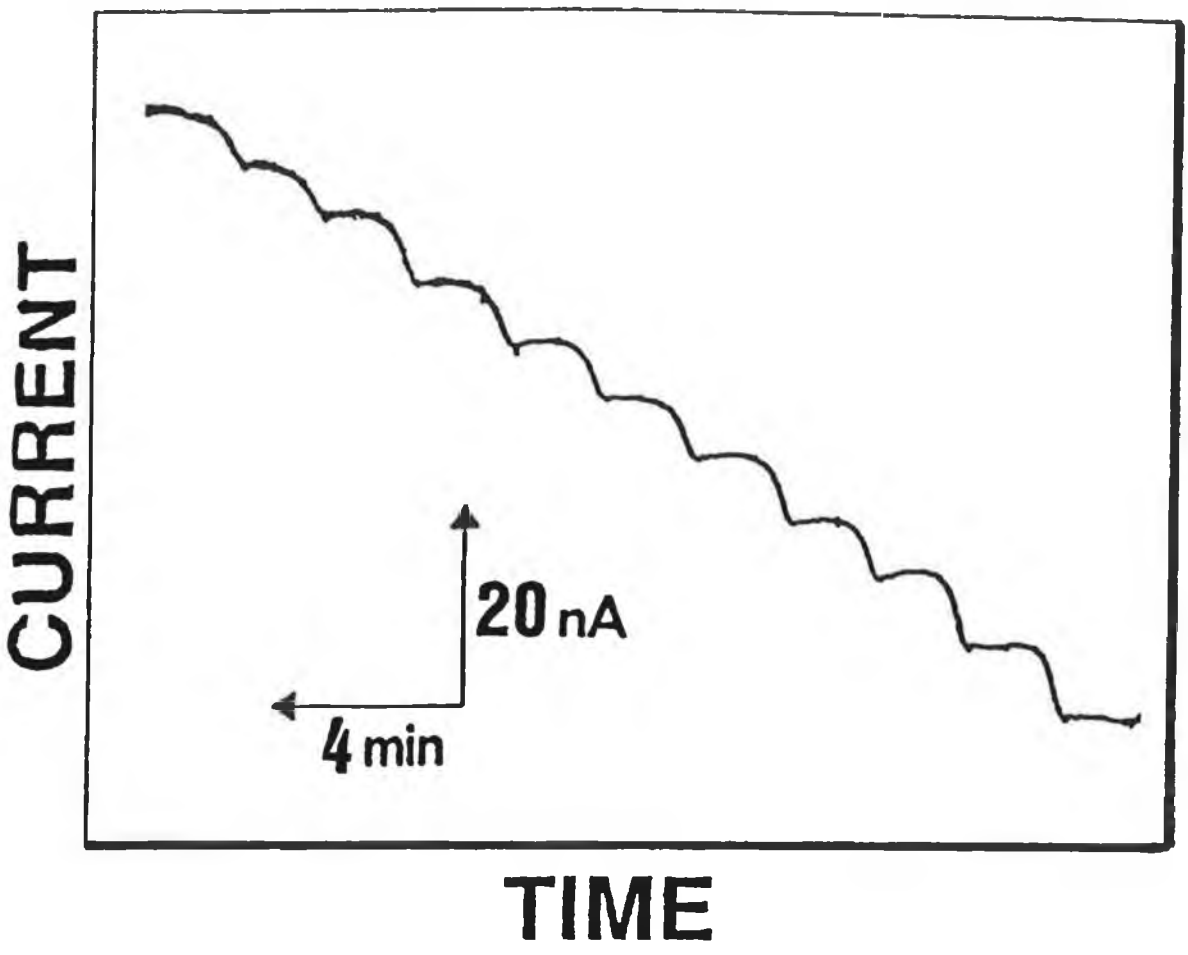


FIGURE 2.16.

Current-time recording for successive injections of 5 x 10^{-6}M lysine. Batch experiment, stirring at 400 rpm, 0.4 V operating potential and 0.83 Units enzyme/electrode employed. Phosphate buffer (0.05 M) pH 9.5 provided the electrolyte solution 1 mM NAD^+ and 2 mM ferricyanide.

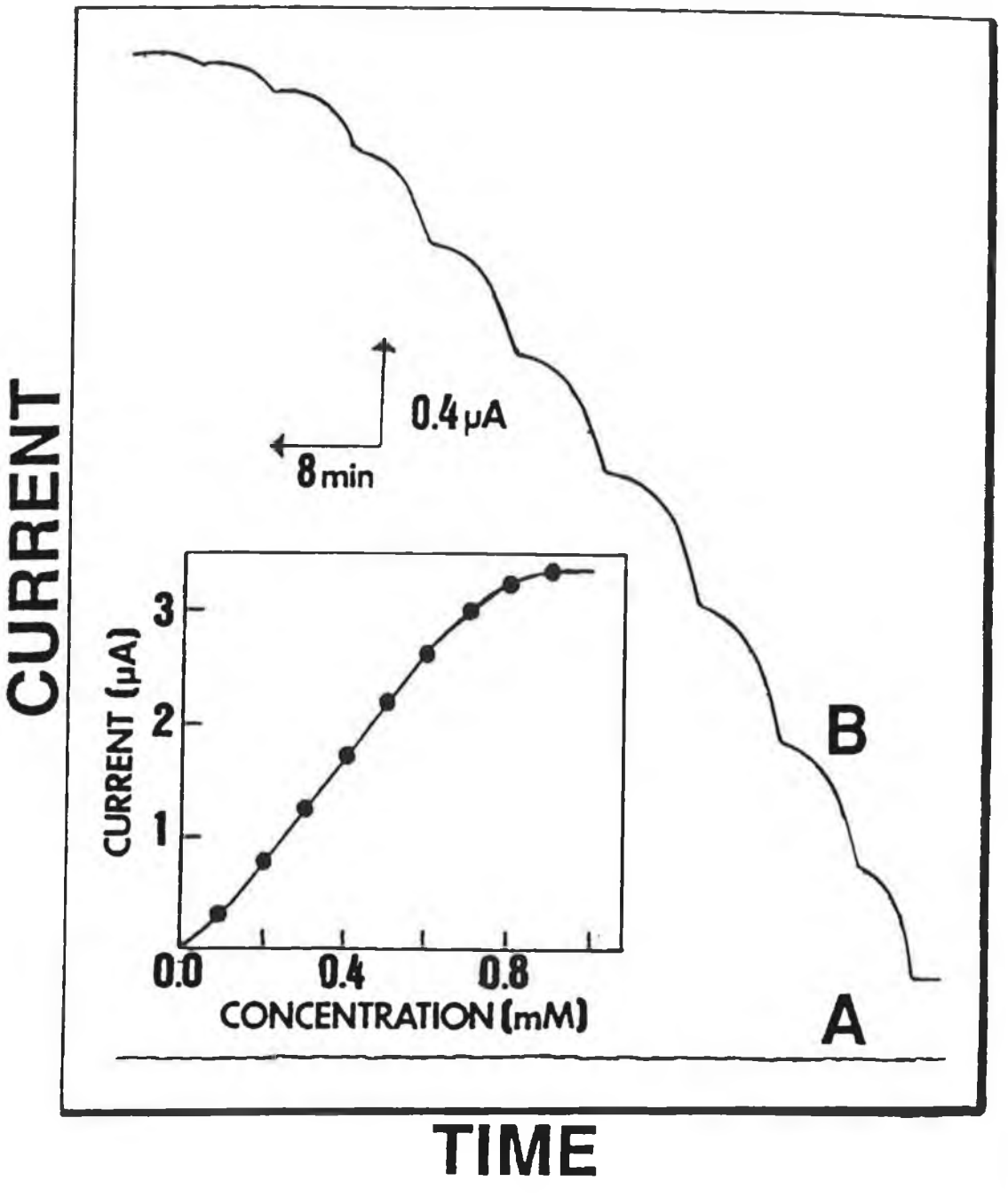


FIGURE 2.17.
Current-time recording at (A) unmodified and (B) enzyme membrane electrode surface for successive additions of 0.1 mM L-lysine. Other conditions as in Figure 2.16.

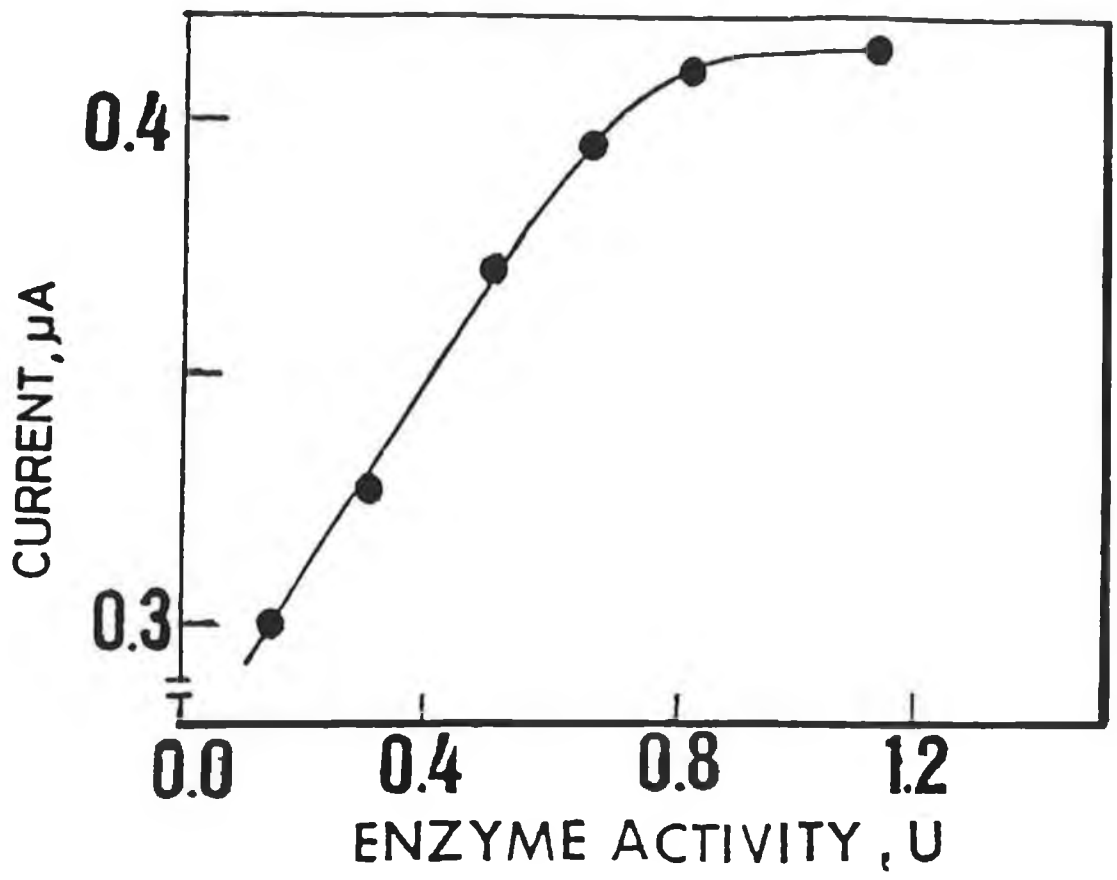


FIGURE 2.18.
Dependence of the response on enzyme loading. Injection of 0.1 mM lysine using 0.125 Units/electrode. Other conditions as in Figure 2.16.

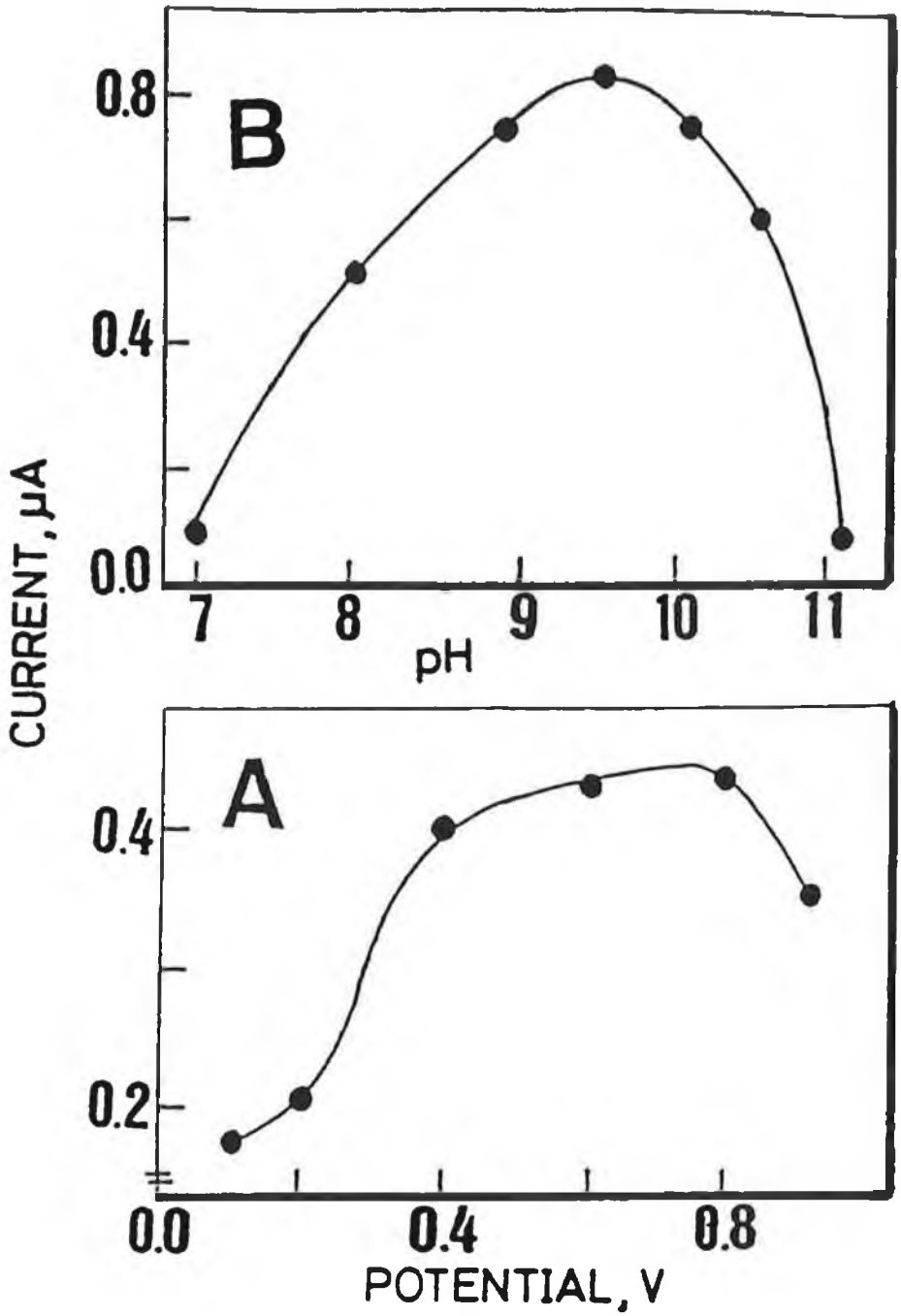


FIGURE 2.19.
Effect of (A) applied potential and (B) solution pH on the response to injection of 0.25 mM and 0.1 mM lysine using 0.125 and 0.83 Units/electrode respectively. Other conditions as in Figure 2.16.

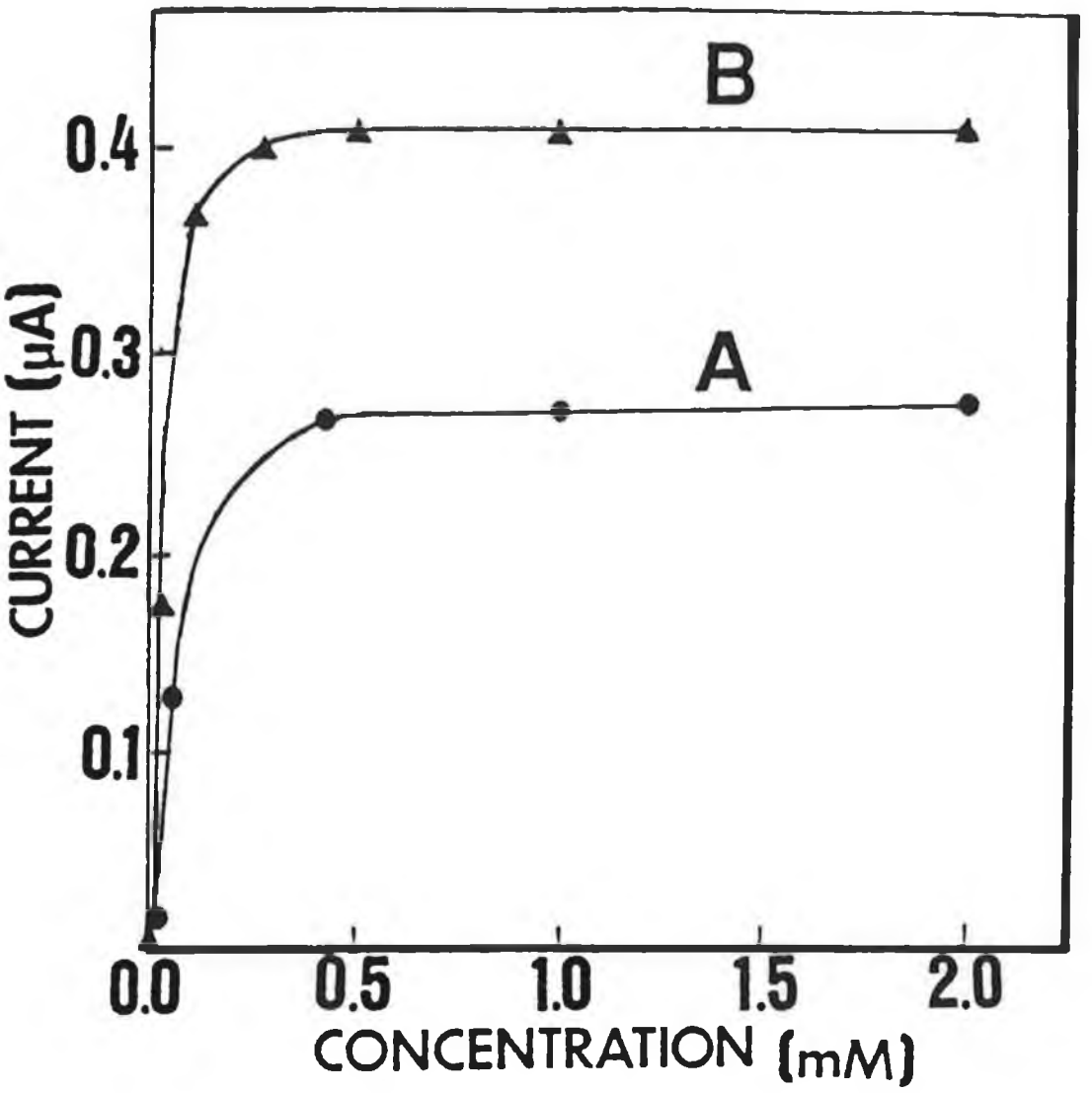


FIGURE 2.20.
Effect of NAD^+ concentration (B) on current response with ferricyanide and the concentration of the mediator (A) in the presence of 0.5 mM NAD^+. Injection of 0.1 mM L-lysine at 0.4 V with 0.125 Units enzyme activity per electrode.

2.4.2.2. *Selectivity Experiments and Flow Injection Analysis*

Perhaps the main advantage of the LysDH-based biosensors is their inherent selectivity towards L-lysine. LysDH is known for its nearly exclusive deamination of L-lysine with no action even on D-lysine [55]. Only L-cysteine is deaminated very slightly (2.9 % relative to L-lysine). Other enzymes employed for biosensing of L-lysine suffer from some interferences, particularly from co-existing amino acids, e.g. 7 % relative activity of L-glutamine, L-histidine and L-asparagine in the case of L-lysine oxidase [56]. The effects of various compounds relevant to the quantitation of L-lysine in fermentation broths, mammalian cell culture preparations or biological fluids was examined. The amperometric response for 0.1 mM L-lysine was not affected by the presence of 0.25 mM L-alanine, L-proline, L-tryptophan, L-lactate, L-glutamate, ornithine, glucose, fructose, sucrose, galactose, methanol. Mixtures of these amino acids, carbohydrates or alcohols also did not affect the response, for L-lysine (Fig. 2.21.). The electrode was found to have an analytically useful range up to 25 days, when stored in 1 mM L-lysine in phosphate buffer (pH 7.4) at 4°C. 20 and 40 % decreases in the activity were reported after 2 and 12 days respectively.

There is a growing demand for a fast, on-line method for monitoring L-lysine during process development, production and recovery operations [57]. The rapid response of the LysDH electrode makes it attractive for such assays of flowing streams. For example, Fig. 2.22. illustrates flow injection peaks for 20 µl samples of increasing L-lysine concentration. A sensitive and rapid response to these dynamic changes in the concentration was observed. The peak width (80 s) allowed an injection rate of 40 samples/hour. Linearity up to 7 mM and a detection limit of 8×10^{-5}M can also be estimated from Figure 2.22. A series of twenty repetitive injections of the 1 mM L-lysine solution resulted in a highly reproducible response (RSD = 1.9 %).

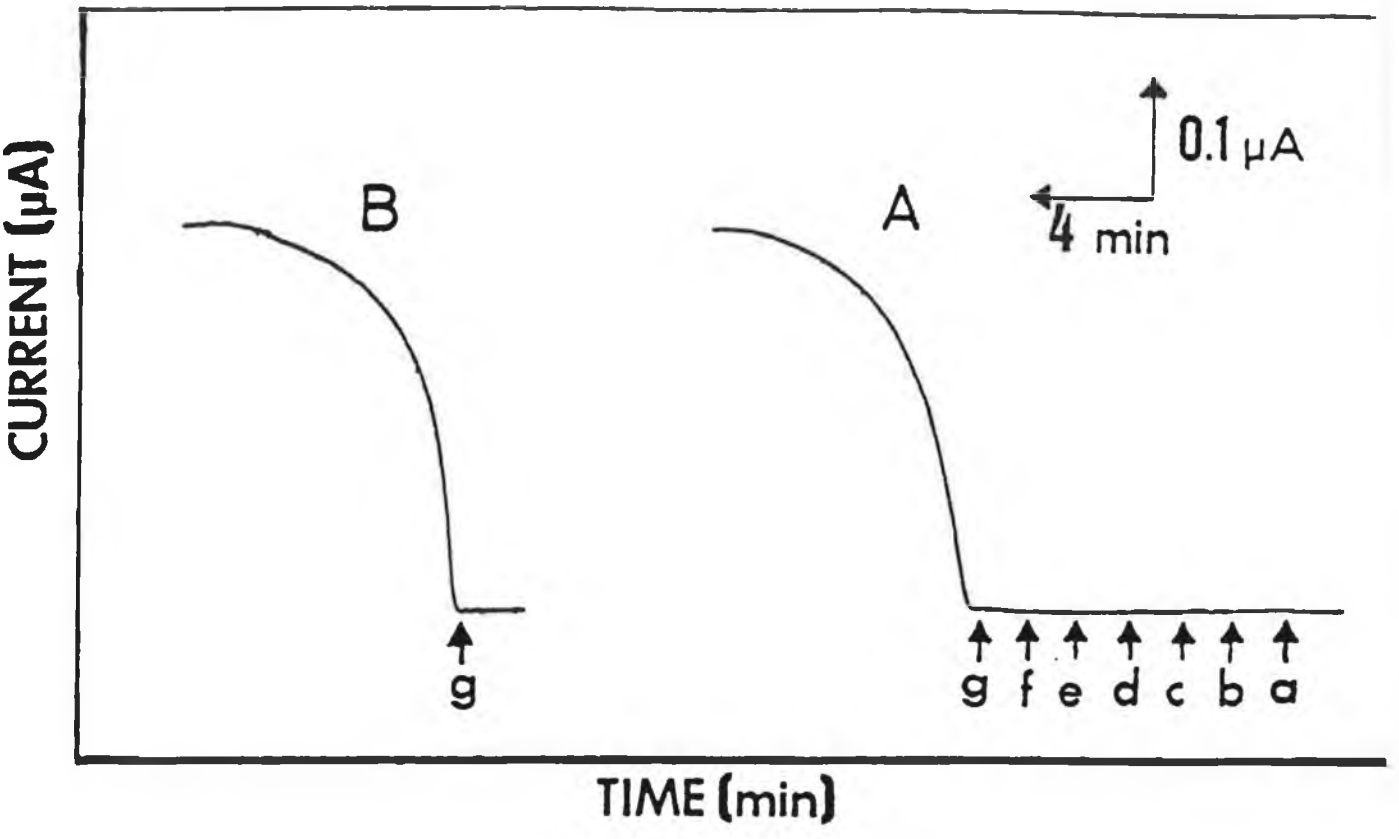


FIGURE 2.21.

(A) shows the electrode response to 0.1 mM L-lysine (g) in the presence of L-ornithine (a), L-proline (b) , glycine (c), L-alanine (d), L-tryptophan (e), L-leucine (f). (B) 0.1 mM L-lysine response without presence of interferences.

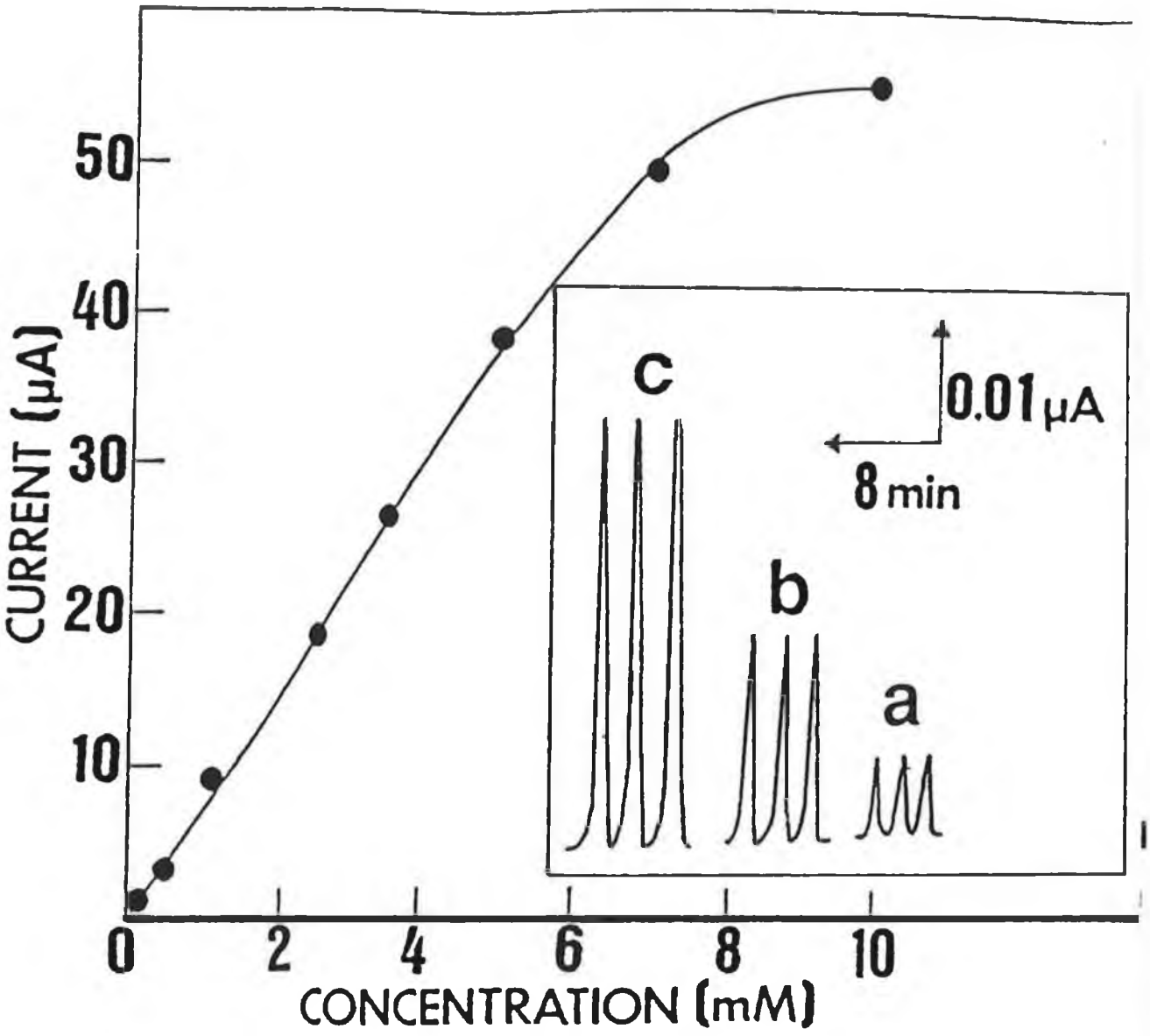


FIGURE 2.22.

Calibration curve for lysine at the lysine dehyrogenase containing gelatine membrane using FIA. Flow rate = 1ml/min, applied potential 0.4 V. Carrier and sample solution contained 0.5 mM NAD^+, 1 mM ferricyanide, in 0.05 M phosphate buffer (pH 9.5). Inset represents injections of (a) 1, (b) 2.5 and (c) 5 mM lysine.

2.4.3. CONCLUSION

An amperometric biosensor for L-lysine based on the recently isolated enzyme lysine dehydrogenase has been described. Immobilisation of the enzyme onto a platinum electrode was achieved via entrapment within a gelatine support on a cellulose membrane. Anodic detection (at 0.4 V vs. Ag/AgCl) was facilitated by the presence of a redox-mediating ferricyanide ion. The effect of experimental variables such as pH, enzyme loading, applied potential, cofactor and mediator concentrations were evaluated in order to optimise analytical performance. A detection limit of $7x10^{-8}M$, and linearity up to $7x10^{-4}M$ were reported. The fast response permitted adaptation for flow injection operation with good precision (RSD = 1.9%) with high sample throughput (40 samples/hour). The high specificity offered by this new enzyme is indicated by the lack of interference by other L-amino acids, alcohols or carbohydrates.

In conclusion, the feasibility and applicability of using lysine dehydrogenase for amperometric sensing of lysine based on its incorporation into a gelatine matrix on a dialysis membrane was assessed. The protein nature of gelatine, its hydrophilicity and strong swelling power make it an attractive matrix for immobilisation of enzymes and decreases the mass transfer resistance to the diffusion of substrate and reaction product [56]. The high selectivity and sensitivity of the amperometric LysDH probe makes it very attractive for routine biotechnological, industrial or clinical applications. Further improvements in the analytical performance (particularly the stability and speed) can be achieved by utilising other immobilisation schemes (e.g. within carbon pastes) or redox mediators (e.g. phenoxazines). Co-immobilisation of the NAD^+ cofactor would result in a reagentless device. In addition to electrochemical measurements, this enzyme may be coupled to other transducing devices (e.g. fiber optics).

2.5. THE DEVELOPMENT OF A LACTATE SENSOR BASED ON IMMOBILISED LACTATE OXIDASE IN AN ELECTROPOLYMERISED O-PHENYLENEDIAMINE FILM

Polymeric coatings have recently become of increasing interest in the modification of electrode surfaces [58]. By appropriate choice of monomer and experimental conditions for film preparation, polymer films with particular desired properties may be achieved. Heineman *et al.* [59] have reported that o-phenylenediamine forms polymeric films upon electrochemical oxidation in solutions of neutral/slightly acidic pH, and that the electrode exhibits a nearly Nernstian response with changes in pH [60, 61]. The selective permeability can be varied with the nature of the ions dissolved in solution [62].

Polymer coated electrodes have several attractive features, such as being a convenient way of immobilising an electrocatalyst near the electrode surface in high concentration. The electrochemical method of immobilisation permits a controlled method of localisation of biologically active molecules at electrode surfaces. Therefore by this approach the spatial distribution of the enzyme may be readily controlled and thickness of the enzyme film easily varied.

