STUDIES OF MEMBRANE-MODIFIED AMPEROMETRIC BIOSENSORS

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ABSTRACT

The project was primarily concerned with the optimisation of membranes for glucose, lactate and cholesterol enzyme electrodes. Membrane permeability, selectivity and biocompatibility properties were also investigated.

Enzyme electrodes using immobilised oxidases were developed for clinical use employing an oxygen-type ("Rank") electrode. The platinum working electrode was polarised at +650 mV with respect to a silver reference electrode for hydrogen peroxide detection. Low substrate permeability external membranes including unmodified and detergent-modified cellulose acetate and 0.01 μm pore size polycarbonate enhanced linearity for both glucose and lactate measurement.

Inner permselective unmodified and detergent-modified cellulose acetate, plasticised polyvinyl chloride, polyethersulphone membranes were studied. The detergent-modified and unmodified cellulose acetate membranes were effective in preventing charged interferents from reaching the electrode and gave improved blood compatibility. The degree of electrode passivation by diffusible electroactive species was dependent on the magnitude of the polarising potential applied during blood/plasma exposure.

The performance of miniature (needle-type) lactate and glucose sensors was assessed *in vitro*. The electrodes consisted of a central Teflon-coated platinum wire as the working electrode which was polarised at +650 mV vs an outer stainless steel pseudoreference electrode. The inner polyethersulphone membrane was optimised and the following casting conditions caused improved selectivity:- (a) a high concentration of the polymer (4% w/v); (b) a 3:1 solvent/non-solvent mixture of dimethylformamide and 2-methoxyethanol. The performance characteristics and stability of various external polyurethane membranes were assessed.

For lactate needle sensors successive dipcoating using the same concentration (2% or 3% w/v in tetrahydrofuran) or increasing concentration of polyurethane gave sensors with the following performance characteristics:- (a) a maximum linear response for lactate levels of up to 24 mM; (b) sensitivity of 0.7 nA/mM; (c) background current of 0.2-1.0 nA; (d) response times of 5-7 minutes. The sensor response was also stir independent and unaffected by pH variation between 6 and 7.5.

DECLARATION

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Sharmistha Ghosh

DEDICATION

This thesis is dedicated to

my parents and my husband, Pradip

without whose perseverance this goal

would not have been achieved.

A C K N O W L E D G E M E N T S

I would like to express my gratitude to Prof. P. M. Vadgama (Dept. of Clinical Biochemistry, Hope Hospital, University of Manchester) for the supervision, guidance and advice in the completion of my study and help in the preparation of the thesis. I am indebted to Dr. G. S. Rigby, Dr. K. Warriner and Mr. S.M. Reddy for their continual guidance during writing of this dissertation. I am grateful to Dr. M. A. Desai for his supervision throughout my project.

I would like to thank the rest of my collegues:- Dr. P. Treloar, and Dr. I. Christie for their help. I would also like to thank the routine laboratory staff at Hope Hospital. Finally, I would like to express my gratitude to the Science and Engineering research council for providing financial support during my project.

ABBREVIATIONS LIST

ITEM	ABBREVIATION
Sodium ascorbate	ASC
P-aminophenol	PAP
Hydroquinone	HQ
Paracetamol	PARA
Catechol	CAT
Urate	URA
Cysteine	CYS
Phloroglucinol	PHLO
Dimethylsulphoxide	DMSO
Dimethylformamide	DMF
Tetrahydrofuran	THF
Ethanol	EtOH
Isopropanol	IPA
Cellulose acetate	CA
Polyvinyl chloride	PVC
Polyethersulphone	PES
Polyurethane	PU
Polycarbonate	PC
Polyphenylenediamine	PPD
Polyvinyl alcohol	PVA
Dioctyl phthalate	DOP
Isopropylmyristate	IPM
7,7,8,8 tetracyanoquinodimethane	TCNQ
Flavin adenine dinucleotide	FAD
Adenosine 5'-diphosphate	ADP
Glucose oxidase reagent	GOD
Lactate oxidase reagent	LOD
Cholesterol oxidase reagent	COD
Bovine serum albumin	BSA
Molecular weight	MW
Michaelis constant	K_m
Scanning electron microscopy	SEM

THE AUTHOR

I obtained a BSc honours degree in Biochemistry from UMIST in July 1989. I did an MSc research project on enzyme-based biosensors under the supervision of Prof. P. M. Vadgama (Hope Hospital, Dept of Clinical Biochemistry, University of Manchester) on July 1991. Since then I continued doing research in this particular field for my PhD. I have several publications related to this research area in collaboration with my collegues and have been cited as * in the reference list.

CHAPTER ONE - GENERAL INTRODUCTION

1.1 Biosensors

1.1.1 Introduction

Spectrophotometric techniques have held their importance in the field of analytical chemistry, however, rapid progress in biosensor research (Turner, Karube and Wilson 1987) has lead to the development of a new technology. A biosensor consists of a biological recognition element (e.g. enzyme, cell, antibody, tissue slices) or biocatalyst in intimate contact with a transducer device which converts the biological signal to a quantifiable electrical signal (Figure 1.1.1). The biocatalytic component e.g. enzyme is immobilised onto the transducer which responds to the products of the biocatalytic process and relays information to an interfacing detector which records or displays the data. Transducers can be of several different types (amperometric, potentiometric, optical or other) (Table 1.1).

1.1.2 Historical Background

The biosensor concept was first described by Clark and Lyons (1962) when the term "enzyme electrode" was adopted. The oxido-reductase enzyme (glucose oxidase) was held next to a Clark O_2 electrode in a semipermeable membrane sandwich. The Pt cathode polarised at -0.6 V vs Ag (anode) responded to oxygen consumed by the enzymic reaction with glucose. The glucose oxidase reaction formed the basis of the first blood glucose analyser (Yellow Springs Instrument model 23YSI Chua and Tan 1978). Here a membrane was interposed between the electrode and enzyme layer which allowed peroxide permeation but prevented access of electroactive interferents including ascorbate. Another membrane between the enzyme layer and sample allowed

Transducer	Biological layer	Analyte
Ion-selective electrode	Enzyme, whole cell, organelle, antibody	Substrate, antigen, drug enzyme inhibitor
Amperometric electrode	Enzyme, whole cell organelle	Substrate, enzyme inhibitor
Thermistor	Enzyme	Substrate
Piezoelectric crystal detector	Antibody, enzyme	Volatile, substrate antigen, whole cell
Optical fibre	Enzyme, affinity molecule	Substrate, ion, gas metabolite
Planar wave guide	Antibody	Drug, antigen
Conductivity detector	Enzyme	Substrate

Table 1.1

Major transducer types used in biosensors.

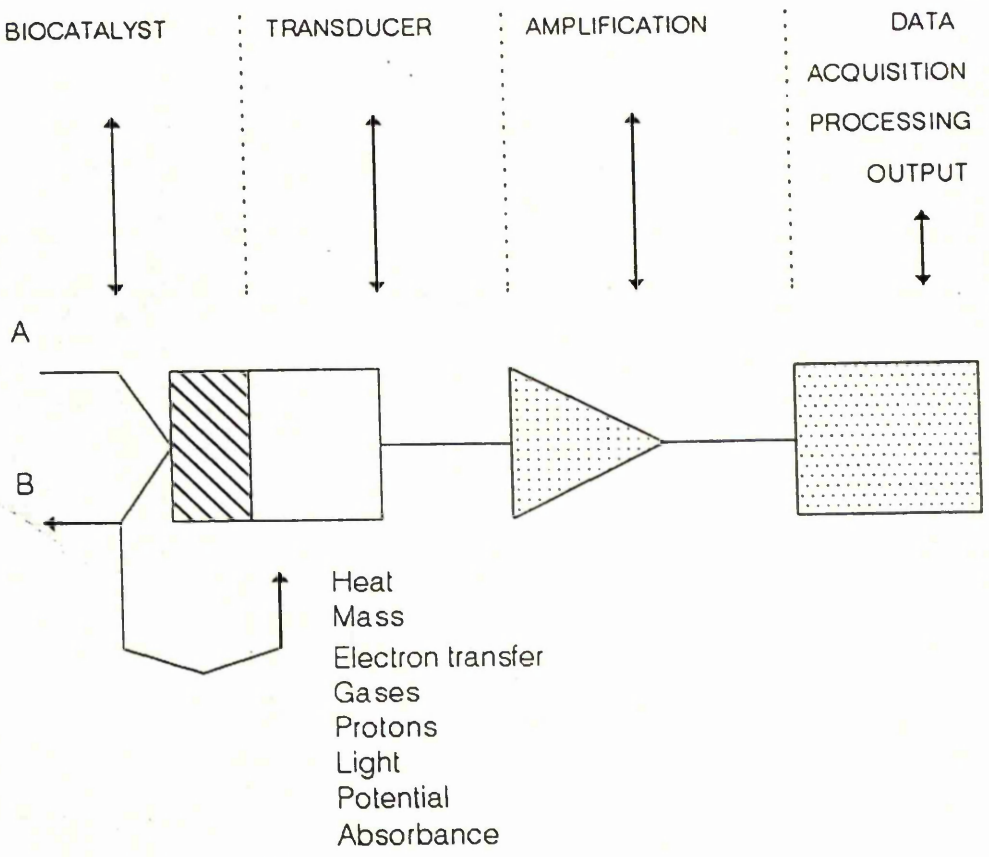


Figure 1.1.1

Diagrammatic representation of the chief components of a biosensor where A = analyte and B = product.

substrate/analyte to enter it. This was done by using a permselective cellulose acetate membrane and a Nuclepore^{R} polycarbonate membrane (Grooms et al 1980). Recently a portable pen-shaped Exactech^{R} glucose meter (Matthews et al 1987) has been developed by Medisense Inc. This device incorporates a ferrocene mediator in the immobilised glucose oxidase layer and shows a rapid response (30 s).

1.1.3 Advantages of enzyme electrodes

The advantages of electrochemical detection over routine clinical measurement of analytes has been reviewed by Vadgama (1988). Enzyme electrodes combine the sensitivity and specificity of enzymic methods of analysis with the selectivity and speed of ion-selective electrode measurements. The sample is not completely destroyed since only a small amount of sample of substrate is consumed. The sensitivity limit of the enzyme is usually suitable for clinical measurement. Therefore a prior sample preparation step e.g., dilution, protein precipitation or dialysis (Trinder 1969) is not required and measurements can be carried out on turbid (e.g. blood) or optically opaque biological fluids. There is also the possibility of continuous monitoring without the need for complex instrumentation, unlike the biochemical systems which involve a spectrophotometric method.

1.1.4 Applications

Biosensors can be used for measuring the concentration of a wide range of substances in various processes with scientific, industrial, environmental, diagnostic and therapeutic applications (Hall 1990).

For basic scientific purposes, for example, enzyme electrodes can be used in studies of reaction kinetics and the study of the characteristics

of enzyme inhibitors (Guilbault 1980). In industry, biosensors can be used for real-time monitoring of carbon sources, dissolved gases, products etc in fermentation processes which lead to an optimised procedure giving increased yields at decreased materials cost. They are also used in environmental monitoring especially in regions where discharge of pollutants is continuous e.g. factory effluent must be regularly checked to determine biological oxygen demand-BOD (a good indicator of pollution levels), atmospheric acidity, river water pH and herbicides and fertiliser content (organophosphates, nitrates etc). They have also been used in the military for the detection of toxic gases e.g. for acetylcholine receptor systems consisting of a matrix of 13-20 proteins there is a 95% certainty of all toxin detection.

The application of enzyme electrodes in Clinical Biochemistry and Medicine has fallen into two categories: clinical analysis and therapeutic medicine. The concept of performing clinical analysis outside the generalised hospital laboratory has stimulated great interest in rapid and simple analytical procedures. Enzyme electrodes make measurement possible in whole blood and also enable monitoring of clinically important metabolites continuously and precisely. Glucose measurements in biological fluids are now made by enzyme electrodes, replacing the use of spectrophotometric techniques in hospital laboratories. Enzyme electrodes for lactate and oxalate determination have also been developed for clinical analysis.

In therapeutic medicine, enzyme electrodes can be used to closely monitor the metabolic state of patients such as diabetics and are also

useful for drug monitoring especially in intensive care units where patients show rapid variations in biochemical levels which require prompt action. Continuous real-time monitoring will eventually be used for treating chronic conditions. Here direct feedback with a controlled drug administration system forms part of a closed loop giving improved patient management. Normally insulin is administered subcutaneously but glucose homeostasis is not achieved completely. In a "closed-loop" system insulin delivery is controlled by the glucose sensor output which is integrated with the insulin infuser ("artificial pancreas").

1.1.5 Electrochemical Techniques

A variety of electrochemical techniques are available for analysis of substances which can undergo an electrochemical reaction. They differ in the type of potential waveform impressed on the working electrode, the type of electrode (planar, dropping mercury or rotating disc electrode), and the state of the solution in the electrochemical cell (quiescent or flowing). However, a common feature is that the electrode processes are all diffusion-related.

1.1.5.1 Voltammetry

Voltammetry is a form of polarography and is a study of current-voltage relationships. A polarogram (or polarographic wave) is obtained which gives information about the nature and concentration of the reacting species. The classical cell used is the dropping mercury electrode (d.m.e) consisting of small droplets of mercury issuing from small bore tubing. This surface is continuously renewed as the drop grows. An external reference electrode completes the electrolysis cell. The external circuit provides a variable voltage source and measures small electrolysis currents (10^{-8}-10^{-4} A). The large mercury pool serves as both

the auxiliary and reference electrodes. As potential is scanned from positive to negative values, the current is measured as a function of potential. More precisely a reference electrode (SCE) and auxiliary electrode may be used and the polarising potential is provided by a potentiostat.

The polarographic current consists of 3 components (diffusion, migration and residual currents). Migration current occurs due to reaction of the sample which arrived at the d.m.e by transference and can be reduced by means of excess inert electrolyte. The residual current is due to the reaction of the supporting electrolyte at the d.m.e and determines the sensitivity of the polarographic technique. It is necessary to correct for the residual current so that only the diffusion current is detected. This arises from reaction of the sample electroactive species at the d.m.e. resulting from diffusion alone.

Ilkovic derived an equation for the diffusion current:-

$$i_d = 607nD^{1/2}m^{2/3}t^{1/6}c \qquad (1.1.5a)$$

where i_d is the average diffusion current (A)
m the average mercury flow rate
t the lifetime of each drop (s)
c is the concentration of the electroactive species (M)

With a reversible reaction, equilibrium is maintained throughout the drop lifetime, and the Nernst equation is followed; the E-i curve or polarogram is called a reversible wave i.e.

$$E_{dme} = E_o' + \frac{0.059}{n_o} \log \frac{C_o^o}{C_o^r} \qquad (1.1.5b)$$

C_o and C_r are the bulk concentrations of oxidised and reduced species, C^o is the concentration at the d.m.e. E_o' the standard electrode potential.

Since the current density is proportional to the concentration gradient across the diffusion layer,

$i = k'(C_o - C_o^\circ) = k''(C_r - C_r^\circ)$

where $k' = i_{d,c}/C_o$ and $k'' = i_{d,a}/C_r$; if k'' is negative then

$$E_{dme} = E_o' + \frac{0.059}{n} \log \frac{k''}{k'} + \frac{0.059}{n} \log \frac{i_{d,c} - i}{i - i_{d,a}} \qquad (1.1.5c)$$

$$= E_{1/2} - \frac{0.059}{n} \log \frac{i - i_{d,c}}{i_{d,a} - i} \qquad (1.1.5d)$$

The half wave potential, $E_{1/2}$ occurs where $i = 1/2\ (i_{d,c} + i_{d,a})$, $i_{d,c}$ and $i_{d,a}$ being the cathodic and anodic diffusion currents respectively. The above equation is called the Heyrovsky-Ilkovic equation and may be used to evaluate $E_{1/2}$ for a reversible wave. The slope of the plot of E_{dme} vs $\log \frac{i - i_{d,c}}{i_{d,a} - i}$ will be $\frac{-0.059}{n}$ V.

Cyclic voltammetry consists of cycling the potential of an electrode over a given range and measuring the resulting current. The potential of the working electrode is controlled vs. a reference electrode e.g a saturated calomel electrode, or Ag/AgCl electrode. A triangular-wave potential is applied across these two electrodes so that the working electrode potential is swept linearly through the voltammetric wave and then back again. Single or multiple cycles can be used depending on the information needed. For an electrode process involving an oxidised species O and a reduced species R and the transfer of n electrons, the reversible electrochemical reaction can be represented as following:

$$O + ne^- \rightleftharpoons R \qquad (1.1.5e)$$

In a typical cyclic voltammogram, on the forward scan, the current

response is the linear potential sweep voltammogram as O is reduced to R, and the current is called the cathodic current. On the reverse scan, the R molecules near the electrode are reoxidised to O and an anodic peak results. The cathodic (anodic) peak in the CV results from the competition of two factors (Evans et al 1983), the increase in the (net) rate of reduction/oxidation as the potential is made more negative/ positive and the electrolysis of the reactant which depletes its concentration near the electrode surface, thus decreasing the rate of mass transport.

The fundamental equations of linear sweep and cyclic voltammetry have been developed by Delahey (1954) and Nicholson and Shain (1964). For an electrochemically reversible system, the peak current is given by the relation:-

$$I_p = 0.4463\ n\ F\ A\ (Da)^{1/2}\ C \qquad (1.1.5f)$$

with $a = \frac{n\ F\ \nu}{R\ T}$ (1.1.5g)

where I_p = peak current, Amps
n = number of electrons involved in the reaction (reduction or oxidation)
F = Faraday constant, 96,485 $Cmol^{-1}$
A = area of the working electrode, cm^2
ν = potential scan rate, V/s
R = gas constant, 8.314 J/K.mol
T = absolute temperature, °K
C = concentration of the bulk species, mol/l
D = diffusion coefficient of the electroactive species, cm^2/s

At 25 °C, in terms of the adjustable parameters, the peak current is given by the Randles-Sevcik equation:

$$I_p = 2.69 \times 10^5 n^{3/2} AD^{1/2} C\nu^{1/2} \quad (1.1.5h)$$

Accordingly, I_p increases with $\nu^{1/2}$ and is directly proportional to concentration and $D^{1/2}$. The relationship to C and $D^{1/2}$ is particularly important in analytical applications and in studies of diffusion processes respectively. The values of the anodic peak I_{pa} and cathodic peak currents I_{pc} should be numerically close for a simple reversible couple. That is

$$\frac{I_{pa}}{I_{pc}} \approx 1 \quad (1.1.5i)$$

However, the ratio of peak currents can be significantly influenced by chemical reactions coupled to the electrode process. In a reversible process, the anodic and cathodic peak potential can be related to the number of electrons in the electrochemical reaction with the equation:

$$E_{pa} - E_{pc} = 2.22 \frac{RT}{nF} = \frac{0.0595}{n} \text{ at } 25\ ^\circ C \quad (1.1.5j)$$

This equation provides a rapid and convenient means of establishing the number of electrons transferred in the redox reaction, and is a good criterion for determining electrode-process reversibility. The difference between the anodic and cathodic peak potentials is affected by the reversibility of the electrode process, due to its dynamic nature (Wang 1988). If the process is fast and the scan rate slow, the process is reversible; if the process is slow and the scan rate fast, the process becomes effectively irreversible and the difference between the anodic and cathodic peak potentials is greater than 0.0595/n volts.

1.1.5.2 Amperometry and the oxygen electrode

Amperometric electrodes rely upon the measurement of current. The detected species (either a product or a co-substrate of the enzymatic reaction) is electrochemically consumed at the working electrode, which is held at a constant polarising voltage relative to a reference electrode, thus resulting in a transfer of electrons and current generation in an external circuit. The current response is given by the Cottrell equation:-

$$i = n\,F\,A\,J \qquad (1.1.5k)$$

Where i is the current, n is the number of electrons transferred per molecule, A is the area of the working electrode and J is the flux of the electroactive species. If a sufficiently high enzyme loading is employed, then the current obtained approximates to that of a non-enzyme amperometric sensor, with a covering membrane of thickness L:

$$i = \frac{n\,F\,A\,D_s\,[S]_b}{L} \qquad (1.1.5l)$$

This yields a simple proportional relationship between substrate concentration in the bulk solution and the current output.

The most well-known sensor is the oxygen electrode. This comprises a noble metal (Pt, Au, Ag) cathode maintained at a negative potential (e.g. -600 mV vs Ag/AgCl) where oxygen molecules are electrochemically reduced to produce a current. The current magnitude is directly related to the oxygen partial pressure, pO_2. To allow proper electrical contact between the cathode and reference electrodes, they require connection via a suitable ion-conducting pathway, using an internal electrolyte solution. Sometimes this is provided by the biological fluid in which the sensor is placed. However, this can lead to problems due to the coating of the electrode surfaces by proteins and

cells. Inclusion of a gas-permeable membrane over the anode and reference could be used in order to alleviate this problem. Suitable materials include:- polyethylene, polyvinyl chloride (PVC), polytetrafluoroethylene (PTFE), silicone rubber, polystyrene or polyurethane. The membrane establishes controlled conditions for the diffusion of oxygen from the sample to the cathode surface, thus ensuring a useful relationship between pO_2 and sensor current according to the simplified equation derived from Fick's first law:-

$$I = k. \frac{nFAP}{V_T a} \qquad (1.1.5m)$$

where F is the Faraday constant, A is the cathode area, P is the membrane oxygen permeability coefficient, a the membrane thickness, n the number of electrons per molecule of oxygen reduced, and V_T is a correction factor for temperature. During sensor operation there is continuous consumption of oxygen. leading to continual removal from the sample. The pO_2 in the region at the interface between the sensor surface and the sample may be reduced unless the consumed oxygen can be replaced immediately by diffusion of oxygen from the bulk to the sample. This is known as the depletion or stirring effect, because it may be eliminated by stirring of the sample, and reduced by modification of sensor design. The current can thus be reduced by, increasing membrane thickness, reduction of the cathode area A, or by using a membrane with a low oxygen diffusion coefficient, D. However the sensor response time is also governed by these parameters:-

$$t_{95} = 3.7\ (a/\pi)^2\ (1/D) \qquad (1.1.4n)$$

Therefore the use of a membrane with a low value of D and/or large value of a, will, in addition to reducing the depletion effect, increase the response time of the sensor. On the other hand, using a small cathode

area A, whilst reducing the depletion error does not adversely affect response time.

1.1.4.3 Potentiometry

An ion-selective electrode (ISE) is a device that develops an electrical potential proportional to the logarithm of the activity of an ion in solution. The response to an ion, i, of activity a_i and charge z, is given by the Nikolsky-Eisenman equation :-

$$E = \text{constant} + \frac{2.303\ RT}{zF} \log\ [a_i + K_{ij}\ (a_j)^{z/y}] \qquad (1.1.5o)$$

in which E is the measured potential, R is the gas constant, T is the absolute temperature, z is the number of electrons involved, F is the Faraday constant, K_{ij} is the selectivity coefficient, j is any interfering ion of charge y and activity a_j. The selectivity coefficient is a numerical description of the preferential response to the major ion, i, in the presence of the interfering ion, j.

These electrodes result in the generation of an interfacial potential with negligible uptake of analyte. They do not suffer as greatly from solution effects since they reach an equilibrium response determined by the ion exchange properties of the sensor surface therefore "fouling" is not a problem. Negligible analyte consumption with these electrodes results in no perturbations in local ion concentrations during measurement and therefore can be deployed in restricted spaces such as the intracellular and interstitial compartments.

An ISE comprises a membrane-based system where the membrane separates an internal reference electrolyte of fixed activity containing

the internal reference electrode, from an external sample solution. An external reference electrode in contact with the sample solution completes the circuit. The reference electrode should maintain a stable potential and is used with an indicator electrode. Overall cell emf is determined by a series of potential steps, membrane, internal/external reference electrode and liquid junction potentials. However "ion selectivity" is determined by membrane properties. If the membrane is selective for a given ion (i) the membrane potential which depends on the ratio of activities of the ion on each side of the membrane:-

$$E_{memb} = \frac{RT}{nF} \ln \frac{(a_i)\ sam}{(a_i)\ int} \qquad (1.1.5p)$$

The analyte solution in which the electrodes are placed imparts an interfacial potential at the indicator electrode because of the difference in activity of the ions on either side of the membrane thereby altering the (difference) potential with respect to the reference. This assumes that the reference potential is unvaried in solutions of quite different types. An interfacial potential is set up at the junction between the reference electrolyte and the sample, called the liquid junction potential, the value of which depends upon the exact composition of the sample solution (Moreno and Zahradnik 1973). Here, interdiffusion of ions takes place and differences in the mobility of interfacial ions lead to charge separation and therefore an electrical potential, the size of which is governed by ionic strength, pH, junction type and temperature. A major drawback of ISEs is that they provide a comparative measurement against a standard solution, and it is difficult to obtain a standard solution which exactly matches the fluid for assay. A reference electrode of high ionic strength is used with cations and anions of

similar mobility (e.g. KCl) in order to reduce variability in the junction potential. In biological samples containing charged colloids or whole cells, a suspension effect arises which further distorts the liquid junction potential, contributing further to the mismatch of sample and standard (Kater et al, 1968). Ion-selective electrodes may be classified as having (1) glass, (2) solid or (3) liquid membranes.

1.1.5.3.1 Glass membrane electrodes

The earliest electrode employed measured pH. It consisted of a glass tube housing a Ag/AgCl electrode and 1M HCl solution with a glass membrane (Eisenman 1966; Buck et al 1974). The glass consisted of a mixture of sodium and calcium silicates and SiO_2. The backbone formed an extensively cross-linked polymer of SiO_4 units with electrostatically bound Na^+ and Ca^{2+} ions. In the hydrated layers on either side of the glass membrane an equilibrium is established between H^+ and Na^+ electrostatically bound to anionic sites in the glass and in solution:-

$$H^+ (aq) + Na^+ (gl) \rightleftharpoons H^+ (gl) + Na^+ (aq) \qquad (1.1.5q)$$

If the concentration of H^+ (aq) is low this equilibrium shifted to the left; Na^+ from the glass migrates into the hydrated region to maintain electrical neutrality. Hydrogen ions on the other side of the glass penetrate a little deeper into the glass to replace the migrated sodium ions. This combination of ion migration gives sufficient electric current that the potential is measurable with a high-impedance voltmeter. Since H^+ ions are intrinsically smaller and faster moving the Na^+, most of the current in the hydrated region is carried by H^+ and the glass electrode behaves as if it were permeable to H^+ therefore acting as an indicator electrode sensitive to pH.

1.1.5.3.2 Solid-state electrodes

These comprise a solid salt in a crystalline form or as a compressed pellet made from powdered materials e.g. the fluoride electrode where a single crystal of lanthanum fluoride is used(Durst and Ross 1974). LaF^{2+} provides cationic fixed charge sites for the ion exchange reaction:-

$$F^-_{(cryst)} + OH^-_{(solution)} \rightleftharpoons F^-_{(solution)} + OH^-_{(cryst)} \quad (1.1.5r)$$

The necessary properties include:- low water solubility, low electrical conductivity, and high ion binding constants. Mobile fluoride anions in a doped crystal are likely to be the charge carriers. The fluoride electrode has similar selectivity properties to the glass pH electrode.

1.1.5.3.3 Liquid ion-exchange electrodes

These are prepared by dissolving an organic ion exchanger in an appropriate solvent. The solution is then held in an inert matrix together with a solvent as plasticiser (e.g., di-octyl sebacate) and an ion-selective complexing agent (ion exchanger or ionophore) e.g., ligand association complexes such as formed by the transition metals with derivatives of 1,10-phenanthroline (Ross 1969), quarternary ammonium salts (anion selective) and ionophore antibiotics including valinomycin (K^+ selective) (Pioda et al 1969; Scholer and Simon 1970). The ion exchanger and solvent may be entrapped in an inert polymer matrix such as PVC or poly(methyl methacrylate) and coated on a platinum wire or graphite rod. The interfacial potential generated at such "liquid" membranes has enabled selective assay of ions.

1.1.5.3.4 Gas and enzyme ISEs

In typical devices a gas-permeable membrane is applied over an ion-selective electrode e.g. for CO_2 or NH_3 detection. The gas diffuses

through the membrane and alters the pH of an internal filling solution which is measured using a glass electrode. The archetypal CO_2 sensor is the Severinghaus electrode (Severinghaus and Bradley 1958) based on a glass pH sensor bathed in a HCO_3^- solution and retained behind a CO_2 permeable membrane. The equation representing the electrode reaction is:-

$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+ \qquad (1.1.5s)$$

The pH of the solution changes by the diffusion of carbon dioxide through the membrane with a consequent change in cell emf proportional to log pCO_2.

Other variables include enzyme loaded membranes with can convert substrates into readily detectable ions e.g. the urea electrode utilised urease to form NH_4^+ (Guilbault and Montalvo 1970). A commercial NH_4^+ selective glass membrane electrode was used in order to detect the pH change.

$$CO(NH_2)_2 + H_3O^+ + H_2O \xrightarrow{\text{urease}} 2NH_4^+ + HCO_3^- \qquad (1.1.5t)$$

However it was liable to interferences from Na^+ and K^+ ions. Guilbault and Hrabankova (1970) carried out a sample pretreatment procedure using cation-exchange granules to remove the interferences. Other approaches used include (a) sensing of the carbon dioxide produced from the urease reaction (Guilbault and Shu 1972); (b) use of a membrane which had a higher selectivity for NH_4^+ ions over Na^+ and K^+ ions. The NH_4^+ ion selective electrode contained the antibiotic nonactin embedded in a silicone rubber matrix (Guilbault and Nagy 1973).

1.1.5.3.5 Ion-selective field effect transistors

A field effect transducer (FET) is a transducer device in which the conductivity of a semiconductor material is controlled by an electrical field. Normally there is a negligible current flow between the "source" and "drain" but a change in voltage of appropriate polarity and magnitude at the "gate" allows current to flow between the source and drain. An ion-selective field effect transistor (ISFET) utilises an ion-selective membrane over the gate region which permits the passage of a single type of ion. The threshold voltage is governed by the analyte pH and the circuit contains a reference electrode contacted with the ISFET amplifier. The operational mechanism of the ISFET originates from the pH sensitivity of the inorganic gate oxide e.g. SiO_2, Al_2O_3, Si_3N_4 or Ta_2O_5 (Sibbald 1986). A functional relationship exists between the drain current i_D and the activity of the primary ion a_i at the surface of the ion-sensitive gate:-

$$i_D = k[(V_G - V_T)V_D + E_{REF} + RT(\ln a_i) - VD^2/2] \qquad (1.1.5u)$$

for $V_D < V_G - V_T$, where k is a constant which is dependent on the materials and geometry of the transducer, V_T is called the turn-on (or threshold) voltage, and V_D is the voltage between the drain D and source C of the transistor. In normal ISFET operation, the drain current is kept constant, therefore change in the membrane potential is compensated by an equal and opposite change of the gate voltage V_G. This is the feedback mode of operation where a Nernstian response is observed (Figure 1.1.5).

ISFETs offer several different advantages over conventional glass membrane electrodes including, planar construction, small dimension,

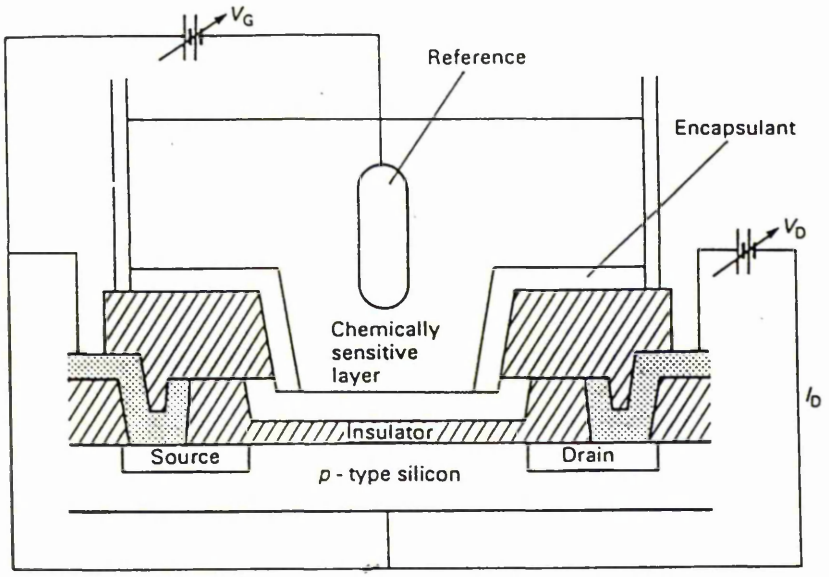


Figure 1.1.5

Diagrammatic representation of the construction of an ISFET (From Hall 1990).

low impedance, fast response, large scale production, ease of multisensor (differential) measurement and can be directly applicable after dry storage. Since it is easier to deposit thin membranes onto flat surfaces ISFETs offer a better control over membrane thickness. During processing a minimum amount of enzyme is usually required since many ISFETs can be made on one silicon wafer.

Caras and Janata (1980) utilised the differential measurement mode for penicillin. Penicillinase (cross-linked with albumin) was placed onto the gate region of one chip and the other gate contained cross-linked albumin only. Analyte buffer capacity had a profound effect on sensor sensitivity and linearity as well as concentration range. A decrease in buffer capacity led to less ion suppression of the membrane pH change, giving greater device sensitivity. Shul'ga et al (1993) showed that the influence of buffer capacity of enzyme coated FETs (ENFETs) for glucose and urea could be reduced by the incorporation of an additional permselective polyvinylbutyral membrane above the enzymic membrane. The negatively charged polyvinylbutyral membrane prevent mobile OH^- ions and other ions from penetration into the gate region while the hydrophobic polyvinylbutyral layer could limit the diffusion of hydrophilic species.

Modified reference field-effect transistors (REFETs) and K^+-selective ISFETs (containing a liquid membrane, polyvinyl chloride and valinomycin) which are pH-insensitive have been prepared (Skowronska et al, 1990). The gate surface was covalently modified using (3-trimethoxysilyl) propyl methacrylate followed by polymer membrane

(polybutadiene, polyacrylate or polyurethane) deposition.

1.2 Enzyme electrodes

1.2.1 Kinetics of enzyme electrodes

Substrate reaches the active site of an immobilised enzyme by diffusion and mass transfer, the substrate concentration therefore may become rate-limiting for the reaction. Therefore (Figure 1.2.1) as enzyme loading is increased the reaction switches from being kinetically controlled (A-B), to being diffusion controlled (C-D); both internal (membrane) and external (bulk) diffusion resistances contribute to the latter. Internal diffusion can be altered by enzyme concentration, and external diffusion by stirring rate variation. At very high enzyme loadings, the barrier due to enzyme protein may lead to a lowered diffusion rate and a reduced electrode signal. The advantage is that the reserve of enzyme is increased and large losses in activity may be sustained without any appreciable loss in response, thus improving the electrode stability. Substrate response is, furthermore, "buffered" against pH, temperature and ionic strength changes and also against activators and inhibitors in solution since a small proportion of the total enzyme is involved in catalysis.

1.2.2 Theory of operation

A mathematical model of a two-substrate enzyme electrode has been proposed by Leypoldt and Gough (1984). It described a glucose-specific sensor in which the enzymes (glucose oxidase/catalase) were immobilised within a membrane attached to an electrochemical sensor for oxygen (co-substrate). The model focused on the reaction and diffusion phenomena that occurred within the enzyme membrane and

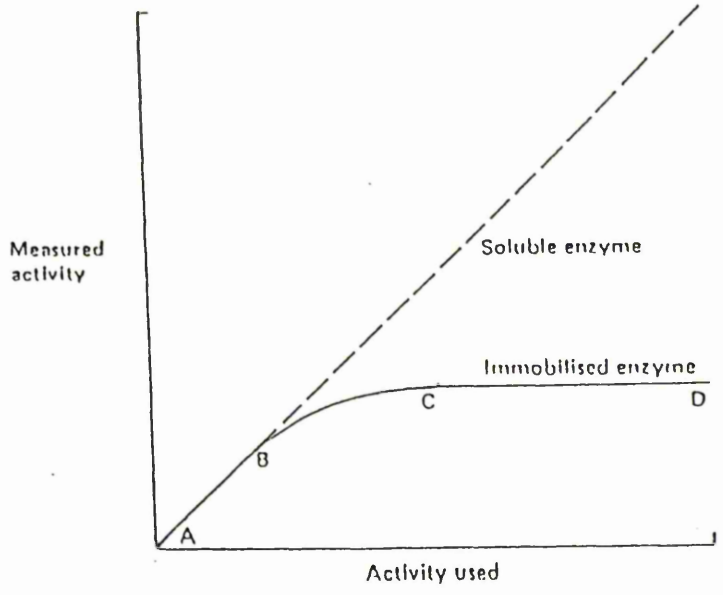


Figure 1.2.1

Comparison of kinetics for soluble and immobilised enzymes (from Vadgama 1989).

provided a mathematical justification for using diffusion-limited membranes.

1.2.2.1 Enzyme kinetics

Binding of the substrate to an enzyme is the prerequisite for enzyme catalysis. The enzyme reaction scheme involves a single substrate, S, which combines with enzyme, E, to give the intermediate enzyme substrate complex, ES. This complex then undergoes reaction to produce the product, P:-

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\Rightarrow} E + P \qquad (1.2.2a)$$

where k_1 and k_{-1} are the forward and reverse rate constants for complex formation and k_2 is the rate constant for complex decomposition into product. From the kinetic scheme it can be shown that:-

$$K_m = \frac{(k_1 + k_2)}{k_{-1}} \qquad (1.2.2b)$$

and the rate, v, of product formation:-

$$v = \frac{k_2 [E]_o [S]}{K_m + [S]} \qquad (1.2.2c)$$

where $[E]_o$ is the total concentration of enzyme and [S] is the substrate concentration. There are two important points to consider from this equation. At lower substrate concentrations, where $[S] << Km$ the rate is proportional to the substrate concentration. At higher substrate concentrations the rate reaches its maximum velocity, $k_2[E]_o$, limited by the amount of enzyme. Therefore with the two step reaction sequence of equation (1.2.2a), two processes can control the overall observed rate. Either the association reaction to form the enzyme-substrate complex or the catalysed reaction step within the complex which results in product formation. A more detailed treatment of this theory was

reported by Carr and Bowers (1980).

1.2.2.2 Mass transport

Three mechanisms of mass transport can occur in solution: diffusion, convection and migration.

1.2.2.2.1 Fick's laws of diffusion

In an arbitrary plane surface within a solution as shown in Figure 1.2.2. Let the solution on one side contain a concentration c of a chemical species and that on the other a concentration c + δc. With equal probability of movement of any given molecule in any given direction, the amount of material crossing the plane surface in either direction will be proportional to the concentrations on the two sides of it. Hence the net flux, j, of material across the surface in a given time will be directly related to the concentration difference, δc, or more precisely to the concentration gradient, $\delta c/\delta x$. This results in Fick's first law of diffusion in one dimension:

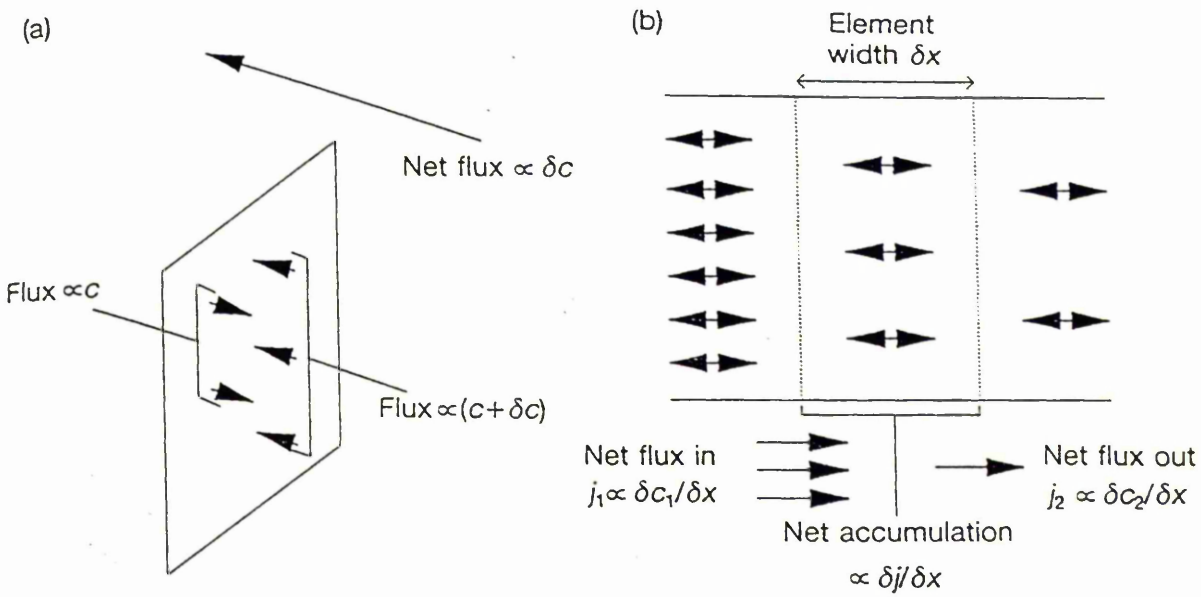
$$j = -D\left\{ \frac{\delta c}{\delta x} \right\} \qquad (1.2.2d)$$

where D, is the diffusion coefficient.

The flux of material from one small volume element to another leads to concentration changes within those volume elements. Fick's second law of diffusion in one dimension can be derived from the concentration change associated with a diffusional flux in the presence of a concentration gradient:-

$$\frac{\delta c}{\delta t} = D\left\{ \frac{\delta^2 c}{\delta x^2} \right\} \qquad (1.2.2e)$$

The rates of transport (i.e. the flux as described by Fick's first Law) of material to the sensor surface and the build up or depletion of material



Flux across surface

$$j = -D\left(\frac{\delta c}{\delta x}\right)$$

Rate of change of concentration

$$\frac{\delta c}{\delta t} = D\left(\frac{\delta^2 c}{\delta x^2}\right)$$

Figure 1.2.2

Schematic representation of (a) diffusional flux across a surface, and (b) the concentration change associated with this diffusional flux in the presence of a concentration gradient (Eddowes 1990).

there, in accordance with Fick's Second Law can be considered. However, additional information is needed such as initial and boundary conditions, which define the initial state of the system and its state at the boundaries of the region of space over which the diffusive mass transport occurs. These boundary conditions are dependent upon factors such as the biochemical and other processes occurring at the sensor surface and the concentration of species in the bulk of solution from which material is transported to the sensor. Also, geometrical aspects of the sensor are important. An expression can then be derived for concentration as a function of time and distance from the surface:-

$$c = c_\infty \, \mathrm{erf} \left\{ \frac{x}{2D^{1/2}t^{1/2}} \right\} \qquad (1.2.2f)$$

where c_∞ is the bulk concentration far from the surface and erf is an error function. This expression describes the increase in concentration with increasing distance, x, away from the surface and the decrease in concentration with increasing time, t. An expression describing the variation of reaction rate with time is obtained by differentiation of equation (1.2.2f) with respect to x with substitution of $x = 0$ to give the concentration gradient at the surface. This is then substituted into the flux expression of Fick's first law to give the Cottrell equation :-

$$j_D = \left\{ \frac{D^{1/2}}{\pi^{1/2} t^{1/2}} \right\} c_\infty \qquad (1.2.2g)$$

where j_D is the diffusion controlled flux per unit area.

1.2.2.2.2 Convective mass transport

Convection refers to the movement of the solution as a whole under the influence of an external mechanical force. Close to a surface, convection can be employed to enhance the rate of mass transport by replacing the

solution close to the surface which has been depleted of reactant. It can also be employed to maintain the reaction at a constant rate provided the steady state flow is maintained.

Over a stationary surface, solution immediately adjacent to the surface will be stationary whilst the next layer of solution will move a little with respect to it, according to its viscosity. For a given force, each subsequent layer of solution moves a little with respect to the previous layer such that the viscosity of the solution with respect to the stationary surface increases with the distance from it. Thus, an essentially stagnant layer of solution exists close to the surface. Where reaction takes place at the surface and species are destroyed or generated, they must diffuse through the stagnant layer, called the diffusion layer, between the surface reaction site and the well stirred bulk of solution. It is convenient therefore to think in terms of the diffusion layer thickness, x_D, which is the thickness of the stagnant surface layer across which the material must diffuse between the well stirred bulk of solution and the surface of the sensor.

1.2.2.2.3 Transport due to migration

Migration is the movement of charged species under the influence of an applied potential gradient. It represents the mechanism by which charge flows through the solution between the two electrodes at which ionic charge is created and destroyed by electron transfer, thereby maintaining the necessary charge balance. However, its contribution to the overall rate of mass transport of an electrochemically generated species is minimal due to the likely presence of a large excess of other ionic species, referred to as an inert supporting electrolyte, which is

responsible for the passage of the required charge and depresses any potential gradients which might arise in solution.

1.2.3 Factors affecting enzyme electrode performance

1.2.3.1 Stability

Enzyme electrode stabilisation is governed by the stability of the immobilised enzyme. This is attributed to the anchoring of the enzyme into active conformations which are then better able to resist the molecular unfolding occurring during denaturation. Stability generally increases in the order soluble < physically entrapped < chemically immobilised. Within gels, steric hindrance reduces enzyme translational and rotational mobility, therefore the enzyme becomes less likely to uncoil and will therefore resist thermal denaturation to a greater extent. Stability will improve if non-covalent (e.g. ionic) interactions exist between the enzyme and gel polymer. A declining enzyme activity could be attributed to a loss of enzyme protein by leaching out from a gel or diffusion across semipermeable membranes. With immobilised enzymes activity decay could also be due to oxidation of constituent amino groups, degradation by microorganisms, loss of prosthetic group (e.g. FAD from amino acid oxidase) or repeated exposure to toxic reagents and reagent products (e.g. hydrogen peroxide).

1.2.3.2 Response Time

Many factors generally affect the speed of response of an enzyme electrode. In order for the electrode to respond the substrate has to:-

(a) diffuse through the bulk solution to the membrane surface;

(b) diffuse through the membrane and react with enzyme active site;

(c) the products formed must diffuse to the electrode surface where they are measured;

1.2.3.2.1 Rate of substrate diffusion

The rate of substrate delivery is dependent on the stirring rate of the bulk solution. This has been shown experimentally by Mascini and Liberti (1974) for the amygdalin electrode. As the stirring rate was increased the substrate quickly reached the membrane surface where the reaction rate was increased hence a much faster (1-2 minute) response time (stirred) was observed compared to 10 minutes for unstirred conditions.

1.2.3.2.2 Substrate and enzyme concentration

An increase in reaction rate and decreased response time are observed with an increase in substrate concentration. The enzyme activity of the gel has a dual effect on enzyme electrode performance:-

(a) it will ensure that a Nernstian calibration plot is obtained for potentiometric electrodes;

(b) it will affect the speed of the response.

This has been demonstrated for the amygdalin electrode by Mascini and Liberti (1974). For optimal enzyme electrode function it is recommended that a highly active enzyme is used to ensure rapid kinetics in as thin membrane as possible.

1.2.3.2.3 pH

Each enzyme has an optimum maximum pH at which it is most active. The immobilised enzyme has a different pH range from the range of the soluble enzyme because of its environment. For fastest responses, the optimal pH range should be used, however this is not always possible e.g. Guilbault and Tarp (1974) used an NH_3 sensor to monitor the urea-urease reaction. At the optimal pH for urease,

7.0-8.5, there is very little free ammonia detectable using the air-gap

electrode. Therefore a decreased pH was used which ensured sufficient sensitivity of the sensor.

1.2.3.2.4 Temperature

Temperature variation has a dual effect on electrode response:- an increase in the rate of reaction resulting in a faster response time as well as a shift in an equilibrium potential by virtue of the temperature coefficient in the Nernst and Van't Hoff equations. The effect of temperature variation (10-50 °C) on glucose electrode response has been demonstrated (Guilbault and Lubrano 1973). Linear plots of the log rate and log current versus 1/T were observed as predicted by the Van't Hoff [$\ln k = \ln C - \Delta H/RT$, k = equilibrium constant] and Arrhenius [$\ln k = \ln A - E_a/RT$, k being the rate constant] equations. Practically, this means that the temperature for enzyme electrode operation should be carefully controlled for best results during kinetic measurements. For equilibrium measurement room temperature or ~25 °C should be used.

1.2.3.2.5 Membrane Thickness

The time required to reach a steady state potential or current is strongly dependent on gel layer thickness. This is dependent on the rate of diffusion of the substrate through the membrane to the enzyme active site, and of diffusion of products through the membrane to the electrode. A mathematical model relating the thickness of the membrane d, the diffusion coefficient D, the Michaelis Constant K_m, and the maximum velocity of the enzyme reaction, V_{max} ($= k_3 E_o$) was prepared by Mell and Maloy (1975):-

$$V = \frac{k_3 E_o d^2}{D K_m} \qquad (1.2.2h)$$

where V compares the rate of chemical reaction in the membrane to the

rate of diffusion through the membrane. The larger the value of V, the faster the enzyme catalysis is compared with diffusion. Guilbault and Montalvo (1970) found the response time was ~26 seconds with a 60 μm thick net of urease and about 59 seconds with a 350 μm thick net for 8.33 x 10^{-2} M urea and an enzyme concentration of 175 mg/cm^2 of gel.

1.3 Glucose monitoring

1.3.1 Why is a glucose sensor required for insulin administration?

Insulin is a polypeptide hormone produced by the beta cells of the pancreas which controls carbohydrate, protein and fat metabolism. In diabetes mellitus there is relative or absolute insulin deficiency leading to the loss of control of blood glucose levels within normal limits (fasting 3.8-5.6 mmol/l). Other manifestations associated with hyperglycaemia include degeneration of blood vessel walls, particularly of the eyes, leading to eventual blindness, kidney and heart disease.

Insulin causes removal of glucose from the blood and therefore excess amounts can cause the blood sugar level to fall too low (hypoglycaemia) and if below 1.1 mmol/l both convulsions and coma may occur. Hence the accurate measurement of blood glucose levels on patients taking doses of insulin is particularly important, with methods involving whole blood being preferable to those requiring serum or diluted blood. Since diabetics perform their own blood determinations, systems for use at home must be accurate, easy to read, portable and quick. A glucose sensor may form part of a closed-loop system, where insulin administration is controlled by the sensor response (i.e. blood glucose concentrations).

1.3.2 Aims of glucose biosensor development

The following demands need to be met during glucose biosensor development (Turner et al 1987):- The sensor needs to be highly specific to substrate. The response should be linear to 30 mmol/l for glucose and measure low glucose levels which in hypoglycaemic cases may fall below 1-2 mmol/l. A suitable response time of 3-5 minutes is usually required. The response should be independent of fluid hydrodynamics in vessels or tissues for implantable sensors, e.g., a stable response may not always be obtained due to stir dependence (i.e. stable mass transfer of the substrate is needed). It should exhibit a low temperature dependence. A signal variation of 5%/°C would be best although 10%/°C is acceptable. It should exhibit low pH dependence, and response should be independent of oxygen variations. The analytical signal should show low baseline drift, especially for implantable sensors when direct zeroing is impossible and have low drift; the slope of the calibration curve should be sufficiently stable not to increase the error above 10%, making a weekly recalibration sufficient.

For implantable sensors, it should show long-term mechanical, chemical and enzymatic stability of glucose oxidase and its support at 37 °C, in whole blood, lymph or tissue. The covering polymeric membrane protects the enzyme layer from mechanical damage. The enzyme protein should not leak out into surrounding fluids and tissues since it is a foreign protein, and its recognition by the immune system would provoke an immune reaction.

The implanted sensor parts should be biocompatible e.g. encapsulated

fibroblasts should not be present. Woodward (1982) suggested that the optimal configuration for a subcutaneously implantable sensor is in the form of wire or filament. Such a structure, if measuring less than about 2 mm in diameter, would evoke a minimal tissue response. Sensor miniaturisation should not modify the geometrical, physical and enzymatic characteristics which control its analytical properties. The sensor should have a prolonged lifetime and be easily replaced as necessary (which demands relative inexpense).

1.3.3 Role of membranes in glucose enzyme electrodes

Membranes play a very important part in enzyme electrodes. The enzyme has to be immobilised in a membrane configuration and needs to be covered by protecting polymeric membranes which prevent access of interferences either to the enzymatic or the electrochemical reaction. They may have effects on electrode stability, lifetime and extend the electrode linear range to well above the enzyme K_m. The key to successful and practical deployment of enzyme electrodes often lies in the combination of the choice of such covering membranes and the immobilisation of enzyme. Polymer membranes also act as diffusion barriers by controlling the passage of analytes to be detected and preventing the permeation of interferents both to the enzymatic reaction and to the electrode surface process.

The classical structure of the prototype immobilised enzyme layer is in the form of a "sandwich" configuration e.g for the H_2O_2-based glucose electrode (Grooms, Clark and Weiner 1980). The laminated enzyme layer usually comprises an internal low molecular weight cut-off cellulosic membrane and an external microporous polycarbonate

membrane, with the chemically crosslinked enzyme sandwiched in between. As shown in the Figure (1.3.3), the external covering membrane allows the diffusion of glucose whilst preventing the permeation of large molecules such as protein. It also protects the enzyme from mechanical disturbances. The underlying membrane acts as a selective barrier, screening out the interferences such as ascorbate while permitting the enzymatic reaction product hydrogen peroxide to pass relatively freely.

1.3.3.1 External membrane

The outer membrane acts as a protective layer for the immobilised enzyme and reduces mechanical and turbulence related distortion of the enzymic layer underneath therefore a robust and rigid membrane matrix is preferable. It should allow substrate to pass through but restrict diffusion of interferents which affect the enzymatic reaction, such as inhibitors and impurities.

Outer membranes are used in order to extend the linear operating range by acting as glucose diffusion barriers between the sample and enzyme (Koochaki et al 1993). Permeability is tailored so that they can restrict glucose access to the enzyme, thereby avoiding saturation of the enzyme kinetics, whilst allowing short diffusional distances to ensure rapid responses. Neutron track-etched polycarbonate membranes consisting of well defined pore sizes of <0.03 μm have been used as external membranes (Koochaki et al 1993). The extension of linear range is assisted by the enhanced permeability of the polymer compared to oxygen (co-substrate); therefore the adverse effects of a low or

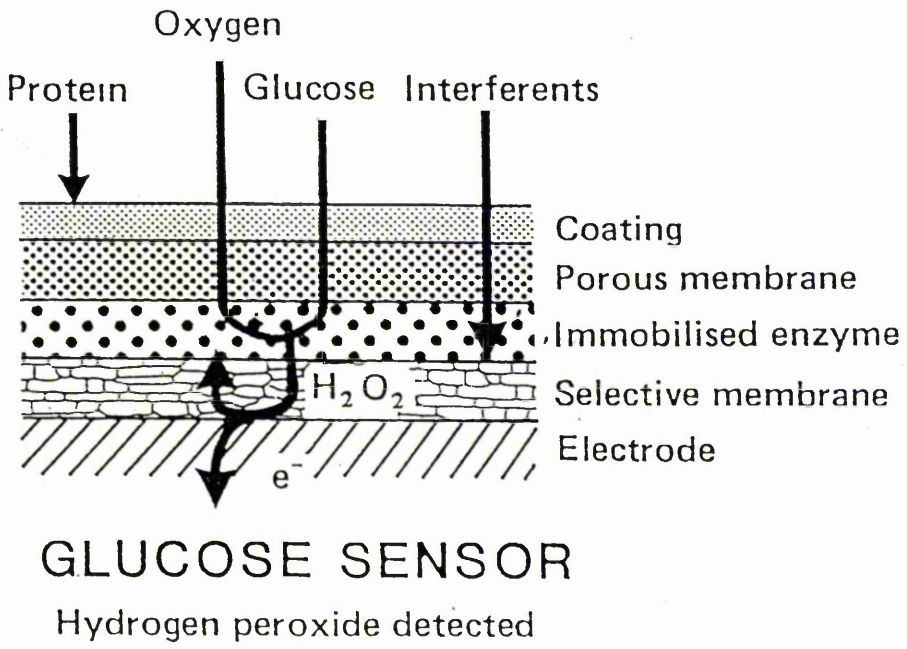


Figure 1.3.3

Membrane configuration of a glucose enzyme "laminate".

fluctuating pO_2 environment are virtually abolished. Further adjustment of the differential glucose:oxygen flux of porous polycarbonate has been achieved through partial coating with organosilane layers (Mullen et al 1986) or lipid (e.g. isopropylmyristate) into the pores (Tang et al 1990) which further reduce glucose:oxygen permeability ratios. Both agents had additionally reduced blood-surface interactions and therefore the surface fouling which destabilises performance (Tang et al 1990).

Tang et al (1990) explained that since isopropylmyristate has been exploited as an analogue of skin lipids it is a biocompatible material. The haemocompatibility of isopropylmyristate may be attributed to its biological inertness and fluidity properties. The glucose electrode was stable in blood for 55 measurements and a correlation coefficient of 0.969 was obtained with a routine method.

Lipid bilayer films have been employed as external layers because they are coherent, fluid and present a surface which is analogous to the natural cell membrane and confer a high degree of biocompatibility. However, inherent deficiency in mechanical strength has limited their use, but this has been overcome by exploitation of liposomes used over an hydrogen peroxide electrode (Rosenberg et al 1991). The diversity of usable lipids with their different glass transition temperatures, additionally give unprecedented control over fluidity and permeability (Nelson 1992).

A recent coating material used for restricting glucose diffusion is diamond like carbon (DLC) (Higson et al 1993). These films are

deposited from the gas phase over a polymer without any damage to the material. The duration of DLC-coating governed haemocompatibility. Greater resistance to biofouling was observed with an increase in deposition of DLC. Membrane porosity was an important factor, smaller (0.01 μm) pore size DLC-coated membranes exhibited enhanced haemocompatibility compared with 0.05 μm or 0.1 μm membranes. Cellulose membranes especially of cellulose acetate have also shown excellent biocompatibility behaviour (Gunasingham et al 1989) with the extension of the electrode analytical linear range. Particular attention has been given to porous polyurethanes which have been used as biocompatible membranes in miniature needle-type sensors (Vadgama et al 1989). Both the materials differ from the previous polymers in that they can be solvent dip-coated and readily take on the geometry of the device.

1.3.3.2 The enzyme layer

Recently Bartlett et al (1992) entrapped glucose oxidase into nonconducting electropolymerised films of six different phenols. Rapid polymerisation of phenolic compounds took place especially $\sim +0.65$ V vs SCE on anodic potential cycling corresponding to poly(phenylene) oxide production. Similarly polymerisation occurred with 4-hydroxybenzenesulphonic acid, 3-nitrophenol, pyragallol and bromophenol at a slower rate. Poly(phenylene) oxide films showed the greatest sensitivity to glucose compared to the other phenolic films due to a higher permeation of hydrogen peroxide.

Various other methods for enzyme immobilisation on sensors have been proposed e.g. enzyme may be covalently attached to a derivatised

membrane (Mutlu et al 1991), or to the inner transducing surface. Immobilisation by covalent attachment within a porous platinum structure has been reported for a micro-sensor (Beh et al 1989). Glucose oxidase has also been co-deposited over conducting carbon fibre (Wang and Angnes 1992). In this case, rhodium had intrinsic catalytic properties which has allowed the potential for hydrogen peroxide measurement to be reduced to ~ +300 mV vs Ag/AgCl. Some of these techniques may supersede the conventional crosslinking of enzyme (with albumin) using a bifunctional reagent such as glutaraldehyde which is toxic and leads to high losses of enzyme activity or uncertain physical entrapment procedures within gels e.g. in polyacrylamide or gelatin. Gamma radiation has been used to achieve immobilisation of glucose oxidase within ultra thin poly-(N-vinylpyrrolidone) (Galiatstatos et al 1990). In this way rapid response electrodes have been formed with at the same time a rigid, stabilised radiation polymerised biolayer being produced.

Glucose oxidase has been co-entrapped in electropolymerised films. Wolowacz et al (1992) utilised enzyme coupled to N-2 carboxyethyl pyrrole via surface lysyl residues with a carbodiimide promoted reaction. The polypyrrole modified surface with 30.2 mol pyrrole/mol of glucose oxidase displayed similar properties to the native enzyme but with a more acidic isoelectric point; the stability was 6-fold higher at pH 7.0 (60 °C). Electropolymerised films are advantageous since they minimise diffusional distances to < 10 nm. Poly(o-phenylenediamine) formed films with one second response times while rejecting ionic solute interference. (Malitesta et al 1990). However, a time-dependent

decrease in response to glucose in the presence of 0.5 mM ascorbic acid was observed (Palmisano et al 1993).

1.3.3.3 Internal membrane

The internal membrane forms an interface between the two environments in which the enzymatic and electrochemical reactions take place. It has a key role in selectivity by allowing the hydrogen peroxide to pass through easily, while simultaneously restricting the passage of other electroactive species present in biofluids (e.g. blood) such as thiols, ascorbate, urate and paracetamol which can also be oxidised at the polarised electrode and give a false signal. Selectivity has been achieved on the basis of charge e.g. anionic polymers for anionic interferents. Membranes used include polyethersulphone (Vadgama et al, 1989), cellulose acetate (Mullen et al 1986) and esterified sulphonic acid polymers (e.g. Eastman AQ-29D) (Wang et al 1991). Recently an anionic charge screening membrane (Nafion) has been incorporated into biocompatible lipid films at a modified carbon electrode (Mitzutani et al 1993). However cellulose acetate still remains an important choice for an inner membrane, since it uses charge repulsion and size exclusion (Mullen et al 1986). A drawback is the additional diffusional barrier that all membranes present leading to prolonged response times.

