

ENZYME ELECTRODES USING
CONDUCTING ORGANIC SALTS

by

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ABSTRACT

The work contained in this thesis is concerned with the use of organic salts as electrode materials for use in enzyme electrodes. It has previously been found that these materials are much more effective than bare metals for the oxidation of certain biological molecules, such as flavoenzymes and NADH. In this thesis the possibility of using organic salts within sensors is explored and the underlying mechanism of the electron transfer between the enzymes and organic salts is investigated.

An enzyme electrode for choline is described in which choline oxidase is entrapped close to the organic salt surface by a semi-permeable membrane. The sensor responds to both choline and betaine aldehyde, and analysis of the response using a theoretical model allows the processes involved in limiting the response of the device to be determined. Conclusions made from this analysis are confirmed by observing changes in the behavior of the sensor when its component parts are altered.

Several methods for the electrosynthesis of NMPTCNQ and TTFTCNQ modified electrodes are investigated and a TTFTCNQ electrode fabricated in this way is used in a membrane electrode which responds to glucose. Rotating disc studies on glucose oxidase using a TTFTCNQ electrode are also described and the experimental data for different enzyme concentrations, rotation speeds and potentials are compared with two theoretical models in an effort to elucidate the mechanism of the system. The results of this analysis are inconclusive, but further conditions that any mechanistic model has to satisfy to predict the overall behavior of the system are determined.

To my parents.

"Man`s got to know his limitations"

C Eastwood

"..what is a man, what has he got?

If not himself, then he has not

To say the things he truly feels

And not the words of one who kneels.."

P Anka

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CHAPTER 1

INTRODUCTION

This thesis is concerned with the development of enzyme electrodes for use as chemical sensors for biological molecules in complex mixtures or in vivo. Enzyme electrodes are devices which utilise enzymes for their high selectivity together with electrochemical detection for its sensitivity and ease of use. In this first chapter, the basic properties of enzymes are described as well as conducting organic salts which are employed as electrode materials in the work contained in this thesis. The next section gives a short overview of the field of biosensors and in particular enzyme electrodes, taking the development of glucose sensors as a case study. Finally, this chapter concludes with a brief description of the rest of this thesis.

1.1 ENZYMES

Enzymes are the essential catalysts of biochemical reactions. Compared to the corresponding non-biological catalysts enzymes exhibit greater catalytic efficiency, higher specificity, and elimination of unwanted side-reactions. The reasons for this lie in their structure and the mechanisms by which they operate. A detailed exposition of these factors is beyond the scope of this review and the interested reader is referred to several excellent texts on the subject^[1-5]; however some of the more basic properties of enzymes that are relevant to this thesis will now be

examined.

All enzymes are proteins, although many contain smaller non-protein molecules known as co-factors or prosthetic groups, depending on whether they are ionically or covalently bound to the protein. When a substrate interacts with the enzyme it will bind at a specially tailored surface known as the specificity site. It is the binding properties of that site that determine the specificity of the enzyme for that particular substrate. Adjacent to, or overlapping with, the specificity site will be a region where the reaction will take place, called the active site. The nature of the interactions between the enzyme and substrate at this site, and at the specificity site, will determine how and at what speed the reaction will take place.

The rate at which an enzyme catalyses a reaction depends on many factors, such as pH, temperature, ionic strength, and enzyme and substrate concentration. In general, enzymes work most effectively at physiological values of the first three parameters and will be deactivated or denatured, possibly irreversibly, under more extreme conditions. Usually the reaction rate is directly proportional to the enzyme concentration; however its dependence on substrate concentration will be more complicated and depends on the exact mechanism of the reaction. Many enzyme/substrate systems have been found, however, to approximately obey the same experimental rate law known as the Michaelis-Menten equation^[6]:

$$v = k_{cat} [E]_t [S] / (K_m + [S]) \quad (1.1)$$

where v is the rate of reaction, k_{cat} is known as the turnover number, $[E]_t$ is the total concentration of enzyme present, $[S]$ is the substrate concentration and K_m is known as the Michaelis constant. This rate law can arise from many different kinetic models and a derivation of this law for a particular kinetic mechanism is given in Appendix 1.

This equation is represented graphically in Figure 1.1.1. It can be seen that at high substrate concentrations ($[S] \gg K_m$) the rate of reaction reaches a maximum, v_{max} which is determined by the turnover number and the enzyme concentration. i.e.

$$v_{max} = k_{cat} [E]_t \quad (1.2)$$

At low substrate concentrations ($[S] \ll K_m$) the rate of reaction is given by:

$$v = (k_{cat} / K_m) [S] [E]_t \quad (1.3)$$

Thus, under these conditions, the rate of reaction is directly proportional to substrate concentration.

When confronted with experimental results, the data can be treated in several ways to find experimental values of the various kinetic parameters. In this thesis the Hanes plot [7] is employed, which relies on expressing equation(1.1) as:

$$[S]/v = [S]/v_{max} + K_m/v_{max} \quad (1.4)$$

Thus plotting experimental values of $[S]/v$ as the ordinate and $[S]$ as the abscissa gives K_m and v_{max} from the gradient and the intercept (see Figure 1.1.2).

There are many different reactions catalysed by

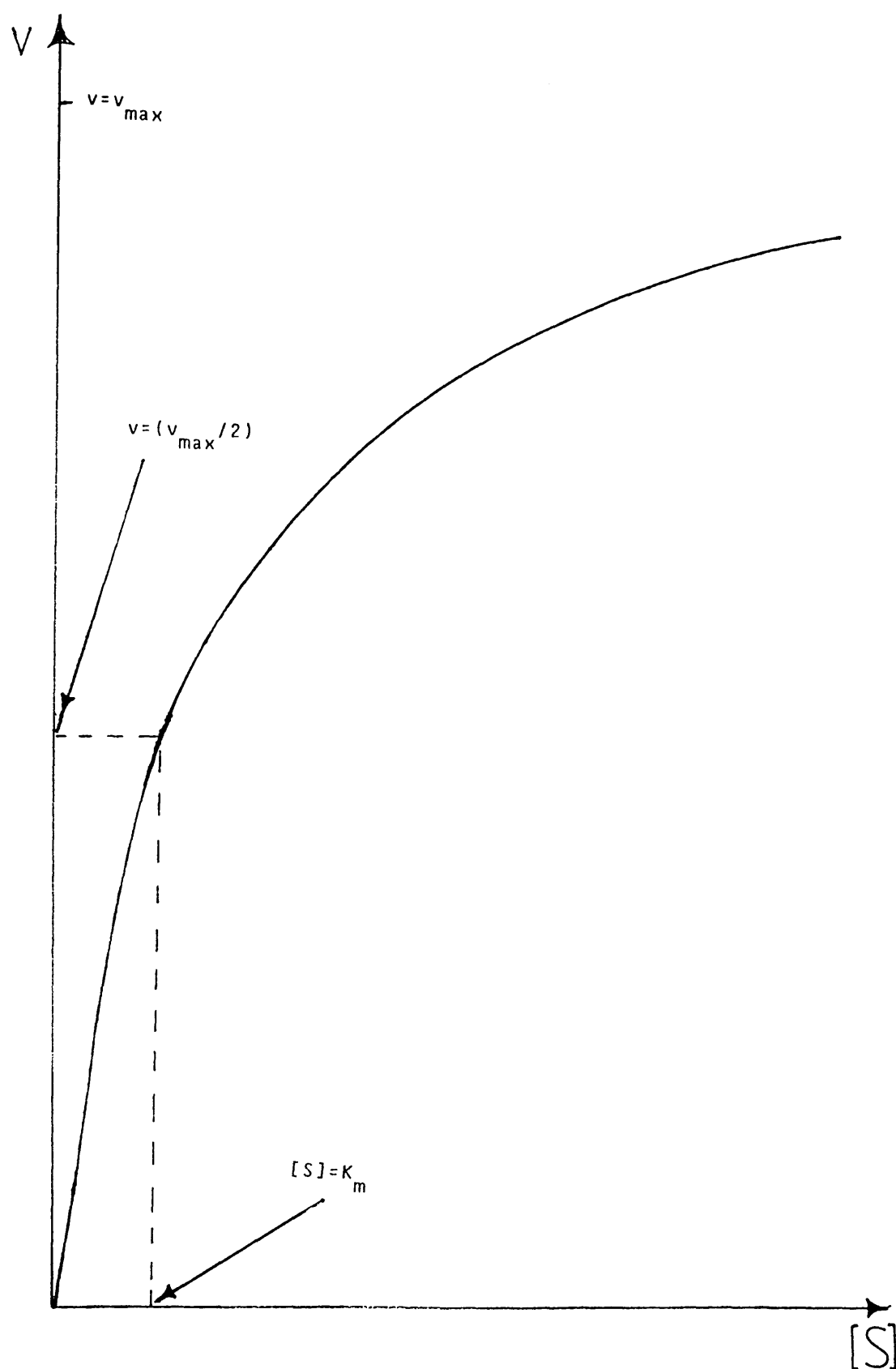


Figure 1.1.1 Dependence of rate with substrate concentration for enzymes displaying Michaelis-Menten kinetics.

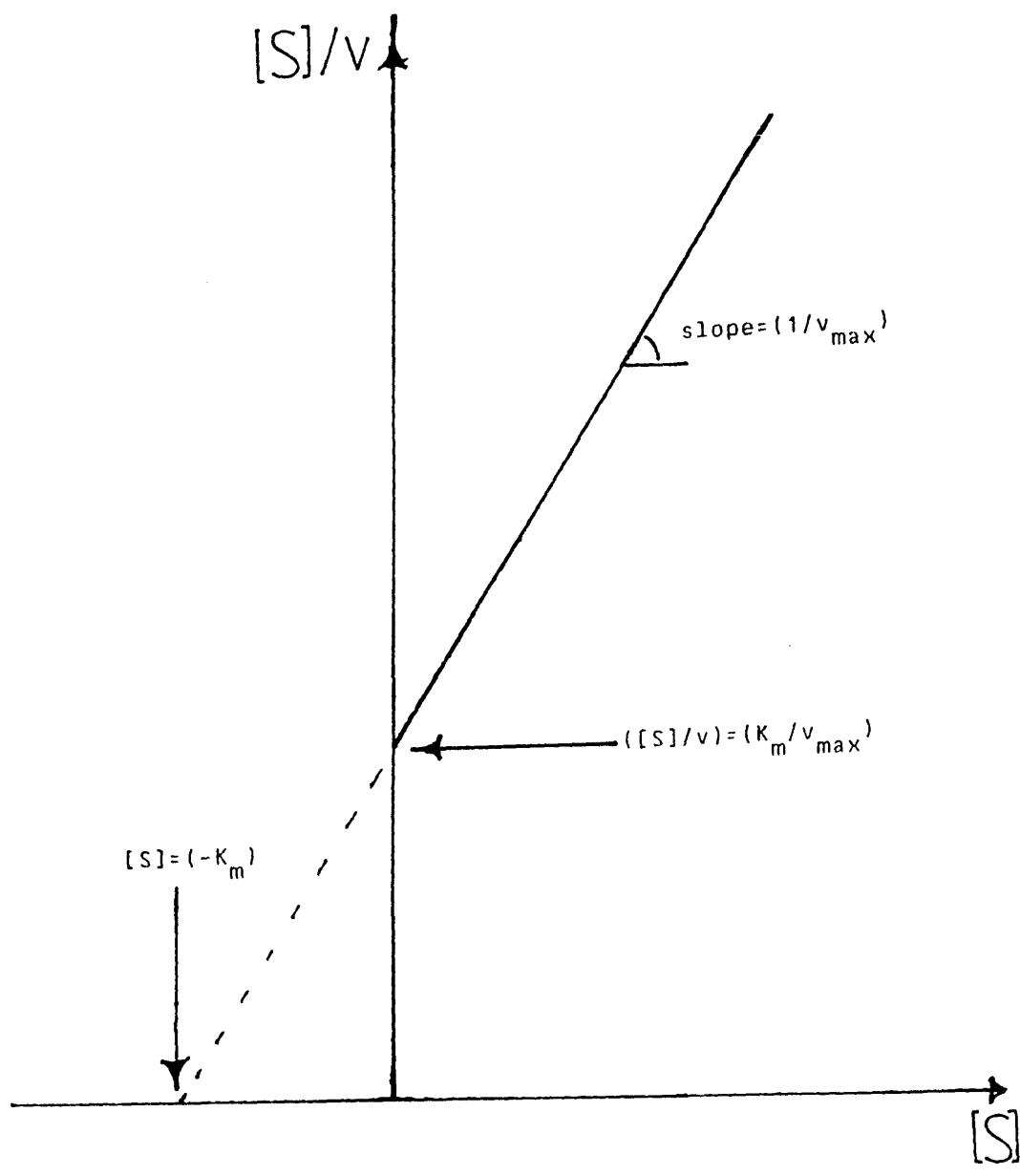
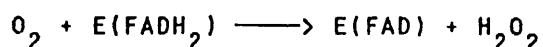
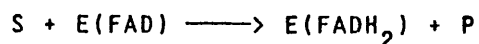


Figure 1.1.2 The Hanes Plot.

enzymes. The enzymes used in the work contained in this thesis catalyse oxidation/reduction reactions and form part of a class of enzymes known as oxidoreductases. Furthermore, they both contain flavin adenosine dinucleotide(FAD, Figure 1.1.3) as a co-factor or prosthetic group and are therefore known as flavoproteins. These will be discussed in more detail in the next section.

1.1.1 FLAVOPROTEINS

As stated above, flavoproteins^[8,9] contain the FAD moiety as a co-factor or prosthetic group. The FAD moiety is normally very tightly bound to the protein by ionic interactions, although in some cases it is bound covalently^[10]. Many flavoproteins perform a two electron transfer directly on the substrate, resulting in a corresponding reduction in the isoalloxine ring of the FAD (Figure 1.1.4). The reoxidation of the enzyme to its substrate active form is then facilitated by a suitable electron acceptor, commonly molecular oxygen in biological systems. In this case, the overall reaction scheme is:



In this work, two flavoproteins are employed, namely glucose oxidase (GOD) and choline oxidase (ChOD). Each will now be examined in turn.

a) Glucose Oxidase

Glucose oxidase(GOD) catalyses the oxidation of B-D-glucose by molecular oxygen to give D-gluconolactone and

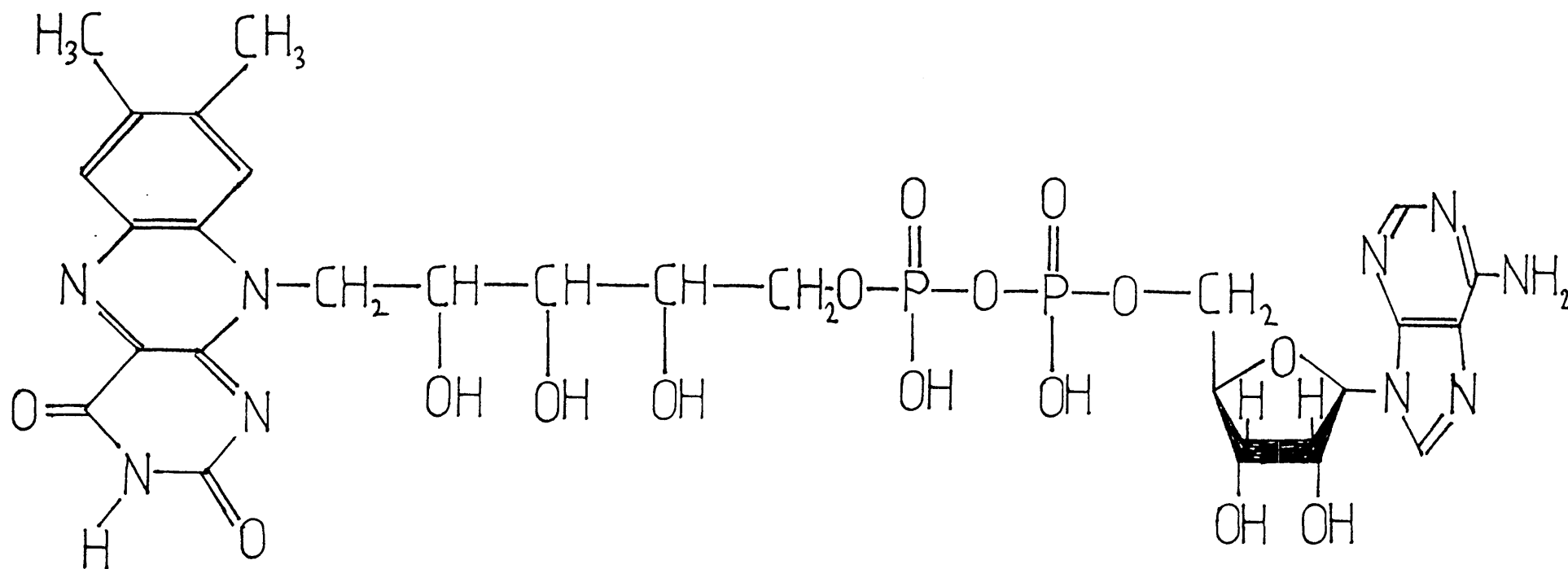


Figure 1.1.3 Chemical structure of FAD

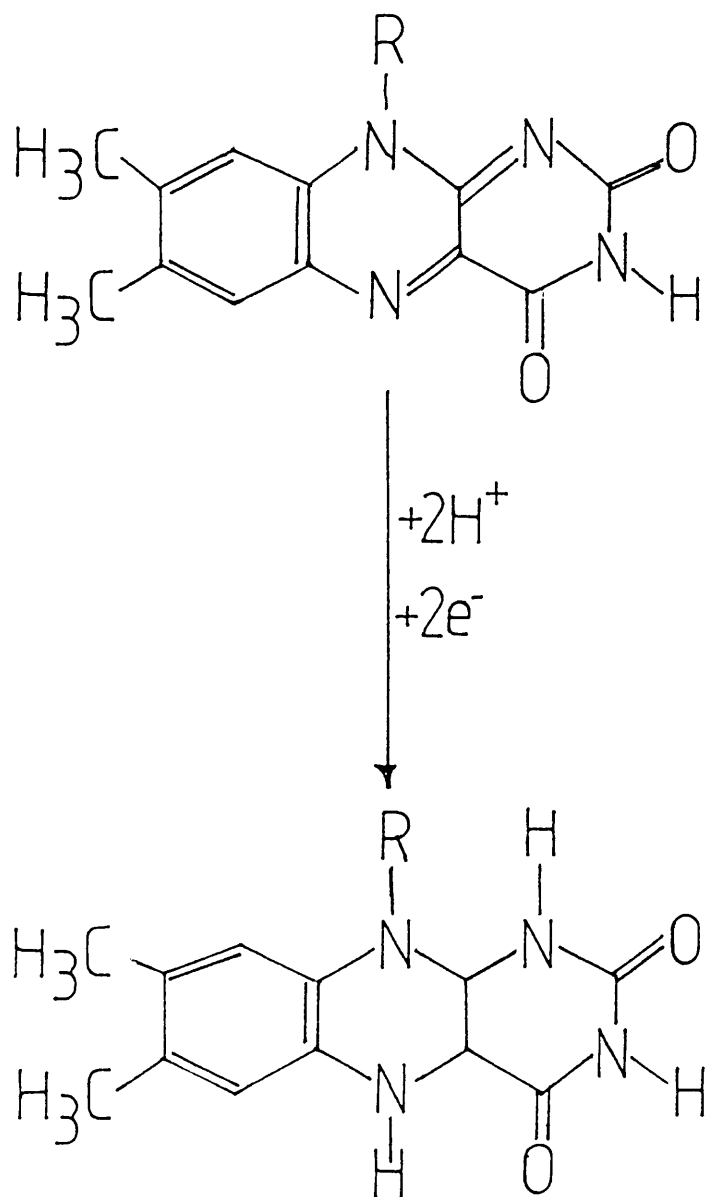
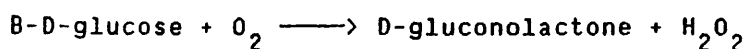


Figure 1.1.4 Reduction of the isoalloxine ring of FAD.

hydrogen peroxide:



The most commonly used sources of the enzyme are Aspergillus niger^[11] and some species of Penicillium^[12]. In both cases the enzyme obtained is a dimer with two ionically bound FAD groups. These groups cannot be removed by normal dialysis, but the enzyme/FAD complex will dissociate in 8M urea solution^[8]. The binding of FAD has been shown to change the conformation of the enzyme from a loose flexible coil to a tight spherical form, thus stabilising the protein^[13]. The enzyme is highly selective towards B-D-glucose, only showing very slight activity to some other sugars such as mannose, xylose and galactose^[14].

GOD has been studied extensively, and many of its kinetic parameters have been determined^[14,15]. Because it is an oxidoreductase, electrochemical methods have been used in an attempt to further elucidate its mechanism. However, conventional techniques have proved to be of little value because of the low detection limits required. Scheller^[16] used differential pulse polarography (DPP) to first demonstrate direct electron transfer between GOD and a mercury electrode. Postulating that electron transfer was facilitated by adsorption of the enzyme onto the electrode, Ianiello^[17,18] deliberately immobilised GOD to a glassy carbon electrode using cyanuric chloride and again employed DPP to observe direct electron transfer. Using (3-amino-phenyl)boronic acid instead of cyanuric chloride Wingard and

Narasimhan^[19] also reported direct electron transfer, although Bourdillon found that direct electron transfer could not be observed when carbodiimide was employed as the immobilising agent^[20]. By employing pre-treated platinum electrodes in thin-layer cells, Durliat and Comtat^[21] reported the electrochemical oxidation of GOD without adsorption or immobilisation of the enzyme onto the electrode; however it is unclear as to whether this reaction was mediated by free FAD in solution and other workers had previously failed to observe this^[22].

b) Choline Oxidase

Choline oxidase (ChOD) has been obtained from several sources including Arthrobacter globiformis^[23], Alcaligenes species^[24] and Cylindrocapsa didymum M-1^[25]. From the latter source the enzyme contains two covalently bound FAD moieties per molecule and is a dimer consisting of two identical sub-units^[26,27]. When obtained from the former two sources, the enzyme is a monomer containing one covalently-bound FAD unit per molecule^[24,28].

ChOD catalyses the reaction between choline and molecular oxygen to give betaine aldehyde, which is further oxidised by molecular oxygen in the presence of ChOD to betaine (see Figure 1.1.5). At physiological oxygen tensions, the $v_{\max}$ for both reactions is the same, although the K_m for betaine aldehyde is approximately 15% of that for choline.

Some activity is also shown by the enzyme towards choline analogues, such as N,N-dimethylaminoethanol and

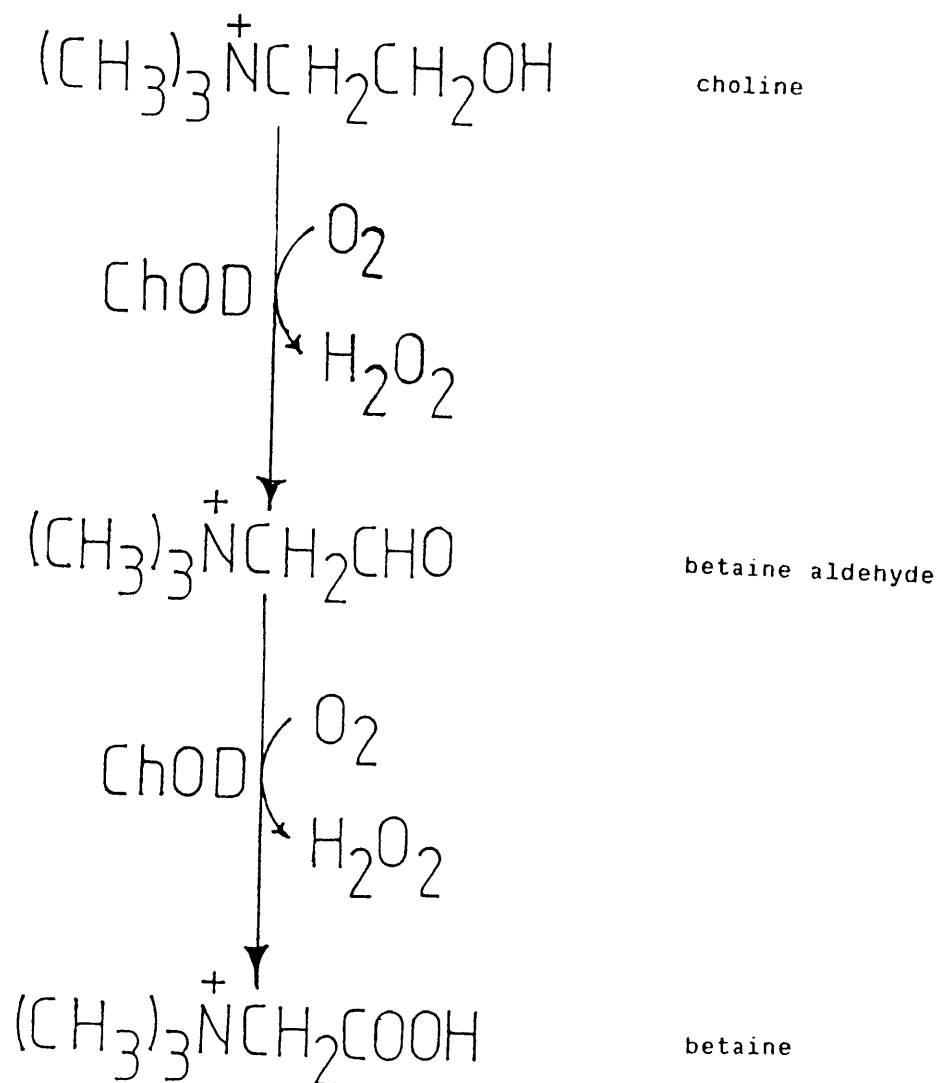


Figure 1.1.5 The action of choline oxidase (ChOD).

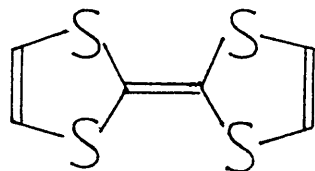
triethanoamine, but not to simple alcohols or aldehydes^[24].
The electrochemistry of ChOD does not seem to have been
investigated, possibly because it is not so well
characterised compared to other flavoproteins due to its
relatively recent discovery.

1.2 CONDUCTING ORGANIC SALTS

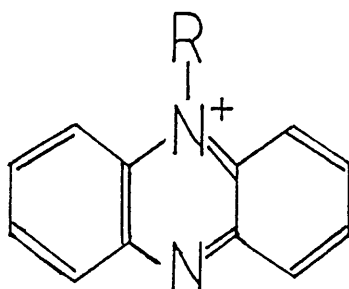
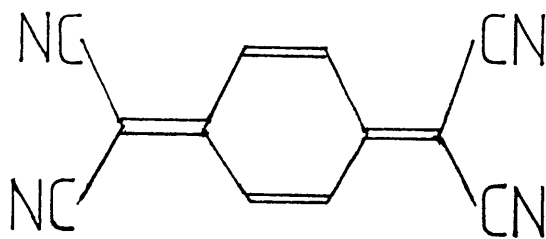
The electrical conductivity of most organic materials is very low. However, many strong π -molecular donor(D) and acceptor(A) molecules often react to form ion-radical salts(D^+X^- or M^+A^-) and charge-transfer complexes(D^+A^-) which have much higher conductivities^[29-31].

Although work began in this field in the 1950's, it was with the discovery of tetracyanoquinodimethane (TCNQ, Figure 1.2.1) in 1960 by Melby^[32] that interest really mounted. This π -molecular acceptor was used to form salts with a variety of cations and electron donors^[33]. With certain of the latter (Figure 1.2.1), TCNQ was found to form salts of unusually high metallic-like conductivities along one of the crystal axes, these materials being referred to as 1-dimensional organic conductors, organic metals or conducting organic salts^[34]. Many of these salts have a 1:2 or 2:3 ratio of cation to TCNQ, such as $Et_3NH(TCNQ)_2$, but some salts form 1:1 complexes such as N-methylphenazinium (NMP)TCNQ and tetrathiafulvalinium (TTF)TCNQ. It is this latter class of salts that this discussion will be restricted to, as they have been characterised much more extensively than complexes with other stoichiometries.

Much work has been carried out to find out why these salts conduct and why seemingly small changes to either the donor or acceptor result in large differences in conductivity. There now seems to be a consensus as to two essential conditions which must be fulfilled for a compound



TTF

NMP⁺ (R=Me)NEP⁺ (R=Et)

TCNQ

Figure 1.2.1 Chemical structures of TTF, NMP⁺, NEP⁺ and TCNQ.

to exhibit high conductivity. First, the crystal structure of the material must be such that there are separate stacks of donor and acceptor molecules^[30]. This explains the anisotropic nature of these compounds, as conduction only takes place along the direction of the stacks. An interesting example to show how the crystal structure can affect conductivity is to compare the TCNQ salts of N-ethylphenazinium (NEP⁺, Figure 1.2.1) and NMP⁺, where the donor species have very similar chemical structures. In NEPTCNQ, the donor and acceptor molecules are arranged alternatively in each stack (Figure 1.2.2); the result being an insulator. On the other hand, in NMPTCNQ separate donor and acceptor stacks are formed, resulting in a conductor.

The second condition concerns the degree of charge transfer between the donor and acceptor molecules, described by the equation



In organic metals, σ has always been found to lie between 0 and 1^[35,36]; otherwise the material is an insulator or semiconductor, even if the crystal structure conforms to the first condition described above (an example of a this is Na⁺TCNQ⁻, Figure 1.2.2).

1.2.1 MODELS FOR CONDUCTION IN ORGANIC METALS

The basic ideas of band theory in solids explain why the above two criteria are necessary for high conductivity. When the crystal structure consists of segregated stacks of donor and acceptor molecules the π -orbitals in each

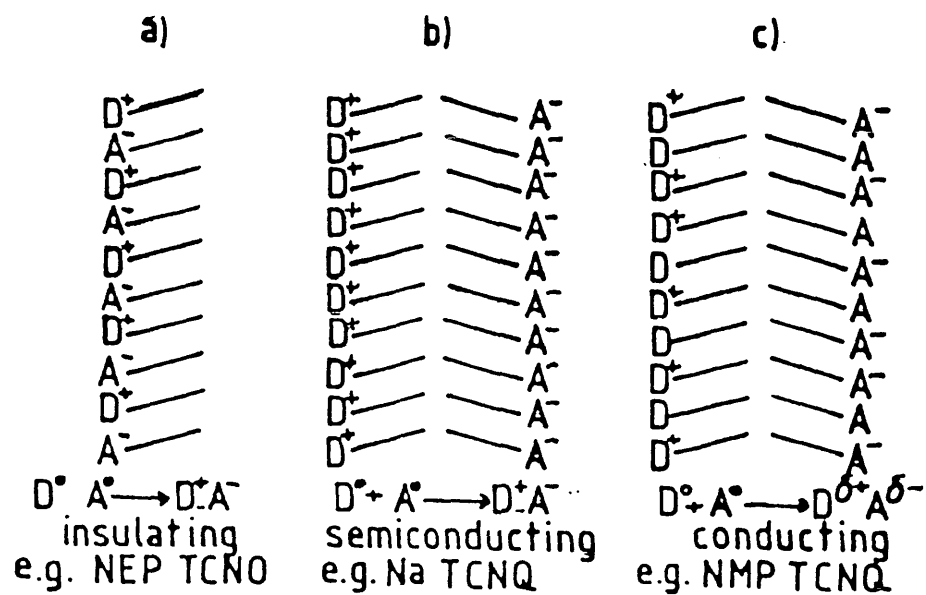
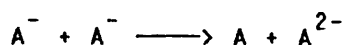


Figure 1.2.2 Schematic view of packing in organic charge transfer crystals.

individual stack combine to form energy bands^[30]. If $\sigma=1$ then the highest-filled band will be completely full, hence a band-gap will exist between the highest-filled energy state (called the Fermi level) and the lowest unoccupied state. As conduction involves promoting an electron between these two states, and this will require an input of energy, an insulator or a semiconductor will probably be the result. However, if σ is less than 1, then empty states will exist close to the Fermi level, as the highest-filled band will not be completely full. Thus the material will probably be a conductor.

An alternative, less rigorous explanation, involves treating the conduction process as an electron hopping mechanism. If $\sigma=1$ then all the molecules on a stack will be ionised; thus conduction can be thought of as involving the following disproportionation process (using the acceptor stack as an example):



A similar process will take part along the donor stack. On the other hand, if σ is less than 1, then some molecules on each stack will be neutral. Electron hopping will then involve the following, more energetically favourable, self-exchange process:

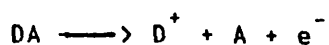


Answering the question as to why segregated stacks are formed in some cases but not in others is quite difficult and does not seem to have been resolved. However the

factors involved in determining σ are better known, and it seems that σ is only less than 1 when the salt is composed of a moderately good donor species combined with a moderately good acceptor species^[37].

1.2.2 ELECTROCHEMISTRY OF CONDUCTING ORGANIC SALTS

Electrodes made of conducting organic salts have been prepared by several methods. Jaeger and Bard^[38] fabricated compacted pellets of several salts of TCNQ, then examined their electrochemical behavior using cyclic voltammetry. In every case, the electrodes were only stable over a certain potential range, being themselves oxidised or reduced outside this range by reactions such as:



Depending on the background electrolyte, subsequent voltammograms may show peaks attributable to insoluble donor or acceptor species or their salts formed on the electrode surface. Employing electrodes prepared by packing a small cavity with a paste made of crystallites of organic salts in a polymer solution, Craston^[39,40] confirmed and extended these results to other organic metals, and concluded that TTFTCNQ was the electrode material of choice because of its relatively large stable range and low background currents.

Electrodes made from single crystals of some organic salts have also been fabricated, where suitably large crystals were available^[39,41]. These show very similar properties to electrodes produced by other methods.

1.2.3 CONDUCTING ORGANIC SALTS AS ELECTRODE MATERIALS

Jaegar and Bard^[42] have shown that TTFTCNQ pellet electrodes behave very much like platinum when used to oxidise or reduce couples such as Cu(II)/Cu(I) and ferricyanide/ferrocyanide. In addition they have shown that copper can be deposited or stripped from pellet NMPTCNQ electrodes without changing the surface^[38].