The usefulness of sensors based on immobilised enzymes depends on several factors such as the immobilisation method, thickness and stability of the entrapped enzyme, the response time and storage conditions required. Despite uncertainties as to the mechanism of entrapment, electrochemical deposition offers advantages over the more traditional methods of enzyme immobilisation, particularly in the simplicity and reproducibility of the one-step construction process. Conventional enzyme electrodes employing discrete macroscopic membranes (to overcome problems associated with interferences and electrode fouling) suffer in lack of reproducibility and response times [63, 64].

It has recently been demonstrated that immobilisation of glucose oxidase with an electrochemically generated o-phenylenediamine film can be used for the amperometric detection of glucose [65]. A fast and permselective response was thus reported. The objective of the present work was to explore the o-phenylenediamine electropolymerisation scheme for effective immobilisation of lactate oxidase.

L-lactate is one of the most important metabolites for which a reliable analytical method is required in blood, where its function is related to muscle diseases, critical care, lymphomas, sarcomas etc., in food analysis e.g. wines and dairy products [66], and biotechnological process control. The determination of

L-lactate is also important in the diagnosis of respiratory insufficiencies, heart disease [67, 68] and in sport medicine.

Previous methods for determination of L-lactate have involved enzyme reactors [69], potentiometric methods [70], co-immobilisation of lactate oxidase and its dehydrogenase in enzymatic amplification schemes [64, 71], entrapment of the enzyme behind a semi-permeable membrane [63]. The immobilisation of L-lactate oxidase in poly(vinyl alcohol) using gamma-irradiation, has also been reported [72]. Some of these methods among others will be discussed further.

An amperometric flow injection system, with a lactate oxidase, lactate dehydrogenase co-immobilised reactor involving amplification by substrate recycling has been used as a specific postcolumn detector system in liquid chromatography, for determination of lactate and pyruvate [73]. Both components were separated on a reversed phase column and recycled enzymatically during the passage through the enzyme reactor in the presence of NADH in the carrier stream. Flow injection analysis of L-lactate with enzymatic amplification and amperometric detection has also been described [74]. The system utilised an oxygen electrode for measurement of changes in the oxygen concentration in the flow system.

A lactate oxidase (LOx) packed enzyme bed system has been reported for the determination of lactate in brain microdialysate [75]. Rapid measurement of this compound together with glucose and glutamate allowed the dynamics of the energy balance of the brain to be studied. The determination of lactate in human saliva with an electrochemical enzyme probe was also described based on immobilised lactate oxidase in a cellulose acetate membrane [76].

Carbon paste electrodes with incorporated lactate oxidase and mediators have also been reported [77]. Several ferrocenes and phenoxazine derivatives were evaluated as mediators, with Meldola Blue exhibiting the best results. Reagentless amperometric L-lactate bioelectrodes based on carbon paste modified with yeast and mediators have also been described [78], with high linear range and long term stability, allowing for L-lactate determination in serum. Another reagentless lactate electrode has been constructed based on the electrocatalytic oxidation of flavocytochrome b_2, which was entrapped on the surfaces of electrodes modified with carbon black [79]. The electrocatalytic oxidation of a reduced enzyme by the electroactive surface groups of carbon black enables the enzyme electrode to be used for the determination of lactate.

Some of these methods however, result in slower response times, interference problems and prove to be more complicated. This research employs a simple and easily controlled enzyme immobilisation procedure that has

application in the development of sensors for this biologically important compound.

2.5.1. EXPERIMENTAL

2.5.1.1. *Apparatus*

Amperometric measurements and electropolymerisation of o-phenylenediamine were carried out in a 3-electrode cell (Model VC-2, BAS), containing 10 ml of 0.05 M phosphate buffer as supporting electrolyte. Reference and counter electrodes were Ag/AgCl (Model RE-1, BAS) and platinum wire, respectively. A platinum disk electrode served as the working electrode and all three joined the cell through holes in its Teflon cover. The three-electrode system was then connected to an EG&G PAR Model 264A polarographic Analyser, the output of which was displayed on a strip chart recorder (Model 4500 Microscribe, The Recorder Co.). A magnetic stirrer and bar provided the convective transport. The flow injection system used was equipped with a carrier reservoir, a platinum thin-layer detector (Model TL-10A, BAS) a Rainin Model 5041 sample injection valve (20 µl loop) and interconnecting tubing. A flow rate of 2.0 ml/min was employed throughout.

2.5.1.2. *Reagents*

All experiments were conducted at room temperature in 0.05 M phosphate buffer pH 7.4, and all solutions were prepared with doubly-distilled water. Lactate oxidase (activity 38 Units/mg), L-lactate, glucose, nicotinamide adenine dinucleotide (reduced form), uric acid and acetaminophen were obtained from Sigma Chemical Co. The ascorbic acid was obtained from Baker and the o-phenylenediamine dihydrochloride from Aldrich. The cellulose dialysis membrane (MW cut-off 12-14k) was purchased from Spectrum Medical Industries (Los Angeles CA). Stock solutions of glucose were allowed to mutarotate at room temperature for 24 hrs before use. The nitrogen used to obtain a controlled atmosphere in the electrochemical cell was of high purity grade.

2.5.1.3. *Electrode Preparation*

Prior to electropolymerisation the platinum disk working electrode was polished firstly with 3 μm alumina followed by 0.05 μm, after which it was sonicated in distilled water for 5 mins to remove residual alumina. Lactate oxidase/o-phenylenediamine films were electrochemically grown on platinum electrodes from a fresh solution of 1 mM o-phenylenediamine and 3.42 Units enzyme in 0.05 M phosphate buffer, (pH 7.4). Electropolymerisation was initiated by applying a constant potential of 0.65 V (vs. Ag/AgCl) in deaerated unstirred solutions for 20 min. The enzyme electrode was thoroughly rinsed with doubly-distilled water after preparation and stored in phosphate buffer pH 7.4 at 4°C when not in use.

2.5.1.4. *Procedure*

Amperometric responses of the lactate oxidase/o-phenylenediamine (LOX/o-PD) electrode to L-lactate were measured in batch and flow experiments by application of a fixed potential of 0.75 V to the enzyme electrodes. The background current was allowed to decay before aliquots of the stock L-lactate solution were added and the oxidation current due to hydrogen peroxide measured.

An estimation of the activity of enzyme entrapped in the polymer film was carried out amperometrically. A clean polished platinum electrode was held at 0.75 V (vs. Ag/AgCl) in stirred solution containing 1.0 mM L-lactate in phosphate buffer, pH 7.4. When a steady state was achieved, a known activity of lactate oxidase was injected and the current response noted for the production of hydrogen peroxide. A calibration curve was constructed and the amount of activity on each LOX/o-PD electrode expressed as equivalent activity in solution. Enzyme stability within the film was ascertained by measuring the current response over a 20 day period. In between measurements the electrode was stored in phosphate buffer pH 7.4 at 4°C.

2.5.2. RESULTS AND DISCUSSION

2.5.2.1. *Batch Experiments*

Lactate oxidase catalyses the reaction shown below and electrochemical biosensing was based on the amperometric monitoring of the hydrogen peroxide produced.

$$\text{L-LACTATE} + O_2 + \text{LOD(FAD)} \Leftrightarrow \text{LOD}(FADH_2) + \text{PYRUVATE} + H_2O_2 \qquad (2.7.)$$

The response of the LOX/oPD electrode to successive additions of L-lactate at the bare and modified surfaces, using both amperometric and chronoamperometric measurements, is shown in Fig. 2.23. Steady state currents were achieved within 15 sec and 2 min respectively, with high sensitivity for μM concentrations of substrate and a detection limit of 2.46×10^{-7}M for both techniques.

The effects of many experimental variables were investigated in order to optimise analytical performance. Optimisation of enzyme loading in the polymer film is shown in Figure 2.24. Linearity and response increased with increasing biocatalytic activity in the film, the optimum activity being 3.42 Units in solution (1.6 mU/electrode). The response to 50 μM additions of L-lactate resulted in a slope of 4.5 nA/μM and a correlation coefficient of 0.9998 when the electropolymerisation solution contained 1 mM o-phenylenediamine and 3.42 units lactate oxidase.

The effect of operational potential (A) and pH (B) for amperometric experiments is shown in Figure 2.25. An increase in oxidation current for hydrogen peroxide, resulting from 0.25 mM injections of L-lactate occurred as the applied potential increased from 0.2 V to 0.8 V with a decrease at higher potentials. An optimum current response to 0.5 mM injections of L-lactate was achieved at pH 7.4 and therefore this pH taken as that providing maximum activity for the operation of this enzyme electrode.

Figure 2.26(A) shows the effect on anodic response with increasing concentration of o-phenylenediamine in the electropolymerisation solution, keeping both enzyme activity and time constant. The greatest current was observed for the film prepared with 1 mM monomer solution. A subsequent decrease in response was observed until 5 mM, followed by an increase once more up to 10 mM.

A thicker film implies a slower response time and a lowering of sensitivity but results in a wider linear range. A thinner film appears to incorporate more enzyme resulting in a greater response. The diffusion of the product hydrogen peroxide through the film may be the limiting factor as the thickness is increased. In a thinner film the hydrogen peroxide may be generated and consumed at the same location at the electrode surface, whereas within a thicker film such generation and consumption may be spatially separated. In the latter case, the enzymatic reaction may occur more readily in the outer portions of the polymer layer near the solution side rather than in the interior of the film.

The effect of potential of electropolymerisation on current response is shown in Fig. 2.26(B). An optimum value was found to be 0.65 V, but as the anodic potential was increased above this value, the amperometric response decreased. A decrease in conductivity in the film and a loss of activity occured at high potentials. Destruction of the polymer may be attributed to irreversible deterioration of the film by formation of electroinactive polymer films on the surface. Fig. 2.26(C) shows the effect of polymerisation time on performance of the sensor. The form of the current vs. time curve implicates a nucleation and phase growth process in the mechanism of deposition of o-phenylenediamine, similar to that of the growth of m-toluidine on gold [80]. The monomer appears to oxidise more readily on the poly(o-phenylenediamine) film than on the platinum itself. Initial growth requires 5-10 min followed by a rapid increase in current with increasing thickness of polymer film. Saturation of enzymatic activities above 20 min may result in the conformational arrangements of the enzyme within the film. Due to release of protons in the course of polymerisation, the acidic environment produced may lead to denaturation of lactate oxidase at longer electropolymerisation times.

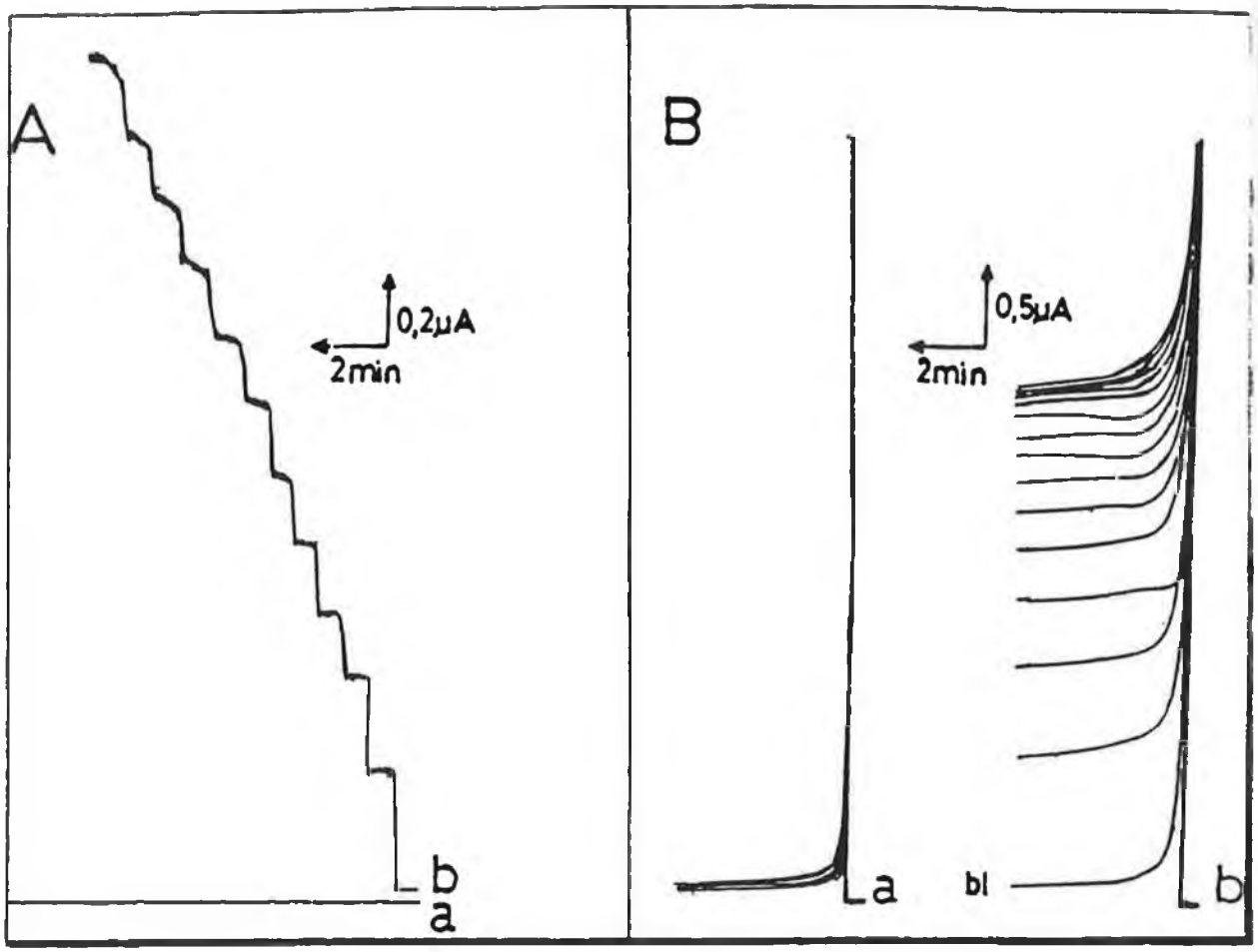


FIGURE 2.23.

(A) Current-time response for successive $5x10^{-5}M$ injections of L-lactate obtained at (a) unmodified and (b) modified enzyme surfaces in batch. Operating potential 0.75 V with 400 rpm stirring rate and electropolymerisation solution containing 1 mM o-phenylenediamine and 3.42 Units enzyme (5 mU/cm^2 electrode) for 20 mins at 0.65 V. Electrolyte, 0.05 M phosphate buffer pH 7.5. (B) Chronamperometric response to $1.25x10^{-4}$ M L-lactate increments again at (a) unmodified and (b) modified surfaces. Other conditions as (A).

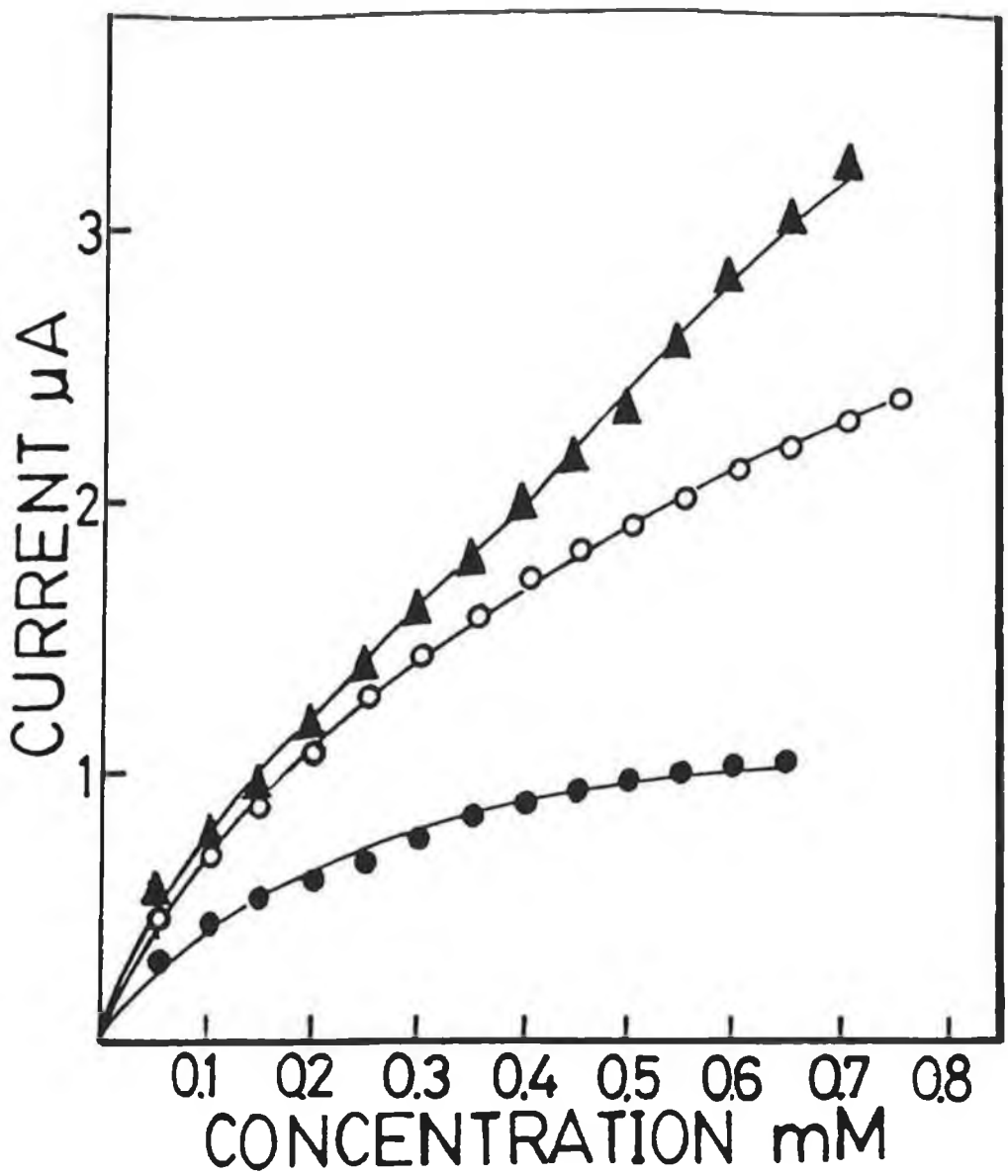


FIGURE 2.24.

Current dependence on L-lactate oxidase loading in the electropolymerised film upon injection of $5x10^{-5}$M L-lactate. Polymerisation carried out in 1 mM o-phenylenediamine and (▲) 3.42 Units, (o) 2.28 Units and (•) 1.14 Units enzyme activity. Other conditions as in Fig. 2.23(A).

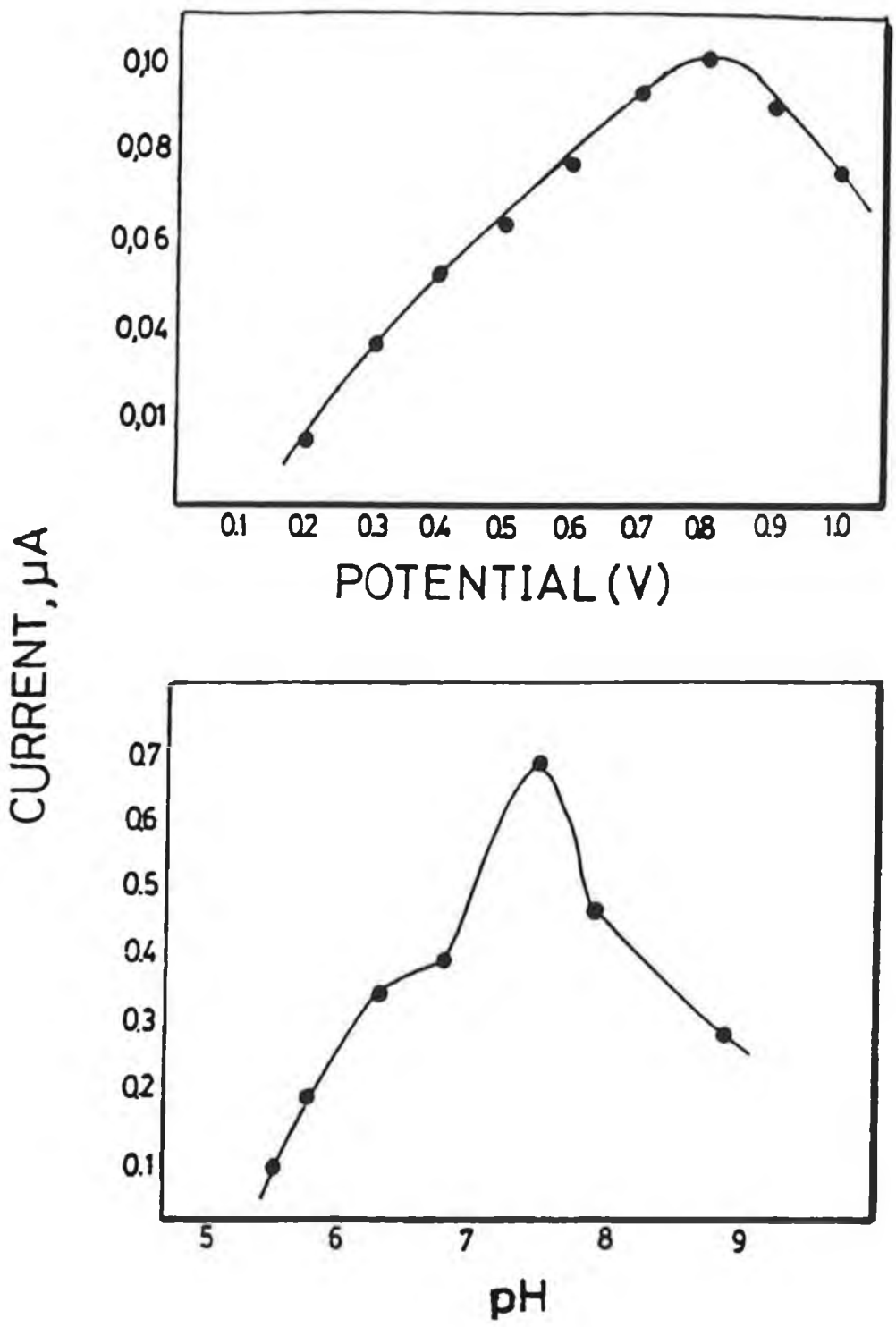


FIGURE 2.25.

Effect of applied potential (A) and solution pH (B) on response to 2.5×10^{-4}M and 5×10^{-4}M injections of L-lactate respectively. Electropolymerisation solution containing 10 mM (A) and 5 mM (B) o-phenylenediamine with 3.04 Units enzyme activity. Enzyme loading - 5 mUnits/cm^2. Other conditions as in Fig. 2.23(A).

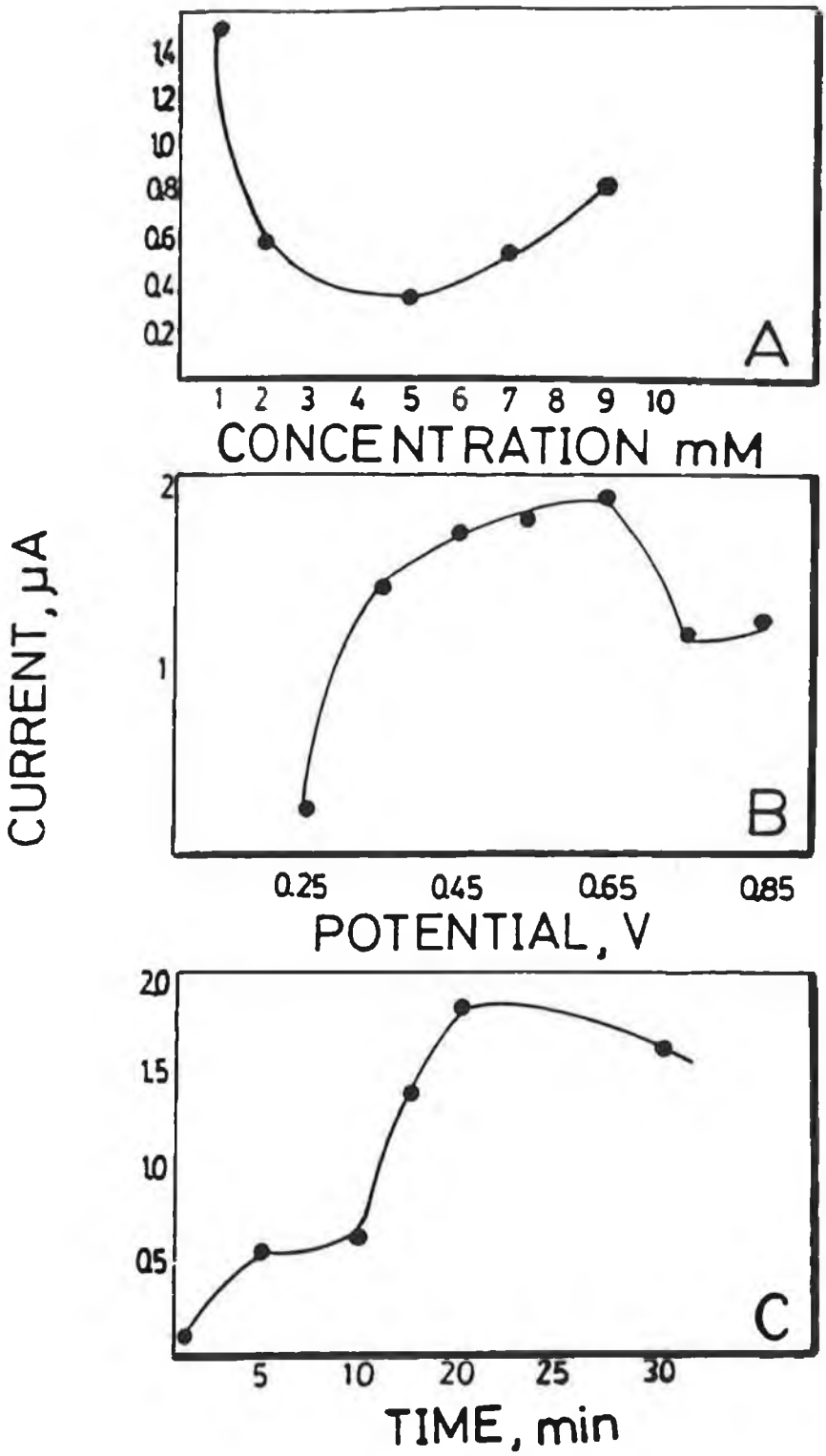


FIGURE 2.26.

(A) Effects of o-phenylenediamine concentration on response to $5x10^{-4}M$ injections of L-lactate. Enzyme activity constant at 5 mU/cm^2 electrode. (B) Dependence of current on electropolymerisation potential in 1 mM o-phenylenediamine and 3 Units enzyme for 20 mins. Injection of $5x10^{-4}M$ L-lactate. (C) Current - electropolymerisation time response to $5x10^{-4}M$ L-lactate. Electropolymerisation solution as in (B) and amperometric detection at 0.75 V.

2.5.2.2. *Flow Injection Analysis*

The fast response and stability of the LOX/o-PD electrode makes it an attractive choice in dynamic flow systems. Fig. 2.27. illustrates flow injection peaks for 20 µl samples of increasing L-lactate concentration. The peak width of 6 seconds allowed a sample throughput of 180 samples/hour, with linearity observed up to 1.5 mM and a limit of detection of $1x10^{-4}$M. 25 successive injections of a 5 mM L-lactate solution allowed a precision of 3.54 % to be obtained (Fig. 2.28.). Under optimum conditions, a precision study employing 10 consecutive separate batch additions of 50 µM L-lactate resulted in a RSD of 3.23 % (not shown).

The potential required to oxidise H_2O_2 was sufficiently anodic that several other interfering compounds may contribute to the signal. However, due to the permselectivity of the electropolymerised o-PD film, access of substances such as ascorbate to the electrode surface were blocked. Similar improvements were reported for analogous sensing of glucose [65].

The selectivity of the LOx/o-PD electrode was studied by monitoring the response to 0.5 mM L-lactate alone and in the presence of relevant compounds at the 1.0 mM level which may interfere by direct electrode oxidation (Table 2.3.). In the case of polypyrrole films [81] anionic species showed enhanced electrochemistry due to the electrostatic interaction with the polymer matrix which contained cationic fixed sites. A similar situation appears here, but the relatively high response to uric acid may be further decreased by an additional cellulose membrane (12-14k MW cut-off) over the electrode. The polymer/membrane electrode proved effective in further reducing the current signal due to uric acid, ascorbic acid, NADH, and glucose (all at the 1.0 mM level) while retaining 90% ((B) with cellulose membrane) and 64.3% ((A) without cellulose membrane), of the L-lactate response (0.5 mM), see Table 2.3.

The oxidation of o-phenylenediamine in phosphate buffer showed a completely irreversible oxidation peak at 0.35 V using cyclic voltammetry, which with continuous scanning between 0.2 V and 0.7 V resulted in a decrease in peak current until eventually no current flowed. This behaviour was indicative of a non-conductive polymeric film coating the electrode until eventually access of the monomer to the surface was blocked resulting in the formation of a very compact and insulating layer [82].

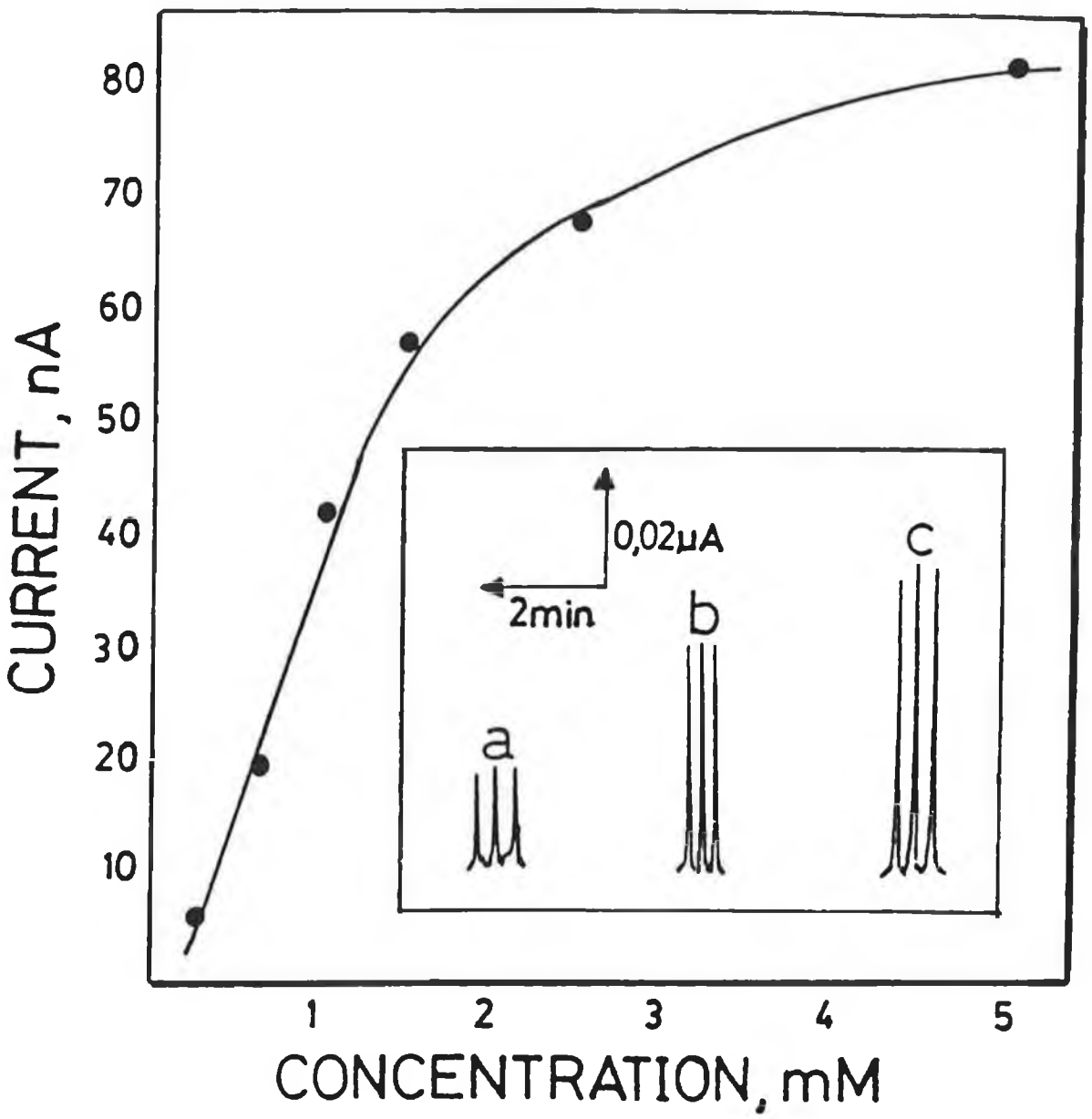


FIGURE 2.27.

