

Cranfield Institute of Technology

Nick A Morris

***Thin Layer
Bioelectroanalysis***

Biotechnology Centre

PhD

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1990-91

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***Thin layer
Bioelectroanalysis***

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August 1991

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ABSTRACT

The suitability of the octagonally coordinated complex, ruthenium (III) hexamine, as a soluble electron mediator for glucose oxidase in the electrochemical capillary fill device (CFD) was investigated. Electrochemical studies revealed highly reversible electrochemistry at gold electrodes with $E_{1/2} = -0.2$ V and a diffusion coefficient of $\sim 1.7 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$ (2 M KCl, pH 7). The second order rate constant, k_g , for the reaction between reduced glucose oxidase and oxidized mediator could not be determined by voltammetric sweep techniques, indicating a value for k_g far inferior to the ferrocenes. A numerical analysis of the difference between the current produced in the presence of the homogeneous catalytic reaction under pseudo first order conditions and the diffusion current yielded a value of k_g for ruthenium (III) hexamine of $\sim 80 \text{ M}^{-1} \text{ s}^{-1}$. The maximum pH-dependent response for the reaction between ruthenium (III) hexamine and glucose oxidase occurred at pH 9. There was no increase in the rate of electron transfer at elevated pH values intrinsic to the ruthenium (III/II) couple, from studies of the heterogeneous rate constant & employing the Marcus relationship. The increased reactivity at pH 9 was thought to be due to enhanced thermodynamic viability, shown by an increase in the difference in between the half wave potentials of the FAD/FADH_2 & $\text{Ru(III/II)(NH}_3)_6$ couples. Exhaustive coulometric determination of glucose in a CFD using ruthenium (III) hexamine and glucose oxidase resulted in a response with a linear range up to 30 mM glucose ($r^2 = 0.999$, $\text{cv} < 5\%$, $n = 100$) and a limit of detection of 0.2 mM.

Strategies for a reduction in the total analysis time were explored. Numerical models were constructed to simulate the behaviour of the catalytic phase of the analysis. Modelling the flux of glucose and reduced mediator to, from and within the catalytic layer showed that the low value for k_g was not flux limiting and suggested that a reduction in the duration of the catalytic phase could not be achieved by increasing the concentration of enzyme in the layer. The models suggested that a dramatic improvement in response time could be achieved by reducing the gap width of the CFD and emphasised the importance of a high fractional diffusion coefficient in the catalytic layer. Correlative parameters based on a cell dimension and diffusion coefficient dependent time constant and the K_m of the enzyme generally applicable to catalytic devices of this type were constructed. The use of a series of potential pulses separated by potential relaxation periods in the CFD to derive analytical information was investigated theoretically using analytical models and a series of numerical models, and practically using manufactured devices. The method allowed a reduction in analysis time in the glucose CFD using ferricyanide as mediator from 60 s to 10 s with an increased limit of detection (to 0.1 mM from 0.5 mM) and a greater precision (cv from 8% to 4%) without adjustments to the reagent layer. The models were also used to solve the flux equations for lateral diffusion, demonstrating the redundancy of the guard electrode using this method of current signal analysis.

The amenability of the CFD design to accommodate complex chemistries was demonstrated for the L-lactate dehydrogenase/NAD/diaphorase/ferricyanide system.

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LIST OF GLOBALLY APPLIED ABBREVIATIONS, SYMBOLS & CONSTANTS

A	area / m ²
C	concentration / moles l ⁻¹ or moles m ⁻³
CFD	Capillary Fill Device
CHEMFET	Chemically Sensitive Field Effect Transistor
cv	coefficient of variance
D	diffusion coefficient of chemical species / m ² s ⁻¹
DPV	Differential Pulse Voltammetry
E	potential / volts / V
EDTA	Ethylenediaminetetraacetic acid
E_o/E_r	oxidized / reduced forms of an oxidoreductase
F	Faraday's constant / 96 485 C mol ⁻¹
FAD	Flavin Adenine Dinucleotide
GODFAD/H₂	oxidized / reduced forms of glucose oxidase
i	current / amperes / A
IRS	Internal Reflectance Spectroscopy
ISE	Ion Selective Electrode
ISFET	Ion Selective Field Effect Transistor
IUPAC	International Union of Pure & Applied Chemistry
k_e	bimolecular or second order rate constant for the reaction between reduced glucose oxidase & oxidized mediator / M ⁻¹ s ⁻¹
K_h	heterogeneous rate constant / cm s ⁻¹
K_m	the Michaelis-Menton constant / M
LDH	Lactate Dehydrogenase
M_o/M_r	oxidized / reduced mediator
n	number of moles of electrons or reacting species involved in a reaction
NAD	Nicotinamide Adenine Dinucleotide
OECD	Organisation for Economic Cooperation & Development
Q	charge / coulombs / C
R	gas constant / 8.314 J ⁻¹ mol ⁻¹ K ⁻¹
SCE	Saturated Calomel Electrode
sd	standard deviation of the mean
t	time / s
T	temperature / °K
v	scan rate / V s ⁻¹
z	ionic charge / coulombs / C

PREFACE

Summary statements of the work in each chapter may generally be found at the end of each chapter under '**Concluding Remarks**'. For **Chapter II**, conclusions are stated at the end of each section, the climax of the work occurs in **Section II.5.5.** In **Chapter III** the salient points are stated in **Section III.3.2.**, for the modelling of the catalytic layer, and in **Section III.4.6.**, for the modelling & experimentation of the application of a series of potential pulses separated by relaxation periods.

The **main body** of the thesis comprises **Chapters II to III** ; electrochemical investigations of the interaction between ruthenium (III) hexamine & glucose oxidase, **Chapter II**, are linked to an exploration of strategies for the reduction of analysis time in the CFD, **Chapter III**, through the development of numerical models to simulate the flux behaviour of reduced mediator and glucose to & within the catalytic layer. **Chapter IV** contains an introductory investigation into the amenability of the CFD design to accept complex chemistries with little modification and is not directly connected to the main body of work

Subdivisions of the thesis have the form; **Chapter.Section.Part.**, Chapters have been assigned Roman numerals & Sections & Parts have been assigned numbers, for example, II.5.2.. All equations, figures & numbers have the form; **Chapter.Section.Number.**; the chapter and section is described by the first two numbers, the third number refers to the order of that equation or figure in that section. Equations are referred to in the text by the application of square brackets '[]' around the number describing the position of the equation in the thesis, elsewhere square brackets surrounding a chemical species refer to the concentration of that species. Where two diagrams occur on the same page, the legends have been placed on the back of the preceding page, opposite the relevant diagram. The list of abbreviations & symbols is not exhaustive, but concentrates on those which occur throughout the thesis, other symbols are defined locally.

Chapter I, overview of the field

1.1.1 THE SIGNIFICANCE OF CHEMICAL SENSING

The more complex the nature of the technological-industrial infrastructure of the most technologically developed societies, the greater the range and diversity of their products. The capacity to sense various chemical components of industrial processes is necessary in order to construct process feedback and control systems that enable those processes to be optimised in terms of product yield. A range of technologies derived from advances in diverse sciences including molecular biology and gene cloning, immunochemistry, biochemistry, fermentation and microbiology have come to constitute the term 'biotechnology'. Various 'biotechnologies' offer the potential to create new products and to substitute new processes to achieve similar ends across a range of industries. The first OECD Report on Biotechnology was published in 1982 (Bull *et al.*, 1982). It identified various chemical processes that may be amenable to improvement by the mediation of a broad range of technologies that are grouped under the name 'biotechnology'. It also identified emergent biotechnologies that would be able to create a new range of products with applications in strategic industrial sectors such as energy, health care and medicine, waste management and environmental monitoring, food manufacture and agriculture. Biosensor technology may be viewed as a product of technological development across a broad range of disciplines in the chemical and biochemical sciences, transducer technology and signal processing. In the context of an emerging biotechnological industrial infrastructure it may be treated as a development necessary to service the information requirements of that infrastructure about its own processes.

In addition to servicing the needs of processes involving a broad range of chemical processes, the new sensor technology has created the possibility for the creation of new products, particularly in the biomedical field (Li, 1985). The incorporation of biosensor technologies into multifunction semi-automated clinical analysers may allow more tests to be completed per patient at a lower overall cost. The reduction in cost is attributable to the fact that a lower skill level is necessary to achieve the same level of certainty with the new technology than formerly, less time is necessary to complete a test and the cost of the materials per test may be less. A greater emphasis on decentralised clinical testing, in the doctor's office rather than at centralised clinical laboratories is also forecast. The market for new sensor technologies may be enhanced by legislative policy such as the implementation of the U.S. government's diagnostic related group reimbursement policy (Jarvis, 1985), which is forcing hospitals and clinical laboratories to reduce the costs of diagnostic testing.

1.2.1. BIOSENSORS

The confluence of a number of technologies in the fields of biochemistry, immunology, molecular recognition, electrochemistry, optics and electronics have facilitated the possibility of constructing low cost devices that are sensitive to industrially or medically important compounds. Several definitions of

a biosensor exist. One of the most acceptable definitions is given by Lowe (1985): "A device comprising a biologically sensitive material immobilised in intimate contact with a suitable transducing system which converts the biochemical signal into a quantifiable and processable electrical signal." This definition is robust because it does not dictate that the biologically sensitive material must be of biological origin. Substitution of the term 'chemical signal' for 'biochemical signal' would extend Lowe's definition to include those sensors that are sensitive inorganic analytes which may be the products of biochemical processes, such as CO₂ and O₂, which are important analytes in medicine or in the fermentative industries. Perhaps the widest definition of a biosensor has been stated by the V.5th commission of IUPAC (Thevenot, 1989); "...a device that acts as a chemical sensor capable of carrying specific analytical information, quantitative or semi-quantitative, about biological processes." This would admit the inclusion of devices such as home use pregnancy testing kits, whose signal response is dependent on a threshold concentration of analyte and is therefore essentially qualitative. For the purposes of this thesis only systems capable of producing analytically quantitative information shall be considered.

The flow of information from the chemistries of interest to an electrical signal is shown schematically in Figure 1.1.1.. The initial chemistry is associated with a specific reaction between the analyte and the 'biologically sensitive material'. Enzymes have been frequently used to specifically catalyse chemical transformation of the analyte. The antigen-antibody reaction (Green, 1987) and the use of ion specific chelators (Dong & Wang, 1989) are both examples of molecular recognition reactions. Generally this type of reaction does not involve any profound intrinsic transformation of the analyte. In addition to these examples, cell organelles, whole cells and sections of tissues have been used as analyte specific elements. The action of one or a set of analyte specific chemistries effects a system perturbation which may be manifested by a change in pH, an oxidation or reduction of a compound, the generation of certain ions or the production of photons. All system perturbations involve a change in the enthalpy of the system.

1.3.1 SIGNAL TRANSDUCTION

The choice of transduction medium is dependent on the nature of the principal system perturbation. All system perturbations involve enthalpy changes but measurement of system enthalpy changes is not always practicable, although advances have been recently made using microfabricated thin-film thermistors (Urban *et al.*, 1991). Danielsson and Mosbach (1987) have reviewed the theory and applications of thermometric sensors for a variety of enzyme catalysed reactions. Other than this potentially universal transduction system, other transduction systems may be divided into three groups; electrochemical, optical and piezo-electric.

Chemistries that involve specific surface analyte binding reactions, such as the incorporation of the

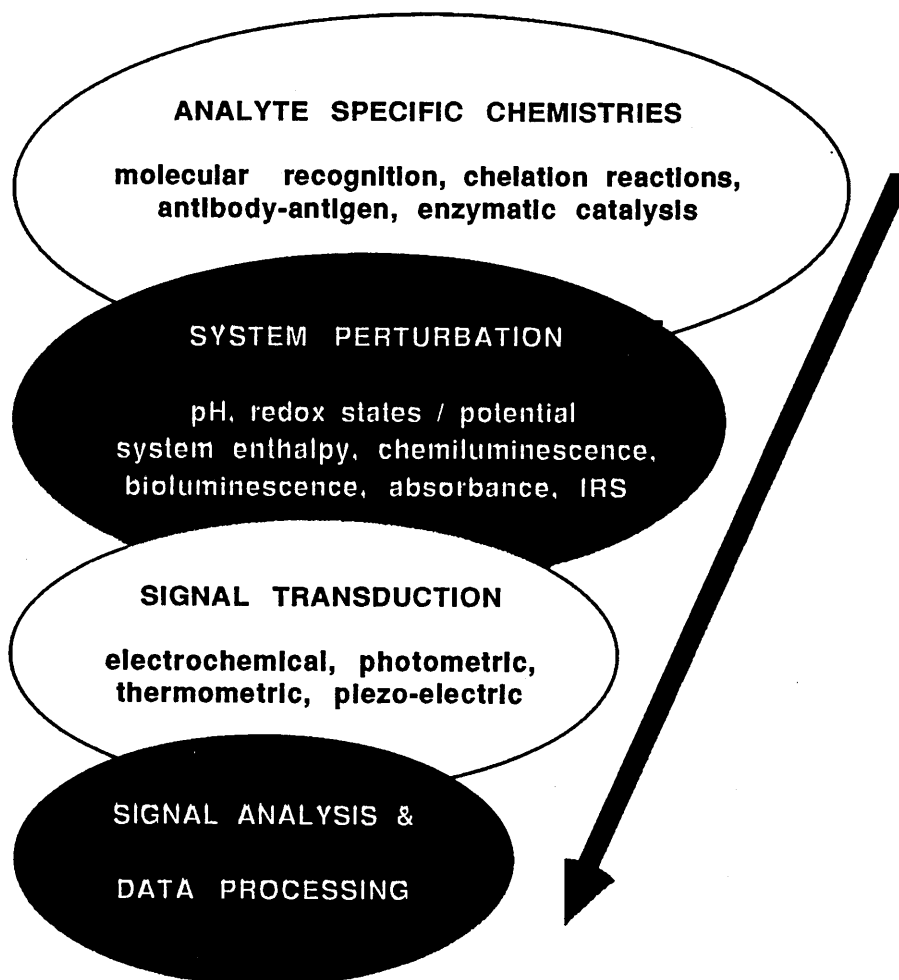


Figure 1.1.1. Schematic diagram representing information flow in a chemical sensor.

antigen-antibody reaction between surface bound antibodies and antigens in solution, lend themselves to piezo-electric determinations or to optical techniques such as surface plasmon resonance. The rationale of piezo-electric technique is based on the change in oscillation frequency of an antibody coated piezo-electric crystal on the binding of antigen. The resonant frequency of the crystal is reduced on the binding of antigen because of an increase in its mass, the change in resonant frequency may be correlated with the concentration of antigen present in solution (Clarke *et al.*, 1987). Measurement of antigen concentration by surface plasmon resonance is based on the change in the thickness of an immunological film on the surface of a waveguide coated with antibody on exposure to solutions containing antigen (Sutherland and Dähne, 1987). The change in internal reflective index may be correlated with the concentration of antigen in solution.

Fibre optic wave guide technology has realised the potential for the development of miniaturised spectrophotometric techniques. A range of sensing chemistries have been incorporated into fibre optic waveguide technology for the analysis of CO₂, O₂, and pH (Seitz, 1987), and for a range of antibody binding reactions involving bioluminescent or chemiluminescent chemistries (McCapra, 1987), or involving spectrophotometric absorbance measurements in solution (Schultz, 1987).

Although the CFD concept has been developed to incorporate both optical sensing strategies (originally Badley *et al.*, 1987, and recently with specific applications in immunodiagnostics, Attridge *et al.*, 1991; Deacon *et al.*, 1991) and an electrochemical format (Birch and Burns, 1987), this thesis was centred on an investigation of electrochemical sensing in the CFD. Electrochemical sensors may be divided into two categories, potentiometric and amperometric.

1.3.2. POTENTIOMETRIC AND AMPEROMETRIC SENSORS.

Potentiometric sensors operate on the principle of measuring the electrode potential after the establishment of a local equilibrium. Amperometric measurements are made of the current that results in the application of a potential difference that perturbs the local equilibrium. Selectivity in potentiometric regimes relies on some ion selective process at the electrode where potential is measured that ensures that the change in measured potential is due to the presence of the analyte. Selectivity in amperometric regimes is conferred by the selection of an appropriate operating potential, often coupled to compound selective membranes over the working electrode. Ion selective electrodes (ISE) are potentiometric devices whose specificity is dependent on the ion selectivity of the glass membrane that covers the electrode surface. The potentiometric response for the ion of interest, *i*, of an electrode operating behind an ion selective interface may be approximated by [1.3.1.].

$$E = E^{\ominus} + (RT/zF) \ln a_i \quad [1.3.1.]$$

Here E is the experimentally measured potential, E^0 is the formal redox potential, T is the temperature in $^{\circ}\text{Kelvin}$, R is the gas constant ($8.314 \text{ J}^{-1} \text{ mol}^{-1} \text{ K}^{-1}$), F is Faraday's constant (96487 C mol^{-1}), z is the charge on the ion of interest and a_i is the activity in solution of the selected ion, i .

The extent of the ionic selectivity of an ISE is dependent on the nature of the glass and the ionic environment of the sample solution. During the operation of an ISE there is no ionic transposition of the ion of interest across the glass membrane. Ionic communication between the test solution and the electrode is mediated by mobile cations present in the glass, frequently Na^+ or Li^+ . The extent to which an ion-selective glass is sensitive to other ions in the test solution is dependent on the ionic radii of those ions and the structure of the glass at the hydrated interface with the test solution. The sensitivity of a glass to a competing ion, j , may be defined by the product of the potentiometric selectivity coefficient, $k_{\text{pot}(a_i, a_j)}$ and the activity of j in the test solution. The potential response of an ISE to the activity of the ion of interest may be more correctly defined by substituting $(a_i + k_{\text{pot}(a_i, a_j)} a_j)$ for a_i in [1.3.1.]. If the sum of the product of the selectivity coefficient and the activity for each competing ion is small compared to a_i then the sensor is effectively selective for i , otherwise the response of the sensor is greater than would be expected from the species of interest alone. The response of an ISE is also dependent on the overall ionic strength of the test solution.

The composition of the glass used to construct an ISE determines the selectivity of the device. It is possible to rank the ionic sensitivity of different forms of glass to different cations: pH electrodes have a selectivity order $\text{H}^+ \gg \text{Na}^+ > \text{K}^+, \text{Rb}^+, \text{Cs}^+ > \text{Ca}^{2+}$ and for sodium sensitive glasses the selectivity order is $\text{Ag}^+ > \text{H}^+ > \text{Na}^+ \gg \text{K}^+, \text{Li}^+ > \text{Ca}^{2+}$. The corollary of the selectivity order for a sodium sensitive glass is that the pH must be stable and low in order to draw analytically significant conclusions from any measurements. The presence of Ag^+ would also elicit erroneous signals.

Potentially useful ISE sensors for the construction of enzyme electrodes have been reviewed by Kuan and Guilbault (1987). Taking the ammonium ion as an example, an examination of enzyme catalysed reactions that involve NH_4^+ reveal that a NH_4^+ sensitive ISE may be constructed to measure the concentration of urea, amino acids, glutamine, glutamic acid, nitrate or nitrite by the selection of the appropriate enzyme. The success of ISE technology in incorporating enzymatically catalysed reactions is dependent on the capacity to produce a glass that is highly selective to the ion of interest. Sample solutions of interest may be of varying ionic strength and may often contain a complex and variable mixture of potentially competitive ions. The problems associated with the operation of ISE technology in complex mixtures explains their limited success.

Transposition of the selective membrane technologies developed for ISEs onto transistor devices has resulted in the creation of the ion selective field effect transistor (ISFET) and the analogous use of chemically selective membranes has resulted in the creation of the chemically sensitive field effect

transistor (CHEMFET), (Blackburn, 1987). Although CHEMFETs and ISFETs are superior to ISEs because of the mass production benefits associated with microfabrication technology, such as device uniformity and unit cost reduction. However, like ISEs, the quality of the signal response obtained from CHEMFET and ISFET sensors is dependent on the selectivity of the the membrane employed. Field effect devices are additionally handicapped by their greater signal response sensitivity to electric fields compared to ISEs.

The differences between the operating principles of potentiometric and amperometric sensors are summarised in Table I.3.1. from Albery *et al.*, 1986.

Table I.3.1. Overview of the operating characteristics of potentiometric and amperometric sensors (from Albery *et al.*, 1986), c represents concentration.

<i>parameter</i>	<i>potentiometric</i>	<i>amperometric</i>
operating principle	measure potential at $i=0$	measure transport limited current
electrode kinetics	must be fast	electrode potential can drive reaction
response	c is an exponential function of EF/RT , good dynamic range, c is sensitive to errors in measurement of E	c is a linear function of current, normal dynamic range and normal response to errors in measurement of i
mass transport	unimportant	must be controlled

The principal errors associated with amperometric sensors arise because of differences in mass transport to the electrode, Table I.3.1.. The CFD obviates errors in variations of material flux at the electrode by integrating the current signal with respect to time and by defining a limited volume of solution where the current signal may proceed to zero. The electrode response is rendered insensitive to variations in material flux provided the current signal is close to zero at the end of the integration period. The current and charge responses of the CFD are described in depth in Section I.4.. The sensitivity of both types of sensor is 10^{-6} M or less (Albery *et al.*, 1986).

1.3.3. OPERATING STRATEGIES FOR AMPEROMETRIC ENZYME ELECTRODES.

Many enzymes which catalyse oxidation and reduction reactions contain redox centres composed of iron or copper complexes or quinone based compounds. For an oxidase, the oxidation of the substrate is associated with a reduction of the redox centre. The redox centre is then re-oxidized by oxygen with the concomitant evolution of hydrogen peroxide. The reduction and re-oxidation of the FAD group of glucose oxidase by di-oxygen is described in equations [1.3.2.] and [1.3.3.]:



The redox centres of enzymes such as glucose oxidase are surrounded by the protein structure of the enzyme. This restricts efficient electron transfer to simple electrodes. The reaction schemes outlined in [1.3.2.] and [1.3.3.] offer two strategies for the amperometric monitoring of the reaction: the oxidation of H_2O_2 at 0.6 V vs. SCE or the reduction of O_2 at -0.6 V vs. SCE. The reduction of O_2 was the first strategy to be applied to the oxidation of glucose by glucose oxidase (Clark, 1956). The electrode surface is covered with a gas permeable selective teflon membrane. The monitoring of oxidoreductase reactions based on the Clarke oxygen electrode (Clark, 1956) suffers from many practical problems. The response of the sensor is intrinsically dependent on the partial pressure of oxygen in the sample, and since the reaction is monitored by measuring the decrease in concentration of oxygen, the signal sensitivity is dependent on the ambient oxygen concentration. The hydrogen peroxide generated by the reaction inactivates the enzyme. The measurement of hydrogen peroxide obviates the problems of sensitivity encountered when measuring oxygen. The electrode reaction is defined in [1.3.4.].



This reaction has been employed by Clark (1970) for sensing glucose using glucose oxidase. This principle has been commercially exploited in the Model 23 Yellow Springs Instrument Analyser in 1973. Despite the development of hydrogen peroxide based sensors for the detection of lactate (Mullen *et al.*, 1986, Weil *et al.*, 1986) and pyruvate (Mascini & Mazzi, 1987), the signal response of sensors based on the detection of hydrogen peroxide produced from an oxidase reaction are dependent on the concentration of oxygen in the sample and its rate of diffusion through the solution. Additionally, many other compounds are oxidized at the oxidation potential of hydrogen peroxide. Compounds eliciting extraneous signals include ascorbate, paracetamol, cysteine and uric acid (Palleschi *et al.*, 1986).

Sensors based on oxidation of hydrogen peroxide have been termed first generation enzyme

electrodes (Albery *et al.*, 1986). Albery *et al.* (1986) also defines second and third generation devices. In second generation devices a compound which undergoes redox changes at a potential compatible with the redox couple of the redox centre of the oxidoreductase is substituted for molecular oxygen in [1.3.1.]. The amperometric signal is derived from the oxidation of the reduced electron mediator at the electrode surface. Second generation devices are superior to sensors based on hydrogen peroxide detection because the rate of oxidation of substrate is not dependent on the concentration of oxygen in the sample and because it is possible to select mediator compounds which are oxidized at a lower potential than hydrogen peroxide. The most successfully applied group of electron mediator compounds are the ferrocenes (Cass *et al.*, 1984; Cass *et al.*, 1985), whose oxidation potentials are between 0.1 V and 0.4 V. This represents a significant advantage over electrode configurations which detect hydrogen peroxide as a means of monitoring a reaction in terms of the reduction of the proportion of the signal derived from extraneous sources. Third generation devices involve modification of the electrode surface by deposition of organic conducting salts such as $\text{NMP}^+\text{TCNQ}^-$ (Albery *et al.*, 1985).

Immunochemistries may be monitored amperometrically by labelling the antibody with an enzyme or with an electrochemically active molecule (Green, 1987). Enzyme labels include glucose-6-phosphate, alkaline phosphatase and glucose oxidase. Enzyme label assays require the presence of a substrate molecule or substrate analogue whose product may be electroactive. An example is the use of alkaline phosphatase with the electrochemically inactive substrate phenyl phosphate (Wehmeyer *et al.*, 1986) the hydrolysis product, phenol, is electroactive. Labelling an antibody of low molecular mass with a ferrocene allows construct to act as an electron acceptor for glucose oxidase. On binding antigen the efficiency of electron mediation of the ferrocene labelled antibody is reduced (di Gleria *et al.*, 1986), resulting in a decrease in catalytically derived current in the presence of glucose and glucose oxidase. Recent advances in electrochemical immunoassays have been reviewed by Jenkins *et al.*, 1991.

1.4.1. THE CAPILLARY FILL DEVICE: FUNDAMENTAL CONCEPTS AND DESIGN.

The CFD was designed to realise bulk electrolysis of a substance within an analytically useful time. In terms of determining the total amount of analyte present in a sample, complete electrolysis of that substance yields a correlation between the amount of charge passed, Q_t/C , in the analysis time, t/s , according to equation [1.4.1.] (Bard and Faulkner, 1980; Kissinger, 1987):

$$Q_t = \int_0^t i dt = nFN \quad [1.4.1.]$$

here F is Faraday's constant, 96485 C mol^{-1} , N is the number of moles undergoing the redox reaction at the electrode and n is the number of electrons transferred to the electrode per mole of substance. Q is also equivalent to the integral of current at the electrode, i/A , over the period t s.

From [1.4.1.] it is apparent that the determination of the amount of analyte ought to be completely unambiguous provided that all of the substance undergoes reaction at the electrode and that there are no extraneous signals. The amount of time needed to achieve complete depletion of an analyte is dependent on the ratio of the volume of solution to the electrode area, a function of cell configuration, and upon the flux of substance to the electrode surface, a function of the mass transfer characteristics of the system, for a quiescent solution principally the diffusion coefficient of the substance in the solution.

Complete depletion of substance is achieved in about 60s for a substance with a diffusion coefficient of about $10^{-9} \text{ m}^2 \text{ s}^{-1}$ in the CFD by making the greatest distance that the substance has to diffuse through very small compared to any dimension of the electrode. This is realised in construction by producing a screen printed electrode and conducting electrolysis in a layer of solution, about $100 \times 10^{-6} \text{ m}$ thick, held directly above the electrode by an electrochemically and chemically inert plate. Figure 1.4.1. shows the dimensions and plan of the electrode configuration employed in the CFD. Any application involving an enzymatically catalysed reaction with an analyte or otherwise chemically transforming an analyte prior to its determination may involve reagents. These may be screen printed onto the top plate above the electrode. Figure 1.4.2. shows an exploded view of the complete device, including the position of a 'reagent' or 'catalytic' layer. Figure 1.4.2. shows a cross-section through the centre of the device. The approximate volume above the surface of the central electrode is about $1.8 \times 10^{-5} \text{ m}^3$ and the principal distance through which most of the analyte diffuses to the electrode surface is $100 \times 10^{-6} \text{ m}$, directly above the electrode and in a direction perpendicular to it. From the Einstein-Stokes equation (Section II.4.) certain timescales associated

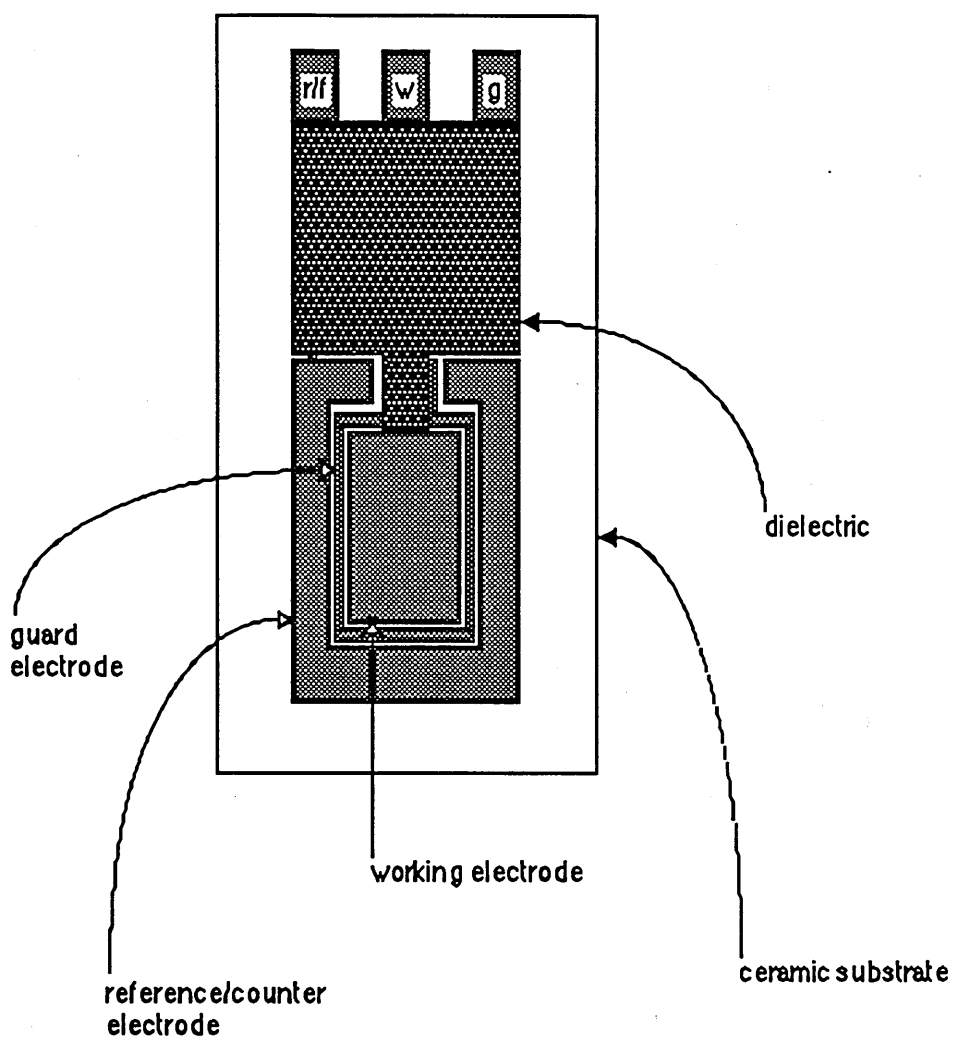


Figure 1.4.1. Planar view of the electrode configuration employed in the CFD. The dimensions of the working electrode are 3 mm x 6 mm. The electrodes are bounded by an area equal to 6 mm x 10 mm. The ceramic substrate has dimensions of 10 mm x 20 mm. The connections from the reference/counter electrode, guard electrode and the working electrode are represented by r/f, g and w respectively.

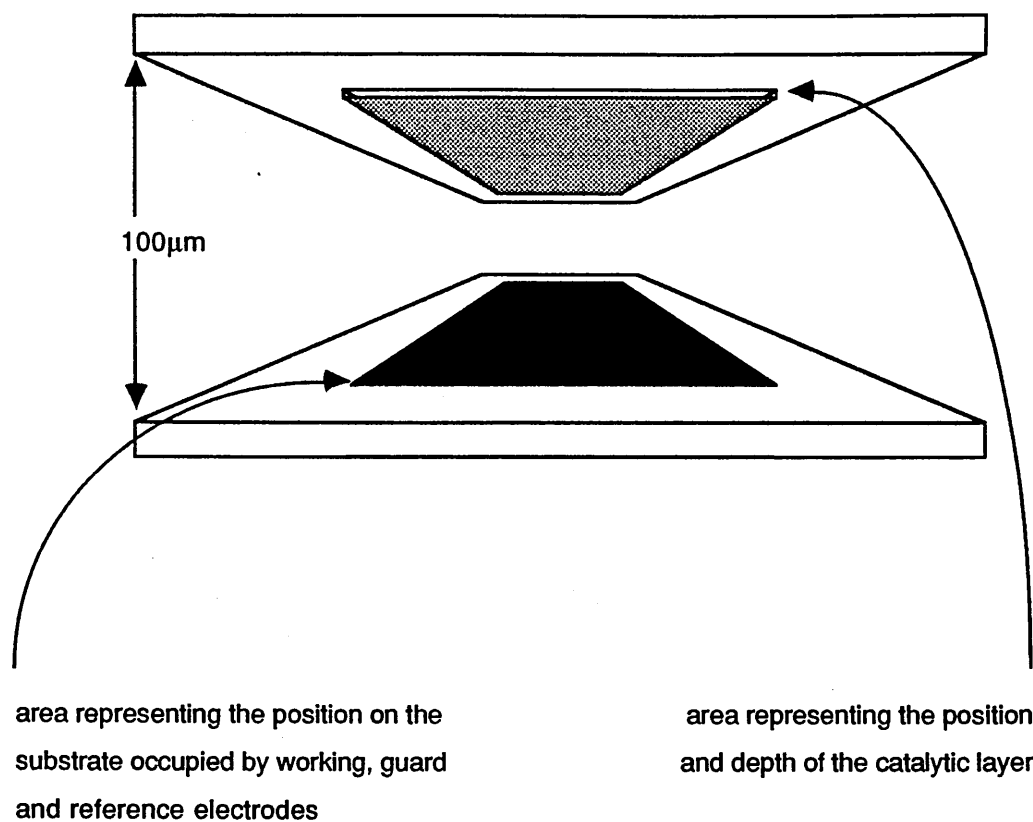


Figure 1.4.2. Exploded view of the CFD. The gap width is defined by optical fibres of diameter $100\mu\text{m}$.

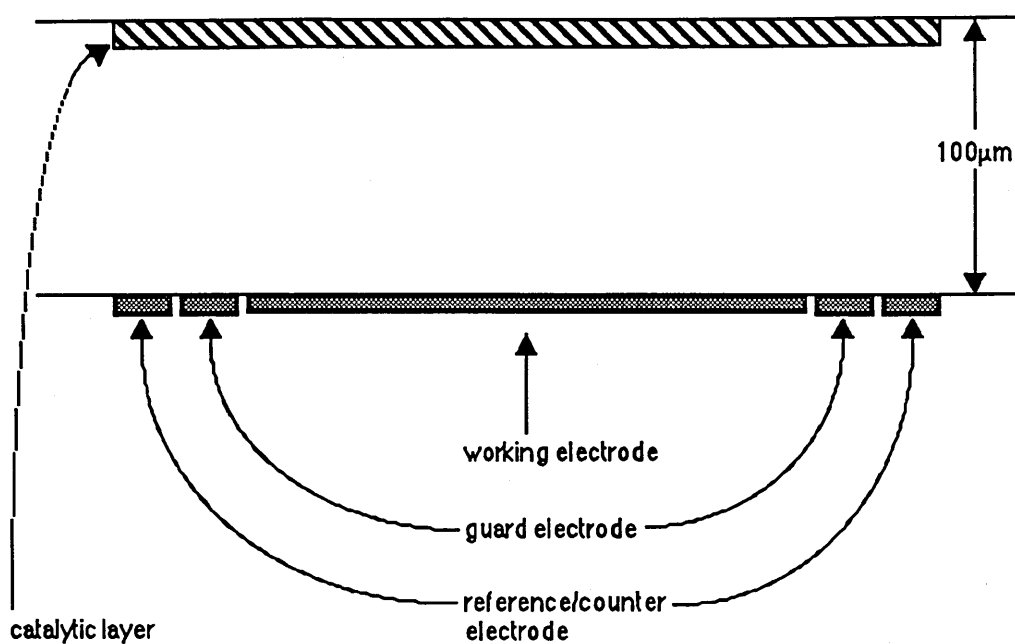


Figure 1.4.3. Schematic representation of a cross-section through the CFD. The distance between the working and guard electrodes and the guard and reference/counter electrodes is 200 μm. The widths of the guard and working electrodes are 0.5 mm and 1 mm respectively. Although the electrodes are printed onto the surface of the substrate, they are represented here by shaded blocks within the substrate. The depth of the printed electrodes is neglected. The catalytic layer has a depth of 10 μm.

with the extent of depletion may be evaluated. For a substance with a diffusion coefficient of about $10^{-9} \text{ m}^2 \text{ s}^{-1}$ the diffusion timescale for a molecule to traverse the cell is about 10 s.

The potential is set at the outer electrode by means of the couple between the oxidized and reduced forms of the electroactive substance as defined by the Nernst equation, [I.4.2.] (Bard and Faulkner, 1980; Kissinger, 1984):

$$E = E^\ominus + (RT/nF) \ln [M_O]/[M_R] \quad [\text{I.4.2.}]$$

where the potential, in volts, E , is defined by the formal potential, $E^\ominus$, plus some function of the ratio of the concentrations of the oxidized, $[M_O]$, and reduced, $[M_R]$, states of the substance. R is the gas constant, $\text{J mol}^{-1} \text{ K}^{-1}$, and T is the temperature of the system in degrees Kelvin. The second term of [I.4.2.] is a measure of the deviance from the formal potential, which describes a potential of the half cell measured against a normal hydrogen electrode at an arbitrary set of conditions including an equivalent concentration of each of the redox states of the substance.

The central electrode acts as a working electrode and the reference electrode also acts as a counter electrode. Provided that the concentration of the oxidized form of the substance predominates during the timescale for application of potential pulse the potential will not drift significantly. Any potential drift may be anticipate by setting an acceptable overpotential.

The boundary is defined in the x-dimension in Figure I.4.1. by the position of the top plate. Diffusion in the z-dimension is limited by the relative dimensions of the cell; the current contribution from lateral diffusion is insignificant during the early period of depletion of the layers of solution above the electrode and only affects the edges of the electrode but becomes predominant when these layers have become depleted (Section I.4.2.). In order to define a boundary in the z-dimension a third electrode, nominated the 'guard electrode' is operated at the same potential as the working electrode, effectively preventing a flux of material from the rest of the volume at a distance of half the gap between the edge of the working electrode and itself, $100 \times 10^{-6} \text{ m}$.

Having defined a volume by the use of a physical barrier and an electrochemical screen it is possible to correlate the amount of charge passed with the concentration of analyte present, [I.4.1].

1.4.2. CURRENT TRANSIENTS AND DIFFUSION IN THE CFD.

Evaluation of the current transients from the flux equations that describe the diffusion of a substance in a thin layer of solution to an electrode perpendicular to the direction of diffusion yield a solution involving an exponentially decaying current of the form $i_t = Ae^{-\beta t}$. The rate of decay is some function of the cell dimensions and the diffusion coefficient of the substance.

The concentration of an electroactive species, $[M_o]$, at a distance x and a time t is described by [1.4.3.]:

$$\partial[M_o]_{(x,t)}/\partial t = D_{Mo}(\partial^2[M_o]_{(x,t)}/\partial x^2) \quad [1.4.3.]$$

where D_{Mo} is the diffusion coefficient of the substance in $m^2 s^{-1}$. The following boundary conditions correspond to the CFD system just prior and for the duration of the application of potential pulse:

$$\text{for } t=0, 0 \leq x \leq l, \quad [M_o]_{(x,0)} = [M_o]_0 \quad [1.4.4.]$$

$$\text{for } t>0, \quad [M_o]_{(0,t)} = 0 \quad [1.4.5.]$$

$$\text{for } t>0 \ x=l, \quad (\partial[M_o]_{(x,t)}/\partial x) = 0 \quad [1.4.6.]$$

Equation [1.4.4.] states that at $t=0$, prior to the application of potential pulse, the electroactive species is homogeneously distributed at all values of x , between $x=0$, the electrode surface, and $x=l$, at the inert boundary, at a concentration $[M_o]_0$. Equation [1.4.5.] states that on application of a potential pulse, the concentration of M_o is zero. The physical boundary of the end plate is represented by [1.4.6.], describing zero flux of M_o at the boundary, l .

According to Bard and Faulkner (1980) the solution of [1.4.3.] results in a time dependent concentration profile involving an exponential involving an exponential constant, β , [1.4.8.]. Substituting the solution to [1.4.3.] into [1.4.7.] results in an approximation of the current at a time, t , solely dependent on the initial current value, i_0 , and B , [1.4.9.]:

$$i_t = nFAD_{Mo}(\partial[M_o]_{(x,t)}/\partial x) \quad [1.4.7.]$$

$$\beta = D_{Mo}\pi^2/l^2 \quad [1.4.8.]$$

$$i_t = i_0 e^{-\beta t} \quad [1.4.9.]$$

In [1.4.7.] A is the area of the electrode in m^2 and all other symbols are as previously stated.

In fact the decay of current in the CFD does not follow this relationship during a sixty second application of potential. There are four kinds of current behaviour over the course of a sixty second, or longer, potential pulse. Current for the first few milliseconds is composed of contributions from the surface concentration of substance and from capacitive effects of the ionic double layer. Thereafter, before the effect of condition [I.4.6.] asserts an influence at the electrode, the flux of material to the electrode approximates the solution of [I.4.3.] for a semi-infinite solution. Solving [I.4.3.] for a dimension perpendicular to the electrode that is large compared to the magnitude of the diffusion coefficient of the substance, i.e. removing condition [I.4.6.], and substituting the solution into [I.4.7.] results in the Cottrell Equation for current due to diffusion at a planar electrode, [I.4.10.]:

$$i_t = nFAD^{0.5}[M_0]/\pi^{0.5}t^{0.5} \quad [I.4.10.]$$

The duration over which the time dependence of current more closely corresponds to [I.4.10.] than [I.4.9.] is dependent on the diffusion coefficient of the substance. It can be shown (Bard and Faulkner, 1980) that the root mean square distance, Δx , traversed by a molecule in time Δt is given by [I.4.11.]:

$$\Delta x = (2D_{Mo}\Delta t)^{0.5} \quad [I.4.11.]$$

for a molecule of ferricyanide, which has a diffusion coefficient of about $5 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$, the time taken to traverse $100 \times 10^{-6} \text{ m}$ is $\sim 8 \text{ s}$. At this point, however, the zero flux condition at I would have affected the flux close to the electrode and the time dependence of current may become transitional between [I.4.9.] and [I.4.10.]. Kissinger (1984) defines the distance from an electrode where the solution is disturbed as about $6(Dt)^{0.5}$. Applying this equation to the same conditions results in a time of about 0.5s. In fact [I.4.10.] is applicable to the current derived from a solution of ferricyanide for approximately 7 s (Section II.4. and Chapter III.).

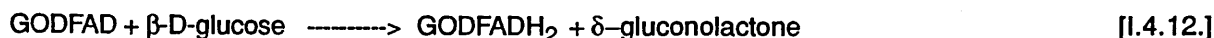
At times after the layers of solution above the electrode have become depleted of substance the current derived from the lateral diffusion of material, in the z-dimension, becomes predominant. Because the distance in the z-dimension is of the order of several millimetres and because the effective area of the electrode exposed to these elements of solution is small, approximating the perimeter of the working electrode, current is once again proportional to $t^{-0.5}$. The guard electrode was devised to obviate the effects of lateral diffusion when performing exhaustive coulometry. At times where the current contribution derived from diffusion in the x-dimension is of the same order of magnitude to the current contribution derived from lateral diffusion the principal source of variation between otherwise identical sample solutions in determination of charge arise from variations in lateral diffusion. Variations in lateral diffusion arise from differences in solution viscosity. The viscosity of the layer of solution within the CFD is primarily dependent on the temperature of the device. The

application of an oxidizing potential at the guard electrode renders the response temperature independent, provided that the potential is applied for a sufficient period such that the current response is allowed to fall to zero. Applying a similar rationale, any variations in the viscosity of the layer of solution that may arise from differences in the constitution between samples ought to be obviated.

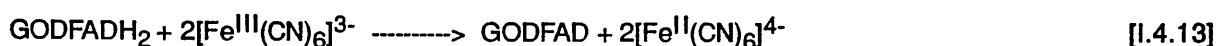
The present mode of operation of the CFD involves complete coulometry of the analyte present in the sample. This strategy makes use of the additive effects of all of the current-time behaviours. Other strategies which utilise one or more of the other time dependent current relationships may be considered. It may be possible to use the information from the first $t^{-0.5}$ period of current dependency using a series of potential pulses separated by potential relaxation periods, thereby inducing an exponential decay of charge response for each of those potential pulse periods due to the fact that the distance through which the principle flux contribution acts is small compared to the time-scale and the diffusional characteristics of the electroactive species, Chapter III.

1.4.3. EMPLOYMENT OF REAGENT LAYERS IN THE CFD FOR THE DETERMINATION OF GLUCOSE.

The oxidoreductase glucose oxidase undergoes reduction of the flavin adenine dinucleotide moiety (FAD) in the presence of β -D-glucose, equation [1.4.12.]:



here GODFAD represents glucose oxidase with an oxidized FAD cofactor, GODFADH₂ represents the reduction of FAD by glucose. In 'nature' GODFADH₂ is oxidized by molecular oxygen. Other chemical entities such as ferricyanide or ferrocene may also act to restore the oxidation state of the reduced enzyme (Chapter II.):



here two moles of ferricyanide ($[\text{Fe}^{\text{III}}(\text{CN})_6]^{3-}$) are reduced to ferrocyanide ($[\text{Fe}^{\text{II}}(\text{CN})_6]^{4-}$) for every mole of GODFADH₂ oxidized. The ferrocyanide may then be oxidized at an electrode poised at the appropriate potential to produce an electroanalytical current, [1.4.14.]. This signal is some function of the original concentration of glucose.



Reactions [1.4.12.] to [1.4.14.] may be incorporated in the CFD by placing ferricyanide and glucose oxidase in the reagent layer above the electrode, Figures 1.4.2. and 1.4.3..

In order to realise the objective of introducing the sample to the sensor without any chemical pretreatment of the sample it is important that other substances be present in the reagent layer to support the operation of the enzyme and to minimise the cell resistance. Glucose oxidase operates at a maximum rate at pH 5.5 with molecular oxygen as the electron acceptor. However, when artificial electron acceptor molecules are used the pH optimum is often shifted from this value (Chapter II). By setting the pH at which the device operates at a value different to pH 5.5 any competitive reaction between molecular oxygen and the mediator molecule is reduced. Because the concentration of mediator is very high, about 3 M in the reagent layer at the start of the reaction, it may be demonstrated that the concentration of glucose at or near the surface of the reagent layer is zero for the duration of the determination (Chapter III.). Considerations of the ease of manufacture and printing of reagent layer and of uniformity of pH between samples dictated that the buffer system should be robust rather than rate optimal. Consequently equimolar di-hydrogen potassium phosphate and hydrogen di-potassium phosphate were used to construct a buffer of pH 7 at a working concentration in the device of 0.5 M. Potassium chloride is also present in the reagent layer to augment the ionic strength

of the solution, resulting in a value for ionic strength of greater than one.

Solutions containing analyte are introduced to the CFD at the edge furthest from the connectors (Figure I.4.1.). This is facilitated by the protrusion of the bottom plate from underneath the top plate by about 1 mm and by the application of a layer of the detergent Triton-X100 on the inside surfaces of both plates during manufacture. Once the device has filled buffer, mediator and potassium chloride diffuse rapidly into the volume beneath the reagent layer. Glucose oxidase is more likely to remain within the reagent layer matrix and since its diffusion coefficient is about 500 times smaller than that of ferricyanide, would not diffuse more than about $10 \times 10^{-6} \text{ m}$ in any case over a 60 s reaction time¹. As previously stated, it is likely that the penetration of glucose into the catalytic layer is not significant, because the rate of reactions [I.4.12.] to [I.4.13.] in the reagent layer are high compared to the diffusion timescale of glucose reduced mediator is generated at the same rate as glucose arrives (Chapter III.).

I.4.4. MANUFACTURE OF THE CFD AND REAGENT LAYER.

The electrodes, conducting tracks and contacts were deposited onto an alumina back plate (Kyocera A4476) using conventional silk screen printing techniques and a gold printing paste (Engelhard T4474). After firing in a high temperature oxidizing furnace, the insulating layer (Dupont 5704 dielectric printing ink) was overlaid onto the ceramic back plate. The distance between the top and bottom plates was defined by 100 μm diameter optical fibres.

The reagent layer manufactured for the glucose CFD was screen printed onto the top plate as an area equal to the area within the bounds of the reference-counter electrode in a position directly matching the perimeter of the reference-counter electrode, Figure I.4.1.. This is important in terms of providing an ionically homogeneous solution prior to the application of the potential pulse. The ink constructed for the glucose CFD employed 5% cellulose acetate in cyclohexanone as carrier, 33% potassium ferricyanide, 12.5% each of di-hydrogen potassium phosphate and hydrogen di-potassium phosphate and 4% glucose oxidase. Given that 2 mg of ink is deposited in each CFD over an area of approximately $6 \times 10^{-5} \text{ m}^2$, at a depth of 10 μm , the final concentrations of glucose oxidase and ferricyanide in the catalytic layer are in the region of 0.8 moles m^{-3} and 3000 moles m^{-3} respectively.

Potassium ferricyanide, di-hydrogen potassium phosphate and hydrogen di-potassium phosphate and all other chemicals used in the manufacture of the glucose CFD were obtained from Aldrich Chemical Co. and used at Analar Grade where available. Glucose oxidase, Type VII from *Aspergillus niger* with a specific activity of 200 units mg^{-1} was obtained from Genzyme Ltd..

¹ substituting a diffusion coefficient of $10^{-12} \text{ m}^2 \text{ s}^{-1}$ into [I.4.11.]

***Chapter II, electron mediators for employment in
the CFD***

II.1.1. INTRODUCTION TO CHAPTER II: ELECTRON MEDIATORS FOR EMPLOYMENT IN THE CFD.

In order to understand the rationale underlying the choice of electron mediator it is necessary to be aware of the principles which govern the operation of the CFD.

Prior to this work Dicks *et al.*, 1987 made a review study of potential soluble electron mediators for GOD and associated oxidoreductases that may be utilised in the CFD. A principal reference cited was the work of Fultz and Durst (1982), who reviewed electrochemical studies of redox compounds which were known to oxidise or reduce oxidoreductases or electron transfer proteins. Dicks identified the following qualities that a compound ought to possess in order to be of use as a mediator in a CFD:

*water solubility ($>0.1\text{M}$) : a minimum solubility in water of 0.1 M is important for two reasons: firstly, because the reference potential of the device is determined by the Nernstian couple at the gold surface, the change in the ratio of the concentrations of the reduced to the oxidized state of the compound during the course of a coulometric determination should be limited so that the potential does not drift below the oxidation potential of the compound. There are three reasons why this is not as important as might first be assumed. a) The ratio of the concentrations of the reduced and oxidized states of the mediator does not change significantly during the application of the potential pulse. b) A high concentration of oxidized mediator is advantageous when considering the rate of oxidation of the reduced oxidoreductase. c) Any effect of a variable concentration of the oxidized form over the working electrode during the coulometric stage is obviated by the effect of the guard electrode; the ratio [oxidized]:[reduced] above the counter-reference electrode is invariant after the application of oxidizing potentials at the guard and working electrodes.

*rapid solubility ($< 60\text{ s}$): if the reaction between the catalytic layer, containing the enzyme, and the bulk solution containing the oxidized mediator and the substrate is allowed to proceed for 60 s prior to coulometric determination, then it is desirable that most of the mediator in the catalytic layer enters solution within 60 s . In fact, as discussed elsewhere (Section I.4.) the local concentration of the mediator at the interface of the catalytic layer and the bulk solution is very much greater than its mean concentration throughout the device. With regard to the maintenance of potential during the coulometric determination, it is important that the concentrations of the reduced and oxidized forms are homogeneous when potential is first applied.

*stability to autoxidation in solution and rapid reaction kinetics compared to di-oxygen: in order to maintain the integrity of the stoichiometry between the number of moles of glucose oxidized and the number of moles of mediator reduced the reduced form ought to be resistant to autoxidation and the rate of oxidation by di-oxygen should be negligible. Both of these factors contribute to a linear correlation between concentration of glucose and concentration of reduced mediator.

*reversible electrochemistry and a redox potential close to 0 V vs. SCE: reversible electrochemistry is important for the establishment and maintenance of potential and for to facilitate the oxidation of the reduced mediator at the electrode. Analytical samples contain substances that may be electroactive within the operational potential range of the device. Examples include ascorbate and aceylsalicylate. The lower the redox potential of the mediator compound the smaller the contribution to the final charge value from extraneous electrochemical sources.

In addition to the criteria discussed above any utilisable compound ought to be pH insensitive within the range pH 4 to 9, stable on long term dry storage, ought not to react electrochemically nor chemically with components in the sample and not elicit enzyme inactivation.

The use of the CFD in its present format requires that the electron mediator should have a high solubility in order that the potential is stable over the period of analysis. The use of mediators that have been successfully employed, such as the ferrocenes (Cass *et al.*, 1984 & 1985) or TTF (Turner *et al.*, 1987) is inappropriate in this context. Ferricyanide has been used as an electron acceptor for reduced glucose oxidase (Birch & Burns, 1987). Ferricyanide fulfils most of the criteria discussed previously although it has an homogeneous rate constant for its reaction with reduced glucose oxidase which is inferior to the ferrocenes and is too small to be determined by cyclic voltammetric studies (Nicholson, 1965). However, this is not a handicap in the CFD since it may be deployed at concentrations greater than 0.3 M. The present study identified ruthenium (III) hexamine as a potentially useful compound for the defined purpose. Ruthenium (III) hexamine is a structurally analogous compound to ferricyanide. The outer electronic structure of ruthenium is $4s^2 4p^6 4f^7 5s^1$, the principal oxidation states of its compounds are two and three.

*Electron transfer properties of ruthenium compounds: Reversible electrochemistry of hexacyano-ruthenium complexes and pyridyl-penta-amine complexes and their use as mediators in the glucose oxidase/glucose system has been documented (Crumbliss *et al.*, 1986). Ruthenium (III) hexamine has been used as a mediator compound for the Mo/Fe enzyme, sulphite oxidase (Coury, 1990). Ruthenium complexed with pyridine has also been reported in several studies of electron transfer from cytochromes and related electron transfer proteins (Norton & Hurst, 1978; Cumins and Gray, 1977). Compounds containing ruthenium have also been used as artificial electron relays to chemically modify electron transfer proteins (Jackman *et al.*, 1988; Sykes, 1988) and to modify redox proteins (Degani & Heller, 1988), as have ferrocenes (Bartlett *et al.*, 1987; Degani & Heller, 1988). The kinetics of the reduction of cytochromes by ruthenium (II) hexamine has been examined (Scott & Gray, 1980) and a mechanism for electron transfer consistent with outer sphere electron transfer proposed (Wherland & Gray, 1976).

The information presented in Part II describes the electrochemistry of ruthenium (III) hexamine and its reaction with glucose oxidase. it also includes an attempt to account for an unusual pH-activity profile

of the ruthenium (III) hexamine/glucose oxidase/glucose system by correlating dependence of the heterogeneous rate constant in different ionic environments with the homogeneous rate constant between ruthenium (III) hexamine, by employing the Marcus relationship. The final section of Part II attempts to describe the comparative electrode kinetics of capillary fill devices employing ferricyanide and ruthenium (III) hexamine.

II.2.1. PROCEDURES USED TO INVESTIGATE THE ELECTROCHEMISTRY OF RUTHENIUM (III) HEXAMINE AT PLANAR GOLD ELECTRODES.

The chloride salt of hexamineruthenium (III) (or ruthenium (III) hexamine, $[\text{Ru}^{\text{III}}(\text{NH}_3)_6]\text{Cl}_3$) was used in all experiments. It is a yellow powder that is air stable at room temperature and has a molecular mass of 309.61. Ruthenium forms a six coordinated octagonal complex with a variety of ligands, including NH_3 (Figure II.2.1.).

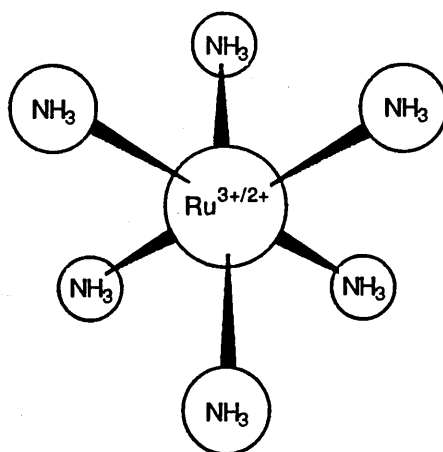


Figure II.2.1. The configuration of ruthenium (III) hexamine illustrating octagonal structure. Ruthenium (II/III) hexamine has an ionic radius of $3 \times 10^{-10} \text{m}$ (Wherland & Grey, 1976).

Chemicals and electrochemicals. Ruthenium (III) hexamine trichloride was obtained from Strem chemicals Inc. of Newbury, M.A., U.S.A.. All other chemicals used were obtained from Aldrich Chemical Company and were of 'Analar' grade.

Electrochemistry. Cyclic voltammetry and linear sweep voltammetry were performed using an EG&G m270 potentiostat and EG&G m270 electrochemical software run on an Amstrad 1612 PC with an Intel Above Board memory expansion giving 1 Mb of RAM. Differential pulse voltammetry (DPV)

was performed using a Bioanalytical Systems model 100 electrochemical analyser (BAS 100) (Bioanalytical Systems Inc., Indiana, U.S.A.). An electrochemical cell comprising two chambers connected by a glass frit was used throughout. A 3 mm diameter gold electrode (Bioanalytical Systems Inc., Indiana, U.S.A.) acted as the working electrode. A platinum wire counter electrode and a SCE reference (Russell pH Ltd., Fife, Scotland) completed the arrangement. Determination of the diffusion coefficient of ruthenium (III) hexamine by chronocoulometry was performed using the BAS 100 electrochemical analyser.

Experimental. As an initial investigation of the electrochemistry of ruthenium (III) hexamine differential pulse voltammetry (DPV) was used to elucidate the reduction and oxidation potential peaks. Ruthenium (III) hexamine was present at a concentration of 0.5 mM in a solution of potassium chloride present at a concentration of 1 M with 50 mM phosphate buffer at pH 7.0. A series of cyclic voltammograms at scan rates from 1 mVs⁻¹ to 900 mVs⁻¹ were performed to determine whether ruthenium (III) hexamine exhibited reversible electrochemistry at gold electrodes. Peak currents were recorded from the fifth and sixth cycles. The reactions were supported in electrolyte consisting of 4 M KCl in 50 mM phosphate buffer at pH 7. The concentration of ruthenium (III) hexamine was 2 mM in all cases.

II.2.2. THE ELECTROCHEMISTRY OF RUTHENIUM (III) HEXAMINE AT GOLD ELECTRODES.

Referring to Figures II.2.2. and II.2.3., DPV revealed cathodic and anodic (oxidation and reduction) potential peaks of -244 mV and -192 mV vs. SCE respectively.

Cyclic voltammetry was performed using identical solutions, except that ruthenium (III) hexamine was present at a concentration of 2 mM. The cathodic potentials at both electrodes were about -250 mV and the anodic potentials were about -185 mV. Typical cyclic voltammograms performed at scan rates between 0.5 V s⁻¹ and 0.9 V s⁻¹ for solutions containing 2 mM ruthenium (III) hexamine are shown in Figure II.2.4.

The cathodic reaction is defined as:



The anodic reaction is defined as:



Three diagnostics were used to determine the extent of reversibility:

(I) a plot of scan rate^{-0.5} vs. anodic or cathodic peak currents has a straight line of positive slope with zero intercept for a reversible couple, Figure II.1.5..

(II) the values of i_{pa} (peak anodic current) and i_{pc} (peak cathodic current) are similar in magnitude for a reversible couple with no kinetic complications; $i_{pa}/i_{pc} \sim 1$. i_{pa}/i_{pc} should be approximately constant with respect to scan rate, Figure II.1.6..

(III) if a system is chemically and electrochemically reversible then relation [II.1.3.] applies;

$$E_p = E_{pa} - E_{pc} \sim 0.057/n \text{ (V)} \quad [\text{II.1.3.}]$$

Where E_p is the peak separation in volts and E_{pa} and E_{pc} are the anodic and cathodic peak potentials respectively.

From inspection of Figure II.1.5. it is apparent that $[\text{Ru(NH}_3)_6]$ exhibits good reversible electrochemistry under these condition. The slopes of i_{pa} and i_{pc} vs. scan rate^{0.5} are linear with

correlation coefficients of 0.9823 and 0.9907 respectively with origins at, or very close to zero.

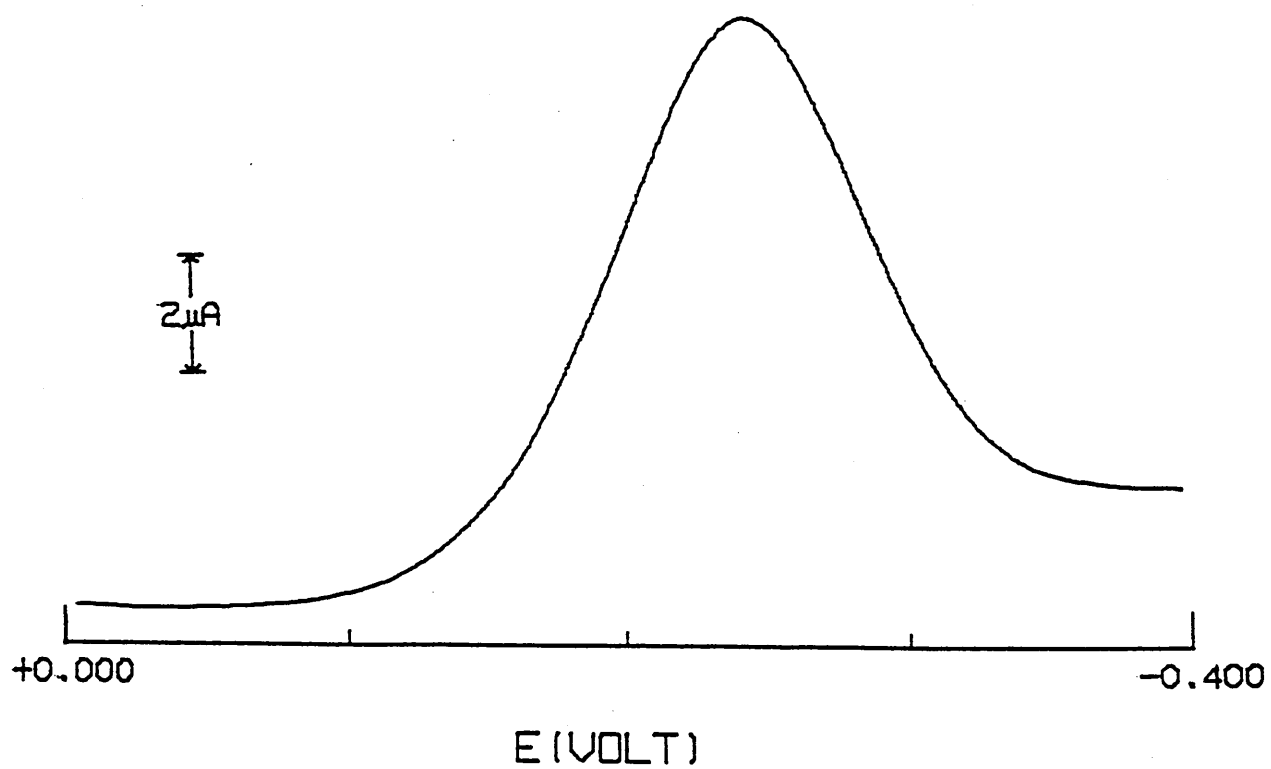
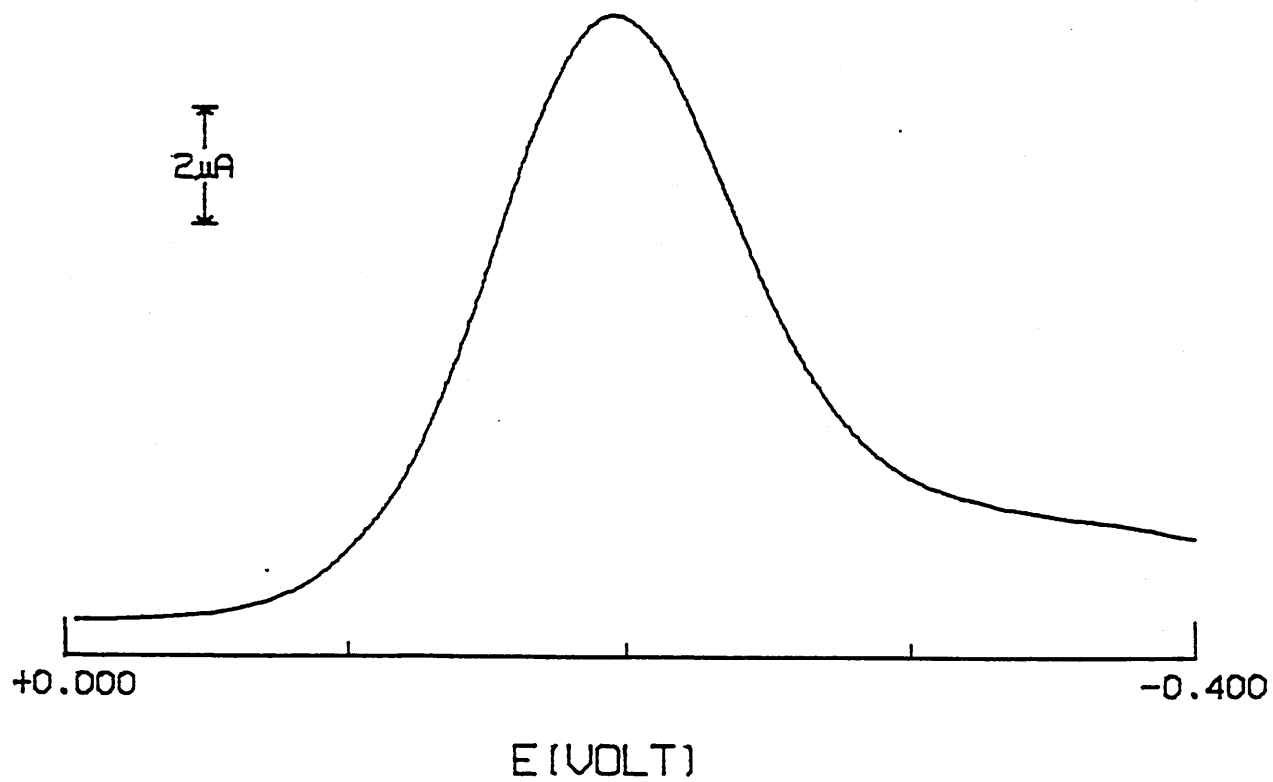
Figure II.1.6. is also indicative of highly reversible electrochemistry (criterion (ii)). The ratio of i_{pa}/i_{pc} is almost invariant and close to unity (mean value is 0.96, $n=10$, $sd=0.018$). From this evidence it may be inferred that some no significant following chemical reaction is occurring.

Ruthenium (II) hexamine is reported to be able to reduce oxygen in solution (Pladziejewicz *et al.*, 1973). Should oxygen oxidize ruthenium (II) hexamine at a significant rate relative to the scan rate an enhancement in amplitude of the cathodic peak and a diminution in amplitude of the anodic peak would be expected, resulting in a value for i_{pa}/i_{pc} of less than one. This result is significant in terms of the use of ruthenium (III) hexamine as an electron acceptor for an oxidoreductase.

Peak potential differences of the order of 60 mV are obtainable by cyclic voltammetry. The theoretical minimum value for E_p is 57 mV (Bard and Faulkner, 1980) The balance of evidence from this set of data is that the ruthenium (II) hexamine/ruthenium (III) hexamine couple is highly reversible under the stated conditions.

Figure II.2.2. Differential pulse voltammogram at a 3 mm diameter planar gold electrode for a solution containing 0.5 mM ruthenium (III) hexamine. Pulse conditions: initial potential -0.4 V; final potential 0 V; scan rate 4 mV s⁻¹; pulse amplitude 50 mV; pulse width 60 ms; pulse period 1 s. Peak potential; -244 mV.

Figure II.2.3. Differential pulse voltammogram at a 3 mm diameter planar gold electrode for a solution containing 0.5 mM ruthenium (III) hexamine. Pulse conditions: initial potential 0 V; final potential -0.4 V; scan rate 4 mV s⁻¹; pulse amplitude 50 mV; pulse width 60 ms; pulse period 1 s. Peak potential; -192 mV.