Electropolymerised phenolic films have been utilised in order to achieve selectivity (Vadgama et al 1991) while ensuring a short diffusion path. A 1,2-diaminobenzene polymer film on platinised reticulated vitreous carbon, served avoided anionic and amino acid interference and also gave a more biocompatible surface in serum (Sasso et al 1990). The platinised RVC (reticulated vitreous carbon) electrode formed part of

a flow injection analysis (FIA) system. It extended the apparent K_m for glucose from 5 mM to 25 mM. The self-limiting nature of the deposition also meant that the layer was uniform over an electrode with complex topography.

1.4 Immobilised enzymes

1.4.1 Advantages of using immobilised enzymes

Routine analysis may require large quantities and expensive amounts of enzymes. However, insolubilisation of enzymes enables reuse many times and therefore reduces the cost. Immobilised enzymes are usually more stable since they are held in an environment more like their natural state. Immobilisation imparts greater enzyme stability, making them more useful over a broader pH and temperature range. They are much less susceptible to normal activators and inhibitors which affect the soluble enzyme. Therefore they can be used in complex biological matrices e.g. blood, urine rather than the soluble enzyme.

1.4.2 Physical methods of immobilisation

1.4.2.1 Adsorption

This technique involves mixing of an enzyme with a solid of a high surface area. Adsorption on insoluble supports results from ionic, polar, hydrogen bonding or hydrophobic or π-electron interactions. Adsorption into water soluble matrices has also been studied e.g. glass, quartz, charcoal, silica gel, alumina, carboxy-methyl cellulose (Wykes 1971), controlled pore ceramics and cellulose (Messing 1975). The major advantage is the simplicity of the method and the enzyme is not denatured since mild conditions are employed. It is difficult to obtain a high enzyme loading since the enzyme is desorbed readily by alterations in pH, solvent, substrate, temperature and ionic strength.

1.4.2.2 Gel entrapment

Here a polymeric gel is prepared in a solution containing an enzyme leading to entrapment of the enzyme within the three-dimensional lattice. Low molecular weight substrates can have access to the enzyme which itself diffuses slowly through the gel lattice. This technique was developed by Bernfield and Wan (1963). In the polyacrylamide gel system, copolymerisation of N,N'-methylenebisacrylamide and acrylamide is done in absence of oxygen. The resulting lattice contains a hydrocarbon backbone with carboxamide side chains which can act as binding sites for other molecules. The reaction is radical catalysed by persulphate and riboflavin, and the enzyme is included in the reaction mixture. Gel permeability and mechanical properties are dependent upon the degree of crosslinking and therefore on the relative concentrations of N,N'-methylenebisacrylamide and acrylamide monomer. This is a mild method of enzyme immobilisation and does not alter the enzyme properties. Polyacrylamide gels have also been used in enzyme electrodes (Updike and Hicks 1967).

However several drawbacks of this technique include:- free radicals (singlet O_2, SO_4^{-}) generated during the polymerisation process cause enzyme denaturation. Gel formation results in large diffusional barriers for transport of substrate and product causing reaction retardation especially for high molecular weight substrates.

1.4.2.3 Microencapsulation

Microencapsulation within thin-walled semipermeable spheres has been achieved by Chang (1966). The enzymes are prevented from diffusing out of the microcapsule while reactants and products can permeate the

surrounding membrane. Polymer deposition around emulsified aqueous droplets takes place by interfacial coactivation or interfacial polycondensation. The main disadvantage is that many of the interfacial polymerisation procedures cause enzyme deactivation. Rony (1971) circumvented this problem by first producing hollow fibres (instead of spheres) in which the enzyme was placed prior to sealing the ends.

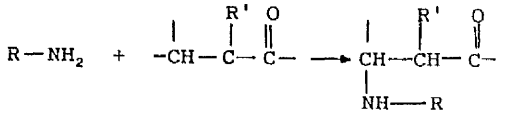
1.4.3 Chemical methods of immobilisation

1.4.3.1 Crosslinking using bifunctional reagents

Chemical crosslinking of an enzyme with a bifunctional reagent ensures total retention of the enzyme protein. The enzyme layers can either be formed directly over an electrode or prefabricated and stored dry before use. This procedure leads to an increase in the average molecular weight of the protein. Bifunctional reagents can be classified as being "homobifunctional" or "heterobifunctional" depending on whether the reagent possesses two identical or two different functional groups. Homobifunctional reagents include:- bisdiazobenzidine-2,2' disulphonic acid, 4,4' difluoro-3,3'-dinitrodiphenyl sulphone, 1,5 difluoro-2,4 dinitro-benzene and glutaraldehyde (Avrameas 1969). A few heterobifunctional reagents are:- toluene-2-isocyanate-4-isothiocyanate, trichloro-o-triazine and 3-methoxydiphenylmethane 4,4,-diisocyanate. The major advantages of this method are (i) simplicity and (ii) chemical binding of the enzyme enables control of the physical properties and particle size of the final product. However many enzymes are sensitive to the coupling reagents so that they lose activity during the process.

Richards and Knowles (1969) proposed a possible mechanism for the glutaraldehyde reaction. This proposed that glutaraldehyde existed as α,ß unsaturated aldehyde oligomers in solution. These react with amino

groups giving rise to Michael type adducts. The adduct is stable to acid hydrolysis:-



Alkylamino derivative (1.4.2)

1.4.3.2 Covalent Binding

Modified enzyme electrodes have been constructed using this technique. Glucose oxidase has been immobilised onto silanised anodised platinum to give a micro-enzyme electrode (Moody et al 1986). It has also been chemically immobilised on a nylon mesh with a lysine spacer and glutaraldehyde following treatment with dimethylsulphate and sodium hydroxide (Hornby and Morris 1975). Collagen membranes containing chemically activable -COOH groups have been used. Untanned films, previously acid-methylated were activated by acyl-azide formation. After removal of reagents by repeating washing, the coupling reaction was initiated by immersion of the activated film in the enzyme solution (Coulet et al 1974).

1.4.4 Properties of immobilised enzymes

Immobilised enzymes show increased long-term and temperature stability which can be increased by proper choice of coupling procedure and insoluble carrier. Alterations in enzyme reactivity, kinetic constants (due to a change in activation energy), pH profile, Michaelis constant or specificity occur on immobilisation. Typically K_m is found to decrease although in some cases no change or an increase is observed. In an ionic matrix, the microenvironment of the active site may be altered by the electrostatic field. The pH within the matrix will be different from the external solution affecting the optimal activity of the enzyme. If the

substrates are also charged there may be an alteration in the apparent Michaelis constant value (Weetall 1974).

Covalent coupling may bring about the effects as well as a change in specificity due to an alteration of the enzyme's net charge, nearest neighbour effects on the active site region, perturbations of intramolecular interactions and conformational changes. By a suitable matrix choice a shift in the pH profile can be effected either to high or to low values. Covalent binding to electrically neutral carriers by nonactive site residues has no effect on their kinetic behaviour towards low molecular weight substrates (Silman and Katchalski 1966). Enzyme immobilisation makes it less susceptible to temperature denaturation thereby allowing greater operational lifetimes at higher temperatures.

1.4.4.1 Alteration in the pH profile

For an immobilised enzyme the optimal pH shifts depending on the nature of the carrier. Goldstein et al (1964) reported that if a carrier is negatively charged, a high concentration of positively charged ions (H^+) will accumulate at the boundary layer between the carrier and the surrounding solution. This will cause the pH at the carrier surface to drop below that of the bulk solution. The enzyme therefore "sees" a pH below that of the bulk solution. Therefore the apparent pH of the immobilised enzyme may be increased. If the carrier is positively charged, the opposite occurs.

1.4.4.2 Kinetics

An alteration in the apparent K_m is observed for immobilised enzymes. This effect is related to the charge on the substrate and/or carrier,

diffusional effects, and, in some cases, tertiary changes in enzyme configuration.

1.4.4.3 Stability

Immobilised enzymes show a lower rate of inactivation/denaturation than the free enzyme. Thermal denaturation of the native protein initiates at a specific structural region where unfolding of the tertiary structure usually begins. Enzyme stabilisation by immobilisation is thought to be achieved as a result of blocking this unfolding nucleus (Schellenberger and Ulbrich 1989). The chemical and thermal stability of soluble and immobilised alpha-amylases from B.licheniformis and A. oryzae has been studied (Ulbrich 1988). The thermolabile alpha-amylase (A. oryzae) as well as thermostable (B. licheniformis) were both stabilised by covalent binding to gamma-aminopropyl silica. Soluble and immobilised alpha-amylases from B.licheniformis were more resistant to guanidine hydrochloride, urea, methanol, ethanol, propanol and t-butanol than the alpha-amylases from A. oryzae. However operational stability is also dependent on carrier durability and organic inhibitor levels, including heavy metals.

1.5 Glucose electrodes

1.5.1 Glucose oxidase - The enzyme and kinetics

Glucose oxidase was discovered in 1923 by Muller in mycelial extracts of Aspergillus niger and Penicillium glaucam (Muller 1926). He showed that the enzyme catalysed the oxidation of glucose by molecular oxygen to give an acidic product (gluconic acid). The enzyme possessed antibiotic activity due to the formation of hydrogen peroxide and was termed Notatin (Penicillin B) (Coulthard et al 1942). Glucose oxidase

can operate in the presence of other hydrogen acceptors including dichlorophenolindophenol (DCPIP) although reaction proceeds at a much reduced rate e.g. 3.3 % for DCPIP compared with oxygen (Keilin and Hartree 1948). Keilin and Hartree (1948) found that the enzyme was stable to one hour incubation at 39 °C (pH 5.6) in :- 0.2 % sodium deoxycholate, 0.2 % sodium dodecylsulphate, 1 % pepsin and 1 % papain (and cysteine),though thermal stability decreased after 40 °C.

The apoenzyme requires the presence of FAD (flavin adenine dinucleotide) (Keilin and Hartree 1946) co– enzyme. These workers denatured glucose oxidase in order to produce the coenzyme which was used to reconstitute activity of another flavoprotein oxidase enzyme called D-amino acid oxidase. Additional studies revealed that the enzyme did not fluoresce in ultraviolet light until denaturation had taken place with release of fluorescent FAD. FAD fluorescence developed rapidly at 70 °C and at pH > 8 or < 2.

The role of FAD during catalysis was studied by Swoboda (1969). FAD was tightly bound to the apoenzyme and could not be removed by dialysis at neutral pH. According to Swoboda (1969) the apoenzyme undergoes a conformational change on binding to FAD from a flexible loose coil to a more rigid, globular form. This gave the much lowered resistance to proteolysis and acid/thermal denaturation of the holoenzyme. Glucose oxidase was subsequently found to be a homodimer of molecular weight 150-180 kDa containing two polypeptide chains linked by a disulphide bond (Swoboda and Massey 1965).

Periodate oxidation of glucose oxidase revealed that the enzyme was a glycoprotein containing 12% carbohydrate by weight (Nakamura et al 1976). Mannose was the major component although glucose, galactose and galactosamine were also present. Periodate treatment gave a time-dependent decrease in carbohydrate content. The catalytic properties, immunological reactivities and conformation were not significantly altered by treatment. The protein moiety remained practically unaltered although minor modification in amino acid composition and absorbance were observed. A crystallographic structure of the enzyme molecule at 2.3 Å resolution has been produced (Hecht et al 1993). The refined model revealed that the enzyme contained 580 amino acid residues, FAD, 6 N-acetylglucosamine residues, 3 mannose residues and 152 solvent molecules.

The substrate binding domain is formed from non continuous segments, characterized by a deep pocket. One side of this pocket is formed by a 6-stranded antiparallel beta-sheet with the flavin ring system of FAD located at the bottom of the pocket on the opposite side. Part of the entrance to the active site pocket is at the interface to the second subunit of the dimeric enzyme and is formed by a 20-residue lid, which in addition covers parts of the FAD-binding site. The carbohydrate moiety attached to Asparagine 89 at the tip of this lid forms a link between the subunits of the dimer. Radioactive tracer experiments using $^{18}O_2$ and $^{18}H_2O$ (Bentley and Neuberger 1949) gave the following reaction scheme for glucose oxidase utilising D-glucose:-

$$C_5H_{11}O_5CHO + H_2O \rightarrow C_5H_{11}O_5.CH.(OH)_2 \qquad (1.5.1a)$$

$$C_5H_{11}O_5.CH.(OH)_2 + FAD \rightarrow C_5H_{11}O_5.COOH + FADH_2 \qquad (1.5.1b)$$

$$FADH_2 + O_2 \rightarrow FAD + H_2O_2 \qquad (1.5.1c)$$

During the reaction an increase in acidity was observed because δ-gluconolactone, the initial reaction product, was spontaneously hydrolysed to gluconic acid. The enzyme is very stereospecific and rapid oxidation takes place with ß-D-glucose rather than α-D-glucose (Keilin and Hartree 1952). Glucose oxidase could however catalyse the mutarotation of the α-glucose to the ß-form prior to oxidation (Bentley and Neuberger 1949). The enzyme reacts to a lesser extent with D-glucosone and 2-deoxy-D-glucose. Substitution of the 1, 2 or 3 hydroxyl groups of glucose causes a complete loss of activity (Keilin and Hartree 1952).

Neuberger and Bentley (1949) proposed that in the initial step glucopyranose loses a proton and an electron to give free radical, $HO_2^{\cdot}$ and a radical derived from glucose; the latter then loses a second electron to form a carbonium ion which was subsequently stabilised by resonance. A prototypic reaction between the oxonium ion and peroxide anion then completes the oxidation.

The oxygen dependency of the reaction has been demonstrated by various workers (Keilin and Hartree 1948; Laser 1952). Keilin and Hartree (1948) showed that the activity of glucose oxidase in pure oxygen was about x1.5 greater than in air. Laser (1952) observed a marked decrease in the rate of substrate oxidation at oxygen tensions of less than 20%. The apparent K_m values were determined at various oxygen tensions for notatin and the crystalline enzyme.
At low oxygen tensions the reaction becomes rate-limiting for glucose. Laser (1952) suggested that the approximately sevenfold increase in K_m

value for glucose with a twentyfold increase in O_2 tension could be explained by application of the Briggs-Haldane treatment according to the following equations:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \qquad (1.5.1d)$$

$$ES \overset{k_3}{\Rightarrow} E + \text{products} \qquad (1.5.1e)$$

With increased oxygen tension, the velocity at infinite glucose concentration correspondingly increases resulting in an elevation of k_3. Since $K_m = k_2 + k_3/k_1$, this is also accompanied by an increase in K_m. However application of the simplifying assumption that k_2 is large compared to k_3, is not possible for glucose oxidase. Ingraham (1955) proposed that two equilibria were present, one between glucose and enzyme (E) and one between oxygen and E:

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_1 \qquad (1.5.1f)$$

$$E_1 + B \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E_2 \qquad (1.5.1g)$$

$$E_2 \overset{k_5}{\Rightarrow} E + \text{products} \qquad (1.5.1h)$$

At low oxygen tensions, K_m is not simply given by $k_2 + k_3/k_1$.

Glucose oxidase reacts by a ping-pong mechanism. In this case one substrate (i.e. glucose) reacts with the enzyme via transfer of a functional group onto the enzyme, leaving a product (gluconic acid). The second substrate (i.e. oxygen) then reacts picking up the functional group forming a second product (hydrogen peroxide).

Evidence for the existence of ping-pong mechanism was provided by Gibson et al (1964). The workers determined each of the individual rate constants as well as the kinetic parameters K_m(gluc) and $K_m(O_2)$ at different temperatures. At 25 °C glucose oxidase possessed a K_m for glucose of 3.3×10^{-2} M and a K_m for oxygen of only 2×10^{-4} M. The Km values for glucose and oxygen were 60 and 0.33 mM respectively from Duke et al (1969). Mikkelson et al (1991) found a K_m of 66 mM for glucose using a rotating disc electrode. The turnover rate (k_{cat}) i.e. the maximum number of substrate molecules which can be converted to products per molecule per unit time is related to the maximum reaction velocity ($V_{max} = K_{cat}[E_o]$) and was calculated as being 0.605 s^{-1} by Mikkelsen et al (1991).

1.5.2 Hydrogen peroxide based glucose detection

These sensors measure hydrogen peroxide with the working (platinum) electrode polarised at +0.65 V with respect to Ag/AgCl reference

Hydrogen peroxide is oxidised at the platinum surface:-

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^- \qquad (1.5.2a)$$

The hydrogen peroxide flux to the anode can be made to directly reflect the glucose concentration. This method is susceptible to interference from other endogeneous electroactive species (e.g. ascorbate, urate and tyrosine). Various methods have been developed in order to increase the selectivity. The response may be compensated by a using a second non-enzymatic sensor (Thevenot et al 1978) or the platinum anode can be covered by selective membranes such as cellulose acetate used in the Yellow Springs electrode (Yellow Springs Instrument Co, 1975). Independence on sample pO_2 for hydrogen peroxide-based detection is

an advantage. The minimum local oxygen level necessary for the enzymatic reaction must be taken into account. Oxygen regenerated during the electrochemical oxidation of hydrogen peroxide at the platinum surface helps to replenish the enzyme layer.

1.5.2.1 In-vivo monitoring

Shichiri et al (1982) developed a needle electrode comprising a platinum wire working and silver-plated stainless steel tube reference electrodes separated by a glass sleeve insulator. Glucose oxidase was immobilised in a cellulose acetate matrix and overlaid with a polyurethane which reduced glucose diffusion relative to that of oxygen. This membrane gave independence from solution variables such as pH, viscosity and stirring (figure 1.5.2a). The electrodes formed part of a wearable closed-loop device ("artificial pancreas") and could be inserted into veins or subcutaneous tissue. Response was used to control blood glucose for over seven days in pancreatectomised dogs. The wearable system consisted of a sensor, a microcomputer system which calculated insulin and glucose infusion rates, and two roller pumps which drove the unit for insulin and glucagon infusions respectively. Continuous glycaemic control with the artificial pancreas was achieved for 6 days, with the sensors being replaced every fourth day. Correlations of the sensor response with routine blood measurements were obtained in the range of 2.7-21.6 mmol/l.

Moussy et al (1993) evaluated the performance of needle type glucose sensor under both in vitro and in vivo conditions (Figure 1.5.2b). Essentially a tri-laminate membrane arrangement consisting of Nafion as the protective outer coating and poly (o-phenylenediamine) as the

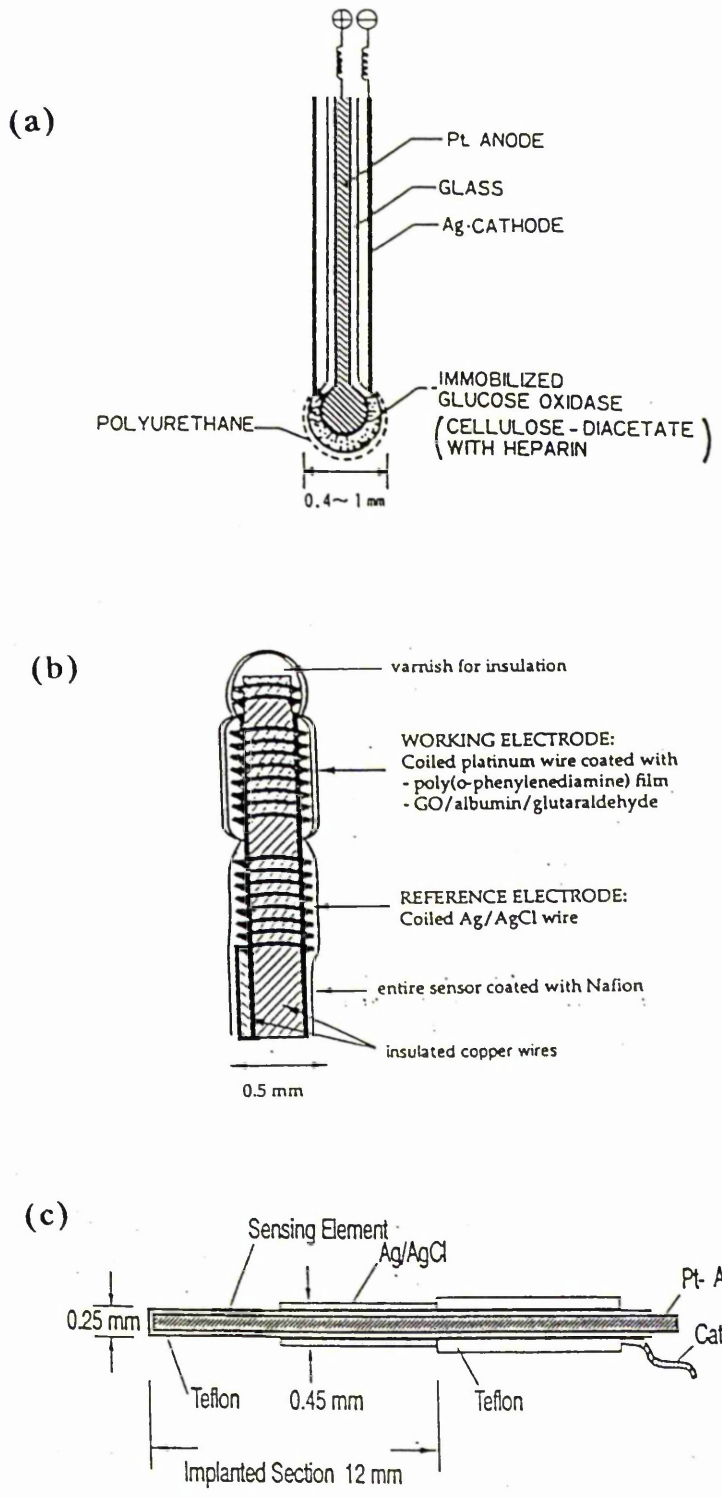


Figure 1.5.2

Representation of glucose needle-type electrode designs from (a) Shichiri et al (1983) (b) Moussy et al (1993) (c) Zhang & Wilson (1993).

permselective inner coating was used. Glucose oxidase immobilised in a bovine serum albumin matrix was sandwiched between these coatings. The sensor was 0.5 mm in diameter and could be inserted under the skin through an 18-gauge needle. Coiled Pt and Ag/AgCl wires were wrapped around a Cu wire and served as working and reference electrodes respectively. Sensor responses *in vitro* were linear to at least 10 mM, and a maximum linearity of 20 mM with the response time being fairly rapid (~33 seconds) compared to polyurethane coated sensors. The time to reach a stable basal current was between 10-30 minutes and was shorter than the 2-hour period reported by Bindra et al (1991). In vivo performance of the sensors were characterised. In an acute experiment subcutaneous implantation in a conscious dog was carried out. After a 30-40 minute stabilisation period an intravenous glucose tolerance test was done. The sensor signal followed a similar pattern to plasma glycaemia. A lag time of 3 minute was consistent with the lag time between subcutaneous and blood glucose levels.

Zhang and Wilson (1993) described an alternate needle-electrode design as shown in Figure 1.5.2c. *In vitro* sensors with a high response size (low linearity) showed a greater dependency on oxygen partial pressure whilst low sensitivity sensors were not affected by pO_2 levels. For in-vivo evaluation glucose and oxygen sensors were implanted through a 20-gauge catheter into the subcutaneous tissue of a rat on the back of the neck. Sensor sensitivity was found to be an important factor in the measurement of tissue pO_2 since it represented the level of oxygen consumption by the sensor. Therefore sensitivity had to be suppressed to such an extent that oxygen consumed by the sensor was

negligible compared to the rate of tissue oxygen supply.

1.5.2.2 Extracorporeal monitoring

Extracorporeal monitoring of blood glucose levels formed the basis of the Biostator^{R} Glucose Controlled Insulin Infusion system (Fogt et al 1978) developed by Miles laboratories. It was a closed-loop system and all of the components were built into a device of dimensions 42 x 46 x 46 cm. A hydrogen peroxide sensor measured the glucose concentrations, and a computer-controlled infusion pump regulated the delivery of glucose or insulin according to the requirements. The initial various biocompatibility problems were overcome when anti-platelet agents were added.

1.5.2.3 Mediator-based enzyme electrodes

Although dioxygen is the natural electron acceptor for glucose oxidase, it may be replaced by an electron transfer mediator. A mediator is usually a low molecular weight redox couple which shuttles electrons from the redox centre of the enzyme to the electrode. The mediator needs to react with the reduced enzyme and then diffuse to a second mediator molecule or the electrode.

In solution

$$\text{Glucose} + \text{FAD} + H_2O = \text{Gluconic Acid} + FADH_2 \qquad (1.5.2b)$$

$$FADH_2 + M_{ox} = FAD + M_{red} + 2H^+ \qquad (1.5.2c)$$

At the electrode

$$M_{red} = M_{ox} \qquad (1.5.2d)$$

The rate at which reduced mediator M_{red} is produced can be measured amperometrically but the mediator is preferentially retained at the transducer surface, (Cass et al 1984) although the biochemical reaction

can take place in the bulk solution with a free diffusible mediator (Wang et al 1990).

The advantages of mediated enzyme electrodes systems have been outlined by Cardosi and Turner (1987). Since the reduced mediator is unreactive with oxygen, measurements can be made independent of pO_2 fluctuations. The working potential is determined by the formal potential (E°) of the mediator couple, which can be advantageous if the mediator has a low E°; this reduces interference from other electroactive species. If the electrode reaction does not involve protons this also makes the enzyme electrode pH insensitive.The essential criteria for a practical mediator system are:- Chemical stability in the oxidised and reduced forms; absence of any side reactions; ready access to the active site of the enzyme; physical properties including net charge must be compatible with enzyme/electrode interaction; lack of toxicity. The most successful class of mediators use ferrocenes (η bis-cyclopentadienyl iron) molecule, a transition metal π-arene complex consisting of an iron sandwich or cyclopentadienyl (Cp) rings.
The reaction scheme for glucose oxidase (flavin-containing glycoprotein) with ferrocene can be represented as follows:-

$$\text{Beta-D-glucose} + \text{GOD(FAD)}_{ox} \rightarrow \text{Gluconolactone} + \text{GOD(FADH}_2)_{red} \quad (1.5.2e)$$

$$\text{GOD(FADH}_2)_{red} + 2\text{Fecp}_2\text{R}^+ \rightarrow \text{GOD(FAD)}_{ox} + 2\text{Fecp}_2\text{R} + 2\text{H}^+ \quad (1.5.2f)$$

$$2\text{Fecp}_2\text{R} \rightarrow 2\text{Fecp}_2\text{R}^+ + 2e^- \quad (1.5.2g)$$

Ferrocene has a low operating potential ($\sim +160$ mV vs SCE), does not react with oxygen rendering the sensor oxygen insensitive and exhibits

rapid reaction kinetics. Ferrocene derivatives can be made relatively insoluble and can therefore be easily retained.

Some disadvantages include:- competition for electrons with oxygen; the rate constant (k) for oxygen is 1.5 x 10^6 $lmol^{-1}s^{-1}$ at 25 °C and pH 7 (Cass et al 1984) but with ferricinium ion derivatives a rate constant of 2.01 x 10^6 $lmol^{-1}s^{-1}$ was recorded. Ferrocenes can also catalyse the oxidation/reduction of interferents; polyvinylferrocene films convert ascorbic acid to dehydroascorbic acid (Dautartas and Evans 1980).

The glucose electrode described by Cass et al (1984) exhibited an excellent linear response for glucose to 30 mM covering the medically relevant range whilst retaining rapid response times (60-90s). These were achieved by the use of a "spongy" carbon-foil electrode which also provided a sufficient diffusional restriction barrier for assay in unstirred samples, without the requirement for an additional membrane. There was a small difference in response when analysis was performed under aerobic versus anaerobic conditions. With an anion exclusion membrane, interference from anionic metabolites was reduced.

With physical adsorption of ferrocene, the water solubility of the ferricinium ion can affect the lifetime of the electrode (Cass et al 1984). Various methods of enzyme/mediator immobilisation have therefore been proposed. Thus the mediator has been mixed with graphite powder and paraffin and retained with a suitable membrane (Gorton et al 1990). With graphite foil mediated electrodes (Cass et al 1984) doped with 1,1'-dimethylferrocene, the enzyme can be immobilised by a

carbodiimide-type reaction with surface carboxyl groups.

Ferrocene has been linked to flexible, siloxane polymers (Gorton et al 1990) thereby allowing efficient electron transfer. Mediator spacing along the polymer and the bridging distances to the polymer were important determinants of electrode current and dynamic response. Ferrocene has also been linked to glucose oxidase (Heller et al 1990). An efficient "electron relay" was obtained by introduction of approximately 12 ferrocene functions into buried sites as well as the external enzyme surface. Without the "internal relay" the glycoprotein shell around FAD formed an insulating layer thereby resulting in an exponential fall in electron transfer as the mutual separation distance between the redox centres increased. A complex between the redox centre [Os (2,2' bipyridine)$_2$ Cl$^-$) $^{1+/2+}$ and poly(vinylpyridine) has been used as an electron wire (Gregg and Heller 1990). The cationic polymer formed an electrostatic complex with the negatively charged glucose oxidase molecule held over working electrodes; this enabled direct charge relay from the enzyme active site to the working electrode.

O- and p-quinoids can also act as electron acceptors for glucose oxidase (Kulys and Cenas 1983). The rate constants of oxidation for each acceptor (k_{ox}) was determined and varied from 1.5 x 10^6 $M^{-1}s^{-1}$ to 3.5 x 10^3 M^{-1} s^{-1}. P-quinoidal compounds oxidised the reduced form of glucose oxidase at rates determined by their redox potentials. A linear relationship was found between the oxidation rate constants and the acceptor potentials. The p-quinoids 1,2-Naphthoquinone and pyocyanin exhibited the greatest reactivity. Introduction of a negatively charged

substituent (sulpho) to the organic acceptor 1,2-Naphthoquinone caused a decreased rate of oxidation. This was because negatively charged reagents had restricted access to the enzyme active site since FAD (cofactor) is usually embedded in hydrophobic surroundings deep inside the external glycoprotein shell.

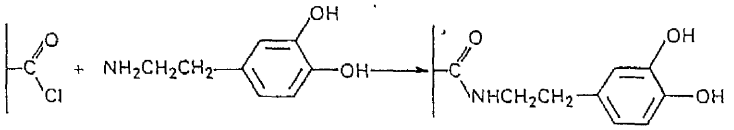
1.5.2.4 Chemically modified electrodes (CMEs)

Numerous reviews and articles have been published regarding the manufacture and use of CMEs (Gorten 1991; Faulkner 1984; Murray 1984). The earliest approaches of electrode modification involved adsorption of a species from solution. This is a reversible process and gives only monolayer or submonolayer coverage. Alternative direct covalent attachment of mediators to the electrode were developed by Murray's group (Moses et al 1975) and Kuwana's group (Lin et al 1977). These methods relied on a direct chemical linking of the redox group to the electrode surface using silanisation of the electrode to produce M-O-Si linkages, the use of cyanuric chloride, or with carbon direct reaction with acidic and carbonyl functionalities at the electrode surface.

Multilayer coverage has been achieved by electrode surface polymeric modification. Methods include electrochemical polymerisation to give redox polymer films, dipcoating of polymer on electrodes, and gas phase plasma polymerisation of monomer. The first approach is advantageous due to the ease of film formation and control over the thickness which can be provided by control of the electrochemistry (Murray 1984).

Electron promoters have been used in CMEs and commonly possess an

electron-rich pi-system with charge delocalisation and ready stabilisation. In a similar fashion, carbon electrodes with their extended pi-system form effective adsorption surfaces. Adsorption of promoter species e.g. 4,4' bipyridyl onto gold electrodes has facilitated electron transfer between the haem site of the redox protein, cytochrome c and the electrode surface (Albery et al 1981). However 4,4' bipyridyl was itself not electroactive in the potential region for this reaction and, therefore was not acting as a charge-transfer mediator. Enhanced charge transfer was dependent upon the bipyridyl adsorption to the electrode surface and rapid on-off binding of cytochrome c at the surface. The protein molecule was held sufficiently close to the electrode surface and in the correct orientation to allow the electron transfer step to take place efficiently. The interaction of cytochrome c with the gold/4,4' bipyridyl electrode was suggested to occur through specific lysine residues, on the protein surface, via hydrogen bonding to one of the nitrogen atoms of the promoter. A major disadvantage of promoter adsorption is its inherent instability, therefore these reagents may be anchored using a covalent bond. Metal oxides or oxidised carbon can be utilised. The latter produces a high density of -COOH groups which can be modified giving -COCl to which the pi-rich promoters can be attached e.g.



(1.5.2h)

Attachment to metal oxides could be achieved via the reactive silane functionality.

(1.5.2i)

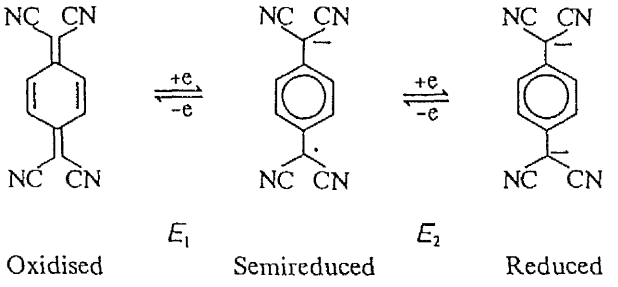
Polymer films have been extensively used to make CMEs since they adhere well to the electrodes to provide multilayer films which may be electrochemically active or inert. Electroactive polymers have ion-exchange, redox or conducting properties. Ion exchange polymer films may become electroactive by doping with electroactive ions. Thus exchange of ClO_4^- with $Fe(CN)_6^{3-}$ in a polyvinylpyridine film gave an electrode showing ferricyanide redox activity (Oyama and Anson 1980). The fluoropolymer, Nafion, is a cation exchanger containing both hydrophilic and hydrophobic zones. This polymer requires a redox cation to act as an electron carrier from the polymer to the electrode (Oyama et al 1980; Oyama and Anson 1980), e.g. $Ru(bpy)_3^{2+}$ (bpy = 2,2′ bypyridine) (White et al 1982) and Cp_2FeTMA^+ ([trimethylammonio) methyl] ferrocene). However, since the ion-exchange reaction is an equilibrium process it will be slowly reversed in the absence of the ion in solution.

Redox polymers containing p- and o- quinone groups have been adsorbed onto the surface of electrodes (Cenas et al 1983 and 1984). These act as oxidants for reduced glucose oxidase, L-lactate oxidase and xanthine oxidase. Cenas (1983 and 1984) found that these enzymes could be re-oxidised in the potential range of 0.05-0.5 V (vs Ag/AgCl) at pH 7. The oxidation of these enzymes was found to occur at a oxidation potential of the polymer modifier suggesting that these polymers acted as mediators. A major drawback associated with these redox polymer electrodes, however, is that they can lose their electrocatalytic activity after a relatively short period to time, typically 5 days (Cenas et al 1984).

Conducting polymers on CMEs may be formed by electrochemical polymerisation include polypyrroles, polyphenoxides and polyanilines. If electropolymerised in the presence of glucose oxidase they can entrap the enzyme. These films exist in both insulating and conducting forms, depending upon the oxidation state. Film structure and redox properties can be altered by choice of derivative, and the electrolyte and solvent of the electropolymerisation reaction e.g. for polypyrrole the conductivity is dependent on the conditions of polymerisation.

Diaz et al (1981) carried out electropolymerisation of pyrrole in the presence of $Et_4N^+BF_4^-$ from an aqueous solution containing glucose oxidase to produce a polypyrrole film containing the enzyme.
Electro-oxidation was believed to proceed via a reactive pi-radical cation, which reacted with neighbouring pyrrole species to produce a chain which was predominantly alpha-alpha' coupled (figure 1.5.2.4). The resultant polymer incorporated anions from the supporting electrolyte. The electrode responded to glucose at an optimal potential of +0.8 V.

Tetracyanoquinodimethane (TCNQ) as a conducting compound has been used in CMEs. The molecule is a very strong electron acceptor forming first the radical anion and then the dianion.



(1.5.2j)

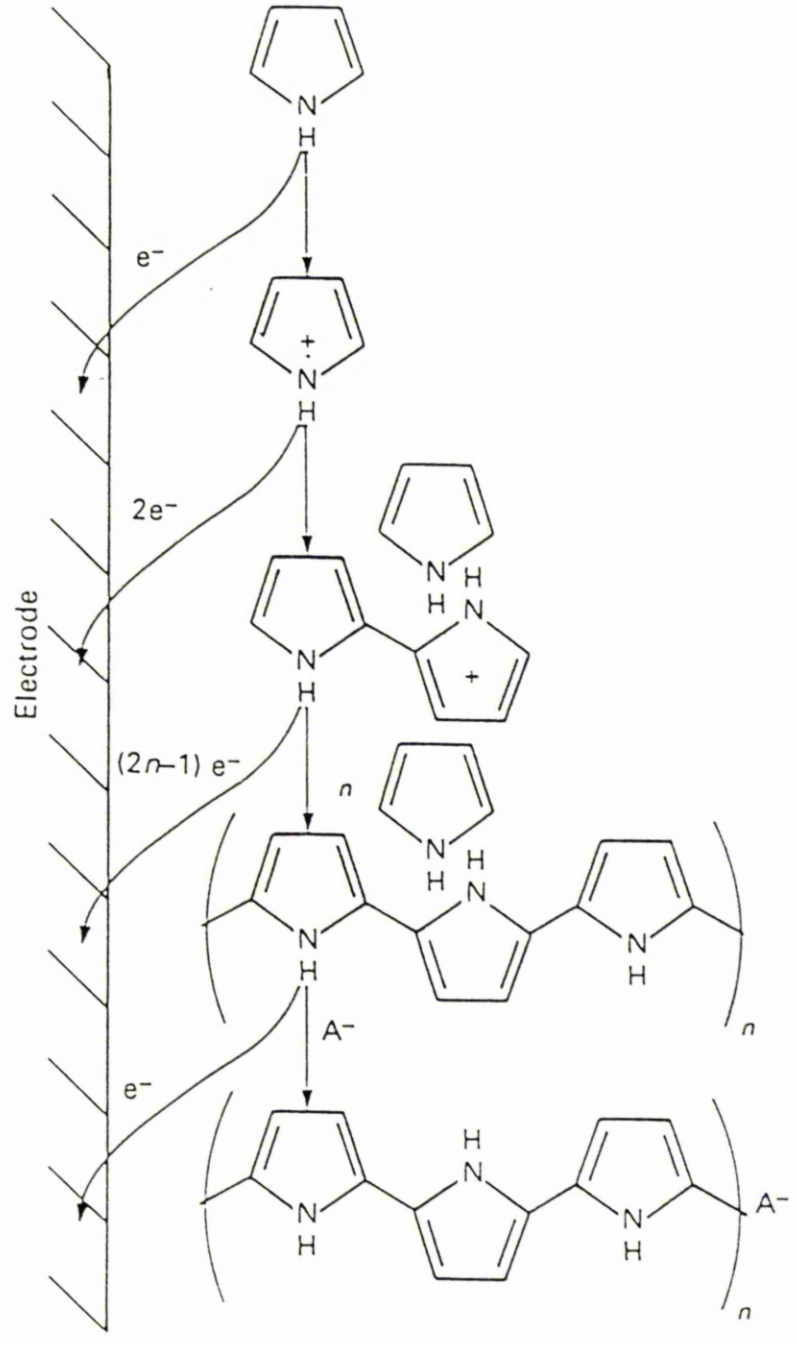


Figure 1.5.2.4

Mechanism of polypyrrole film generation by electrolytic oxidation of pyrrole (From Hall 1990).

In electrochemical terms the interconversions of the system of neutral molecule, radical anion and dianion are characterised by redox potentials E_1 = 0.127 V and E_2 = -0.219 V. This gave an indication of energy differences between the oxidation states. The semireduced form is in fact stabilised from the relatively unstable quinoid structure to aromatic which allows extensive delocalisation of the pi-electrons over the carbon skeleton. Electrodes utilising glucose oxidase based on Charge Transfer complexes, NMA^+TCNQ^- or NMP^+TCNQ^- have been reported. The electrodes responded to glucose at potentials greater than 0.1 V relative to Ag/AgCl; i.e. the peak oxidation potential of NMP^+ and NMA^+. The electrodes showed a good linear range and remained stable for at least 100 days (Cenas et al 1981).

The mechanism by which charge transfer to the electrode occurs has been under question. It has been argued that the enzyme can be oxidised by direct electron transfer rather than by mediator reaction. It has been claimed that for flavin containing oxidases the mechanism of transfer is mediation (Kulys and Samalius 1982). These workers suggested that mediators are formed near the electrode surface during slight dissolution of the organic metal. This mechanism was suggested because substrate oxidation only proceeded at potentials corresponding to the "mediator redox potential". Alternatively, Albery et al (1985) proposed that direct electron transfer to glucose oxidase with NMP^+TCNQ^-. This mechanism was proposed since conducting salts were too insoluble and the homogeneous kinetics was far too slow for mediated transfer to occur. Since the conducting salt possessed stacks of negative and positive ions it could form a strong attachment to the

enzyme and was therefore an electrocatalyst.

1.5.3 Oxygen-based glucose detection

Oxygen-based glucose measurement involves the detection of oxygen consumption due to glucose oxidase a polarising voltage of -0.6 V (Pt cathode) versus Ag/AgCl reference electrode:-

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O \qquad (1.5.3)$$

Use of hydrophobic teflon or polypropylene membranes allows selective permeation of oxygen and excludes electroactive interferents. Clark and Lyons (1962) described the concept of the glucose enzyme electrode. Soluble glucose oxidase enzyme was retained behind a polymer (Cuprophan)^R membrane and an oxygen electrode estimated the local decrease of pO_2. However the response was very sensitive to partial pressure of oxygen within the fluid in contact with the electrode. This problem was solved by the addition of a second electrode associated with a glucose oxidase membrane, forming a differential system (Updike and Hicks 1967). Sample pre-equilibration with oxygen by air bubbling was carried out by some workers.

Romette et al (1979) solved the problem of sample oxygen variability by using an enzyme membrane which had a much higher solubility (x20) for oxygen than water. The active membrane consisted of gelatin and enzyme over a polypropylene film, with the enzyme/gelatin mixture subsequently crosslinked using glutaraldehyde. A three-part measurement protocol was carried out. Glucose was firstly injected into the measurement cell, this was followed by a rinsing stage using isotonic

phosphate (0.05 M) buffer and finally rinsing using air. During measurement, oxygen was rapidly consumed at the enzyme layer however this was compensated by equilibration in air.

A differential method of glucose measurement was tested in dogs by Bessman et al (1981). The sensor consisted of two galvanic oxygen electrodes covered by polypropylene membranes with a circular (15 mm diameter) plastic housing. The sensor response was linear over the 0-20 mmol/l range and responses decreased substantially by a lowered oxygen tension. Electrodes implanted in the subcutaneous tissue of dogs recorded glucose levels which were approximately half those of blood. A closed-loop system consisting of a sensor and reciprocative insulin pump also failed to maintain euglycaemia in diabetic dogs in spite of using a differential mode of measurement (Bessman 1977). The underestimation of glucose was explained by the lowered tissue pO_2 levels.

Kondo et al (1982) utilised a differential glucose sensor for a Clark-type oxygen electrode. An oxygen-permeable hydrophobic Teflon membrane covered the electrode which was overlaid by a nylon Millipore filter and an outer polyester membrane. The latter ensured sufficient oxygen reached the enzyme layer but reduced the glucose flux. The sensor was tested in dogs where an external arterio-venous shunt was created between the carotid artery and the jugular vein and the electrodes placed in the shunt. Four out of eleven experiments failed due to thrombosis or electrical problems, but in other studies there was a good correlation between sensor output and blood glucose levels.

1.6 Polymer chemistry

1.6.1 Basic definitions and classification

A polymer is a large molecule consisting of repeating structural units joined by covalent bonds. A structural unit comprises a simple group of atoms joined by covalent bonds in a specific spatial orientation. Polymers can be classified as condensation and addition type depending on the method of synthesis. In a condensation polymer the structural unit contains fewer atoms than the monomer (or monomers) from which the polymer is derived. In an addition polymer the structural unit has the same molecular formula as the monomer.

Polymerisation reactions can also be classified according to mechanism. The processes are divided into stepwise and chain polymerisations. In a stepwise polymerisation the polymer is built up slowly by a sequence of discrete reactions; the initiation, propagation and termination reactions are kinetically similar. The monomer can react with another monomer or polymer molecule with equal ease, therefore there is rapid disappearance of monomer at an early stage. In chain polymerisation there is rapid polymer chain growth once initiation takes place. The initiation, propagation and termination reactions are significantly different in rate and mechanism. The monomer cannot react with another molecule but only with an active end group on a polymer radical or ion. Therefore high molecular weight polymer and monomer are present throughout the reaction.

1.6.2 Structural unit variety

Polymers can be classified as being either **homopolymers** (where the polymer consists of repeating structural units of the same kind) or as

copolymers containing more than one structural unit. Most important commercial copolymers contain 2-kinds of structural unit whilst a few have 3 units (terpolymers). Depending on the monomers utilised and the experimental techniques used, various distributions of structural units within the polymer as illustrated in Figure 1.6.2.

1.6.3 Structural unit orientation and tacticity

In vinyl polymerisation, three different types of linkages can be formed because each monomer molecule can assume either of two orientations as it adds to the preceding unit. They are identified by referring to the substituted end of the monomer molecule as the "head" and to the unsubstituted end as the "tail". Three arrangements are therefore possible in the polymer; the linkages may be all head-to-tail, they may be alternately head-to-head and tail-to-tail or they may be mixed. All vinyl polymers are predominantly head-to-tail in their orientation (Figure 1.6.3a). Further variability in structure arises when the backbone of the polymer molecules contains a carbon atom attached to two different side groups. These polymers may have various configurational arrangements or tacticity. Polymers with a regular arrangement are known as **tactic** whilst those with a random arrangement are **atactic** (Figure 1.6.3b).

1.6.4 Physical structure

Polymers can be both linear and branched. The bulk polymer state (also known as condensed or solid state) includes both amorphous and crystalline regions. In amorphous materials the molecules are highly kinked in an irregular fashion (randomly coiled) whilst in crystalline materials the molecules are generally spiral or zigzag and do not possess crystalline regions. " Crystalline " polymers are

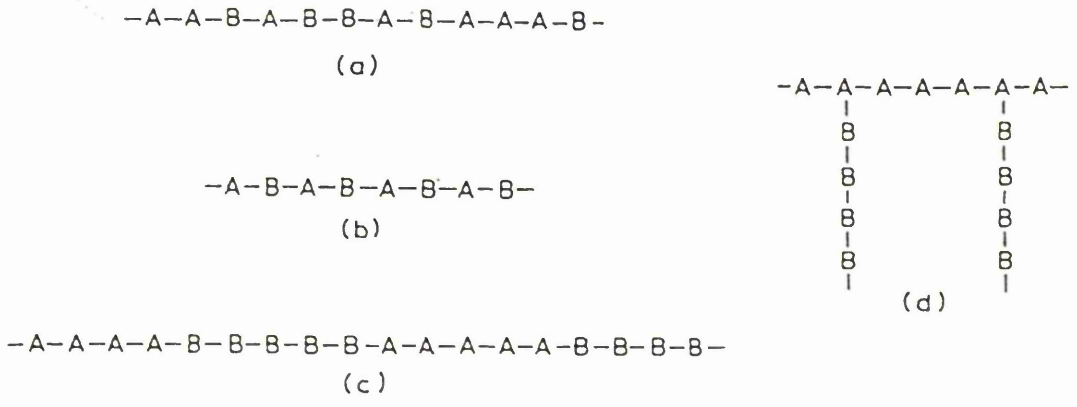
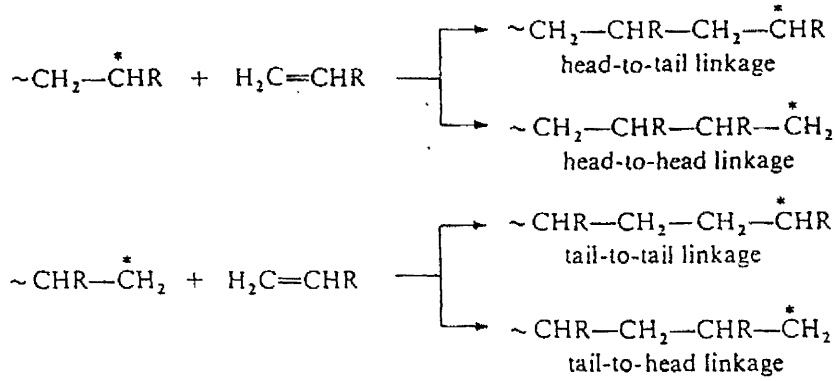


Figure 1.6.2

Representation of the different copolymer types:- (a) Random copolymer, (b) Alternating copolymer, (c) Block copolymer (d) Graft copolymer

(a)



(*signifies a radical, anion or cation)

(b)

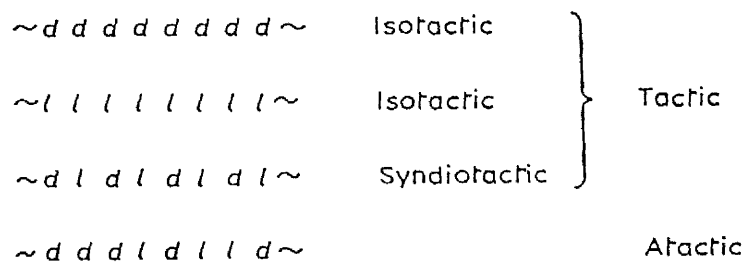


Figure 1.6.3

Representation of polymer (a) structural unit orientation (b) tacticity.

generally semicrystalline containing appreciable quantities of amorphous material and the melted state is amorphous. It is therefore essential to consider the kinetics of transformation from the amorphous to crystalline state and vice versa.

Amorphous polymers exhibit widely different physical and mechanical behaviour patterns depending on temperature and structure. At low temperatures, amorphous polymers are glassy, hard and brittle. As the temperature is increased they go through a glass-rubber transition. The glass-transition temperature (T_g) occurs when the polymer softens to a rubbery state. The polymer chain conformation in the amorphous state has been considered. X-ray and mechanical studies have led to the proposed random-coil model. Here, polymer chains are permitted to wander about in a space filling way as long as they do not pass through themselves or another chain (Flory 1979).

X-ray diffraction can be done for crystalline polymers where they also undergo the first order transition known as melting. Polymers crystallized in the bulk state are not totally crystalline due to their long chain nature. The melting (fusion) temperature, T_f exceeds the glass transition temperature. Therefore a polymer can be hard and rigid or flexible. Crystallinity is dependant on the structural regularity. Generally isotactic and syndiotactic polymers e.g., polyamides and polyesters usually crystallize whereas atactic polymers do not. Non-regularity firstly decreases the melt temperature and then prevents crystallinity. Melting temperature is governed by polarity, hydrogen bonding and packing capability.

1.7 Plasticisers

A plasticiser is a nonvolatile, high boiling, non-separating substance which when added to another material, alters the physical and chemical properties of that material. Plasticiser and plasticised material are held together by intermolecular forces to give complexes called **molecular aggregates.**

Boyer (1951) considered a plasticiser to decrease the intermolecular forces along polymer chains. Tensile strength and softening temperature are reduced, but flexibility, tensile strength, adhesion, gloss, water resistance, fire resistance, oil resistance and electrical properties may be altered.

In polymer production plasticisers are usually liquids, miscible with the polymers to at least 10-20% or preferably to 100% by weight and endow a polymer resin with enhanced thermoplasticity and rubber-like properties. In polar polymers plasticisers mask polar sites and reduce hydrogen bonding. In all polymers, they tend to force chains apart and reduce Van der Waals forces giving them greater freedom of movement.

1.7.2 Types of plasticisers

Solvent, nonsolvent and polymeric type plasticisers exist. Each decrease intermolecular forces in slightly different ways. The solvent type is miscible in all proportions of the polymer. Molecules are dispersed throughout the polymeric system and action is restricted to a small local region, determined by the Brownian motion of that molecule. The non-solvent type has limited miscibility. Dispersed molecules behave as the solvent-type while non-dispersed forms create clusters which can disrupt

inter-chain forces over long distances.

The polymeric type plasticisers have molecular weights from a few per cent up to 100 per cent of the base polymer. Whether compatible or incompatible, they form large continuous regions which separate the main polymer chains from each other. The most widely used plasticisers are the ester type ones (solvent type). These are of high molecular weight and can possess more than one functional group, and include organic phthalates and phosphates. Plasticisers can be categorised as being primary or secondary (Partridge and Jordan 1952). The primary type contains high dispersing power and compatible with polymer in all useful proportions. Secondary plasticiser exhibits limited compatibility and has to be used in conjunction with a primary plasticiser in order to achieve the desired properties.

1.7.3 Mechanism of plasticisation

Several theories have been proposed regarding the mechanism of plasticiser action:- viscosity theory, thermodynamic theories and mechanistic theories.

1.7.3.1 Viscosity theory

The viscosity theory states that the viscosity of the plasticiser determines the behaviour of the plasticised polymer (Leilich 1943). The proposed theory is dependent on the viscosity-temperature behaviour of miscible plasticisers. Jones (1947) states that in general plasticisers of lower viscosity give softer plastics than those of higher viscosity. Also plasticisers with low temperature coefficients of viscosity give polymers which are in turn less sensitive to temperature change. This temperature dependence is generally greater than that of polymers.

Therefore compounds of low plasticiser-to-polymer ratios are less temperature sensitive. Viscosity theory cannot explain how polymers are plasticised by other substances, and can only relate the physical property to the viscosity-temperature behaviour of the plasticiser.

1.7.3.2 Thermodynamic theories

Doty and Zable (1946) classified plasticisers for polyvinyl chloride on the basis of X_1-values. X_1 can be defined as the coefficient characteristic of the interaction between polymer and solvent from the equations derived by Flory (1953) and Huggins (1947). It can also give a quantitative criterion of compatibility and an index of the solvent power of a liquid for a particular polymer.

The thermodynamic properties of polymer/diluent mixtures may be summarised using the following equation:-

Polymer + diluent ⇌ solution (1.7.3a)

In general thermodynamic terms this process will be at equilibrium if the free energy change ΔG is zero. With negative values of ΔG the process will proceed from left to right, and in reverse for positive values. The free energy change in any process can be related to the respective enthalpy and entropy changes (ΔH and ΔS) by the equation:

$$\Delta G = \Delta H - T\Delta S \qquad (1.7.3b)$$

Where T is the absolute temperature. If mixing is to occur it follows that the balance of the enthalpy change and entropy changes of mixing must be negative. Therefore, an exothermic process, i.e. negative values of ΔH, or increase in the entropy of the system, will tend to favour mixing. Entropy of mixing may be considered as the increase in randomness resulting from the formation of a mixture. According to the

Boltzmann equation, if W_1 and W_2 are respectively the probabilities of a random mixture and a state of complete segregation of two components, then:-

$$\Delta S = k\log_e(W_1/W_2) \qquad (1.7.3c)$$

where k is the Boltzmann constant.

Flory and Huggins have approached the problem of evaluating W_1/W_2 for polymer-liquid systems by considering constituent molecules of a solution to be arranged in a regular lattice. Each solvent molecule occupies one site whilst the polymer molecule occupies a succession of nearest neighbour sites. The resultant equation expresses entropy change associated with increase in a variety of ways of arranging solvent and polymer molecules when a solution is formed i.e., the configurational entropy change, as follows:-

$$\Delta S^* = R[\log_e(1-v_2) + v_2(1-1/m)] \qquad (1.7.3d)$$

where ΔS^* is the configurational entropy of mixing
v_2 is the volume fraction of polymer in the mixture
and m is the ratio of the molar volumes of polymer and diluent.

By the addition of the semi-empirical heat of mixing term X_1 to the above equation the free energy of mixing may be expressed as:-

$$\Delta G = -RT[\log_e(1-v_2) + v_2(1-1/m) + X_1v_2^2] \qquad (1.7.3e)$$

X_1 can also be expressed as:-

$$X_1 = X_S + X_H/RT \qquad (1.7.3f)$$

where X_S and X_H represent the entropy and heat contributions to the value of X_1. From the equations it can be stated that the greater the negative value of free energy of dilution of a polymer solution (Δ G) the further the system is removed from the point of phase separation i.e. the

greater the solvent power.

Frith and Tuckett (1945) assumed that polymer and plasticiser were in thermodynamic equilibrium and plasticiser compatibility was explained according to the Flory-Huggins theories of polymer solutions. They stated that phase separation should occur when the free energy of dilution becomes zero. For a plasticised polymer two phases are formed, one is the swollen polymer and the other almost pure plasticiser. If the temperature is increased there is a decrease in free energy permitting more plasticiser to enter the plasticiser-polymer phase. Therefore at a given temperature, the composition of the swollen phase represents the maximum quantity of plasticiser the can be incorporated without leaching out. A quantitative measure of phase separation (X_c) has been provided using the following equation:-

$$X_c = (1 + m^{1/2})^2/2m \qquad (1.7.3g)$$

For a polymer and solvent of molar volumes of 20 000 and 200 respectively, m = 100, so that $X_c = 0.605$. With an increase in the value of m, X_c decreases reaching a limiting value of 0.50 when m is infinitely large. Therefore if X_1 for a particular system is greater then X_c the polymer and liquid will be incompatible. If X_1 is less then X_c they will be compatible. A small value of X_1 indicates a large degree of interaction between polymer and liquid. Therefore for a given polymer, small or negative values of X_1 are indicative of liquids with high solvent power, larger values up to X_c indicating a low degree of solvent power. The contribution of the thermodynamic theory for elucidation of the mechanism of plasticisation is at present limited. The theory only deals with ideal solutions involving simple molecules.

1.7.3.3 Mechanistic theories

According to the "lubricity theory" the plasticiser is said to act as a lubricant reducing intermolecular "friction" between the polymer molecules. Different workers have proposed slight variations of the lubricating mechanism, but the basic idea was the same.

According to the "gel theory" (Manfred and Obrist 1927) plasticisation occurs due to the separation or disaggregation of the polymer molecules followed by oriented aggregation. Doolittle (1947) published work which supported the theory derived from studies on the mechanism of solvent action whereby polymer molecules are brought into solution by organic solvent. These are attracted to each other by forces originating form the "active centres" along the polymer chains. In solution thermal agitation tends to separate polymer molecules, whereas intermolecular attractive forces operate to reunite them. A dynamic equilibrium exists at every concentration and temperature. Therefore there will always be a fixed fraction of these active centres or cross-linking points binding the polymer molecules together at any time. The formation and breakdown of these polymer-polymer contacts is known as the aggregation-disaggregation equilibrium.

While the polymer molecules are constantly making and breaking close contacts with each other, solvent molecules are simultaneously attaching themselves to the "active centres" on the polymer chains (polar side groups, for example) and being dislodged by collisions with other molecules. The overall result is that for a given temperature and concentration, a certain fraction of the active centres on the polymer

chains is solvated. Although the duration of any given polymer-solvent contact may be momentary, the overall result is to eliminate a definite fraction of the active centres on the polymer as potential crosslinking points. This second equilibrium is known as the solvation-desolvation equilibrium and it operates simultaneously with the aggregation-disaggregation equilibrium. Doolittle (1947) applied these ideas to explain the mechanism of plasticisation. The rigidity of the unplasticised polymer mass was considered to be caused by an internal three-dimensional honeycomb or gel structure formed by contacts between polymer molecules at various points along the chains. By the above solvation-desolvation mechanism, plasticisation reduced the number of polymer-polymer contacts, thereby decreasing the rigidity of the three-dimensional structure allowing deformation without rupture.

1.8 Synthetic membranes

1.8.1 Structure and classification

Membranes separate two solutions or phases and differ in chemical composition from both of them which results in a differential permeability to various solution components. Those through which ions permeate with different ease are termed electrochemical. They regulate movement of solute, participate in the development of (not necessarily static) hydrostatic pressures and in achieving partial or complete separation of solutes from solvent. With electrolytes additional effects occur e.g. static or dynamic membrane potentials, anomalous osmosis, movement of third ions against concentration gradients, electroosmosis etc. In all cases they transform the free energy of the adjacent phases (or energy applied through them) into other forms of energy

(mechanical, concentration, electrical etc) sometimes in a reversible but mostly in an irreversible manner. They function in an analogous manner to mechanical machines because they regulate energetic processes without being changed, exhausted or consumed.

Membranes can be classified according to their pore structure:- homogeneous phase membranes ("oil membranes") and heterogeneous phase membranes (membranes of porous character) shown in Figure 1.8.1. Homogeneous phase membranes exert their function by selective, differential solubility. Heterogeneous membranes act as sieves which screen out various solute particles according to their different adsorption capabilities, and ions, also depending on size and magnitude of their charge e.g polyvalent ions are readily excluded since they have a larger size and high charge. This prevents them by electrostatic repulsion from entering narrow pores which are more readily accessible to univalent ions of the same sign.