Flow injection detection of L-lactate. Calibration plot over the 2.5×10^{-4}M to 5×10^{-3}M range with typical peaks for (a) 0.5 mM (b) 1.0 mM (c) 1.5 mM L-lactate. Flow rate 2 ml/min. Sample and carrier - phosphate buffer pH 7.5. Other conditions as in Fig. 2.23(A).

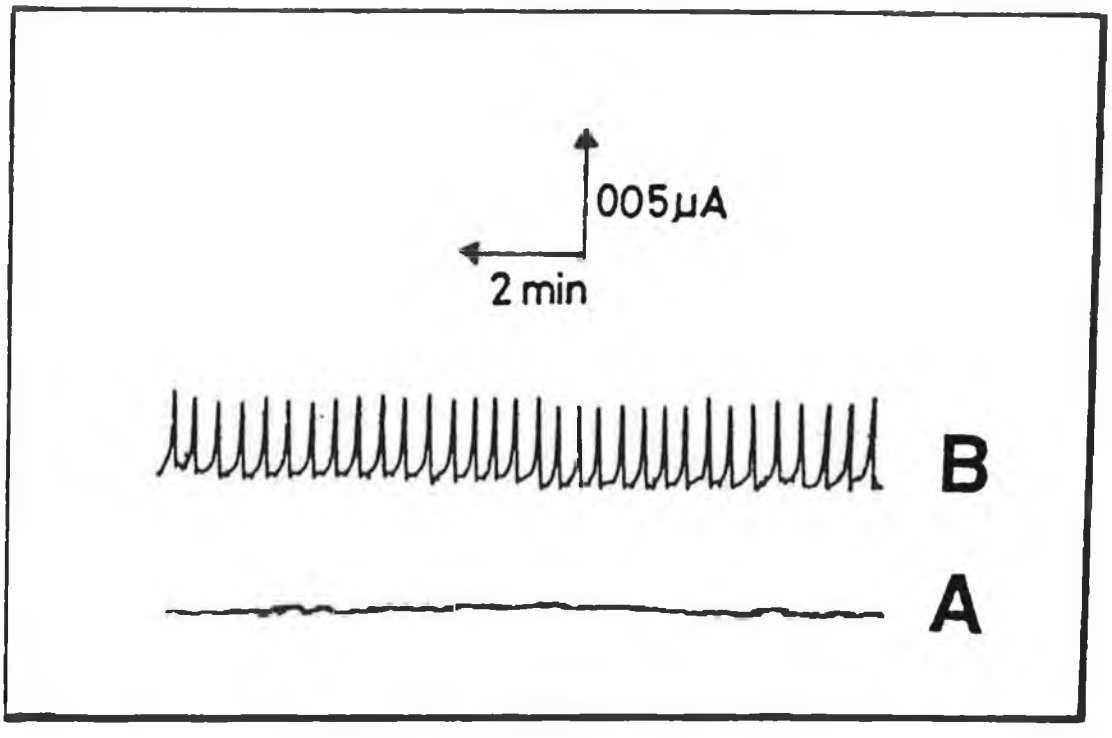


FIGURE 2.28.
Flow injection peaks for 5 mM injections of L-lactate at (A) enzyme containing surface and (B) polymer only. Sensitivity 500 nA full scale.

INTERFERENCE	A%	B%
Glucose	0	0
NADH	5.5	4.1
Ascorbate	19.0	11.0
Uric acid	94.0	50.0

TABLE 2.3.
Percentage interference response compared to that of analyte (A) without membrane and (B) with cellulose membrane. All interferences at the 1.0 mM level in presence of 0.5 mM L-lactate. Electropolymerisation solution containing 4.54 Units and 10 mM o-phenylenediamine. All other conditions as in Fig. 2.23(A).

The mechanism by which the enzyme is entrapped is uncertain; however adsorption of the lactate oxidase at the platinum surface prior to electropolymerisation could play an important part. Initial film formation is that of a conducting organic film with subsequent formation of a non-conducting film which eventually insulates the electrode [82]. o-Phenylenediamine forms polymeric films during oxidation at almost all pH values. Most organic electrochemical oxidations of this type form a monocation as the initial electrolysis product, which is involved in the follow-up chemical reaction. The insulating film could then possibly be formed by the polymerisation of the dication formed upon disproportionation. Poly(o-phenylenediamine) contains phenazine rings as electroactive sites. The ladder polymer is partially ring opened and involves moieties of the oxidised forms of the quinone-imine type [83].

Under optimum conditions, 5 mUnits/cm^2 electrode surface, which is equivalent to 1.52 Units/ml in the electropolymerisation solution may be incorporated into the film. The estimation of enzyme activity on the surface was

carried out by comparison to the rate of H_2O_2 production when the enzyme was in solution in the presence of excess substrate. This is assuming that the solution kinetics are equivalent to those when the enzyme is in the immobilised form, which is not true due to mass transfer limitations resulting from immobilisation. The activity within the film however, may be estimated however from the plot of current vs. enzyme activity in solution.

The upper limit of the electrode responses may be improved by placement of a cellulose membrane onto the LOX/oPD film. This restricts the diffusion of L-lactate to the underlying enzyme layer and as a result extends linearity. The improvement in linear response was accompanied by a significant decrease in the noise level for injections of μM quantities of substrate. In the presence of the cellulose membrane the response has mass transfer as opposed to kinetic limitations, the latter resulting in a levelling off at lower substrate concentrations. This restricts the diffusion of L-lactate to the underlying enzyme layer and as a result extends linearity. The improvement in linear response was accompanied by a significant decrease in the noise level for injections of μM quantities of substrate. A LOX/o-PD polymer electrode prepared from 1.14 Units enzyme activity and 1 mM o-phenylenediamine resulted in an I_{max} of 1.236 μA and apparent K_m of 117.3 μM. The same electrode covered with cellulose membrane showed an I_{max} of 1.3528 μA and K_m of 342.2μM.

The additional diffusional restrictions imposed as a result of the membrane cause alteration of basic catalytic properties and shifts in apparent K_m. The increase in K_m(app) in the case of the membrane indicates that the immobilisation process generates a loss of affinity to the substrate due to the additional environmental constraints placed on the enzyme [84]. However, this method is useful from the analytical point of view due to extension of the upper limit.

An improvement in linearity was also observed using 10 mM monomer in the electropolymerisation solution, and after film formation, polymerisation once more in the same solution. Linearity in this case was extended up to 1.5 mM again at the expense of sensitivity and resulting in a slower response time.

The stability of the LOX/o-PD electrode with storage at 4°C in phosphate buffer (pH 7.5) was also investigated. Responses were measured as the oxidation of resultant H_2O_2 caused by the addition of 0.5 mM L-lactate at 24 hourly intervals. A 50% and 93% decrease in response was observed after 5 and 20 days respectively.

2.5.3. CONCLUSION

The immobilisation of L-lactate oxidase at a platinum electrode was achieved by entrapping the enzyme within an o-phenylenediamine film at 0.65 V (vs. Ag/AgCl). Anodic detection of the product of the enzymatic reaction, i.e. hydrogen peroxide, at 0.75 V (vs. Ag/AgCl) was employed for the quantification of L-lactate using amperometric batch and flow injection methods. This technique allowed the enzyme to be entrapped in a strongly adherent thin membrane. The sensor exhibited a very fast response time, an active enzyme loading of 5 mU/cm^2 electrode surface, and high sensitivity with a detection limit of 2.46×10^{-7} M. A sample throughput of 180/hr with precision of 3.53 % for 25 injections and linearity up to 1.5 mM was obtained in flow injection analysis studies. The one-step procedure for sensor preparation requires 20 min, and the discriminative properties of the polymer film showed great promise as a means of excluding interfering compounds commonly found in serum.

Electrochemically initiated polymerisation has recently received great attention in the modification of electrode surfaces. Polymer film electrodes offer higher inherent chemical and physical stability, higher surface activity and a very sensitive electrochemical response. The electropolymerisation of o-phenylenediamine provides simultaneous immobilisation of lactate oxidase and offers the advantage of producing a very thin and self insulating film that can be coated on any conducting surface. The sensor was found to reject physiologically important compounds e.g. ascorbic acid while incorporating a significant amount of lactate oxidase. The very fast response time, together with the high sensitivity and high sample throughput, are further distinctive features of the described sensor. The stability and dynamic behaviour of the film allows for application in flowing streams.

2.6. CRITICAL ASSESSMENT OF IMMOBILISATION TECHNIQUES FOR AMPEROMETRIC ENZYME SENSORS AND THEIR APPLICATION TO THE CLINICAL FIELD

The construction of modified electrodes with biological enzymes has proven to be a formidable challenge. Electroanalytical problems associated with the development of such electrochemical transducers include the need for increased sensitivity and specificity, a decrease in biological matrix interferences, more rapid response times, improved miniaturisation and more reliable calibration procedures. The main analytical problems may be improved by using chemically modified and genetically engineered enzymes. The direct communication between the biocomponent and the electronics will lead to substantial advancement. In this respect, direct electron transfer from redox enzymes to electrodes, or coupling of neuronal networks will result in optimised signal transfer. The ability of neurons to convert binding events into frequency-coded digital signals offers microprocessor-compatible responses.

Many different strategies and variations of strategies have been used to immobilise and to communicate electrically with enzymes on electrode surfaces. The ultimate enzyme-based sensor would not only immobilise the enzyme at the sensing electrode but would also incorporate an electrocatalyst capable of mediating the enzymes own redox process.

This chapter has dealt with the analysis of some biologically important compounds using enzyme modified amperometric electrodes in aqueous media. The first method of immobilisation described, i.e. coating with Nafion, for the theophylline and choline sensors, forms a stable film for enzyme entrapment and results in sensors with fast response times, but the considerable interference problem needs to be addressed further. An additional lipid layer was required in the case of the theophylline sensor, as the negatively charged Nafion polymer alone does not serve to eliminate biologically important anionic compounds. The stability of the choline sensor was found to be less due to co-immobilisation of the two enzymes. The gelatine method of immobilisation for lysine proved stable but the response time was slower due to the thick gelatine layer and additional cellulose dialysis membrane. The problem of interferences was partially solved in this case by the molecular weight cut-off ability of the cellulose membrane, allowing only small analyte molecules access to the surface. The lactate sensor exhibited reasonable stability with the thin film resulting in very fast response times though interferences again proved to be a problem. The presence of an additional cellulose membrane considerably reduced the signal due to these

compounds.

In order to alleviate the problems encountered in the analysis of such analytes in biological fluids and to develop sensors more amenable to *in vivo* measurements, future work may involve differential measurement to compensate for signals due the effects of fouling. This method consists of subtracting a signal at a control electrode from the signal of the working biosensor. Also promising, are computer controlled sensor arrays, based on electrodes coated with different permselective films and operated in connection with statistical recognition methods [85,86].

Further work on the ability of lipid layers such as phosphatidylcholine to prevent protein fouling may be examined using Scanning Tunnelling Microscopy (STM), which will allow information to be achieved as to the extent of adsorption of proteins on the surface of the electrode [87].

New polymers/derivatives in addition to the well documented, commonly used few, may provide additional properties/qualities advantageous to biocatalyst immobilisation. STM may also prove useful here to give information as to the optimum pore size for rejection of interferences.

Additional anti-interference layers/membranes between the sample and immobilised biocatalyst may serve to eliminate easily oxidisable compounds. The measurement of lactate in microsamples of spinal fluid has been reported by Clark *et al.* [88]. The electrodes are prepared by placing a glutaraldehyde solution of the enzyme between a cellulose acetate and polycarbonate membrane. The membranes serve to support the oxidase, they prevent the diffusion of soluble electroactive species, e.g. uric acid, ascorbate and acetaminophen and form a diffusion-limited path for the lactate.

A tissue bioelectrode for eliminating protein interferences has been described recently [89]. The rich biocatalytic activity of tissue e.g. papaya containing surfaces was exploited for *in situ* enzymatic digestion of interfering proteins. The presence of the protease enzyme papain in the papaya tissue effectively eliminates protein interferences. The smaller peptides resulting from the cleavage of the proteins do not passivate the surface.

Co-immobilisation of mediators and cofactors provide reagentless devices which then may be fabricated as screen printed strip type electrodes, thereby becoming more readily applicable in the clinical field [90]. A range of sensing systems have been described, using electrochemical techniques for the measurement of various analytes, these have been demonstrated to be applicable to the manufacturing methods required for single-use disposable tests [91]. Such reagentless devices may also prove useful in the development of enzymes on

microelectronic substrates.

Biosensors may form the electrochemical detector of a HPLC system with the column providing separation prior to detection at the biologically modified electrode surface [92-95].

Additional studies on the electrochemical activation processes for conditioning the surface of glassy carbon electrodes, may allow for better substrates for enzyme immobilisation [96]. Glassy carbon amperometric flow detectors were shown to exhibit a substantial improvement in their stability following a simple preanodisation procedure. The stability enhancement is attributed to the formation of an oxide layer on the surface. Prevention of electrode passivation was observed for repetitive injections of NADH, albumin and serum solutions [97].

The transition to *in vivo* and clinical use necessitates studies that address materials and interfacing problems [98].

In the majority of the biosensors described here, a compromise has been necessary between sensitivity, speed of response and stability. In FIA, enzyme films are generally less stable due to the continuous flowing stream causing slow leaching of the enzyme layer.

All of the compounds studied here are water soluble (with the exception of theophylline, being only partly soluble in water), but numerous similar analytes are soluble only in non-aqueous solvents. The next chapter therefore deals with the application of enzyme based biosensors in organic media, where otherwise inaccessible analytes may be determined by amperometric means. Together with the numerous applications of organic-phase biosensing, fundamental studies of enzyme kinetics give vital information as to the differences of enzyme action in non-aqueous as opposed to aqueous media.

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CHAPTER 3

DEVELOPMENT OF TYROSINASE AND PEROXIDASE-BASED AMPEROMETRIC SENSORS FOR INHIBITING COMPOUNDS AND A STUDY OF THE KINETICS OF TYROSINASE IN ORGANIC MEDIA

3.1. INTRODUCTION

Enzymes retain catalytic activity in certain organic solvents as a consequence of a hydrated shell engulfing the enzyme molecule. The internally bound water acts as a plasticiser to provide the enzyme molecule with the flexibility necessary for catalysis [1-3]. Insolubility in organic solvents removes the enzymes conformational flexibility, thereby preventing them from acquiring conformations in organic solvents that are different from those in water. Thus, they remain frozen in the conformation corresponding to the aqueous solution from which they were isolated, due to the high kinetic barriers required in the transition from an aqueous to a non-aqueous solvent.

The solvent can influence the enzymatic reaction in different ways; first, the solvent can influence the partitioning of water in the system and thereby indirectly affect the enzymatic activity; second, the solvent can influence partitioning of substrate and product and thirdly, the solvent can directly influence the enzyme e.g. by acting as inhibitor.

Recent developments in biotechnology demand wider applications of biocatalysts. Important applications of organic phase enzymology may be found in lipid conversions and peptide synthesis [4]. It is usually desirable to find a solvent in which the enzyme is not only thermodynamically stable but also catalytically active. In some cases, the catalytic activity of enzymes is even enhanced by organic solvents. The basis for this enhancement of enzyme activity and/or stability may be the restoration of conditions more closely resembling those in the cellular environment [5].

Enzymes in organic solvents offer great potential for the biocatalysis of a wide range of chemical processes that do not occur in water, for example

- enzymatic conversion of petrochemicals
- polymerisation
- transformation of oils and fats
- enzymatic analysis of organic compounds extracted using apolar solvents
- high thermostability and sometimes altered substrate specificity in anhydrous organic solvents.

It would be better to carry out enzymatic conversions in organic solvents instead of water due to the following reasons:

- organic substrates dissolve better in organic media and enzymes are insoluble in organic media
- water often participates in undesirable side reactions, e.g. hydrolysis of acid anhydrides
- thermodynamic equilibria of many processes are unfavourable in water
- product recovery from aqueous solutions is often difficult and expensive.

As long as the essential water is localised around the enzyme molecule, replacement of the rest of the water with an organic solvent should be possible without adversely affecting the enzyme. The most hydrophobic solvents e.g. hydrocarbons are best suited for this purpose as there is no incentive for the essential water to partition into them and thus it remains on the enzyme [1]. Fundamental, therefore, to the catalytic activity of the enzyme is the selection of a compatible organic phase, i.e. one that does not interact strongly with the essential hydration.

A simple theoretical model was suggested to describe quantitatively the effect of percentage water and nature of organic solvents on the catalytic behaviour of enzymes suspended in low-water media [6]. The hydrophobicity of the organic solvent can be expressed as log P, where P is the partition coefficient of the solvent in a water/octanol biphasic system. The log P values for different solvents are well documented [7]. It has been suggested that solvents with log P values < 2 are not suitable as they distort the biocatalyst-water interaction. Those with log P values from 2-4 affect the activity in an unpredictable way, whereas solvents with log P values > 4 are normally biocompatible. This trend, however, has been established with relatively few systems.

Since water participates in many of the reactions that lead to irreversible loss of enzyme activity, a non-aqueous medium may greatly enhance enzyme stability. The non-covalent interactions that determine the stability of a folded protein will reach a new equilibrium in an organic solvent that may result in loss of catalytic activity and unfolding. Thus they remain frozen in the conformation corresponding to the aqueous solution from which they were isolated. The enzyme remembers the pH of the latest aqueous solution to which it has been exposed [8]. Apparently all chemical and structural changes that lead to irreversible thermal inactivation have one thing in common - they require water. If enzymes are placed in an essentially water-free environment, they will become more thermostable. This increased thermostability would allow reactions to operate at elevated temperatures, where faster reaction rates are generally advantageous.

The activities of enzymes in organic solvents appears to be directly controlled by four factors:

- ground state stabilisation of substrate and/or product and the enzyme itself
- active site flexibility/polarity controlled via the water content of the active site
- water stripping from an enzyme that maintains its native, active structure
- direct solvent-induced perturbation of the enzyme

Understanding how these factors control enzyme structure and function in non-aqueous media provides a framework for proposed methods to improve enzyme function in organic media [9].

In all systems studied, the value of K_m exhibited a steep rise when the water content of the solvent increased. The effect of organic solvents and/or water content on catalytic behaviour of enzymes in low-water media can be adequately assessed only in terms of full kinetic description based on determined V_{max} and K_m values [10, 11].

The advantages that accrue from performing biocatalytic and electrochemical reactions in non-aqueous systems have been discussed in relation to possible applications in medicine, pharmaceuticals, petrochemicals, the food industry, environmental monitoring and defence [12]. The ability of biocatalysts to function in extreme environments such as organic solvents has important implications for the implementation of biosensor technology in formerly inaccessible environments. It also extends the number of detectable analytes to include poorly water soluble organic species.

An organic phase enzyme electrode therefore permits analyses to be carried out in clinical and industrial samples by partitioning of a very low concentration analyte from a large aqueous volume into a small organic volume. The phenol electrode described in the following examples could be used in this way for the detection of phenolic contamination of waste water.

The enzyme polyphenol oxidase is capable of catalysing ortho-hydroxylation of monophenols and oxidation of the corresponding o-dihydroxyphenols to their corresponding quinones [13]. Polyphenol oxidase in chloroform catalyses the regioselective oxidation of substituted phenols to the corresponding o-quinones [14]. This reaction cannot be done using water as a solvent because the quinones rapidly form the polyaromatic pigments which also serves to inactivate the enzyme.

One of the first approaches to the construction of organic phase amperometric biosensors was described by O'Connor *et al.* [15], and was based

on the incorporation of this enzyme polyphenol oxidase (tyrosinase) in silicone grease and using the grease to fill the micropores on a graphite surface. The method resulted in a self-supported (membrane-free) enzymatic layer, that was in close proximity to the sensing graphite sites.

The first report of an organic phase enzyme electrode was described by Hall *et al.* where tyrosinase was used to detect p-cresol via the electrochemical reduction of the product of the enzyme reaction at a graphite foil electrode, in chloroform [16].

Incorporation of the 'fragile' enzyme glutamate dehydrogenase into the carbon paste matrix allowed good performance and high operational stability, with the use of two redox mediators, ferricyanide and phenazine methosulphate [17]. The hydrophobic environment of the carbon paste may be of special interest when there is a risk of enzyme inactivation by chemicals or by hydrophilic solvents. A 'fragile' enzyme as opposed to a robust one was purposely employed, in order to point out the protective effect of the carbon paste matrix.

An enzyme electrode has been described for the determination of cholesterol dissolved in chloroform/hexane. The enzyme electrode was shown to be applicable to the determination of cholesterol in samples of butter and margarine. Cholesterol displays excellent solubility in this system and it is sufficiently hydrophobic to support cholesterol oxidase activity [18]. Peroxidase was coupled with cholesterol oxidase and the bienzyme system was successfully used for accurate, reliable and reproducible determination of cholesterol in toluene [19].

An amperometric enzyme electrode incorporating horseradish peroxidase was described for the determination of hydrogen peroxide in organic solvents [20]. The enzyme was co-adsorbed with an electron mediator, potassium hexacyanoferrate (II) on the surface of a graphite foil electrode to allow for reagentless measurements. The adsorption of mediators dissolved in organic media on carbon electrodes and the subsequent or parallel immobilisation of enzymes to such electrodes has proved to be a powerful method for the construction of relatively stable enzyme sensors with good performance in the aqueous environment. Horseradish peroxidase catalysed polymerisation of phenols is another example of the practical use of newly developed technology of enzymatic catalysis in non-aqueous media. The use of organic solvents as a medium for enzymatic transformation eliminates the drawback of the poor solubility in water of the phenols. A rapid procedure for determining phenol in olive oils based on an organic-phase enzyme electrode has also been described [21]. Direct assays in chloroform solutions which support the tyrosinase activity

The utility of the Eastman AQ films for entrapping enzymes onto electrodes for organic-phase biosensing have been reported [22]. Such a scheme relies on the stability of the Eastman AQ films in various organic solvents. Discrimination against anionic interferences has also been achieved. Laccase was entrapped within an AQ Eastman polymer film, to construct an electrode which responds rapidly to low concentrations of catechol and hydroquinones in various alcohols [23]. The dynamic properties of the sensor allowed convenient monitoring of flowing organic streams.

The organic-phase operation enabled a simple immobilisation procedure for quantifying phenols and peroxides in chloroform based on spreading a thin layer of the tissue containing tyrosinase and horseradish peroxidase onto a rough graphite disc [24]. Mushroom, banana and horseradish root layers were thus immobilised and employed for this purpose.

Electrochemical monitoring of such organic-phase reactions can be greatly improved through the use of microelectrodes. The negligible ohmic drop distortion characterising such electrodes permits reliable amperometric measurements of the corresponding substrates in the absence of deliberately added supporting electrolyte [25]. The utility of on-line biomonitoring of organic streams has also been illustrated [26]. The enzymes horseradish peroxidase and tyrosinase were immobilised by simple adsorption onto an electrochemically pre-treated carbon fibre surface. The resulting detectors respond very rapidly to dynamic changes in the concentrations of organic peroxides and phenolic compounds in flowing chloroform and acetonitrile solutions.

Clinically relevant analytes such as cholesterol and bilirubin are examples of poorly water soluble analytes that may be amenable to detection using organic phase enzyme electrodes. Aldehydes in fats and oils or cholesterol in butter/margarine may be detected by the use of appropriate oxidoreductases. Monitoring of edible oils is also important e.g. the continuous hydrolysis of olive oil by immobilised lipase in organic solvents has been studied [27]. Some enzymes in non-aqueous media exhibit very high stereospecificity and therefore may be used to determine optically active products in food processes. Steroids and steroid-based drugs are amenable to analysis using these electrodes. Analytes in the petrochemical industry e.g. methane, alcohol, aldehydes and esters, may have suitable biocatalysis for their detection in the organic phase.

The use of enzymatic transformations instead of traditional chemical methods have many potential advantages in the fine and speciality chemical industries. Some systems are now close to commercialisation and will have applications in the resolution of chiral compounds, lipolytic modifications of fats

and oils, biosynthesis of peptides and enzymatic polymerisation [28]. Recent developments in protein crystallography and genetic engineering have significantly increased understanding of the structure, catalytic mechanisms, stability and substrate specificity of enzymes.

Enzymes may be redesigned to permit catalysis in non-aqueous solvents by engineering their amino acid sequences, thereby altering their physical and chemical properties to suit the new solvent environment. The introduction of internal crosslinks in the form of new disulphide bridges, H bonds and certain interactions should all lead to improved stability in non-aqueous solvents. Some design rules for engineering non-aqueous solvent stable enzymes are listed below [29].

Compatibility of the surface with solvent

- remove surface charges
- remove or satisfy unfilled H bonding sites

Conformational stability

- internal cross-links
- increase Van der Waals interactions, to maintain or improve tight packing
- maximise internally fulfilled H-bonds; high degree of ordered secondary structure; use side chains to fulfil main-chain H bonding sites
- electrostatic interactions; interaction in proteins interior are likely to stabilise enzyme surface ion pairs and decrease the stability in non-aqueous solvents

Substitutions must also be chosen to minimise disruption of function and introduction of an unfavourable nonbonded interactions.

The activity of polyethyleneglycol (PEG) modified horseradish peroxidase in organic solvents has been studied [30]. Its activity in water-immiscible solvents was found to be enhanced up to 16 times by increasing the enzyme hydrophobicity. The PEG-modified enzymes form micro-particulate suspensions rather than solutions in organic solvents, and can trap water on its surface and exert their activity in water-immiscible solvents. The elimination of almost a half of the carbohydrates from the enzyme molecule improved its k_{cat} value by ten times in methylene chloride and by two times in diethylether.

The activity and flexibility of alcohol dehydrogenase in organic solvents has also been investigated [31]. The reactions of α-chymotrypsin and related proteins

with ester substrates have been examined in non-aqueous solvents [32]. In addition Miyabayashi *et al.* [33] described a potentiometric organic phase enzyme electrode that monitored chymotrypsin-catalysed ester synthesis in organic media .

In the following section organic-phase enzyme electrodes will be employed in the investigation of the effects of inhibitors on the activity of such enzymes that function in organic media.

3.2. ORGANIC PHASE BIOSENSING OF INHIBITORS OF TYROSINASE AND PEROXIDASE

Organic phase biosensors may be applied in new environments or towards additional substrates and can offer simple immobilisation schemes, together with reduced side reactions. The immobilisation of tyrosinase and peroxidase has already been evaluated in connection with the biosensing of phenolic and peroxide compounds in acetonitrile solutions [16,34].

An important factor for expanding the scope of organic phase enzyme electrodes is the identification of new enzymes suitable for bioassays in non-aqueous environments. New substrates, while exploiting the same well documented enzymes in organic media, may be achieved via inhibition studies. The objective of this study was to expand the concept of organic-phase biosensors towards measurements of important enzyme inhibitors. Little attention has previously been paid to the methods for determination of organic compounds that act like inhibitors particularly in the organic phase.

Any substance that decreases the velocity of an enzyme catalysed reaction may be considered to be an inhibitor. Inhibition studies along with important analytical applications, give us more information as to the specificity of an enzyme, the physical and chemical architecture of the active site and the kinetic mechanism of the reaction. The mechanism of inhibitor action on the enzyme may be divided into the irreversible and reversible mode of operation. In the former case enzyme activity cannot be recovered, however with the latter, activity returns upon merely removing free inhibitor, and an equilibrium exists between free inhibitor and enzyme.

$$E + I \underset{}{\overset{K_i}{\Leftrightarrow}} EI \qquad (3.1.)$$

The dissociation constant for EI combination is known as K_i, which is the reciprocal of the E-I affinity. Reversible inhibitors may act on apparent K_m or V_{max}. Those which act only by increasing the K_m(app) while V_{max} remains unchanged are called competitive inhibitors, while those having no effect on the K_m but decrease the V_{max} are known as non-competitive. Competitive inhibition occurs when the substrate binding occurs at a position other than the active site. This combination of the inhibitor with the enzyme causes a change in the conformation that distorts the substrate site and thereby prevents the substrate from binding. Non-competitive inhibition does not affect the combination of S and E but affects the rate (V_{max}), by binding to the ES complex [35].

Enzyme activity decreases owing to the presence of these inhibiting substances that bind to the enzyme. This effect has been used for the determination of numerous species, and work in this field is of interest from both the clinical and toxicological viewpoints. An increase in use of pesticides all over the world has generated a growing interest for pesticide environmental monitoring, and as a great number of pesticides act as inhibitors of certain enzymes, this may be used as a method for their determination. A major problem of analytical chemistry is the determination of sulphur-containing organic compounds present as impurities in various industrial materials and forming part of many pesticides, which also may be analysed based on their inhibition effects.

Monitoring of organophosphorous and carbamate pesticides is of considerable importance. Previous amperometric methods for the detection of these pesticides using disposable biosensors based on their inhibition of cholinesterase have been described [36,37].

In the following sections inhibitor measurements utilising organic-phase biosensors are described. The organic-phase operation may greatly effect the mechanism of inhibition. According to Zaks [38], the replacement of water with an organic solvent results in a reversal of the inhibitor specificity of the enzyme. These changes, coupled with other improvements inherent to biosensors in non-aqueous environments, should greatly enhance the power of enzyme-inhibition based biosensors.

Eastman AQ films allow the development of organic phase enzyme electrodes, which couple the entrapment of high enzyme loadings with discrimination against anionic interferences, short response times and rapid stabilisation of the baseline. Due to the highly resistive nature of organic solvents

the supporting electrolyte, tetraethylammonium p-toluenesulphonate was used in all work. Tetraethyl ammonium salts are excellent supporting electrolytes in solvents such as acetonitrile etc. and there are no excessive solution resistance problems to overcome. The feasibility and applicability of using these tyrosinase and peroxidase modified electrodes in organic phase for amperometric sensing of a variety of inhibiting compounds is assessed in the following section.

3.2.1. EXPERIMENTAL

3.2.1.1. *Apparatus*

Amperometric measurements were performed using a CV-27 voltammograph (Bioanalytical Systems, BAS), the throughput of which was displayed on a X-Y-t recorder (Model RXY, BAS). The 5 ml cell (Model VC-2, BAS) joined the working electrode, reference electrode (Ag/AgCl, Model Re-1, BAS), and platinum wire auxiliary electrode through holes in its Teflon cover. The flow injection system consisting of a carrier reservoir, a Rainin Model 5041 sample valve (20 µl loop), interconnecting Teflon tubing, and a glassy carbon thin-layer detector (Model TL-5, BAS).

3.2.1.2. *Reagents*

Horseradish peroxidase (EC 1.11.1.7, 100 mg), tyrosinase (EC 1.14.18.1, 2400 U/mg), benzoic acid, 2,4- dichlorophenoxyacetic acid (Sigma), acetonitrile, diethyldithiocarbamate sodium salt, hydroxylamine sulphate, dimethylmercury, 1,2-phenylenediamine (Aldrich), thiourea (Fisher), tetraethylammonium p-toluenesulphonate (TEATS) (Fluka) were used as received. The poly(ester-sulphonic acid) polymer (Eastman AQ_{55D}, Eastman Kodak Co.) was obtained dissolved (28 % w/v) in water.