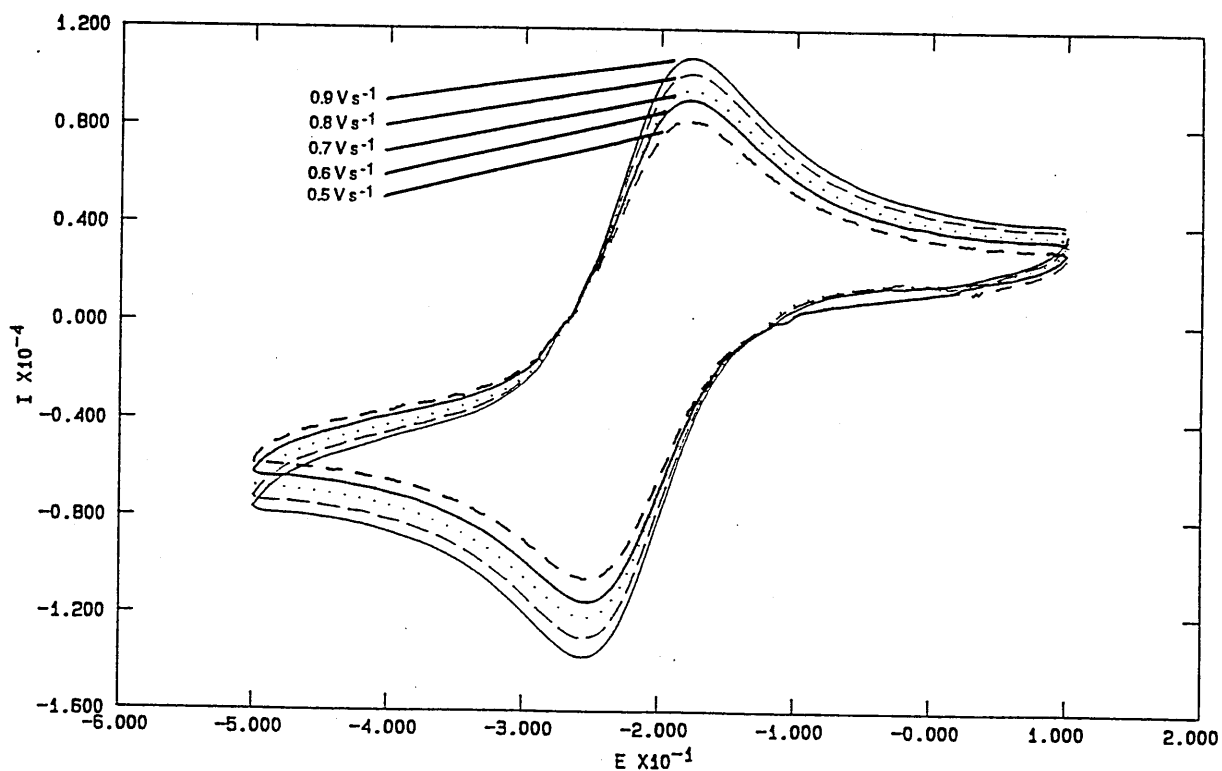
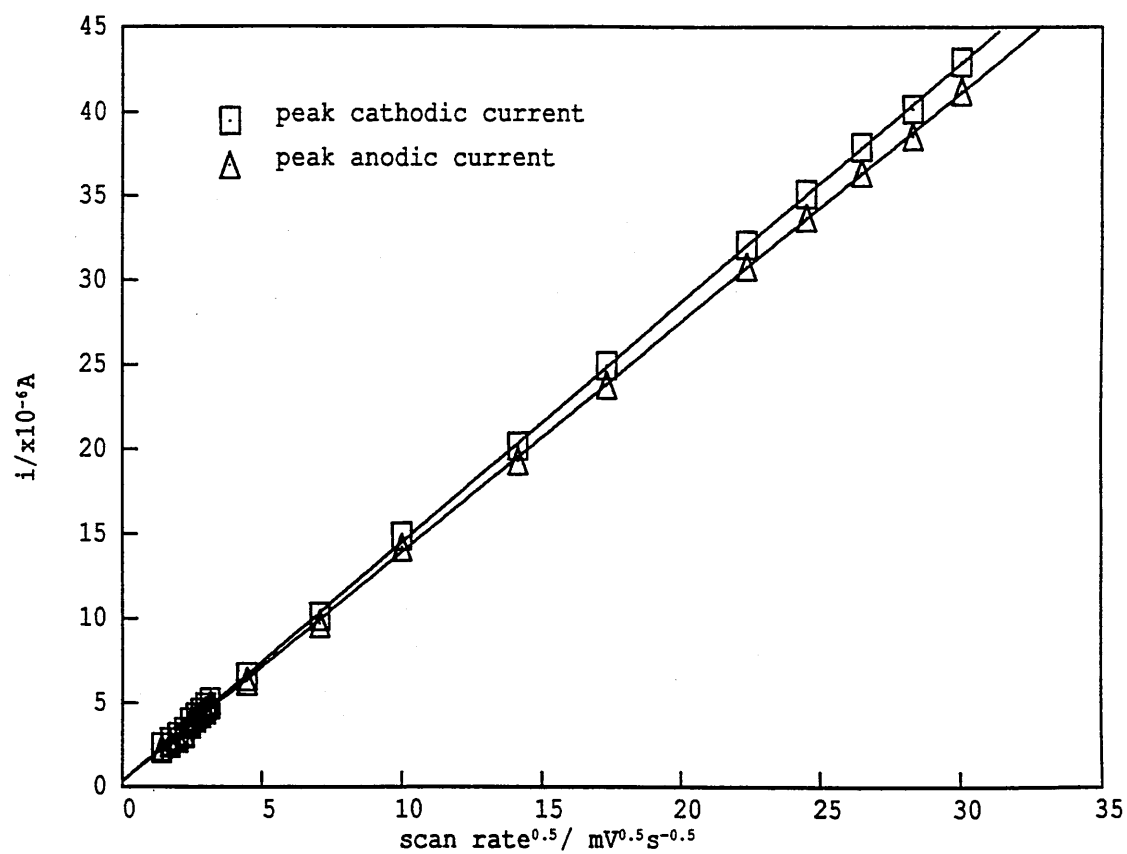
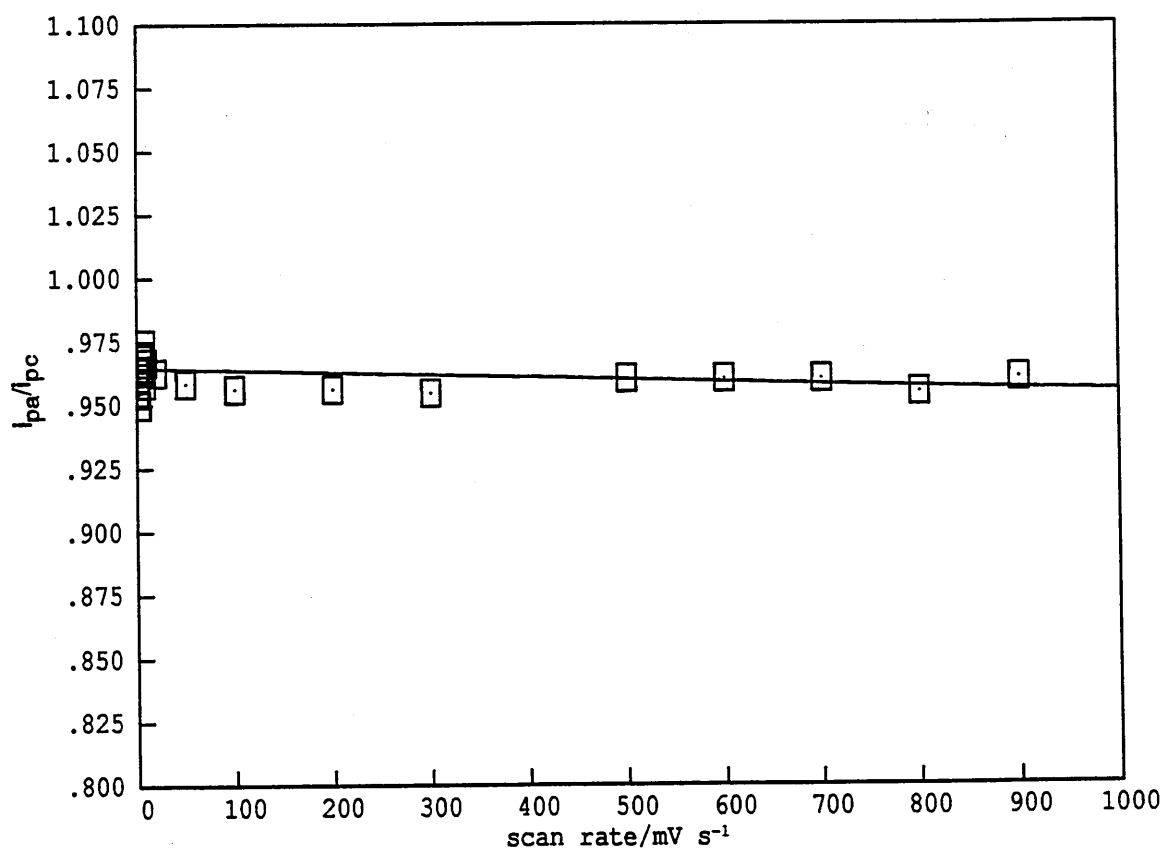


Figure II.2.4. Typical cyclic voltammograms for a solution containing 2 mM ruthenium (III) hexamine. (initial potential 0.1 V; minimum potential -0.5 V; $0.5 \text{ V s}^{-1} \leq v \leq 0.9 \text{ V s}^{-1}$).

Figure II.2.5. Plot of scan rate^{0.5}/ mV^{0.5}s^{-0.5} vs. i_{pa} and i_{pc} at a planar gold electrode for a solution containing 2 mM ruthenium (III) hexamine and 1 M KCl, buffered at pH 7 with 50 mM phosphate,

Figure II.2.6. Plot of scan rate/ mV s⁻¹ vs. i_{pa}/i_{pc} at a planar gold electrode for a solution containing 2 mM ruthenium (III) hexamine and 1 M KCl, buffered at pH 7 with 50 mM phosphate.



II.2.3. DETERMINATION OF THE DIFFUSION COEFFICIENT OF RUTHENIUM (III) HEXAMINE BY CHRONOCOULOMETRY.

The diffusion coefficient of ruthenium (III) hexamine was determined using chronocoulometry at a 3 mm diameter planar gold electrode. The area of the electrode was determined using the integrated Cottrell equation [II.1.4.] for the electrode reaction : Ox + ne $\rightarrow$ Re. Where Ox and Re are the oxidized and reduced forms respectively of the electroactive species.

$$Q_{\text{total}} = \int_0^t i dt = \frac{2nFAD_0^{0.5}C_0 t^{0.5}}{\pi} + nFAI_0 + Q_{\text{dl}} \quad [\text{II.1.4.}]$$

Where $F=96485 \text{ C mol}^{-1}$, C_0 =concentration of oxidized species /mol m⁻³, D_0 =diffusion coefficient of oxidized species / m² s⁻¹, A =area of electrode / m², I_0 =surface concentration of adsorbed oxidized species /mol m⁻², n =number of moles of electrons involved in the oxidation of one mole of the species (here $n=1$).

The term $nFAI_0$ refers to the surface concentration of species adsorbed to the electrode and Q_{dl} is the charge contribution made by double layer charging.

Ferricyanide has a diffusion coefficient of $6.26 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ at pH 3 in 2 M KCl. Chronocoulometry was performed using the BAS 100 electrochemical analyser. The charge contribution due to adsorption and double layer charging was determined from the value of the intercepts for charge vs. time^{0.5}. The surface of the electrode was abraded using 3 μm alumina oxide and exposed to ultrasound to remove adhered particles.

The cathodic peak potential for ferricyanide is +304 mV under these conditions. Double step coulometry was performed on a solution containing 2.2 mM ferricyanide using an initial potential of -0.1 V vs. SCE, a final potential of 0.5 V vs. SCE and a pulse width of 250 ms. The Faradaic contribution can be separated from $Q_{\text{adsorbed}} + Q_{\text{dl}}$ by locating the intercept at $t=0$ for a plot of charge vs. $t^{0.5}$. The BAS 100 locates the intercept and determines the slope of the line for an Anson plot automatically. A typical value for $Q_{\text{adsorbed}} + Q_{\text{dl}}$ under these conditions is $0.7962 \times 10^{-6} \text{ C}$. The total charge value, comprising Q_{adsorbed} , Q_{dl} and the Faradaic charge contribution, is $21.8 \times 10^{-6} \text{ C}$. The faradaic charge contribution is, therefore, $21.0 \times 10^{-6} \text{ C}$. Applying equation [II.1.4.] produces a value for electrode area of $7 \times 10^{-6} \text{ m}^2$, using a value for the diffusion coefficient of ferricyanide of 6.26×10^{-10}

$\text{m}^2 \text{s}^{-1}$. The fact that the electrochemically calculated electrode area ($7 \times 10^{-6} \text{ m}^2$) approximates the geometrically calculated area ($7.07 \times 10^{-6} \text{ m}^2$) is not surprising considering the polished nature of the surface.

Using the value for area previously generated, double step chronocoulometry was performed on a solution containing ruthenium (III) hexamine at a concentration of 5 mM and potassium chloride at a concentration of 2 M. The initial and final potentials were, respectively, -0.5 V and 0 V vs. SCE. The pulse width was 250 ms. The faradaic charge contribution was separated from the total charge contribution from the BAS 100 derived intercept value at $t=0$, Figure I.1.7.. The faradaic charge value was calculated as $80.14 \times 10^{-6} \text{ C}$. Applying equation [II.1.4.] generates a value for the diffusion coefficient of ruthenium (III) hexamine under these conditions of $1.8 \times 10^{-9} \text{ m}^2 \text{s}^{-1}$.

Conduction of several trials for the determination of the diffusion coefficient of ruthenium (III) hexamine resulted in a value of about $1.77 \times 10^{-9} \text{ m}^2 \text{s}^{-1}$ ($n=10$: $\text{sd}=9 \times 10^{-11}$). According to Wherland & Gray (1976) ruthenium (III) hexamine and ferricyanide have a radial ratio of 3:5 ($3 \times 10^{-10} \text{ m}$ and $5 \times 10^{-10} \text{ m}$ respectively). Since the drag on a object moving through a medium is directly proportional to its cross-sectional area, the diffusion coefficient ought to be inversely proportional to the cross-sectional area of the object. The ratio of the areas of their respective centre cross-sections is 0.36 (ruthenium (III) hexamine:ferricyanide), which approximates the inverse ratio of the diffusion coefficients of the two species ($6.26 \times 10^{-10} / 17 \times 10^{-10} \sim 0.36$), supporting the experimental conclusion.

These exploratory investigations illustrate in broad terms the electrochemical behaviour of ruthenium (III) hexamine. As will be shown in Section II.3., the oxidation and reduction potentials and peak separation, a function of the heterogeneous rate constant, are highly sensitive to the ionic nature of the solution. From this study one potential advantage of ruthenium (III) hexamine over ferricyanide for use in an oxidoreductase based CFD has been illuminated; namely, because the oxidation potential is less than 0 mV vs. SCE the proportion of the analytical signal due to extraneous electrochemical oxidation is likely to be less in any system employing ruthenium (III) hexamine rather than ferricyanide.

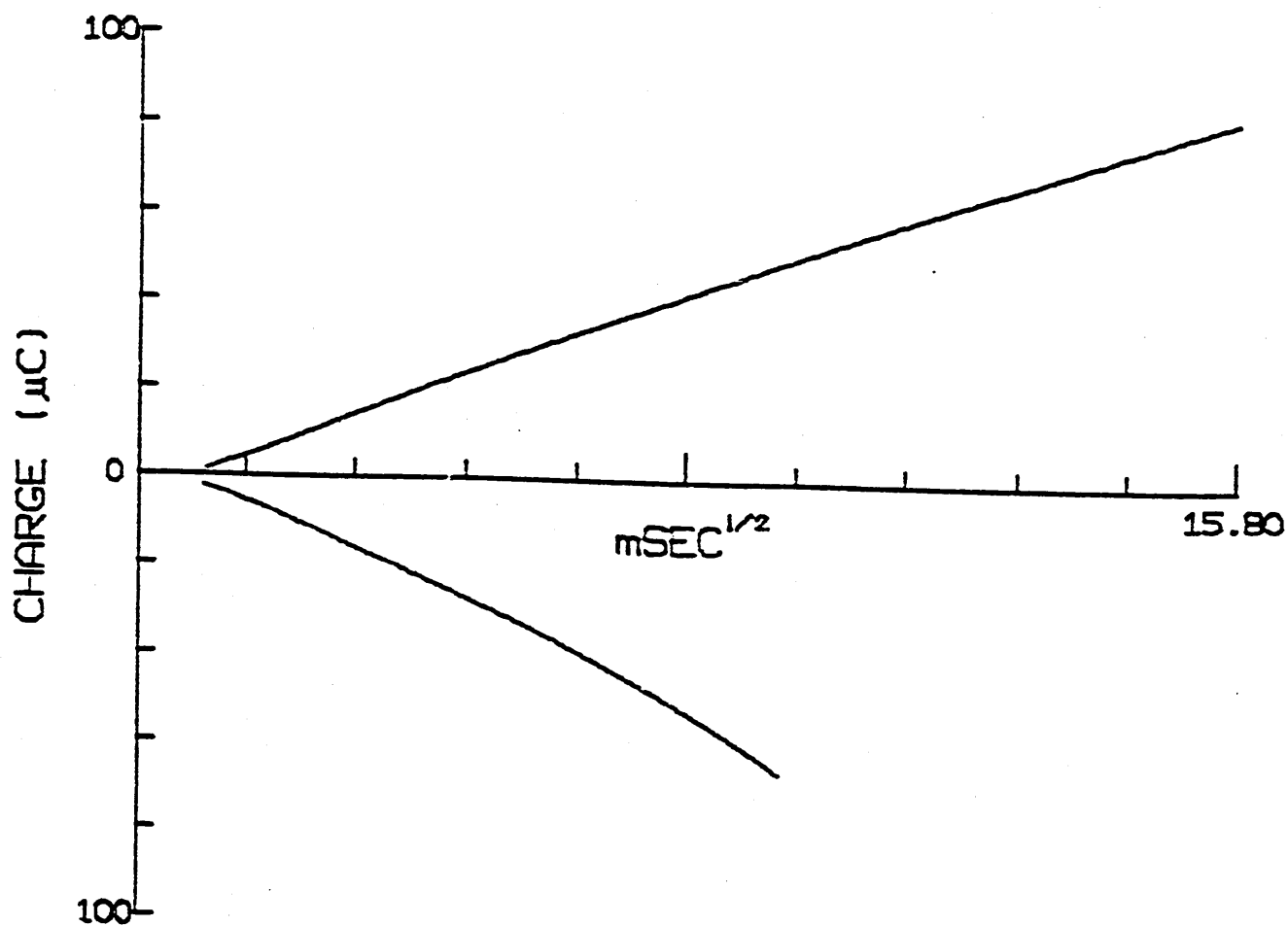


Figure II.2.7. Charge vs. $t^{-0.5}$ for a solution containing 5 mM ruthenium (III) hexamine in 2 M KCl. Conditions: initial potential=-0.5 V, final potential=0 V, pulse width=250 ms.

II.3.1. ELECTROCHEMICALLY COUPLED OXIDOREDUCTASE REACTIONS.

An awareness of the importance of electron transfer between molecules of various redox states in energy transduction in living cells is well documented (Allen & Usterwood, 1984; Gutman and Keyzer, 1986).

The substitution of artificial electron mediators for molecular oxygen for the oxidation of oxidoreductases such as glucose oxidase enables analysis of various aspects of reactions involving the enzyme, provided that the electron transfer molecule may be oxidized or reduced at an electrode. One of the most frequently used methodologies for ascertaining whether a particular candidate redox molecule is capable of undergoing coupled redox changes with an oxidoreductase involves analysis of the enhancement of diffusion current achieved during linear sweep voltammetry or cyclic voltammetry of a candidate molecule, in the presence of reduced oxidoreductase, that is known to undergo reversible electrochemistry (Nicholson and Shain, 1964). Certain analyses apply the same principles to a chronoamperometric response (Feinberg and Ryan, 1981).

A mediator molecule may undergo coupled reduction to an oxidoreductase but if the diffusion coefficient of the mediator molecule is high compared to the homogeneous rate constant then no enhancement of diffusion current during potential sweep methods is apparent. Additionally, it may not be possible to establish the pseudo-first order conditions necessary to apply these analyses. In the following analysis linear sweep voltammetry is applied to a system containing ruthenium (III) hexamine, glucose and glucose oxidase. Although there was an enhancement of peak current the system remains under mixed diffusional and kinetic control. The application of Nicholson (1965) theory to determine the suitability of potential electron mediator compounds to couple oxidoreductase transitions may not always be appropriate. Ferricyanide is capable of oxidizing reduced glucose oxidase but the associated homogeneous rate constant is insufficient to elicit a catalytic current. In the CFD it has an operating concentration of about 0.3 M. The high concentration compensates for the low value of k_e . The strategy embodied in the CFD therefore admits the potential utilisation of a wider range of electron mediator molecules than other systems.

II.3.2. PROCEDURES USED TO INVESTIGATE THE REACTION OF RUTHENIUM (III) HEXAMINE AND GLUCOSE OXIDASE.

Chemicals, electrochemicals and equipment. All chemicals were obtained from previously stated sources and at previously stated grades. Except for the assessment of the coulometric

response of the system, which was conducted in empty CFDs, all of the investigations in this section were conducted using a one compartment electrochemical cell supplied by EG&G with integral water jacket. A 3 mm gold disc electrode was employed as the working electrode coupled with a platinum wire counter electrode and a SCE acted as reference. All electrochemistry was conducted using an EG&G potentiostat and EG&G m270 electrochemical software. Glucose oxidase from Type VII from *Aspergillus niger* (EC 1.1.3.4.) with a specific activity of 200 units mg^{-1} was obtained from Sigma Chemical Co.. Reagents were prepared on a daily basis in ultra pure water and filtered through 0.2 μm filters immediately prior to use. Glucose oxidase solutions were prepared in bacterial free pH 7.0 phosphate buffer and kept in sealed containers at 4°C until needed. The concentration of glucose oxidase expressed in terms of catalytically active FAD was determined spectrophotometrically at 450 nm and assuming a molar extinction coefficient of $1.3 \times 10^4 \text{ l mol}^{-1} \text{ cm}^{-1}$.

Linear sweep voltammetry. Linear sweep voltammetry was performed at a scan rate of 1 mV s^{-1} in a solution containing final concentrations of ruthenium (III) hexamine, glucose and glucose oxidase were 10 mM, 70 mM and 20 mg ml^{-1} (100 μM) respectively. Linear sweep voltammetry was performed in oxygen free solution ebulliated with 'white spot' nitrogen.

Current - potential response. The temperature of the solution was maintained at $25^\circ \text{C} \pm 0.5^\circ \text{C}$. The solution was stirred at a rate of 200 revolutions min^{-1} and the surface of electrode was placed parallel to the surface of a cylindrical stirring flea, approximately 3 mm above its surface. In order to compare the values obtained between different experiments it is important that the boundary layer between the surface of the electrode and the bulk solution is uniform over those experiments. Initially the electrode system was allowed to reach steady state in 1.8 ml of solution containing the ruthenium salt and glucose buffered at pH 7.0 using equimolar potassium di-hydrogen phosphate and dipotassium hydrogen phosphate to a final concentration of phosphate of 0.1 M. Once steady state current had been achieved 0.2 ml of a solution containing glucose oxidase was added to the cell and the current response was assessed in two ways: by the rate of change of current with respect to time or by the final steady state current. The final concentrations of ruthenium (III) hexamine, glucose and glucose oxidase in the system were 50 mM, 50 mM and 0.5 mg ml^{-1} respectively. The current response was measured at fixed potential values between -0.5 V and + 0.4 V. Five trials were performed at each potential and their arithmetic means used for analysis.

The pH dependency of the reaction rate of the system. The rate of change of current with respect to time was measured at an oxidizing potential of 0.1V on addition of a solution containing glucose oxidase to a solution containing ruthenium (III) hexamine and glucose. Procedures and final concentrations of all chemicals were as described for the determination of current potential response. The rate of change of current was measured at pH values between 4.6 and 12.2. Potassium phosphate buffer was used between pH values of 7.5 and 12.2 and sodium citrate buffer was used between pH 4.6 and 6.8. Final concentration of all buffers was 0.1 M. The solution containing

glucose oxidase was buffered at pH 7 using phosphate buffer at a concentration of 20 mM. On addition of this solution to the solution containing ruthenium (III) hexamine and glucose the final concentration of the phosphate buffer in this solution was 2 mM.

The rate of oxidation of glucose by ruthenium (III) hexamine without the addition of glucose oxidase was assessed using the same procedure as described previously except that the initial system consisted only of ruthenium (III) hexamine in the appropriate buffer and a solution containing glucose was substituted for the glucose oxidase solution.

Charge response of the system vs. glucose concentration. The assessment of the performance of ruthenium (III) hexamine as an electron mediator between reduced glucose oxidase and a printed gold electrode was performed in empty CFD cells. Mixtures of the three components were prepared to give final concentrations of ruthenium (III) hexamine of 0.3 M and of glucose oxidase of 1 mg ml^{-1} ($5.5 \times 10^{-6} \text{ M}$)¹. The concentration of glucose ranged between 0 M and 40×10^{-3} M. The system was buffered at pH 7.0 using equimolar concentrations of sodium di-hydrogen phosphate and di-sodium hydrogen phosphate, at a concentration of phosphate of 0.2 M. After mixing an aliquot of the mixture was introduced to the CFD. Sixty seconds lapsed between the completion of the mixture and the application of an oxidizing potential of 0.05V. The potential pulse was applied for 60s. The current signal was integrated over the full period of pulse application to give a charge value.

II

III.3.3. LINEAR SWEEP VOLTAMMETRY OF RUTHENIUM (III) HEXAMINE WITH GLUCOSE OXIDASE AND GLUCOSE.

The homogeneous rate constant for the reduction of ruthenium (III) hexamine by reduced glucose oxidase was not high enough to enable a kinetic determination using cyclic voltammetry (Nicholson and Shain 1964) or by the use of any technique involving chronoamperometry (Feinberg and Ryan, 1981) that relies on the generation of a stable catalytic current. Linear sweep voltammetry was performed at a scan rate of 1 mV s^{-1} . Inspection of Figure II.3.1. reveals, however, that although limiting current was not obtained a significant current enhancement was achieved. The persistence of a peak current indicates that the electrode process is under mixed kinetic and diffusional control (Albery, 1975). Although there is an increase in the concentration of reduced mediator close to the electrode, the rate of production of reduced mediator is not competitive with the rate of diffusion to the electrode surface, so that the current peaks and as the potential becomes more positive the current flux becomes dependent on Fick's laws of diffusion. Qualitative analysis of the linear sweep voltammograms imply that the homogeneous rate constant is probably higher than for ferricyanide but is inferior to ferrocene derivatives.

¹ Using a value for the molecular mass of glucose oxidase of 180 kd (Bouin *et al.*, 1982).

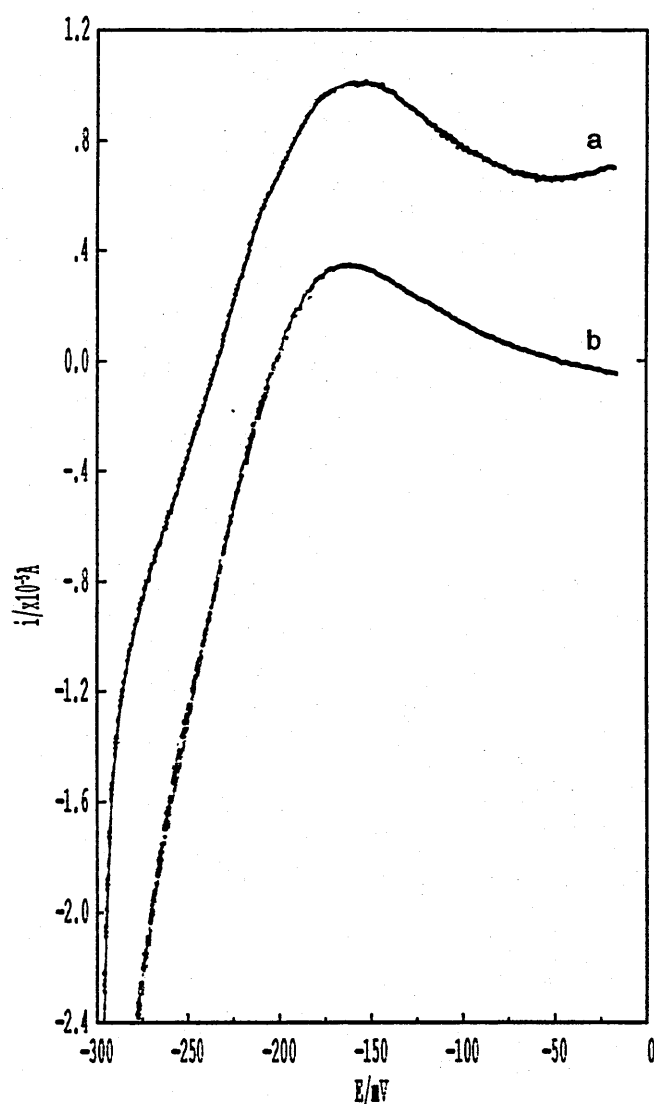


Figure II.3.1. Linear sweep voltammograms of solutions containing ruthenium (III) hexamine glucose and glucose oxidase. Conditions: scan rate 1 mVs^{-1} ; initial potential -0.35 V ; final potential 0 V .

Key:

- a** 10 mM ruthenium (III) hexamine, 50 mM glucose, $100 \mu\text{M}$ glucose oxidase, 0.1 M KCl , 50 mM phosphate buffer at pH 7.
- b** 10 mM ruthenium (III) hexamine, 50 mM glucose, 0.1 M KCl , 50 mM phosphate buffer at pH 7.

II.3.4. CURRENT-POTENTIAL CURVES FOR THE RUTHENIUM (III) HEXAMINE / GLUCOSE OXIDASE / GLUCOSE SYSTEM AT GOLD DISC ELECTRODES.

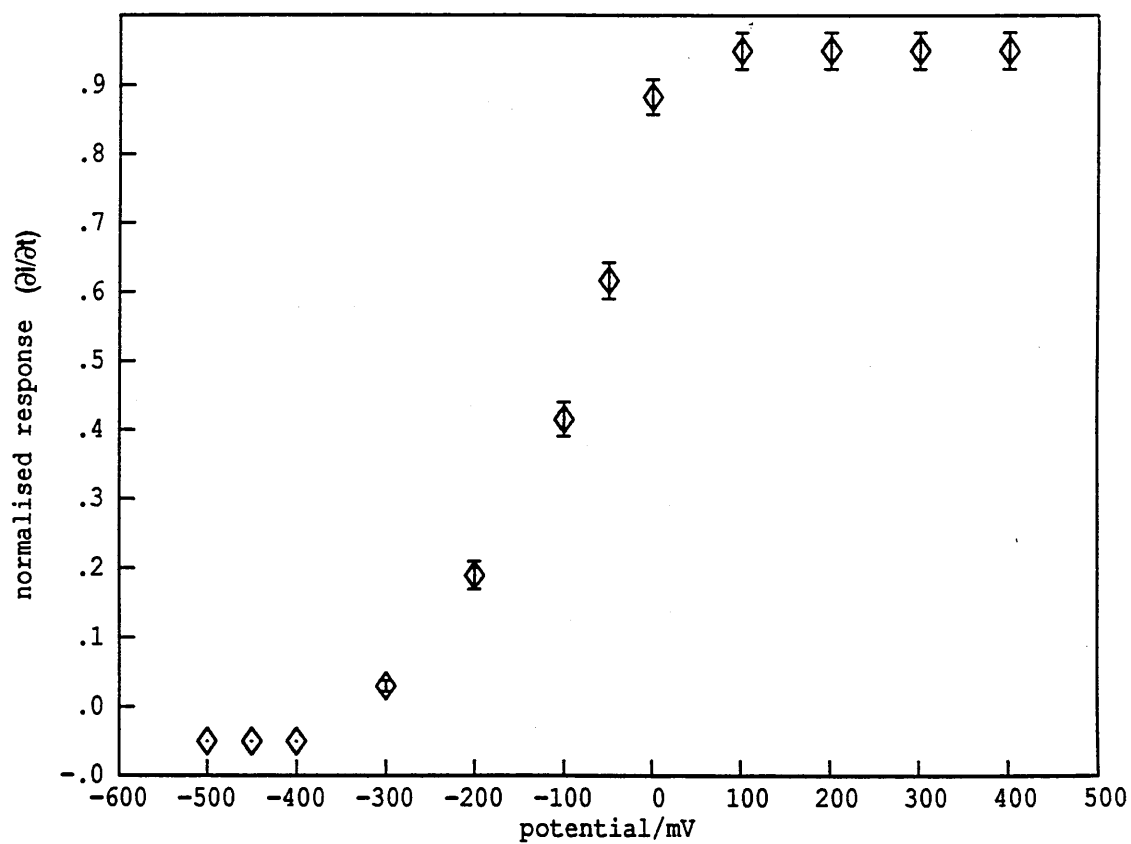
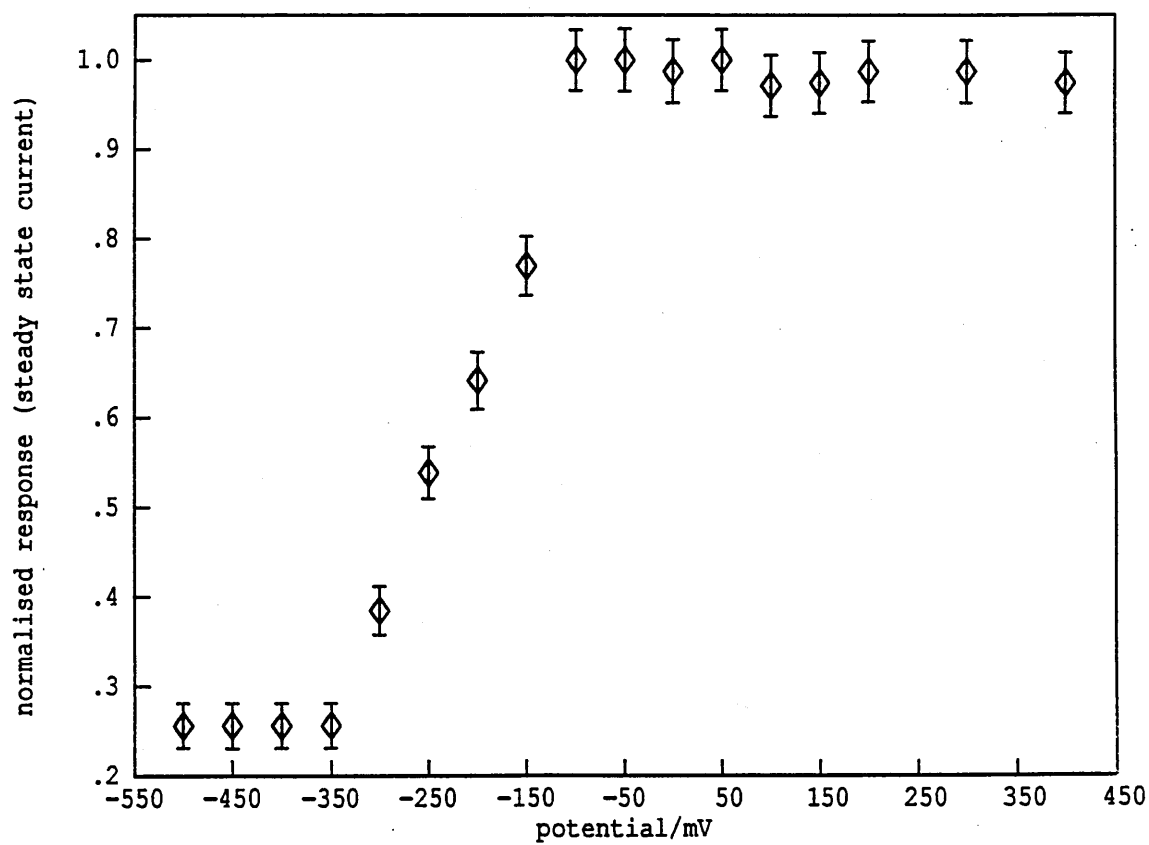
Normalised current-potential curves for the ruthenium mediated glucose oxidase/glucose system were derived from the initial rate of oxidation of ruthenium (II) hexamine (the maximum gradient of the current time response immediately after addition of glucose oxidase) at the gold disc electrode in stirred solution, Figure II.3.2., and from the steady state current achieved in each case, Figure II.3.3.. Values for Figure II.3.2. were normalised against a maximum value of $7.8 \times 10^{-7} \text{ A s}^{-1}$ and for Figure II.3.3. against a maximum value of $7.5 \times 10^{-4} \text{ A}$. This second value represents the maximum change in steady state current response, from a value of $-6 \times 10^{-4} \text{ A}$ at -0.5 V to $+1.5 \times 10^{-4} \text{ A}$ at 0.2 V vs. SCE.

It is apparent from these two analyses that the current curves produced show that any CFD configured sensor would have certain advantages in terms of the electrochemical interference, especially when analysing samples of biological origin. The current-potential response measured by the initial flux of reduced mediator at the electrode on introduction of glucose oxidase (Figure II.3.2.) reveals that the flux begins to plateau at -50 mV vs. SCE. The steady state current tends to plateau from between 0 mV to $+50 \text{ mV}$ vs. SCE (Figure II.3.3.). This concurs with the cyclic voltammetric studies conducted previously.

The plateau region comes into being as the current flux at the electrode changes from limitation due to the heterogeneous rate constant and becomes solely determined by the diffusional flux of electroactive material to the electrode surface (Albery, 1975). At potentials within the plateau region small changes in electrode potential have no significant effect on current response. In order to achieve reproducibility of response of an amperometric sensor its applied potential should be poised in this plateau region.

Figure II.3.2. Normalised rate of change of current response ($\partial i/\partial t$) vs. potential curves for the ruthenium mediated glucose oxidase/glucose system derived from the initial rate of oxidation of ruthenium (II) hexamine (the maximum gradient of the current time curve immediately after addition of glucose oxidase) at the gold disc electrode in stirred solution.

Figure II.3.3. Normalised current-potential curves for the ruthenium mediated glucose oxidase/glucose system derived from the steady-state current attained at each potential on the addition of a solution containing glucose oxidase to a solution containing ruthenium (III) hexamine and glucose.



II.3.5. THE PH DEPENDENCY OF THE REACTION RATE OF THE RUTHENIUM (III) HEXAMINE / GLUCOSE OXIDASE / GLUCOSE SYSTEM.

Figure II.3.4. shows the normalised current response for the reaction between ruthenium (III) hexamine, glucose oxidase and glucose in the system described in Section II.3.2.. The response was assessed by measuring the maximal gradient of the current-time response immediately after the addition of glucose oxidase, or, in the absence of glucose oxidase, on the addition of glucose. The values were normalised against a maximal value of $170 \times 10^{-7} \text{ A s}^{-1}$. No current response was recorded at pH values below pH 5.7. Figure II.3.5. shows the normalised current response for the system in the absence of glucose oxidase. Here all values were normalised against a maximal value obtained for the system employing glucose oxidase, $170 \times 10^{-7} \text{ A s}^{-1}$. The maximal response obtained for the direct oxidation of glucose by ruthenium (III) hexamine was $1.2 \times 10^{-7} \text{ A s}^{-1}$. No current response was recorded at values below pH 9.0. Each response represents the arithmetic mean of five trials.

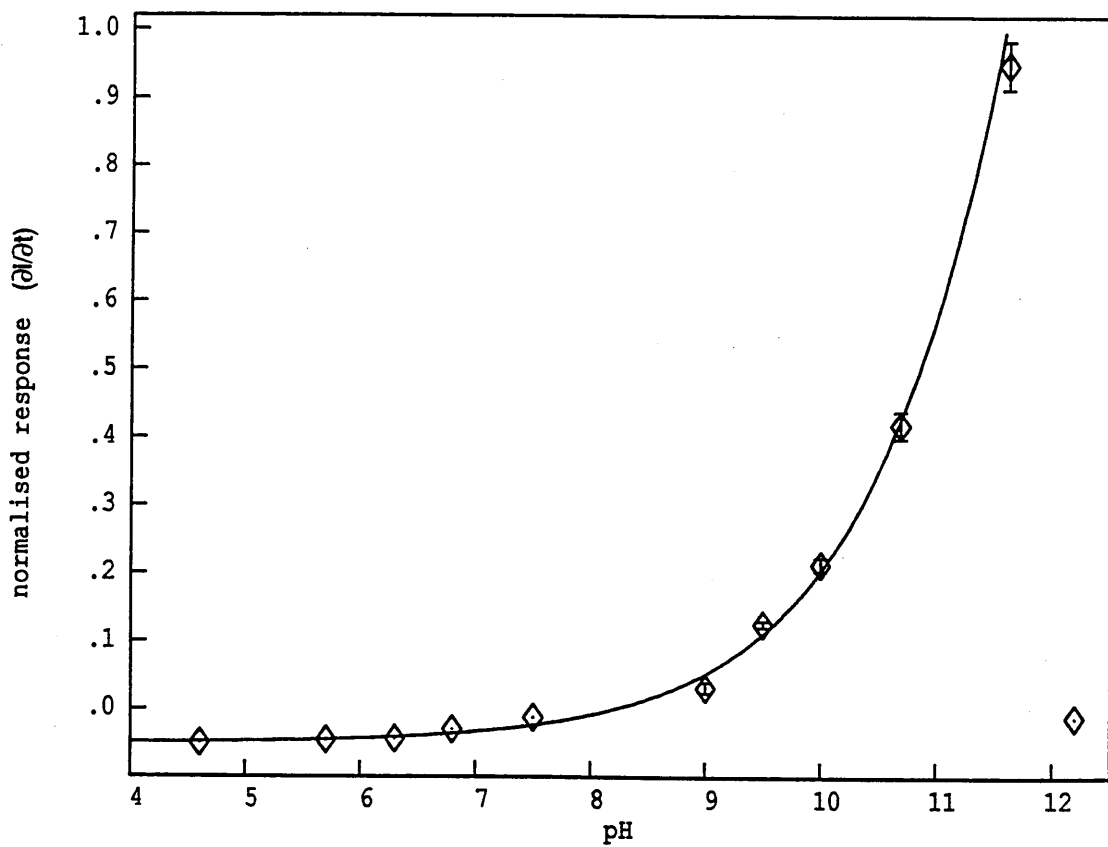
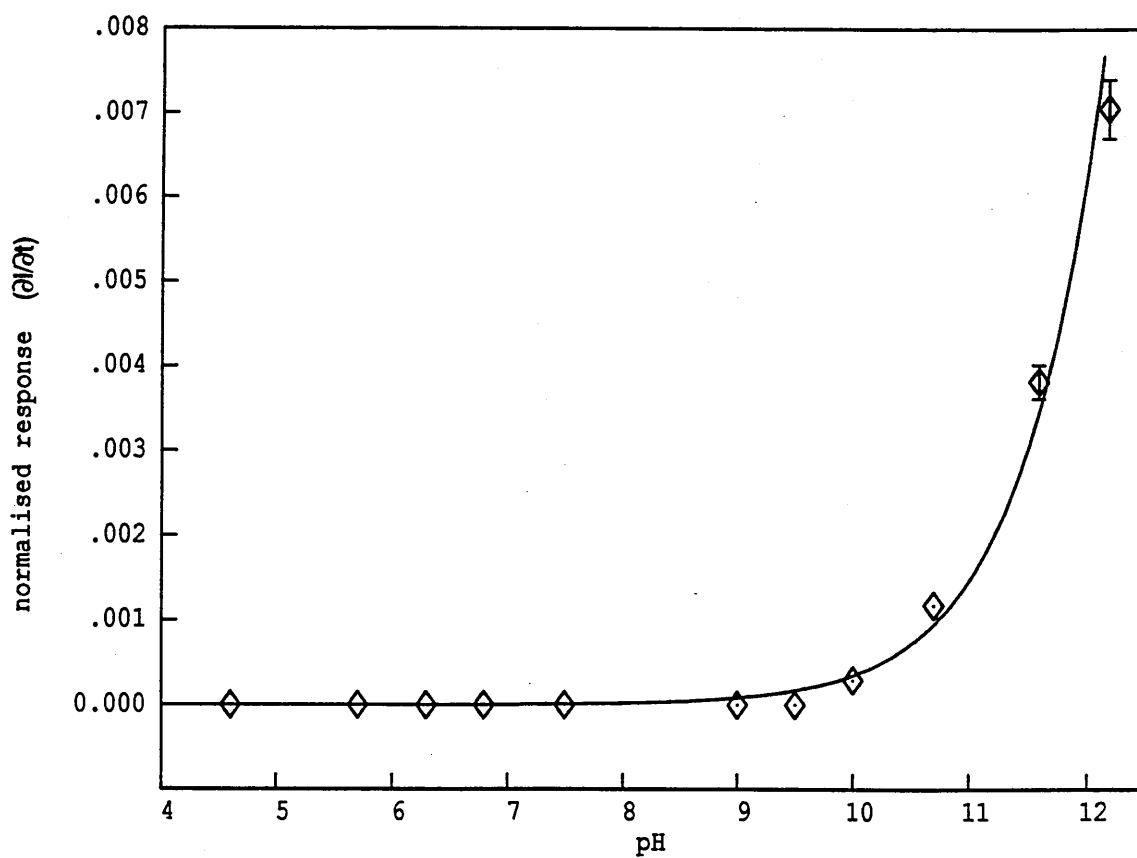
Clearly although there is some oxidation of glucose by ruthenium (III) hexamine in the absence of glucose oxidase it is maximally 100 orders of magnitude less than when glucose oxidase is present. In order to explain the current response dependency on pH it is necessary to discuss the reaction mechanism of glucose oxidase.

The definitive description of the reaction mechanism of glucose oxidase is given by Duke (Duke *et al.*, 1969). The reduced glucose oxidase molecule may only be oxidized by the electron receptor when the D-glucono- δ -lactone is liberated on the arrival of another molecule of glucose. The molecule of glucose causing the release cannot itself react with the reduced glucose oxidase (Duke *et al.*, 1969). Although some glucose oxidase quinones may be the oxidant of glucose oxidase in some natural systems (Szklarz and Leonowicz, 1986) di-oxygen is most prevalent. The maximum rate of reaction between glucose oxidase and molecular oxygen is quoted as occurring at pH 5.5. At pH 5.5 the reduction of oxygen involves the formation of a 4a-hydroperoxide adduct with FAD. The release of this adduct into solution occurs when it is protonated by the apoprotein. The pKa value of the protein group responsible for protonation of the adduct is 6.9 (Bright & Appleby, 1969). At higher pH values it is likely that the protonation of the adduct will limit the rate of reaction of the enzyme. Unprotonated adducts released into solution at high pH values would exist as superoxides. Wilson & Turner (1991) described a series of experiments that demonstrate the production of superoxides at high pH values, supporting this model.

Wilson & Turner (1991) have classified four major groups of electron acceptors for reduced glucose oxidase on the basis of their pH optima:

Figure II.3.4. Normalised current response for the reaction between ruthenium (III) hexamine, glucose oxidase and glucose vs. pH in the system described in Section II.3.2.. The response was assessed by measuring the maximal gradient of the current-time response ($\partial i/\partial t$) immediately after the addition of glucose oxidase, or, in the absence of glucose oxidase, on the addition of glucose. The curve represents a least squares exponential regression of the data at each pH except the value at pH 12.2 ($r^2=0.9928$, $n=40$, $sd\sim 5\%$ of value).

Figure II.3.5. Normalised current response for the reaction between ruthenium (III) hexamine and glucose vs. pH in the system described in Section II.3.2.. The response was assessed by measuring the maximal gradient of the current-time response ($\partial i/\partial t$) immediately after the addition of glucose oxidase, or, in the absence of glucose oxidase, on the addition of glucose. The curve represents a least squares exponential regression of the data at each pH value, except the value at pH 12.2 ($r^2=0.9901$, $n=40$, $sd\sim 5\%$ of value).



i). Electron acceptors with a pH optimum of 5.6 in citrate buffer. The mechanism of action is as discussed above for di-oxygen. Quinones also behave in a similar fashion, forming an adduct with FAD whose protonation becomes increasingly difficult with increasing pH due to the pKa of 6.9 of the protein group responsible for protonation.

ii). Electron acceptors with a pH optimum of 7.5 in phosphate buffer. Wilson & Turner (1991) quote electron tunnelling from the reduced FAD of glucose oxidase to the electron transfer compound as the reaction mechanism for this group of compounds. The group includes methylene blue and toluidine blue and ferrocene derivatives, ferrocenemonocarboxylic acid and 1,1-dimethylferrocene, TTF and TCNQ and benzyliologen. Compounds in this group are supposed to be unable to form adducts with the FAD directly.

iii). Electron acceptors with a high charge density and an acidic pH optimum. At pH 7.5 the active site of glucose oxidase is hydrophobic (Kulys and Cenas, 1983). In consequence it is reasonable to suppose that a highly polarised molecule such as ferricyanide is less likely to enter the active site than at lower pH values, where the active site tends to acquire a positive charge. At lower pH values then, due to increased hydrophilicity and electrostatic attraction, the rate of reaction between the reduced glucose oxidase and ferricyanide increases. Wilson (personal communication, 1990) conducted charge shielding experiments to investigate the charge dependence on the rate of reaction between ferricyanide and reduced glucose oxidase at pH 7.5 and at pH 3.0: shielding of the mutual charges by potassium sulphate resulted in an increase in the rate of reaction at pH 7.5 and a decrease in the reaction rate at pH 3.0.

iv). Electron acceptors having an acidic pH optimum but low charge density. Indolphenols like DCIP (dichloroindolphenol) form a covalent adduct with the FAD group of reduced glucose oxidase. DCIP has a pKa value of 5.8 and consequently is not readily protonated at the pH optimum of compounds in group i). Progressive decreases in the pH result in an increasing rate of reaction between the electron acceptor and the reduced glucose oxidase.

Examination of the points presented above with regard to the elucidation of any universal reaction mechanism between reduced glucose oxidase and electron acceptors reveals no consensus and some anomalies. The mechanism of electron transfer between mediator molecules and reduced glucose oxidase, for example, has been described in terms of electron tunnelling from the active site of glucose oxidase to the electron mediator (Degani and Heller, 1988). The observed pH-rate dependency may be explained in terms of the likelihood of the electron acceptor entering the active site on the basis of the mutual charges of mediator and active site at different pH values (Section II.3.6).

With regard to the apparently shifted pH optimum of glucose oxidase with ruthenium (III) hexamine, Hendry (1989) found that the pH optima for poly(ferrocenylenemethylene) and methoxyphenyl-

substituted polyferrocene mediated glucose oxidase electrodes are shifted to alkaline pH values, of pH 9.0 or greater. Changes in the quaternary structure of glucose oxidase due to changes in pH were offered as a possible explanation for the increase in the rate of reaction between glucose oxidase and these polyferrocene derivatives at increasing pH values. At alkaline pH values the glucose oxidase molecule becomes more relaxed, resulting in a reduction of the distance between the FAD moiety and the surface of the enzyme. Hendry argued that the polyferrocene derivatives were increasing likely to be able to interact with the FAD group at increasing pH, due to the progressively more open structure of glucose oxidase, whereas, at lower pH values the access of the electron acceptor to the FAD moiety was limited due to steric restrictions. A similar argument was used by Wilson & Turner (1991) to explain the pH optimum for ferricyanide and glucose oxidase, although he cites electron tunnelling from the FAD through the enzyme as the mechanism of electron transfer: at pH 7 the distance between the active site, where the FAD moiety resides, and the surface of the enzyme is between 111 nm and 130 nm, whilst at pH 4 the distance is 80 nm. This reduction in distance may be small enough to facilitate electron tunnelling from the FAD through the structure of the enzyme (Degani and Heller, 1988).

II.3.6. IONIC ENVIRONMENT AND PH DEPENDENCY OF CHANGES IN THE RATE OF OXIDATION OF GLUCOSE BY GLUCOSE OXIDASE IN THE PRESENCE OF RUTHENIUM (III) HEXAMINE.

None of the speculations raised formerly discussed in isolation can explain the pH profile obtained with ruthenium (III) hexamine and glucose oxidase. Clearly for ruthenium (III) hexamine decreasing steric hindrance on quaternary structure relaxation is not likely to be significant. It has an ionic radius of about 30 nm a similar order to glucose. An understanding of the pH profile may be attained by examining the nature of the amino acid residues in the vicinity of the active site and the FAD molecule. It is known that there is a histidyl residue proximal to the active site. It has functional significance in the oxidation of glucose due to its stabilisation of the semi-quinone form of FAD (Nakanishi 1984). Histidine is a basic amino acid, carrying a net positive charge at pH 6.0. Histidine has three pKa values: pKa1=1.82, pKa2=6.0 and pKa3=9.17 (Leninger 1979). There is some evidence that variation of net protein charge as a function of pH can be mapped from calculations of the degree of ionization using standard pKa values (Feinberg and Ryan, 1981). For the study on 'high potential iron-sulphur protein' (HiPIP) (Feinberg and Ryan, 1981), an electron transfer protein from *Chromatium vinosum*, all the amino acid residues were known. Assuming some local correspondence between the ionic behaviour of free histidine and the histidyl residue, at pH values above 6.0 the histidyl residue becomes increasingly more negative. Approaching pH 9.17 this residue attains a net negative charge.

Conversely, at pH values less than 6.0 the active site ought to become progressively more positive. The pH profile for ferricyanide, a small high charge density negatively charged molecule, and for

ruthenium (III) hexamine, a small high charge density positively charged molecule, may be able to be explained in terms of mutual electrostatic affinity of the active site and the electron acceptor at various pH values. This hypothesis may be tested by changing the ionic environment in which the electron acceptor and reduced glucose oxidase interact. The concentrations of ruthenium (III) hexamine, glucose and glucose oxidase were the same as described in Section II.3.5. The value of the gradient of the current-time response was used to gauge the system response as described previously at pH 11.5 and at pH 5.0 but with progressively increasing concentration of Cl^- ions. The concentration of chloride ions was varied between 0.1 M and 0.6 M by varying the concentration of potassium chloride in the system. If the hypothesis is at all correct charge shielding at pH 5.0 should enhance the rate of reaction whilst at pH 11.5 it ought to have the opposite effect. Figure II.3.6. shows the normalised values obtained for system response on addition of solution containing glucose oxidase at varying concentrations of Cl^- at pH 11.2 and at pH 5.0. Each value represents the arithmetic mean derived from five trials. The values at pH 11.2 were normalised against a system response of $84 \times 10^{-7} \text{ A s}^{-1}$. The values at pH 5.0 were normalised against a system response of $2.54 \times 10^{-7} \text{ A s}^{-1}$.

Examination of Figure II.3.6. reveals that the system responded as predicted with regard to the change in system response upon increasing concentration of chloride anions. This data supports the hypothesis that the increase in the reaction rate of the system on increasing pH is some function of the increase in electrostatic attraction between the mediator and the active site of glucose oxidase. This is further supported by Wilson (personal communication), who found the converse was true for ferricyanide when the active site ought to have been polarised on increasing concentration of sulphate ions.

The functional relationship between the concentration of hydroxy anions and $\partial i / \partial t$ appears to be linear. Referring to Figure II.3.4. , discarding the data points at pH 12.2 and at 4.5 where presumably the enzyme is not capable of functioning, the application of a least squares exponential regression to this data results in a correlation coefficient of 0.9928 for the data derived from the enzyme catalysed oxidation of glucose.

Similar analysis of the data derived from the direct oxidation of glucose by ruthenium (III) hexamine results in a correlation coefficient of 0.9901.

Since pH is defined as $-\log_{10}[\text{H}^+]$, there seems to be a direct linear dependence of system response on the activity of hydroxy anions. Since this relationship appears to apply to the direct oxidation of glucose by ruthenium (III) hexamine and to the enzymic catalysed reaction it seems pertinent to ask whether, in addition to the possible mechanism discussed above, there are changes in the electrochemical behaviour of the ruthenium complex on changes in pH. Specifically, are there any changes in the rate of electron transfer of the complex at different pH

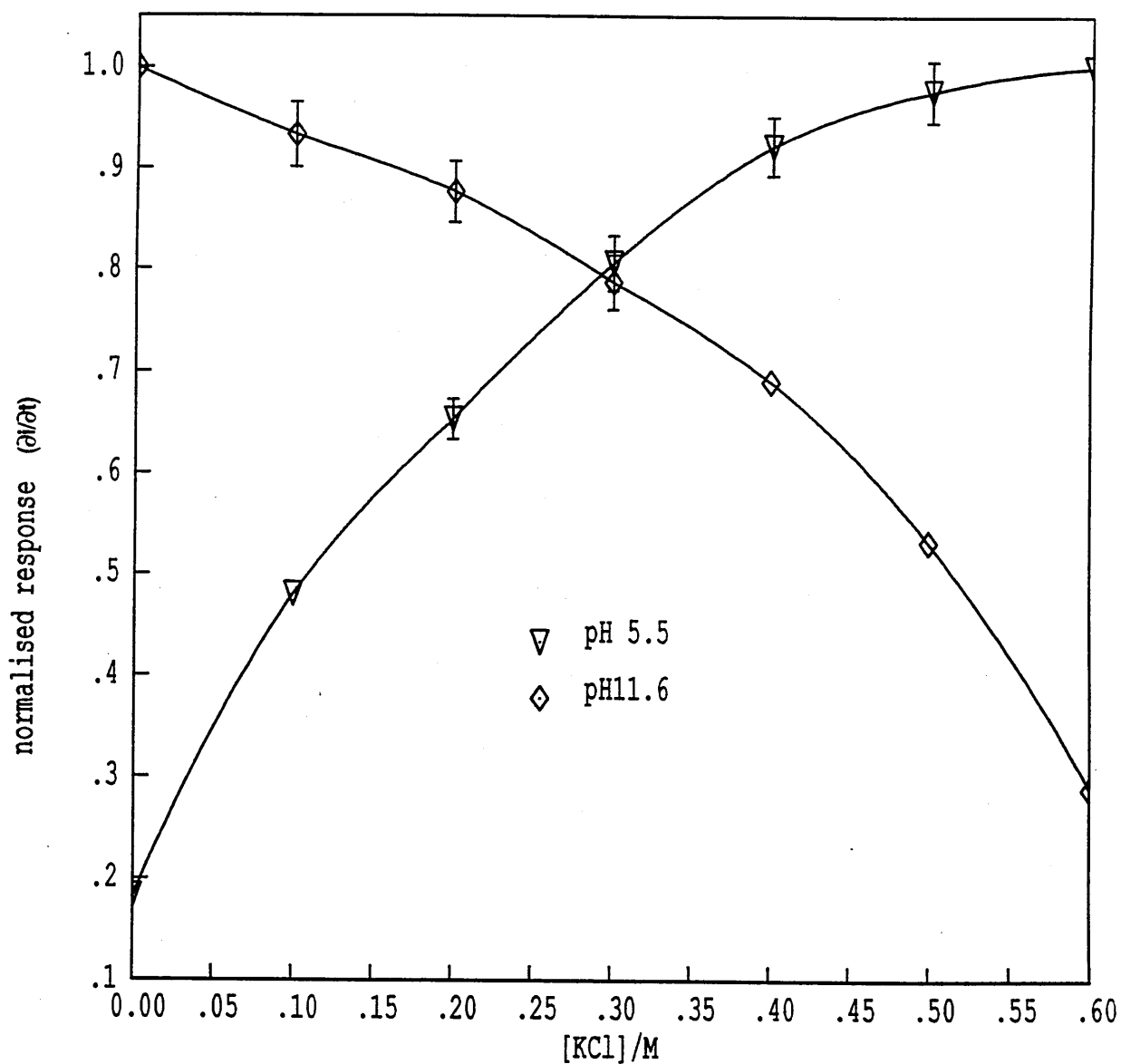


Figure II.3.6. Normalised values obtained for system response ($\partial i/\partial t$) on addition of solution containing glucose oxidase at varying concentrations of Cl^- at pH 11.2 and at pH 5.0.

values ? This was investigated using cyclic voltammetry and the correlative kinetic parameter ψ , to determine the heterogeneous rate constant, K_h , according to Nicholson's method (Nicholson ,1965). The heterogeneous rate constant may be related to the homogeneous rate constant for electron transfer by the Marcus relationship. This investigation is presented in the next section.

At first sight the analysis discussed above may seem at odds with the kind of explanation of the effect of pH on enzyme kinetics in solution given by Bailey and Ollis (1977). They argue that for the catalytic site of an enzyme to be active it must exist in the appropriate ionisation state, which is pH dependent. If E^- is the enzyme active state and E and E^{2-} are inactive forms derived from protonation and deprotonation respectively (Bailey and Ollis 1977):



Acid base equilibrium between E , H^+ and E^- necessitates that:

$[H^+][E^-]/[E]=K_1$

[II.3.2.]

Similarly for K_2 :

$[H^+][E^{2-}]/[E^-]=K_2$

[II.3.3.]

It is apparent that from [II.3.2.] and [II.3.3.] that the proportion of the active form of the enzyme is directly dependent on $[H^+]$ and therefore upon pH.

This is true if, as the model assumes, the first ionization shift in either direction completely deactivates the enzyme. Whilst for glucose oxidase it is known that the histidyl residue is functionally important in the oxidation of glucose. The pH optimum for the activity of glucose oxidase with molecular oxygen as electron acceptor is 5.5, closely corresponding to the pK_{a2} value for histidine. So why is glucose oxidase able to function at such eccentric pH values using artificial electron acceptors ?

Applying the argument of Bailey and Ollis (1977) the glucose oxidase molecule ought not to be able to function at all at the pH values obtained in this study with ruthenium (III) hexamine. There are two reasons why their argument should, perhaps, not be applied rigorously to the present study: i) since

the ruthenium complex substitutes for di-oxygen as electron acceptor the concentration of hydrogen peroxide in the environment of the active site may be reduced, resulting in a surfeit of hydronium ions (H_3O^+). Accordingly the active site micro-environment may be more acidic than the bulk solution, where the pH is monitored. ii) although the enzyme kinetics for the oxidation of glucose may become less favourable at elevated pH values, the effect of increased probability of electron transfer between mediator and reduced glucose oxidase at elevated pH values, perhaps due to mutual electrostatic attraction, may have resulted in increased current flux. Thermodynamic and kinetic considerations for electron transfer reactions between reduced glucose oxidase and oxidized mediators are discussed in Section II.5..

II.3.7. CORRELATION OF CHARGE RESPONSE TO THE INITIAL CONCENTRATION OF GLUCOSE FOR THE CFD EMPLOYING RUTHENIUM (III) HEXAMINE AND GLUCOSE OXIDASE.

It is possible to define an effective operational volume within the CFD corresponding to the complete electrolysis of an electroactive compound of a known concentration. By using Faraday's constant, [II.3.4.], the total number of moles undergoing electrolysis, N , may be calculated:

$$Q = nFN \quad \text{[II.3.4.]}$$

here Q is the quantity of charge passed, C ; $F = 96485 \text{ C mol}^{-1}$, n is an integer representing the number of moles of electrons transferred for each mole of compound electrolysed. Calculation of a working volume for the CFD associated with coulometry for a defined time period may be facilitated by the application of [II.3.4.] to a solution of a known concentration of an oxidizable species. Ferrocyanide (hexacyanoferrate (II)) at a concentration of 10 mM and 15 mM in a solution of 0.3M ferricyanide underwent oxidation in empty CFDs for 60s. The potential at the working electrode was poised at +0.2V. At 60 s the rate of charge accumulation is close to zero, Figure II.3.7.. The charge passed at 60s was 2.25 mC for solutions containing 10 mM ferrocyanide and 3.35 mC for solutions containing 15 mM ferrocyanide. Applying [II.3.4.] yields an effective volume of $2.3 \times 10^{-9} \text{ m}^3$.

In an equilibrated solution of D-glucose the β isomer constitutes 63% of the total number of moles present. In the time available to the reaction the rate of mutarotation from α to β forms is not significant. Consequently the concentration of glucose available for oxidation is reduced by 37%. From the calculated working volume of the CFD and the estimation of the number of moles available for oxidation it is possible to define a theoretical maximum response in coulombs, for a given concentration of glucose. The complete oxidation of one mole of glucose is equivalent to two moles of ferrocyanide.

Figure II.3.8. shows the correlation between the concentration of glucose in the sample solution and the amount of charge passed during the application of the potential pulse. The linear regression coefficient is 0.9921. One hundred values were used to make the correlation, 9 at each concentration. Over the normally accepted range for diabetics (2 to 30 mM) the correlation between theoretical and experimental charge values is very good ($r=0.999$). The precision of the response has an average coefficient of variation of less than 8% over the entire range, Table II.3.1.. The limit of detection, calculated at the y-intercept for 3sd at a concentration of zero glucose, was determined as 0.2 mM.

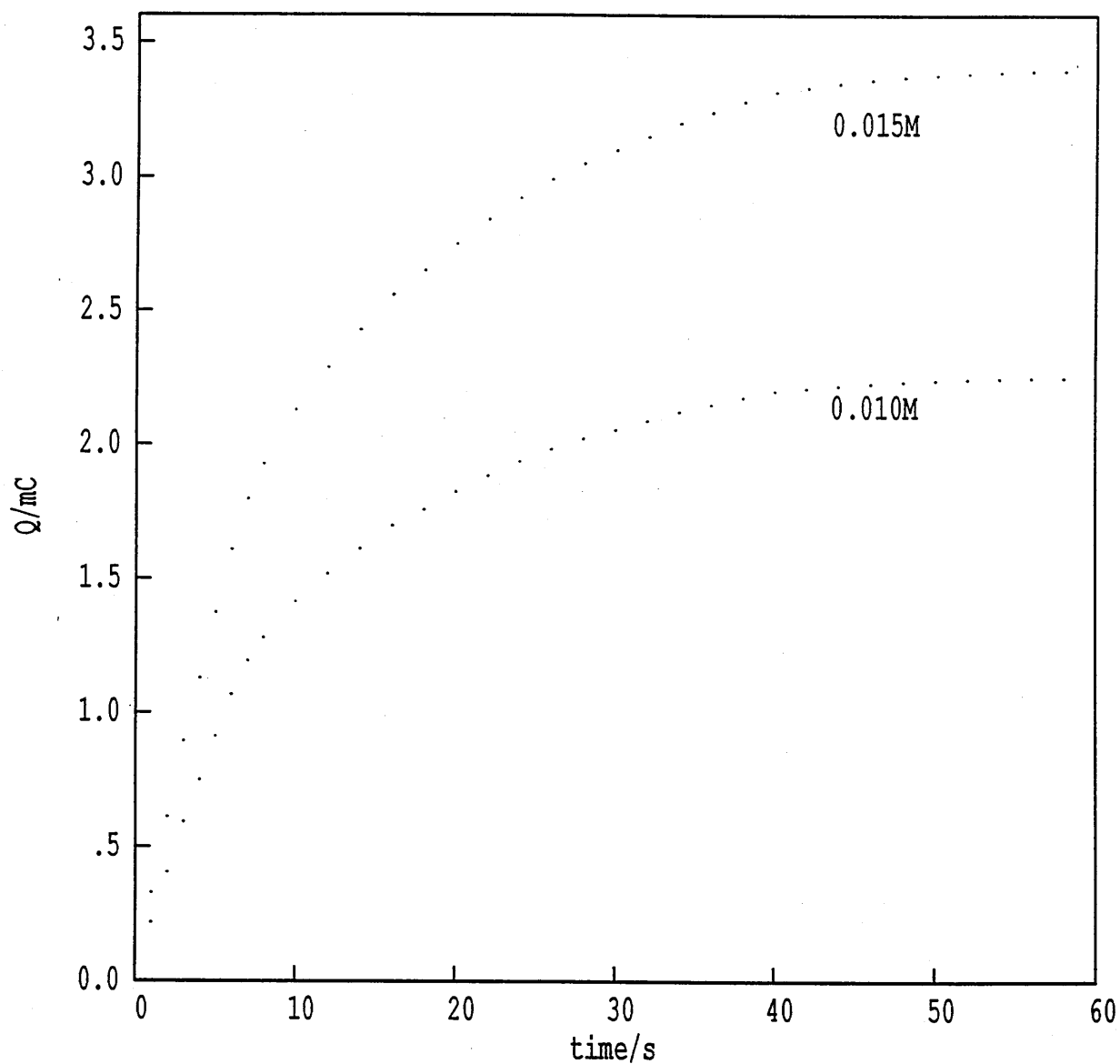


Figure II.3.7. Coulometric response of a CFD containing ferrocyanide at concentrations of 10 mM and 15 mM on the application of a potential pulse of 0.2 V of duration 60 s.

Figure II.3.8. The correlation between the concentration of glucose in the sample solution and the amount of charge passed during the application of the potential pulse. The least squares linear regression coefficient is 0.9921. One hundred values were used to make the correlation, 9 at each concentration.

Figure II.3.9. The correlation between the theoretical and experimental charge values at each concentration of glucose. The linear regression coefficient is 0.9911. Certainly at concentrations up to about 20 mM the correlation between the theoretical and experimental data is close to one; the outer lines represent 95% confidence limits.

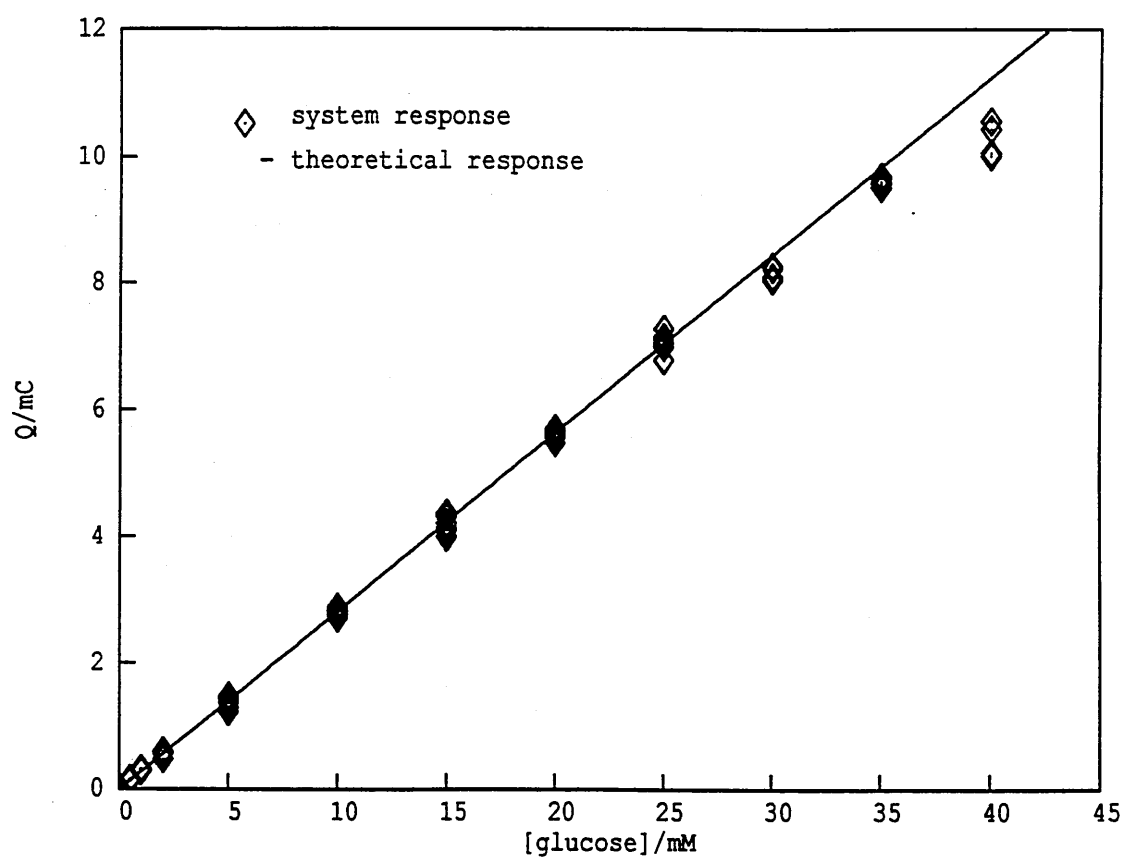
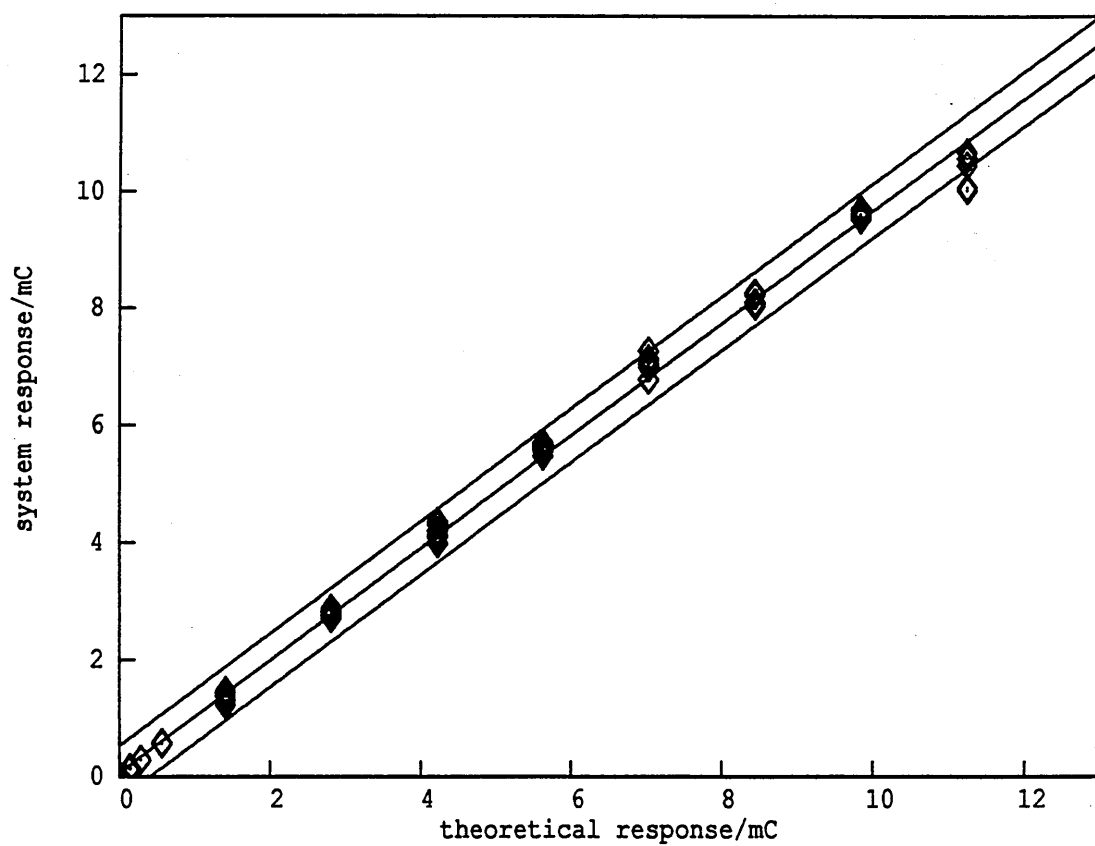


Table II.3.1. Accuracy precision profile for the coulometric determination of β -D-glucose in the CFD using ruthenium (III) hexamine as electron acceptor for glucose oxidase. (sd=standard deviation, cv=coefficient of variance).

[glucose] /mM	expected charge /mC	actual charge /mC	sd /mC	cv (%)
0.0	0.0	0.025	0.0014	5.0
0.5	0.14	0.15	0.011	7.5
1.0	0.28	0.29	0.0072	2.5
2.0	0.56	0.57	0.0072	1.3
5.0	1.41	1.39	0.08	5.7
10.0	2.82	2.78	0.054	1.9
15.0	4.22	4.18	0.133	3.2
20.0	5.63	5.60	0.067	1.2
25.0	7.03	7.02	0.160	2.3
30.0	8.44	8.16	0.095	1.2
35.0	9.85	9.62	0.061	0.6
40.0	11.26	10.36	0.260	2.6

Figure II.3.9. shows correlation between the theoretical and experimental charge values at each concentration of glucose. The linear regression coefficient is 0.9911. Certainly at concentrations up to about 20 mM the correlation between the theoretical and experimental data is close to one. The significance of the agreement between the theoretical maximum charge passed and the system response is that the oxidation of β -D-glucose in the sample seems to have proceeded to its maximal extent below about 25 mM glucose. The deviation from the expected charge response at high values of glucose is probably due to an incomplete biochemical reaction.

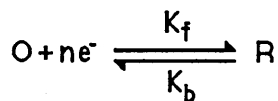
It is advantageous with regard to the specificity of the analytical signal that the oxidation potential of a candidate mediator compound lies below that of any substance that might exist in a sample of biological origin, such as blood or a food product. The half wave potentials of several substances that are present in blood were determined by Palleschi *et al.*, 1986. Of the substances mentioned only ascorbate may exist in blood and in a food product and has a half wave potential of +0.05 V or less. In spite of the quoted half wave potential the current response on applying linear sweep voltammetry to solutions containing ascorbate and 1 M KCl begins to become significant at potentials greater than

about +0.270V (Hendry, 1989).

Since the half wave potential for ruthenium (III) hexamine is about -0.2 V vs. SCE, about 0.25 V more negative than the half wave potential for ascorbate, ascorbate present in samples is unlikely to contribute to extraneous signals, either by their direct oxidation, or by oxidation or reduction of ruthenium (III/II) hexamine. Of the other electrochemical interferents mentioned by Palleschi *et al.*, 1986; hydrogen peroxide, uric acid, cysteine, acetaminophen; all have quoted half wave potentials above +0.15 V and are therefore unlikely to contribute to extraneous signals.

II.4.1. DETERMINATION OF HETEROGENEOUS RATE CONSTANT FOR RUTHENIUM (III) HEXAMINE BY CYCLIC VOLTAMMETRY.