Heterogeneous membranes consist of a solid matrix in which there are well defined pores. These vary from the **coarsely porous**, with pore diameters of 5 nm upto several μm (e.g. glass frits and track etched polycarbonate membranes), to **finely porous** (ion sieve) with pore diameters reaching 1 nm-5 nm (e.g. some ion exchange and cellulosic membranes). Heterogeneous membranes may be symmetric or asymmetric. The latter possess a well defined "skin" layer above a highly porous region. The "skin" layer participates in the function of permselectivity, while the porous zone provides mechanical support. Asymmetric membranes could be a composite with a different polymer

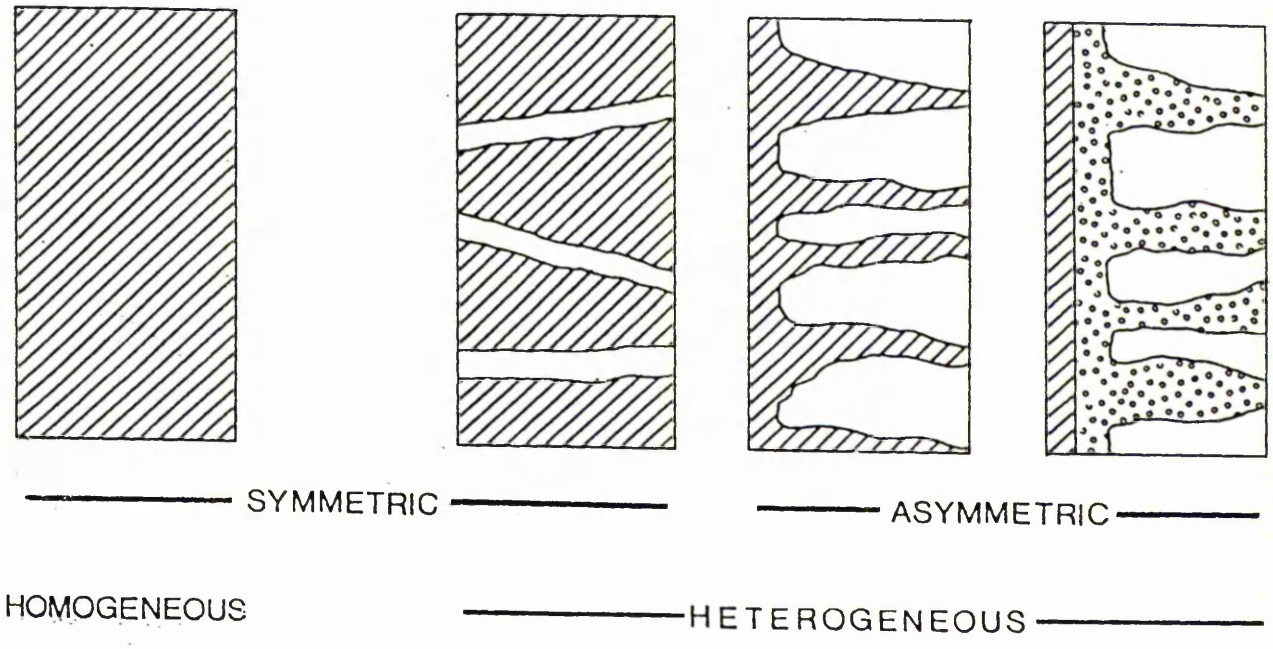


Figure 1.8.1

Schematic diagram of synthetic membrane structure (From McDonnell & Vadgama 1989).

providing the porous support layer and the skin layer itself may be microporous or homogeneous. Homogeneous membranes consist of a continuous, non-porous phase on a micro-scale.

1.8.2 Fabrication procedures

1.8.2.1 Phase-inversion

This process involves dissolving a polymer in an appropriate solvent, followed by altering the phase to form the polymer membrane by varying conditions such as temperature. The simplest procedure involves complete evaporation of the solvent of a polymer solution under ambient conditions, with the resulting condensation of the polymer on a solid surface or substrate. Rea, Rolfe and Goddard (1985) developed a dip-coating method for implantable needle-type sensors. Initially the electrode tips were dipped into a suspension of glucose oxidase (200 mg/ml) made in cellulose acetate (2.5% w/v in acetone or a 1:1 mixture of ethanol and acetone) and after 45 minutes (room temperature) a layer of 6 g 100 ml^{-1} polyurethane (Estane 5701f1) in a 1:18 (v/v) mixture of dimethylformamide and tetrahydrofuran was applied. They were left to dry in air at room temperature for 12-18 hours prior to use.

Another method requires the polymer to be dissolved in a water immiscible solvent and by pouring the solution into water or a suitable gelation medium as described by Sourirajan (1972) for preparation of reverse osmosis asymmetric cellulose acetate membranes. Taylor et al (1977) developed a similar method for casting cellulose acetate membranes. A solution consisting of 1 part cyclohexanone, 1/24 part Eastman CA resin powder and 1/23 part isopropanol (by weight) were dissolved in a stoppered flask and stirred for several hours. An aliquot

of the casting solution was poured onto dust-free water to form a circular pool (~15 cm in diameter). After the curing process (5-10 minutes) the membrane was picked up onto a polyethylene sheet. The problem lies in solvent and non-solvent selection because suitable solvents may not be volatile under normal conditions. Therefore the evaporation step may require extended periods at elevated temperatures in order to remove the high boiling point solvent.

An alternative solvating system may be comprised of two liquids which individually are solvents for the polymer under specified conditions of concentration and temperature. However, when combined in the proper proportions, the mixture of these liquids may dissolve the polymer and form an asymmetric membrane.The solubility can be defined according to the following relationship:-

$$\delta^2 = \delta_d^2 + \delta_p^2 + \delta_h^2 \qquad (1.8.2a)$$

where δ = usual solubility parameter defined by Hildebrand and Scott (1949) and δ_i = corresponding (dispersion, polar or hydrogen bonding) component of the solubility parameter.The three parameters, δ_d, δ_p and δ_h can be plotted in a triangular graph (Teas 1968).

$$F_i = [\delta_i / (\delta_d + \delta_p + \delta_h)] \ (100\ \%) \qquad (1.8.2b)$$

Each of these three terms represent an axis on the triangular diagram. The region of solubility known as the "solubility envelope" is defined by those solvents which dissolve the particular polymer. The solubility envelope dimensions are governed by temperature and polymer concentration.

For solvent mixtures, the partial parameters for the mixture (δ_{dm}, δ_{pm} and

δ_{hm}) can be calculated from:-

$$\delta_{im} = \phi_1\delta_{i1} + \phi_2\delta_{i2} \qquad (1.8.2c)$$

ϕ_1 and ϕ_2 are the volume fractions of components 1 and 2. It is possible to choose a mixed, good solvent (referred to as cosolvent) composed exclusively of liquids which individually are non-solvents and are located on opposite sides of the region of solubility. The two solvents are selected so that the tie line joining the two pure liquids passes through the solubility envelope. The exact location of the initial cosolvent system in the solubility envelope may be adjusted by variation of the volume ratio of the two nonsolvents.

Kinzer et al (1985) utilised the above system for the preparation of sulphonated polysulphone membranes using a cosolvent system consisting of tetrahydrofuran and formamide and a gelation medium of isopropylalcohol. The proportions of cosolvent used were dependent on the 3-component solubility parameter concept of Hansen (1967).

Microfiltration polysulphone membranes were prepared using a solvent/non-solvent system. The solvent possessed high water affinity and moderate solution power. Casting solution viscosity increased by inclusion of a polymer additive which also influenced porosity and pore size. Generally solutions in highly swollen states were made with increasing non-solvent content. A non-solvent/solvent ratio above 0.35 caused the solution to become unstable and phase separation occurred (Stengaard 1988). A further modification could be by inclusion of a small amount of water and water soluble salt in the original polymer solution. This forms channels or pores as the salt leaches out following

contact of the polymer solution with water to form a porous structure. Magnesium perchlorate has been included during casting of cellulose acetate membranes (Lonsdale et al 1962).

1.8.2.2 Nucleation track etching

A polycarbonate or polyester film of 10-20 μm thickness is exposed to collimated, charged high density fission products of a nuclear reactor with the resultant formation of discrete tracks of damaged membrane material. This is produced due to breakage of chemical bonds of the original polymer. Ionising radiation e.g. ultraviolet light (Gesner and Kelleher 1968), high energy electrons, neutrons, X-rays or γ-rays induces crosslinking or degradation of the polymer. Here hydrogen atoms are first split out with the formation of free radicals which may lead to primary crosslinking or to chain breaking and the formation of carbon-carbon double bonds (Figure 1.8.2a). The film is then placed in an alkali etch bath where the tracks become preferentially etched, leaving mainly cylindrical pores (Figure 1.8.2b). Pore density is controlled by the residence time in the reactor, while pore size is determined by the duration of the etching process.

1.8.2.3 Mechanical stretching

This has been used mainly for homogeneous polymers such as polyenes, e.g. when extruded annealed isotactic polypropylene, containing equal proportions of crystalline and amorphous regions are stretched, pores can be formed in the amorphous domain.

1.8.2.4 Melt extrusion

Preformed thermoplastic polymers can be melted and extruded in the

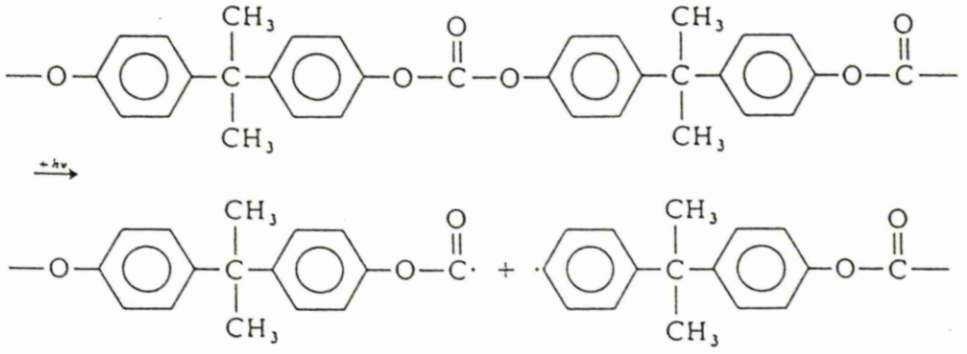


Figure 1.8.2a

Representation of the breakdown of polycarbonate by ultraviolet irradiation.

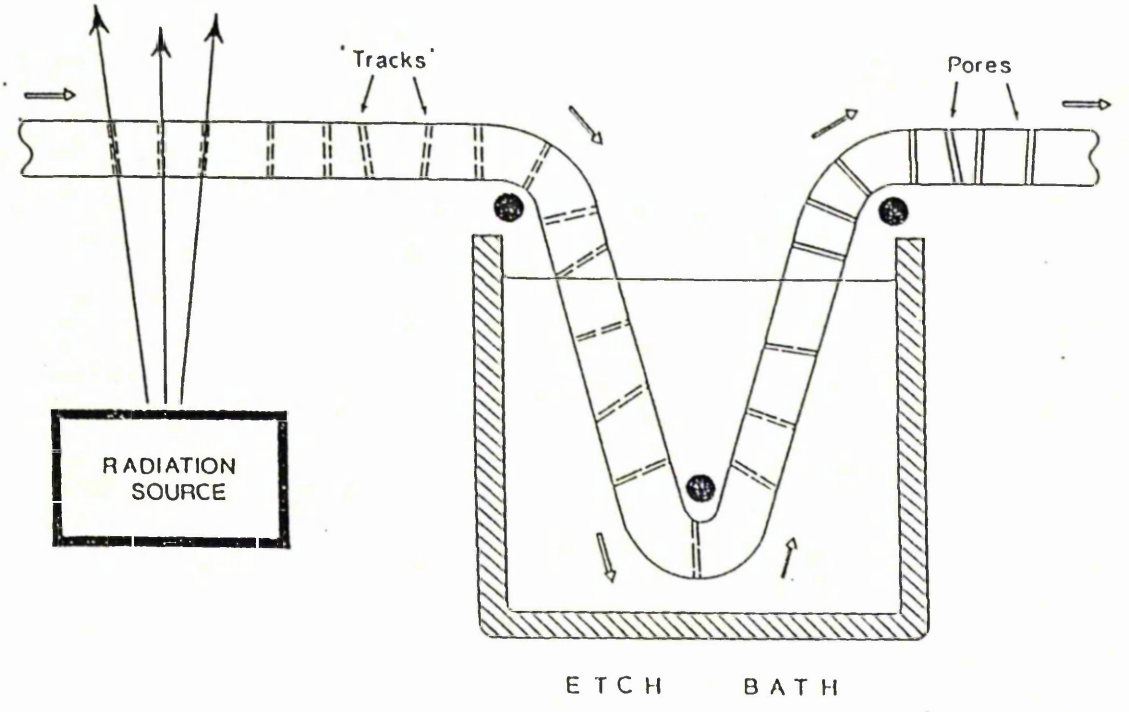


Figure 1.8.1b

Schematic diagram of the track-etch process used for the manufacture of polycarbonate (Nuclepore^{R}) membrane filters (From McDonnell & Vadgama 1989).

form of membrane sheets. Therefore by forcing a hot bulk polymer slab through a slit die and into a water bath e.g. polyethylene gas permeable membranes may be created in sheet form by this method.

1.8.2.5 In-situ formation by polymerisation

Polymer membranes can be formed directly from their monomers or oligomers by polymerisation in situ. Many gel-type membranes are prepared in this way e.g. polyvinyl alcohol (PVA) which is formed into a gel from its low molecular weight oligomers and polyacrylamide gels are formed from acrylamide monomers.

1.8.3 Ion exchange membranes

Ion exchange membranes contain ionogenic groups fixed to a resin or polymer matrix ($-SO_3^-$, $-COO^-$, $-NH_3^+$, NH_2^+). These membranes can be made by dispersion of ion-exchange resin within a polymer matrix, by chemical modification of an existing non-ionic polymer, or casting a film from an ionomeric polymer (Helfferich 1962; Flett 1983). Permselectivity relies on the ability to allow passage of ions with opposite charge to that of the fixed groups (counter-ions) whilst excluding ions with the same charge (co-ions) by electrostatic repulsion. The degree of repulsion depends on electrolyte concentration. At low concentrations, coions are almost absent in the membrane phase but as concentration is increased coions enter together with counterions in order to maintain electrical neutrality. The number of coions in the membrane phase will be less than the number of counterions by an amount equal to the number of ionogenic groups in the membrane.

There is a need to produce ion exchange membranes with ever-increasing selectivity. In the main, membranes are produced from derivatives of divinylbenzene copolymer (e.g. styrene-divinylbenzene and chloromethyl-styrene-divinylbenzene). Others have been produced by grafting monomers onto polymeric fibres, such as poly(tetrafluoroethylene) and poly(propylene). Here the ion exchange groups have a less uniform distribution in the film (Hegazy et al 1984). Charged groups have been introduced by sulphonation and amination of polyethersulphone and polyvinylchloride respectively.

Composite ion-exchange membranes and allowed modification of basic ion-exchange properties. Thus, amphoteric membranes have presented mixed surface charge in a single membrane structure. In mosaic membranes, discrete arrays of anion and cation exchange sites are presented; such membranes have enhanced permeability to electrolytes (Weinstein and Caplan 1968) and have been used to separate salts from low relative molecular mass non electrolytes and amino acids.

1.8.4 Membrane transport

Membrane transport can be classified as being active or passive. During passive transport, the driving force for mass transfer is a chemical or potential gradient across the membrane. It may be "facilitated" where mobile carriers in the membrane phase combine reversibly with permeant molecule to increase its solubility in the membrane phase and increase its rate of transfer. In active transport, the driving force is provided by a specific chemical reaction taking place within the membrane phase or the opposite bulk phase.

There is a close correlation between the type of membrane used and the transport mechanism (Lakshiminarayanah 1969). Models for transport in coarsely porous membranes assume sieve-like behaviour, with particles passing through the pores by mainly a convective mechanism, with little discrimination and with little interaction with the polymer matrix. Finely porous membranes show transport by both convection and diffusion with molecules moving in the voids as well as the membrane matrix. However in order to cross a homogeneous membrane, permeants have to be soluble in the polymer matrix and transport is determined mainly by diffusion.

1.8.5 Membrane separation processes

Membrane separation processes may be divided into three classes according to their characteristics e.g. pore size and charge distribution (McDonnell and Vadgama 1989; Van den Berg and Smolders 1992):-

(i) Sieve effect

In ultrafiltration (UF) and microfiltration (MF) separation is based on the differential size/molecular weight distribution of the permeating species, with a pressure gradient as the driving force; the membranes used in ultrafiltration can have pore sizes of 1-50 nm, while for microfiltration the pore dimensions are from 0.05-10 μm.

(ii) Electrochemical effect

Separation is based on the differences in the charge of the permeating molecules e.g. in electrodialysis. This allows the separation of charged molecules from uncharged ones by using ion exchange membranes. The ions are transported by a migrational mechanism, as a result of an

applied potential difference.

(iii) Solubility effect

This involves a separation process based on the different solubility of the permeants in the membranes e.g. reverse osmosis, gas separation and pervaporation.

The primary requirement for separation is the use of dense membrane structures (pores < 1 nm). It makes use of a difference in affinity between several feed components and the membrane, and of the difference in diffusivity through the membrane; the driving force is a pressure difference for reverse osmosis and gas separation. For pervaporation the driving force is a concentration gradient.

Membrane separation processes can be classified according to the smallest particle or molecule which is retained. Thus, microfiltration membranes retain particulates, organisms, colloids and viruses; ultrafiltration membranes retain macromolecules in solution such as proteins and oligosaccharides; reverse osmosis membranes retain small ionic species such as sodium chloride. Other membrane processes include:- facilitated transport by liquid and fixed site charge carrier membranes (Noble 1991; Lonsdale 1982; Toher et al 1977; Duax et al 1972.)

1.8.5.1 Microfiltration

Microfiltration membranes are usually microporous and rely on the sieve effect with discrimination based on size and shape. Particles of larger dimension than the largest pore are retained smaller particles pass through and degree of separation depends on pore size

distribution. Typically substances ranging in MW, from 300 to 300 000 are retained and low operating pressures can be used. Examples of microfiltration membranes include:- track-etched polycarbonate filters, PTFE and polyolefins.

1.8.5.2 Ultrafiltration

Ultrafiltration membranes are generally asymmetric. They possess a thin (0.5-1.5 μm) skin layer of finely porous material on a thicker (50-250 μm) non porous layer. The dense pellicle layer confers high permeability with selectivity and reduces blockage of pores. Early filters were cellulose-based but more recently polysulphone, polyesters, polyamides and polycarbonates have been used. Materials may be used as copolymers and may need to resist strong acids and bases, solvents and high temperatures.

Separation is based on sieving, with species ranging in size from 1-10 nm (molecular weight 300 and 300 000). Operating pressures are 2-10 x 10^5 Nm^{-2}. At lower pressures, filtrate flux rises linearly with pressure, but a plateau is reached at which no further increments in flux occur. This is due to a zone of increased solute concentration in the feed side of the membrane due to solvent removal at this point. This phenomenon of concentration polarisation leads to the back transport of solute away from concentrated zone into the bulk feed solution. Additional increase in applied pressure may lead to gel formation at the membrane creating an additional filtration barrier. Mass transfer into and out of the boundary layer establishes a hydraulic resistance which is therefore greater than that of the membrane.

1.8.5.3 Reverse osmosis

Reverse osmosis membranes can separate small organic solutes such as NaCl or $MgSO_4$ which are otherwise freely permeable through microfiltration and ultrafiltration membranes. During the process pressure is applied in the reverse direction to that of normal osmotic flow across a semipermeable membrane. Water flux varies linearly with the net pressure gradient ($\Delta P - \Delta\pi$) where ΔP is the applied pressure gradient and $\Delta\pi$ is the osmotic pressure gradient. While solute flux is independent of applied pressures, at a sufficiently high pressure, water can be made to flow from the concentrated to the dilute solution side. Desalting of water ($\Delta\pi \sim 25$ atm) and brackish water ($\Delta\pi \sim 1$-4 atm) can be carried out at operating pressures of 10-100 x 10^5 Nm^{-2}. Reverse osmosis membranes are asymmetric, and are usually made of cellulose acetate or aromatic polyamides comprising a homogeneous skin and microporous support.

Composite cellulose acetate membranes have been made by Lonsdale et al (1971). In their work, a thin film of cellulose triacetate (0.04-8 μm) was applied to a porous support by vertically withdrawing the support membrane at a constant speed from a solution of cellulose triacetate. These "integral" composite membranes gave good reverse osmosis membranes. Subsequently Dave et al (1992) have used a cellulose acetate/cellulose diacetate blend in order to improve reverse osmosis characteristics. Separation is based on a solution-diffusion mechanism, determined by differences in solubilities and diffusivities. It is assumed that each component is sorbed by the membrane at one interface, transported by diffusion across the membrane and desorbed

at the other interface. The rate-limiting step is taken to be diffusion. The transport of solute and solvent are also assumed to be independent. Lonsdale et al (1965) derived the following equation for water transport:-

$$J_w = \frac{-D_w C_{wm} V_w}{RT} \cdot \left[\frac{\Delta P - \Delta \pi}{\Delta x} \right] \qquad (1.8.5a)$$

where J_w = water flux (gram/cm^2.sec)
D_w = water diffusion coefficient (cm^2/sec)
C_{wm} = concentration of water in the membrane (gram/cm^3)
Δx = membrane thickness (cm)

For solute transport:-

$$J_s = -D_s \frac{\Delta C_{sm}}{\Delta x} \qquad (1.8.5b)$$

where J_s = salt flux (gram/cm^2 sec), D_s = salt diffusion coefficient (cm^2/sec) and C_{sm} = solute concentration in the membrane.

The molar distribution coefficient K, may be expressed as the ratio of the mass of solute (g) present per unit volume (cm^3) of membrane to the mass of solute (g) dissolved per unit volume (cm^3) in solution.

Assuming $\bar{K}$ is constant over the concentration range of interest:-

$$J_s = D_s K \frac{C_{sb}}{\Delta x} \qquad (1.8.5c)$$

where C_{sb} = concentration of salt in the bulk solution

Species of molecular weight greater than 150 are rejected but also larger organics may permeate through cellulose acetate and some phenols permeate more readily than water. Matsuura and Sourirajan (1971) proposed that permeation of organics through cellulose acetate was affected by pKa and hydrogen bonding by undissociated species enhanced permeation. Electrostatic repulsion of the ionic

form led to rejection. Reverse osmosis has also been used for the concentration of food products and solutions in chemical and pharmaceutical industries.

1.8.5.4 Membrane distillation

This is a non-pressure driven process. When a hydrophobic microporous membrane of appropriate thermal conductivity (e.g. poly(propylene), PTFE, poly(vinylidene) fluoride is placed between two aqueous compartments maintained at different temperatures, water vapour transfers through the membrane from the high to the low temperature side, with condensation of vapour at the low temperature interface. The driving force is the vapour pressure difference across the membrane. Such membranes may be usable for desalination of seawater and have the advantage that a higher solute rejection is achievable than for reverse osmosis membranes, which are limited by osmotic pressure of the concentrated feed solution.

1.8.5.5 Electrodialysis

Here an ion-exchange membrane is used to separate ions from electrolyte solution using an external electrical field as the driving force (Solt GS 1976; Tanaka 1991). A series of compartments separated alternately by cation-selective and anion-selective membranes is used (50-200 pairs). The terminal compartments are bounded by two electrodes employed to pass current through the entire stack (Figure 1.8.5). A dilution stream of e.g. Na^+ and Cl^- is fed into the alternating dilution compartments; Na^+ ions then migrate towards the cathode, permeating the cation exchange membrane, and collect in the adjacent

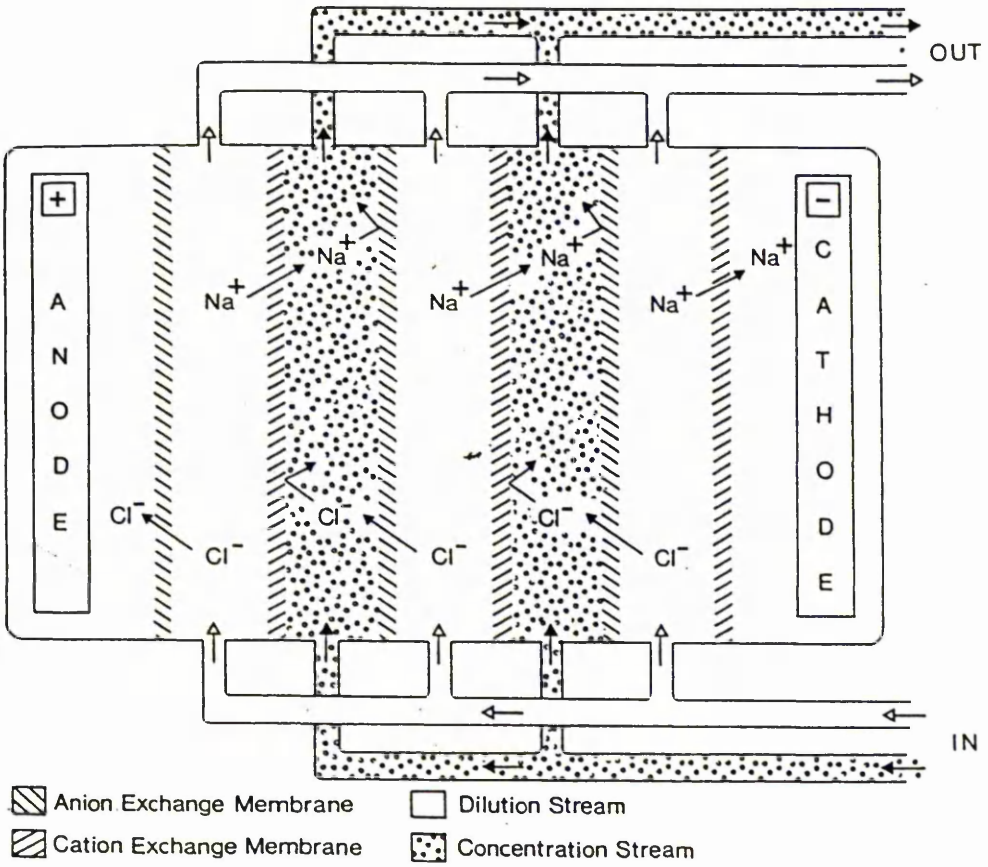


Figure 1.8.5

Schematic diagram of the electrodialysis process (From McDonnell & Vadgama 1989).

concentration compartment, blocked from further passage by the anion exchange membrane. At the same time Cl^- ions migrate towards the anode, permeating the anion exchange membrane, collecting in the concentration compartments on the opposite side, being then trapped by a cation exchange membrane. Ion-exchange membranes used in electrodialysis need to have good selectivity for the counter ion, a high ionic permeability (low electrical resistance) to minimise power requirements and high chemical and mechanical stability. They are used in the production of salt and fresh water from brackish and sea water, reduction of acidity in citrus juices and processing of galvanic waste waters.

1.8.5.6 Pervaporation

This process separates a liquid mixture by removing the permeate in the form of a vapour. The partial pressure of the permeate on the downstream side is lower than its saturation vapour pressure. The process can be made continuous; using a vacuum or carrier gas on the vapour side. Although liquid is in contact with the membrane on the feed side, the downstream face is dry, and anisotropic membrane swelling may occur; this could lead to time dependent changes in membrane characteristics. Differences in solution-diffusion determines selectivity.

1.9 Project aims

The major aim of the project is to develop membranes for enzyme electrodes used for glucose determination in clinical samples. Direct and rapid measurement of glucose and lactate for example is preferable

in undiluted biological fluids especially at the bedside. Elevated blood lactate levels can occur in the intensive care unit. A glucose sensor will be of use in the treatment/management of diabetes and diabetic ketoacidosis. Combined lactate and glucose monitoring would be potentially useful in diabetics as part of controlling insulin delivery. This is because rapid rises of lactate levels in the absence of exercise usually indicate increased glucose turnover due to excess insulin administration. Cholesterol measurement is essential in the determination of cardiovascular disease. Biosensors provide an alternative to routine methods of analysis in the Clinical Biochemistry laboratory. Routine enzymatic methods of analysis often prove to be labour intensive and require skilled technicians. Laboratory analyzers are expensive and cumbersome. Therefore these techniques are impossible or impractical for continuous monitoring or implantation and may require continual blood withdrawal from the patient.

One of the major drawbacks with the use of hydrogen peroxide sensors is that other oxidisable compounds present in blood including ascorbate, urate and paracetamol can also give rise to responses. Additionally, diffusible electroactive species produced from platelet degranulation may passivate the platinum working electrode surface. These problems have been solved during the project by the use of various screening internal membranes including:- cellulose acetate, plasticised polyvinyl chloride and polyethersulphone. Also the permselectivity/biocompatibility properties of surfactant-incorporated cellulose acetate have been considered.

A second major problem is that the linear range of a sensor response limited by the enzyme K_m (Michaelis constant) . In clinical conditions analyte levels may extend far beyond this particular value e.g. in diabetics glucose levels may increase up to 30 mM. Substrate diffusion-limiting membranes employed include:- low pore size (0.01 μm) polycarbonate, surfactant-incorporated and unmodified cellulose acetate and polyurethane were used. Miniature needle-type glucose and lactate sensors could be useful for continuous trend monitoring of the metabolites *in vivo*. The needle-shaped devices are designed to minimise trauma and clot formation when implanted and may form part of a closed-loop system which could control insulin administration according to measured glucose levels in future. The electrode response should show independence from external variables such as pH, temperature and stirring rate. The needle electrodes should be biocompatible and platelets aggregation near the sensor surface should be avoided. The properties are governed by the external polyurethane membrane. The sensor should be easily sterilisable prior to implantation. The response should exhibit low oxygen dependency since subcutaneous tissue is often is the chosen site of implantation for glucose determination in diabetics. However poor oxygen tissue perfusion in conditions such as hypoglycaemia and ketoacidosis make measurement difficult.

CHAPTER TWO - EXPERIMENTAL AND METHODS

2.1 List of Chemicals

The following reagents (of analytical reagent quality) were all obtained from BDH Chemicals, Poole, Dorset:- polyvinyl chloride (molecular weight 100,000 and 200,000), glutaraldehyde (50% v/v), hydrogen peroxide (approximately 30% v/v hydrogen peroxide),
D-glucose, paracetamol, catechol, uric acid, polyvinyl alcohol (molecular weight 125,000), dimethylsulphoxide and cholesterol. Cellulose acetate (39.8% acetyl content), and the nonionic surfactants Triton X-100, dioctyl phthalate were purchased from Aldrich Chemical Co., Milwaukee USA.

The following enzymes were obtained from Sigma Chemicals, Fancy Road, Poole, Dorset:- glucose oxidase (EC 1.1.3.4 from **Aspergillus niger**, type XS, 138 U/mg), Lactate oxidase (EC 1.1.3.2 from **Pediococcus Sp.**,38 units/mg). The following chemicals were also purchased from Sigma Chemicals:- lithium L(+) lactate, Bovine serum albumin fraction (V), Tween-80, hydroquinone and sodium deoxycholate. The enzymes lactate oxidase (EC 1.1.3.2 from **Pediococcus species**, 200 units of specific activity ~55 U/mg protein) and Cholesterol oxidase (EC 1.1.3.6 from **Nocardia erythropolis**, 1 mg of specific activity 45 U/mg protein) were obtained from Boerhinger Mannheim, Diagnostics and Biochemicals Ltd, Bell Lane, Lewes, East Sussex. Isopropylmyristate, L(+) ascorbic acid (sodium salt), dimethylformamide, p-aminophenol were purchased from Fluka, Glossop. Tetrahydrofuran and acetone were purchased from FSA

Laboratory supplies, Bishop Meadow Road, Loughborough.

The Na^+ and H^+ polyethersulphone polymers containing different ratios of sulphated aromatic to non-sulphated aromatic constituents (10:1, 20:1) and S=5 (polyetherethersulphone) were the kind gift of Dr Graham Scott, ICI Chemicals and Polymers Group, Runcorn, UK. "Estane 5754f1" (polyesterurethane) was generously donated by BF Goodrich, Belgium. The medical grade polyetherurethane "Pellethane" 2363-90AE, produced by Dow Chemical Co (No L81541 10/90TL) and was manufactured by Fronline Filmbashing, Norkopping, Sweden (Batch 910428 CENTR) and supplied by the EC Biomaterials Research Coordination Secretariat, Berlin. The precursor Polyurethane Trixene SC 762 was a gift from Baxenden Chemical Co. Ltd, Accrington, Lancashire.

2.1.1 Buffers and Stock Solutions

Isotonic phosphate buffer (Yellow Springs Buffer, Yellow Springs, Ohio) (Chua and Tan 1978) comprised disodium hydrogenphosphate (52.8 mmol/l), sodium dihydrogenphosphate (15.6 mmol/l), sodium chloride (5.1 mmol/l) and dipotassium EDTA (0.15 mmol/l) (Reagents were from BDH Chemicals).

The following quantities were employed for isotonic buffer preparation :- NaH_2PO_4 12.2 g, Na_2HPO_4 37.47 g, NaCl 15 g, and K_2EDTA 3 g. These materials were dissolved in approximately 800 ml of distilled water and the pH was adjusted to 7.4 by dropwise addition of concentrated sodium hydroxide solution (5 M). The buffer solution

was made up to 1 litre in a volumetric flask and diluted by a factor of five to yield the assay buffer.

Stock solutions were prepared by dissolving the appropriate chemical in a suitable volume of isotonic phosphate buffer. These were stored at 4 °C when not required, and allowed to attain room temperature before use. A 1.0 mol/l glucose stock solution was prepared by initially dissolving 18 g of D-glucose in approximately 90 ml of phosphate buffer, which was made up to 100 ml in a volumetric flask. The solution was left overnight to allow the equilibrium between alpha and beta anomers to be attained (Pileggi and Scustkiewics 1974).

A 100 mmol/l lactate stock solution was prepared by dissolving 0.098 g of lithium L(+) lactate into 10 ml of isotonic phosphate buffer. A 5.3 mmol/l cholesterol standard solution was made up by addition of 208 mg of solid in 10 ml sodium deoxycholate solution (containing 1 mg sodium deoxycholate in 10 ml distilled water) according to the method described by Clark et al (1981).

Solutions of other solutes were prepared on a daily basis to avoid autooxidation. A 10 mmol/l hydrogen peroxide solution was prepared by dilution of 11.2 μl of 100 volume concentrated hydrogen peroxide solution into 10 ml of buffer. A 10 mmol/l solution of L-ascorbic acid (sodium salt) was prepared by addition of 19.8 mg of the solid into 10 ml phosphate buffer. Similarly an approximately 1 mmol/l uric acid solution was made by dissolving 4.5 mg uric acid into 25 ml phosphate buffer. Catechol and paracetamol solutions (10 mmol/l)

were made by dissolving 11 mg catechol and 15 mg paracetamol respectively in 10 ml phosphate buffer. Hydroquinone and para-aminophenol solutions (10 mmol/l) were made up by addition of 11 mg hydroquinone and 10.9 mg p-aminophenol into 10 ml buffer.

2.1.2 Other Materials

The following commercial membrane sources were used throughout the study:- Cuprophan^{R} dialysis membranes were obtained from haemodialysis cartridges (Gambro, Lund, Sweden). Porous track-etched polycarbonate membranes (pore sizes of 0.01 μm, 0.03 μm, 0.05 μm, 0.4 μm and 1.0 μm) were obtained from Poretics, California, USA.

Stainless steel tubes (internal diameter 0.25, 0.50 mm with wall thicknesses 0.05, 0.08 mm respectively) were purchased from Goodfellow Metals Ltd, Cambridge, UK. Teflon-coated platinum wires (bare diameter 0.05 mm or 0.125 mm) were obtained from Clark Electromedical Instruments, Pangbourne, Reading, UK. Quick-set epoxy resin and hardener were supplied by RS Components, Corby. Aluminium oxide (alpha-Al_2O_3) polishing powder (particle size 0.3 μm) was obtained from BDH Chemicals Ltd. Serum and whole blood samples (stored in fluoride/oxalate tubes containing blood specimen were used in order to prevent glycolysis) were obtained from the Dept of Chemical Pathology NHS laboratory at Hope Hospital, Salford.

2.2 Apparatus

2.2.1 Electrochemical measurement systems

The measurement system comprised 2-electrode electrochemical cells (either an oxygen electrode manufactured by Rank Brothers, Bottisham,

(a)

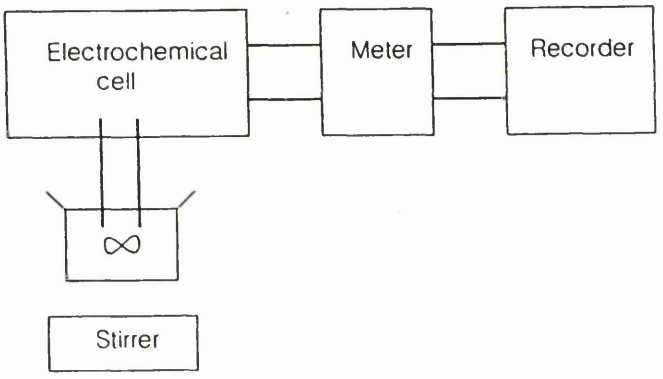


Figure 2.2.1a

Schematic representation of the electrode measuring system.

(b)

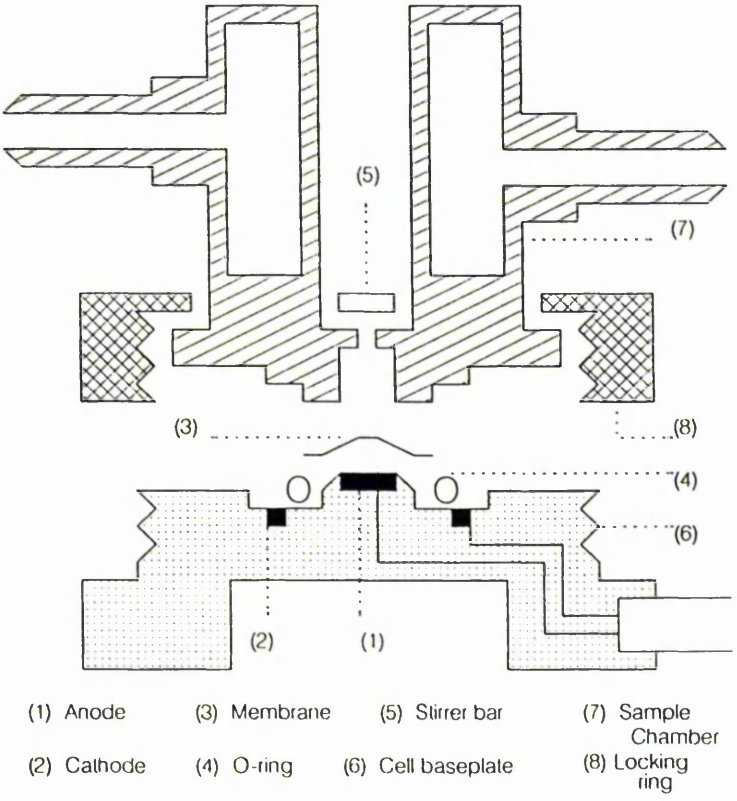


Figure 2.2.1b

Diagram of the cross-section of an oxygen-type "Rank" electrochemical cell.

(c)

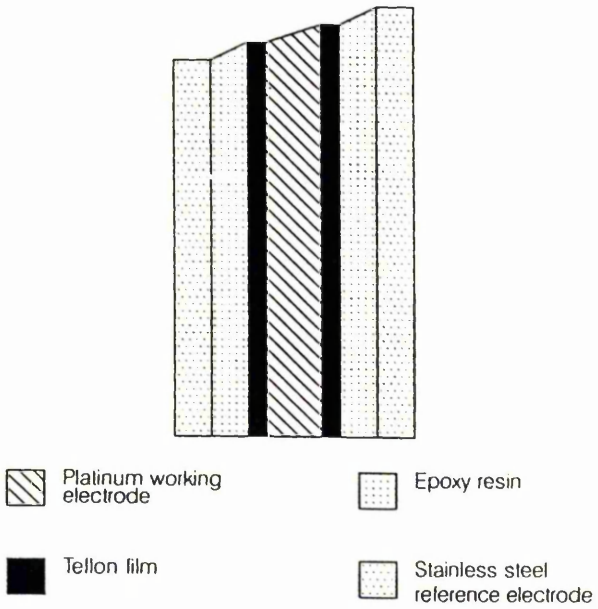


Figure 2.2.1c

Longitudinal section of a bare needle electrode.

Cambridge or a needle electrode), a voltage source/current meter (potentiostat) and a chart recorder. The potentiostat provided a variable polarising voltage at the working electrode while this electrode effected the electrochemical reaction. The resultant current was digitally displayed and reproduced at a strip chart recorder as a pen deflection (shown in Figure 2.2.1a).

(i) Amperometric oxygen electrode

This was manufactured by Rank Brothers, Bottisham, Cambridge, England and consisted of a 2.0 mm diameter central platinum disc (working electrode) and a concentric outer 12.0 mm diameter silver ring as the reference electrode, both of which were embedded in a Perspex body. The assembled cell was clamped using a locking nut.
The arrangement is illustrated in Figure 2.2.1b.

(ii) Needle electrode

This was composed of a central Teflon-coated platinum wire of diameter 0.05 or 0.125 mm employed as the working electrode and an outer stainless steel tube (diameter 0.25 or 0.50 mm) acting as a pseudoreference electrode (shown in Figure 2.2.1c). Epoxy resin sandwiched between the two materials provided electrical insulation.

(iii) Potentiostat

This was manufactured by the School of Chemistry Workshops, University of Newcastle-Upon-Tyne. The instrument had the capacity to vary of the applied polarising potential (range +/- 2.0 volts) and

provide a current readout on a digital display (range 20 nA-2mA).

(iv) Chart recorder

Two types of strip chart recorder were used including a J J Lloyd Model CR652S (J J Lloyd Instruments Limited, Brook Avenue, Warsash, Southampton, SO3 6HP, England) which had a maximum sensitivity of 0.5 mV (full scale deflection), input impedance of 20 M Ohm/V and a chart speed from 0.02 mm/min to 20 mm/s. A Linseis Chart recorder (supplied by Electroplan, Royston in Hertfordshire) was otherwise employed. A sensitivity scale of 50, 100 or 200 mV full scale deflection was mostly used although 20 or 10 mV scales were utilised for high sensitivity work.

2.2.2 SCANNING ELECTRON MICROSCOPY

The polymer membrane surfaces were analyzed using this technique. This was carried out at the Electron Microscopy Unit, School of Biological Sciences, University of Manchester using the Cambridge model S360. Specimen preparation prior to SEM examination was required to obtain the optimal visual image. The procedure as detailed below consisted of drying the membrane, mounting it on a 10 mm diameter metal stub, earthing (using a silver paint) and coating with a conductive film of gold (less than 100 μm thick).

Air-drying was used for all of the membranes. The dried membrane was transferred onto a stub and coated with gold by a sputtering technique in a vacuum. An even metallic covering of was achieved by mounting the

(a)

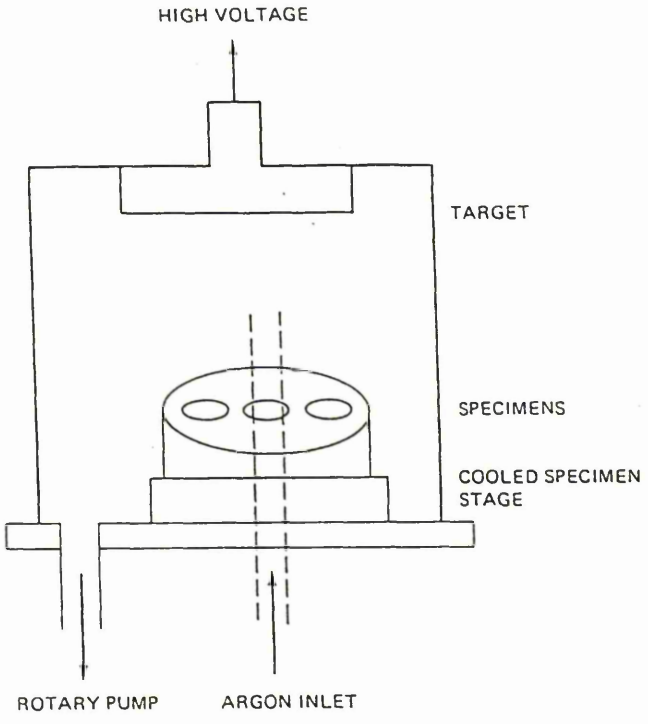


Figure 2.2.2a

Diagram of the gold sputtering apparatus.

(b)

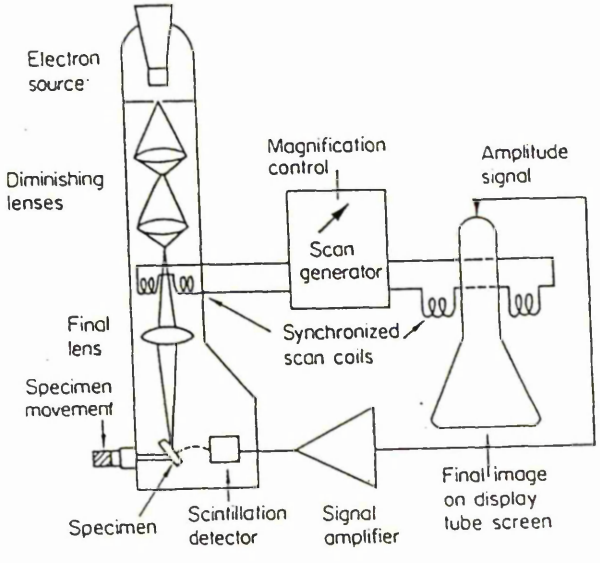


Figure 2.2.2b

Schematic representation of the components of a Scanning Electron Microscope.

stub on a rotating turntable. During the process metal (gold) atoms were eroded from a target material by argon. A transfer of momentum from the argon ions to the gold surface resulted in ejection of gold atoms. A general method involved evacuation of the specimen chamber to the 10^{-2} torr range and removal of residual vapours. A high voltage of ~2.5 KeV was then applied and argon was admitted to a plasma discharge current of ~20 mA for ~3 minutes (Figure 2.2.2a)

SEM operation is based on the following:- an electron beam is emitted by a heated hairpin filament positioned inside a cathode assembly behind a small aperture. Electrons are drawn off the tip of the filament due to the high negative voltage on the cathode and earth potential at the anode. The subsequent beam diameter is regulated through use of two condenser lenses. The electron beam is bent backwards and forwards by scan coils to form a raster over the membrane surface, and is focused by a lens onto the surface to produce a sharp image. The electrons scattered from the surface are attracted to the collector which is positively biased. The collector surface is coated with phosphor which emits light when an electron collides with the surface, and the photons of light are passed to photomultiplier which produces an electronic signal, which, in turn is displayed on the monitor (Figure 2.2.2b).

2.2.3 Needle electrode - fabrication and membrane dipcoating

The method of needle electrode construction according to Churchouse et al (1986) was utilised initially. A stainless steel tube (of diameter 0.25 or 0.50 mm) was cut to a 3-4 cm length and Teflon-coated platinum wire (0.05 or 0.125 mm bare diameter) to a length of 5 cm. Both ends

of the stainless steel tube were filed with coarse emery paper to open the tube holes and to make them burr-free. The Teflon-coated platinum wire was carefully inserted into the steel tube so that approximately 1 cm protruded at one end. A small amount of epoxy resin was applied to the wire and the wire was gently pulled backwards and forwards in the tube so that the resin on hardening firmly fixed it in place. Epoxy resin was applied to the other end of the tube thereby securing the platinum. The thin Teflon coating was stripped from the connection end of the platinum wire and the bare wire and steel tube at this end were soldered to a subminiature coaxial cable. The soldered connection was protected with a sleeve cut from a micropipette tip and embedded in epoxy resin. The working end of the needle was polished with coarse then fine emery paper and the tip was checked under a binocular microscope to ensure that a smooth, polished surface was produced.

For needle electrodes, a dip-coating method (Vadgama et al 1989) was employed in order to affix the polymer membrane over the needle tip. A single step procedure was used in which the needle tip was immersed in the polymer solution (e.g. 5% w/v polyurethane in tetrahydrofuran) for 1-2 seconds, taken out and allowed to dry in an upright position. For polyethersulphone membranes there was an absolute requirement to use a vacuum oven because the solvent employed (dimethylsulphoxide) was not sufficiently volatile at room temperature and atmospheric pressure; a pressure reduced to +700 mmHg was applied for 3-4 hours at 40 °C.

2.3 Membrane casting methods (for oxygen electrode)

2.3.1 Polyvinyl chloride

PVC (high molecular weight, 200,000), 0.12 g was slowly dissolved in 10 ml tetrahydrofuran at room temperature. The dissolved polymer solution was poured into a flat glass Petri-dish of diameter 10 cm (precleaned with acetone to ensure that it was clean and dust-free). Controlled evaporation was achieved by leaving the dish covered using the glass lid for 2 days.

2.3.2 Plasticised polyvinyl chloride

A similar method of casting as for unplasticised PVC was used (Christie 1988), however 300 μl or 150 μl isopropylmyristate (or dioctyl phthalate) was added to the polymer solution prior to casting.

2.3.3 Detergent-modified polyvinyl chloride

Polyvinyl chloride (0.06 g of high or low molecular weight) was slowly allowed to dissolve in 5 ml tetrahydrofuran. A 100 μl aliquot of Tween-80 or Triton X-100 was added to this and thoroughly mixed. The resultant polymer solution (2 ml) was poured into a flat glass petri-dish (8 cm diameter) and left covered in a horizontal position overnight.

2.3.4 Cellulose acetate

The appropriate masses of cellulose acetate (39.8 % acetyl content) were taken in order to make 1.2%, 1.6%, 2.0% and 2.4% w/v solutions in acetone. Membranes were cast either on a glass plate or into a Petri-dish. The following procedure was employed, adapted from (Tsuchida and Yoda 1981) for casting onto a glass plate:- a one ml aliquot of the

polymer solution was applied dropwise to the centre of a 60 cm x 60 cm glass plate and spread evenly using a plastic pasteur pipette or "Gilson" pipette tip. The solution was allowed to evaporate at room temperature until a membrane gel layer had formed after 2-3 minutes. During this procedure the plate was slowly rotated manually in a horizontal position in order to facilitate a uniform covering layer. The resultant membrane was then left to dry at room temperature for at least one hour. The previous steps were conducted in a fume cupboard. In some cases, the glass plate was pre-treated with dimethyldichlorosilane (2% v/v in 1, 1, 1 trichloroethane) to provide a hydrophobic casting surface to allow easier separation of the membranes. A membrane was removed from the glass plate using a pair of fine tweezers or was alternately pre-soaked in buffer and allowed to float off.

The following protocol was utilised when casting cellulose acetate membranes in glass Petri-dishes:- the appropriate cellulose acetate solution was made up into 10 ml acetone. The polymer solution was poured into a clean dust-free petri-dish (8 cm diameter). Controlled evaporation was carried out at room temperature by covering the dish overnight.

2.3.5 Solvent-modified cellulose acetate

A 2% (w/v in acetone) cellulose acetate solution was made using a total volume of 5 ml acetone. To the resulting polymer solution 50 μl of dimethylformamide, formamide or ethanol was added. After thorough mixing, the solution was poured into a flat glass Petri dish (diameter 7 cm) and was left covered in a horizontal position at room temperature overnight.

2.3.6 Detergent-modified cellulose acetate

A 5% (w/v in acetone) solution of cellulose acetate was made by dissolving 0.25 g cellulose acetate into 5 ml acetone. A fixed aliquot (100 μl) of detergent/surfactant (Triton X-100 or Tween-80) was added to this making a 2% v/v solution. Two ml of the resulting mixture was pipetted into the centre of a glass petri-dish (10 cm diameter). The solution was spread evenly and the dish rotated slowly in a horizontal position so that a uniform gel layer was produced. The solvent was allowed to evaporate at room temperature from the uncovered dish for at least 2-3 hours before use.

2.3.7 Polyethersulphone

Polyethersulphone (0.4 g) dissolved slowly into a solvent mixture of 7.5 ml dimethylformamide and 2.5 ml 2-methoxyethanol (or 10 ml dimethylformamide alone). The dissolved polymer was poured into a flat glass Petridish (diameter 10 cm) and covered. The dish was left in a horizontal position for at least two days.

2.3.8 Polyurethane

Solutions of the polyurethane "Pellethane" (0.5-1.5% w/v in tetrahydrofuran) were made up into a total volume of 5 ml solvent. The solution was poured into a glass Petridish (diameter 7 cm) and left covered overnight.

2.4 Enzyme electrode construction

2.4.1 Amperometric oxygen electrode

Initially the plastic electrode top was removed by loosening the screw

nut as depicted in Figure 2.2.1b. The surface of the working electrode was moistened with buffer in order to provide proper electrical contact with the surrounding silver/silver chloride reference electrode. The prepared laminate (incorporating chemically crosslinked enzyme) was rinsed using isotonic buffer and carefully layered over the working and reference electrode surfaces, so that no air bubbles were trapped underneath. A securing rubber "O" ring was applied over the laminate and finally the whole assembly was tightened by means of the screw nut. Prior to any measurement a stable current reading for the given voltage polarisation source was required. This was primarily achieved by placing a few drops of buffer under constant stirring onto the laminate. The apparatus used was an oxygen electrode construction but a polarisation appropriate for hydrogen peroxide detection was employed.

2.4.2 Enzyme immobilisation

The main procedure in the construction of an enzyme electrode was the immobilisation of the enzyme. When a layer of immobilised enzyme was prepared and employed over a oxygen electrode, the main parts of the enzyme electrodes were thus formed. Variation of the external covering membrane and the underlying membrane resulted in the production of enzyme electrodes of varying practical performance; different analytes (substrates) could be determined by alteration of the enzyme in the immobilised layer.

The enzyme membrane employed over the oxygen electrode was of a "sandwich" structure, with the immobilised enzyme layer between the polymer membranes. Permselective inner membranes were cellulose

acetate, polyvinyl chloride or polyethersulphone) and usually included Cuprophan in contact with the working electrode. Commercial pre-formed polymeric membranes e.g. of polycarbonate were generally used as external covering membranes.

For glucose or lactate electrodes, chemical immobilisation of the glucose/lactate oxidase were used. The procedure for preparing a crosslinked enzyme layer (Tang et al 1990) was as follows:- two pieces of polymer membrane approximately 10 mm x 10 mm were placed onto separate glass microscope slides. Six μl of the enzyme reagent solution containing 30 mg/ml glucose or lactate oxidase and 200 mg/ml bovine serum albumin were placed onto a glass microscope slide using a 20 μl "Gilson" automatic micropipette. The enzyme solution was mixed with the crosslinking reagent, glutaraldehyde (5% and 1% w/v for glucose and lactate oxidase respectively) on another slide and the entire mixture immediately transferred onto the central portion of one of the membranes. The second membrane was placed on top and the two membranes were pressed between the two glass slides under hand pressure for five minutes. The slides were then prised apart and the membrane/enzyme sandwich layer ("laminate") was left to dry in air at room temperature for at least five minutes. The laminate was washed with buffer and placed over the oxygen electrode.

For cholesterol electrodes, two methods of enzyme immobilisation were used:- chemical cross-linking using glutaraldehyde and physical entrapment using polyvinyl alcohol solution. The crosslinking method was similar to the preparation of the immobilised glucose oxidase layer.

The polyvinyl alcohol entrapment procedure involved the following steps:- Initially, a 8% (w/v) polyvinyl alcohol was made up by dissolving 0.8 g of polyvinyl alcohol into 10 ml buffer. Three μl of polyvinyl alcohol solution (stirred) was mixed with 6 μl cholesterol oxidase enzyme reagent and spread evenly over a polycarbonate membrane square onto a silanised glass slide. A second polycarbonate membrane was placed over the first and the "laminate" was allowed to set in air for approximately 10 minutes.

2.4.3 Needle electrode

Unlike oxygen type ("Rank") enzyme electrodes, the immobilised enzyme layer and the membranes were formed **in situ** on the needle tip by using a dipping, coating and drying method. Initially a permselective polyethersulphone membrane (as described in section 2.2.3) was applied. Then the needle was coated with a very thin layer of immobilised enzyme GOD or LOD by applying the needle tip into a viscous mixture of enzyme, albumin and glutaraldehyde for 1-2 seconds and allowing it to dry at room temperature for at least 10 minutes. Finally the diffusion-limiting polyurethane membranes were applied. These were prepared by sequential dip-coating using polyurethane dissolved in tetrahydrofuran (5%, 10% w/v for the Goodrich "Estane 5724f1", 8%, 16%, 33%, 50% v/v for the Baxenden SC 762 prepolyurethane and 1-3% for "Pellethane") (Vadgama et al 1989). The sensor was dipped once into the relevant polyurethane solution for 1-2 seconds, kept in a vertical position to allow solvent to evaporate in air for 30 minutes before the next polyurethane layer was applied. The fully-coated needle was stored dry in air overnight before use. The

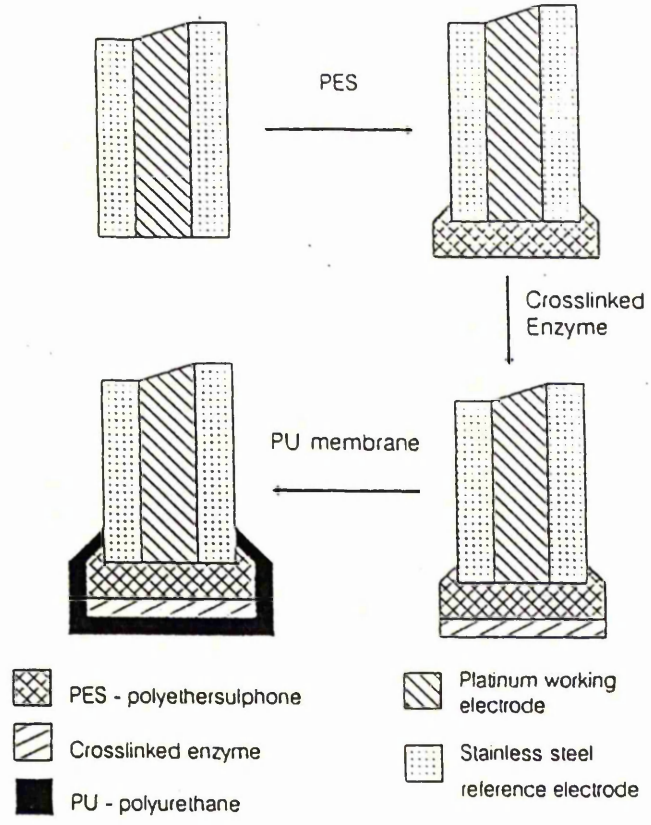


Figure 2.4.3

Schematic diagram of the enzyme membrane structure of needle electrodes.

prepolyurethane (Trixene SC 762) polymerised on exposure to atmospheric water vapour (Figure 2.4.3).

2.5 Analysis using enzyme electrodes

2.5.1 Measurement in aqueous solution

The protocol for setting up a 2-electrode hydrogen peroxide detection system has been described previously (section 2.4.1). In all cases, steady state measurements were made. Calibration curves were obtained by adding increasing amounts of stock substrate standard to a fixed volume of assay buffer solution (stirred) up to final volume additions of less than 0.02%. This avoided electrical transients due to complete replacement of solutions. For discrete measurements the assay volume was 5000 μl for either the electrode cell or needle electrodes.

2.5.2 Measurement in whole blood

For glucose measurements in undiluted blood the electrode was initially calibrated using aqueous glucose standards. A series of heparinised whole blood samples stored in fluoride/oxalate tubes, were obtained from the NHS laboratory, Hope Hospital. Samples were measured without further recalibration or manipulation. For biocompatibility evaluation, aqueous glucose standards were tested between blood exposure and the percentage change of electrode response was determined to assess blood effects on membranes and therefore electrode performance. An alternative method monitoring the drift in signal during continued blood exposure without a rinsing stages using buffer for recalibration was also carried out. To examine the possibility of Pt working electrode passivation the following procedure was employed; the response of a bare Pt electrode to 0.04 mM hydrogen

peroxide in buffer was first recorded, then after two-hour indirect blood exposure behind a particular specific membrane laminate, the laminate was removed, the electrode rinsed thoroughly and the stirred response to H_2O_2 re-recorded.

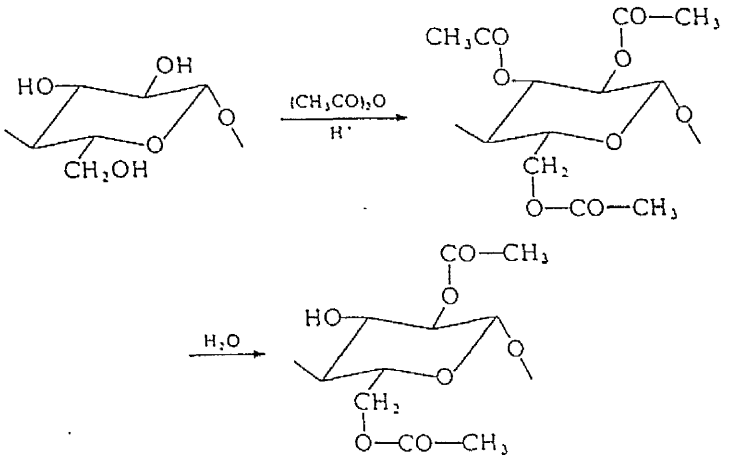
2.6 Solute partitioning in polyvinyl chloride

The following experimental protocol was followed in order to demonstrate solute partitioning:- a 300 μl aliquot from a solution of electroactive species (5 mM) was tested on a oxygen type ("Rank") amperometric cell covered with a dialysis membrane. Plasticiser (IPM/DOP - 10 ml) was mixed with 10 ml of the 5 mM standard solution of the electroactive compound into a separating funnel. After vigorous shaking (10 minutes) a 300 μl aliquot from the aqueous phase was tested and the solute was assayed electrochemically.