Most attention, however, has been focussed on the ability of organic metal electrodes to oxidise biological molecules at lower overpotentials than on conventional metal electrodes. In 1980, Kulys showed that NMPTCNQ, NMP(TCNQ)₂, and N-methylacridinium (NMA)TCNQ could be used to oxidise GOD, peroxidase and cytochrome b₂^[43]. Albery extended this work to show that several other organic salts could be used to oxidise GOD^[40], and in particular used TTFTCNQ electrodes to oxidise some other flavoproteins^[44]. The oxidation of NADH has also been studied at NMPTCNQ electrodes^[45], and this system has been used to produce enzyme electrodes for ethanol^[46] and bile acids^[41].

The mechanism by which biological molecules are oxidised at organic salt electrodes is by no means clear, and is the subject of Chapter 4 of this thesis. It is discussed in detail in that chapter.

Some work has also been performed by Sharp in the use of organic salts within ion-selective electrodes for cations such as Ag⁺, Cu⁺ and Pb²⁺^[47]. Although very good calibration plots were obtained, these systems do not seem

to have offered significant advantages over existing devices.

1.3 BIOSENSORS

The analysis of many biochemical substances in complex sample matrices is becoming increasingly important in areas such as medical diagnosis and research, process control and the food industry^[48,49]. Conventional methods of analysis for these substances usually involve the use of complex and expensive instruments, skilled personnel and time-consuming protocols. Furthermore, samples are usually analysed in batch mode and continuous on-line or in-vivo measurements are not possible; this is a serious limitation for automating process control or monitoring seriously ill patients. There is a need, therefore, for devices which can selectively measure the concentration of a given substance in a complex mixture and which are inexpensive, easy-to-use, able to operate in a continuous mode and give rapid results. To meet this requirement the concept of biosensors has evolved and an enormous amount of work has been carried out over the past few years to realise their potential.

All biosensors operate by the same basic principles. They can be defined as devices which transduce a selective biochemical reaction into an electrical signal^[49], and always consist of a biochemical receptor (bioreceptor) immobilised close to a physical transducer, in addition to some associated electronics which manipulate the electrical signal from the physical transducer into the desired form (Figure 1.3.1). Usually the biosensor will be encapsulated in a semi-permeable membrane; this may perform several

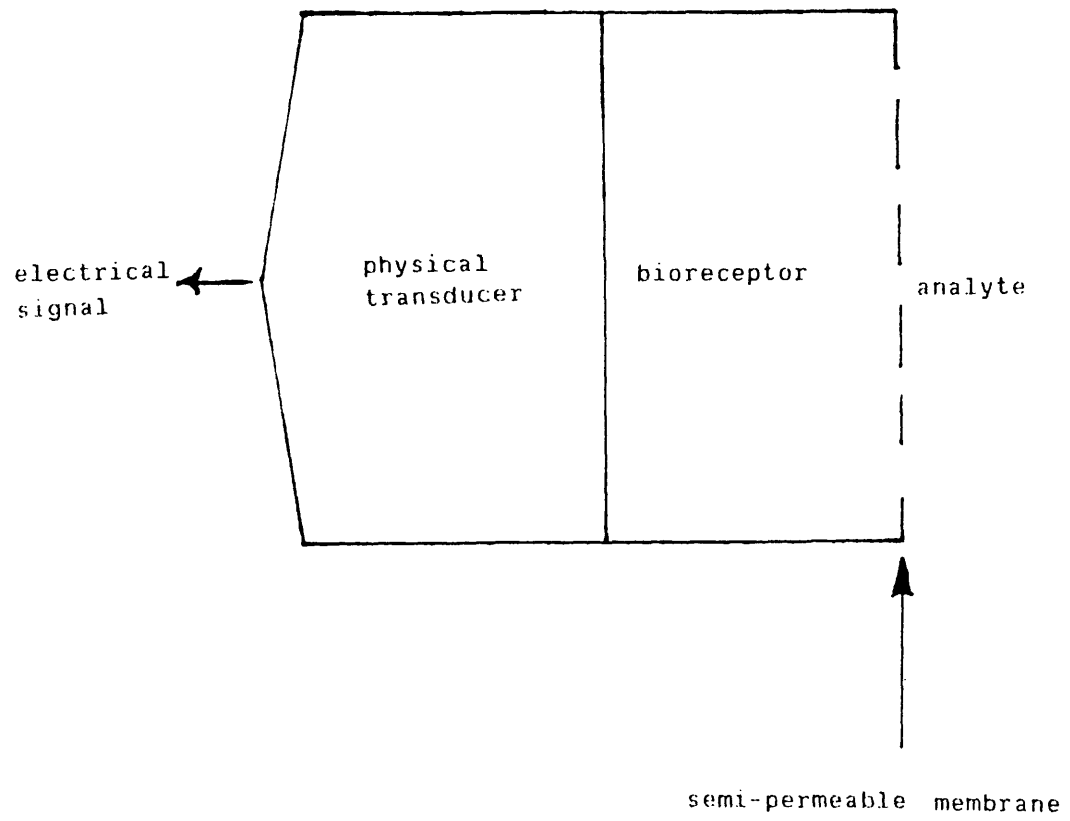


Figure 1.2.3 Schematic diagram of a biosensor.

functions, such as increasing biocompatibility, immobilisation of the bioreceptor and reducing interferences from other substances in the sample.

The bioreceptor should be chosen so as to react as selectively as possible with the analyte of interest; examples of suitable bioreceptors being enzymes, bacteria, antibodies and DNA or RNA strands^[50]. The physical transducer must then be chosen to measure a specific physicochemical change which occurs on the reaction between the analyte and bioreceptor. Table 1.3.1 lists the main types of physical transducers used in biosensors together with the physicochemical change which they measure. Electrochemical devices will now be examined in more detail; the reader interested in other types of biosensors is directed to a selection of books and reviews on the subject^[49-51].

1.3.1 ELECTROCHEMICAL DEVICES

Many biological molecules exhibit poor electrochemical behavior on metal electrodes, thus precluding conventional methods of electrochemical analysis^[52,53]. To overcome this problem, an electrochemical detector can be combined with a system which selectively reacts with the molecule of interest to give a product which is much more amenable to electrochemical detection. In this way a specific sensor can be produced for use in complex mixtures.

Electrochemical biosensors fall into two major categories: potentiometric or amperometric. In the former type of device the potential where no net current flows is

TABLE 1.3.1

Physical transducers employed in biosensors.

Transducer	Physicochemical Quantity Measured
Electrode	Potential Current Conductivity
Thermistor	Temperature
Piezo-electric crystal	Weight
Optical sensor	Absorbance Luminescence
Semiconductor	Current/Voltage

measured; conversely in an amperometric device the current is monitored at some chosen, fixed potential.

a) Potentiometric Biosensors

As stated, these devices measure potential at the point where there is no net flow of current. Under these conditions the potential E , is related to the concentration of the electroactive species c , by the following equation^[54]:

$$\ln(c) = \text{constant} + nEF/RT \quad (1.5)$$

where n is number of electrons involved per molecule in the reaction of interest, F is the Faraday constant, R is the gas constant and T is the temperature.

Although direct potentiometry of a number of biological molecules has been attempted, in general their electrochemical rate constants are too low to allow them to establish a stable potential. Because of this, most interest in this field has centred on producing potentiometric biosensors based on immobilising an enzyme layer over a conventional ion-selective(ISE) or gas-sensing electrode(GSE). The ISE or GSE monitors the appearance of a product of the enzyme/substrate reaction, or possibly the disappearance of a cofactor such as O_2 . An example of the former is a urea sensor based on using the enzyme urease combined with an ammonium ISE^[55]. Here the enzyme catalyses the hydrolysis of urea to give ammonium and bicarbonate ions; by monitoring the increase in concentration of ammonium the amount of urea in the sample

can be determined.

A more recent development of ISE's are ion-selective field effect transistors (ISFET's)^[56], which can be thought of as an ISE and its potentiometer integrated into a small solid-state device. ISFET's have several potential advantages over ISE's. Miniaturisation of the device is possible, together with the possibility of multiple sensor configurations on one 'chip'. However, there are still technological problems to be resolved before ISFET's can begin to replace ISE's^[56]. In addition, immobilisation of sufficient amounts of enzyme to an ISFET to produce a viable biosensor may be difficult due to the small size of the device. Given these limitations can be overcome, enzyme-based ISFET's may become an important class of biosensors.

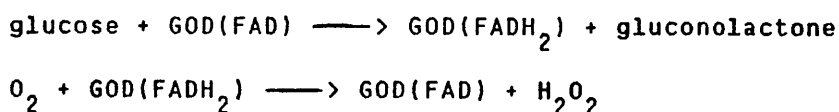
b) Amperometric Biosensors

Amperometric sensors work under non-equilibrium conditions, where an appropriate potential is selected and faradaic currents are measured. In general the potential is chosen such that the electrode kinetics are relatively fast and the response is determined by the rate of mass transport to the electrode surface. Operating in this mode, the current is usually directly proportional to the concentration of the target species. As with potentiometry, direct amperometry of biological molecules is not usually feasible, in this case because of the high overpotentials required and possible poisoning of the electrode surface. However, in an analogous way to potentiometric biosensors,

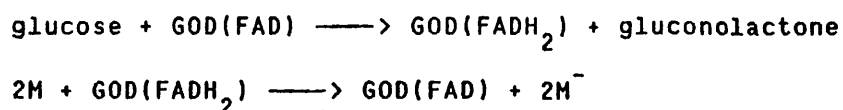
an amperometric detector can be combined with a bioreceptor which selectively reacts with the molecule of interest to consume a co-factor or give a product which is much more amenable to electrochemical methods of analysis.

There are many examples of amperometric biosensors reported in the literature^[57]. Much work has centred on developing a sensor for glucose, especially for use in diabetes treatment and research, and we will take the various attempts to produce a viable biosensor for glucose as an illustration to the strategies involved.

Most glucose biosensors employ the enzyme GOD as the bioreceptor. This enzyme was described in Section 1.1.1. It catalyses the oxidation of B-D-glucose by molecular oxygen in the following manner:



The first enzyme electrode for glucose was described by Clarke in 1962^[59]. Here the depletion of oxygen consumed in the enzymic reaction was followed amperometrically. Following this, in 1970 a similar device was developed in which the accumulation of hydrogen peroxide was measured^[60]. However both devices suffer the same problem, which is that the ambient concentration of oxygen can affect the response of the sensor. Because of this limitation, redox mediators(M) have been used to replace oxygen in the second step of the enzymic reaction^[61]:



The mediator must have the following properties:

i) it should be stable in both oxidised and reduced forms;

ii) it should extract electrons rapidly from the reduced enzyme(GOD(FADH₂));

iii) the reduced form of the mediator should be unreactive towards oxygen;

iv) the reduced form of the mediator should be oxidised at the electrode at sufficiently low potentials to obviate interferences from the oxidation of extraneous species in the sample.

It is also preferable to use a mediator which can be immobilised onto the electrode surface so that it does not have to be added to the sample solution in order to make the analysis. Examples of mediators that have been employed to couple electrochemically with GOD are benzoquinone^[62], phenazine methosulphate^[63], ferricyanide^[64] and ferrocene derivatives^[65]. The latter has actually been used in the commercial production of a glucose sensor by Medisense Ltd^[66].

Another type of 'reagentless' enzyme electrode (so-called because all the reagents necessary for the analysis are contained in the sensor itself) has been developed by Boutelle et al^[67], who employed GOD absorbed on TTFTCNQ microelectrodes to produce glucose sensors that have been used to measure glucose levels in the brains of freely-moving rats. These sensors operated for several days.

without loss of activity, and compared well with results obtained simultaneously from blood samples.

Although the above examples relate to the development of a biosensor for a particular substrate, they illustrate that probably the major problem encountered in the field of biosensors is in the interface between the bioreceptor and the physical transducer. In this case the electrode surface was modified in order to produce reagentless biosensors, which were smaller, cheaper and easier to operate than existing devices. Another route would be to modify the bioreceptor itself, and this possibility has been demonstrated by attaching electron relays to GOD to enable it to transfer electrons to a glassy carbon electrode^[68,69]. Again, this may offer the possibility of producing a reagentless biosensor based on this technology in the future.

c) Comparison of Amperometric and Potentiometric Sensors

One of the main differences between the two types of device lies in the relationship between response and concentration; generally this is linear in the case of amperometric devices but logarithmic for potentiometric sensors. The consequence of this is that while the former type of device generally has a smaller dynamic range, the latter is much more prone to errors in measurement^[54].

A second major difference is that amperometric devices will consume the reactant and thus usually mass transport to the electrode has to be controlled, whereas in potentiometric

devices there is no net current flow and mass transport is unimportant as the reactant is not consumed.

Finally, it should be noted that the development of potentiometric biosensors is limited by the availability of suitable enzymes which catalyse reactions that can be monitored by the ISE's and GSE's available at present. Amperometric detectors are much more versatile as the potential can be chosen to monitor many other species, thus increasing the number of enzyme systems that can be used as bioreceptors and so potentially making many more different biosensors feasible.

1.4 THE ORGANISATION OF THIS THESIS

The rest of this thesis is divided into three main chapters. In Chapter 2, an amperometric enzyme electrode for choline is described which uses a conducting organic salt electrode with choline oxidase. Parameters governing the response of the sensor are determined with a view to facilitating its development as a biosensor for choline.

In Chapter 3 a number of methods for the electrosynthesis of TTFTCNQ and NMPTCNQ are described with the object of producing thin-film organic-salt electrodes for use in biosensors and mechanistic studies.

The mechanism by which flavoenzymes are oxidised at organic salt electrodes is very important, and this is the subject of Chapter 4. Theoretical models are compared with experimental results in order to resolve this question.

CHAPTER 2

INITIAL DEVELOPMENT OF AN ENZYME ELECTRODE FOR CHOLINE

2.1 INTRODUCTION

This chapter concerns the initial development of an enzyme electrode for choline, based on oxidising choline oxidase (ChOD) at a TTFTCNQ electrode. A schematic diagram of the system is shown in Figure 2.1.1. ChOD is entrapped close to the organic salt electrode by a semi-permeable membrane. When the device is interrogated with a sample containing choline, the latter will pass through the membrane to react with the ChOD contained in the sensor, producing a reduced form of the enzyme. This can be oxidised at the electrode back to its substrate-active form, thus producing a current from which the concentration of choline in the sample can be determined.

Although the measurement of choline itself is not so important, the detection of choline is an essential part of systems which measure acetylcholine (a neurotransmitter)^[70] and phospholipids (an indicator of liver function)^[71]. In both cases the analyte is an ester of choline, and their determination involves enzymic hydrolysis which releases choline. A further use for a choline biosensor would be in a device to measure the activity of the enzyme acetylcholinesterase (AChE), important in neurophysiology and in the investigation of the action of nerve gases and pesticides. AChE catalyses the hydrolysis of acetylcholine to acetic acid and choline. By interrogating a sample with

electrode enzyme/electrolyte layer bulk solution

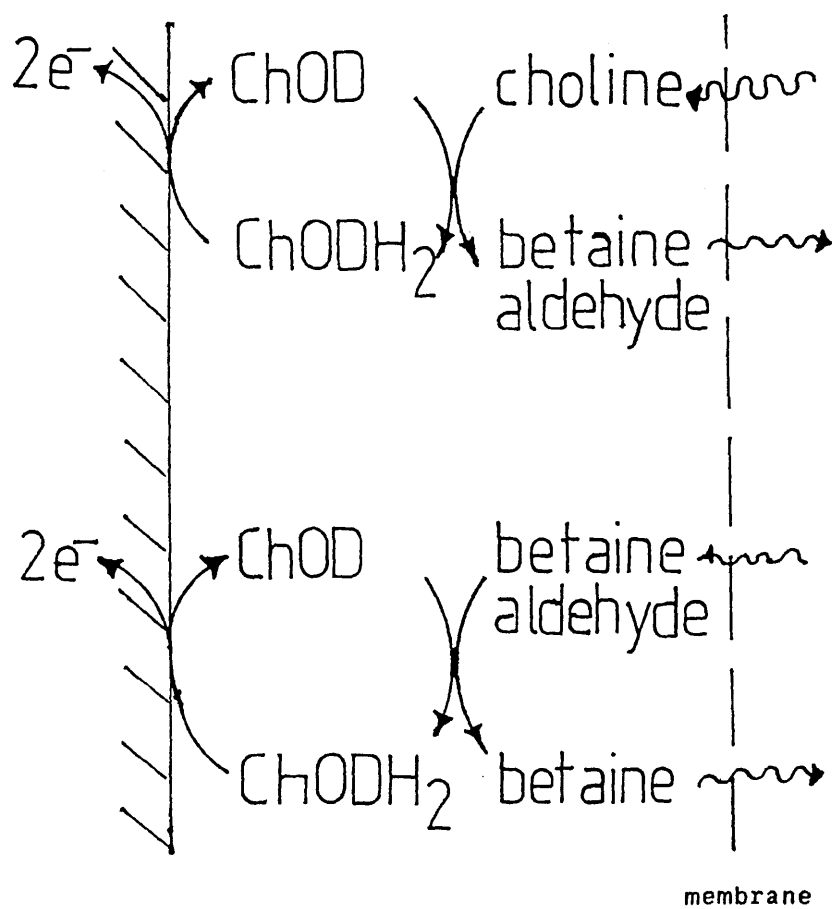


Figure 2.1.1 Schematic diagram of the choline enzyme electrode.

excess acetylcholine and monitoring the rate of choline produced, the enzyme's activity can be determined.

In the past, the assay of choline has been performed by a variety of methods based on several different enzymes. Usually these involve separation processes together with the use of radiolabels or spectrophotometric techniques^[70]. When ChOD has been used, H_2O_2 production has been detected by a coupled chemiluminescence reaction^[72] and by amperometry^[73]. However, both these techniques require the presence of oxygen as a co-factor, and a reagentless sensor would be more desirable, especially for in-vivo applications. Furthermore, it was hoped that by incorporating AChE into this device a multiuse sensor for acetylcholine could be produced, for on-line or in-vivo applications.

This work is concerned only with the initial development of a choline enzyme electrode, to be optimised by other workers for their particular application. To this end, in this chapter the essential characteristics of the electrode are determined, including the factors controlling the sensitivity and response time of the electrode. This is carried out by comparing experimental with theoretical results and by empirical methods. By knowing how the various experimental parameters affect the electrode, its subsequent development for any particular use is then facilitated.

2.2 EXPERIMENTAL

2.2.1 CHEMICALS AND SOLUTIONS

Details of the three preparations of choline oxidase used are given in Table 2.2.1. All chemicals were commercially obtained of AnalaR grade, and both these and the enzymes were used without further purification. TTFTCNQ was synthesised by the following method^[74]:

Separate solutions of equimolar amounts of TTF and TCNQ were made in the minimum amounts of hot acetonitrile. The two solutions were then quickly mixed and allowed to cool slowly to room temperature. Small black crystals of TTFTCNQ were then collected by filtration. The crystals were washed several times with cold acetonitrile, allowed to air-dry overnight, and stored before use at room temperature in the dark. As TTFTCNQ is unstable in solution, no further purification was attempted.

All solutions were made up freshly each day using a stock pH7.4 buffer containing 0.1 mol dm^{-3} sodium hydrogen orthophosphate and 0.15 mol dm^{-3} sodium chloride, the pH being adjusted to 7.4 using concentrated hydrochloric acid. It was prepared using doubly deionised water (DDW) which had been purified by passage through an ion-exchange resin (Houseman) followed by circulation through a Milli Q system (Millipore). The resistance of the water obtained by this procedure was less than $10 \text{ M}\Omega \text{ cm}^{-1}$.

Prior to electrochemical measurement, all solutions were deoxygenated by passing nitrogen (BOC, "white spot")

TABLE 2.2.1

Properties of the three preparations of choline oxidase
employed in this work.

Company	Sigma	BCL	Finn Sugar
Supplied as	Lyophilised powder	50 units/ml solution	Lyophilised powder
Specific activity	15 units/mg	10 units/mg	16 units/mg
K_m betaine aldehyde	6.2 mmol dm^{-3}	8.7 mmol dm^{-3}	8.7 mmol dm^{-3}
choline	$0.87 \text{ mmol dm}^{-3}$	1.2 mmol dm^{-3}	1.2 mmol dm^{-3}
Source	Alcaligenes species	Arthrobacter globoformis	Arthrobacter globoformis
Molecular weight	70,000	83,000	83,000
Stored at	$<0^\circ\text{C}$	$+4^\circ\text{C}$	$<0^\circ\text{C}$

through them for about half an hour. This nitrogen was pre-treated by passage through an aqueous solution containing reduced anthraquinone-2-sulphonate and zinc amalgam, to deoxygenate it further.

All glassware was cleaned by soaking overnight in a dilute solution of Decon 90 (BDH), then rinsing thoroughly with DDW.

2.2.2 MEMBRANES

All membranes were obtained from Cole-Palmer and were supplied in the form of dialysis tubing. They were pretreated to soften and remove impurities by boiling in a 10 g dm^{-3} aqueous solution of sodium carbonate for 10 minutes, then prepared for use by cutting the tubing into strips about three centimetres long. Membranes were stored in a solution of 10 mmol dm^{-3} Tris containing 1 mmol dm^{-3} EDTA at 4°C .

2.2.3 ELECTRODES

Working electrodes were fabricated in-house by Mr M Pritchard and Mr D Dyer. A platinum disc connected to a brass contact was press-fitted into teflon with a recess (depth 1 mm), to produce a cavity electrode. This electrode was contained in an outer sleeve of teflon which could be adjusted so that the inner could be retracted a short, known distance (0.25–5.00 mm) (see Figure 2.2.1). A conducting salt electrode was then produced as follows:

A mixture of TTFTCNQ and polyvinyl chloride (9:1 w/w)

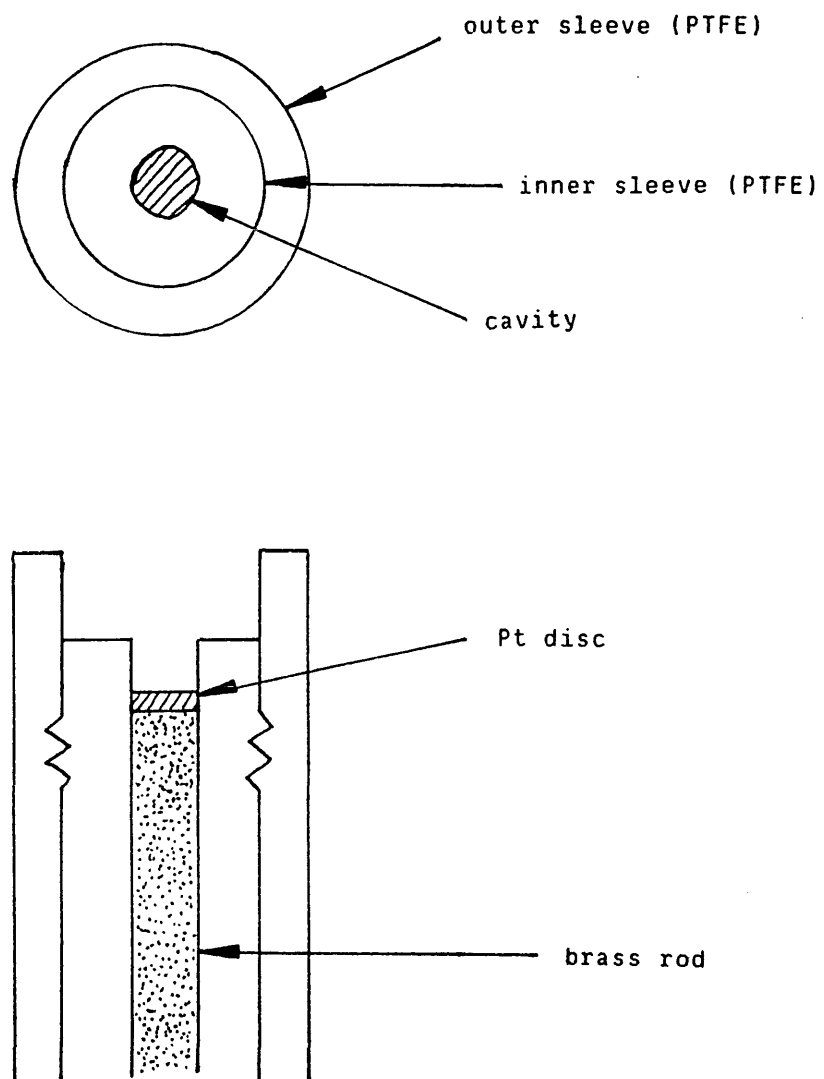


Figure 2.2.1 Cross-section and plan of the cavity electrode.

was made up into a thick slurry using the minimum amount of tetrahydrofuran. The resulting slurry was then carefully dropped into the cavity of the electrode, ensuring that any bubbles of air were removed using a Pasteur pipette, and this slurry was allowed to dry inside the cavity. By successive applications of this mixture the amount of TTFTCNQ in the cavity was increased until the cavity was completely filled. The electrode was now left to dry in air at room temperature for at least one hour, after which the surface of the organic salt was trimmed with a scalpel to produce a flat surface that was level with the rest of the electrode. The electrode was pre-treated before use by electrochemically cycling it overnight between -200 and +400 mV (relative to the saturated calomel electrode) in pH7.4 phosphate buffer. Finally the resistance of the electrode was checked using a standard multimeter, being repacked with organic salt if the measured resistance was above 500 Ω .

Saturated calomel electrodes made in-house were used as reference electrodes, and all quoted potentials are with respect to this. Counter electrodes were made of platinum gauze, being cleaned regularly by immersion overnight in concentrated nitric acid, followed by washing with copious amounts of DDW.

2.2.4 APPARATUS

Experiments were carried out using a modular system of electronics made in these laboratories by Mr J Hooper^[75].

Bryans series 60000 or 29000 chart recorders were used to display experimental results.

All experiments were carried out in a special glass cell shown in Figure 2.2.2. The solution in the cell was kept at a constant temperature of 25 °C by pumping water from an external thermostatted source through the water jacket.

2.2.5 EXPERIMENTAL PROCEDURES

a) Preparation of Enzyme Electrodes

A drop of enzyme electrolyte solution was placed onto an organic salt electrode so that the whole of the surface of the electrode was covered. A membrane was then placed over it and secured by means of an "o"-ring (see Figure 2.2.3). Care was taken to avoid trapping air bubbles underneath the membrane; if this occurred then the membrane was removed and the procedure repeated. Excess enzyme solution was then removed by washing with copious quantities of DDW. The electrode was stored before use in pH7.4 phosphate buffer.

b) Measurement of Current/Time Transients

The electrochemical cell was filled with a known amount of buffer, generally 20 ml. This buffer was deoxygenated (see above) and then the enzyme electrode was placed in the cell and poised at the appropriate potential, which is +50 mV unless otherwise stated. The background current was then monitored until it reached a steady value. The response of the electrode was then measured by introducing small

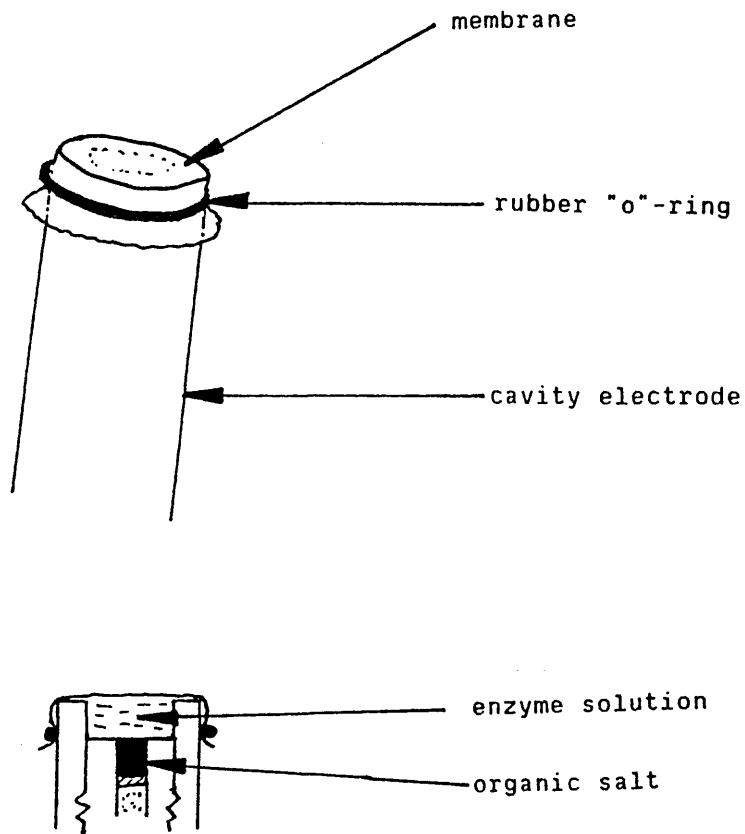


Figure 2.2.2 Diagram of a membrane enzyme electrode.

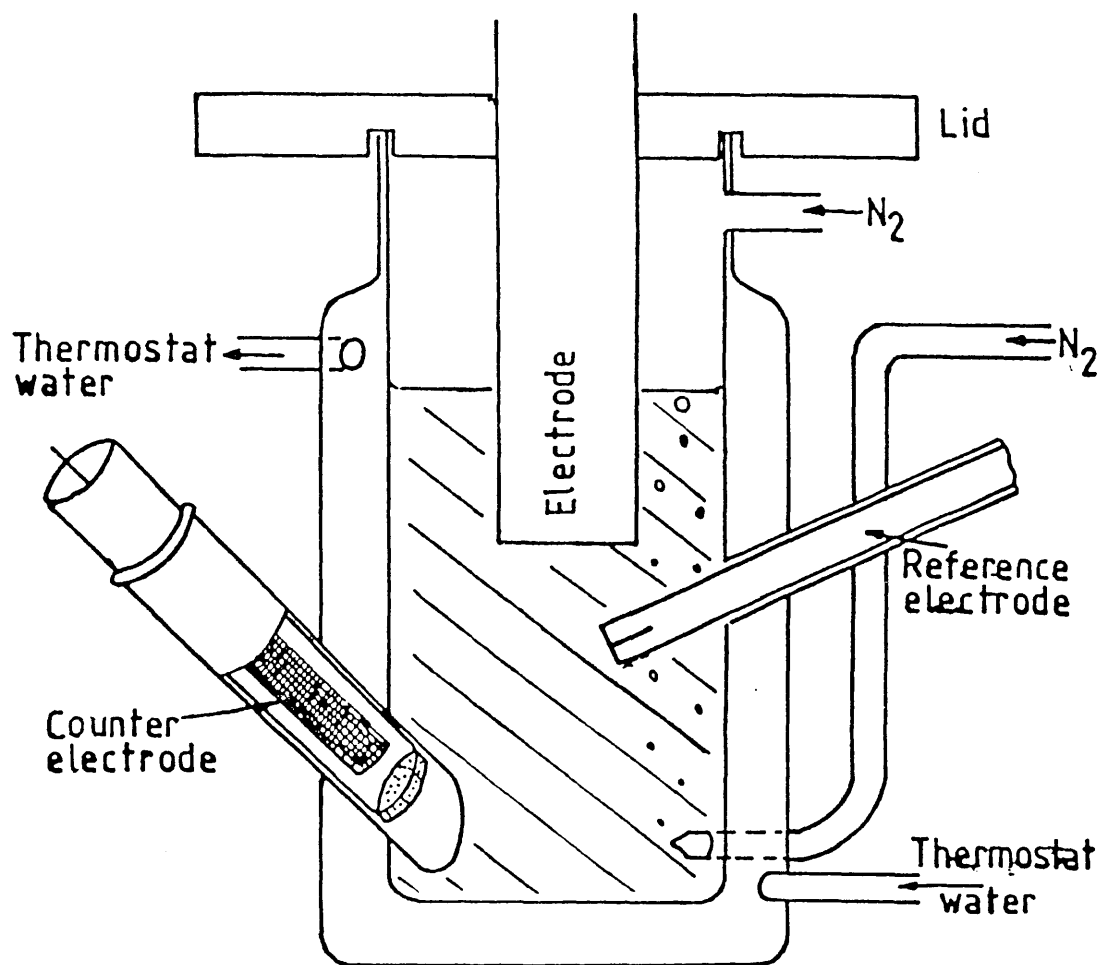


Figure 2.2.3 The electrochemical cell.

aliquots of a stock solution of substrate, allowing the current to reach a steady value after each addition.

2.3 THEORY

This section is divided into two parts. The first part compares two kinetic models which could apply to an organic salt enzyme electrode, depending on the rate-determining process at high substrate concentrations. The second part deals specifically with the response of the choline enzyme electrode to choline individually in the limiting cases of low and high substrate concentrations, which cannot be treated by the above two models because the oxidation product of choline is itself a substrate of the enzyme. In the following theory flux, j , is used throughout to describe the rate at which ChOD is oxidised at the electrode. Although experimentally the current, i , is usually measured, it is related to j by the simple expression $i = nFAj$ (where n is number of electrons transferred per molecule, F is the Faraday constant and A is the area of the electrode) and i can be substituted for j in any expression provided allowance is made for the constant of proportionality.