For a simple reversible charge transfer reaction at an electrode, [II.4.1.]:



[III.4.1.]

Where O and R are the oxidized and reduced forms respectively of the species, k_f & k_b are the forward and reverse rate constants for the reaction, and n is the number of electrons transferred, at low scan rates an electrochemical equilibrium may always be maintained at the electrode. When this is the case the separation of anodic and cathodic potentials is $57/n$ mV at 298K (Nicholson 1965). In this case no kinetic information about the electron transfer reaction can be obtained. If the scan rate is increased the kinetics of electron transfer may become competitive with the rate of potential change. Under these conditions it is possible to relate the rate of potential change to the separation of peak potentials and the kinetics of the electrode reaction.

A paper by Nicholson, 1965, presents a theoretical correlation between peak potential separation, ΔE_p , the standard heterogeneous rate constant, K_h , and the scan rate, v . These elements are correlated in an expression that defines a kinetic parameter, ψ , [II.4.2.]:

$$\psi = (D_o/D_r)^\alpha K_h / (\pi D_o n F v / RT)^{0.5} \quad [II.4.2.]$$

Where F =Faraday's constant 96485 C mol^{-1} , $R=8.314 \text{ J mol}^{-1} \text{ K}^{-1}$, v =scan rate V s^{-1} and D_o and D_r are the diffusion coefficients of the oxidized and reduced forms respectively. A working curve relating the kinetic parameter, ψ , to $n\Delta E_p$ may be constructed from Nicholson's paper (Figure II.4.1.). From the working curve experimental data of the peak potential separation at various scan rates may be used to find the value of ψ at these scan rates, the value of K_h may be calculated from [II.4.2.].

A major limitation of this method is that there is some dependence of ψ on the charge transfer coefficient, α . Nicholson has demonstrated qualitative differences in the form of the curves produced by cyclic voltammetry dependent on the value of α : for $\alpha < 0.5$ the cathodic peak is more rounded than the anodic peak which also results in a lowering of peak height. For $\alpha > 0.5$ the converse is true. Additionally there is an effect on peak position: as α decreases the cathodic peak is displaced cathodically, as is the anodic peak. So the effects of variations in the value of α tend to negate each other with respect to the differences in peak potential, E_p . In using this method to determine

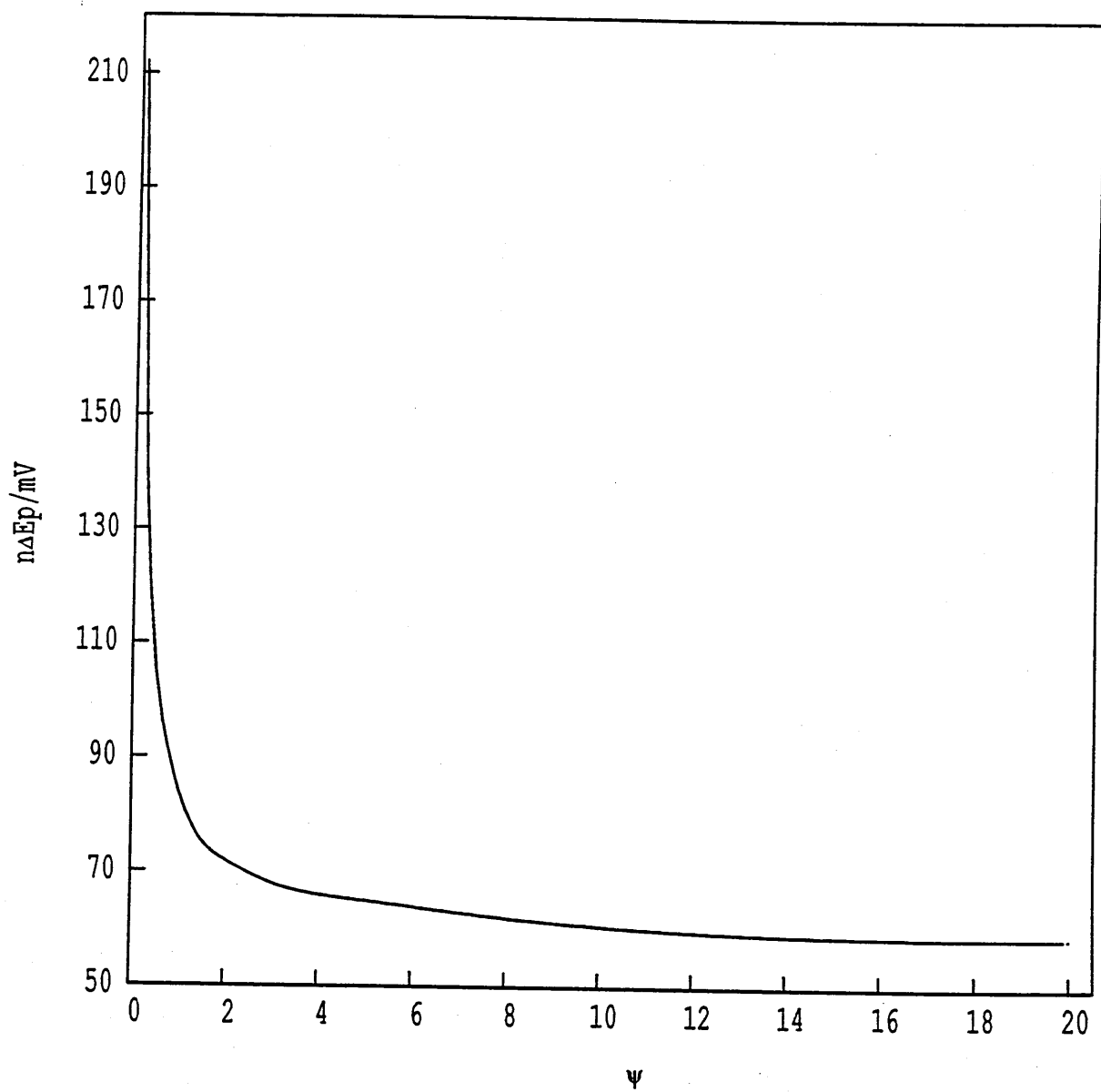


Figure II.4.1. The working curve for the evaluation of K_h by cyclic voltammetry (from Nicholson, 1965).

the magnitude of K_h one of the critical assumptions made is that the values of E_p are virtually independent of α in the range $0.3 < \alpha < 0.5$. For $\alpha > 0.5$ there is about a 5% variation of E_p for changing α from 0.3 to 0.7. When applying [II.4.2.] to experimental data $(D_o/D_r)^\alpha$ is very close to unity except in cases where there is a very large difference between D_o and D_r . The upper limit for the value of the electron transfer constant is between 1 and 5 cm s⁻¹.

II.4.2. THE EFFECT OF THE IONIC ENVIRONMENT ON THE VALUE OF THE HETEROGENEOUS RATE CONSTANT.

Noel and Anantharaman (Noel and Anantharaman 1985) have performed detailed voltammetric studies on the variation of the rate of electron transfer for the ferricyanide (FRO)/ferrocyanide (FRI) system at solid electrodes in the presence of cations and anions having different properties. Ideal behaviour assumes that the anionic redox species undergoes a single electron transfer process, [II.4.3]:



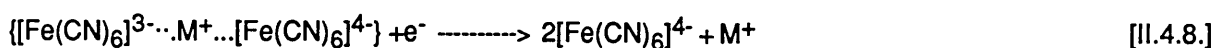
Many workers have noted the variation of K_h due to the influence of the supporting electrolyte. In the FRO/FRI system Peter *et al.*, 1976 have shown that an activated complex is involved in the electron transfer that consists of an ion pair and at least one supporting electrolyte cation. The process may be represented as:



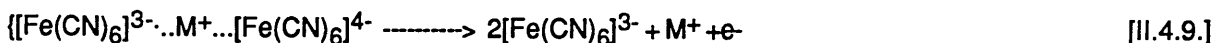
Supporting electrolyte anions were reported to have some effect on K_h and this was attributed to the field effect.

Sohr *et al.*, 1975 have suggested that anionic dimers involving FRO/FRI adsorbed on the electrode function as electroactive species:





or



One purpose of the present study was to investigate whether an analogous behaviour exists for $[\text{Ru}^{\text{III}}(\text{NH}_3)_6]^{2+/3+}$ under variations in the nature of the anions present in the electrolyte or their concentration.

Any evidence of changes in the rate of electron transfer at the electrode due to an increase in anion concentration may illuminate the mechanism involved in the apparent increase in the rate of reaction between the ruthenium complex and glucose oxidase in a forced convective system with increasing pH up to about pH 9. The electron transfer rate in solution may itself be dependent on the supporting electrolyte since, according to the Marcus relationship (Marcus 1965) the homogeneous electron transfer rate constant, K_{ex} , is related to the heterogeneous rate constant.

A number of hypothetical reaction schemes for ruthenium hexamine may be written which are analogous to the proposals discussed previously, for example:



from Peter *et al.*, 1976. The mechanism proposed by Sohr *et al.*, 1975 for the FRO/FRI system may also be applied to ruthenium hexamine.

II.4.3. PROCEDURES EMPLOYED FOR THE DETERMINATION OF THE HETEROGENEOUS RATE CONSTANT FOR RUTHENIUM (III) HEXAMINE.

Chemicals & electrochemicals. Ruthenium (III) hexamine trichloride was obtained from Strem Chemicals Inc. of Newburyport, MA, USA. All other chemicals used were obtained from Aldrich Chemical Company and used at 'Analar' grade.

Electrochemistry. Cyclic voltammetry was performed on EG & G m270 Electrochemistry software which was run off an Amstrad 1640 with Expanded Memory Manager (giving a memory of 1 Mb) and used to drive an EG & G. m270 potentiostat and electrometer. A one compartment electrochemical cell with integral water jacket from EG & G was used for all cyclic voltammetry experiments. A 3 mm diameter gold disc electrode from BAS (Indiana, U.S.A.) functioned as the working electrode, a platinum wire counter electrode and a SCE completed the arrangement.

Experimental. Electrode pretreatment. An oxide layer rapidly forms at the surface of gold at potentials >0.5 V vs. SCE or in an oxidising environment. In an attempt to standardise the state of the electrode surface prior to performing cyclic voltammetry the working electrode was pulsed ten times at a reducing potential of -1.0 V vs. SCE, each pulse having a period of 1 s. In the study of the effect of different ionic media on the electrode kinetics of the FRI/FRO system on the glassy carbon electrode (Noel & Anantharaman, 1985) the state of the electrode surface was critical in obtaining reproducible cyclic voltammograms. Gold rather than glassy carbon was used in this set of experiments because gold electrodes were used in the study of the effect on the rate of reaction between the ruthenium salt and pH and the desire to find a correlation for the unusual pH/rate profile. Additionally, the procedures recommended by Noel and Anantharaman for creating a surface for a glassy carbon electrode that results in reproducible cyclic voltammograms involved potentiodynamic sweeps at oxidising potentials for 15-30 min., or the use of a strongly oxidising environment. The procedure adopted in this study takes less than 30 s to execute and cyclic voltammetry may be performed immediately afterwards with exposure of the electrode to the atmosphere. The cell temperature was maintained at $25\text{ }^{\circ}\text{C} \pm 0.5\text{ }^{\circ}\text{C}$. All solutions were purged with nitrogen prior to cyclic voltammetry being performed. Following the application of the pulsed reducing potential the solution was allowed to equilibrate for 30 s prior to the execution of cyclic voltammetry.

Cyclic voltammetry. In order to apply Nicholson's method for the determination of heterogeneous rate constants the instrumentation must be capable of resolving the value of the anodic and cathodic peaks to 1 mV. Using a zoom facility of the m270 software it is possible to determine the value of each peak to ~ 0.5 mV. This enables an estimate of the error in the determination of the value of K_h to be performed. Nicholson's paper also discussed the need to compensate for IR drops in the cell: the effects of uncompensated IR drop are qualitatively similar to kinetic effects. In the system used this was not always necessary as the ionic strength of most of the solutions used was greater than one. In

consequence IR compensation, using a facility of the m270 software, had to be performed in only a few cases. The potential was swept from +0.1 V to -0.58 V.

Concentration effects. The peak separation of an electroactive species is partially determined by the concentration of that species. The effect on the peak separation of concentrations of the ruthenium salt between 10 mM and 1.5 mM was investigated to determine the concentration at which the peak separation was reduced no further. Concentrations of ruthenium (III) hexamine of 10, 6, 3, 2 and 1.5 mM were used in 1M KCl with a scan rate of 0.2 V s^{-1} (Figure II.4.2.). From Figure II.4.2. it was determined that a concentration of 2 mM was optimal for use in further experiments.

Use of different ionic environments. The following anions in concentrations from 0.5 M to 4 M (where appropriate) were used to measure the value of the heterogeneous rate constant: Cl^- (NaCl & KCl), PO_4^- ($\text{K}_2\text{H}_2\text{PO}_4$) and citrate (di-sodium citrate). The variation in K_h at different pH values was determined from pH 3.0 to pH 10.0 using concentrated sodium hydroxide and hydrochloric acid to adjust the pH, and sodium chloride such that the concentration of chloride ions was approximately uniform at a value of 1 M.

Determination of K_h . Cyclic voltammetry was performed at appropriate scan rates to elicit values for ψ in the region $\psi \geq 0.5$ (Section II.4.1. and Figure II.4.1.). The value of K_h was calculated by making measurements of ψ at different scan rates for each determination. The values of ψ obtained were then plotted against $v^{0.5}$, K_h may be derived from the value of the slope, [II.4.2.], all the other constants being known or determined previously. A value of D of $1.77 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ was used for the calculation of K_h determined previously.

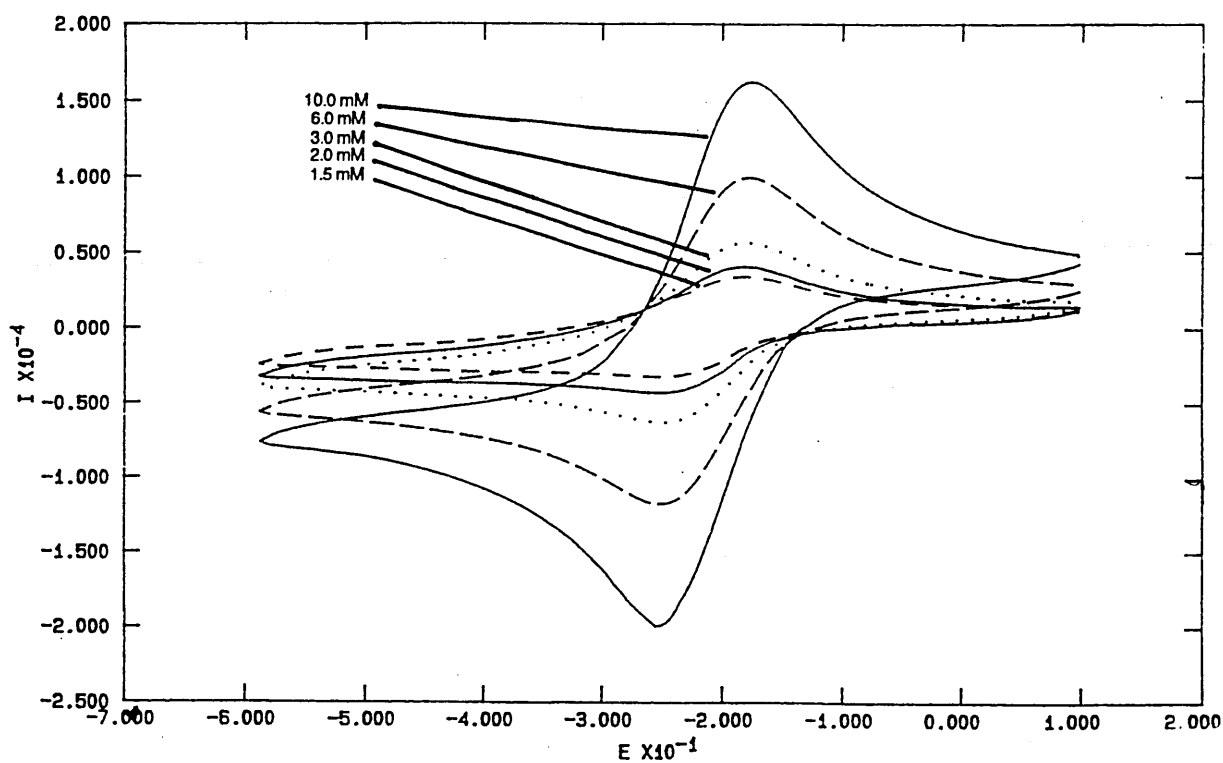


Figure II.4.2. The effect of concentration of ruthenium (III) hexamine ($1.5 \text{ mM} \leq [\text{Ru}(\text{NH}_3)_6] \leq 10 \text{ mM}$) on the peak separation obtained during cyclic voltammetry. All cyclic voltammograms conducted at 0.2 V s^{-1} in 1 M KCl (initial potential 0.1 V; minimum potential -0.6 V).

II.4.4. CALCULATION OF THE HETEROGENEOUS RATE CONSTANT IN DIFFERENT MEDIA.

Examples of the variation of peak separation at different scan rates are shown in Figure II.4.3. for a solution containing 2 M KCl and 2 mM ruthenium (III) hexamine. The peak separations obtained from cyclic voltammograms such as those presented in Figure II.4.3. were used to find the corresponding value of ψ from the working curve (Figure II.4.1.).

The values for ψ were plotted against the reciprocal square root of scan rate ($V^{-0.5}/s^{-0.5}$); the value of K_h may be calculated from the slope of this line, from equation [II.4.1.], all other values being known. In all cases the slope used to derive K_h is the result of a linear regression analysis that was performed on the data. An example of this calculation is given below and corresponds to Figure II.4.4., which shows a set of data obtained for ruthenium hexamine in 4M NaCl.

Equation [II.4.1.] may be rearranged to yield an expression that describes the gradient, $\psi/v^{-0.5}$, in Figure II.4.4., [II.4.13.]:

$$\psi/v^{-0.5} = (D_o/D_r)^\alpha K_h / (\alpha D_o n F v / RT)^{0.5} = 0.927 \quad [\text{II.4.13.}]$$

assuming that $(D_o/D_r)^\alpha \sim 1$, then an expression may be written for K_h , [II.4.14.]:

$$K_h = \psi / v^{-0.5} (\alpha D_o n F / RT)^{0.5} \quad [\text{II.4.14.}]$$

Using the value for the diffusion coefficient of ruthenium (III) hexamine previously determined then the value of K_h determined for the data presented in Figure II.4.4. is 0.0135 cm s^{-1} .

Assuming that the value of α is 0.5 for all experiments, the principal cause of error in the determination of the value of K_h arises from the uncertainty of determining the peak separation. The instrument has a resolving power of 0.5 mV. Using this as the basis for a calculation of error the typical value obtained for the maximum variation in the value of K_h is about 25% of its value.

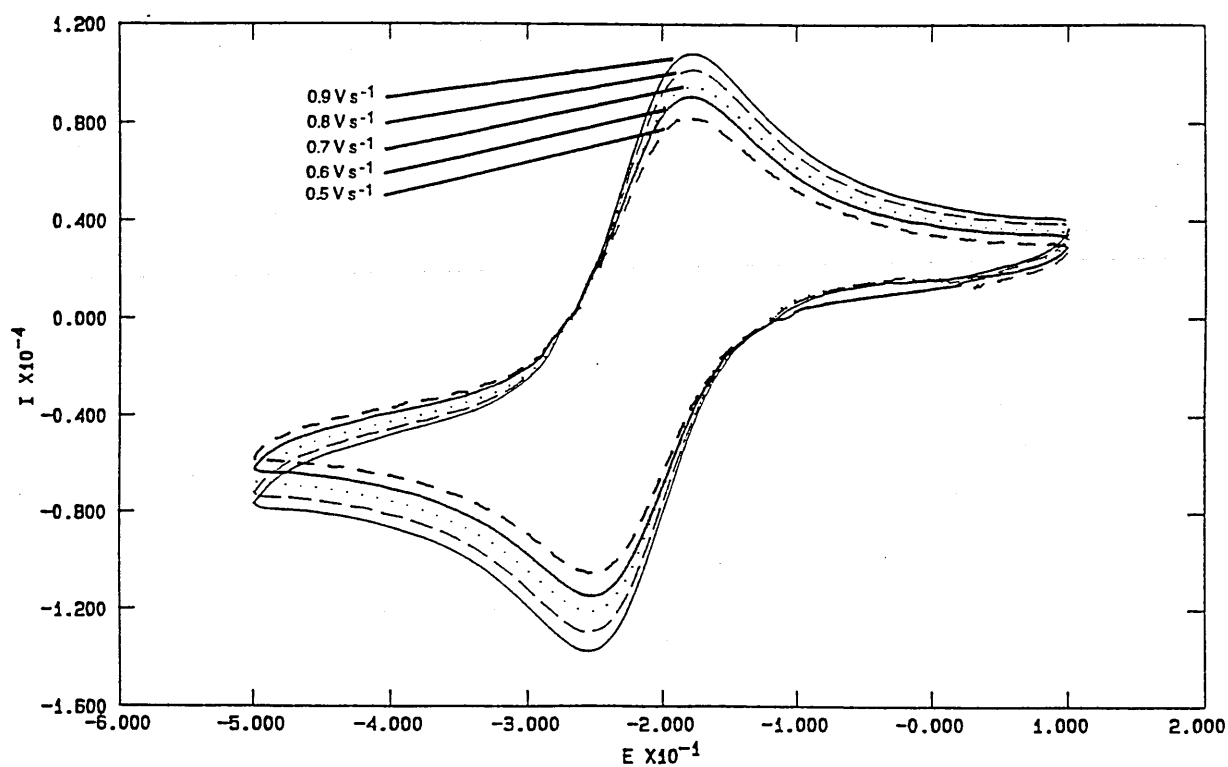


Figure II.4.3. The effect of scan rate ($0.5 \text{ V s}^{-1} \leq v \leq 0.9 \text{ V s}^{-1}$) on peak separation for a solution containing 2 mM ruthenium (III) hexamine and 2 M NaCl (initial potential 0.1 V; minimum potential -0.5 V).

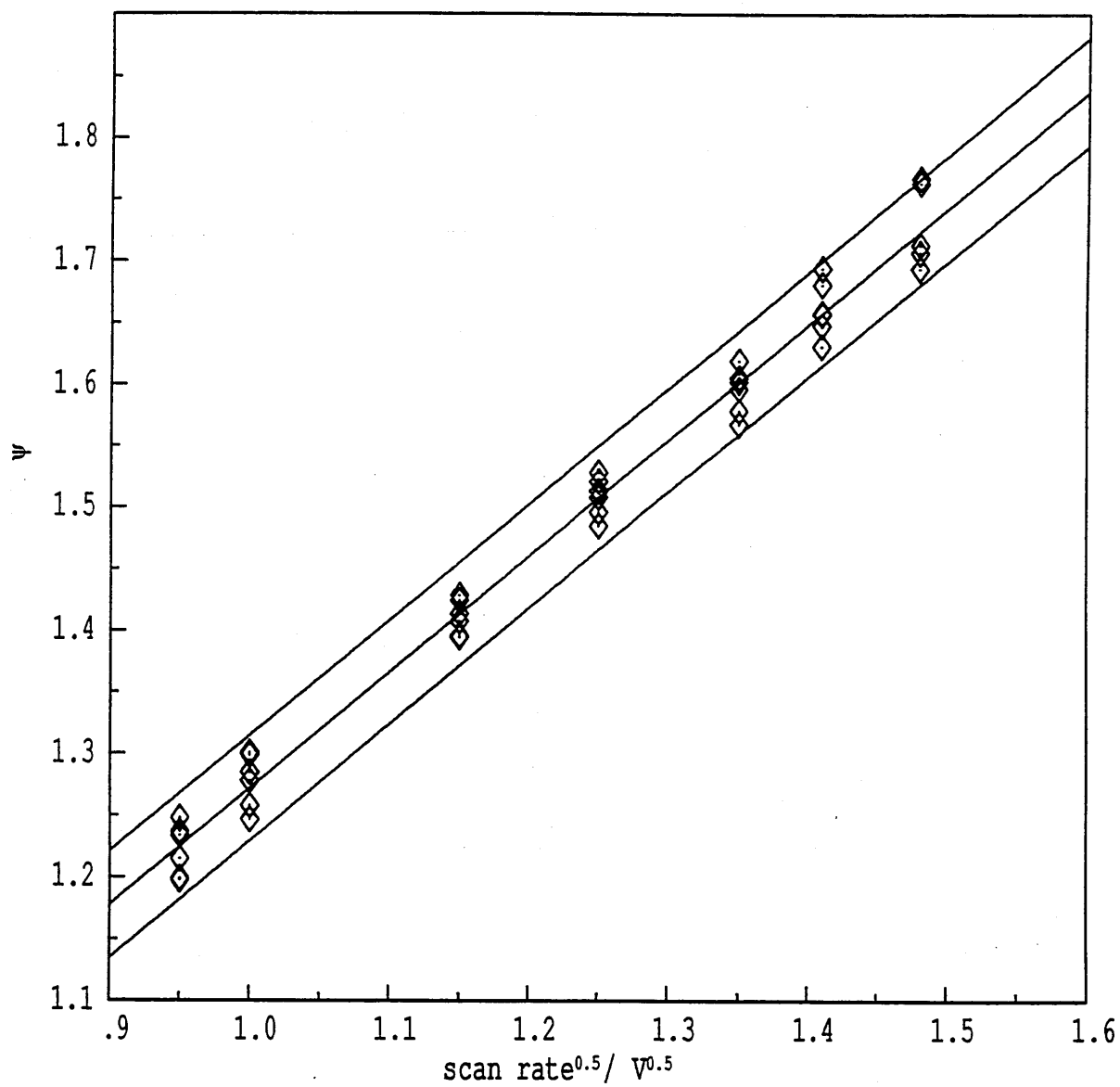


Figure II.4.4. The variation in the value of the kinetic parameter ψ with $v^{0.5}$ for a solution containing ruthenium (III) hexamine at a concentration of 2 mM and NaCl at a concentration of 2 M. Outer lines represent 95% confidence limits ($n=6$ at each scan rate).

Referring to Figure II.4.4., at a ψ value of 1.8, corresponding to a peak separation of 73 mV, the maximum variation in the value of ψ corresponds to peak separations of 73.5 mV and 72.5 mV: ψ values of 1.9 and 1.75 respectively from the working curve, Figure II.4.1.. Applying the same procedure to the lowest value of ψ results in a maximum slope of 1.2 and a minimum slope of 0.78. Substituting these gradient values into II.4.14. yields upper and lower limits for the value of K_h of 0.0176 and 0.0114 cm s⁻¹ respectively. The same procedure was applied to K_h values derived from different media.

The effect on K_h of changing the concentration of different ionic environments is shown in Figures II.4.5. to II.4.9.

Variation in the value of K_h with increasing concentration of Cl⁻, Figures II.4.5. and II.4.6., both show that K_h apparently increases with increasing concentration of chloride ions up to some limiting value. This is most likely attributable to the well documented bridge charge complex arrangement that occurs at the surface of gold electrodes in the presence of chloride.

The value of K_h decreases with increasing concentration of phosphate or citrate ions or with increasing pH (increasing concentration of hydroxy ions). In contrast to the increase in the value of K_h on addition of chloride ions, in both cases K_h fell from a value of about 0.014 cm s⁻¹ to about 0.005 cm s⁻¹ at the maximum anion concentration (4 M for phosphate and 1 mM for hydroxy ions). The relationship between the homogeneous electron transfer rate constant (K_{ex}) and the heterogeneous rate constant (K_h) according to the Marcus equation [II.4.14.]:

$$K_h \sim (K_{ex}/1000)^{0.5} \quad \text{[II.4.14.]}$$

implies that the increase in the rate of the reaction with pH for the ruthenium (III) hexamine, glucose oxidase and glucose system cannot be explained in terms of a change in the homogeneous electron transfer kinetics intrinsic to the mediator couple. Having eliminated this possibility what possible mechanisms remain? It was postulated that there may have been an increase in K_{ex} on increasing pH as the active site of glucose oxidase becomes more negatively charged and this particular mediator is positively charged. If this could have been shown to be the case it would correlate well with the observation that the reaction rate between the negatively charged hexacyanoferrate (III) and glucose oxidase is at a maximum at pH <5. If the Marcus equation holds, K_h and K_{ex} are positively correlated, then one might expect to derive some information on the changes in the kinetics in solution from investigating the heterogeneous kinetics.

Figure II.4.5. The variation of K_h for the oxidation and reduction of ruthenium (III/II) hexamine system at a gold electrode with increasing concentration of KCl. The central line represents the value determined from measurements of ΔE_p , the outer lines describe the error boundaries associated with each value of K_h and were determined from consideration of the resolution of the analytical instrument and the shape of the working curve, Figure II.4.1..

Figure II.4.6. The variation of K_h for the oxidation and reduction of ruthenium (III/II) hexamine system at a gold electrode with increasing concentration of NaCl. The central line represents the value determined from measurements of ΔE_p , the outer lines describe the error boundaries associated with each value of K_h and were determined from consideration of the resolution of the analytical instrument and the shape of the working curve, Figure II.4.1..

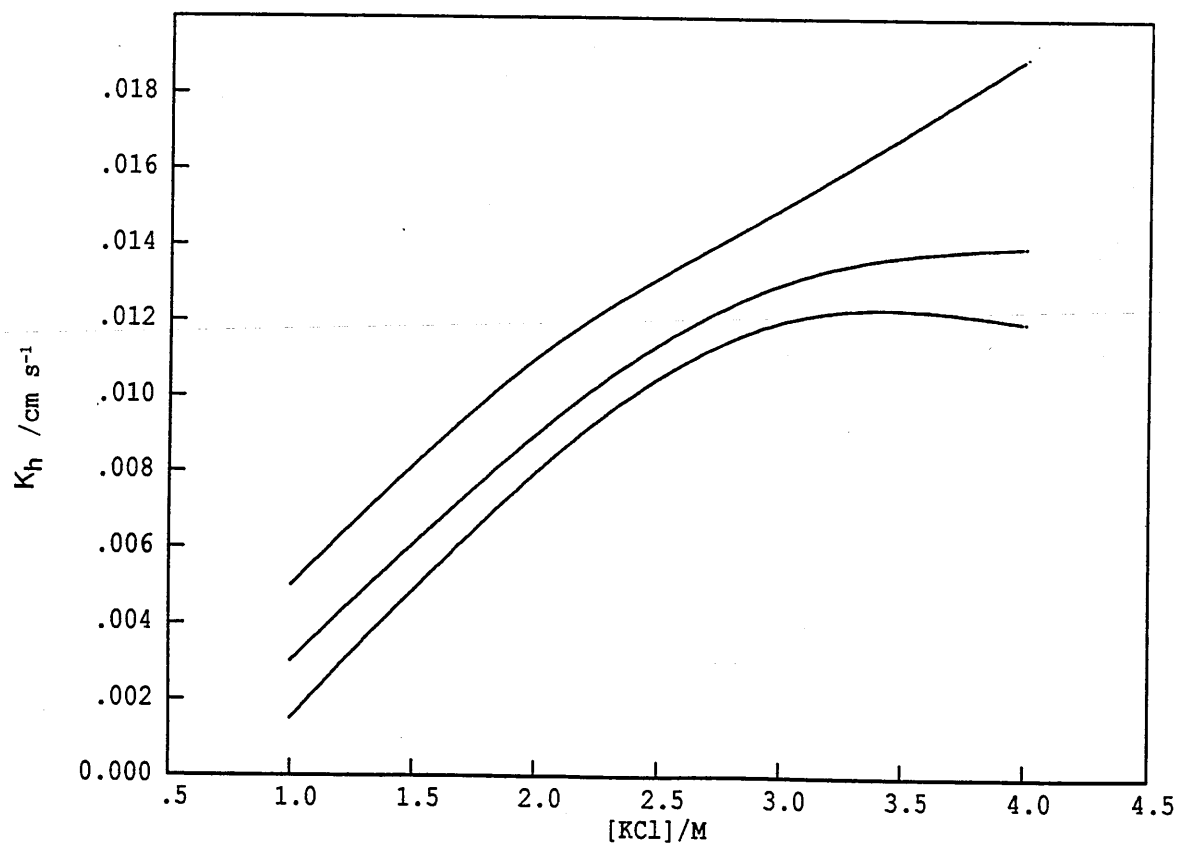
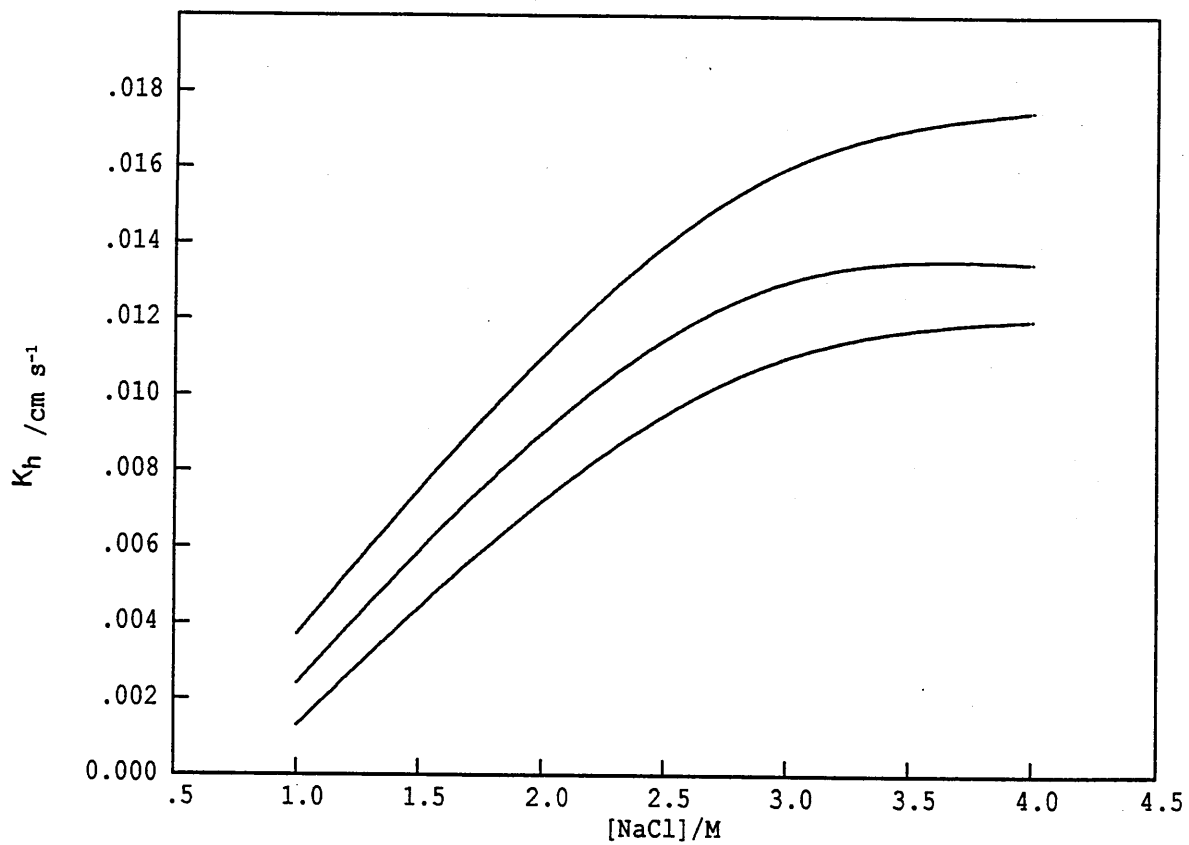
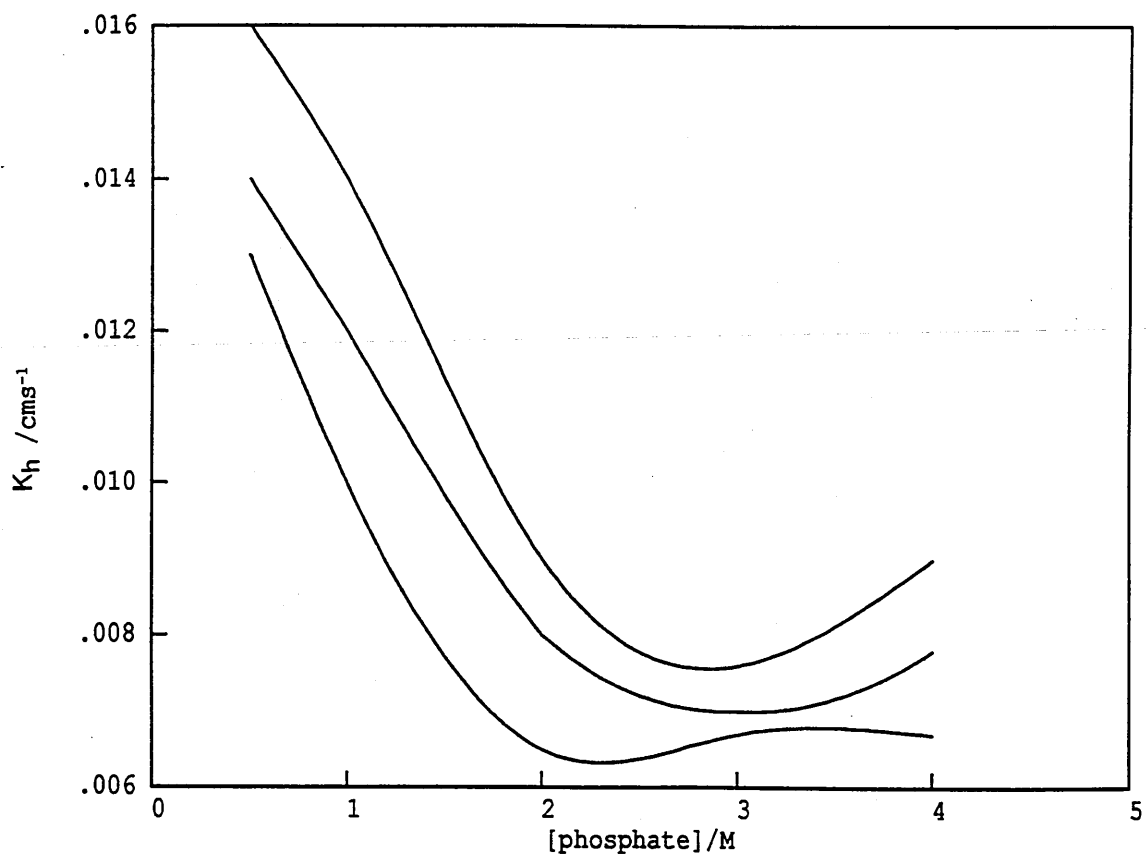
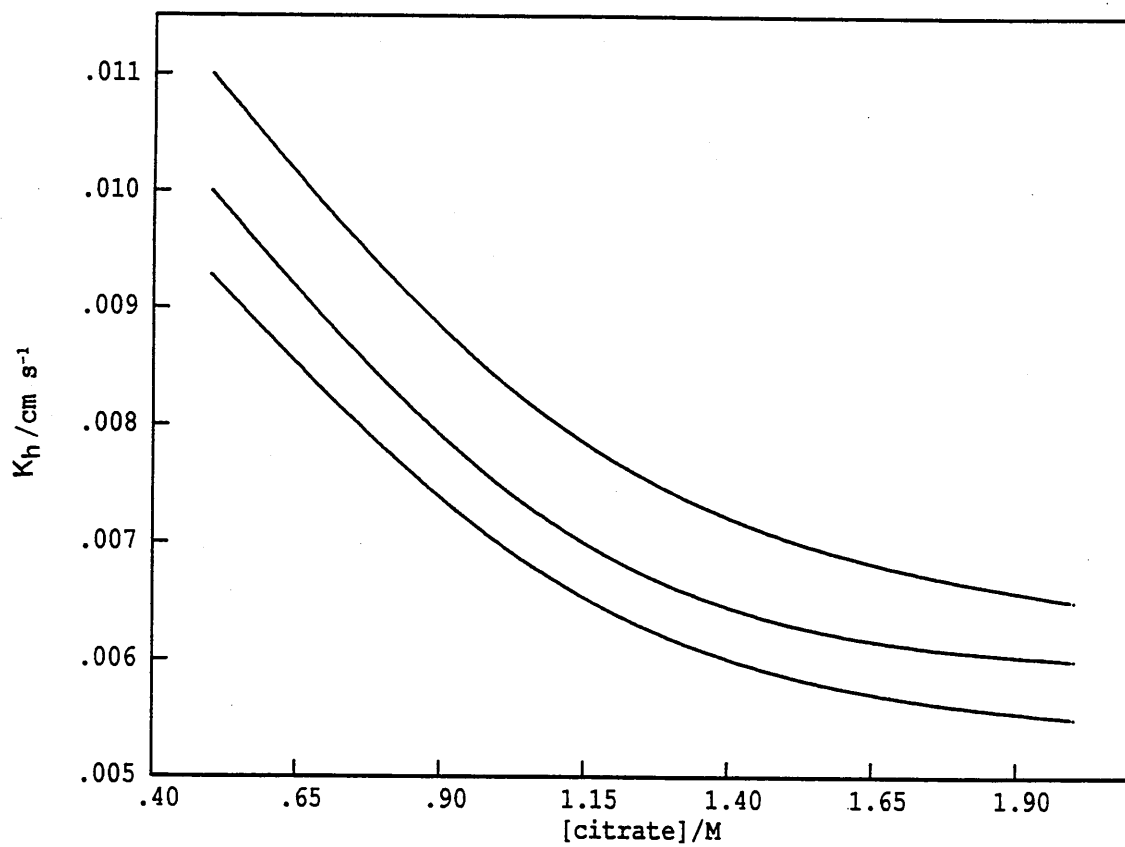


Figure II.4.7. The variation of K_h for the oxidation and reduction of ruthenium (III/II) hexamine system at a gold electrode with increasing concentration of phosphate ions. The central line represents the value determined from measurements of ΔE_p , the outer lines describe the error boundaries associated with each value of K_h and were determined from consideration of the resolution of the analytical instrument and the shape of the working curve, Figure II.4.1..

Figure II.4.8. The variation of K_h for the oxidation and reduction of ruthenium (III/II) hexamine system at a gold electrode with increasing concentration of citrate. The central line represents the value determined from measurements of ΔE_p , the outer lines describe the error boundaries associated with each value of K_h and were determined from consideration of the resolution of the analytical instrument and the shape of the working curve, Figure II.4.1..



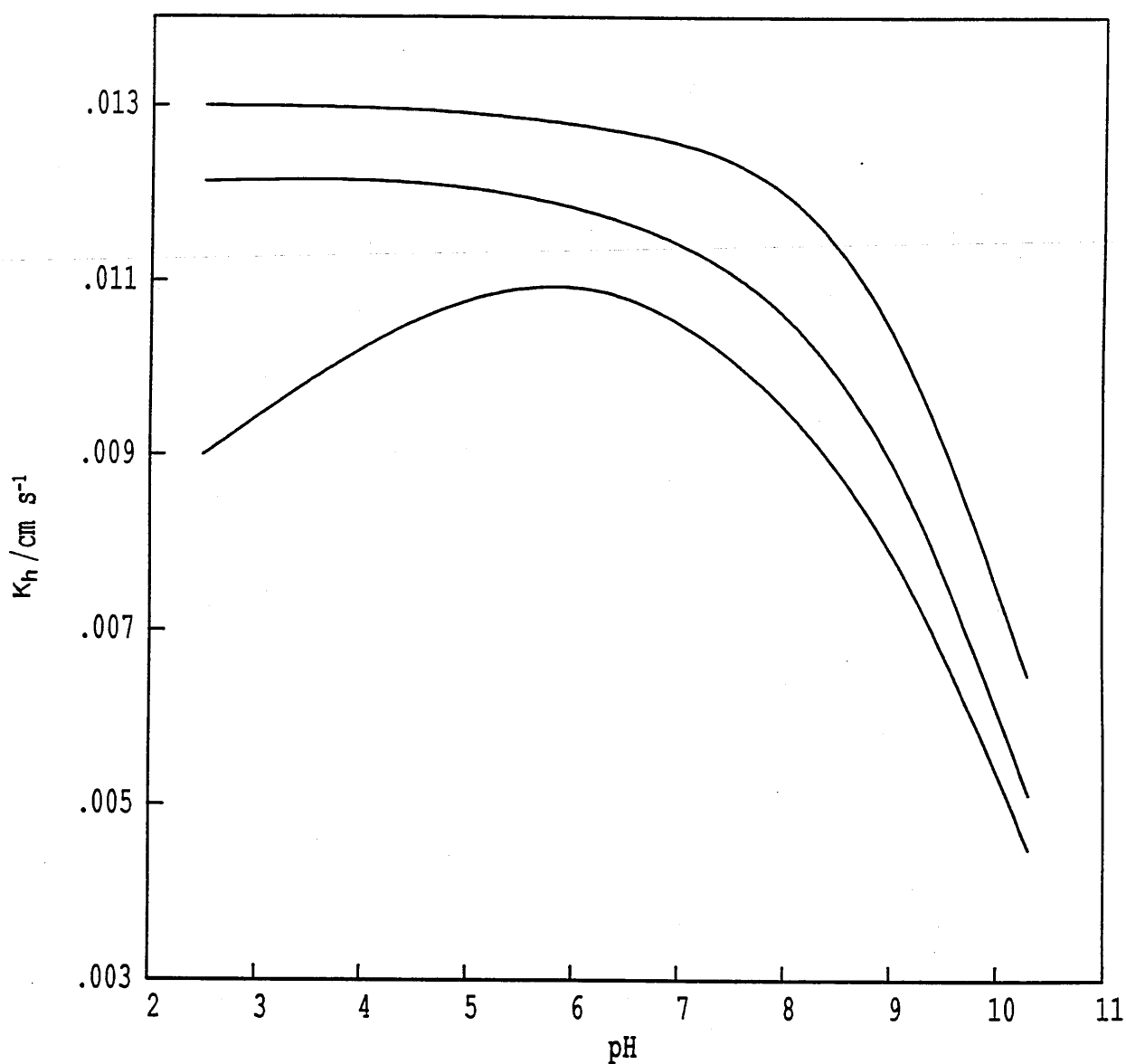


Figure II.4.8. The variation of K_h for the oxidation and reduction of ruthenium (III/II) hexamine system at a gold electrode with pH. The central line represents the value determined from measurements of ΔE_p , the outer lines describe the error boundaries associated with each value of K_h and were determined from consideration of the resolution of the analytical instrument and the shape of the working curve, Figure II.4.1..

Phosphate and hydroxy ions do not appear to function in the same way as chloride ions. It may be envisaged that if an anion does not contribute to any charge transfer complex at the surface of the electrode then it would be likely to have a detrimental effect on the kinetics of electron transfer due to charge shielding effects of the redox centre.

If enhancement of the kinetics of electron transfer intrinsic to the ruthenium (III/II) hexamine couple are not the cause of the observed increase in the reaction rate of the mediator/glucose oxidase/glucose system then the other places to search for an explanation are in the structural changes of the glucose and glucose oxidase that accompany increasing pH or differences in the thermodynamics of electron transfer at elevated pH values (Section II.5.).

II.5.1. KINETIC ANALYSIS OF ELECTRODE CONFIGURATIONS INCORPORATING FERRICYANIDE AND RUTHENIUM (III) HEXAMINE AS ELECTRON MEDIATORS.

Previous theories that describe the current response of amperometric sensors apply to cases where the catalytic layer is placed in intimate contact directly over the electrode surface. Usually an electron mediator compound is co-entrapped with the enzyme within this layer (Senda *et al.*, 1986; Ikeda *et al.*, 1988) or the sensor may utilise the oxidation of hydrogen peroxide. Sensors employing enzyme entrapment within an electrically conducting polymeric film such as polypyrrole behave in an analogous fashion (Anson, 1980; Andrieux *et al.*, 1980; Andrieux & Saveant, 1982; Bartlett & Whitaker, 1987). In several of these cases a membrane between catalytic layer and the bulk solution limits the flux of analyte to the catalytic layer to a value that is below the rate of catalysis and electron transfer per unit area of the catalytic layer (Albery & Hillman, 1984). Here the concentration of analyte is linearly correlated to the value of steady state current response. Reduction of the analyte flux to the catalytic layer reduces the steady state current response but extends the linear range of the device. Several kinetic analyses attempt to demonstrate that the current response is limited by the rate of diffusion of analyte rather than the rate of reaction in the catalytic layer (Albery & Bartlett, 1985), which is the most desirable condition for this type of sensor with respect to consistency of response between different production runs of the sensor. The variability of characteristics of manufactured diffusion limiting membranes being less than for the catalytic layer. Additionally, by selecting the appropriate membrane, dependency of the chemical reactions occurring in the catalytic layer, and of the diffusion coefficient of the analyte on differences in temperature are obviated.

There are other differences between the present manufactured format of the enzyme CFD and the examples quoted above. As sample containing analyte is introduced to the CFD (Section I.4.) there is an instantaneous large flux of soluble mediator and buffer salts from the catalytic layer into the bulk solution, a flux of analyte towards the catalytic layer concomitant with a flux of reduced mediator from the catalytic layer towards the electrode surface. This dynamic condition is very complex and cannot be modelled by any of the methodologies described in the papers quoted above. The principal difference between previously developed systems and the present one is that the assumption of steady state flux of analyte to the surface of the catalytic layer within which it is assumed that there is also a steady state cycling of redox mediator between its oxidized and reduced states. In the CFD the concentration of reduced mediator never reaches a steady state concentration during the analysis time but approaches an end point equilibrium concentration at the end of the analytical potential pulse. Yokoyama *et al.*, 1989, has modelled the flux of a soluble electron mediator into a catalytic layer containing glucose oxidase situated above an electrode. In this system, once within the catalytic layer however, the concentration of reduced mediator approaches a steady state as the flux of oxidized mediator generated at the electrode surface approaches the flux of reduced mediator towards the electrode.

As has been demonstrated by the correlation curves for the coulometric sensor response to glucose, because the enzymatically catalysed reaction has approached an end point at the beginning of the analysis stage, any attempt to characterise sensor kinetics in terms of previously developed theory of steady state current response is meaningless. In contrast to the steady state current that is achieved in the examples discussed above, during the first sixty seconds after the introduction of sample containing analyte, the system tends towards an end point equilibrium, where the concentration of β -D-glucose tends to zero. In order to achieve a stoichiometric equivalence between charge and moles of analyte present, oxidizing potential is best applied when the equilibrium has been reached. In this system it is pertinent to ask whether the equilibrium may be approached more rapidly by the substitution of ruthenium (III) hexamine for ferricyanide.

One route to an understanding of the operation of the catalytic layer of a CFD lies in developing a method of deriving the second order rate constants for the reaction between reduced glucose oxidase and oxidised mediator, and then using the values obtained to model the reaction of the glucose within the catalytic layer, and the dependent fluxes of glucose to the catalytic layer and of reduced mediator from the catalytic layer. The use of Nicholson & Shain theory (Nicholson & Shain, 1965) is only applicable to cases where the second order rate constant is $\geq 10^3 \text{ M}^{-1} \text{ s}^{-1}$. As has been demonstrated previously, Section II.3.3., catalytic limiting currents were not produced using linear sweep voltammetry and the second order rate constant for the reaction involving ferricyanide has not been elucidated using Nicholson & Shain theory. This implies that the second order rate constant for the reaction involving these two mediators is far inferior to that obtained for the ferrocenes. Therefore an analysis was developed based on the difference between amperometric current decay in the presence and absence of an homogeneous catalytic reaction. That analysis is presented here and the development of a model of the catalytic layer incorporating the results of this analysis is described in Section III.3.2..

II.5.2. PROCEDURES EMPLOYED TO DETERMINE THE SECOND ORDER RATE CONSTANTS FOR THE REACTION BETWEEN RUTHENIUM (III) HEXAMINE OR FERRICYANIDE AND REDUCED GLUCOSE OXIDASE.

Solutions of glucose and ruthenium (III) hexamine or ferricyanide were added to a solution of glucose oxidase. The final concentrations of glucose and oxidized mediator were 60 mM and 0.3 M respectively. The final concentration of glucose oxidase was 1 mg ml^{-1} ($5.5 \times 10^{-6} \text{ M}$). The system was maintained at pH 7.0 by employing equimolar quantities of sodium di-hydrogen phosphate and disodium hydrogen phosphate for a concentration of phosphate of 0.1 M.

Instantaneous mixing of the constituent solutions was assumed. This assumption was reasonable as the final volume of each mixture was $100 \times 10^{-6} \text{ m}^3$. An aliquot of the mixture was introduced to the CFD and the current transient was measured for 4 s commencing 20 s after the realisation of the complete mixture. Experimental current transients were recorded using a computer controlled PSTAT10 potentiostat with GPES3 electrochemical software (eco chemie, Slough, U.K.) at a sampling frequency of 20 Hz. Oxidizing potentials of 0.4V and 0.1 V were applied for the analysis of ferricyanide and ruthenium (III) hexamine respectively. The simulated catalytic current, $i_{k(\text{sim})}$ (equation [II.5.16.]), was evaluated numerically using a program written in BASIC.

Chemicals & electrochemicals. All chemicals were from the same sources as described previously and were obtained at the same grade. Glucose oxidase from Type VII from *Aspergillus niger* (EC 1.1.3.4.) with a specific activity of 200 units mg^{-1} was obtained from Sigma Chemical Co.. Reagents were prepared on a daily basis in ultra pure water and filtered through 0.2 μm filters immediately prior to use. Glucose oxidase solutions were prepared in bacterial free pH 7.0 phosphate buffer and kept in sealed containers at 4°C until needed. The concentration of glucose oxidase expressed in terms of catalytically active FAD was determined spectrophotometrically at 450 nm and assuming a molar extinction coefficient of $1.3 \times 10^4 \text{ l mol}^{-1} \text{ cm}^{-1}$. All solutions were ebulliated with 'white spot' nitrogen prior to use.

II.5.3. EVALUATION OF THE SECOND ORDER RATE CONSTANTS FOR THE REACTION BETWEEN RUTHENIUM (III) HEXAMINE OR FERRICYANIDE AND REDUCED GLUCOSE OXIDASE.

Information about the rate of reduction of mediator by reduced glucose oxidase may be derived from many features of the current time transient. It is important to remember that the oxidation of glucose does not reach an equilibrium before the application of the potential pulse. Rather, the reaction proceeds at a certain rate depending on the concentration of glucose and of oxidized mediator, and continues for the duration of the potential pulse. Therefore it is possible to define a theoretical current response based on an estimate of the concentration of reduced mediator at a defined time, and to contrast this with the actual current transient. The difference between the experimentally obtained current and the theoretically derived diffusion current contains information related to the overall rate of the oxidation of glucose and the reduction of mediator. In certain cases, where the concentration of glucose is very much greater than the K_m of glucose oxidase for the duration of the transient examined, such that the concentration of reduced enzyme, $[E_r]$, approaches the total concentration of enzyme, $[E_t]$, it is possible to derive the first order rate constant associated with the

reduction of oxidized mediator, M_O , and to make an estimate of the second order rate constant for the oxidation of E_r by M_O under these conditions.

The validity of the assumption of a Cottrell diffusion current for the first seconds of a potential pulse applied to a solution of electroactive compound in a CFD is demonstrated in Figure II.5.1. Figure II.5.8. is a plot of current vs. $\text{time}^{-0.5}$ for a CFD containing 0.295 M ferricyanide and 5 mM ferrocyanide on the application of an oxidizing potential of 0.2 V. A least squares linear regression analysis of this data yields a correlation coefficient of 0.9978 ($n=949$). The pulse was applied for 1 s. According to Bard and Faulkner, 1980, the mean diffusion distance, x , in time t , of a molecule with a diffusion coefficient of D , is given by [II.5.1.]:

$$x = (2Dt)^{0.5} \tag{II.5.1.}$$

Applying [II.5.1.] to this analysis system it is possible to estimate the duration of the linear dependence of current on time in a CFD. The distance between the electrode surface and the end plate is $100 \times 10^{-6} \text{m}$. The time values for ruthenium (III) hexamine and for ferricyanide are about 4 s and about 8 s respectively.

It is important to establish the validity of assumptions of quasi-first order reaction with respect to the concentration of oxidized mediator before embarking on a determination of rate constants.

The following analysis is based on the assumption that for the duration of the analysis the enzyme should be predominantly in the reduced form. Equations [II.5.2.] and [II.5.3.] describe the reactions occurring in the glucose sensing CFD, and may be applied to amperometric sensors employing an electron mediator generally (Lam, 1981; Eddows, 1989). G and DL represent α -D-glucose and the product of its oxidation, β -gluconolactone. E_OG is the enzyme-substrate complex. E_O , E_r , M_O and M_r have been previously defined. The constants k_1 and k_{-1} are the association and dissociation constants that characterise the formation of the enzyme-substrate complex and k_2 is the rate constant for the dissociation of the enzyme-sustrate complex after oxidation of glucose has occurred. The constant k_e is the bimolecular rate constant that characterises the oxidation of reduced oxidoreductase by oxidized mediator. The Michaelis-Menton constant, K_m , may be defined by $(k_{-1} + k_2)/k_1$.



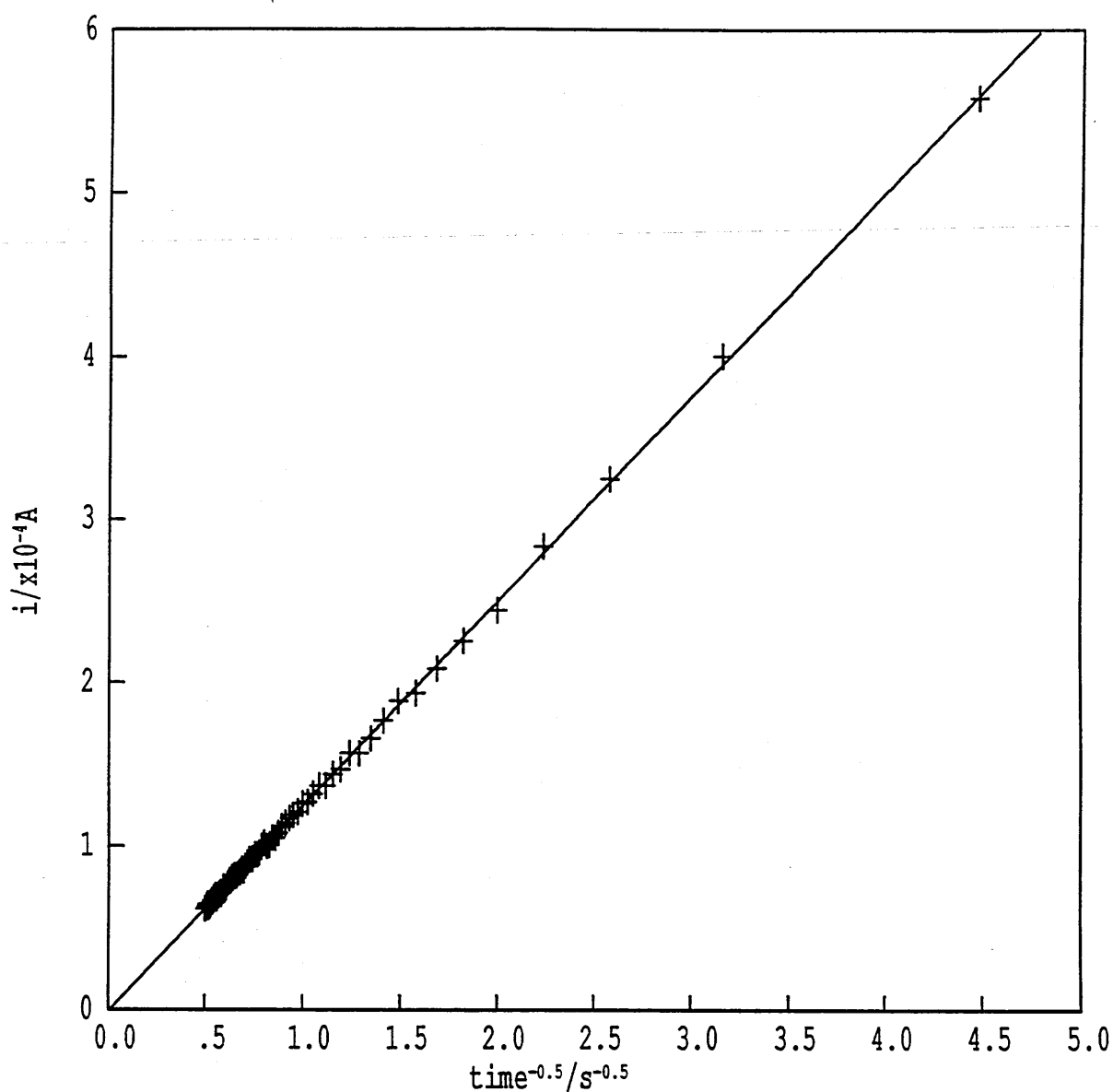


Figure II.5.1. A plot of current vs. $time^{-0.5}$ for a CFD containing 0.295 M ferricyanide and 5 mM ferrocyanide on the application of an oxidizing potential of 0.4 V of duration 4 s. Demonstrating the applicability of the Cottrell current equation for semi-infinite solution to diffusion in the CFD over this timescale (line represents linear regression for i vs. $t^{-0.5}$; $r^2 > 0.99$).



For ferricyanide and ruthenium (III) hexamine, and for other electron mediator compounds exhibiting a redox state change of one, such as the ferrocenes (Cass *et al.*, 1984), $n=2$. The reaction at the electrode surface is associated with an heterogeneous rate constant K_h :



The rate of reaction described by [II.5.4.] is assumed to be fast compared to the homogeneous catalysed reaction. The current response to the reaction that occurs in bulk solution is dependent on the diffusion coefficient of the mediator compounds, and in a thin layer device is characterised at times greater than between about 4 s and 8 s by an exponential constant with respect to time (Section I).

In order to determine the first order rate constant for the rate of change in concentration of oxidized mediator it is necessary to establish quasi-first order conditions with respect to the concentration of oxidised mediator. If the total concentration of enzyme is E_t , then;

$$[E_\text{t}] = [E_\text{r}] + [E_\text{O}] + [E_\text{O}G] \quad [\text{II.5.5.}]$$

at high concentrations of glucose, $[E_\text{r}] \gg [E_\text{O}]$.

The rate of change of $[M_\text{O}]$ due to reaction [II.5.3.] is:

$$-\partial[M_\text{O}]/\partial t = 2k_\text{e}[M_\text{O}][E_\text{r}] \quad [\text{II.5.6.}]$$

for $n=2$ (Liaudet *et al.*, 1990). However, as $[G] \gg K_\text{m}(\text{glucose oxidase})$, $[E_\text{r}]$ is assumed to be constant throughout the course of the reaction, and therefore the reaction may be considered to be quasi-first order with respect to $[M_\text{O}]$. Because $[M_\text{O}] \gg [E_\text{t}]$ and $[E_\text{r}]$ throughout the course of the reaction the significance of any subsequent reaction between E_r and electrode generated oxidized mediator may be neglected. It is possible, then, to write a differential equation for the rate of change of $[M_\text{O}]$ incorporating a first order rate constant, K_1 , for the reduction of M_O :

$$-\partial[M_\text{O}]/\partial t = K_1[M_\text{O}]_0 \quad [\text{II.5.7.}]$$

where $[M_\text{O}]_0$ is the initial concentration of M_O . Integration of [II.5.7.] yields:

$$[M_\text{O}]_\text{t} = [M_\text{O}]_0 e^{-K_1 t} \quad [\text{II.5.8.}]$$

here $[M_o]_t$ is the concentration of M_o at a time, t . $[M_r]$ at a time t may also be defined from [II.5.8.]:

$$[M_r]_t = [M_o]_0 - [M_o]_0 e^{-K_1 t} \quad [\text{II.5.9.}]$$

From the current transient close to the application of the potential pulse it is possible to closely approximate the concentration of M_r present at the start of the potential pulse. This allows an estimate to be made of the concentrations M_r at the start of the potential pulse, provided its concentration is known at the point where the reaction was initiated. A diffusion current, i_d , may be calculated by using the Cottrell equation. The diffusion current is an evaluation of the current transient that would result from an initial concentration of M_r calculated at the start of the potential pulse, in the absence of any homogeneous catalytic reaction. The diffusion current response, i_d , may be defined as:

$$i_d = nFA[M_r]_0(D/\pi t)^{0.5} \quad [\text{II.5.10.}]$$

The current difference between the experimentally derived current, i_e , and the diffusion current, i_d , is some function of the rate of the homogeneous catalytic reaction. A catalytic current, i_k , may be defined that is dependent on the rate of generation of M_r from M_o that is separate from the current contribution derived from the concentration of M_r present at the start of the potential pulse, $[M_r]_0$:

$$i_k = i_e - i_d \quad [\text{II.5.11.}]$$

In order to derive an expression for i_k it is necessary to treat the current response as a population of superimposed finitely small Cottrell decays. All times in this explanation relate to the 'time zero' obtained on the application of the potential pulse. Consider that the time between '0' and about 4 s, the period of the transient under analysis, is divided into a number of finite elements. The duration between each of these elements is Δt s. For a total time period of t s there are n elements.

$$t = n\Delta t \quad [\text{II.5.12.}]$$

The concentration of M_r at $t=0$ is zero, so the current at $1\Delta t$ s is zero. The initial current response would occur at $2\Delta t$ s; the current here would be a function of the change in concentration of M_r over the time interval between $t=0$ and $1\Delta t$ s. If the change in concentration of M_r over this period is $\Delta[M_r]_1$ and making $a = nFA(D/\pi)^{0.5}$, then the catalytic current response at $2\Delta t$ s may be evaluated;

$$i_{k(2\Delta t)} = a (\Delta[M_r]_1 / \Delta t^{0.5}) \quad [\text{II.5.13.}]$$

The current at $3\Delta t$ s will have contributions from the change in concentration of M_r over the

preceding time period ($\Delta[M_r]_2$ for $3\Delta t-2\Delta t$) and also from the decay, according to the Cottrell equation, from the concentration of M_r present at $1\Delta t$ s, for the time interval $3\Delta t-\Delta t=2\Delta t$ s. The catalytic current at $3\Delta t$ s may be written;

$$i_{k(3\Delta t)} = a((\Delta[M_r]_2/\Delta t^{0.5}) + (\Delta[M_r]_1/2\Delta t^{0.5})) \quad [II.5.14.]$$

Generally, for the j^{th} catalytic current element;

$$i_{k(j\Delta t)} = a((\Delta[M_r]_{(j-1)}/\Delta t^{0.5}) + (\Delta[M_r]_{(j-2)}/2\Delta t^{0.5}) + \dots + (\Delta[M_r]_{(1)}/(j-1)\Delta t^{0.5})) \quad [II.5.15.]$$

The value of $\Delta[M_r]$ over each time interval is the same over the interval investigated, [II.5.10.]. The common factor $(\Delta[M_r]/\Delta t^{0.5})$ may be placed in front of the parentheses, revealing a series of the form $(1 + 1/2^P + 1/3^P + \dots + 1/(n-1)^P + 1/n^P)$, for the j^{th} catalytic current response, [II.5.17];

$$i_{k(j\Delta t)} = a(\Delta[M_r]_{\Delta t}/\Delta t^{0.5})(1 + 1/2^{0.5} + 1/3^{0.5} + \dots + 1/(j-1)^{0.5}) \quad [II.5.16.]$$

For a time t (equation [II.5.12]) taking $n \rightarrow \infty$ then $\Delta t \rightarrow \delta t$, this series does not converge (Stroud, 1981). This means that the series in [II.5.16.] must be evaluated numerically.

Equation [II.5.16.] predicts that i_k is a positive linear function of $t^{0.5}$, because as n increases linearly, the value of the series increases by a factor of $t^{0.5}$, whilst the factor $a(\Delta[M_r]_{(j-1)}/\Delta t^{0.5})$ is invariant. The shape of the i_k vs. t curve and operation of [II.5.16.] is illustrated in Figure II.5.2. in terms of a set of current decay curves whose form is identical but whose absolute value is a function of the summation of the decay currents from all previous current elements as determined by the series in [II.5.16.].

If [II.5.16.] is correct then the value of K_1 may be evaluated by correlating the experimentally derived catalytic current response, $i_{k(e)}$, with the simulated response, $i_{k(sim)}$. The starting values for K_1 used to generate $i_{k(sim)}$ may be obtained from an evaluation of the current response at $t=0.05$ s using the Cottrell equation and expression [II.5.9.]. The value of $\Delta[M_r]_{\Delta t}$ in [II.5.16.] may be obtained by evaluating the slope of $[M_r]_t$ vs. t from [II.5.9.], and applying the time interval used in the simulation, Δt . If the reaction proceeds at the same rate over the pre-potential period as during the current transient the application of the value obtained for K_1 obtained by this means to [II.5.16.] should produce a simulated catalytic response that closely corresponds to $i_{k(e)}$.

The bimolecular, or second order, rate constant, k_e , may be derived by equating [II.5.6.] and [II.5.7.] as:

$$k_{\theta} = K_1/2[E_r]$$

[II.5.17.]

assuming that as $[G] \rightarrow \infty$, then $[E_r] \rightarrow [E_t]$.

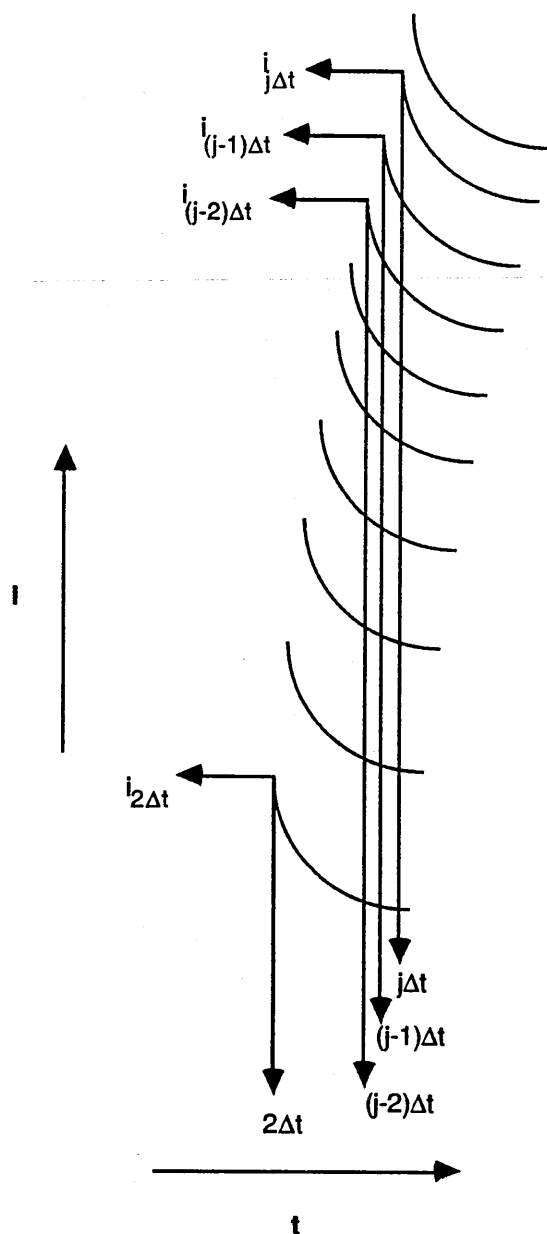


Figure II.5.2. Schematic diagram illustrating the operation of [II.5.16.]. Each of the identical current decay curves represent the Cottrell current derived from the change in concentration of reduced mediator over the previous time element, but their absolute current value at any time is a summation of the current decay contributions from all preceding decays. Current elements are temporally separated by Δt s and differ in magnitude as determined by the series in [II.5.16.]. The first current response occurs at $2\Delta t$ s, the current at $j\Delta t$ s is a summation of the current decay contributions between $(j-1)\Delta t$ s and $2\Delta t$ s plus the current contribution derived from $\Delta[M_r]_{\Delta t}$.

II.5.4. ANALYSIS OF DATA FOR EVALUATION OF THE SECOND ORDER RATE CONSTANT FOR THE INTERACTION BETWEEN ELECTRON MEDIATOR AND GLUCOSE OXIDASE.

Figures II.5.3. and II.5.4. show the experimentally derived current and the calculated diffusion current for ruthenium (III) hexamine and for ferricyanide. Analysis of the current at $t=0.05$ s using the Cottrell equation yields an initial concentration of reduced mediator of 4.4 mM for ruthenium (III) hexamine and 3.55 mM for ferricyanide. Application of equation [II.5.9.] yields values for K_1 of $9.0 \times 10^{-4} \text{ s}^{-1}$ for ruthenium (III) hexamine and $5.9 \times 10^{-4} \text{ s}^{-1}$ for ferricyanide. These values were used to evaluate $\Delta[M_r]_{\Delta t}$ in [II.5.16.] from [II.5.9.], which yielded values for $\partial[M_r]/\partial t$ of $0.266 \text{ moles m}^{-3} \text{ s}^{-1}$ for ruthenium (III) hexamine and $0.175 \text{ moles m}^{-3} \text{ s}^{-1}$ for ferricyanide. Evaluation of $i_{k(\text{sim})}$ was performed using an algorithm incorporating the series factor in [II.5.16.] for $\Delta t=0.05$ s & $n=70$ ($n\Delta t=3.5$ s).