CHAPTER THREE - CELLULOSE ACETATE MEMBRANES

3.1 INTRODUCTION

The cellulose acetate polymer is made by acetylation of cellulose with acetic anhydride in the presence of sulphuric acid as catalyst. If acetylation is interrupted before esterification is complete a mixed product consisting of cellulose triacetate and cellulose are produced. Hydrolysis of cellulose triacetate lead to "primary cellulose acetate" production which hydrolyses to form cellulose diacetate as illustrated below:-



(3.1)

The physical and chemical properties of the polymer are determined by the degree of esterification e.g. cellulose acetate (52-54% acetyl content) undergoes decomposition well below its softening point. Therefore plasticiser incorporation e.g. dimethylpthalate or triphenyl phosphate (25-35%) is required. Plasticiser type and concentration greatly affect polymer physical properties for instance cellulose acetate (54-56% acetyl content) is used for film, fibre and lacquer production due to its enhanced water resistance. Acetone is the most important solvent for cellulose acetate. All types of cellulose acetate are readily hydrolysed in aqueous acids and alkalis.

Dense asymmetric cellulose acetate membranes have been used for salt removal from seawater (reverse osmosis). Loeb and Sourirajan (1962) showed that the membranes consisted of a relatively dense surface layer followed by a gradual transition to a macroporous structure. The solvent composition used to dissolve the polymer determines whether asymmetric membranes are formed e.g. ternary cellulose acetate-plus-acetone solutions containing other solvents such as tetrahydrofuran, acetonitrile or p-dioxane mixtures do not lead to asymmetric membrane formation. Additional, $Mg(ClO_4)_2$ (Loeb and Sourirajan 1962), formamide (Manjikian 1967) or additives (Vaughan 1959; Elford 1937) require incorporation to form resultant asymmetric structures.

Cellulose acetate (CA) membranes have been widely used for biosensors based on glucose oxidase because of their high selectivity for hydrogen peroxide. Prefabricated cellulose acetate films may be placed over the electrode (Mullen et al, 1986) or a dip-coating technique may be employed for miniature needle-type sensors (Shichiri et al 1982; Bindra et al 1991). Additionally the electrode surface could be modified using cellulose acetate by droplet evaporation or spin-coating.

Sittampalam and Wilson (1983) demonstrated that a cellulose acetate-coated bare electrode is able to prevent electrode poisoning by the protein bovine serum albumin (BSA) in a standard hydrogen peroxide solution. BSA is a high molecular weight protein and is therefore blocked by the cellulose acetate from diffusing to the electrode. Lonsdale et al (1971) showed that only small molecules (H_2O_2, O_2) and ions (Na^+, Cl^-) could permeate cellulose acetate. The polymer has been

utilised commercially in the Yellow Springs Instrument Glucose analyzer (Chua and Tan 1978). The membrane allows hydrogen peroxide permeation whilst excluding other electrochemically active species from blood including ascorbate, urate, certain amino acids and small peptides e.g. glutathione (Palleschi et al 1986). Wang and Hutchins (1985) showed that film permeability could be controlled by using base hydrolysis. As the hydrolysis period was extended to 30 minutes permeation of paracetamol (MW 151) and oestriol (MW 288) gradually increased. Larger species e.g. NADH or ferrocyanide penetrated the film after 40 minutes of alkaline hydrolysis. However coated cellulose acetate membranes could lose adhesion to the polished electrode surface with prolonged use due to swelling and seepage of water in between the membrane and electrode. This occurs partly because of the hydrophilic nature of the platinum surface as well as of cellulose acetate.

The following investigations were carried out:-

(1) Application of cellulose acetate films in both inner and outer configurations of glucose electrodes; (2) Determination of the impact of polymer concentration on permeability of hydrogen peroxide, phenolics and ascorbate; correlation of structure to permeation characteristics.(3) Solvent effects on permeation (4) Film modification using surfactants (Tween-80 and Triton-X100).

3.2 Unmodified cellulose acetate

3.2.1 Cellulose acetate surface structure

Surface examination of the cellulose acetate film (Figure 3.2.1)

essentially homogeneous, uniform and no pores are visible. SEMs of phase-inversion cellulose acetate membranes have been shown by Frommer and Doron (1972). A magnified cross-section of the acetone-cast membrane made by phase inversion (in water) revealed the presence of many 1 μm or less diameter pores (Type B membranes). Non-porous homogeneous membranes were formed with acetic acid or triethylphosphate (Type A membranes). The structure of acetone-cast membranes which were air dried was also shown to be completely structureless (Riley et al 1964). This was believed to be the outer dense layer from the phase-inversion modified membranes cast by Loeb and Sourirajan (1962). However the modified membranes also contained a porous interior of pore size (0.1 μm).

3.2.2 Cellulose acetate concentration effects on permeability

The transport of electroactive species at 0.04 mM through acetone-cast membranes using increasing concentrations of the polymer (w/v) were determined. Increased polymer concentration caused a decreased permeation of hydrogen peroxide although maintaining selectivity against ascorbate. However the flux of phenolic compounds were not dependent on polymer concentration which was especially valid for catechol and acetaminophen (paracetamol) measurement (Figure 3.2.2).

3.2.3 Inner membrane of glucose electrode

Interposition of a permselective non-porous cellulose acetate membrane between the working electrode and the crosslinked enzyme layer produced a pronounced diminution in signal size in comparison to the 0.03 μm (control) polycarbonate membrane (Figure 3.2.4). At 2 mM

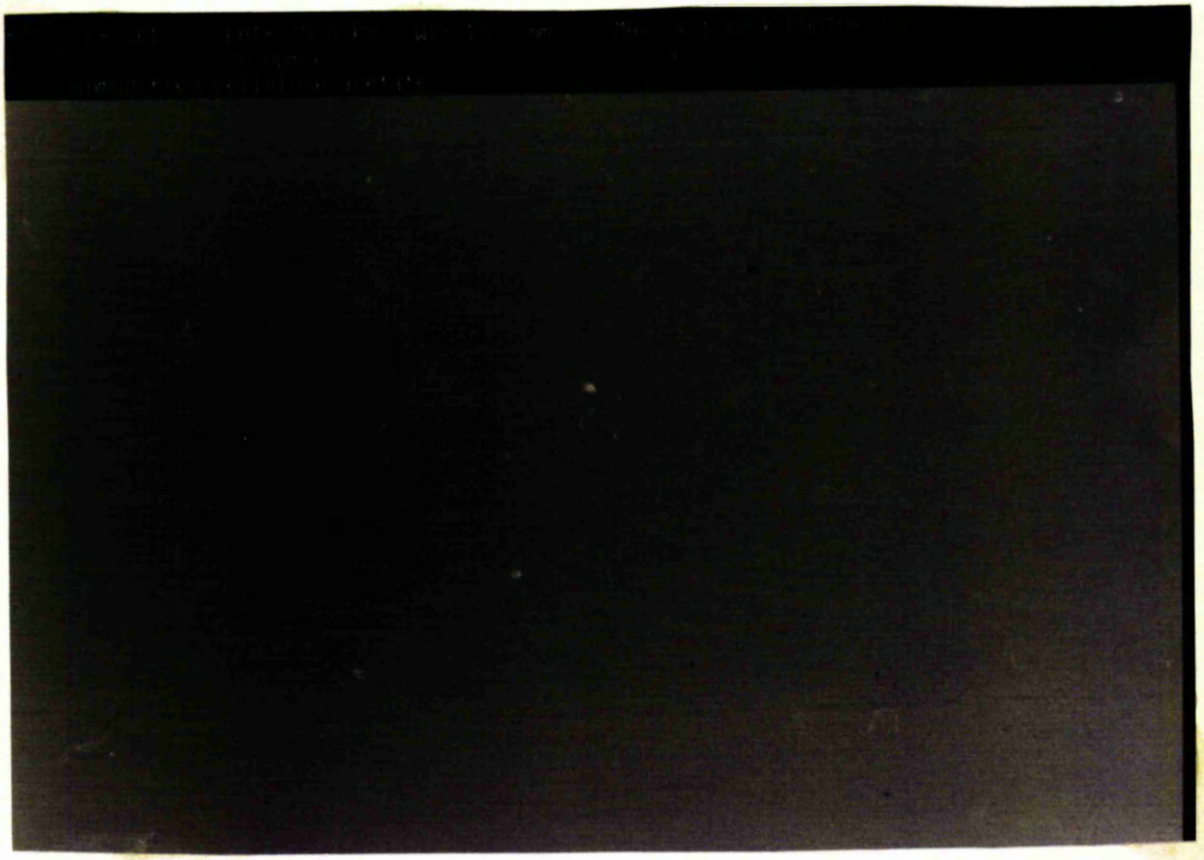


Figure 3.2.1

Scanning electron micrograph of a cellulose acetate (5% w/v in acetone) membrane.

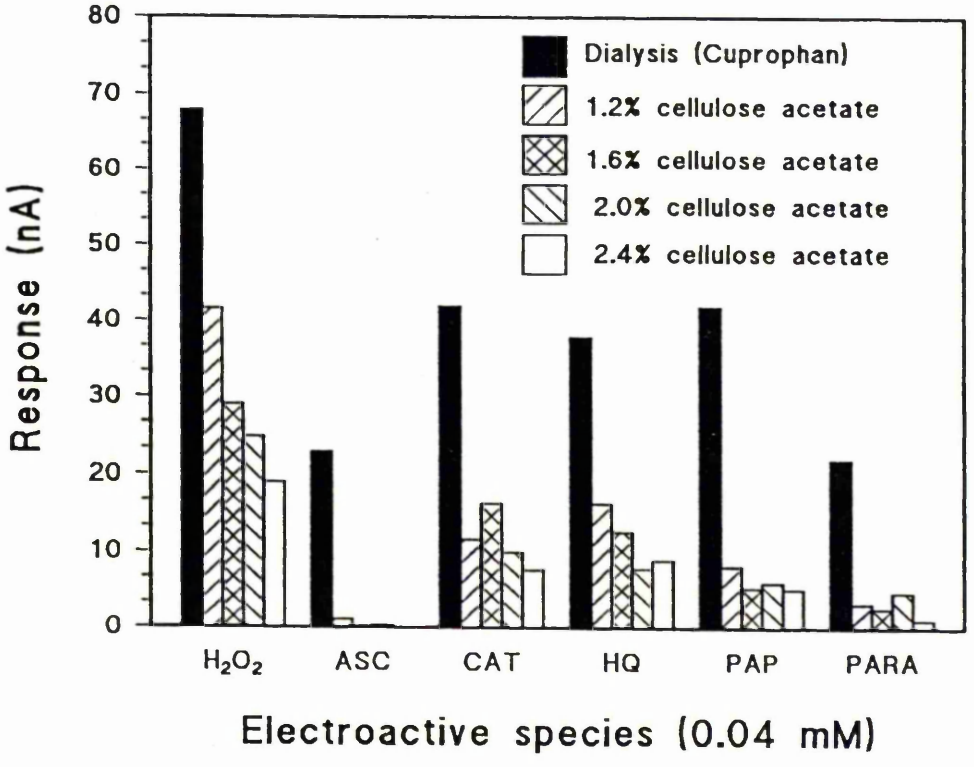


Figure 3.2.2

Variation of permeability with cellulose acetate concentration (1.2%-2.4% w/v in acetone) for electroactive species at 0.04 mM for individual electroactive species.

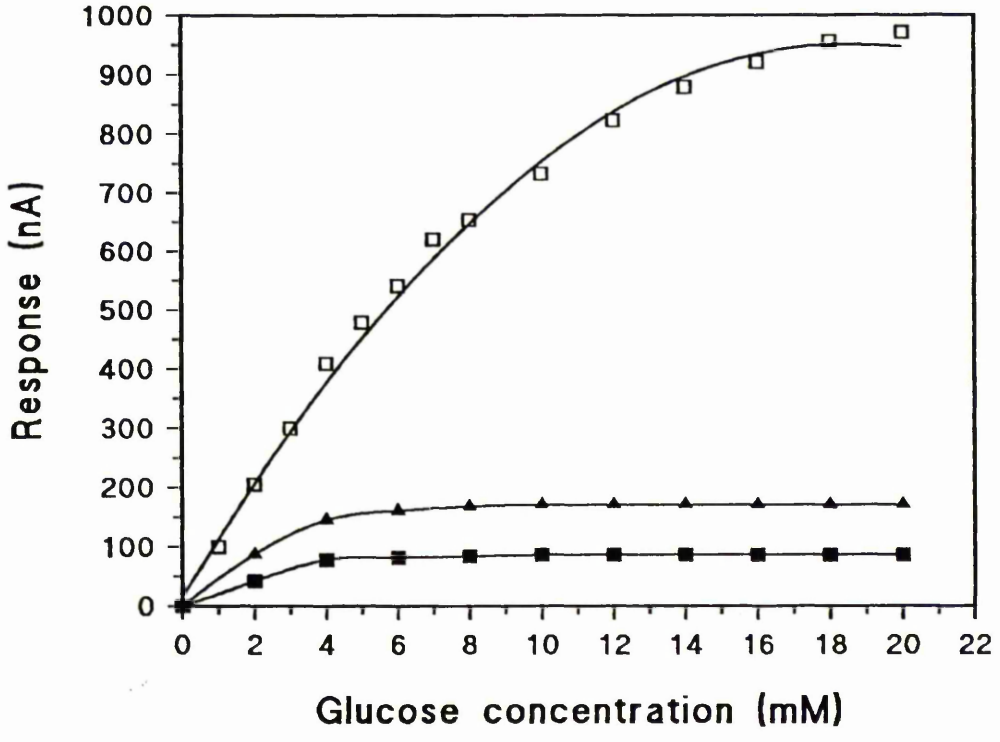


Figure 3.2.3

Inclusion of cellulose acetate as internal membranes of glucose enzyme laminates. Inner membranes:- (□) 0.03 μm polycarbonate, (▲) 2% CA (w/v in acetone) and (■) 5% CA (w/v in acetone).

glucose, response with a 2% w/v cellulose acetate membrane was ~50% of the polycarbonate control and a 25% for the 5% w/v cellulose acetate membrane. Both of the laminates also showed a limited linear range (maximum linearity of 2-3.5 mM), and response times went from 30-60 s (0.03 μm polycarbonate membrane) to 1.5-2 minutes (cellulose acetate).

3.2.4 Outer membrane of glucose electrode

Inclusion of acetone-cast 2% and 5% w/v cellulose acetate as an external membrane caused a dramatic increase in the linear range for glucose to ~100 mM (Figure 3.2.4). However this was accompanied by a sharp decline in the sensor sensitivity (~0.5 nA/mM for 5% cellulose acetate, 0.8 nA/mM for 2% cellulose acetate and 100 nA/mM for the 0.03 μm polycarbonate membrane) and response time extended to 3-5 minutes. Signal size was reproducible to +/- 0.9% for the entire glucose range.

3.3 Modified solvent casting

3.3.1 Permeability study

Two % cellulose acetate showed a greater permeation of H_2O_2 than to phenolics (catechol, paracetamol, hydroquinone and p-aminophenol) at a concentration of 0.04 mM. However, almost complete exclusion of anionic interferences (ascorbate and urate) was observed. Dimethylformamide in the casting solution produced a slight elevation in the response to phenolic species especially catechol, hydroquinone and para-aminophenol, however alteration in H_2O_2 : charged interferents permeability ratios was not seen. Ethanol in the casting

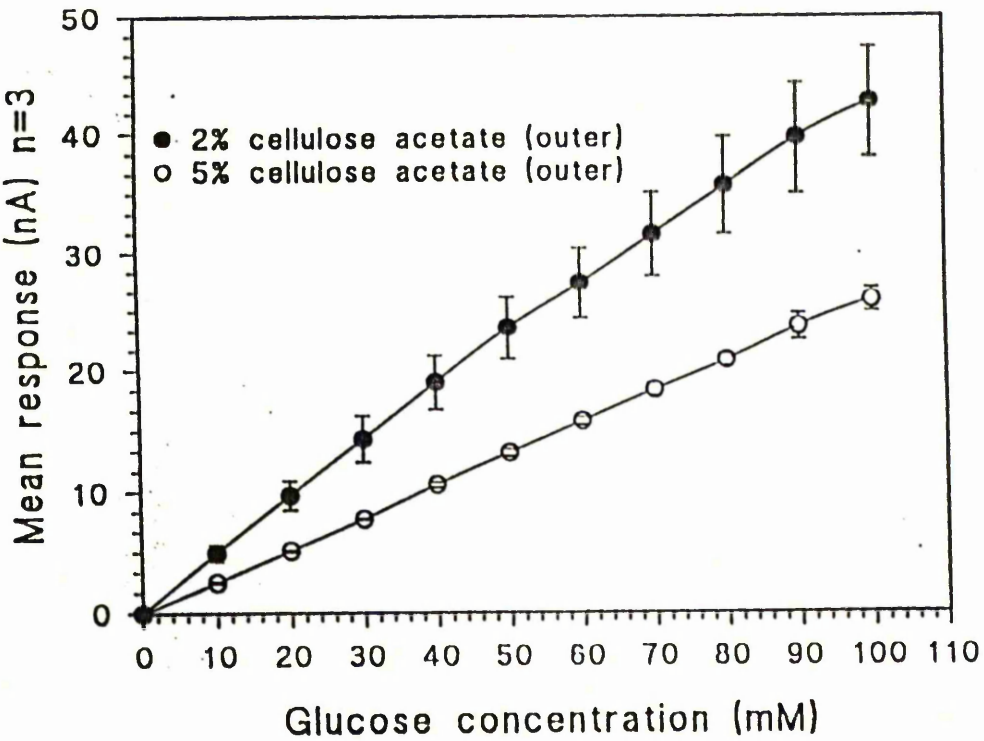


Figure 3.2.4

Determination of the effect of inclusion of external cellulose acetate membranes (●) 2% w/v in acetone and (○) 5% w/v in acetone on glucose electrode response (duplicate values are given as SEMs).

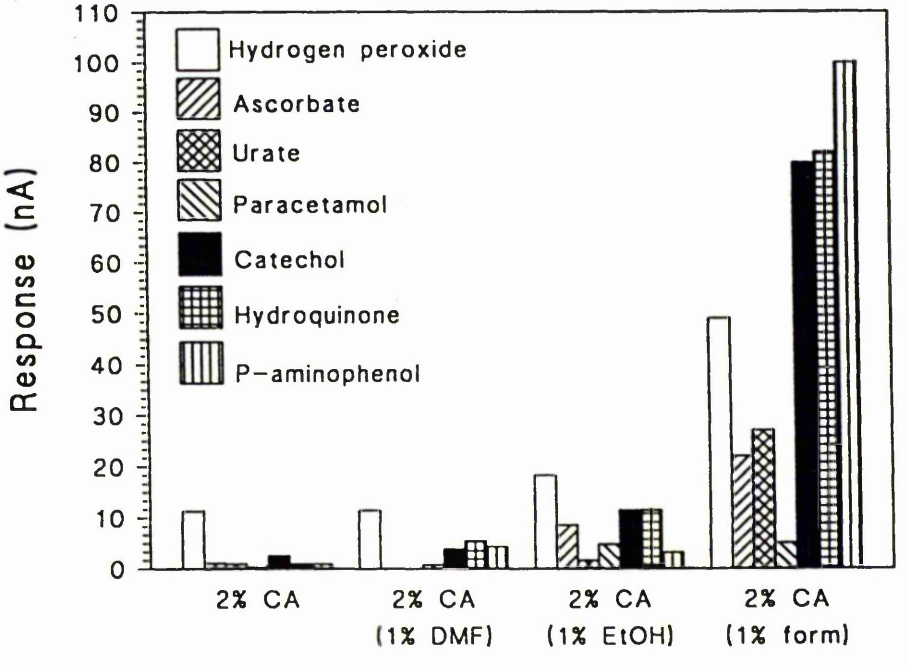


Figure 3.3.1

Casting solvent variation for cellulose acetate on permeability properties for individual membranes.

mixture increased response to H_2O_2, but also a general increased flux for the other species except urate. Formamide incorporation resulted in a considerably greater rise in permeation for all species with no evidence of any selectivity (Figure 3.3.1).

3.3.2 Mixed solvent membranes in glucose electrode

Diminution in current was observed for all of the membranes accompanied also with a loss in linear range. Inclusion of acetone, acetone-ethanol and acetone-dimethylformamide during membrane casting resulted in a 80% current decline, and the effect was less (50%) using the acetone-formamide mixture (Figure 3.3.2).

3.4 Detergent-modified cellulose acetate

3.4.1 Surface examination

The scanning electron micrographs in Figure 3.4.1 show that Tween and Triton-modified cellulose acetate films are non-porous, homogeneous structures. A much smoother surface is noticeable for the Triton-modified compared to the Tween-modified membrane.

3.4.2 Permeability properties of Tween-80 and Triton-X100 modified cellulose acetate

Detergent-incorporation increased permeability to all electroactive species compared to unmodified cellulose acetate. Also selectivity against ascorbate was enhanced. Significant elevations in response were especially observed for catechol and p-aminophenol. Triton-modified membrane showed an enhanced effect compared to Tween-modified cellulose acetate, where the flux was twice the magnitude of

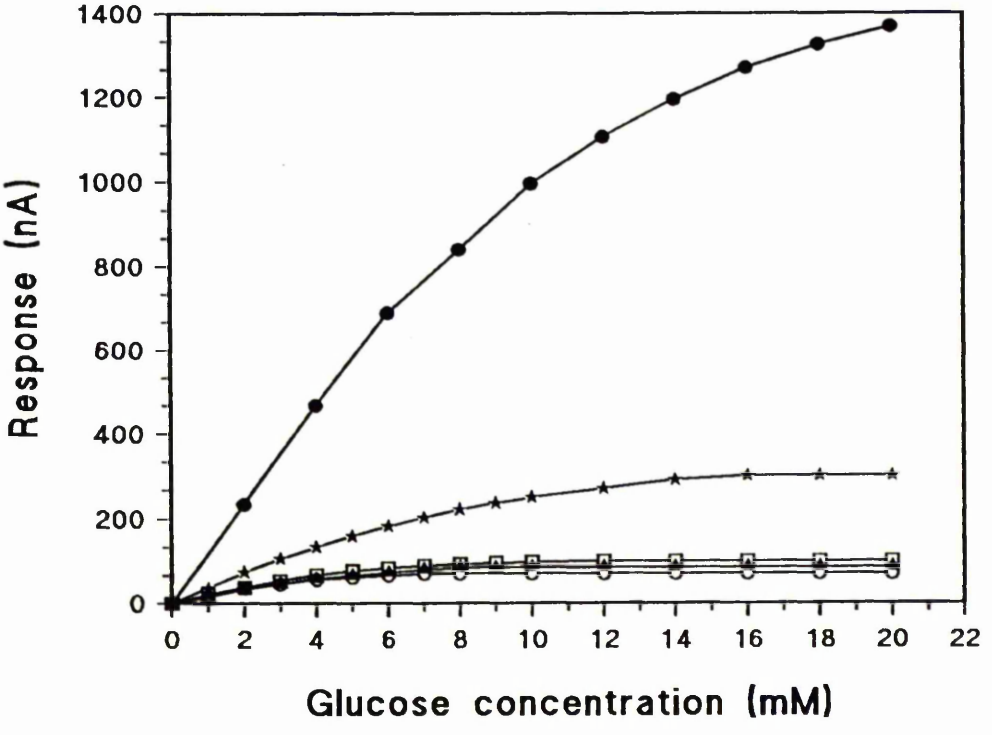
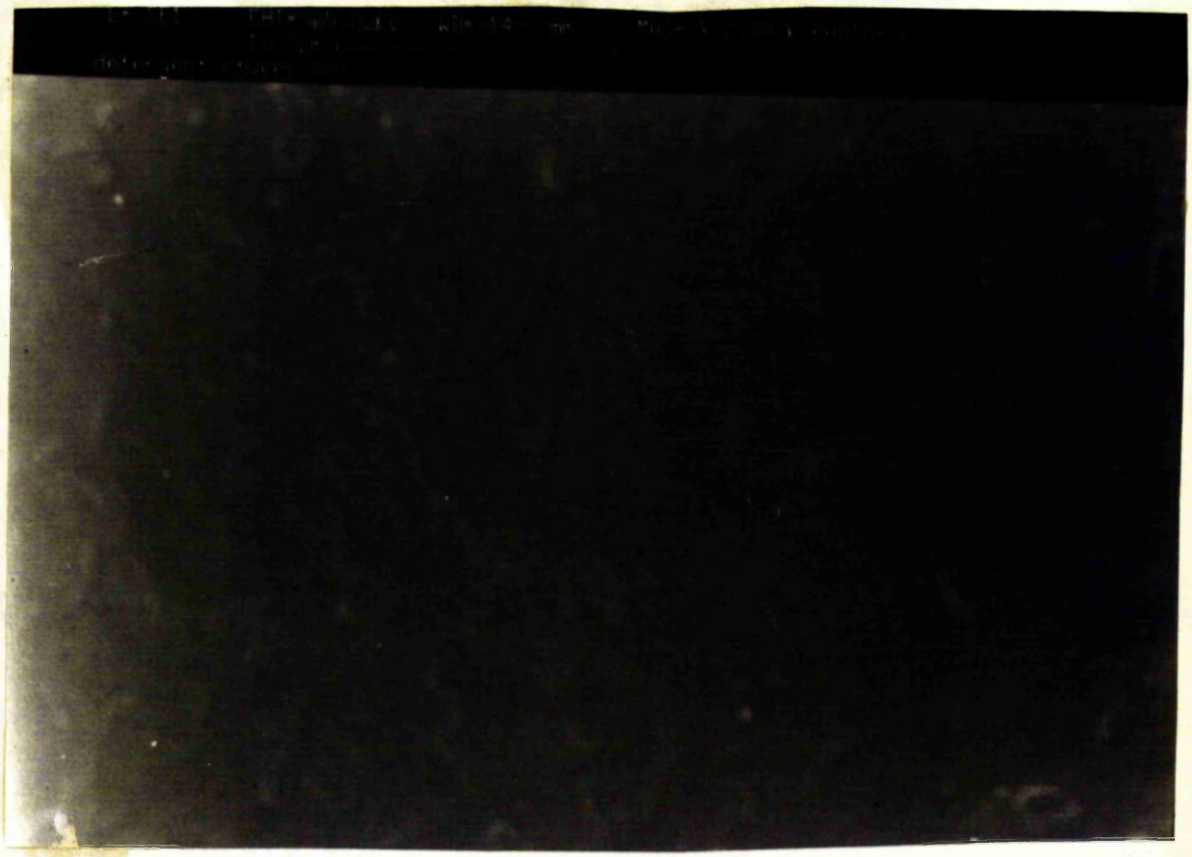


Figure 3.3.2

Effect of using casting solvent modified cellulose acetate internal membranes on glucose electrode response.
Inner membranes:- (●) 0.03 μm pore size polycarbonate, (*) 2% CA (1% formamide v/v in acetone), (□) 2% CA (1% ethanol v/v in acetone), (▲) 2% CA (1% dimethylformamide in acetone) and (○) 2% CA w/v in acetone alone. (An external 0.03 μm pore size polycarbonate membrane was present in each laminate).

(a)



(b)

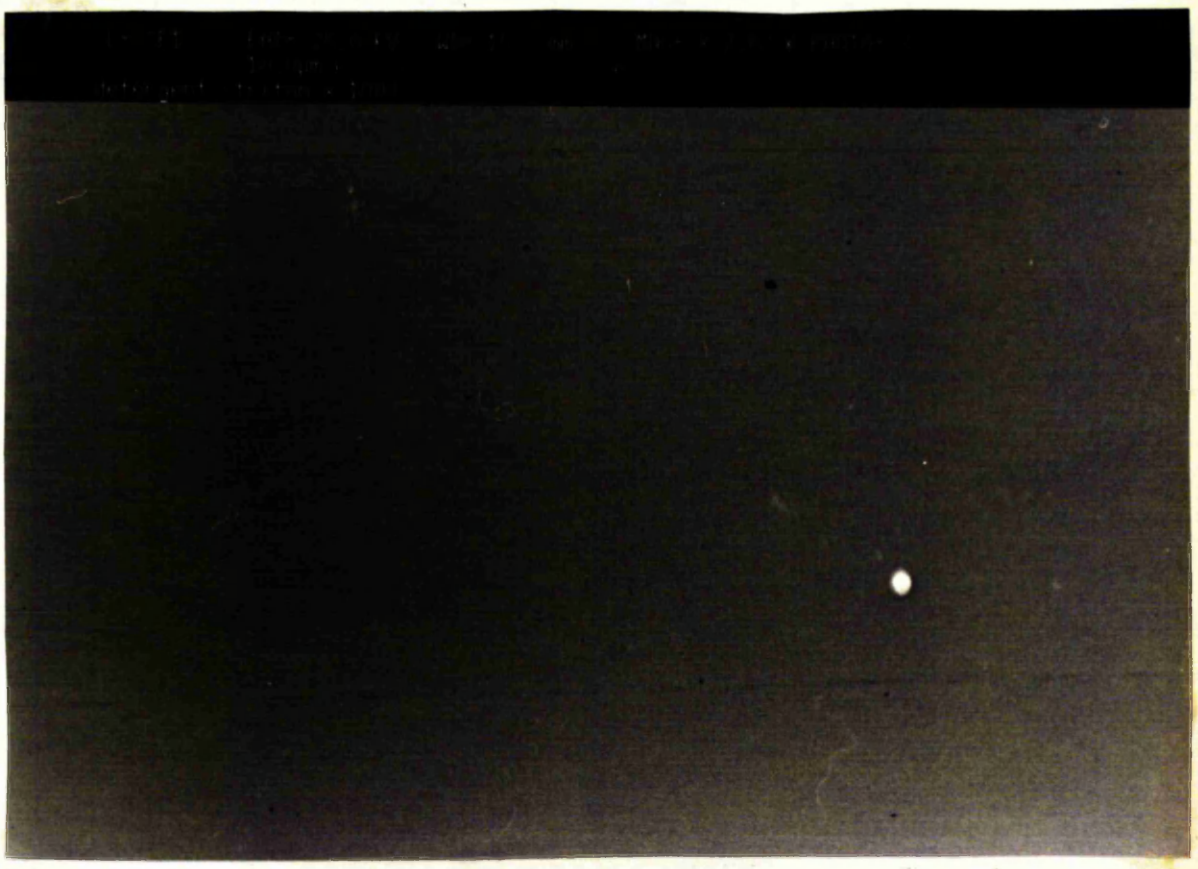


Figure 3.4.1

Scanning electron micrographs of (a) Tween-80 and (b) Triton X100 modified cellulose acetate membranes.

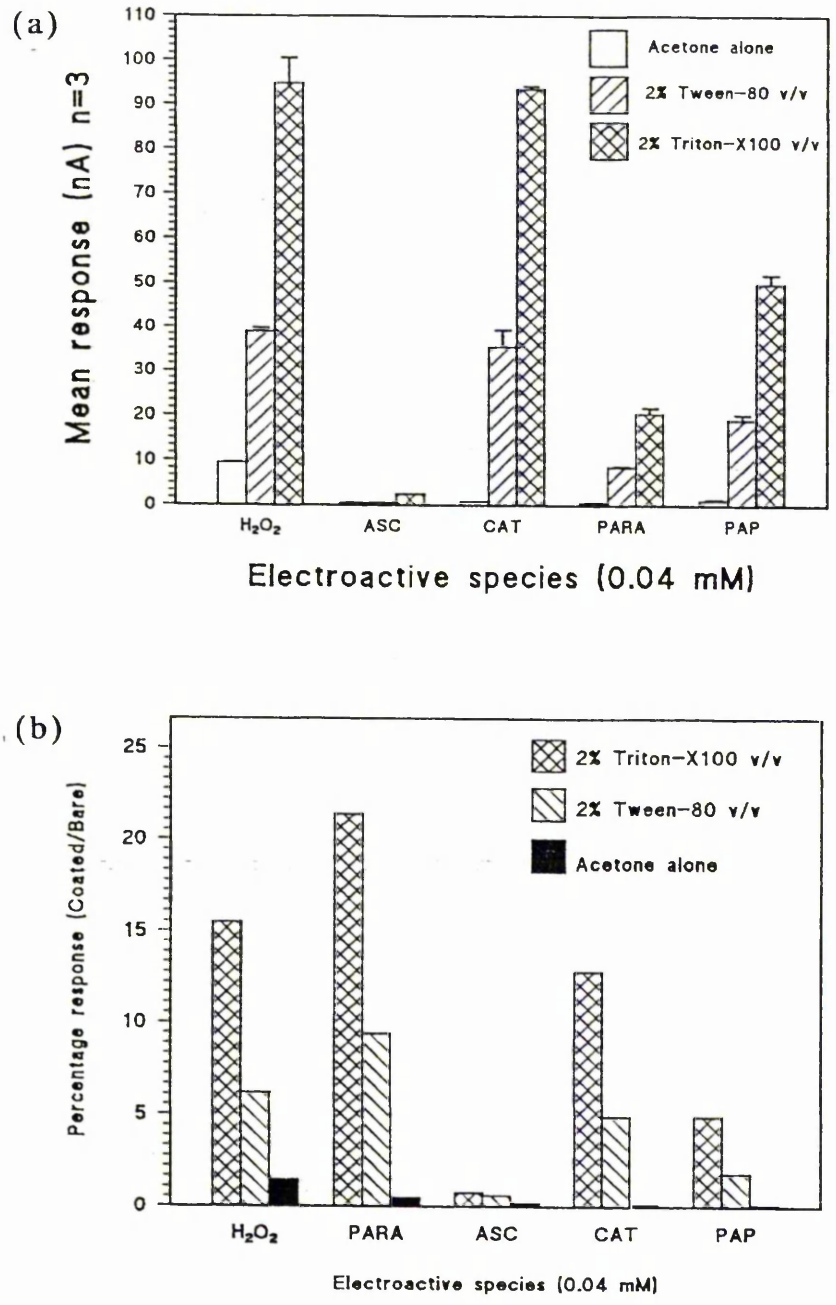


Figure 3.4.2

Permeability characteristics of unmodified as well as modified (using 2% v/v Tween-80 or Triton-X100) cellulose acetate membranes for various electroactive species in aqueous buffer solution (pH 7.4) at a concentration of 0.04 mM (a) actual responses (duplicate values are given as SEMs) and (b) percentage residual signal with the membrane in place compared to the bare electrode response (i_m/i_b x 100).

Tween-modified membrane (Figure 3.4.2a). Since both surfactants were pure liquids the molar content in the casting solution differed. Triton-X100 had a lower molecular weight of 650 compared to Tween-80 (1300) therefore a greater amount was incorporated per unit volume. Membranes were also assessed by determining the percentage residual signal, compared with a bare electrode (i_m/i_b x 100). A generalised reduction in permeability for all species was confirmed, though hydrogen peroxide and paracetamol permeation were reduced to a lesser extent compared to other species (Figure 3.4.2b).

3.4.3 Response to H_2O_2 and phenolics

Results for Tween-modified and Triton-modified cellulose acetate membranes were similar. Electrode calibration for H_2O_2, phenolic species and urate showed a linear dependence of concentration to at least 0.1 mM. Although catechol permeation showed earliest saturation and p-aminophenol did not appear to saturate at 0.4 mM. This interesting phenomenon could be shown with both membranes (Figures 3.4.3a and 3.4.3b).

3.4.4 Permeability effects of detergent

A general trend of gradual increased permeation of hydrogen peroxide and paracetamol with increased detergent incorporation (1-4% v/v in acetone) was seen for both Tween and Triton-modified cellulose acetate membranes, while rejection of ascorbate was maintained for the Tween-modified cellulose acetate membranes (Figures 3.4.4a and 3.4.4b).

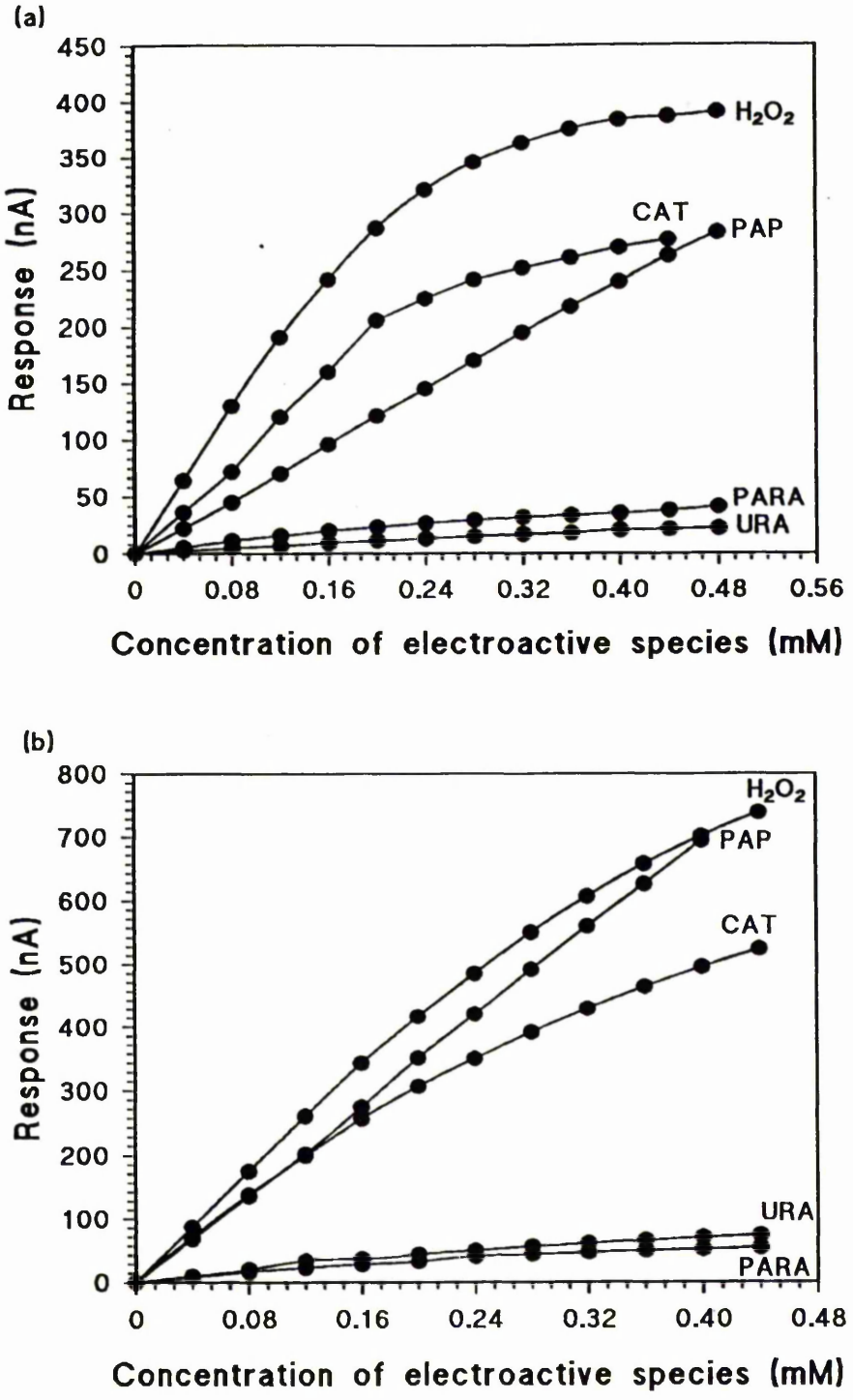


Figure 3.4.3

Determination of the linear range of permeation using solutions of hydrogen peroxide, phenolic compounds as well as urate for (a) Tween-80 and (b) Triton-X100 modified cellulose acetate membranes.

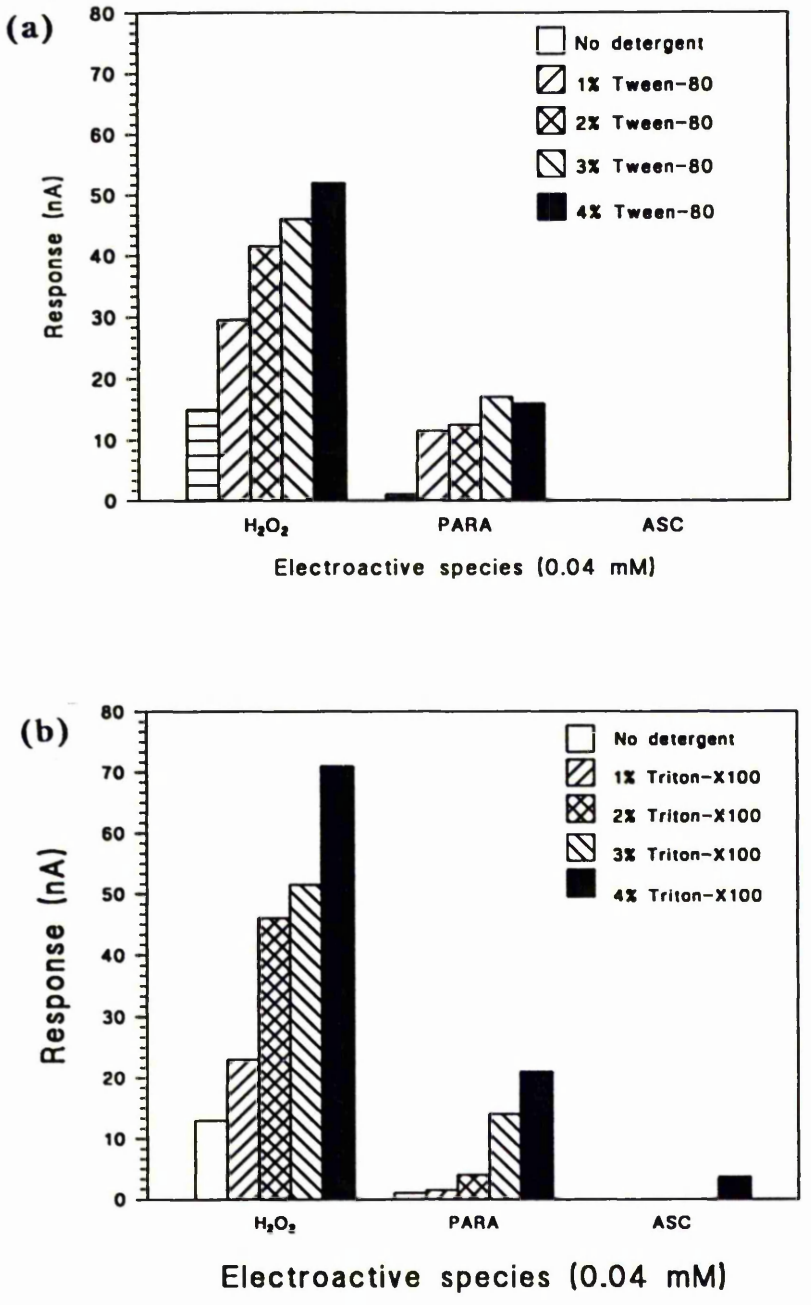


Figure 3.4.4

Variation of detergent concentration (1-4% v/v in acetone) during casting on permeation behaviour to 0.04 mM hydrogen peroxide, paracetamol and ascorbate for (a) Tween-80 and (b) Triton-X100 modified membranes.

3.4.5 Use as external membranes

Detergent modified cellulose acetate external membranes resulted in elevations in response to glucose with sensitivities of 0.4 nA/mM for Tween-modified and 0.7 nA/mM for Triton-modified compared to 0.2 nA/mM for unmodified cellulose acetate. This is despite maintained linear ranges to at least 100 mM (Figure 3.4.5).

3.4.6 Inner membrane of glucose electrode

Interposition of cellulose acetate between the working electrode and crosslinked enzyme resulted in less of a decline in sensor sensitivity, and the linear range was also less affected compared with the unmodified membrane. Tween-modified response was 25 nA/mM, Triton-modified was 40 nA/mM and unmodified cellulose acetate was ~16 nA/mM. (Figure 3.4.6). Response times were similar in all cases (1-3 minutes).

3.4.7 Stability of glucose electrodes

Repeated aqueous calibration using 2 and 4 mM standard solutions for enzyme laminates using Tween or Triton-modified (internal) cellulose acetate membranes were carried out over a 6-day period. The Tween-modified membrane showed a general stabilisation of electrode response throughout the 6-day period (Figure 3.4.7a). For the Triton-modified cellulose acetate all responses were less stable showing a general upward trend in output signal (Figure 3.4.7b). Variation in response is more evident for the 4 mM glucose calibration.

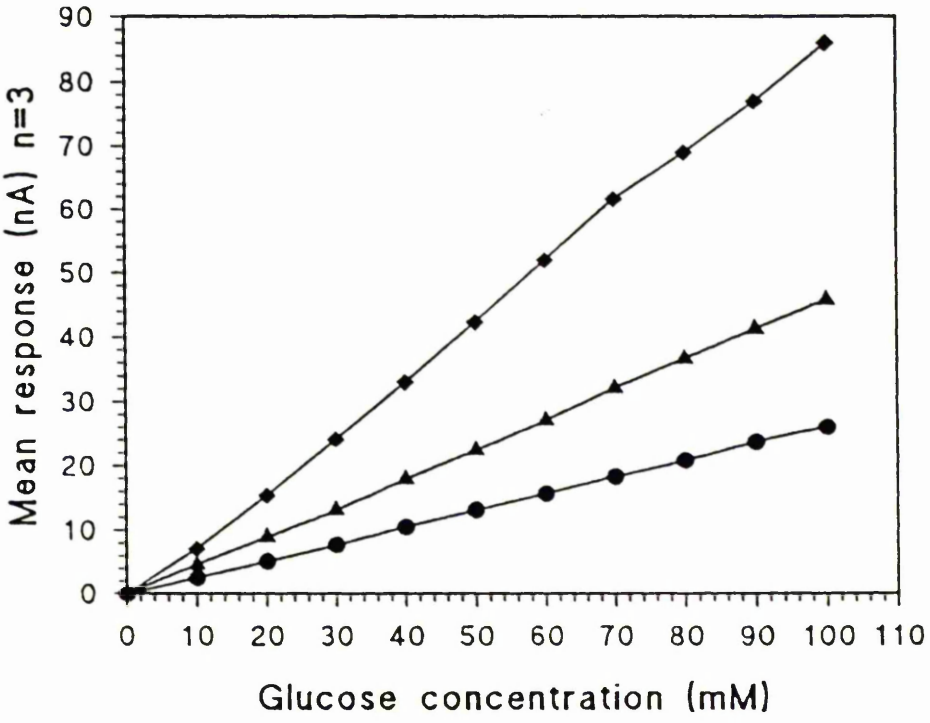


Figure 3.4.5

Effect of using non-ionic surfactant modified external cellulose acetate membranes in glucose enzyme electrodes.
External membranes:- (♦) Triton-X100 modified, (▲) Tween-80 modified and (●) unmodified cellulose acetate. (An internal 0.03 μm pore size polycarbonate membrane was present in each laminate).

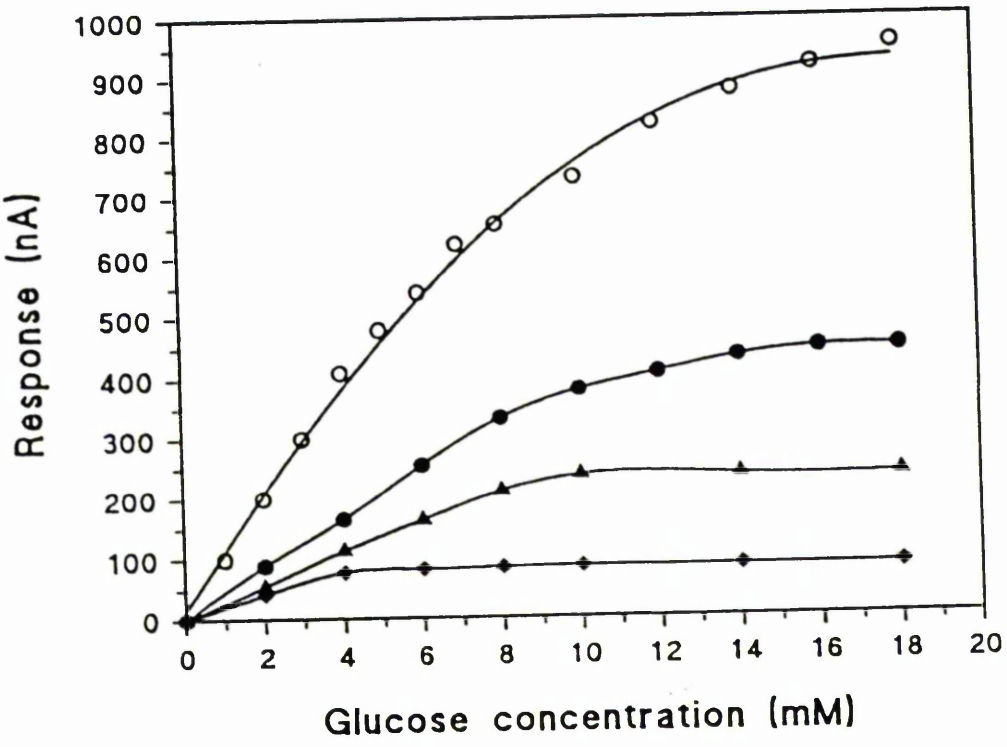


Figure 3.4.6

Determination of the effect of inclusion of internal cellulose acetate membranes (unmodified as well as detergent-modified) on glucose electrode calibration.
Internal membranes:- (○) 0.03 μm polycarbonate, (●) Triton X100 modified, (▲) Tween-80 modified and (♦) 5% CA (unmodified). (An external 0.03 μm pore size polycarbonate membrane was included in each laminate).

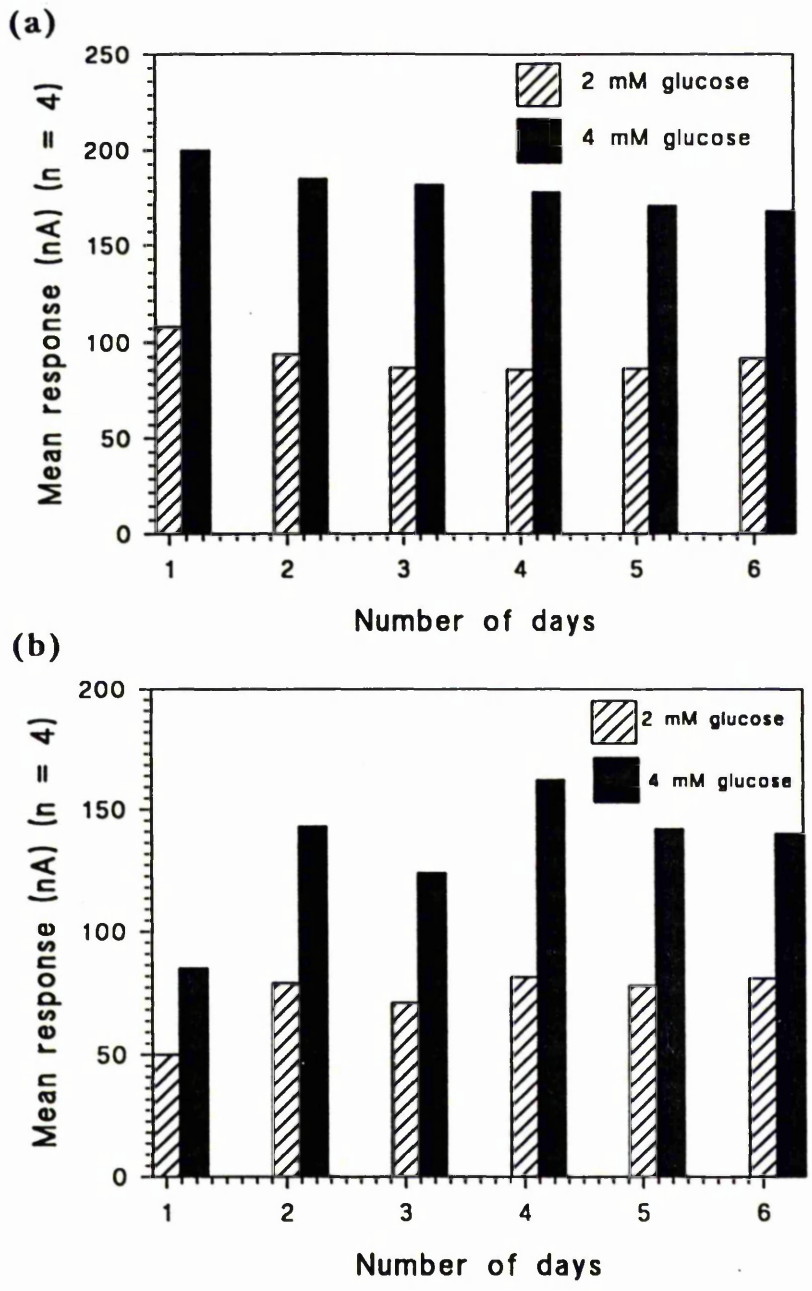


Figure 3.4.7

Operational stability of glucose enzyme laminates containing (a) Tween-80 and (b) Triton X100 modified internal cellulose acetate membranes over a 6-day time period.

3.5 Discussion

Cellulose acetate membranes are apparently non-porous on the basis of scanning electron micrograph evidence. The membranes thus consist of a dense homogeneous phase with permeation governed by close solute membrane polymer interactions. The cast membranes were structurally distinct from the asymmetric Loeb-Sourirajan type reverse osmosis membranes which usually consist of two distinguishable layers. The anisotropic membranes consist of a salt-rejecting "skin" layer and a highly porous supporting structure with relatively little resistance to water flow and no selectivity to low molecular weight solutes. The thickness of the "skin" layer is difficult to define. Electron micrographs done by Riley et al (1964) show that the skin blends into the porous structure through a region of graded porosity. The effective membrane thickness is dependent on the casting conditions and subsequent treatment. The porous substructure of the membranes contains ~60% water by weight. If the membrane is allowed to dry without special treatment it shrinks and distorts markedly and the water permeability is reduced. Apparently the pores collapse increasing resistance to flow.

The selectivity properties were determined using 0.04 mM standard solutions (Figures 3.2.2a and 3.2.2b). A low concentration of phenolics prevented possible fouling by generation of phenoxy radicals from phenol which undergo a polymerisation reaction (Koile and Johnson 1979). The observed results were similar to the acetone cast membranes (1.5% w/v) of Koochaki et al (1991). An increase in polymer concentration caused a decline in permeability. The results are consistent with those of Kuhn et al (1989). Here cyclic voltammetry of

a ferrocene derivative showed an associated decline in the diffusion controlled current with increased cellulose acetate incorporation. The permselective behaviour of the polymer has been explained on the basis of charge (Lonsdale et al 1965) and size (Wang and Hutchins 1985) exclusion. Species e.g. ascorbate (pK_a = 4.04) which are anionic at the working pH of 7.4 are rejected by cellulose acetate by charge repulsion. This is consistent with findings of Koochaki et al (1991) and Wang and Hutchins (1985). Ferricyanide was also rejected by cellulose acetate since the polymer has proton acceptor character. Therefore anionic species are rejected preferentially to undissociated phenolics and H_2O_2. Since p-aminophenol is cationic it is not rejected by cellulose acetate. It has been theoretically proposed that ions are excluded from permeating by Coulombic forces arising from the pore walls in ultrafiltration membranes (Lonsdale et al 1965). A similar hypothesis can be applied to cellulose acetate. For reverse-osmosis type membranes Matsuura and Sourirajaan (1972) correlated the permeation of phenolic and organic species with pk_a and hydrogen bonding ability.

Wang and Hutchins (1985) found a rapid decline in permeation with molecular weight upto ~140. This was followed by a gradual decrease in permeation associated with further increases in solute molecular weight. A similar trend was observed in Figures 3.2.2a and 3.2.2b. A decrease in permeation with paracetamol (MW 151) obtained compared to catechol (MW 110) which may be attributed to the bulky acetamide substituent present in paracetamol.

Inclusion of cellulose acetate internal membrane caused a decreased

sensitivity to glucose compared to the 0.03 μm pore size polycarbonate (Figure 3.2.3). This was due to the increased mass transport barrier to hydrogen peroxide presented by cellulose acetate with a resultant decreased flux to the working electrode and hence a reduced current. A greater polymer concentration in an equivalent solvent volume, (5% w/v in acetone) membrane presented a greater barrier to hydrogen peroxide diffusion and hence showed a greater diminution in current compared to the 2% w/v membrane; the increase in response time is consistent with this possibility.

Inclusion of cellulose acetate (external membrane) in a glucose laminate presented a substrate diffusional barrier. This is indicated by the extended linear range as well as a decline in response magnitude (Figure 3.2.4). The results indicate that anionic (from dissociated acetyl groups) cellulose acetate is essentially permeable to uncharged glucose. The results are consistent with the findings of Gorten et al (1991) using another "anionic" membrane. Here a carbon paste glucose electrode coated with polyestersulphonic acid barrier gave an extension of the apparent K_m from 50-70 mM (uncoated) to 200 mM (coated) was obtained.

When ethanol/formamide non-solvents are used (Figures 3.3.1a and b) The probable mechanism for increased permeability is pore formation as discussed by Kesting et al (1964) for dense reverse osmosis membranes. Generally there is rapid removal of solvent (acetone) followed by entry of nonsolvent with resultant pore formation. Chawla and Chang (1975) showed that increased ethanol incorporation resulted

in an enhanced dialysis rate for creatinine. A direct relationship between ethanol incorporation and membrane water content was found. Also Frommer et al (1970) investigated two-component mixtures of cellulose acetate and several solvents including acetone, dioxane, acetic acid, triethylphosphate, dimethylformamide and dimethylsulphoxide. Here solvent movement out of the cast layer and of water into the layer depended on the solvent used and therefore determined the porosity of the resultant membranes. Since formamide is a nonsolvent for cellulose acetate at room temperature it also acts as a secondary plasticiser (Daane and Barker 1964) and lowers the polymer glass transition temperatures from the normal values of 50 °C and 115 °C. Formamide incorporation results in increased polymer chain flexibility and the spacing between chains increases with a resultant increased flux for all membranes.

Solvent-modified and unmodified cellulose acetate internal membranes (Figure 3.3.2) both resulted in a diminution in glucose electrode response. Hydrogen peroxide flux to the electrode surface was reduced due to the barrier presented by the membranes. However with formamide response was comparatively high presumably because of the plasticiser effect of formamide.

By SEM, detergent incorporated cellulose acetate membranes revealed non porous structure. Therefore permeation behaviour occurred via partitioning through a homogenous membrane phase. Permeability increased, but there was also some in selectivity (Figures 3.4.2a and b). The mechanism of action of the two nonionic surfactants is unclear but

they probably increased interchain spacing. Also, they would promote both hydrophilic and hydrophobic interactions with solute and facilitate solvent partitioning, though this needs to be tested. Differences between the plasticising effects can therefore be due to polarity differences. A further advantage for biosensor use is that the membranes as well as being transparent are more pliable and physically tougher (Desai et al 1993).

Electrode calibration for detergent-modified cellulose acetate (Figures 3.4.3a and b) are similar to the findings of Koochaki et al (1991) for unmodified cellulose acetate. However, p-aminophenol responses were linear beyond 0.1 mM for both modified membranes unlike the case with the basic membrane. This property could be of use for PAP detection, of value in the determination of alkaline phosphatase activity based on p-aminophenol phosphate substrate (Christie et al 1992).

A general increased permeation for hydrogen peroxide and paracetamol with ascending detergent concentrations was observed although selectivity against ascorbate was maintained (Figures 3.4.4a and b). Such results were obtained by Christie et al (1992) for polyvinyl chloride membrane plasticised with dioctyl phthalate; a rise in catechol flux was seen but the membrane remained impermeable to ascorbate. The results suggest that surfactants also acted as plasticiser without compromising base polymer selectivity.

While general decrease in current magnitude compared to a 0.03 μm polycarbonate membrane was obtained with cellulose acetate internal

membranes (Figure 3.4.6), the effect was less pronounced with the plasticised structures.

For practical use, the stability of glucose electrodes incorporating surfactant-modified cellulose acetate (internal) membranes revealed that responses were relatively constant within experimental error over a 6-day period (Figures 3.4.7a and b). These show that there is no apparent leaching of membrane incorporated surfactant for both Tween-80 and Triton X100 within this time period.

3.6 Summary

This chapter was concerned primarily with the development of cellulose acetate membranes for enzyme-coated sensors. The permselectivity properties of these particular anionic membranes were investigated. The ability to screen out anionic interferents was not markedly affected by polymer concentration, although the permeability to other species especially hydrogen peroxide was concentration dependent. It was not particularly advantageous to employ cellulose acetate as the internal membrane of a glucose enzyme "laminate". However, an extended linear response to glucose was reproducibly produced with cellulose acetate as the external membrane. The use of solvent mixtures during membrane casting was not advantageous. Although formamide incorporation resulted in much elevated hydrogen peroxide permeation, a generalised loss of selectivity was observed. Therefore this casting procedure was impractical for sensor applications. Surfactant (Tween-80 or Triton-X100) incorporation resulted in membranes with enhanced permeation of hydrogen peroxide as well as of phenolics compared to unmodified cellulose acetate although selectivity against charged species

including ascorbate and urate was maintained. These permeation properties are beneficial for the development of oxidase-based sensors utilising hydrogen peroxide detection as well as for drug detecting (e.g. paracetamol) sensors. A membrane surfactant concentration-dependent permeation pattern was obtained for hydrogen peroxide, paracetamol and ascorbate. From these results an optimised surfactant (detergent) concentration of 2% v/v in acetone was used. Surfactant incorporated cellulose acetate (external) membranes of glucose electrodes proved to be advantageous especially with regard to the amplified response to hydrogen peroxide and linearity of <100 mM compared to the unmodified membrane. Stability studies with glucose electrodes using surfactant incorporated internal membranes in aqueous solution showed that stable responses were obtained over a 6-day period. This preliminary investigation has shown that it is feasible to employ cellulose acetate, and in particular surfactant-incorporated membranes in practical biosensors.