2.3.1 THE ONE-SUBSTRATE MODEL

The kinetic scheme of this model is shown in Figure 2.3.1. In the case of the choline enzyme electrode this model applies when betaine aldehyde is used as the substrate. The electrode will exhibit different behavior depending on which kinetic steps are rate-limiting. At low concentrations of substrate the current will be determined by the transport of substrate through the membrane, by the

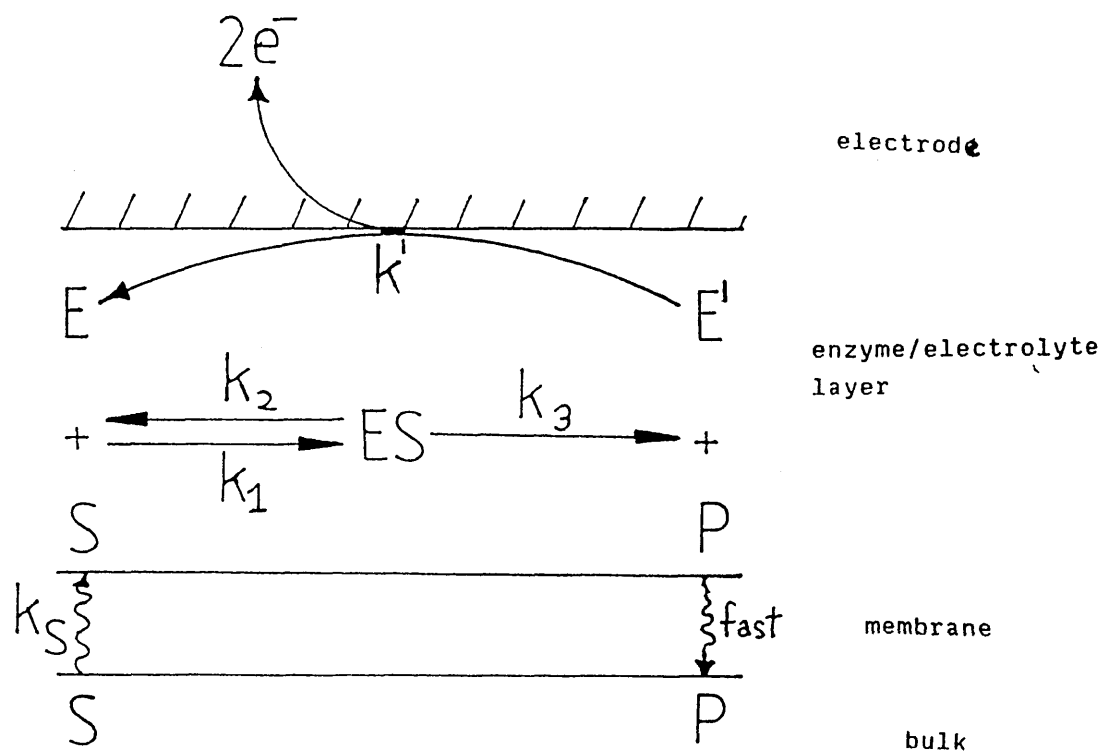


Figure 2.3.1 Reaction scheme for the one-substrate model.

reaction of substrate with the enzyme, or by a combination of these two processes. At high substrate concentrations the current will be determined either by the breakdown of the most kinetically important enzyme intermediate (ES), by the oxidation of E' at the electrode, or as above, by a combination of these two processes. For this kinetic scheme we have the following equations:

$$j = Lk_1[E][S] - Lk_2[ES] \quad (2.1)$$

$$j = Lk_3[ES] \quad (2.2)$$

$$j = k'[E']^{1/2} \quad (2.3)$$

$$j = k_s([S]_{\infty} - [S]) \quad (2.4)$$

$$[E]_t = [E] + [ES] + [E'] \quad (2.5)$$

where $[S]$ and $[S]_{\infty}$ denote the concentration of substrate in the enzyme/electrolyte layer and in the bulk respectively.

It should be noted at this point that k' may represent a collection of kinetic constants, and equation 2.3 is based on the observation by Bartlett (supported by the results in Chapter 4 of this thesis) that except at very low enzyme concentrations the observed flux of electrons is proportional to $[E']^{1/2}$ [41]. If the flux is linear with respect to $[E']$, then k_{cat} may also be a composite apparent rate constant made up from enzymic and/or electrochemical contributions. We also have to assume that there is no concentration polarisation of the enzyme. If this is not the case, L must be replaced by L_R , the enzyme reaction layer thickness, which will depend on substrate concentration.

We will first find a general equation to link flux, j

with bulk substrate concentration, $[S]_{\infty}$, and then examine two cases of the equation, depending on the rate-limiting step at high values of substrate concentration. From equations 2.2 and 2.3, we can define $[ES]$ and $[E']$ in terms of j , L , k' and k_3 . By substituting for $[ES]$ in equation 2.1 we can find $[E]$. Combining these results with equation 2.5, we find:

$$[E]_t/j = (K_m/Lk_{cat}[S]) + 1/Lk_{cat} + j/(k')^2 \quad (2.6)$$

where $K_m = k_1/(k_2+k_3)$ and $k_{cat} = k_3$. But from equation 2.4,

$$[S] = (1/k_s)(k_s[S]_{\infty} - j) \quad (2.7)$$

Substituting this result into equation 2.6 and rearrangement gives:

$$\frac{[S]_{\infty}}{j} = \frac{1}{k_{me}} + [S]_{\infty} \left[\frac{1}{Lk_{cat}[E]_t} + \frac{j}{(k')^2[E]_t} \right] \left[1 - \frac{j}{k_s[S]_{\infty}} \right] \quad (2.8)$$

where $(1/k_{me}) = (1/k_s + K_m/Lk_{cat}[E]_t)$. Lk_{cat}/K_m is in fact the effective heterogeneous second order rate constant for the reaction of S with E , so k_{me} is really a combination of this rate constant and k_s . This equation is too complicated to be treated analytically in full but we can simplify it, depending on whether the enzyme or electrode kinetics control the response at high substrate concentrations. However, we will first examine its behavior for the extreme cases of high and low substrate concentrations.

At low substrate concentrations $[S]_{\infty}$ tends to zero, hence the equation reduces to

$$j = k_{me}[S]_{\infty} \quad (2.9)$$

The value of k_{me} will generally be equal to either k_s or

$Lk_{cat}[E]_t/K_m$ depending on whether the membrane or enzyme kinetics are rate limiting.

At high substrate concentrations, as $[S]_\infty$ becomes very large, the flux will reach a maximum value, j_{max} given by

$$1/j_{max} = 1/Lk_{cat}[E]_t + j_{max}/(k')^2[E]_t \quad (2.10)$$

Remembering that $[ES]=j_{max}/Lk_{cat}$ and $[E']=(j_{max}/k')^2$, it can be seen that j_{max} depends on which of these enzyme forms are important under these conditions, and that, in general only one of the terms on the right hand side of this equation will be important.

We can now simplify equation 2.8 by treating the two cases of enzyme and electrode control at saturated conditions separately, assuming that one or the other of the terms on the right hand side of equation 2.10 will be unimportant, depending on the case.

a) Control by Enzyme Kinetics at High Substrate Concentrations

In this case at high substrate concentrations $[ES] \gg [E']$. Thus $1/Lk_{cat} \gg j_{max}/(k')^2$, where j_{max} is the maximum flux of the enzyme electrode and will be equal to $Lk_{cat}[E]_t$. Equation 2.8 will now be reduced to:

$$\frac{[S]_\infty}{j} = \frac{1}{k_{me}} + \frac{[S]_\infty}{Lk_{cat}[E]_t} \left[1 - \frac{j}{k_s[S]_\infty} \right] \quad (2.11)$$

This equation, in a slightly different form has already been examined by Alberly and Bartlett^[76], and what follows is a summary of their work. The first stage of the kinetic

analysis will be to find k_{me} by plotting $[S]_{\infty}/j$ against $[S]_{\infty}$. We can note that this is similar to the Hanes plot mentioned in Chapter 1, section 1.1. Although it may be a curve, the limiting value as $[S]_{\infty}$ tends to 0 gives $\{[S]_{\infty}/j\}_0 = 1/k_{me}$. Next we calculate the parameter ϱ , where:

$$\varrho = (j/[S]_{\infty})/k_{me} \quad (2.12)$$

Substitution into equation 2.11 gives:

$$y = \frac{\varrho^{-1} - 1}{[S]_{\infty}} = \frac{k_{me}}{Lk_{cat}[E]_t} \left[1 - \frac{\varrho k_{me}}{k_s} \right] \quad (2.13)$$

A plot of y against ϱ (which we shall call a normal ϱ -plot, as this is the plot used by Albery and Bartlett) will then give $k_{me}/(Lk_{cat}[E]_t)$ from the intercept at $\varrho = 0$ and k_s/k_{me} from the intercept at $y = 0$. The former ratio can be used to find j_{max} , as $j_{max} = Lk_{cat}[E]_t$. The relative importance of the transport and enzyme kinetics at low substrate concentrations can be obtained from k_s/k_{me} . It will be unity when transport across the membrane is rate-limiting and very large when the unsaturated enzyme kinetics dominate.

We shall now turn to the case where the electrode kinetics dominate at high concentrations. It will be seen that the treatments are quite similar, although the equations describing the kinetic behavior are different.

b) Control by Electrode Kinetics

In this case, at high substrate concentrations $[E'] \gg [ES]$, or in other words, $(j_{max}/(k')^2) \gg (1/Lk_{cat})$.

Here, $j_{\max}$ is given by $k'([E]_t)^{1/2}$. Hence equation 2.8 reduces to:

$$\frac{[S]_{\infty}}{j} = \frac{1}{k_{me}} + \frac{[S]_{\infty} j}{(k')^2 [E]_t} \left[1 - \frac{j}{k_s [S]_{\infty}} \right] \quad (2.14)$$

The value of k_{me} will be the same as in the previous treatment, and can be found as above. Defining ϱ as before, we can rearrange equation 2.14 to give:

$$y' = \frac{\varrho^{-1} - 1}{[S]_{\infty} j} = \frac{k_{me}}{(k')^2 [E]_t} \left[1 - \frac{\varrho k_{me}}{k_s} \right] \quad (2.15)$$

This is a modified ϱ -plot as this equation differs from equation 2.13 only in that $y' = y/j$ and that $(k')^2$ replaces Lk_{cat} . Thus the intercept at $y' = 0$ gives $k_{me}/((k')^2 [E]_t)$ (in other words, $k_{me}/(j_{\max})^2$), and the intercept at $\varrho = 0$ gives k_s/k_{me} .

In analysing the experimental results, the data will be treated to test for both the above cases, by using both types of ϱ -plot. The most satisfactory fit should then tell us which kinetic step is rate limiting at high substrate concentrations, and from this plot the rate limiting step at low substrate concentrations should be identified.

2.3.2 RESPONSE OF THE ENZYME ELECTRODE TO CHOLINE

Treatment of a kinetic model to describe the behavior of the response of the electrode is too complicated to be worked out in full, but at low and high substrate concentrations we can simplify the model considerably, by

assuming the concentration of some of the different forms of the enzyme will be negligible. We will first consider the response of the electrode to low concentrations of choline (ie unsaturated conditions) and then consider the limiting response of the electrode under saturated conditions.

a) Response of the Electrode Under Unsaturated Conditions

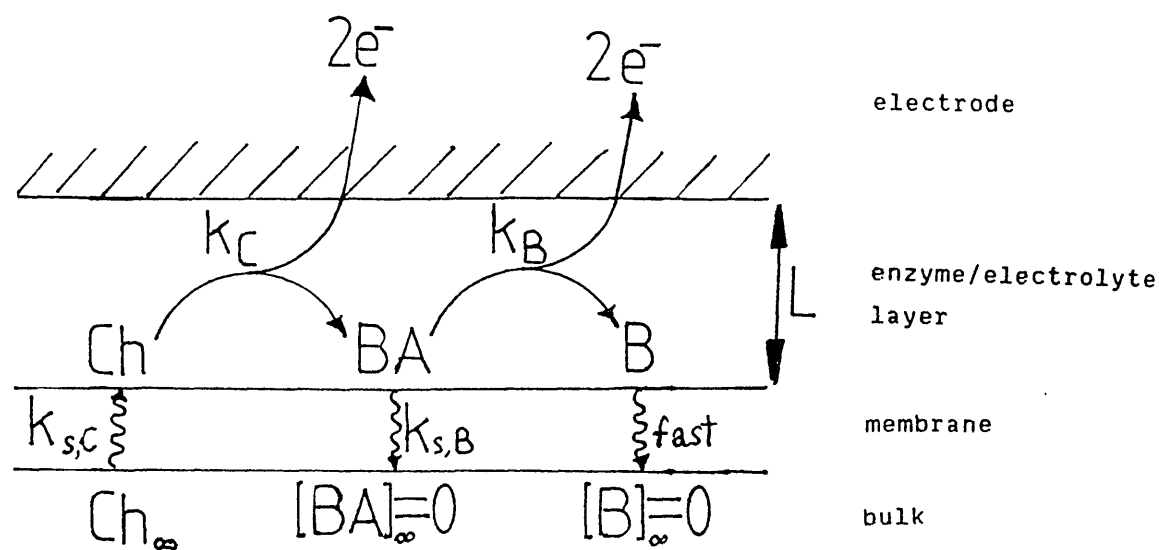
In this model, we can assume that the enzyme is almost completely in its free form, E, as it is working under unsaturated conditions. Secondly we only have to consider the unsaturated kinetics of the enzyme, the transport of choline through the membrane, and the loss of betaine aldehyde through the membrane to the bulk. Here the electrode is merely measuring the overall flux of E being converted to E', and the kinetics of this reaction will be unimportant. The kinetic scheme is therefore shown in Figure 2.3.2. The second order rate constants for the reaction of the free enzyme with each substrate (k_B and k_C) are given by the respective values of $(k_{cat}[E]_t)/K_m$. We can now define the fraction of betaine aldehyde, ϕ_B that reacts with the enzyme:

$$\phi_B = Lk_B / (Lk_B + k_{s,B}) = k_{me,B} / k_{s,B} \quad (2.16)$$

The overall flux, j is then given by:

$$j = (1 + \phi_B)Lk_C[C] \quad (2.17)$$

where $[C]$ is the concentration of choline in the enzyme electrolyte layer. We can also equate the net flux of choline transported across the membrane with that of choline reacting with the enzyme. If $[C]_\infty$ is the bulk concentration



Ch = Choline

BA = Betaine
Aldehyde

B = Betaine

Figure 2.3.2 Reaction scheme for choline under unsaturated conditions.

of choline then

$$Lk_C [C] = k_s ([C]_\infty - [C]) \quad (2.17)$$

Combining this equation with equation 2.17 after rearrangement we find:

$$\frac{[C]_\infty}{j} = \frac{1}{(1+\phi_B)} \left[\frac{1}{k_{s,C}} + \frac{1}{Lk_C} \right] = \frac{1}{(1+\phi_B)k_{me,C}} \quad (2.18)$$

Remembering that this equation applies at low substrate concentrations, the value of the right-hand side of the expression can be obtained from plotting $[C]_\infty/j$ against $[C]_\infty$ and finding the intercept at $[C]_\infty = 0$. This intercept will be an apparent value of $1/k_{me}$ for choline. From the Hanes and g-plots for betaine aldehyde we can calculate ϕ_B , and find Lk_B . The value of Lk_C can be calculated from Lk_B and the ratio of the apparent second order rate constants for the two substrates (this ratio is known from previous work on the enzyme^[23]). Thus $k_{s,C}$ can be calculated using equation 2.19. This will allow us to find out about the interplay between the unsaturated enzyme kinetics and the transport through the membrane for choline alone.

b) Response of the Electrode Under Saturated Conditions

In this case, the concentration of the free form of the enzyme will be negligible. The enzyme will be mostly in its reduced form, E' or in a complex with either choline, EC or betaine aldehyde, EB . We also have to consider the loss of betaine aldehyde to the bulk. The kinetic model is therefore shown in Figure 2.3.3.

The overall flux, j is given by a sum of the fluxes of the reduction of choline and betaine aldehyde, j_C and j_B

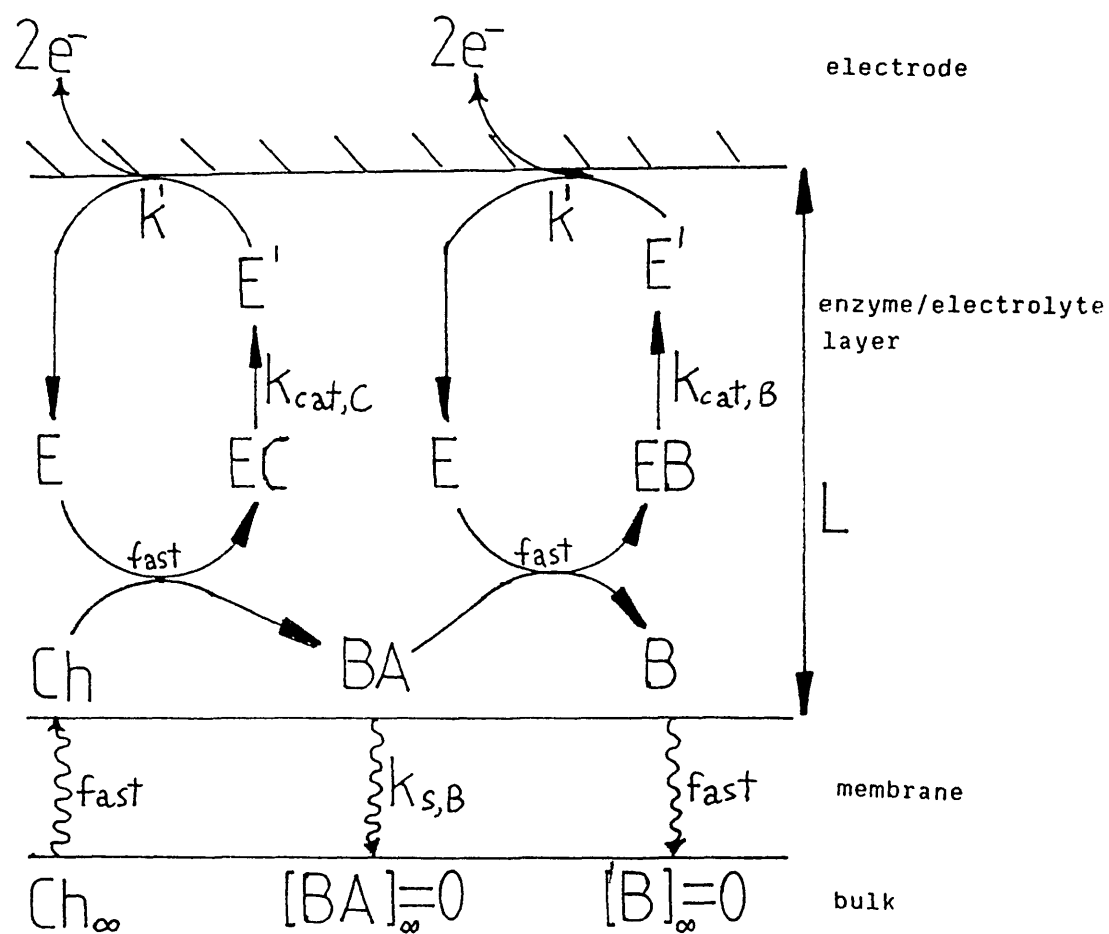


Figure 2.3.3 Reaction scheme for choline under saturated conditions.

respectively:

$$j = j_C + j_B \quad (2.20)$$

Looking at the kinetic model we can now find expressions for the concentrations of each of the enzyme forms in terms of the above fluxes and the rate constants:

$$[EC] = j_C / Lk_{cat,C} \quad (2.21)$$

$$[EB] = j_B / Lk_{cat,B} \quad (2.22)$$

$$[E'] = (j/k')^2 \quad (2.23)$$

Again, the flux is assumed to be proportional to the square root of $[E']$. The sum of these enzyme forms must be equal to the total enzyme concentration, $[E]_t$. Hence,

$$[E]_t = j_C / Lk_{cat,C} + j_B / Lk_{cat,B} + (j/k')^2 \quad (2.24)$$

We can note that as betaine aldehyde is a product of the reduction of choline then $j_C \gg j_B$ at all times. Thus, from equation 2.20, $1 \gg (j_C/j) \gg 1/2$. Introducing the parameter ψ for j_C/j and using the result $j_B = j - j_C$ we obtain:

$$\frac{[E]_t}{j} = \psi \left[\frac{1}{Lk_{cat,C}} - \frac{1}{Lk_{cat,B}} \right] + \frac{1}{Lk_{cat,B}} + \frac{j}{(k')^2} \quad (2.25)$$

Although this equation appears to be complicated, each term is associated with a specific enzyme form, and thus usually only the one term will be important. Now, the parameter ψ describes whether the enzyme kinetics are fast enough to reduce the betaine aldehyde before it can escape through the membrane to the bulk. If the enzyme kinetics are fast then most of the betaine aldehyde reacts with the enzyme. In this case $j_B \sim j_C$ and so $\psi \sim 1/2$. Conversely, if transport through the membrane is fast then $j_B \sim 0$ and so

$\psi \sim 1$. We are now able to predict the limiting flux, $j_{\max}$ depending on which enzyme form is most predominant under these conditions. The results are summarised in Table 2.3.1. An interesting case arises when $k_{\text{cat,C}} \sim k_{\text{cat,B}}$. In this case, no matter what value ψ takes $j_{\max}$ remains the same, and comparison of equation 2.25 with equation 2.10 shows that this is equal to the maximum response to betaine aldehyde when it is used as the substrate instead of choline. Finally, we will examine what happens if the flux is linear with $[E']$, not $[E']^{1/2}$ as assumed. Here, the equations for limiting flux will only differ from the above treatment if E' is the predominant form of the enzyme at saturation. Under these conditions $j_{\max}$ is then given by $k_{\text{app}}[E]_t$, where k_{app} is the apparent electrochemical rate constant for E' .

TABLE 2.3.1

Summary of kinetic equations describing the response of the choline enzyme electrode under saturated conditions.

Ψ	Important Form of Enzyme		
	EB	EC	E'
1	-	$Lk_{cat,C}[E]_t$	$k'([E]_t)^{1/2}$
1/2	$2Lk_{cat,B}[E]_t$	$2Lk_{cat,C}[E]_t$	$k'([E]_t)^{1/2}$

2.4 RESULTS

2.4.1 CHOICE OF ENZYME

The decision as to which source of choline oxidase to use is not an easy one as the three commercially available preparations of enzyme are supplied in different purities and forms. All three preparations can be employed in a choline enzyme electrode (see Figure 2.4.1 and 2.4.2), though for some experiments or applications one or other of the preparations will be preferred. For most of this study the BCL enzyme was used, because this is supplied as a 50 Units/ml solution which can be used directly to make up the enzyme electrode (1 Unit is that amount of enzyme that catalyses the formation of 1 μmol of H_2O_2 per minute). The Sigma and Finn Sugar enzymes are supplied as powders; the preparation of small amounts of enzyme solutions accurately is difficult and there will be relatively large errors in the concentration of the enzyme. Attempts to make up larger amounts of stock solutions of these enzymes failed, as the enzymes quickly lost activity even when stored at $+4^\circ\text{C}$. Because of this, unless otherwise stated all results presented use the BCL enzyme.

The powder preparations of enzyme were preferred when it was wished to vary the concentration of the enzyme, as the BCL enzyme could only be diluted from its maximum concentration as supplied of 50 units/ml. Although the Sigma preparation was much cheaper than that of Finn Sugar, it contained approximately 30% by weight bovine serum

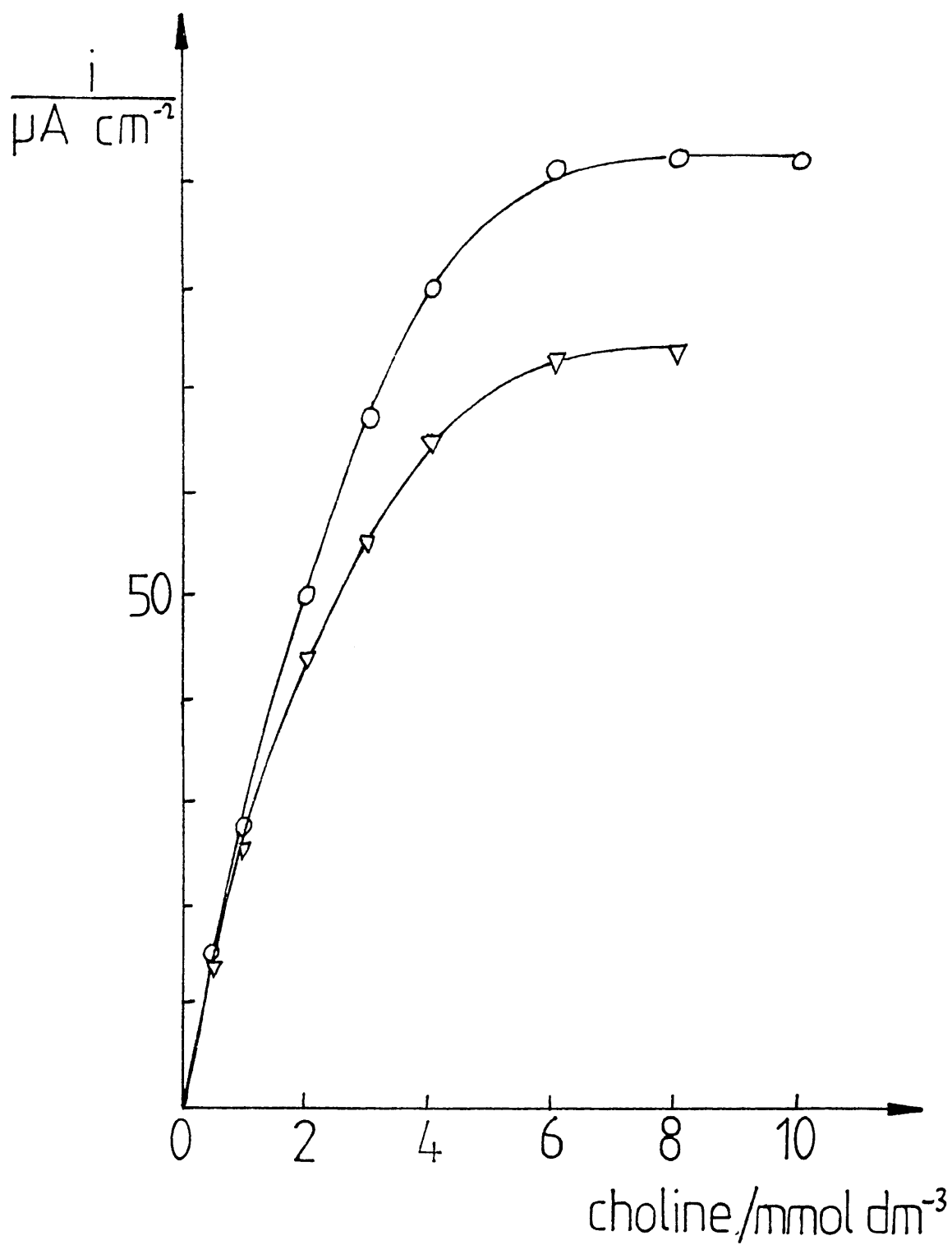


Figure 2.4.1 Response of membrane enzyme electrode to choline using Finn Sugar ($\circ$, 50units/ml) and BCL (∇ , 50units/ml) enzymes.

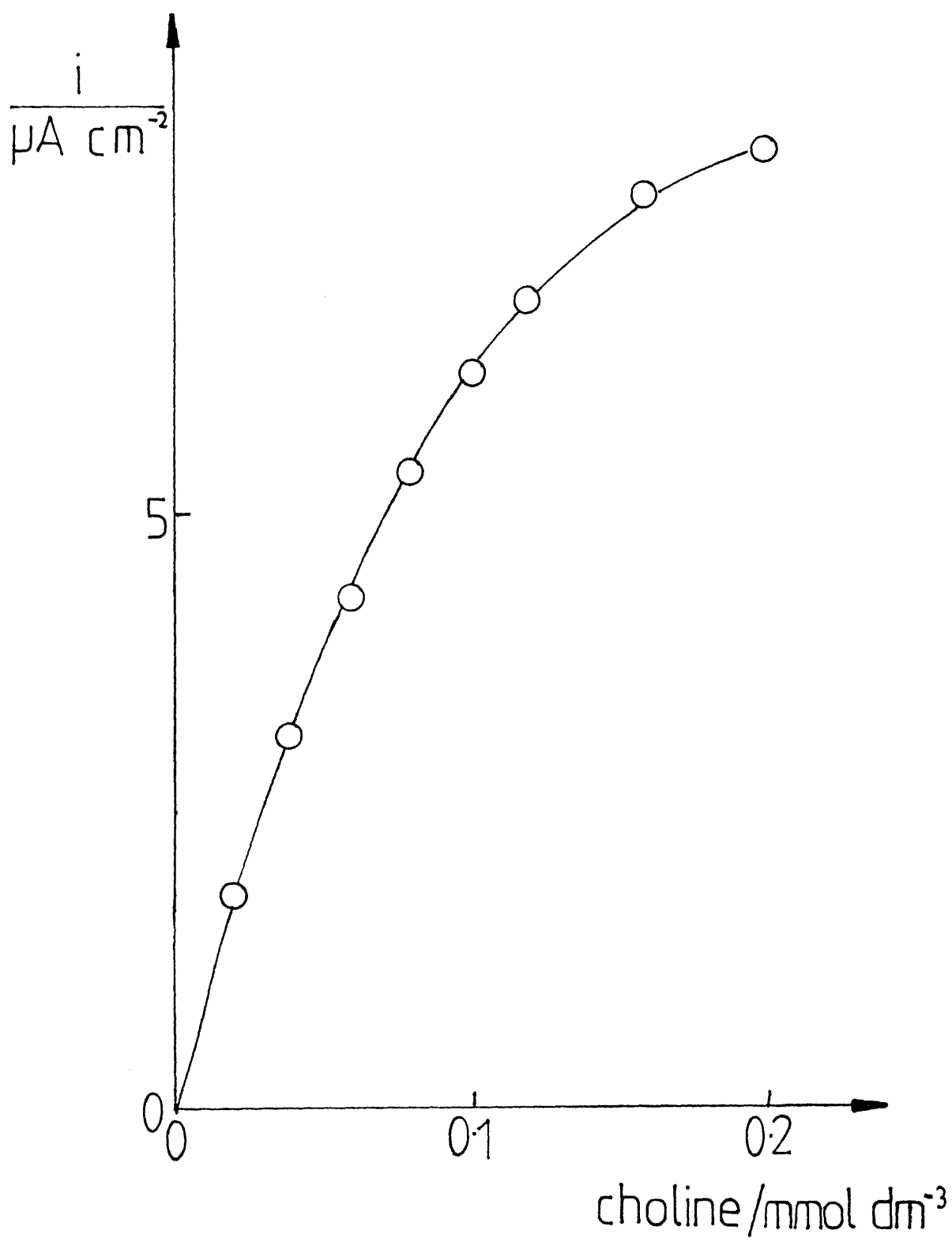


Figure 2.4.2 Response of membrane enzyme electrode to choline using Sigma (15units/ml) enzyme.

albumin (BSA). Additionally, it was found that unlike both the BCL and the Finn Sugar enzymes, the Sigma enzyme did not adsorb to the electrode surface, possibly because the BSA adsorbed preferentially, blocking sites on the electrode, or because it came from a different source. For these reasons, the Finn Sugar preparation was employed in preference to that of Sigma.

2.4.2 REPRODUCIBILITY AND LIFETIME OF ELECTRODES

The reproducibility of electrodes can be divided into two main factors. First, the ability of the organic salt electrodes to oxidise ChOD differed from one electrode to another. One of the main reasons for this is the variability in the actual surface area and porosity of these electrodes. On average, ChOD showed satisfactory activity on about 25% of the organic salt electrodes fabricated. For this reason, comparative results are shown only where the same organic salt electrode was used. Unfortunately, electrodes fabricated by other methods, such as pressed pellet, proved unsuitable for use in enzyme electrodes as the currents obtained were too low.

The lifetime of the enzyme electrode tended to be limited by the lifetime of the enzyme. When stored at room temperature, the response of the enzyme electrode to choline deteriorated quite quickly, and sometimes the electrode showed no activity towards choline after two days. However, when loaded with fresh enzyme solution the electrode again responded to choline, showing that the enzyme had lost its

activity rather than the electrode. This conclusion is supported by the work of Dr Sally Durrant, who showed that by storing the electrodes at 4°C their lifetime was much improved and they still showed activity towards choline after 7 days^[77].

2.4.4 RESPONSE OF THE ENZYME ELECTRODE TO BETAIN ALDEHYDE

When choline is used as a substrate for ChOD it is first oxidised to betaine aldehyde and then to betaine. The choline enzyme electrode should therefore also show activity towards betaine aldehyde. The response of the electrode to this substrate is shown in Figure 2.4.3, compared to the response to choline under the same conditions.

To find what kinetic step determines the response of the electrode to betaine aldehyde, we can analyse the results according to the theory given above. The Hanes plot, shown in Figure 2.4.4, gives a value for k_{me} for betaine aldehyde of $5.6 \times 10^{-5} \text{ cm s}^{-1}$. Using this result, the normal and modified ρ plots are shown in Figures 2.4.5 and 2.4.6. It can be seen that the normal ρ plot is a straight line with a negative gradient, as would be expected if the rate limiting step at high substrate concentrations was due to the k_{cat} term in equation 2.10. The modified ρ plot is curved with a positive gradient. If the k' term in equation 2.10 limited the response at high substrate concentrations then this plot would be straight with a negative gradient. For these reasons we can therefore conclude that at high

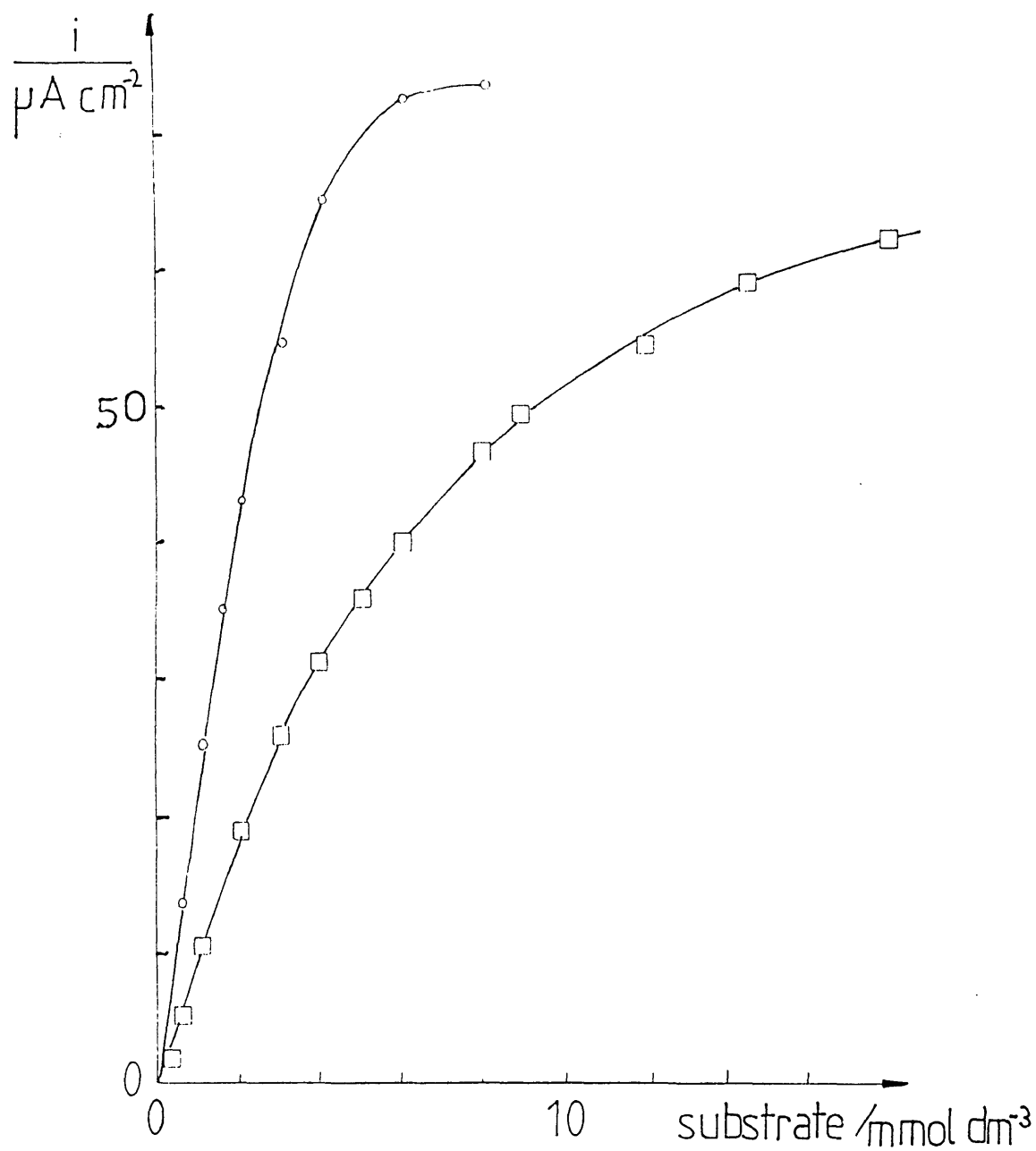


Figure 2.4.3 Response of membrane enzyme electrode to choline (O) and betaine aldehyde (□).