If the assumption of pseudo first order reaction over the duration of the experiment was valid then the values obtained for K_1 from the pre-potential period should produce values for $\partial[M_r]/\partial t$ which, when substituted into [II.5.16.], should generate a simulated catalytic current response which closely corresponds to the experimentally derived catalytic current. Consideration of the final concentration of glucose suggests that the assumption of pseudo first order reaction conditions for the duration of the experiment is valid since the concentration of glucose changes by less than 5% for the duration of the experiment, from 60 mM to about 57 mM, as determined from the initial current response and neglecting the change in glucose concentration over the course of the potential pulse. Thus the concentration of glucose is always significantly greater than the K_m of glucose oxidase for β -D-glucose (for $K_m(\text{glucose})=7$ mM (Mell & Maloy, 1975), $[G]:K_m$ of ~ 8).

Figures II.5.5. & II.5.6. show $i_{k(e)}$ and $i_{k(\text{sim})}$ vs. time for ruthenium (III) hexamine and ferricyanide respectively. Figures II.5.7. & II.5.8. show $i_{k(e)}$ and $i_{k(\text{sim})}$ vs. $\text{time}^{0.5}$ for ruthenium (III) hexamine and ferricyanide respectively. The values for $i_{k(e)}$ were derived from [II.5.12.] and $i_{k(\text{sim})}$ was generated as described previously.

Although the values for $\Delta[M_r]_{\Delta t}$ derived from the initial current response could have been used as the starting values for an evaluation of the catalytic current in [II.5.16.] (using a least squares error algorithm to find the value of K_1 at which the differences between $i_{k(\text{sim})}$ and $i_{k(e)}$ for $t>0$ s were minimised) since the least squares linear regression for $i_{k(\text{sim})}$ vs. $i_{k(e)}$ using the starting values for $\Delta[M_r]_{\Delta t}$ generated from [II.5.9.] were > 0.99 , the application of such a procedure was considered redundant. This further supports the assumption of pseudo first order reaction conditions. The high degree of correspondence between $i_{k(\text{sim})}$ and $i_{k(e)}$ and the fact that $i_{k(e)}$ vs. $t^{0.5}$ is linear for $t > 0$ strongly suggest that the series in [II.5.16.] is a good representation of the time dependence of the catalytic current.

The bimolecular rate constant, k_{θ} , may be estimated from [II.5.17.]. Based on the assumption that $[E_r] = 5.5 \times 10^{-6} \text{M}$, k_{θ} was calculated as $80 \text{ M}^{-1} \text{ s}^{-1}$ for ruthenium (III) hexamine and $52 \text{ M}^{-1} \text{ s}^{-1}$ for ferricyanide. The example shown here matches the mean value obtained for k_{θ} over a number of trials ($n=10$, $cv \sim 5\%$).

The principal intrinsic error of this strategy for calculating k_{θ} is the negation of the catalytic current contribution for the period between $t=0 \text{ s}$ and $t=0.05 \text{ s}$. Negation of the catalytic current contribution over this period is necessary since the exact relationship between i_{θ} and t over this interval is unknown, but for 0.05 s the deviation between i_d and $i_{\theta} \rightarrow 0$ as $k_{\theta} \rightarrow 0$.

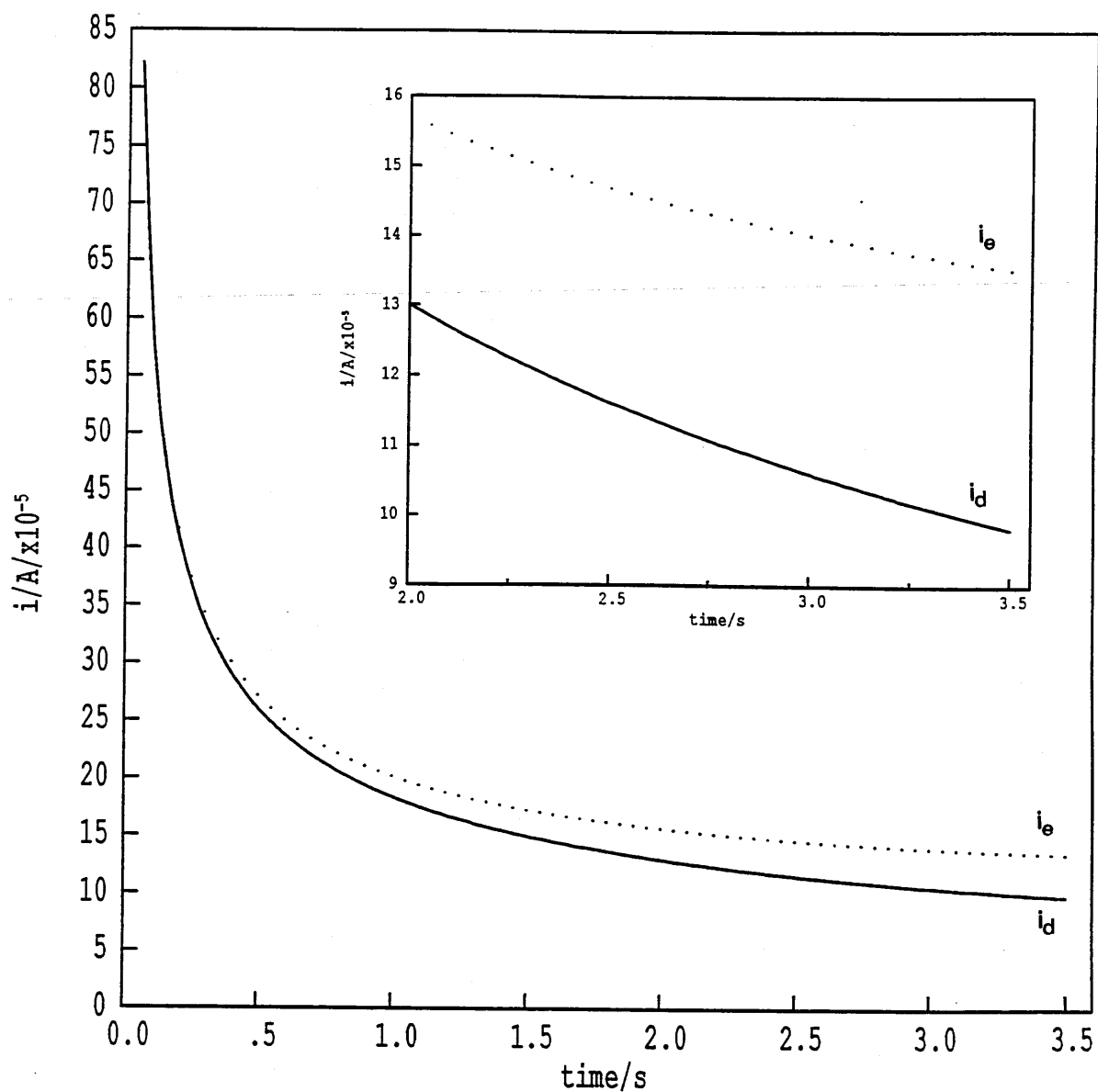


Figure 11.5.3. Experimentally derived current transient, i_e , involving an homogeneous catalytic reaction, and calculated diffusion current transient, i_d , for a system containing ruthenium (III) hexamine, glucose oxidase and glucose. Inset; expansion of the current responses for $t > 2$ s.

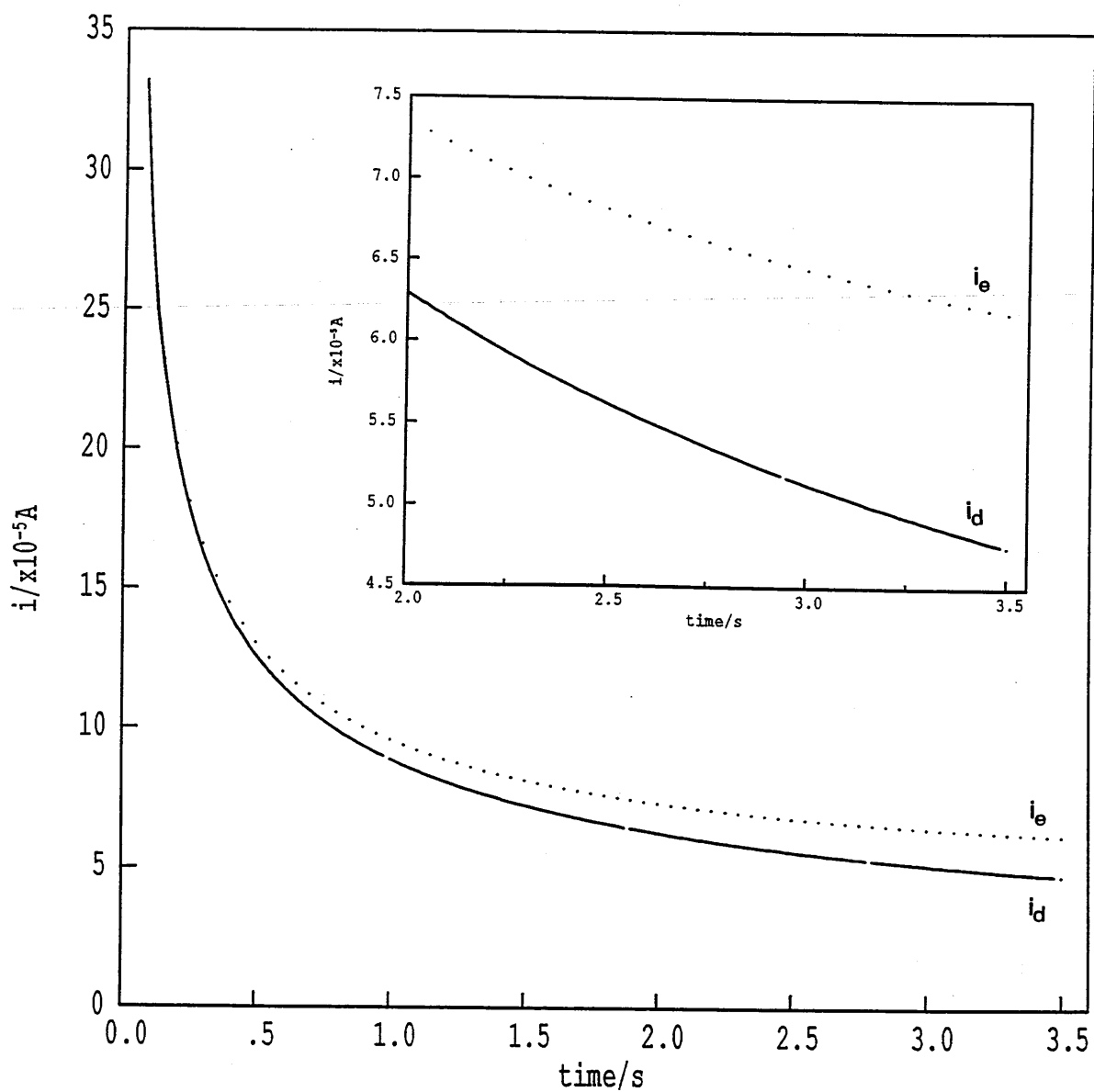
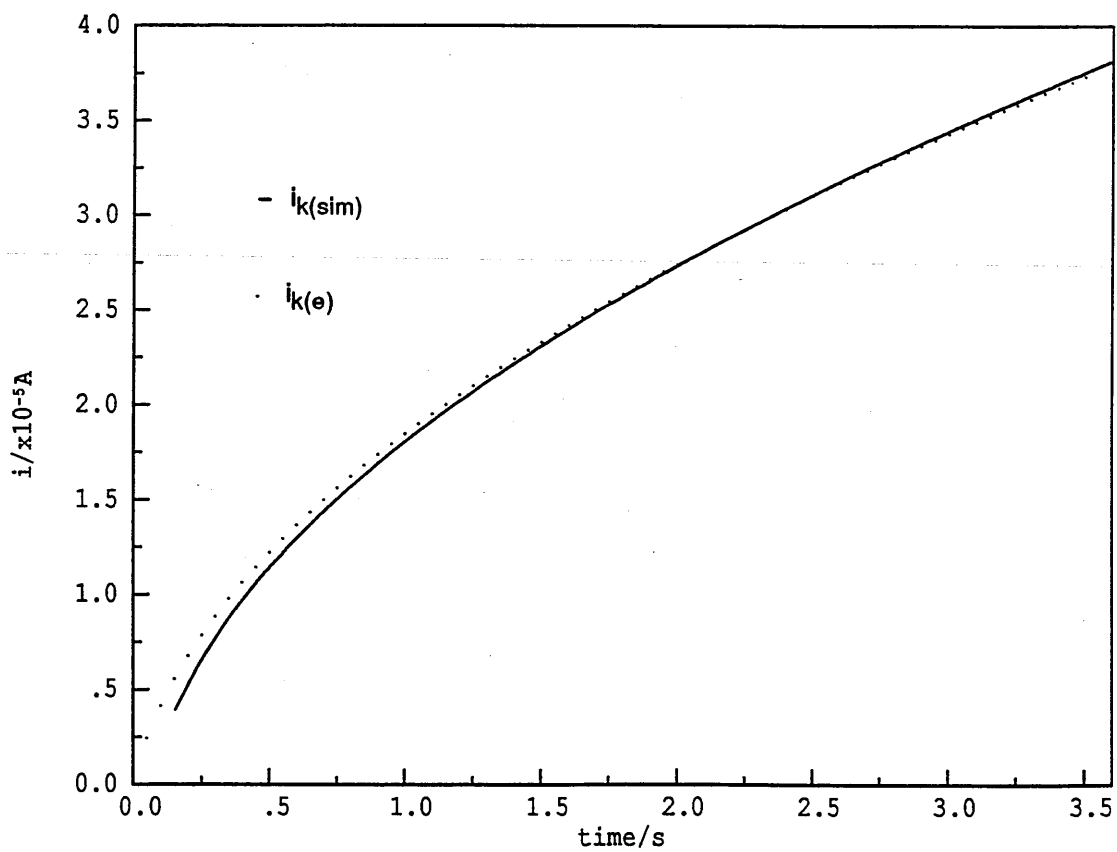
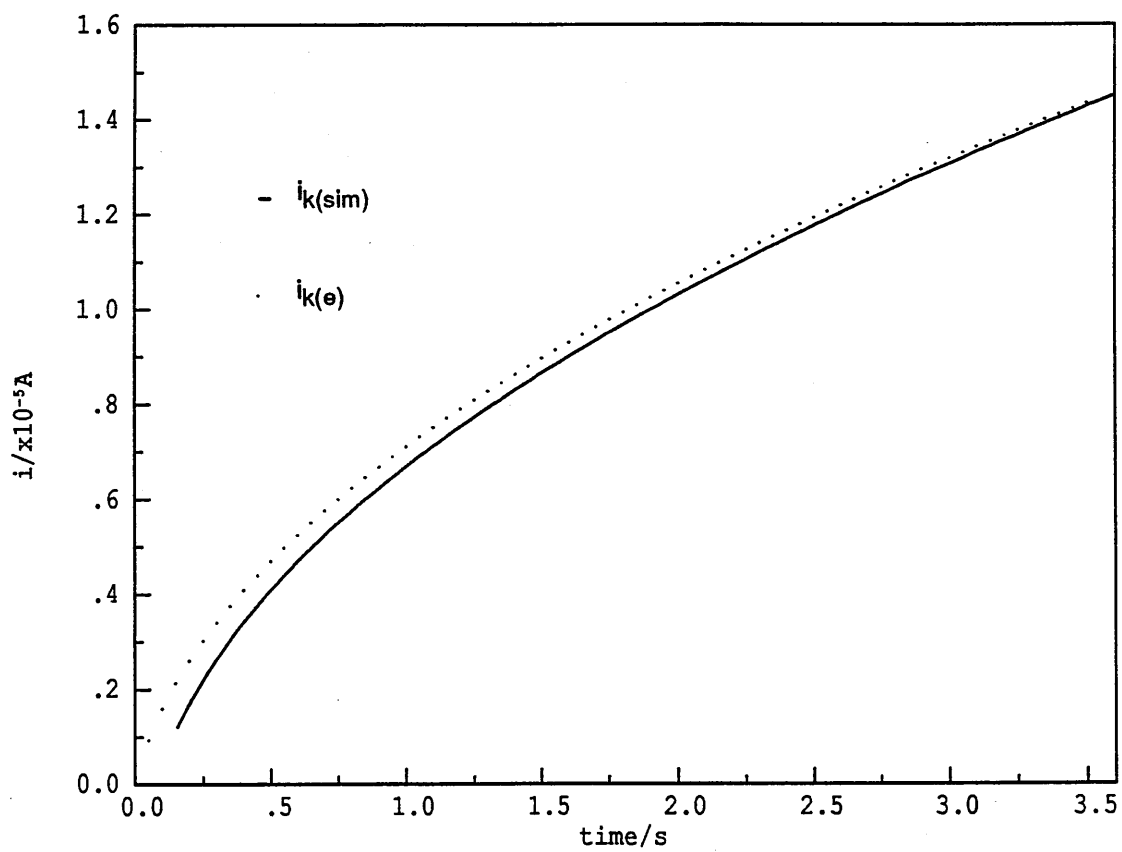


Figure 11.5.4. Experimentally derived current transient, i_e , involving an homogeneous catalytic reaction, and calculated diffusion current transient, i_d , for a system containing ferricyanide, glucose oxidase and glucose. Inset; expansion of the current responses for $t > 2 \text{ s}$.

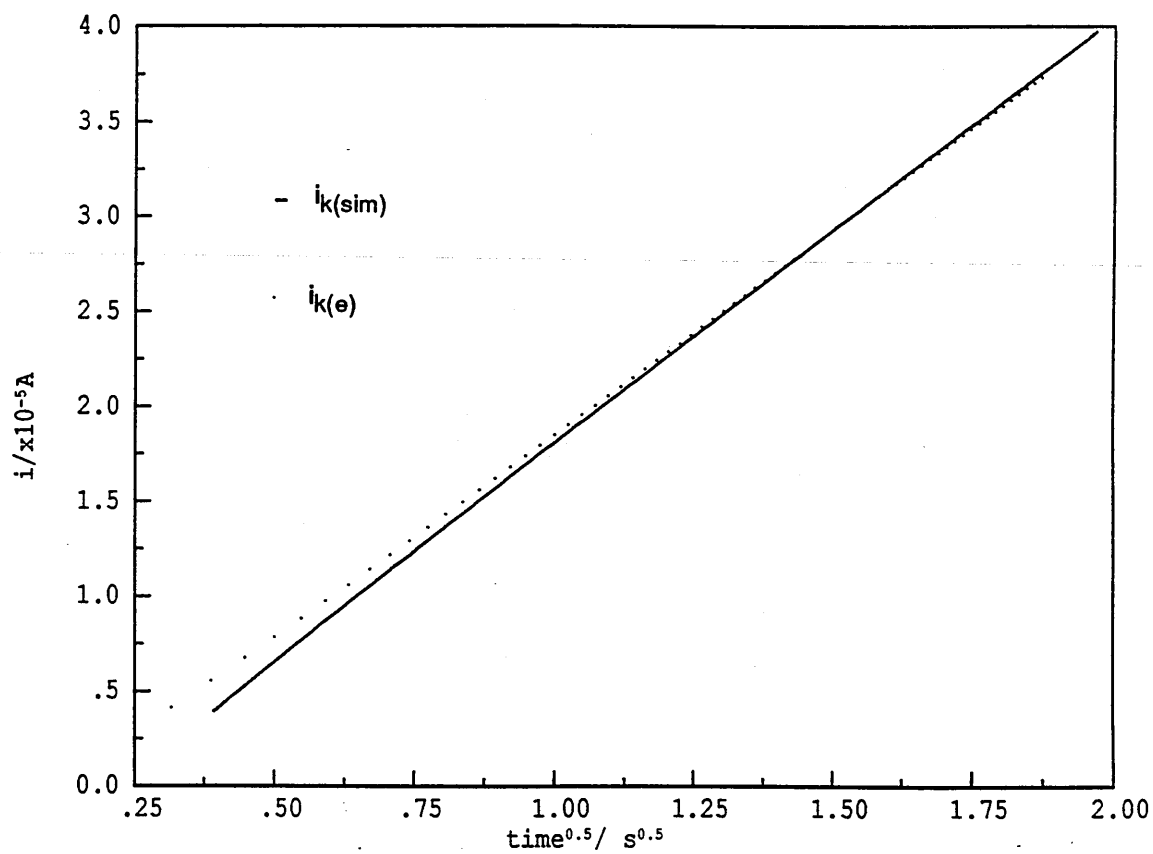
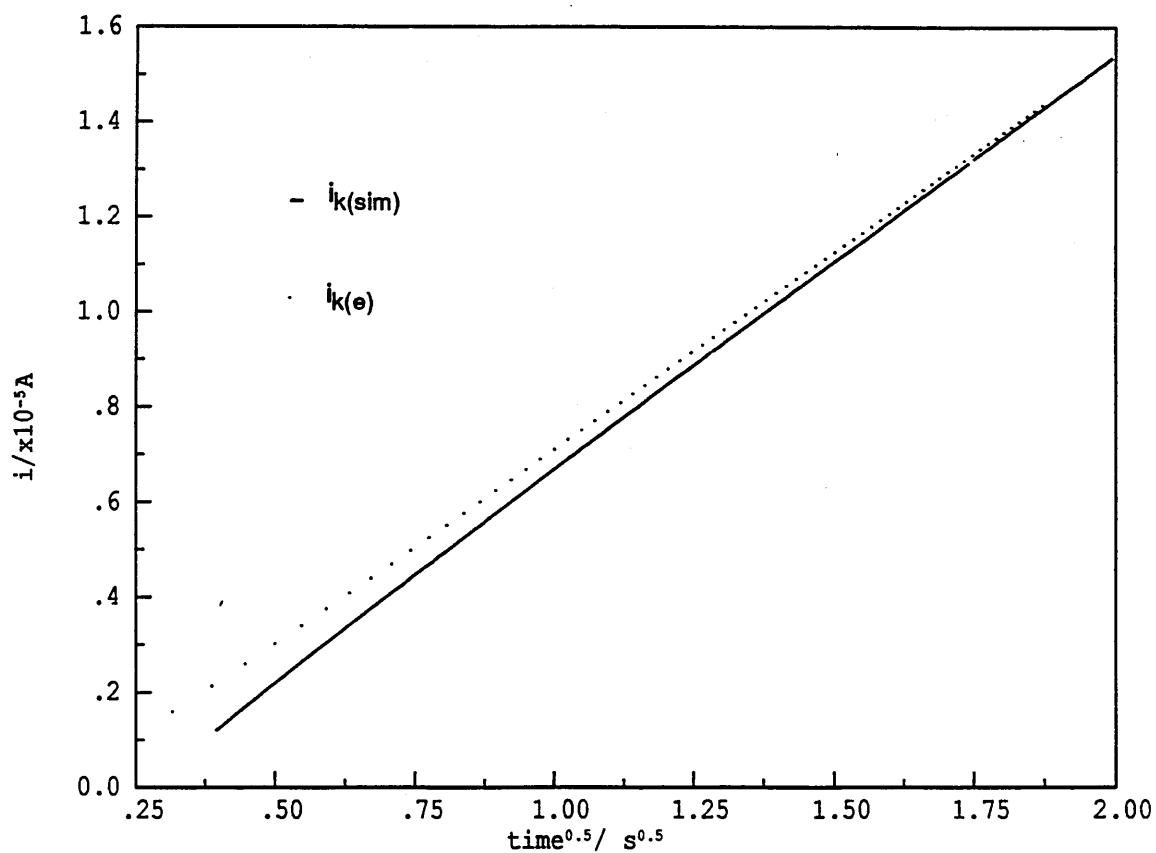
Figure II.5.5. $i_{k(e)}$ and $i_{k(sim)}$ vs. time for ruthenium (III) hexamine.

Figure II.5.6. $i_{k(e)}$ and $i_{k(sim)}$ vs. time for ferricyanide.



Figures II.5.7. $i_{k(e)}$ and $i_{k(sim)}$ vs. $time^{0.5}$ for ruthenium (III) hexamine. As predicted from [II.5.16.], i_e exhibits a linear correspondence to $t^{0.5}$.

Figure II.5.8. $i_{k(e)}$ and $i_{k(sim)}$ vs. $time^{0.5}$ for ferricyanide. As predicted from [II.5.16.], i_e exhibits a linear correspondence to $t^{0.5}$.



II.5.5. DISCUSSION OF ELECTRON TRANSFER KINETICS BETWEEN GLUCOSE OXIDASE AND REDOX MEDIATORS AND THE SIGNIFICANCE OF THE KINETIC AND PHYSICAL CHARACTERISTICS OF THE TWO MEDIATORS WITH RESPECT TO THE OPERATION OF THE CFD.

It is interesting to speculate on the reasons for the existence of large variations in the kinetic data for different redox mediators. Modern theories of electron transfer (Feinberg & Ryan, 1981) predict that the re-oxidation rate of reduced glucose oxidase by a mediator is governed by:

(I). The thermodynamic driving force of the reaction, i.e., the difference between the reversible potentials of the enzyme bound FAD/FADH₂ and the electron acceptor couple. Clearly, the greater this difference, then the greater the rate coefficient, k_e .

(II). The contribution to the Gibbs energy of activation from the intrinsic reorganisation energy arising from reaction [II.5.4.].

(III). The Gibbs energy barrier, $\Delta G^\ddagger$, for the reaction of ionic species, which may be written as:

$$\Delta G^\ddagger = \Delta G_i^\ddagger + \Delta G_{es}^\ddagger + \Delta G_\mu^\ddagger \quad [\text{II.5.18.}]$$

Here $\Delta G_i^\ddagger$ is the intrinsic contribution (reorganisation plus thermodynamic driving force), $\Delta G_{es}^\ddagger$ the contribution due to the electrostatic work involved in bringing the charged reactants together and $\Delta G_\mu^\ddagger$ is the ionic atmosphere contribution.

At pH 7, glucose oxidase is a negatively charged glycoprotein (Duke *et al.*, 1969). Since ruthenium hexamine is positively charged, the electrostatic interaction between the two should be favourable. The redox potential of the ruthenium (III) hexamine couple ($E_{1/2} = -215$ mV vs. SCE) is low, and consequently the thermodynamic driving force for the reaction with reduced glucose oxidase is inferior to that experienced by the ferrocenes ($E_{1/2}$ between 0.1 V and 0.4 V vs. SCE, Cass *et al.*, 1984), or for the reaction between ferricyanide and reduced glucose oxidase ($E_{1/2} = 350$ mV vs. SCE). The low redox potential for ruthenium (III) hexamine has advantages in terms of reduction in the extraneous charge contribution, but accounts for its slow redox kinetics with reduced glucose oxidase. Although ferricyanide has a higher redox potential than ruthenium (III) hexamine, it is likely that the slow reaction kinetics of its reaction with reduced glucose oxidase are due to their mutually repulsive charges, the $\Delta G_{es}^\ddagger$ term in [II.5.18.] is likely to be greater than for ruthenium (III) hexamine. Examination of the literature values for the redox potential of reduced glucose oxidase at pH 5.3 ($E_{1/2} = -0.307$ V vs. SCE) and pH 9.3 ($E_{1/2} = -0.584$ V vs. SCE) (Stankovich *et al.*, 1978) may provide an insight into the unusual pH-reaction rate profile obtained with ruthenium (III) hexamine and glucose

oxidase, Section II.3..

All techniques of analysis yield similar values for K_1 and for k_0 for each system. It is significant that the values for k_0 associated with each mediator is a factor of 100 less than calculated values for mediators such as ferrocene (Cass *et al.*, 1985). To date it has not been possible to characterise k_0 for glucose oxidase and ferricyanide or for ruthenium (III) hexamine using cyclic voltammetry. This, presumably is because the value of k_0 is not great enough with respect to the diffusion coefficients of the mediator molecules; this results in mixed diffusional and kinetic control for current response for linear potential sweep experiments as discussed with respect to ruthenium (III) hexamine in Section II.3.3.

With respect to the analysis of the kinetic parameters for ruthenium (III) hexamine and ferricyanide and their significance when making a comparative evaluation for their deployment in the CFD, although the calculated values for K_1 and k_0 are marginally superior for ruthenium (III) hexamine than for ferricyanide the principal arguments for the preferential employment of the ruthenium complex lie in its greater diffusion coefficient, which has implications for coulometric response time, and its lower oxidation potential, which is significant for the exclusion of extraneous electrochemical signals.

Ruthenium (III) hexamine may have certain advantages over ferricyanide in terms of a reduction in time taken to complete a coulometric analysis. The response time constant, T_c , for a thin layer cell operating in a coulometric mode may be defined by the Einstein-Stokes equation (Kissinger and Heineman, 1984), [II.5.19.]:

$$T_c = l^2/D \quad \text{[II.5.19.]}$$

where D is the diffusion coefficient of the molecule and l is the distance between the electrode and the end plate. [II.5.19.] defines the response time to complete 93% electrolysis of analyte in the sample. Application of [II.5.19.] the present case results in a ratio of response time for ferricyanide:ruthenium (III) hexamine of 2.824, in direct inverse relation to the values of their diffusion coefficients. Ruthenium (III) hexamine has direct perceived advantages in terms of the response time in the coulometric mode of operation for a CFD. This may be true for the completion of the determination by oxidation of ruthenium (II) hexamine produced by the catalytic reaction, but the use of ruthenium (III) hexamine is unlikely to result in a reduction in the catalytic phase of a determination, Section III.3.2.. Ruthenium (III) hexamine may also be superior to ferricyanide in any amperometric mode: for a given concentration of ruthenium (III) hexamine the signal would be a factor of 1.68 greater than for an equivalent concentration of ferricyanide.

***Chapter III, strategies for the reduction of analysis
time of thin layer electroanalytical devices***

III.1.1 STRATEGIES FOR THE REDUCTION IN THE ANALYSIS TIME FOR A CFD INCORPORATING A CATALYTIC LAYER USING CONTROLLED POTENTIAL TECHNIQUES.

The present strategy employed for the determination of the concentration of a substance in a CFD is described in Section I.4.1.. The total time taken for the analysis is 180 s, 60 s each for the conduction of the reaction in the catalytic layer and for the oxidation of reduced mediator at the electrode. Reduction of the total analysis time may be achieved by addressing the processes occurring in either the reaction phase or the analysis phase or both.

It may be shown that a tenfold increase in the concentration of glucose oxidase in the catalytic layer would probably result in a marginal decrease in the time taken to complete the reaction, Section III.3.2.. This conclusion was arrived at using the simplifying assumption that the fractional diffusion coefficients of all the substances diffusing to the catalytic layer are the same in solution and throughout the catalytic layer. It was demonstrated that the rate of reduction of glucose in the catalytic layer is unlikely to be limited by the rate of oxidation of reduced glucose oxidase by oxidized mediator, despite the low bimolecular rate constant evaluated for ferricyanide or ruthenium (III) hexamine for their reaction with reduced glucose oxidase. The flux of glucose to the catalytic layer is initially controlled by the rate of the enzyme catalysed reaction occurring in the catalytic layer because the system is dynamic the boundary conditions that determines the glucose flux undergo a transition during the course of the reaction from a catalytic reaction limitation to a diffusion limitation. Because the rate of evolution of reduced mediator at the surface of the catalytic layer is determined by the flux of glucose at the solution/catalytic layer interface, no reduction in the time allowed to complete the reactive stage of the analysis may be achieved by the substitution of a compound with a higher diffusion coefficient, such as ruthenium (III) hexamine, for ferricyanide.

Diffusion timescales associated with both phases may be shortened by simply reducing the x-dimension. By reducing the distance between the end plate and the electrode from 100×10^{-6} m to 50×10^{-6} m the diffusion timescale for a molecule with a diffusion coefficient of $10^{-9} \text{ m}^2 \text{ s}^{-1}$ would change from 5 s to 1.25 s, by applying the relation for root mean square distance traversed, equation [I.4.10.]. Such a change in configuration would have a dramatic effect in reducing the timescales necessary to complete both phases of the analysis. However, since the error associated with the manufacture of the gap width in the device would become more significant at smaller gap widths, and because the gap width is pivotal in the reproducibility of signals derived from the CFD, a reduction of the gap width would be associated with an increase in the uncertainty of determination of concentration. Additionally, the current contribution derived from lateral diffusion would become proportionately greater.

Since modification of the configuration of the catalytic layer seems unlikely to result in a reduction in the analysis time, and any reduction of the distance between the electrode and the endplate using

present technology may lead to greater variability in the signal response, a third strategy involving a novel analysis of the current transient response was devised and investigated in an attempt to reduce analysis time whilst retaining signal reproducibility. In the following pages a novel analytical strategy will be presented that reduces the time taken to determine the concentration of reduced mediator from 60 s to about 10 s, whilst reducing the uncertainty in determination of the concentration of reduced mediator.

III.2.1. USING A PULSED POTENTIAL SIGNAL TO DEFINE A VOLUME IN A CFD.

This text presents an analysis of the current response to an application of series of potential pulses at the working electrode of the CFD separated by open circuit periods, allowing relaxation of the solution elements. The current-time or charge-pulse number information derived may be correlated with the concentration of reduced mediator present in the device. These correlations result in a reduction in the analysis period of the device by a factor of about six. In addition to a significant reduction in the analysis time the analysis obviates the construction of a guard electrode, simplifying manufacture and electronics.

The following analysis makes use of the period after the application of the potential pulse where the current response is proportional to time^{-0.5}, Section I.4.1.. If a potential pulse is applied for a duration where this time dependency of current applies the following approximation for the root mean distance diffusion by a molecule should be appropriate, [III.2.1.]:

$$\Delta x = (2D_{Mr}\Delta t)^{0.5} \quad \text{[III.2.1.]}$$

here Δx is distance traversed, m, in the time Δt , s, and D_{Mr} is the diffusion coefficient of the reduced electroactive species, M_r , $m^2 s^{-1}$. The period where [II.2.1.] may be applied is dependent on the diffusion coefficient of the molecule, Section I.4.1..

Consider that prior to the application of the first potential pulse the concentration of the reduced form of a substance that undergoes reversible electrochemistry is homogeneous throughout the volume defined by the CFD. Applying an oxidizing potential at the electrode results in a concentration of zero for the substance at the electrode surface, Section I.4.1.. Substituting values for Δt of 2 s and for D of $6.26 \times 10^{-10} m^2 s^{-1}$ results in a value for Δx of about $50 \times 10^{-6} m$. It may be considered that all of the electroactive species in a volume equivalent to the product of area of the electrode and Δx has undergone oxidation at the electrode. This is about half the gap width of a CFD, Figure I.4.3.. Further application of the pulse would result in a time dependent exponential decaying current signal. A period of relaxation, however, during which the potential is not applied removes the zero concentration boundary condition at $x=0$, Section I.4.1.. If the relaxation period is sufficient the concentration of M_r throughout the volume elements between the electrode and the end plate

should become homogeneous. During this relaxation period most of the substance diffusing into the space above the working electrode is derived from diffusion in the x-dimension, the contribution from the z-dimension is negligible, Section III.2.2.. The current response to an application of a second potential pulse to the resulting solution may therefore be treated in the same way as the first.

Consider the application of a potential pulse of frequency L Hz, at the working electrode of a CFD. Prior to the first pulse there is a fixed concentration of reduced mediator in the device. Referring to Figure I.4.2., the following equations describe the changes in concentration of the reduced mediator, $[M_r]$. The concentration of reduced mediator prior to the application of the first potential pulse is assumed to be homogeneous with respect to all the dimensions of the CFD and is defined by [III.2.2.]:

$$[M_r] = NM_r/V \quad \text{[III.2.2.]}$$

NM_r is the number of moles of M_r in the volume V , m^3 .

A series of potential pulses with equivalent relaxation times has a frequency L Hz, a period of L^{-1} s. For a working electrode of area A m^2 the approximate volume, Δv , swept out during the application of a potential pulse of period t s is:

$$\Delta v = A(2D_{Mr}\Delta t)^{0.5} \quad \text{[III.2.3.]}$$

If the initial concentration of reduced mediator is $[M_r]_0$, then during the application of the first pulse the number of moles of M_r that undergo oxidation, NM_{r0} , may be written:

$$NM_{r0} = [M_r]_0 A(2D_{Mr}\Delta t)^{0.5} \quad \text{[III.2.4.]}$$

The charge passed during the first pulse, Q_0 , will be $nFNM_{r0}$, n and F are as defined previously.

The change in concentration between the application of the first and second pulses is in direct relation to the ratio of the volume 'swept out', Δv , and the volume of the elements of solution directly above the working electrode, V . Generally the ratio of concentrations between successive pulses is described by [III.2.5.], where p is the number of pulses, the first pulse being designated as $p=0$, then for any pulse after $p=0$:

$$[M_r]_p/[M_r]_{p-1} = 1 - (\Delta v/V)^p \quad \text{[III.2.5.]}$$

Neglecting any effect of lateral diffusion over a large number of pulses the concentration of the solution above the electrode at the start of the p^{th} pulse may be expressed as:

$$[M_r]_p = [M_r]_0 (\Delta v/V)^p \quad \text{[III.2.6.]}$$

Expression [III.2.6.] may be used to estimate the amount of charge passed for each pulse of a series of pulses. The change in charge between pulses may be expressed in the same form as [III.2.6.]:

$$Q_p = Q_0 (\Delta v/V)^p \quad \text{[III.2.7.]}$$

and the charge passed is given by [III.2.8.]:

$$Q_p = nF\Delta v[M_r]_p \quad \text{[III.2.8.]}$$

where $[M_r]_p$ is the concentration of M_r present on the application of the p^{th} pulse.

Q_p may also be estimated by substitution of the concentration evaluated from [III.2.6.] into the integrated Cottrell equation, [III.2.9.]:

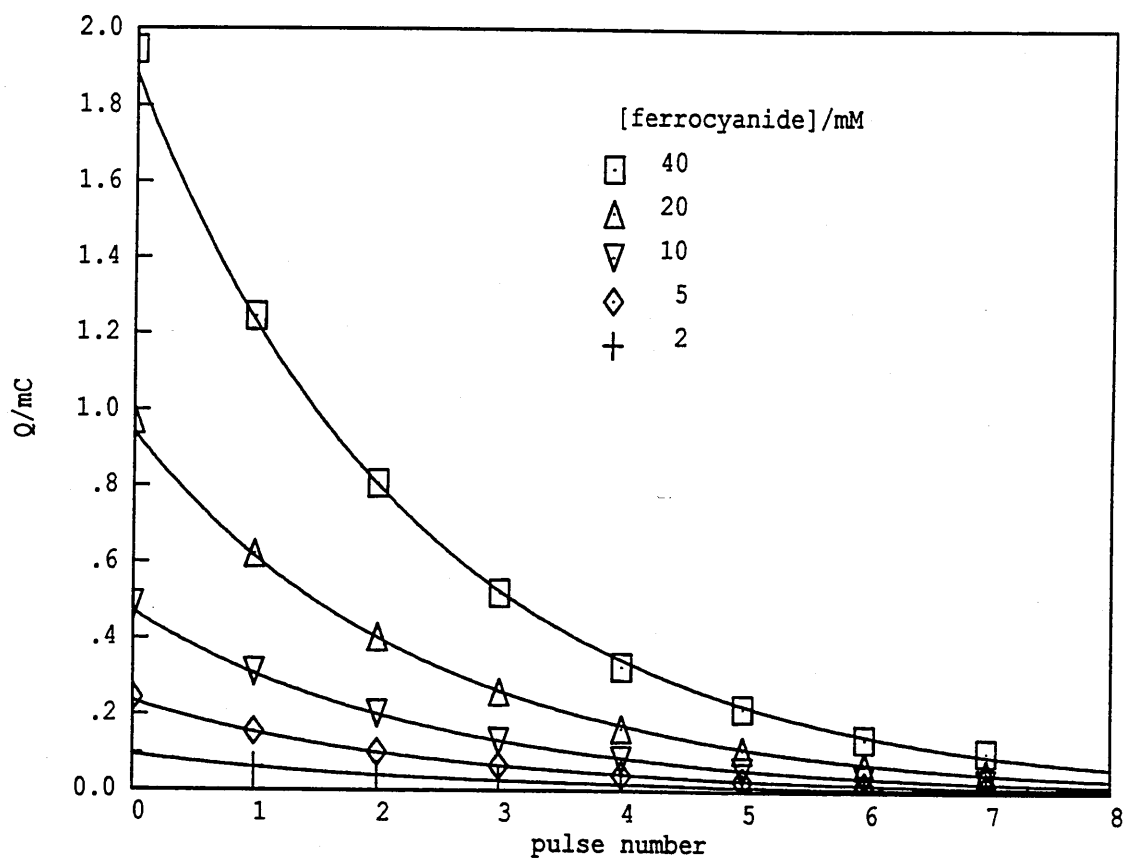
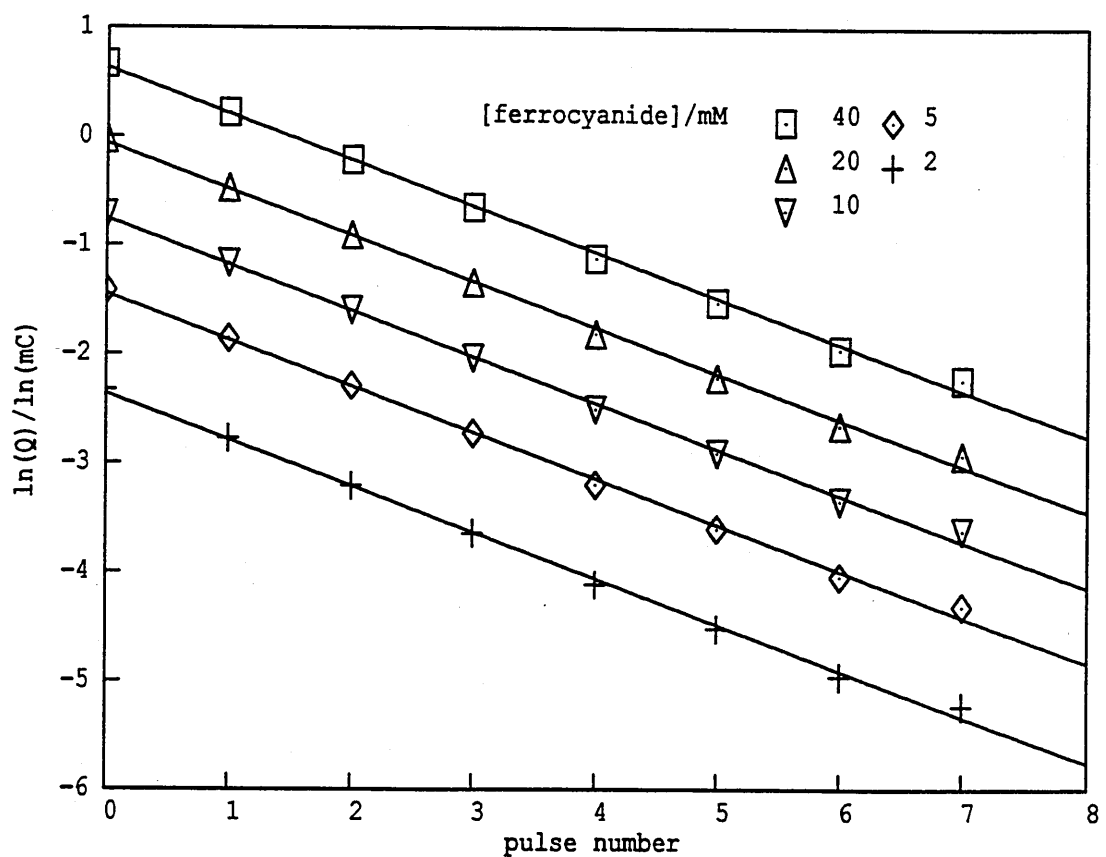
$$Q_p = (2nFAD_{M_r}^{0.5}[M_r]_p t^{0.5})/\pi^{0.5} \quad \text{[III.2.9.]}$$

Since the proportional change in concentration of M_r is the same between each pulse a plot of $\log(Q)$ vs. pulse number should yield a straight line with a slope that is a function of the pulse frequency and the diffusion coefficient of the reduced mediator and a charge-axis intercept that is proportional to the concentration of reduced mediator. A similar analysis may be performed on the current response to the single pulse of duration 60 s that is presently used to make the coulometric determination of concentration of substance. However, the period where the $\log(i)$ is directly proportional to time may only be applied after about 10 s in the case of ferricyanide, Section I.4.1..

The projected charge response for a solution containing ferrocyanide at concentrations of 2 mM, 5 mM, 10 mM, 20 mM, 30 mM and 40 mM to a series of eight pulses of frequency 1 Hz is shown in Figure III.1.1. A diffusion coefficient of $6 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ was used to determine Δx and $[M_r]_p$. The charge response was calculated by substituting the value obtained for $[M_r]_p$ into the integrated Cottrell equation. Lateral diffusion was neglected. A plot of $\ln(Q)$ vs. pulse number, p , results in a straight line of equal slope at each concentration, Figure III.1.2.. The slope of the line is dependent on the pulse frequency, the intercept at $p=0$ is dependent on the concentration of reduced mediator. Whilst theoretically it is possible to make a determination on the basis of the first pulse alone, in terms of error reduction it may be better to take several pulses and discard the charge value from the first and extrapolate back to $p=0$: the first pulse being likely to have the maximum variation associated with charging current effects.

Figure III.2.1. The projected charge-pulse response for the working electrode of a CFD for a series of solutions containing ferrocyanide at concentrations between 2 mM and 40 mM for a pulse/relaxation frequency of 1 Hz.

Figure III.2.2. Logarithmic ($\ln(Q)$) charge response for the working electrode of a CFD for a series of solutions containing ferrocyanide at concentrations between 2 mM and 40 mM for a pulse/relaxation frequency of 1 Hz.



III.2.2. ANALYSIS OF THE EFFECT OF LATERAL DIFFUSION ON CURRENT AND CHARGE RESPONSE.

Figure III.2.3. represents a cross-section through a CFD. The boundary at $z=0$ represents the edge of the electrode. The space outside the working electrode is defined by the region $z>0$ and the space over the electrode is defined by $z<0$. The x -dimension represents the gap width; the electrode surface is at $x=0$ and the end plate at $x=l$. The effect of lateral diffusion over the edge of the electrode into the space above the working electrode may be approximated to the diffusion of a front into a semi-infinite space. Consider the diffusion of a substance with diffusion coefficient D , present at all points as defined by $z>0$ and $l \leq x \leq 0$ at a constant concentration $[M_r]$, into the space bounded by $z \leq 0$ and $l \leq x \leq 0$, where the concentration of the substance is zero.

According to Eddowes (1990), the solution to this problem is described by III.2.10.:

$$[M_r]_z = \text{erfc}(z/(2Dt)^{0.5}) \quad \text{[III.2.10.]}$$

where $\text{erfc}(f)$ is the complementary error function of some function, f . The complementary error function of the expression in parentheses describes the way in which the concentration of M_r changes in the space bounded by $z \leq 0$ and $l \leq x \leq 0$ over a time, t . Figure III.2.4. describes the concentration profile of a substance with diffusion coefficient of $10^{-10} \text{ m}^2 \text{ s}^{-1}$ after a period of 1 s. The initial conditions, at $t=0$, were that the substance was present at a normalised concentration of one at $z>0$ and $l \leq x \leq 0$ and present at a concentration of zero at $0 \leq z$ and $l \leq x \leq 0$.

A mean concentration for the substance over the electrode, $[M_r]_m$ may be described by integrating [III.2.10.] between $z=0$ and $z=-\Delta z$, [III.2.11.]:

$$[M_r]_m = 1/\Delta z \int_0^{-\Delta z} [M_r]_z \partial z \quad \text{[III.2.11.]}$$

where $[M_r]_z$ is the concentration of M_r at distance z .

If an electrode has dimensions a and b then the perimeter, P , of the electrode is given by [III.2.12.]:

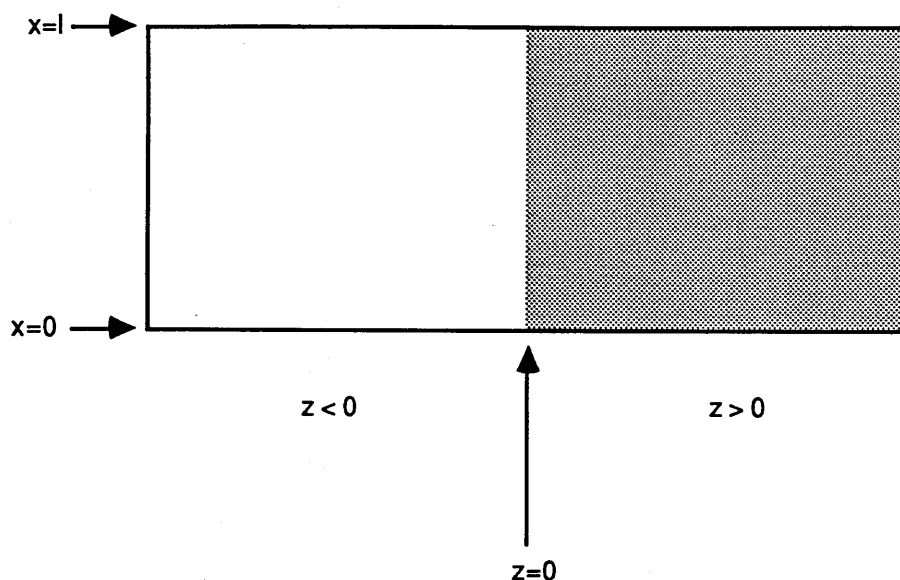


Figure III.2.3. Schematic diagram illustrating the initial conditions for an evaluation of the charge contribution derived from lateral diffusion over the application of a potential pulse of duration t s. $z=0$ corresponds to the edge of the electrode, $z>0$ describes the region beyond the electrode edge, where the concentration of reduced mediator has a normalised concentration of one at $t=0$. $z<0$ describes the region over the edge of the electrode where the reduced mediator has a concentration of zero at $t=0$.

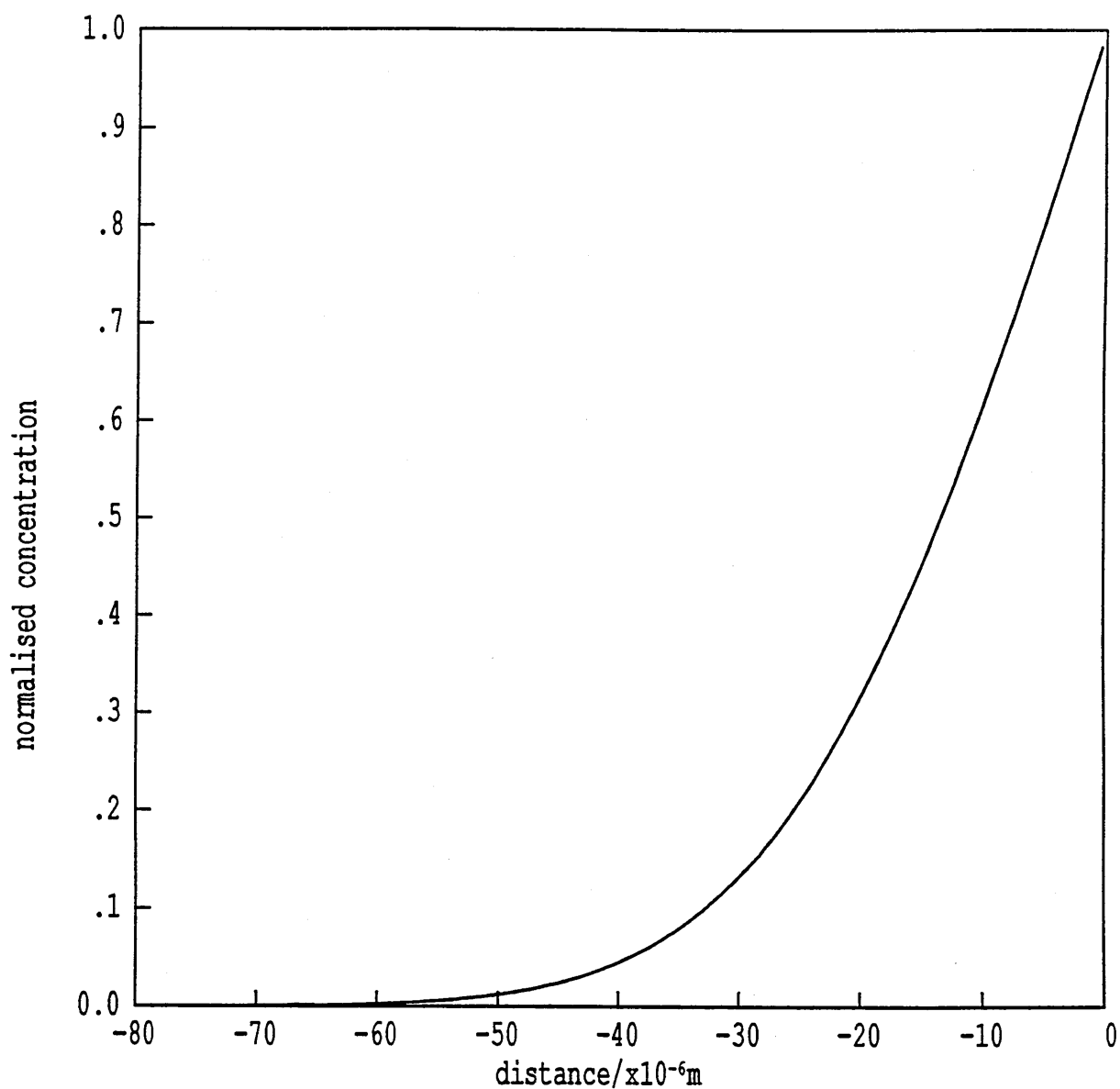


Figure III.2.4. The normalised concentration profile over the electrode edge of a substance with a diffusion coefficient of $10^{-10} \text{ m}^2 \text{ s}^{-1}$ after a period of 1 s from the initial conditions stated in the text and described in Figure III.2.3..

$$P=2(a+b)$$

[III.2.12.]

The area of the electrode affected by lateral diffusion, A_l , may be approximated by [III.2.13.]:

$$A_l = \Delta z P$$

[III.2.13.]

[III.2.13.] approximates the perimeter area affected by considering the perimeter as a strip of length P and width Δz . Corner effects are neglected. The product of the area affected and the mean concentration may be equated with the perimeter multiplied by the integral in [III.2.11.] by substitution of [III.2.13.] into [III.2.11.]:

$$A_l [M_r]_m = P \int_0^{-\Delta z} [M_r]_z dz$$

[III.2.14.]

Finally, an estimate of the charge derived from the flux of material into the space above $z \leq 0$ may be made by substituting [III.2.14.] into the integrated Cottrell equation for semi-infinite solution, [III.2.9]:

$$q_l = \frac{2 n F D^{0.5} t^{0.5} P \int_0^{-\Delta z} [M_r]_z dz}{\pi^{0.5}}$$

[III.2.15.]

where q_l is the charge derived from lateral diffusion for the application of a potential pulse of duration t s. Here $t=1$ s and n and F are as defined previously.

The value of q_l was evaluated under these conditions using a mathematical spreadsheet package, Mathcad (Aston Scientific, 49 Long Plough, Aston Clinton, Bucks, HP22 5HD, UK). Charge due to lateral diffusion was calculated to have a value of 1.53×10^{-7} C for an electrode of width 3×10^{-3} m and

length $6 \times 10^{-3} \text{ m}$. The area affected by lateral diffusion was evaluated as $6.3 \times 10^{-7} \text{ m}^2$. The total area of the electrode, $1.8 \times 10^{-5} \text{ m}^2$, was used to evaluate the charge value due to diffusion of substance to the electrode in the x-dimension using the integrated Cottrell equation, [III.2.9.]. The charge value due to diffusion in the x-dimension, q_x , was determined as $1.939 \times 10^{-5} \text{ C}$. The value of q_x/q_l is about 126.

This cursory examination has shown that the value of lateral diffusion is at least two orders of magnitude less than that derived from diffusion of substance in the x-dimension for the duration of the first pulse applied to a CFD which contains an homogeneously distributed concentration of reduced mediator. This is an over estimate because the exercise assumes that the diffusion characteristics of a front of material moving into a space where it is present at a concentration of zero are the same as for the real case. The net flux of a substance between two points is proportional to the concentration difference of the substance between those two points. In this evaluation the concentration difference is equal to the value of the concentration at all values of x at $z > 0$. Prior to the application of the potential pulse in the real system the concentration of substance is homogeneous for all values of x and z. On application of the potential pulse the concentration of M_r at $x=0$ is zero and during the pulse a concentration profile is established in the x-dimension as described in Section I.4.1.. Consequently the rate of lateral diffusion into $z \leq 0$ for values at and close to $x=0$ is greater than at higher values of x. Close to $x=l$ the concentration of substance above the working electrode may not change during the course of the pulse, consequently the contribution of lateral diffusion here is minimal if at all. Effectively then, the only elements of solution that experience conditions approximating the diffusion of a substance into a semi-infinite space where it is present at a concentration of zero are those elements of solution close the surface of the electrode. It is likely then, that the charge contribution derived from lateral diffusion over the edge of the electrode is more than two orders of magnitude less than the charge contribution derived from diffusion perpendicular to the electrode.

The amount of substance diffusing laterally across $z=0$ during the relaxation periods will be less than during the application of the potential pulse. This is because the mean concentration difference across $z=0$ is less than when the boundary condition of $[M_r]=0$ at $x=0$ and $z \leq 0$ are applied. Over several pulses, as the amount of material directly above the electrode becomes depleted, it may be assumed that the lateral flux of material becomes proportionately greater compared to the flux in the x-dimension. Using the approximation that the concentration of M_r at $z > 0$ is always $[M_r]_0$ for the duration of a series of pulses, within a pulse and over the course of relaxation period, then the contribution of lateral diffusion would be approximately the same for each pulse. In order to evaluate the flux of material at the electrode it is necessary to use a technique that takes account of the concentration gradient between adjacent elements of solution, such as finite difference analysis, or to find an analytical solution to a second order differential equation for concentration in two dimensions by applying the stated boundary conditions, Section III.3.1..

This analysis has demonstrated that the charge contribution from lateral diffusion is at least two orders of magnitude less than that derived from diffusion in the x-dimension. Consequently, the conclusions about the flux and current response to a series of potential pulses and relaxation periods applied to a CFD containing a homogeneously distributed concentration of material are fundamentally correct, Section III.1.1..

III.3.1. FINITE DIFFERENCE MODELS TO SIMULATE DIFFUSION AND CURRENT RESPONSE DURING THE APPLICATION OF A SERIES OF POTENTIAL PULSES SEPARATED BY POTENTIAL RELAXATION PERIODS.

The arguments presented in Section III.2. yields a cursory analysis of the behaviour of the system. In order to generate a better approximation of the diffusion within a CFD during the application of a series of potential pulses interrupted by a series of open circuit relaxation periods a number of finite difference models were constructed.

Finite difference methods for the simulation of diffusion of dissolved substances through a solution or to simulate heat transfer problems are well established (Hambling, 1975; Leibmann, 1953; Maloy, 1987; Bard & Faulkner, 1980). The first stage in the construction of finite difference models to solve such problems is to write the diffusion equations in standard mathematical notation that represent the rate of transfer of material between regions where the substance is present at different concentrations. For problems involving heat or mass transfer some form of Fick's Laws of diffusion apply. Firstly, considering diffusion in the x-dimension perpendicular to the electrode, Fick's first Law may be written as:

$$J_{(x,t)} = -D_{Mr} \partial[M_r]_{(x,t)} / \partial t \quad [III.3.1.]$$

$J_{(x,t)}$ is the flux at a position x and at a time t , all other symbols are as stated previously. Equation [III.3.1.] states that the flux is proportional to the concentration gradient $\partial[M_r]/\partial t$ at x and t . Flux is the net mass transfer rate of M_r and has units of $\text{mol s}^{-1} \text{m}^{-2}$.

The finite difference form of [III.3.1.] may be written as:

$$J_{(x,t)} = \lim_{(\Delta x \rightarrow 0)} -D_{Mr} ([M_r]_{(x+\Delta x,t)} - [M_r]_{(x,t)}) / \Delta x \quad [III.3.2.]$$

where Δx is the incremental distance between different elements of solution perpendicular to the electrode. This is illustrated in Figure III.3.1..

An implicit assumption in finite difference analysis is that the incremental differences in time or distance dimensions may be made small enough so that [III.3.1.] may be approximated by [III.3.3.]:

$$J_{(x,t)} = -D_{Mr} ([M_r]_{(x+\Delta x)} - [M_r]_{(x,t)}) / \Delta x \quad [III.3.3.]$$

Considering any node in Figure III.3.1., at a distance x from the electrode, [III.3.3.] may be written in terms of the interval $\pm \Delta x$ around a node at distance x , [III.3.4.]:

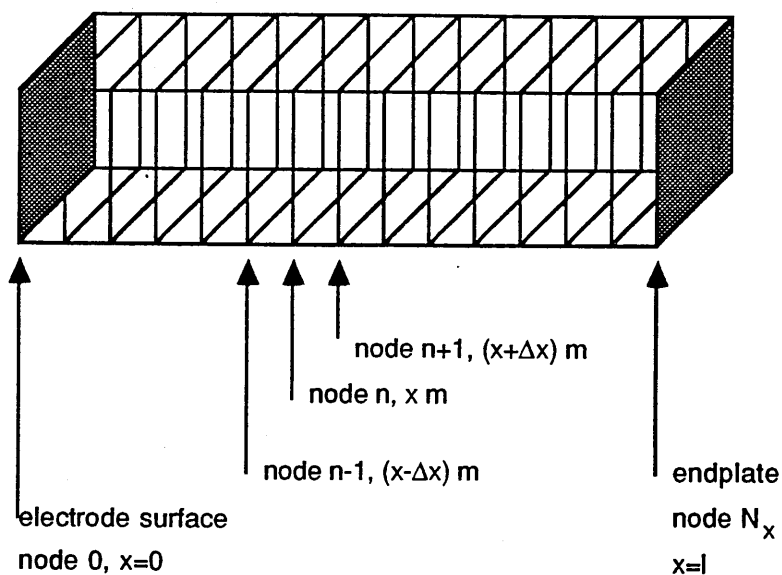


Figure III.3.1. Schematic diagram illustrating the operation of a series of finite difference equations to represent one dimensional material flux through a solution. The solution consists of $n-1$ elements defined by n nodes. Each node is associated with an area, A , equal to the electrode area. For all nodes except those at $x=l$ and $x=0$ each volume element is defined by $A\Delta x$. The nodes at $x=l$ and at $x=0$ have volumes of $A\Delta x/2$.

$$J_{(x,t)} = -D_{Mr}/\Delta x ([M_r]_{(x+\Delta x/2,t)} - [M_r]_{(x-\Delta x/2,t)}) \quad [III.3.4.]$$

Considering Fick's second law, [III.3.5.]. Equation [III.3.5.] states that the rate of change of $[M_r]$ with time at (x,t) is a function of the rate of change of the concentration gradient of M_r at (x,t) with respect to distance and its diffusion coefficient, D_{Mr} .

$$\partial[M_r]_{(x,t)}/\partial t = -D_{Mr} \partial^2[M_r]_{(x,t)}/\partial x^2 \quad [III.3.5.]$$

Substituting [III.3.1.] into [III.3.5.] the second law may be written in terms of flux, [III.3.6.]:

$$\partial[M_r]_{(x,t)}/\partial t = -\partial J_{(x,t)}/\partial x \quad [III.3.6.]$$

the finite difference form of [III.3.6.] may be written as in [III.3.7.] having already applied the limits; $\lim \Delta t \rightarrow 0$ and $\lim \Delta x \rightarrow 0$.

$$([M_r]_{(x,t+\Delta t)} - [M_r]_{(x,t)})/\Delta t = -(J_{(x+\Delta x/2,t)} - J_{(x-\Delta x/2,t)})/\Delta x \quad [III.3.7.]$$

Substitution of [III.3.4.] for the fluxes in [III.3.7.] results in a finite difference expression of [III.3.5.]:

$$([M_r]_{(x,t+\Delta t)} - [M_r]_{(x,t)})/\Delta t = -D_{Mr}/\Delta x^2 ([M_r]_{(x+\Delta x,t)} - 2[M_r]_{(x,t)} + [M_r]_{(x-\Delta x,t)}) \quad [III.3.8.]$$

rearranging [III.3.8.] a finite difference expression for the concentration of M_r at $(x,\Delta t)$ may be written, [III.3.9.]. Notice that [III.3.8.] describes the rate of change of concentration with time for the all nodes except for the 0th and the nth nodes. Specific boundary conditions must be applied for these nodes which will be dealt with later.

$$[M_r]_{(x,t+\Delta t)} = [M_r]_{(x,t)} + D_{Mr}\Delta t/\Delta x^2 ([M_r]_{(x+\Delta x,t)} - 2[M_r]_{(x,t)} + [M_r]_{(x-\Delta x,t)}) \quad [III.3.9.]$$

This is the form of the finite difference expression of Fick's second law that was used to calculate the flux of material in the simulation. However, the model system consists of a simulation of material flux in a cross-section of the CFD and therefore may be represented by a second order differential equation in two dimensions that describes the rate of change of concentration of M_r at a position and time described by (x,z,t) , [III.3.10.].

$$\partial[M_r]_{(x,z,t)}/\partial t = - (D_{Mr} \partial^2[M_r]_{(x,t)}/\partial x^2 + D_{Mr} \partial^2[M_r]_{(z,t)}/\partial z^2) \quad [III.3.10.]$$

Equation [III.3.10.] may not be solved analytically since one of the boundary conditions required for an evaluation of the second order differential equation describing flux in the z-dimension is $[M_r]_{(x,z,t)}$.

which necessitates solution of [II.3.10.] itself. Equation [II.3.10.] may be solved using finite difference expressions. Each of the second order expressions in [III.3.10.] may be written in finite difference form as described for the case of diffusion in one dimension. The finite difference form of [III.3.10.] is expressed in [III.3.11.].

$$[M_r]_{(x,z,t+\Delta t)} = [M_r]_{(x,z,t)} + M_x([M_r]_{(x+\Delta x,t)} - 2[M_r]_{(x,t)} + [M_r]_{(x-\Delta x,t)}) + M_z([M_r]_{(z+\Delta z,t)} - 2[M_r]_{(z,t)} + [M_r]_{(z-\Delta z,t)}) \quad [III.3.11.]$$

Where M_x and M_z are defined by [III.3.12.] and [III.3.13.] and constitute the model diffusion coefficients or mass transfer coefficients.

$$M_x = \lim_{x \rightarrow 0} \lim_{t \rightarrow 0} D_{Mr} \Delta t / \Delta x \quad [III.3.12.]$$

$$M_z = \lim_{z \rightarrow 0} \lim_{t \rightarrow 0} D_{Mr} \Delta t / \Delta z \quad [III.3.13.]$$

Figure III.3.2. is very much like Figure III.2.1. but specifically describes this model system. The space bounded by $-6(Dt)^{0.5} \leq z \leq +6(Dt)^{0.5}$ and $0 \leq x \leq l$ was subdivided into a number of nodes where the finite difference forms of the diffusion equation under certain boundary conditions are applied. By defining the diffusion coefficient of the species, the potential pulse duration and the number of time increments desired the number of nodes in the model were defined as explained in the following description. For a defined pulse duration of PD s and number of time increments N_i , the time interval may be calculated from [III.3.14.].

$$\Delta t = PD / N_i \quad [III.3.14.]$$

The time interval, Δt , has units of s. The internodal distance in the z-dimension was calculated by substituting this time interval into the expression for root mean distance diffusion, Δx , equation [III.2.1.]. Notice that this does not correspond to the expression for model diffusion coefficient defined in the finite difference equations previously stated. It is however, a useful approximation as the value of any mass transfer coefficient in a finite element analysis must have a value of less than 0.5. Mass transfer coefficients exceeding this value for diffusion in any one dimension would result in an unstable simulation where the concentration of substance in certain nodes would apparently increase at a greater rate than the flux balances would indicate, leading to flux oscillations at connected nodes on subsequent iterations. The overall width of the z-dimension is defined by boundaries at $\pm Z_{lim}$, where Z_{lim} is defined by:

$$Z_{lim} = 6(Dt)^{0.5} \quad [III.3.15.]$$

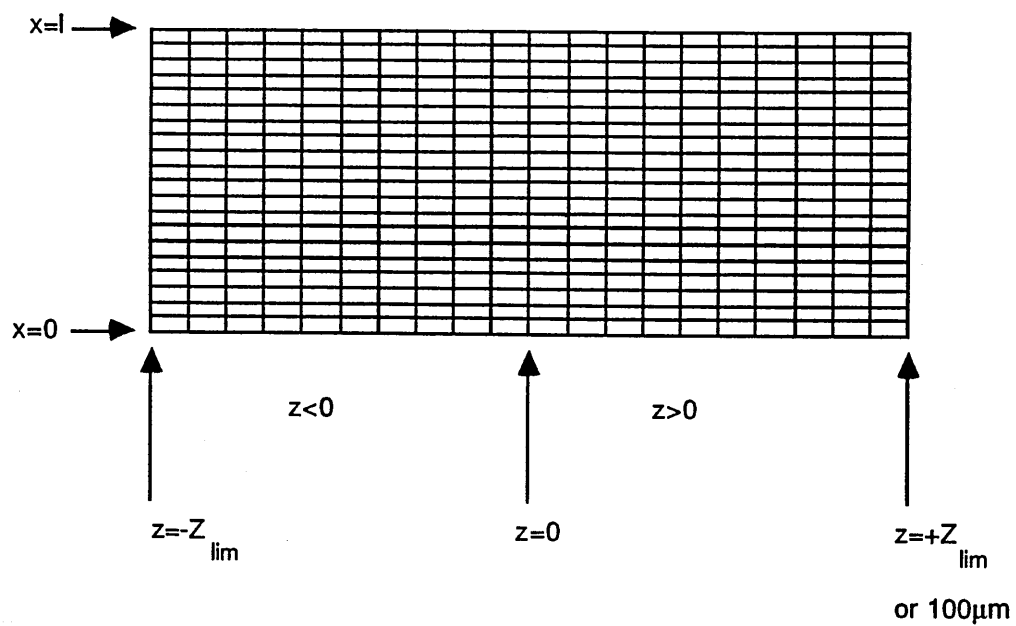


Figure III.3.2. Schematic diagram illustrating the subdivision of the model space into a number of nodes; $z=0$ represents the electrode edge and the limits $-Z_{lim}$ and $+Z_{lim}$ or $100 \times 10^{-6} m$ represent the edge of the diffusion front; l represents the distance between the electrode surface and the endplate.

This distance is an approximation of the furthest concentration perturbation encountered on the application of a boundary condition of zero concentration perpendicular to a column of solution (Bard and Faulkner, 1984). Here it is used to define the outer boundaries of a column of solution where the substance is diffusing into a region where the concentration gradient is principally derived by diffusion in a perpendicular dimension as described by [III.2.4.]. Dividing this distance by Δz results in the number of nodes in the z-dimension that is unlikely to be an integer. By rounding down this value to an integer value and then redefining Δx by dividing the distance in the z-dimension by the integer value, $N_z(\text{int})$, a value for Δz , is obtained that corresponds with the number of nodes and the boundary distance exactly, [III.3.16.] and [III.3.17.]:

$$N_z(\text{int}) = (2Z_{\text{lim}} / \Delta z) + 1 \quad [\text{III.3.16.}]$$

the revised value for Δz is given by:

$$\Delta z = 2 Z_{\text{lim}} / N_z(\text{int}) \quad [\text{III.3.17.}]$$

from henceforth any reference to Δz shall mean the value defined in [III.3.17.] unless otherwise stated and the number of nodes in the z dimension, N_z , shall refer to the integer value defined in [III.3.16.].

The number of nodes in the x-dimension, N_x , and the internodal distance in the x-dimension, Δx , were calculated in a similar fashion by applying the boundary distance at $x=l$.

The mass transfer coefficient used in the models for the x- and z- dimensions were calculated from [III.3.12.] and [III.3.13.] respectively by substitution of the revised values for Δx and Δz .

In the program the intervals Δt , Δx and Δz are replaced by integers between zero and N_t , N_x and N_z respectively. The boundary conditions and the flux equations for the pulse phase are written below.

Prior to the application of the first potential pulse the following boundary condition applies.

$$\text{For } t=0, \quad 0 \leq x \leq l, \quad -Z_{\text{lim}} \leq z \leq +Z_{\text{lim}}$$

$$[M_r]_{(x,z,t)} = [M_r]_0 \quad [\text{III.3.18.}]$$

Here $[M_r]_0$ is the initial concentration of reduced mediator.

After the application of an oxidizing potential pulse the concentration of M_r at the surface of the electrode becomes zero. This is stated in the boundary conditions stated in [III.3.19.].

For $t > 0$, $x = 0$, $z \leq 0$

$$[M_r]_{(x,z,t)} = 0 \quad [\text{III.3.19.}]$$

For all of the nodes except for those on the boundaries of the system, defined by [III.3.20.].