3.2.1.3. *Procedures*

The peroxidase - AQ_{55D} film was prepared by mixing 20 µl of 5 mg/ml horseradish peroxidase solution (100 U/mg) together with 20 µl AQ_{55D}. 20 µl of this solution was then placed on the surface of a previously polished glassy carbon electrode, resulting in 5 U/electrode surface. The film was dried (30 min) using a heat gun.

The preparation of the tyrosinase electrode was achieved by covering the electrode surface with a 10 µl aliquot of the tyrosinase - AQ mixture, prepared by dissolving 2 mg of the enzyme in 200 µl of 1.4 % AQ polymer, resulting in 240 U/electrode surface. The electrode was dried as before.

Prior to use the enzyme electrode was immersed in a 0.2 mM phenol solution in acetonitrile (0.1 M TEATS) for 10 min. This procedure allowed for rapid stabilisation of the baseline upon use and also allowed for regeneration of the enzyme electrode after addition of reversible inhibitors.

All experiments were carried out at room temperature in acetonitrile (containing 2 % phosphate buffer and 0.1 M TEATS). The amperometric response to 0.2 mM phenol and 1.0 mM 2-butanone peroxide was measured after application of -0.2 V (vs. Ag/AgCl), in order to allow detection of the o-quinone product and the reduction of the 1,2-phenylenediamine mediator for tyrosinase and horseradish peroxidase respectively. Batch experiments were carried out with a magnetic stirrer providing convective transport at 300 rpm.

3.2.2. RESULTS AND DISCUSSION

3.2.2.1. *Amperometric Batch Inhibition Measurements*

The concept of organic-phase inhibitor biosensors is illustrated in the following sections for monitoring inhibitors of horseradish peroxidase and tyrosinase, in connection with the Eastman-AQ/enzyme immobilisation approach. Figure 3.1(A) shows the response of the tyrosinase immobilised electrode to 0.2 mM phenol (S) followed by successive additions of diethyldithiocarbamate (In), each addition affecting a 20 µM increase in concentration and a subsequent proportional decrease in enzyme activity. The steady state current response to 0.2 mM phenol was achieved at the tyrosinase electrode by application of -0.2 V and allowing the charging current to decay prior to inhibitor injection. Figure 3.1.(B) represents the same case for thiourea injections, where again it may be seen that the enzyme electrode responds rapidly to µM changes in inhibitor concentration with no need for an incubation period. 100 % inhibition of enzyme activity occurred when saturation was achieved at 0.08 mM diethyldithiocarbamate and 45.5 % of enzyme activity remained at 0.5 mM thiourea.

The low noise levels allowed limit of detections of 1.4 and 27.2 µM to be achieved for diethyldithiocarbamate and thiourea respectively, with linearity prevailing up to 0.08 mM and 0.5 mM. Sensitivity of the sensor to each inhibitor was calculated as the slope of the linear portion of the curve and found to be 250

nA/mM and 127.5 nA/mM, with correlation coefficients of 0.9785 and 0.9829 for diethyldithiocarbamate and thiourea respectively. The fast response time was due to the excellent stability of the AQ polymer in organic media. The inhibitors were dissolved in ethanol and controls (dotted lines for Fig. 3.1.(A) and (B)) were performed by injecting the same volume of ethanol only. Steady-state signals were achieved within 5-15 sec.

Thiourea was found to have a reversible effect on the enzyme activity. In the case of the carbamate pesticides, e.g. diethyldithiocarbamate, the breaking of the enzyme-inhibitor complex takes a long time; however, gradual recovery of the enzyme activity results after a period of time, therefore this inhibitor may be called slowly reversible [39].

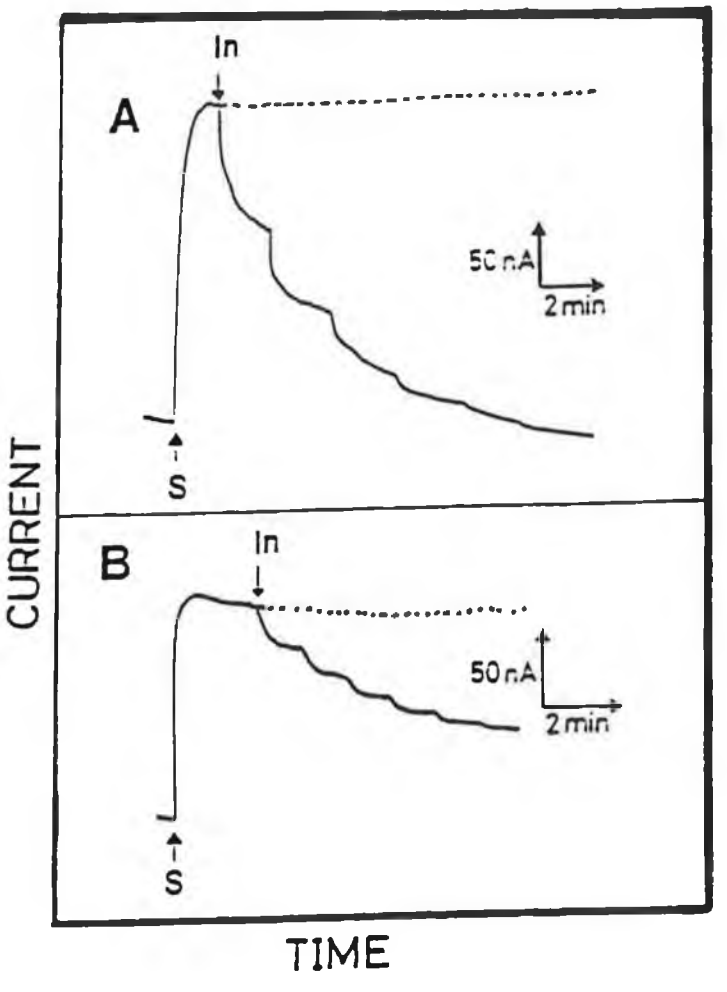

FIGURE 3.1.

(A) Tyrosinase inhibition by diethyldithiocarbamate sodium salt (in ethanol). Electrode - 240 U/surface in 1.4 % Eastman AQ polymer. Electrolyte - acetonitrile 2% phosphate buffer with 0.1 M TEATS. E_{app}= -0.2 V; 2.5 mV/cm, 0.01 mA/V, giving 25 nA/cm. Injection of 0.2 mM phenol (S) followed by 20 μ M additions of the pesticide (In). Control - ethanol injections only (dotted line).
(B) Tyrosinase inhibition by 0.1 mM thiourea additions.

Figure 3.2.(A) shows an example of horseradish peroxidase inhibition by the irreversible inhibitor 2-mercaptoethanol (in ethanol), after injection of 1.0 mM 2-butanone peroxide (not shown), with the control represented by the dotted line as shown in Fig. 3.1. Here linearity was found to extend up to 1.5 mM with sensitivity of 649.6 nA/mM, a limit of detection of 10.7 μM and correlation coefficient of 0.9988. The type of inhibition exerted by 2-mercaptoethanol proved irreversible, with the compound acting on the sulphur groups of the enzyme amino acids, resulting in the formation of disulphide linkages, hence bringing about the denaturation of the enzyme. Fig. 3.2. (B) shows the peroxidase electrode response to additions of the inhibitor hydroxylamine sulphate. Again excellent sensitivity and fast response was obtained, with linearity up to 0.1 mM, sensitivity of 0.93 nA/min with limit of detection 1.375 μM and r = 0.9899. Steady-state signals were achieved within 5-15 sec. Obviously in the absence of biocatalytic activity, an unmodified electrode, was found to be unresponsive to additions of either substrate for both enzymes examined.

Calibration curves for all inhibitor compounds in the case of (A) peroxidase and (B) tyrosinase are shown in Figure 3.3. Fig. 3.3.(A) (a) shows the sigmoidal type response curve for thiourea. This compound resulted in very slow inhibition initially, with further inhibitor addition resulting in a faster decrease in enzyme activity. The sensitivity in this case was found to be - 2260 nA/mM with limit of detection of 15 μM and correlation coefficient of 0.93375. Fig. 3.3(A) (b) shows the response to 2- mercaptoethanol (full response not shown, as Fig. 3.2. (A)). Fig. 3.3(A)(c) shows the response to diethyldithiocarbamate, with linearity extending up to 0.45 mM, sensitivity of 565 nA/mM, limit of detection of 5 μM and correlation coefficient 0.9882. The hydroxylamine sulphate response may be seen in Fig. 3.3(A) (d) as Fig. 3.2.(B) (selective only for peroxidase).

Fig. 3.3(B) shows the response to inhibitors of tyrosinase with (a) corresponding to thiourea additions, resulting in a different (linear) type of inhibition when compared to that of horseradish peroxidase - see Fig. 3.1.(B), (b) showing the 2- mercaptoethanol response; linearity in this case was observed only up to 0.1 mM as opposed to 1.5 mM in the case of tyrosinase with sensitivity 2.37 nA/μM, limit of detection 6.66 μM and correlation 0.9856. Fig 3.3(B) (c) shows the diethyldithiocarbamate response as Fig. 3.1. (A), and (d) the benzoic acid response (selective only for this enzyme), with linearity up to 8 μM, sensitivity of 8.38 nA/μM, limit of detection 0.25 μM and r = 0.9736.

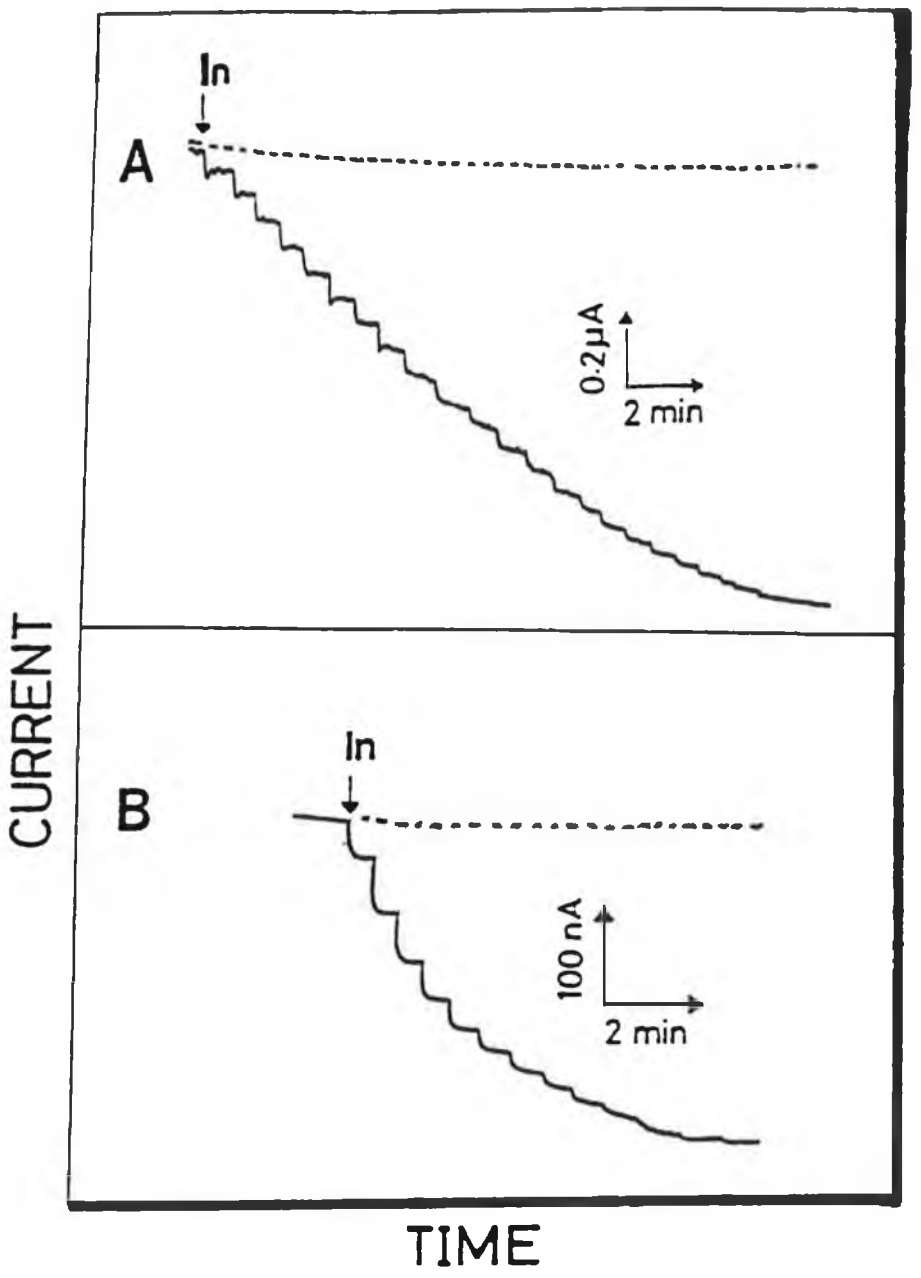

FIGURE 3.2.

(A) Peroxidase inhibition by 2-mercaptoethanol (in ethanol). Electrode containing 5 U/electrode surface in 1.4 % Eastman AQ polymer. Electrolyte - acetonitrile 2% phosphate in 0.1 M TEATS containing 0.2 mM o-phenylenediamine. E_{app} - 0.2 V; 10 mV/cm, 0.01 mA/V, giving 0.1 µA/cm. Injection of 1.0 mM 2-butanone peroxide (not shown) followed by 0.1 mM 2-mercaptoethanol. Control (dotted line) - injection of ethanol only.

(B) Peroxidase inhibition by hydroxylamine sulphate. As (A) except 5 mV/cm. Injection of 1.0 mM 2-butanone peroxide (not shown) followed by 20 µM additions of hydroxylamine sulphate. Control - as above.

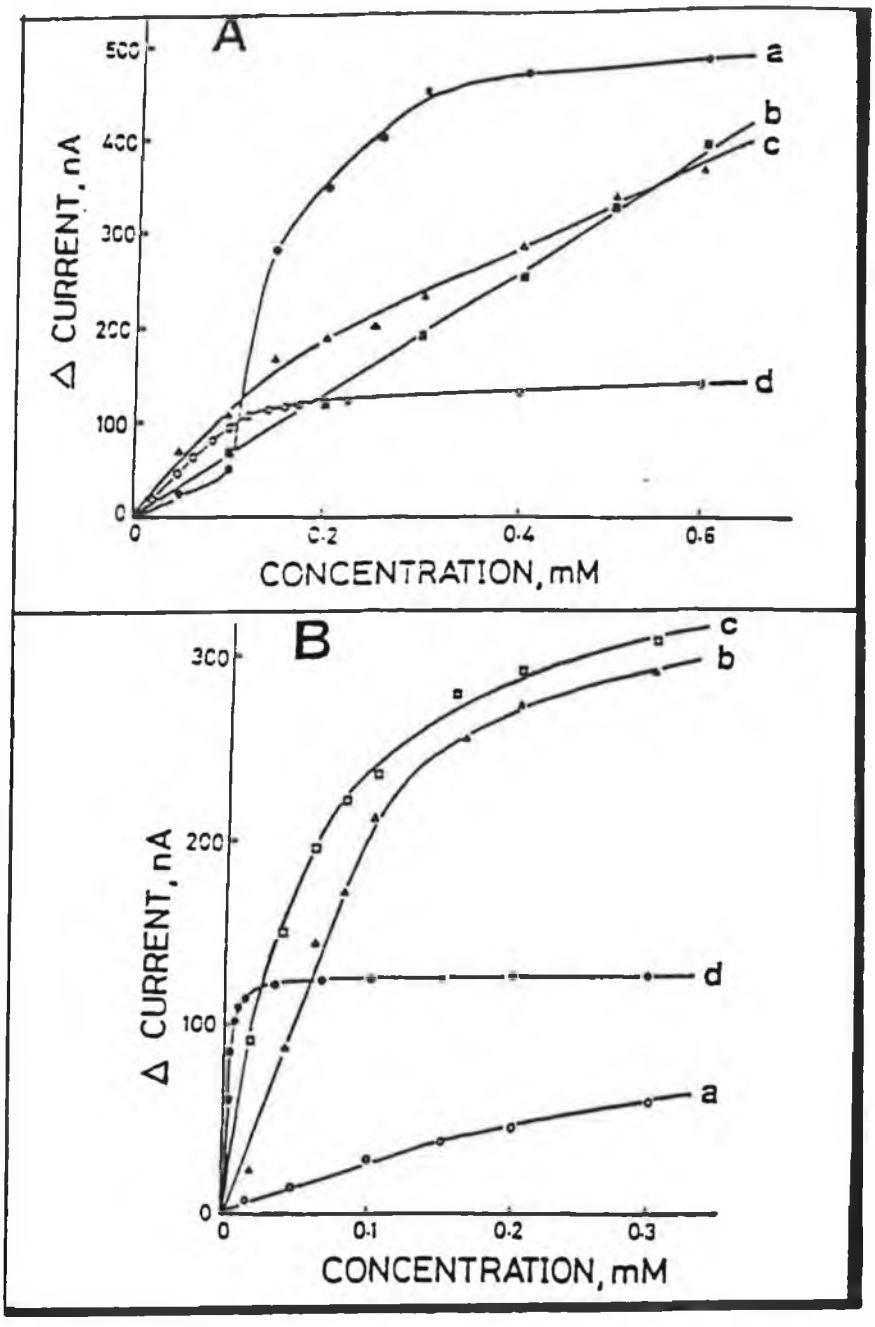

FIGURE 3.3.

(A) Calibration curves for all inhibitors of peroxidase, (a) thiourea, (b) 2-mercaptoethanol, (c) diethyldithiocarbamate, (d) hydroxylamine sulphate. Injection of 1.0 mM 2-butanone peroxide followed by each inhibitor. E_{app} -0.2 V; 5mV/cm, 0.01 mA/V, giving 50 nA/cm. Other conditions as before.

(B) Calibration curve for all inhibitors of tyrosinase, (a) thiourea, (b) mercaptoethanol (c) diethyldithiocarbamate (d) benzoic acid. All other conditions as (A) except 2.5 mV/cm and injection of 0.2 mM phenol followed by injection of inhibitors (dissolved in ethanol).

The linear range and sensitivity differ from inhibitor to inhibitor. For example, for thiourea, mercaptoethanol and diethyldithiocarbamate (at the tyrosinase electrode) linearity prevailed up to 0.15, 0.10 and 0.01 mM, respectively. For hydroxylamine sulphate, mercaptoethanol and diethyldithiocarbamate (at the peroxidase electrode) linearity prevails up to 0.10, 0.60, and 0.12 mM respectively. Overall, the profiles shown in Figure 3.3. reflect the kinetics and mechanism of the enzyme inactivation in the organic media.

Benzoic acid has been found to be a potent competitive inhibitor with interaction occurring at the copper active site of the enzyme [40]. Sulphydryl compounds are also widely known to be among the most powerful inhibitors of tyrosinase with 2- mercaptoethanol giving rise to an enzymatically inactive complex [40]. Thiourea appears reversible and diethyldithiocarbamate slowly so. Upon incubation of the enzyme electrode in 0.2 mM phenol after thiourea inhibition and before testing once more it was found to retain a large percentage of initial activity.

Examples of two inhibitors which exhibit very slow inhibition responses on peroxidase and tyrosinase respectively, may be seen in Fig. 3.4. Fig. 3.4(A) shows the response to successive additions of thiourea at the peroxidase modified electrode. Increasing negative slopes of 78.0 nA/min, 54.0 nA/min and 28.66 nA/min were calculated for (b) 0.025, (c) 0.05, and (d) 0.1 mM injections respectively, following injection of 1.0 mM 2-butanone peroxide (a). The enzyme activity decreased with time in proportion to the concentration of inhibitor, with 75.7 %, 46.6 % and 32.0 % remaining activity, corresponding to (b) 0.04, (c) 0.2 and (d) 1.0 mM thiourea additions respectively. This type of inhibition in the case of peroxidase however, involves two steps: an initial rapid reversible competitive inhibition followed by irreversible enzyme inactivation with time [41].

Figure 3.4. (B) shows again the slow tyrosinase inhibition exhibited by dichlorophenoxyacetic acid. Slopes of -7.567 nA/min, -4.428 nA/min, -2.143 nA/min were observed for (b) 0.04 mM, (c) 0.20 mM and (d) 1.00 mM additions respectively. A limit of detection of 8.57 μM was calculated. The percentage inhibition due to selected levels of inhibition were calculated for different concentrations, with (b) 81.11 %, (c) 43.82 %, (d) 19.10 % enzyme activity remaining. Environmental monitoring of this pesticide in e.g. food crops is of interest. The analysis of this pesticide is also important in toxicological studies.

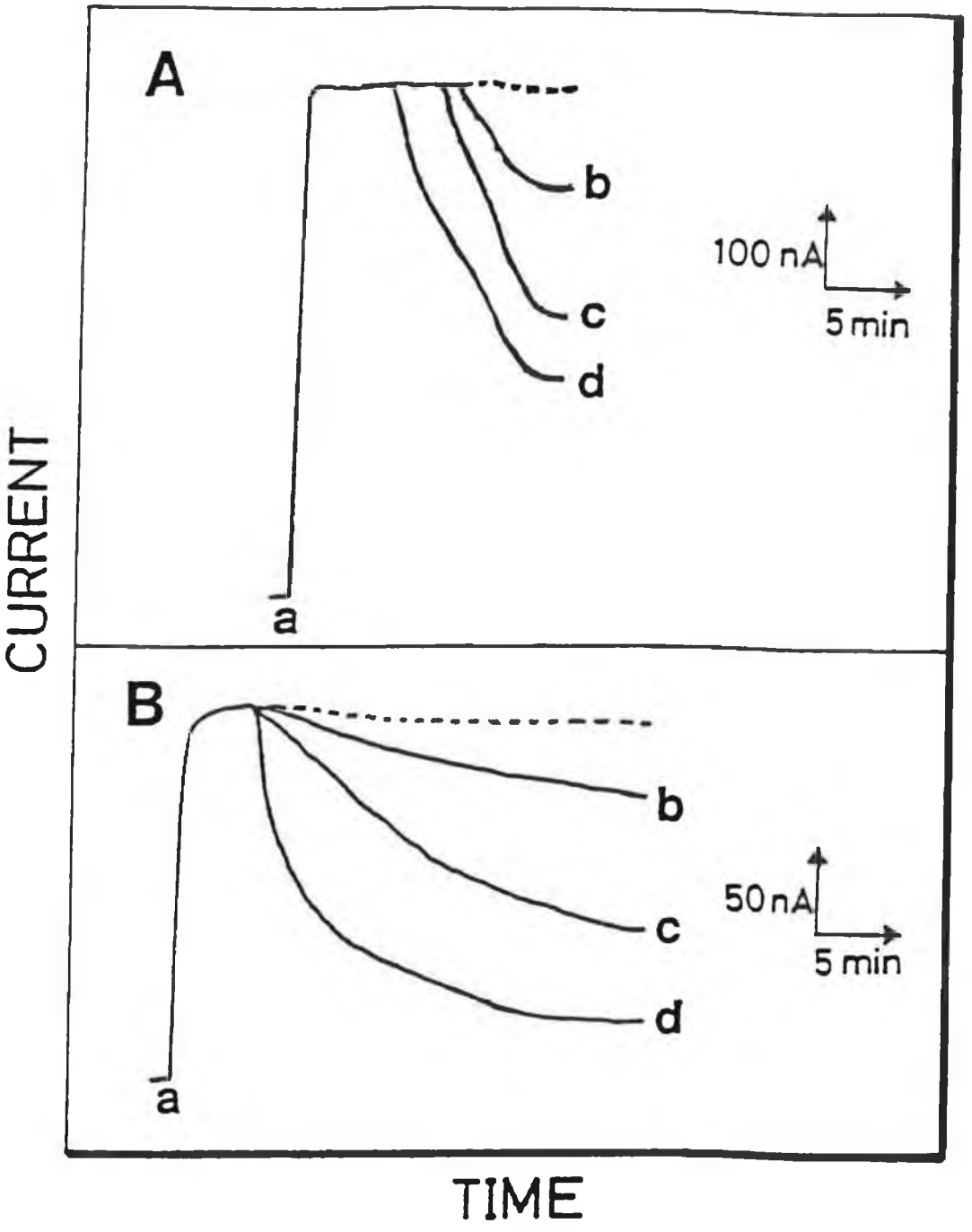

FIGURE 3.4.

(A) Peroxidase inhibition by thiourea. Injection of (a) 1.0 mM 2-butanone peroxide, followed by (b) 0.025 mM (c) 0.05 mM (d) 0.10 mM additions of thiourea. 50 nA/cm.

(B) Tyrosinase inhibition by dichlorophenoxyacetic acid, after (a) 0.1 mM phenol injection, and then (b) 0.04 mM (c) 0.2 mM (d) 1.0 mM additions of the pesticide. 2.5 mV/cm. All other conditions as before.

3.2.2.2. *Estimation of the Effect of Substrate Concentration and Calculation of Relevant Kinetic Parameters*

The effect of the substrate concentration upon the diethyldithiocarbamate response at the tyrosinase electrode was examined, in the presence of constant inhibitor concentration. The % inhibition (I%) vs. substrate concentration plot was found to be constant with a slight increase in enzyme activity at high substrate concentrations (not shown). This proves that I% is essentially the same for a given concentration of phenol and the behaviour is consistent with a non-competitive process [42]. However at high substrate concentration the inhibition process may be slightly hampered.

The data shown in Figures 3.1 to 3.4. may be used in order to calculate the various kinetic parameters relevant to the inhibition process. For example, Table 3.1. shows the coefficient of inhibition, C_{50}, i.e. the concentration corresponding to 50 % inhibition and the rate constant k (nA/min) calculated from the initially linear portion of the progress curves, i.e the first order decay of the remaining enzyme activity, for each inhibitor of both enzymes.

The K_i, which corresponds to the concentration of inhibitor that doubles the slope of the 1/rate vs. [S] plot, may be calculated from the equation :

$$K_i = C_{50}/(1+[S]/K_m) \text{ [35]} \qquad (3.2.)$$

The lower the value of K_i the greater the degree of inhibition at any given substrate and inhibitor concentration. The K_m for tyrosinase was found to be 1.066×10^{-6} M.

The K_i values for each inhibitor of tyrosinase were calculated using the above equation, and found to be 324.9 μM for thiourea, 25.0 μM for diethyldithiocarbmate, 2.19 μM for benzoic acid, 57.989 μM for 2-mercaptoethanol, 227.95 μM - dichlorophenoxyacetic acid and 1199.77 μM for dimethylmercury.

In addition to this method, a method described by Dixon [43] was employed using the diethyldithiocarbamate inhibition of tyrosinase as an example. This provides an accurate and convenient means of determining K_i values. The velocity (i.e. current) was determined with a series of diethyldithiocarbamate concentrations while keeping the substrate concentration constant. A plot of $1/i_{ss}$ vs [I] gave a straight line. If a second series of points are determined at another substrate concentration, a second straight line (s_2) will be obtained which cuts the first, this point of intersection being K_i. If competitive inhibition occurs the

V_{max} (intercept on the Y axis) will be he same for s_1 and s_2. In this case however the diethyldithiocarbamate was found to be a non-competitive inhibitor with two different intersections and therefore V_{max} values, and the two lines s_1 and s_2 joining each other on the baseline at K_i = 180 μM. This value differs from that obtained using the previous method but as FIA was employed here a different value may be expected.

INHIBITOR	TYROSINASE		PEROXIDASE	
	C_{50}(mM)	k(nA/min)	C_{50}(mM)	k(nA/min)
Thiourea	0.325	13.75	0.065	37.50
Diethyldithiocarbamate	0.025	19.58	0.750	30.43
Hydroxylamine	No effect		0.160	16.66
Benzoic acid	0.002	8.21	No effect	
2-Mercaptoethanol	0.058	7.79	0.900	66.66
Dichlorophenoxyacetic acid	0.228	8.00	No effect	
Dimethyl mercury	1.200	7.53	No effect	

TABLE 3.1.
Coefficient of inhibition and rate constants for each inhibitor of both enzymes horseradish peroxidase.

It has been suggested [38] that the organic media can affect the binding of inhibitors to the enzyme sites, thus altering the apparent kinetic parameters of the inhibition process. The data shown in Table 3.1. reflect these changes in the inhibition specificity, and illustrate the utility of organic-phase enzyme electrodes for elucidating the inhibition process.

Each inhibitor examined in this study has specific solubility in organic/aqueous solvents (see Table 3.2.). Thiourea was found to be an irreversible inhibitor and was dissolved in ethanol for tyrosinase and acetonitrile for peroxidase. Diethyldithiocarbamate is a slowly reversible inhibitor, and was used dissolved in ethanol for tyrosinase and acetonitrile for peroxidase. It is soluble in both water and alcohol. Hydroxylamine sulphate is an irreversible inhibitor, and as it is water soluble, was used dissolved in 10 % water, 90 % acetonitrile for peroxidase. Benzoic acid is a reversible inhibitor, dissolved in ethanol for tyrosinase, soluble in chloroform, ether and benzene. 2-mercaptoethanol exhibits irreversible action, is miscible with water, alcohol and ether. In this case it was dissolved in acetonitrile. Dichlorophenoxyacetic acid was used, dissolved in acetonitrile for tyrosinase. It is insoluble in water and soluble in organic solvents only. Dimethylmercury is insoluble in water, but soluble in alcohol and ether, and in this case was dissolved in ethanol.

INHIBITOR	TYPE	SOLUBLE IN WATER	SOLUBLE IN ORGANIC
Thiourea	IR	-	+
Diethyldithiocarbamate	R	+	+
Hydroxylamine	IR	+	-
Benzoic acid	R	-	+
2-Mercaptoethanol	IR	Miscible	+
Dichloro-phenoxyacetic acid	IR	-	+
Dimethyl mercury	IR	-	+

TABLE 3.2.

Solubility of each inhibitor examined in water/organic; also shown type of inhibitor IR = irreversible and R = reversible.

3.2.2.3. *Flow injection Experiments*

The attractive dynamic properties of the organic-phase inhibitor enzyme electrode can be exploited for on-line applications, as desired for continuous monitoring of flowing organic streams. An organic-phase flow detector for monitoring phenolic and peroxide substrates was developed recently [44]. The fast response times of these electrodes allow application to flowing streams. Figure 3.5. shows the effect of three inhibitors for tyrosinase with (a) 0.5 mM phenol injections in the absence of inhibitor, (b) 0.5 mM phenol spiked with 0.1 mM diethyldithiocarbamate, (c) 0.5 mM phenol with 0.1 mM thiourea, (d) 0.5 mM phenol with 0.1 mM benzoic acid, and (e) 0.5 mM phenol again showing the renewal of enzyme activity after inhibition. 70.84 % of the initial phenol response was obtained after addition of diethyldithiocarbamate and full activity was recovered after both thiourea and benzoic acid inhibitors. Limits of detection of 33.80 μM, 16.48 μM and 22.6 μM were obtained for thiourea, benzoic acid and diethyldithiocarbamate, respectively with R.S.D. of 2.28 % for n = 7 injections. Such on-line monitoring relies on the decrease of the peak for the phenol substrate (vs. those observed without the inhibitor (A, E)). The fast response to these changes results in a rate of 60 samples/hr. The well-defined response to these 0.5 mM inhibitor solutions indicates convenient flow-injection quantitation of submillimolar and micromolar concentrations. Note also that complete renewal of the enzymatic activity, as indicated from comparison of the substrate peaks before (A) and after (E) this series.