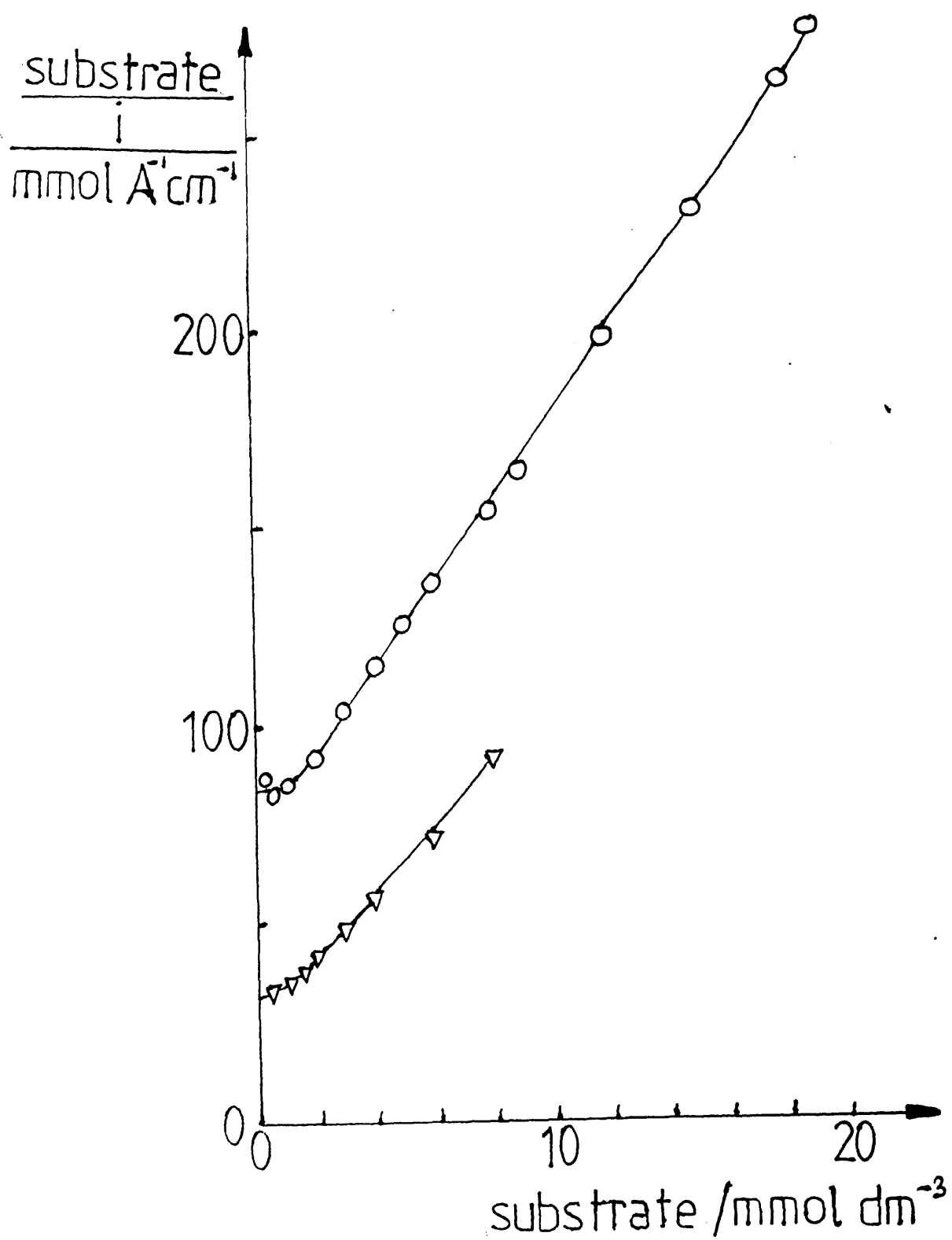


Figure 2.4.4 Hanes plot of data shown in Figure 2.4.3. (∇) choline, ($\circ$) betaine aldehyde.

$$\frac{\frac{\rho^{-1}-1}{[\text{BA}]}}{(\text{mmol dm}^{-3})^{-1}}$$

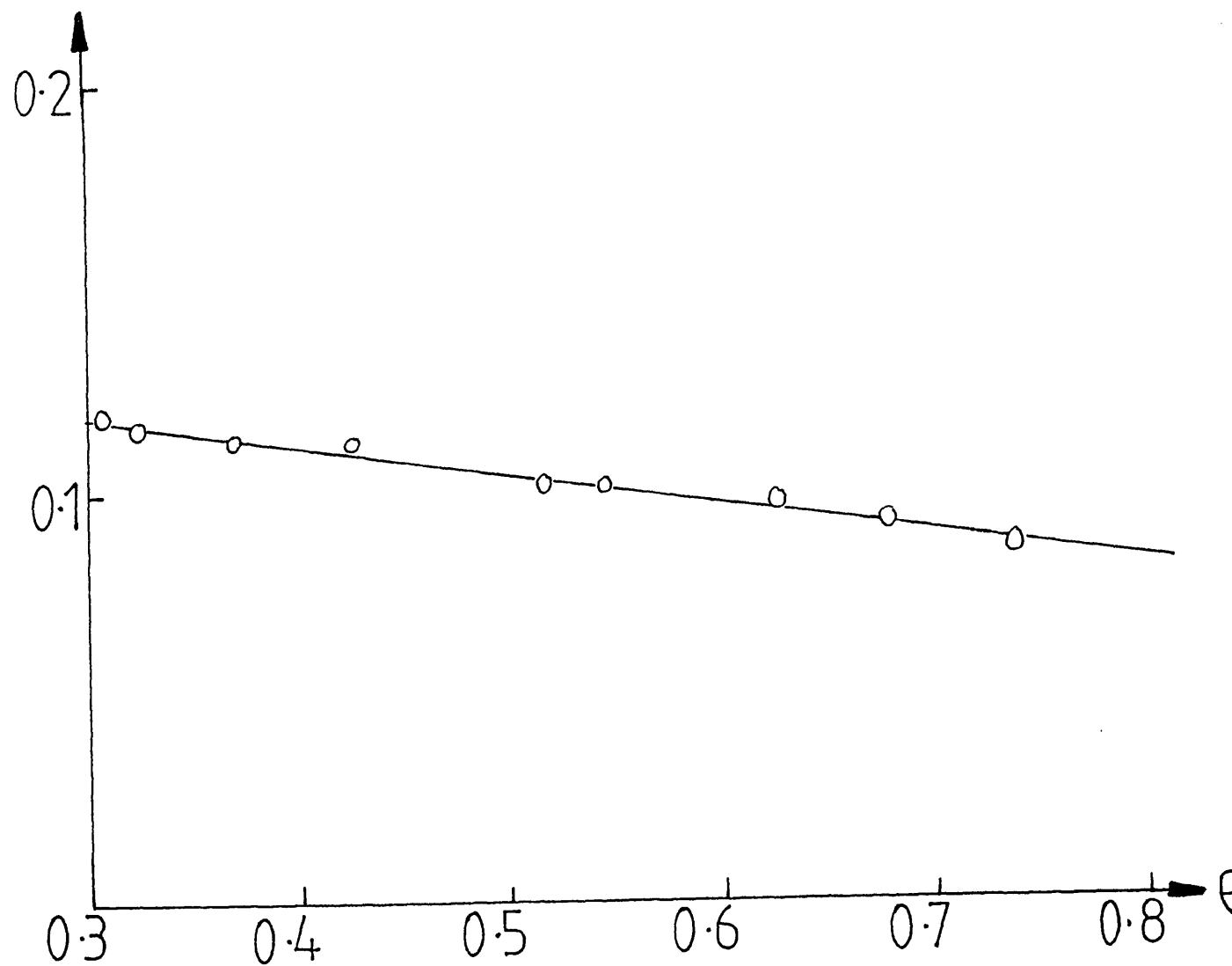


Figure 2.4.5 Normal g-plot for betaine aldehyde of data shown in Figure 2.4.3.

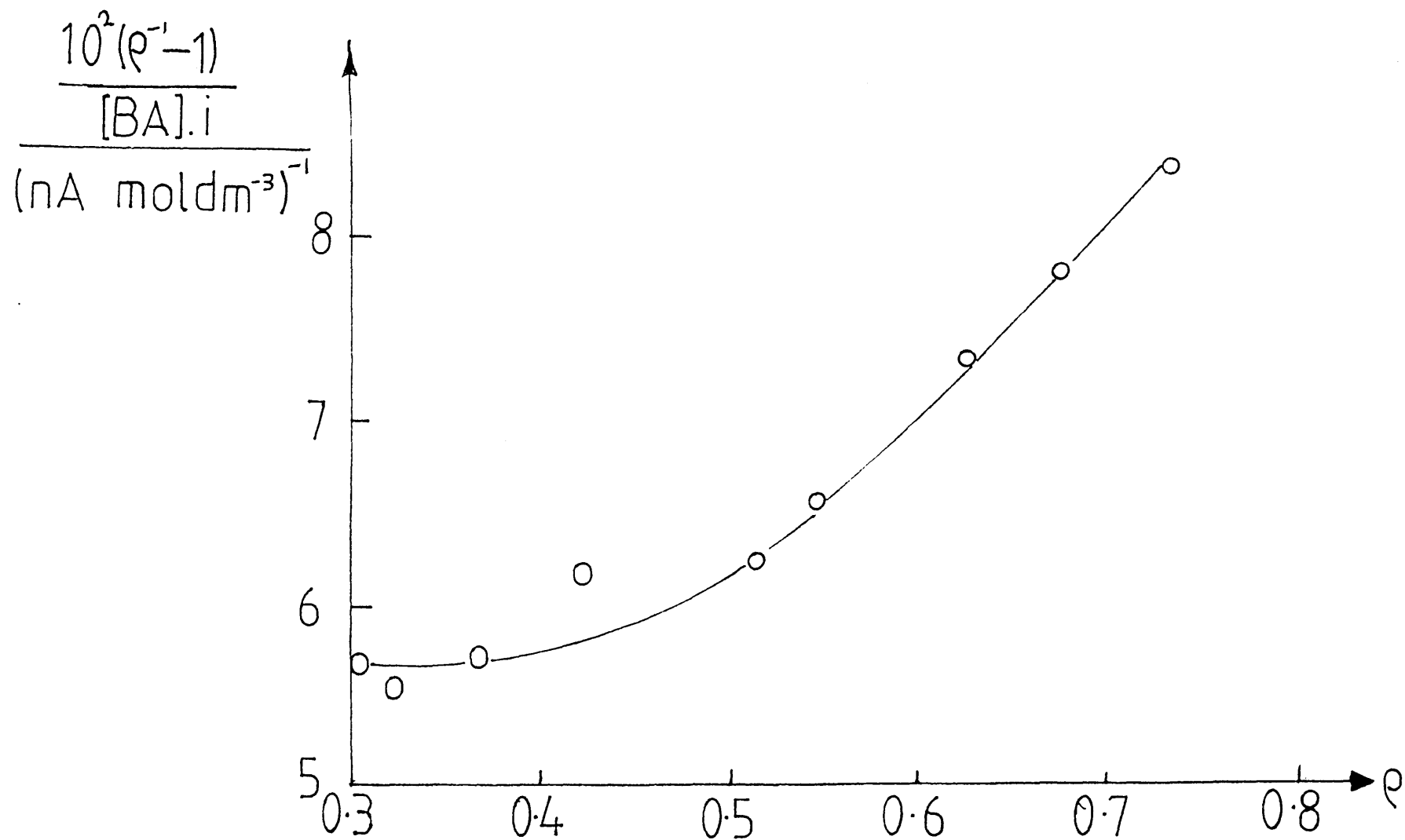


Figure 2.4.6 Modified g -plot for betaine aldehyde of data shown in Figure 2.4.3.

concentrations the rate limiting step is probably the enzyme reaction with substrate although we cannot yet rule out the electrode kinetics in case k_{cat} is a composite constant containing contributions from the latter.

Turning to the response of the electrode at low substrate concentrations, we find from the normal p-plot that $k_s \sim (Lk_{cat}[E]_t)/K_m$ (see Table 2.4.1). This means that the response is limited by enzyme kinetics and transport through the membrane almost equally.

We have now obtained a value for $Lk_B (= (Lk_{cat}[E]_t)/K_m)$, the effective second order heterogeneous rate constant for the reaction of betaine aldehyde with choline oxidase. The value of Lk_B/j_{max} is an apparent K_m for betaine aldehyde for this system, and from the experimental data is equal to 3.8 mmol dm^{-3} . The similarity between this value and the true value for the K_m for betaine aldehyde of 8.7 mmol dm^{-3} [23] strongly suggests that the rate limiting step at high substrate concentrations is the enzyme kinetics. In the case where the electrode kinetics are slow, the apparent K_m would be expected to be much smaller than the true value.

If we now compare the response to betaine aldehyde with that to choline, it can be seen first of all that both substrates have approximately the same i_{max} . From the theoretical analysis this implies that either the electrode kinetics are rate-limiting or that $k_{cat,C} \sim k_{cat,B}$. The analysis of the data for betaine aldehyde rules out the former possibility. It is interesting to note that in free solution using oxygen as a co-factor the k_{cat} 's for both

TABLE 2.4.1

Values of various kinetic parameters and constants for the
choline enzyme electrode

Substrate	Betaine Aldehyde	Choline
$k_{me} / \text{cm s}^{-1}$	5.6×10^{-5}	1.6×10^{-4}
Intercept at $q=0 \times \text{mM}$	0.14	
i_{max} / nA	2550^+	$2430^\ddagger$
Intercept at $y=0$	2.1	
$k_s / \text{cm s}^{-1}$	1.2×10^{-4}	1.3×10^{-4}
$(Lk_{cat}[E]_t)/K_m / \text{cm s}^{-1}$	1.1×10^{-4}	7.7×10^{-4}

$^+$ calculated from $i_{max} = (nFAk_{me})/(q)_{y=0}$

‡ maximum observed current

substrates are the same. In this case it may be thought that this is due to the reaction with oxygen being rate limiting; however the results shown above indicate that the true k_{cat} 's for both substrates are similar, possibly because the reaction is limited by the breakdown of a common enzyme intermediate to give the reduced form of the enzyme.

Looking at the response of the electrode to low concentrations of choline, we now know ϕ_B and Lk_B . From published data, we can calculate that $k_C = 7.3k_B^{[23]}$. Hence from the apparent k_{me} for choline (see Figure 2.404) we find that $k_{s,C} = 1.3 \times 10^{-4} \text{ cm s}^{-1}$. This is very close to the value of $k_{s,B}$, which is to be expected as the molecules are very similar in structure, size and charge. This supports the conclusions presented above and shows the results to be consistent. From Table 2.401 it can also be seen that k_s is approximately one quarter of the second order enzyme rate constant for choline, showing that the flux of choline at low concentrations is determined mainly by transport across the membrane.

The conclusions made above can be confirmed qualitatively by varying some of the experimental parameters and examining the effect on the response of the electrode. This proved to be difficult for betaine aldehyde as it is very expensive, therefore only choline was used in these experiments. The remainder of this chapter describes attempts to do this.

2.4.4 ADSORBED ENZYME ELECTRODES

Experiments using the Sigma enzyme showed that it did not adsorb to the organic salt electrode. When an electrode was dipped into the BCL enzyme solution for 10 minutes, and then washed with DDW, it showed a response to choline as shown in Figure 2.4.7, compared to the response of the same electrode with a membrane over it. As can be seen, the $i_{\max}$ is decreased in the absence of the membrane, showing that the enzyme in the electrolyte layer gives some contribution to $i_{\max}$. At low concentrations of choline, the response is increased when no membrane is present, showing that the membrane must at least partially control the response in this region.

In the case of the Finn Sugar enzyme, a different approach was taken, as the the enzyme was supplied in powder form. One unit of enzyme was placed onto the electrode by allowing 10 μ l of 100 units/ml aqueous enzyme solution to air-dry on the electrode surface. The electrode was then washed with DDW. The response of the electrode to choline is shown in Figure 2.4.8.

The following conclusions can be made from this result about the nature of the adsorption process. First, the enzyme must adsorb strongly or else it would be washed off or desorb during the experiment, causing a downward drift in the current, which did not occur. Secondly, the enzyme is robust enough to survive air-drying on the electrode. This could be important in the manufacture of these electrodes commercially.

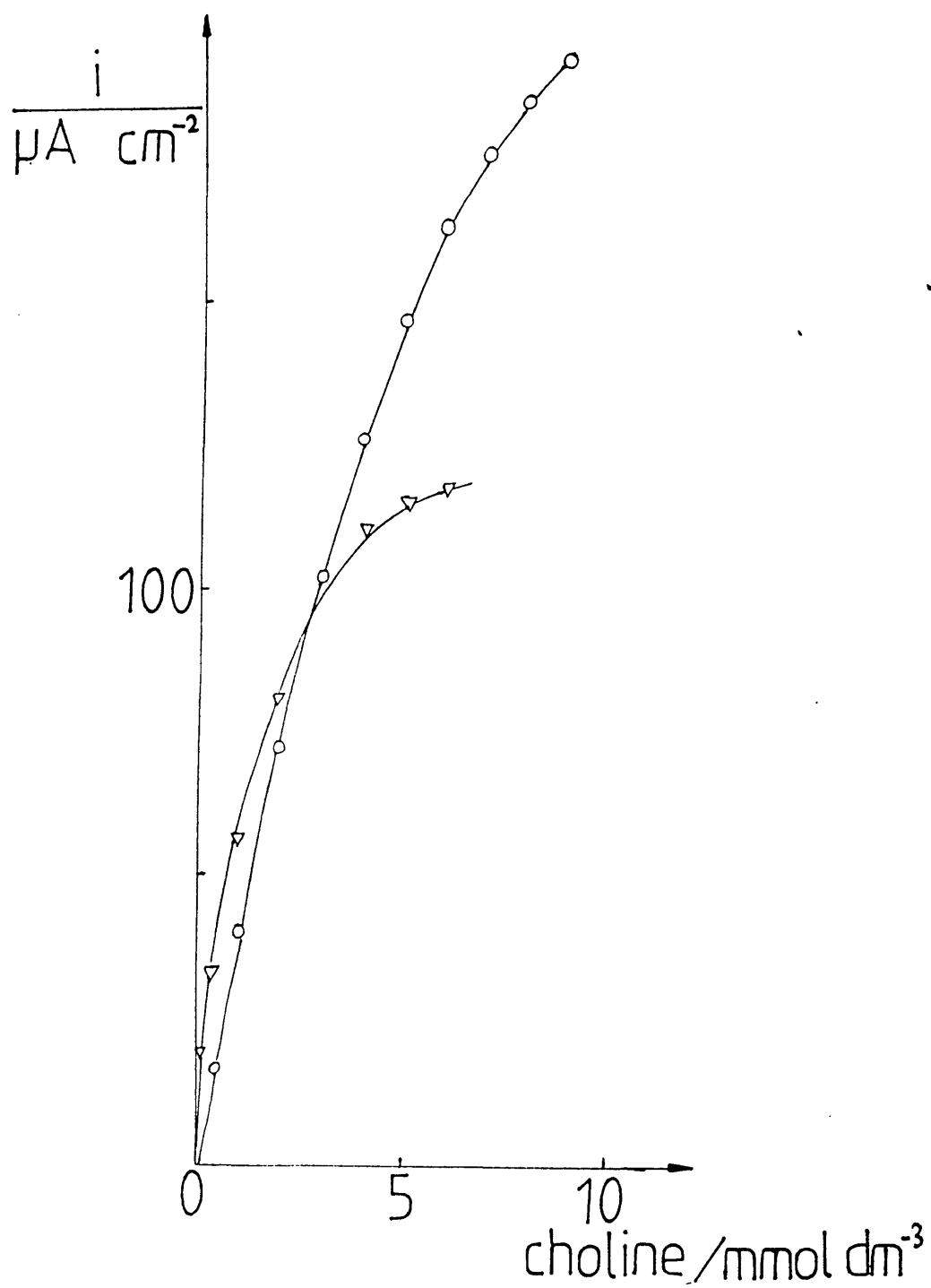


Figure 2.4.7 Comparison of the response of adsorbed (∇) and membrane ($\circ$) enzyme electrodes to choline.

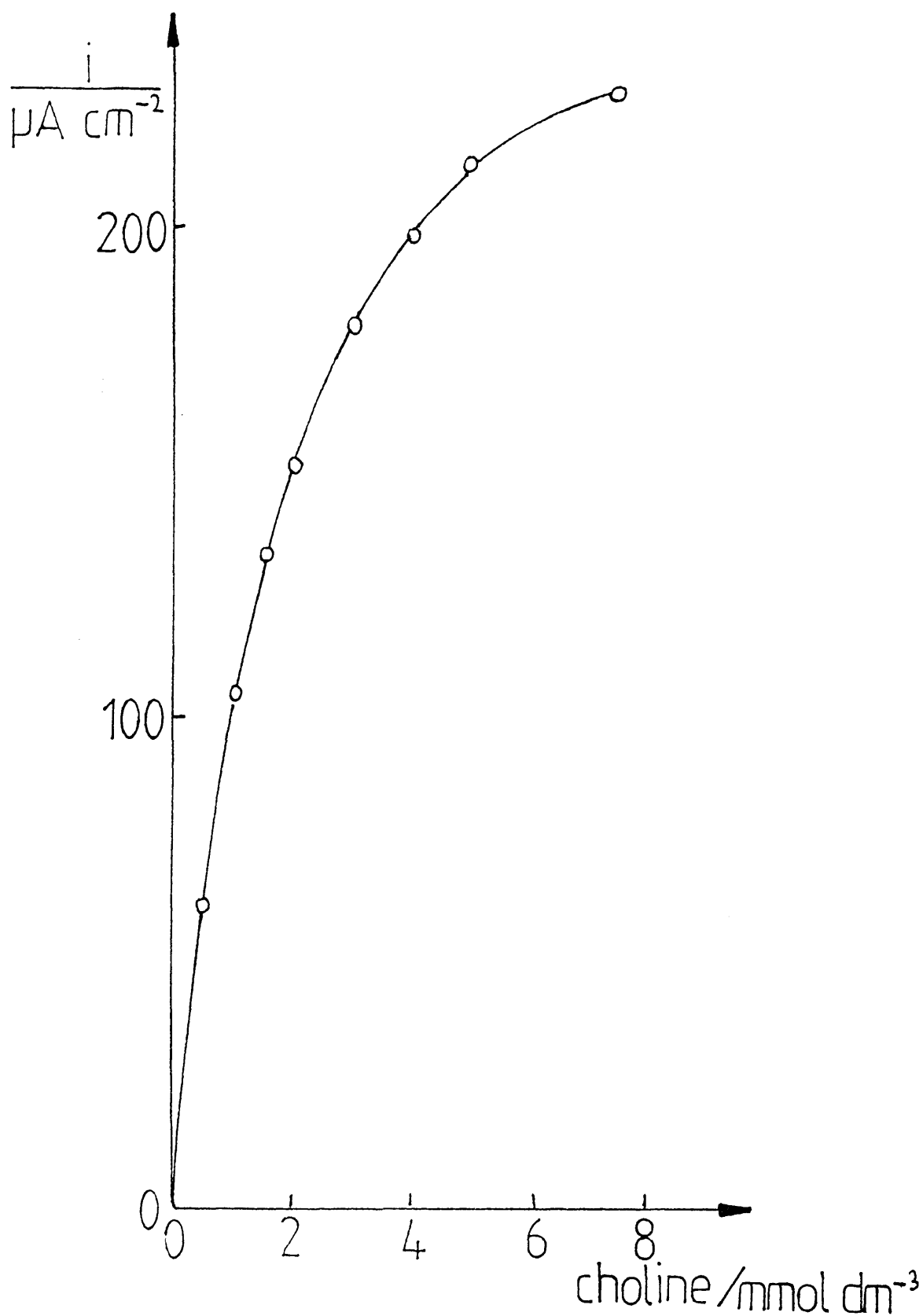


Figure 2.4.8 Response of air-dried adsorbed enzyme electrode to choline.

2.4.5 EFFECT OF SEMICARBAZIDE

In order to try to eliminate the effect of betaine aldehyde on the response of the electrode, semicarbazide was added to the bulk solution. This should react with betaine aldehyde to produce a semicarbazone, to which the enzyme would be inactive. In this way the current due to the turnover of choline should be isolated. The effect of adding semicarbazide to the bulk solution is shown in Figure 2.4.9. On Day 1 no semicarbazide was present. On Days 2 and 3, the bulk solution contained 72 mmol dm^{-3} semicarbazide. On Days 4 and 5 conditions were as Day 1. It can be seen that semicarbazide does alter the response of the electrode to a given concentration of choline. However, the response of the electrode to choline was lower on Day 3 than Day 2. Furthermore, although on Day 5 the electrode shows a similar response to choline as Day 1, on Day 4 the response is decreased, even though no semicarbazide was present. The effect of semicarbazide is therefore not as simple as may first be thought; a possible explanation of this phenomenon may be that it poisons the electrode in some way and blocks sites for the enzyme to adsorb. This would account for the decreases in i_{max} that occur when semicarbazide is present. For this reason, the use of semicarbazide was discontinued and quantitative analysis of results in which semicarbazide was added to the bulk solution was not attempted.

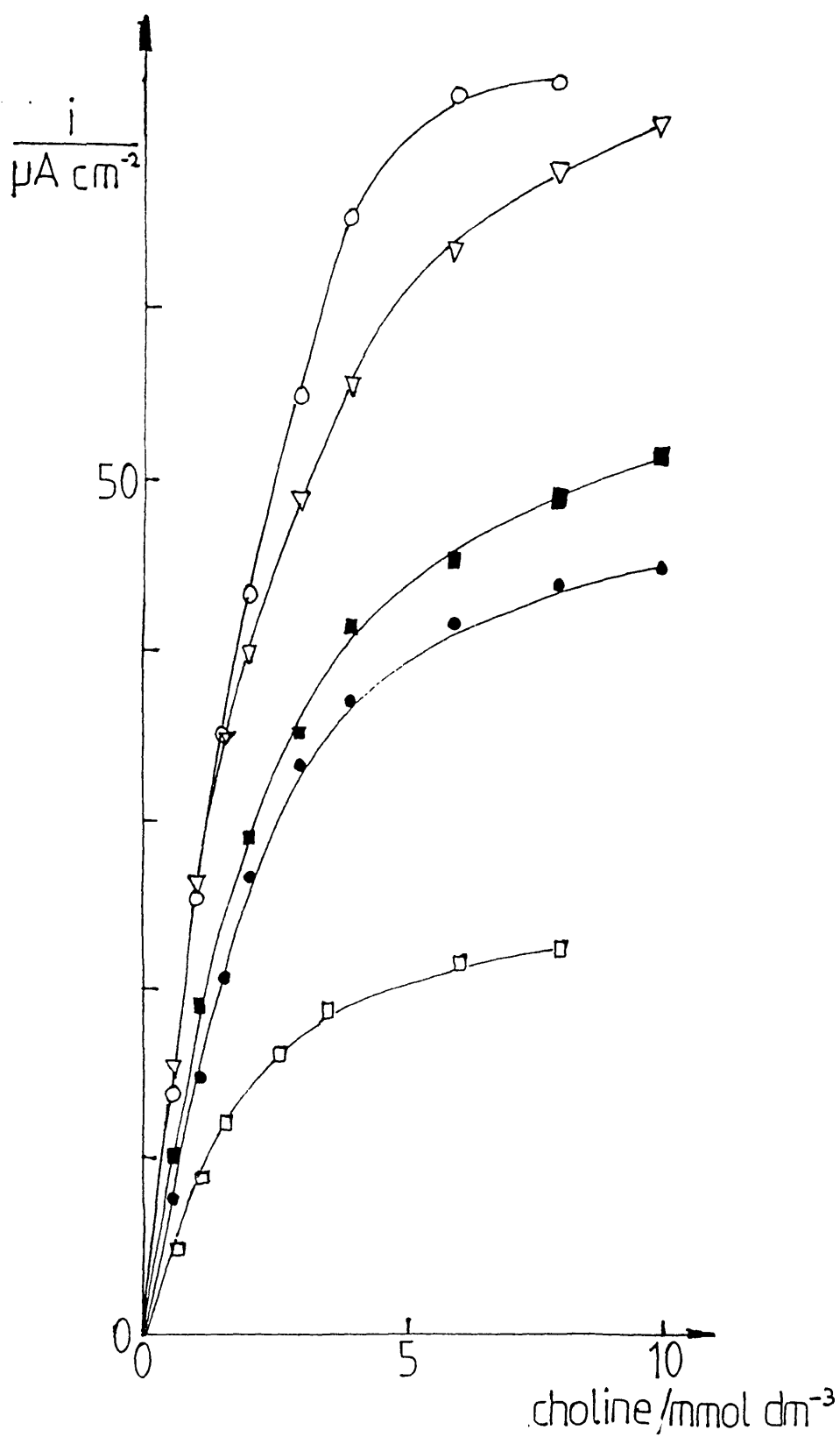


Figure 2.4.9 Effect of semicarbazide on the response of the membrane enzyme electrode to choline. Days 1 (○), 4 (■) and 5 (▽): no semicarbazide present. Days 2 (●) and 3 (□): 72 mmol dm^{-3} present in bulk solution.

2.4.6 EFFECT OF CHANGING MEMBRANE THICKNESS

One of the ways to vary the experimental parameters would be to change k_s . If the response of the electrode was partly or fully controlled at low concentrations by the membrane kinetics then a small change in k_s would bring about a change in response, and hence a change in the apparent k_{me} for choline. Considering the conclusions made so far, however, it can be seen that the relationship between k_s and apparent k_{me} for choline is not a simple one. If we decrease k_s then $k_{me,C}$ should decrease, resulting in a lower response. However, ϕ_B will increase, as less betaine aldehyde will escape into the bulk, and this will tend to compensate a little for the loss of response. In order to make conclusions from results we therefore have to change k_s in a known manner and to do this we have to examine how k_s is determined.

The rate constant, k_s , is controlled by the membrane thickness L_m , the diffusion coefficient, D_m and the partition coefficient of S, K_s in the following way^[40]:

$$k_s = (K_s D_m) / L_m \quad (2.26)$$

The easiest way to change k_s is to simply use a different membrane. However, in this case K_s , D_m and L_m could all be altered, with unpredictable results. A better approach is to try to double L_m by using two identical membranes instead of one. Comparing the results obtained with those where a single membrane was used would then be easier, as L_m would be approximately doubled and D_m

and K_s would be constant. The value of k_s for a double membrane would therefore be approximately half that of a single membrane, although some error may creep in from the dead space in between the membranes.

Figure 2.4.10 shows the response of the electrode when this procedure is carried out, and the corresponding Hanes plots are shown in Figure 2.4.11. The latter give values of apparent k_{me} of choline as $1.6 \times 10^{-4} \text{ cm s}^{-1}$ where a single membrane was employed, and $0.8 \times 10^{-4} \text{ cm s}^{-1}$ where a double membrane was used. If we assume k_s is decreased by a factor of two, then equation 2.19 would predict a value for the apparent k_{me} of $0.77 \times 10^{-4} \text{ cm s}^{-1}$ for the double membrane. After allowing for experimental error and the assumptions involved, this agrees with the experimental value quite well. The most important conclusion however, is that the apparent k_{me} is changed substantially on changing k_s ; thus membrane transport must be the most significant factor in determining the response of the electrode at low substrate concentrations.