For $t > 0$, $0 < x < l$, $-Z_{\text{lim}} < z < +Z_{\text{lim}}$

$$\begin{aligned} [M_r]_{(x,z,t)} &= [M_r]_{(x,z,t-1)} + \\ M_x ([M_r]_{(x+1,z,t-1)} - 2 [M_r]_{(x,z,t-1)} + [M_r]_{(x-1,z,t-1)}) &+ \\ M_z ([M_r]_{(x,z+1,t-1)} - 2 [M_r]_{(x,z,t-1)} + [M_r]_{(x,z-1,t-1)}) & \end{aligned} \quad [\text{III.3.20.}]$$

For $t > 0$, $x = 0$, $0 < z < Z_{\text{lim}}$

$$\begin{aligned} [M_r]_{(x,z,t)} &= [M_r]_{(x,z,t-1)} + \\ M_x ([M_r]_{(x+1,z,t-1)} - [M_r]_{(x,z,t-1)}) &+ \\ M_z ([M_r]_{(x,z+1,t-1)} - 2 [M_r]_{(x,z,t-1)} + [M_r]_{(x,z-1,t-1)}) & \end{aligned} \quad [\text{III.3.21.}]$$

For $t > 0$, $x = l$ $-Z_{\text{lim}} < z < +Z_{\text{lim}}$

$$\begin{aligned} [M_r]_{(x,z,t)} &= [M_r]_{(x,z,t-1)} + \\ M_x ([M_r]_{(x+1,z,t-1)} - [M_r]_{(x,z,t-1)}) &+ \\ M_z ([M_r]_{(x,z+1,t-1)} - 2 [M_r]_{(x,z,t-1)} + [M_r]_{(x,z-1,t-1)}) & \end{aligned} \quad [\text{III.3.22.}]$$

For $t > 0$, $0 < x < l$ $z = +Z_{\text{lim}}$

$$\begin{aligned} [M_r]_{(x,z,t)} &= [M_r]_{(x,z,t-1)} + \\ M_x ([M_r]_{(x+1,z,t-1)} - 2 [M_r]_{(x,z,t-1)} + [M_r]_{(x-1,z,t-1)}) &+ \\ M_z ([M_r]_{(x,z-1,t-1)} - [M_r]_{(x,z,t-1)}) & \end{aligned} \quad [\text{III.3.23.}]$$

For $t > 0$, $0 < x < l$ $z = -Z_{\text{lim}}$

$$\begin{aligned} [M_r]_{(x,z,t)} &= [M_r]_{(x,z,t-1)} + \\ M_x ([M_r]_{(x+1,z,t-1)} - 2 [M_r]_{(x,z,t-1)} + [M_r]_{(x-1,z,t-1)}) &+ \\ M_z ([M_r]_{(x,z+1,t-1)} - [M_r]_{(x,z,t-1)}) & \end{aligned} \quad [\text{III.3.24.}]$$

For the nodes at each corner may be generally described by [III.3.25.]:

$$[M_r]_{(x,z,t)} = [M_r]_{(x,z,t-1)} + M_x(A - [M_r]_{(x,z,t-1)}) + M_z(B - [M_r]_{(x,z,t-1)}) \quad [\text{III.3.25.}]$$

Where A and B describe different nodes at t-1. Other boundary conditions may be described by substituting the expressions for A and B (equations [III.3.26] to [III.3.29]) into [III.3.25].

For $t > 0$, $x = 0$, $z = +Z_{\text{lim}}$

$$A = [M_r]_{(x+1,z,t-1)} \quad B = [M_r]_{(x,z-1,t-1)} \quad [\text{III.3.26.}]$$

For $t > 0$, $x = 0$, $z = -Z_{\text{lim}}$

$$A = [M_r]_{(x-1,z,t-1)} \quad B = [M_r]_{(x,z+1,t-1)} \quad [\text{III.3.27.}]$$

For $t > 0$, $x = 0$, $z = -Z_{\text{lim}}$

$$A = [M_r]_{(x+1,z,t-1)} \quad B = [M_r]_{(x,z+1,t-1)} \quad [\text{III.3.28.}]$$

For $t > 0$, $x = l$, $z = +Z_{\text{lim}}$

$$A = [M_r]_{(x-1,z,t-1)} \quad B = [M_r]_{(x,z-1,t-1)} \quad [\text{III.3.29.}]$$

Equations [III.3.18] to [III.3.29.] fully describe the concentration at $t=0$ and the flux at all nodes in the simulation for the duration of the applied potential pulse. On the commencement of the open circuit relaxation period the boundary condition at $x=0$ and $-Z_{\text{lim}} < z \leq 0$, [III.3.19.], transforms to the form expressed in [III.3.21.]. So for a series of potential pulses interrupted by open circuit relaxation periods the boundary conditions and flux equations are as described in equations [III.3.18.] to [III.3.29.], with the boundary condition at $x=0$ and $-Z_{\text{lim}} < z \leq 0$ oscillating between [III.3.19.] and [III.3.21.].

The models were also constructed so that the duration of the relaxation and pulsed periods may be different. In this case the same value for time increment and distance increments in both dimensions were used. This ensures that each phase of the model has similar definition.

From applying the boundary conditions, [III.3.18.] to [III.3.29.], to [III.3.10.] it should be clear that the flux in the z-dimension is significantly less than the flux in the x-dimension. This feature of the system allows models to be constructed that have less definition in the z-dimension than in the x-dimension. This is reasonable because the rate of change of concentration with respect to time and distance dimensions is less in the z-dimension than in the x-dimension. In order to facilitate a more rapid simulation the models were constructed so that the distance in the z-dimension could be divided, if desired, by a predetermined number of nodes such that the number of nodes would be less than would be determined by [III.3.16.]. The number of nodes chosen was 21. Since the internodal distance is invariably larger than as determined by [III.3.16.] a new value for the mass transfer coefficient in the z-dimension was determined from [III.3.13.].

Each node over the electrode is associated with a certain area of the electrode. For an electrode of dimensions p and q the central area, A_c , where the effect of lateral diffusion was assumed to be zero is defined by [III.3.30].

$$A_c = (p - 2Z_{lim})(q - 2Z_{lim}) \quad [III.3.30.]$$

Where Z_{lim} has been previously defined.

Figure III.3.3. considers the calculation of the strip area associated with nodes lying at a_1 and a_2 . Referring to Figure III.3.3., if the electrode has a centroid at o, then the area of the inner area, A_i , is defined by [III.3.31.]. The outer area, A_o , in this case equal to the total area of the electrode, is defined by [III.3.32.].

$$A_i = 2oa_2 \ 2ob_2 \quad [III.3.31.]$$

$$A_o = 2oa_1 \ 2ob_1 \quad [III.3.32.]$$

Consider that the width of the strip is equal to the internodal distance in the simulation, Δz , and that y and z are positioned equidistantly between a_1 and a_2 . Positions y and z correspond to the distance of node under examination. Then [III.3.31.] and [III.3.32.] may be restated:

$$A_i = 2(oz - \Delta z/2) \ 2(oy - \Delta z/2) \quad [III.3.33.]$$

$$A_o = 2(oz + \Delta z/2) \ 2(oy + \Delta z/2) \quad [III.3.34]$$

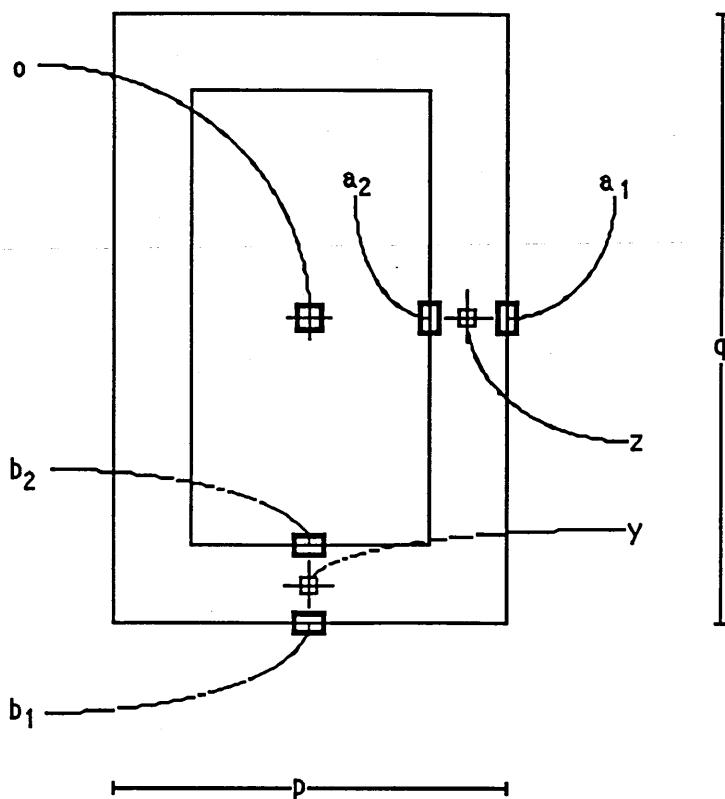


Figure III.3.3. Schematic diagram denoting the positions on the electrode surface used in the derivation of the electrode strip area associated with each node, equations [III.3.30] to [III.3.36].

The area of the strip, A_s , is described by [III.3.35.]:

$$A_s = A_o - A_i \quad [\text{III.3.35.}]$$

Substitution of the expanded forms of [III.3.33.] and [III.3.34.] into [III.3.35.] results in an expression for the area of a strip in terms of the internodal distance in the z-dimension and the position of the node under examination, [III.3.36.].

$$A_s = 4\Delta z(oz + oy) \quad [\text{III.3.36.}]$$

The values of oz and oy may be evaluated from the position of the node in relation to the perimeter of the electrode. For the node at $z=0$ the area of the strip is given by $2\Delta z(oz + oy)$.

The number of moles of reduced mediator, N_{Mr} diffusing to each node of the electrode over each time increment was determined by the product of the electrode strip area associated with that node, A_s , the internodal distance Δx and the concentration change between $n=0$ at the electrode surface and $n=1$, [III.3.37.]:

$$N_{Mr} = M_x [M_r]_{(1,z,t)} A_s \Delta x \quad [\text{III.3.37.}]$$

The current signal at each node, $i_{(0,z,t)}$, was determined by multiplying the product of [III.3.37.] by Faraday's constant and the reciprocal of the time increment, Δt , n has its usual meaning, [III.3.38.]:

$$i_{(0,z,t)} = n N_{Mr} F \Delta t^{-1} \quad [\text{III.3.38.}]$$

Charge calculations were made by taking the summation of the product of the time increment, Δt , and each calculated current value over the period of the application of the pulse.

The models produce five types of graphical representation of the simulation: a current-time plot, for the periods of potential pulse and concentration of substance throughout the duration of the simulation in the x-dimension at chosen positions in the z-dimension, and in the z-dimension at chosen positions in the x-dimension. A plot of concentration of substance in both distance dimensions at a chosen time is also available.

III.3.2. CONSTRUCTION OF FINITE DIFFERENCE MODELS TO SIMULATE THE REACTION IN THE CATALYTIC LAYER AND THE FLUX OF MEDIATOR AND GLUCOSE TO AND FROM THE CATALYTIC LAYER.

The evaluation of the second order rate constant for the reaction between reduced glucose oxidase and ferricyanide or ruthenium (III) hexamine enables a determination of the likely rate limiting steps occurring in the catalytic layer. Consideration of the reactions described by equations [II.5.2.] to [II.5.4] leads to several possible rate limiting conditions. The rate of association of the enzyme-substrate complex, with a rate constant k_1 (equation [II.5.2.]) may be negated in a simplified treatment by comparison of the time constant for the catalytic layer with an effective pseudo first order rate constant dependent on the concentration of the enzyme in the catalytic layer. For a catalytic layer of depth $10\mu\text{m}$, a rate constant for the transport of substrate across the catalytic layer, τ , may be defined by D/l^2 , s^{-1} , from [II.5.19.]. For glucose ($D=6.5\times 10^{-10} \text{ m}^2 \text{ s}^{-1}$) τ is 5 s^{-1} . Considering that k_1 for the reaction between glucose oxidase and glucose, equation [II.5.2.], is $\sim 10^4 \text{ M}^{-1} \text{ s}^{-1}$ (Eddowes, 1990) a pseudo first order reaction rate constant may be defined as $k_1[E]$. The concentration of glucose oxidase in the catalytic layer is about 0.8 mM , giving a value for the pseudo first order rate constant of 8 s^{-1} . Because the diffusion coefficient of glucose in the catalytic layer is likely to be some fraction of its value in solution the difference between the value of $k_1[E]$ and τ is probably even greater.

Taking the lower limit of the evaluated second order rate constant for ferricyanide and for ruthenium (III) hexamine it may be demonstrated that the rate of oxidation of glucose is probably determined by its flux to the surface of the catalytic layer and by the catalytic steps in the catalytic layer. If the concentration of glucose oxidase in the catalytic layer is equivalent to about 0.8 moles m^{-3} . The minimum concentration of oxidized mediator in the catalytic layer and throughout the device is 300 moles m^{-3} , when the oxidized mediator initially present solely in the catalytic layer has become homogeneously distributed throughout the device, from an initial concentration of $3000 \text{ moles m}^{-3}$. Using the lower limit for the value of the second order rate constant evaluated for ferricyanide of about $50\times 10^3 \text{ m}^3 \text{ mole}^{-1} \text{ s}^{-1}$, Section II.5., the minimum velocity, v_m , of the reaction that involves the oxidation of reduced glucose oxidase is $12\times 10^6 \text{ moles m}^{-3} \text{ s}^{-1}$ (from $v_m=k_e[M_o][E_r]$). If glucose is assigned a diffusion coefficient of $6.5\times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ (Treybal,1968), then the root mean distance traversed in a defined time may be estimated from [III.2.1.]. An interval of 0.01s yields a distance traversed of $4.5\times 10^{-6}\text{m}$. Using this value as the width of an element the number of moles of glucose diffusing to such an element in a time interval of 0.01 s may be estimated. For a concentration of glucose of 40 moles m^{-3} , 40 mM , considering that the area of the catalytic layer over the working electrode is $1.8\times 10^{-5} \text{ m}^2$, the volume affected is about $6.5\times 10^{-11} \text{ m}^3$; $2.6\times 10^{-9} \text{ moles}$ of glucose would diffuse into such a volume element in 0.01 s . Applying the value for the velocity of reaction

calculated previously, $12 \times 10^6 \text{ moles m}^{-3} \text{ s}^{-1}$, 1.9×10^{-5} moles of reduced glucose oxidase would be oxidized in that element over 0.01 s. Thus the maximum rate of diffusion of glucose to the catalytic layer is always several orders of magnitude less than the minimum rate of oxidation of glucose in the catalytic layer. Noting that when the flux of glucose is at a maximum value the concentration of oxidized mediator is also at a maximum and that the rate constant used in this estimate was the lowest value obtained from Section II.5. it is possible that the rate of oxidation of glucose within the catalytic layer exceeds its rate of diffusion to the catalytic layer by a value greater than the estimate given here, assuming the value for k_6 is identical in the catalytic layer and in the solution.

For any element within the catalytic layer the concentration of glucose will be dependent on the flux of glucose into that element over the preceding time interval Δt , and the rate of the oxidation of glucose by glucose oxidase. From the previous considerations it is likely that the velocity of this reaction may be expressed in terms of k_2 .

The constant k_2 (equation [II.5.2.]) describes the rate of dissociation of the enzyme substrate complex. The velocity of an enzyme catalysed reaction, v , may be described by [III.3.39.]:

$$v = k_2 [E_o] [S] / (K_m + [S]) \quad \text{[III.3.39.]}$$

The rate of change of glucose concentration in the catalytic layer is described by [III.3.40.]:

$$\partial[G]/\partial t = D_G (\partial^2[G]/\partial x^2) - v \quad \text{[III.3.40.]}$$

Assuming the arguments concerning the rate of oxidation of reduced glucose oxidase by oxidized mediator are correct, then the rate of change of reduced mediator concentration in the catalytic layer may be described by [III.3.41.]:

$$\partial[M_r]/\partial t = D_{Mr} (\partial^2[M_r]/\partial x^2) + nv \quad \text{[III.3.41.]}$$

for a single electron transfer reaction (ruthenium (III) hexamine and ferricyanide), from the stoichiometry of reaction [II.5.3.] $n=2$.

Previous treatments have been applied for the case of an enzyme layer entrapped behind a membrane permeable to substrate molecules, Section II.5.1., where the concentration of substrate inside the permeable membrane was the function of a partition coefficient between the solution and the membrane. The present system is treated as if the enzyme reaction is zero order with respect to mediator concentration, as a consequence of the evaluation of the second order rate constant.

Finite difference models were constructed to demonstrate the differences in glucose concentration-time response and reduced mediator concentration-time response between glucose-CFDs constructed using ruthenium (III) hexamine and ferricyanide.

Unlike the previous models lateral diffusion was neglected, the model consists of a series of nodes in the x-dimension only. In order to solve the flux equations in the catalytic layer it is necessary to solve the flux equations in the elements of solution between the electrode and the surface of the catalytic layer. The catalytic layer was assigned a depth of 10 μm for an overall gap width of 100 μm . Two models were constructed: the first was assigned equivalent internodal distances in the solution and in the catalytic layer of 10 μm ; in the second the internodal distances in the solution and in the catalytic layer were assigned values of 10 μm and 1 μm respectively. One model has a catalytic layer consisting of two nodes ($\Delta x=10 \mu\text{m}$), the other, which should yield a more accurate description of the concentration-time profiles within the catalytic layer, has a catalytic layer consisting of ten nodes ($\Delta x=1 \mu\text{m}$). Although the ten node catalytic layer model is a better representation of the system, the cost in terms of time of generating the whole of the concentration-distance-time surface for the solution and the catalytic layer for a 60 s simulation were thought to be excessive. As will be demonstrated, the difference in performance between the two models with respect to the generation of reduced mediator and consumption of glucose does not alter the conclusions of this study.

Information about the diffusion of substances through the catalytic layer was not available but it is reasonable to assume that the diffusion coefficients of substances within the layer would be some fraction of their value in solution. Consequently the simulation allowed a fractional diffusion coefficient, α , to be set for fluxes within the catalytic layer and between the catalytic layer and the solution.

The mass transfer coefficients were defined for the solution elements and for the catalytic layer elements separately to take account of the difference in the internodal distance of the catalytic layer and the solution elements in the more complex model.

The mass transfer coefficients for mediator, M_{ms} (solution) and M_{mc} (catalytic layer), and glucose, M_{gs} and M_{gc} were defined in a similar fashion to M_x and M_z in Section III.2.0, but with predetermined values for Δx . Oxidized and reduced forms of the mediator were assumed to have the same value of diffusion coefficient. The value of Δt was determined from $\Delta x^2/(2D)$, using the highest diffusion coefficient value, glucose or mediator. For the model incorporating ten nodes in the catalytic layer the internodal distance within the catalytic layer, 1 μm , was used to determine Δt . The values of the appropriate mass transfer coefficients were calculated by substitution of the value obtained for Δt with the associated distance into equations of the form of [III.3.12.]. These coefficients are modified for exchanges within and between the catalytic layer by multiplication by the fractional diffusion coefficient, α , where $0 < \alpha \leq 1$.

The initial boundary conditions were set for a uniform concentration of β -D-glucose, $[G]_0$, between the electrode surface and the interface of the solution and the catalytic layer, [III.3.44.]. Glucose was assumed to be initially absent throughout the catalytic layer, [III.3.45.].

$$\text{For } t=0, \quad 0 \leq x < l_{\text{cat}} \quad [G]_{(x,t)} = [G]_0 \quad [\text{III.3.42.}]$$

$$\text{For } t=0, \quad l_{\text{cat}} \leq x \leq l \quad [G]_{(x,t)} = 0 \quad [\text{III.3.43.}]$$

For $t > 0$ for those elements in solution the flux equations are the one dimensional form of those described in Section III.3.1..

$$\text{For } t > 0, \quad x = 0$$

$$[G]_{(x,t)} = [G]_{(x,t-1)} + M_{\text{gs}}([G]_{(x+1,t-1)} - [G]_{(x,t-1)}) \quad [\text{III.3.44.}]$$

$$\text{For } t > 0, \quad 0 < x < (l_{\text{cat}} - 1)$$

$$[G]_{(x,t)} = [G]_{(x,t-1)} + M_{\text{gs}}([G]_{(x+1,t-1)} - 2[G]_{(x,t-1)} + [G]_{(x-1,t-1)}) \quad [\text{III.3.45.}]$$

but for the solution node adjacent to the catalytic layer (at $x = l_{\text{cat}} - 1$ and $t > 0$) [III.3.46.] is modified to [III.3.47.]:

$$\text{For } t > 0, \quad x = l_{\text{cat}} - 1,$$

$$[G]_{(x,t)} = [G]_{(x,t-1)} + M_{\text{gs}}([G]_{(x-1,t-1)} - [G]_{(x,t-1)}) + \alpha M_{\text{gs}}([G]_{(x+1,t-1)} - [G]_{(x,t-1)}) \quad [\text{III.3.46.}]$$

Equation [III.3.47.] separates the flux between the solution and the catalytic layer; assuming that the oxidation of glucose proceeds at $x = l_{\text{cat}}$ the fractional diffusion coefficient must be incorporated into the expression governing the flux of glucose between the surface the catalytic layer and the adjacent solution element.

For $l_{\text{cat}} \leq x \leq l$ the velocity of the enzyme catalysed oxidation of glucose, v ([II.3.39.]), for any element is obtained by substituting the value of $[G]$ at that node at $t = t - 1$ into [II.3.39.], and in the following equations is denoted by $v_{(x, t-1)}$. The change in the concentration of glucose in each element over the interval Δt is the product of the time interval and the reaction velocity. To avoid negative

concentrations of glucose in any element, the maximum allowed value of $\Delta tv_{(x, t-1)}$ was set as equal to the value of the flux into that element over the previous time period.

For $t > 0$, $x = l_{cat}$

$$[G]_{(x,t)} = [G]_{(x,t-1)} + \alpha M_{gs} [G]_{(x-1,t-1)} - [G]_{(x,t-1)} + \alpha M_{gc} ([G]_{(x+1,t-1)} - [G]_{(x,t-1)}) - \Delta tv_{(x, t-1)} \quad [III.3.47.]$$

For $t > 0$, $l_{cat} < x < l$

$$[G]_{(x,t)} = [G]_{(x,t-1)} + \alpha M_{gc} ([G]_{(x+1,t-1)} - 2[G]_{(x,t-1)} + [G]_{(x-1,t-1)}) - \Delta tv_{(x, t-1)} \quad [III.3.48.]$$

For $t > 0$, $x = l$

$$[G]_{(x,t)} = [G]_{(x,t-1)} + \alpha M_{gc} ([G]_{(x+1,t-1)} - [G]_{(x,t-1)}) - \Delta tv_{(x,t-1)} \quad [III.3.49.]$$

The initial concentration of reduced mediator was set at zero throughout the device, [III.3.50.].

$$\text{For } t=0, \quad 0 < x < l \quad [M_r]_{(x,t)} = 0 \quad [III.3.50.]$$

For $t > 0$ for those elements in solution the flux equations are the same form as those describing the flux of glucose, [III.3.44.] to [III.3.46.], but M_{ms} is substituted for M_{gs} and M_{mc} is substituted for M_{gc} . The models incorporated the assumption that 0.63 of the initial concentration of glucose consisted of β -D-glucose.

Those elements within the catalytic layer are subject to the same conditions that determine glucose flux but the rate of generation of reduced mediator is determined by $2\Delta tv_{(x, t-1)}$ as described in [III.3.41.].

For $t > 0$, $x = l_{cat}$

$$[M_r]_{(x,t)} = [M_r]_{(x,t-1)} + \alpha M_{ms} [M_r]_{(x-1,t-1)} - [M_r]_{(x,t-1)} + \alpha M_{mc} ([M_r]_{(x+1,t-1)} - [M_r]_{(x,t-1)}) + 2\Delta tv_{(x, t-1)} \quad [III.3.51.]$$

For $t > 0$, $l_{cat} < x < l$

$$[M_r]_{(x,t)} = [M_r]_{(x,t-1)} + \alpha M_{mc} ([M_r]_{(x+1,t-1)} - 2[M_r]_{(x,t-1)} + [M_r]_{(x-1,t-1)}) + 2\Delta t v_{(x,t-1)} \quad [III.3.52.]$$

For $t > 0$, $x = l$

$$[M_r]_{(x,t)} = [M_r]_{(x,t-1)} + \alpha M_{mc} ([M_r]_{(x+1,t-1)} - [M_r]_{(x,t-1)}) + 2\Delta t v_{(x,t-1)} \quad [III.3.53.]$$

Because of the assumption of fast kinetics for the reaction between reduced glucose oxidase and oxidized mediator (from considerations of the minimum concentration of oxidized mediator and the value of k_0) the concentration of oxidized mediator, M_o , present in any element of the catalytic layer plays no role in the simulation of the flux of glucose and reduced mediator. A simulation of the flux of oxidized mediator from the catalytic layer to the solution was performed for illustrative purposes using the two node catalytic layer model. The mediator was considered to be initially absent throughout the device except in the catalytic layer, where it was present at a concentration of $[M_o]$; [III.3.54.] & [III.3.55.].

$$\text{For } t=0, \quad 0 \leq x \leq l_{cat}-1 \quad [M_o]_{(x,t)} = 0 \quad [III.3.54.]$$

$$\text{For } t=0, \quad l_{cat} \leq x \leq l \quad [M_o]_{(x,t)} = [M_o]_0 \quad [III.3.55.]$$

For $t > 0$, the equations which govern the flux of M_o throughout the device ($0 < x < l$) are the same form as those describing the flux of glucose in the solution and throughout the catalytic layer, equations [II.3.44.] to [III.3.49.], with the deletion of the kinetic term, $\Delta t v_{(x,t-1)}$.

The arrangement of the model and the operation of the flux equations in the solution and the catalytic layer is illustrated in Figure III.3.4..

Simulations of the flux response for a system incorporating ferricyanide used a diffusion coefficient of $6.26 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ and those incorporating ruthenium (III) hexamine used a diffusion coefficient of $1.77 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$. Glucose was assigned a diffusion coefficient of $6.5 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$. Glucose oxidase was assigned a K_m value for glucose of 7 mM (Mell & Maloy, 1975). The lower estimate for k_2 of $\sim 100 \text{ s}^{-1}$ was employed (Eddowes, 1991). Finite difference programs incorporating the expressions developed in this Section were written in BASIC and were executed on a PC with a 286 microprocessor and a maths co-processor.

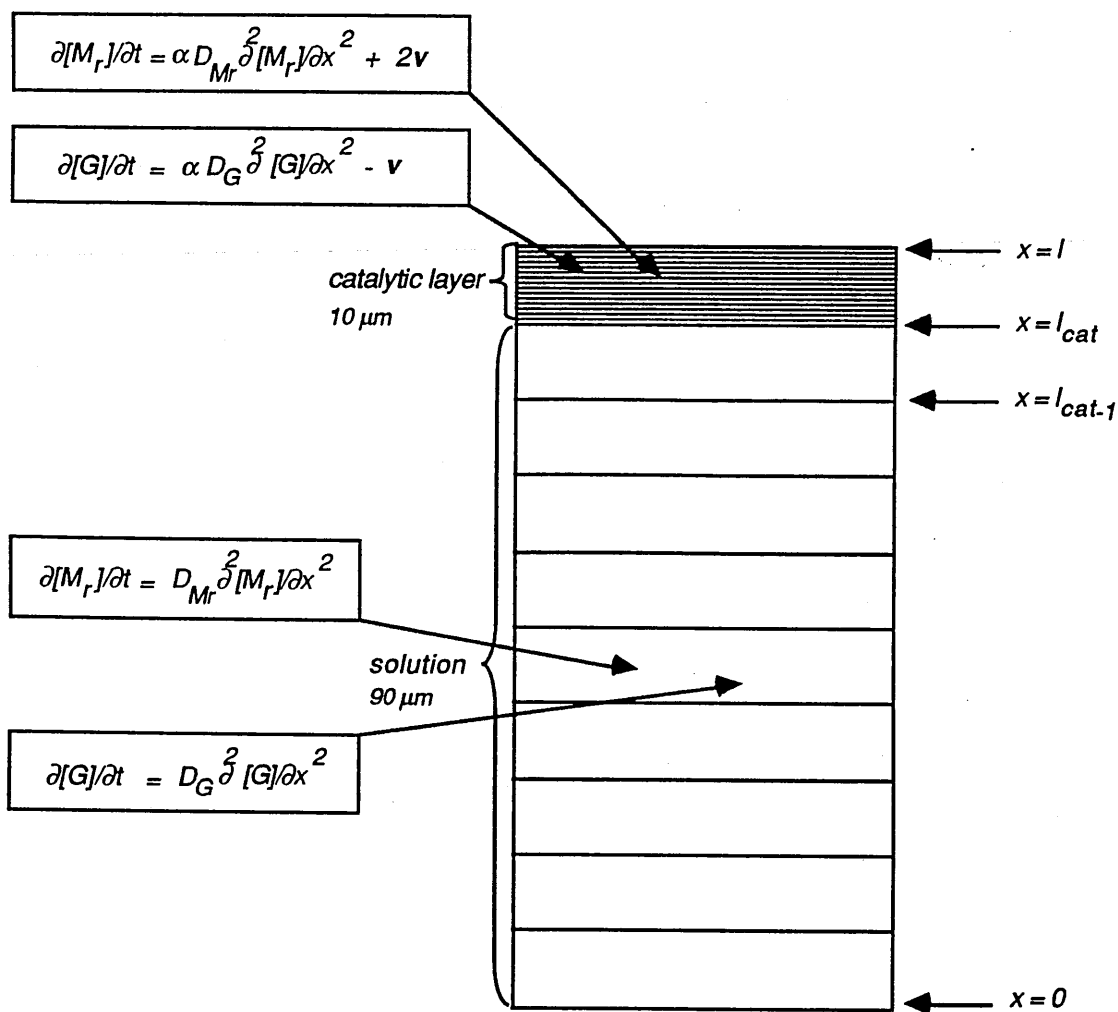


Figure III.3.4. Schematic representation of the model used to simulate the catalytic phase of the operation of the glucose-CFD for glucose and reduced mediator. Equations refer to the sector (arrows & brackets) where they operate, α is the fractional diffusion coefficient of substances within the catalytic layer and between catalytic layer and solution and v is defined by equation [III.3.39].

Comparative performance of ruthenium (III) hexamine and ferricyanide. The response of the system was initially assessed using the two node catalytic layer model incorporating a value for α of 0.5. Figure III.3.5. shows the change in concentration of glucose in the solution in the x-dimension over the course of sixty seconds. The concentration profile describing the diffusion of oxidized mediator from the catalytic layer to the solution is shown in Figure III.3.6. for ruthenium (III) hexamine. The corresponding evolution profiles for ruthenium (II) hexamine and for ferrocyanide are shown in Figures III.3.7. and III.3.8. respectively. The diffusion profiles of glucose and reduced mediator within the catalytic layer were not shown here because of the low fractional concentration relative to the concentration in solution, typically < 5%, in the case of glucose, and because concentration polarisation of reduced mediator occurs only at very short times.

Examination of Figures III.3.5. and III.3.6. reveal that, as predicted, for the catalytic layer-solution interface, the glucose flux maximum coincides with the flux maximum for the oxidized mediator. The significance of this observation is that the actual maximum rate of oxidation of reduced glucose oxidase in the catalytic layer is probably several orders of magnitude higher than the estimate made prior to the construction of the finite difference model. This analysis assumes a similar value for second order rate constant in the catalytic layer as in solution.

Analysis of the evolution profiles of ferrocyanide and ruthenium (II) hexamine (Figures III.3.7. and III.3.8.) lead to the conclusion that the advantages of using ruthenium (III) hexamine rather than ferricyanide in terms of a reducing the total analysis time are marginal. This is not surprising since the rate of generation of reduced mediator is the same for both systems, being dependent only on the flux of glucose to the catalytic layer. The concentration of ferrocyanide is greater at the catalytic layer-solution interface than the concentration of ruthenium (II) hexamine at any time until concentration equilibrium is attained. The marginal difference in time for each system to attain an homogeneous concentration of mediator in the x-dimension is solely due to the difference in the diffusion coefficients of each mediator and does not represent a significant advantage in terms of overall analysis time.

The fact that the diffusion coefficient of ruthenium (III) hexamine is about three times as great as the diffusion coefficient of ferricyanide would only be significant in terms of a reduction of duration of the catalytic phase if the diffusion coefficient of glucose were significantly greater than the diffusion coefficient of either mediator.

The degree of correspondence between the response of the two node model and the system response does not affect the validity of the comparative performance of the two mediators; the value

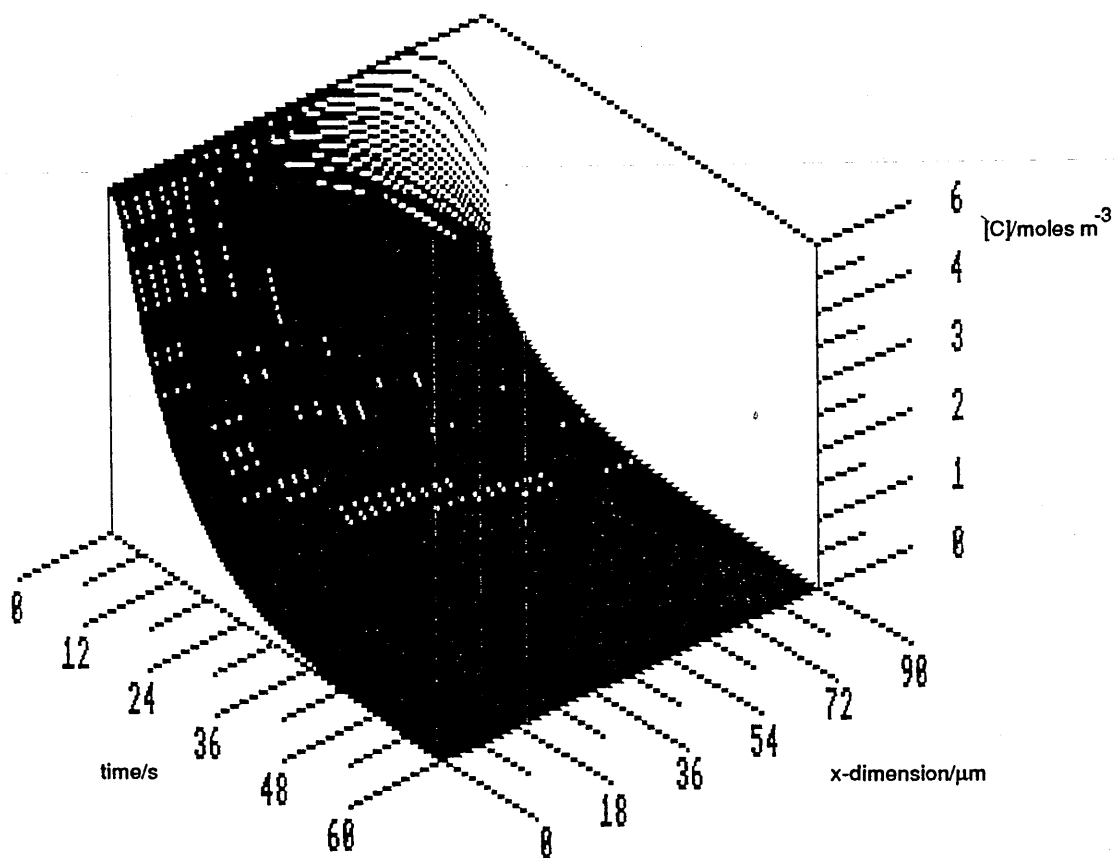


Figure III.3.5. The simulated change in the concentration of glucose in the x-dimension over the course of a sixty second catalytic reaction. Glucose was assigned a diffusion coefficient of $6.5 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$; α was set at 0.5. Figure III.3.4. represents the concentration profiles of glucose for the catalytic phase when ruthenium (III) hexamine or ferricyanide are employed as mediator compounds.

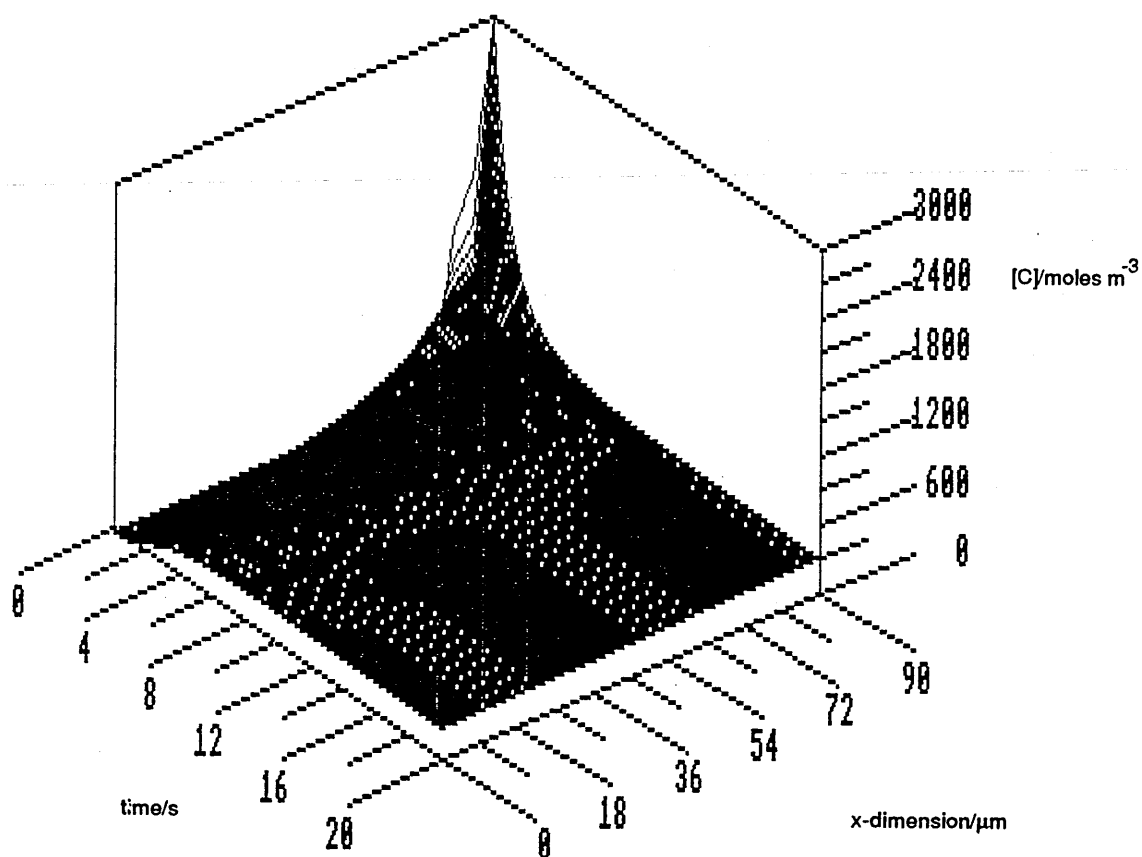


Figure III.3.6. The simulated change in the concentration of oxidized mediator, ruthenium (III) hexamine, over the course of a sixty second catalytic reaction. Ruthenium (III)hexamine was assigned a diffusion coefficient of $1.7 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$; α was set at 0.5.

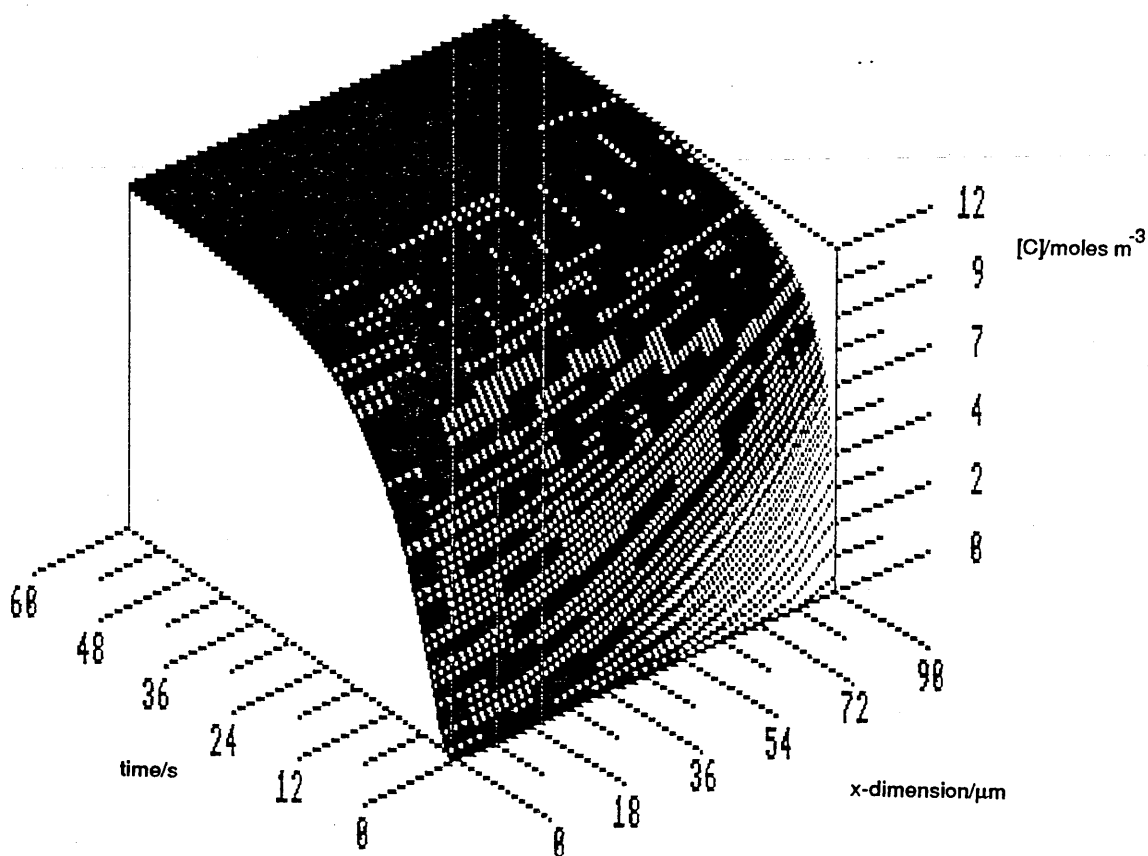


Figure III.3.7. Simulation response of the evolution of ferrocyanide at the surface of the catalytic layer. Ferrocyanide was assigned a diffusion coefficient of $6.26 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$; α was set at 0.5.

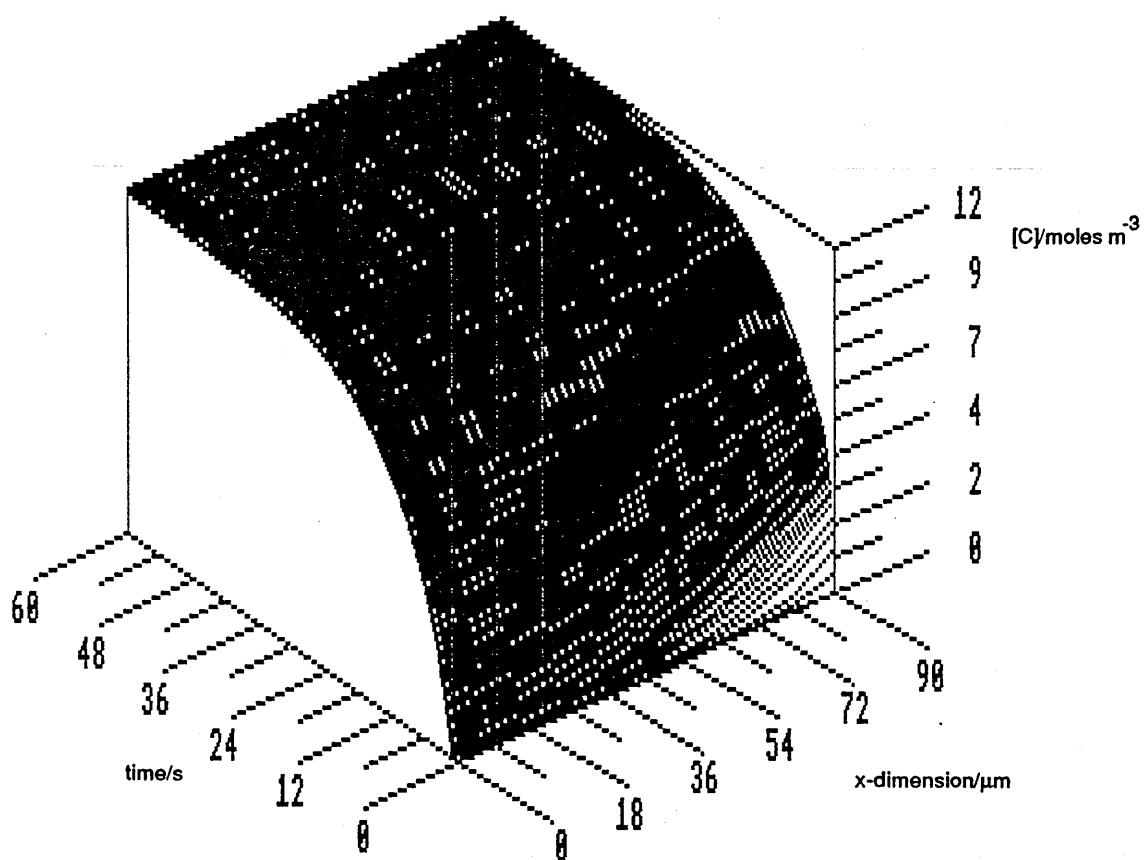


Figure III.3.8. Simulation response of the evolution of ruthenium (II) hexamine at the surface of the catalytic layer. Ruthenium (II) hexamine was assigned a diffusion coefficient of $1.7 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$; α was set at 0.5.

for α was arbitrarily chosen but if the assumptions used to construct the model are correct, then the relative performance of each mediator is the same, irrespective of the value of α .

The response of the model to variations in concentration of glucose. The glucose concentration dependence of the response was explored for $1 \text{ mM} \leq [\text{G}] \leq 100 \text{ mM}$ for $\alpha=1.0$ using the ten node catalytic layer model. The change in concentration of glucose at the electrode surface ($x=0$) throughout the course of the simulation may be used to assess the point of reaction completion. All responses within this concentration range may be represented by the normalised glucose concentration at $x=0$ - time profile in Figure III.3.9., denoted $n[\text{G}]_{(0,t)}$. Taking $[\text{G}]_0$ as the initial concentration of β -D-glucose the time taken for the concentration of β -D-glucose at $x=0$ to reach an arbitrary fraction of this value may be used as an index of the extent of the reaction. The fractional concentration used was 0.01 as an approximation of zero glucose concentration ($n[\text{G}]=0$), or reaction completion, with an associated time for reaction completion, $t_{n[\text{G}]=0}$. On the basis of fractional concentration there is no significant difference between the time taken to complete the reaction. However, using a concentration of glucose of 10^{-2} mM at $x=0$ as an approximation of $[\text{G}] \rightarrow 0$, significant differences in the time taken to achieve reaction completion ($t_{[\text{G}]=0}$) become apparent. Figure III.3.10. shows $[\text{G}]_{(0,t)}$ at initial concentrations of glucose between 1 mM and 100 mM. The value of $t_{[\text{G}]=0}$ varies between 28 s and 55 s for $1 \text{ mM} \leq [\text{G}] \leq 100 \text{ mM}$ for $\alpha=1$.

Unlike the steady state responses of enzyme layers retained behind diffusion membranes characterised by other workers, the present system is dynamic: the flux of glucose into the catalytic layer is a function of the initial concentration of glucose and the concentration difference between the catalytic layer and the adjacent solution. Initially, at $t=0$ s, the concentration difference is equal to the initial concentration of glucose, since glucose is initially absent in the catalytic layer. Consequently the initial glucose flux is maximal and is a function of the concentration difference and the value of α . At this point the boundary conditions that determine glucose flux to the surface of the catalytic layer are identical to the boundary conditions that determine the flux of reduced mediator to the surface of an oxidizing electrode, Section III.3.1.. Thereafter the flux of glucose at I_{cat} is a function of v and α .

It may be shown that for a fixed value of α , that the concentration of glucose across the catalytic layer is approximately the same fractional concentration of the initial concentration of glucose present in solution at any time, so that the fractional flux of glucose (with respect to $[\text{G}]_0$) to the catalytic layer at any time is the same. Consequently the fractional response of $[\text{G}]_{(0,t)}$ is likely to be more sensitive to the value of α than to $[\text{G}]_0$. Figure III.3.11 shows the fractional ($[\text{G}]_{(x,t)}/[\text{G}]_0$) concentration profiles of glucose through the catalytic layer for solutions containing 1 mM glucose and 100 mM glucose at 0.1 s and 1.0 s.

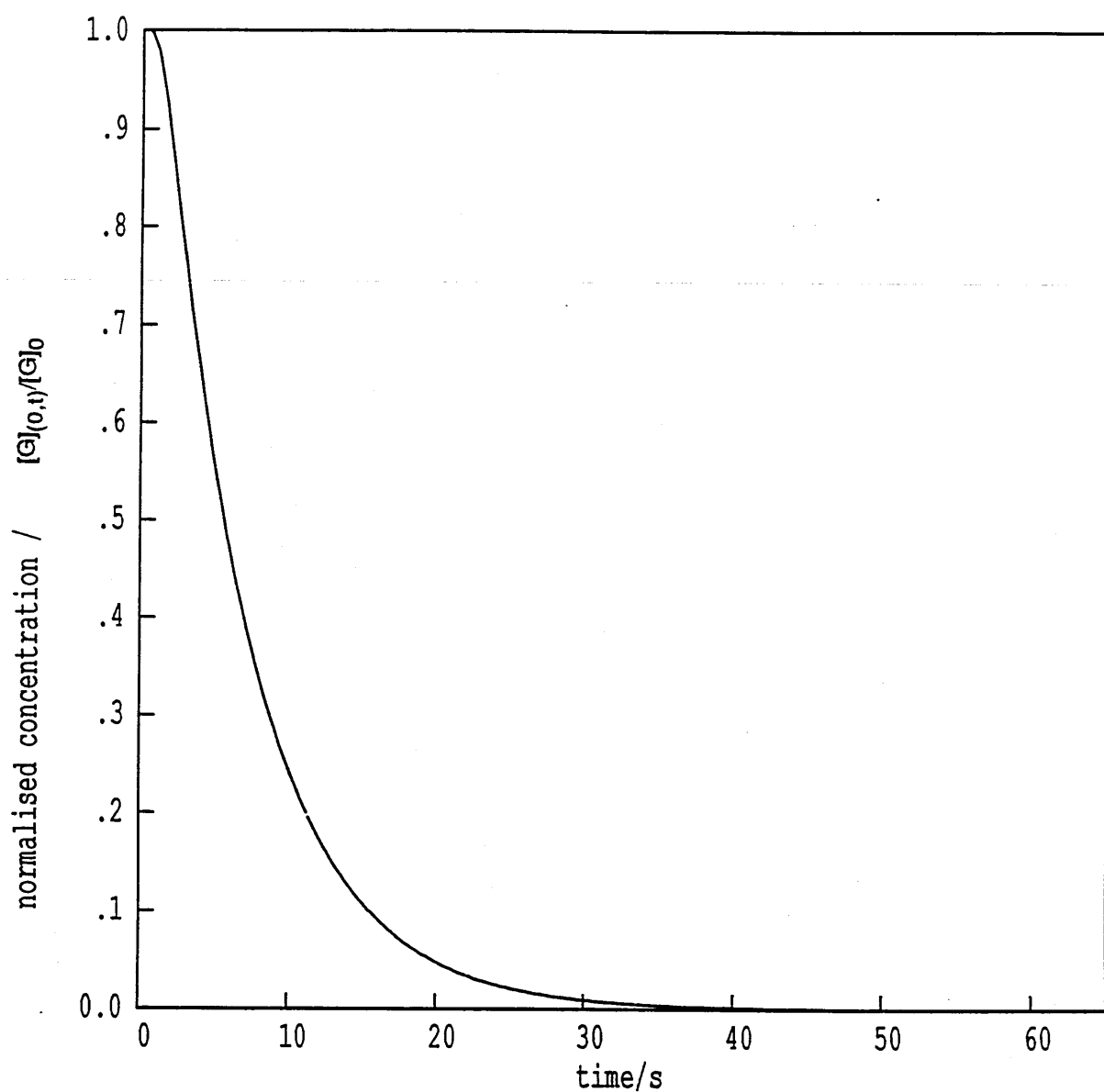


Figure III.3.9. The normalised concentration-time profile of glucose at $x=0$ for the glucose-CFD. The functional relationship between $[G]_{(0,t)}$ and t is of the form $[G]_t=[G]_0e^{-\beta t}$, and at this value of $[E]$ is applicable for substrate in the range $[\beta\text{-D-glucose}] \leq 63 \text{ mM}$. ($\alpha = 1.0$, $[E]=0.8 \text{ moles m}^{-3}$, $l=100 \mu\text{m}$).

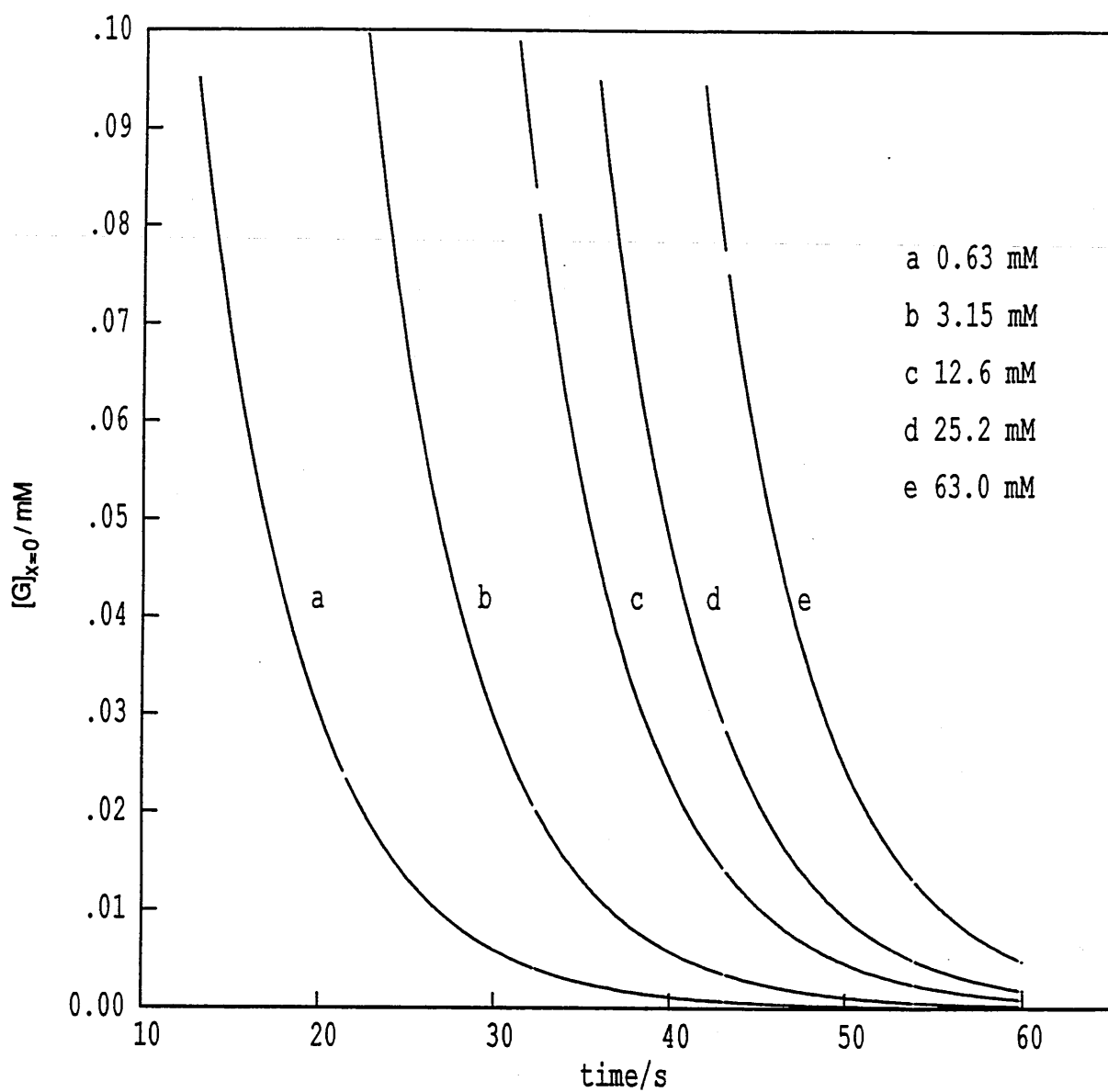


Figure III.3.10. The actual concentration-time profile of glucose at $x=0$ for the glucose-CFD for a-e; $0.63 \text{ mM} \leq [\beta\text{-D-glucose}] \leq 63 \text{ mM}$. ($\alpha = 1.0$, $[E]=0.8 \text{ moles m}^{-3}$, $l=100 \text{ }\mu\text{m}$).

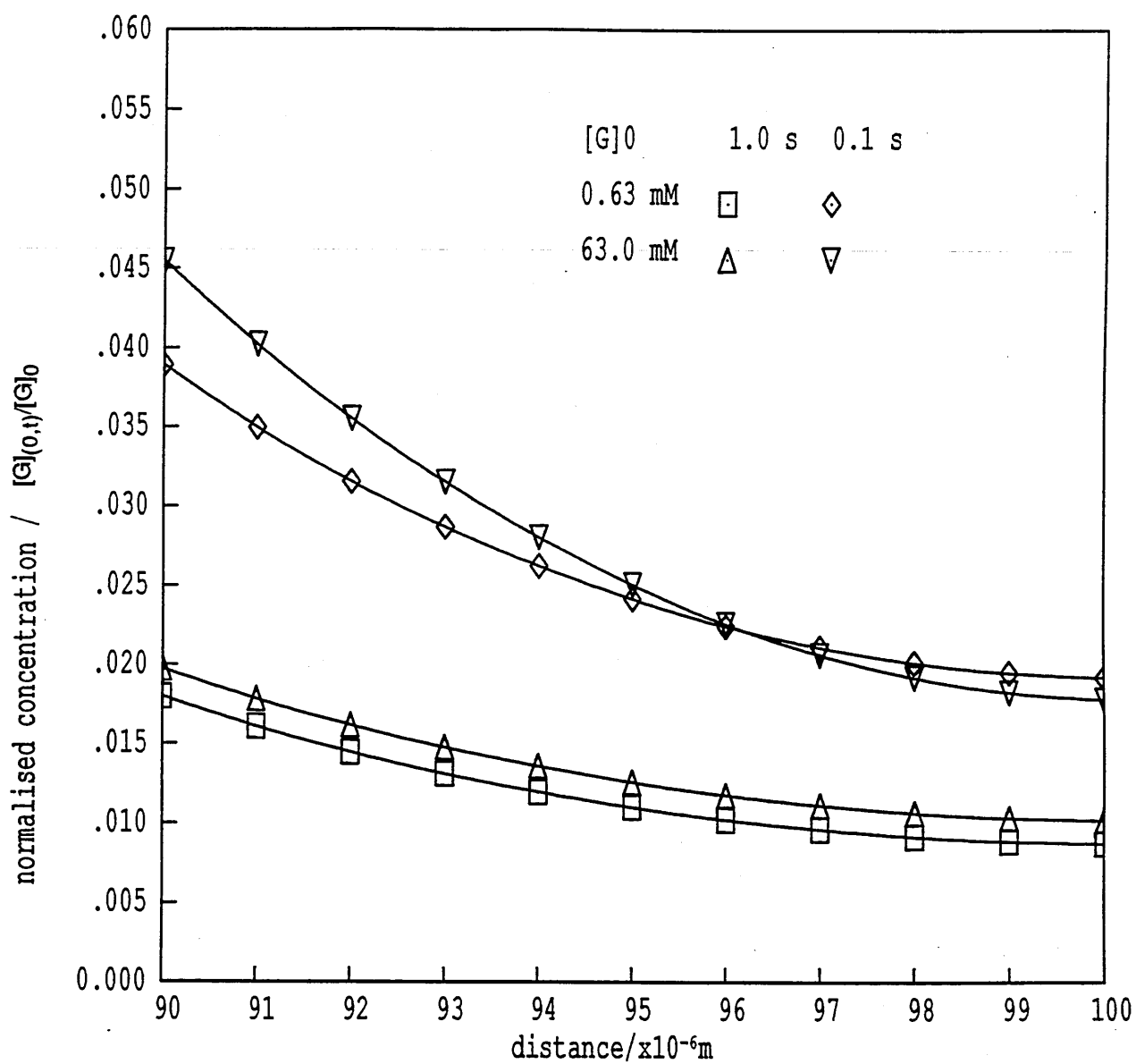


Figure III.3.11. The concentration profiles for glucose throughout the catalytic layer, normalised against $[G]_0$ values of 63 mM and 0.63 mM for $t=0.1$ s and $t=1.0$ s. ($\alpha = 1.0$, $[E]=0.8$ moles m^{-3} , $l=100$ μm).

The sensitivity of $[G]_{(0,t)}$ to α . The sensitivity of the model glucose flux response at the surface of the electrode ($x=0$) to variations in the value of α were explored for $0.1 \leq \alpha \leq 1.0$. Figure III.3.12. shows the normalised concentration of glucose present at $x=0$ vs. time for model solutions using a diffusion coefficient for glucose of $6.5 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ (Treybal, 1968) for $0.1 \leq \alpha \leq 1.0$. Using a fractional concentration of glucose of 10^{-2} as an approximation of $[G] \rightarrow 0$, for $0.5 \leq \alpha \leq 1.0$ the model predicts that the reaction is completed within 60 s.

The sensitivity of $[G]_{(0,t)}$ to overall gap width. The effect of reducing the gap width on the time taken to complete the catalytic reaction, discussed in Section III.1.1., was tested for $30 \mu\text{m} \leq l \leq 100 \mu\text{m}$ at $\alpha=1.0$, assuming a catalytic layer of thickness $10 \mu\text{m}$. Figure III.3.13. shows normalised $[G]_{(0,t)}$ response over a catalytic reaction of duration 60 s. A reduction in l from $100 \mu\text{m}$ to $40 \mu\text{m}$ results in a reduction in the value of $t_{n[G]=0}$ from 28 s to 10 s. Using present manufacturing techniques such reduction in gap width would almost certainly be associated with higher fractional variability in the gap width and therefore greater variability of the overall coulometric response, but the application of the model does demonstrate the relative advantages associated with a reduction in the value of l , irrespective of α . The lower value of l is likely to be determined by the surface tension of the sample, provided that capillary effects are used to create a thin film of solution.

The sensitivity of $[G]_{(0,t)}$ to α and to $[G]_0$. A performance profile for the sensor may be constructed by combining the analyses of the $[G]_{(0,t)}$ response of the models to α and $[G]_0$. Restating the time constant, T_c , for a thin layer electrochemical cell of width l m, for a molecule with a diffusion coefficient of $D \text{ m}^2 \text{ s}^{-1}$ (the Einstein-Stokes equation, [II.5.19.], [III.3.56.];

$$T_c = l^2/D \quad \text{[III.3.56.]}$$

The time constant, T_c , is proportional to the square of the cell width. Therefore the response of T_c may be linearised with respect to l by raising T_c to the power 0.5. The concentration of glucose at $x=0$ decays exponentially with respect to time, Figure III.3.9. which may be described by an equation of the form $[G]_{(0,t)} = Ae^{-\beta t}$. This equation has the same form as the equation used to describe the decay of current or flux at the surface of a thin layer cell, Section I., but in this case the exact relationship between β and D and l is not known since the boundary conditions for the concentration of glucose at the catalytic layer-solution interface are not known. Application of a least squares exponential regression to the curve in III.3.9. results in the identification of the decay constant, β , and the constant A . These constants were used to find the time at which the fractional concentration had decayed to 10^{-2} of its initial concentration.

Using this procedure it can be demonstrated that there is a constant relationship between $T_c^{0.5}$ and

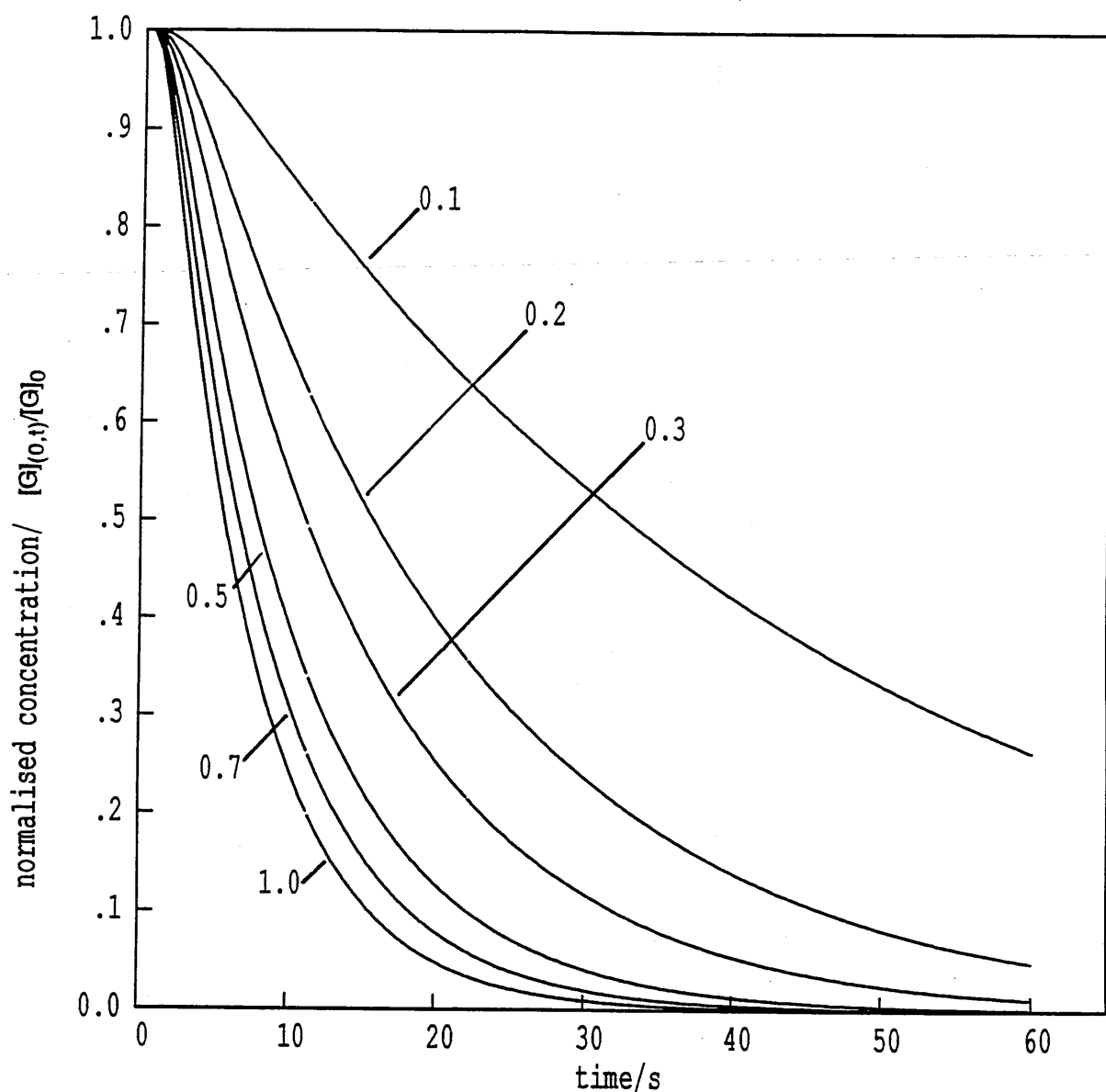


Figure III.3.12. The normalised concentration-time profiles for $[G]_{(0,t)}$ for $0.1 \leq \alpha \leq 1.0$ (numbers & arrows). ($0.63 \text{ mM} \leq [G] \leq 63 \text{ mM}$, $[E]=0.8 \text{ moles m}^{-3}$, $l=100 \text{ }\mu\text{m}$).

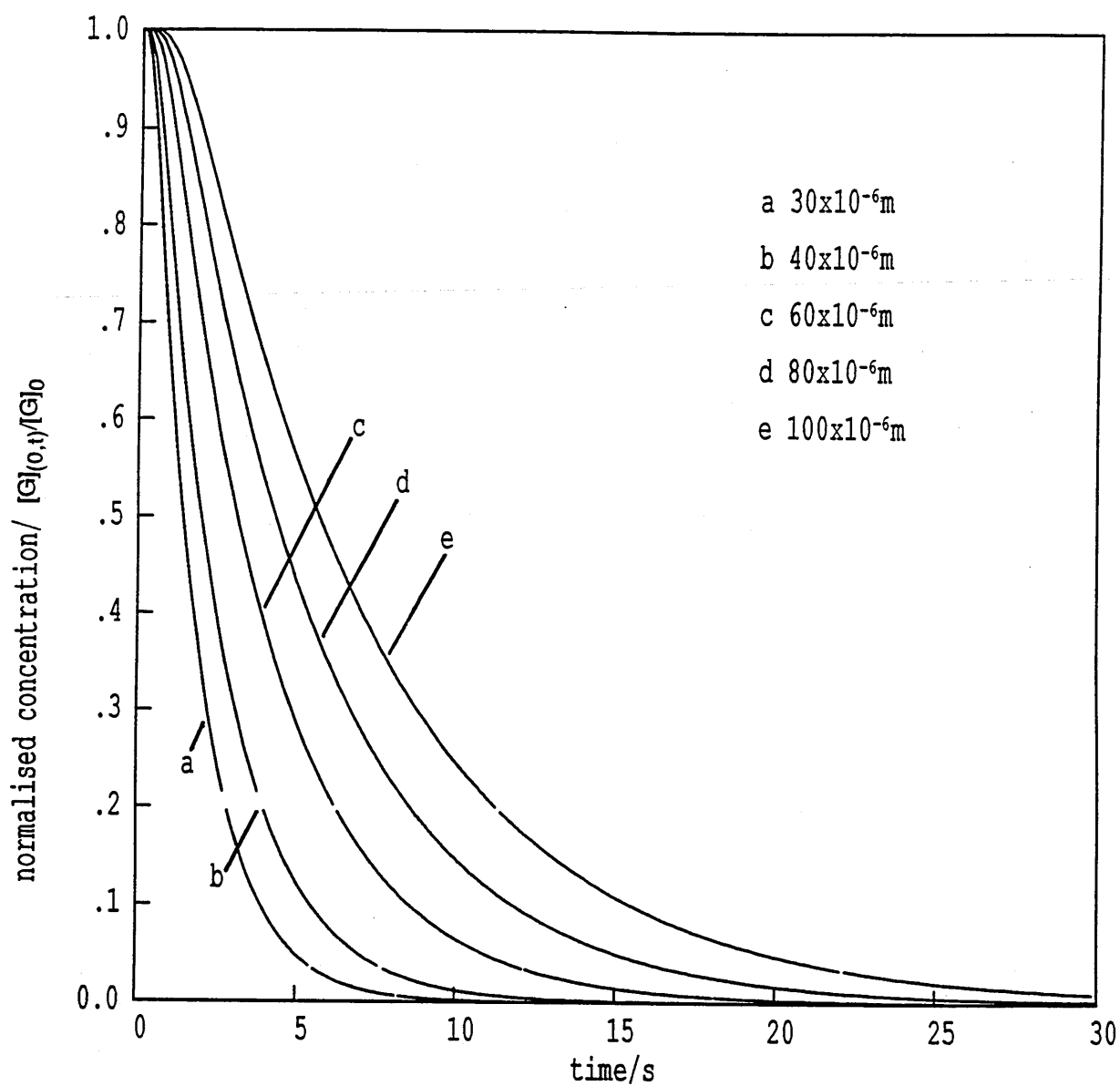


Figure III.3.13. The normalised concentration-time profiles for $[G]_{(0,t)}$ for an overall gap width in the range a-e; $30 \mu\text{m} \leq l \leq 100 \mu\text{m}$. ($0.63 \text{ mM} \leq [G] \leq 63 \text{ mM}$, $[E]=0.8 \text{ moles m}^{-3}$, $\alpha=1.0$).

$t_{n[G]=0}$, for cells of various gap widths, defined by [III.3.57.].

$$\chi_n = T_c^{0.5} / t_{n[G]=0} \quad [\text{III.3.57.}]$$

The constant χ_n is associated with the time taken for the concentration of β -D-glucose at $x=0$ to attain 10^{-2} of $[G]_0$ and is applicable over a range of gap widths.

$T_c^{0.5}$ was evaluated using the distance between the catalytic layer-solution interface and the electrode as an approximation of l . Using results obtained for a value of α of 1.0, the parameter, χ_n , varies from $0.119 \text{ s}^{0.5}$ at $l=90 \text{ }\mu\text{m}$ to $0.110 \text{ s}^{0.5}$ at $l=20 \text{ }\mu\text{m}$, or a variation of $\sim 7\%$ (the variation is $< 4\%$ between $90 \text{ }\mu\text{m}$ and $30 \text{ }\mu\text{m}$). Therefore it is possible to approximately represent the flux behaviour of thin layer devices of varying gap widths for a given value of α by normalised change in concentration of glucose at $x=0$ vs. the parameter, χ_n . Similarly, the parameter $T_c^{0.5}/t$ ($\text{s}^{-0.5}$) represents the time-time constant behaviour for each cell. Figure III.3.14. shows the normalised response of the model for $0.1 \leq \alpha \leq 1.0$ using a catalytic layer containing glucose oxidase at a concentration of 0.8 moles m^{-3} .

Using this approach it is possible to construct relationships between the parameter χ_n , α and the values for initial concentration of substrate and the K_m value of the enzyme that are representative of any thin layer enzyme electrode of this configuration provided that certain constraints with respect to enzyme concentration in the catalytic layer and the enzyme mechanism are realised. Taking $t_{[G]=0}$, Figure III.3.10., as an approximation of $[G]_{(0,t)=0}$, $T_c^{0.5}/t_{[G]=0}$, denoted χ_a , represents the time taken to complete a reaction at different concentrations of glucose for any overall gap width ($30 \text{ }\mu\text{m} \leq l \leq 100 \text{ }\mu\text{m}$) at a certain value of α . By plotting the χ_a response vs. the dimensionless parameters $[G]_0/K_m$ (Figure III.3.15.) or $K_m/[G]_0$ (Figure III.3.16.) the response profiles for the system may be produced which incorporate all of the response dependent variables except enzyme concentration.