Figure 3.6. displays a flow-injection calibration experiment for diethyldithiocarbamate over the 0-400 μM concentration range. The tyrosinase-based flow detector responds in a non-linear fashion to these changes in the pesticide concentration, showing a decrease in current for constant phenol injections, with solutions spiked with increasing concentration of diethyldithiocarbamate. Also shown in Fig. 3.6. (inset) are the actual peaks in the presence of 0 (a), 80 (b) and 160 (c) μM diethyldithiocarbamate. Linearity extended up to 160 μM with precision of 3.37 % for n = 5 injections and limit of detection of 22.6 μM.

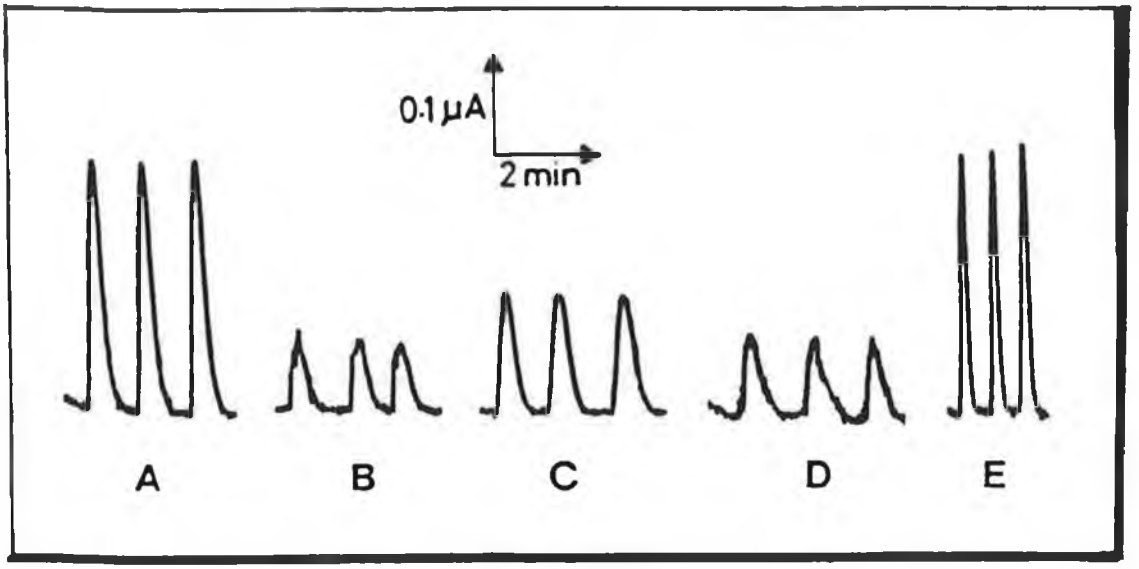

FIGURE 3.5.

Examples of reversible inhibitor effect on 0.5 mM phenol responses, (a) 0.5 mM phenol only, (b) 0.5 mM phenol plus 0.5 mM benzoic acid, (c) 0.5 mM phenol plus 0.5 mM thiourea (d) 0.5 mm phenol plus 0.5 mM diethyldithiocarbamate, (e) 0.5 mm phenol only. Carrier - 0.05 M TEATS in acetonitrile, 2 % water with flow rate 1 ml/min. E_{app} -0.2 V, 1 µA full scale: 0.05 µA/cm, 1.0 min/cm (except (e) 2.5 min/cm),

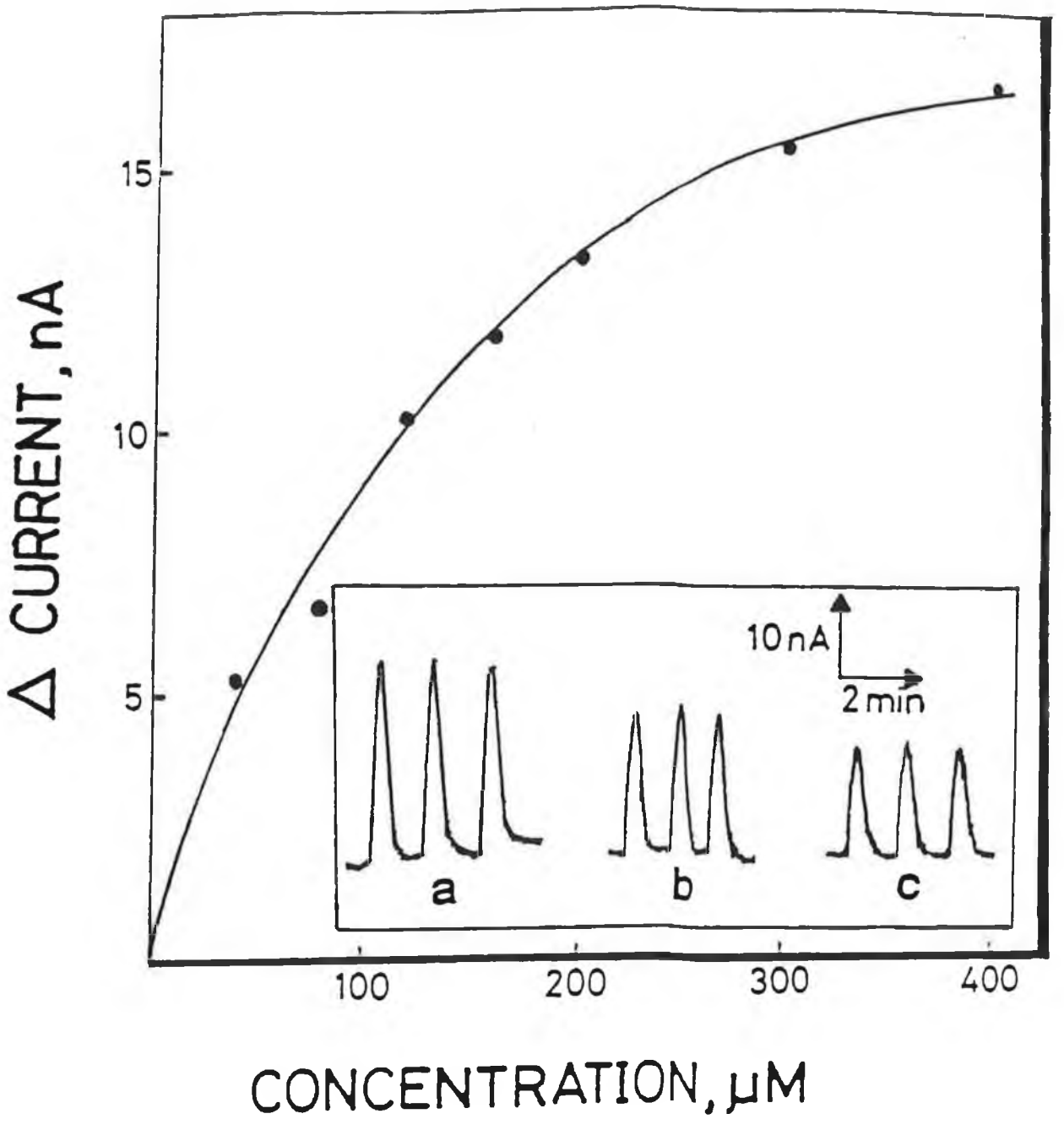

FIGURE 3.6.

Calibration curve for flow injection analysis of diethyldithiocarbamate, with (a), (b) and (c) corresponding to 0.0 mM, 0.12 mM and 0.4 mM inhibitor in the presence of 0.5 mM phenol. 100 nA full scale, 5 nA/cm. Other conditions as Figure 3.5.

In the case of the peroxidase electrode, the solution pH was found to have a profound effect on the stability of the HRP/AQ film. When the aqueous enzyme solution was prepared in phosphate buffer pH = 5.0, more stable films were obtained. This is due to the fact that horseradish peroxidase is positively charged at this pH, and this therefore will result in a more stable interaction between the negatively charged Eastman AQ polymer and the enzyme. It has been reported elsewhere that the enzyme remembers the pH of its last aqueous solution before its application in organic phase [2], and hence retains charge and activity at this pH.

In addition to their promise for exploring enzyme inhibition processes in non-aqueous environments, the organic-phase electrodes may offer several analytical advantages. For example, a judicious choice of the solvent may be useful for enhancing the sensitivity and expanding the linear range of inhibitor sensors (through manipulation of kinetic parameters). Second, the organic-phase operation greatly expands the scope of inhibitor sensors toward previously inaccessible inhibitors. For example, several of the inhibitors examined in this study (e.g. dichlorophenoxyacetic acid, thiourea, benzoic acid or dimethyl mercury) are poorly soluble in water.

3.2.3. CONCLUSION

Organic phase biosensors, suitable for monitoring low levels of enzyme inhibitors in non-aqueous media, have been described. The inhibition of tyrosinase and horseradish peroxidase by various organic compounds was exploited for highly sensitive amperometric measurements in organic media. Eastman organic AQ_{55D} polymeric films were used to entrap the enzymes on the surface of a glassy carbon electrode, resulting in films with great stability in organic media. The cathodic (-0.2 V vs. Ag/AgCl) detection allows the determination of the o-quinone product and the reduction of the oxidised 1,2 phenylenediamine mediator, for tyrosinase and horseradish peroxidase respectively. The different types of inhibitors investigated may be categorised according to their mechanism of action on the enzyme. The coefficients of inhibition, rate constants and the K_i values were calculated for each inhibitor compound. Selectivity may be achieved by selective action of some of the inhibitors on either enzyme alone. Good linearity and limits of detection as low as 1.4 μM, were achieved with applications to flowing streams, employing the enzyme-inhibition based detector and an acetonitrile carrier solution, which allowed fast on-line monitoring of various inhibitors. Sensing advantages accrue from such operations, particularly the applicability to inhibitors with poor water solubility. Solvent-induced changes in the mechanism of inhibition, are also discussed. The latter opens the possibility of tuning the response of inhibitor biosensors.

A major problem encountered in inhibition studies was the non-selectivity exerted by inhibiting compounds. A means of overcoming this problem may involve the use of different tissues e.g. those rich in tyrosinase, banana, mushroom and apple, each containing a slightly different form of the enzyme polyphenol oxidase. Such tissues may exhibit specific individual responses towards inhibiting compounds and can be incorporated into a sensor array system [45]. The mushroom tyrosinase has been studied, and it was demonstrated that enzymes can exist in more than one form within different sections of the mushroom tissue [46]. These forms can display different enzymatic and kinetic characteristics, and using the sensor array, future work could employ electrodes constructed using tissue taken from various parts of the mushroom, in order to investigate differences in activity, substrate and inhibitor specificity in different areas.

An additional extension of this work may be the application of a similar system, employing graphite epoxy electrodes, where the tissues are incorporated

into the resin, allowing for selective inhibition studies to be carried out in the organic phase. In order to detect inhibiting compounds in the presence of metals (which will also exhibit inhibitory action), an additional complexing agent such as EDTA may be added to the sample, in order to complex the metals and to allow for the selective determination of the pesticide of interest. Thiourea has been found to exhibit not only its own inhibitory action but promotes emergence of the inhibitory action of metals e.g. Hg, Cu and Zn [47].

In conclusion, therefore it has been demonstrated for the first time that organic-phase enzyme electrodes may be employed for monitoring inhibitors in non-aqueous environments. A new class of analytes has thus been added to those measured by organic-phase biosensors. The new devices extend the scope of inhibitor biosensors toward many inhibitors with poor water solubility, and offer insights into the inhibition action in organic solvents. Applicability to inhibitor measurement in previously difficult samples is also anticipated. The solvent-induced changes in the inhibitory action holds a great promise for manipulating the response of inhibitor biosensors. While the concept was illustrated with inhibitors of peroxidase or tyrosinase, it could be extended to other enzymes and inhibitors. Analogous organic-phase measurements of enzyme activators should be accomplished in a similar manner.

The increased solubility of organic analytes in organic solvents and their subsequent detection suggest that far more industrial areas can benefit from biosensor technology, e.g. petrochemical industry and the chemical industry. By partitioning an analyte present at very low concentration from a large aqueous volume (e.g. water sample for pesticide analysis) into a small organic volume, many challenging analytes may be determined. In addition, the $K_{m(app)}$ value of the enzyme may be altered, by manipulating the polarity of the enzymes micro environment, and thus affecting the sensitivity and linearity of the inhibitor sensor.

3.3. INVESTIGATION OF THE EFFECTS OF VARIOUS ORGANIC SOLVENTS ON THE ACTIVITY OF IMMOBILISED TYROSINASE USING THE ROTATING DISC ELECTRODE

Interest in biocatalytic conversions in non-aqueous environments continues to grow [9, 39, 48]. The remarkable discovery that enzymes can operate in organic media offers unique opportunities for synthetic organic work together with biotechnological applications, as well as the design of novel biosensors [12]. A key factor for such applications is understanding the biocatalytic behaviour of enzymes in non-aqueous media. Despite numerous studies aimed at obtaining such knowledge, little is still known about the exact role of important factors such as solvent polarity, immiscibility with water and interaction with active site water. Quantitative analysis of the effects of organic solvents on the catalytic activity and substrate specificity are crucial for the development of kinetic models that may be used to predict optimal conditions for enzymatic reactions in organic phase. An improved understanding of the factors influencing enzyme activity in non-aqueous media should facilitate the construction of suitable enzyme electrodes for various organic-phase/substrate systems.

Proper immobilisation of the enzyme on the electrode surface is critical in obtaining correct and reproducible results. However, in organic phase enzymology, this procedure is simplified as the water soluble enzyme will remain firmly adsorbed on the glassy carbon surface. Enzymes in nonpolar solvents are 'rigid'. This rigidity is a result of stronger internal electrostatic interactions and hydrogen bonding and can control not only the accessibility of bulky substrates into the active site but also the positioning of such substrates within the active site [11]. Advantages of carrying out the experiments in organic solvents include no complicated immobilisation schemes, therefore resistance to transport of small molecules through the immobilised enzyme matrix is decreased.

Kinetic models of enzyme-catalysed reactions involving enzymes immobilised by different strategies on rotating disc electrode (RDE) surfaces have been reported [49-54]. These reports have established the mechanism of the enzyme-catalysed reactions in the film together with a kinetic analysis of the process. Shu and Wilson [55] used the RDE to study the mass transfer/reaction kinetics of GOx covalently immobilised on carbon paste with I^-/I_2 as mediator. Kamin and Wilson [56] applied a similar technique to determine diffusion and rate coefficients with a GOx-based biosensor on platinum discs, where they employed direct amperometric measurement of H_2O_2 at the electrode.

Bourdillon *et al.* [57] used GOx and the RDE to study the combined rates of diffusion and reaction by amperometric measurement of the H_2O_2 produced.

These previous reports have dealt with enzyme electrodes in the aqueous phase only. The goal of the present study was to demonstrate the utility of the RDE for *in-situ* probing of biocatalytic conversions in organic media. The well-defined hydrodynamics of the RDE [58,59] allows accurate control of the substrate transport towards the immobilised enzyme layer. By varying the rotation speed (ω) it is possible to make mass transport competitive with the enzyme catalysis rate. Such control of the rate limiting process allows investigation of both surface-catalysed and substrate mass transport controlled reactions. Studies in organic media, as reported in the following sections, allow very useful information to be obtained with respect to biocatalytic processes in non-aqueous environments.

3.3.1. EXPERIMENTAL

3.3.1.1. *Apparatus*

Batch amperometric electrochemical studies were performed with an EG&G Princeton Applied Research Model 264A analyser and a XYT recorder (Model RXY, BAS). The 50 ml glass cell contained the rotating glassy carbon working electrode, and potentials were applied relative to an Ag/AgCl reference, with platinum wire used as auxiliary electrode.

3.3.1.2. *Reagents*

Electrochemical measurements were conducted at room temperature in the respective solvent, containing 0.05 M tetraethylammonium-p-toluenesulphonate (Fluka). Tyrosinase (EC.114.18.1 2400 U/mg) was purchased from Sigma. Phenol was purchased from Baker with chloroform, 2-butanol, 1-propanol and hexane from Aldrich.

3.3.1.3. *Procedure*

The glassy carbon rotating disc electrode was first polished with 0.05 μM alumina and rinsed with distilled water prior to use. 25 μl of 5 mg/ml tyrosinase (170 Units/cm^2 electrode surface) was adsorbed and dried using a heat gun (30 mins). Before use the dried enzyme electrode was immersed in a 0.5 mM

phenol solution (in the appropriate solvent) for 5 mins. This procedure allowed for rapid stabilisation of the baseline and resulted in activation of the surface. After each experiment a new electrode surface was prepared. Upon application of -0.2 V, the charging current was allowed to decay and once steady - state was achieved, the phenol substrate was injected with cathodic detection of the o-quinone product achieved at this constant potential.

3.3.2. RESULTS AND DISCUSSION

3.3.2.1. *Enzyme Model*

The kinetic model applied in this study involves a uniform distribution of the substrate with both diffusion and reaction occurring in the same region within the enzyme layer. Under steady-state conditions, the amount of substrate reaching the region by diffusion is equal to the amount consumed by the enzyme reaction. Here it will be assumed that the concentration of the enzyme on the electrode is uniform and that Michaelis - Menten kinetics describe the enzymatic reaction:

Scheme I

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_{cat}}{\Rightarrow} P$$

$$K_m = \frac{k_{-1} + k_{cat}}{k_1} = \frac{[E][S]}{[ES]} \qquad (3.3)$$

$$\frac{d[P]}{dt} = k_{cat}[ES] = \frac{k_{cat}C_e}{K_m/[S] + 1} \qquad (3.4)$$

where C_e (= [E] + [ES]), is the concentration of active enzyme immobilised within the enzyme layer, and $k_{cat}C_e$ represents the maximum rate of product formation.

Tyrosinase (polyphenol oxidase), which catalyses the ortho-hydroxylation

reactions of mono-phenols and oxidation of the corresponding o-hydroxyphenols to quinones, was selected for this study. This enzyme is well known for its activity in various organic media [60-62]. Scheme II shows the catalytic conversion of the phenol substrate by dissolved oxygen, to catechol (Rxn 1) and then to the corresponding quinone product (Rxn 2). Upon application of -0.2 V vs. Ag/AgCl the quinone product may be reduced back to catechol at the electrode surface (Rxn 3) [60, 63].

Scheme II

$$\text{(1)}\quad \text{Phenol} + O_2 + 2H^+ \xrightarrow{\text{Tyr}} \text{Catechol}$$

$$\text{(2)}\quad \text{Catechol} + O_2 \xrightarrow{\text{Tyr}} \text{o-benzoquinone}$$

$$\text{(3)}\quad \text{o-Benzoquinone} \xrightarrow{\text{-0.2 V}} \text{Catechol}$$

The response of such an immobilised tyrosinase electrode is dependent on the rate at which electroactive species reach the underlying electrode surface. This is determined by mass transfer of substrate and product within the enzyme layer. By using forced convection techniques mass transfer is well defined and under experimental control.

The concentration polarisation profiles for the substrate and product are determined by the balance between diffusion of species within the layer and the kinetics of the enzyme reaction. Here, there is no concentration polarisation of substrate within the enzyme layer. This is due to the fact that the enzyme film is thin and the diffusion of phenol is therefore assumed to be fast.

The fact that Scheme II involves three steps complicates the steady-state kinetics. However, since the quinone product is generated and subsequently reduced at the electrode surface (Scheme II, Rxn 3), the regenerated catechol rejoins the enzymatic sequence (Rxn 2). Being an electron transfer process the reduction of quinone is fast and non rate-limiting at the applied voltage [17]. Moreover, as the amount of enzyme adsorbed on the electrode surface is small (170 U/cm^2 electrode surface), all reactions in Scheme II take place close to the electrode surface and the mobility of reaction species is not limited by thickness

of the enzyme layer or by the immobilisation matrix. Under these conditions, the response of the simple tyrosinase electrode depends on the rate of Rxn (1) of Scheme II. Therefore Scheme I can be used to characterise the sensors.

Our experimental strategy was to determine the tyrosinase steady state kinetic constants I_{max}, K_m', and $dk_{cat}C_e/K_m'$ (catalytic efficiency) in the presence of different organic solvents with varying degrees of hydrophobicity. Chloroform was found to be the best choice of solvent in this case as it is a good phenoxy radical quencher [64]. In aqueous environments polar products tend to remain in the vicinity of the enzyme and can cause product inhibition or can undergo unwanted side reactions, in this case spontaneous polymerisation of the quinones.

3.3.2.2. *Rotating Disc Experiments at Constant Potential*

Figure 3.7(A) and (B) show calibration curves for phenol using the tyrosinase immobilised electrode in the solvents chloroform and 2-butanol respectively. Each point represents the average of three measurements at each speed. We can see that as rotation speed increases after 900 rpm for chloroform and 1600 rpm for 2-butanol, a decrease in current (i_{ss}) occurs.

Under mass-transfer rate-limiting conditions, the maximum rate of solute diffusion to the surface occurs when the difference in concentration of solute between the bulk solution and the electrode surface is at a maximum. This occurs when the concentration at the surface is essentially zero. Under these diffusion-controlled conditions for a rotating immobilised enzyme electrode, the steady state current as defined by Shu and Wilson [55] is given by the following equation.

$$i_{lim} = 0.62nFACD^{2/3}\nu^{-1/6}\omega^{1/2} \qquad (3.5)$$

In eqn. (3.5), ν is the kinematic viscosity of the solvent (3.91×10^{-3} cm^2 s^{-1} and 5.22×10^{-2} cm^2 s-1) for chloroform and 2-butanol, respectively), C is the substrate concentration (1.6 and 5.0 mM phenol for chloroform and 2-butanol, respectively), the limiting current (I_{lim}) is 1.35 μA (chloroform) and 0.2 μA (2-butanol), ω represents the optimum rotating speed (900 rpm for chloroform and 1600 rpm for 2-butanol), A represents the electrode area (0.4418 cm^2) and the other terms have their usual meaning. The expression will hold at low substrate concentrations under diffusion limited conditions. The diffusion coefficient values calculated from eqn. (3) were found to be higher in chloroform (1.49×10^{-9} cm^2 s^{-1}) than in 2-butanol (5.61×10^{-12} cm^2 s^{-1}). This result shows a correlation between D values and the type of response shown in Fig. 3.7. 2-

Butanol (Fig. 3.7(B)) with a lower D value than chloroform exhibits lower currents at all rpm.

According to Scheme II catechol is regenerated (in Rxn 3) at the same time as it is produced in Rxn (1). This leads to an increase in catechol concentration. The positive deviation of the low rpm currents from linearity, with increasing substrate concentration in Fig. 3.7(B), suggests that the accumulated catechol undergoes conversion to o-benzoquinone. However, at high speed the catechol is lost to the bulk solution so that the catechol concentration is only a fraction of that due to Rxn (1) Scheme II. Thus the experimental (steady-state) current decreases, and approaches linearity as ω increases. This applies to 2-butanol in which catechol is expected to have a low diffusion coefficient, (since the diffusion coefficient for phenol of comparative molecular weight is low).

The diffusion coefficient of phenol in chloroform is by more than two orders of magnitude higher than that in 2-butanol. Consequently, the accumulation of catechol observed for 2-butanol does not occur in chloroform, so that the controlling factor at high substrate concentration is enzyme saturation. At higher speed more catechol is lost to the bulk solution resulting in the lowering of i_{ss} and an extension of linearity.

Figure 3.8 shows the Lineweaver-Burk plots for the data of Fig. 3.7. Table 3.3 shows the calculated K_m' and I_{max} values in the various solvents employed. As may be seen K_m' values are higher in the case of 2-butanol and in both solvents K_m' values decrease as ω increases.

Figure 3.9 shows the Levich plots (eqn (3.5)) of limiting current vs. $\omega^{1/2}$ for enzyme electrode operation in chloroform (A) and 2-butanol (B). The dependence of current on ω reflects the relative effects of diffusion and catalysis. The current passes through a maximum at a characteristic rotation speed. The position of this maximum is dependent on $k_{cat}C_e/K_m'$, which is a measure of the catalytic efficiency [55]. When catalysis is slow the value of $k_{cat}Ce/K_m'$ is small, and as it increases $k_{cat}C_e/K_m'$ also increases. Operating at the maximum rotating speed for each solvent improves the sensitivity of the electrode. After 900 rpm in chloroform and 1600 rpm in 2-butanol the current decreases with increasing rotation speed. As ω increases most of the total product within the enzyme layer escapes to the bulk solution bringing about a decrease in the concentration of quinone at the electrode surface. At low speed the reaction is not limited by concentration of product but by the diffusion of substrate, because any quinone formed is consumed in the fast electron transfer reactions.

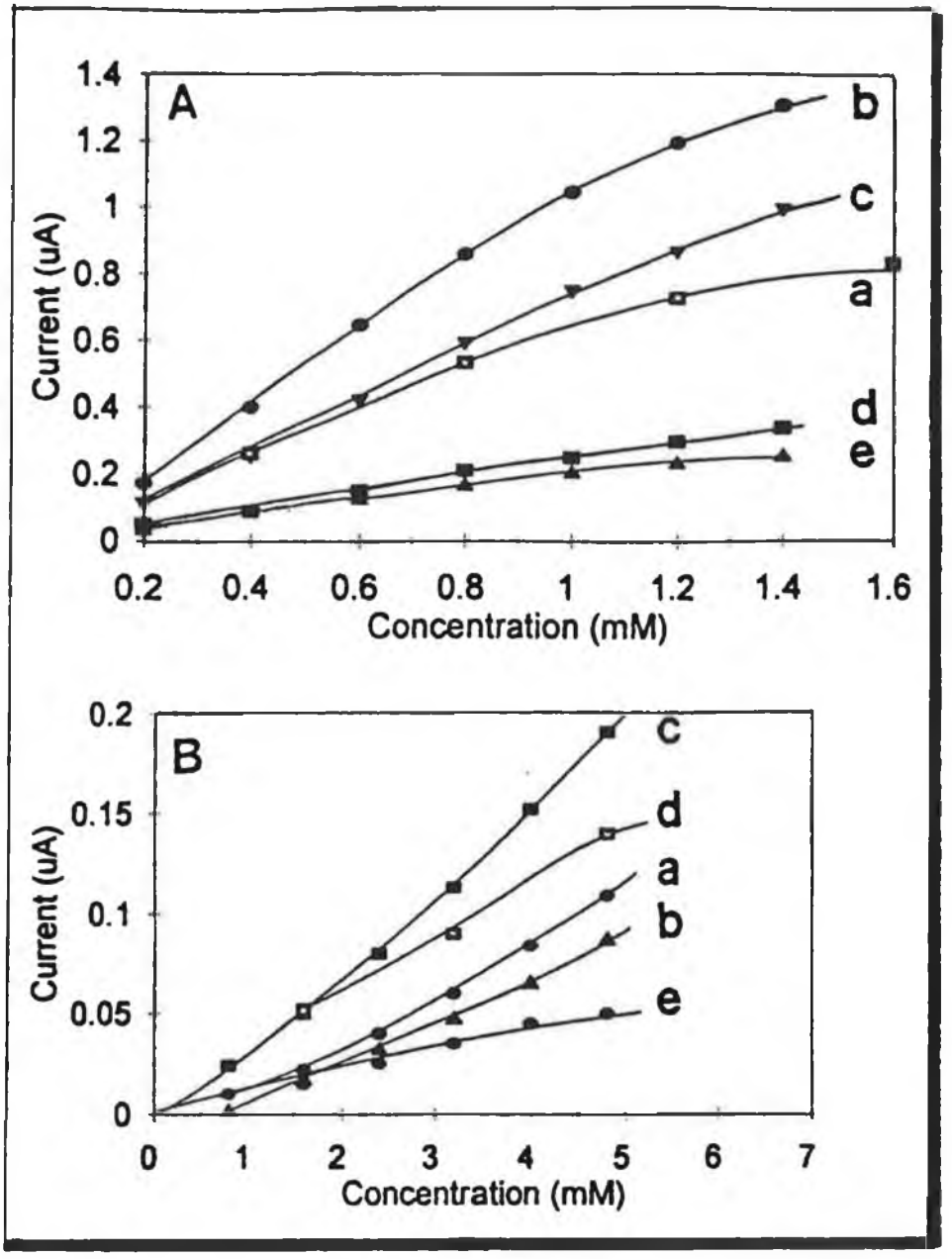

FIGURE 3.7.

(A) Calibration curves for successive 0.2 mM phenol additions at (a) 400, (b) 900, (c) 1600, (d) 2500 and (e) 3600 rpm in chloroform at the tyrosinase modified electrode. Electrolyte : phosphate buffer saturated chloroform containing 0.05 M TEATS. Enzyme activity on surface - 170 U/cm^2. Eapp -0.2 V, sensitivity 20 μA full scale (0.1 V/cm), 2.5 min/cm.

(B) Calibration curves for successive 0.8 mM phenol additions in 2-butanol containing 0.05 M TEATS, again for different ω (a) 400 - (e) 3600 rpm. Sensitivity - 2 μA full scale (0.1 V/cm). Other conditions as (A).

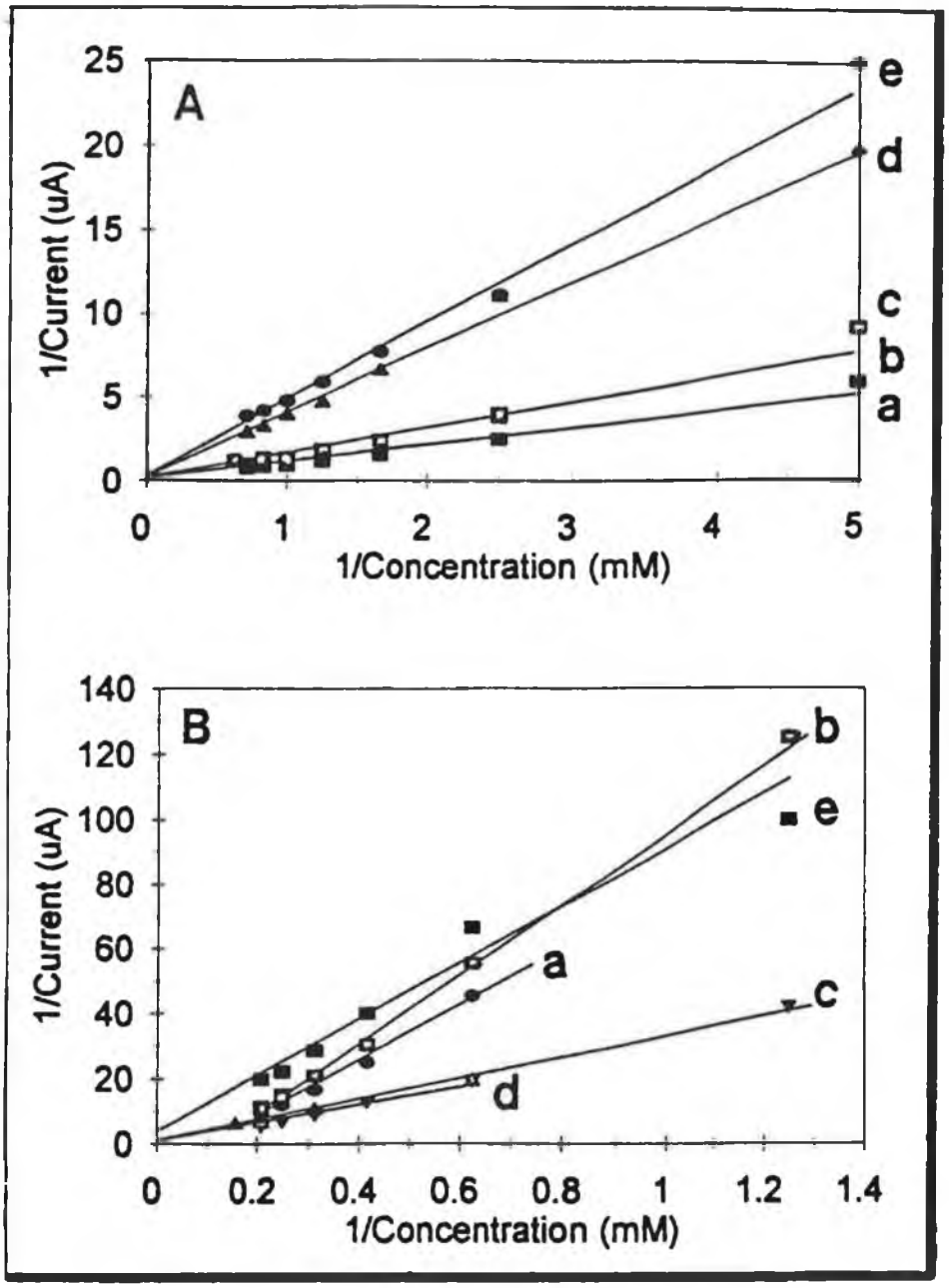

FIGURE 3.8.