2.4.7 EFFECT OF CHANGING ENZYME/ELECTROLYTE LAYER THICKNESS

Looking at equation 2.8, it is clear that a change in the electrolyte layer thickness, L , will result in a change in the terms which involve the homogeneous kinetics of the enzyme. Figure 2.4.12 shows the effect of changing L to 0.25 mm. At first sight it seems strange that there is not an appreciable change in response. This may seem to

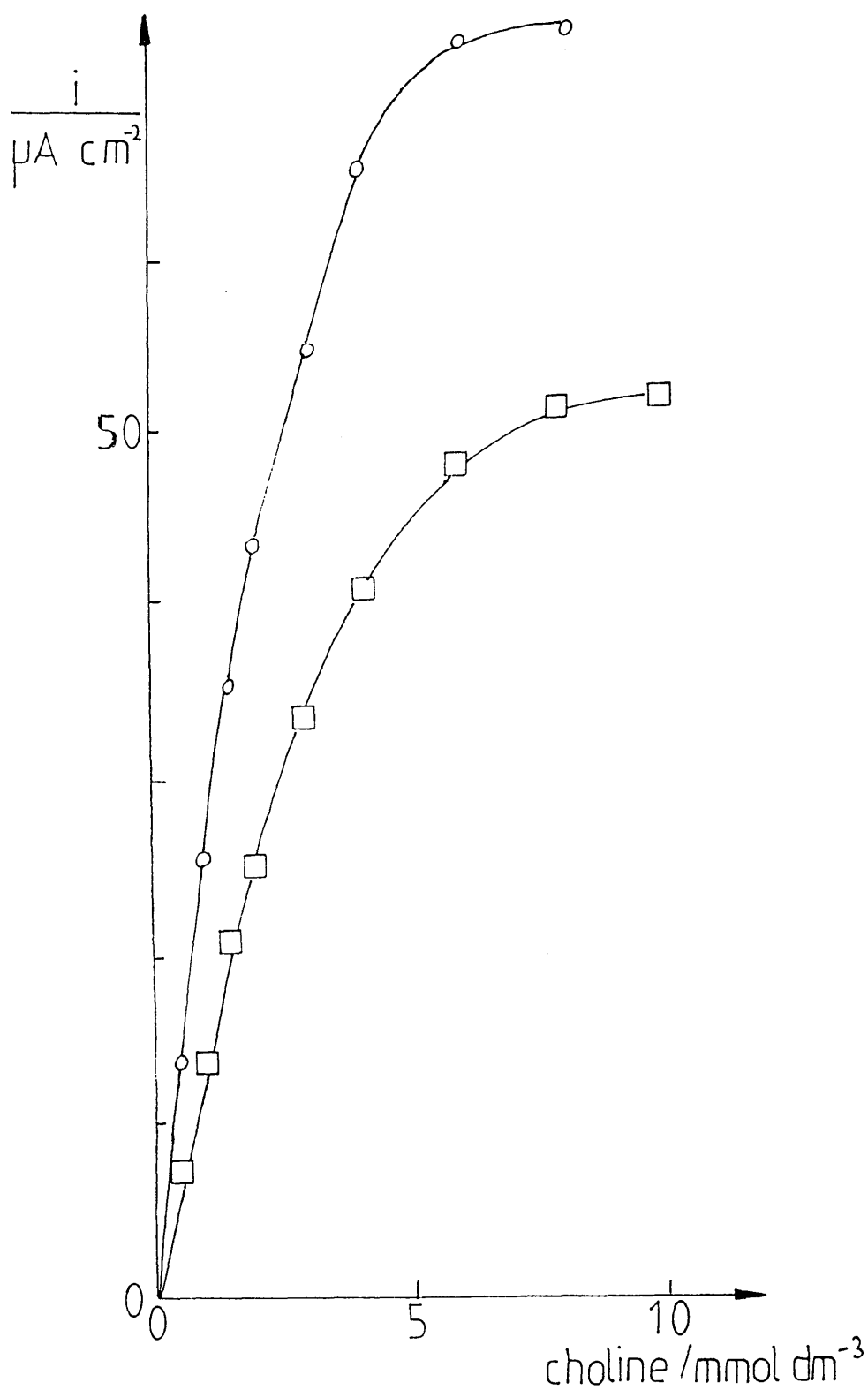


Figure 2.4.10 Comparison of the response of single membrane ($\circ$) and double membrane ($\square$) enzyme electrodes to choline.

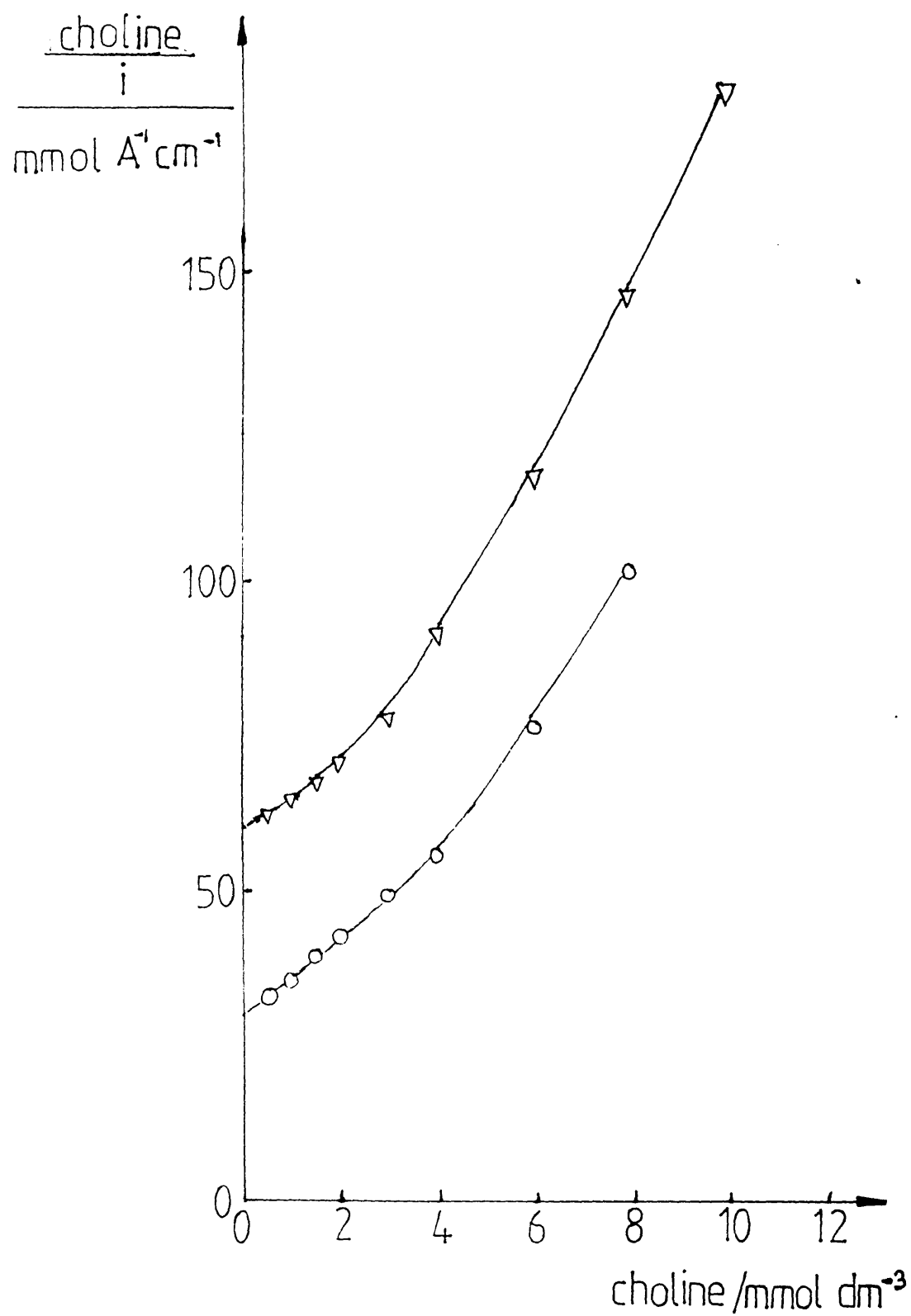


Figure 2.4.11 Hanes plots of data presented in Figure 2.4.10. (O) Single membrane, (∇) double membrane.

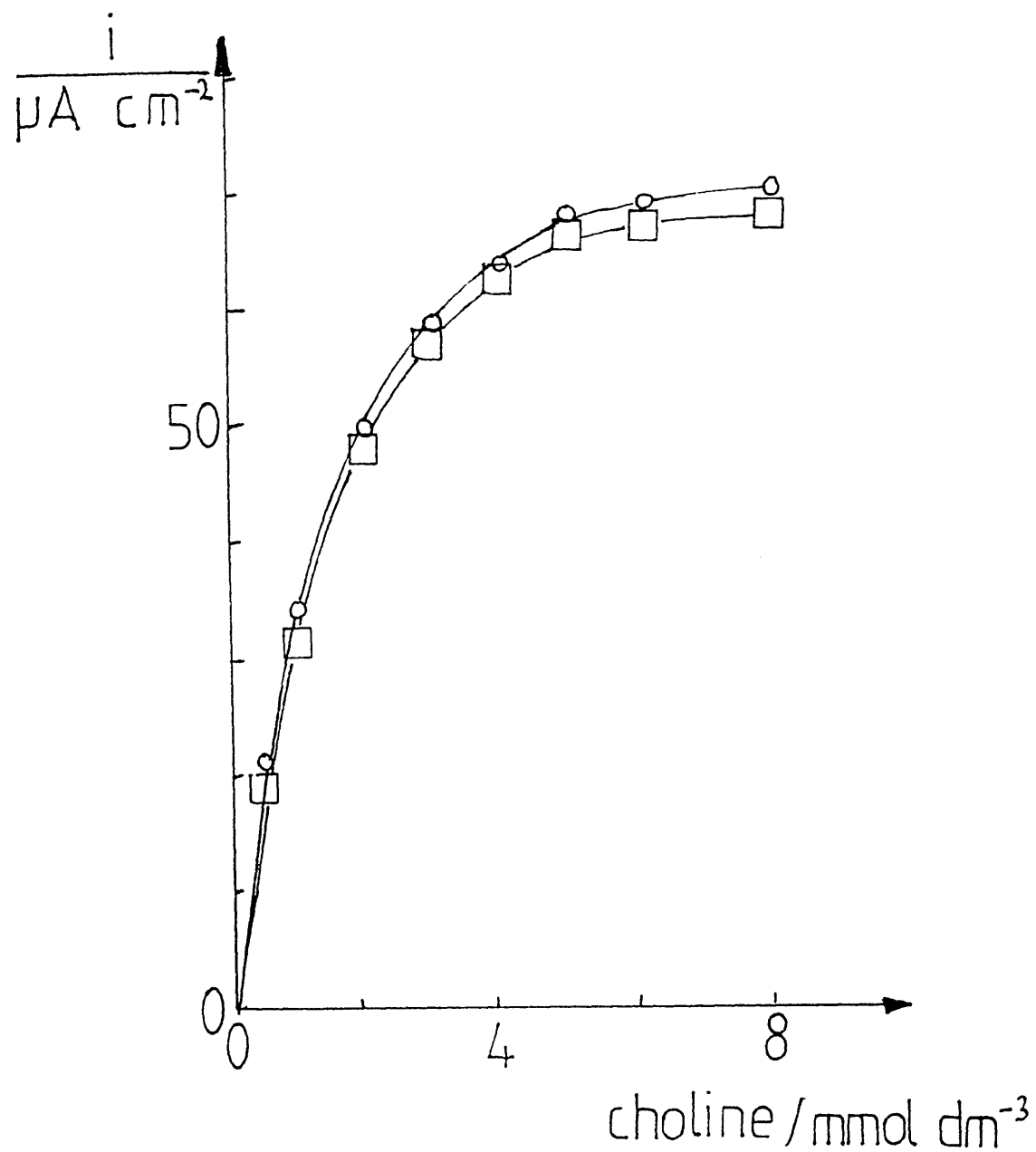
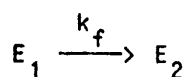


Figure 2.4.12 Comparison of the response to choline of membrane enzyme electrodes having a minimum value of L (□), and a value of L of 0.25 mm (○).

indicate that our original conclusions were wrong and that the response is limited solely by membrane transport at low choline concentrations and the electrode kinetics at high concentrations. However, the results can be explained by looking at the reaction layer of the enzyme and comparing it with the electrolyte layer thickness. Because the solution behind the membrane is unstirred only the enzyme in a thin reaction layer will turnover and thus there will be less enzyme effectively taking part in the reaction.

Albery and Bartlett^[76] have examined this phenomenon and we shall look at a simplified version of their scheme. We shall assume the enzyme exists mainly in two forms, E_1 and E_2 . We shall assume no product inhibition. Thus



In these circumstances the reaction layer of the enzyme, L_r is given by:

$$L_r = (D_E/k_f)^{1/2} \quad (2.27)$$

where D_E is the diffusion coefficient of the enzyme. Now, at saturation, $E_1=ES$, $E_2=E'$ and so k_f is given by k_{cat} , whose value is approximately 10 s^{-1} [23]. A reasonable value for D_E is $5 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$. Thus, $L_r \sim 2 \times 10^{-4} \text{ cm}$. For the results shown, $L = 250 \times 10^{-4} \text{ cm}$. We can therefore conclude that under these circumstances, the enzyme is concentration polarised and does not all take part in reaction.

Additionally, adsorbed enzyme will give the same

contribution to i_{max} whatever value L takes and so the

effect of altering L will be attenuated.

At lower substrate concentrations, the treatment is more complicated as k_f will depend on the concentrations of choline and betaine aldehyde in the electrolyte layer, as well as the unsaturated enzyme kinetic rate constants. The situation is also complicated by the presence of adsorbed enzyme. However, given that the membrane kinetics mainly control the response in this region, and that the adsorbed enzyme will contribute substantially to the current, the observation that there is little change in response on altering L is not surprising.

2.4.8 EFFECT OF CHANGING ENZYME CONCENTRATION

The effect of changing $[E]_t$ should be twofold; first $i_{\max}$ should be proportional to $[E]_t$ and secondly there should be an effect on k_{me} if the unsaturated enzyme kinetics are important. If the enzyme concentration is decreased both ϕ_B and Lk_C will also decrease; hence the apparent k_{me} for choline will be lower. The converse will be true at higher concentrations of enzyme, although the upper limit for the apparent k_{me} will be $2k_s$, when ϕ_B is equal to unity and $k_s \ll Lk_C$.

Unfortunately the situation is more complicated than this because enzyme adsorbs onto the electrode, thus the effective enzyme concentration will be larger than would otherwise be thought. To test for this, an organic salt electrode was dipped in 50 units/ml ChOD solution for 30

minutes. The electrode was then washed with DDW and a wetted membrane fixed over it. The response of this electrode to choline is shown in Figure 2.4.13, along with the response of electrodes containing 50 units/ml and 500 units/ml of entrapped enzyme solution. It can be seen that the response of the adsorbed enzyme electrode is approximately 50% of an electrode containing 50 units/ml enzyme. Furthermore, when the enzyme concentration is increased to 500 units/ml the response of the electrode is increased considerably at high substrate concentrations. The results therefore confirm that $i_{\max}$ increases with enzyme concentration. They also show that the dynamic range of the electrode increases with enzyme concentration as well. By looking at the variation of $i_{\max}$ with concentration we can also check that enzyme kinetics limit the response at high substrate concentrations. If this is the case, then $j_{\max}$ will be related to enzyme concentration in the following way:

$$j_{\max} = Lk_{\text{cat}}[E]_t + j_{\max, \text{surf}} \quad (2.28)$$

where $j_{\max, \text{surf}}$ is the contribution to $j_{\max}$ from the adsorbed enzyme. In the preceeding section we have shown that the enzyme may be concentration polarised under these conditions. In this case we can substitute L_r for L . We have here assumed that the surface is saturated with enzyme. This can be justified because the enzyme binds to the electrode strongly. Experimentally, the enzyme is measured in units. From the definition of the unit:

$$[E]_u = (10^{-6}/60)k_{\text{cat}}[E]_t \quad (2.29)$$

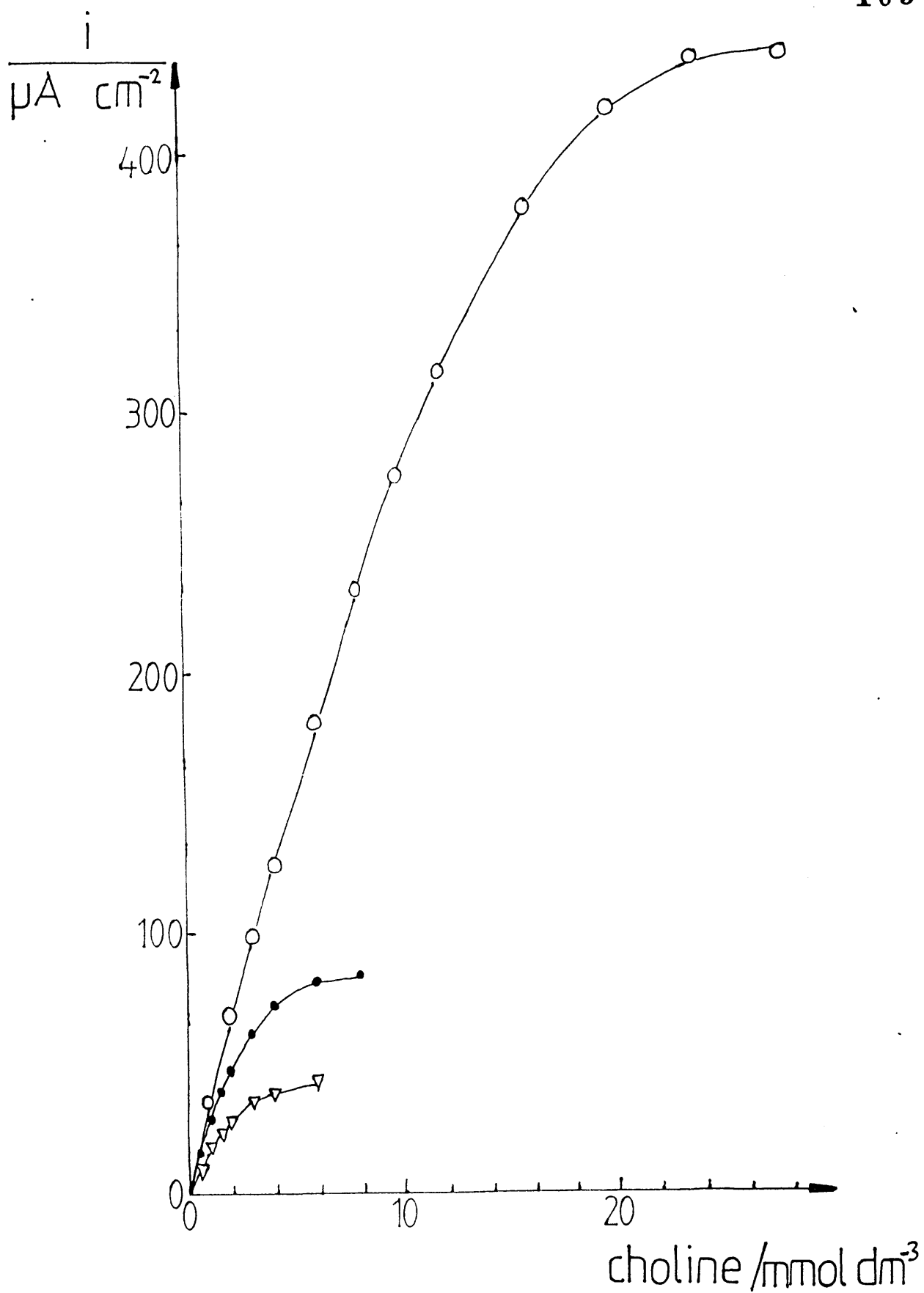


Figure 2.4.13 Comparison of the response to choline of membrane enzyme electrodes with different values of enzyme concentration. Enzyme concentration: (O) 500 units/ml, (●) 50 units/ml, (▽) adsorbed enzyme layer only.

where $[E]_u$ is the concentration in units/ml. Hence, after converting flux to current:

$$i_{\max} = nFA(10^{-6}/60)L[E]_u + i_{\max, \text{surf}} \quad (2.30)$$

Although $i_{\max}$ cannot be obtained exactly, it can be estimated from the response of the electrode to the highest concentration of choline used. When $i_{\max}$ is plotted against $[E]_u$ we obtain the results in Figure 2.4.14. It can be seen that the points lie approximately on a straight line. From the gradient we can calculate L to be 2.3×10^{-4} cm, a value that is similar to the previous estimate for L_r . This provides more evidence for concluding that the response is controlled by the enzyme kinetics at high concentrations of substrate.

An interesting question now arises as to why the q -plot is a straight line if the enzyme is concentration polarised. The electrolyte layer thickness has been previously estimated as being of the order of microns. This is approximately the same value as that calculated for L_r . Because of this, concentration polarisation may only be important at the highest substrate concentrations, and so a non-linearity in the q -plot may be difficult to detect.

At low concentrations of substrate, the response of the sensor does increase a little with enzyme concentration. However, the increase is not as marked as the differences in $i_{\max}$. This can be explained because the response is already mainly controlled by the membrane kinetics in the unsaturated region. Even altering the enzyme concentration substantially

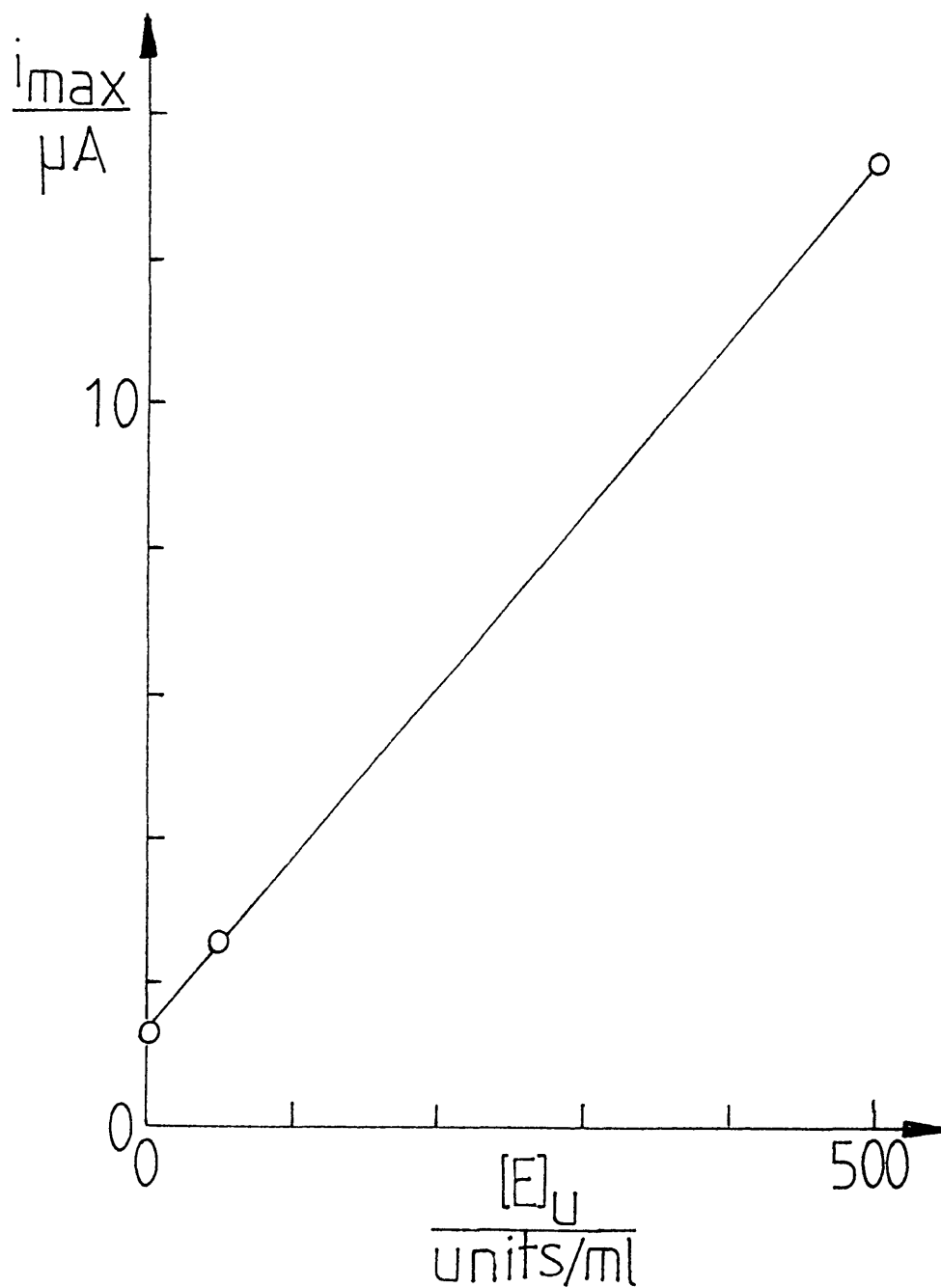


Figure 2.4.14 Plot of observed maximum current to choline against enzyme concentration for data shown in Figure 2.4.13.

will therefore have only a small effect on response.

2.4.9 FACTORS INVOLVED IN DETERMINING THE RESPONSE TIME OF THE ENZYME ELECTRODE

In order to understand how to optimise the response time of the sensor, one must look at the current/time transients when the concentration of choline in the bulk is changed. To compare transients we must first normalise the response of the electrode by dividing the change in current, Δi by the total change in current, Δi_{lim} . Typical transients are shown for low concentrations of choline in Figure 2.4.15, and for high concentrations in Figure 2.4.16. The following points can be noted:

- i) the response time is faster at lower concentrations;
- ii) increasing the electrolyte layer thickness, L increases the response time;
- iii) at high concentrations the response of the sensor shows an initial drop on addition of substrate;
- iv) after saturation the response of the sensor decreases dramatically.

We will first examine points iii) and iv). The same phenomena occur for adsorbed enzyme electrodes as well as membrane electrodes; they occur in both the presence and absence of semicarbazide; and when betaine aldehyde is used as a substrate iv) does not occur and there is little drop in response when the concentration of betaine aldehyde is changed. We can therefore rule out the cause of this effect to be due in any way to the membrane, betaine aldehyde or

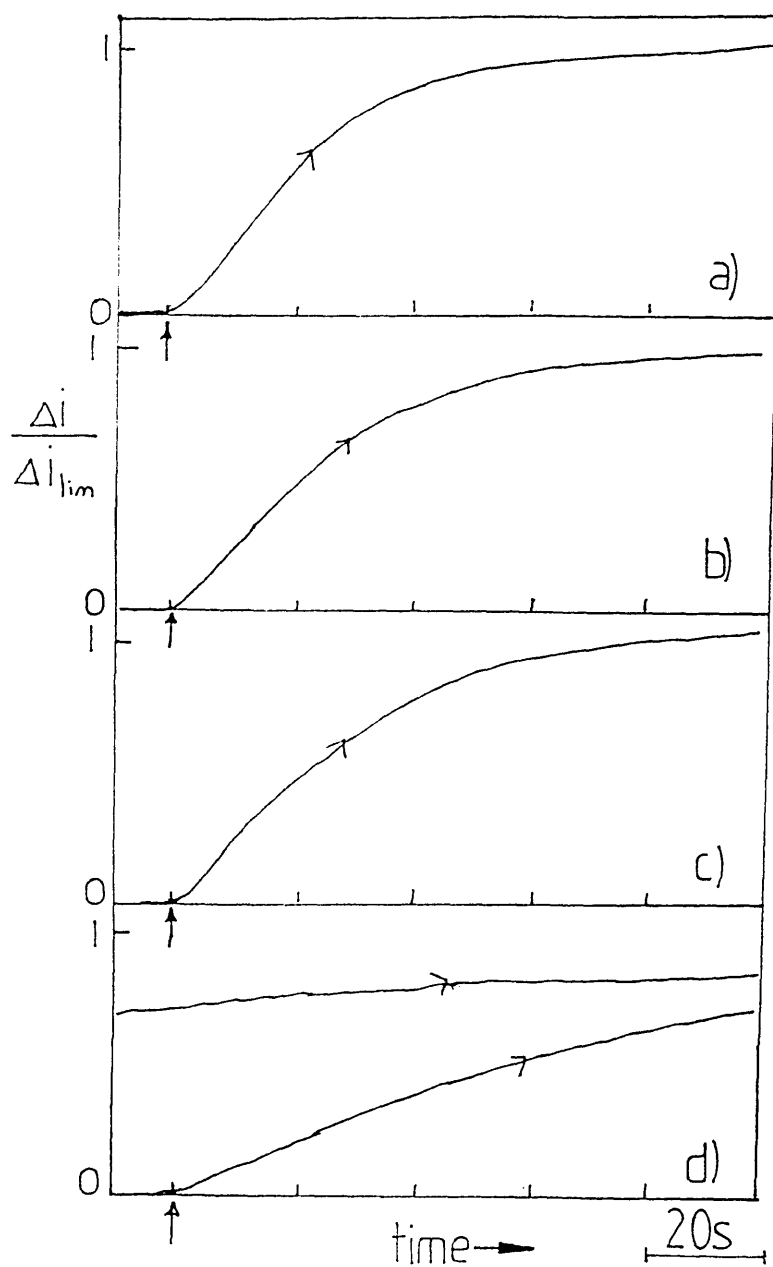


Figure 2.4.15 Transient responses of different enzyme electrodes to low concentrations of choline: a) adsorbed enzyme only, no membrane; b) 50 units/ml enzyme, single membrane, minimum value of L ; c) 500 units/ml enzyme, single membrane, minimum value of L ; d) 50 units/ml enzyme, single membrane, $L=0.25$ mm.

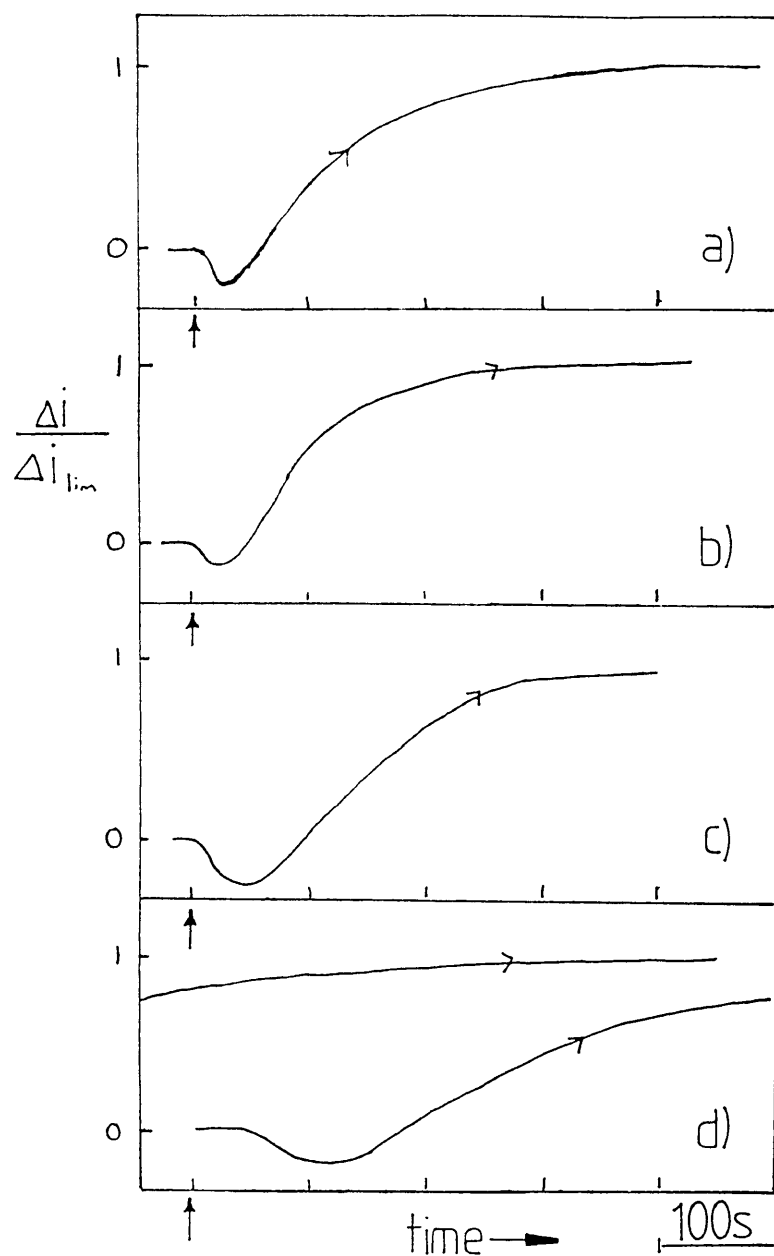


Figure 2.4.16 Transient responses of different enzyme electrodes to high concentrations of choline: a) adsorbed enzyme only, no membrane; b) 50 units/ml enzyme, single membrane, minimum value of L ; c) 500 units/ml enzyme, single membrane, minimum value of L ; d) 50 units/ml enzyme, single membrane, $L=0.25$ mm.

product inhibition. Furthermore, the presence of choline seems to have little effect on the performance of a glucose oxidase conducting salt enzyme electrode in its response to glucose. This excludes the possibility that choline poisons the electrode surface. A question must also be asked as to whether the two phenomena are related or independent.

A definitive explanation for this behavior probably requires further work on the enzyme itself. One possibility which could account for the decrease in the response is that choline oxidase is inhibited by high concentrations of choline. This has been observed in a recent study of the enzyme^[77], but was not been mentioned by previous workers.

Another intriguing possibility is that the k_{cat} for choline is slightly less than, although comparable to, that of betaine aldehyde. A pseudo-saturation region could be reached where all the enzyme is complexed with either choline (EC) or betaine aldehyde (EB). In this case, a sharp increase in choline concentration would result in the concentration of EC being increased at the expense of EB. Due to the difference in the k_{cat} 's this would result in an initial fall in current, which could later be compensated for as the concentration of betaine aldehyde increased to its steady state value and restored the balance between the two complexes.

Point ii) is probably explained by looking at the dead space behind the membrane. When $L = 0.25$ mm this will be comparatively very large and this will lead to high response

times.

In use the sensor will be operating under unsaturated conditions (ie. low concentrations of substrate), and the kinetic analysis of the steady-state data shows that in these circumstances the steady-state response is controlled mainly by the membrane kinetics. The response time under these conditions can either be governed by the membrane kinetics or the unsaturated enzyme kinetics. To test whether it is the former we can compare the transients when a double membrane is used compared to a single membrane. For an increase in bulk choline concentration of 0 to 0.5 mmol dm⁻³ these are shown in Figure 2.4.17a. It can be seen that the double membrane electrode has a substantially longer response time than that with a single membrane, showing that the membrane is at least partially determining the response time. Quantitatively, if the response time, τ is controlled solely by the membrane kinetics then

$$\tau \propto D_s / (L_m)^2$$

If the transport kinetics determined τ , for the double membrane electrode τ should be a quarter of that of the single membrane, as L_m has been doubled and D_s remains the same. This can be tested by plotting $\Delta i / \Delta i_{lim}$ against time, t for the double membrane electrode and against $4t$ for that of the single membrane. If the membrane solely controls the response time then the two plots should coincide. These plots are shown in Figure 2.4.17b. The agreement between the curves is quite good, and shows that the response time of the sensor is mainly limited by the membrane kinetics.