Performance limits for the sensors at different values of α and over different concentration ranges of substrate may be estimated from Figures III.3.15. and III.3.16. Using $t_{[G]=0}$ as the criterion for reaction completion, the maximum concentration of β -D-glucose that may be completely oxidized within 60 s is 63 mM at $\alpha=1.0$, 22 mM at $\alpha=0.7$, 6 mM at $\alpha=0.5$; all at $l=100 \text{ }\mu\text{m}$. The use of an arbitrary definition of reaction completion, either in terms of normalised response, to 1% of $[G]_0$ at $x=0$ ($t_{n[G]=0}$), or of an absolute value, 10^{-2} mM β -D-glucose at $x=0$ ($t_{[G]=0}$), illustrates an important aspect of sensor design; the compatibility of the performance characteristics of the sensor with its analytical demands. The use of $t_{[G]=0}$ to define the reaction performance implies the requirement to maintain the same absolute error in determination over the selected concentration range, the use of $t_{n[G]=0}$ implies the less

¹ A similar parameter based on the decay constant, β , and the initial concentration of glucose, $[G]_0$, may be constructed; $T_c^{0.5}/([G]_0/\beta)$, the term $([G]_0/\beta)$ is proportional to $t_{[G]=0}$. Analogous application of this parameter results in similar relationships for the sensor response as assessed using $[G]_{(0,t)}$, but of a more obscure nature with regard to interpretation.

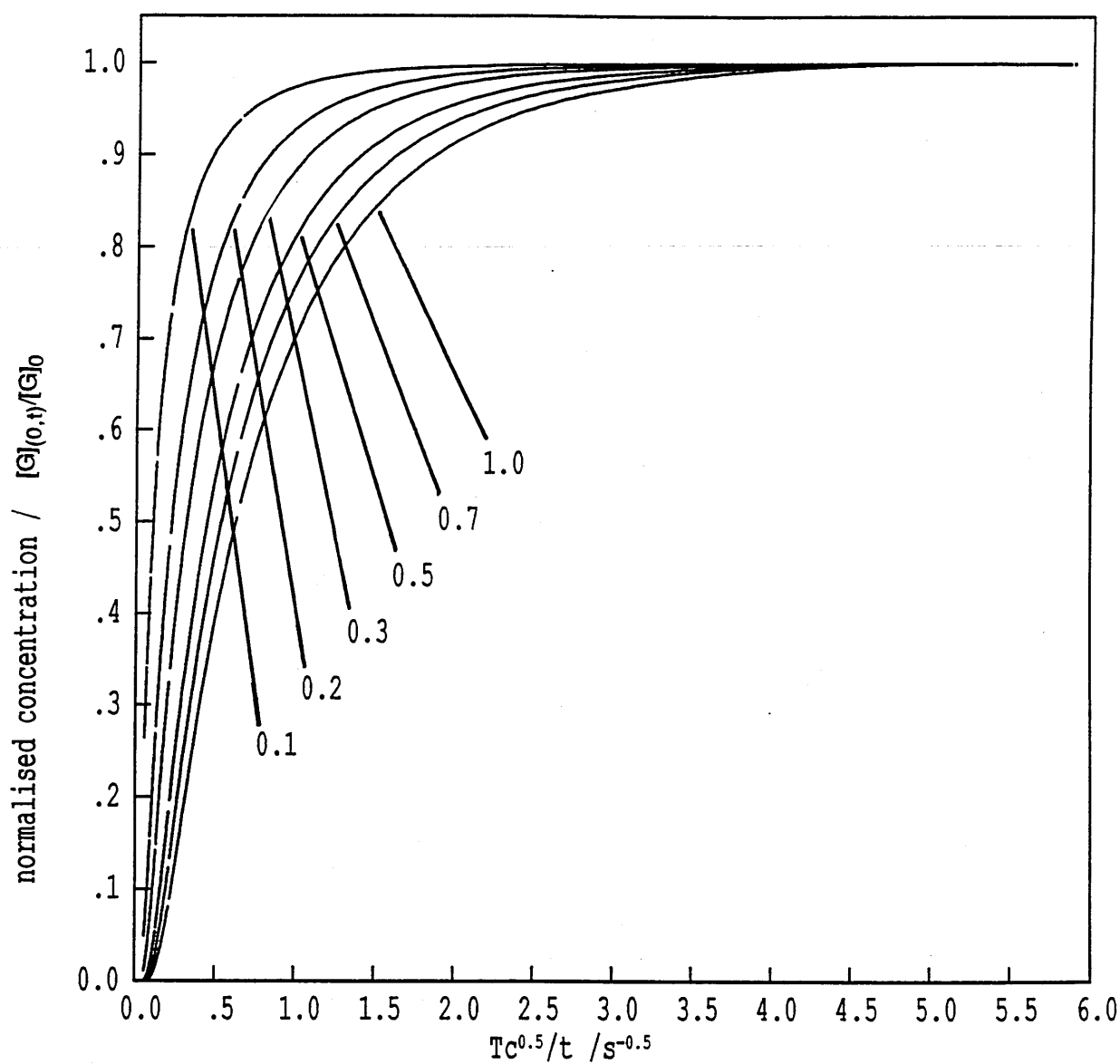


Figure III.3.14. Normalised concentration- $(T_c^{0.5}/\text{time})$ profiles for $[G]_{(0,t)}$ for $0.1 \leq \alpha \leq 1.0$ (numbers & arrows). ($0.63 \text{ mM} \leq [G] \leq 63 \text{ mM}$, $[E]=0.8 \text{ moles m}^{-3}$, $30 \text{ }\mu\text{m} \leq l \leq 100 \text{ }\mu\text{m}$).

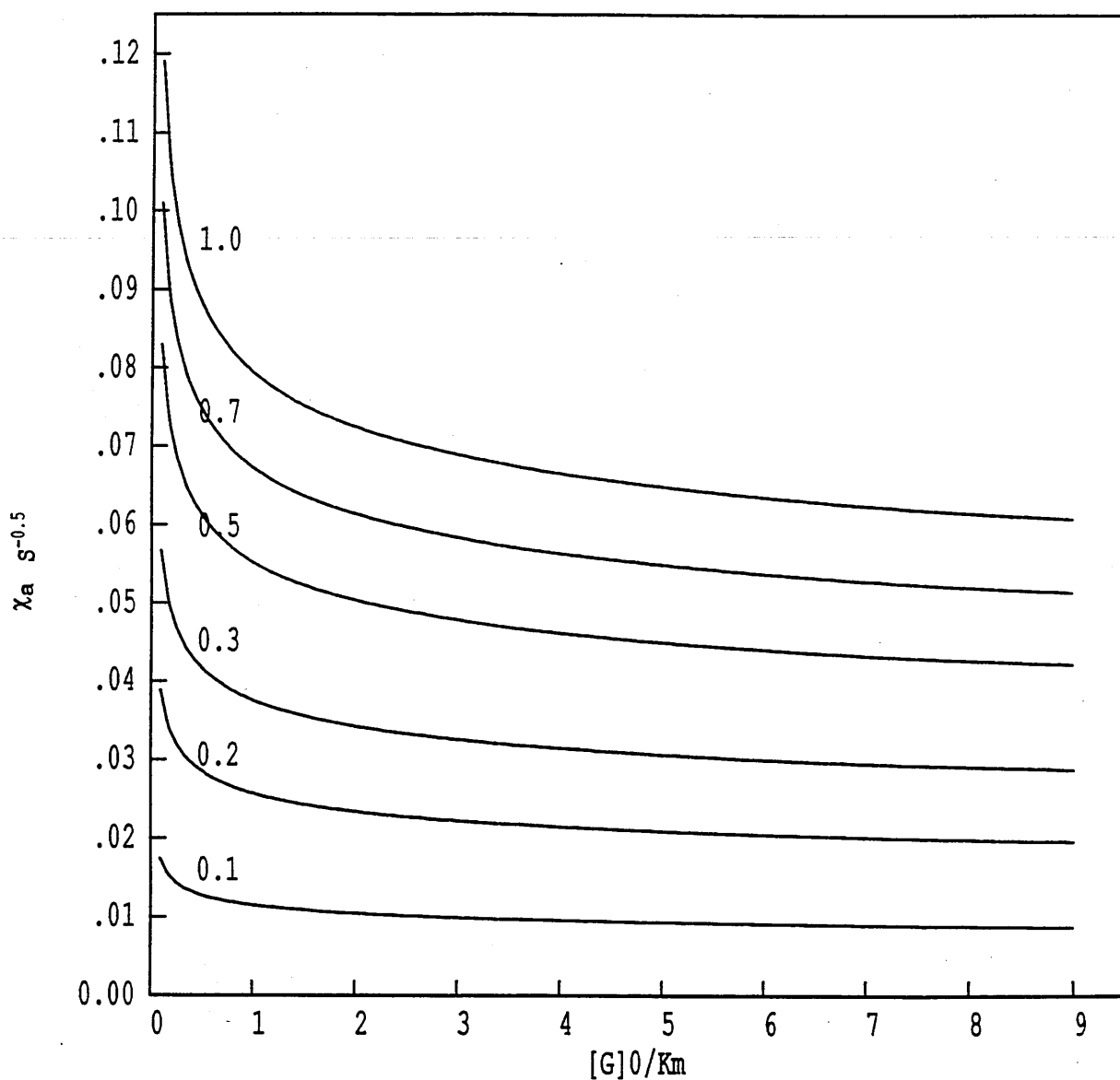


Figure III.3.15. The parameter χ_a vs. $[G]_0/K_m$ for $0.1 \leq \alpha \leq 1.0$. ($[E]=0.8$ moles m^{-3} , $30 \mu m \leq l \leq 100 \mu m$).

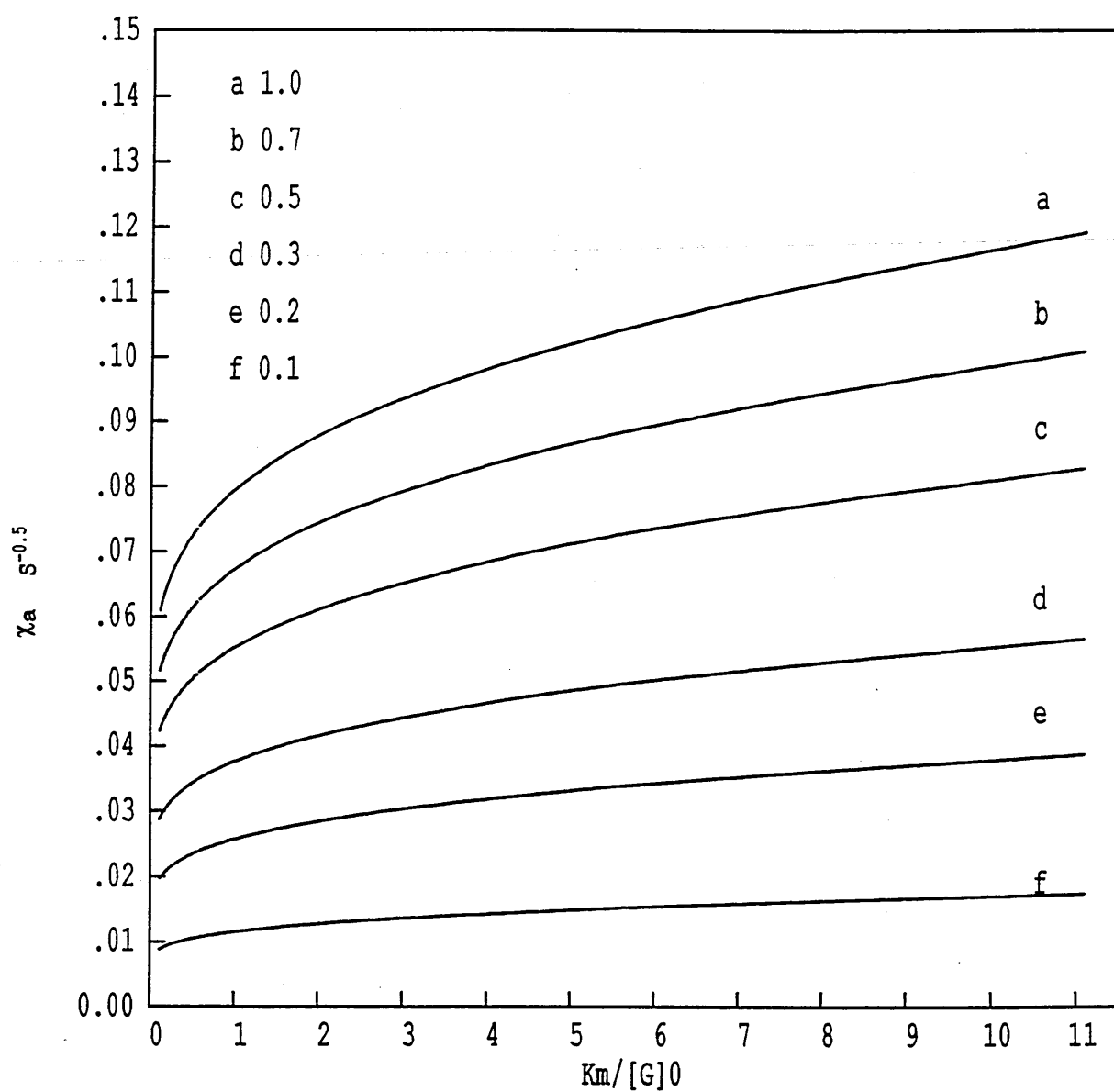


Figure III.3.16. The parameter χ_a vs. $K_m/[G]_0$ for $0.1 \leq \alpha \leq 1.0$ (a-f). ($[E]=0.8$ moles m^{-3} , $30 \mu m \leq l \leq 100 \mu m$)

stringent requirement that a constant relative error be maintained over the specified concentration range. The definition of $t_{[G]=0}$ (i.e. 10^{-2} mM at all values of $[G]_0$) used here is probably very stringent for the envisaged functions of the glucose-CFD in the context of the food industry.

Assessment of the dependency of reaction time on enzyme concentration in the catalytic layer. Considering that the initial concentration of glucose in the catalytic layer is zero, the greatest possible rate of decay of $[G]$ at $x=0$ would occur if the velocity of the oxidation of glucose by glucose oxidase was superior to the flux of glucose under this boundary condition i.e. the boundary condition for glucose concentration for $t > 0$ at l_{cat} would be $[G]_{l_{cat}}=0$ (Case A). This boundary condition is identical to that for reduced mediator at the surface of the electrode during the application of an oxidizing potential. The value for χ_n obtained for the system operating under these conditions may be compared to the value of χ_n obtained from simulations conducted using the more complex and variable boundary conditions at l_{cat} that arise throughout the reaction from the presence of the catalytic reaction (Case B, equation [III.3.40.]). Using a value for enzyme concentration of 0.8 moles m^{-3} the reaction rate due the enzyme catalysed reaction relative to the theoretical maximum reaction rate obtained using $[G]_{l_{cat}}=0$ was tested for $0.1 \leq \alpha \leq 1.0$, Figure III.3.17. The difference between the values of χ_n obtained at the two boundary conditions translates to a value of $t_{n[G]=0}$ of 27.2 s for Case A and 29.6 s for Case B (at $\alpha=1.0$ for $l=100 \text{ }\mu\text{m}$, $D_G=6.5 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$). Figure III.3.16. illustrates the dynamic nature of the flux equations which describe the operation of the catalytic phase: all reactions, at all levels of α , are a mixture of Case A and Case B behaviour. The point of transition between Case B, catalytically determined boundary conditions for glucose concentration at l_{cat} and flux to l_{cat} , and Case A, diffusion limited flux to l_{cat} , is dependent on the value of v in equation [III.3.40.], the initial concentration of β -D-glucose and the value of α . The value of χ_a for the two cases converges as $\alpha \rightarrow 0$, Figure III.3.17., with complete convergence at $\alpha=0.1$. Convergence would occur at higher values of α with an increase in $[E]$ and therefore v .

In electrode configurations involving the correlation of a current transient obtained from operation in conditions of semi-infinite solution it is usually advantageous in terms of linearisation of electrode response to maintain α such that Case A is predominant. In the absence of any chemical amplification of the signal this results in a decrease in the sensitivity of the response relative to the response obtained when Case B predominates. The CFD operates in a volume controlled micro-environment and therefore it is advantageous to operate under Case B in order to obtain the highest decay constant for flux of glucose at $x=0$. This implies the maximisation of α and $[E]$ are desirable for the minimisation of reaction duration.

The scheme considers only the reaction for the condition where the concentration of glucose in the catalytic layer is determined by a combination of the influences of k_2 and by the flux equation itself. For $[E] > 0.8 \text{ moles m}^{-3}$ the models constructed made the assumptions that the reaction rate due to k_1 was fast compared to the flux of glucose into the catalytic layer and that the reaction rate due to k_6 and

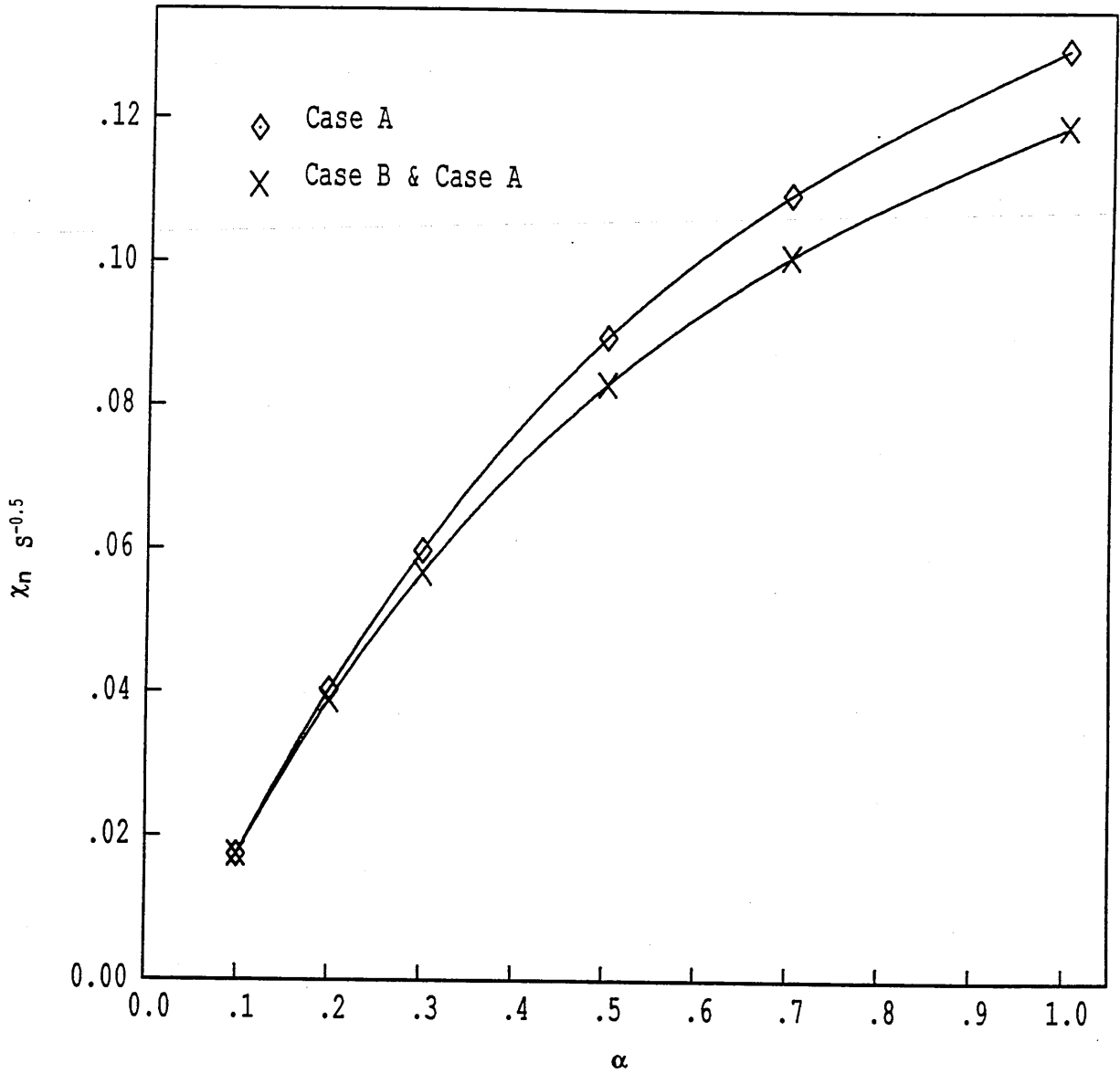


Figure III.3.17. The parameter χ_n , defined by the normalised concentration response, Figure III.3.9., vs. α at boundary conditions for the concentration of glucose at I_{cat} determined by the velocity of the enzyme catalysed reaction at $x \geq I_{cat}$ (Case B & Case A), and by a maximum theoretical reaction rate obtained for the boundary condition of $[G]_{I_{cat}}=0$ (Case A). ($[E]=0.8 \text{ moles m}^{-3}$, $30 \mu\text{m} \leq l \leq 100 \mu\text{m}$, $0.63 \text{ mM} \leq [G] \leq 63 \text{ mM}$).

the concentration of oxidized mediator throughout the device was also fast compared to the glucose flux. Consideration of the probable effect on reaction time of increasing the concentration of enzyme in the catalytic layer may be made using the models for $[E] \geq 0.8 \text{ moles m}^{-3}$, so that the assumptions regarding the contribution of k_1 were not invalidated. The parameter ρ , defined by the ratio of χ_n using the boundary condition of $[G]_{\text{cat}}=0$, designated χ_{n0} , and the value derived from the boundary conditions derived from the catalytic activity of the enzyme layer, designated χ_{nv} , is an index of the reaction duration relative to a theoretical maximum value, [III.3.58.].

$$\rho = \chi_{nv} / \chi_{n0} \quad \text{[III.3.58.]}$$

ρ was evaluated for $0.8 \leq [E] \leq 10 \text{ moles m}^{-3}$ for $\alpha=1$, Figure III.3.18.. For a tenfold increase in $[E]$ from 0.8 moles m^{-3} to 8 moles m^{-3} , ρ increases by $\sim 5\%$, equivalent to a decrease in $t_{n[G]=0}$ of about 2 s (29.6 s to 27.7 s: $l=100 \mu\text{m}$; $\alpha=1.0$; $0.63 \text{ mM} \leq [G] \leq 63 \text{ mM}$). From Figure III.3.18. It is apparent that, according to the model, the $[G]_{(0,t)}$ response is $\sim 93\%$ of the theoretical response, an increase in enzyme concentration becomes very cost ineffective for $[E] \geq 2 \text{ mM}$ (a change in $t_{n[G]=0}$ of 1.5 s; 29.6 s to 28.2 s). Considering the marginal effect of increasing the concentration of glucose oxidase in the catalytic layer on the reduction in $t_{n[G]=0}$, any attempts to reduce the duration of the catalytic phase of the analysis should concentrate on ensuring a consistently high value of α , and perhaps exploring manufacture of devices with reduced gap widths.

Concluding remarks. The use of mathematical models to simulate the operation of the catalytic phase of the analysis has illuminated which strategies should be avoided and which are the most promising in terms of a potential reduction in analysis duration.

(I) A significant reduction of the duration of the catalytic phase may not be achieved by the substitution of a mediator with a superior diffusion coefficient to ferricyanide, assuming equivalent fractional diffusion coefficients in the catalytic layer. It is also unlikely that the substitution of compounds which exhibit a higher k_0 value for the oxidation of reduced glucose oxidase would achieve the stated objective.

(II) Any increase in the concentration of glucose oxidase in the catalytic layer is unlikely to be cost-effective.

(III) When operating using the present configuration, with the electrode reaction situated on the opposite side of the cell to the catalytic reaction, it is important to maximise α . In order to achieve complete depletion within 60 s using $t_{[G]=0}$, α must be maintained in the range $0.5 \leq \alpha \leq 1.0$ for $0.63 \text{ mM} < [G]_0 \leq 63 \text{ mM}$. Any further reduction in the catalytic phase as dictated by $t_{[G]=0}$ may be achieved

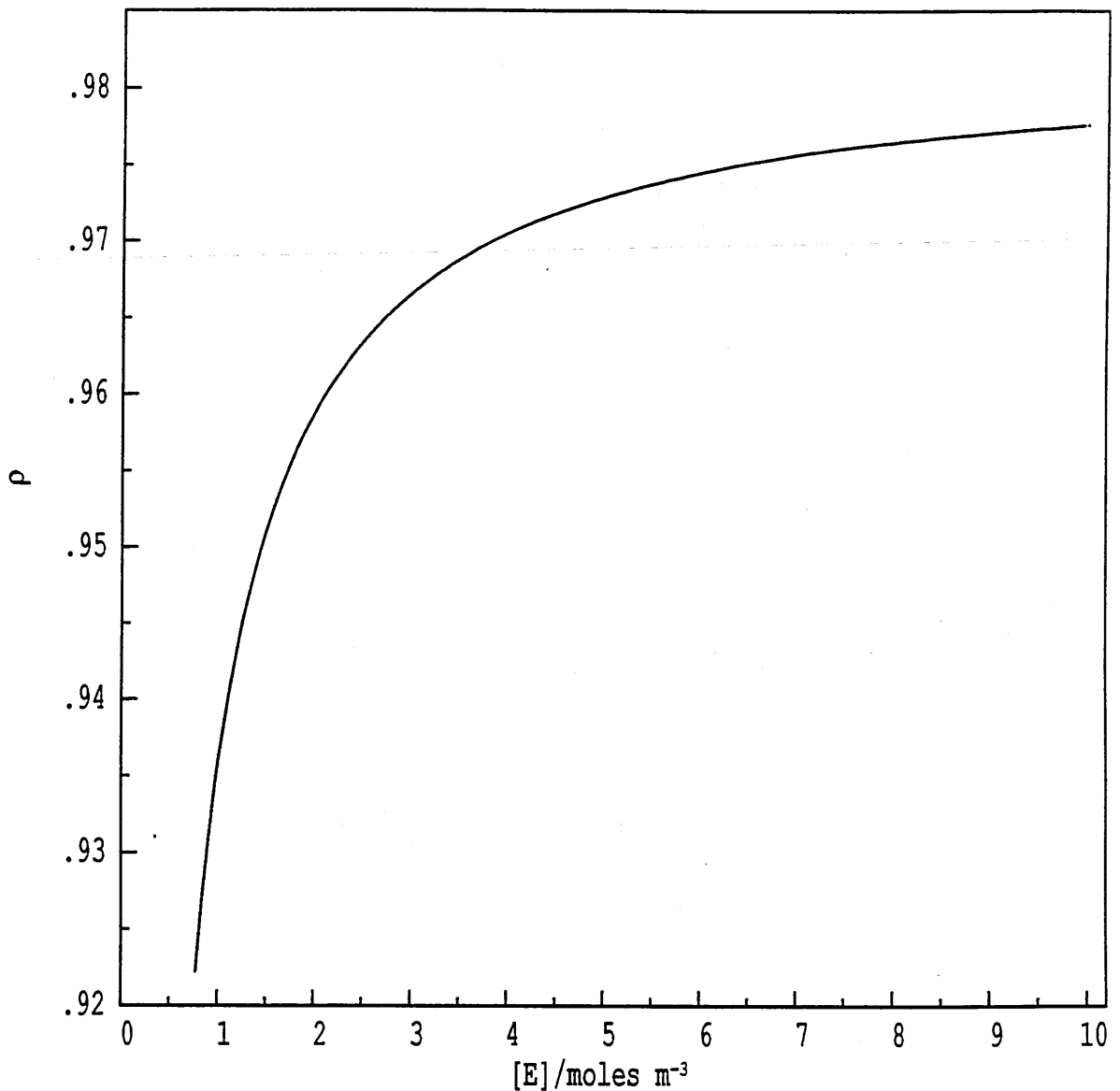


Figure III.3.18. The parameter ρ vs. $[E]$ for the normalised concentration response, Figure III.3.9.. ρ is an index of the time taken to complete the reaction at boundary conditions for the concentration of glucose at l_{cat} determined by the velocity of the enzyme catalysed reaction at $x \geq l_{\text{cat}}$ (Case B & Case A) relative to a maximum theoretical reaction rate obtained for the boundary condition of $[G]_{l_{\text{cat}}=0}$ (Case A). ($0.8 \text{ mM} \leq [E] \leq 10 \text{ mM}$, $30 \text{ }\mu\text{m} \leq l \leq 100 \text{ }\mu\text{m}$, $0.63 \text{ mM} \leq [G] \leq 63 \text{ mM}$).

$$(\rho = \chi_{nv} / \chi_{n0}).$$

using a more stringent range for α , or by lowering the upper limit of detection for linear response.

(iv) The potential effect on a reduction of analysis time of reducing the gap width of the device is significant. Using Figure III.3.15., a change in overall gap width from 100 μm to 50 μm has benefits in terms of a reduction in reaction time; for 63 mM β -D-glucose, $t_{[G]=0}$ changes from 56 s to 26 s at $\alpha=1.0$, and from 123 s to 55 s at $\alpha=0.3$. This benefit has two manifestations; firstly, a direct reduction in time necessary to complete the catalytic phase; secondly, for any defined analysis time a greater robustness of response given variations in the viscosity between samples, or variations in the compositions of the catalytic layer between batches, due to the insensitivity of the response for $0.3 \leq \alpha \leq 1.0$ ($[G]_0=63$ mM, response time < 60 s). If the precision in the manufacturing technique that defines the gap width remained unchanged, the cost of reducing the gap width would be apparent in terms of a greater absolute error in the determination of glucose concentration.

In order to test the models for glucose and reduced mediator flux, and the assumptions of the controlling rate constant in the enzyme layer, it would be necessary to construct CFD devices with the catalytic layer printed over the electrode surface and to modify the models to give a current response at $x=l$. This could be achieved by setting a boundary condition of $[M_r]_{(l,t)}=0$ in the same fashion as in Section III.3.1., and to operate the model potential and the device potential from $t>0$. Although the current responses obtained in this way would be different to the transients obtained from the CFD in the present mode of operation, achieving a high degree of correspondence between the model and device responses would enable a more confident assessment of the validity of the assumptions used to construct the models. Nevertheless this exercise has illuminated the limited scope for a reduction in analysis time by manipulation of the configuration of the catalytic layer.

III.4.1. PROCEDURES USED TO GENERATE SYSTEM AND MODEL CURRENT-TIME AND CHARGE-PULSE RESPONSES.

Equipment. Capillary fill devices as described in Chapter I were used throughout. Section III.4.4. comprises a comparison of a coulometric determination of the concentration of glucose with the pulse-relaxation method. Manufactured glucose-CFDs were used throughout this investigation, other experiments were conducted using CFDs constructed without a catalytic layer. The potential at the working electrode was controlled by a program written in 'C' through an 'Artek' interface (Artek, 8D Marsh, Carlton, Bedfordshire, UK). Current data points were sampled at a rate of 200 s^{-1} . The charge over the period of potential pulse was then calculated by the same rationale as described for the simulation charge (Section III.3.1.). The charge values for each pulse were stored in the form of ASCII files on a floppy disc. The program allowed the selection of potential pulse and relaxation periods of different durations and up to 20 pulse-relaxation cycles. The charge response of the system incorporating the 'Artek' interface was correlated against the charge responses derived from measurements made upon identical solutions using the PSTAT10 potentiostat with GPES3 electrochemical software (eco chemie, Slough, Berkshire). Finite difference programs incorporating the expressions developed in Section III.3.1. were written in BASIC. The data acquisition program & the simulation programs were executed on a PC with a 286 microprocessor and a maths co-processor.

Chemicals &electrochemicals. Potassium hexacyanoferrate (III/II) were obtained from the Aldrich Chemical Co., and were used at 'Analar' grade. The materials used in the construction of the glucose-CFD were as described in Section I.4.4..

III.4.2. THE CORRELATION OF SIMULATION RESPONSES AGAINST CURRENT TIME TRANSIENTS AND THE CORRELATION OF THE CHARGE RESPONSE OBTAINED FROM THE ARTEK INTERFACE WITH THAT OBTAINED FROM THE PSTAT10 POTENTIOSTAT.

A series of solutions containing ferrocyanide and ferricyanide simulating the working system of the glucose CFD after the completion of the catalytic reactions were used to assess the efficiency of the model at simulating the current transients produced in the working system and to correlate the integrated charge values produced by the interface to the values derived from a commercially available system, Section III.4.1.. The concentration of ferrocyanide was varied between 0 mM and 40 mM and the concentration of ferricyanide was varied between 0.3 M and 0.26 M to give a total concentration of 0.3 M. The guard electrode was inactivated in all experiments and was passive in all simulations.

Figures III.4.1. to III.4.4. show typical results obtained for a 1 s potential pulse at 0.2 V applied to solutions containing 5, 10, 20, and 40 mM of ferrocyanide respectively. The results of conducting a simulation using 100 time iterations, the same rate at which data was acquired by the interface in this case, and a diffusion coefficient of $6.2 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ are shown at the appropriate concentrations.

Figures III.4.5. to III.4.8. show the current response of the system and the model plotted against $t^{-0.5}$ and Figures III.4.9. to III.4.12. show the current values at each time of the model and the system plotted against each other.

On inspection of the series of plots at each concentration it is immediately apparent that the model seems to be a good representation of the system at concentrations up to about 20 mM but between 20 mM and 40 mM ferrocyanide there is some non-linear behaviour of initial current response. The least squares linear regression of system current response against model current response (lines) tends towards one only after about 0.7 s at 40 mM ferrocyanide while at lower concentrations the correspondence occurs after about 0.2 s or less, Figures III.4.9. to III.4.12.. This cannot be explained in terms of an overall concentration dependent diffusion coefficient where the diffusion coefficient is some inverse function of concentration since this would merely result in a reduction of the current at all durations of the pulse, notice that at 40 mM ferrocyanide the model and system responses do show a high degree of correspondence after 0.8 s.

Figure III.4.13. shows the model charge response and the system charge responses for the Artek interface and the PSTAT10 potentiostat obtained for a pulse of duration 1 s. The model used a diffusion coefficient for ferricyanide/ferrocyanide of $6.2 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ and a time interval of 0.01 s. The increasing deviation of the model response from the system responses at concentrations of ferrocyanide above about 20 mM may be due to the limitations of both of the interfaces to recognise high currents at early times after the stepping of the potential pulse. The system response in Figure III.4.13. comprises the data from both interfaces; there is a high degree of correspondence between

the two, the lines on either side of the symbols represent 95% confidence limits for data from the combined sources.

The sensitivity of the model charge response to the value of the diffusion coefficient was investigated for $1 \times 10^{-10} \text{ m}^2 \text{ s}^{-1} \leq D \leq 10 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$. Figure III.4.14. shows the normalised deviation from the model charge response obtained using a diffusion coefficient of $6.2 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$. Since all of the model parameters are constructed on the basis of the user supplied diffusion coefficient Figure III.4.14. represents a complete description of the sensitivity of the model to the input variables, above a certain number of time iterations ($\sim 100 \text{ s}^{-1}$, or a time interval of 0.01 s) all of the model responses converge.

Figure III.4.1. Simulation and system current transients obtained for a 1 s potential pulse in a solution containing 5 mM ferrocyanide.

Figure III.4.2. Simulation and system current transients obtained for a 1 s potential pulse in a solution containing 10 mM ferrocyanide.

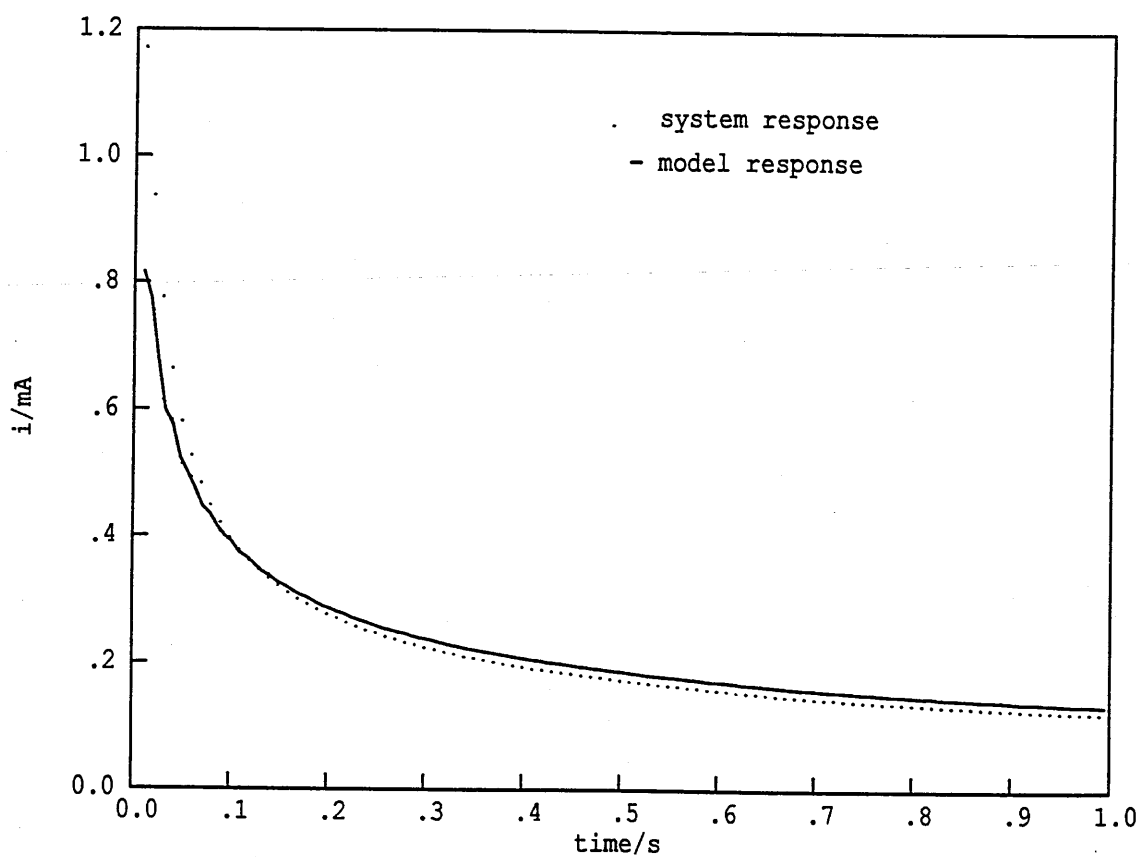
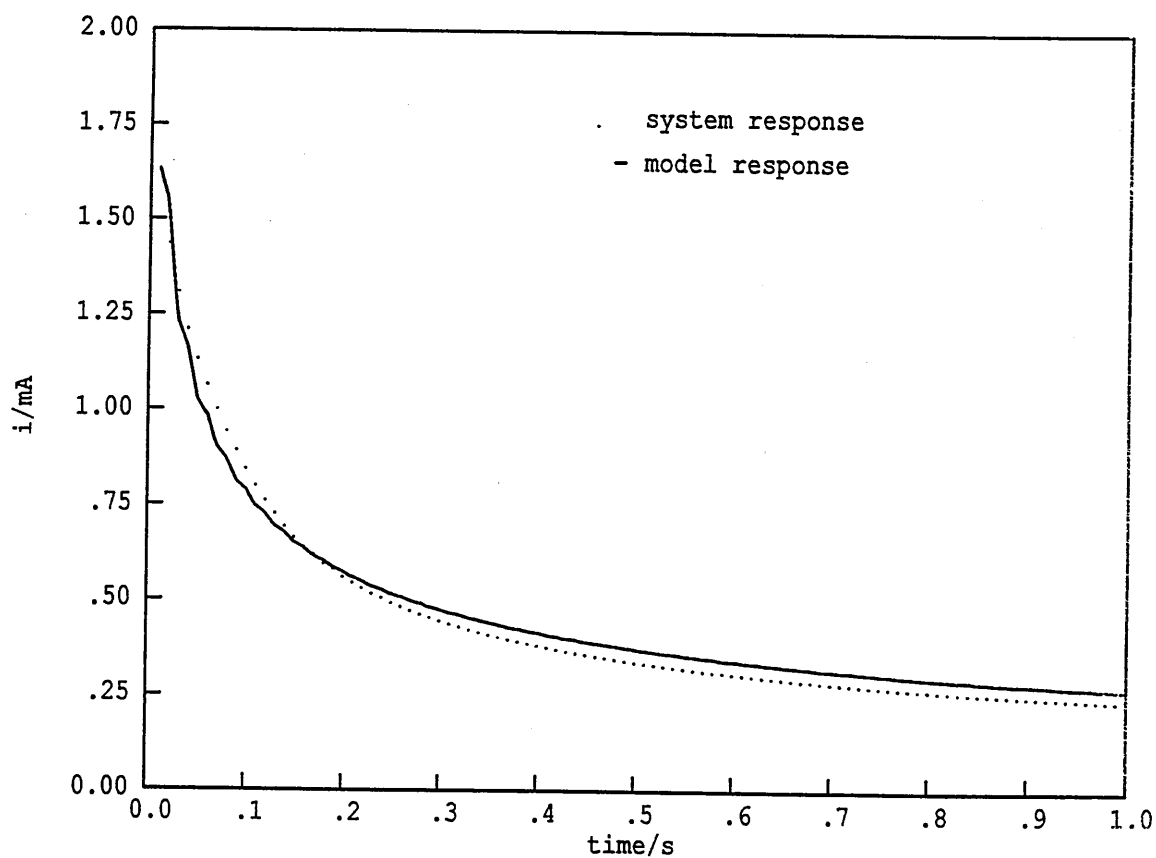


Figure III.4.3. Simulation and system current transients obtained for a 1 s potential pulse in a solution containing 20 mM ferrocyanide.

Figure III.4.4. Simulation and system current transients obtained for a 1 s potential pulse in a solution containing 40 mM ferrocyanide.

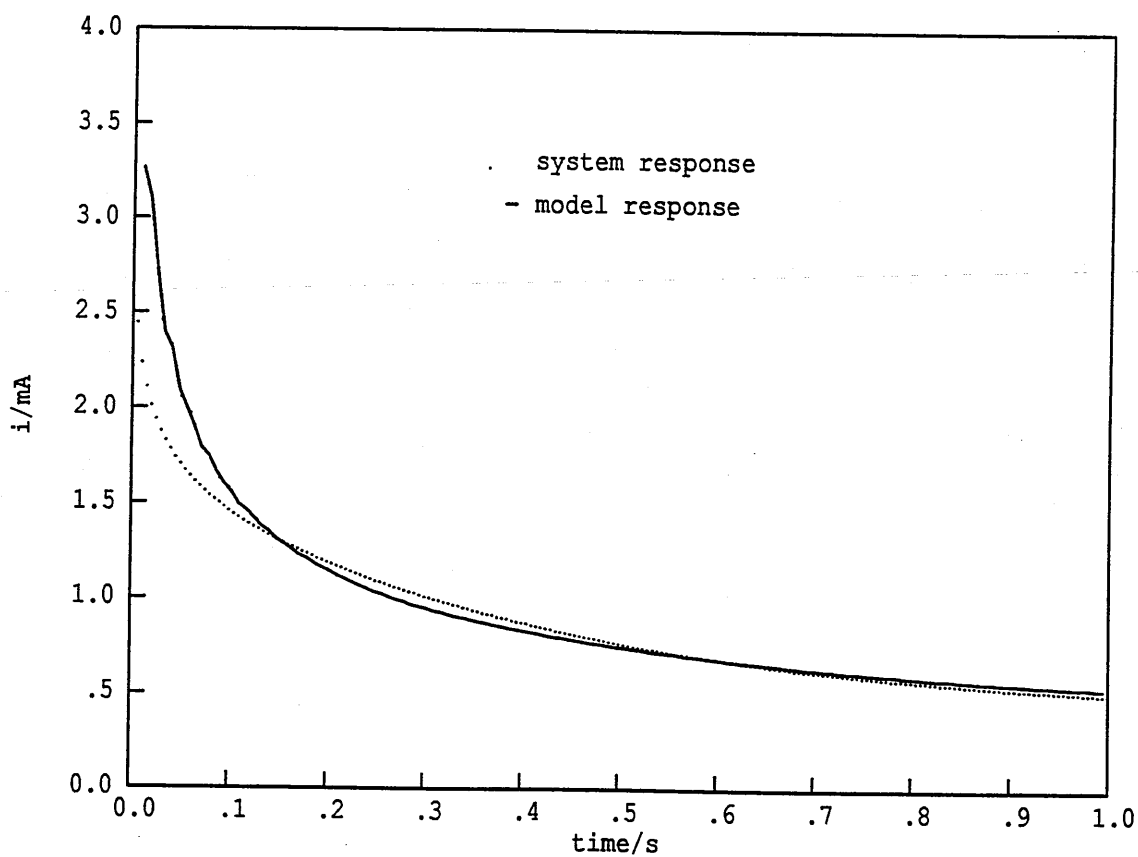
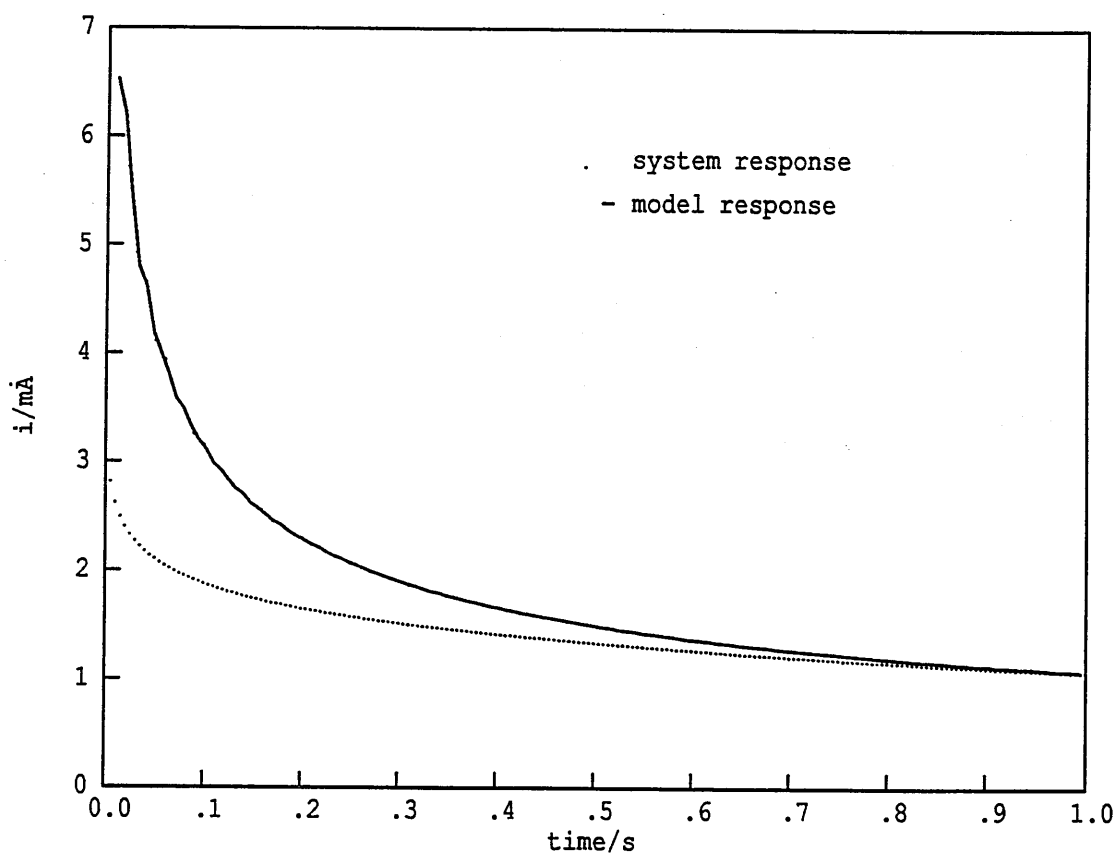


Figure III.4.5. Simulation and system current responses vs. $t^{-0.5}$ obtained for a 1 s potential pulse in a solution containing 5 mM ferrocyanide (*cf.* Figure III.4.1.).

Figure III.4.6. Simulation and system current responses vs. $t^{-0.5}$ obtained for a 1 s potential pulse in a solution containing 10 mM ferrocyanide (*cf.* Figure III.4.2.).

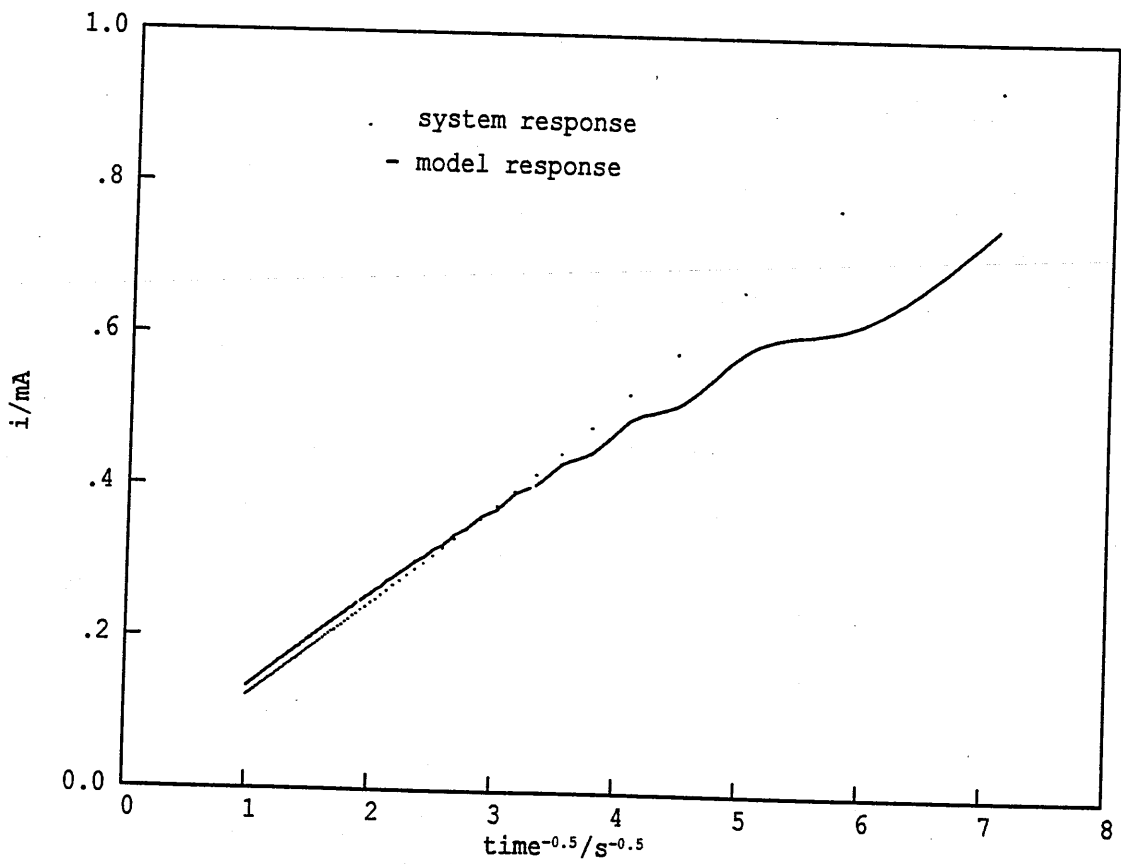
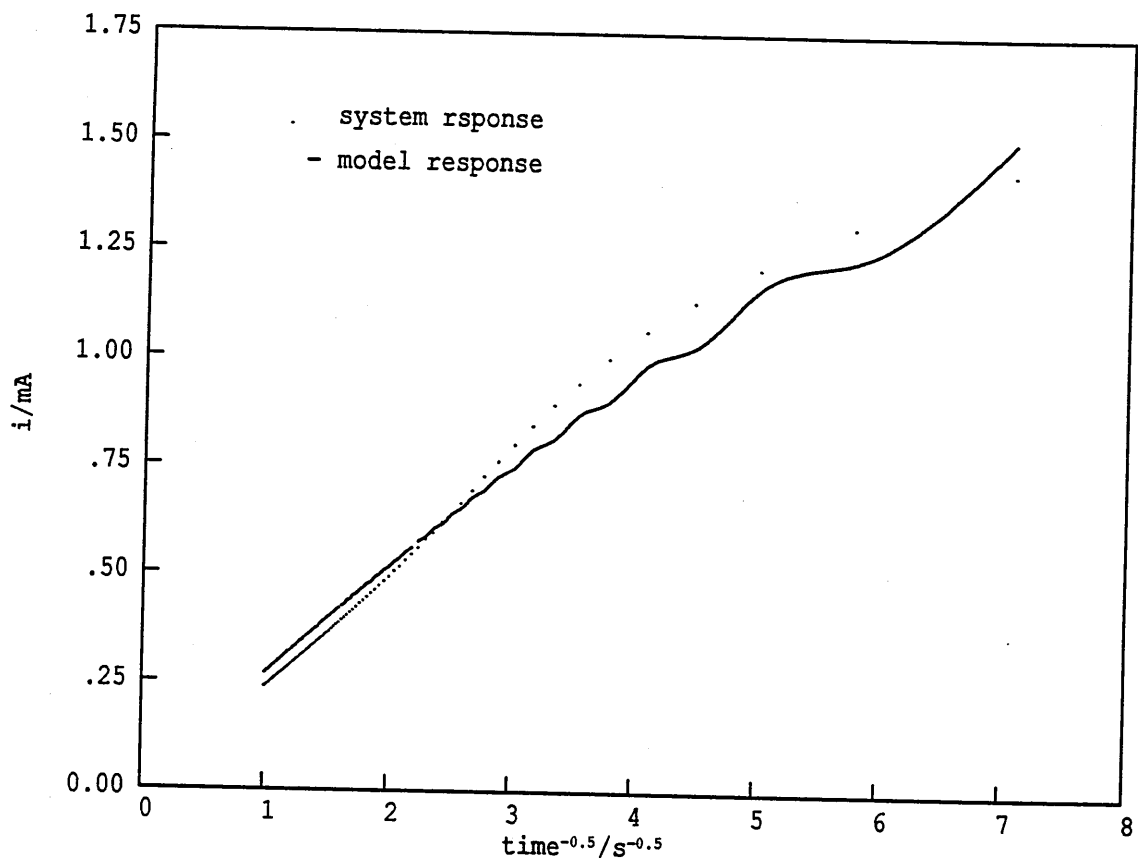


Figure III.4.7. Simulation and system current responses vs. $t^{-0.5}$ obtained for a 1 s potential pulse in a solution containing 20 mM ferrocyanide (cf. Figure III.4.3.).

Figure III.4.8. Simulation and system current responses vs. $t^{-0.5}$ obtained for a 1 s potential pulse in a solution containing 40 mM ferrocyanide (cf. Figure III.4.4.).

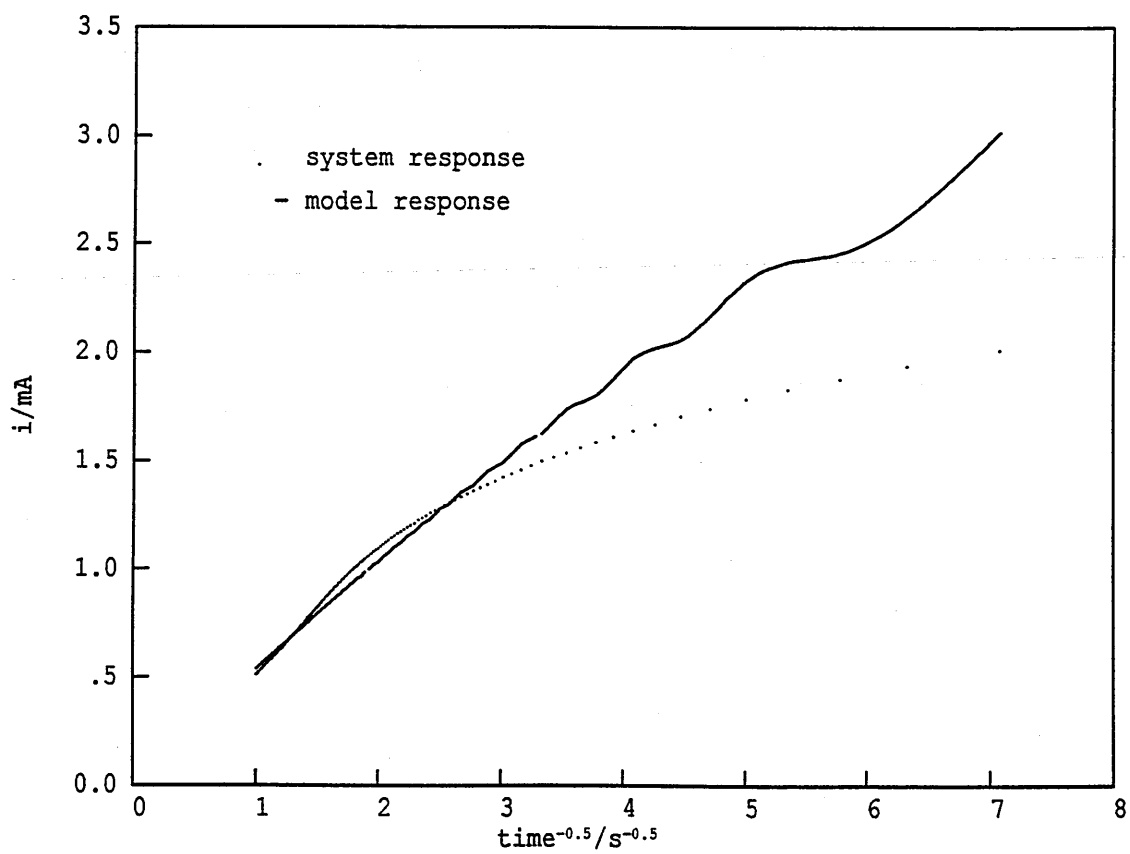
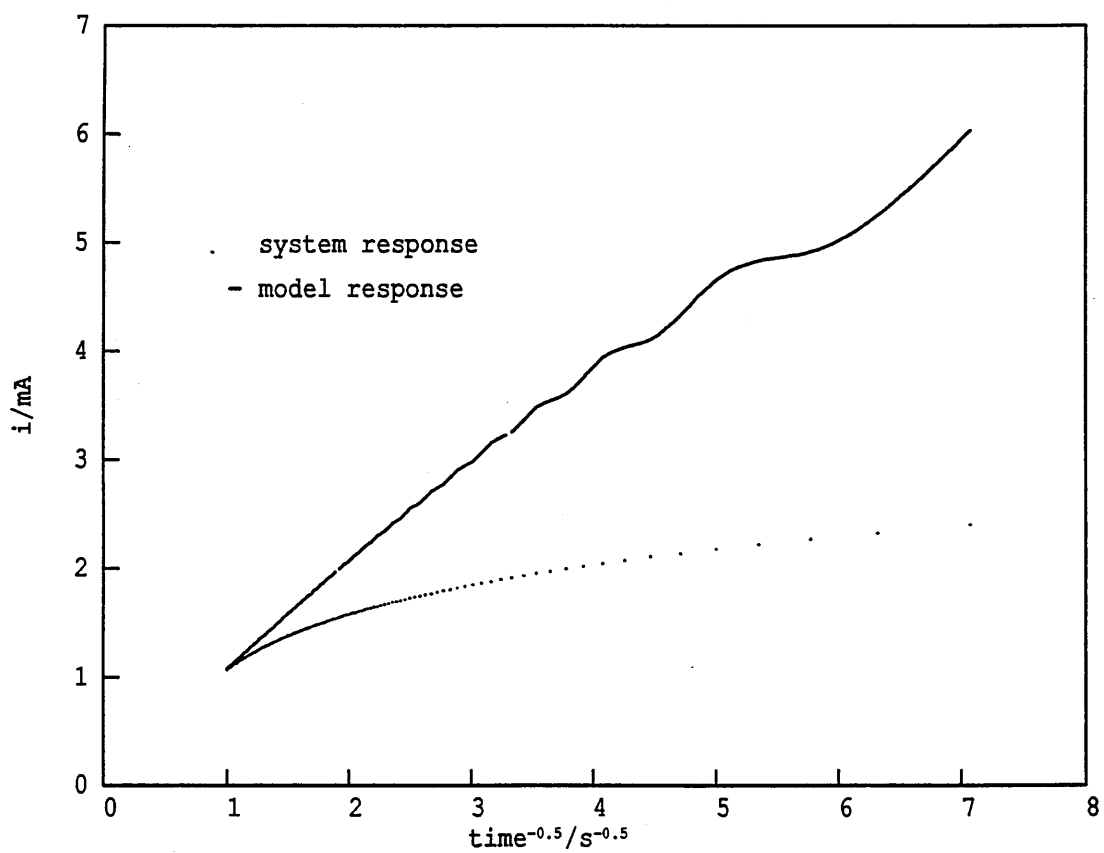


Figure III.4.9. Simulation vs. system current responses at equivalent time values obtained for a 1 s potential pulse in a solution containing 5 mM ferrocyanide (*cf.* Figure III.4.1.).

Figure III.4.10. Simulation vs. system current responses at equivalent time values obtained for a 1 s potential pulse in a solution containing 10 mM ferrocyanide (*cf.* Figure III.4.2.).

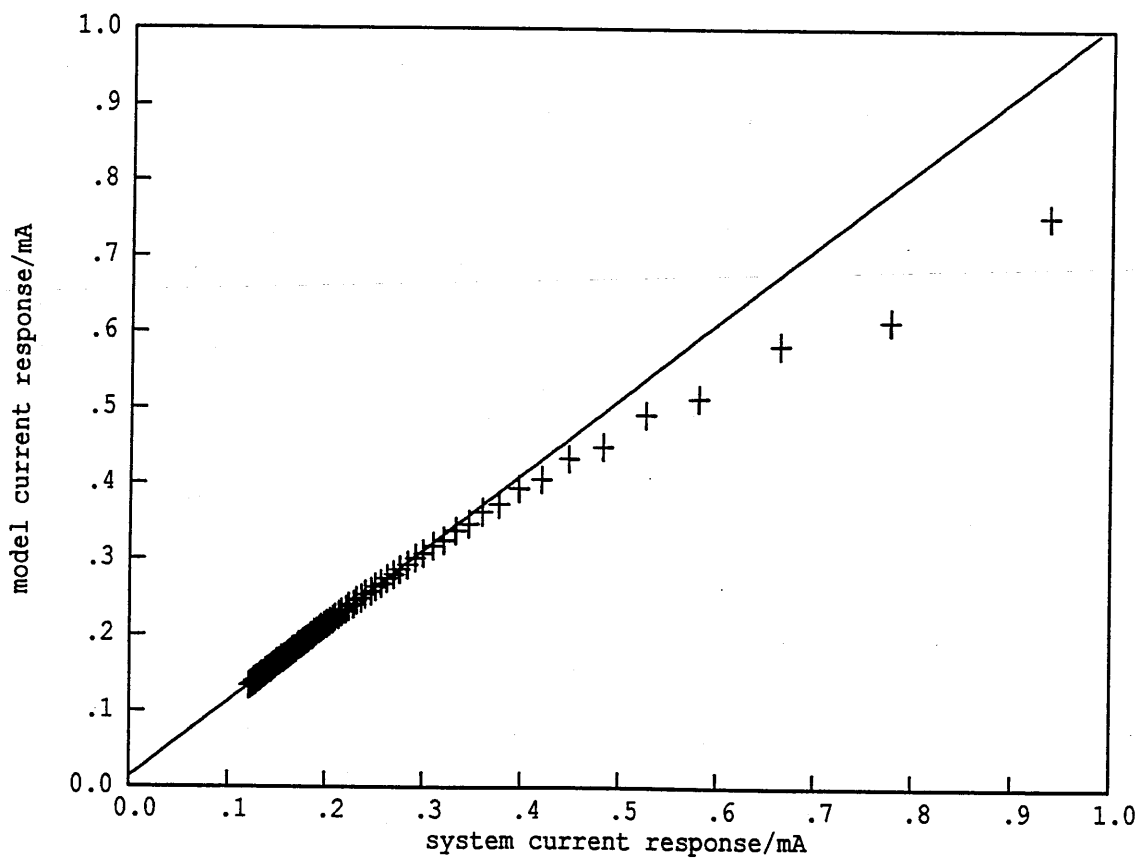
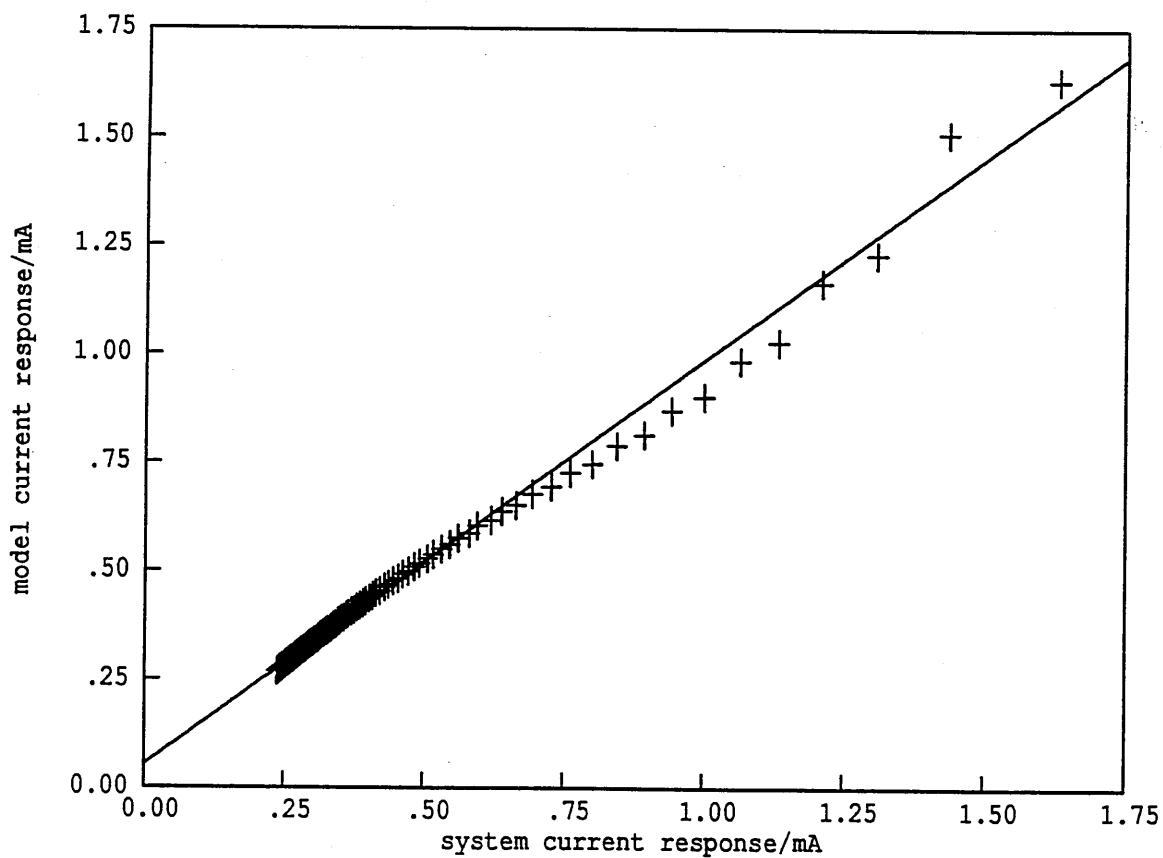
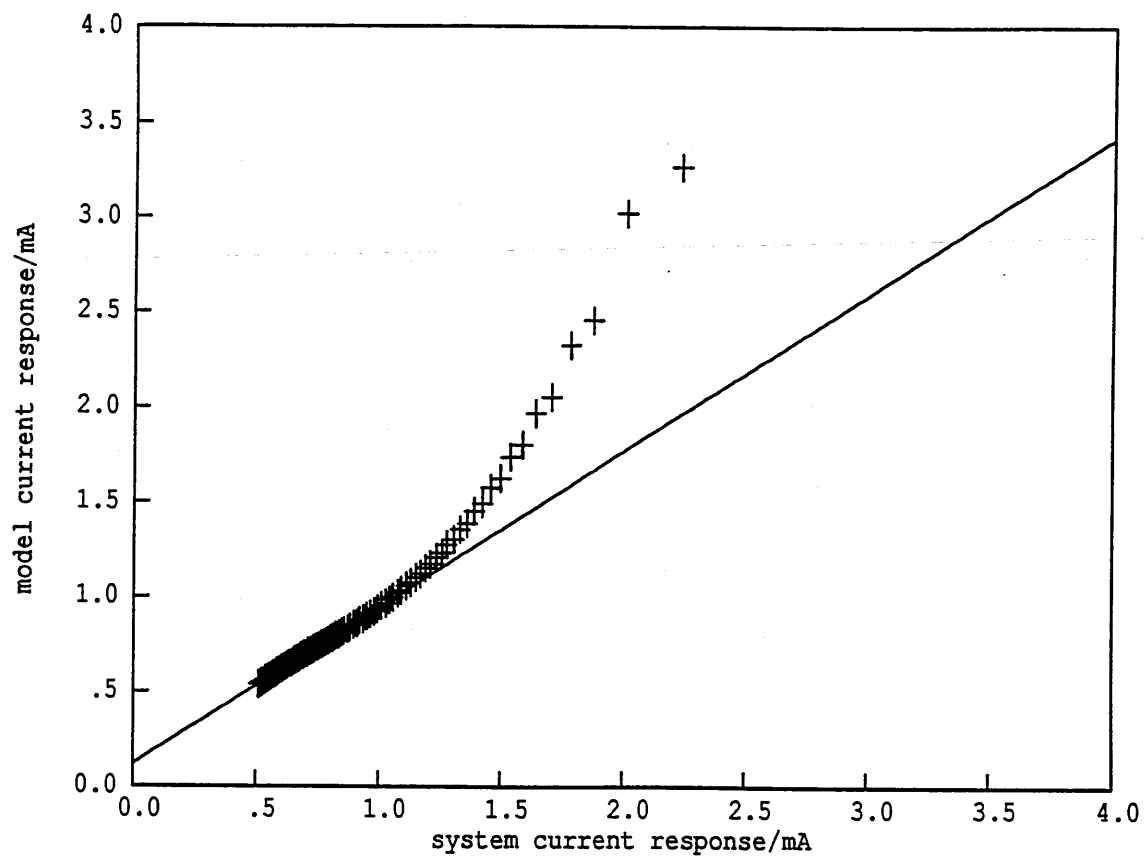
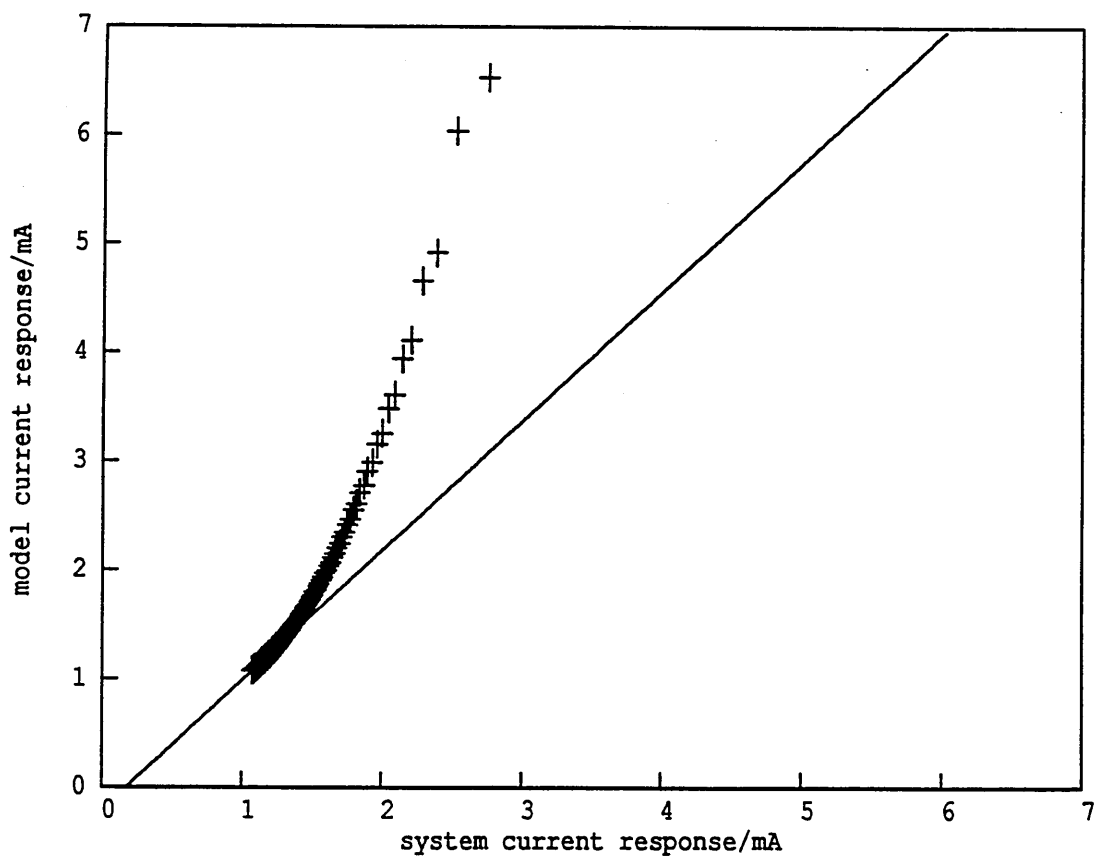


Figure III.4.11. Simulation vs. system current responses at equivalent time values obtained for a 1 s potential pulse in a solution containing 20 mM ferrocyanide (cf. Figure III.4.3.).