Lineweaver - Burk plots for electrode response at different speeds in (A) chloroform and (B) 2-butanol, from same data as Fig. 3.7. Conditions as in Figure 3.7.

SOLVENT	SPEED (rpm)	K_m' (mM)	I_{max} (μA)
Chloroform	400	2.78	2.0
log P = 2.0	900	11.75	10.0
	1600	9.44	5.0
	2500	7.80	2.0
	3600	8.96	1.8
2-butanol	400	87.17	1.0
log P = 0.8	900	84.83	0.8
	1600	27.98	0.8
	2500	29.86	1.0
	3600	15.55	0.2
Hexane	400	0.14	0.07
log P = 3.5			
1-propanol	400	78.25	1.33
log P = 0.05	900	37.50	0.80

TABLE 3.3.

The solvent and rotation speed effects on the K_m' and I_{max} of the immobilised tyrosinase. Conditions as Fig. 3.7.

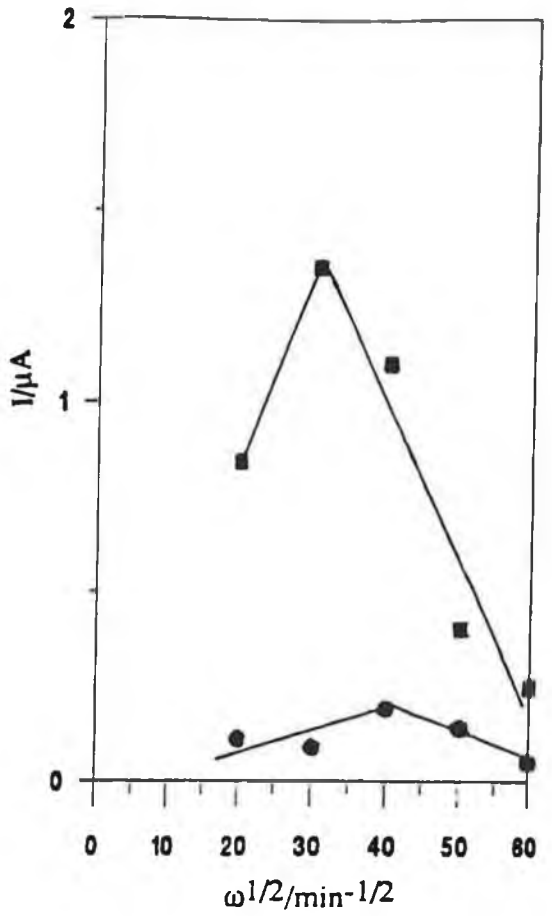

FIGURE 3.9

Levich plots for limiting currents vs. $\omega^{1/2}$ in solvents, (■) chloroform, (●) 2-butanol. Currents taken at 1.6 mM and 5.0 mM phenol respectively. Other conditions as Figure 1. (Lines represent the trend between current and $\omega^{1/2}$).

3.3.2.3. *Comparison of activity in various organic solvents*

Figure 3.10 shows a comparison of phenol calibration curves for the different solvents employed at constant rotating speed (900 rpm). Chloroform as reaction medium exhibited the greatest sensitivity, but a very narrow linear range. 2-butanol showed extended linearity but was less sensitive. 1-propanol was found to exhibit a similar response to that of 2-butanol.

It has been suggested that the activity of enzymes in organic media may be correlated to the polarity of the solvent. A common measure of solvent polarity is the log P value [66], defined as the ratio of equilibrium concentrations of the solvent in octanol and water phases of the biphasic system. Higher log P values reflect increasing solvent hydrophobicity. The effect of solvent polarity on the catalytic efficiency ($dk_{cat}C_e/K_m'$) may be seen in Figure 3.11. A sigmoidal type curve is obtained with levelling off occurring at high solvent hydrophobicity (e.g. in solvents such as chloroform). This agrees with the results of Laane *et al.* who showed that catalytic efficiency increases as hydrophobicity of the solvent

increases [66]. Solvents with high log P values are therefore more suitable for the tyrosinase activity. The reason that enzymes suspended in hydrophobic solvents appear to exhibit greater activity than those in hydrophilic ones, may be due to the fact that there is a difference in the distribution of water between the enzyme and the medium in viscous solvents. Hydrophilic solvents do not give up water easily to the enzyme, whereas enzymes can readily strip water from hydrophobic solvents. Hence depending on the hydrophobicity of the solvent, the same water content in it results in entirely different amounts of water on the enzymes. One may conclude therefore that the enzymatic activity in organic media is primarily determined not by interactions of the solvent with the enzyme per se but by those with the water on the enzyme [66]. Other solvents investigated (including toluene, ethyl acetate, tetrahydrofuran, dioxane, acetonitrile, methanol and dimethylsulphoxide) did not allow for any biocatalytic activity.

The water content in the solvent may also have a profound effect upon organic phase biocatalytic reactions. Figure 3.12(A) shows the effects of percentage water in the solvent 2-butanol on the mean steady state current response. A gradual increase in current was observed followed by a sharp decrease after 3 % water. Our results suggest that a minimal amount of water is required by an enzyme for maximal activity. In its undiluted form 2-butanol which is a hydrophilic organic solvent is expected to strip active site water from the enzyme, exhibiting a lower current compared to that of chloroform (see Fig. 3.7).

I_{max} for the enzyme electrode reaction which follows Scheme I can be shown [67] to be related to k_{cat} as follows:

$$I_{max} = 1/2\ nFAdk_{cat}C_e \qquad (3.6)$$

$dk_{cat}C_e$ values for different 2-butanol/water mixtures calculated from I_{max} values are shown in Fig. 3.12(B). As water content of the solvent increases its ability to adsorb active-site water reduces, leading to an increase in $dk_{cat}C_e$ of the sensor. The largest $dk_{cat}C_e$ (see Fig 3.12(B)) value is shown at a dilution level of the solvent after which water starts forming H-bonds with the phenol reducing the amount available for the enzyme catalysis. This drastically reduces the tyrosinase biosensor catalytic efficiency.

Since high K_m' values indicate greater tendency of the ES complex in Scheme I to dissociate into enzyme and substrate, then $dk_{cat}C_e/K_m'$ may be used as an indicator of the catalytic or kinetic efficiency of the sensor. According to Fig. 3.12(C), the tyrosinase biosensor shows maximum response in 2-butanol

containing 3 % water; after which the catalytic efficiency drops sharply. It is important to mention here that d and C_e were kept constant throughout the experiment and tyrosinase was stable for several days in all the butanol/water mixtures used for this study. Our results suggest that a minimum amount of water is necessary for response of the sensors (see Fig 3.12(A)). The catalytic efficiency of each enzyme studied in a similar experiment by Zaks and Klibanov [11] was found to increase as the percentage water in the solvent increases.

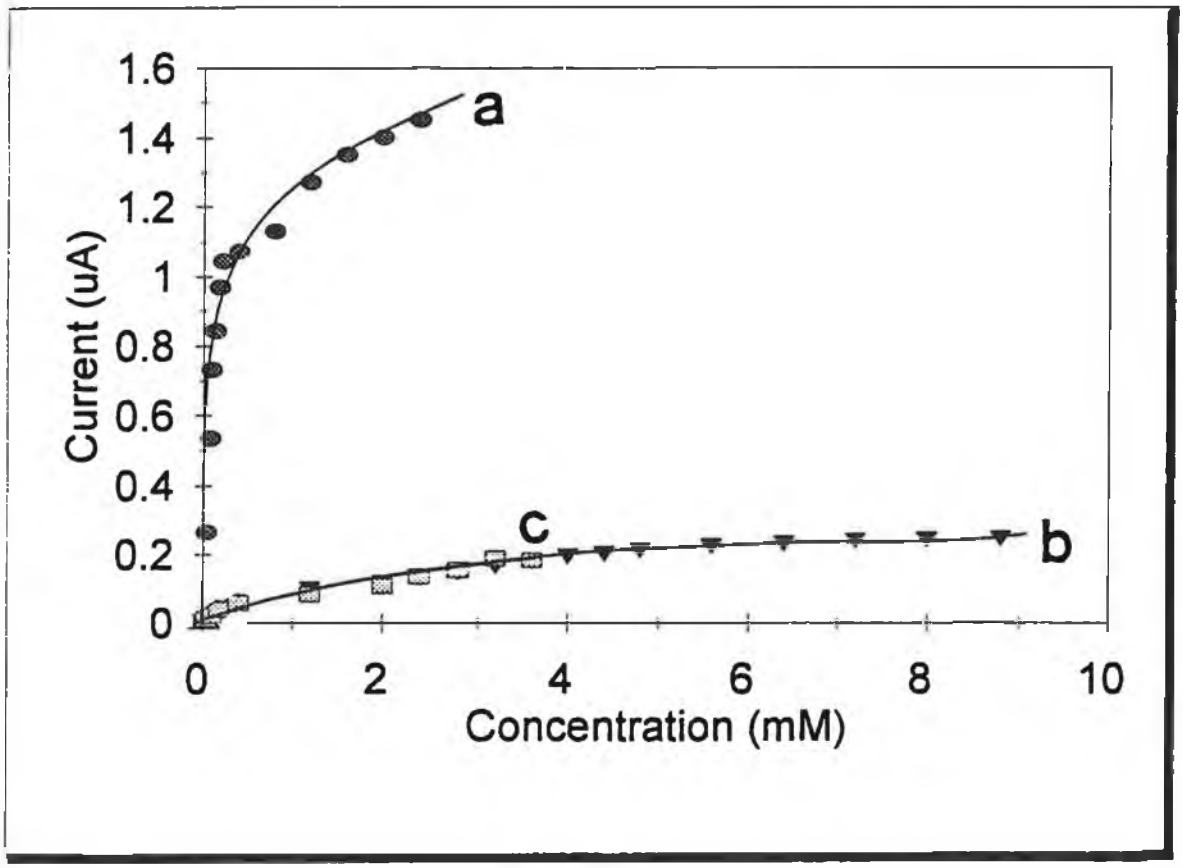

FIGURE 3.10.

Comparison of current responses for the different solvents at constant rotating speed (900 rpm) in (a) chloroform, (b) 2-butanol and (c) 1-propanol. Injection of (a) 0.2 mM phenol, (b) and (c) 0.8 mM phenol.

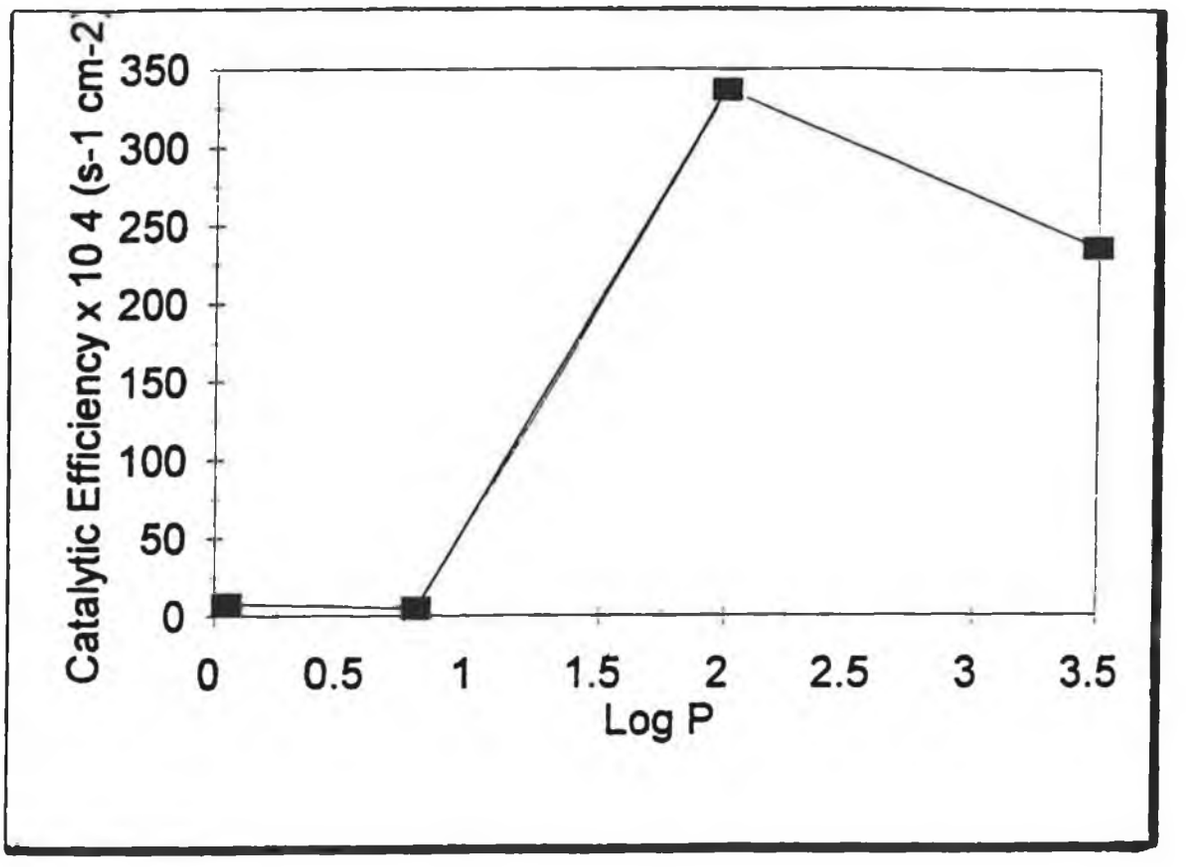

FIGURE 3.11.

Plot of catalytic efficiency ($dk_{cat}C_e/K_m'$) for increasing solvent hydrophobicity (log P). $dk_{cat}C_e/K_m'$ values were determined from Lineweaver-Burk plots at 400 rpm in four different solvents. Log P values are: 1-propanol 0.05, 2-butanol 0.8, chloroform 2.0, hexane 3.5. Conditions as Fig. 3.7.

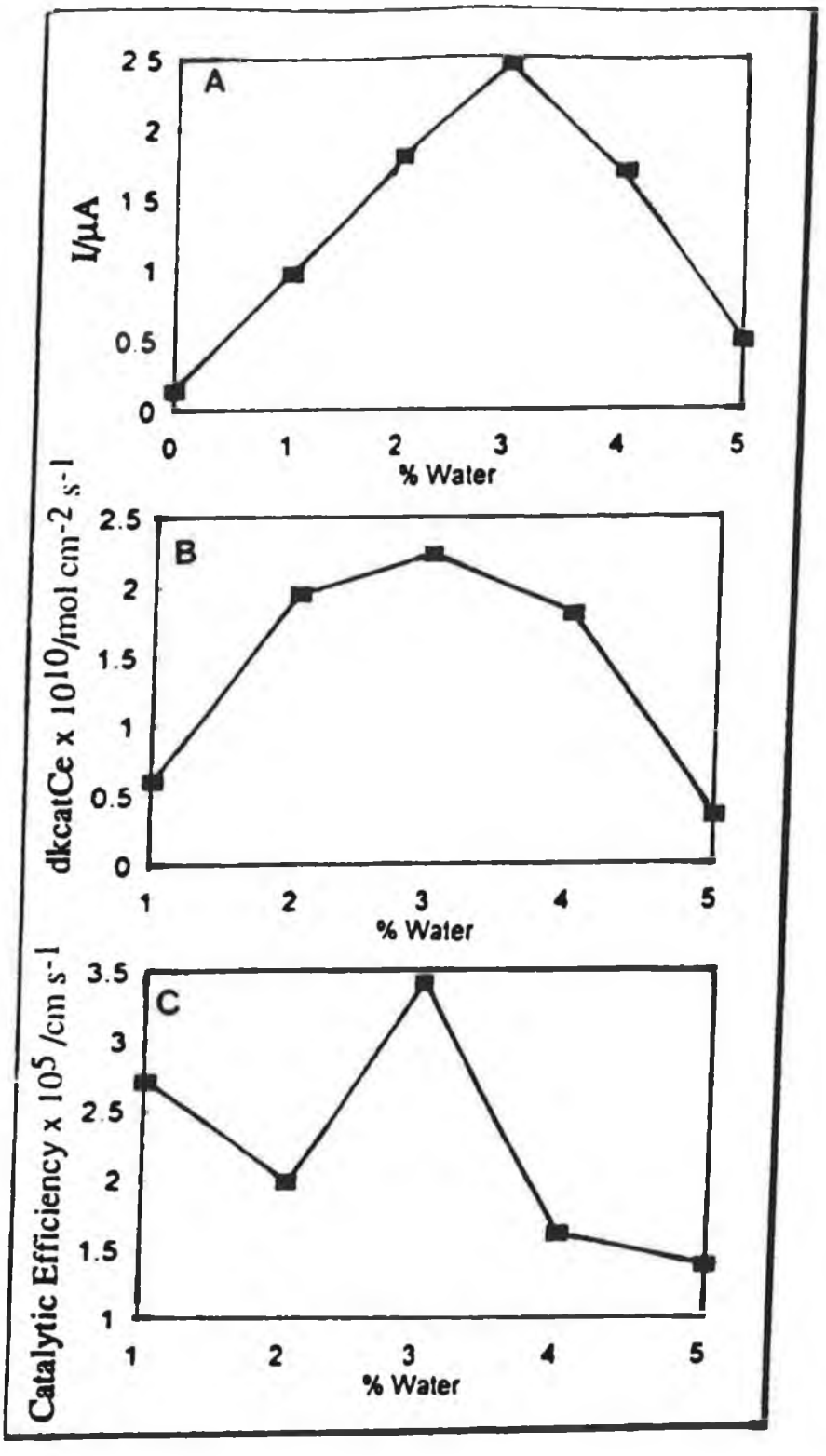

FIGURE 3.12.

The effect of increasing the 2-butanol percentage water content on :

(a) current response at 6.4 mM phenol, (b) $dk_{cat}C_e$, and (c) catalytic efficiency $dk_{cat}C_e/K_m'$. Electrolyte 0.05 M TEATS, ω = 400 rpm, sensitivity 50 μA full scale, 0.1 V/cm, 2.5 min/cm, injection of 0.8 mM phenol.

3.3.3. CONCLUSION

A tyrosinase immobilised rotating disc electrode was evaluated in terms of catalytic performance in various organic solvents. Immobilisation was achieved via adsorption of the enzyme onto the glassy carbon surface. The amperometric detection of the o-quinone product served to measure the rate of the reaction. The effects of ω on sensitivity and linearity were investigated. Kinetic parameters including K_m' and catalytic efficiency were compared in various solvents. The effects of both solvent hydrophobicity, and the extent of 2-butanol hydration, on the enzyme activity were also evaluated.

The major factor in determining enzymatic activity in non-aqueous media stems from the ability of a particular solvent to partition the essential water from the enzyme. The loss of the essential water has a detrimental effect on enzymatic activity, but when replenished the catalytic activity is restored. In hydrophobic solvents water tends to partition into the enzyme such that even very small concentrations of water in the solvent yield up to 30 % water on the protein. However additions of water to hydrophilic organic solvents results in relatively moderate increases in the concentration of water on the protein [68].

In order to take advantage of the novel opportunities afforded by non-aqueous enzymology it is imperative to understand such basic features and characteristics of this phenomenon as the dependence of enzymatic properties on the nature of solvent, amount of water required for catalysis, catalytic parameters and conformational stability of enzymes in organic solvents. Elucidation of these issues in addition to its biotechnological importance should provide profound insights into such questions as protein folding, dynamics, role of water in enzymatic catalysis, stability and protein intramolecular interactions and mobility.

Our objective in this research has been to determine I_{max}, K_m' and $dk_{cat}C_e/K_m'$ values in the presence of different organic solvents, each with varying degrees of hydrophobicity. The highest catalytic efficiency was found to be achieved when using the solvents chloroform and 2-butanol. The more hydrophobic chloroform exhibits the greatest current response but a narrow linear concentration range, with the more hydrophilic 2-butanol giving a wide linear range but reduced current response. K_m' values were lower in chloroform, reflecting the greater affinity of the substrate for the enzyme in a more hydrophobic medium.

Using the rotating disc electrode it is possible to measure the rate of catalytic reaction and to extract pertinent kinetic parameters. The technique provides a convenient method for separating mass transport and kinetic control of

surface catalysed reactions by well-defined variations of hydrodynamic conditions. Mass transport can always be made sufficiently rapid such that the observed currents will depend on the catalysis rate. The principal advantage of the rotating disc electrode for the study of surface bound catalysts is the ability to control the rate of external substrate mass transfer to the catalytic layer, both reproducibly and under well-defined conditions. By varying the ω the effect of substrate mass transfer on enzymatic catalysis may be investigated. The RDE provides an ideal system for investigating the kinetic behaviour of an immobilised enzyme.

Finally therefore we have studied the effect of mass transport on the cathodic current response of the immobilised tyrosinase to substrate (phenol) additions. Lineweaver-Burk plots allow for calculation of corresponding kinetic data when the following conditions were varied, ω, solvent hydrophobicity and percentage water in the solvent. Applicability to other enzyme and solvent systems is anticipated.

3.4. FUTURE POSSIBILITIES FOR ORGANIC-PHASE ENZYME ELECTRODES

The early approach by Connor *et al.* [69] involved the incorporation of tyrosinase in silicone grease, which was used to fill the surface micropores on a graphite surface. An extension of this work may be had using a similar immobilisation technique but employing the open-pore carbonaceous material known as Reticulated Vitreous Carbon (RVC). Because of it attractive electrochemical, mechanical and hydrodynamic properties, RVC has been widely used as a working electrode material [70]. Enzyme electrodes, based on RVC supports have also been reported [71,72] with two dimensional bioelectrode surfaces obtained by filling the voids of RVC with the biocomponent.

High quality polypyrrole films were first achieved by Diaz *et al.* [73] using the tetraethylammonium tetrafluoroborate (TEATS)/acetonitrile electrolyte under galvanostatic conditions. Polymerisation from aqueous solutions were successful but films grown from acetonitrile were of a higher quality. Such films may be employed for immobilisation of enzymes in organic media. The film may either be grown in non-aqueous solvents and the electrode used in aqueous media or *vice versa*. The use of enzymes in organic solvents is a new and developing area and new methods of stable immobilisation are required.

A comparison of the activity of enzymes in aqueous and non-aqueous

solvents is required as sometimes an enhancement in response may be observed. In addition new enzymes which retain activity in non-aqueous solvents may be discovered; of particular interest are those which exist in the body within lipid layers/bound by membranes and act on hydrophobic substrates.

Investigation of the enhancement of thermal stability of enzymes in organic media is necessary. Water is required for the inactivation processes at high temperatures and as enzymatic catalysis in organic media obeys the conventional property of enhanced reaction rates at elevated temperatures, this factor is therefore a useful advantage in obtaining optimum enzyme activity.

The relationship between log P values and/or dielectric constants of solvents and the action of specific enzymes may be examined; additional factors which may influence the choice of solvent include solvent density and viscosity, surface tension and water content.

The use of enzymes in organic synthesis will continue to focus on novel methods of regioselective and asymmetric synthesis, particularly in the areas of hydrolytic reversals, and general redox reactions; stereochemical effects of enzymes in organic media may be studied, which are important for regioselective reactions and resolution of racemic mixtures.

Alteration of observed substrate specificity may be investigated e.g. in the case of transesterification, here only the wet lipase catalyses the transesterification between tributyrin and bulky tertiary alcohols, whereas smaller primary and secondary alcohols were found to be substrates for either enzyme preparation; organic solvents also affect the binding of substrates to the active site by altering the $K_{m'}$ e.g. chymotrypsin prefers amino acid substrates, in aqueous solutions these compounds partition into the hydrophobic active site. In non-polar organic media, this partitioning decreases and K_m increases.

Diffusional limitations must be eliminated and the intrinsic kinetics of enzymatic catalysis in various solvents measured; it is through this approach that direct comparisons between various organic solvents and water can be made and interferences to alterations in protein structure, substrate binding, catalytic efficiency and reaction specificity in organic media can be developed. A kinetic and mechanistic description of enzymatic catalysis in organic solvents with different water content is needed. Electron Spin Resonance is a useful analytical tool for examining conformational changes that occur in going from aqueous to non-aqueous media.

The term 'organic solvent' now includes gaseous and supercritical fluids; application of new biocatalysts in these medias, either through recent isolation or genetic and protein engineering in organic media, will open up new areas of research.

With all the diversity in structure and function in proteins many similarities in their behaviour in non-aqueous media have been observed. They require only a small amount of water to retain activity, they are thermostable when placed in a suitable organic solvent, they possess the same unique features in non-aqueous environments as in aqueous solutions, including pH dependence, strict substrate specificity, stereo and regioselectivity. Enzymatic catalysis in organic solvents is not limited to a number of obscure proteins, but in fact is a general phenomena which will significantly facilitate the understanding and utilisation of biological catalysts in the future.

The research discussed so far has involved the use of enzymes in aqueous and non-aqueous media. The next chapter will deal with the application of antibodies in the development of highly specific sensors.

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CHAPTER 4

DIFFERENTIAL PULSE VOLUMETRIC DETERMINATION OF 7-HYDROXYCOUMARIN IN HUMAN URINE AND DEVELOPMENT OF AN ANTIBODY-BASED AMPEROMETRIC BIOSENSOR TO STUDY ITS REACTION WITH THE SPECIFIC ANTIBODY

4.1. INTRODUCTION

Coumarin (1,2-benzopyrene) is of significant clinical importance due to its use in the treatment of many disease states [1,2]. It has been used in clinical practise, in post-thrombotic syndromes for treatment of varicose veins and in post-traumatic edema. Coumarin has also been shown to activate macrophages *in vitro*. This property has also been utilised clinically in the treatment of high protein lymphodemas. Activation of macrophages gives rise to an augmentation of the immuno response. This has resulted in the use of coumarin in cancer therapy and has also been found to have an inhibitory effect on the induction of cancer [3,4].

Coumarin is metabolised initially by a specific cytochrome P-450 system resulting in hydroxylation at positions 7 or 3. In primates, man and baboons, he major metabolite formed is 7-OH-coumarin. Moran *et al.* [5] showed that on average 63 % of a total dose of 200 mg coumarin was recovered as 7-OH-coumarin in the urine of volunteers over a 24 hour period. It may be inferred from this fact that a very large proportion of the dose is excreted in the urine within 24 hours of administration.

Because of its therapeutic significance it has recently been the focus of intense bioanalytical study. Many methods have therefore been developed for the determination of coumarin and its derivatives. These 3- and 7-hydroxycoumarins are known to fluoresce in alkaline solution and are usually analysed by spectrofluorimetry [6]. A sensitive liquid chromatographic method was also developed for the analysis of 4-hydroxycoumarin anticoagulant rodenticides in blood with fluorescence detection [7]. A rapid and sensitive HPLC assay was also developed for the determination of coumarin, 7-OH-coumarin and its glucuronide conjugate in urine [8]. TLC has been used together with GC [9], HPLC [10] and spectrophotofluorimetry [11] for the separation and analysis of coumarins. Pingarron *et al.* described an electroanalytical study of the oxidation processes of umbelliferone at a glassy carbon electrode in micellar solution and emulsified medium by different voltammetric techniques [12].

As discussed in Chapter 1 antibody based biosensors provide a sensitive and highly selective alternative to enzyme-based systems when specific enzymes are unavailable. In this study an antibody based biosensor will be employed for the amperometric determination of 7-OH-coumarin. There are numerous means of studying antibody-antigen interactions and antibody specificity. These include immunodiffusion, ELISA (enzyme-linked immunosorbent assay), EMIT, radio immunoassay, dialysis studies and blotting techniques [13]. The development of

the Biacore system [14] offers a dynamic 'real-time' method of monitoring antibody-antigen reactions. Recently, many other sensor systems have been described which use antibody-based methods [15].

In biosensor technology, specific recognition is based upon chemical binding of a single analyte to a biological molecule, such as an antibody or an enzyme [15]. An antibody, being a protein, has, in an aqueous environment, a net charge associated with it. The polarity and magnitude of this charge depends on the isoelectric point of that protein and on the ionic composition of the solvent. If both antibody and antigen have a net electrical charge, and antibody-antigen binding is by Van der Waals, hydrogen bonding and electrostatic forces, the electrical charge of the resulting complex will be different from that of the antibody alone. Therefore, the means to detect this binding process by electrochemical methods, may be achieved either by measuring the changes in charge at the electrode surface, the changes in potential arising from the antibody-antigen interaction [16-18], or by monitoring the decrease in current of the antigen (or antibody) upon binding to the corresponding antibody (or antigen) [19-23].

The first section of this work describes an alternative method for the determination of 7-OH-coumarin based on its oxidative behaviour at a glassy carbon electrode. In the second section, the same voltammetric method is employed to study the interaction of 7-OH-coumarin with its specific antibody, based on the decrease in the current observed for the metabolite at a glassy carbon electrode containing the antibody entrapped behind a cellulose dialysis membrane.

4.2. DIFFERENTIAL PULSE VOLTAMMETRY OF 7-HYDROXYCOUMARIN IN HUMAN URINE

4.2.1. EXPERIMENTAL

4.2.1.1. *Apparatus*

A conventional three electrode system, with platinum gauze as counter, glassy carbon as working and saturated calomel as reference electrodes were connected to an EG&G PAR Model 174A polarographic analyser and the output currents measured using a Philips Model PM 8251 recorder. Cyclic voltammetry was performed using an EG&G PAR Model 264A polarographic analyser connected

to a JJ Instruments Model PL4 recorder. A batch mode of operation was employed throughout the differential pulse and amperometric experiments with a magnetic stirrer and bar providing the convective transport. During the temperature controlled studies, the cell was immersed in a thermostatically controlled water bath.

4.2.1.2. *Electrode Preparation*

Before use the bare glassy carbon electrode was polished firstly with alumina slurry, after which it was sonicated in distilled water, followed by electrochemical pretreatment (cycling between -1.5 V and +1.5 V for 4 min at 50 mV/sec).

4.2.1.3. *Reagents*

Batch experiments were conducted at room temperature in 0.1 M phosphate buffer (pH 7.4) and all solutions were prepared using deionised water obtained by passing distilled water through a Milli-Q water purification system. 7-OH coumarin (Umbelliferone) was purchased from Sigma. Potassium dihydrogen phosphate and dipotassium hydrogen phosphate were obtained from Riedel-de-Haen. Diethyl ether and methanol (HPLC grade) was obtained from Lab Scan Ltd. Dublin, Ireland.

4.2.1.4. *Procedures*

4.2.1.4.1. *Electrochemical Behaviour of 7-OH-Coumarin*

The electrochemical behaviour of the metabolite was first investigated at the bare glassy carbon electrode (previously activated as described above), by immersing the electrode in 20 ml phosphate buffer (pH 7.4). All measurements were performed in quiescent solution with the exception of the DC voltammetric experiments. In the case of differential pulse voltammetry, the potential range was set between 0.0 V and 1.5 V with a scan rate of 5 mV/sec. An oxidation peak for 7-OH-coumarin was observed under these conditions at 0.66 V vs. SCE. Cyclic voltammetry confirmed the irreversible oxidation peak at this potential. For DC experiments a constant potential of 0.66 V was applied to the electrode and transient currents allowed to decay prior to injection of the analyte into the solution.