CHAPTER FOUR

POLYVINYL CHLORIDE MEMBRANES

4.1 Introduction

The polyvinyl chloride (PVC) polymer possesses a head-to-tail structure. X-ray studies have shown that commercial PVC is amorphous although a low degree (~5%) of crystallinity is present. NMR studies (Tonelli et al 1979) suggest that PVC is ~55% syndiotactic. The vinyl chloride may be prepared by addition of HCl to acetylene,i.e.

$$HC\equiv CH \quad + \quad HCl \text{-------}\rightarrow H_2C\text{-}CHCl \qquad (4.1)$$

The reaction is carried out in the vapour phase with a mercuric chloride catalyst. Alternatively, vinyl chloride may be manufactured by the addition of chlorine to ethylene to give ethylenedichloride which is then pyrolysed at 500 °C and 25 atms pressure. Suspension polymerisation of vinyl chloride is carried out to produce polyvinyl chloride. In this technique, vinyl chloride monomer is dispersed in water in small droplets of 10^{-2}-10^{-1} cm diameter under vigorous stirring conditions. A monomer soluble initiator (di-2 ethylhexyl) peroxydicarbonate is added and polymerisation occurs within each droplet. Generally a material e.g. polyvinyl alcohol is included in order to provide a protective coating for the droplets; it also prevents the droplets from cohering when they are at a stage of being composed of a sticky mixture of monomer and polymer. The contaminant-free polymer is obtained in a small bead form.

Polyvinyl chloride is a colourless, rigid material of relatively high density and low softening point. It has a high dielectric constant due to the presence of the polar C--Cl bond. PVC is a weak proton donor and is soluble in the solvents, dioxan, tetrahydrofuran and ethylene

dichloride Plasticisers (40-60 parts per 100 parts of polymer) may be incorporated in order to convert it to a flexible rubber-like material. PVC is chemically inert and resistant to acids, alkalis and strong oxidising agents. However the polymer is degraded at elevated temperatures (< 70 °C) and during ultraviolet light exposure.

Polyvinyl chloride has been widely used as a membrane matrix material for potentiometric ion-selective electrodes (ISEs) (Masadome et al 1992; Yim and Meyerhoff 1992). Here it is employed as a continuous separate phase and is impermeable to ions. A typical ISE membrane may contain 33% PVC, 66% plasticiser and 1% carrier w/v. The selectivity properties have been achieved by inclusion of appropriate plasticisers (e.g. o-dinitrophenylether, dioctylsebacate), ion exchangers and neutral carrier molecules. A combination of the ionophore, valinomycin and ion-exchanger, tetraphenylborate (included in order to reduce lipophilic anionic interferences e.g. Cl^-, SCN^- and perchlorate) has been used for K^+ selective electrodes (Morf et al 1974). Even without the presence of ion-exchangers, ISEs respond to lipophilic ions e.g. thiocyanate and phenols (Morf and Simon 1978; Christie 1988) due to possible ion partitioning in the organic polymer phase.

The following investigation examined the role of plasticiser (dioctylpthalate and isopropylmyristate) in controlling the permselectivity behaviour of polyvinyl chloride (in the absence of any ion exchanger) especially in relation to exclusion of charged interferents. The PVC membrane formed an integral part of an oxidase enzyme based amperometric glucose detection system. The possible

application in clinical measurement of whole blood/serum glucose has been exploited. The effect of detergent incorporation on flux behaviour was considered.

4.2 Unplasticised polyvinyl chloride

4.2.1 Permeability properties

The membrane showed limited permeability to all electroactive species at 1 mM (Figure 4.2). All species including ascorbate and urate permeated through the membrane. Selectivity ratios of 4.4:1, 3.8:1, 3.7:1 and 0.69:1 were obtained for hydrogen peroxide : ascorbate, urate, paracetamol and catechol respectively. However the membranes were less permeable to phenolics than plasticised polyvinyl chloride (section 4.3.1).

4.3 Plasticised polyvinyl chloride

The cast plasticised PVC membranes were transparent, colourless and possessed slight elasticity. Even slow solvent evaporation resulted in incomplete incorporation of isopropylmyristate (IPM). There was some residual liquid plasticiser on the membrane surface. However dioctylphthalate incorporation was complete and produced uniform membranes. A dialysis (Cuprophan^{R}) supporting membrane was used for all measurements. The Cuprophan^{R} membrane maintained a stable inner electrolyte film and allowed reliable, reproducible responses. Also the problem of PVC insulating the electrodes and preventing the completion of the electrochemical cell was avoided.

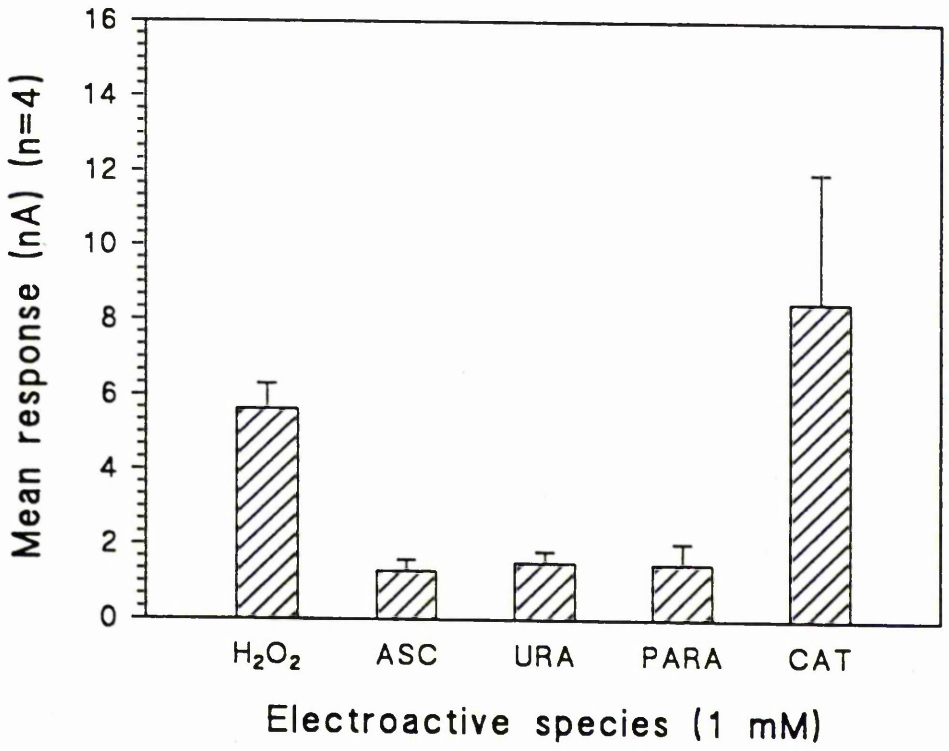


Figure 4.2

Permeability of unplasticised polyvinyl chloride membranes to electroactive species at 1 mM (duplicate values are given as SEMs).

4.3.1 Permeability effects of isopropylmyristate (IPM) and dioctylpthalate (DOP)

A generalised enhancement in selectivity for hydrogen peroxide against charged interferants was observed compared to unplasticised polyvinyl chloride e.g. H_2O_2 : ascorbate permeability ratios were 168.5 : 1 (DOP as plasticiser), 720.8 : 1 (IPM as plasticiser) and 4.4 : 1 (unplasticised). However an increased permeation of phenolics compared to unplasticised polyvinyl chloride was observed as shown in Figure 4.3.1. This was especially noticeable for catechol where the response was increased 148 times (IPM) and 75 times (DOP). There was an increase in response with IPM compared to DOP-incorporated polyvinylchloride membranes.

4.3.2 Solute partitioning

The results in Figure 4.3.2 showed that aromatic species readily partitioned into the organic phase e.g. catechol, hydroquinone, p-aminophenol and paracetamol. However charged species e.g. ascorbate did not partition. The degree of partitioning was greater in IPM than DOP especially for p-aminophenol and paracetamol.

4.3.3 Glucose electrode response with polyvinyl chloride inner membrane

Interposition of a plasticised polyvinyl chloride-dialysis composite between the working electrode and the crosslinked enzyme layer produced a considerable decline in the response. However an increase in the linear range was obtained with various external membranes (Figures 4.3.3a-d). The degree of linearity was governed initially by the

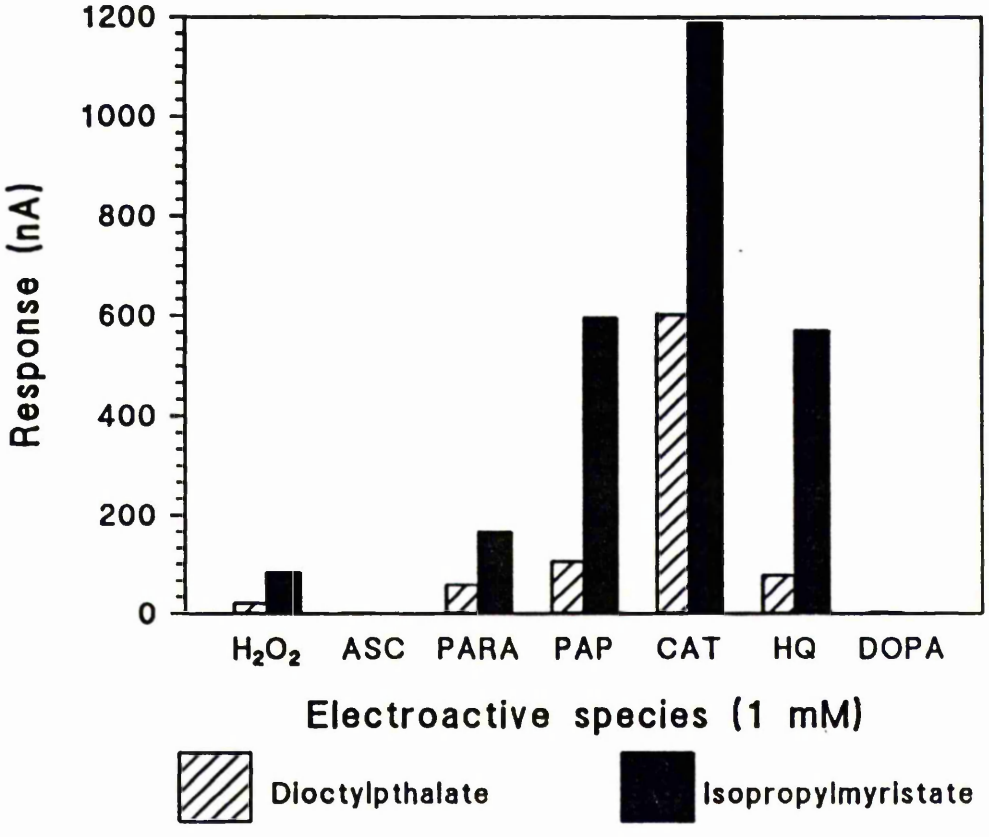


Figure 4.3.1

Permeability of plasticised polyvinyl chloride membranes using dioctylphthalate or isopropylmyristate to electroactive species at 1 mM.

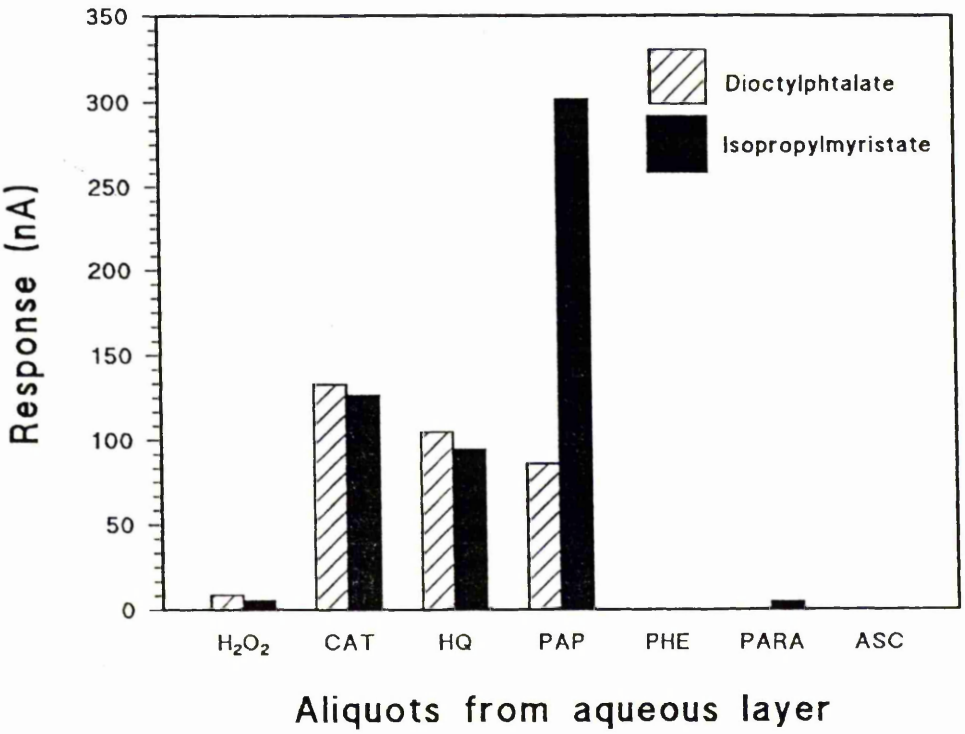
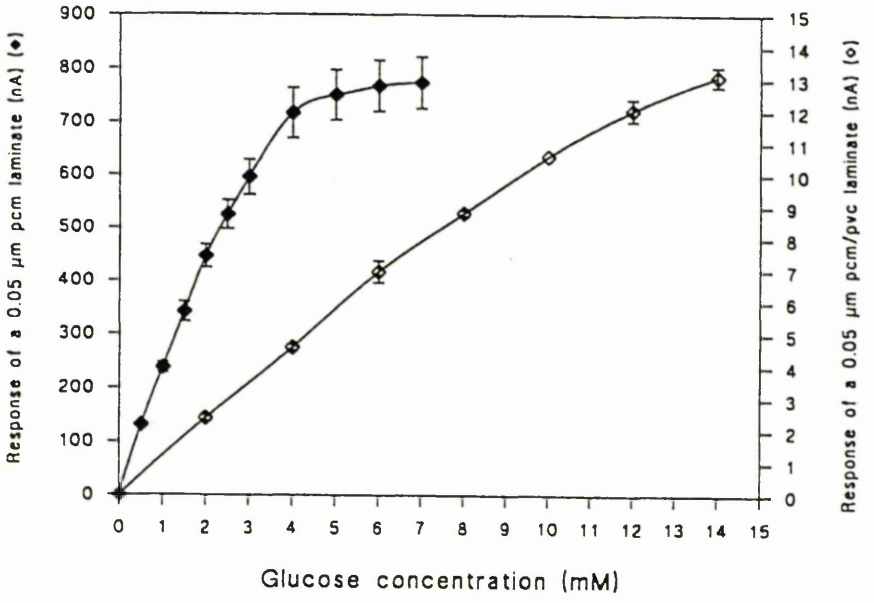


Figure 4.3.2

Demonstration of solute partitioning through isopropylmyristate and dioctylpthalate.

(a)



(b)

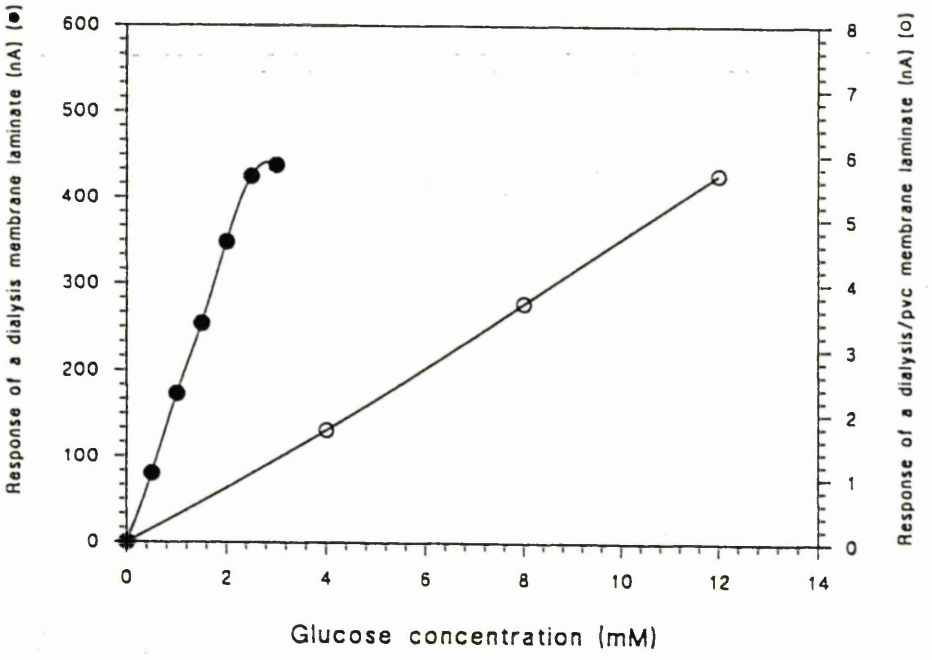


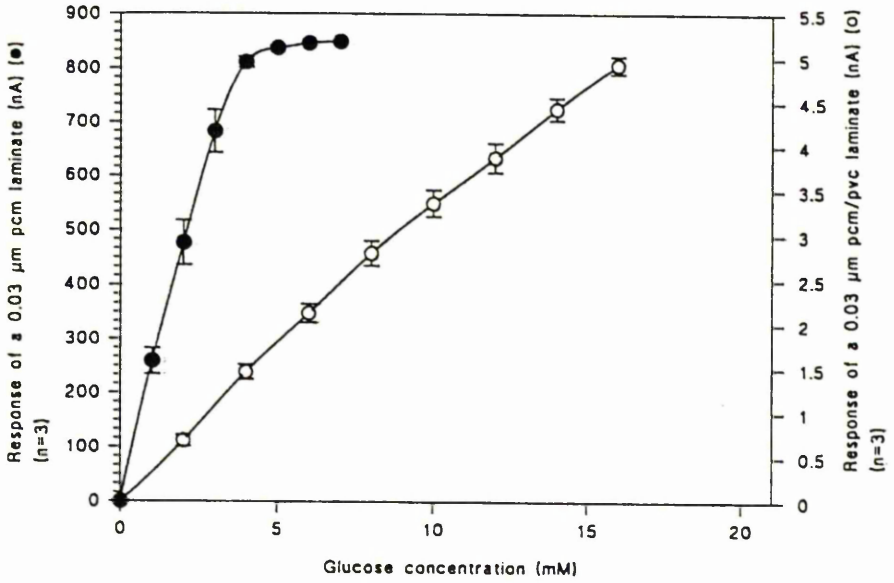
Figure 4.3.3

Glucose electrode responses with plasticised polyvinyl chloride (PVC) internal membrane.

(a) (♦) 0.05 µm/0.05 µm polycarbonate membrane (PCM) laminate
(◇) 0.05 µm PCM/PVC laminate
(error bars indicate SEM values)

(b) (●) dialysis/dialysis laminate
(○) dialysis/PVC laminate

(c)



(d)

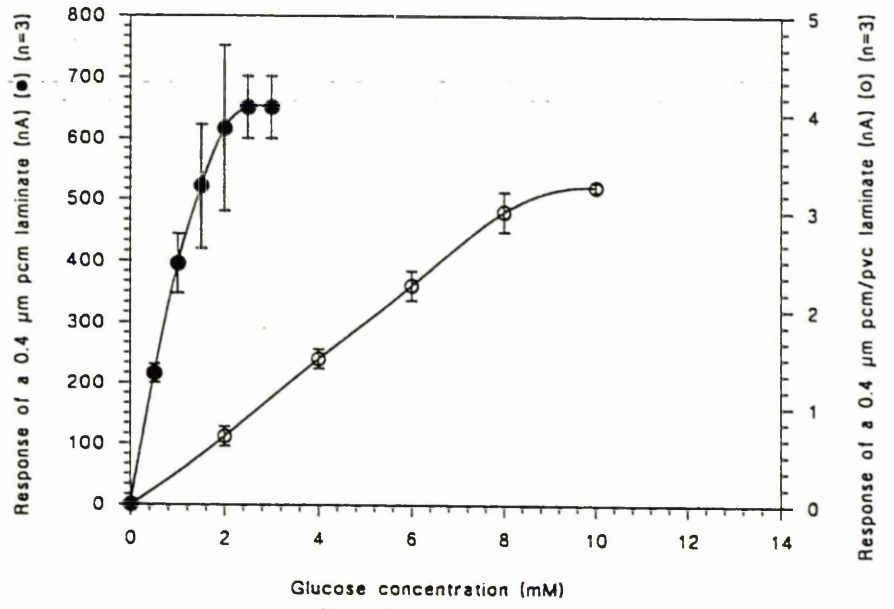


Figure 4.3.3

Glucose electrode responses with plasticised polyvinyl chloride (PVC) internal membrane.

(c) (●) 0.03 µm/0.03 µm polycarbonate membrane (PCM) laminate
(○) 0.03 µm PCM/PVC laminate

(d) (●) 0.4 µm/0.4 µm polycarbonate membrane (PCM) laminate
(○) 0.4 µm PCM/PVC laminate

(error bars show SEM values)

porosity of the outer polycarbonate (PCM) membrane, i.e. a linear range of ~8 mM was recorded for a 0.4 μm outer PCM and linearities of 8-10 mM and 15-25 mM were obtained for 0.05 μm and 0.03 μm PCMs respectively. The dialysis (control) external membrane produced a similar effect. In all cases linear ranges increased with the use of impermeable inner polyvinyl chloride membranes. A control experiment using dialysis (Cuprophan) as the inner membrane showed a worsening in the linear range as well as a decline in response.

4.3.4 Serum glucose measurement

Serum samples (n=20) were measured using an enzyme laminate containing plasticised polyvinyl chloride as the inner membrane. A correlation coefficient of r = 0.99 was obtained when the results were compared with those obtained using a Yellow Springs glucose analyser (as in Figure 4.3.4). There was no evidence of fouling during the electrochemical assay.

4.3.5 Measurement of whole blood glucose

Whole blood samples (n=19) were assayed using a glucose enzyme laminate containing plasticised polyvinyl chloride as the internal membrane. A correlation coefficient of r = 0.96 was obtained when the results were compared with measurement using a routine clinical determination (Yellow Springs glucose analyser) as illustrated in Figure 4.3.5.

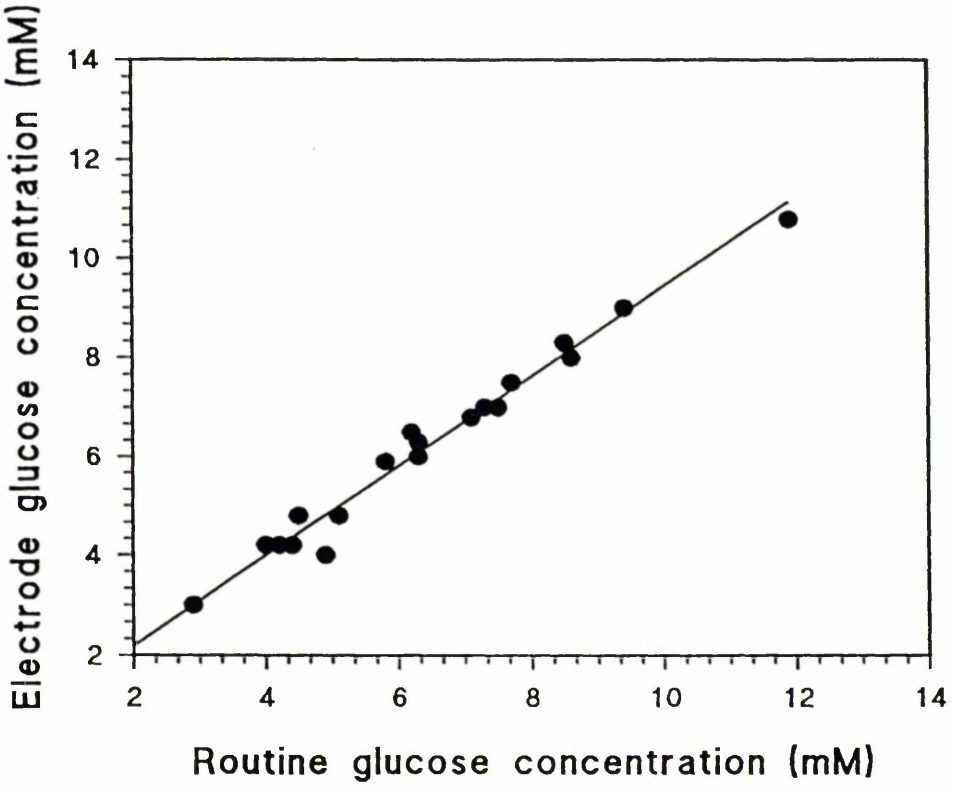


Figure 4.3.4

Serum glucose assay using a 0.03 μm polycarbonate/polyvinyl chloride membrane laminate and correlation with routine determination (Yellow Springs Instrument analyzer).
y = 0.908512(x) + 0.382433, r = 0.99088, n = 20

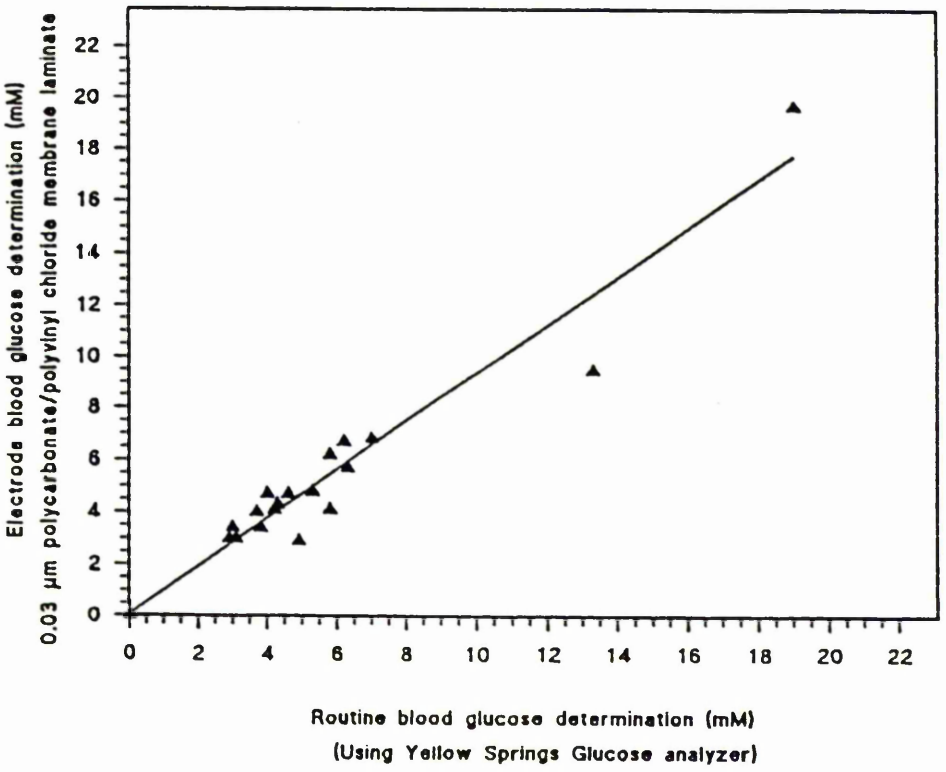


Figure 4.3.5

Measurement of whole blood glucose using a 0.03 μm polycarbonate/polyvinyl chloride membrane laminate and correlation with routine determination (Yellow Springs Instrument analyzer).
$y = 0.932732(x) + 0.05317$, $r = 0.964$, $n = 19$

4.4 Detergent-modified polyvinyl chloride membranes

Cast, detergent-incorporated membranes were homogeneous, transparent and possessed flexible rubber-like characteristics. This was in sharp contrast with the rigid unplasticised polyvinyl chloride membranes; the membranes were also mechanically robust and could therefore be handled easily.

4.4.1 Permeability with Tween-80 and Triton-X100 incorporation

The membranes were highly permeable to all species (at 0.04 mM) and responses were enhanced compared to unplasticised polyvinyl chloride. However there was no selectivity for H_2O_2 over ascorbate for both detergent-incorporated membranes. However the Tween-modified membrane showed greater permeation of H_2O_2 to ascorbate than the Triton-X100 modified membrane. Selectivity ratios obtained for H_2O_2 : ascorbate were 15:1 and 4:1 for Tween and Triton-modified polyvinyl chloride (high molecular weight) membranes respectively. Both detergent-modified membranes exhibited varying permeability to phenolic species (Figure 4.4.1a). Detergent-modified membranes were also cast using low molecular weight polyvinyl chloride. The permeability properties were essentially similar to the previous modified membranes (Figure 4.4.1b) although the signal sizes were approximately increased by 2-fold.

4.4.2 Use as internal membranes of glucose electrodes

Incorporation of detergent-modified polyvinyl chloride membranes between the working electrode and the external enzyme membrane

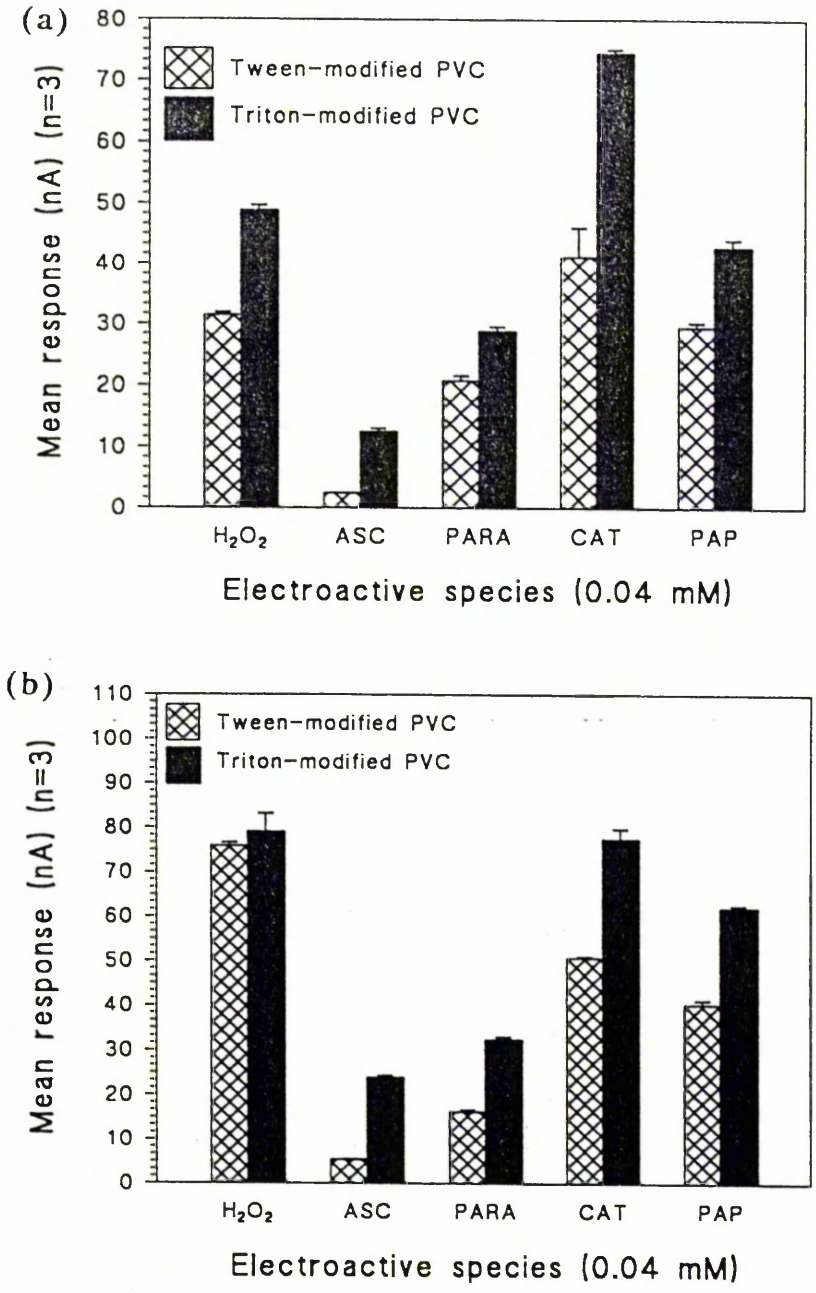


Figure 4.4.1

Permeability of detergent-incorporated (Tween-80 and Triton X-100) polyvinyl chloride (a) high molecular weight (MW 200 000); (b) low molecular weight (MW 100 000) membranes to electroactive species at 0.04 mM (error bars indicate SEM values).

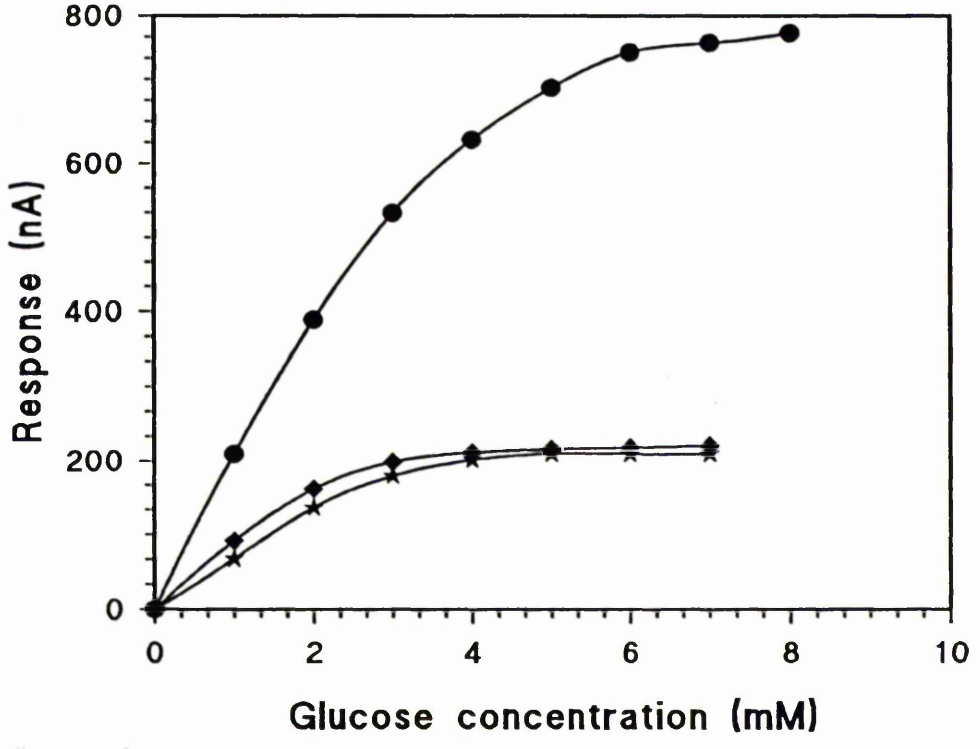


Figure 4.4.2

Use of detergent-incorporated polyvinyl chloride (PVC) as internal membranes of a glucose electrode. (A 0.03 μm pore size polycarbonate external membrane was present in all cases).
Internal membranes:- (●) Dialysis (Cuprophan)
(◆) Triton X-100 modified PVC
(★) Tween-80 modified PVC

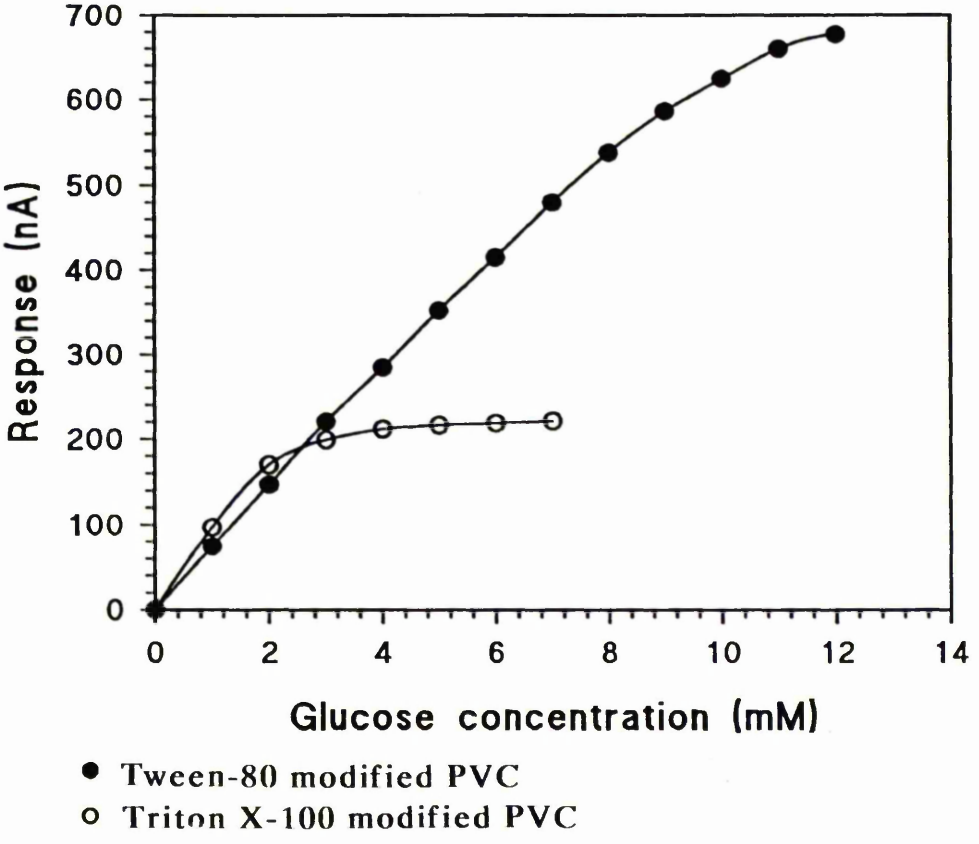


Figure 4.4.3

Use of detergent-modified polyvinyl chloride (PVC) outer membranes of a glucose enzyme electrode. (A 0.03 μm polycarbonate inner membrane was employed in both cases).

produced a lower response compared to the dialysis (control). At 2 mM the response was 41% and 35% for the Triton and Tween-modified membranes respectively. However the linear range of the glucose calibration curve was unchanged (Figure 4.4.2).

4.4.3 Use as external membranes of glucose electrodes

A 4-fold increase in linear range was observed for the detergent-modified polyvinyl chloride membranes as compared to the use of a 0.03 μm polycarbonate (control) membrane . The linearity increased from 2mM to 9 mM for both of the modified membranes, the magnitude of the electrode responses remained high (Figure 4.4.3). The Triton-modified polyvinyl chloride membrane gave higher responses than the Tween-modified membrane.

4.5 Discussion

The magnitude of the electrode responses of plasticised polyvinyl chloride were considerably enhanced compared to unplasticised membranes, probably the plasticiser imparts an alteration in the ordered 3-dimensional conformation of the rigid unplasticised polymer network (Boyer 1951). Plasticiser incorporation lead to an effective reduction in intermolecular forces causing a net increase in polymer chain spacing. There is also increased chain flexibility and the polymer is converted to an elastic state. A more detailed description on the mechanism of plasticisation has been considered in chapter one (section 1.7).

Polyvinyl chloride (PVC) membranes (containing dioctylphthalate or isopropylmyristate as plasticisers) showed extreme permselective behaviour and excluded charged species to a great extent (especially ascorbate and urate). Uncharged aromatic species (at pH 7.4) permeated readily especially catechol, hydroquinone and paracetamol. Para-aminophenol although charged at pH 7.4 managed to permeate PVC possibly due to its aromatic character which may have facilitated partitioning into the organic phase of the polymeric membrane (figure 4.3.1). The results agree with the findings of Christie et al (1992). According to their data polyvinyl chloride gave a H_2O_2 : Ascorbate selectivity ratio of 1500 : 1 whereas it was 200 : 1 for cellulose acetate. Other workers used polyestersulphonic acid (Wang et al, 1990), Nafion (Bindra and Wilson 1989; Harrison et al 1988), and cellulose acetate (Bindra et al 1991). However, complete exclusion of ascorbate was not achieved with any of the membranes.

Further evidence showed that permselectivity behaviour was based on solute charge discrimination (Christie et al 1992). At elevated pH values beyond the pK_a of catechol (9.46) there was a pronounced attenuation in signal size. Therefore at a high pH, dissociation of the hydroxyl substituents of catechol took place causing the charged species to predominate which could not permeate through polyvinyl chloride. Almost complete exclusion of catechol was observed at pH 11.0. Since catechol was uncharged and aromatic at pH 7.4 it showed high permeation behaviour from my data. However ascorbate possessed a pK_a of only 4.04 therefore it was charged at pH 7.4 therefore it could not permeate polyvinyl chloride.

The results may be explained on the basis of structure of the cast membranes. They are essentially non-porous liquid membranes containing a solid membrane phase. A scanning electron micrograph of the membrane showed that the external surface was free from perforation or cavities (Rosenberg 1992). The permeability properties were analogous to isopropylmyristate-coated polycarbonate membranes (Tang et al 1990). These may be attributed to the presence of the liquid lipid isopropylmyristate (plasticiser for PVC). Transport through the polyvinyl chloride membranes appears to rely mostly on partition. The aromatic (uncharged) species were readily soluble in the liquid lipid, isopropylmyristate and could permeate into it. Non-lipophilic (polar) species e.g. ascorbate and urate could not partition into the lipid and were therefore excluded. Hydrogen peroxide is still a polar molecule therefore limited permeation occurred.

Membranes plasticised using isopropylmyristate (IPM) exhibited greater permeability to all species including H_2O_2 compared to the dioctylpthalate (DOP) incorporated membranes (Figure 4.3.1). Theoretically the efficiency of plasticisation was governed by the size and concentration of plasticiser and independent of molecular structure (Wurstlin and Klein 1955). It is inherently related by the ability of the particular plasticiser to shield specific polar groups along the polymer chains and reducing interchain bonding (Zhurkov 1945). Therefore since IPM is a smaller, aliphatic (polar) molecule compared to bulkier DOP (containing aromatic and ester chains) it has a greater ability to shield polar groups in the polymer. Due to the greater response size, IPM was used the plasticiser for further experiments. However

dioctylphthalate is more commonly used for plasticisation in membranes employed for ion-selective electrodes.

Solute partitioning studies confirmed that this was an important route for solute transfer into the organic phase (Figure 4.3.2). The results essentially followed the same pattern as the permeability experiments. Partition coefficients were determined by Rosenberg (1992) who found that at a pH of 7.4, catechol (uncharged) had a partition coefficient of 1.8 which exceeded other partition coefficients including ascorbate (1.1).

Inclusion of plasticised PVC as an internal membrane of a glucose electrode produced a significant extension of linearity as well as a marked decrease in the magnitude of the electrode response (Figures 4.3.3 a-d). The trend in linearity was also dependent on the porosity of the external membrane containing crosslinked enzyme. This is expected because diffusional limitation of substrate (glucose) meant that enzyme would be saturated with substrate less rapidly leading to an increased apparent K_m value hence an increased linear range. The results essentially agree with previous data (Vadgama et al 1989) using various polycarbonate membranes of different porosities to construct glucose enzyme electrodes.

The extension of linearity was also thus due to the internal polyvinyl chloride membrane. This was confirmed by a control experiment where the linearity was unaffected using a Cuprophan^R (dialysis) internal membrane. The results could be explained on the basis that the H_2O_2

reaction product could diffuse back into the bulk solution or could be electrochemically oxidised at the working electrode to generate the oxygen cosubstrate for the enzymic reaction. Therefore the rate of H_2O_2 oxidation at the electrode to regenerate oxygen could be a critical step in the operation of the electrode. If the O_2 permeability properties of the inner membrane governed the amount of O_2 which could thereby be replenished, then the polyvinyl chloride internal membrane possibly increased pO_2 in the enzyme layer. This may be attributed to the enhanced oxygen solubility of the liquid lipid, IPM (Thews 1960).

The practical use of polyvinyl chloride as an internal membrane has been illustrated by subsequent glucose analysis performed in whole blood and serum (Figures 4.3.4 and 4.3.5). Excellent correlation coefficients were obtained in both biological matrices. With an improved r value ($r = 0.99$) in serum and $r = 0.96$ in whole blood. The results essentially confirm the findings of Christie et al (1992). There is no PVC membrane fouling in serum when used to assay alkaline phosphatase and the results correlated well with spectrophotometric measurement ($r = 0.99$). Kihara et al (1984) entrapped pyruvate oxidase in a polyvinyl chloride matrix for amperometric determination of pyruvate. These workers also found good correlation coefficients in blood/serum.

Detergent-incorporation caused an analogous effect on flux of electroactive species to the previous plasticised (IPM/DOP) membranes (Figure 4.4.1). Non-ionic surfactants (Tween-80 and Triton X-100) probably increased the chain spacing/flexibility of the rigid polymer

network and charged species such as ascorbate managed to permeate the membrane. This was because surfactants could not prevent ions from permeation. The selectivity properties were therefore not improved compared to previous cellulose acetate (Koochaki et al 1991) or polyestersulphonic acid (Wang 1990) membranes.

No generalised impact on the linear range of glucose calibration curves with detergent-modified PVC inner membranes was observed, although a decrease in signal size was obtained due to the diffusional barrier to H_2O_2 presented by the modified membranes (Figure 4.4.2). An elevated linear range was obtained with detergent-incorporated PVC (external) membranes. However the linearity did not correspond with the clinically relevant range of 2-20 mM (Figure 4.4.3). From the magnitude of the responses it may be concluded that the detergent-modified membranes were highly permeable to glucose.

4.6 Summary

Plasticised polyvinyl chloride membranes similar to those used in ion-selective electrodes but without any specific affinity molecules (e.g. ion exchangers or neutral carriers) have been successfully exploited as permselective barriers in amperometric enzyme electrodes. Fluxes of specific classes of molecule investigated, especially uncharged aromatic compounds gave enhanced electrode responses coupled with extreme selectivity against charged species. Particularly important is the virtual exclusion of the interferents found in biological matrices (e.g. blood, serum) which pose problems during amperometric biosensor development. The partitioning behaviour of the incorporated plasticisers has supported the selectivity characteristics of the

membrane.

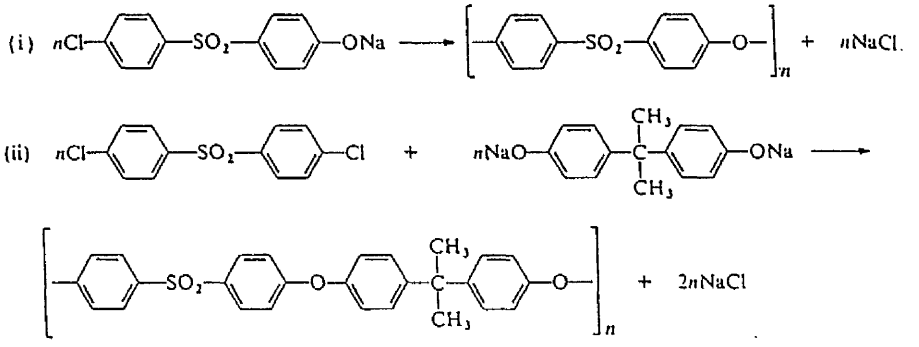
Plasticised polyvinyl chloride has been successfully employed in internal membranes of amperometric electrodes. The enhanced permselectivity together with the improved linear range in aqueous solution meant that the sensors could be used for glucose determination. The resulting electrodes also showed little or no fouling in serum and therefore could be used to measure glucose in biological matrices accurately. The selectivity of plasticised polyvinyl chloride was much improved compared to conventional permselective membranes. Therefore polyvinyl chloride may be used for future clinical biosensor development.

Detergent-modified polyvinyl chloride did not give the desirable selectivity properties. A generalised increase in permeation of all electroactive species was found including ascorbate. No particular effect on linearity of glucose response was observed when employed as an internal membrane. An extension of the linear range was noticeable when the membranes were utilised externally although this was not within the relevant clinical range.

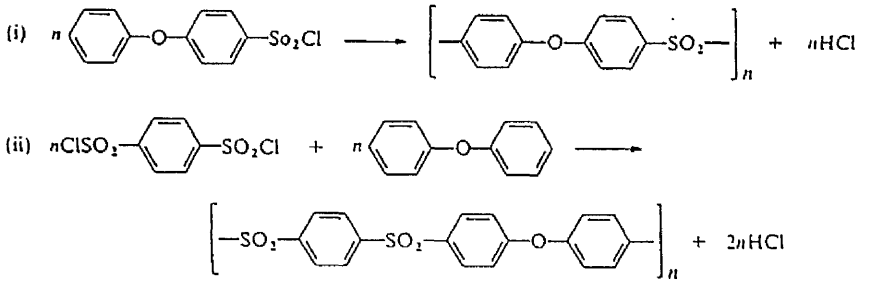
CHAPTER FIVE - POLYETHERSULPHONE MEMBRANES

5.1 INTRODUCTION

Polysulphones generally contain sulphone ($-SO_2$) groups in the main polymer chain. They are polysulphide derivatives and can be prepared by oxidation of polysulphides with hydrogen peroxide. Polyethersulphone constitutes one type of polysulphone. However polyarylsulphones and polysulphones are the other main groups (Figure 5.1). Two general methods for polysulphone preparation are used, including polyetherification and polysulphonylation. Polyetherification involves nucleophilic substitution of aromatic halogen by phenoxy ions. The reaction is carried out in 2 stages at 130-250 °C in chlorobenzene or dimethylsulphoxide (solvents).



Polysulphonylation involves electrophilic substitution in 2 steps of aromatic halogen by sulphonylium ions, i.e.



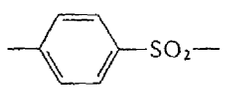
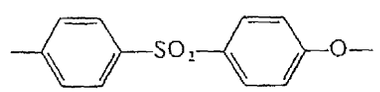
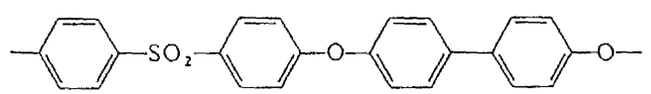
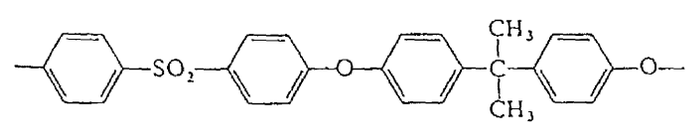
Structural unit	*Tg*	*Designation*
A. 	Tm = 520°C	—
B. 	230°C	Polyethersulphone
C. 	290°C	Polyarylsulphone
D. 	190°C	Polysulphone

Figure 5.1

Different types of polysulphones (Tg = glass transition temperature).

The reactions are done in nitrobenzene or tetrachloroethylene at 100 °C to 250 °C in the presence of a Friedel-Crafts catalyst e.g. ferric chloride.

The sulphone group is highly polar therefore the polymers possess high softening temperatures. However ether linkages may be introduced to reduce chain stiffness. The polymers are soluble in organic solvents e.g. aniline, dimethylacetamide, pyridine and are resistant to dilute acids and alkalis. Polyethersulphone membranes have been widely used for ultrafiltration (Kotezelski et al 1991; Kim et al 1992), microfiltration and reverse osmosis (Kinzer et al 1985). They are generally prepared by using a phase-inversion technique (discussed in chapter one). These membranes provide a screening barrier in biosensors for the passage of anionic interferents including ascorbate, and permeation of paracetamol is also reduced. They are mechanically robust and can be easily deposited into needle-type sensors (Vadgama et al 1989; Churchouse et al 1986).

Polysulphone has been sulphonated to varying degrees by using a 2:1 mixture of sulphur trioxide and triethylphosphate as the sulphonating complex. The reaction was done at room temperature in 1,2-dichloroethane. The resulting polysulphone sulphonic acid was neutralised with sodium methoxide to give the sodium salt (SO_3^- Na^+) (Noshay and Robeson 1976). The salt form was only soluble in polar organic solvents such as dimethylformamide, dimethylsulphoxide, methylpyrrolidone and diethylene glycol monoethylether. Polysulphone membranes of intermediate sulphonation content exhibited greater hydrophilicity allowing enhanced water flux and improved salt rejection

during reverse osmosis compared to unmodified polysulphone.

The following study was aimed at evaluating the selectivity of both dipcoated and "cast" membranes. The properties of three different polymers of varying sulphation content were considered.

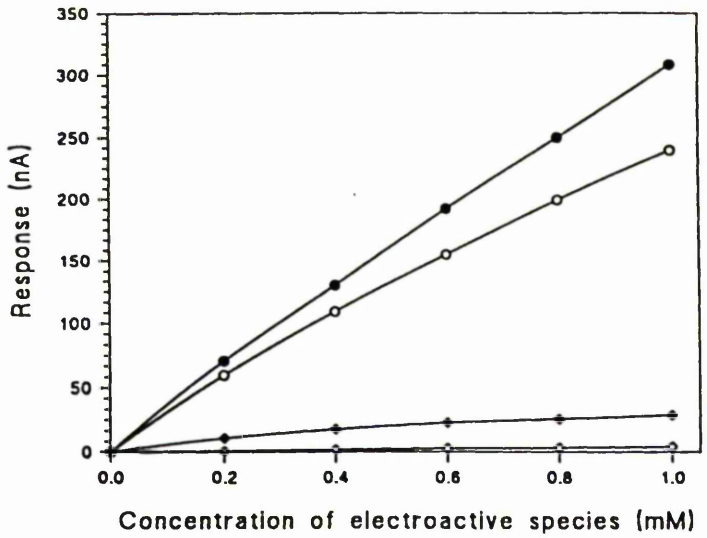
5.2 Needle electrodes

5.2.1 Comparison of bare and coated responses

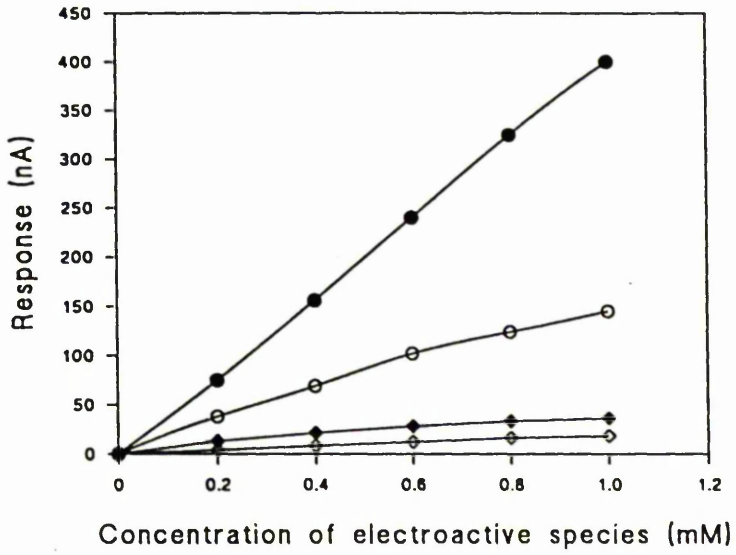
Calibration curves for hydrogen peroxide and ascorbate (0-1.0 mM) were constructed as shown in Figure 5.2.1a-f and Table 5.2.1a-c. For the 20:1 (sulphonated ratios) polymer a significant reduction in response to hydrogen peroxide occurs on dipcoating although the ascorbate response remains essentially unaltered. However if dimethylformamide (DMF) or a mixture of DMF and 2-methoxyethanol are used, the coated response to ascorbate diminishes to a greater extent.

For the S=5 (with a sulphonation ratio of 5:1) polymer a marked decline in H_2O_2 response does not occur but there is an accompanied diminution in ascorbate flux (Figure 5.2.1a and d). The results are also indicated by the Bare:Bare and Coated:Coated selectivity ratios for H_2O_2 : Ascorbate (Table 5.2.1a). For the 10:1 polymer there is a diminution in H_2O_2 response on coating which is not as pronounced as the S=5 polymer although there is an accompanied decrease in ascorbate response (Figure 5.2.1b and e). The Coated (H_2O_2):Coated (Ascorbate) (in dimethylsulphoxide) selectivity ratios are improved compared to the 20:1 polymer (Table 5.2.1c).In general selectivity for H_2O_2 over ascorbate worsens for each polymer if dimethylsulphoxide

(a)



(b)



(c)

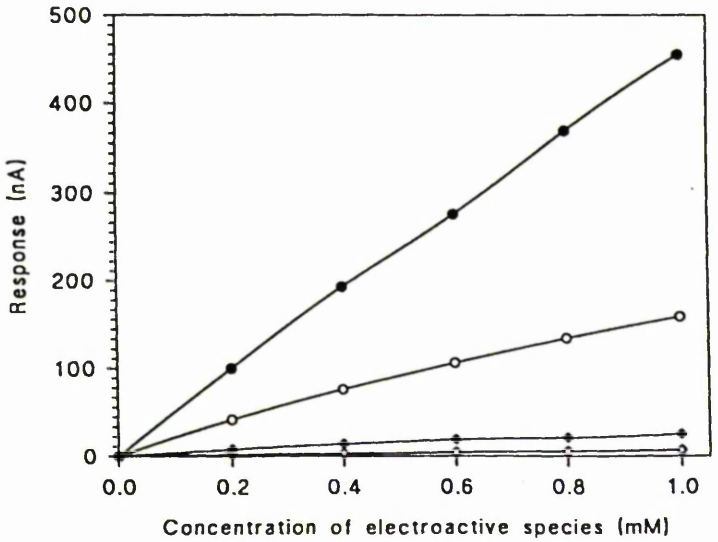
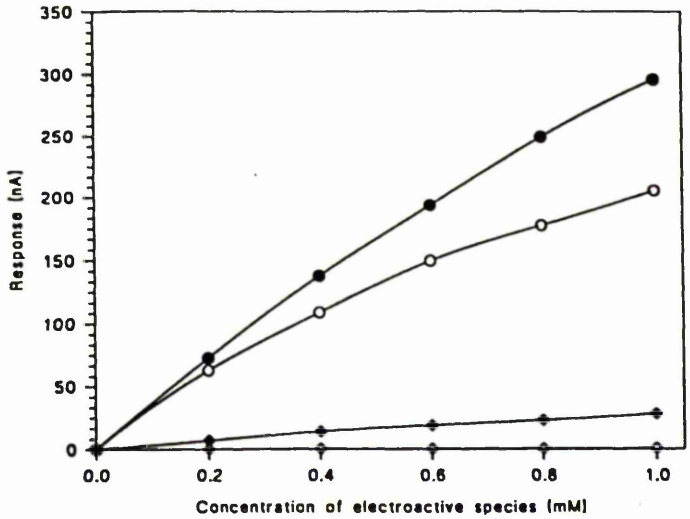
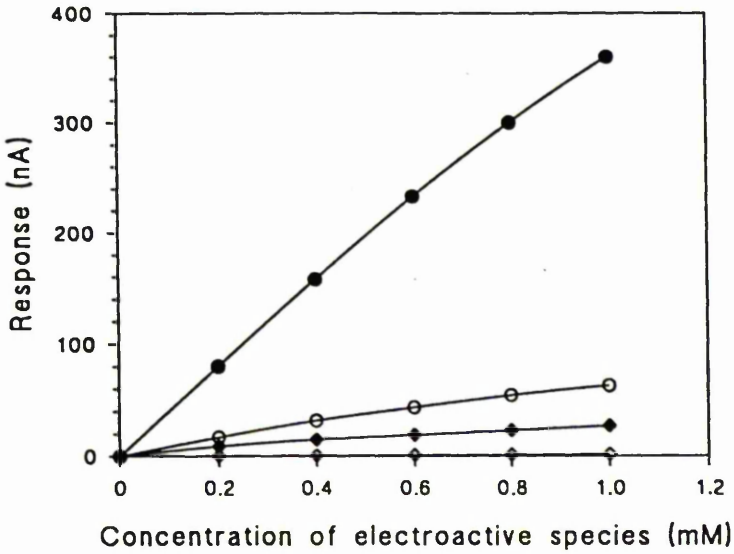


Figure 5.2.1 a-c
Comparison of bare H_2O_2 (●) and ascorbate (♦) with coated H_2O_2 (○) and ascorbate (◇) responses for needle electrodes dipcoated with polyethersulphone membranes (a) S=5 (b) 20:1 and (c) 10:1 in dimethylsulphoxide.