Figure III.4.12. Simulation vs. system current responses at equivalent time values obtained for a 1 s potential pulse in a solution containing 40 mM ferrocyanide (cf. Figure III.4.4.).



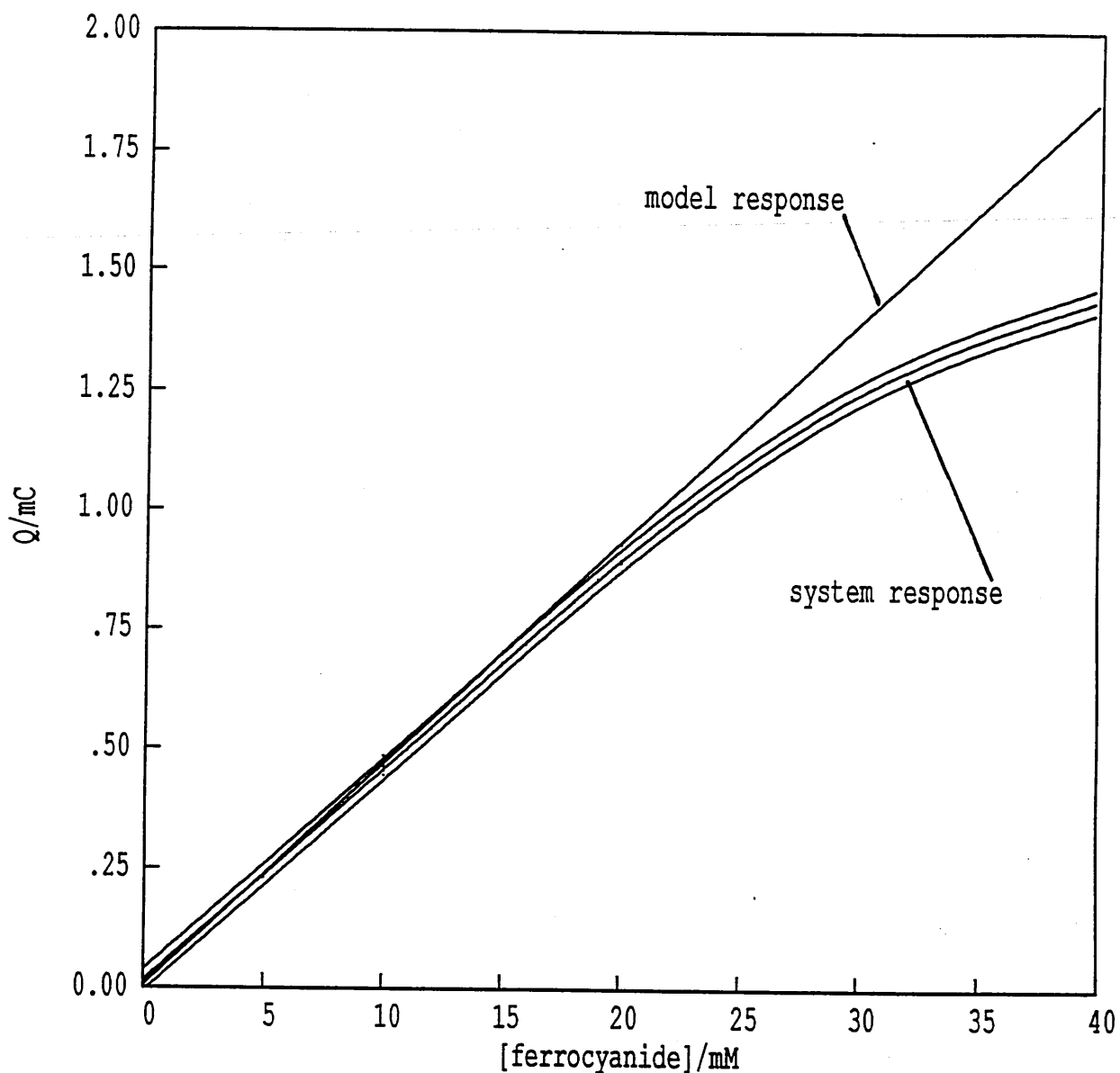


Figure III.4.13. The integrated current response of data derived from the Artek interface, the PSTAT10 potentiostat and the simulation. From Figure III.4.13. it is clear that the data derived from the Artek interface and the PSTAT10 potentiostat show a high degree of correspondence (the lines surrounding the system response represent 95% confidence limits for pooled charge responses from the PSTAT10 and the Artek interface) while both deviate from the simulation response between 20 mM and 40 mM ferrocyanide.

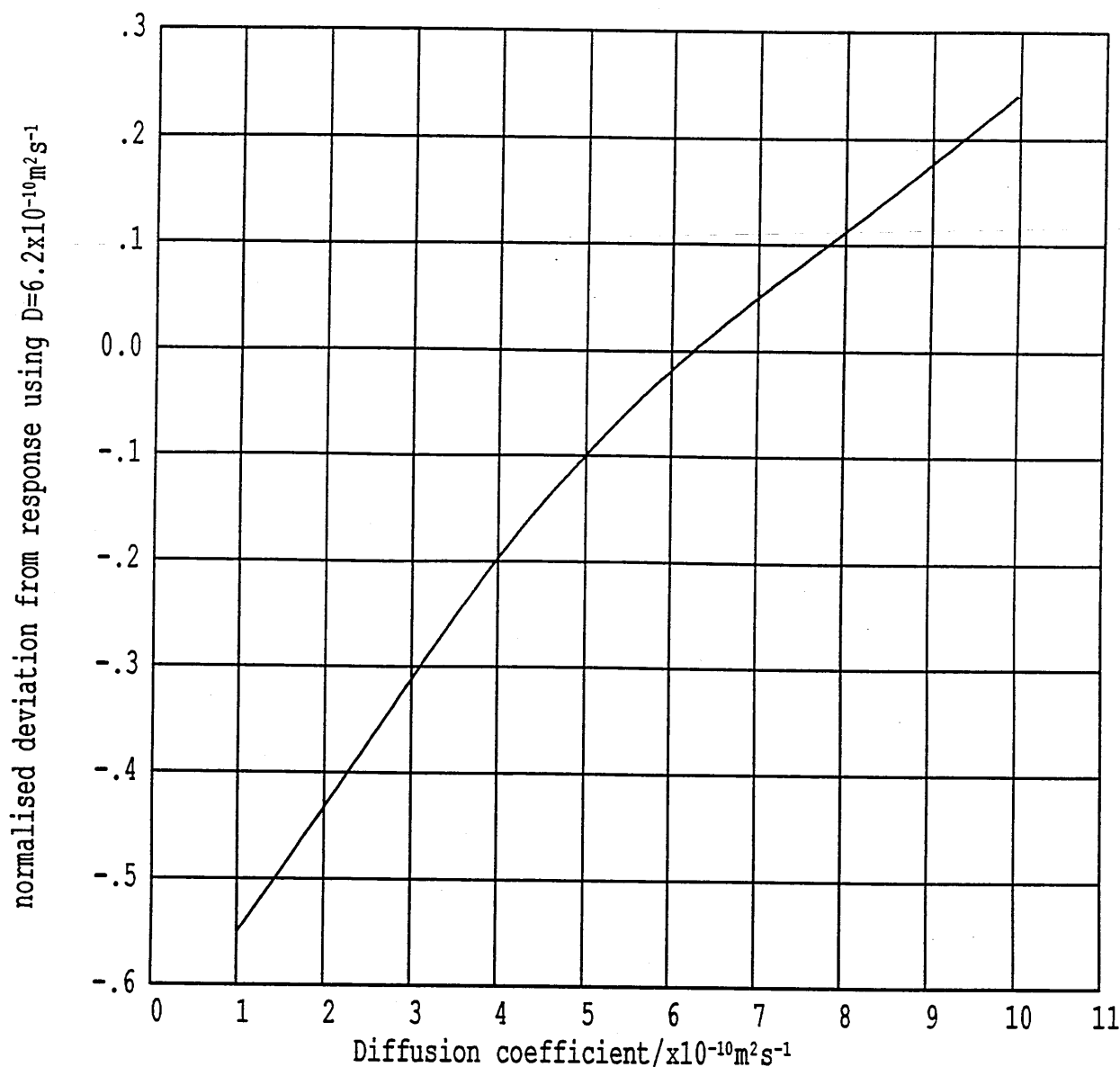


Figure III.4.14. The sensitivity of the model charge response to the value of the diffusion coefficient for $1 \times 10^{-10} \text{ m}^2 \text{ s}^{-1} \leq D \leq 10 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ represented by the normalised deviation in charge response from the model charge response obtained using a diffusion coefficient of $6.2 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$.

III.4.3. THE CORRELATION OF SIMULATION RESPONSE AGAINST CHARGE PULSE DATA DERIVED FROM SOLUTIONS CONTAINING FERROCYANIDE.

A series of eight potential pulses and relaxation periods each of duration 1 s were applied to the working electrode of CFDs containing solutions of ferrocyanide and ferricyanide. The concentration of ferrocyanide was varied between 0 mM and 40 mM and the concentration of ferricyanide was adjusted to give a total concentration of the two forms of 0.3 M. The working electrode was poised at 0.2 V. The guard electrode remained passive throughout the experiments and their simulations.

Figure III.4.15. shows the simulation response over the series of pulses at different concentrations of ferrocyanide using a diffusion coefficient of $6.2 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ and 100 time iterations. Figure III.4.16. shows the system response at different concentrations of ferrocyanide over eight pulse and relaxation periods. The principle of using a series of potential pulses and relaxation periods as discussed in Section III.1. predicts an exponential decrease in charge with respect to pulse number. This relationship is not supported by either the experimental data nor the results derived from the numerical simulation. Figure III.4.17. shows a comparison between the numerically derived results and the system response and the predicted charge-pulse response for an initial concentration of ferrocyanide of 10 mM using the integrated Cottrell equation and some of the equations developed in Section III.1.. The application of a least squares exponential regression to all of the data derived from the analytical solution yields a correlation coefficient of one. This is equivalent to a least squares linear regression of logarithmic charge values with respect to pulse number and has the form $Q_p = Ae^{\beta p}$, where Q_p is the charge passed over the p^{th} pulse, β is the exponential constant and A is the intercept at $p=0$.

The same analysis cannot be applied to the system response and the simulation response due to the disproportionately high magnitude of the charge passed over the first pulse compared to the subsequent pulses. The arguments generated in Section III.1. assumed that during the relaxation period the mediator attained an homogeneous concentration across the cell. An examination of simulation generated diffusion profiles of reduced mediator in the x-dimension between during the first pulse and during the first relaxation period reveals that an homogeneous concentration is not achieved at the end of the relaxation period, Figures III.4.18. and III.4.19. respectively. It is reasonable to assume that these profiles are good representations of the actual state of the system at any time since there is a high degree of correspondence between the simulation response and the system response with respect to the current transient during the pulse and the charge passed over that pulse. Examination of the diffusion profiles generated on subsequent pulse and relaxation periods reveals that after the first pulse the system attains a stable oscillatory state with respect to the concentration of reduced mediator in the x-dimension. Figures III.4.20. and III.4.21. show the

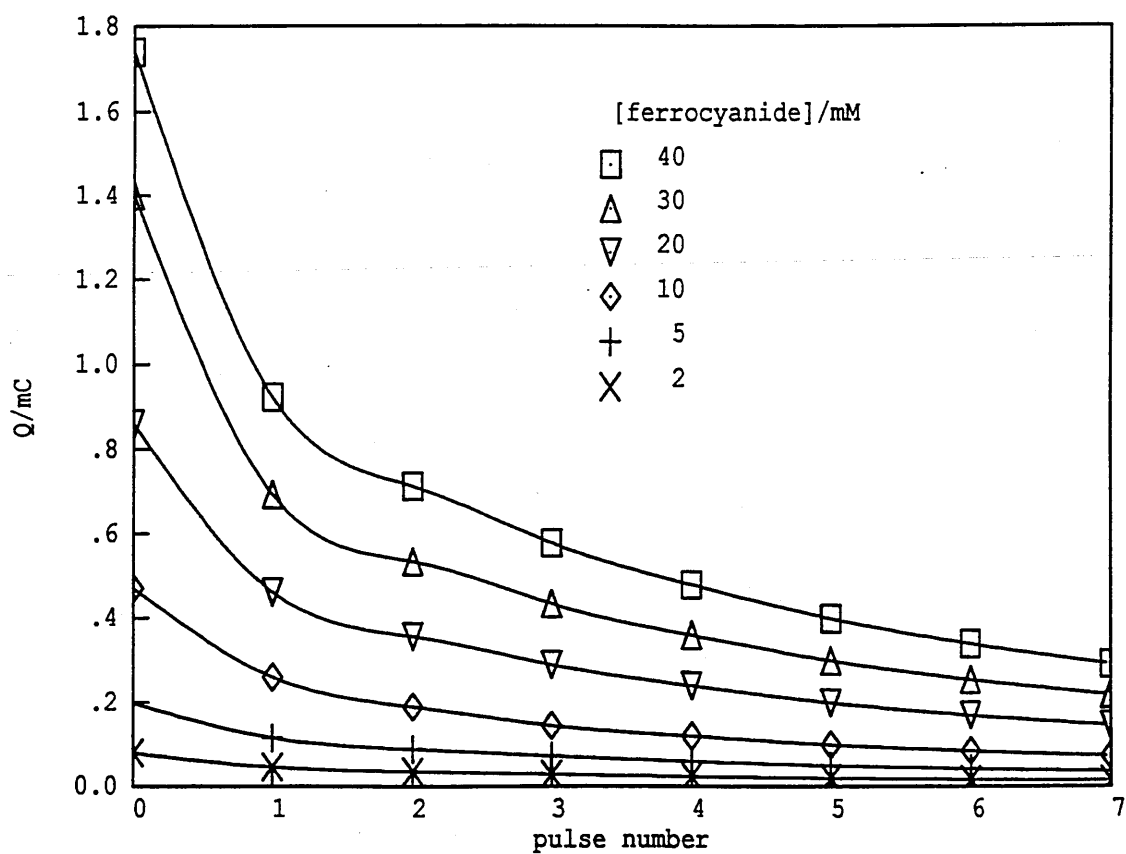
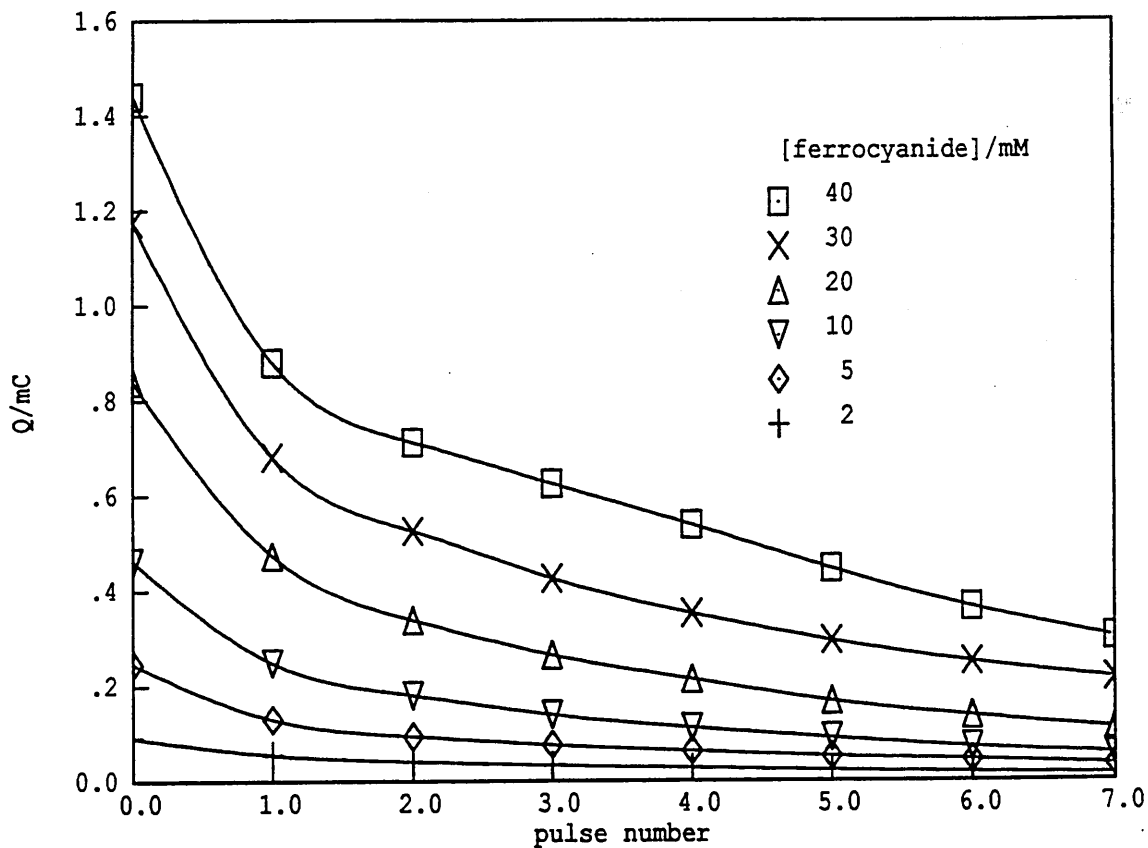
diffusion profiles for the reduced mediator in the x-dimension during the third and fourth relaxation periods.

The failure of the system to attain equilibrium over the same duration that the perturbation was applied may be explained in terms of the flux equation. Neglecting lateral diffusion, for the duration of the applied potential pulse the system would only approach a diffusion equilibrium when all of the reduced mediator had become oxidized, the situation that exists when conducting coulometry in a CFD. Since the flux between adjacent volume elements is proportional to the difference in the concentrations present in those elements it is self evident that the flux at the electrode during the relaxation period is always less in magnitude than the flux during the application of the potential pulse. This may be practically demonstrated by changing the ratio of relaxation period to pulse period. Figure III.4.22. shows the charge response at a concentration of ferrocyanide 10 mM for a pulse duration of one second and a relaxation duration of four seconds. The charge-pulse response does not conform to the prediction of total relaxation, Figure III.4.22., and examination of the diffusion profiles derived from the simulation response show that the relaxation state prior to the application of the second pulse is not in equilibrium, Figure III.4.23. In terms of a reduction of the analysis time an increase of the relaxation period would be counter productive.

The non-linearity of the charge response for the first potential pulse with respect to concentration is not carried over into subsequent pulses, even at a concentration of 40 mM ferrocyanide. The application of the exponential least squares fitting routine to the charge-pulse data in Figure III.4.16. neglecting the first two pulses, results in a correlation coefficient of greater than 0.998 for all the concentration values, Figure III.4.24.. The original concentration may be correlated to the value of the constant A, calculated at each concentration, Figure III.4.25.. The correlation has a straight line and an intercept very close to zero. This may be taken as the basis for making a correlation between charge passed over a series of pulses and the concentration of ferrocyanide present.

Figure III.4.15. The simulated charge response for model solutions with different model concentrations of ferrocyanide for a pulsed potential-relaxation system where the pulse duration and relaxation duration periods were equal at 1 s. The model incorporated a diffusion coefficient for ferrocyanide of $6.2 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$.

Figure III.4.16. The charge response for solutions of ferricyanide containing ferrocyanide at concentrations between 2 and 40 mM, (PD=RD=1 s).



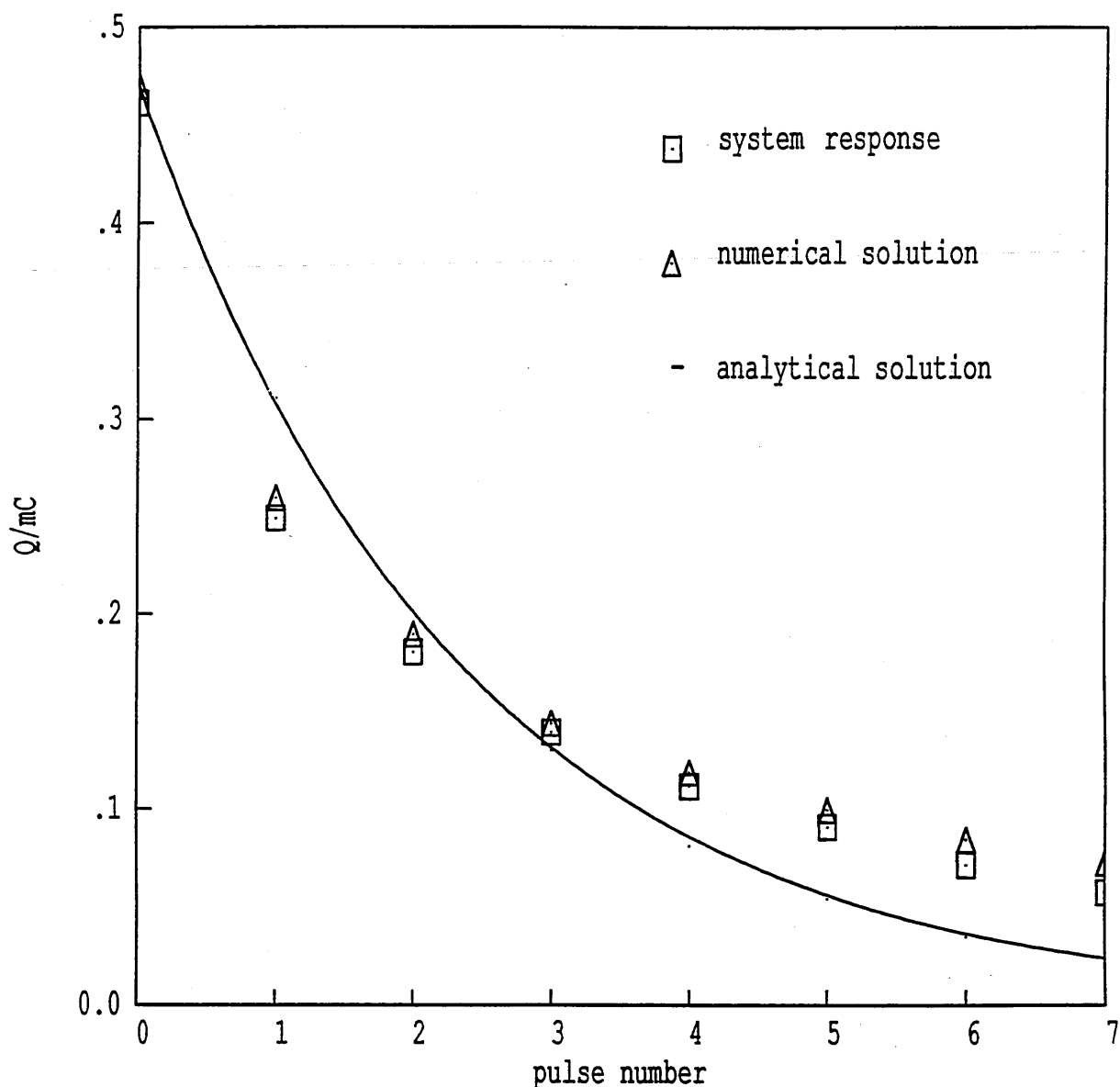


Figure III.4.17. A comparison between numerically derived charge response data, the system charge response and the charge response based on expressions generated in Section III.2. and the integrated Cottrell equation. All are for solutions or model solutions containing 10 mM ferrocyanide with PD=RD=1 s. The solid line represents a least squares exponential regression of the analytically derived response. This response assumes total solution relaxation in between the periods of potential pulse. The numerical model and the system response do not express this relationship between charge passed and pulse number until the system has achieved a stable oscillatory state with respect to concentration gradients in the x- and z- dimensions (quantitatively the z-dimension is negligible, and in the system response there are three spatial dimensions to consider). At 10 mM ferrocyanide this occurs after the first pulse but at 40 mM there is some perturbation of the charge response of the second pulse. The stabilisation of charge response may be explained in terms of failure of the solution to attain complete relaxation during the interval between pulses (Figures III.4.18. and III.4.19.).

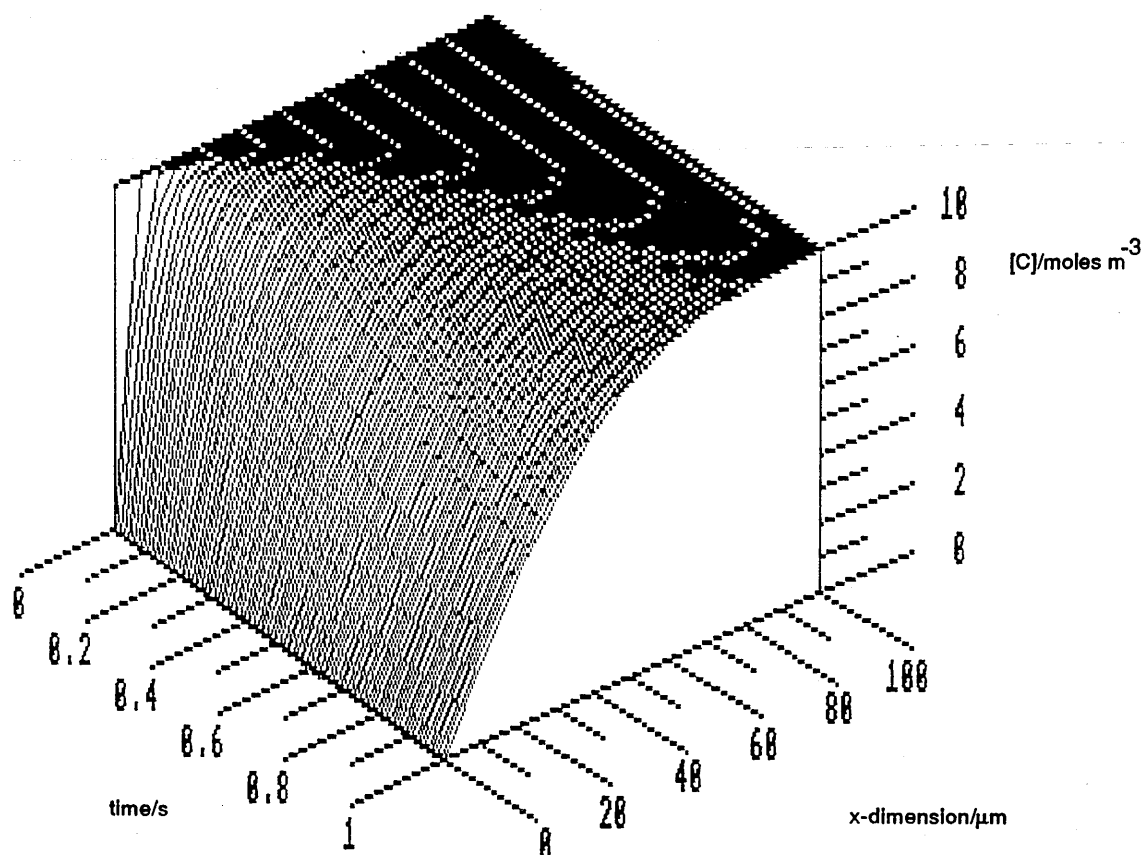


Figure III.4.18. The simulated concentration profiles of ferrocyanide in the x-dimension over the duration of the first potential pulse applied to a model solution containing 10 mM ferrocyanide for a potential pulse of duration 1 s.

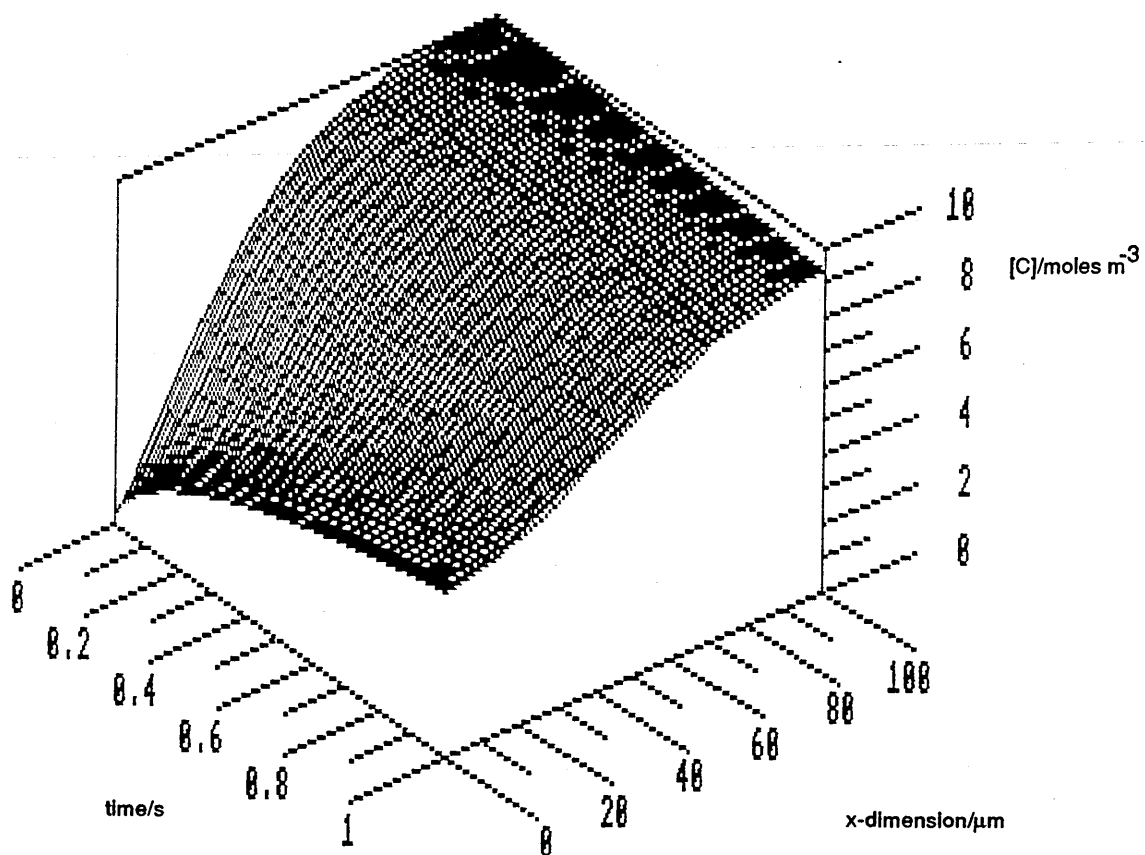


Figure III.4.19. The simulated concentration profiles of ferrocyanide in the x-dimension over the duration of the first relaxation period for a model solution containing 10 mM ferrocyanide for a relaxation period of duration 1 s.

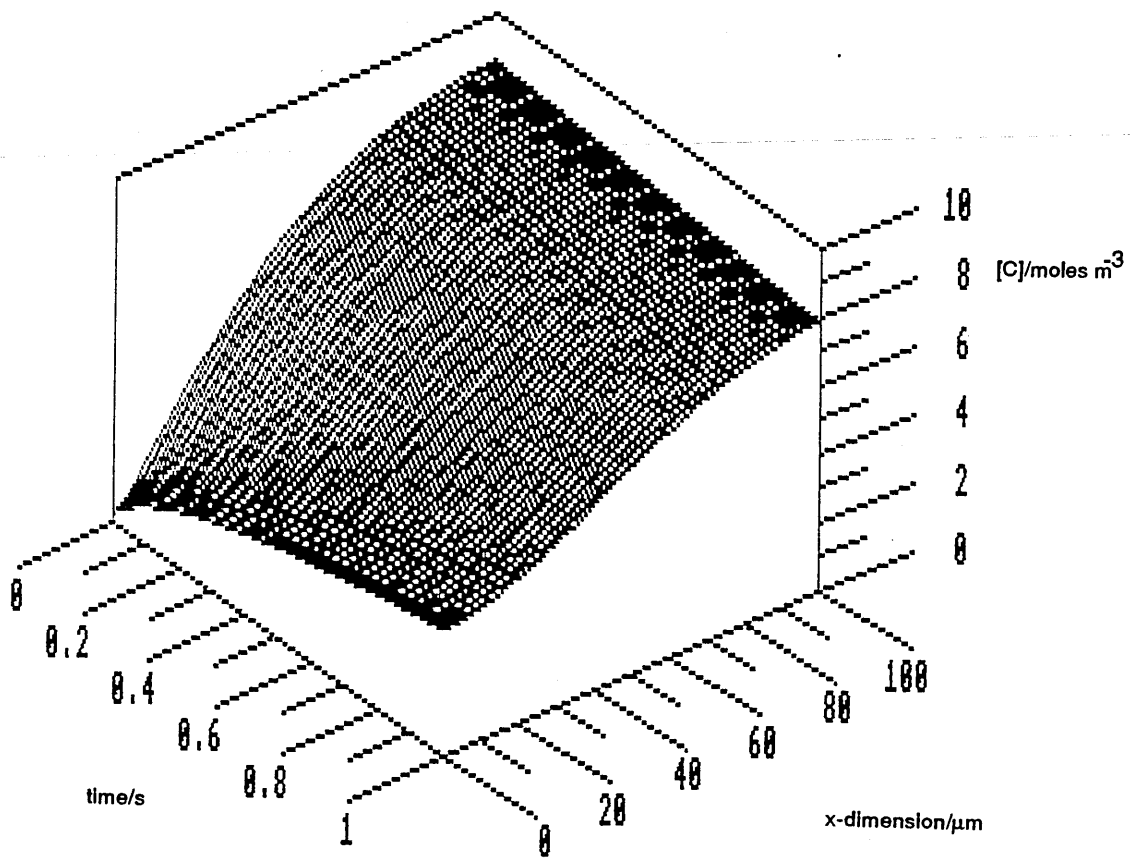


Figure III.4.20. The simulated concentration profiles of ferrocyanide in the x-dimension over the duration of the third relaxation period for a model solution containing 10 mM ferrocyanide for a relaxation period of duration 1 s.

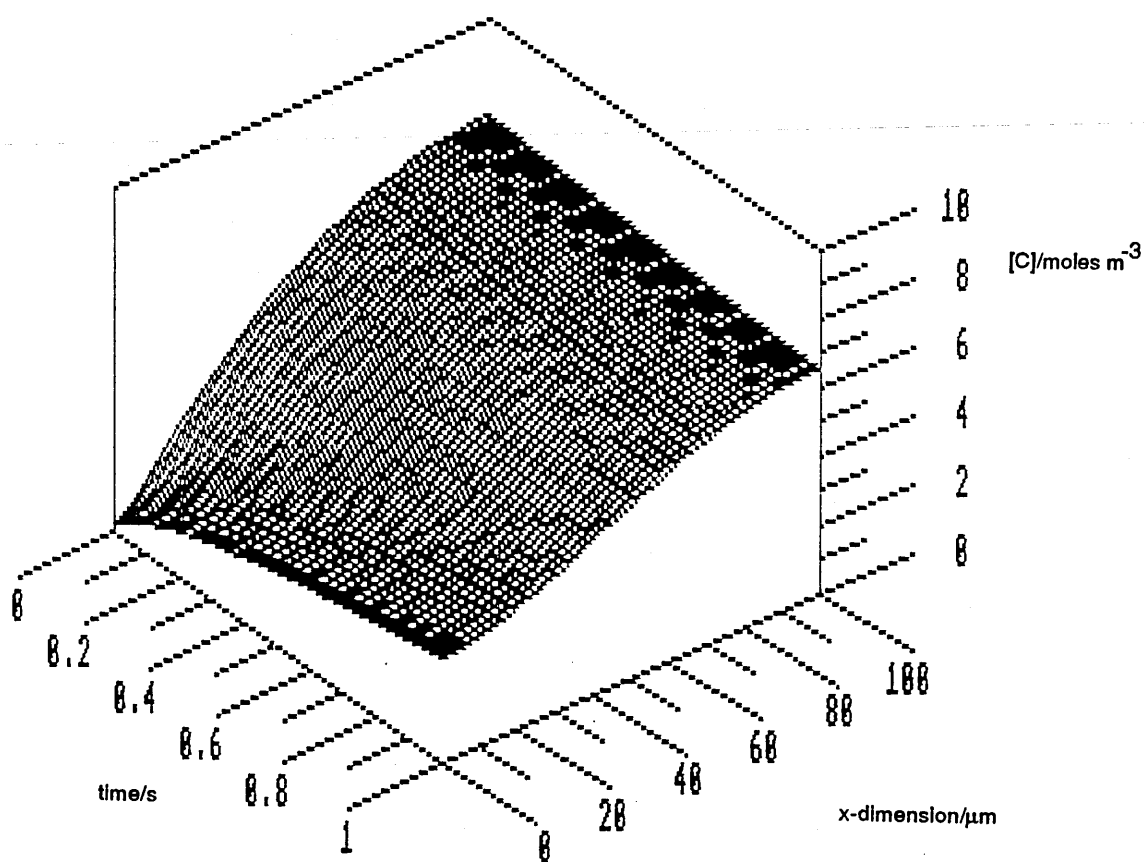


Figure III.4.21. The simulated concentration profiles of ferrocyanide in the x-dimension over the duration of the fourth relaxation period for a model solution containing 10 mM ferrocyanide for a relaxation period of duration 1 s.

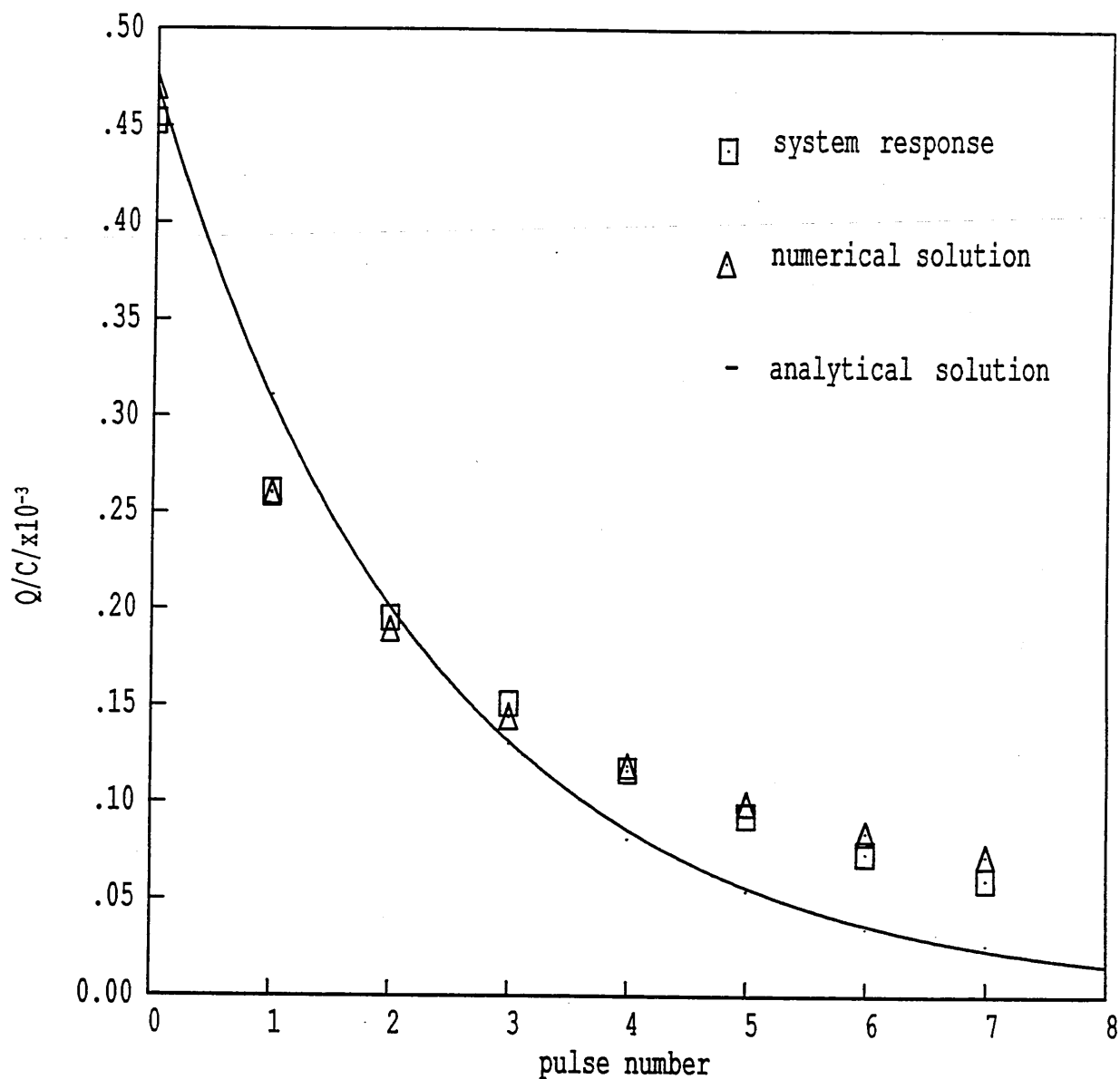


Figure III.4.22. A comparison between numerically derived charge response data, the system charge response and the charge response based on expressions generated in Section III.2. and the integrated Cottrell equation. All are for solutions or model solutions containing 10 mM ferrocyanide and with PD=1 s and RD=4 s. The solid lines represent a least squares exponential regression of the analytically derived response and of the numerical and system responses for all pulses excluding the first two pulses, which do not conform to this behaviour. The system and numerical responses illustrate the deviation from total relaxation behaviour

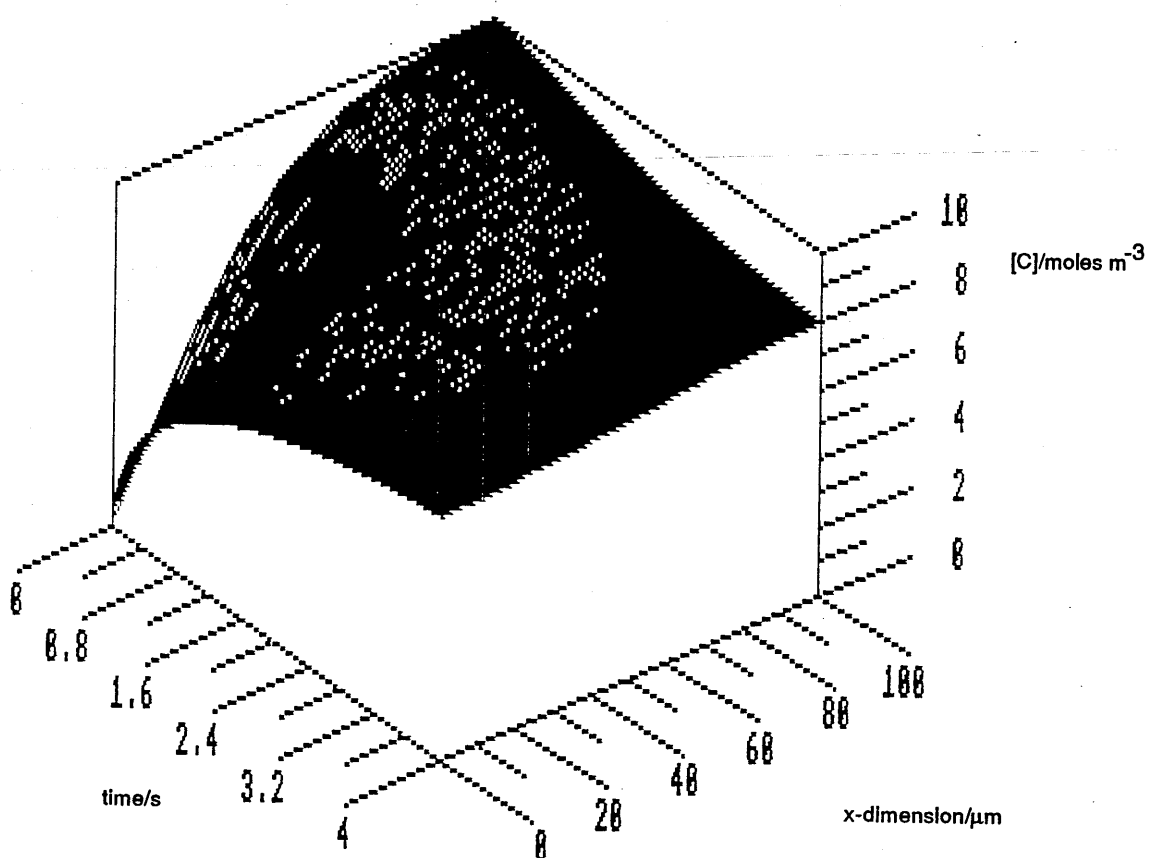
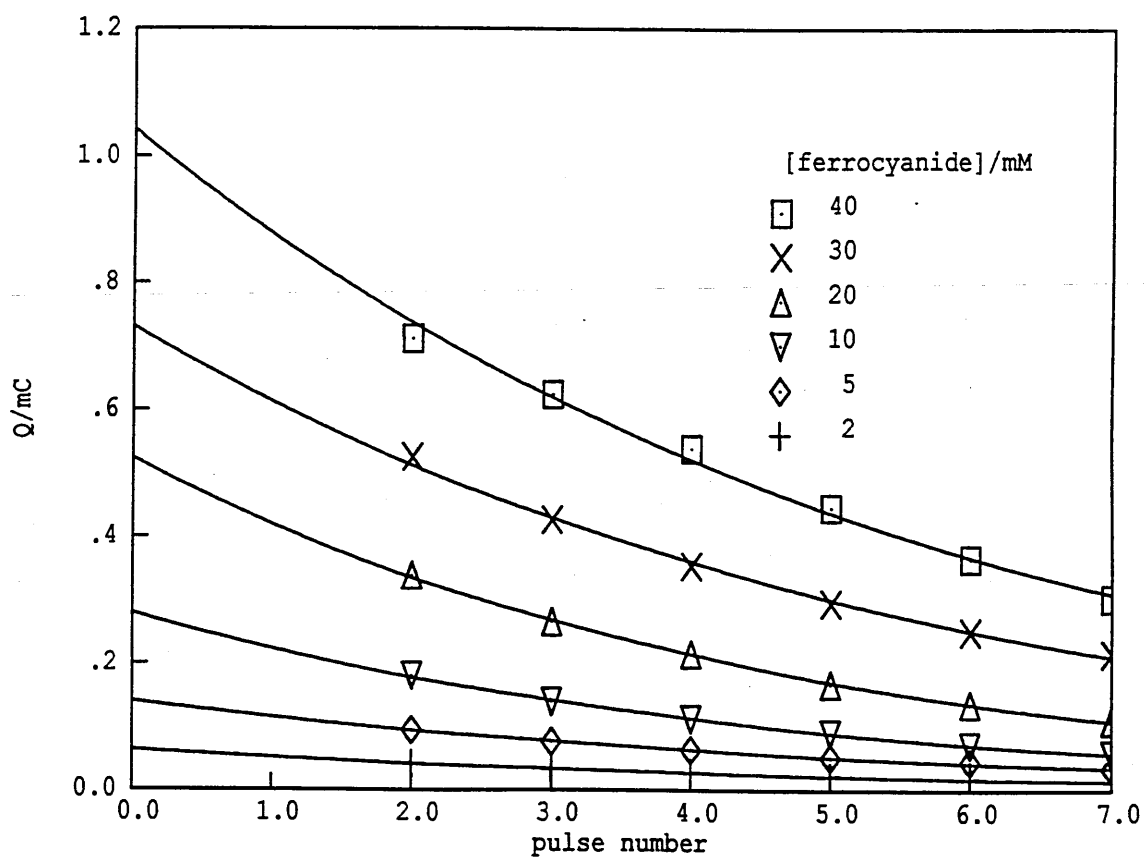
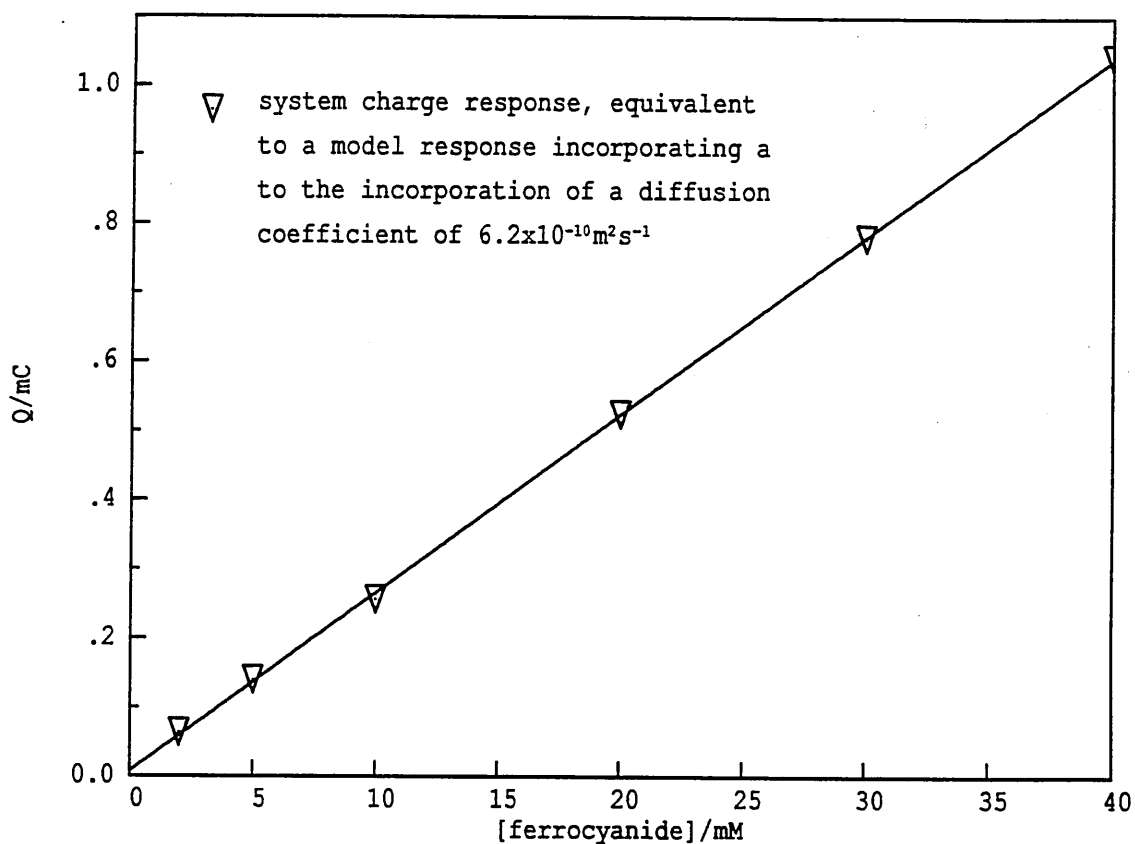


Figure III.4.23. The simulated concentration profiles of ferrocyanide in the x-dimension over the duration of the first relaxation period for a model solution containing 10 mM ferrocyanide with PD=1 s and RD=4 s.

Figure III.4.24. Application of an exponential regression least squares fitting routine to the charge-pulse data in Figure III.4.16. to all pulses except the first two pulses. All regressions have correlation coefficients greater than 0.998.

Figure III.4.25. Correlation of the intercept at $p=0$ for the data in Figure III.4.24. with the concentration of ferrocyanide present.

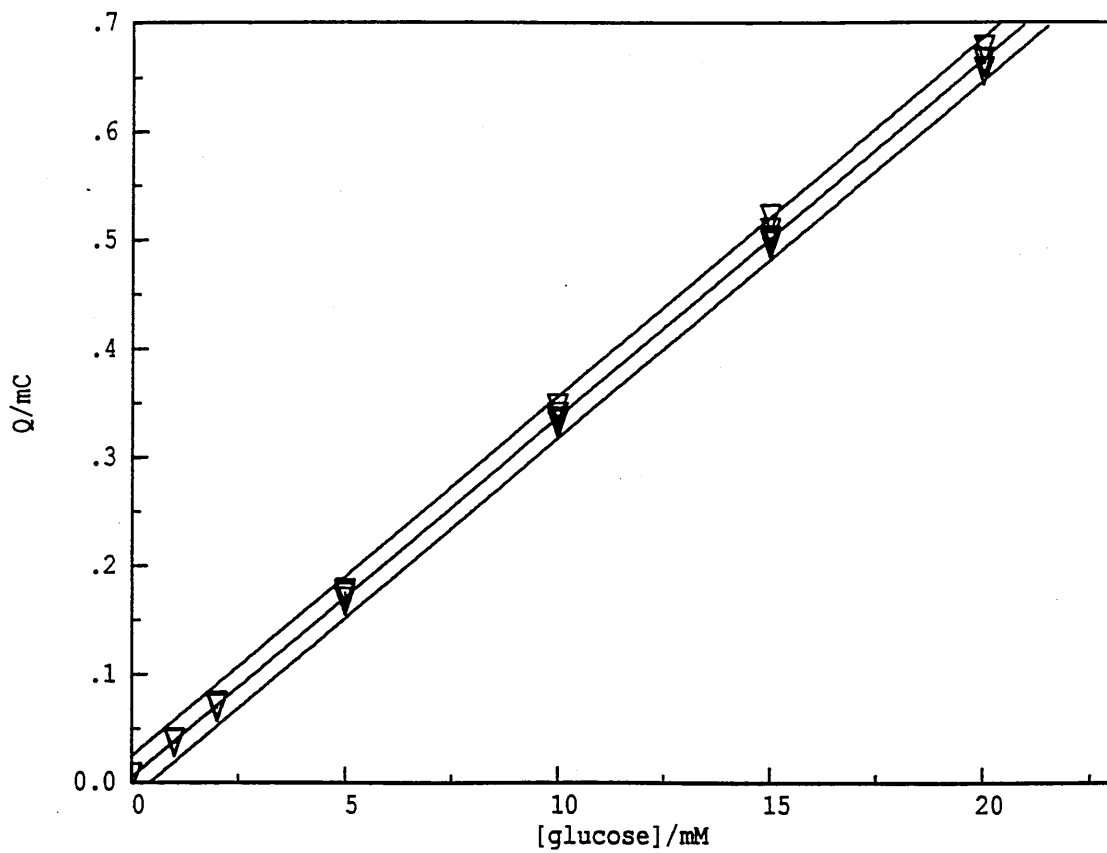
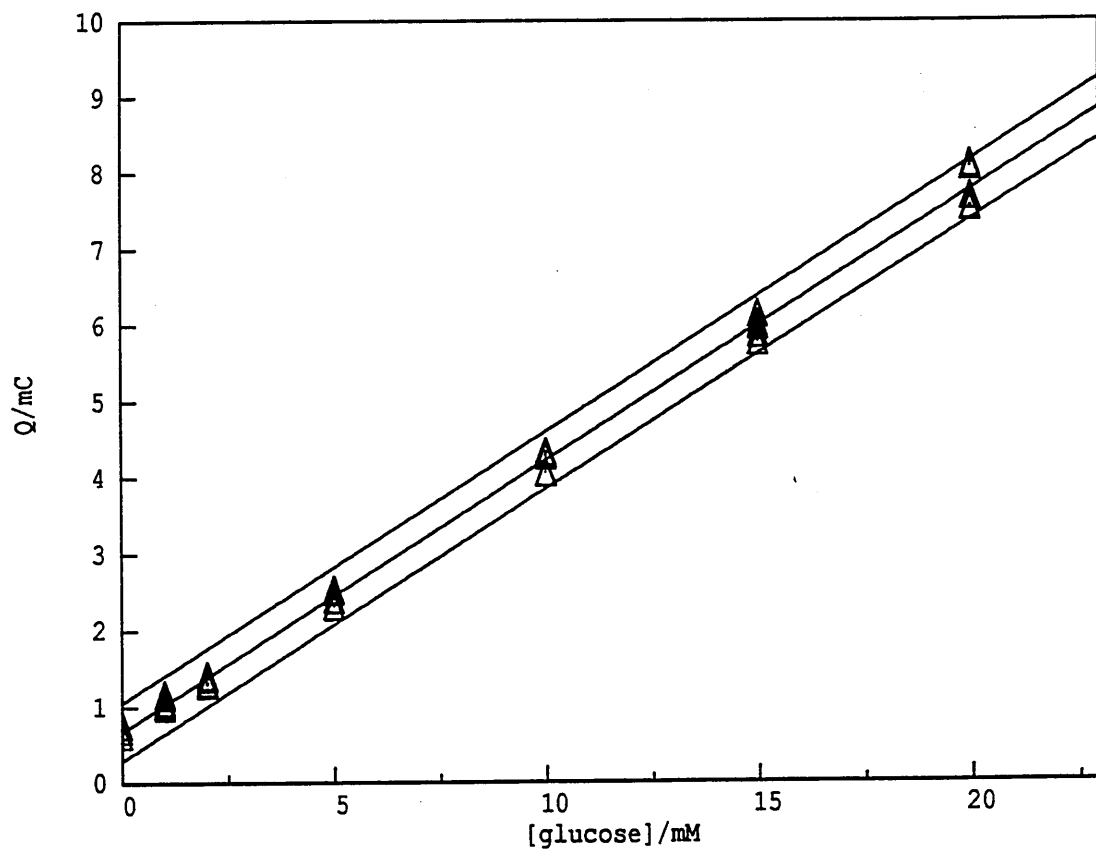


III.4.4. COMPARISON OF COULOMETRY AND THE PULSED-POTENTIAL RELAXATION METHOD FOR THE DETERMINATION OF GLUCOSE IN THE GLUCOSE-CFD.

The performance of the pulsed-potential relaxation method of analysis in glucose-CFDs was assessed for $0 \text{ mM} \leq [\text{glucose}] \leq 20 \text{ mM}$. Figure III.4.26. shows the correlation obtained using the same procedure used to generate Figure III.4.25., six measurements were taken at each concentration. The application of a least squares linear regression to the data in Figure III.4.26. yields a correlation coefficient of 0.9938 (centre line, 95% confidence limits - outer lines). The standard deviation at a concentration of glucose of zero is $4 \times 10^{-4} \text{ mC}$, giving a limit of detection of 0.2 mM. The coefficient of variance at each concentration falls between 4% and 5% and the response is linear for all of the range tested. Figure III.4.27. shows the coulometric response for the application of an oxidizing potential pulse of duration 60 s to aliquots taken from the same solutions used to construct Figure III.4.26. and utilised the CFDs from the same batch of electrodes. The high value of the intercept (0.67 mC) obtained in the absence of glucose and the greater coefficient of variance (~6%; from a standard deviation of $4 \times 10^{-2} \text{ mC}$, and a mean response of 0.67 mC) gives a limit of detection of ~0.6 mM using the definition applied to the analysis of the correlation between charge response and the concentration of glucose for ruthenium (III) hexamine, Section II.3.. The performance of the pulse-relaxation method is superior to the coulometric analysis in terms of the limit of detection (0.2 mM vs. 0.6 mM) and analysis time ($\leq 15 \text{ s}$ vs. 60 s - for a pulse/relaxation frequency of 1 Hz & using the intercept derived from charge values from pulses 2 to 5 an analysis time of ~10 s is possible at this frequency with little loss in precision, Figure III.4.24.), but is inferior in sensitivity (0.033 C M^{-1} vs. 0.35 C M^{-1}).

Figure III.4.26. The charge response correlation obtained using the pulse-relaxation technique using glucose-CFDs for $0 \text{ mM} \leq [\text{glucose}] \leq 20 \text{ mM}$. The intercept at a concentration of glucose of zero is close to zero, giving a limit of detection of 0.2 mM (cv=4.5%) and a linear range that extends beyond 20 mM glucose. The outer lines represent 95% confidence limits (n=6 at each concentration).

Figure III.4.27. The charge response correlation obtained by coulometry using glucose-CFDs for $0 \text{ mM} \leq [\text{glucose}] \leq 20 \text{ mM}$. The intercept at a concentration of glucose of zero is 0.67 mC, giving a limit of detection of ~0.6 mM (cv=6%) and a linear range that extends beyond 20 mM glucose. The outer lines represent 95% confidence limits (n=6 at each concentration).



III.4.5. THE EFFECT OF LATERAL DIFFUSION AND GUARD ELECTRODE ACTIVATION ON CHARGE & FLUX RESPONSE.

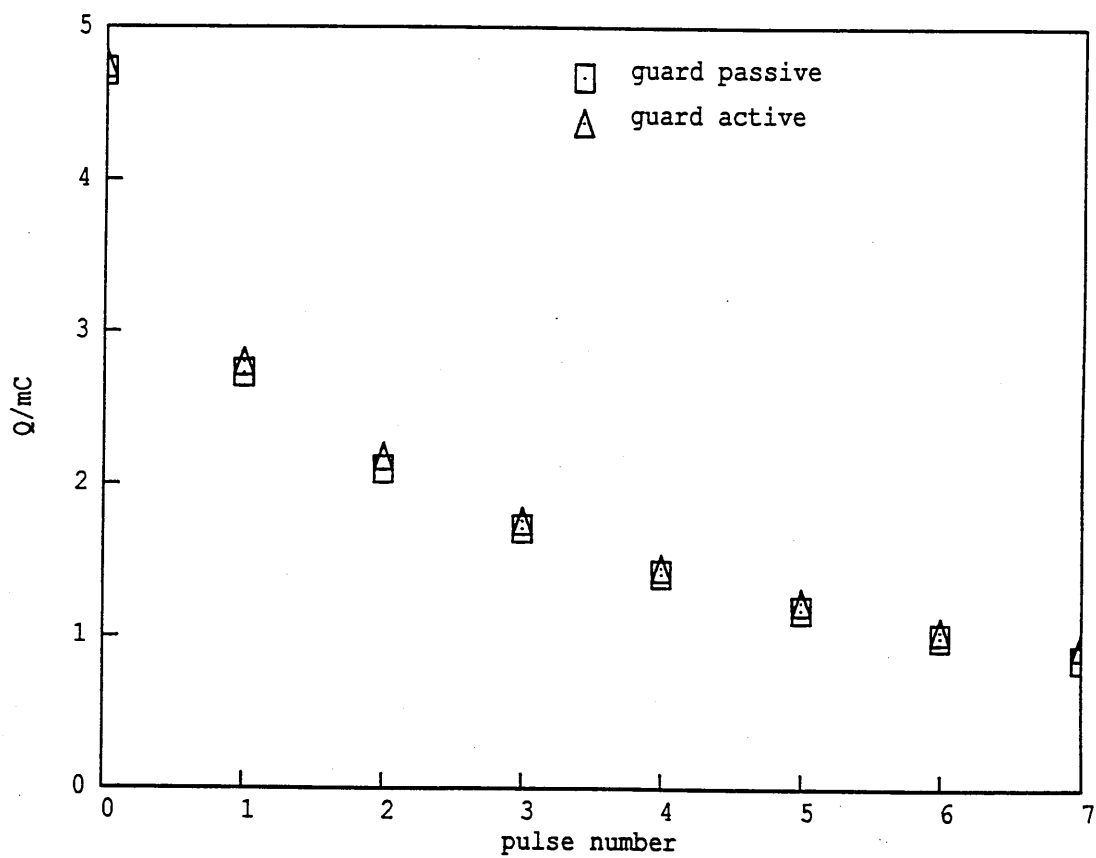
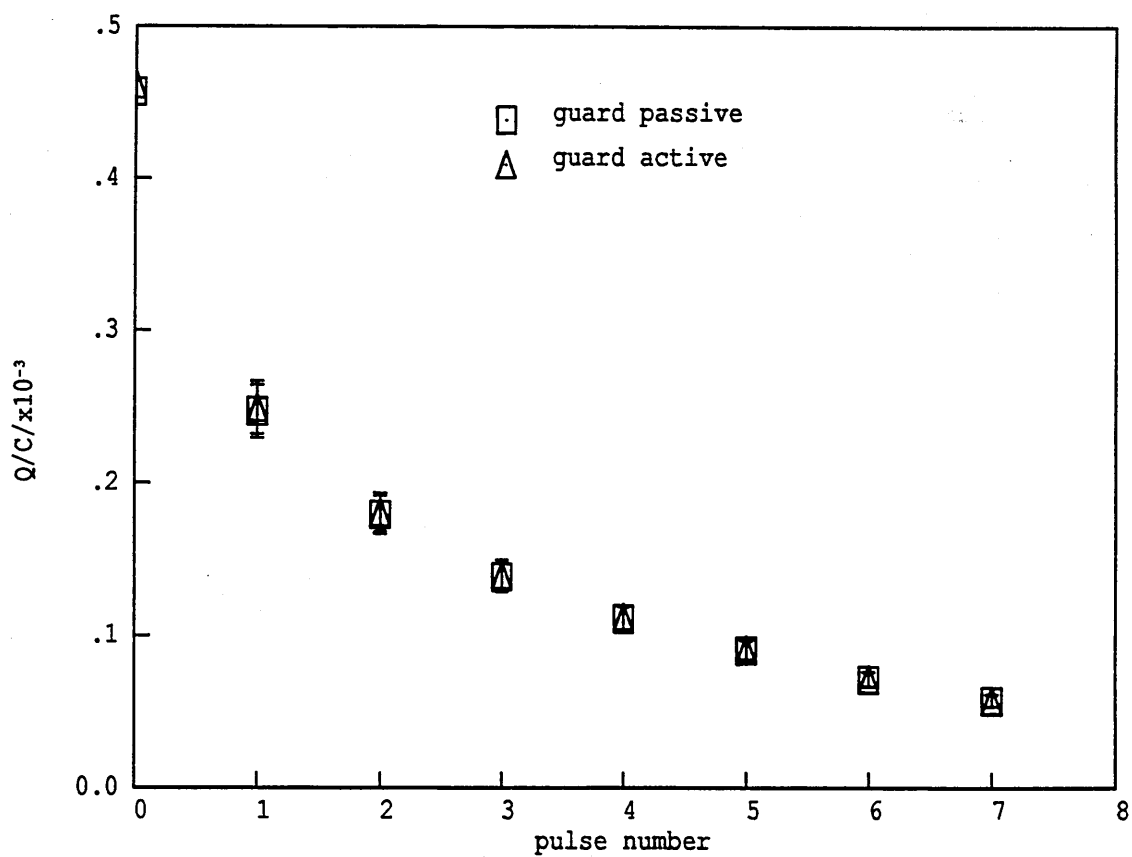
In a system of potential pulses of duration 1 s the effect of lateral diffusion cannot be neglected whether the guard electrode is active or passive. When conducting exhaustive coulometry the effects of lateral diffusion approaches the magnitude of diffusion perpendicular to the electrode towards the end of the analysis. The guard electrode was introduced to reduce the variability in charge passed. In the pulsed system the maximal permitted extent of the diffusion front in the z-dimension is about 150×10^{-6} m for a one second pulse approximating the diffusion front to $6(Dt)^{0.5}$, Section III.3.1. The effect on charge passed of running the models with active and passive guard electrodes was investigated. Figures III.4.28. shows the model charge response over several pulses derived for an initial concentration of ferrocyanide of 10 mM and for one second pulse and relaxation periods. The only difference between the two models was the designated extent of the diffusion front in the z-dimension in the region $z > 0$, beyond the electrode edge (Figure III.3.2.). This parameter was determined by $6(Dt)^{0.5}$ when the guard electrode was passive and was assigned a value of 100×10^{-6} m when the guard electrode was active. This is half the distance between the working and the guard electrodes and has implications for the validity of the models as is discussed below. The diffusion front in the region $z < 0$, Figure III.3.2., was governed by $6(Dt)^{0.5}$ in both cases. The charge-pulse values are slightly greater when the simulation was run with an active rather than with a passive guard electrode. The charge value obtained over any pulse with a passive guard electrode was about 0.97 of the value obtained when the guard was active. Analogous experiments were conducted, Figure III.3.29.. Analysis of the differences in charge magnitudes over each pulse for the experimentally derived data on guard activation reveal smaller differences than the model would suggest; using the mean charge value for each pulse derived from 6 trials, for any pulse the charge passed when the guard electrode was passive is about 0.99 of the value for when the guard was active. Figure III.3.29. shows the error bars representing the standard deviation for the data at each pulse value.

This analysis implies that the operation of the guard electrode during the application of a series of potential pulse and relaxation periods has a marginal effect on the charge passed over each pulse and as has been demonstrated practically in Section III.4.4., since the magnitude of lateral diffusion over the electrode edge during the pulsed analysis is always negligible compared to the contribution in the x-dimension, any variation in signal response obtained with coulometry operating without a guard electrode ought to be obviated in this system.

Although the difference in the simulated charge response may be due to the fact that the calculated mass transfer coefficients in the z-dimension do not change continuously with Δz , but are discretized, there are reasons for thinking that the small difference that exists may be due to different values for the concentration gradient of the reduced mediator in the z-dimension. The model actually represents

Figure III.4.28. Simulated charge response for a system involving a pulse/relaxation frequency of 1 Hz applied to a model solution containing 10 mM ferrocyanide with either active or passive guard electrodes.

Figure III.4.29. Experimentally derived charge-pulse response for a pulse/relaxation frequency of 1 Hz applied to a solution containing 10 mM ferrocyanide with either active or passive guard electrodes.(Error bars represent the sd at each charge value; n=5).



concentration in the z-dimension to a distance over the edge of the electrode that is dependent on the diffusion coefficient of the mediator and the duration of the relaxation or pulse as defined by $6(Dt)^{0.5}$. The model is able to achieve a reasonable simulation to the diffusion profiles in the z-dimension without solving the flux equations for those solution elements between the edge of the guard electrode and the midpoint between the guard and working electrodes by assuming flux symmetry in the x-axis at the midpoint of the two electrodes, Figure III.4.30. Application of the idea of symmetrical flux about the midpoint is equivalent to a zero flux boundary condition as would exist at an inert physical boundary. The form of the finite difference equations governing the flux of material at the end plate and at the z-dimension limits of the simulation are identical, Section III.3.1.. The validity of using this simplifying idea stems from an expectation of identical flux behaviour at the edge of the working and the guard electrodes. This expectation is valid given the relative dimensions of the permitted diffusion front in the z-dimension and the widths of the working and guard electrodes; maximally about 150 μm for the diffusion front and 3000 μm and 500 μm respectively for the electrodes. The idea of flux symmetry as represented in Figure III.4.30. is also applied to the lines of symmetry of the electrodes. This ensures that the inner areas (Section III.3.1.) of the working and guard electrodes are subject to flux in the x-dimension only.

When considering the validity of the model where the maximum allowed distance in the z-dimension in the region $z > 0$ is determined by $6(Dt)^{0.5}$ it should be remembered that this value is an approximation of the extent of the diffusion front after a time, t , in a direction perpendicular to an electrode. Although this value may be a good estimate of the maximum distance from a concentration perturbation that a volume of solution may be affected through diffusion, the magnitude of the flux in the z-dimension only approaches the magnitude in the x-dimension at $x=0$ and at nodes very close to the edge of the electrode. The concentration perturbation experienced at $+Z_{\text{lim}}$ for $x=0$ at the end of several pulse-relaxation cycles is less than 0.1 of the value of the initial concentration of reduced mediator; Figure III.4.31 shows the concentration - distance - time surface of ferrocyanide in the z-dimension at $x=0$ over the fourth potential pulse of a series of pulse-relaxation cycles of equal period with the guard electrode passive. With the guard electrode inactivated the model simplifies the real system by setting a limit for the maximal boundary distance in the z-dimension which is probably exceeded in the actual system, since the rules regarding flux symmetry between the edge of the working electrode and the edge of the guard electrode cannot be applied when the guard electrode is passive, but the quantitative effect of this simplification on flux at the edge of the working electrode is probably negligible. Concentration profiles for the reduced mediator from the edge of the working electrode to the boundary of the simulation are shown for guard electrode active and inactive states (Figure III.4.32.) at the end of the fourth pulse of a series of pulse and relaxation states corresponding to the charge data in Figure III.4.28. The concentration of reduced mediator is shown at $x=0$, the position in the x-dimension where the lateral diffusion flux is maximum. Examination of Figure III.4.32. reveals that the concentration gradient of ferrocyanide in the z-dimension is greater when the guard is active than when passive, as to be expected from the marginally higher charge values obtained when the guard was active.

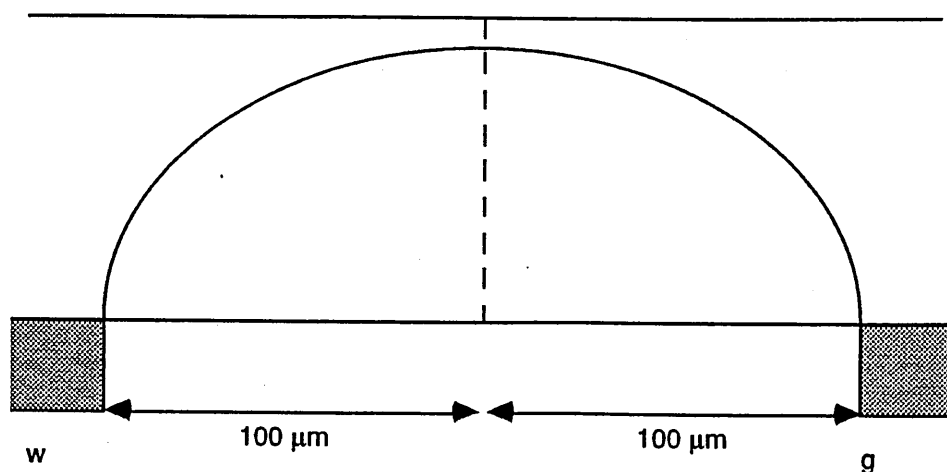


Figure III.4.30. Schematic diagram illustrating the concentration profile of reduced mediator between the edges of the working electrode (w) and the guard electrode (g) during the application of a potential pulse. The models were constructed to incorporate the concept of flux symmetry about three lines in the x-dimension; when the guard electrode is active flux symmetry is assumed at the mid-point between the working and guard electrodes for all values of z at values of $t > 0$. This assumption rests upon the flux symmetry at the mid-point of each of the electrodes at all z values for $t > 0$.