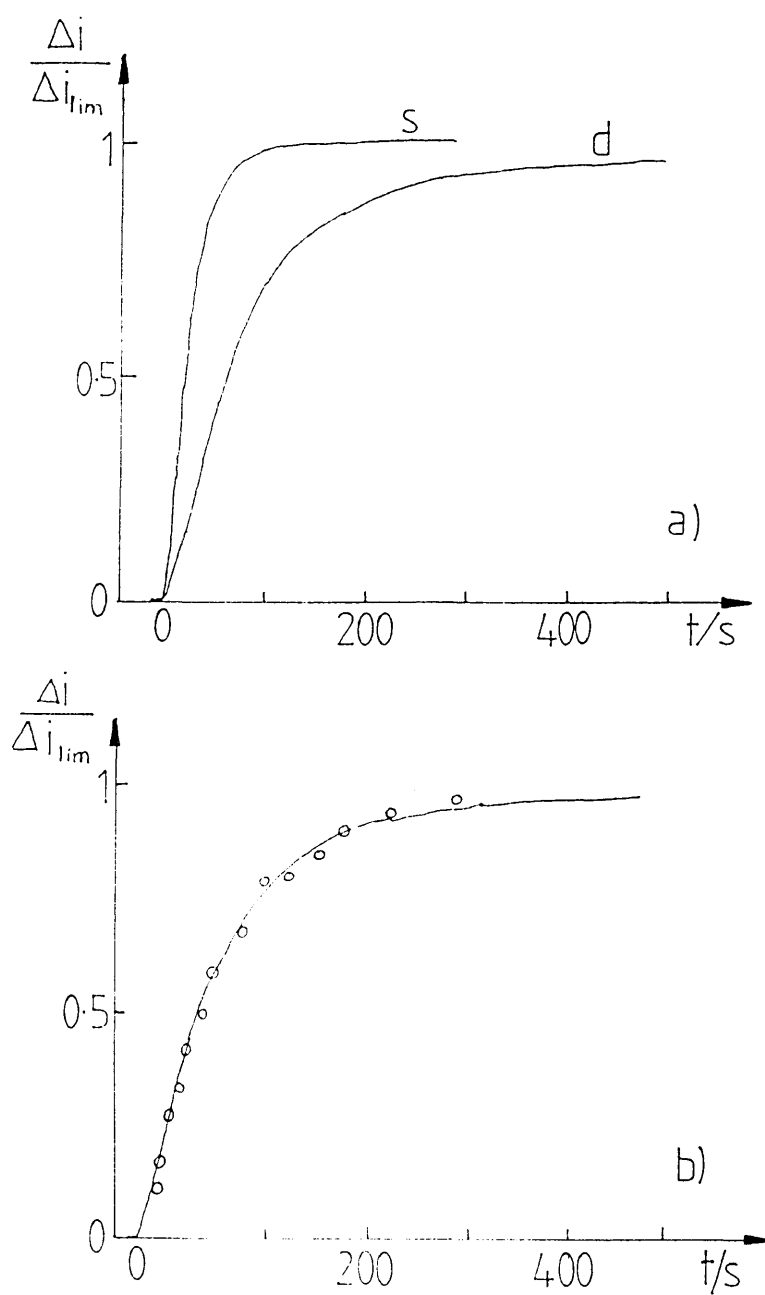


Figure 2.4.17 Transient response of membrane enzyme electrodes to choline: a) s, single membrane; d, double membrane; b) the same data, comparing the transient for the double membrane (—) with the transient for the single membrane replotted against $4 \times$ time ($\circ$).

Furthermore, under these circumstances it has previously been shown that $t_{1/2}$, the time taken to reach half the steady state response is given by^[78]:

$$t_{1/2} = (L_m)^2 / (7.199 D_s) \quad (2.31)$$

For a single membrane $t_{1/2}$ was about 15 s and L_m was measured to be approximately 6×10^{-3} cm using a micrometer. This gives D_s to be 3×10^{-7} cm² s⁻¹, which agrees with a value found using independent techniques^[79], thus supporting the conclusions above.

2.5 DISCUSSION

The results presented above allow us to form several conclusions concerning the processes which determine the steady state response of a conducting salt choline electrode and its response time. Using kinetic analysis, we have shown that at low concentrations of choline, the response is mainly limited by transport across the membrane. This was confirmed empirically by altering the enzyme concentration, the enzyme/electrolyte layer thickness and the thickness of the membrane. Only changing the latter parameter had a substantial effect.

At high concentrations of choline, the electrode kinetics are relatively fast and so the enzyme kinetics are rate limiting, although they are fast enough to cause concentration polarisation of the enzyme at saturation. This is confirmed using kinetic analysis and by comparing the experimentally determined reaction layer thickness with that obtained using standard theory.

The response time of the sensor is determined by the membrane. Again, both qualitative results and theoretical analysis confirms this.

In this study, alteration of the experimental parameters produced enzyme electrodes with different characteristics. It is suggested that the electrode which made the best sensor contained 500 units/ml enzyme solution, used a single membrane and was made up with a minimal value of L . This had the best dynamic range and smallest response time. It should be

possible to increase the former if required by increasing the enzyme concentration. The response time could be altered by changing the membrane, although of course this could change the steady state response.

These experiments have also given some insight into the kinetics of the enzyme itself. They show that the true k_{cat} for the enzyme reduction step both for choline and betaine aldehyde are the same. They also show that choline probably inhibits the enzyme at high concentrations.

Because of the unresolved problem concerning the reproducibility of the organic salt electrodes, it was decided that it would be desirable to find an alternative method of fabrication for these electrodes. The next chapter describes attempts to produce these electrodes electrochemically. The reader interested in following the development of these choline electrodes to detect acetylcholine is directed to the thesis of Dr Sally Durrant^[77].

CHAPTER 3

THE ELECTROCHEMICAL SYNTHESIS OF ORGANIC SALTS

3.1 INTRODUCTION

Several methods have been used to produce conducting organic salt electrodes. In the previous chapter, electrodes fabricated using a polymer paste of the organic salt were employed. These gave high currents but were irreproducible, probably due to their varying porosity. In this laboratory, pressed pellets have also been used. However, these electrodes are fragile, give poor currents with flavoenzymes, and require a high degree of technical expertise to construct them. Another method of fabrication has been to prepare paste electrodes using silicone oil as the binder. These electrodes have much better reproducibility, but again show smaller currents with flavoenzymes (see Chapter 4). Dr P N Bartlett has used single-crystal electrodes for the oxidation of GOD^[41], but again these show lower currents than polymer paste electrodes, require high quality crystals and are much less versatile in their application.

There is therefore a requirement for a method of producing organic salt electrodes that is reproducible, capable of being applied to the construction of electrodes of differing configurations (such as the rotating disc) and relatively easy to produce. A potential answer to this problem is to produce thin-film organic salt modified

electrodes by electrochemical methods. If this was possible, any surface made of a suitable electrode material could be coated with a thin, even layer of a known amount of organic salt using the appropriate conditions. Furthermore, by producing thin-films of the organic salt, the use of techniques such as surface enhanced Raman and UV-reflectance spectroscopy to investigate their properties would be facilitated.

In this chapter, the synthesis of organic salt electrodes by electrochemical techniques is carried out using several methods. Because TTFTCNQ and NMPTCNQ have become the materials of choice in this laboratory, these salts were chosen as targets for synthesis in this manner. Previous work has shown that these salts can be produced electrochemically, and the strategies available will be discussed in the next section. However, in these previous syntheses the object was to make crystals of the salt, not a thin film. Thus these methods had to be modified in order to try to make suitable electrodes for use in sensors or mechanistic studies.

3.1.1 THE ELECTROCHEMICAL SYNTHESIS OF ORGANIC SALT ELECTRODES

The electrosynthesis of conducting organic salts is, in principle, very simple. If D denotes the donor and A the acceptor then the oxidation of a solution containing D^0 and A^- or the reduction of a solution containing D^+ and A^0 should yield the salt DA. However, there are several

problems to be overcome before this approach can be used. Firstly, both D and A in all the relevant oxidation states must be soluble in the solvent employed but DA must not; secondly the donor cation and acceptor anion must not form an insoluble salt with any background electrolyte used; and finally many donors and acceptors are unstable to both air and light, thus if the preparation takes place over a timescale of hours it must be carried out in the dark under an inert gas, such as nitrogen.

Addressing these problems to the specific cases of TTF⁰TCNQ and NMPTCNQ, first we must note that the commercially available forms of their components are TTF⁰, TCNQ⁰ and NMP⁺ (as its methosulphate salt). The neutral form of NMP⁺ is very unstable in solution and therefore cannot be used in an electrochemical synthesis^[80]. LiTCNQ can be easily synthesised in bulk^[33] and other salts of the same anion can be produced from this by metathesis. The simplest way to produce salts of TTF is electrochemically in acetonitrile; separation can be difficult if the salt is soluble in that medium and so if a TTF⁺ salt is to be used for electrosynthesis in acetonitrile it is best made in-situ. The solubilities of TTF⁰, TCNQ⁰, NMPMeSO₃ and some of their salts in water and acetonitrile are shown in Table 3.1.1. No stable salt of TTF⁺ has been found to be soluble enough in water to be used as a background electrolyte. It can be seen that as TCNQ⁰ and TTF⁰ are only soluble in acetonitrile and the neutral form of NMP⁺ is unstable, this

TABLE 3.1.1

Solubilities of TTF^0 , TCNQ^0 , NMPMeSO_3 and some of their salts in water and acetonitrile.

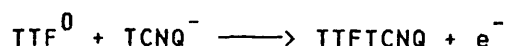
Material	Water	Acetonitrile
TTF^0	X	S
$\text{TTF}^+\text{CH}_3\text{COO}^-$	S ^[42]	
TTFC1	SS	X
TTFC1O_4		S
TCNQ^0	X	S
LiTCNQ	S	SS
$\text{Et}_4\text{N}^+\text{TCNQ}^-$	X	S ^[39]
NMPMeSO_3	S	S

(Results of spot tests carried out in this work unless otherwise indicated.

S=soluble, SS=slightly soluble, X=insoluble)

must be the solvent of choice for any electrosynthesis based on this method.

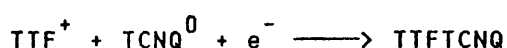
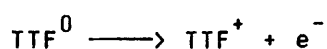
Nearly all the previous work in this area has been directed at producing TTFTCNQ, the most popular synthetic route to date being the oxidation approach. This is probably because salts of TCNQ^- are easier to prepare than those of TTF^+ . In 1976 Wheland and Gillson oxidised a solution of TTF, TTFTCNQ and NMPTCNQ in acetonitrile to produce crystals of TTFTCNQ^[37]. Presumably the overall reaction was:



However, the reaction took 2 weeks to complete, and the apparatus had to be encased in a nitrogen glove bag. In this case, NMPTCNQ served as both the background electrolyte and source of TCNQ^- .

The next attempts at the electrochemical synthesis of organic salts were made by Kathirgamanathan^[81,82], who produced salts of the form TTF_nX^- (where n may be non-integral) by oxidising solutions of TTF^0 in the presence of the appropriate anion. The crystals took 9-15 hours to produce and again had to be grown under nitrogen. Approximately 20 salts were made altogether, including TTFTCNQ, although the exact salt of TCNQ employed is not recorded. When this synthesis was repeated by Dr Derek Craston in these laboratories using tetraethylammonium(TEA)TCNQ, TTFTCNQ was produced but in very low electrochemical yield. This was attributed to the ion-pairing of TEATCNQ causing a high solution resistance and poor supply of TCNQ^- to the electrode^[39].

Finally, El Kacemi and Lamache have produced TTFTCNQ by a two stage, "one pot" method by first producing a solution of TTF^+ via the oxidation of TTF^0 in acetonitrile using tetrabutylammonium perchlorate as a background electrolyte^[83]. Adding TCNQ^0 to this solution, they then reduced the mixture to give TTFTCNQ. Thus the two-step preparation was therefore as follows:



Part 1 of this chapter explores electrosyntheses based on the above general approach. The methosulphate salt of NMP^+ is very soluble in acetonitrile and so for initial studies it was decided to attempt a synthesis of NMPTCNQ by reducing a solution of NMPMeSO_3 and TCNQ^0 in acetonitrile, using the NMP^+ salt as the background electrolyte. This is described in Section 3.3(1).1. As this approach failed to give satisfactory electrodes, a second method was tried which involved oxidising a solution of TTF^0 and LiTCNQ in an acetonitrile/water mixture (described in section 3.3(1).2). Finally, the El Kacemi/Lamache method was repeated using slightly different conditions calculated to give the best chance of producing a thin-film electrode rather than crystals. This is described in Section 3.3(1).3.

Following the limited success of the above methods a completely new, two step approach was adopted which is described in Part 2 of this chapter. Work on the electrochemistry of TTF and TCNQ salts showed that it is

very easy to make reproducible thin-film modified electrodes of either TTF^0 or TCNQ^0 . In the case of a modified TTF^0 electrode, it could be postulated that TTFTCNQ could then be generated by oxidation in the presence of the TCNQ anion. In an analogous way, if a modified TCNQ electrode was employed, then the organic salt could be generated by reduction in a solution of the donor cation. Furthermore, by choosing the appropriate salts of the donor and acceptor, all the electrochemistry could be carried out in water, and for the second step no additional background electrolyte needed to be added. This approach tended to be more successful than previous attempts, and TTFTCNQ electrodes made by this method were employed for some mechanistic studies of the $\text{TTFTCNQ}/\text{GOD}$ system described in Chapter 4.

3.2 EXPERIMENTAL

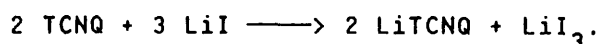
3.2.1 CHEMICALS AND SOLUTIONS

All chemicals were commercially obtained of AnalaR or similar grade, and were used without further purification. To allow equilibration of the different anomers of glucose, solutions of this substrate were made up 24 hours before use and stored at 4 °C. Details of the glucose oxidase (GOD) employed are given in Table 3.2.1. Acetonitrile (Dried Distilled grade) was obtained from Fisons Ltd, and DDW was produced as given in Section 2.2.1. LiTCNQ, NMPTCNQ (in bulk) and TTFC1 were synthesised as below.

a) Preparation of LiTCNQ

LiTCNQ was prepared by the method of Melby et al^[33].

It employs the following reaction:



Three equivalents of lithium iodide in hot acetonitrile were mixed with two equivalents of TCNQ in hot acetonitrile. The mixture was allowed to cool to room temperature and the purple solid was collected and washed on the filter with acetonitrile until the washings were bright green. The solid was then washed with a large volume of ether.

b) Preparation of NMPTCNQ (in Bulk)

This material was prepared by the method of Melby^[33].

A boiling solution of one equivalent of NMPMeSO₃ in ethanol was mixed with a boiling solution of one equivalent of LiTCNQ in ethanol. After the solution had cooled to room temperature, the black solid was collected on a filter and

TABLE 3.2.1

Properties of the glucose oxidase employed in this work.

Company	Sigma (Type VII-S)
Supplied as	Lyophilised powder
Specific Activity	146 Units/ml
K_m	33 mmol dm ⁻³
Source	<u>Aspergillus niger</u>
Molecular Weight	186,000
Stored at	<0 °C

washed with ethanol.

c) Preparation of TTFCl

A degassed solution containing 10 mmol dm^{-3} TTF⁰ and 100 mmol dm^{-3} tetraethylammonium chloride in acetonitrile was electrolysed at +300 mV (vs SCE) at a platinum disc electrode rotated at 4 Hz. Black crystals grew from the electrode surface, occasionally falling to the bottom of the cell. When the solution was almost clear the electrolysis was stopped, the black crystals were collected on a filter and washed with acetonitrile.

3.2.2 MEMBRANES

Details of the membranes employed and their pretreatment are given in Section 2.2.2.

3.2.3 ELECTRODES

Working electrodes were fabricated in-house by Mr M Pritchard. Both disc and ring-disc electrodes were employed, made of gold, platinum or glassy carbon. Gold and platinum electrodes were hand-polished using $0.3 \mu\text{m}$ alumina. Glassy carbon electrodes were hand-polished using jeweller's rouge.

Both saturated calomel (SCE, usually for aqueous and mixed solvent work) and Ag/Ag^+ (for work in acetonitrile) reference electrodes were employed. These were made in-house. All potentials are given relative to the actual reference electrode used for that experiment.

Counter electrodes were fabricated from platinum gauze, and were cleaned as described in Section 2.2.3.

3.2.4 APPARATUS

The rotation system was made in these laboratories and has been described previously^[84].

Non-aqueous work was carried out in a 10 ml beaker, using 5 ml of solution. The experimental set-up is shown in Figure 3.2.1.

All other apparatus is as described in Section 2.2.4.

3.2.5 EXPERIMENTAL PROCEDURES

Other than below, these are given in Section 2.2.5.

All solutions were degassed prior to and during use by bubbling deoxygenated nitrogen through them (see Section 2.2.1).

Because NMP^+ is unstable in solution to light, the electrochemical cell was wrapped in aluminium foil whenever this cation was employed.

Glucose oxidase membrane electrodes were made in the same way as those for choline, described in section 2.2.5, except that a solution of 500 units/ml GOD was employed instead of a choline oxidase solution.

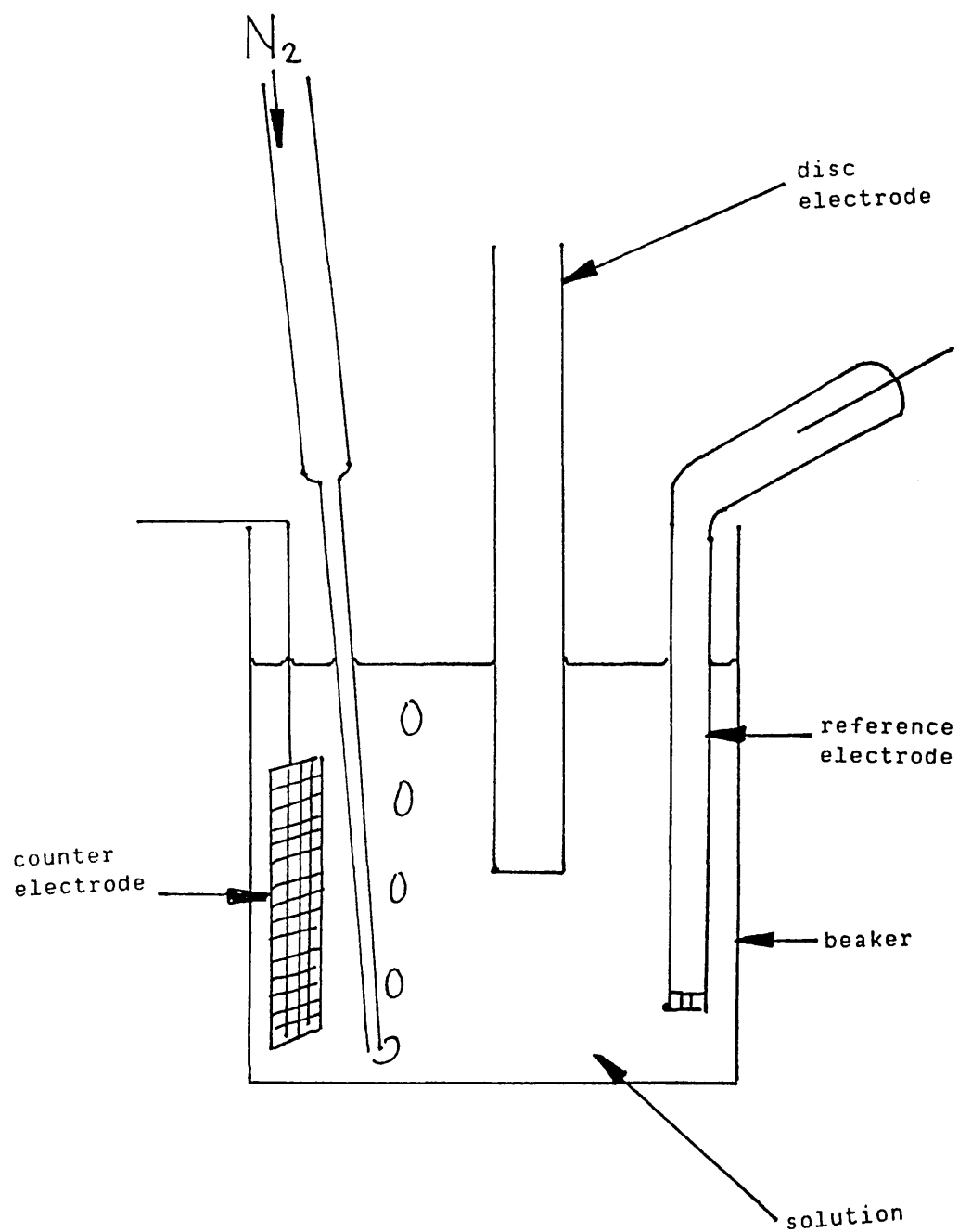


Figure 3.2.1 Set-up for non-aqueous experiments.

3.3 RESULTS

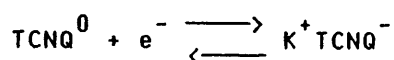
Before we can attempt to produce modified organic salt electrodes we must first devise some means to characterise the electrode once it has been made. Failure to produce an electrode made up of the organic salt may result in electrodes modified by some kind of donor or acceptor species, or uncomplexed mixtures of the two. To differentiate between these possibilities, three primary methods of characterisation were used in this work: cyclic voltammetry, visual inspection of the electrode and response of the electrode to GOD and NADH.

Taking the latter method first, blank experiments show that bare metal electrodes do not respond at potentials such as +50 mV when exposed to NADH or reduced GOD. Previous work has shown that NMPTCNQ electrodes are able to oxidise both GOD and NADH quite well at these potentials^[40,45]. TTFTCNQ electrodes respond well to GOD, although their response to NADH seems to be rather irreproducible^[85]. Care should be taken with these results, however, as electrodes made up of the uncomplexed donor or acceptor molecules may oxidise these species, so we cannot say that the electrode must therefore consist of the pure organic salt.

We will now turn to a discussion of the former two means of characterisation.

a) Electrochemistry of TTF and TCNQ Modified Electrodes

In this study modified TTF or TCNQ electrodes were made by two methods. Both gave similar results. The simplest method is to carefully drop an acetonitrile solution of the donor or acceptor onto the surface of a platinum electrode and allow the solvent to evaporate, leaving the species of interest adsorbed on the electrode. The second method, described more fully in Section 3.3(2), was by oxidation of LiTCNQ or reduction of TTFCl at a bare metal electrode. Here, TCNQ^0 or TTF^0 respectively are precipitated at the electrode surface. Figures 3.3.1 and 3.3.2 show the cyclic voltammograms in 100 mmol dm^{-3} KBr for TCNQ and TTF electrodes modified by the latter method. For both electrodes one cathodic and one anodic peak are apparent, corresponding to the reactions.



With both of these reactions, the modified electrode changes colour, as the oxidation state of the species on the surface changes. These colour changes are given in Table 3.3.1.

It should be noted that Jaegar and Bard^[42] have also studied the electrochemistry of TTF and TCNQ in their solid state, using impregnated carbon paste electrodes. The results contained in their work are in agreement with this study. They have also shown that the peak potentials can change significantly, depending on the nature of the background electrolyte.

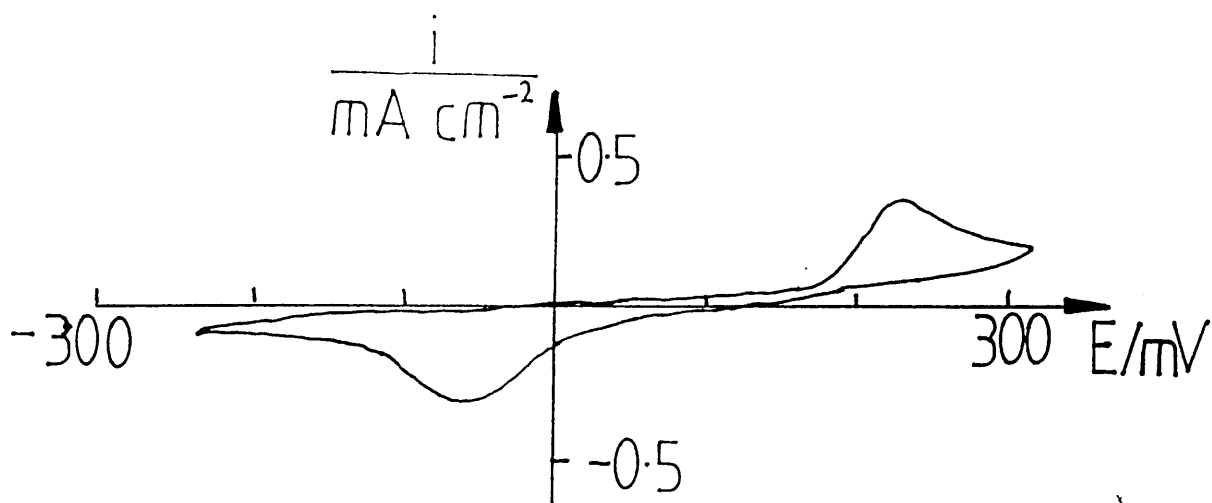


Figure 3.3.1 Cyclic voltammogram of a TCNQ modified electrode (50 mV/s vs SCE)

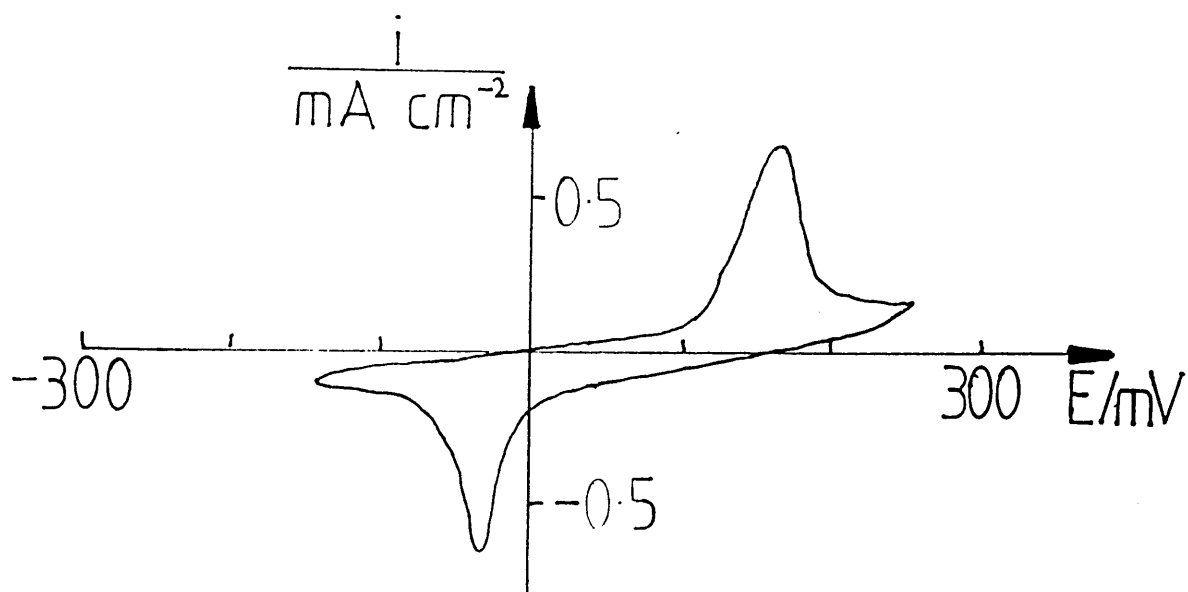


Figure 3.3.2 Cyclic voltammogram of a TTF modified electrode (50 mV/s vs SCE)

TABLE 3.3.1

Colour of TTF and TCNQ modified electrodes.

TTF ⁰	Orange/Yellow
TTFBr	Light Purple
TCNQ ⁰	Green
KTCNQ	Deep Purple

b) Electrochemistry of TTFTCNQ and NMPTCNQ Electrodes

The electrochemistry of the above organic salts has been previously studied by Jaeger and Bard^[38] and has already been discussed in Section 1.2.2 of this thesis. Both salts have a stable range of potential beyond which they are reduced or oxidised. Figures 3.3.3 and 3.3.4 show cyclic voltammograms obtained for carefully produced polymer paste TTFTCNQ and NMPTCNQ electrodes respectively. It can be seen that if swept within the stable range, the TTFTCNQ electrode does not exhibit any peaks. Reduction of this electrode leads to four new peaks. These peaks can be attributed to the electrochemical couples $\text{TTF}^0/\text{TTF}^+$ and $\text{TCNQ}^0/\text{TCNQ}^-$ occurring on the surface of the electrode. This can be shown by comparison of the cyclic voltammograms of TTF and TCNQ modified electrodes with that of the TTFTCNQ electrode. Although the peak potentials have shifted a little, probably due to resistive effects, they appear in approximately the same places.

In the case of NMPTCNQ, the donor is soluble in most aqueous electrolyte solutions. Like TTFTCNQ, the material shows no peaks within its stable range. However when reduced only one stable cathodic and one stable anodic peak appear, corresponding to the $\text{TCNQ}^0/\text{TCNQ}^-$ couple. A reduced, unstable form of NMP^+ is also produced in the reduction process (" NMP^0 "), and this may give rise to a broad anodic peak at -135 mV on the reverse sweep which disappears after oxidation as the product dissolves into solution. It should be noted however, that reduction of the organic salt

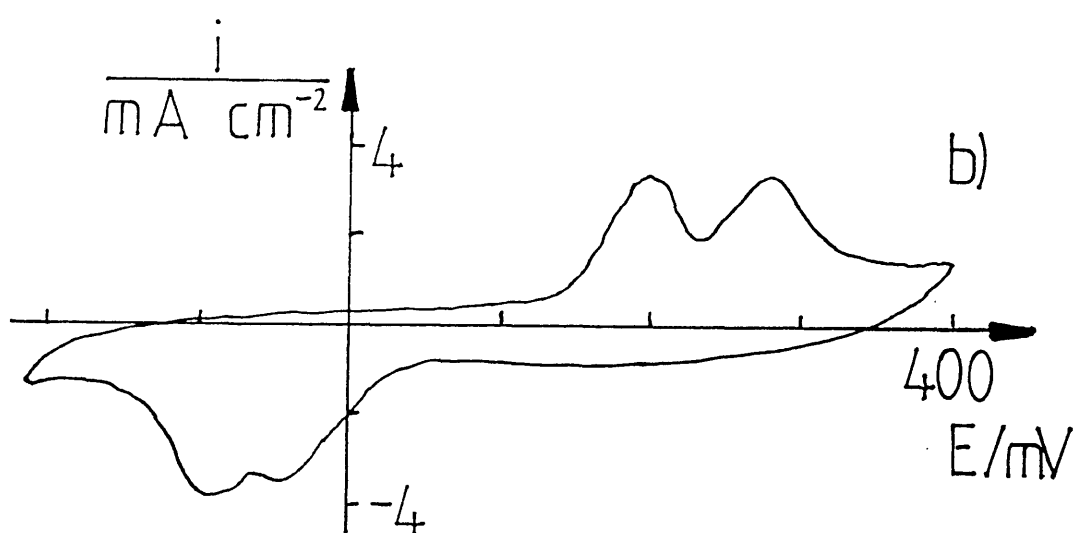
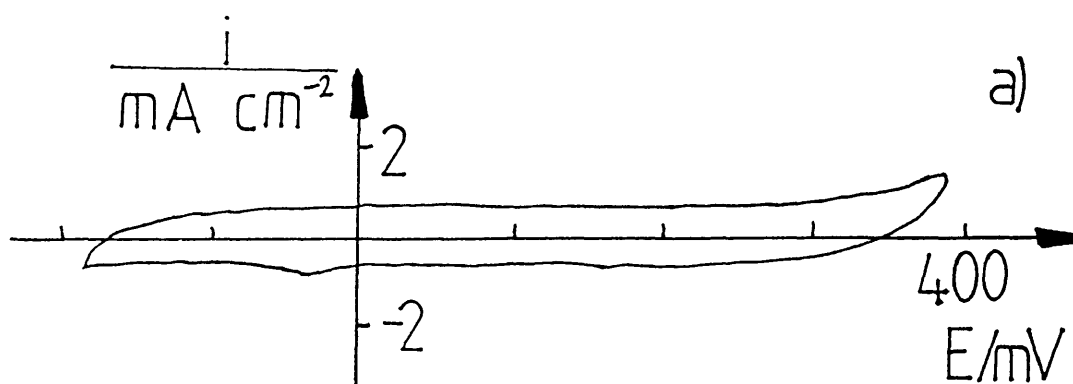


Figure 3.3.3 Cyclic voltammogram of a TTFTCNQ polymer paste electrode (50 mV/s vs SCE): a) before reduction; b) after reduction.