4.2.1.4.2. *Analysis of 7-OH-Coumarin in Urine samples*

The determination of this metabolite in urine was investigated using a modification of the extraction technique developed by Egan *et al.* [8]. I ml of urine was spiked with the appropriate volume of 7-OH-coumarin and the sample extracted with 3.5 ml diethyl ether by inversion for 10 mins. The mixture was then centrifuged at 3,000 rpm for 10 min, 2.2 ml of the organic layer removed, evaporated to dryness under nitrogen, and reconstituted with 200 µl methanol. This was then centrifuged at 3,000 rpm for 5 min and 120 µl of the supernatant transferred to a clean glass tube. 50 µl of the final sample was then injected in to a 5 ml cell for electrochemical analysis. The initial spiking range of 0 to 15 mM resulted in a final concentration in the cell of 3.3×10^{-5} to 4.9×10^{-4} M after extraction.

4.2.2. RESULTS AND DISCUSSION

4.2.2.1. *Anodic Voltammetric Behaviour of 7-OH-Coumarin*

The main reason for the relative unpopularity of solid electrodes among electroanalytical chemists is the fact that it is normally difficult to obtain reproducible results owing to the different number of active sites available at the electrode surface for successive experiments. Electrochemical conditioning of the glassy carbon electrode results in the formation of electroactive surface compounds which enhance the electrode response via unspecified redox mediation processes [24,25]. Cycling the potential from -1.5 V to +1.5 V appears to produce a relatively non-specific enhancement of electrode response for a wide range of irreversibly oxidised compounds [26,27]. Electrochemical pretreatment of the electrodes was found to improve both the stability and reproducibility of the response due to the anodic oxidation of the 7-OH-coumarin which occurred at 0.66 V vs. SCE in 0.1 M phosphate buffer, pH 7.4.

The cyclic voltammetric behaviour of 7-OH-coumarin at the bare activated glassy carbon electrode is shown in Fig. 4.1. From this it can be seen that 7-OH-coumarin exhibits an irreversible oxidation peak. The effect of scan rate on current was examined with a plot of i (µA) vs. scan rate$^{1/2}$ (mV/sec), showing linearity over the range investigated (0 to 100 mV/sec), proving the diffusion controlled rate of reaction.

A typical calibration curve for the oxidation of 7-OH-coumarin at the bare glassy carbon electrode using differential pulse voltammetry, is shown in Fig. 4.2. It may be seen that the current measured for the oxidation process is a linear function of concentration up to 0.5 mM with sensitivity 1.1 nA/mM, r = 0.9979 and a L.O.D. of 10 µM. The R.S.D. for n = 10 batch injections was 3.2 %.

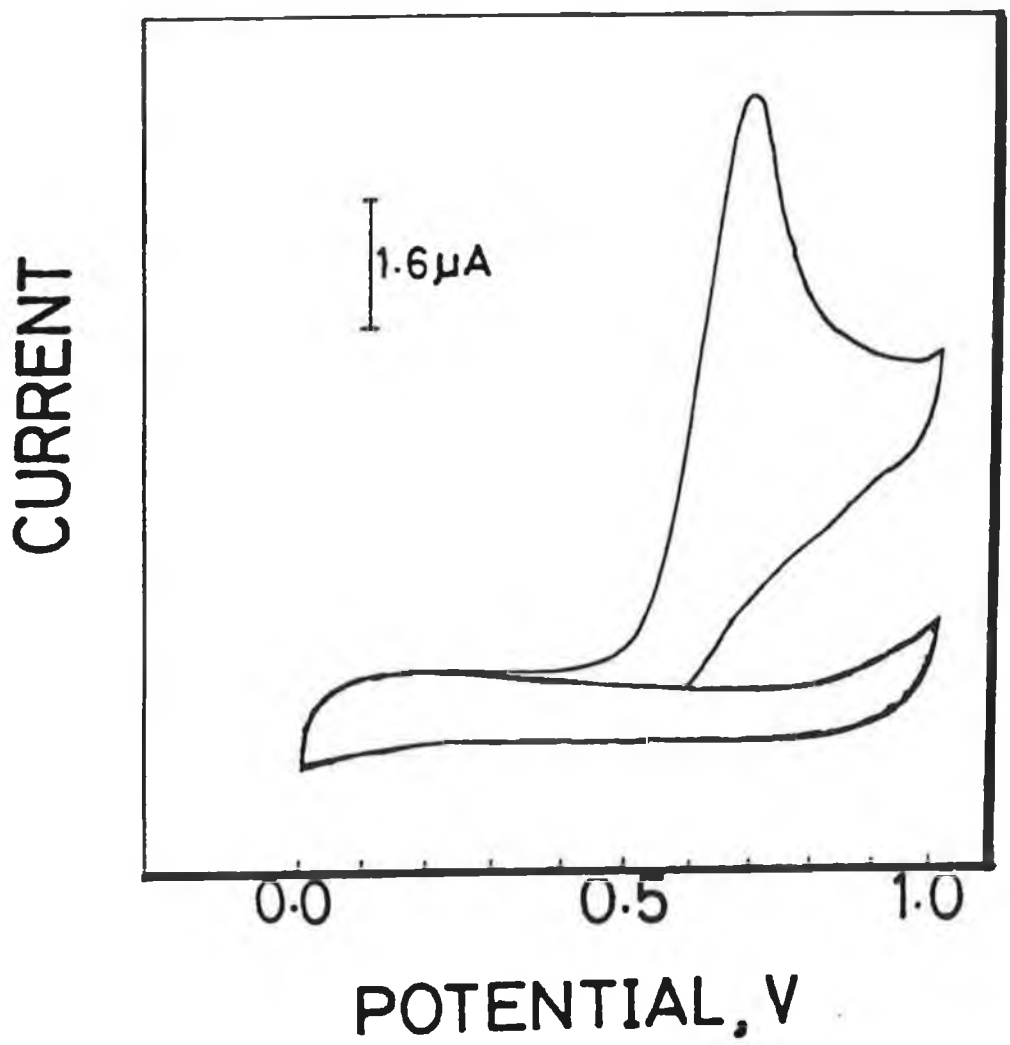

FIGURE 4.1.
Cyclic voltammogram for 0.2 mM 7-OH-coumarin, on 0.1 M phosphate buffer, at the electrochemically pretreated glassy carbon electrode. Sensitivity, 20 µA full scale, scan rate 20 mV/sec, scan range 0.0 V to 1.0 V.

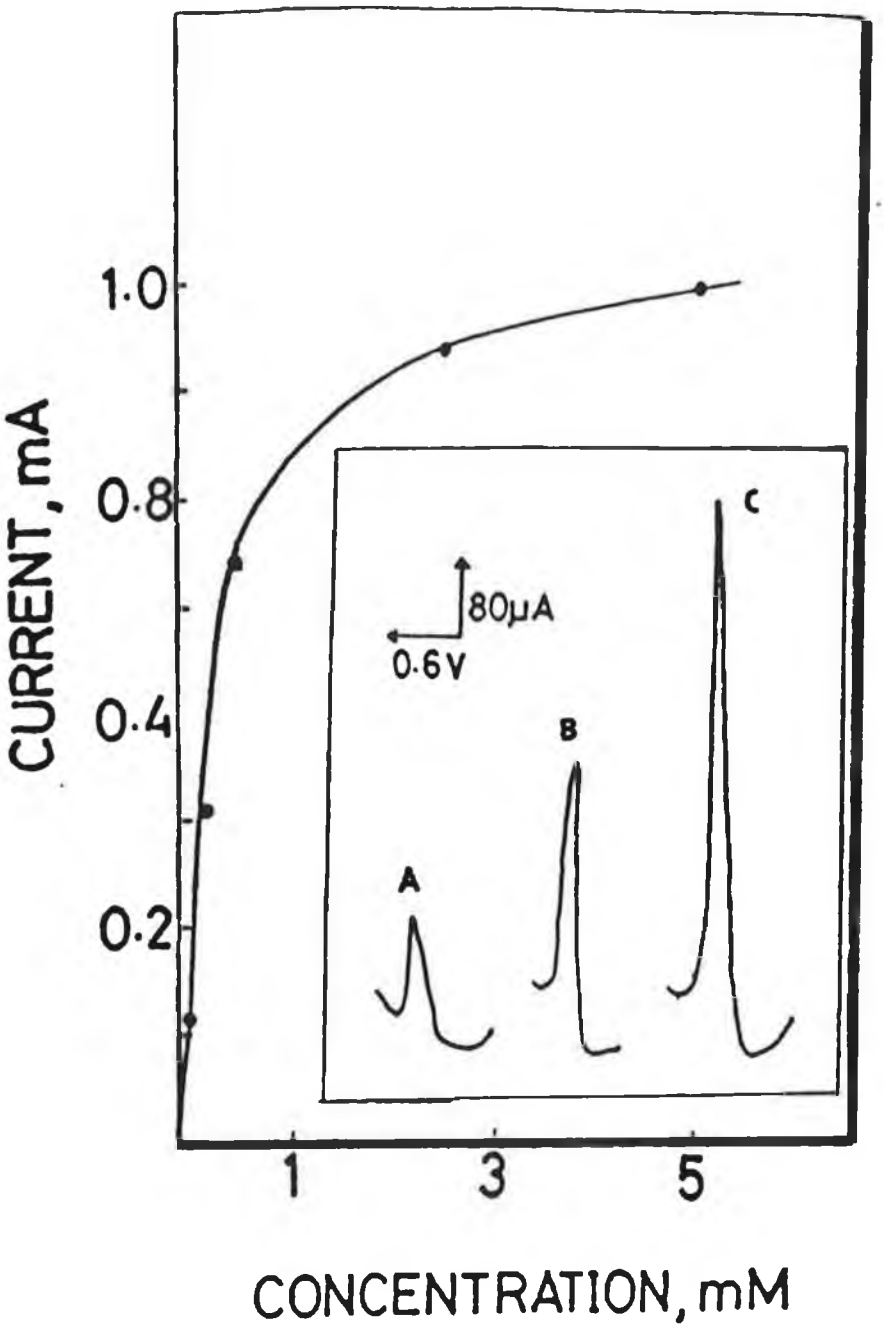

FIGURE 4.2.

Calibration curve for the oxidation of 7-OH-coumarin at the bare glassy carbon electrode, (previously activated) using differential pulse voltammetry. Scan range 0.0 to 1.5 V and 5 mV/sec with A, B, and C representing 0.1, 0.25 and 0.5 mM injections respectively. Sensitivity 1 mA full scale, pulse amplitude 50 mV.

4.2.2.2. *Voltammetric Determination of 7-OH-Coumarin in Human Urine*

Clinical pharmaceutics is a health science discipline which deals with the application of pharmokinetics to the safe and effective therapeutic management of the individual patient. Drugs are eliminated from the body either in the unchanged form usually via renal excretion or as metabolites. Drug metabolites are often more polar than their parent compound and thus are readily excreted in the urine. Many drugs, including coumarin, are converted into their corresponding glucuronide conjugate by the cytochrome P450 enzyme system.

Most errors in drug analysis occur during extraction, subsequent evaporation of organic solvents and derivitisation. The commonest way of isolating drugs from a biological medium is by solvent extraction. Selection of a suitable solvent depend on extraction efficiency (recovery), and selectivity with respect to co-extraction of interferents. The optimum is one that extracts all or at least a large proportion of the drug but does not extract endogenous compounds/metabolites.

Using the extraction method employed by Egan *et al.* [8], a series of control urine samples (1 ml) were spiked with 7-OH-coumarin in the range 0 to 15 mM, and 50 µl of the final methanol extract injected into a 5 ml cell for electrochemical analysis at a bare glassy carbon electrode. The amount of metabolite present in extracted urine samples, as compared to a series of authentic standards in methanol (concentration in the cell from the final methanol extract was in the range 3.29×10^{-5}M to 4.94×10^{-4}M) describes the percentage recovery. The peak heights of extracted and authentic standards were compared at each concentration, taking into account the dilution factors (see Table 4.1.). Linearity for both standard and sample calibration curves extended from 0 to 15 mM spiking concentration (0.49 mM final conc.) 7-OH-coumarin, with r = 0.9998 and 0.9995 with a sensitivity of 1.3×10^{-1} mA/mM and 1.2×10^{-1} mA/mM respectively. In the HPLC method developed by Egan *et al.* [8] which involved the separation of coumarin and 7-OH-coumarin following solvent extraction from a urine sample, linearity in this case extending up to 100 µg/ml (0.617 mM), with a correlation coefficient of 0.9978, a L.O.D. of 2.0 µM 7-OH-coumarin and a precision for inter-and intra- day assay variability of <10 % for n = 5. Percentage recoveries were in the range 84-114 % for 7-OH-coumarin.

SPIKING CONC. (mM)	STANDARD (mA)	SAMPLE (mA)	% RECOVERY
1	0.126	0.119	94.18
2	0.256	0.241	93.24
5	1.080	1.000	92.59
10	1.250	1.193	95.46
15	1.930	1.880	97.40

TABLE 4.1.
Percentage recovery determined by comparing the peak height of spiked control urine samples (in the range 0 to 15 mM) with authentic methanol standards of the same final concentration after extraction.

Analysis of urine samples before and after drug administration in one human volunteer who had received a single oral dose of coumarin (100 mg) was then carried out. The concentration of free 7-OH-coumarin excreted was determined at regular time intervals (0, 4, 8, 12, 16, 20, 24 h) (Fig. 4.3) from the standard curve of spiked extracted urine standards. The free 7-OH-coumarin excreted over the period studied was found to be 5.07 mM (821 µg/ml). The 7-OH-coumarin conjugated to glucuronide was determined following treatment of 1 ml of sample urine with 1 ml β-glucuronidase (10,000 Units/ml) in sodium acetate buffer, incubated at 37°C for 30 min. The conjugated 7-OH-coumarin could be calculated from the difference between the value obtained after hydrolysis and the free 7-OH-coumarin concentration. The total 7-OH-coumarin determined was found to be 918.16 µg/ml (5.67 mM), of which 821.0 µg/ml (5.07 mM) was free and 97.16 µg/ml (0.6 mM) was conjugated. The total represents 82.8 % of the coumarin excreted as the 7-OH-coumarin metabolite. It is now known that individuals have very major differences in their ability to metabolise coumarin to 7-OH-coumarin (Pelkonen O., personal communication). Since 7-OH-coumarin may in fact be the main active pharmacological agent, it is

important to monitor its production in the urine. The procedure described offers a novel approach for such determinations.

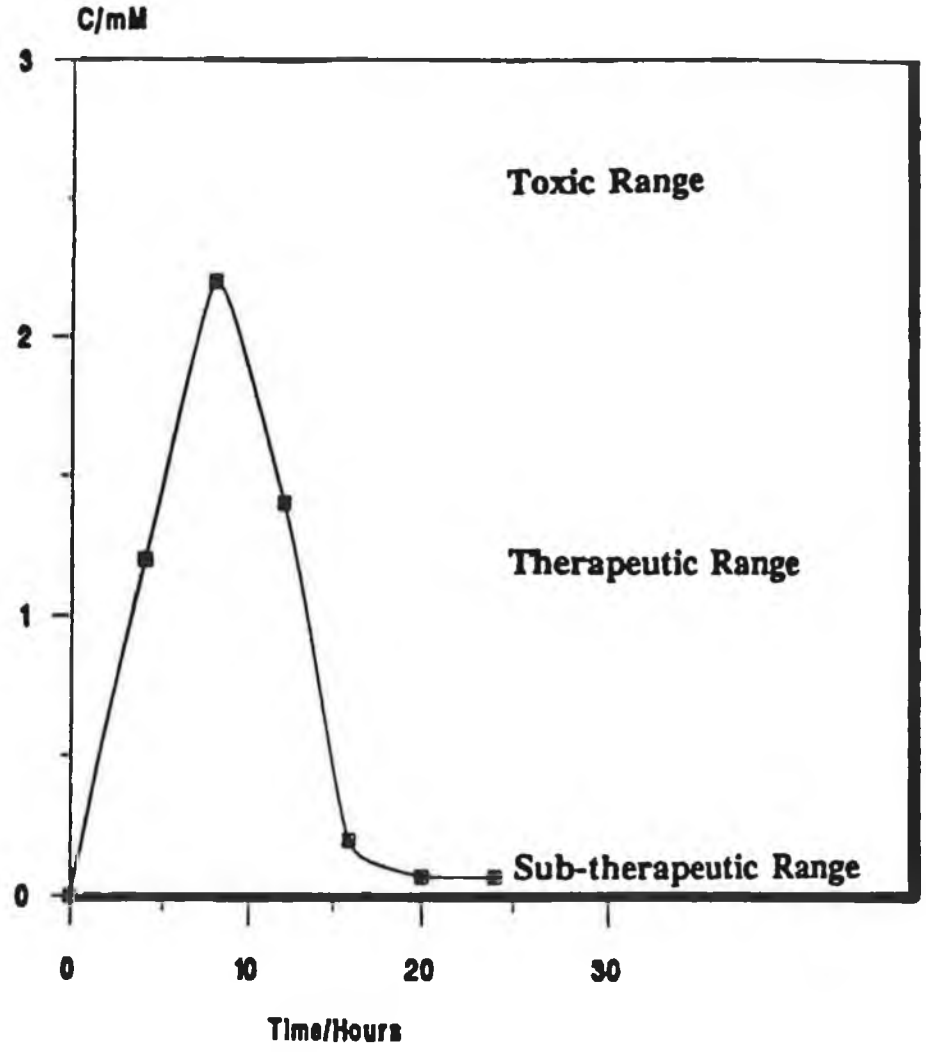

FIGURE 4.3.

Pharmokinetic profile for a human volunteer. Total 7-OH-coumarin determined in extracted urine samples (taken every 4 hours) at the bare glassy carbon electrode.

4.3. DEVELOPMENT OF AN ANTIBODY-BASED AMPEROMETRIC BIOSENSOR TO STUDY THE REACTION OF 7-OH-COUMARIN WITH ITS SPECIFIC ANTIBODY

4.3.1. EXPERIMENTAL

4.3.1.1. *Apparatus*

A conventional three electrode system, with platinum gauze as counter, glassy carbon as working and saturated calomel as reference electrodes were connected to an EG&G PAR Model 174A polarographic analyser and the output currents measured using a Phillips Model PM 8251 recorder. Cyclic voltammetry was performed using an EG&G PAR Model 264A polarographic analyser connected to a JJ Instruments Model PL4 recorder. A batch mode of operation was employed throughout the differential pulse and amperometric experiments, with a magnetic stirrer and bar providing the convective transport. During the temperature controlled studies, the cell was immersed in a thermostatically controlled water bath.

4.3.1.2. *Electrode Preparation*

Before use the bare glassy carbon electrode was polished firstly with alumina slurry, after which it was sonicated in distilled water, followed by electrochemical pretreatment (cycling between -1.5 V and +1.5 V for 4 min at 50 mV/sec). Modification of the electrode was achieved via drop coating of a 10 µl aliquot of the appropriate concentration of anti-7-OH-coumarin antibody (in phosphate buffered saline (PBS) pH 7.4) to the surface of the electrode, which was subsequently covered with a cellulose dialysis membrane (MW cut-off 12-14k) and held in place using an O-ring.

4.3.1.3. *Reagents*

Batch experiments were conducted at room temperature in 0.1 M phosphate buffer (pH 7.4) and all solutions were prepared using deionised water by passing distilled water through a Milli-Q water purification system. 7-OH-coumarin (umbelliferone) was purchased from Sigma and the anti-7-OH coumarin antibody was raised in rabbits [10]. A 12 mg/ml solution of antibody was diluted in PBS (Dulbecco 'A') obtained from Oxoid. Potassium dihydrogen phosphate and

dipotassium hydrogen phosphate were obtained from Riedel-de-Haen and the cellulose dialysis membrane form Sigma (MW cut-off 12-14 KDaltons). Diethyl ether and methanol (HPLC grade) was obtained from LabScan Ltd. Dublin, Ireland.

4.3.2. RESULTS AND DISCUSSION

4.3.2.1. *Electrochemical Studies on the Behaviour of 7-OH-Coumarin*

The electrochemical behaviour of 7-OH-coumarin was investigated at the bare glassy carbon electrode (as described in 4.2.2.), and these conditions were applied to the DPV determination of 7-OH-coumarin in human urine samples [19].

4.3.2.2. *Development of an Antibody-Based Biosensor*

The development of an amperometric biosensor based on an anti-7-OH-coumarin antibody is reported in thiis study. The antibody has a high affinity for 7-OH-coumarin and the presence of an hydroxyl group in the 7-position appears to play an important role in determining the antibody specificity. When similar experiments were conducted using the antibody sensor compared to the bare glassy carbon electrode, a decrease in the oxidation current of the drug was observed, as predicted, as the binding of antibody to the antigen occurred at the electroactive site i.e. the 7-OH group in the molecular structure. The influence of pH and temperature on this reaction were then studied, together with an investigation of the kinetics parameters involved for various loadings of antibody at the electrode surface.

The effect of pH on peak current is shown in Fig. 4.4.(a), where the plateau in this graph at pH 7.4 reflects the optimum pH for reaction of this metabolite with its specific antibody. Fig. 4.4(b) shows the effect of temperature on the antibody activity with an optimum temperature being found at 33°C i.e. the point at which the greatest decrease in electroactivity of the drug was observed, with greater electroactivity noted at both higher and lower temperatures.

This represents a shift from the expected optimum value of 37°C, but this may be attributed to the fact that the antibody was not in its natural environment, as the experiments were carried out *in vitro* therefore resulting in a lowering of the optimum temperature for activity of this antibody. Analysis of the Arrhenius-

type plot of log i(mA) vs. 1/Temp.(K^{-1}) resulted in an activation energy value of 25.79 kJ/mol.

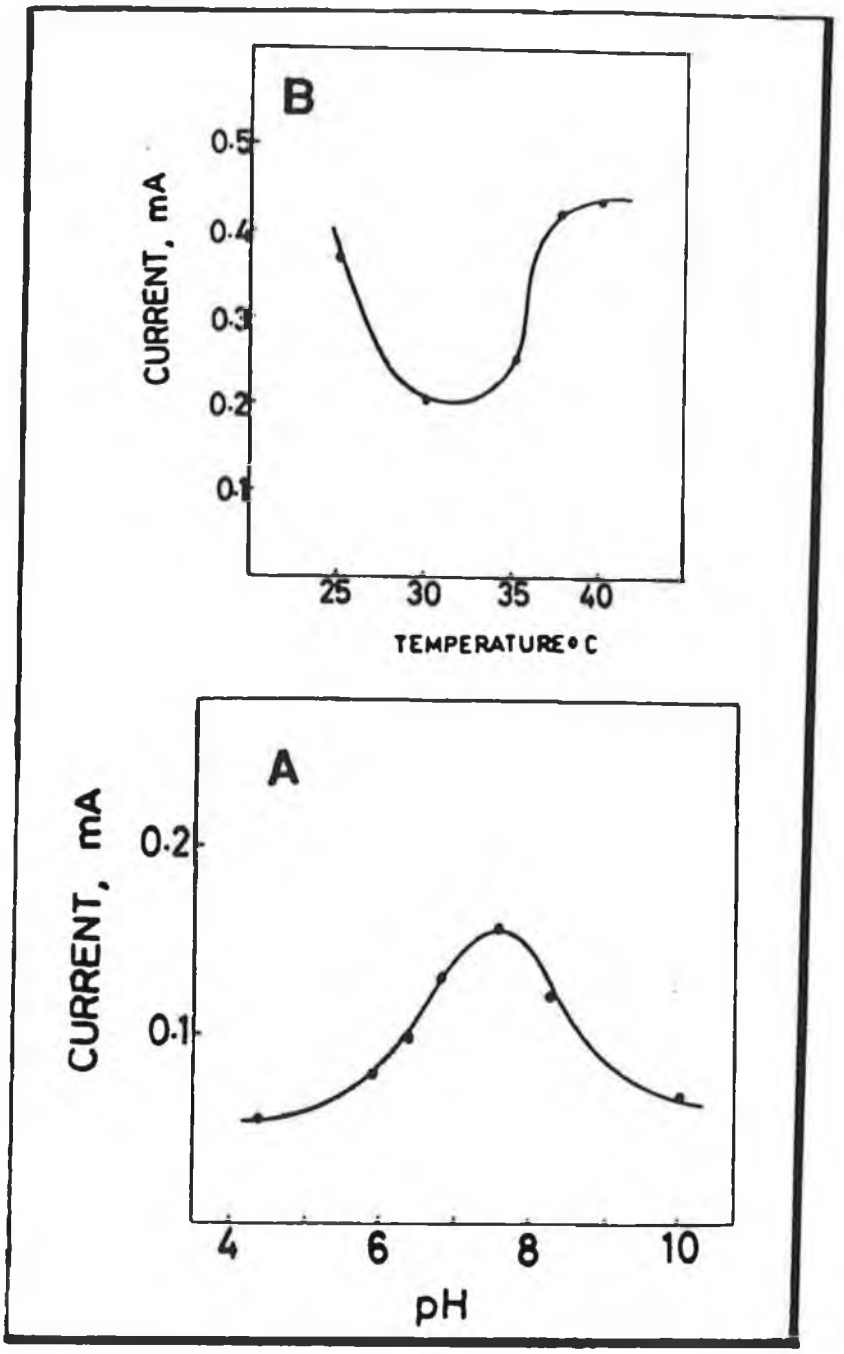

FIGURE 4.4.

(A) Effect of pH on the oxidation current for 0.1 mM 7-OH-coumarin. (B) Temperature controlled study for investigation of the optimum activity of the anti-7-OH-coumarin antibody in a thermostatically controlled cell. 0.1 mM 7-OH-coumarin antibody in a thermostatically controlled cell. 0.1 mM 7-OH-coumarin and $2x10^{-4}$mg/ml antibody in solution for 5 min before scanning.

Fig. 4.5.(A) and (B) show the DPV and DC amperometric responses at the bare and modified electrodes, respectively, and show the decrease in electroactivity resulting from the bonding process. Studies of the antibody-antigen reaction in solution at the unmodified glassy carbon electrode were carried out in order to confirm this. The results exhibit the expected decrease in electroactivity of the 7-OH-coumarin when the concentration of the latter was held constant (at 0.1 mM in solution) while increasing antibody concentration was injected into the electrolyte. The current response was measured after increasing time intervals. A greater slope was observed when measurements were taken after t = 10 min., as opposed to t = 0 mins.

Fig. 4.6. shows the effect of increasing antibody activity behind the cellulose membrane. At low surface antibody concentrations, the slowest decrease in electroactivity of the metabolite was observed over a period of 60 min, with the first order rate constant increasing from 9.136×10^{-3} min^{-1} to 1.966×10^{-2} min^{-1} with increased loading from 0.1 mg/ml to 0.4 mg/ml antibody, after which a levelling off occurred at higher antibody loadings. All readings were taken in triplicate by injecting the appropriate volume of 7-OH-coumarin to give a final concentration of 0.1 mM in the 20 ml cell, with readings every 10 min.

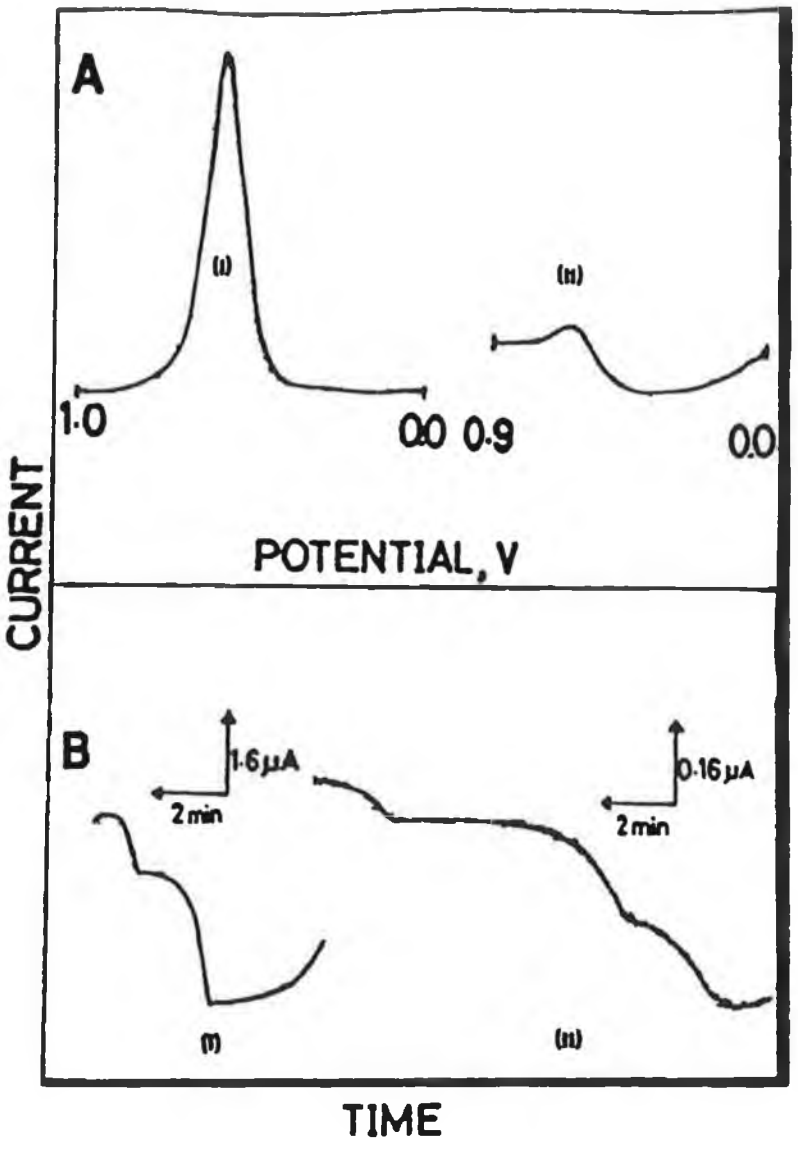

FIGURE 4.5.

(A) (i) DPV scan of 1 mM 7-OH-coumarin at the GCE covered with cellulose membrane and (ii) that at the surface with antibody immobilised behind the membrane and left to equilibrate at 4°C for 2 hours. 90 % decrease in electroactivity of the drug observed over this time.

(B) (i) DC amperometric experiment at the cellulose covered GCE with E_{app} = 0.66 V for 1 mM injections of 7-OH-coumarin and (ii) same as (i) except antibody present behind membrane showing ca. 90 % decrease as above.