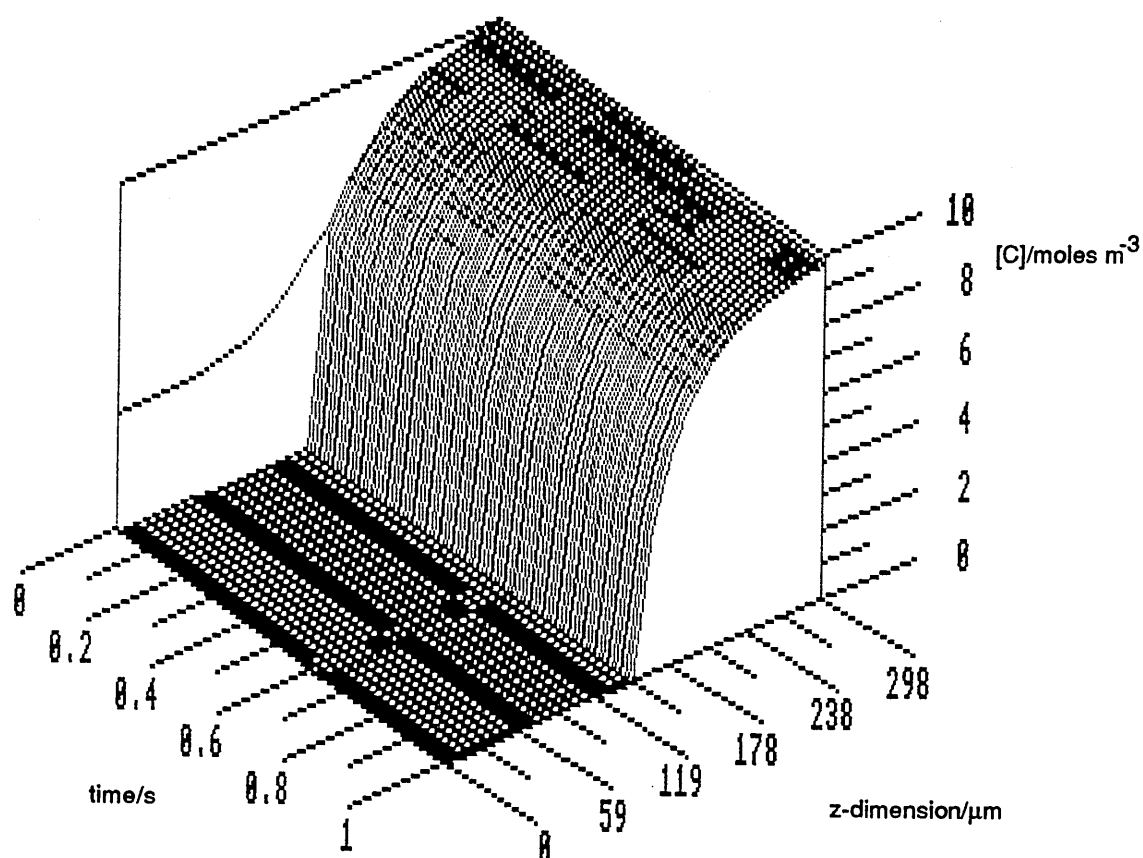


Figure III.4.31. The simulated concentration profiles of ferrocyanide in the z-dimension at $x=0$ over the duration of the potential pulse for a model solution containing 10 mM ferrocyanide ($PD=RD=1$ s, guard electrode inactive). The z-dimension is represented from the inner most electrode strip ($0\ \mu\text{m}$) to the edge of the diffusion coefficient defined solution boundary ($298\ \mu\text{m}$). The line at $t=0$ s represents the concentration of ferrocyanide for all values of z at $x=0$ at the end of the previous relaxation period.

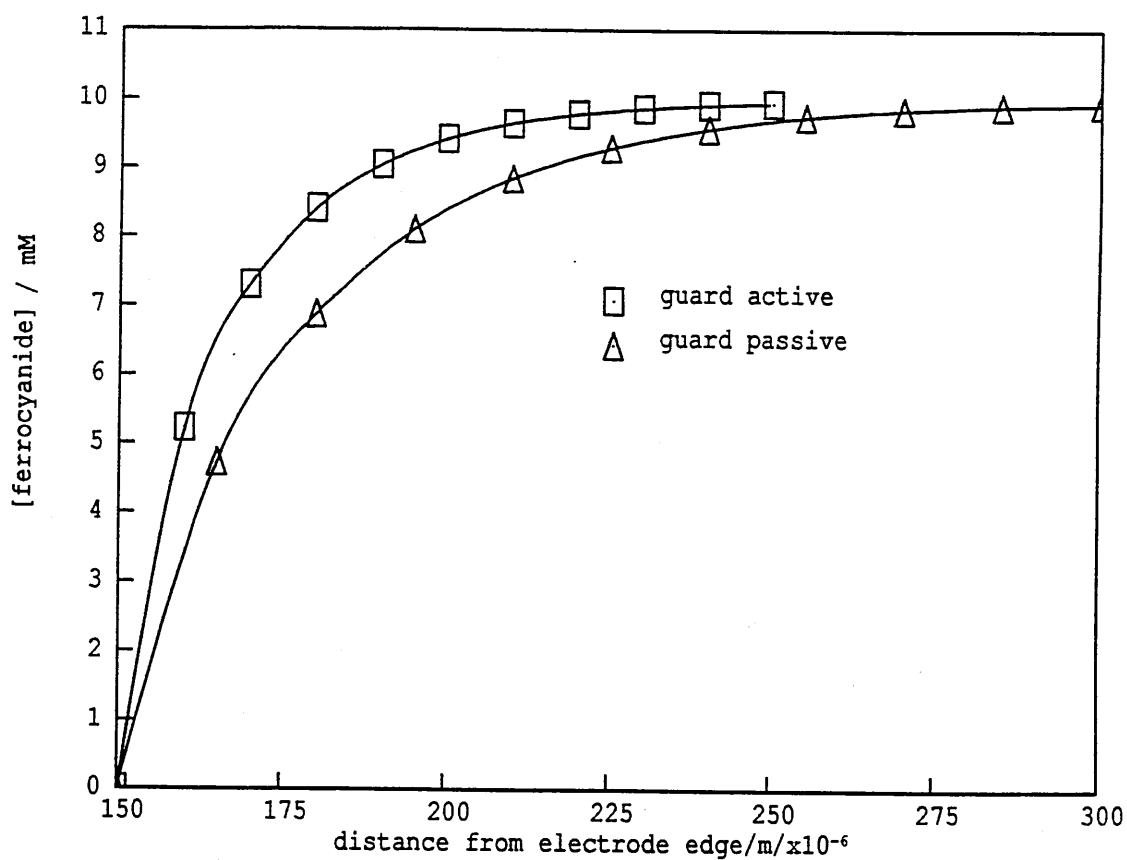


Figure III.4.32. Simulated concentration gradient of ferrocyanide beyond the edge of the working electrode at $x=0$ at the end of the fourth potential pulse for an applied frequency of 1 Hz with active and passive guard electrodes.

III.4.6. CONCLUDING REMARKS.

These exploratory numerical simulations of the behaviour of the catalytic layer have suggested that any manipulation of the catalytic layer will probably not result in a significant reduction in the overall analysis time, provided that the fractional diffusion coefficient of species in the catalytic layer compared to their diffusion coefficients in solution is close to one. The investigations presented in Section III.3.2. did illustrate the theoretical reduction in analysis time which may be achieved on a reduction in the overall gap width, but at the cost of a greater relative error in the definition of volume at the same level of precision in defining the gap width. Section III.3.2. contains a more detailed summary of these findings.

The modelling of the pulse-relaxation technique for determination of initial concentration of reduced mediator accurately predicted;

(I) the incomplete relaxation of the solution in-between each of the potential pulses and the high correlation between the charge value at $p=0$ evaluated from the exponential decrease in charge passed over all pulses excluding the first or second pulses.

(II) the negligible contribution of charge derived from lateral diffusion.

The analytical application of a pulse-relaxation frequency of 1 Hz to glucose-CFDs challenged with solutions containing glucose in the range $0 \text{ mM} \leq [\text{glucose}] \leq 20 \text{ mM}$ suggested superior characteristics compared to coulometry over the same concentration range in terms of limit of detection and reduction in analysis time (0.2 mM vs. 0.6 mM; ~10 s vs. 60 s), but inferior response sensitivity (0.033 C M^{-1} vs. 0.35 C M^{-1}). Since the coefficient of variance for the response - glucose concentration correlation is of the same order for both methods the pulse-relaxation technique has no intrinsic disadvantages compared to a coulometric analysis.

'Review, comments & further work' contains an overview of the findings generated by Sections III.3.2. and III.4..

Chapter IV, the development of an L-lactate CFD

IV.1.1. THE ANALYTICAL IMPORTANCE OF LACTATE.

The sensing of L-lactate is significant for a wide range of industrial and clinical applications. In the clinical arena the principal diagnostic use of L-lactate sensing is to determine the extent to which the blood flow to body tissues has been impaired. It is possible to correlate the extent of restriction of adequate blood flow to tissues to the concentration of L-lactate in the blood because a restriction of blood flow is associated with a tissue oxygen deficit that is metabolically bridged by the reduction of pyruvate to L-lactate. This phenomenon is called metabolic acidosis and is associated with several pathological conditions including diabetes, leukemia, and glycogen storage disease (Oliva, 1970). Blood L-lactate concentration may also be monitored to assess the degree of tissue damage where, having undergone surgery, a patient has suffered a reduction in cardiac output. It should be noted that sensing of D-lactate has a separate clinical significance associated with the diagnosis of bacterial infections (Smith *et al.*, 1989), or where high concentrations of abnormal gut flora result in an accumulation of D-lactate (Stolberg *et al.*, 1982). This illustrates that the development of a sensor for lactate should focus on the application envisaged: frequently it is important to specifically sense one of the optical isomers, although it may be possible to correlate the concentration of either or both optical isomers with the desired function. The construction of a sensor for L-lactate implies the capacity to construct a sensor for the activity of the certain NAD-dependent lactate dehydrogenases (Dix Smith & Olson, 1974) that are of clinical importance in the diagnosis of myocardial infarction (Rishpon & Rosen, 1989; Tietz, 1987). All present determinations of lactate concentration in samples of blood require the immediate removal of erythrocytes from the sample. This is imperative as anaerobic glycolysis within the erythrocytes after the sample is taken generates additional lactate to that which was present *in vivo*.

Lactate sensing is also important in monitoring the quality of a number of food products (Moo-Young, 1985). The process of cheese making is mediated by the metabolic activity of lactic acid bacteria of the genera streptococcus and lactobacillus. The action of cultures of these bacteria on milk converts lactose to lactate. The concentration of lactate in this process significantly influences the processes involved in the development of the final texture of the cheese and its flavour. The monitoring of the concentrations of lactate is also important in the manufacture of yoghurt. The cultures produce a mixture of both of the optically isomeric forms of lactate. Here the optimum concentration of lactate for the desired taste of the product is between 4 mM and 6 mM and the optimal ratio of L-lactate:D-lactate is greater than 0.75. Lactate sensing has an analogous role in monitoring the development of 'ripeness' in various fermented sausages. Additionally monitoring and control of the concentration of L-lactate has important implications in terms of efficiency in several industrial fermentations involving lactic acid bacteria or where lactate is a constituent of the feedstock.

IV.1.2. DETERMINATION OF LACTATE USING NON-ENZYMATIC TECHNIQUES.

Lactate may be determined by numerous analytical techniques such as the oxidation of lactate by ceric sulphate to acetaldehyde followed by the spectrophotometric quantification of coloured derivatives (Hochella & Weinhouse, 1965). Several other analogous techniques exist, involving ferric chloride, potassium thiocyanate or p-hydroxydiphenyl (Snell & Snell, 1953). All rely on the correlation of the concentration of lactate to a spectrophotometrically sensitive colour change. Many are highly sensitive to the presence of certain metal ions and may involve significant sample preparation.

Determination of lactate may also be realised by using techniques of gas-liquid chromatography (Ansari *et al.*, 1979), high performance liquid chromatography (Molnar & Horrath, 1977) or gas chromatography (Jones-Owen & Lechocki, 1974).

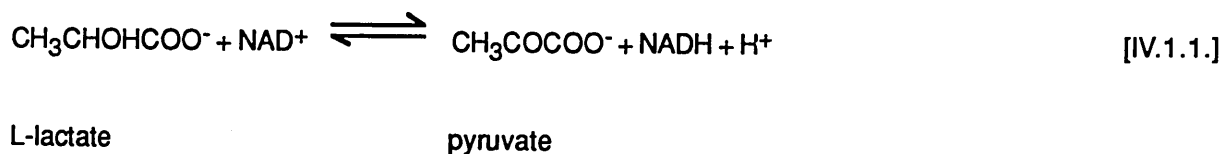
None of the methods presented are optically isomeric specific, all are labour intensive, involve expensive instrumentation and may require centralised analysis facilities.

IV.1.3. ANALYTICAL STRATEGIES EMPLOYING L-LACTATE OXIDIZING ENZYMES AND THEIR INCORPORATION INTO AMPEROMETRIC SENSORS.

Four different commercially available lactate oxidizing enzymes have been identified that have been employed for the construction assays for the determination of L-lactate. Some have been incorporated into commercially available diagnostic kits based on spectrophotometric determination of a chromophore and all have been incorporated into various amperometric sensor configurations.

NAD-dependent L-lactate dehydrogenase (lactate dehydrogenase) (EC 1.1.1.27).

Lactate dehydrogenase catalyses the oxidation of L-lactate with the concomitant reduction of NAD, [IV.1.1.] and is commercially available in preparations derived from a variety of mammalian tissues:



the equilibrium constant for [IV.1.1.] at pH 7.0, reading left to right, is 2.76×10^{-6} (Barman, 1969). The forward reaction is favoured at pH values greater than about 9.5. It is facile to state that since a proton is always formed or absorbed in a pyridyl-linked reaction that a change in pH value will elicit a change in equilibrium constant. An examination of the mechanism of reduction of NAD by lactate dehydrogenase reveals the reasons for the equilibrium shift at pH values greater than 9.5. At physiological pH values lactate dehydrogenase plays a crucial role in the glycolytic pathway (Leninger, 1979), reducing pyruvate to L-lactate. The structure of the active site of lactate dehydrogenase has been elucidated and the functions of the amino-acid residues in the active site have been determined by protein engineering techniques (Clarke *et al.*, 1989). At physiological pH values the reaction that converts pyruvate and NADH to L-lactate and NAD proceeds by the donation of a proton from the active site His195 residue to the carbonyl oxygen atom and of a hydride ion (H^-) from the dihydronicotinamide ring of NADH to the carbonyl carbon atom. Reading [IV.1.1.] right to left, it is essential for His195 to be protonated in forming the enzyme-NADH-pyruvate complex but, left to right, unprotonated in forming the enzyme-NAD⁺-lactate complex. Since the carboxyl group of histidine is negatively charged above pH 9.3, provided that all substrates and cofactors are present at equal concentration, the oxidation of L-lactate is favoured over the reduction of pyruvate. NAD dependent lactate dehydrogenase is commercially available in freeze dried preparations derived from numerous animal and bacterial sources. All NAD dependent lactate dehydrogenases contain four structurally and functionally identical subunits, each of which binds one molecule of substrate and one molecule of cofactor.

In addition to performing the assay at pH values greater than 9.5, any assay designed to detect L-lactate the initial concentration NADH and pyruvate would be zero, and there would be a surfeit of NAD. By Le Chatelier's Principle, the equilibrium state in [IV.1.1.] would be shifted in favour of L-lactate oxidation. Despite the shift in the equilibrium that may be achieved by using these starting conditions the increase in the concentration of pyruvate prevents complete the oxidation of L-lactate. The conversion of pyruvate to some other chemical entity during the course of the assay prevents any equilibrium state from ever being attained. This strategy has been adopted by Leroux (1974). Here the pyruvate formed in [IV.1.1.] undergoes a nucleophilic addition reaction with hydrazine resulting in the formation of pyruvate hydrazone and the elimination of water (Morrison & Boyd, 1983), [IV.1.2.]:



pyruvate hydrazine pyruvate-hydrazone

the concentration of L-lactate is correlated to the change in concentration of NADH determined spectrophotometrically at 340 nm. Unfortunately NAD and hydrazine interact at high pH values resulting in erratic determinations. This may be obviated by the inclusion of EDTA (Engel & Jones, 1978). Sigma Diagnostics (Sigma Chemical Co. Ltd., Fancy Road, Poole, Dorset, BN17 7NH)

manufacture a diagnostic kit for L-lactate based on NAD-dependent lactate dehydrogenase and the hydrazone-pyruvate reaction (Pyruvate/Lactate Procedure No. 726-UV/826-UV). Other strategies that prevent the system from ever reaching equilibrium by transforming pyruvate to another species include the addition of L-glutamate and glutamate-pyruvate transaminase (EC 2.6.1.2.) to the assay (Calbiochem-Behring Laboratories Ltd., Bishop Stortford, Herts.). Here pyruvate and L-glutamate undergo a transaminase reaction, resulting in the formation of L-alanine and 2-oxoglutarate. The concentration of L-lactate is determined spectrophotometrically.

The NADH produced by reaction [IV.1.1.] may also be linked to the reduction of a redox compound by reactions mediated by the enzyme diaphorase (NADH:lipoamide oxidoreductase EC 1.6.4.3.), [IV.1.3.]:



here M_{ox} and M_{red} are the oxidized and reduced forms of the redox compound and n is the number of moles of M_{ox} required to oxidize one mole of NADH. One mole of NADH is completely oxidized by two moles of electrons. The change in concentration of the redox compound correlated with the initial concentration of L-lactate and may be assessed spectrophotometrically or electrochemically, depending on the characteristics of the species.

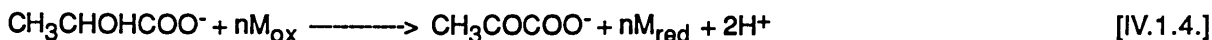
Many of the reaction sequences involving NAD-dependent lactate dehydrogenase may be incorporated into an amperometric sensor configuration. The most direct route of linking the oxidation of L-lactate by NAD-dependent lactate dehydrogenase to an amperometric signal is to poise the electrode at or above the oxidizing potential of NADH. Direct oxidation of NADH at solid electrodes occurs at +0.8 V vs. Ag/AgCl at carbon and +0.6 V vs. Ag/AgCl at platinum electrodes (Yon Hin & Lowe, 1987). The use of electron mediator compounds with lower oxidizing potentials have been employed to transfer electrons from NADH to the electrode. Oxidation of NADH at +0.2 V vs. Ag/AgCl has been reported at hexacyanoferrate-modified nickel electrodes (Yon Hin & Lowe, 1987). Electrode fouling on oxidation of NADH has been reported due to the generation of radical intermediates (Moiroux & Elving, 1978). Other strategies for the oxidation of NADH at reduced potentials by the use of chemically modified electrodes include electrode surface immobilised quinones which facilitate the oxidation of NADH at between 0.1 V and 0.3 V vs. SCE (Ueda *et al.*, 1982) or by the use of the conducting polymer salt $\text{NMP}^+ \text{TCNQ}^-$ (Albery & Bartlett, 1984).

Diaphorase may be used to mediate the oxidation of NADH and the reduction of a redox compound such as Bindschedler's Green (Dix-Smith & Olson, 1974) or of the redox dye p-iodo-nitrotetrazolium violet (Bergel *et al.*, 1989) or of ferricyanide (Bergel *et al.*, 1989; Yao & Wasa, 1985). All of these compounds may be detected spectrophotometrically or electrochemically. The use of such a compound as an electron mediator between the homogeneous chemistries of the redox reaction and an electrode may enable the electrode potential to be poised at a value less than the oxidation

potential of NADH: +0.056 V vs. SCE for Bindschedler's Green and +0.3 V vs. SCE for ferricyanide.

Cytochrome b_2 (L-lactate:ferricytochrome C oxidoreductase EC 1.1.2.3.).

Cytochrome b_2 , obtained from *Saccharomyces cerevisiae*, oxidises L-lactate to pyruvate in the presence of electron acceptors which include ferricinium carboxylate (Cass *et al.*, 1985) ferricyanide and molecular oxygen (Labeyrie *et al.*, 1974), [IV.1.4.]:



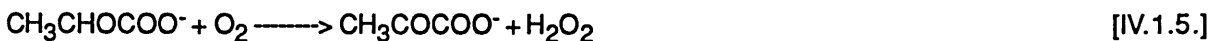
L-lactate

pyruvate

here n , M_{ox} and M_{red} have their usual meaning. In the case of ferricyanide and of cytochrome b_2 itself n is two. This system is obviously highly amenable to incorporation into either spectrophotometric (Morton *et al.*, 1964) or amperometric detection regimes (Williams *et al.*, 1970; Durlait *et al.*, 1990; Cass *et al.*, 1985; Preneta, 1987). This system has been commercially exploited using ferricyanide as an oxidant for reduced cytochrome b_2 in the Roche lactate analyser (Roche Products Ltd., Welwyn Garden City, Herts.). Cytochrome b_2 has also been adsorbed onto the surface of NMP+TCNQ⁺ modified electrodes (Kulys & Svirnickas, 1980) where the concentration of L-lactate was able to be correlated against an amperometric signal when the electrodes were poised between -0.2 V and +0.5 V vs. SCE.

Lactate oxidase (EC 1.1.3.2.).

Lactate oxidase from *Pediococcus pseudomonas* catalyses the oxidation of L-lactate to pyruvate by molecular oxygen, [IV.1.5.].



The most direct route of producing an amperometric sensor using lactate oxidase is to detect the increase in concentration of hydrogen peroxide or the decrease in concentration of oxygen. Lactate oxidase has a K_m value for L-lactate of 0.8 mM. The design of sensors incorporating L-lactate therefore have involved the use of membranes over the electrode surface to limit the rate of diffusion of L-lactate to the electrode surface. This extends the linear range of the sensor but increases the response time and reduces the sensitivity. Several types of membrane and enzyme immobilisation regimes may be employed; cellulose acetate (Fonong, 1987), giving a linear range between 50 μM and 300 μM ; glutaraldehyde immobilisation on polycarbonate membranes (Battersby, 1988) elicited a linear response up to 16 mM. The use of lactate oxidase in an amperometric sensor

configuration is frequently associated with high potentials necessary to oxidise hydrogen peroxide, +0.65 V vs. SCE. Composite electrodes such as the palladium/gold sputtered on carbon may oxidise hydrogen peroxide at potentials as low as +0.4 V vs. SCE (Scheller, 1986).

L-lactate-2-monooxygenase (EC 1.13.12.4.)

L-lactate-2-monooxygenase is derived from *Mycobacterium smegmatis* and catalyses the decarboxylation of L-lactate in the presence of oxygen to yield acetate (CH₃COOH), carbon dioxide and water, [IV.1.6.].



The concentration of L-lactate in solution may be correlated to the decrease in the concentration of oxygen monitored at an oxygen electrode (Mascini, 1984; Weigelt, 1987). The operating linear range of such a sensor may be as high as 25 mM (Makovas, 1985).

In addition to these commercially available enzymes, sensors have also been constructed incorporating quinoprotein-based lactate dehydrogenases derived from *Propionibacterium pentosaceum* (Preneta, 1985), which are not currently available commercially.

IV.2.1. L-LACTATE SENSING IN THE CFD.

Consideration of the published work in the field of electrochemical L-lactate sensing with respect to the characteristics of the CFD lead to the exclusion of certain of the strategies adopted by other workers, Section IV.1. When considering the type of assay system to be adopted several criteria were applied to inclusively or exclusively screen the host of potential systems. The proposed application of a sensor for L-lactate was to monitor the state of 'ripeness' in fermented sausages or possibly other food products containing L-lactate such as yoghurt. Presently the 'ripeness' of this product is assessed by measuring the pH of a slurry of the sausage. There are indications that the activity of the bacteria responsible for the development of the desired taste in the sausage may be correlated with the concentration of L-lactate in the sausage (Dr. Brian Birch, personal communication, 1989). A number of criteria must be satisfied by an industrially useful L-lactate sensor.

Specificity. Any proposed assay design should be able to specifically sense L-lactate up to a concentration of 15 mM in a sample of food product. Of all the non-enzymatic analytical techniques which may be used to determine the concentration of lactate the only ones which may be compatible with electrochemical determination in a CFD involve ligand binding reactions between lactate and metal centred compounds such as ferric chloride (Snell & Snell, 1953). Here it is possible to demonstrate that a shift in the reduction potential of ferric chloride occurs on reaction with lactate at pH values less than two. However, all of the reactions described in Section IV.1.2. are overall, inferior to enzymatic based methods since they are non optically isomeric specific and their use frequently demands extensive pretreatment.

Cost. Since the proposed design was to be incorporated into a manufacturing context consideration of unit cost was of paramount importance. Examination of the commercial sources of reagent components constituting the examples presented in Section IV.1. lead to the immediate exclusion of lactate oxidase, lactate 2-monooxygenase and cytochrome-b₂. PQQ lactate dehydrogenase was excluded because it is not commercially available and is difficult to purify (Preneta, 1985). Perhaps surprisingly the projected unit cost incorporating NAD-dependent L-lactate dehydrogenase, NAD and diaphorase was the most favourable of those options examined.

Compatibility. The assay components should be highly water soluble and ideally should be able to undergo oxidoreductive reactions with ferricyanide or a functionally analogous molecule. Lactate oxidase and lactate 2-monooxygenase are both excluded because of the need to monitor the reaction by amperometric detection of oxygen or hydrogen peroxide, which occurs at a high potential relative to ferricyanide, and by their rate dependence on the concentration of dissolved oxygen, since the rate of diffusion of oxygen into the CFD is severely limited because of the limiting ratio of the surface of solution in contact with the atmosphere to the volume of solution within the CFD. Additionally since the value for K_m of lactate oxidase for L-lactate is 0.8 mM any system incorporating

lactate oxidase operating within the CFD would entail the construction of membranes over the catalytic layer to limit the rate of diffusion into the catalytic layer so that the concentration of L-lactate would always be inferior to the K_m value. The K_m value of NAD-dependent L-lactate dehydrogenase for L-lactate is 17 mM (Barman, 1969) and for cytochrome b_2 is 1.4 mM (Morton & Sturtevant, 1964). Although systems incorporating either of these enzymes would be compatible with the existing CFD format by virtue of their capacity to be linked to the reduction of ferricyanide, the superior K_m value of lactate dehydrogenase for the L-lactate is highly advantageous considering the design objectives of the assay with respect to concentration. The selection of a pH that is optimal for the rate of any enzyme based reaction system is not intrinsically problematic with respect to the design of the CFD, provided that an appropriate buffer exists, Section IV.2.3..

Complexity. It would generally be expected that an increase in the complexity of a system would be associated with a reduction in the reliability of the system. Using minimum complexity as a favourable criterion for the development of an assay would favour the selection of an assay employing cytochrome- b_2 and ferricyanide over any of the other enzymatic detection systems. Further simplicity may be obtained by attempting to correlate the chelation of L-lactate by ferric chloride with some electrochemical parameter such as a shift in reduction potential, but perhaps at the cost of reduced specificity. Stability of the system components is most important when storage of the complete devices is considered. There is no reason to believe that any of the enzyme systems have intrinsically superior robustness on storage when incorporated into a catalytic layer similar to that developed for the glucose CFD.

Of all the systems examined it was decided that an assay system employing a NAD-dependent lactate dehydrogenase, NAD, diaphorase and ferricyanide best fulfilled the principle considerations with respect to cost and specificity and compatibility with existing CFD configuration and manufacture.

Consideration of the incorporation of an assay for L-lactate based on NAD-dependent dehydrogenase into a CFD obviates many of the problems associated with this strategy that have been noted by previous workers. Since the CFD is a disposable device designed for a single determination high stability of the system over several analyses is not required. In terms of reproducibility over a series of trials under the same conditions all that is demanded is that the manufacturing process is consistent within and between each production run. Here the primary concern would be for the viability of the reagents in the manufactured device whilst in storage. Since the only substance diffusing to the electrode surface is ferrocyanide the problems of variability in the electroanalytical signal associated with the oxidation of NADH do not arise. Additionally, because the oxidation potential of ferrocyanide is about 0.15V under the conditions of the assay, compared to between 0.6V to 0.8V vs. Ag/AgCl for NADH, the proportion of signal derived from extraneous sources in the sample is likely to be reduced. Unfortunately those assay systems employing hydrazine to prevent the system from reaching equilibrium (Section IV.1.) are inadmissible in an amperometric determination due to the reduction potential of hydrazine, +0.1 V vs. Ag/AgCl

(Ravichandran & Baldwin, 1983). Additionally, because of the cost of procurement from a commercial supplier, the use glutamate-pyruvate transaminase for the same purpose was initially avoided in the assay design.

The development of an assay system for the detection of L-lactate based on an NAD dependent lactate dehydrogenase compatible with the CFD format illustrates the capacity of the CFD concept to accommodate a series of complex biochemical reactions with little modification.

IV.2.2. PROCEDURES USED IN THE DEVELOPMENT OF THE L-LACTATE CFD.

NAD-dependent L-lactate dehydrogenase (E.C.1.1.1.27. from rabbit muscle; 500 u mg⁻¹), diaphorase (E.C.1.6.99.2. from *Clostridium kluyveri* ; 8 u mg⁻¹) and β -nicotinamide adenine dinucleotide (from yeast) were obtained from Genzyme Biochemicals Ltd. (Springfield Mill, Maidstone, Kent, ME14 2LE, UK). The lithium salt of L-lactate was obtained from Sigma Chemical Co. (Fancy Road, Poole, Dorset, BH17 7GT, UK). All were in the form of freeze dried powder.

All other components were obtained from previously stated sources and prepared as described in preceding sections.

The L-lactate oxidizing ink was prepared in the same way as described for the glucose-CFD, Section I.4.4., except that pyrophosphate and potassium phosphate buffers were used to give a final pH value in the device of ~9.5.

IV.2.3. ELECTRODE OPTIMISATION CONSIDERATIONS.

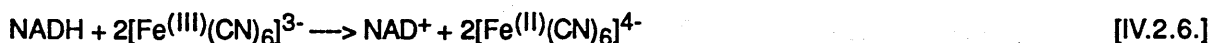
When considering which assay optimisation techniques to employ, it was decided that enough pre-information existed about the reaction rate for the oxidation of lactate by NAD-dependent lactate dehydrogenase with respect to pH that the use of non-linear model techniques such as simplex algorithms could be eschewed. Although the use of simplex and other techniques are well documented for the solution of complex problems in analytical chemistry (Deming & Morgan, 1973; Krause & Lott, 1974; Beh *et al.*, 1989) and process control technology (Himmelblau, 1970) their use in assay development would be more appropriately applied when less information was known about the processes and when less constraints were apparent. Consideration of the system variables; pH, concentrations of lactate dehydrogenase, diaphorase, ferricyanide and NAD, implies inclusion of invariant values for pH and the concentration of ferricyanide. The concentration of ferricyanide is invariant, because of the potential referencing demands of the CFD. Since the reaction is thermodynamically unfavourable at pH values of less than about 9.5, it is reasonable to assume that the system would have to be buffered at or above this pH value.

The remaining variables should be considered in terms of the likely rate limiting steps in the reaction sequence and the assay specification. The reactions which constitute the oxidation of L-lactate and the reduction of NAD by lactate dehydrogenase, [IV.1.1.], are well understood (Clarke *et al.*, 1989). Lactate dehydrogenase binds lactate only in the presence of coenzyme, implying an ordered reaction mechanism. The reaction sequence of the oxidation of L-lactate is summarised by [IV.2.1.] to [IV.2.5.]. The reaction scheme actually represents the sequence of events associated for one of the four identical subunits of lactate dehydrogenase (LDH).



The oxidation of L-lactate to pyruvate is fast, [IV.2.3.]. The slowest steps in the reaction sequence are the dissociation of pyruvate [IV.2.4.] and, even slower, the dissociation of NADH, [IV.2.5.].

The remainder of the reaction sequence is defined in [IV.2.6.] and [IV.2.7.].



Reaction [IV.2.6.] may be catalysed by diaphorase. Whether [IV.2.6.] would become rate limiting in any assay configuration depends on the concentration of ferricyanide, and diaphorase if present, and on the rate of evolution of NADH. The rate of the electrode reaction, [IV.2.7.], is assumed to be very fast compared to the preceding steps.

It is likely that the rate limiting step between [IV.2.1.] and [IV.2.7.] is the dissociation of NADH from LDH. The preceding step may also be influential since the K_i value of pyruvate for LDH is 10^{-4} M (Barman, 1969). From previous work it seems that the removal of the inhibitory effects of pyruvate generation is necessary for the reaction to proceed to a point close to completion (Sigma assay, Section IV.1.). Further, that the rate of oxidation of NADH by ferricyanide is fast compared to the preceding steps (Dix-Smith & Olson, 1974). The specifications of the proposed assay are that the sensor should give a linear response between 5 mM and 15 mM L-lactate, the approximate sensing range of L-lactate in the slurries, dependent on the extent of the fermentation and the extent of the dilution. The assay should also have a resolution of about 1 mM and a response time of 60 s for the catalytic reaction. The requirements of this sensor are quite different from the majority of lactate sensors that have been previously constructed. It should be able to respond to lactate concentrations of up to 15 mM, compared previous systems, often designed to measure blood lactate concentrations. Previous sensors were designed to respond typically to concentrations of L-lactate of between 0.1 mM (Arnold, 1988) and 2 mM (Yao & Wasa, 1985). In systems employing an oxidant for NADH, the concentrations of ferricyanide and Bindschedler's Green reported are 1 mM (Yao & Wasa, 1985) and 0.07 mM (Dix-Smith & Olson, 1974) respectively. The former of these systems employed diaphorase as a catalyst for the oxidation of NADH by ferricyanide. These systems employ concentrations of NAD between 0.1 and 1 mM, presumably because of cost considerations. The micro volume of the CFD allows the use of concentrations of NAD which are stoichiometrically equivalent or greater to the concentration of L-lactate.

Consideration of the previous discussion led to a rationale for the construction of an assay based on a ranking of the different strategies on the basis of complexity: the system of highest complexity being attributed the lowest ranking. The order of construction is described in Table IV.2.1..

Table IV.2.1. The order of priority for the construction of an electrochemical assay for L-lactate.

<u>system components</u>	<u>comments</u>
	low complexity, high ranking
LDH, ferricyanide, NAD ⁺ .	minimum number of components without direct oxidation of NADH.
LDH, ferricyanide, NAD ⁺ , diaphorase.	catalyst for oxidation of NADH by ferricyanide.
LDH, ferricyanide, NAD ⁺ , diaphorase, L- glutamate, glutamate-pyruvate transaminase	enzymatically catalysed pyruvate - transamination reaction, inhibitory effect of pyruvate obviated.
	high complexity, low ranking

IV.2.4. OPTIMISATION OF THE RATE OF REACTION WITH RESPECT TO THE CONCENTRATIONS OF LDH, DIAPHORASE AND NAD.

The route for the development of the assay inherent in Table IV.2.1. was initiated by constructing a pH - reaction rate profile for the least complex system, Figure IV.2.1.. The final concentrations of reagents were 0.3 M for ferricyanide, 30 mM for L-lactate, 50 mM NAD⁺ and 4.8 mg ml⁻¹ of lactate dehydrogenase. The catalytic reaction was allowed to proceed for 60 s before the application of a ferrocyanide oxidizing potential of duration 60 s. The pH - reaction rate response was constructed using a mixture of potassium phosphate, pyrophosphate, and glycine buffers, Section IV.2.2.. The concentration of L-lactate and NAD⁺ binding sites present in the lactate dehydrogenase in solution was equivalent to about 3.4x10⁻⁴ M (four subunits per molecule / molecular mass 140 kd). Comparison between the maximum charge passed, about 5 mC, and the amount of charge that would be passed on oxidation of all of the L-lactate present, 13.3 mC (from considerations of the working volume of a CFD for a potential pulse of duration 60 s and [II.3.4.], Section II.3.7.) reveals that the reaction had progressed to about 0.4 of its maximal extent.

Inspection of Figure IV.2.1. reveals that the reaction proceeds the furthest at pH values of 9.5 or higher. The relationship between the concentration of LDH and the extent of reaction in the time period was investigated using L-lactate at a concentration of 30 mM, Figure IV.2.2., twice the upper value of the specified concentration range. The system was buffered at pH 9.7 and contained 50 mM NAD. The response is not linearly proportional to the increase in the concentration of binding sites initially available for L-lactate and NAD⁺. This implies that the turnover number of the enzyme is such that an adequate number of binding sites exist for the reaction to proceed at its maximal rate under these conditions. The non-linear response on the increase in the ratio of the number moles of binding sites to the number of moles of L-lactate in each case indicates that the number of binding sites available is not rate limiting.

Inspection of the extent of reaction dependence on the concentration of NAD⁺, Figure IV.2.3., implies that the reaction is not limited by the concentration of NAD⁺ used previously. The values for Figure IV.2.4. were obtained using concentrations of L-lactate, LDH, and ferricyanide of 30 mM, 3 mg ml⁻¹ and 0.3 M respectively. The system was buffered at pH 9.7.

Finally the dependence of the response on the rate of oxidation of NADH by ferricyanide was investigated by using the catalyst diaphorase. The conditions which elicited maximum response in the previous experiments were used: 30 mM L-lactate, 0.3 M ferricyanide, 35 mM NAD⁺, and LDH at a concentration of 4.8 mg ml⁻¹. The system was buffered at pH 9.7. The system response to diaphorase is shown in Figure IV.2.4.. The extent of reaction is limited to about 0.5 of the calculated maximal extent.

These studies implied that in order to construct a system that allows the reaction to attain an end point the use of a pyruvate trapping mechanism, involving glutamate pyruvate transaminase, should be explored. Initial investigations using glutamate pyruvate transaminase and L-glutamate indicated that does have some effect on the extent of reaction over the time-scale explored, at 30 mM L-lactate the charge response over the analysis period increased by a factor of ~1.2, probably due to a diminution of the influence of K_i, but the effect on charge response in the time scale was considered ineffective relative to the resources involved in incorporation of this pyruvate trapping chemistry into the catalytic layer. The primary cause of the failure to reach an end point may be due to the rate of dissociation of NADH. Because of the marginal effect of pyruvate trapping over the timescales involved it was decided to construct charge - concentration correlations using diaphorase, ferricyanide, LDH and NAD as specified.

Figure IV.2.5. shows the charge response of the system at concentrations of L-lactate between 0 mM and 25 mM for a ferrocyanide oxidizing potential of duration 60 s, applied 60 s after the initiation of the reaction. The linear range in Figure IV.2.5. is between about 0 mM and 10 mM (with the exception of the response at 0 mM lactate, at each concentration, n=8, cv ≤ 6%, r²=0.9910). In this region the

Figure IV.2.1. The normalised coulometrically determined pH-response curve for the LDH/NAD/ferricyanide/L-lactate system. Conditions; L-lactate, 30 mM; NAD, 50 mM; LDH, 4.8 mg ml⁻¹ (500 u ml⁻¹; 3.4 x10⁻⁵ M); ferricyanide, 0.3 M; t_{react}=t_{analysis}=60 s. Mean values normalised against a maximum mean response of 5.12 mC (n=5, cv ≤ 10%).

Figure IV.2.2. The normalised coulometrically determined [LDH]-response curve for the LDH/NAD/ferricyanide/L-lactate system. Conditions; L-lactate, 30 mM; NAD, 50 mM; pH, 9.7 (phosphate-pyrophosphate buffer); ferricyanide, 0.3 M; t_{react}=t_{analysis}=60 s. Mean values normalised against a maximum mean response of 5.1 mC (n=5, cv ≤ 10%).

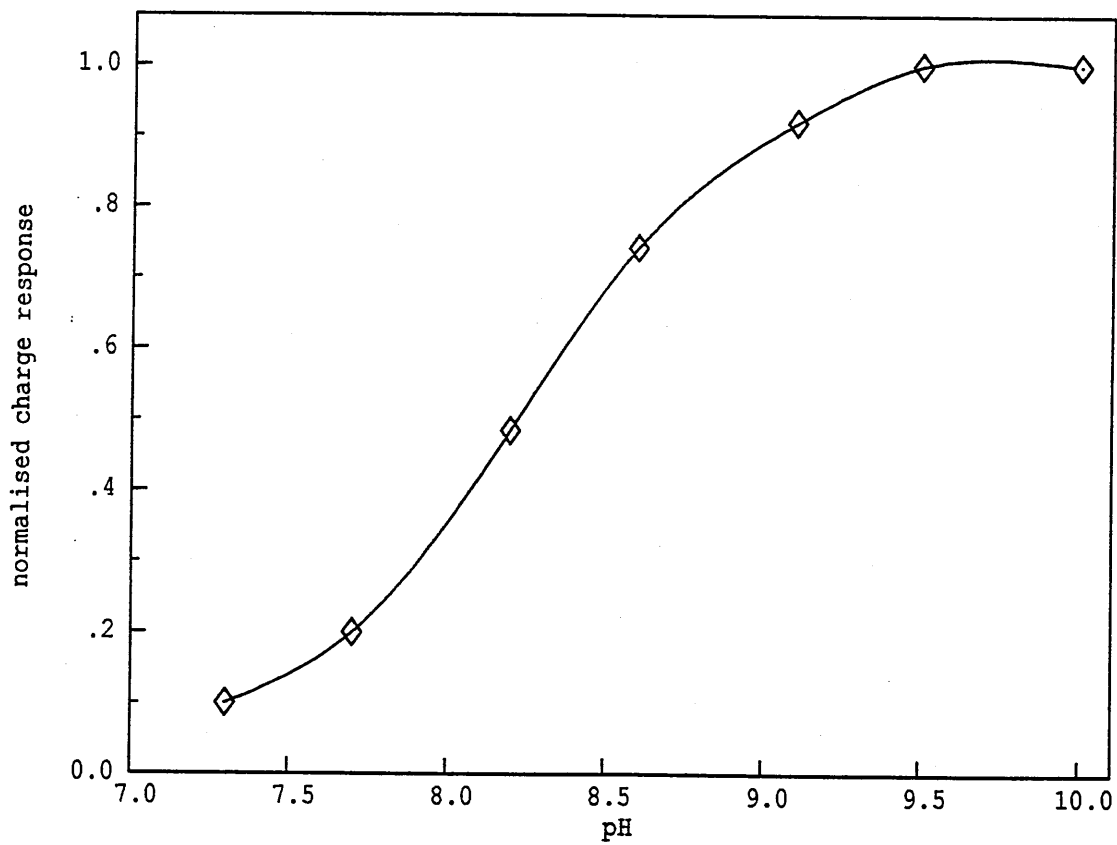
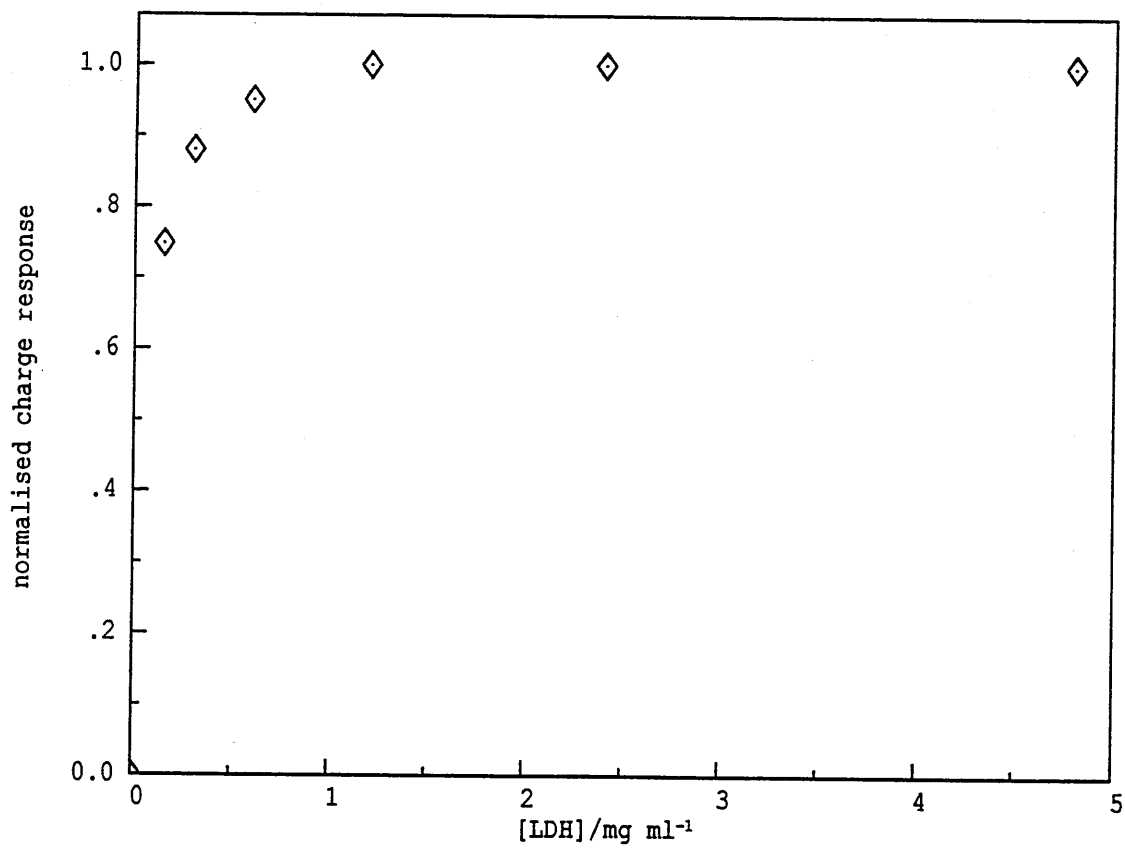
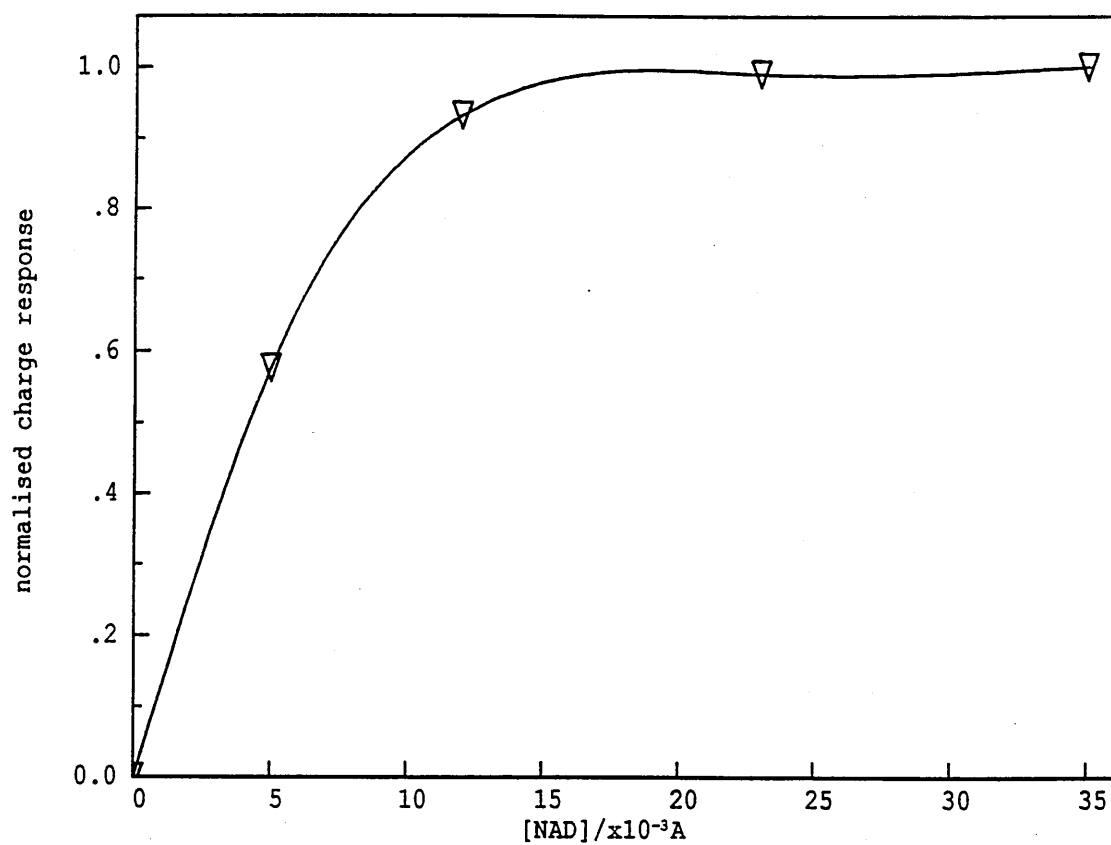
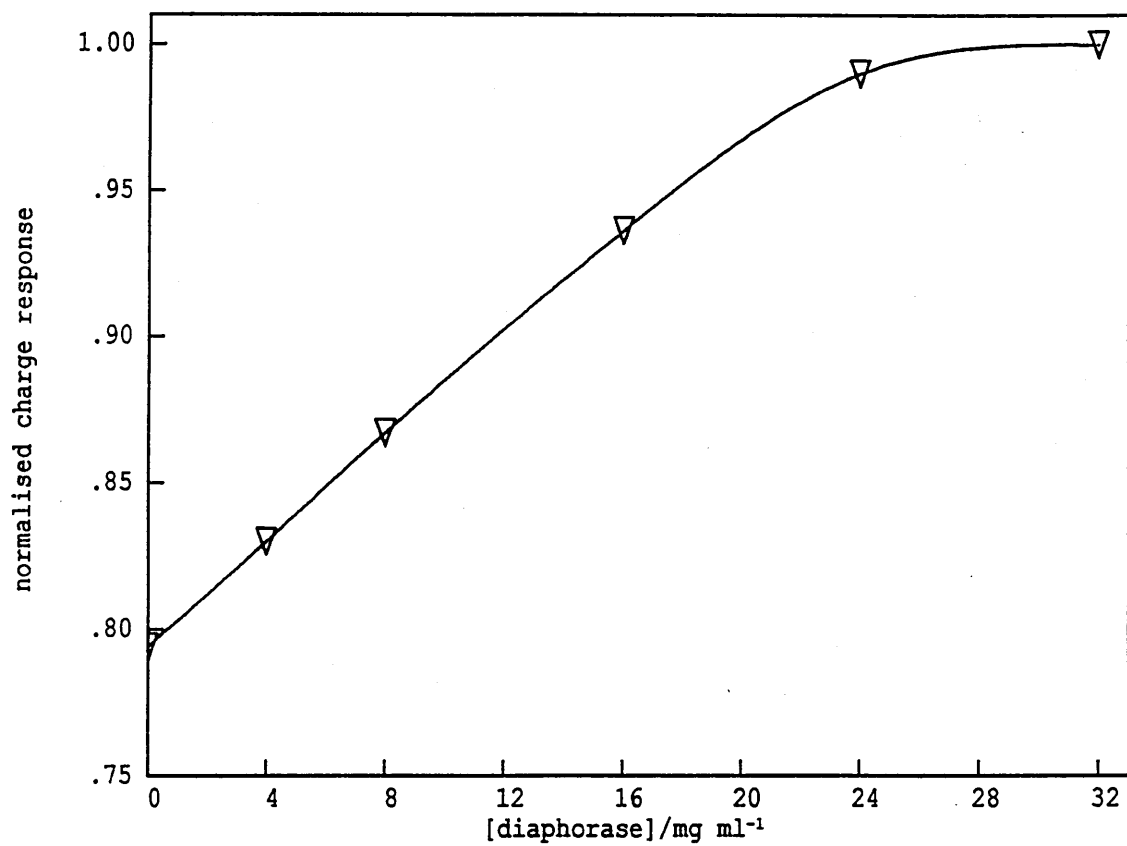


Figure IV.2.3. The normalised coulometrically determined [NAD]-response curve for the LDH/NAD/ferricyanide/L-lactate system. Conditions; L-lactate, 30 mM; LDH, 3 mg ml⁻¹; pH, 9.7 (phosphate-pyrophosphate buffer); ferricyanide, 0.3 M; $t_{\text{react}}=t_{\text{analysis}}=60$ s. Mean values normalised against a maximum mean response of 5.14 mC (n=5, cv~10%).

Figure IV.2.4. The normalised coulometrically determined [diaphorase]-response curve for the LDH/NAD/ferricyanide/L-lactate-diaphorase system. Conditions; L-lactate, 30 mM; LDH, 3 mg ml⁻¹; NAD, 35 mM; pH, 9.7 (phosphate-pyrophosphate buffer); ferricyanide, 0.3 M; $t_{\text{react}}=t_{\text{analysis}}=60$ s. Mean values normalised against a maximum mean response 6.41 mC (n=5, cv~10%).



slope of the line is 0.3 C M^{-1} , the intercept $[\text{L-lactate}]=0$ is 0.51 mC ($\text{sd}=0.09 \text{ mC}$, giving a limit of detection of $\sim 1 \text{ mM}$). The response is non-linear at concentrations greater than about 10 mM L-lactate, and whilst the reaction is between 60% and 70% complete for analyte concentrations below this value, the extent of reaction falls to about 40% of its theoretical maximum. The response over the whole of the concentration range $0 \text{ mM} \leq [\text{L-lactate}] \leq 25 \text{ mM}$ may be fitted to a second order polynomial, Figure IV.2.5., with a correlation coefficient of > 0.99 (95% confidence limits represented by outer lines). The coefficient of variance decreases with increasing concentration of analyte (cv at 0 mM L-lactate $\sim 15\%$; cv at 10 mM L-lactate $\sim 6\%$). The principle cause of absolute error at each concentration, probably originates from the high background concentration of ferrocyanide.

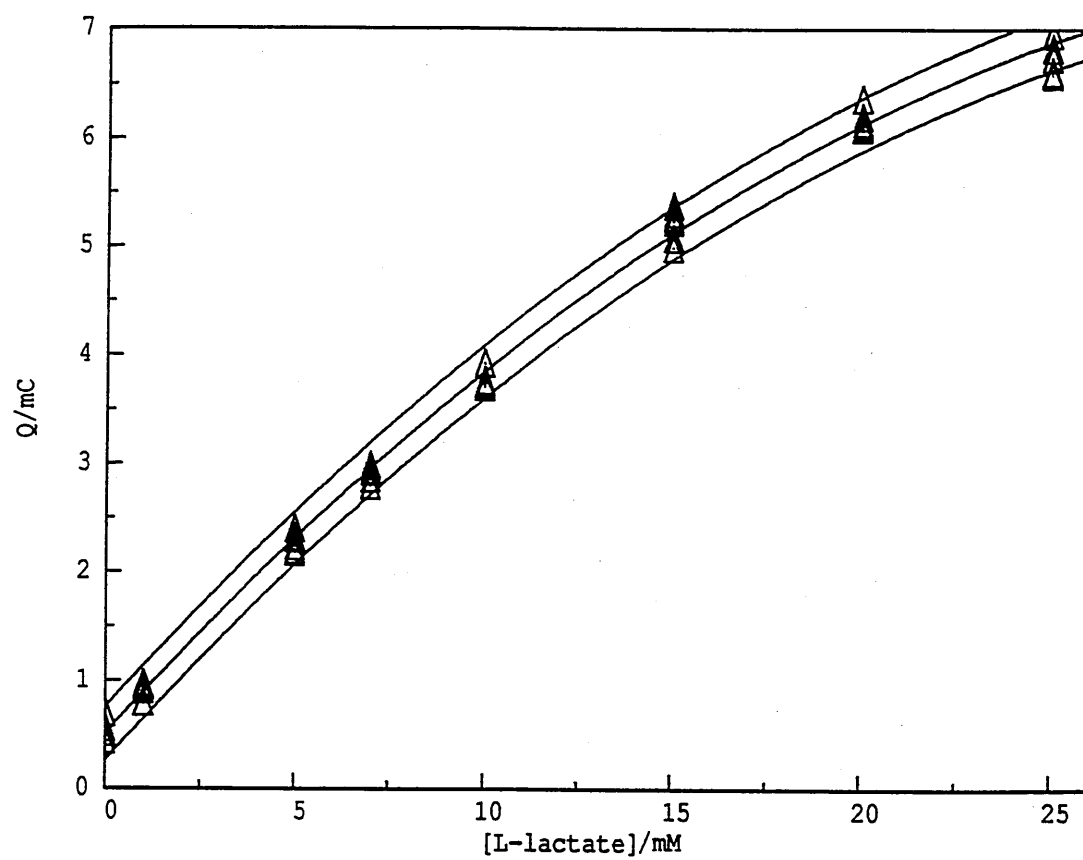
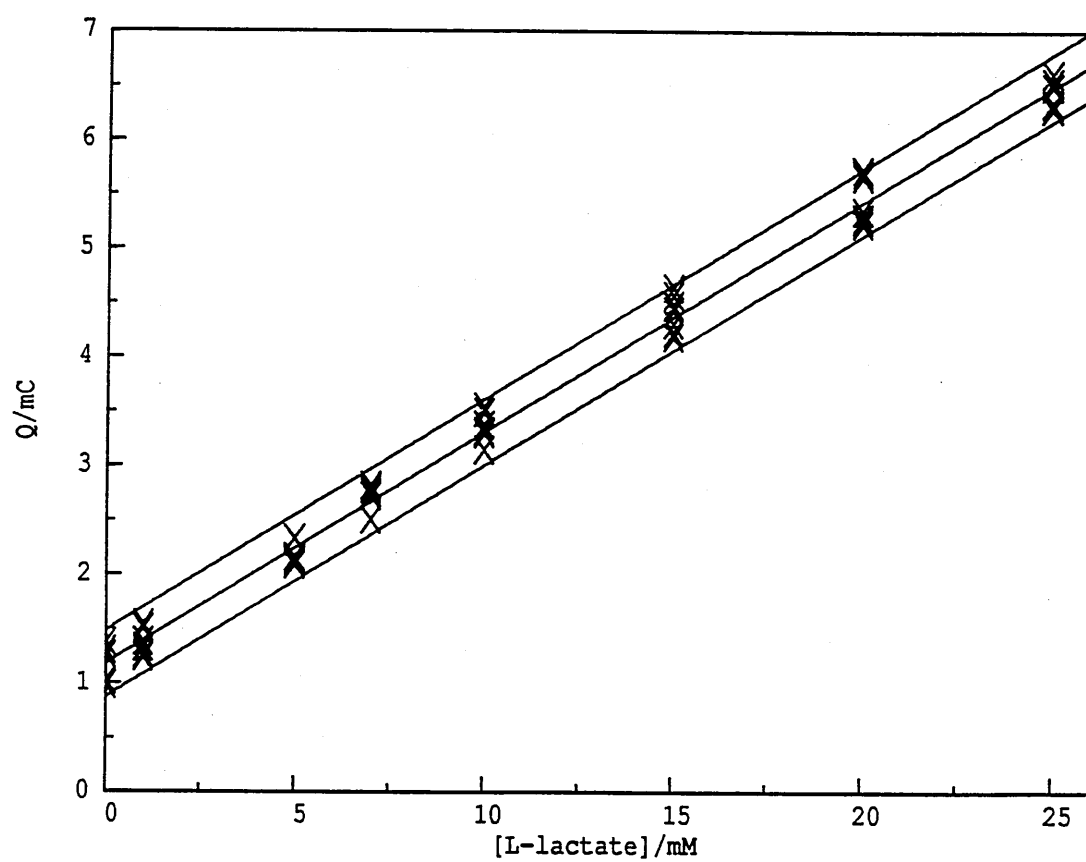
IV.2.5. THE CONSTRUCTION & PERFORMANCE OF AN L-LACTATE OXIDIZING INK INCORPORATING LDH, NAD, DIAPHORASE AND FERRICYANIDE.

Previous studies (Sections IV.2.3. and IV.2.4.) suggest that the reaction rate dependence on pH is identical for phosphate-based buffers and for glycine buffer systems. The glycine buffer system was unsuitable for incorporation into an ink of the type developed for the glucose CFD, Section I.4., because of an interaction between the glycine, ferricyanide and cellulose acetate that resulted in extensive reduction of the ferricyanide. In preference to commencing the design of a carrier medium suitable for incorporation of glycine and the other components and printing, phosphate and pyrophosphate buffers were substituted. The final mean concentrations of the components throughout the CFD incorporating the lactate ink were identical to the soluble system.

The response of the sensor to solutions containing L-lactate between 0 mM and 25 mM is shown in Figure IV.2.6.. The slope of the line is 0.2 C M^{-1} , indicating that the response is less sensitive than the linear region of Figure IV.2.5., but the linear range is extended to more than 20 mM (at each concentration $n=8$, $\text{cv} \leq 14\%$, $r^2=0.990$). The intercept at 0 mM L-lactate is 1.18 mC . The extent of reaction in all cases is about 40%. The extension of the range of the linear response compared to the soluble system may be explained in terms of the diffusional rather than kinetic rate limitation, evident in the response for the soluble system. The greater coefficient of variance compared to the soluble system is a manifestation of the greater variability in the concentration of ferrocyanide present in the catalytic layer prior to the introduction of the sample (sd at zero analyte concentration is 0.17 mC , giving a limit of detection of about 2 mM). The higher variability at 0 mM L-lactate coupled to the decreased sensitivity of the response is responsible for the doubling in the limit of detection compared to the soluble system. The increased variability at 0 mM L-lactate may be caused by the change in pH composition of the catalytic layer compared to the layer constructed for the glucose-CFD. Although the catalytic layer is composed of constituents based in an organic matrix, Section I.4.4., absorption of water by the layers would result in some expression of pH in the layer prior to use, which may accelerate the reduction of ferricyanide.

Figure IV.2.5. The coulometric response curve to L-lactate for the soluble LDH/NAD/ferricyanide/L-lactate-diaphorase system. Conditions; LDH, 3 mg ml⁻¹; NAD, 35 mM; diaphorase, 4 mg ml⁻¹; pH, 9.7 (phosphate-pyrophosphate buffer); ferricyanide, 0.3 M; $t_{\text{react}}=t_{\text{analysis}}=60$ s. The inner line represents the least squares second order polynomial of the charge response, Q; $Q=0.51 + 0.3867[\text{L-lactate}] - 5.27 \times 10^{-3}[\text{L-lactate}]^2$. The outer lines represent 95% confidence limits for all of the data using this routine. The response is linear for the concentration range $0 \text{ mM} \leq [\text{L-lactate}] \leq 10 \text{ mM}$; $Q=0.51 + 0.3[\text{L-lactate}]$. Both regression analyses yield a correlation coefficient of > 0.99 , and coefficient of variance at each concentration of $5\% \leq cv \leq 10\%$. The limit of detection, determined at 0 mM L-lactate, is ~ 1 mM.

Figure IV.2.6. The coulometric response curve to L-lactate for the matrix-bound LDH/NAD/ferricyanide/L-lactate-diaphorase system. Conditions; LDH, 3 mg ml⁻¹; NAD, 35 mM; diaphorase, 4 mg ml⁻¹; pH, 9.7 (phosphate-pyrophosphate buffer); ferricyanide, 0.3 M; $t_{\text{react}}=t_{\text{analysis}}=60$ s. The inner line represents the application of a least squares linear regression analysis for $0 \text{ mM} \leq [\text{L-lactate}] \leq 25 \text{ mM}$, outer lines represent 95% confidence limits. The charge response may be represented by... $Q=1.17 + 0.21[\text{L-lactate}]$. The coefficient of variance over the concentration range falls between 6% and 10%. The limit of detection, determined from the standard error at 0 mM L-lactate is ~ 2 mM.



IV.2.6. CONCLUDING REMARKS.

These introductory explorations into the amenability of the CFD to accommodate a set of L-lactate oxidizing chemistries imply that it should be possible to construct a sensor to meet the requirements of the assay provided that the problem of variability of charge response at 0 mM L-lactate is solved. The limit of detection of the CFD incorporating the L-lactate oxidizing ink is ~2 mM (the limit of detection for the glucose-CFD, Section III.4.4. is ~0.6 mM). It is thought that this is due to the an increase in the concentration of ferrocyanide in the catalytic layer perhaps caused by absorption of water by the ink and layer during preparation and manufacture. Approaches to a solution to this problem include the separation of the printed layers containing the buffer salts from the rest of the components, either by printing a layer containing buffer salts over the catalytic layer or over the electrode. Alternatively the buffer salts may be completely excluded from the CFD and appropriately buffered solutions may be used to make the slurries for analysis.

review, comments & further work

REVIEW, COMMENTS & FURTHER WORK.

A theme that has touched several aspects of the work presented in this thesis is the nature of the interaction between electron mediators and oxidoreductases. Initial investigations using ruthenium (III) hexamine as a soluble electron mediator for glucose oxidase revealed an unusual pH-response profile. Further probing revealed that the reason for the apparent increase in the reactivity of ruthenium (III) hexamine with glucose oxidase in the presence of glucose was probably not associated with an increase in the electron transfer constant with increasing pH intrinsic to the ruthenium (III/II) hexamine couple (investigation of K_{ex} using K_h - K_{ex} correlation, Section II.4.). The reason underlying the increased reactivity at high pH values was thought to be associated with an electrostatic Gibbs energy term and an enhanced difference between the redox potential of the FAD/FADH₂ and ruthenium (III/II) hexamine couples (Section II.5.). The second order rate constant, k_e , for the reaction between reduced glucose oxidase and the electron mediators ruthenium (III) hexamine and ferricyanide was evaluated on the basis of differences in the current decay in the presence and absence of an homogeneous catalytic reaction (Section II.5.). The values of k_e for the interaction of both mediators with reduced glucose oxidase was found to be far inferior to that exhibited by the ferrocenes ($80 \text{ M}^{-1} \text{ s}^{-1}$ for ruthenium (III) hexamine, $50 \text{ M}^{-1} \text{ s}^{-1}$ for ferricyanide and $\leq 10^5 \text{ M}^{-1} \text{ s}^{-1}$ for ferrocene derivatives, Section II.5.).

The use of ruthenium (III) hexamine as an oxidant for reduced glucose oxidase for the analysis of glucose concentration using coulometry resulted in a linear response for $0 \text{ mM} \leq [\text{glucose}] \leq 30 \text{ mM}$. The coefficient of variance for the response at each concentration of glucose tested was $\leq 5\%$. The limit of detection was significantly enhanced compared to analysis using ferricyanide as mediator (0.2 mM for ruthenium (III) hexamine vs. 0.6 mM for ferricyanide, Sections II.3.7. and III.4.4.).

Both phases of the analysis were analysed using a series of numerical models in order to explore routes for the reduction of overall analysis time. The value of k_e for ruthenium (III) hexamine and ferricyanide was used to construct models of the operation of the catalytic layer, Section III.3.2.. Despite the low k_e of these mediators compared to the ferrocenes all of the modelling investigations suggested that the rate limiting step probably does not involve k_e , due to the high concentration of mediator present (0.3 M). For the concentration range of glucose oxidase in the catalytic layer investigated ($[E] \geq 0.8 \text{ moles m}^{-3}$) and using the simplifying assumption that $k_1[E] > \tau$, the rate constant associated with the dissociation of the enzyme-substrate complex, k_2 , was determinant in setting the boundary conditions for flux of glucose to the surface of the catalytic layer. Unlike previous kinetic analyses of conventional enzyme electrodes it was found that the flux of glucose to the surface of the catalytic layer in the CFD operates under transitory boundary conditions. Two cases were identified; Case A, where the concentration of glucose at the catalytic layer-solution interface was zero, the rate of the enzyme catalysed reaction being greater than the maximum diffusion controlled flux of glucose; Case B, where the concentration of glucose at the catalytic layer-solution interface was non-

zero and its flux to the catalytic layer-solution interface was determined by the rate of the enzyme catalysed reaction. The boundary conditions in Case A are essentially the same as for a fast heterogeneous oxidation or reduction reaction and the flux of glucose is therefore determined by the diffusion characteristics of the species in that solution. During the course of a determination of glucose the boundary conditions make a transition from Case B behaviour to Case A behaviour. The point of that transition is determined by the concentration of glucose oxidase in the catalytic layer and by the diffusion characteristics of glucose in the catalytic layer as defined by its fractional diffusion coefficient in the catalytic layer, α ; the greater the value of α and $[E]$ the sooner Case A behaviour occurs. Explorations of the sensitivity of the model response to α suggest that maintenance of a value of α close to one is important in terms of reducing the determination time. The sensitivity of the model response to increasing the concentration of glucose oxidase above its present value implied that only a marginal decrease in the duration of the catalytic phase may be achieved even by raising the concentration of glucose oxidase by an order of magnitude. The effect of gap width on the model response was incorporated into overall sensor performance profiles by using a time constant that related the cell dimensions, the diffusion coefficient of glucose and a function related to the exponential decay constant for glucose concentration at the electrode surface. These profiles indicate that very significant reductions could be made in overall analysis time by reducing the gap width, but would have to be associated with improvements in the precision of gap definition; for a reduction in gap width by a factor of two, the precision of determination of the gap width would have to double in order to maintain the same level of relative error in volume definition.

The analysis phase was also modelled numerically to explore the perceived advantages in terms of a reduction in analysis time of applying a series of potential pulses separated by relaxation periods to solutions containing reduced mediator. The model solved the flux equations for reduced mediator to the surface of the electrode perpendicular to the electrode surface, and laterally across the edges of the electrode. For times dependent on the gap width of the cell and the diffusion characteristics of the diffusing species the models predicted a time^{-0.5} - dependent current decay, suggesting essentially semi-infinite solution characteristics, Sections II.5. & III.2..

Initial considerations of the charge response, Section III.2., predicted an exponential decay of charge values with respect to pulse number for all pulses, founded on the assumption of an homogeneous concentration of reduced mediator in the x-dimension at the end of each relaxation period. The numerical models, Sections III.3.1 & III.4.3., generated a similar charge-pulse response, but only after the second or third pulse. Comparison of the diffusion profiles in the x-dimension for the first pulse with those obtained from subsequent pulses revealed that the concentration of reduced mediator does not attain an homogeneous concentration across the cell during the relaxation periods. This has the effect that the fractional change in concentration at the surface of the electrode between the first and the second pulses is greater than that obtained for subsequent pulses. Therefore the charge response for the first pulse does not conform to the exponential decay observed for subsequent pulses. After the first and second relaxation periods, however, the distribution of reduced mediator in

the x-dimension conforms to a similar fractional value of that obtained at the end of the previous relaxation period. Consequently the exponential least squares regression of the charge decay for the charge response, excluding the first pulse, may be used to obtain linear correlations with intercepts close to zero for the concentration of reduced mediator present prior to the application of the first potential pulse. The exclusion of the charge passed over the second potential pulse results in a superior linear correlation between initial concentration of reduced mediator and charge response.

Using glucose-CFDs, for $0 \text{ mM} \leq [\text{glucose}] \leq 20 \text{ mM}$, the performance of the pulse-relaxation method was favourable compared to exhaustive coulometry. The principal advantages of the pulse-relaxation method over coulometry appears to be an improved limit of detection and reduced analysis time (0.2 mM vs. 0.6 mM; ~10 s vs. 60 s; for a pulse-relaxation frequency of 1 Hz).

As predicted from initial considerations of the effects of lateral diffusion, Section III.2., for pulse and relaxation periods of 1 s duration the contribution to the charge response from lateral flux of reduced mediator was found to be insignificant compared to the charge response derived from flux in the perpendicular direction. For potential pulses of duration $\leq 1 \text{ s}$ the concentration perturbation occurring in the region of the the guard electrode is negligible (maximum concentration change $150 \mu\text{m}$ from edge of the working electrode is $< 10\%$ of original value) the activation of the guard electrode has little effect on the charge passed over any of the pulses. This obviates the inclusion of the guard electrode in the sensor design, which has advantageous consequences in terms of reducing the complexity of the manufacturing process.

On a practical level, the crude incorporation of a set of lactate oxidizing chemistries taken directly from biological systems has been demonstrated to be of some analytical utility with little modification to the sensor design, Chapter IV.

The use of numerical models in the development of new analytical techniques and for exploring the performance characteristics of catalytic layers has been demonstrated but the potential for the use of analogous techniques extends beyond the bounds of this thesis. Numerical models could be constructed to explore various sensor configurations prior to the construction of any devices. The use of models in this context would allow the rejection of unfavourable designs prior to the investment of the resources necessary for their manufacture, thereby concentrating effort on the most promising designs. Examples that may be of interest in the present context include the incorporation of different discrete chemistries on the same device, to create a multi-sensor, or modelling the current response for working electrodes whose minimum dimension is such that the flux contributions from at least one lateral dimension may be, after a short time (~8 s for ferricyanide), of the same order as the flux contribution in the x-dimension. The former may be achieved by incorporating different substrate specific chemistries on discrete working electrodes, surrounded by guard electrodes to separate the reduced mediator generated from one analysis from the reduced mediator produced on another working electrode. The role of modelling the system prior to

construction would be to determine the minimum distances between the sites for the different chemistries that could be allowed without the product from one set of reactions to interfere with another set, assuming that the guard electrodes remained passive during the catalysis phase.

The effects of reducing the width of the working electrode such that the flux derived from lateral diffusion predominates the current response after a certain time may have analytical advantages analogous to the use of ultramicroelectrodes (Fleischmann *et al.*, 1987), such as increasing the ratio of faradaic to non-faradaic current response. Although the current response for an electrode strip in a CFD would not conform to the steady state current response expected from a circular ultramicroelectrode, it should contain a steady state component that would be derived from lateral diffusion which should predominate any other contributions after a time determined by the diffusion characteristics of the electroactive species. An optimal design may be approached by modelling the current response arising from various electrode conformations.

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