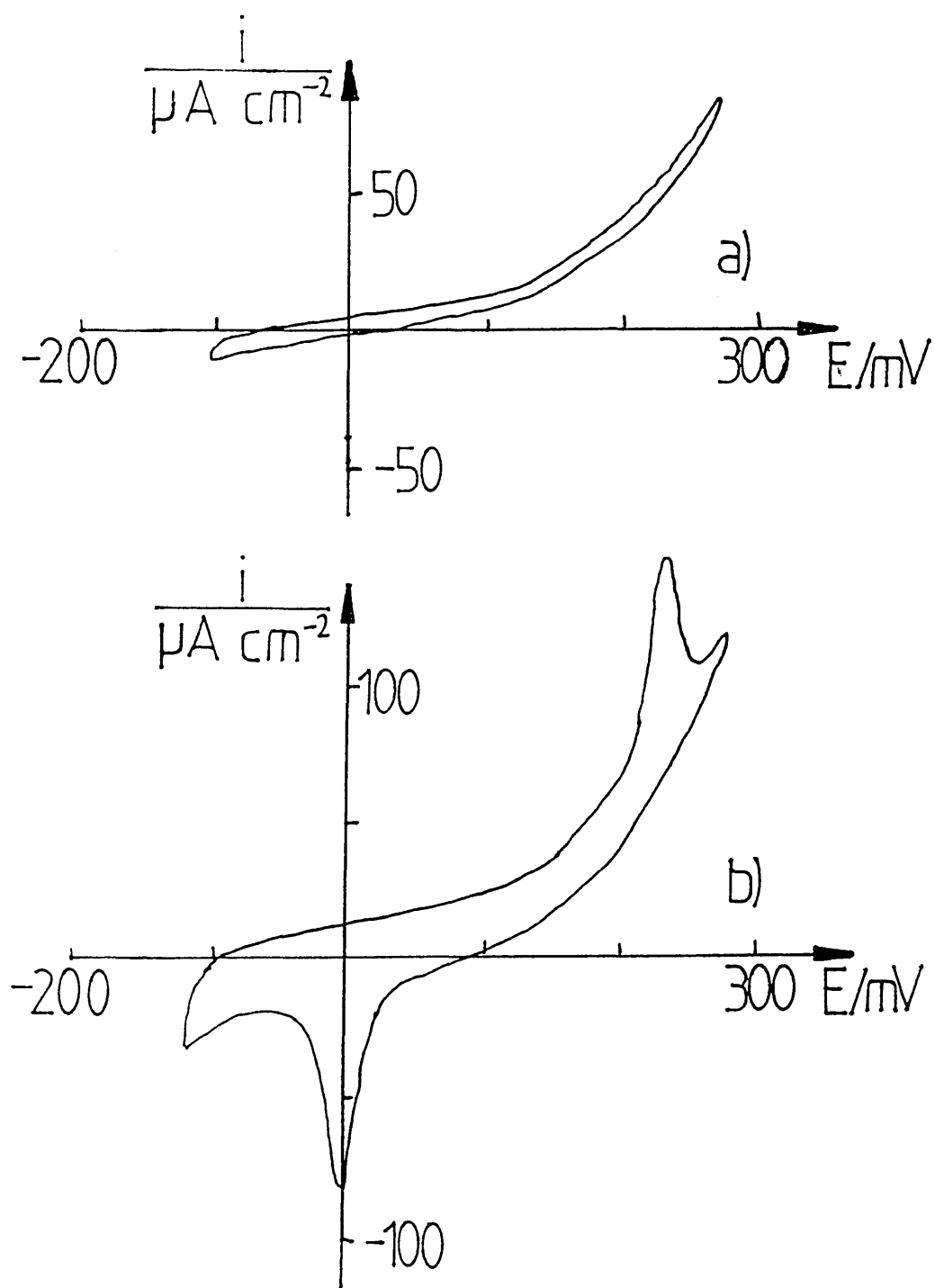


Figure 3.3.4 Cyclic voltammogram of a NMPTCNO polymer paste electrode (50 mV/s vs SCE): a) before reduction; b) after reduction.

occurs at higher reducing potentials than that of TCNQ^0 .

We have now shown that reduction of both organic salt electrodes results in their decomposition into the constituent donor and acceptor molecules, leading to new peaks in their cyclic voltammograms. This behavior can be utilised to identify a newly modified electrode as the desired organic salt. First the newly modified electrode is swept within its stable range. If the cyclic voltammogram displays no peaks then we can eliminate the presence of free donor or acceptor species that are electrochemically active within that range. The electrode is then reduced. In the case of TFTCNQ , new peaks should appear in the cyclic voltammogram attributable to non-complexed TTF and TCNQ . In the case of NMPTCNQ , peaks corresponding to the TCNQ couple should appear. An unstable peak corresponding to the oxidation of " NMP^0 " may also be apparent; however experience in this laboratory shows this can be too broad to detect.

Unfortunately, it must be noted that reduction of the organic salt electrode will irreversibly alter at least its surface, and in the case of a thin-film electrode may destroy it altogether. Thus, this procedure was employed for initially determining that the modified electrode produced using each synthetic route was the desired salt. Whenever a modified electrode was needed to be intact for further studies (ie, in determining its response to GOD or NADH), this method of characterisation was employed to check the electrode consisted of the organic

salt only after any other experiments on it were completed. To screen electrodes which were to be subsequently used for experiments, a non-destructive method of identification was developed which depended on visual inspection. The organic salts are black in colour, whereas the individual donor and acceptors are coloured as given above, in Table 3.3.1. Thus the colour of the electrode is characteristic of the nature of the material modifying it. Although this method appears crude, it is actually quite effective as each of the individual donor and acceptor species absorb light very strongly and can easily be seen even in very small amounts.

c) Calculating the Surface Coverage of a Modified Electrode

An important parameter used to characterise modified electrodes is the amount of material adsorbed onto the electrode. The method employed in this study to determine this parameter utilises the cyclic voltammograms which have already been described above. The area under a peak in cyclic voltammetry is equal to the total charge passed during the oxidation or reduction of the species to which it corresponds to. If the species concerned is a thin-layer on the surface of the electrode which is entirely oxidised or reduced during the experiment, and all other species present in the system are not significantly electroactive at that potential, then we can obtain an estimate of the total number of moles of adsorbed material by dividing the charge passed by the Faraday and n , the number of electrons passed per molecule in the electrochemical reaction^[86].

In the case of NMPTCNO and TTFTCNO, reduction of the

electrode leads to the formation of free donor and acceptor species. Peaks corresponding to the $\text{NMP}^+/\text{NMP}^0$ couple rapidly disappear from the cyclic voltammogram. Experience shows that peaks corresponding to the $\text{TTF}^+/\text{TTF}^0$ couple are metastable and can also disappear, although much more slowly. The $\text{TCNQ}^0/\text{TCNQ}^-$ couple gives rise to stable peaks and from the stoichiometry of the salt, the number of moles of TCNQ remaining on the electrode after the organic salt is reduced must be equal to the original number of moles of the salt. Thus, cyclic voltammetry of the organic salt modified electrode can be used to determine the original surface coverage of the electrode, by measuring the amount of remaining TCNQ. If Q is the amount of charge (in Coulombs) attributable to the conversion of TCNQ^0 to TCNQ^- , or vice versa, and A is the area of the electrode (in cm^2), then the surface coverage η (in mol cm^{-2}) is given by

$$\eta = Q/(FA) \quad (3.1)$$

as in this case $n=1$.

To obtain the average number of monolayers N_m , we must divide η by number of moles which make up 1 monolayer. This has been previously estimated at $1.3 \times 10^{-10} \text{ mol cm}^{-2}$ [45]. Hence,

$$N_m = \eta / (1.3 \times 10^{-10}) = Q / (1.3 \times 10^{-10} FA) \quad (3.2)$$

A prerequisite of this method is that no TCNQ is lost into the solution on reducing the organic salt. This is ensured by using a background electrolyte containing K^+ or Na^+ . The solubility products of KTCNQ and NaTCNQ are small

enough to ensure that the amount of TCNQ^- lost to the bulk is negligible^[47]. The solubility of TCNQ^0 in water has also been shown to be so small that loss of this molecule into the bulk is not important^[87].

Knowing Q , we can also easily calculate the electrosynthetic yield of the experiment. In all the syntheses employed in this work, one electron should be required to synthesise one molecule of the organic salt. Thus the total charge passed during the synthesis Q_T , divided by the Faraday should give us the theoretical yield of organic salt (assuming the contribution to the current by other reactions is negligible). But Q/F is the total amount of organic salt on the surface of the electrode. Hence,

$$\text{Electrosynthetic yield} = (Q/Q_T) \times 100\%. \quad (3.3)$$

3.3(1) "ONE-POT" METHODS

3.3(1).1 PREPARATION OF NMPTCNQ ELECTRODES FROM NMP^+ AND TCNQ^0

In this synthesis a solution containing NMPMeSO_3 and TCNQ^0 was reduced in acetonitrile. NMPMeSO_3 is present at high concentration and serves as the background electrolyte, as well as a substrate for the synthesis. Cyclic voltammograms for NMPMeSO_3 and TCNQ^0 in acetonitrile are shown in Figures 3.3.5 and 3.3.6, and their peak potentials are given in Table 3.3.2. In the case of NMP^+ , its reduction appears to be very irreversible (the peaks are drawn out with a large separation). TCNQ^0 shows four peaks; two cathodic, corresponding to its reduction to TCNQ^- at low reducing potentials and further to TCNQ^{2-} at higher reducing potentials; and two anodic, which can be interpreted as the reverse of these two reactions.

On reducing a mixture of NMP^+ and TCNQ^0 there arises the interesting question of what reaction actually occurs at the electrode. The peak potential for the reduction of TCNQ^0 to TCNQ^- is -190 mV. As given in the footnote to Table 3.3.2, a polarogram of NMP^+ under the same conditions shows that the onset of a measureable reducing current occurs at about -300 mV. In the electrosynthesis, a reducing current is employed which is substantially below the diffusion limited currents for these two reactions. One would therefore expect that under these conditions TCNQ^0 will be reduced at the electrode and should then combine

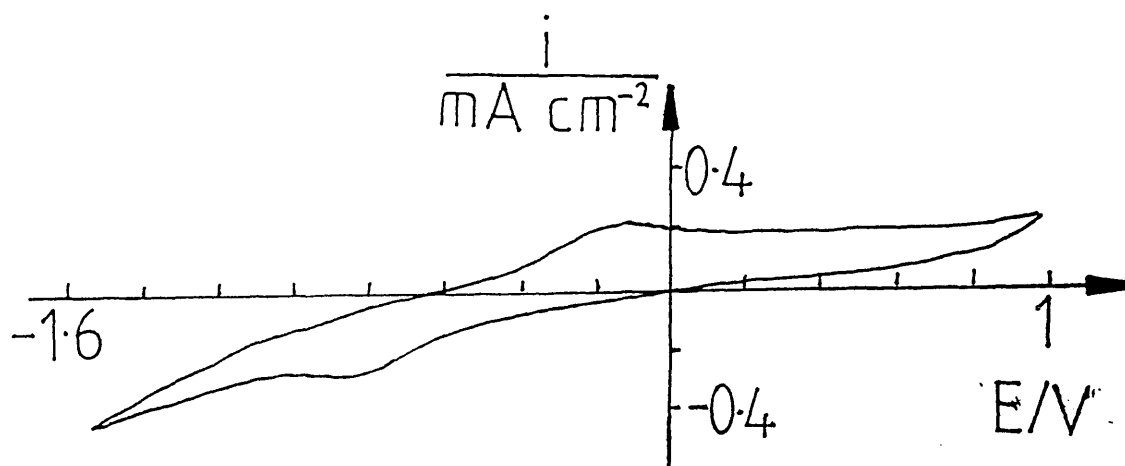


Figure 3.3.5 (above) Cyclic voltammogram of 0.8 mmol dm^{-3} NMPMeSO₃ in acetonitrile (100 mV/s vs Ag⁺/Ag).

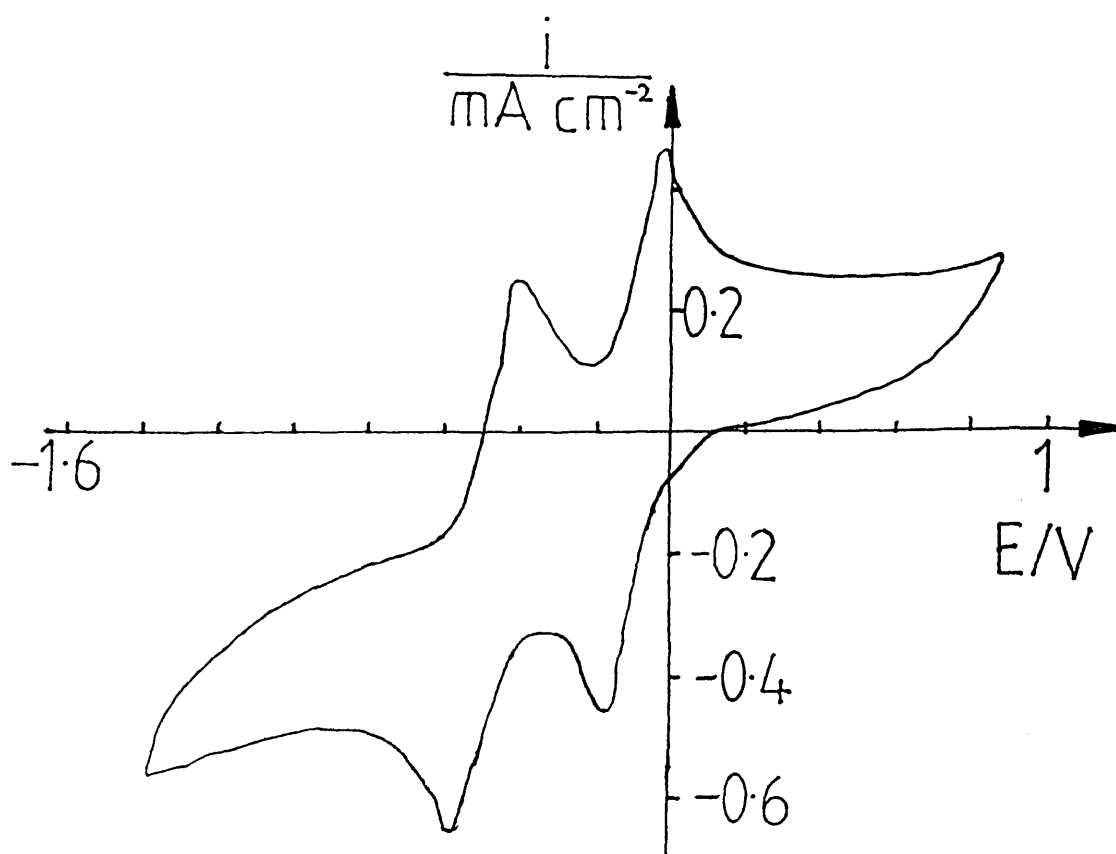


Figure 3.3.6 Cyclic voltammogram of 1.0 mmol dm^{-3} TCNQ⁰ in acetonitrile (100 mV/s vs Ag⁺/Ag).

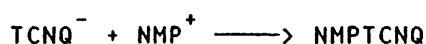
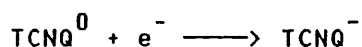
TABLE 3.3.2

Peak potentials (vs Ag/Ag^+) from the cyclic voltammetry of TCNQ^0 and NMPMeSO_3 in acetonitrile.

Couple	Anodic Peak (mV)	Cathodic Peak (mV)
$\text{TCNQ}^0/\text{TCNQ}^-$	-190	-20
$\text{TCNQ}^-/\text{TCNQ}^{2-}$	-580	-400
$\text{NMP}^+/\text{NMP}^0$	-840 [*]	-120

^{*} onset of faradaic current from rotating disc polarogram at -300 mV

with NMP^+ to produce the salt, ie,



Details of the electrosynthetic procedure and the results will now follow.

a) Initial Synthesis

A solution was prepared containing 1 mmol dm^{-3} TCNQ^0 with saturating amounts of NMPMeSO_3 and NMPTCNQ in acetonitrile. A platinum disc electrode (radius 3.5 mm) was placed in the solution and rotated at 4Hz. A reducing current of $10 \mu\text{A}$ was forced through the electrode for 22 minutes. The electrode was washed carefully with DDW.

b) Appearance and Electrochemical Behavior of the Electrode

The electrode appeared to consist of the original bare metal, covered in small black speckles. A cyclic voltammogram of the electrode in pH9.0 Ches buffer containing 0.1 mmol dm^{-3} NaCl is shown in Figure 3.3.7a. Cycling between 50 and 300 mV reveals there is no TCNQ^- present on the electrode; however after reduction peaks corresponding to the $\text{TCNQ}/\text{TCNQ}^-$ couple appeared (see Figure 3.3.7b). It should be noted that the reduction peak for TCNQ^0 occurs at -15 mV, but the initial reduction of the electrode occurred at lower potentials. Thus, although no peaks for pure NMP can be seen, the electrode probably consists of NMPTCNQ and not some TCNQ species.

c) Response of the Electrode to GOD and NADH

Blank experiments show that bare platinum electrodes do

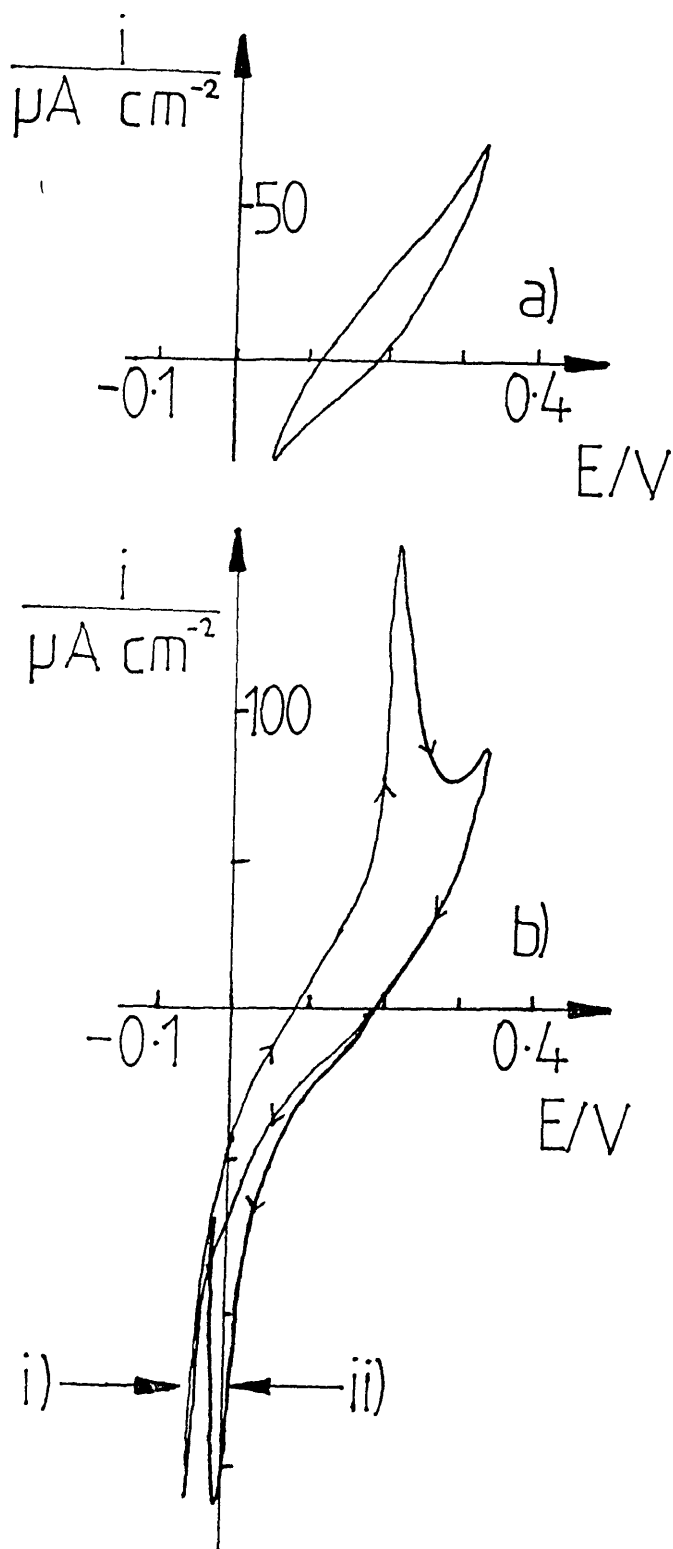


Figure 3.3.7 Cyclic voltammogram of the electrosynthesised NMPTCNO electrode (20 mV/s vs SCE): a) before reduction; b) after reduction, i) first sweep, ii) second sweep.

not respond to either NADH or reduced GOD at +50 mV. The electrosynthesised NMPTCNQ electrode responded to these species as shown in Figures 3.3.8 and 3.3.9. In both cases the current shows some drift. Furthermore it is very poor for GOD, compared to polymer paste electrodes. The response to NADH is approximately $3 \mu\text{A cm}^{-2}$ per mmol dm^{-3} NADH, which is approximately 25-50% of the typical value for polymer paste electrodes^[88]. In the case of GOD, the electrode is saturated at relatively low glucose concentrations (on increasing the concentration of glucose from 6 mmol dm^{-3} to 9 mmol dm^{-3} there was no significant increase in current), and the maximum current is approximately 4% of the comparable value for typical polymer paste electrodes.

These results can be explained by assuming the modified electrode consisted of islets of NMPTCNQ, separated by the bare platinum metal. The rate of oxidation of NADH is dependent on the geometrical area of the electrode, as would be expected, but is relatively insensitive to the electrode's porosity or roughness. Evidence for this statement comes from the observation that NMPTCNQ pellet electrodes (which have a smooth surface) have approximately the same response to NADH as polymer paste electrodes (which have a high roughness factor)^[45]. On the other hand, the current obtained from reduced GOD has been shown to depend significantly on the porosity of the electrode (in other words, its actual surface area)^[41]. If the electrode consisted of separate microcrystallites of NMPTCNQ, the actual surface area of NMPTCNQ would be very small compared

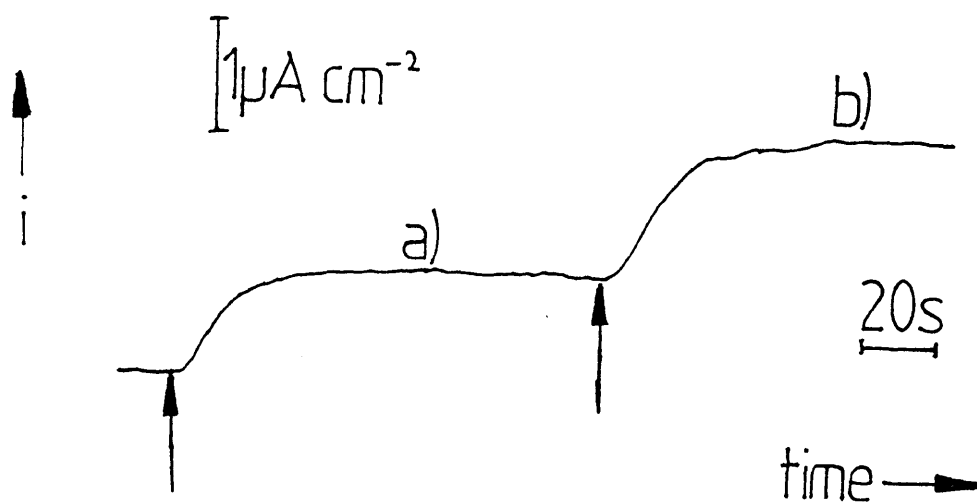


Figure 3.3.8 Response of the electrosynthesised NMPTCNQ electrode to total NADH concentrations of : a) $0.41 \text{ mmol dm}^{-3}$ and b) $0.82 \text{ mmol dm}^{-3}$.

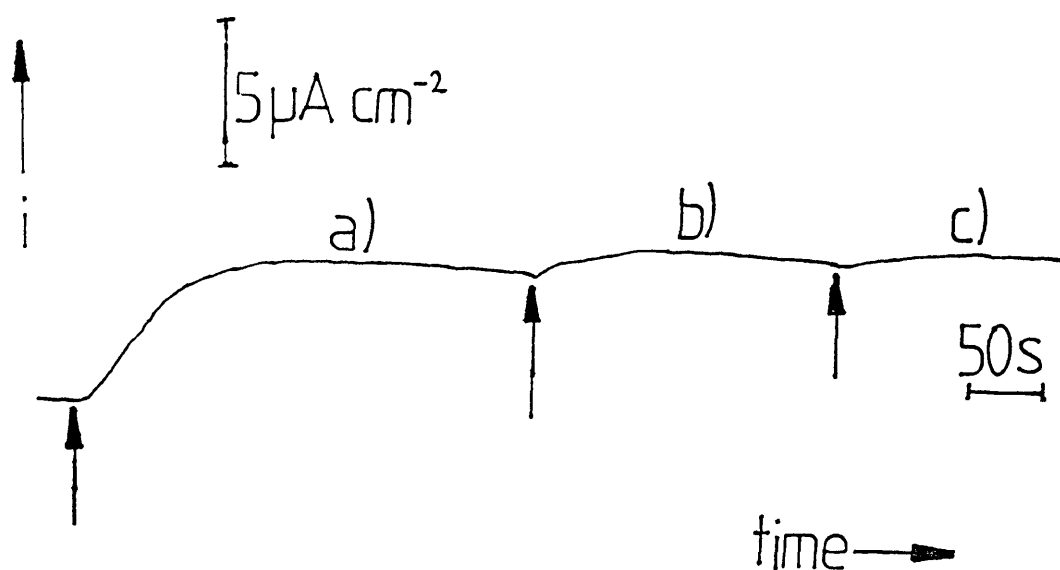


Figure 3.3.9 Response of the electrosynthesised NMPTCNQ/GOD membrane electrode to total glucose concentrations of: a) 3 mmol dm^{-3} , b) 6 mmol dm^{-3} and c) 9 mmol dm^{-3} .

to a polymer paste electrode, although its geometrical area would be relatively similar. Thus one would expect its response to GOD to be poor, but to be similar for NADH. This agrees with the experimental observations presented above.

d) Surface Coverage of the Electrode

Using the formula given in Section 3.1, the surface coverage of the electrode is about 1.5 nmol cm^{-2} NMPTCNQ. This corresponds to about 11-12 monolayers and an electrosynthetic yield of approximately 0.4%. The very low yield probably indicates that either NMPTCNQ is precipitated in solution or that nucleation of NMPTCNQ occurs very slowly on the electrode.

Repeating this experiment using different plating currents produced electrodes with similarly poor surface coverages and electrosynthetic yields. Substituting glassy carbon for platinum as the electrode material did not significantly alter the quality of the modified electrodes produced by this technique. The conclusion must be that this method of synthesis is unsuitable for producing NMPTCNQ thin layer electrodes reproducibly or of sufficient quality for further experimental use.

3.3(1).2 PREPARATION OF TTFTCNQ ELECTRODES FROM TTF^0 AND TCNQ^-

It is difficult to synthesise salts of TTF that are soluble in the same solvent as TTF itself or TCNQ. The same

is true for salts of TCNQ. This is a prerequisite for this type of synthesis, as otherwise the resulting modified electrode may consist of the insoluble donor or acceptor, and not the desired organic salt. To overcome this problem, in the following synthesis a mixed solvent was employed, namely a 1:1 mixture (by volume) of acetonitrile and water. Simple spot tests showed that TTF^0 , TCNQ^0 and LiTCNQ were all soluble to at least 1 mmol dm^{-3} in this medium and their salt, TTFTCNQ proved to be insoluble. Lithium acetate was chosen as the background electrolyte as the acetate salt of TTF^+ is known to be soluble in water^[42]. To see if TTF^+ was soluble in this mixed solvent system electrochemical methods, described below, were employed.

The cyclic voltammetry of LiTCNQ and TTF^0 in a 1:1 water/acetonitrile mixture containing 50 mmol dm^{-3} lithium acetate is shown in Figures 3.3.10 and 3.3.11, as well as polarograms of the two materials in the potential range of interest. Peak potentials appear in Table 3.3.3. In the cyclic voltammogram TTF^0 exhibits two anodic peaks, which can be attributed to its reduction first to TTF^+ and then to TTF^{2+} . There are two corresponding cathodic peaks for the reverse of these two reactions. Although the peak for the reduction of TTF^+ occurs at +130 mV in the cyclic voltammogram, it does not appear in the rotating disc polarogram. This implies that either it is soluble in the solvent system and is swept away by convection at the rotating disc, or that in the time scale of the experiment it undergoes a chemical reaction and so becomes

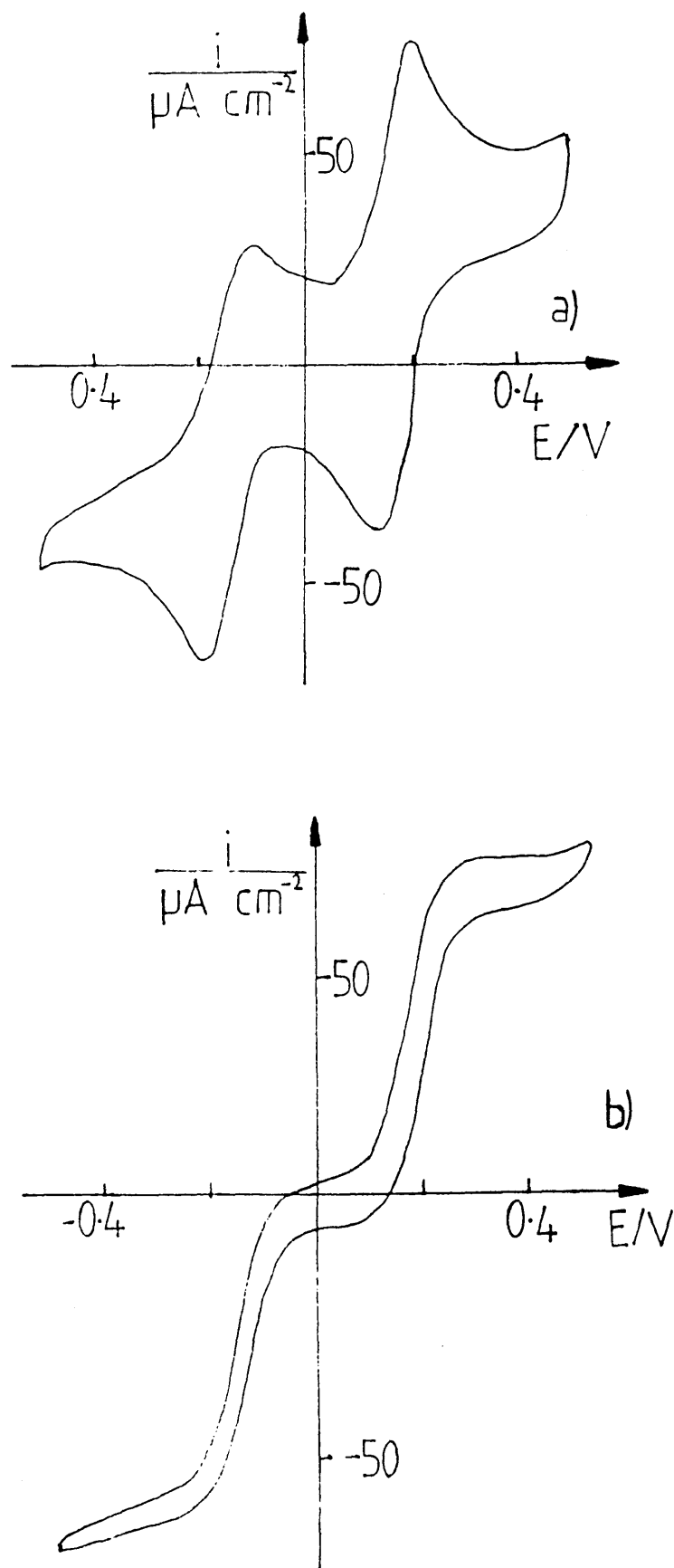


Figure 3.3.10 Electrochemical behavior (vs SCE) of 0.5 mmol dm⁻³ LiTCNQ in a 1:1 mixture of water and acetonitrile: a) cyclic voltammogram at 50 mV/s; b) rotating disc polarogram at 20 mV/s and 4 Hz.

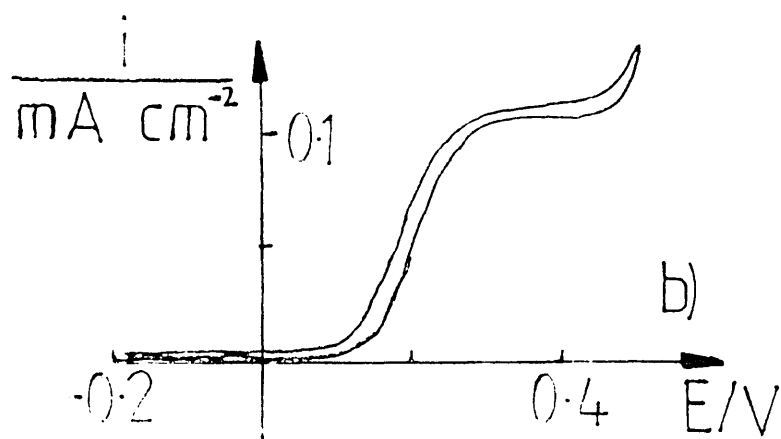
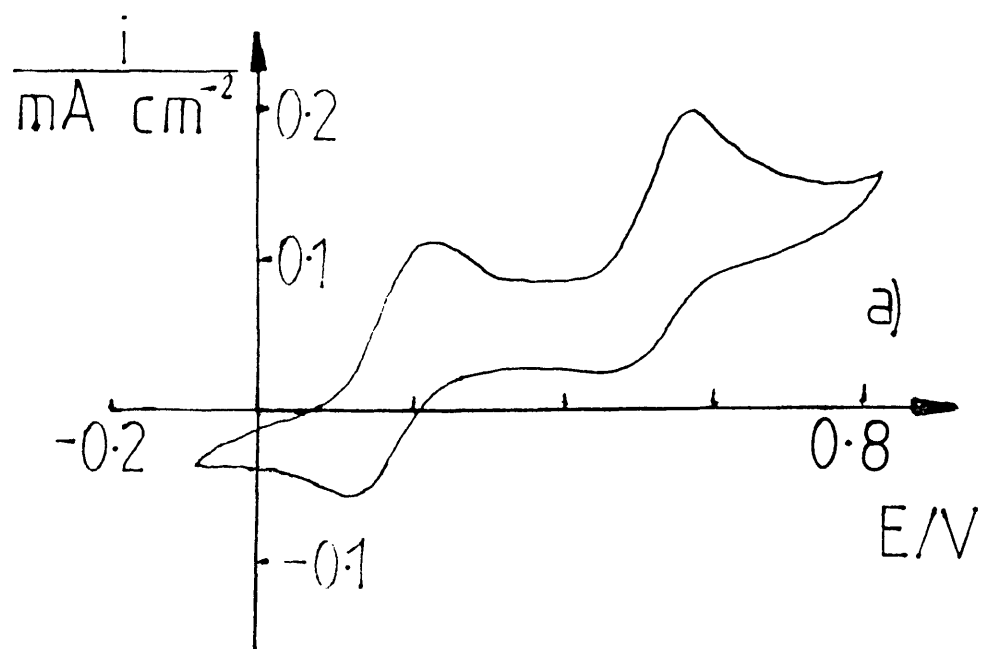


Figure 3.3.11 Electrochemical behavior (vs SCE) of $1.0 \text{ mmol dm}^{-3} \text{ TTF}^0$ in a 1:1 mixture of water and acetonitrile: a) cyclic voltammogram at 50 mV/s ; b) rotating disc polarogram at 20 mV/s and 4 Hz .

TABLE 3.3.3

Peak potentials (vs SCE) from the cyclic voltammetry of TTF^0 and LiTCNQ in acetonitrile.