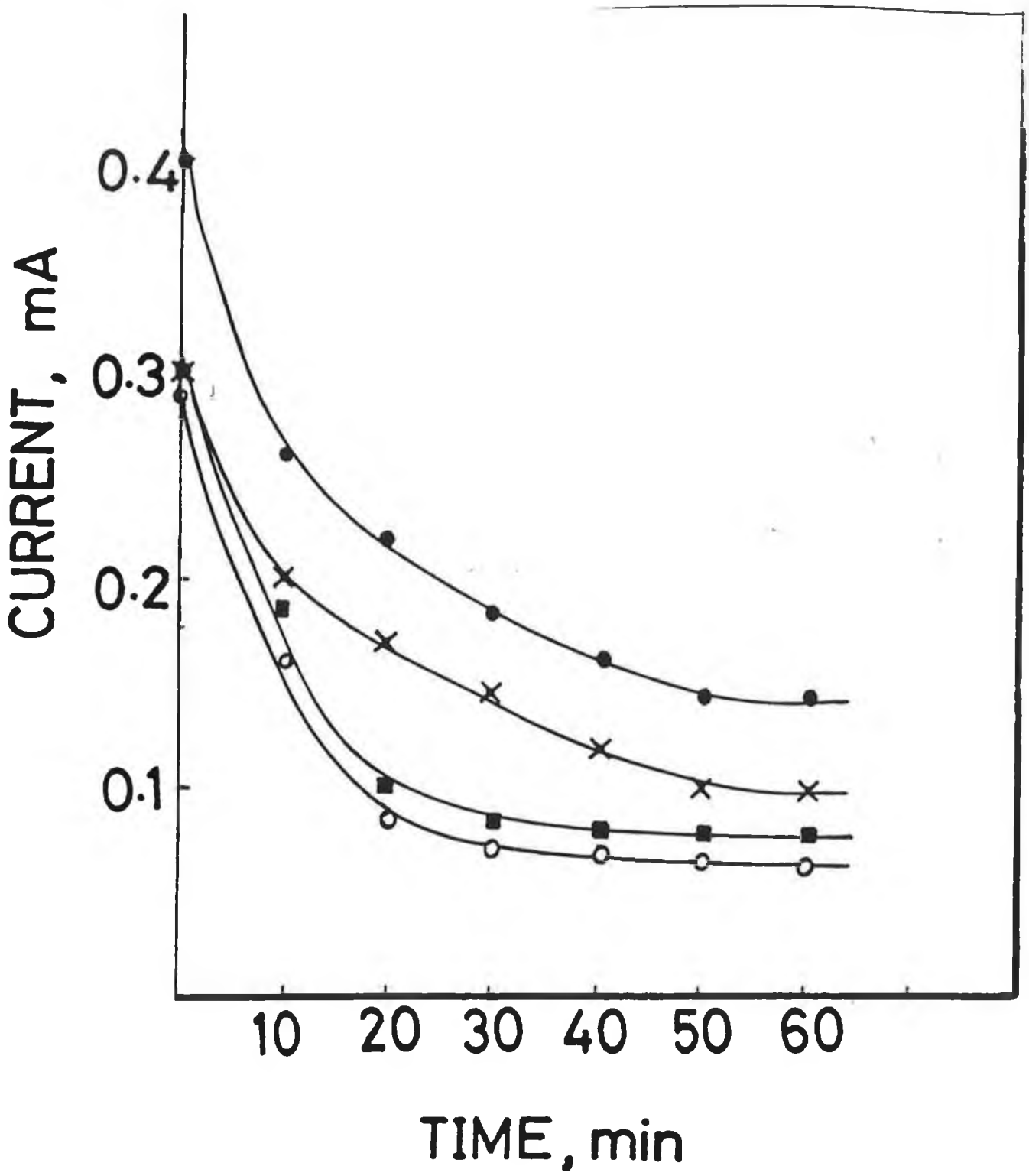

FIGURE 4.6.
Loading study for optimum antibody surface activity, investigated for 10 µl of membrane. Injection of 0.1 mM 7-OH-coumarin (●) 0.1 mg/ml, (×) 0.2 mg/ml,(■) 0.3 mg/ml, (O) 0.4 mg/ml, immobilised behind the cellulose membrane. Injection of 0.1 mM 7-OH-coumarin and readings taken every 10 mins.

4.4. CONCLUSION

The electrochemical behaviour of 7-OH-coumarin at the bare glassy carbon electrode was studied using differential pulse voltammetry, and based on anodic detection of this metabolite at 0.66 V (vs. SCE) using DC amperometry. A method has been developed for the determination of 7-OH-coumarin levels in urine samples, and a pharmaceutical profile established. An amperometric biosensor based on anti-7-OH-coumarin antibody immobilised at the surface of a glassy carbon electrode, behind a cellulose dialysis membrane has also been described. The electrochemical behaviour of this metabolite at the bare glassy carbon electrode was found to be well defined using differential pulse voltammetry, and a 90 % decrease was observed in peak height upon binding of the antibody to the antigen, which occurs at the electroactive site of the 7-OH-coumarin. The kinetics of the antibody:antigen reaction were investigated and a first order reaction observed with k = 0.0329 mA/min. This system provides a novel method for studying antibody specificity and the kinetics of such antibody-antigen interactions.

4.5. FUTURE DEVELOPMENTS IN THE AREA OF ANTIBODY-BASED BIOAFFINITY SENSORS

It is advantageous to achieve a particular orientation of the biomolecules at the surface of the transducer in order to obtain maximum signals. For immobilisation of antibodies, the binding to a prefixed layer of e.g. protein A allows a uniform orientation with the antigen binding fragment to the solution side. This results in an increase in the overall reaction rate and hence a higher signal. Langmuir Blodgett films as discussed in Chapter 1 have great application here.

In addition to the potential of monoclonal antibodies, the modification of the antibody molecule opens up new horizons. This is true for bifunctional antibodies which react with two different species, catalytic enzymes which are a functional hybrid of genetically engineered fragments, and decrease the effect of non-specific binding.

The formation of the immuno antibody-antigen complex is permanent and essentially irreversible. The lack of product formation in an immunoreaction simplifies its possible mode of operation in an organic phase environment by eliminating the need for both substrate and product to be capable of rapid

diffusion at the reaction site in a soluble form. No such examples of immunosensors operating in organic environments have so far been reported. Unlike organic-phase enzymology, it appears that a hydrophilic environment is preferential. However, immunotechnology may, in the future be extended into non-aqueous environments. As with enzyme systems, the operation of immunoreactions in an organic environment could have a number of advantages. Application of organic solvents will distort the quaternery structure of the antibody-antigen complex. This may be used to an advantage, in order to address the problem of an immunosensor's irreversibility by creating a delicate balance between binding destabilisation and denaturation, and allowing perhaps greater stability and wider operational temperature range.

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CHAPTER 5

APPLICATION OF LICHEN-MODIFIED CARBON PASTE ELECTRODES FOR VOLTAMMETRIC DETERMINATION OF METAL IONS IN MULTIELEMENT AND SPECIATION STUDIES.

5.1. INTRODUCTION

Lichens are plants formed by the symbiotic association of an algae and a fungus. Their general structure is that of an upper cortex, a protective central fungal medulla (of loosely packed hyphae), and a lower cortex. The algae may form a distinct layer beneath the upper cortex, or they can be dispersed throughout. Most lichens have an extracellular matrix which is a gelatinous secretion containing polysaccharides such as lichenan, and isolichenan together with glucans, galactomannose and lichen acids.

For years, lichens have been known to accumulate metal ions and have been used extensively as biomonitors of environmental pollution [1,2]. The diversity of lichen species close to a suspected pollution source has been used to assess the levels of gaseous air pollutants, with fewer lichen species being found closer to the emission source [3,4]. These plants are considered to be useful biomonitors of sulphur dioxide [5], acid rain [6], radionucleotides [7], chlorinated hydrocarbons [8] and ozone [9]. Both lichens and mosses have been used as monitors of uranium concentration contamination, by Boileau *et al.* [10] around two centres of uranium mining in Canada. Samples were analysed by X-ray fluorescence spectroscopy for metals such as titanium, iron, nickel, lead and uranium. Richardson and co-workers have also investigated the uptake of lead and uranium by lichens [11].

Lead in the thalli of *Stereocaulon vesuvianum* was found to be located on the hyphae and as discrete particles in the intracellular spaces. Metals may also accumulate in the rhizinae, the fungal strands that anchor the thallus to the substratum. Rhizinae were found to be an important site for mineral accumulation in *Peltigera canina* and *Parmellia omphalodes* [12].

A major mechanism of metal accumulation in lichens involves biosorption in which the metal ions interact with the polyfunctional metal binding groups on the cell walls or within the cellular matrix of these plants [13].

It has been demonstrated that the uptake of strontium by *Cladonia alpestris* involved an ion-exchange process [14]. Uptake was found to be strongly pH dependant, Puckett *et al.* [15] confirmed the ion exchange model and the assumption that the functional groups were composed of a pair of bonding sites that appeared to be O and O-N donors. They noted the remarkable similarity of the metal ion selectivity coefficients between the lichens and the chelating resin DOWEX A-1. Thus cation binding in lichens most likely involves carboxylic-acid-containing ligands, which form part of the fungal cell wall. In addition to the

ion exchange process, a slower and more selective uptake into the cells may also take place.

A review by Richardson [16] has described the pollution sensitivity of lichens along with the mechanism of elemental accumulation by these plants. Beck and Ramelow [17] used lichens enclosed in porous polyvinyl chloride (PVC) tubes as monitors of dissolved metal in natural waters. The PVC tubes were suspended at different sampling points along a river. After 2 weeks, the lichens were analysed by atomic absorption spectroscopy for a wide variety of metal ions including Pb(II) and Cu(II).

Wang *et al.* [18], reported on experiments at algae-modified electrodes to investigate the incorporation of anionic and cationic metal complexes by algae. Gardea-Torresday *et al.* [19] then reported on voltammetric measurements at algae-modified electrodes capable of preconcentrating Cu(II) and Au(I). These studies suggested that the development of lichen-modified electrodes may open up a new area of sensor development based on the non-electrochemical bioaccumulation of metal ions.

Recently, lead and copper uptake at a lichen-modified electrode were investigated using differential pulse voltammetry [20]. The aim of the present work is to report on further developments with these electrodes and to examine the mechanism of metal uptake by these plants.

5.2. EXPERIMENTAL

5.2.1. *Reagents*

All solutions were prepared with double distilled water and electrochemical experiments were conducted at room temperature with 0.02 M sodium acetate (BDH Chemicals) providing the supporting electrolyte. Metal standard solutions (stored in polyethylene bottles) were obtained from BDH Spectrosol Ltd. Hydrochloric acid, EDTA and sodium chloride were obtained form Sigma, and graphite powder, Nafion and Nujol oil were purchased from Aldrich Chemical Co.

5.2.2. *Apparatus*

Electrochemical experiments were performed using an EG&G Princeton Applied Research Model 264 Polarographic Analyser/Stripping Voltammeter, the output of which was displayed on a PL3 X-Y recorder. The cell consisted of a carbon

paste working electrode, a silver/silver chloride reference and a platinum wire auxiliary electrode, all of which joined the cell through holes in its Teflon cover. Provision was also made for passing oxygen-free nitrogen through the solution prior to commencing the experiment. A magnetic stirrer and bar provided the convective transport. The lichens *Cladonia* and *Lobaria* were collected from Killarney, Co. Kerry, Ireland. Roccella was collected from the north coast of Tenerife, Canary Islands.

5.2.3. *Electrode Preparation*

A 40:60 (w/w) mixture of mineral oil and graphite powder was packed into a 3 mm i.d. glass tube with a copper wire providing electrical contact. Modified paste (20 % loading w/w) was prepared by thorough mixing of 0.2 g dried ground lichen with 0.4 g of both oil and graphite powder. This modified paste was then packed as a thin layer at the end of the working electrode and the surface smoothed on a deck of weighing paper. Surface renewal was achieved by replacement of the superficial material with a small amount of fresh modified paste.

5.2.4. *Procedures*

Voltammograms were obtained at lichen-modified electrodes immersed for a set period of time (under open circuit conditions) in a separate accumulation cell containing a 1 mM copper(II) solution. It was important to ensure reproducible conditions with respect to the vessel, the stirring rate (400 rpm) and the geometrical arrangement of the working electrode, relative to the stirrer bar and container wall. After accumulation, the electrode was rinsed with deionised water and placed in the electrochemical cell containing the supporting electrolyte. The initial potential was applied to the working electrode and scanning commenced following a 15 sec resting period, which ensured that the copper was in the complexed form prior to stripping and oxidation to copper(II).

Two surface renewal techniques were investigated. The electrode was soaked for 5 min in 0.5 M HCL, or alternatively it was treated for 2 min after accumulation in a solution of 4 M HCl and 0.1 M EDTA, in order to free the bound metal and reactivate the surface.

Investigation of the binding mechanism involve using four extraction procedures as described by Richardson *et al.* [21]. Samples of dried lichen were oven dried at 70°C for 12 hours. Further samples were immersed in deionised

water maintained at 60°C for 309 min and left to air-dry after filtration. Acetone treatment was achieved by soaking the lichen in 15 ml acetone for 30 min and drying once more. Finally samples were shaken with 30 ml 7.5 % w/v 'Ariel' detergent solution for 24 hours, filtered once more and left to air dry. Modified pastes were then prepared and the copper uptake for each species compared to that of the total lichen.

Simultaneous uptake of a mixture of 1 mM copper(II), lead(II) and mercury(II) was investigated using both sodium acetate and phosphate buffers as electrolytes.

5.3. RESULTS AND DISCUSSION

The application of lichen-modified carbon paste electrodes for determination of lead(II) and copper(II) using differential pulse anodic stripping voltammetry has previously been investigated by Connor *et al.* [20], who optimised parameters such as pH, time of accumulation and loading. The performance of the lichen-modified electrodes was therefore further evaluated with respect to multi-element accumulation and determination, new surface renewal techniques, extraction of various lichen components, and speciation studies.

5.3.1. *Multicomponent Analysis*

The response obtained for simultaneous determination of lead(II), copper(II) and mercury(II) at the *Lobaria* modified electrode is shown in Fig. 5.1. Accumulation of the mixture using phosphate buffer as electrolyte (pH = 5.0) exhibits an increase in the lead response (not shown), but the copper and mercury responses were greater when sodium acetate was used as electrolyte.

The percentage decrease or increase in response when the metal was accumulated in the mixture, as opposed to that when accumulated separately, is shown in Table 5.1. Connor *et al.* showed that accumulation of 1 mM copper(II) in the presence of 0.5 M lead(II), as opposed to accumulation alone, resulted in a 75 % decrease in the copper response due to competition between the binding sites. In this work, however, only a 38 % decrease in the copper(II) response resulted when accumulation was carried out in the presence of 1 mM lead(II) and 1 mM mercury(II). Formation of a mercury layer on the lichen electrode surface may preconcentrate the copper as an amalgam, and therefore the competition between the metal ions is somewhat suppressed. The copper(II) and lead(II)

responses were found to decrease in the mixture due to competition for the binding sites. Lead is preferentially bound over copper and may actually displace bound copper.

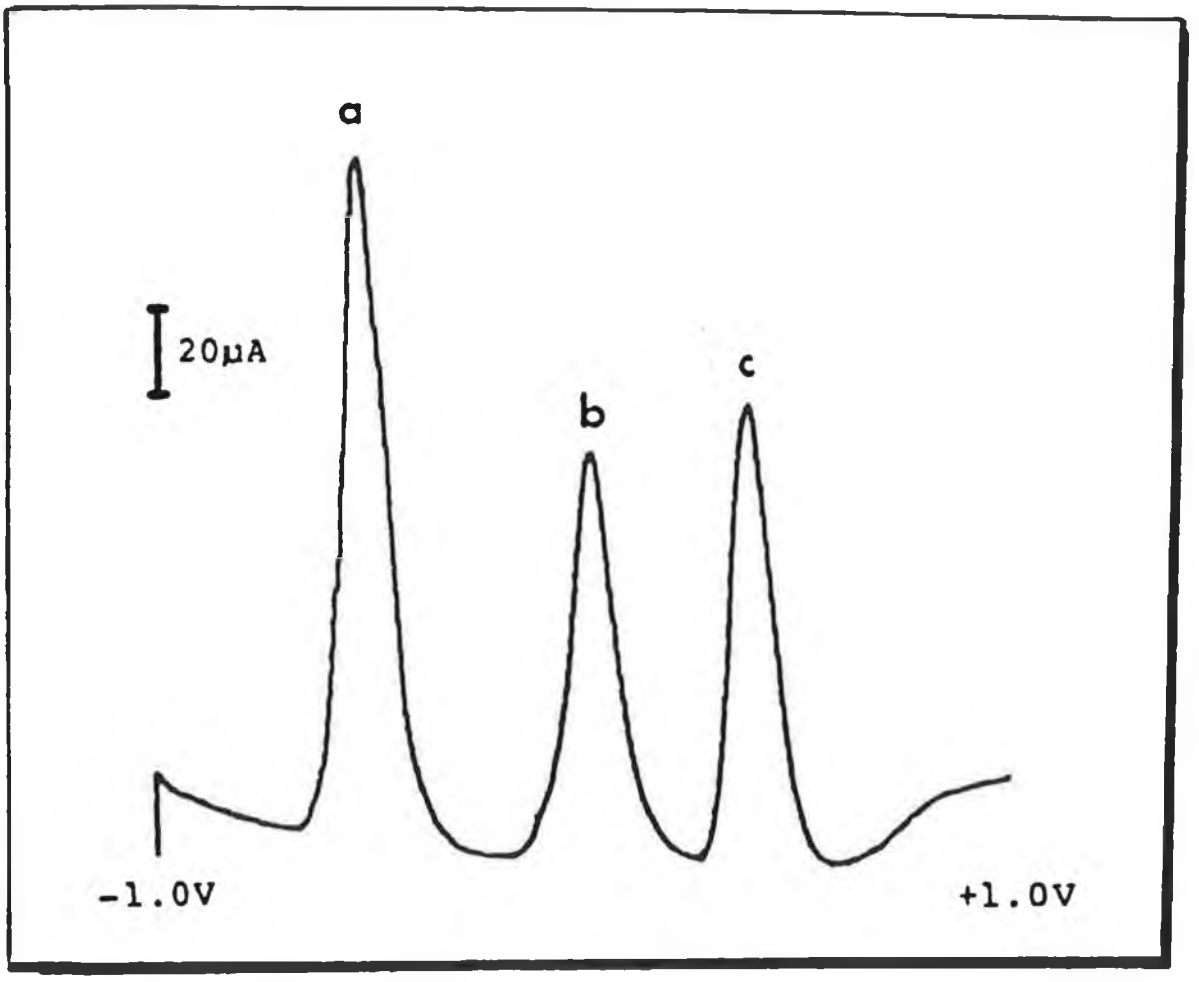

FIGURE 5.1.
Voltammograms (current (µA) vs. potential (V)) showing responses for 1 mM solutions of (a) lead(II), (b) copper(II) and (c) mercury(II) at the *Lobaria* modified electrode (20% loading). Scan range -1.0 V ⇒ +1.0 V.

METAL	% DEVIATION
Copper	-38.80
Mercury	+13.21
Lead	-24.65

TABLE 5.1

Percentage change in metal uptake for a mixture of metal ions compared to that with separate accumulation at the *Lobaria* modified electrode (20 % loading). Electrolyte - 0.02 M sodium acetate pH 5.0. The accumulation solution contained 1 mM copper(II), lead(II), mercury(II), accumulation time - 2 min. Scan rate 10 mV/min, pulse amplitude 100 mV ramp, scan rate -0.5 V $\Rightarrow$ + 0.5V. Current range 500 μA full scale.

5.3.2. *Surface Renewal*

Three treatments were used to renew the electrode surface and hence improve stability:

- manual replacement of modified paste
- acid renewal
- treatment with NaCl/EDTA

After metal accumulation the electrode was soaked in 0.5 M HCl for 5 min, which was found to remove almost all the bound copper, although after further accumulation, an increase of 8 % confirmed the presence of some remaining

bound copper. However, the precision improved over a series of accumulations (Table 5.2 (B)), compared with the reproducibility resulting from manual resurfacing (Table 5.2(A)). The use of NaCl/EDTA instead of acid was then studied, Na^+ acting in the same way as H^+ to compete with the analyte for binding sites, and the EDTA serving to complex the released copper. This treatment removed all the bound copper in the case of the *Lobaria*-modified electrode, but the copper uptake decreased by 70 % over a series of six accumulations. In order to alleviate this problem and improve stability, a thin layer of Nafion (in aliphatic alcohols and 10 % water) was applied to the surface. However, following the Nafion treatment, 23 % of the copper still remained after cleaning. It seems that the release of metal ions from the binding sites was blocked by the presence of the anionic polymer coating. The recommended protocol for surface renewal therefore is manual resurfacing.

SPECIES	(A)	(B)
Lobaria	7.77 %	2.82 %
Cladonia	10.60 %	2.12 %
Roccella	3.82 %	1.00 %

TABLE 5.2

Percentage coefficient of variation of a series of 10 accumulations using :

(A) Manual resurfacing

(B) Acid renewal.

The accumulation solution contained 1 mM copper(II) in sodium acetate (0.02 M) pH 5.0. Other conditions were as Table 5.1.

5.3.3. *Extractions*

A series of extractions were then performed on the dead dry lichen material. Electrodes were prepared from the resulting material with various components removed (Table 5.3). Metal accumulation proved to be reproducible upon consecutive accumulation and cleaning steps, exhibiting a precision of < 7.3 % for n = 5 and response time of 3 min. The hot water treatment is assumed to remove isolichen and other water-soluble polysaccharides, and the increase in response obtained for all three species of lichen examined following this treatment, indicates that these compounds are unimportant in metal binding.

The heat treatment causes destruction of the cell membranes and cracking of the surface of the lichen particles, which allows solutions easier access to all the fungal tissue, exposing sites on macromolecules which are normally protected. The effect of this treatment was to cause a small increase in copper uptake.

Acetone treatment removes lichen substances which are deposited on or in the fungal cell walls of the lichen [22]. It was also found to destroy membranes by dissolving the lipid components. An increase in uptake was observed following the removal of these substances, which therefore do not appear to participate significantly in metal binding.

Extractions with the detergent 'Ariel' (containing a proteolytic enzyme preparation derived form *Bacillius subtilis*) removes the extracellular matrix of the cortical regions of the lichen thalli [23]. This resulted in the maximum metal uptake.

Richardson and Nieboer [24] suggested that a proportion of the metal binding sites might be located on the extracellular matrix that is deposited on and between the hyphae of the upper cortex in lichens. It is composed of polymers such as lichenan. The fact that the removal of much or all of this matrix did not did not decrease the metal binding capacity either in this study or in the study on lead uptake by lichens from solution [21] (see comparison in Table 5.3), confirmed that metal binding sites are not primarily located on this component. As treatment with hot water, acetone and detergent increased the uptake of copper by lichen modified electrodes, it appears that metal binding is primarily an interaction between metal ions and sites (probably carboxylic acid groups), located on or within the cell walls of the fungal component.

EXTRACTION METHOD (A)	ROCCELLA	CLADONIA	LOBARIA
Oven dried	3.7 %	2.0 %	17.2 %
Hot Water	20.3 %	8.2 %	26.7 %
Acetone	42.2 %	28.4 %	91.2 %
Detergent	174.3 %	281.8 %	58.0 %

EXTRACTION METHOD (B)	PELTIGERA	HYPOGYMNIA	LOBARIA
Oven dried	28.8 %	48.6 %	24.0 %
Hot water	34.4 %	22.0 %	30.4 %
Acetone	37.2 %	27.0 %	75.4 %
Detergent	126.2 %	210.0 %	104.1 %

TABLE 5.3.

A comparison of increased metal ion uptake by modified electrodes following various pretreatments, with uptake by lichen samples from solution, having been similarly pre-treated.

(A) Increased percentage Cu(II) uptake by lichen modified electrodes. Conditions as in Table 1.

(B) Increased percentage Pb(II) uptake from solution by crushed lichen (Richardson *et al.* [21].

5.3.4. *Speciation of Copper(I) and Copper(II)*

As the percentage loading of lichen in the modified paste decreased, a second peak on the voltammograms was obtained at a higher potential (0.3 V) than the copper(II) potential (0.18 V). The size of the former peak decreased as the initial potential was made more negative. A slower scan rate resulted in more

time for the oxidation of copper(0) to copper(II). The second peak is therefore considered to be copper(0) oxidised to copper(I).

$$Cu(0) \Rightarrow Cu(I) + 1e^- \Rightarrow Cu(II) + 1e^- \qquad (5.1.)$$

A 1 mM copper (I) solution was accumulated at the *Lobaria* modified electrode, resulting in a peak at 0.28 V. The potential difference between the two responses was the same as the standard electrode potential difference for these two reactions. Therefore the results prove that copper(I) is also accumulated by the lichen material.

In the case of the *Roccella* modified electrode, the total response at a 9.85% loading showed a main peak at 0.3 V, and the effect of the detergent treatment was to increase the copper(II) response as shown in Fig. 5.2 (I) The hot water treatment also increased the copper(I) uptake for *Roccella*. The *Cladonia*-modified electrode response for copper(I) increased as a result of the 'Ariel' treatment Fig 5.2(II), and all treatments except hot water. For the *Lobaria* modified electrode, the effect of acetone, and especially the detergent treatment, was to allow greater uptake of copper(I), as may be seen by the pronounced shoulder at 0.3 V, (see Fig 5.2(III)).

It is possible that the 'Ariel' and acetone treatment exposed more sulphur binding sites to which copper(I) would exhibit more affinity, being of the Class B nature [25]. The three lichen species used had different algal components, and the variations exhibited, particularly when combined with cell wall modification using 'Ariel' or acetone treatments, may allow electrodes with particular metal binding preferences to be developed.

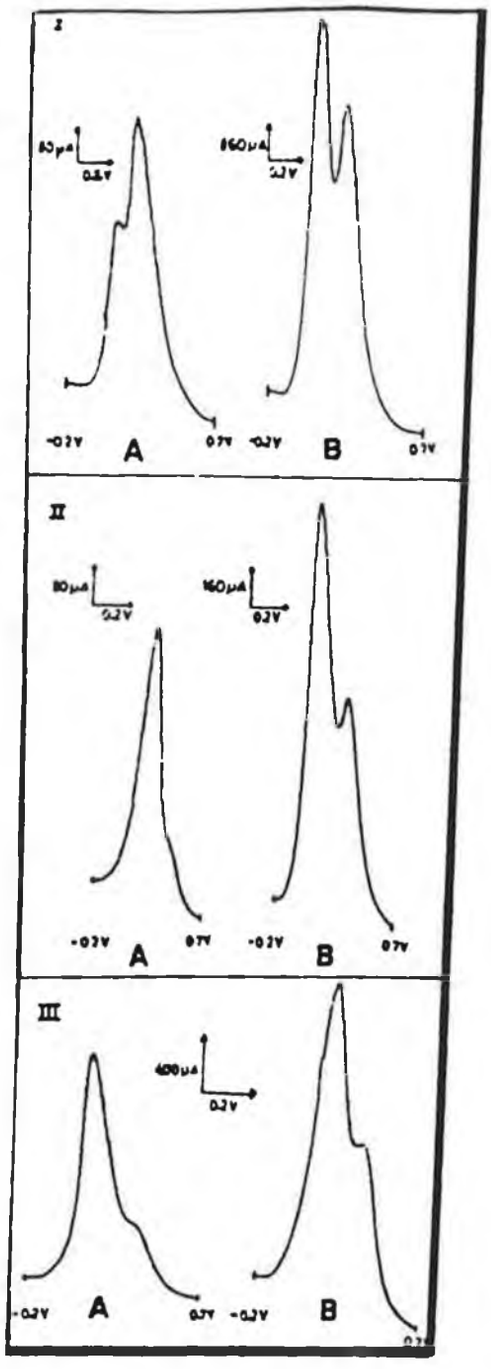

FIGURE 5.2.

Voltammograms of copper(II) and copper(I) at 0.15 V and 0.25 V respectively at

(I) (A) Total *Roccella* (9.75 % loading) modified electrode.
(B) 'Ariel' extracted *Roccella* electrode (9.75 % loading). Scan range -0.2 V ⇒ 0.7 V, 1 mA full scale.

(II) (A) Total *Cladonia* (20 % loading)
(B) 'Ariel' extracted *Cladonia* electrode (20 % loading). Conditions as in (I)

(III) (A) Total *Lobaria* (13.44 % loading) modified electrode
(B) 'Ariel' extracted (13.44 % loading) modified electrode. Conditions as in (I) except 5 mA full scale.

5.4. CONCLUSION

Following the recent development of new lichen-modified electrodes for voltammetric quantitation of some metal ions, further studies have been carried out to investigate the effect of removal of various cell wall components of the lichen material on the responses obtained. Electrodes were constructed using cell wall material from three different lichen species (*Cladonia portentosa, Lobaria pulmonaria and Roccella*), and the ability of these modified electrodes to detect a mixture of three metal ions i.e. lead(II), copper(II) and mercury(II), as well as Cu(II) in the presence of Cu(I) was demonstrated. Advantages of the lichen-modified electrodes include stability, low cost, fast response times and simple preparation. They may form a new class of modified electrodes that utilise the unique properties of lichen cell walls to provide electrodes with specific and useful characteristics.

A mixture of three metal ions were found to be well resolved on one voltammogram with limit of detection 15 μM, 6 μM and 9 μM for copper, lead and mercury respectively. Future work could extend the range of detectable metal ions along with the study of speciation for other metal ions. The use of isolated lichen fungi and algae [25], or lichen cell wall material treated with specific degradative enzymes to remove uptake specificity, may also prove valuable. The selectivity of different lichen species to remove and recover metal ions from industrial wastewater/contaminated groundwaters may be utilised in the future by packing lichen material into columns which function as an ion-exchange column [26]. It has already been shown that lichen material packed into perforated tubes and submerged in contaminated waters, can be useful monitors for various metals [17]. Following these studies the extracted lichen material may give information as to the speciation of various metals in contaminated water.

Although the sensitivity of these sensors is not remarkable the excellent resolution of a mixture of metals, together with the ease of preparation makes them a very promising on-site sensor for metal collection. These newly developed lichen biosensors have the further advantages of useful analytical properties with low cost, and may augment the existing ways in which lichens are used for biological monitoring.

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Appendix

List of Publications

[1] 'Amperometic Enzyme Electrode for Theophylline', Joseph Wang, Eithne Dempsey, Mehmet Ozsoz and Malcolm R. Smyth, *Analyst*, (1991) 116, 997.

[2] 'A Lysine Dehydrogenase-Based Electrode for Biosensing of L-Lysine', Eithne Dempsey, Joseph Wang, Ulla Wollenberger, Mehmet Ozsoz and Malcolm R. Smyth, *Biosensors and Bioelectronics*, (1992) 7, 323.

[3] 'Electropolymerised o-Phenylenediamine Film as means of Immobilising L-Lactate Oxidase for a L-Lactate Biosensor', Eithne Dempsey, Joseph Wang and Malcolm R. Smyth, *Talanta*, (1993) 40, 3, 445.

[4] 'Development of an Amperometric Sensor for Choline, Acetylcholine and Arsenocholine', Beatriz Lopez Ruiz, Eithne Dempsey, Chi Hua and Malcolm R. Smyth, *Anal. Chim. Acta*, (1993) 273, 425.

[5] 'Development of a Flow-Through Electrochemical Detector for Glucose Based on Glucose-Oxidase Microelectrode Incorporating Redox and Conducting Polymer Materials', Ellen Rohde, Eithne Dempsey, Malcolm R.Smyth, Johannes G. Vos and Hendrik Emons, *Anal. Chim. Acta*, (1993) 278(1), 5.

[6] 'Determination of Some Metal Ions using Lichen Modified Carbon Paste Electrodes', Michelle O'Connor, Eithne Dempsey, Malcolm R. Smyth and David H.S. Richardson, *Electroanalysis*, (1991) 3, 331.

[7] 'Application of Lichen-Modified Carbon Paste Electrodes for Voltammetric Determination of Metal Ions in Multielemental and Speciation Studies', Eithne Dempsey, Malcolm R. Smyth and David H.S. Richardson, *Analyst*, (1992) 117(9), 1467.

[8] 'Development of an Antibody-Based Amperometric Biosensor to Study the Reaction of 7-Hydroxycoumarin with its Specific Antibody', Eithne Dempsey, Ciara O'Sullivan, Malcolm R. Smyth, Denise Egan, Richard O'Kennedy and Joseph Wang, *Analyst*, (1993) 118, 411.

[9] 'Differential Pulse Voltammetric Determination of 7-Hydroxycoumarin in Human Urine', Eithne Dempsey, Ciara O'Sullivan, Malcolm R. Smyth, Denise Egan, Richard O'Kennedy and Joseph Wang, *J. Pharm. Biomed. Applic.*, (1993) 11(6), 443.

[10] 'Organic-Phase Biosensing of Enzyme Inhibitors', Joseph Wang, Eithne Dempsey, Arkadi Eremenko and Malcolm R. Smyth, *Anal. Chim. Acta*, (1993) *in press*.

[11] 'Investigation of the Effects of Various Organic Solvents on the Kinetics and Activity of Immobilised Tyrosinase using the Rotating Disc Electrode', Eithne Dempsey, Jie Liu, Joseph Wang and Malcolm R. Smyth, submitted.