(d)



(e)



(f)

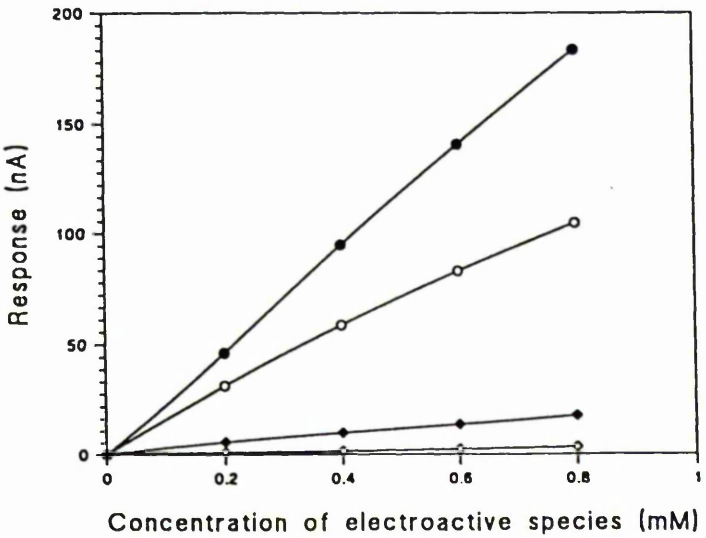


Figure 5.2.1 d-f
Comparison of bare H_2O_2 (●) and ascorbate (♦) with coated H_2O_2 (○) and ascorbate (◇) responses for needle electrodes coated with polyethersulphone membranes (d) S=5 (e) 20:1 and (f) 10:1 in a 3:1 mixture of dimethylformamide and 2-methoxyethanol.

(a)

Selectivity ratios	DMF alone	A 3:1 solvent ratio of DMF:2 methoxy-ethanol	DMSO alone
Bare : Coated H_2O_2 : H_2O_2 ASC : ASC	 2.6 : 1 150 : 1	 1.3 : 1 70 : 1	 1.2 : 1 9 : 1
Bare : Bare H_2O_2 : ASC	8.2 : 1	10.4 : 1	6.4 : 1
Coated : Coated H_2O_2 : ASC	470 : 1	545 : 1	59.5 : 1

(b)

Selectivity ratios	DMF alone	A 3:1 solvent mixture of DMF:2 methoxy-ethanol	DMSO alone
Bare : Coated H_2O_2 : H_2O_2 ASC : ASC	 8.9 : 1 17.5 : 1	 4.7 : 1 25 : 1	 1.97 : 1 3.25 : 1
Bare : Bare H_2O_2 : ASC	6.7 : 1	8.9 : 1	5.76 : 1
Coated : Coated H_2O_2 : ASC	13 : 1	53 : 1	9.5 : 1

Tables 5.2.1a and b

Comparison of bare and coated responses for hydrogen peroxide and ascorbate for dipcoated polyethersulphone membranes (a) S=5 and (b) 20:1 on needle electrodes.

(c)

Selectivity ratios	A 3:1 solvent mixture of DMF : 2-methoxyethanol	Dimethylsulphoxide alone
Bare : Coated H_2O_2 : H_2O_2 ASC : ASC	 1.7 : 1 13 : 1	 2.9 : 1 4 : 1
Bare : Bare H_2O_2 : ASC	 6 : 1	 8 : 1
Coated : Coated H_2O_2 : ASC	 28 : 1	 11 : 1

Table 5.2.1c

Comparison of bare and coated responses for hydrogen peroxide and ascorbate for a needle electrode dipcoated with a polyethersulphone membrane (10:1).

instead of dimethylformamide or a solvent mixture (dimethylformamide and 2-methoxyethanol) is used as the casting solvent. This is clearly evident from the Coated (H_2O_2): Coated (Asc) response ratios (Table 5.2.1a-c). A generalised selectivity pattern of S=5 > 10:1 > 20:1 is clearly demonstrated with the S=5 polymer having the greatest sulphonation content.

5.2.2 Effect of casting solvent composition

Table 5.2.2a illustrates the selectivity ratios for a needle electrode coated with the S=5 polymer using three different casting solvent compositions. The results indicate that selectivity for hydrogen peroxide over ascorbate, paracetamol, urate or cysteine remain invariant of dimethylformamide or dimethylsulphoxide inclusion and interference from paracetamol still remains. However a dramatic increase in selectivity for H_2O_2 over all of the electroactive species occurs for the solvent mixture. For the 20:1 polymer (Table 5.2.2b) the selectivity ratio for H_2O_2 : ascorbate is invariant of the casting solvent composition. The inclusion of 2-methoxyethanol does not improve selectivity for H_2O_2 over ascorbate, paracetamol and cysteine compared to the solvents dimethylformamide or dimethylsulphoxide.

5.2.3 Effect of polymer concentration

Generally the permselectivity of each membrane is improved by increasing the concentration of polyethersulphone from 2-4% (w/v) in dimethylformamide (Table 5.2.3a and b). However for the S=5 polymer membrane the H_2O_2 : paracetamol selectivity ratio remains unaffected. Figure 5.2.3 illustrates a gradual decline in flux for all species

(a)

Solvent	DMF alone	A 3:1 solvent ratio of DMF:2 methoxy-ethanol	DMSO alone
H_2O_2:ASC	107:1	575:1	116:1
H_2O_2:PARA	37:1	383:1	42:1
H_2O_2:URA	120:1	575:1	78:1
H_2O_2:CYS	87:1	383:1	58:1

(b)

Solvent	DMF alone	A 3:1 solvent mixture of DMF:2 methoxy-ethanol	DMSO alone
H_2O_2:ASC	16:1	10:1	14:1
H_2O_2:PARA	28:1	7:1	27:1
H_2O_2:URA	220:1	50:1	59:1
H_2O_2:CYS	95:1	45:1	35:1

Tables 5.2.2a and b

Effect of solvent variation on the selectivity ratios of a number of electroactive species for polyethersulphone membranes (a) S=5 and (b) 20:1 on needle electrodes.

(a)

Selectivity ratios	2% PES (w/v in DMF)	4% PES (w/v in DMF)
H_2O_2 : Ascorbate	32 : 1	107 : 1
H_2O_2 : Urate	177 : 1	120 : 1
H_2O_2 : Cysteine	55 : 1	87 : 1
H_2O_2 : Paracetamol	41 : 1	37 : 1

(b)

Selectivity ratios	2% PES (w/v in DMF)	4% PES (w/v in DMF)
H_2O_2 : Ascorbate	5 : 1	16 : 1
H_2O_2 : Urate	14 : 1	220 : 1
H_2O_2 : Cysteine	12 : 1	94 : 1
H_2O_2 : Paracetamol	16 : 1	28 : 1

Tables 5.2.3a and 5.2.3b

Effect of variation of the concentration of polyethersulphone (a) S=5 and (b) 20:1 in dimethylformamide on permselectivity.

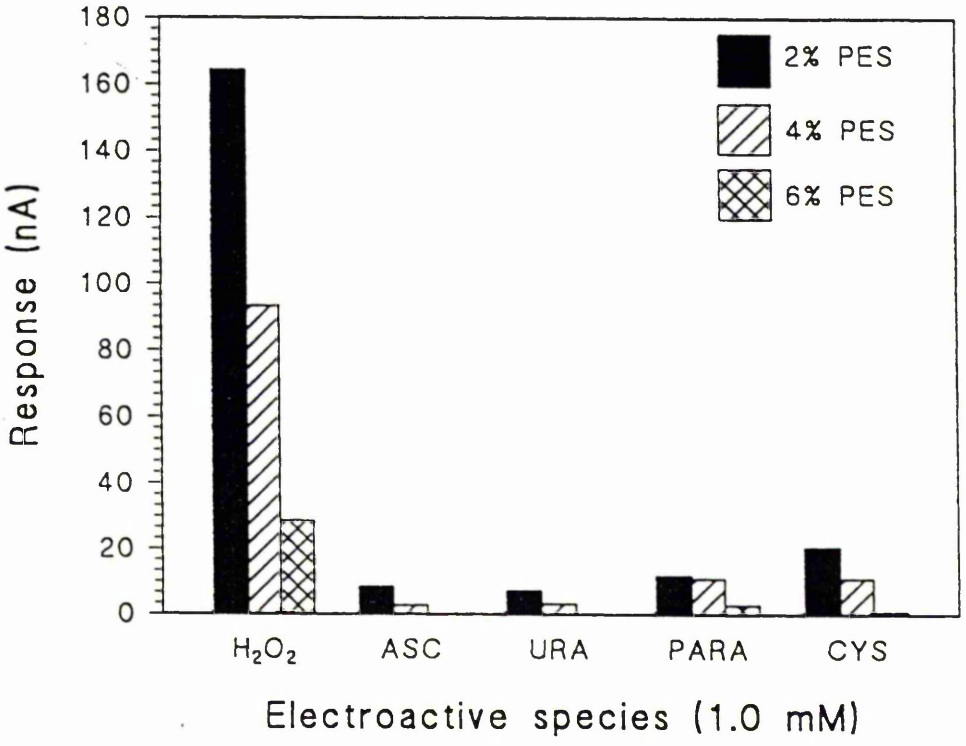


Figure 5.2.3

Variation of polymer concentration on permeability properties of dipcoated polyethersulphone membranes.

associated with increments in polymer concentration. Interference is still noticeable from anionic species (ascorbate and urate), paracetamol and urate using 2% polyethersulphone (w/v in dimethylformamide). A significant loss in hydrogen peroxide response occurs on dipcoating using 6% (w/v) polyethersulphone. An optimal concentration of 4% (w/v) shows an improved selectivity for hydrogen peroxide over all of the species compared to 2% (w/v).

5.3 Oxygen-type electrode

5.3.1 Permeability of cast polyethersulphone

Both polymer membranes (10:1 and S=5) demonstrate a high degree of permselectivity for hydrogen peroxide (Figure 5.3.1a and b). They are also capable of rejecting charged as well as aromatic species. This is particularly true for the 10:1 polymer where a high degree of exclusion is observed. For the S=5 polymer a certain amount of interference from catechol and paracetamol is clearly seen.

5.3.2 As the inner membrane of glucose electrodes

Interposition of polyethersulphone (4% w/v) between the enzyme layer and working electrode results in a marked diminution in response compared to the 0.03 μm polycarbonate membrane e.g., at 2 mM there is a 85-90% decline in hydrogen peroxide flux. The linear range remains essentially unaltered (5-6 mM) for polyethersulphone compared to polycarbonate internal membranes (Figure 5.3.2).

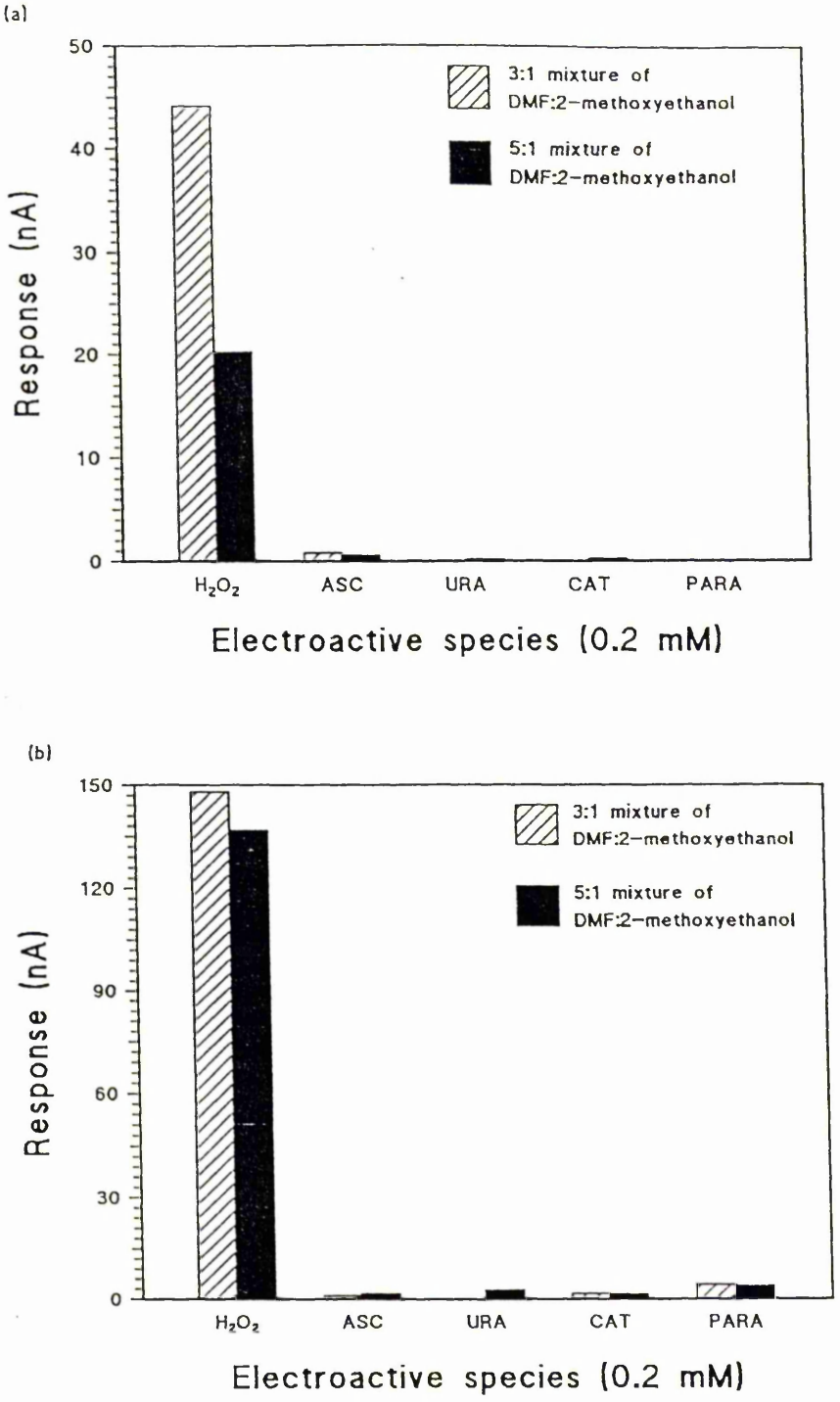
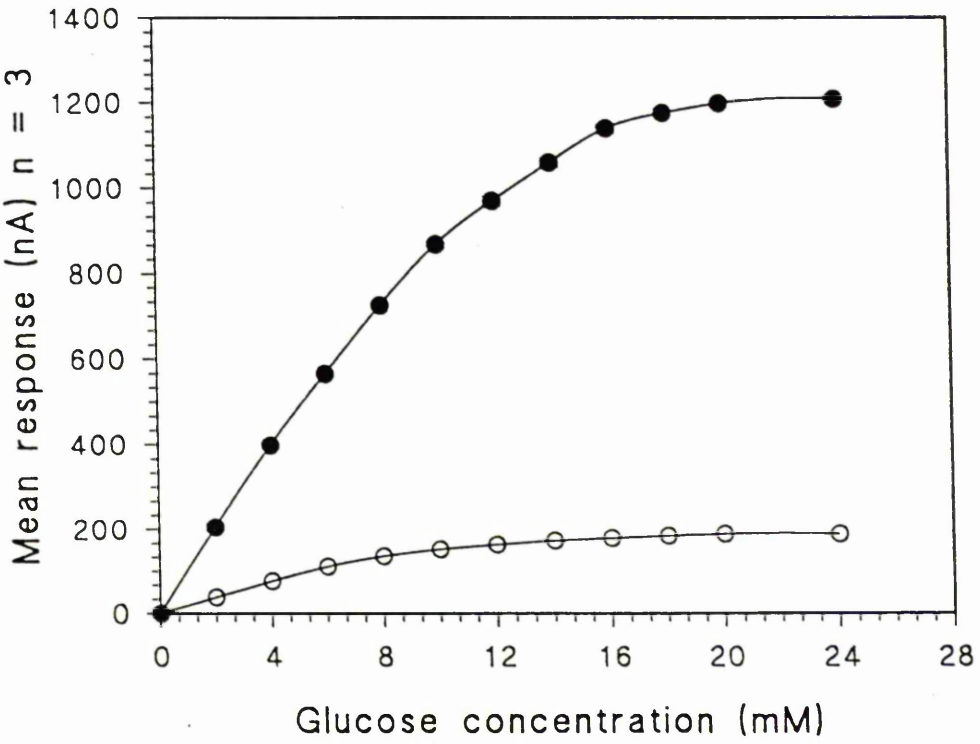


Figure 5.3.1

Selectivity of "cast" polyethersulphone membranes (a) 10:1 (b) S=5.



● 0.03 µm pcm/0.03 µm pcm o 0.03 µm pcm/4% PES

Figure 5.3.2

Effect of polyethersulphone inclusion as the internal membrane of a glucose electrode.

5.4 Discussion

It is clearly evident from Table 5.2.1a and b that each polymer possesses unique permselectivity properties. The S=5 membrane selected against ascorbate to a much greater extent that the 20:1 polymer. This may be due the high concentration of pendant sulphonate (anionic) groups in the S=5 polymer. Therefore the variability in permselective properties can be accounted for by knowledge of the molecular structure/organisation and dispersion of sulphonate groups present in the membranes. A decrease in response size for hydrogen peroxide and ascorbate was obtained after dipcoating using each polymer solution (Figure 5.2.1a-f). This is probably because the presence of an additional film (barrier) across the electrode which lead to a reduction in the steady-state concentration gradients and hence decreased responses. Polyethersulphone cation exchange membranes have been used for electrodialysis and can be prepared by including ion-exchangers into polymer solutions (Zschoke & Quellmalz 1985) with subsequent immersion into water.

From Table 5.2.2 it appears that polyethersulphone membranes selected against permeation of ascorbate, urate and cysteine. The mechanism of permselectivity may be partly dependent on difference in molecular weight but this does not appear to be the case here where ascorbate (MW 198) was rejected although paracetamol (MW 151) was not. Therefore discrimination between various electroactive compounds was also achieved by the presence of negatively charged (sulphonate) groups in the polymer matrix. This results in the formation of ion (cation) exchange membranes which allow permeation of neutral hydrogen

peroxide but prevent access of negatively charged species (co-ions) e.g. ascorbate. A similar mechanism of permselectivity exists for other cation exchange membranes including:- cellulose acetate, poly(ester-sulphonic) acid (Wang et al 1991) and Nafion (Harrison et al 1988).

Poly(ester-sulphonic) acid has a similar molecular structure to polyethersulphone consisting of a hydrophobic polyester backbone and sulphonated exchange sites. The selectivity against ascorbate for these membranes was determined by measuring the response ratios (membrane-coated : bare) and was found to be 0.03 according Wang et al (1991). In comparison the polyethersulphone (S=5) polymer gave a ratio of 0.0066 and cellulose acetate gave a value of 0.03 (Koochaki et al 1991). Therefore polyethersulphone selected against ascorbate to a much greater extent than the other membranes. Also, according to Churchouse et al (1986) polyethersulphone membranes were much more robust and could reject a greater amount of paracetamol than cellulose acetate.

From Table 5.2.2 it can be seen that membrane structure/permeability can be altered by using solvent/non-solvent mixtures containing dimethylformamide and 2-methoxyethanol during casting. This could lead to pore formation due to the presence of the non-solvent pore former (2-methoxyethanol). Therefore these membranes may act as molecular sieves as well as having ion-exchange character leading to increased permselectivity for hydrogen peroxide. An analogous membrane was formed by the inclusion of ethanol (pore-former) in the

casting solution containing cellulose acetate and acetone (Chawla and Chang 1975). Yasuda and Tsai (1974) also suggested that the mechanism of pore formation is similar to that in cellulose acetate. The important factors are the rate of solvent removal and rate of water or nonsolvent entry during membrane formation. Porosity is increased by either increasing the temperature of the water coagulation bath or decreasing polymer concentration. Scanning electron micrographs of the resultant membranes (Zchocke and Quellmalz 1985) revealed an asymmetric structure consisting of a 12 μm thick homogeneous layer supported by a 40 μm thick porous substructure. A detailed study into the phase separation which occur in the polyethersulphone/solvent/non-solvent has been undertaken (Lau et al 1991). Non-solvent inclusion into the polymer solution resulted in a reduction in the solvating ability of the solvent leading to eventual polymer precipitation. Polyethersulphone itself was shown to be hydrophilic and formed a close association with water during membrane formation. Hence it may be possible that 2-methoxyethanol, also a polar molecule could form a close interaction with the polymer in an analogous manner.

The permselectivity of polyethersulphone has been shown to increase with a higher polymer concentration (Table 5.2.3a-b and Figure 5.2.3). This is possibly due to the thicker diffusion limiting barrier presented at a concentration of 6% w/v and there is an associated higher level of sulphonate groups.

The permselectivity of "cast" polyethersulphone is shown in Figure 5.3.1a and b. The permeation features are similar to dipcoated

membranes and show cation exchange character. As expected a decrease in hydrogen peroxide flux occurs when polyethersulphone is included as the inner membrane of a glucose electrode (Figure 5.3.2).

5.5 Summary

Results have demonstrated the permselectivity properties of dipcoated and "cast" polyethersulphone membranes which are able to reject negatively charged species. Selectivity for hydrogen peroxide was different for each polymer. The S=5 polymer exhibited the highest whilst the 20:1 polymer showed the lowest selectivity for hydrogen peroxide. An increase in polymer concentration lead to improved permselectivity which was also governed by the solvent composition used for casting. The ideal casting solution composition consisted of a mixture of dimethylformamide and 2-methoxyethanol.

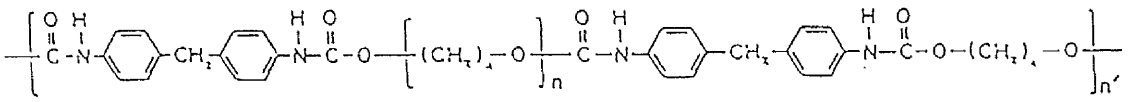
CHAPTER SIX - POLYURETHANE MEMBRANES

6.1 INTRODUCTION

Polyurethane polymers also known as polycarbamates (from carbamic acid) contain urethane groups (NH-CO-O-) in the main polymer chain. These groups are derived from the addition interaction of an isocyanate and a hydroxyl compound:-

$$R\text{-}NCO + HO\text{-}R' \rightarrow RNHCOOR' \qquad (6.1a)$$

When a diisocyanate and a diol react together a linear polyurethane is formed although a branched polymer is formed if the diol is replaced by a polyhydric compound (polyol). The type of polyurethanes called "Thermoplastic elastomers" are commonly used in biomedical applications, and are prepared by reaction of linear hydroxyl-terminated polyesters (mainly adipates) or polyethers (commonly polyoxytetramethylene glycol), diisocyanates (4,4' diisocyanate) and glycols (ethylene glycol and 1,4 butanediol). The resultant block polymer is made up of alternating polyester or polyether blocks (soft segments) and polyurethane blocks (hard segments) e.g. the polyetherurethane "Pellethane^{R}" is a block copolymer consisting of a long linear polyether segment comprising the urethane and aromatic groups (Bouvier et al 1991).



Polyurethane has been mainly used as an external membrane for needle-type sensors (Sternberg et al 1988; Shichiri et al 1982; Vadgama

et al 1989; Rea et al 1985). The material was utilised because it could be dipcoated easily from a solution in order to form thin, robust membranes, and additional layers could be applied once the initial membrane was deposited. One of the major problems associated with enzymatic biosensors is that most enzymes obey classical Michaelis-Menten kinetics. Therefore the enzymic reactions are non-linear with respect to substrate as manifested by a finite K_m (Michaelis constant) which if small results in a poor linear dynamic range. The membrane characteristically maintains oxygen (cosubstrate for oxidases) flux to the enzyme active site relative to substrate transport. Therefore the enzyme does not become saturated with substrate rapidly and the oxygen supply is no longer rate-limiting. A combination of these effects results in an increased apparent K_m at the expense of reduced sensitivity and prolonged response times. A fine-tuning mechanism exists for control of substrate/cosubstrate transport in order to optimise the linear range and sensitivity of substrate detection. Generally if the enzyme activity is high, thick membranes may be required to limit substrate diffusion although thinner dense membranes are preferable. Polyurethane also reduces the dependence of sensor response on external variables e.g. mass transfer rate, pH, temperature and oxygen variations (Bindra et al 1991; Vadgama et al 1989; Weiss and Camman 1988; Kerner et al 1988) since the response is no longer kinetically controlled. A combined hydrogel (polyhydroxyethylmethacrylate) and polyurethane has also been employed as a diffusion-limiting barrier (Shaw et al 1991). Here sensor response was unaffected by a reduction in oxygen tension from 20 to 5 kPa. This is partly attributed to a greater permeability to oxygen

compared to glucose of the outer polyhydroxymethacrylate/polyurethane membrane. Therefore the sensor should operate in subcutaneuous tissue where fluctuations in oxygen levels often occur.

Low permeability polyurethane may be used to reduce the rate of hydrogen peroxide production at the enzyme layer which is a known denaturant of glucose oxidase (Kleppe 1966). Hydrogen peroxide was particularly effective in denaturing the reduced form of glucose oxidase ($E\text{-}FADH_2$). It was suggested that hydrogen peroxide reacted with methionine residues in the enzyme protein close to the active site. It was also proposed that hydrogen peroxide reacted with glucose oxidase to give semiquinones and hydroxyl radicals.

$$E\text{-}FADH_2 + H_2O_2 \rightarrow E\text{-}FAD^{\bullet} + OH^{\bullet} + H_2O \qquad (6.1b)$$

The hydroxyl radicals could promote oxidation of the semiquinone form to produce oxidised glucose oxidase.

In this part of the work, the preformed polyurethane (Pellethane 2393-90AE) for the fabrication of glucose or lactate needle biosensors will be explored. The suitability of this material was assessed following its incorporation over enzyme electrodes and an evaluation of electrode performance in aqueous buffer made and compared against other polyurethanes. The effect of hydrochloric acid treatment of polyurethane on storage stability will be determined. An additional study using preformed "Pellethane" films in 2-electrode amperometric electrodes was carried out. The selectivity of these particular membranes was determined and the possibility of using these as internal membranes also explored.

6.2 Results

6.2.1 Polyurethane surface structure

From Figures 6.2.1a and b for dipcoated polyurethane ("Pellethane"). It is evident that a smooth external surface is provided by the electrode containing an internal polyethersulphone screening membrane. No evidence of pores is seen (Figure 6.2.1b). The surface of the electrode coated with polyurethane alone was not uniform and exhibited a pitted surface (Figure 6.2.1a).

6.2.2 Polyurethane comparative stabilities and diffusional limitation

From Table 6.2.2 the optimised performance of a glucose or lactate sensor with "Pellethane" as the outer diffusion-limiting membrane was compared to sensors using the polyurethanes "Trixene" or "Estane". A variation in the number of applied coats and the concentration of polyurethane was required to construct sensors exhibiting similar response times (T_{90}), extensions in linearity and responses (nA/mM) to substrate. The "Estane" appeared to be the most diffusion limiting, followed by "Pellethane" and "Trixene" respectively although Trixene gave the most stable sensors. Stability studies for enzymic (Figures 6.2.2a and b) and non-enzymic (Figure 6.2.2c) for the polyurethane "Pellethane" indicated that the responses stabilised after at least 4 days. An initial increase in response was observed in all cases followed by a steady value. Dry storage between measurements led to more stabilisation of sensor performance than storage under wet conditions buffer at 24 °C.

(a)

(b)

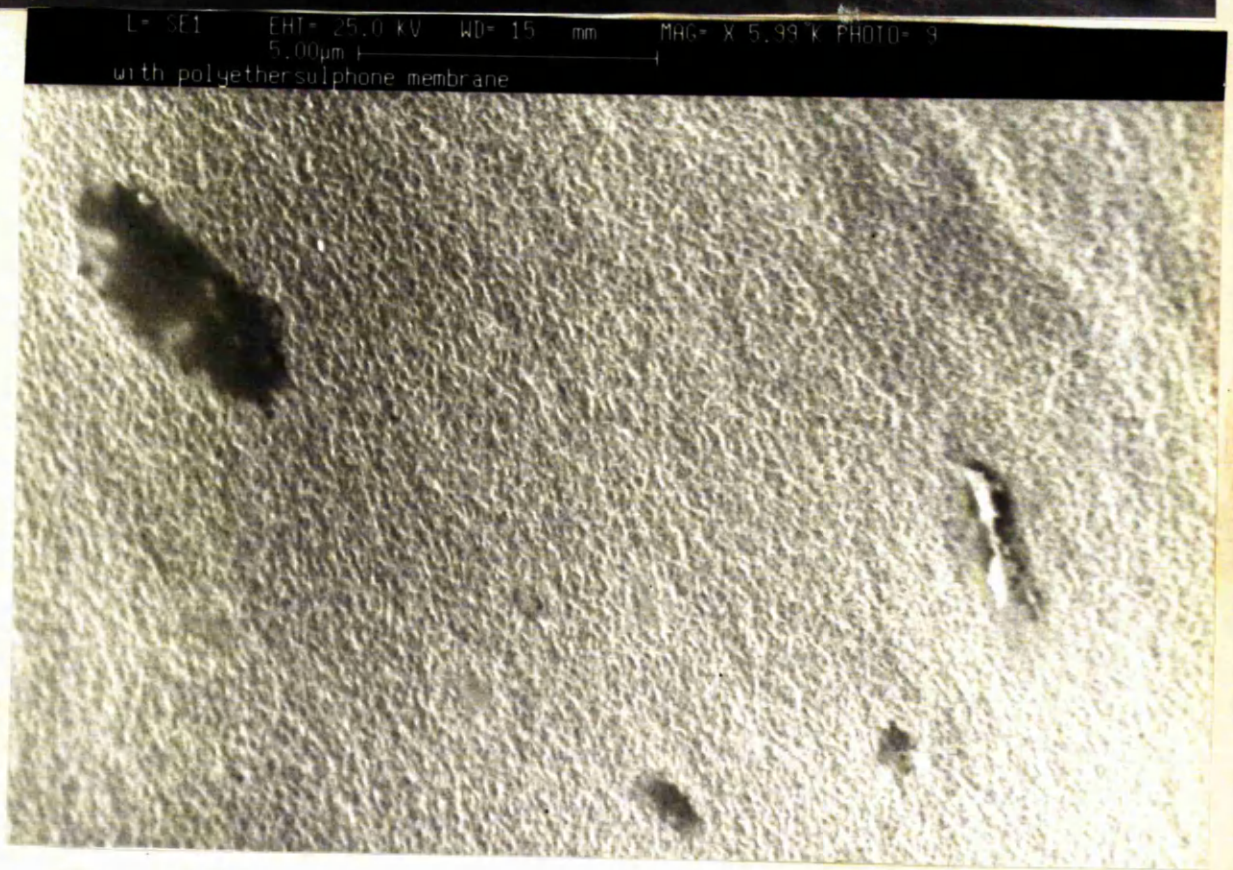


Figure 6.2.1

Scanning electron micrograph of a dipcoated polyurethane membrane of a needle electrode,(a) without an internal polyethersulphone layer and (b) with a polyethersulphone membrane

Polyurethane	Maximum linear range (mM)	Response Time T_{90} (minutes)	Number of coats required	Initial Slope (nA/mM)	Stability
Pellethane (3% $^{w}/_{v}$ in THF)	30 (glucose)	5-7	2	0.5	Acceptable after 4-6 days (dry/ wet) and 5-8 days (wet)
	24 (lactate)	5-7	5	1.0	
Trixene SC 762 (10%, 16%, 33% 50% $^{v}/_{v}$ in THF)	40 (glucose)	5-7	4	0.4	Stable for >5 days dry/wet
	12-14 (lactate)	5-7	4	1.0	
Estane 5724f1 (5% $^{w}/_{v}$ in THF)	38 (glucose)	3-4	1	1.8	Stable for only a few hours (dry/ wet)

Table 6.2.2

Comparison of the in vitro properties of three different polyurethanes in aqueous solution.

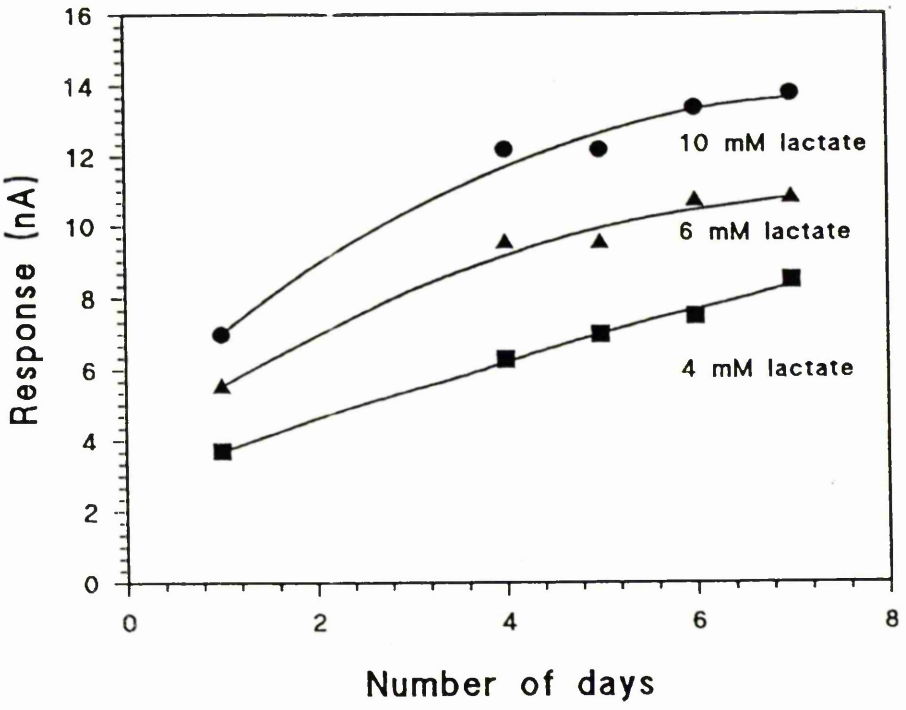


Figure 6.2.2a

Stability of a lactate needle enzyme electrode coated with multiple external polyurethane "Pellethane" membranes.

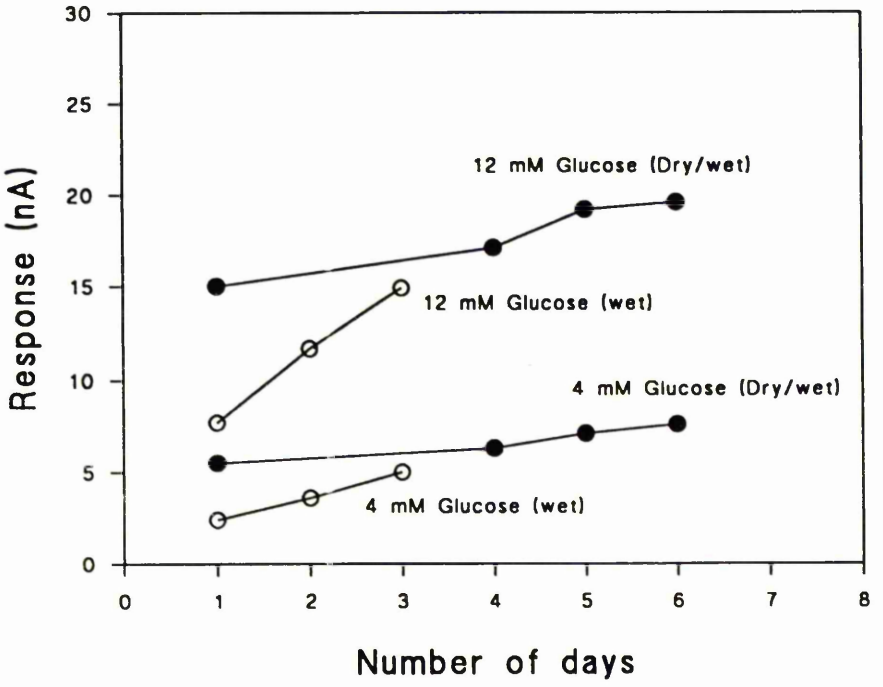


Figure 6.2.2b

Impact of a wet and dry combination and wet storage on response of glucose needle sensors coated with the polyurethane "Pellethane".

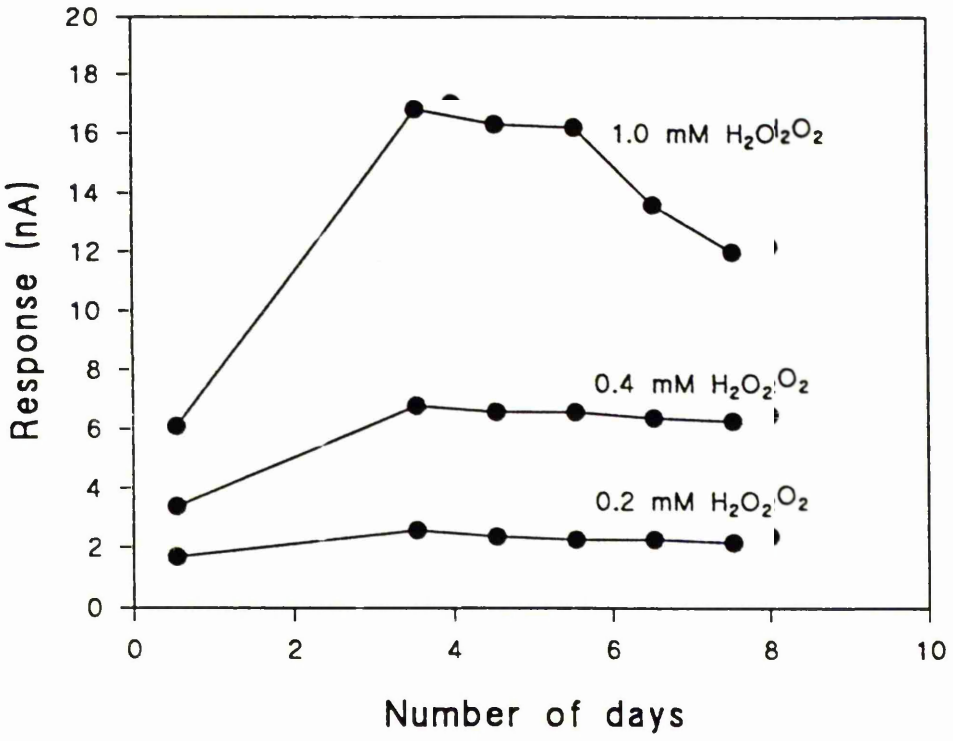


Figure 6.2.2c

Stability of hydrogen peroxide responses for a needle electrode containing a single coat of 3% (w/v in tetrahydrofuran) polyurethane "Pellethane" and stored in aqueous buffer solution.

6.2.3 Hydrochloric acid treatment

The impact of hydrochloric acid treatment on polyurethane (Estane 5724f1) is illustrated in Figure 6.2.3. There was an initial amplification in sensor response however no stabilisation occurred.

6.3 Polyurethane "cast" membranes

6.3.1 Surface examination

The scanning electron micrographs of cast polyurethane membranes (Figures 6.3.1a and b) indicated the presence of a non-porous almost uniform structure.

6.3.2 Selectivity of "Pellethane" membranes

"Pellethane" (1-2% w/v) cast from tetrahydrofuran exhibited limited but measurable solute permeability when placed on the 2-electrode amperometric cell system in 0.2 mM test solutions of H_2O_2, ascorbate, urate and paracetamol. Pellethane membranes (0.5% w/v) were very fragile and showed no preferential selectivity towards ascorbate, urate or paracetamol compared to hydrogen peroxide (figure 6.3.2). The following selectivity ratios were obtained for H_2O_2 : ascorbate, paracetamol, urate respectively (3.7:1, 2.3:1, 2.3:1) for 0.5% polyurethane (w/v in tetrahydrofuran) coated membranes.

6.3.3 Inner membrane of glucose electrode

Utilisation of polyurethane as an internal membrane resulted in a marked diminution in peroxide response, accompanied by an extension of the linear range from the 3 mM value found with polycarbonate films to 8 mM (Figure 6.3.3). However a maximal sensitivity of only 1.2 nA for a glucose concentration of 16 mM was only achieved.

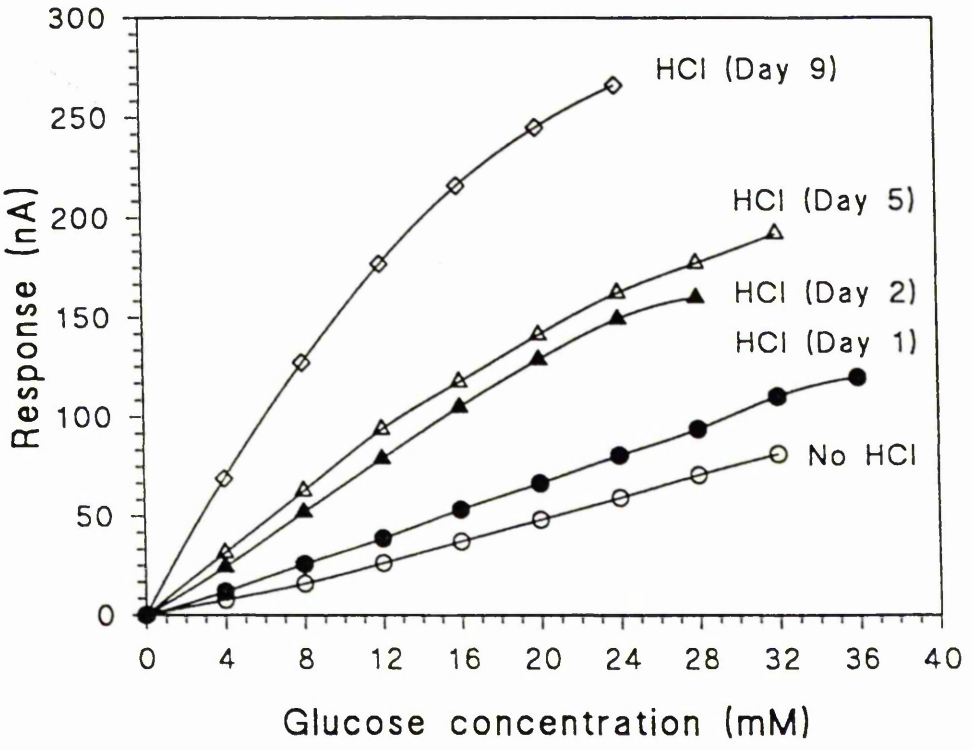


Figure 6.2.3

Effect of hydrochloric acid treatment of the polyurethane "Estane 5724f1" on sensor response stability.

(a)

(b)

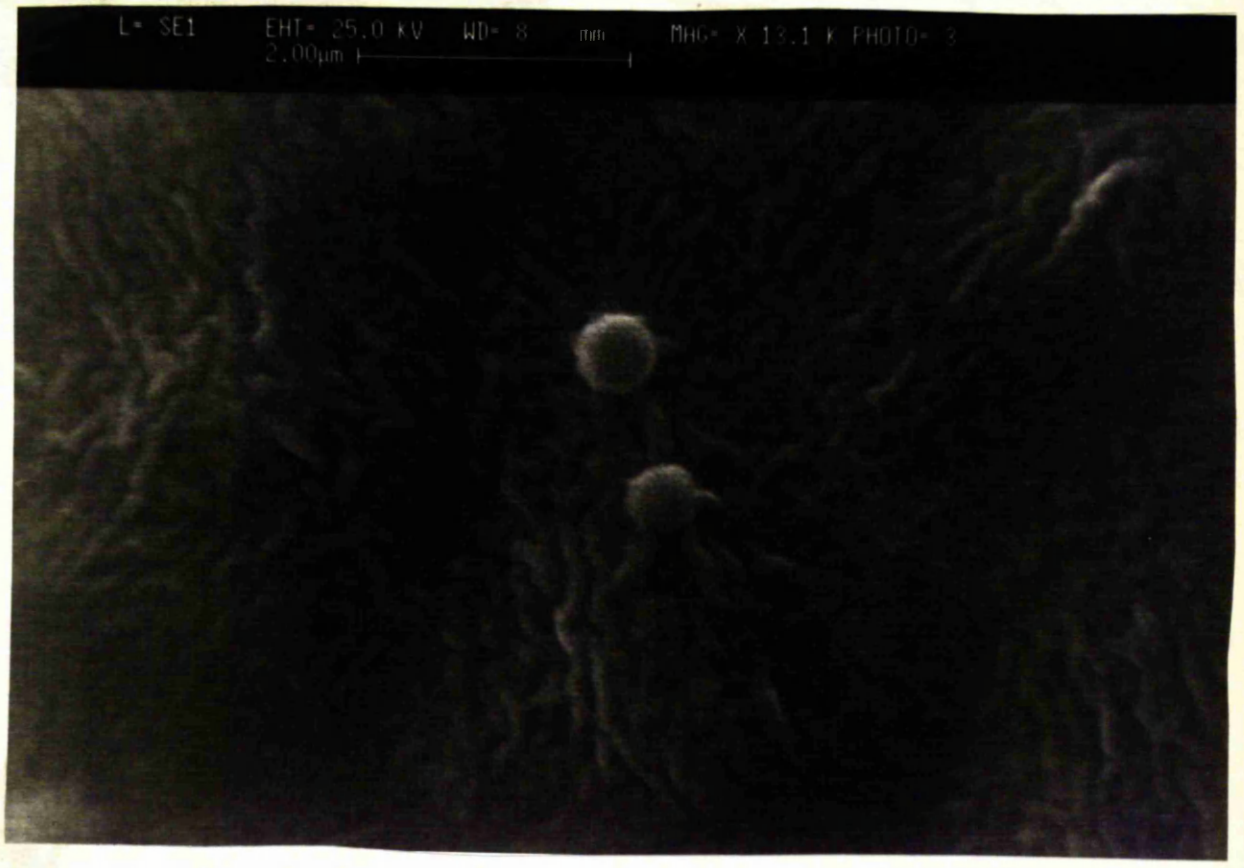


Figure 6.3.1

Scanning electron micrographs of cast polyurethane "Pellethane" membranes.

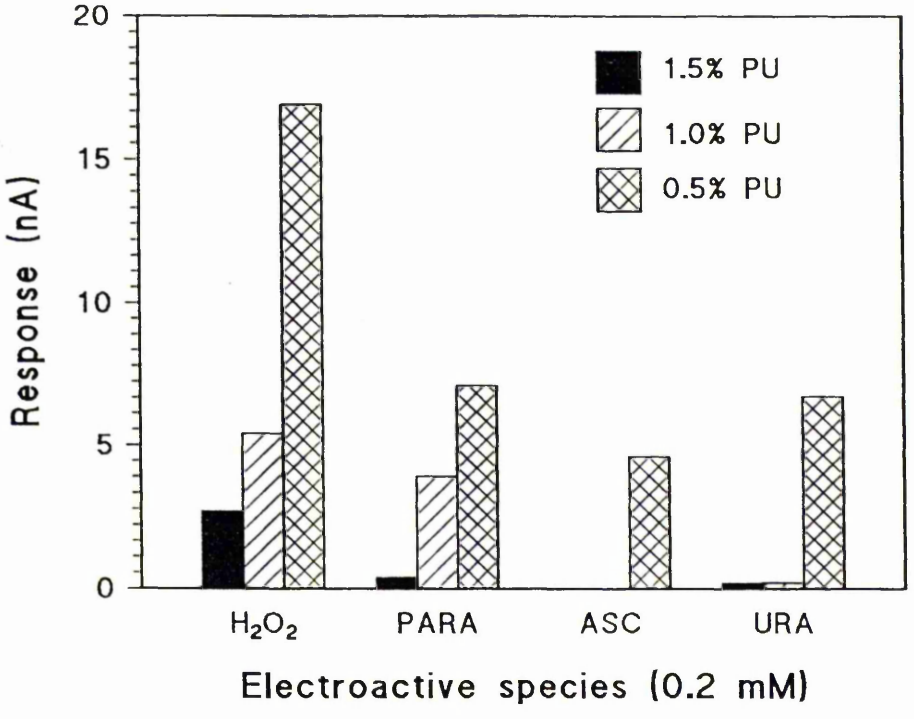


Figure 6.3.2

Permeability properties of a tetrahydrofuran-cast (0.5%-1.5% w/v) polyurethane "Pellethane" membranes tested on an amperometric oxygen cell.

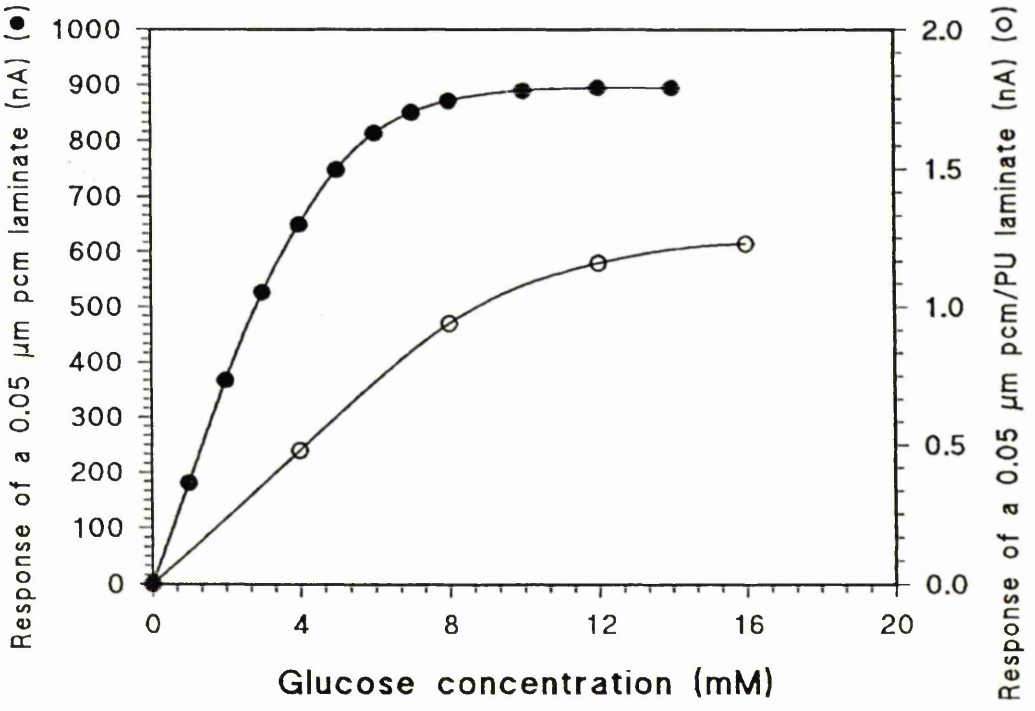


Figure 6.3.3

Impact of using internal polyurethane (0.5% w/v in tetrahydrofuran) membranes in glucose electrodes.
Internal membranes:- (●) 0.05 μm PCM; (○) 0.5% PU.

6.4 Discussion

The polyurethane "Pellethane" cast onto a glass surface appeared to be impermeable to most solutes including glucose. Scanning electron micrographs of the surface (Figure 6.3.1a and b) showed a distinct absence of pores which must restrict solute transport. This is in accordance with previous results obtained by Churchouse et al (1989) and Lelah et al (1985). In contrast films dipcoated onto needle electrodes were showed definite permeability to glucose although actual "pores" were not visible from the SEM of Pellethane (Figure 6.2.1). However earlier SEM analysis of dipcoated Trixene SC762 (Churchouse et al 1986) demonstrated the presence of pores which should facilitate diffusion to the electrode.

A comparison between the different diffusion-limiting properties of the 3 polyurethanes was carried out (Table 6.2.2) because of the apparent number of polyurethane coats required to produce sensors with similar characteristics. Sequential dipcoating using increasing concentrations of polyurethane (Trixene SC762 and Pellethane) lead to a progressively increased linear range. The results agree with those of Vadgama et al (1989) for the Trixene SC672 prepolyurethane. The results signify that increasing membrane thickness caused enhanced substrate diffusion leading to an extension in enzyme apparent K_m hence increased linearity. It was advantageous to use "Pellethane" since the number of dipcoats required was reduced to 2 or 3 and a low concentration was needed. The "ideal" polymer was "Estane 5724f1" since a single application of the membrane (5% w/v in tetrahydrofuran) in conjunction with an internal polyethersulphone membrane resulted in

a linearity of 38-40 mM with enhanced sensitivity and short response times, though Rea et al (1985) only obtained a maximum linearity of 12-13 mM using the same polymer but an internal cellulose acetate membrane.

According to Vadgama et al (1989) repeated coating using Trixene SC762 was ineffective in extending linearity for glucose beyond 20 mM. However 2-3 coats of "Pellethane" (3% w/v in tetrahydrofuran) resulted in enhanced linearity for glucose (~30 mM). The results are a considerable improvement from those obtained by Bindra et al (1991) where a maximal range of ~14 mM for glucose was achieved using polyurethane. Sternberg et al (1988) also found multiple dipcoats of 3.5% polyurethane (in 98% tetrahydrofuran, 2% dimethylformamide) gave an extended linear range.

From table 6.2.2 it is evident that storage stability was affected by the type of polyurethane used. Pellethane (polyetherurethane) and Estane (polyesterurethane) membranes were from formed polyurethane, had a lower initial stabilty than Trixene. Trixene is a polymer precursor which polymerised over the electrode and appeared to be more solvent (tetrahydrofuran) resistant and was preferable as multilayers, giving a more rigid structure. The hydrolytic stabilities closely correlate with the polymer diffusion-limiting properties according to Pavlova et al (1984). It was found that polyurethane oligomers based on polyetherpolyols (similar to Pellethane) possessed a lower diffusion velocity than polyesterpolyol based polyurethanes (similar to Estane) and showed enhanced stability in aqueous/alkaline media. The different diffusion

velocities can be deduced from the initial slope measurements from Table 6.2.2, where Pellethane gave a sensitivity of 0.5 nA/mM and Estane 1.8 nA/mM. Gunatitallake et al (1993) suggested that hydrolytic stability was dependent on the hydrophobic nature of the particular polymer which in turn is governed by the carbon:oxygen ratio of the macrodiol used for polyurethane synthesis.

Pellethane performance may be preferable for practical use because electrodes fabricated using the material gave a more steady response after a 4-8 day period (Figures 6.2.2a-c). An increase in current followed by stabilisation was observed in each case. A similar pattern of stabilisation was observed for enzymic and non-enzymic sensors therefore signal drift due to the gradual inactivation of enzyme by hydrogen peroxide during the first few days could be ruled out. Also Sternberg et al (1988) assessed enzyme activity of electrodes using radiolabelled ^{125}I-glucose oxidase. No significant release of radiolabelled enzyme occurred during the first two weeks of sensor use and the enzymatic response also remained constant. Signal drift could have occurred because of hydrolytic and swelling processes of polyurethane membranes as the residual solvents were replaced by water during the conditioning period thereby leading to an increase in substrate permeability. The results are consistent with Bindra, Zhang et al (1991) and Weiss and Camman (1988). The sensor could therefore be used after 4-5 days storage in buffer.

Bindra, Zhang et al (1991) used their sensors after 3-days buffer storage. According to these workers buffer treatment was required for

removal of leachable toxic substances such as residual organic solvents used for membrane dipcoating and excess glutaraldehyde. Storage under aqueous conditions caused hydrolytic/swelling reactions of membranes and lead to a larger response size compared with sensors under dry storage. Hydrochloric acid hydrolysis of polyurethane (Estane 5724f1) did not result in a stabilisation of response (Figure 6.2.3). However it was expected from evidence (Pavlova et al 1984) that polyurethane containing polyester groups possessed a compact cross-linked structure which was resistant to acidic hydrolysis.

The selectivity studies of preformed polyurethane membranes demonstrate a general decrease in flux of all electroactive species, although they were not selective. An increased polymer concentration resulted in decreased permeation as shown in Figure 6.3.2. This may have been due to the denser, diffusion-limiting polymer matrix presented by the membranes. The results are in accordance with Sternberg et al (1988) where the glucose : ascorbate selectivity ratio was 220 : 1 in the absence of polyurethane and subsequently reduced to 11 : 1 on polyurethane coating.

Polyurethane inclusion as an internal membrane of a glucose enzyme laminate caused a marked diminution in response which is expected since it reduces H_2O_2 flux as shown in Figure 6.3.2. An improvement in the linear range was obtained (Figure 6.3.3) possibly due to the enhanced oxygen back permeability from the platinum electrode (Ziegel 1971). Therefore electrochemically generated oxygen (co-substrate) could be retained in the enzyme layer so that the oxygen level was not

rate-limiting.

6.5 Summary

The results have shown the surface morphological features of polyurethane membranes. Polyurethane incorporation as external membranes in needle electrodes was attempted. The 3 different polymers tested showed very variable diffusion-limiting characteristics as determined by the polymer concentration and the number of layers required. Sensor stability in aqueous solution was also governed by the type of polyurethane, in the order Trixene > Pellethane > Estane. Stability was also determined whether wet or dry storage conditions were used. For long-term use the polyurethane "Pellethane" was suitable after a 3-4 stabilisation period. Hydrochloric acid treatment of Estane 5724f1 did not have any appreciable impact on sensor storage stability.

Preformed polyurethane membranes did not show any preferential selectivity towards H_2O_2. Also membranes cast using a low concentration of polymer proved to be fragile. An improvement in linearity was found when included as internal membranes although there was a considerable decline in hydrogen peroxide flux. It may be concluded that preformed polyurethane will have limited application for sensors.

CHAPTER SEVEN

LACTATE AND CHOLESTEROL ENZYME ELECTRODES

7.1 INTRODUCTION

During anaerobic glycolysis, glucose is converted to pyruvate resulting in the formation of NADH from NAD^+ (a continual supply of the latter is essential for glycolysis to proceed). If conditions remain anaerobic (anoxia) the pyruvate equilibrium is disturbed and it becomes readily converted to lactate via the lactate dehydrogenase reaction which then regenerates the supply of NAD^+.

$$CH_3COCOOH + NADH_2 \rightleftharpoons CH_3CH(OH)COOH + NAD^+ \quad (7.1a)$$

In aerobic conditions pyruvate is transferred across the mitochondrial membrane and instead oxidised via the tricarboxylic acid cycle to form acetyl Coenzyme A (catalysed by the enzyme pyruvate dehydrogenase). In this case the production of lactate is prevented. Pyruvate and lactate are also important precursors of gluconeogenesis in the liver and kidney, and these tissues are therefore responsible for lactate/pyruvate reutilisation to glucose. Lactate measurement therefore has value in clinical practice.

Major tissues responsible for lactate production include the skeletal muscle, skin and erythrocytes. Normal lactate levels in venous blood are in the range 0.4-1.3 mM (Alberti and Nattrass 1977), and if above 2 mM lactic acidosis is considered to occur.

Type A lactic acidosis occurs due to poor oxygen delivery to tissues in cardiovascular shock or heart surgery, septicaemia, severe anaemia or left-ventricular failure. Measurement of lactate is therefore especially

useful in critically ill patients, and relates to the severity and type of metabolic disturbance. The metabolic status of a diabetic patient can be determined since insulin infusion leads to altered lactate levels. Therefore a combined, continuous blood/tissue glucose and lactate measurement system may permit the development of physiologically important biofeedback systems e.g. "artificial pancreas" for insulin delivery and therefore better metabolic regulation (Mascini et al 1985). Peripheral blood lactate levels will increase during poor oxygen utilisation by tissues (Type B lactic acidosis); this can occur in many disease states e.g. cancer, leukaemia and diabetes mellitus, and also after exposure to ethanol, phenformin and fructose which often results in an elevated [lactate]/[pyruvate] ratio.

Lactate level assessment could also be useful during training of athletes (Palleschi et al 1990) e.g. the anaerobic threshold of lactate production can be determined. This occurs for muscle when a certain work rate is exceeded resulting in a steep increase in the lactate level. Also lactate measurement in the cerebrospinal fluid could help to distinguish between viral and bacterial meningitis and perhaps indicate any cerebral oxygen insufficiency.

Noy et al (1979) employed a fluorometric method which utilised diluted blood and used lactate dehydrogenase measuring the production of reduced NAD^+ (excitation wavelength = 340 nm; emission wavelength = 465 nm). Here hydrazine was included in the assay medium to trap pyruvate and pull the reaction equilibrium towards NADH formation.

Amperometric methods have also relied on sample dilution e.g. the Roche lactate analyzer (Soutter et al 1978), here the enzyme cytochrome b_2 (L-lactate:ferricytochrome c oxidoreductase EC 1.1.2.3) was used and hexacyanoferrate (III) was oxidised at a platinum electrode polarised at 0.28 V with the production of a current:-

$$\text{Lactate} + \underset{\text{(III)}}{\text{hexacyanoferrate}} \xRightarrow{\text{Cytochrome } b_2} \text{pyruvate} + \underset{\text{(II)}}{\text{hexacyanoferrate}} \quad (7.1b)$$

$$2Fe(CN)_6^{4-} \rightarrow 2Fe(CN)_6^{3-} + 2e^- \quad (7.1c)$$

Blaedel and Jenkins (1978) described reagentless electrochemical lactate measurement using lactate dehydrogenase with coentrapped cofactor (NAD^+). Reagentless assay is also possible using lactate 2-monooxygenase (LOD) from *Mycobacterium smegmatis* which catalyses the reaction:-

$$\text{lactate} + O_2 \rightarrow \text{acetate} + CO_2 + H_2O \quad (7.1d)$$

Mascini et al (1984) proposed an oxygen based electrode for discrete analysis. Problems arose due to background pO_2 variation and necessitated the use of a second oxygen sensor or pre-equilibration of sample to constant pO_2. Subsequently Weaver and Vadgama (1986) described a continuous flow system in which sufficient air equilibration occurred during sample dilution avoiding the requirement for manual pre-equilibration.

Lactate partitioning between red cells and plasma is unequal because insufficient lactate leaks out of normal red blood cells. Therefore any dilution which disturbs the partitioning equilibrium may result in serious errors in lactate measurement (Piquard et al 1980; Weaver and

Vadgama 1986). It is therefore necessary to permeabilise red cells or haemolyze blood prior to measurement.

A current optimum method of substrate detection utilises the enzyme lactate oxidase (from *Pediococcus Sp.*):-

Lactate + O_2 → pyruvate + H_2O_2 (7.1e)

Hydrogen peroxide oxidation at +650 mV vs AgCl reference electrode again allows determination of lactate levels. The following investigation was aimed to study diffusion-limiting outer membranes to extend the linear range of sensor output above the enzyme K_m of 0.8 mM so that direct clinical determination of lactate could be carried out. The influence of external variables and internal parameters such as enzyme concentration were studied. These experiments were aimed at assessing the needs for invasive lactate monitoring.

The second part of the chapter deals with the assay of cholesterol. Cholesterol belongs to the steroid family; these compounds have a characteristic multi-ring structure the cyclopentaperhydrophenanthrene nucleus. Synthesis is in the liver and then esterified with fatty acids (phosphatidyl choline) is then transported in the body as lipoproteins. Non-esterified cholesterol is present in high concentration in brain and nerve tissue. It is also a chemical precursor of steroid hormones, cholic acid and vitamin D (cholecalciferol) and is a component of gallstones. Normal total blood levels are between 3.1-5.7 mM. Elevated blood cholesterol is associated with atherosclerosis. In this pathological condition, cholesterol accumulation takes place in blood vessels causing wall occlusion and eventual heart disease. A close relation between

total blood levels and low density lipoprotein cholesterol is seen. Measurement of cholesterol is thus of general screening value.

Enzymic spectrophotometric assay utilising cholesterol esterase and cholesterol oxidase has been proposed (Karube 1982). The assay end point involves the reaction of hydrogen peroxide (product) with phenol and 4-aminoantipyrine in the presence of peroxidase to form a coloured derivative. However this and other methods are complicated and involve multiple steps. Assay times are therefore often prolonged, and cost is high due to expensive soluble enzyme use. Measurements may also be influenced by interfering substances e.g., bilirubin and ascorbic acid.

Huang et al (1975) described a fluorometric technique to measure total cholesterol in serum. Hydrogen peroxide evolved from the cholesterol ester hydrolase/cholesterol oxidase reaction was used to oxidize a nonfluorescent to a highly fluorescent compound in the presence of peroxidase i.e.,:-

$$H_2O_2 + \underset{\text{(fluorophor)}}{\text{homovanillic acid}} \xrightarrow{\text{peroxidase}} \underset{\text{compound HVA)}}{\text{fluorescent (oxidised}} \qquad (7.1f)$$

These workers found that a calibration curve of fluorescence change per 5 minutes (Δ f/5 min) against cholesterol concentration was linear from 0-4g of total cholesterol per litre in serum. The method was specific, precise and accurate with results being comparable to current colorimetric enzymatic methods including the Liebarmann-Burchard method ($r=0.98$).

Electrochemical methods of cholesterol detection involve amperometric measurement of hydrogen peroxide decay at a platinum anode polarised at +650 mV or the consumption of oxygen at a platinum working electrode polarised at -650 mV vs a silver/silver chloride reference electrode. Esterified cholesterol is firstly hydrolysed using the enzyme, cholesterol ester hydrolase (esterase):-

Cholesterol ester + H_2O → Cholesterol + fatty acid (7.1g)

The cholesterol oxidase then dehydrogenates the hydroxyl group at the 3-beta position of free cholesterol in the presence of detergent with the concomitant formation of hydrogen peroxide or the consumption of an equivalent amount of dissolved oxygen.

Cholesterol + O_2 → Cholest-4-en-3-one + H_2O_2 (7.1h)

Clark et al (1981) used a cellulose acetate permselective inner membrane in conjunction with an outer porous polycarbonate membrane in their hydrogen peroxide-cholesterol sensor. Standard solution solubilised using sodium deoxycholate was injected directly into a heated (50 °C), thermostatted, cuvette containing soluble enzymes, cholesterol oxidase and esterase. The major disadvantage of the method was that it could not be used in whole blood, as dissolved hydrogen peroxide was destroyed by erythrocytic catalase as soon as it was formed.

Karube et al (1982) immobilised cholesterol oxidase and esterase in an agarose gel while Bertrand et al (1981) reported the immobilisation of cholesterol oxidase onto collagen films. This arrangement allowed cholesterol to be detected at concentrations of about 10^{-8} M and produced a linear calibration curve linear over the range of 10^{-7}-10^{-5} M.

A second electrode containing no enzyme was used to compensate for electrochemical interferences found in serum. Free cholesterol was determined in reconstituted sera using this electrode.

Mascini et al (1983) described an enzyme electrode which relied on oxygen consumption. Cholesterol oxidase was immobilised onto a nylon net and fixed onto a Clark type oxygen electrode. This procedure allowed the determination of unesterified and total cholesterol in human bile samples. A reasonable correlation coefficient of 0.99 was obtained when the results were compared with a spectrophotometric procedure. The lifetime of the electrode was more than 2 weeks during which more than 300 determinations were made.

Dietschy et al (1976) developed a method for free and esterified cholesterol using an oxygen electrode in a modified glucose analyzer. Soluble cholesterol oxidase and cholesterol ester hydrolase were used and cholesterol in all the major lipoprotein fractions (VLDL, LDL, HDL and chylomicrons) was assayed. This procedure was utilised for the assay of sterols in subcellular fractions of cells. Here a high concentration of Triton X100 (0.5-2.0% v/v) inclusion in buffer caused a marked decrease in cholesterol ester hydrolysis by the decrease in cholesterol ester hydrolase activity. Very large triglyceride particles (chylomicrons) which could not solubilised by detergent interfered with the assay, possibly by adsorption onto the teflon membrane covering the oxygen electrode and interfering with oxygen diffusion.

Kameno et al (1977) also repoorted a method for cholesterol

determination using an oxygen electrode. Two procedures were employed; an "end-point method" in which the consumed oxygen at the end point of the reaction was measured until all the cholesterol was used up, and a "rate method" in which the oxygen consumption at the onset of reaction was measured. There were several problems associated with the former method of detection e.g., the chamber had to be of a closed type in order to isolate the reaction medium from the air and also long response times were obtained. For the latter, a non-linear relationship was found between the oxygen consumption rate and the concentration of cholesterol. This was addressed by the use of a competitive inhibitor and by the use of a low substrate concentration in the reaction medium. The cholesterol ester for the release of free cholesterol had to be pre-hydrolysed prior to measurement. This was done using lipoprotein lipase instead of cholesterol esterase. Reported and Christian (1977) measured total cholesterol in serum or plasma by measuring the rate of oxygen consumption. These workers pre-hydrolysed serum cholesterol esters to form free cholesterol by saponification using ethanolic KOH.

Recently total cholesterol was determined by co-entrapment of cholesterol esterase and oxidase in a polypyrrole film (Hin and Lowe, 1992). In situ entrapment of cholesterol oxidase (COD) was realised by poising the platinum working electrode at +800 mV vs a KCl saturated calomel electrode in a nitrogen purged supporting electrolyte (0.1 M sodium phosphate with added sodium perchlorate) containing 0.1 M pyrrole and 5 mg/ml COD. The apparent K_m for cholesterol oxidase was compared to 14-33 μM for soluble COD (Richmond 1973).

7.2 Needle lactate electrodes

7.2.1 Current-voltage relationships

Lactate oxidase was crosslinked with glutaraldehyde (1% w/v) onto a needle electrode and the response to substrate at various applied polarising potentials recorded. All three current-potential curves follow a similar response pattern. At the lowest polarising potential (+100 mV vs stainless steel) the amperometric current is minimal. This is followed by a sharp elevation in current as the potential is increased from +200-+500 mV (Figure 7.2.1). The magnitude of the maximal diffusion limited current is governed by substrate concentration. The highest current is obtained with 1.5 mM lactate.

7.2.2 Effect of polyurethane on response

Successive application of increasing concentration of polyurethane ("Pellethane^{R}", 2% and 3% w/v in tetrahydrofuran) causes a progressive decrease in sensor sensitivity with an accompanied increase in response time of 5-7 minutes (fully-coated) from 20s (uncoated). Simultaneously an increase in the linear range is obtained (Figures 7.2.2a and 7.2.2b). The maximal linear range of >28 mM is seen with the application of 4 or 5 coats. Sensor sensitivities of 0.8 nA/mM and 2 nA/mM for the fully coated electrodes is obtained using 3% w/v and 2% w/v polyurethane membranes respectively. Sequential application of increasing concentration of polyurethane causes a dramatic increase in linearity and a decreased current amplitude even after 3 or 4 coats. The fully-coated electrode (4 coats of "Pellethane") gave a sensitivity of 0.5 nA/mM (Figure 7.2.2c) and 1.0 nA/mM (Trixene SC762) (Figure 7.2.2d).

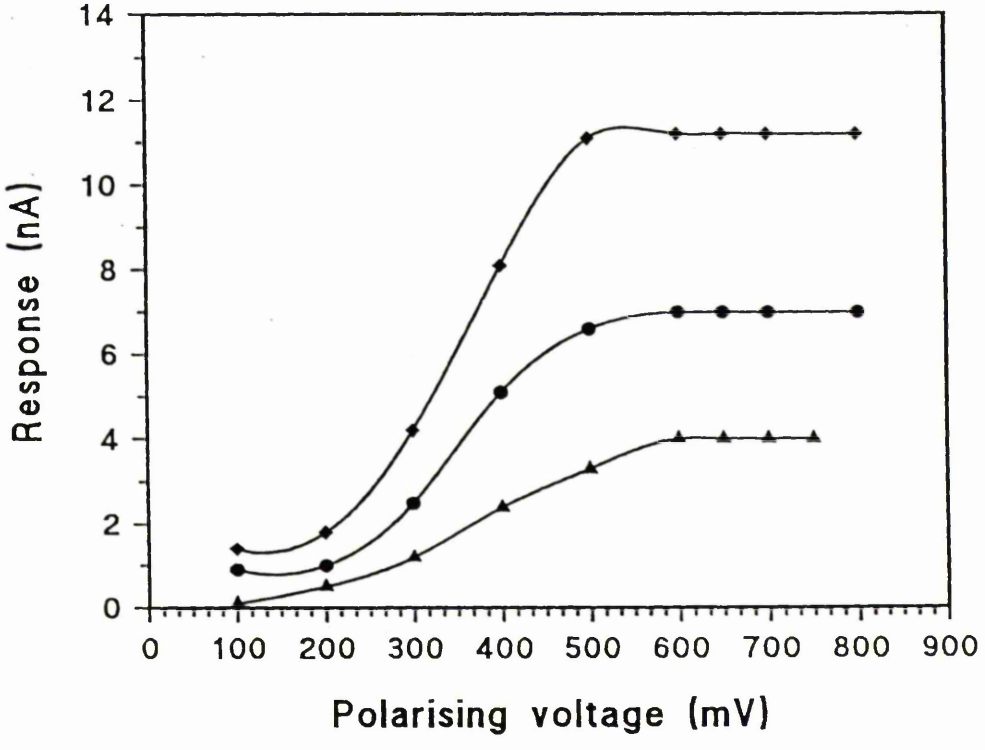
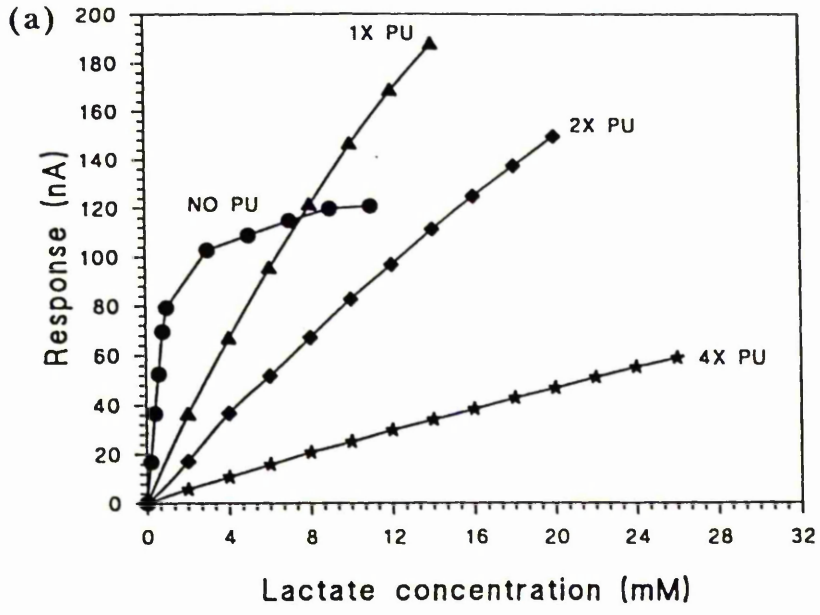


Figure 7.2.1

Voltammograms for crosslinked lactate oxidase on a needle electrode using (▲) 0.5 mM, (●) 1.0 mM and (◆) 1.5 mM lactate.



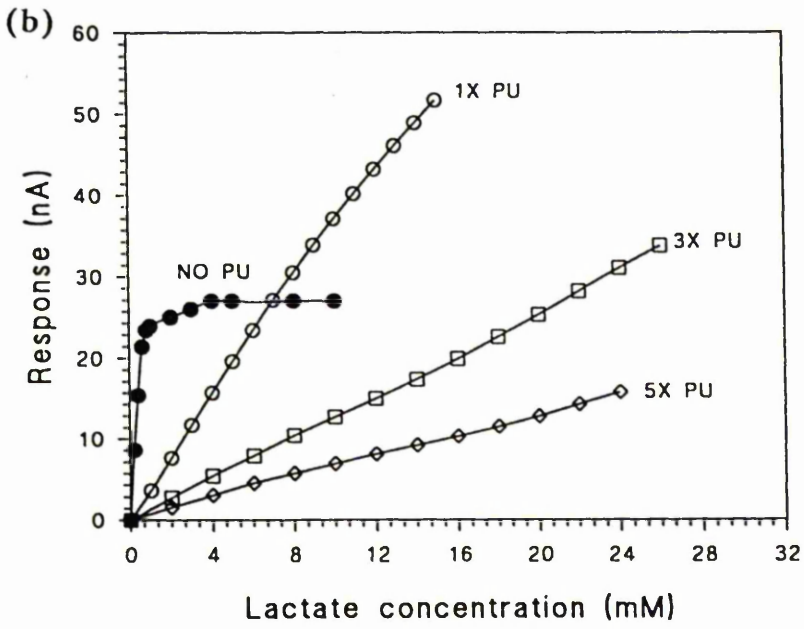


Figure 7.2.2

Effect of multiple polyurethane, PU ("Pellethane") membranes on electrode response for (a) 2% w/v in tetrahydrofuran; (b) 3% w/v in tetrahydrofuran.

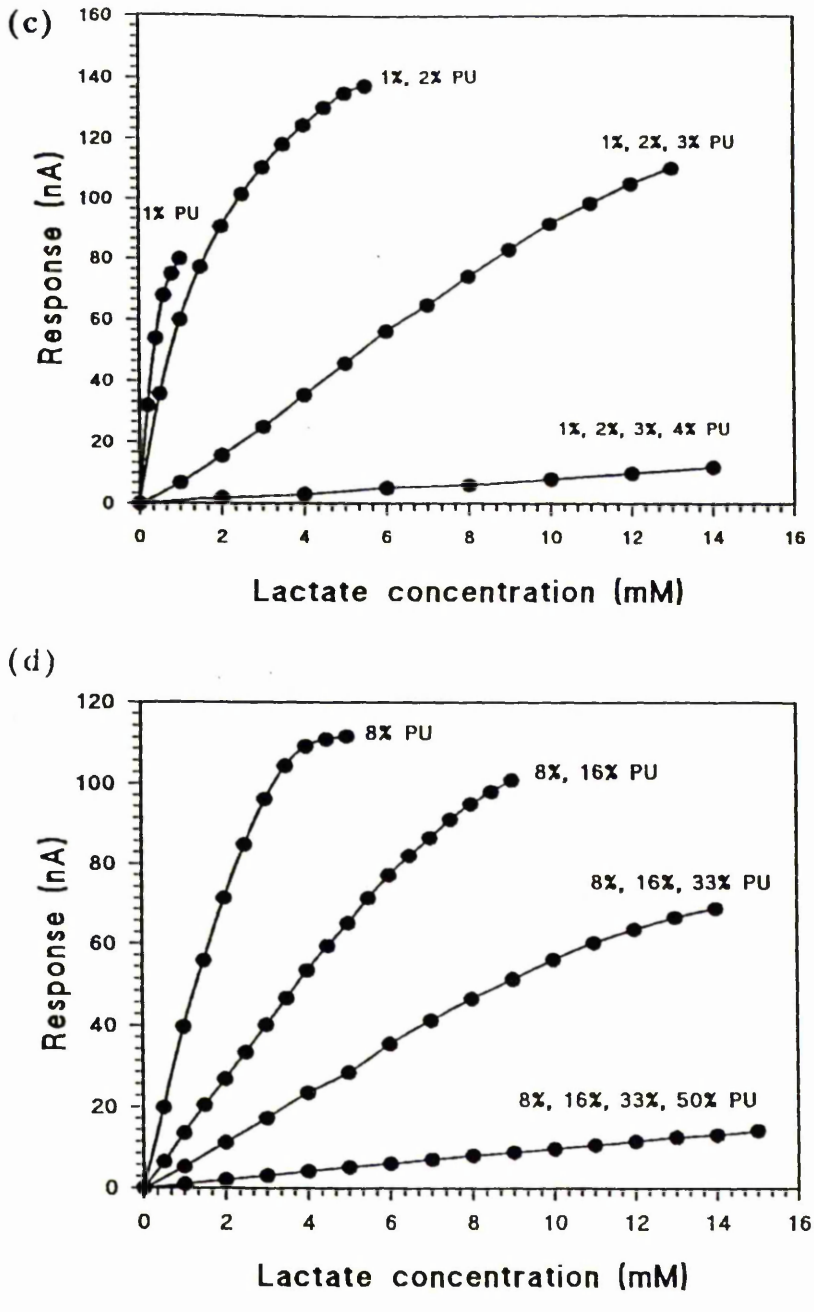


Figure 7.2.2

Sequential dipcoating using increasing concentration of polyurethane, PU for (c) Pellethane and (d) Trixene SC762.

7.2.3 Stir dependence

The response of an optimised (fully-coated) lactate electrode is stir independent over the 0-22 mM concentration range as shown in Figure 7.2.3.

7.2.4 pH dependence

An optimised needle gives pH-independent responses between 6.5 and 7.5 (Figure 7.2.4) at lactate concentrations between 2 and 6 mM. The lowest response is obtained at a pH of ~5.3 and is followed by a gradual rise until pH 5.8.

7.2.5 Temperature dependence

For an optimised needle electrode an increase in response with temperature elevations from 25 to 38 °C is seen (Figure 7.2.5). A temperature dependence of 0.9 nA/°C (2 mM lactate) and 1.0 nA/°C (4 mM lactate) is observed as in Figure 7.2.5.

7.3 Oxygen-type 2-electrode cell for lactate

7.3.1 Effect of polycarbonate membrane porosity

An increase in linear range and decreased response magnitude is apparent as polycarbonate pore size is reduced from 0.4 μm to 0.01 μm. The highest initial response is obtained for a 0.4 μm polycarbonate laminate although saturation kinetics are displayed above 1 mM. Similarly loss of linearity occurs at porosities of 0.05 μm (2.5 mM) and 0.03 μm (3 mM). At a porosity of 0.01 μm a linear range of ~8 mM at a sensitivity of ~50 nA/mM are obtained and saturation is incomplete for the useful clinical range (Figure 7.3.1).

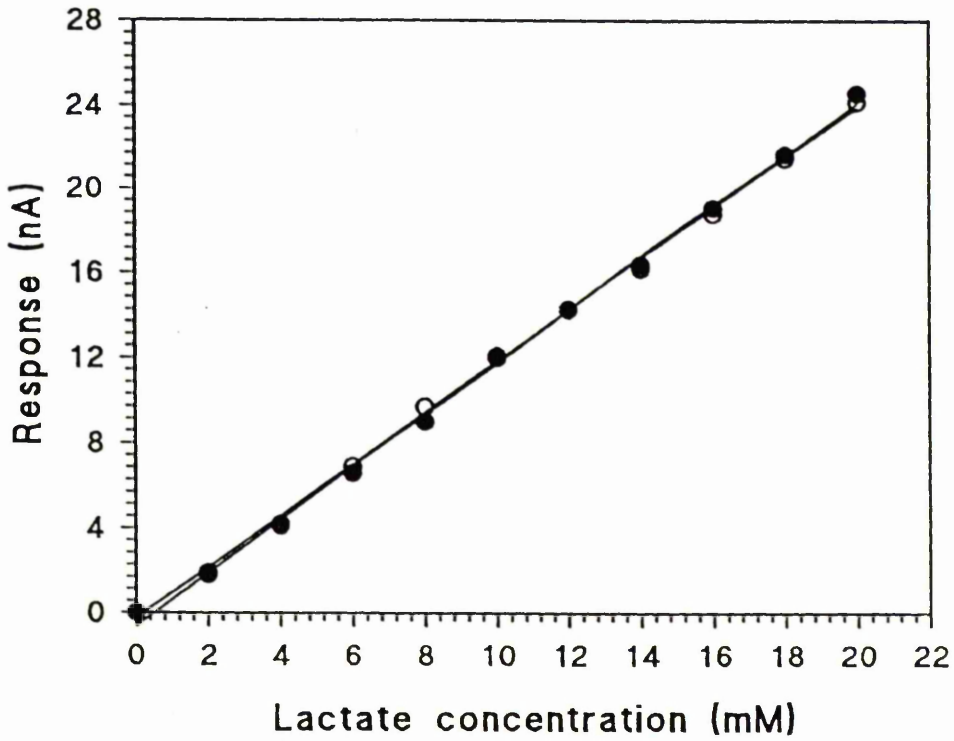


Figure 7.2.3

Response of an fully-coated needle enzyme electrode consisting of and internal polyethersulphone (4% w/v in dimethylsulphoxide) and 4 external polyurethane "Pellethane" (3% w/v in tetrahydrofuran) membranes under (●) stirred and (○) unstirred conditions.

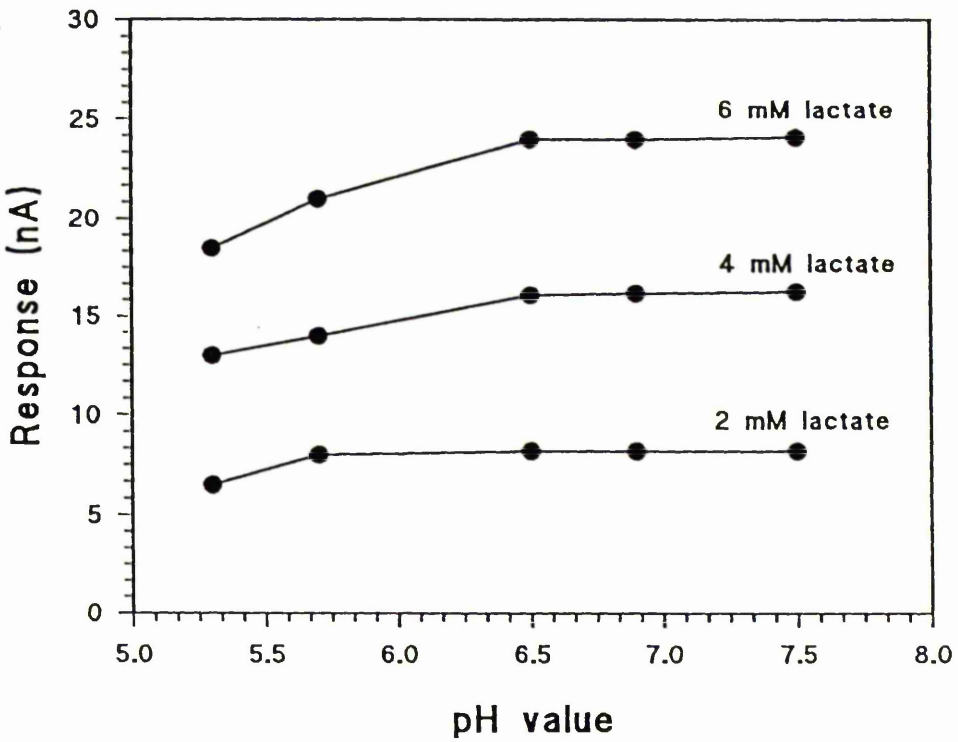


Figure 7.2.4

Effect of pH variation on the response of a fully-coated needle enzyme electrode consisting of an internal polyethersulphone (4% w/v in dimethylsulphoxide) and 4 external polyurethane "Pellethane" (3% w/v in tetrahydrofuran) membranes.

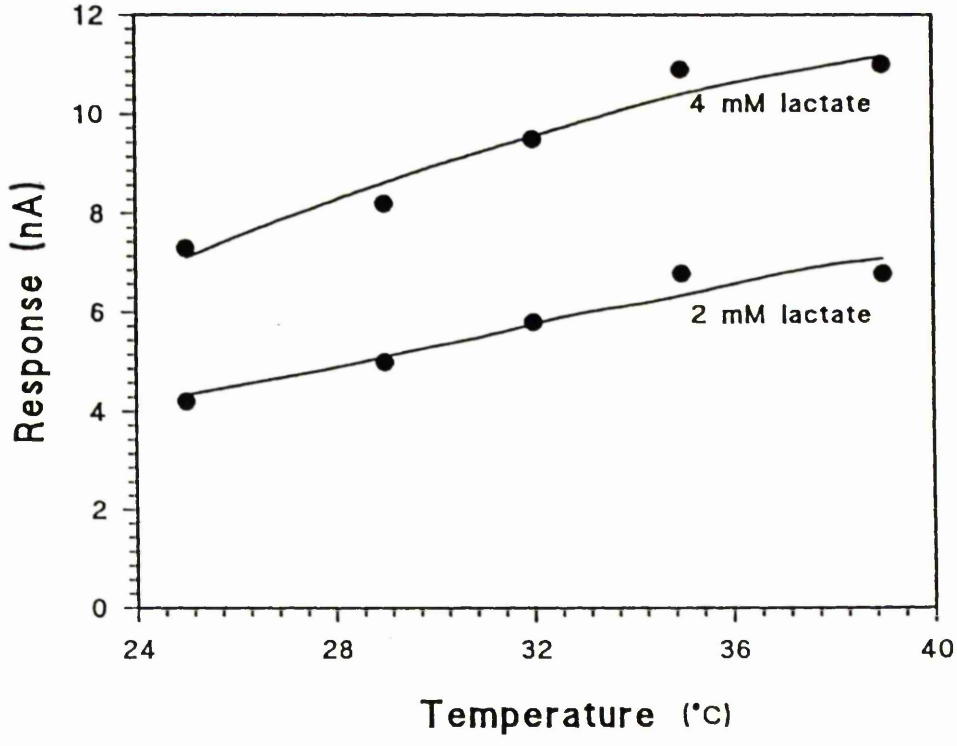


Figure 7.2.5

Temperature dependence on the response of a fully-coated needle electrode consisting an internal polyethersulphone (4% w/v in dimethylsulphoxide) and 4 external polyurethane "Pellethane" (3% w/v in tetrahydrofuran) membranes.

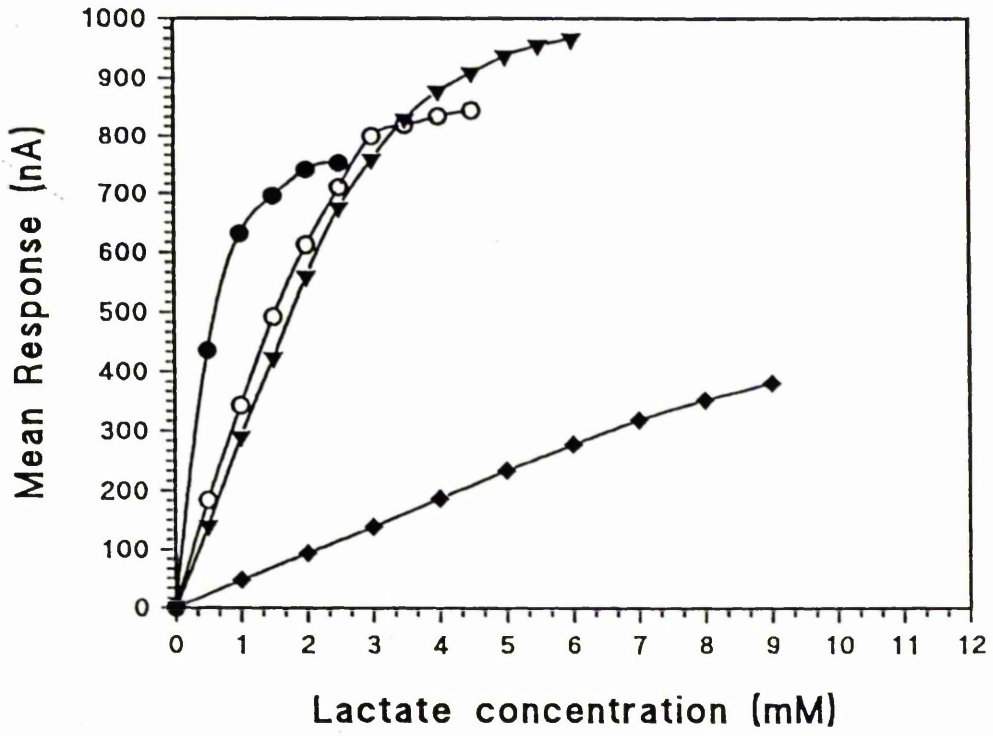


Figure 7.3.1

Effect of polycarbonate membrane pore size variation on lactate electrode response for (●) 0.4 μm/0.4 μm, (○) 0.05 μm/0.05 μm, (▼) 0.03 μm/0.03 μm and (♦) 0.01 μm/0.01 μm enzyme laminates.

7.3.2 Effect of pH

The influence of pH on lactate response is seen in Figure 7.3.2. A sharp rise in current between 3 and 5 occurs for both 1 and 2 mM lactate solutions. Between pH 5.5 and 7.0 maximal current is obtained, however this is followed by a decline in magnitude at pH 8-10.

7.3.3 Stir dependence

No significant difference between stirred and unstirred responses is obtained for a 0.01 μm polycarbonate membrane (outer)/0.05 μm polycarbonate membrane (inner) laminate (Figure 7.3.3).

7.3.4 Enzyme loading variation

An increase in current with increasing loading in the initial enzyme reagent solution (LOD) from 15 mg/ml to 30 mg/ml and 60 mg/ml (Figure 7.3.4) occurs. Response sensitivities of 2 nA/mM and 25 nA/mM are observed for the 15 mg/ml and 60 mg/ml loadings respectively.

7.3.5 Stability

The aqueous calibration of a lactate electrode is relatively stable for 3 days, but declines after this. The response is still relatively acceptable until 5 days (Figure 7.3.5).

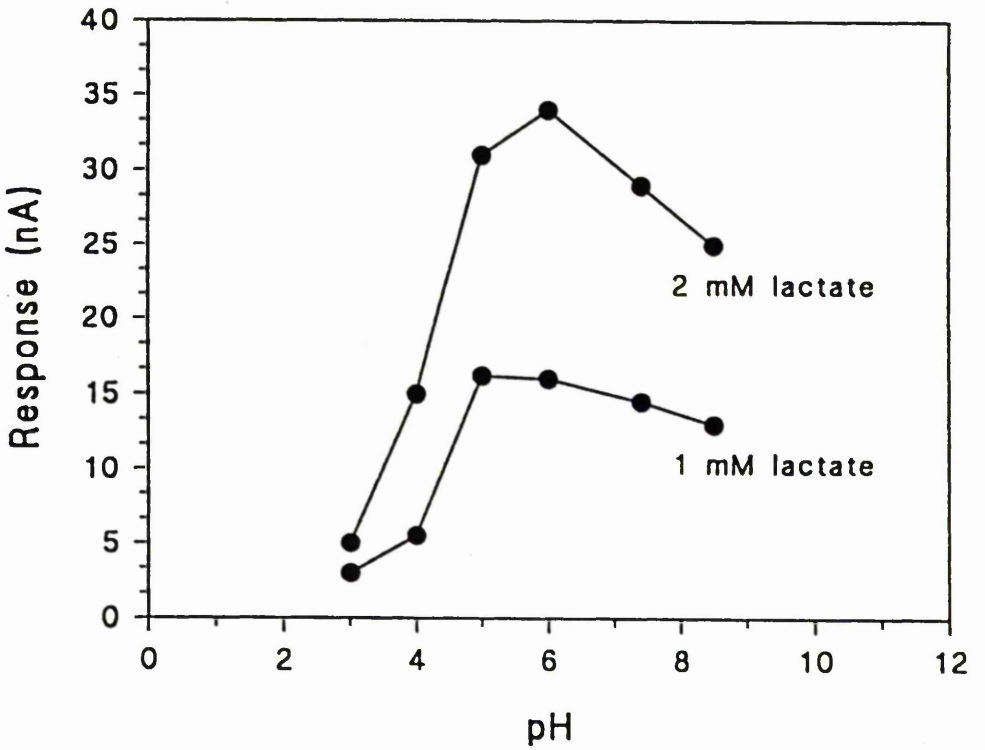


Figure 7.3.2

Effect of pH variation on the response of a 0.01 μm/0.01 μm pore size polycarbonate laminate.

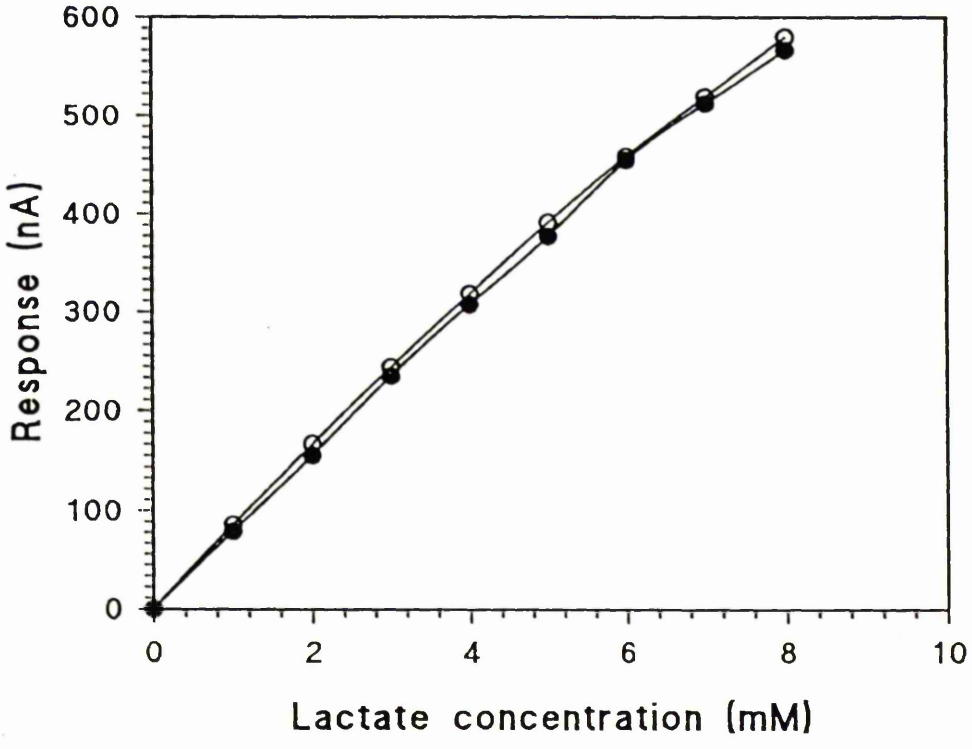


Figure 7.3.3

Response of a 0.01 μm polycarbonate (external) 0.05 μm (internal) under stirred (●) and unstirred (○) conditions.

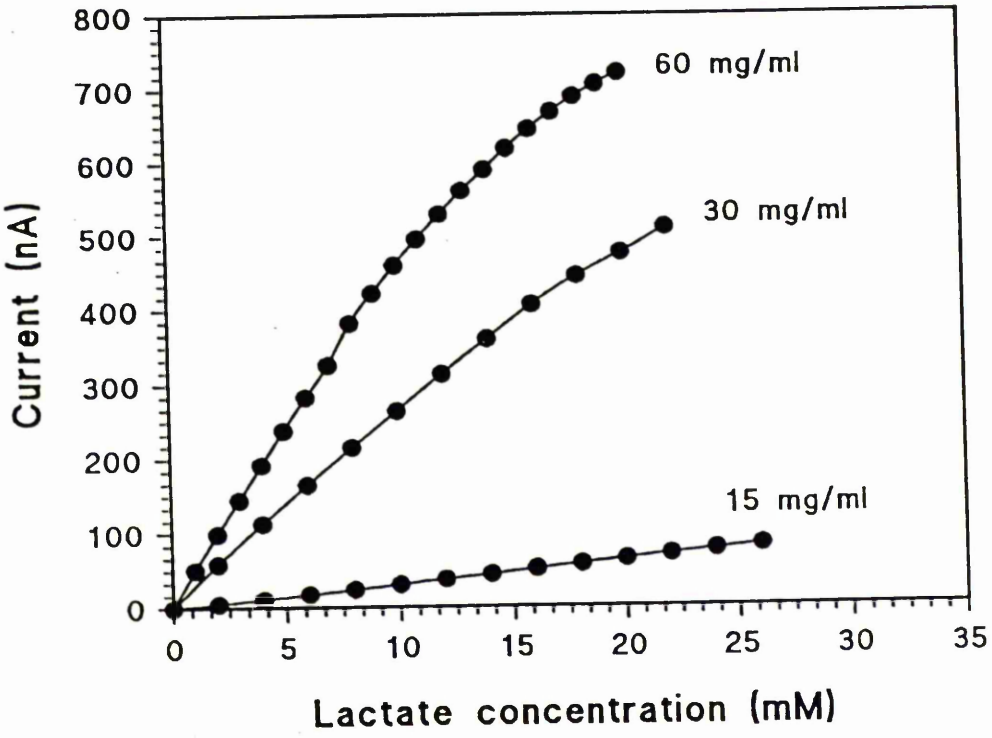


Figure 7.3.4

Effect of enzyme loading variation for a 0.01 μm/0.01 μm polycarbonate laminate.

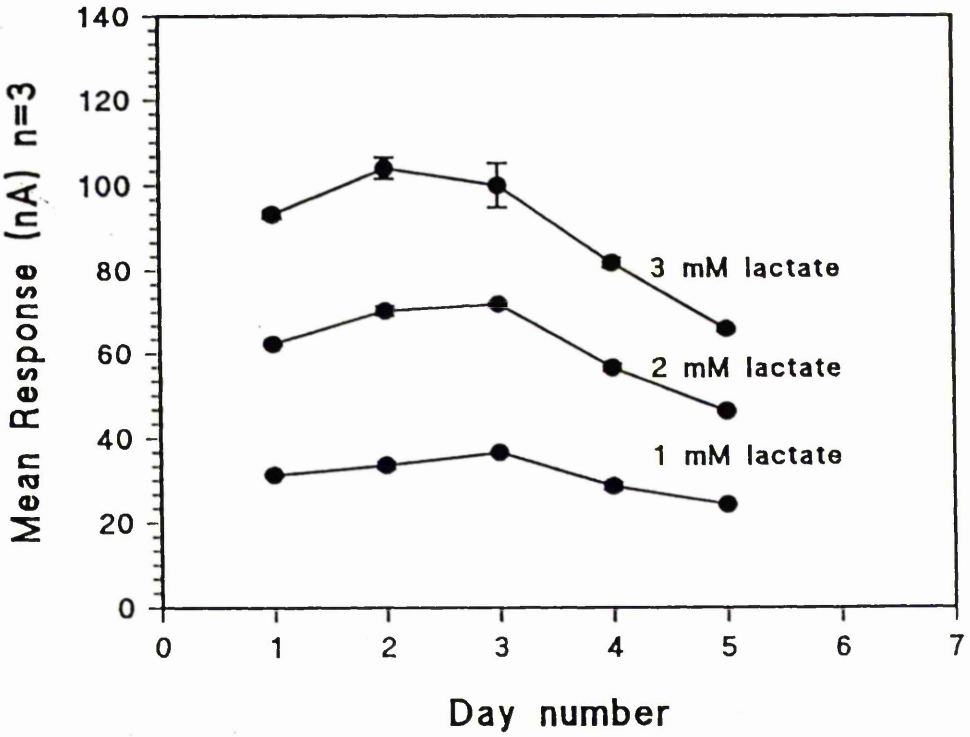


Figure 7.3.5

Stability over a 0.01 μm/0.01 μm polycarbonate membrane laminate in aqueous over five days.

7.4 Cholesterol enzyme electrodes (oxygen electrode)

7.4.1 Solubilisation of cholesterol

Cholesterol is essentially insoluble in water, therefore it was solubilised using ethanol. Since ethanol is electroactive and can be anodically oxidised to the aldehyde or carboxylic acid (Allen MJ 1958; Parker 1973) concentration dependent responses to cholesterol solutions in ethanol were obtained without enzyme (Table 7.4.1a). Ethanol dilution in buffer caused a reduction in background current due to decreased level reaching the electrode. As an alternate, isopropanol was used but amperometric responses without enzyme due to the possible electroactive nature of isopropanol were still noticeable (Table 7.4.1b). Eventually a solubilisation procedure employed by Clark et al (1981) was used. Cholesterol (208 mg) was dissolved into a solution of sodium deoxycholate (1 g in 10 ml water) for which no background interference in the absence of enzyme was recorded.

7.4.2 Cholesterol oxidase immobilisation

Polyvinyl alcohol entrapment produced an improved signal to cholesterol compared to glutaraldehyde crosslinking (Figure 7.4.2). However the current with polyvinyl alcohol entrapment was not linear above 0.2 mM and complete loss of signal was observed after 90 minutes. The responses were stable for long periods for crosslinked enzyme.

7.4.3 Enzyme loading variation

An increase in the level of COD (Cholesterol oxidase + BSA) relative to glutaraldehyde caused an initial amplified response however rapid

(a)	[Cholesterol] (mM) in 100% ethanol	Response (nA)
	0.4	0.7
	0.8	1.2
	1.4	1.7
	1.8	2.5
	2.2	3.0
	2.6	3.4
	3.0	3.8
	3.4	4.2

(b)	[Cholesterol] (mM) in isopropanol	Response (nA)
	0.2	0.4
	0.4	0.7
	0.6	1.1
	0.8	1.5
	1.0	1.9
	1.2	2.2
	1.4	2.6

Tables 7.4.1 a and b

Electrode responses to a nonenzymic 0.03 μm polycarbonate laminates to cholesterol solutions made up in (a) ethanol (b) isopropanol.

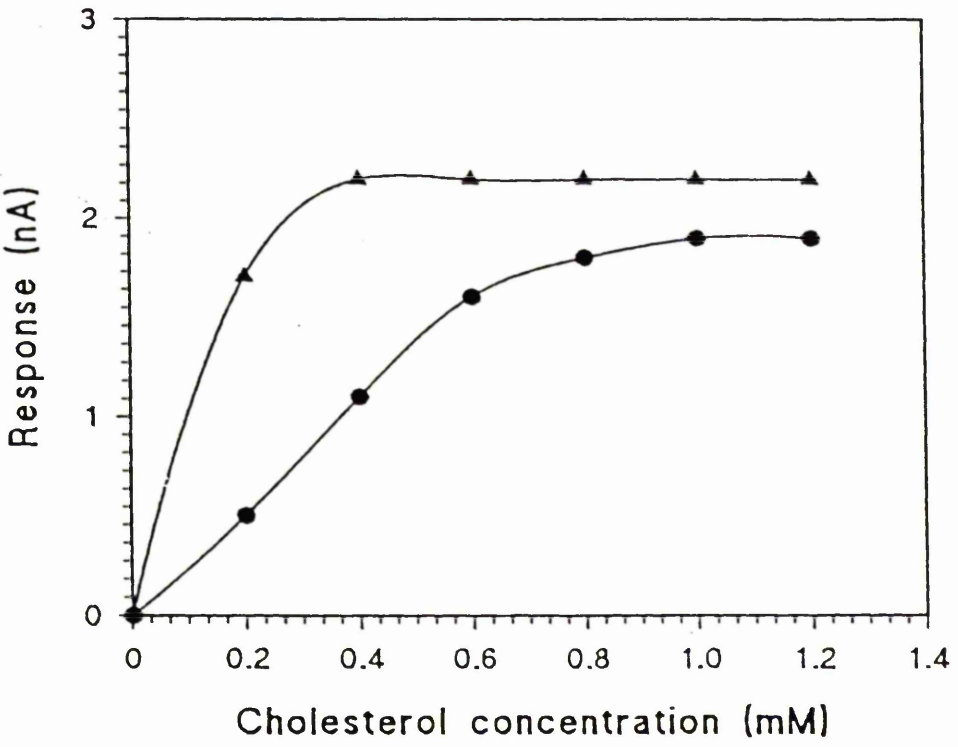


Figure 7.4.2

Effect of enzyme immobilisation on cholesterol electrode response using:- (▲) polyvinyl alcohol entrapment (•) glutaraldehyde crosslinking.

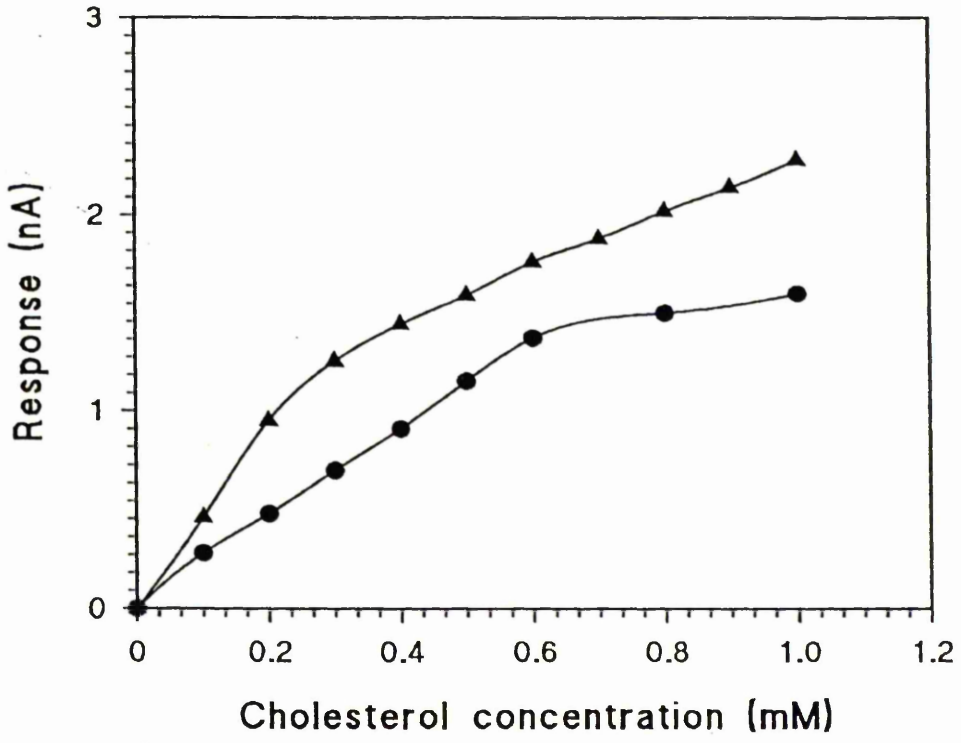


Figure 7.4.3

Effect of enzyme loading variation on cholesterol response using:- (▲) 2 μl glutaraldehyde and 7 μl COD (●) 3 μl glutaraldehyde and 6 μl COD.

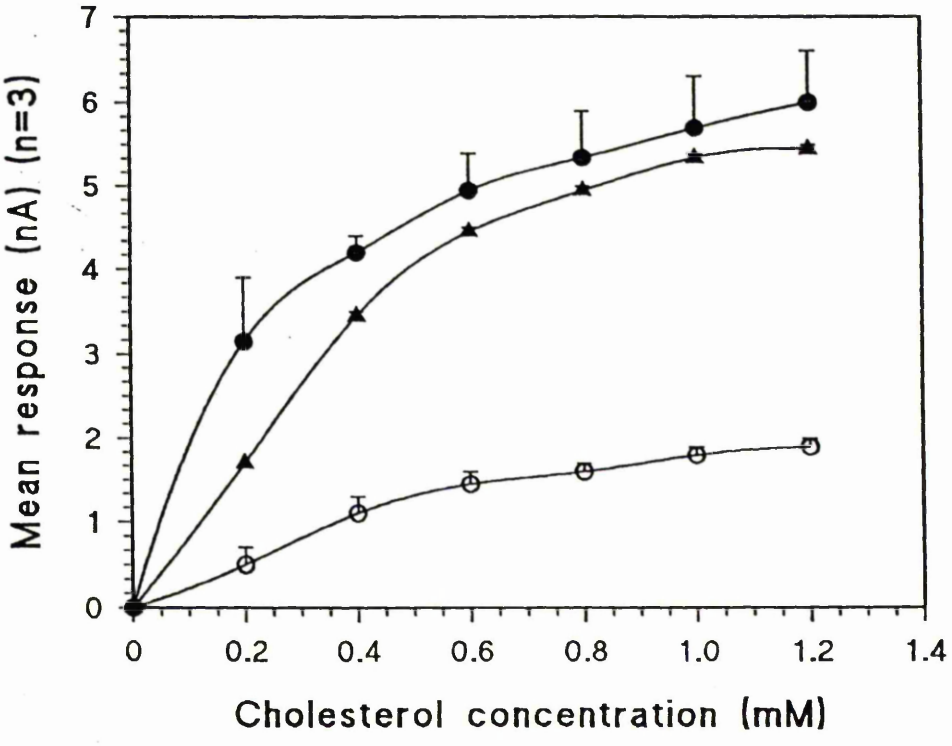


Figure 7.4.4

Effect of pore size variation on cholesterol electrode respones for the following polycarbonate membrane laminates:-
(•) 1.0 μm/1.0 μm, (▲) 0.4 μm/0.4 μm and (o) 0.03 μm/0.03 μm.

loss of linearity for cholesterol was observed after 0.2 mM. A 2:1 loading of COD:Glutaraldehyde caused a decrease in sensor signal accompanied by an increased linear range to at least 0.6 mM (Figure 7.4.3).

7.4.4 Effect of polycarbonate membrane pore size

A reduction in membrane porosity to 0.03 μm did not result in any alteration in linearity (0.4 mM) compared to the 0.4 μm laminate. However the magnitude of the response at 0.2 mM was approximately reduced by 75% compared to the 0.4 μm laminate. The 1.0 μm laminate yields the greatest initial response accompanied by a rapid loss of linearity at 0.2 mM (Figure 7.4.4).

7.5 DISCUSSION

The polarographic waves obtained in Figure 7.2.1 show that quite adequate enzyme loading can be achieved with the low activity lactate oxidase enzyme. Previously the enzyme was also crosslinked using glutaraldehyde onto cellulose acetate (Tsuchida et al 1985). Stability studies indicated that the immobilised enzyme electrode retained 107% of its original activity over 13 days and LOD K_m was relatively unaffected.

The polarographic waves in Figure 7.2.1 show a sharp current rise is observed followed by a plateau region which occurs at the same polarising voltage (600-800 mV) regardless of the substrate (lactate) concentration. Therefore a polarising voltage of +650 mV vs stainless

steel can again be used so that the maximum stable lactate response is obtained using the minimal power requirement.

The effect of addition of external diffusion-limiting polyurethane membranes (Figures 7.2.2a-d) was studied. Here the response is again limited by the rate of lactate diffusion to the enzyme layer rather than by the kinetics of the enzyme itself. This presumably results in a lowered concentration of lactate within the enzyme layer, enabling a linear response to be obtained. The low permeability of polyurethane to lactate as with glucose allowed less substrate entry to the enzyme. Successive application of multiple polyurethane coatings caused the barrier thickness to increase. This multiple membrane application technique has been successfully exploited by Vadgama et al (1989) and Sternberg et al (1988).

Recently Hu et al (1993) described a needle-type lactate sensor consisting of a single outer polyurethane membrane. However a maximal linear range of 28 mM lactate was achieved and large variabilities in the linearity characteristics were obtained between each sensor. However consistent elevations of linearity of above 28 mM (Figures 7.2.2 a and b) were seen in the presented data. Alternatively other workers have reduced the flux of lactate by using low permeability organosilane treated external polycarbonate membranes (Mullen et al 1986; Battersby and Vadgama 1988). However linearities of only 8-11 mM were found.

With outer polyurethane membrane stirring would be expected to have

no influence on signal output (Figure 7.2.3); these results are consistent with those obtained by Hu et al (1993). The pH dependency study of lactate oxidase showed that responses were independent of pH between 6.5 and 7.5 (Figure 7.2.4); This is in accordance with the results reported by Hu et al (1993), Mullen et al (1986) and Tsuchida et al (1985). According to Tsuchida et al (1985) the soluble enzyme shows similar pH dependence characteristics to the immobilised one. Temperature dependence studies revealed a signal increase of 0.9 nA/°C over the 30-40 °C range. However a smaller current variation of 0.03 nA/°C was reported by Mullen et al (1986), 0.08 nA/°C according to Hu et al (1993). A more similar result of 0.6 nA/°C was reported by Faridnia et al (1993). These workers also found that even with the glutaraldehyde immobilised enzyme irreversible denaturation occurred at elevated temperatures.

Lactate oxidase has an absolute requirement for oxygen. The internal membrane permeability will affect H_2O_2 and O_2 flux, this in turn will alter the rate of amperometrically produced oxygen returning to the enzyme layer. Measurements of permeability coefficients for H_2O_2 and O_2 for microporous polycarbonate membranes have been for glucose electrodes (Higson et al 1993). This work suggests that polycarbonate membranes of decreasing pore size progressively show higher O_2 : H_2O_2 permeability coefficient ratios which was associated with extended linearity although sensitivity diminished so it is possible here also that as well as sustaining enzyme layer oxygen levels from the surrounding solution, the entry of electrochemically generated oxygen will also be prevented by small pore membranes.

The extended linear for Figure 7.3.1 could be due to the relative flux of oxygen. However for the larger pore size membranes oxygen possibly which could not be replenished adequately at the higher lactate levels. The problem of oxygen rate-limitation for glucose oxidase based sensors has been previously addressed by Lobel and Rishpon (1979). The electrode system used consisted of a Millipore filter containing immobilised enzyme overlaid with a dialysis membrane. Here linear calibration curves of up to 15 mM glucose were obtained when oxygen was allowed to diffuse into the system and the linearity was restricted to 5 mM when oxygen diffusion was prevented by application of a polyethylene film over the anode. If nitrogen was bubbled into the test solution no responses to variation in glucose concentration were observed.

Response times increased from 10s (0.4 μm laminate) to 50s (0.01 μm laminate) due to restricted lactate flux. An additional factor of enzyme layer thickness has to be considered, however. Even with large pore size laminates a thick enzyme layer will cause prolonged response times. Response time is proportional to d^2/D (where d = enzyme layer thickness; D = substrate diffusion coefficient) a reduction in enzyme layer thickness gave faster response times. Guilbault and Montalvo (1970) obtained a response time of 26 s at a thickness of 60 μm which increased to 59 s at 350 μm.

The pH dependency of immobilised lactate oxidase is governed by the presence of ionizable groups of the enzyme protein. An optimal pH of ~5.5-7.0 was observed with a decline above pH 8.0 (Figure 7.3.2).

This indicates that predominantly basic amino groups with a pK_a of ~6.3 are present. Michaelis (1926) described a simple model for the pH behaviour of many enzymes. The presented results (Figure 7.3.2) agree with to those of Mullen et al (1986) and Tsuchida et al (1985). However a shift in the pH optimum to 8-9 for lactate oxidase was found by Katakis and Heller (1992). Here the redox centre flavin adenine mononucleotide (FMN) of the enzyme was attached to the electrode via a redox polymer (polyvinylpyridine) N-derivatised with bromoethylamine and $Os(bpy)_2Cl_2$ to act an relay for electron transfer. This shift occurred because the enzyme acquires a greater negative charge at high pH when its bases are deprotonated and acids ionized, and it forms a close association with a polycationic redox polymer enabling electron transfer to occur.

Stirring did not influence electrode response (Figure 7.3.3). Similarly stir independence was observed by Battersby and Vadgama (1988) a polycarbonate membrane mounted lactate needle electrodes. The needle responses in aqueous solution was unaffected by stirring rate variation. Stirring does however appear to affect response times which increased by 20 s under unstirred conditions.

A proportional relationship between enzyme loading and lactate response was obtained (Figure 7.3.4) with increased linearity at lower loadings. This could have been due to oxygen (cosubstrate) flux being more adequate at lower enzyme activities. The impact of enzyme loading variation from 0.2-1.0 Ucm^{-2} for lactate oxidase was studied (Pfeiffer et al 1993). A linear increase in current with increased loading

between 0.1-0.8 Ucm^{-2} was obtained. At higher loadings a saturation value was attained when no further increase in current occurred. This shows that the reaction is under diffusional control at high enzyme concentrations. The stability study for the lactate electrode indicated that responses were fairly stable over a 5-day period especially at 1 mM (Figure 7.3.5).

Cholesterol was found to be soluble in non-aqueous solvents e.g. ethanol and isopropanol. However the electroactive nature of these alcohols resulted in constant background interference and therefore were not used. Eventually sodium deoxycholate was used to solubilise cholesterol. Firstly the method of cholesterol oxidase immobilisation was varied (Figure 7.4.2). Physical entrapment using polyvinyl alcohol resulted in enhanced sensitivity although the responses were unstable possibly due to leaching of the enzyme protein out of the matrix. Glutaraldehyde crosslinking produced much diminished responses presumably due to denaturation of the enzyme by forming direct covalent bonds with the enzyme leading to distortion of the active enzyme conformation possibly involving the active site. Therefore this method for cholesterol oxidase may not be suitable due to the low initial activity ~45 U/mg protein compared to 138 U/mg for glucose oxidase. However the responses were stable and linear to 0.4 mM (Figure 7.4.2). Mascini et al (1983) described an enzyme immobilisation procedure onto a nylon net and the usable lifetime was reported to be two weeks. It was advantageous because the net permitted free diffusion of substrate/product and a large amount of activity remained after the process. Variation of the enzyme:glutaraldehyde ratio did not produce

any significant improvement in sensitivity or linearity (Figure 7.4.3).

The porosity of the polycarbonate membranes did not have a great impact on linear range (Figure 7.4.4). However a considerable diminution in signal size was observed e.g. at 0.2 mM cholesterol the responses were 0.4 nA and 30 nA for 0.03 μm and 1.0 μm pore size polycarbonate membrane laminates respectively. The limited linearity was possibly due to the low inherent K_m, 14-33 μM of the enzyme. The incomplete solubilisation of the total cholesterol by micelle formation could possibly be an additional factor. Previously it has been shown that cholesterol oxidase activity could be increased by utilisation of short chain lecithins for solubilisation which were more effective than Triton X-100 (Burns et al 1981). This was due the larger interfacial surface area presented to the enzyme.

7.6 Summary

The in vitro performance of implantable lactate needle enzyme electrodes was investigated. Polarographic analysis confirmed that hydrogen peroxide was the enzymic product detected at +650 mV vs stainless steel. The resultant current was dependent on substrate levels. Effective extensions in the clinically relevant dynamic range were obtained by successive application of semi-permeable polyurethane ("Pellethane" and Trixene SC762) membranes. A maximal linear range of <28 mM was observed although the response sensitivity was reduced to 0.8-2.0 nA/mM ("Pellethane"). The diffusion-limiting properties of polyurethane was illustrated by the relative independence of the electrode response from external solution variables e.g. pH, temperature and stirring rate.

Lactate enzyme electrodes in a oxygen cell configuration as a prerequisite of a benchtop analyzer was studied. The impact of polycarbonate membrane porosity on lactate response was determined. The results showed that the diffusion-limiting characteristics of a 0.01 μm pore size membrane "laminate" was sufficient for clinical analysis. An exploration on the influence of enzyme loading showed that at 15 mg/ml measurable electrode responses were produced. Diffusional limitation of a 0.01 μm pore size "laminate" was illustrated by the relative independence of the response to pH and stirring rate variation. Operational stability in aqueous solution was demonstrated over a 5-day period.

A feasibility study in the development of a cholesterol enzyme electrode did not give any promising results. After the problem of substrate solubilisation was overcome, the maximal linear range of the electrode response only reached 0.6 mM. This was primarily due to the low apparent K_m of cholesterol oxidase. Furthermore the sensitivity of cholesterol detection was not sufficient for a more complete analysis to be made. Alternative immobilisation techniques e.g. polyvinylalcohol entrapment were ineffective due to the inherent instability of the electrode response.

CHAPTER EIGHT

BLOOD COMPATIBILITY

8.1 INTRODUCTION

The term "blood compatibility" may be interpreted in different ways and is dependent on whether the blood is static or flowing as well as the length of time in contact with the foreign surface. For clinical purposes an ideal biocompatible material should not give rise to thrombosis, cancer, inflammatory, toxic or allergic reactions. There should be neither destruction of the cellular elements of blood, depletion of electrolytes, damage to adjacent tissues, nor adverse immune responses. When a sensor comes into contact with a biofluid there is immediate surface fouling which is inevitable with any artificial material. The outer biosensor surface is therefore a critical component since it is in contact with the biological environment. Surface fouling occurs because blood plasma is a fluid containing the plasma proteins fibrinogen and globulin as the chief constituents. Cuypers et al (1977) studied fibrinogen attachment to chromium surfaces using ellipsometry and demonstrated fibrinogen deposition reached thicknesses of ~50 Å within five minutes at both hydrophilic and hydrophobic surfaces.

The sequence of events which occur when an artificial surface comes into contact with blood has been considered by Vroman et al (1977). Within the first few seconds there is unavoidable and competitive adsorption of various plasma proteins (Kim et al 1974) which consist of albumin, gamma-globulin, fibrinogen and prothrombin. The rapidly thickening protein film may reach 50 Å in 5 seconds. The nature of the surface created dictates the preponderance of proteins in the bound

layer, which in turn determine the intensity and magnitude of further interactions; albumin tends to passivate the surface whereas fibrinogen provokes further surface deposition (Young et al 1982; Brash et al 1974). Therefore the limited protein binding layer alters the surface texture, charge and chemistry. After one minute and when the protein layer thickness reaches 100-200 Å, platelet adhesion occurs. This is mediated by various plasma proteins including IgG, thrombin, fibronectin, factor VIII or Von Willebrand factor (Hantgan 1991). The first platelets to arrive flatten, degranulate and become sticky. Platelet degranulation leads to release of adenine dinucleotide phosphate (ADP), 5-hydroxytryptamine, serotonin and phosphoglycerides (Ito et al 1990) which could passivate the working electrode (Higson et al 1993).

The extent of platelet adhesion is determined by the interaction between enzymes produced by platelets and membrane bound oligosaccharide chains of the glycoproteins, fibrinogen and IgG (Kim et al 1974). Platelet aggregation onto polymeric surfaces including, plasticised poly(vinyl chloride), polyurethane and poly(vinyl alcohol) coated poly(vinyl chloride) has been visualised by scanning electron microscopy (Fujimoto et al 1993). The morphology of the adhered platelets were governed by the type of polymer surface, time of blood exposure and blood flow rate.

Subsequently platelets adhere to the degranulated platelets and aggregates are formed. These grow into pyramidal shapes, and within ~4 minutes, white cells are chemotactically stimulated to migrate towards the surface. The white cells become attached to the platelets

forming microthrombi which seriously disturb the flow field near the surface. The flow perturbations along with the chemical modifications of the degranulated platelets, accelerate the coagulation process (Turitto et al 1991). Fibrin strands are formed which trap cells to form a clot (Jackson and Nemerson 1980).

The surface coagulation process is complex and involves two stages:- (i) activation, in which prothrombin is converted to thrombin, its active form and (ii) conversion, where the proteolytic enzyme thrombin converts fibrinogen to a fibrin monomer and subsequent polymerisation to form a clot. Blood clotting relies on an "enzyme cascade", where one enzyme partially hydrolyses the proenzyme of another, resulting in an active enzyme which in turn activates another proenzyme and so on (Figure 8.1). The cascade can be triggered by two independent routes:- (i) by factors existing in the blood, the intrinsic system or (ii) by the presence of substances not normally present in blood, the extrinsic system. Surface activated platelets can either initiate the extrinsic cascade by activating factor VII or the intrinsic pathway via factor XII or XI activation.These sequence of events may alter the permeability characteristics of the sensor surface and cause serious diffusional restriction of the substrate. The dynamic electrode response is critically dependent on the rate of substrate mass transfer to the enzyme therefore signal size and stability are affected.

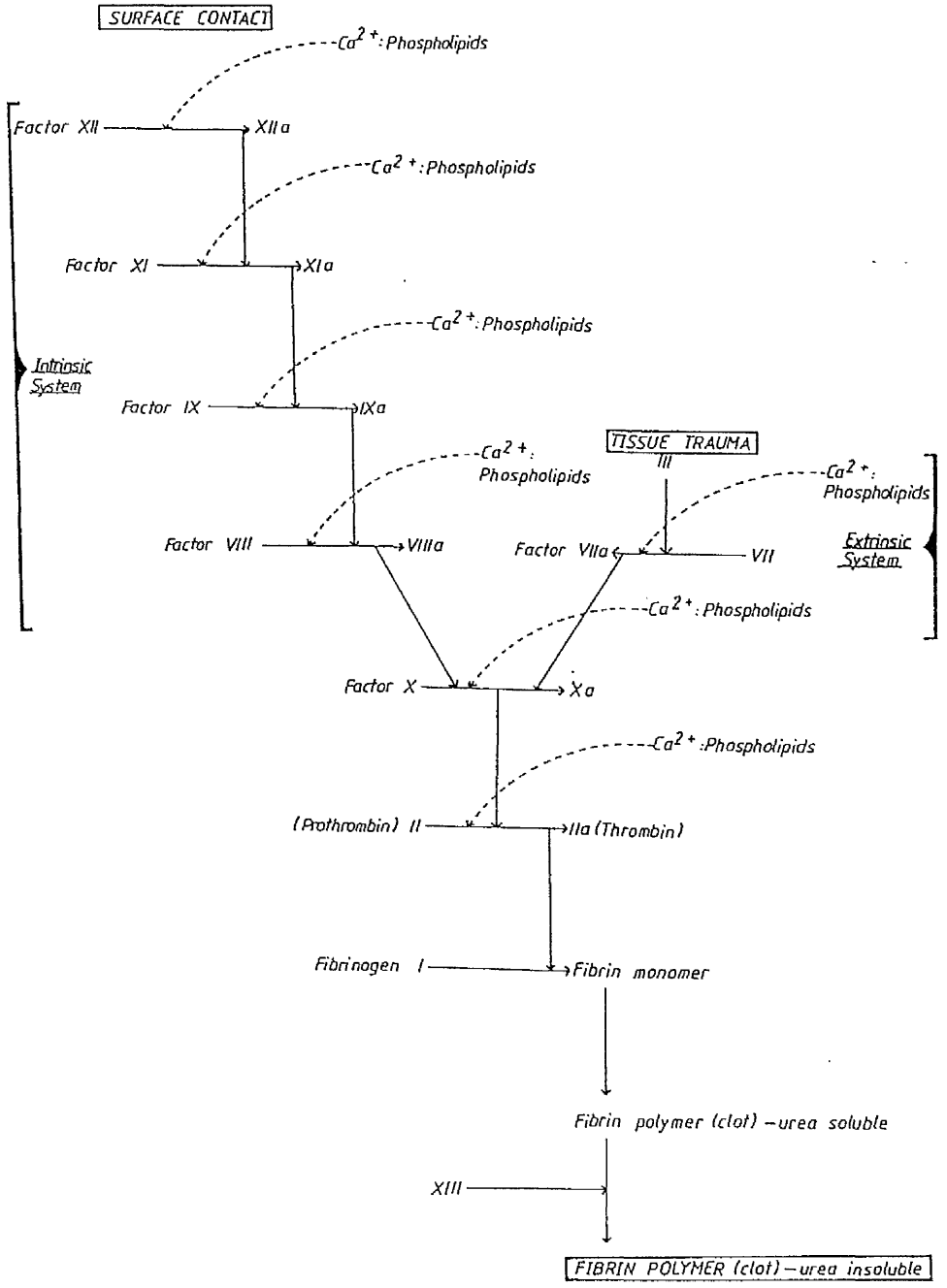


Figure 8.1

Representation of the extrinsic and intrinsic blood coagulation cascades (Keedy 1986).

Surface topography, charge and hydrophobicity govern surface/blood interactions (Vroman 1972) e.g. surface smoothness is important in order to avoid surface coagulation. Merrill (1977) showed that roughness even at the scale of 10 nm favours protein deposition and triggers the coagulation cascade. Polymers usually show varying degrees of crystallinity and protein adsorption at an ordered crystalline surface may reach a stable level. However at a non-crystalline surface both continued and surface adsorption and diffusion into the bulk polymer may cause problems. Surface charge can additionally influence haemocompatibility and a net negative charge at insulators and conductors reduce surface coagulation. Sawyer and Srinavasan (1967) demonstrated a protective effect on platinum wires maintained at +0.3 V to -1 V (vs NHE).

Since biocompatibility is mainly an interfacial problem, attempts to improve membrane compatibility concentrated on modification of membrane surface properties e.g. Hanning et al (1986) demonstrated improved stability and reduced platelet and fibrin/fibrinogen deposition on the external dialysis (Cuprophan^{R}) membrane of an extracorporeal glucose electrode with the membrane previously treated with dimethyldichorosilane. By contrast, in the same system, inclusion of anticoagulant (heparin), antiplatelet agents (adenosine, caffeine, salicylate) in large quantity or the modification of the blood matrix using added albumin, dextran, or high dilutions did little to ameliorate surface fouling.

Internal membranes in H_2O_2-based oxidase sensors have been mainly

used to exclude low molecular weight electroactive interferents from reaching the working electrode (Mullen et al 1986; Churchouse et al 1986; Desai et al 1993). The current study was conducted to determine the relevance of inner membranes in the determination of blood compatibility. Inner membrane barriers of different types as well as different electrode polarisation voltages were tested with glucose enzyme electrodes on exposure to whole blood. The role of internal membranes in determining passivation of the working electrode was investigated. Effects of continuous compared to intermittent blood exposure to glucose enzyme laminates were assessed.

8.2 Needle electrode

8.2.1 Continuous blood exposure with/without inner polyethersulphone membranes

The results in Figures 8.2.1a and b demonstrate that stability of a glucose needle to continuous exposure to whole blood is greatly enhanced by the presence of an inner permselective polyethersulphone membrane. There is minimal drift in electrode response observed in both cases regardless of the external polyurethane ("Trixene SC762" or "Pellethane") membrane. If the polyethersulphone membrane is absent the response of the sensor is reduced quickly on continuous blood exposure. A signal diminution by ~80% is found after 2 hours in both cases.

8.3 Oxygen-type electrode

8.3.1 Effects of continuous blood exposure with cellulose acetate, modified cellulose acetate and polycarbonate

The signal drift pattern during continuous 2 hour blood exposure with the electrode polarised at +650 mV vs. Ag/AgCl enzyme laminates

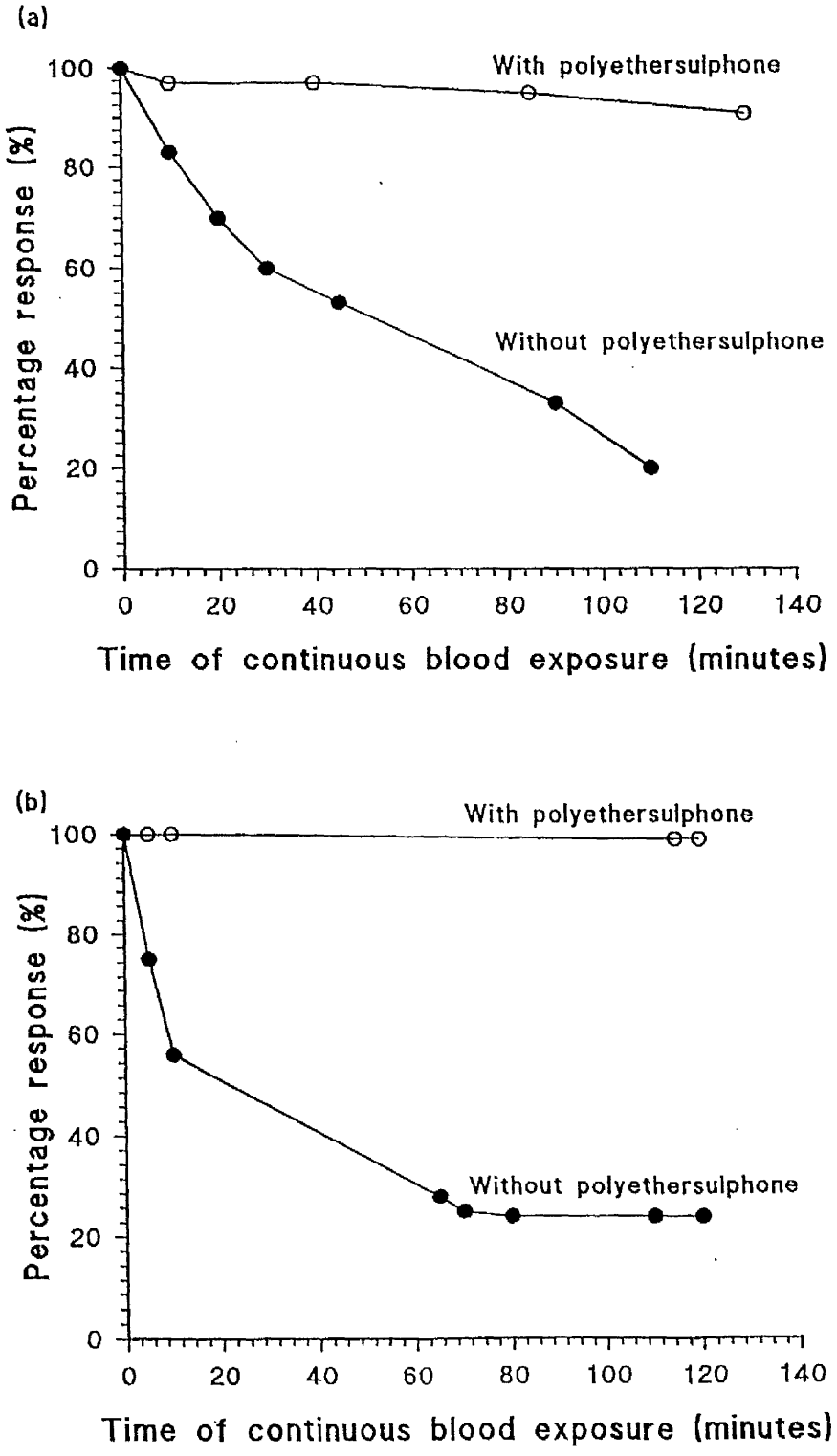
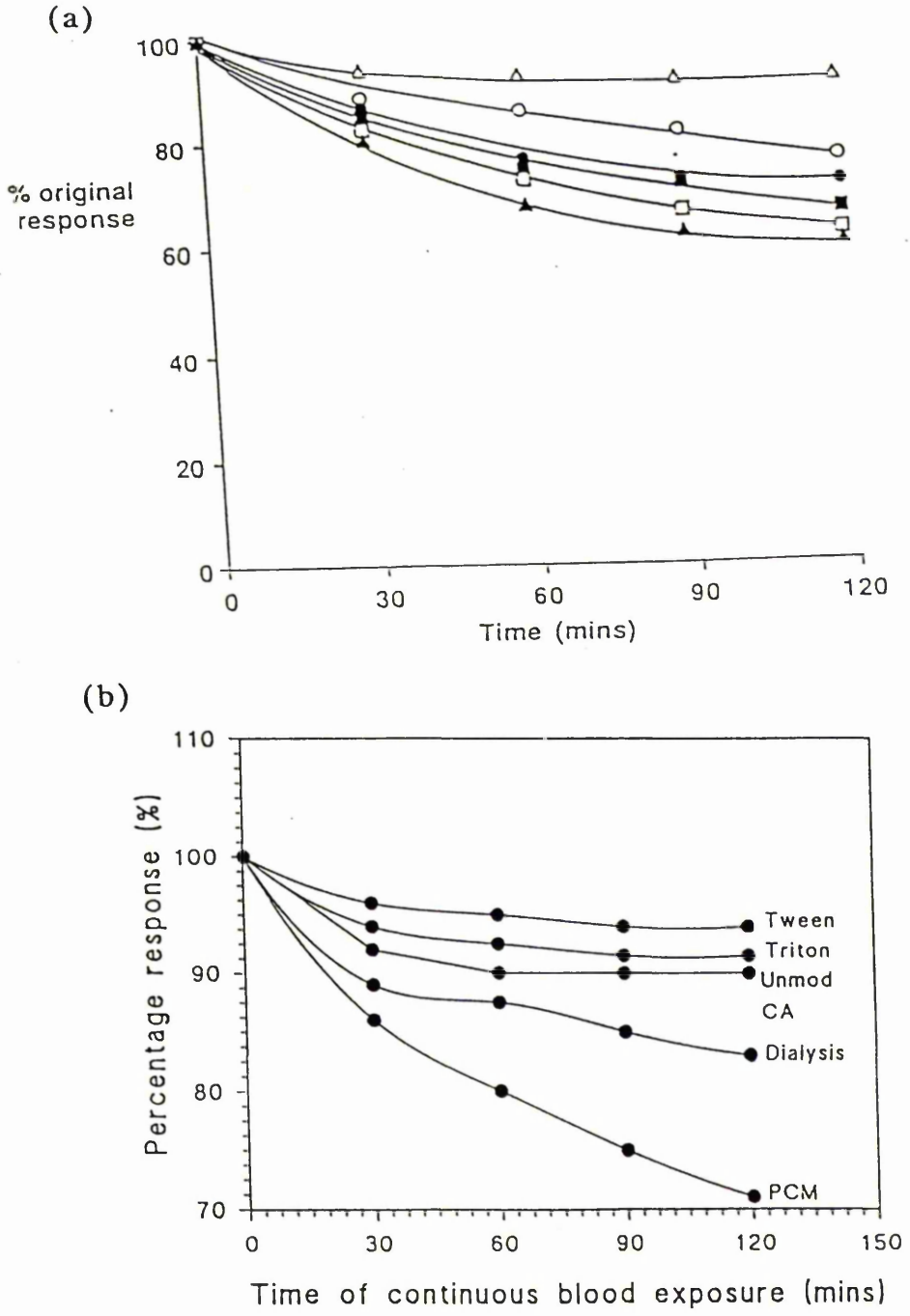


Figure 8.2.1

Effect of continuous blood exposure of needle electrodes with and without internal polyethersulphone membranes. Outer polyurethane membranes: (a) 10%, 16% and 33% v/v Trixene SC762 (in tetrahydrofuran) (b) 3% w/v Pellethane (in tetrahydrofuran)



8.3.1a and b
Percentage loss of signal response to continous whole-blood exposure with time. Electrodes were polarised at +650 mV vs. Ag/AgCl.(a) Inner membranes: (△) 0.01 μm, (■) 0.03 μm, (□) 0.1 μm, (▲) 1.0 μm pore size polycarbonate: (●) 2% w/v cellulose acetate and (○) 5% w/v cellulose acetate.
(b) surfactant modified and unmodified cellulose acetate, 0.03 μm pore size polycarbonate and dialysis. A dialysis membrane was interposed between the working electrode and enzyme laminate.

containing different internal membranes is illustrated in Figures 8.3.1a and b. In a control experiment, aliquots of the same blood specimen were assayed separately by independently calibrated glucose sensors. This was done in order to show that the signal drift observed was only related to biofouling phenomena and not with substrate consumption. In Figure 8.3.1a a general trend for downward drift in signal magnitude follows the order:- 0.01 μm polycarbonate (PC) < 5% cellulose acetate (CA) < 2% CA < 0.03 μm PC < 0.1 μm PC < 1.0 μm PC. The trend in the drift pattern correlates with the H_2O_2 permeability properties of the inner membrane and there appears to be a quantitative time-dependent deposition of diffusible solute which then compromises signal size. In Figure 8.3.1b surfactant (Tween-80 and Triton-X100) incorporated cellulose acetate membranes appear to show less drift during continual blood exposure compared to unmodified cellulose acetate, dialysis (Cuprophan) and 0.03 μm polycarbonate membranes.

8.3.2 Effect of varying electrode polarising potential during blood exposure

In these experiments new enzyme electrodes polarised at +200, +400, +600 and +800 mV vs. Ag/AgCl were exposed to whole blood over 2 hours in the same blood samples. Signal drift for the polycarbonate inner membrane electrodes (Figures 8.3.2a-d) follows the same trend against pore size as seen previously (Figure 8.3.1a). However, signal drift increases as the magnitude of electrode polarising voltage is increased. For unmodified cellulosic membranes (2% and 5% w/v in acetone) the passivation is reduced except in the case of the highest polarising voltage (+800 mV vs. Ag/AgCl), where a paradoxically

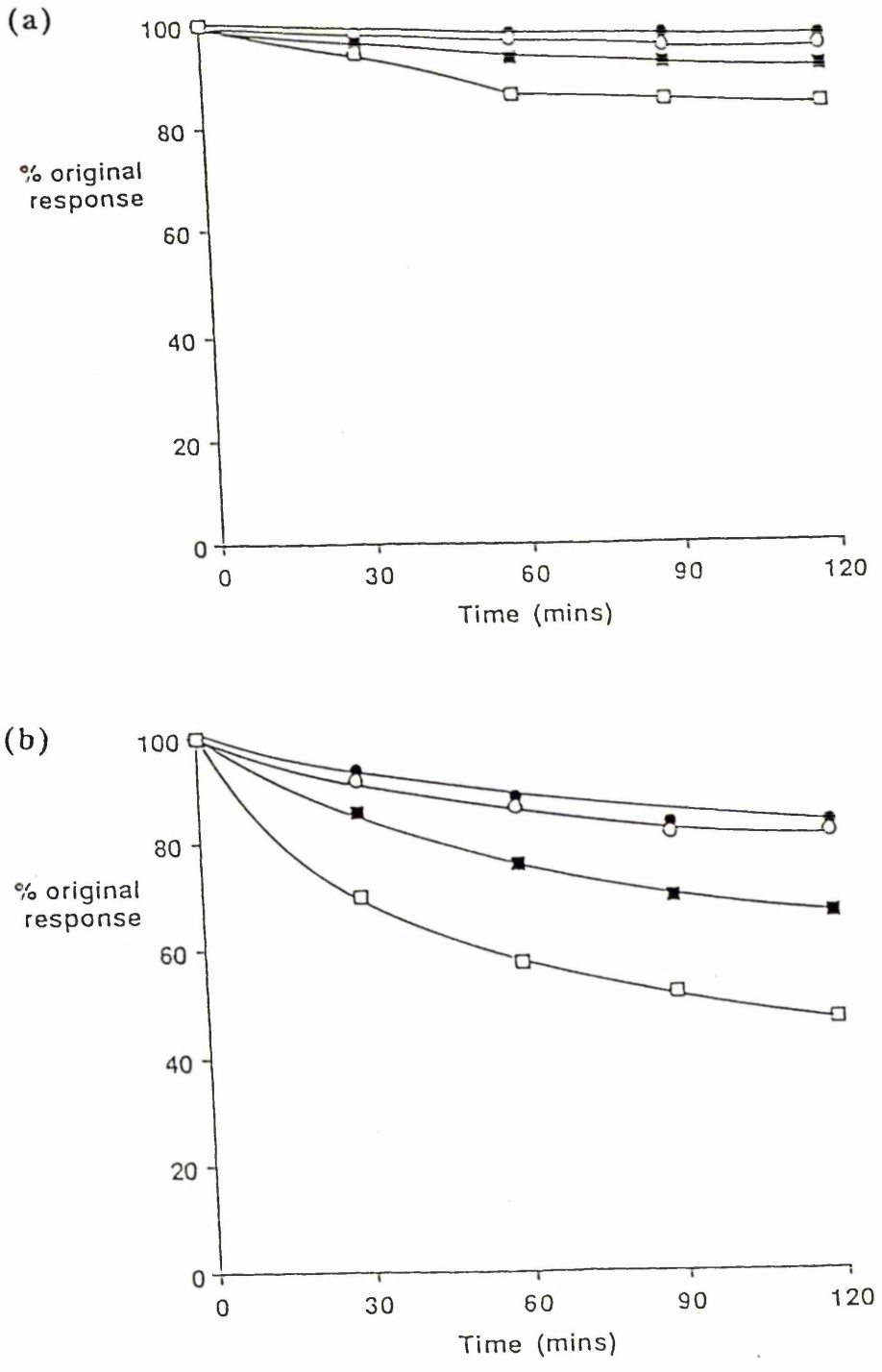


Figure 8.3.2a and b

Loss of signal responses due to continuous exposure to whole blood with time at different polarising potentials. The outer membrane was a 0.03 μm pore size polycarbonate. A dialysis membrane was interposed between the working electrode and enzyme laminate. Inner (a) 0.01 μm (b) 0.03 μm polycarbonate membranes were used.
(●) +200 mV, (○) +400 mV, (■) +600 mV and (□) +800 mV.

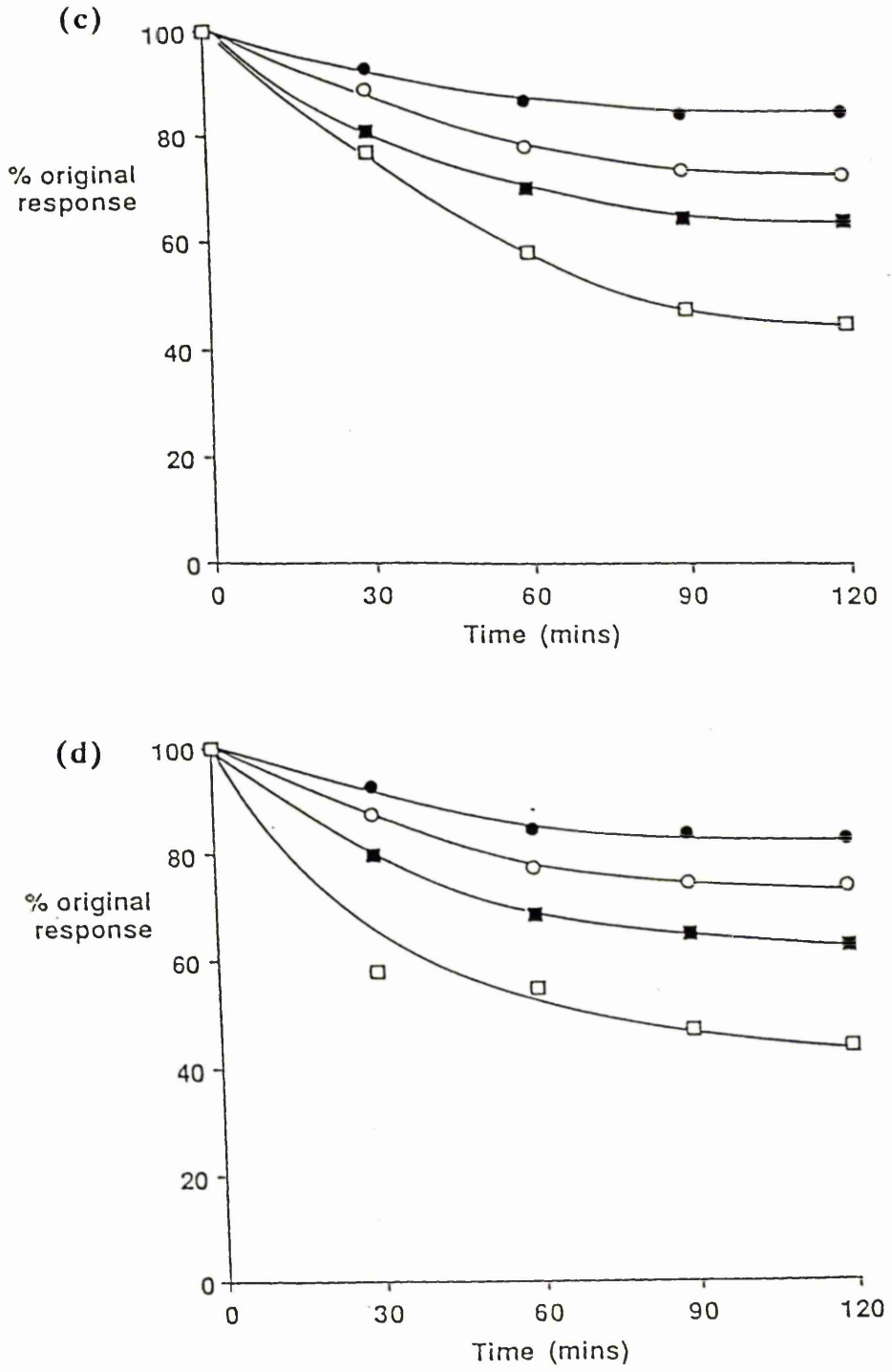
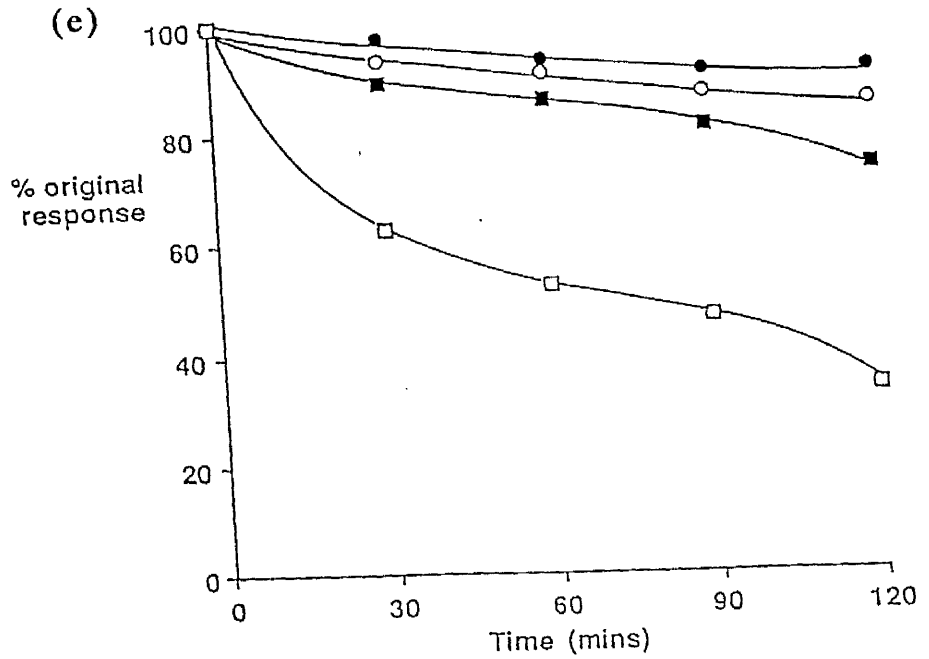


Figure 8.3.2 c and d

Loss of signal responses due to continuous exposure to whole blood with time at different polarising potentials. The outer membrane was a 0.03 μm pore size polycarbonate. A dialysis membrane was interposed between the working electrode and enzyme laminate. Inner (c) 0.1 μm (d) 1.0 μm polycarbonate membranes were used.
(●) +200 mV, (○) +400 mV, (■) +600 mV and (□) +800 mV.



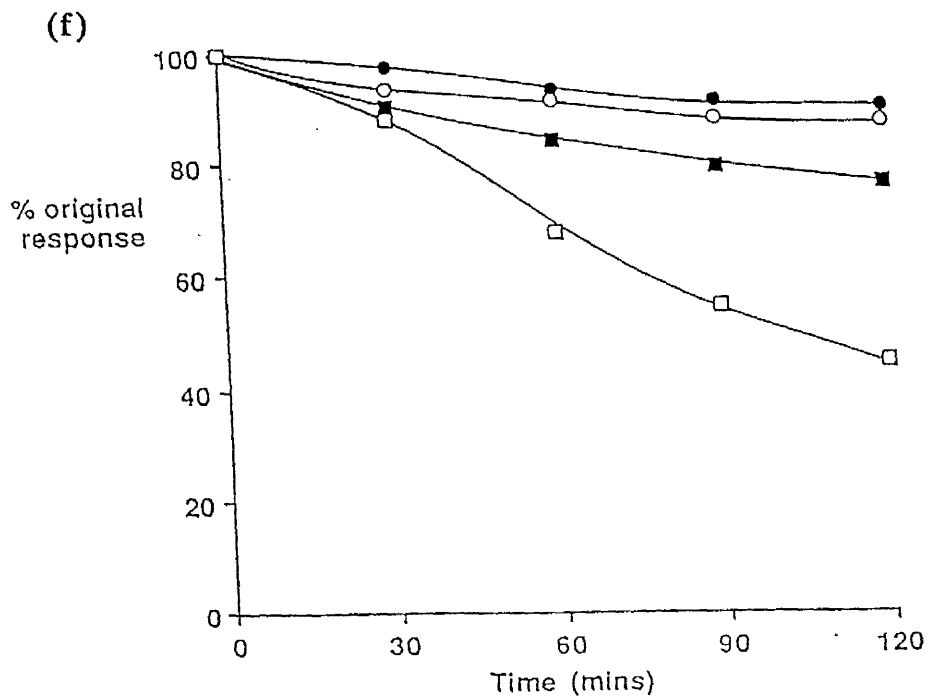
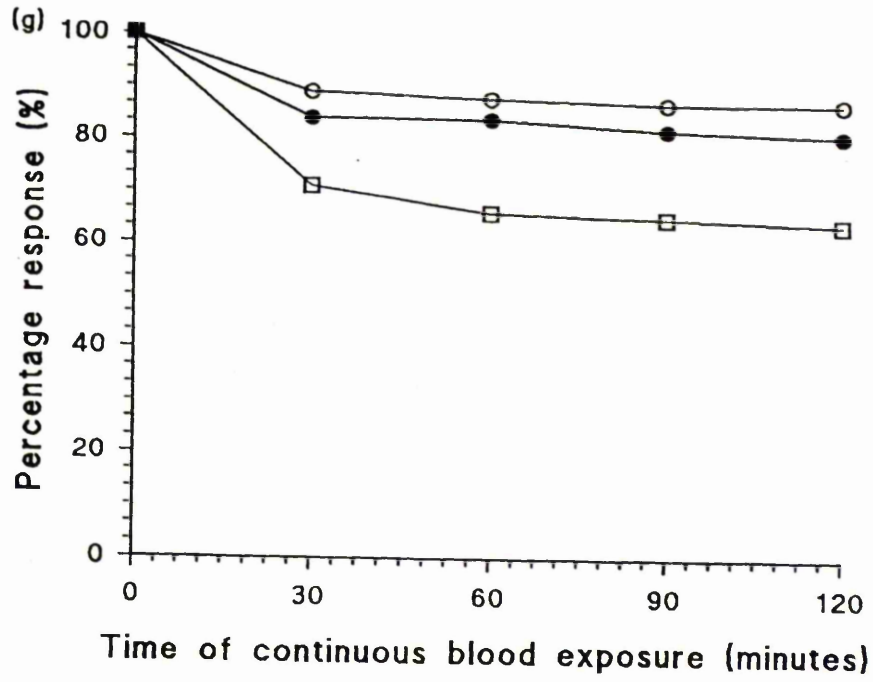


Figure 8.3.2e and f

Loss of signal responses due to continuous exposure to whole blood with time at different polarising potentials. 0.03 μm pore size polycarbonate as outer membranes. Dialysis membrane interposed between the working electrode and enzyme laminate. Internal (e) 2% w/v and (f) 5% w/v cellulose acetate membranes were used.
(●) +200 mV, (○) +400 mV, (■) +600 mV and (□) +800 mV.



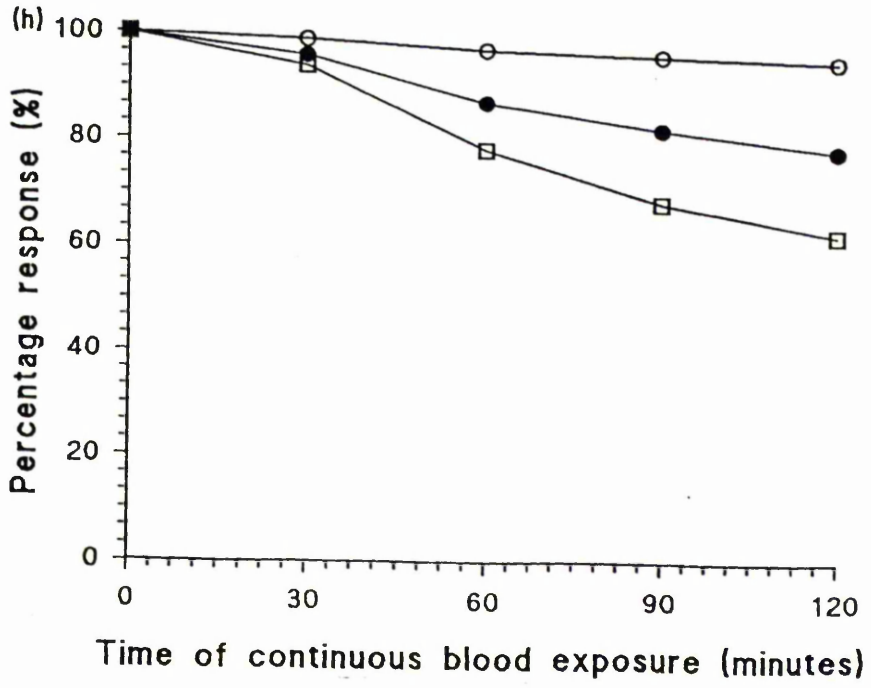


Figure 8.3.2g and h

Loss of signal responses due to continuous exposure to whole blood with time at different polarising potentials. 0.03 μm pores size polycarbonate as outer membranes. Dialysis membrane interposed between the working electrode and enzyme laminate. Internal (g) Tween-80 and (h) Triton X-100 cellulose acetate membranes were used.
(○) +400 mV, (●) +600 mV and (□) +800 mV.

greater deterioration is observed than with any of the other membranes (Figures 8.3.2e-h). A less pronounced deterioration in response at +800 mV is seen with surfactant-incorporated compared to unmodified cellulose acetate membranes.

8.3.3 Passivation effects at platinum electrode

In order to differentiate between inner/outer membrane biofouling effects and electrode passivation, bare electrode responses to a standard 0.04 mM H_2O_2 solution were recorded following careful removal of the entire enzyme laminate after each blood exposure (Figure 3.8.3a). The observed loss in response shows a similar general trend to those corresponding intact enzyme electrodes, with responses remaining unaltered where less permeable membranes had been used. Also, the percentage loss of response incurred is greater in all cases; this is especially true for the large pore size (0.1 μm and 1.0 μm) polycarbonate membranes.

A subsequent study of passivation with surfactant-incorporated cellulose acetate was carried out by determining bare electrode responses to ascorbate, catechol and H_2O_2 (Figure 3.8.3b). The results indicate again that cellulose acetate gives better protection against electrode passivation compared to 0.03 μm polycarbonate. However enhanced biocompatibility is observed for both Tween-80 and Triton-X100 modified cellulose acetate membranes, most notable with catechol (0.04 mM).

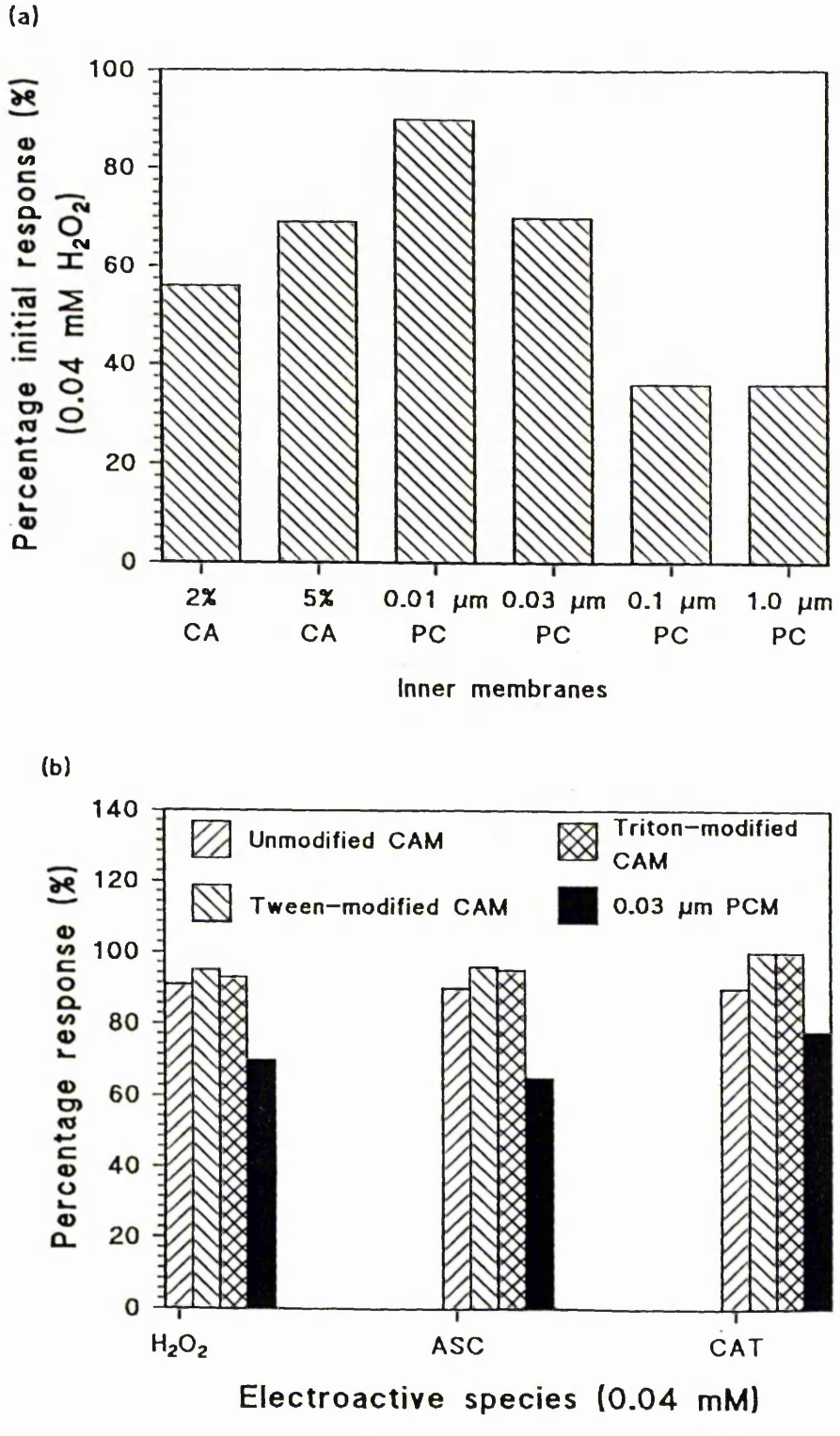


Figure 8.3.3a and b

Loss of bare-electrode responses to (a) 0.04 mmol l^{-1} H_2O_2 and (b) 0.04 mmoll^{-1} H_2O_2, ascorbate and catechol following continuous exposure of enzyme electrodes with various inner membranes to whole blood. The working electrode was polarised at +650 mV vs. Ag/AgCl. A dialysis membrane was interposed between working electrode and enzyme laminate.

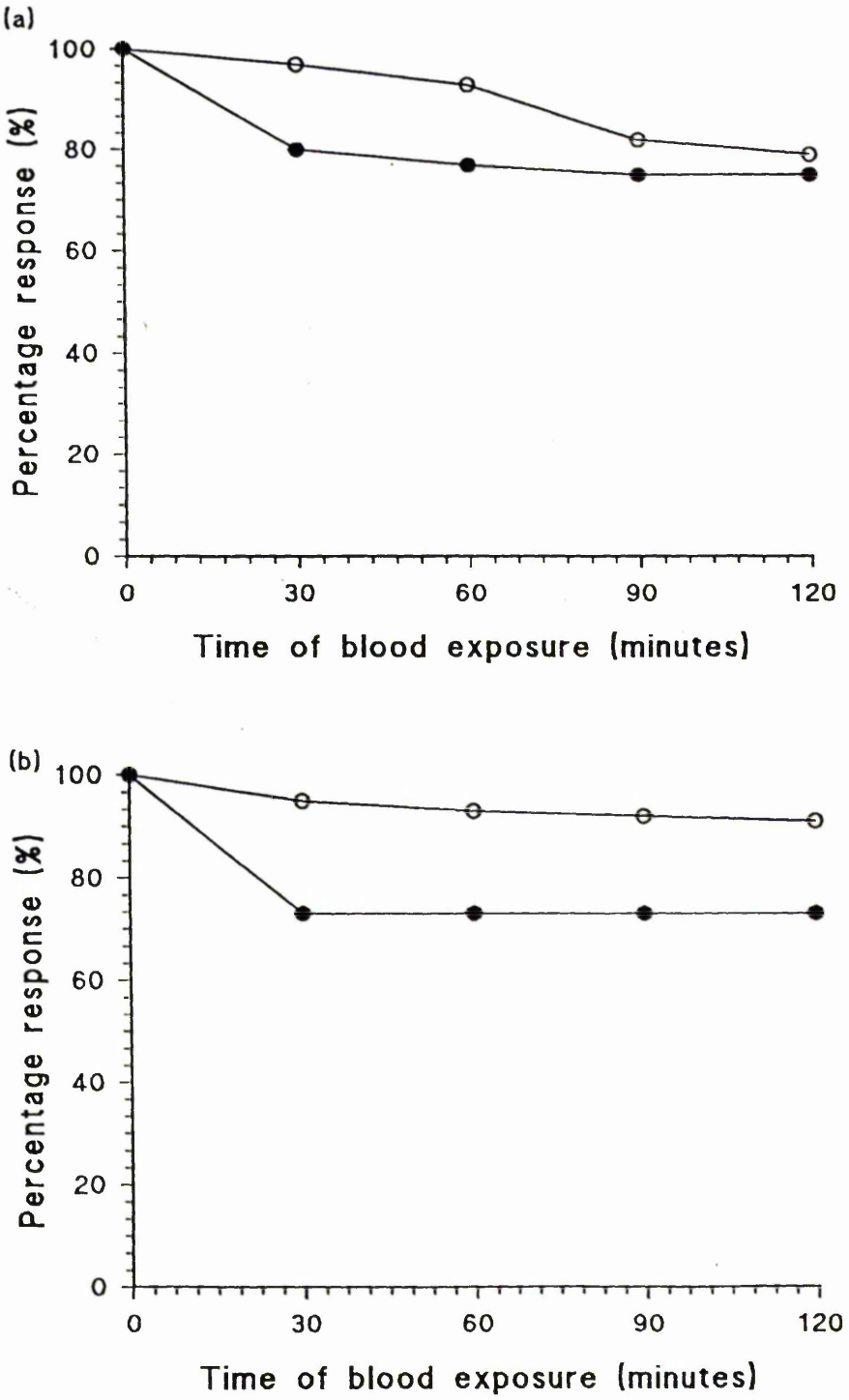


Figure 8.3.4

Comparison of intermittent (●) and continuous (○) blood exposure on the response of a glucose electrode laminate containing an internal (a) 4% polyethersulphone (S=5) membrane (w/v in dimethylformamide); (b) 5% cellulose acetate (w/v in acetone).

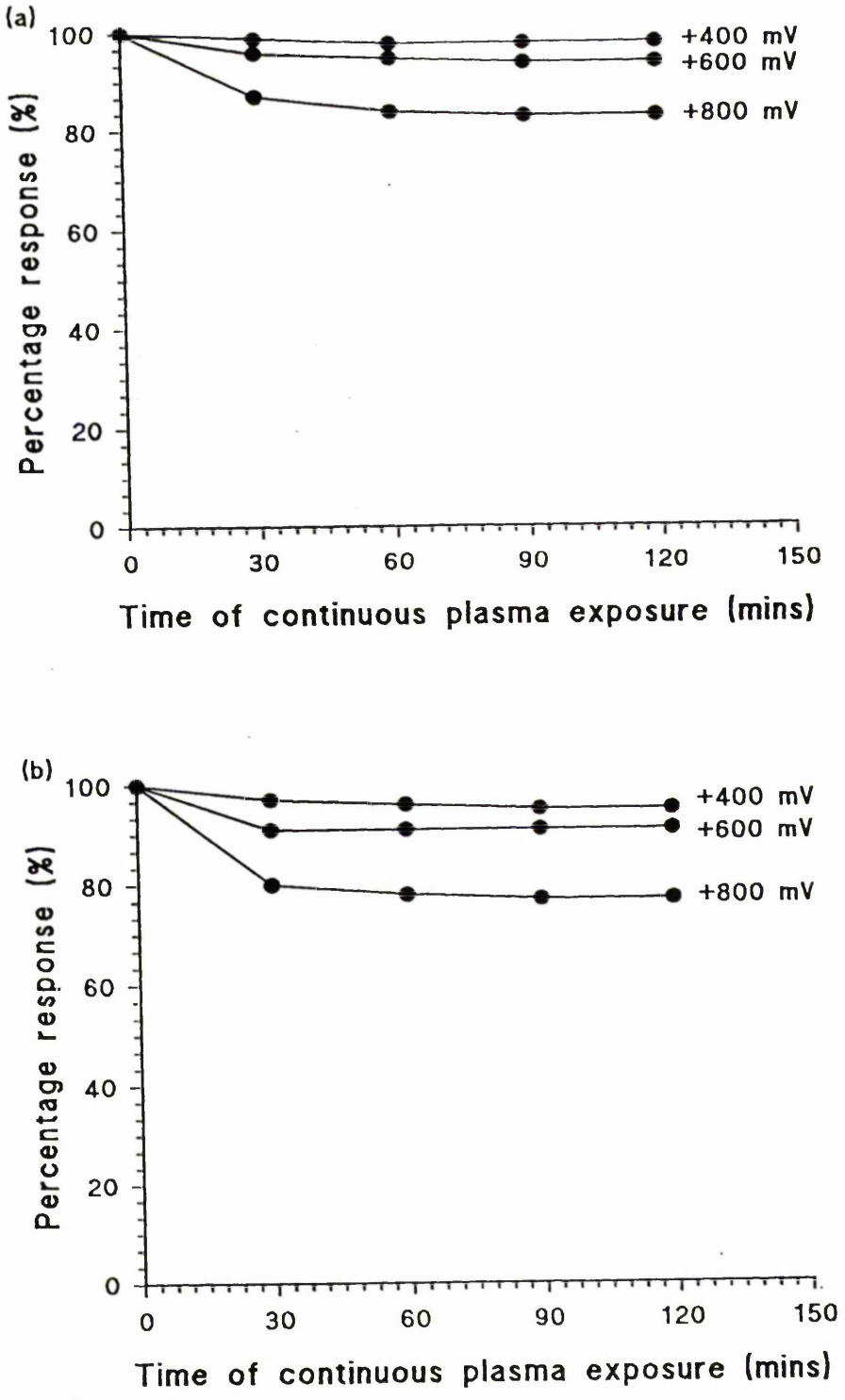


Figure 8.4a and b

Loss of signal responses due to continuous exposure to plasma with time at different polarising potentials. 0.03 μm pore size polycarbonate as outer membranes. Dialysis membrane interposed between working electrode and enzyme laminate. Inner membranes (a) Tween-80 and (b) Triton-X100 modified cellulose acetate.

8.3.4 Effects of discontinuous vs continuous blood exposure

The results for polyethersulphone and cellulose acetate internal membranes are shown in Figures 3.8.4a and b respectively. In both cases intermittent rinsing of the electrode with phosphate buffer after 30-minute blood exposure intervals was done which was followed by recalibration with aqueous 2 mM glucose. As a comparison the signal drift in response to continual blood exposure was carried out. Both results demonstrate that a greater deterioration occurs on discontinuous blood exposure. An initial 20% decline was found after 30 minutes (discontinuous) compared to only 3% (continuous) exposure for polyethersulphone. The initial signal decline was ~25% for cellulose acetate (discontinuous) in comparison with 5% (continuous) exposure.

8.4 Variation of applied polarising potential during continuous plasma exposure

The results (Figures 8.4a and b) show a similar amount of drift at +400 and +600 mV for modified cellulose acetate membranes. However there is pronounced signal deterioration for both internal membranes at +800 mV. The signal drift however is less than seen with whole blood (Figures 8.3.2 e-h).

8.5 Discussion

The results shown in Figures 8.2.1a and b suggest a requirement for an internal polyethersulphone membrane. This confirms previous findings of Churchouse et al (1986) where poor correlation coefficients with routine method were obtained in the absence of a polyethersulphone membrane. Also Desai et al (1993) and Crump et al (1991) found a

greater signal drift for needle electrode without polyethersulphone.

Although the outer polyurethane membranes result in enhanced biocompatibility according to Churchouse et al (1986) and Vadgama et al (1989), the polyethersulphone membrane has an important role in determining blood compatibility. In the absence of an inner membrane released diffusible solutes from blood components stimulated by contact with the outer polyurethane membrane are able to reach the inner (Pt) electrode to passivate it. It is also possible that the diffusible solutes are negatively charged since they are rejected by the polyethersulphone ion-exchange membrane. One additional factor could be that the polyurethane dipcoated over a sulphone layer gives a smoother surface layer as shown by SEM (Chapter 6) than does a direct dip-coat, and this could help reduce surface fouling problems. It is interesting that the presence of the inner membrane rather than the type of polyurethane covering determines apparent haemocompatibility.

Recently Moussy et al (1993) used an inner electrodeposited poly (o-phenylenediamine), PPD in conjunction with a Nafion (perfluorinated ionomer) outer coating for needle type sensors. The PPD layer prevented access of diffusible interferents which penetrated the external Nafion film. The electrodes showed considerable stability in whole blood although exhibiting a slight decrease in sensitivity which was due to the adsorption of blood protein on the Nafion (Harrison et al 1988). This work shows that the inner permselective membrane whether PPD or polyethersulphone has an important role in protection of the working electrode.

The results for continuous blood exposure using various glucose enzyme laminates demonstrates a decay in sensor sensitivity (Figures 8.3.1a and b). This is attributable to some form of either internal or external fouling but the membrane dependence effects support the possibility that fouling/deactivation occurred within the plane of the inner membrane layer. Thus external or protein fouling or enzyme denaturation by blood components cannot be sole explanations for the results. The possibility is supported by the fact that the inner membrane permeability and time of exposure were relevant.

Fouling of the inner membrane itself by diffusible microsolute is most unlikely, since this would actually be worst at the smallest pore size membrane (0.01 μm). However the greater overall deterioration seen with cellulose acetate (Figure 8.3.2a) could be due to such solute entrapment in the dense hyperfiltration membrane structure (Pusche 1982).

Surfactant-incorporated and unmodified cellulose acetate membranes used as internal membranes gave less signal drift in blood (Figure 8.3.1 b). This further suggests that the diffusible solutes were negatively charged and non-aromatic because they were rejected by cellulose acetate which agrees with the findings of Desai et al (1993). From chapter three it has been shown that both modified and unmodified cellulose acetate were capable of rejecting negatively charged species but allowed the permeation of aromatics. The basis of this permselectivity behaviour may rely on electrostatic repulsion between the carboxyl groups of cellulose acetate and negatively

charged species (Lonsdale 1965).

The effects of varying the applied polarising potentials during blood/plasma exposure are illustrated in Figures 8.3.2a-h and 8.4a-b. In all cases signal drift increased as the electrode polarisation increased. This strongly suggests that during exposure there was either enhanced migration of a negatively charged passivating component due to the electrical field set up in the vicinity of the working electrode, or at higher voltages electrochemical degradation and deposition of the passivating solute increased similar to polyphenolic deposition at solid electrodes (Wang et al 1989). An electrical double layer exists at the electrical interface between two sheets of charge, one on the electrode and other in the solution. This arises because the platinum working electrode is positively charged and draws out from the randomly dispersed ions in solution a counterlayer of charged of opposite (negative) sign. The charge densities on the two layers are equal in magnitude but opposite in sign resembling a parallel plate capacitor. This double layer may extend upto 5 Å from the electrode. Beyond this a diffuse (outer) layer extends a further 50-100 Å in the solution (Rifi and Covitz 1974). In this region there is random arrangement of both positively and negatively charged ions due to their mobility. At some point the species become free to move in response to bulk motion of the electrolyte. A potential difference (zeta) exists between this shear layer and a region which extends considerably further away (up to several cm). The effective distance over which this potential is imposed is dependent upon the electrode and the relative permittivities (ε) of the electrolyte plus additional interposed barriers. The permittivity can be

derived from Coulomb's Law which states that the magnitude of the force, F between two electrical charged bodies is inversely proportional to r^2 (separation) and proportional to the products of their charges (Q_1 and Q_2), i.e., $F = k.Q_1.Q_2/r^2$. The value k depends on the medium in which the charges are present, which in turn is determined by permittivity (ε), and can be alternatively represented by $1/4\pi\varepsilon$. Therefore, ionic species may migrate in response to the zeta potential, significantly contributing to the mass transport to a polarised electrode (Kulesza and Galus 1992; Verbrugge and Baker 1992). It is possible that the cellulose acetate membranes are capable of rejecting anionic passivating species until a high voltage threshold is exceeded, beyond which migration becomes more dominant.

From the passivation experiments (Figures 3.8.3a-b) the deterioration in response is greater than with continuous blood exposure. It would have been expected that elimination of an external biofouled layer in contact with blood should have improved the stability of the electrochemical system. A likely cause of this additional deterioration is the arrival and deposition of additional passivating solutes at the platinum working electrode following its use in an enzyme electrode. During prolonged whole blood exposure, the outer membrane will have acquired a layer of cellular elements from blood (leucocytes, platelets) as well as plasma proteins (Vadgama 1990). This cellular layer constitutes a bioactive surface still able to react to changes in environmental conditions. In particular, platelets have been shown to respond following change to their solution environment (Hellem 1960) e.g. platelet adhesiveness increased from 0-60% for plasma treated with

trisodium citrate as temperature decreased from 24 °C to 4 °C. This "cold-induced" adhesiveness disappeared if the plasma was further incubated for 24 hours at 20 °C. Incubation of blood in the calcium chelating agent, EDTA (ethylene diamine tetracetate dihydrate), heparin or oxalate caused a decrease in adhesiveness. Also addition of Triton WR-1339 to the blood sample and silicone coating of the glass test surface reduced adhesiveness.

Physical triggering of platelets has been implicated under turbulent conditions (Turitto et al 1991). Here at physiologically encountered shear conditions increases in local shear rate promoted attachment of platelets and enhanced growth of platelet aggregates on adherent platelets. Release of beta-thromboglobulin has been shown to occur from platelets permanently adhered to a biomaterial surface (Haycox and Ratner 1991) suggesting that the platelets can remain active and release solutes after adherence.

The washing step (using buffer) to purge the electrode cell of blood prior to dismantling the enzyme electrode for H_2O_2, ascorbate and catechol determination, amounts to an environmental change, and could have led to the local release of a range of diffusible solutes including adenine nucleotides (adenosine diphosphate) and 5-hydroxytryptamine (Mills 1968), some of which would have been able to diffuse to the working electrode. Mills (1968) showed that platelets could be stimulated to release these substances by addition of ADP and adrenaline. This possibility is consistent with the finding that the additional deterioration of response at a dismantled electrode was worst

with high-porosity (0.1, 1.0 μm polycarbonate) membranes. A similar explanation for signal drift can be applied when intermittent rinsing stages were carried out during exposure for enzyme laminates containing polyethersulphone/cellulose acetate inner membranes (Figures 8.3.4a and b).

8.6 Summary

The experiments clearly show that the choice of the inner membrane strongly influences signal drift at glucose sensors during exposure to whole blood. An essential requirement for an internal membrane has been clearly shown using polyethersulphone. Also the sensor sensitivity deteriorates further if the blood in contact with the surface is washed or perturbed in any way. This is shown by the results for discontinuous/continuous blood exposure and the passivation studies. The membranes investigated provided protection against signal drift in the order 1.0 μm polycarbonate (PC) < 0.1 μm PC < 0.03 μm PC < 2% w/v CA < 5% w/v CA < 0.01 μm PC. In a second experiment, detergent-modified cellulose acetate caused less drift compared to unmodified cellulose acetate, 0.03 μm polycarbonate or dialysis. The effect of polarising overpotential with increased signal drift at higher overpotentials suggests the passivating species may be charged, and with the contrasting effects on microporous polycarbonate and cellulose acetate membranes, suggests that the dielectric properties of the inner membrane may modify the domain of influence of the polarised electrode. The results demonstrate that the inner phases of multilayer H_2O_2-based amperometric enzyme electrodes can be of equal importance to sensor stability as the events taking place at the interface in direct contact with the biological fluid.

CHAPTER NINE - CONCLUSIONS

The aim of the project was to develop membranes for both lactate and glucose sensors. Various polymeric membranes investigated include:- polyvinyl chloride, cellulose acetate (unmodified and surfactant-modified), polyethersulphone, polyurethane and polycarbonate.

Polyvinyl chloride (PVC) plasticised using isopropylmyristate showed improved permselectivity compared to cellulose acetate. Inclusion of PVC as an internal membrane of a glucose electrode led to a considerable increase in the linear range of the response making measurement of serum/blood levels possible. This is an entirely new finding since it was presumed that the external membrane itself controlled the linear range.

Newly developed non-ionic surfactant-incorporated cellulose acetate membranes exhibited improved permselectivity to H_2O_2 in comparison to unmodified cellulose acetate. A considerable amplification in electrode response to H_2O_2 and phenolic species was observed. They were also permeable to glucose and could be used as external membranes. A more detailed investigation into the diffusion characteristics of these membranes and a study into the possible leaching of these particular surfactants needs to be assessed. An additional study using cationic and anionic surfactant-modified membranes can be carried out.

Studies in whole blood have revealed that the internal membrane is

important in determining blood compatibility. This is an entirely new finding, and the magnitude of signal decay in whole blood was also governed by the applied polarising potential. A detailed investigation of the exact mechanism of electrode passivation needs to be done. A comparative study in plasma needs to be carried out.

The internal polyethersulphone membrane achieved complete exclusion of negatively charged species and aromatic compounds to a certain degree. Selectivity for hydrogen peroxide varied according to the particular polyethersulphone polymer employed. The optimal casting solution composition was a solvent/non-solvent mixture of dimethylformamide and 2-methoxyethanol. Polyethersulphone membrane-coated sensors exhibited a negligible degree of drift on continuous whole blood exposure.

The outer (diffusion-limiting) polyurethane membrane extended linearity for both lactate and glucose needle electrodes. Response stability was determined by the type of polyurethane as well as the storage conditions employed. The ultimate aim of these devices is for continuous *in vivo* monitoring, therefore a detailed study of solution variables such as temperature, oxygen and viscosity on response needs to be done.

This has been a preliminary investigation of novel polymeric membranes for amperometric enzyme electrodes as a means of controlling permselectivity, as well as the biocompatible nature of the sensor-sample interface.

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