Couple	Anodic Peak (mV)	Cathodic Peak (mV)
$\text{TTF}^0 / \text{TTF}^+$	+230	+130
$\text{TTF}^+ / \text{TTF}^{2+}$	+580	~ +460
$\text{TCNQ}^0 / \text{TCNQ}^-$	+200	+120
$\text{TCNQ}^- / \text{TCNQ}^{2-}$	-90	-200

electrochemically inactive although perhaps still remaining on the electrode surface. Inspection of the electrode after the experiment showed no material adsorbed onto its surface. The conclusion must therefore be that TTF^+ is soluble in the solvent system chosen. Turning to LiTCNQ , it can be reduced to TCNQ^{2-} or oxidised to TCNQ^0 , both reactions showing as peaks in the cyclic voltammogram, together with peaks corresponding to their conversion back to TCNQ^- . As expected, the polarogram shows both species to be soluble. The polarograms for LiTCNQ and TTF^0 show that the onset of a measureable oxidation current occurs at a lower potential for TCNQ^- than TTF^0 . Given that in the electrosynthesis a current is employed which is much smaller than the diffusion limited currents for both these species, we can conclude that TCNQ^- should be oxidised at the electrode in preference to TTF^0 . The product, TCNQ^0 should then form the organic salt by combining with TTF^0 in the solution, viz,

$$\text{TCNQ}^- \longrightarrow \text{TCNQ}^0 + \text{e}^-$$

$$\text{TCNQ}^0 + \text{TTF}^0 \longrightarrow \text{TTFTCNQ}.$$

a) Synthesis

A solution containing 1 mmol dm^{-3} TTF^0 , 1 mmol dm^{-3} LiTCNQ , and 50 mmol dm^{-3} lithium acetate in a 1:1 mixture of acetonitrile and DDW was degassed using deoxygenated nitrogen. A gold disc electrode (radius 3.5 mm) was placed in the solution and rotated at 4 Hz. An oxidising current of $3 \mu\text{A}$ was passed through this electrode for 100 seconds. The electrode was carefully removed from the solution and

washed with DDW.

b) Appearance and Electrochemical Behavior of the Electrode

The electrode appeared to be covered with patches of a brownish/black material, separated by the bare electrode surface. The colour of the patches indicated that they may have consisted of the black organic salt with a brownish or yellowish impurity (such as TTF^0) or that the layer of the organic salt was too thin to completely obscure the gold electrode surface underneath.

A cyclic voltammogram of the electrode (see Figure 3.3.12a) within the stable limits of TTFTCNQ reveals two small peaks, at approximately +120 mV and -80 mV. We can assign these tentatively to the $\text{TTF}^0/\text{TTF}^+$ couple. Reduction of the electrode leads to these peaks becoming much larger, and the appearance of two new peaks at +300 mV and +20 mV (Figure 3.3.12b). This behavior is very characteristic of TTFTCNQ, and the two new peaks can be attributed to the $\text{TCNQ}^-/\text{TCNQ}^0$ couple. Thus this result confirms that the electrode consists mainly of TTFTCNQ, together with a very small amount of TTF.

c) Response of the Electrode to GOD and NADH

The electrode appeared to have very little activity towards NADH. However the electrode was found to respond to GOD as shown in Figure 3.3.13. Saturation occurs at about 3 mmol dm^{-3} GOD, with a current of about $10 \mu\text{A cm}^{-2}$. A TTFTCNQ polymer paste electrode under similar conditions would be expected to saturate at about 25 mmol dm^{-3} glucose

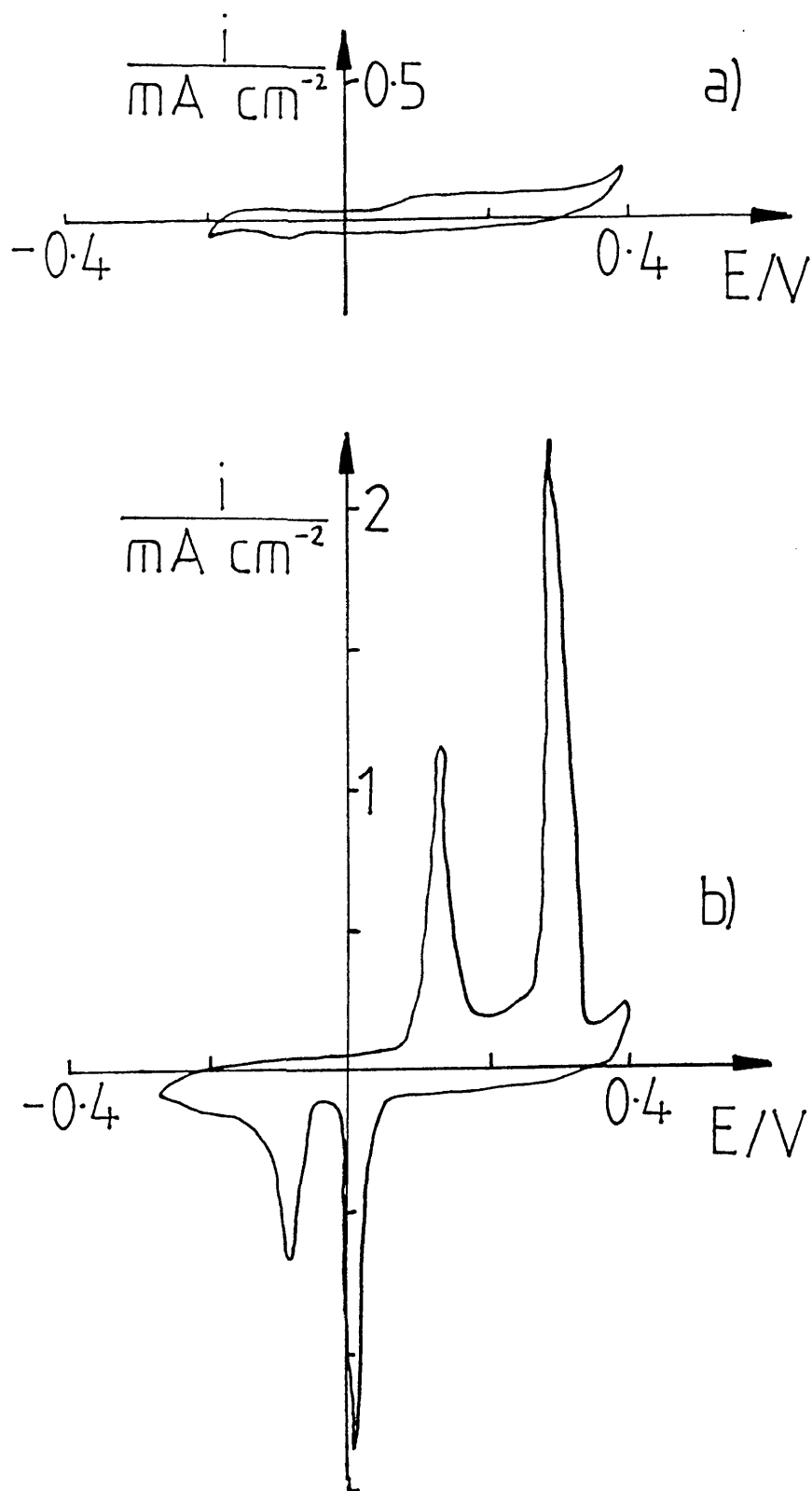


Figure 3.3.12 Cyclic voltammogram of the electrosynthesised TTFTCNQ electrode (100 mV/s vs SCE): a) before reduction; b) after reduction.

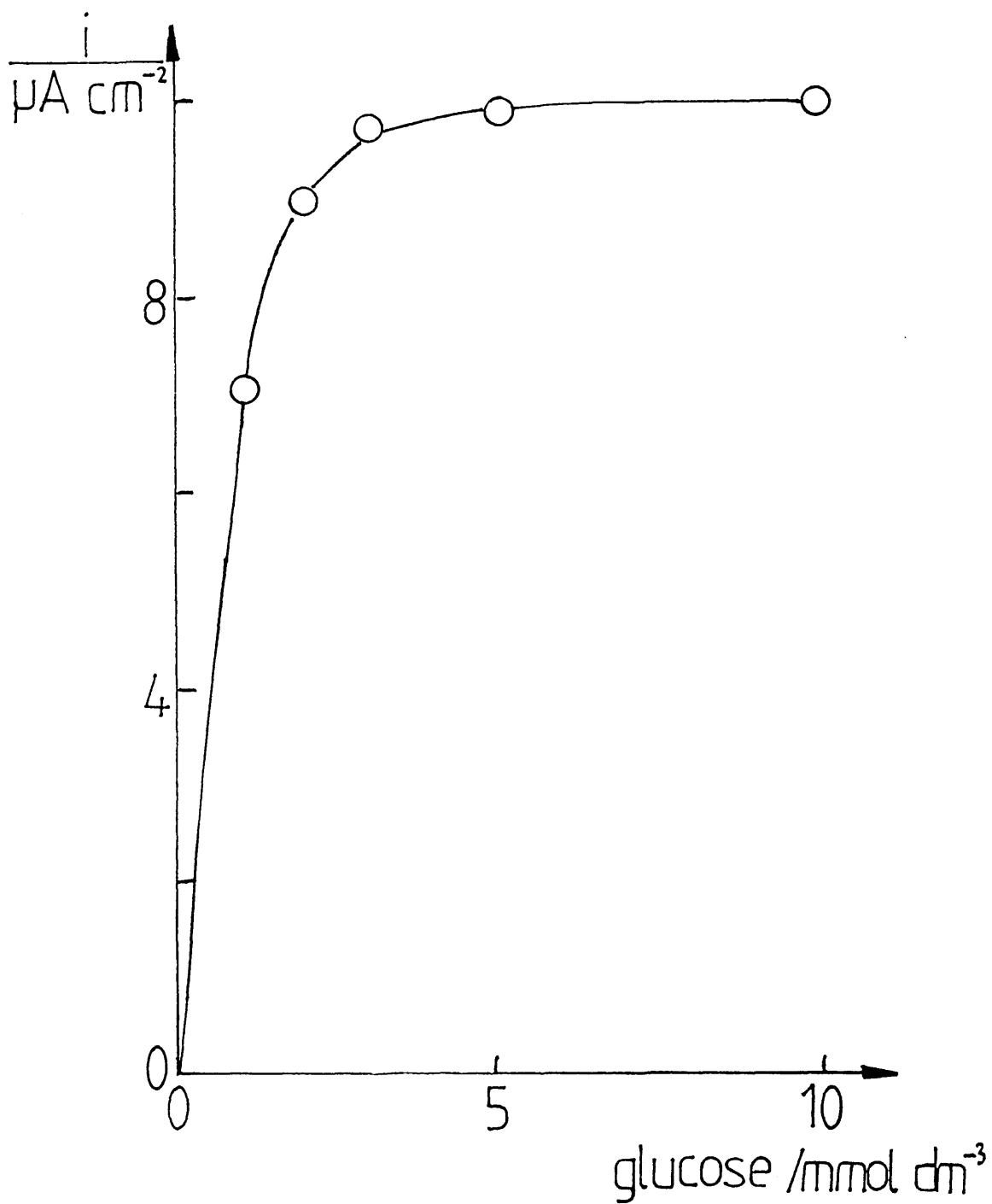


Figure 3.3.13 Response of the electrosynthesised TTFTCNQ/GOD membrane electrode to glucose.

with a current of about $170 \mu\text{A cm}^{-3}$ [40]. Using a similar argument to that presented in Section 3.3(1).1, the comparatively low saturation current is probably due to the low porosity or surface roughness of the electrosynthesised electrode.

d) Surface Coverage of the Electrode

Using the cyclic voltammogram shown in Figure 3.3.12b and the formula given in Section 3.1.1, the surface coverage of the electrode is about 2.5 nmol cm^{-2} , which corresponds to about 19 monolayers. This gives an electrochemical yield of 33%. Although this is a much better yield than the that for the NMPTCNQ synthesis described in Section 3.3(1).1, the surface coverage is not greatly increased. A possible reason for the difference in the yields could therefore be due the large difference in the charge passed during plating.

To investigate this question, the plating time was varied and the resulting surface coverages measured. A plating current of $2.8 \mu\text{A}$ was chosen as this corresponded to the formation of approximately one monolayer per second. Table 3.3.4 shows the results. There is some variability in the surface coverages obtained, but it can be seen that increasing the plating time leads to slightly increased surface coverages but lower yields. This probably indicates that once the electrode is covered with the organic salt the rate of crystal growth slows down, leading to precipitation of the salt in solution instead of at the electrode surface.

TABLE 3.3.4

Surface coverages and electrochemical yields for TTFTCNQ obtained with different plating times.

Current (μ A)	Time (s)	Average Coverage (monolayers)	Range of Coverage (monolayers)	Average Electro- chemical Yield (%)
2.8	60	28	25-32	47
2.8	120	46	29-66	39
2.8	300	55	36-96	17

TABLE 3.3.5

Surface coverages and electrochemical yields obtained for TTFTCNQ at different plating times and currents with a constant total plating charge.

Current (μ A)	Time (s)	Surface Coverage (monolayers)	Electrochemical Yield (%)
0.28	3000	21	7.1
2.8	300	45	15
28	30	28	9.5

Another explanation would be that the mechanical integrity of the layer of organic salt breaks down after a certain surface coverage is reached and so although organic salt still precipitates at the electrode surface it is soon lost into solution.

In a second experiment the total charge passed during plating was kept constant and the plating current (and hence plating time) was varied (see Table 3.3.5). In this case, there does not seem to be great changes in surface coverage, and the differences in surface coverage are probably explained as normal experimental variation. This supports the conclusion reached above that it becomes difficult to increase the surface coverage after it attains a certain level.

The activity of the electrode towards GOD, its visual appearance and especially the cyclic voltammograms all support the conclusion that TTFTCNQ is produced by this synthetic route. However, its utility as a technique for fabricating thin-layer TTFTCNQ electrodes can not be regarded as satisfactory because the surface coverages obtained were low and the bare metal electrode substrate was not completely covered with the salt. For this reason, work was continued on trying to find other synthetic routes towards organic salt electrodes.

3.3(1).3 PREPARATION OF TTFTCNQ ELECTRODES FROM TTF^+ AND TCNQ^0

Because of the very limited success of the previous

methods to produce satisfactory organic salt electrodes, it was decided to attempt to synthesise TTF⁺TCNQ⁰ by the method of El Kacemi and Lamache, outlined in Section 3.1.1. The method consists of initially preparing a solution of TTF⁺ in acetonitrile, adding TCNQ⁰, and then reducing the mixture to produce TTF⁺TCNQ⁰. The object of the original synthesis was to make crystals of the organic salt, not a thin-layer, and so in the second step they produced the salt at a relatively slow rate, presumably at a stationary electrode. In the synthesis described in this work, a rotating disc electrode was employed. It was hoped that in this way, the metal substrate would be uniformly accessible to the plating materials^[89] and nucleation would take place over the whole of its surface, thus resulting in a layer of the organic salt instead of a number of crystals.

a) Synthesis

TTF⁺ was synthesised by oxidising a solution of 20 mg (100 μ moles) of TTF⁰ in 20 ml acetonitrile at +500 mV until the current was 20% of its initial value. 50 mmol dm⁻³ ^tBu₄NC10₄ was employed as background electrolyte. To the resulting solution was added 20 mg TCNQ⁰ (100 μ moles), and this mixture was reduced using a platinum electrode held at +50 mV and rotated at 4 Hz for 4 hours. The resulting modified electrode was carefully washed using DDW.

b) Appearance and Electrochemical Behavior of the Electrode

The modified electrode consisted of many black crystals

which appeared to have grown perpendicular to the electrode surface. These crystals were approximately 0.25 mm long, with a cross-section about 1/5 of the size of their length. Bare platinum metal was visible between the crystallites, which were estimated by eye to cover about 60-70% of the electrode surface.

Cyclic voltammograms of the electrode in 0.1 mol dm^{-3} KBr are shown in Figure 3.3.14. Initially, between -100 mV and +400 mV only very small peaks (possibly for $\text{TCNQ}^0/\text{TCNQ}^-$) can be observed at 0 mV and +180 mV. When the electrode is reduced however, these peaks become much bigger and the anodic peak shifts potential slightly to +215 mV, probably due to resistive effects (see Figure 3.3.14b). In addition two new peaks attributable to the TTF/TTF^+ couple appear at -270 mV and +130 mV. This is characteristic of TTFTCNQ and shows the electrode is made from this material.

c) Response of the Electrode to NADH

The response of the electrode to NADH was approximately $7.5 \mu\text{A cm}^{-2}$ for 1 mmol dm^{-3} NADH. This is comparable to NMPTCNQ polymer paste electrodes. The response to GOD was not tested as the act of placing a membrane on the electrode would have damaged the crystals on its surface.

d) Surface Coverage of the Electrode

From the cyclic voltammogram in Figure 3.3.14b we can calculate that about 63 nmol cm^{-2} of TTFTCNQ had been reduced in the experiment. This corresponds to about 530 monolayers. As inspection of the electrode showed that

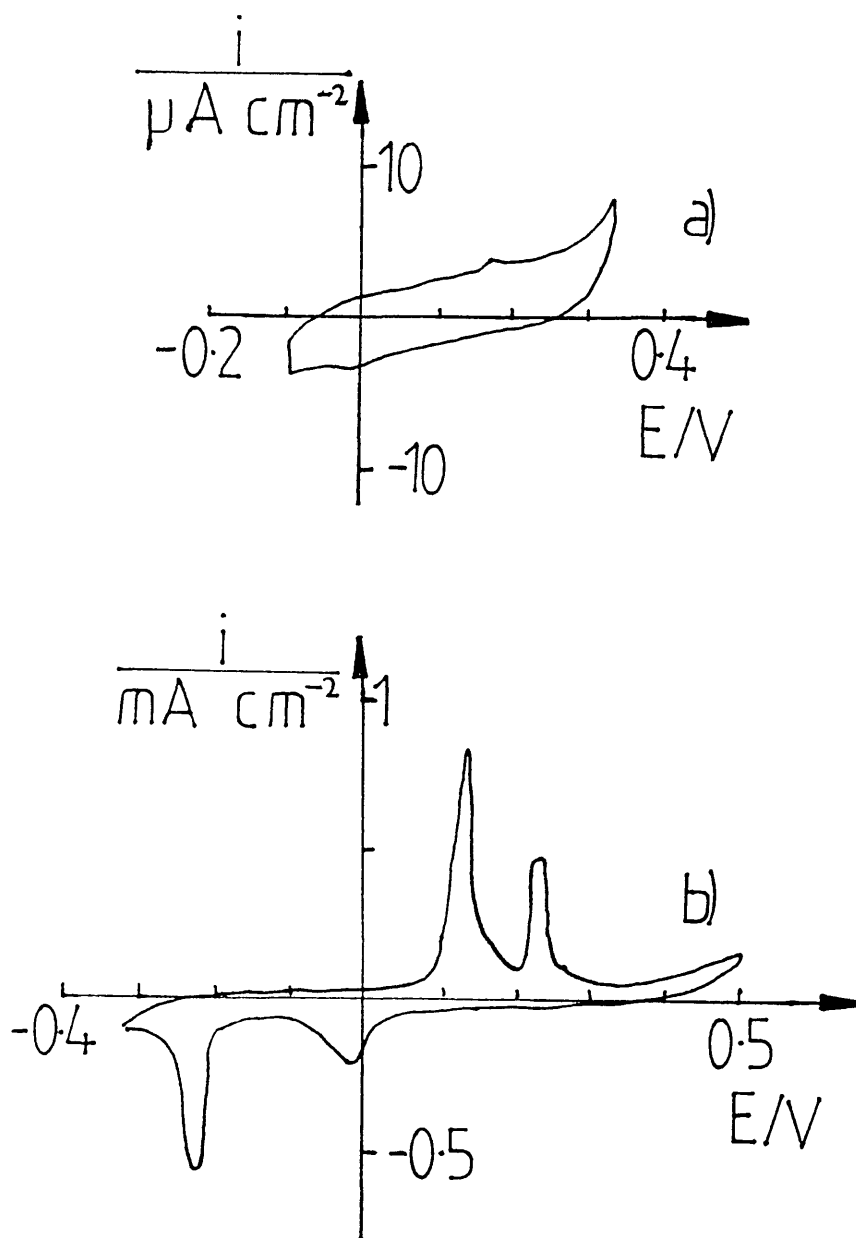


Figure 3.3.14 Cyclic voltammogram of the electrosynthesised TTFTCNQ electrode (vs SCE): a) before reduction, 10 mV/s; b) after reduction, 2 mV/s.

there was obviously much more material than this on the electrode, we must conclude that this value represents an upper limit to the amount of TTF⁺TCNQ that could be reduced before the build up of insoluble species on the organic salt's surface prevented further electrochemistry on the salt itself. This conclusion is supported by the work of Jaeger and Bard, who found that oxidation or reduction of organic salts resulted in the formation of donor or acceptor species on the surface corresponding to of the order of "hundreds of monolayers"^[42].

Although this particular synthesis seems to produce the highest surface coverages out of the methods described so far in this thesis, it does suffer from several disadvantages. This method takes several hours, due to the in-situ preparation of the TTF⁺ salt. This increases the likelihood of the material reacting with any oxygen present. TTF is required for the synthesis in relatively large quantities. As this is expensive compared to NMP⁺ and TCNQ this is obviously not desirable. However, perhaps more importantly, although a high surface coverage was obtained the organic salt did not completely cover the electrode and bare platinum still showed between the crystals. For this reason, no further work was carried out on this synthetic route.

3.3(2) METHODS EMPLOYING MODIFIED DONOR OR ACCEPTOR ELECTRODES

A common feature of the previous three syntheses is the failure of the organic salt to cover the bare metal substrate completely. A possible explanation of this problem is that the organic salt has difficulty "keying" onto the metal and once there are nucleation sites on the surface it has a preference for growing at these sites rather than on the metal. The organic salts are conductive so there would be no problems connected with the newly formed crystals being resistive and thereby preventing further growth. As the crystals grew out into the bulk one could envisage that donors or acceptors diffusing to the electrode would become more and more likely to encounter a crystal rather than the metal electrode and so precipitate at the former site. In this way growth would become centred at existing crystals and would not take place at the electrode surface. Strong evidence for this hypothesis is provided by the synthesis described in Section 3.3(1).3, where despite the fact that relatively large amounts of TTFTCNQ were produced, individual crystals grew at the electrode surface and bare metal was still visible between them.

One way to overcome this difficulty would be to pretreat the electrode in order to create enough nucleation sites to facilitate complete coverage of the metal surface by the salt. Work in these laboratories showed that it was possible to produce TCNQ and TTF modified electrodes quite

easily using electrochemical techniques (see below). These electrodes are relatively stable in reduced and oxidised forms, and form a smooth layer over the metal substrate that appears to completely cover the electrode when seen under a microscope. Ions can easily be incorporated into these electrodes by simple reduction or oxidation in the appropriate background electrolyte. An example of this would be the conversion of a TCNQ^0 electrode to K^+TCNQ^- by reduction at about 0 mV in an electrolyte such as KBr. A cyclic voltammogram showing this process has already been presented in Figure 3.3.1. Although organic donor and acceptor molecules are much larger than simple ions such as K^+ , it was hoped that by using an electrolyte containing the donor or acceptor, the organic salt could be produced by incorporation of the species in solution into the appropriate donor or acceptor modified electrode. Although this may sound unlikely because of the size of these molecules, it should be noted that molecules as large as p-toluene sulphonate have been incorporated in thionine modified electrodes (thionine is similar in size to the NMP^+) [90].

Turning specifically to the procedure for synthesising NMPTCNQ and TTFTCNQ, it must first be noted that NMP modified electrodes are unstable and therefore cannot be used. As background electrolytes in water, NMPMeSO_3 and LiTCNQ appear suitable, but as stated previously TTF salts that are soluble enough in water for this purpose are very

difficult to prepare. Acetonitrile cannot be used as a solvent because TTF^0 and TCNQ^0 are soluble in this medium and modified electrodes made of these materials would dissolve. Thus to prepare NMPTCNQ electrodes one must first produce a TCNQ^0 electrode and reduce it in an aqueous solution of NMPMeSO_3 . To prepare TTFTCNQ electrodes, one must use a TTF^0 modified electrode and oxidise it in the presence of LiTCNQ .

In the remainder of this introductory section ways of producing modified TCNQ and TTF electrodes will be discussed. The use of these electrodes in the electrosyntheses of NMPTCNQ and TTFTCNQ will be described in the following two sections.

a) Preparation of TCNQ Modified Electrodes

As already stated, TCNQ electrodes can be prepared by simply dropping a solution of TCNQ onto the electrode surface and allowing the solvent to evaporate or by the electrochemical oxidation of LiTCNQ in an aqueous medium. The latter method must be preferred and was always used in this work as the amount of TCNQ deposited on the electrode can be controlled by monitoring the amount of charge passed during the plating procedure.

A rotating disc polarogram of LiTCNQ in 100 mmol dm^{-3} aqueous lithium acetate at 4 Hz is shown in Figure 3.3.15. At oxidising potentials a polarographic wave corresponding to the production of insoluble TCNQ^0 is apparent. As the potential is swept towards reducing potentials a peak appears corresponding to stripping of this material off the

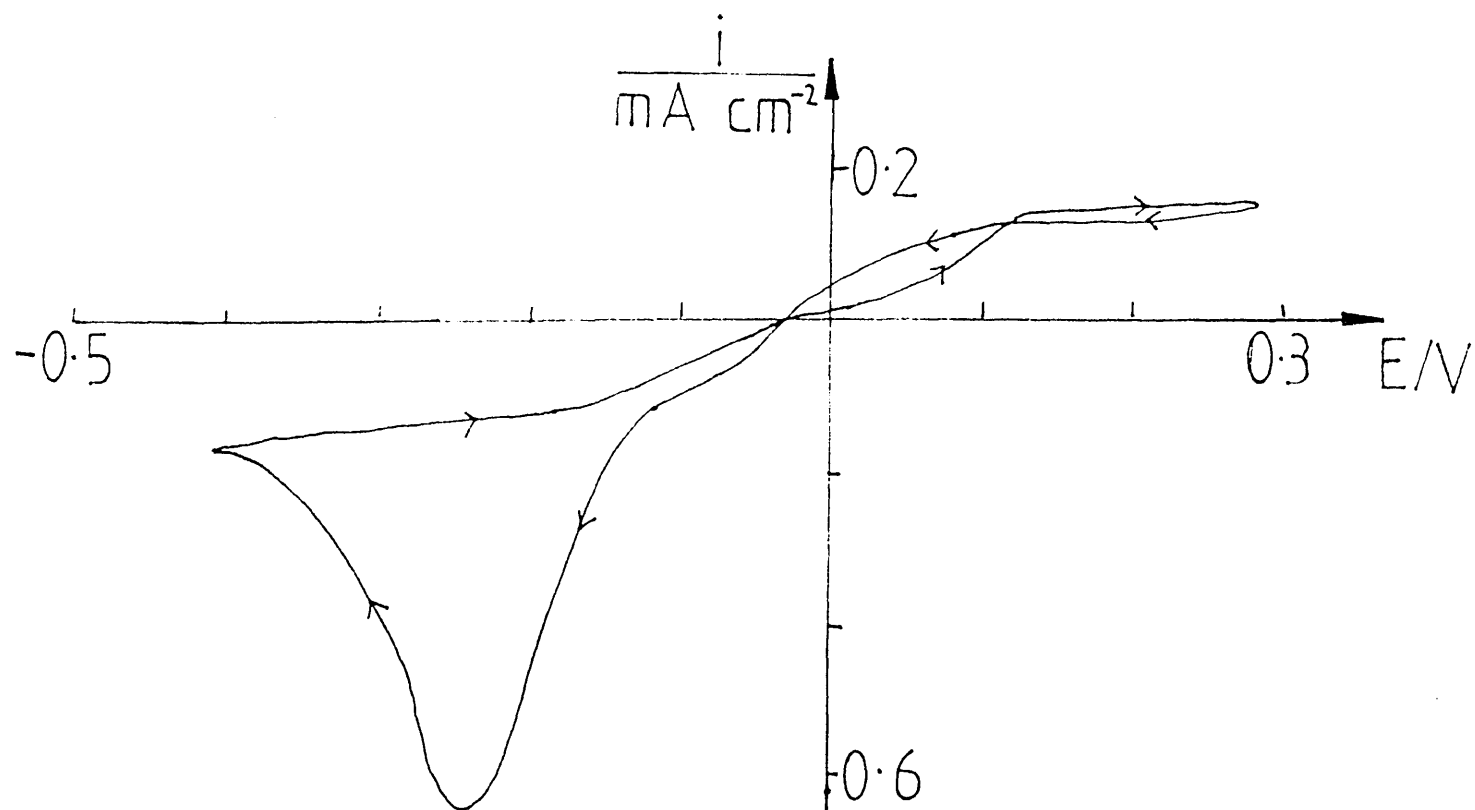


Figure 3.3.15 Rotating disc polarogram of 1.8 mmol dm^{-3} aqueous LiTCNQ (10 mV/s vs SCE).

electrode. This peak is superimposed on a cathodic wave due to the reduction of TCNQ^- to TCNQ^{2-} . Measurement of the total charge passed for the production of TCNQ^0 and its subsequent stripping suggests that within experimental error all the TCNQ^0 produced in the anodic wave remains on the electrode until it is stripped off. This is important as it implies that by monitoring the charge passed in production of a TCNQ^0 electrode we have an accurate measure of the amount of material adsorbed to the surface of the electrode.

In this work, TCNQ^0 electrodes were produced both by potentiostatic and galvanostatic means. As long as in the latter case the forcing current was kept below the diffusion controlled limit, the resulting electrodes did not significantly differ in their properties and both methods were equally satisfactory.

b) Preparation of TTF Modified Electrodes

As with TCNQ, TTF modified electrodes can be produced by simple evaporation of a TTF solution placed on the electrode or by electrochemical methods. Again the latter method is preferred as being more reproducible and controllable.

A rotating disc polarogram of TTFCl in 100 mmol dm^{-3} lithium acetate is shown in Figure 3.3.16. A cathodic wave attributable to the reduction of TTFCl to TTF^0 as well as an anodic wave corresponding to the oxidation of the former species to TTF^{2+} are apparent. There is also an anodic peak at +215 mV which can be attributed to the reoxidation of

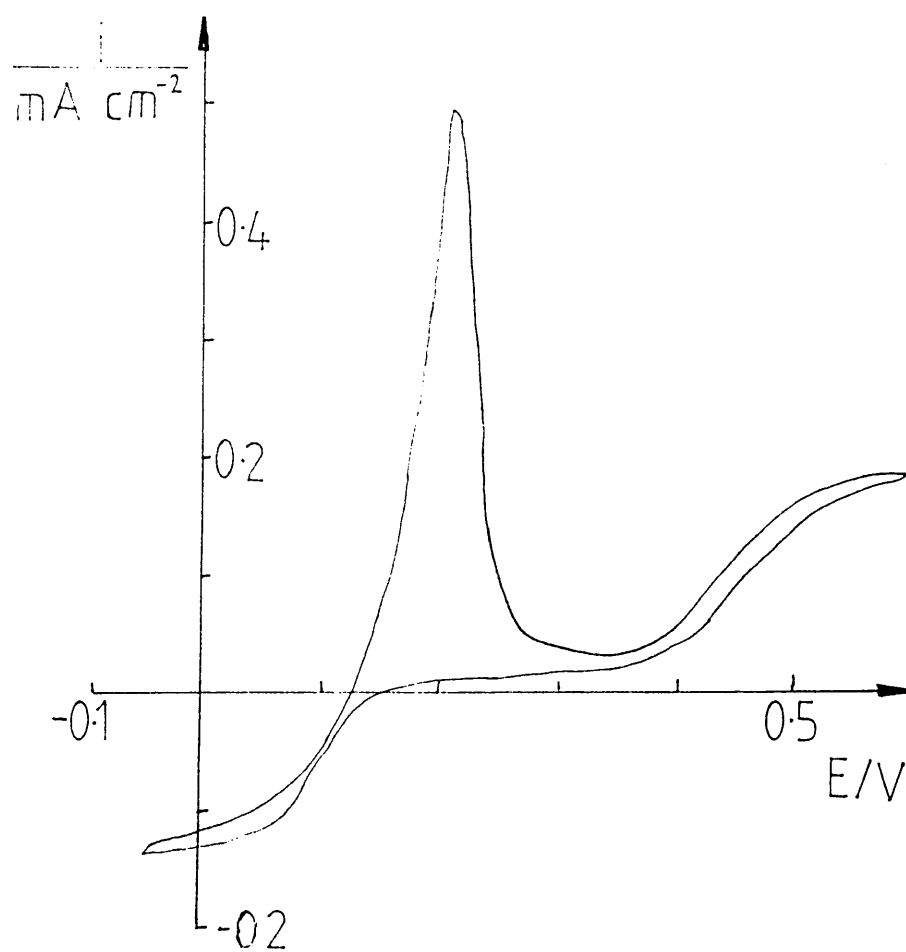


Figure 3.3.16 Rotating disc polarogram of 1.0 mmol dm^{-3} aqueous TTFC1 (5 mV/s vs SCE).

TTF⁰ adsorbed onto the electrode. Comparison of the total charge passed shows that at least 85% of the TTF⁰ produced at reducing potentials is adsorbed onto the electrode and appears in this peak. This result shows that the amount of TTF⁰ adsorbed on the modified electrode can be controlled within satisfactory limits.

Galvanostatic or potentiostatic methods can both be employed to prepare TTF⁰ modified electrodes. In this work the latter method was preferred as TTFCl tends to be unstable in solution and so by setting the potential to give a diffusion controlled current the electrodes could be produced at the fastest rate possible.

3.3(2).1 ATTEMPTED SYNTHESIS OF NMPTCNQ ELECTRODES FROM TCNQ MODIFIED ELECTRODES

In this synthesis a TCNQ⁰ modified electrode is reduced in the presence of NMPMeSO₃ as the background electrolyte. Figure 3.3.17 shows a rotating disc polarogram of the latter on a platinum electrode in 100 mmoldm⁻³ aqueous lithium acetate at 4 Hz. A cathodic wave is clearly apparent corresponding to reduction of NMP⁺ to "NMP⁰", which is adsorbed on the surface of the electrode and is subsequently reoxidised in the reverse sweep, giving rise to a peak at -20 mV. The onset of a measurable Faradaic current for the reduction of 4 mmol dm⁻³ NMP⁺ occurs at approximately -40 mV. The reduction of a TCNQ⁰ electrode normally occurs at about 0 to -50 mV. Thus it is possible that, given the high concentration of NMP⁺ employed, "NMP⁰" may be produced in

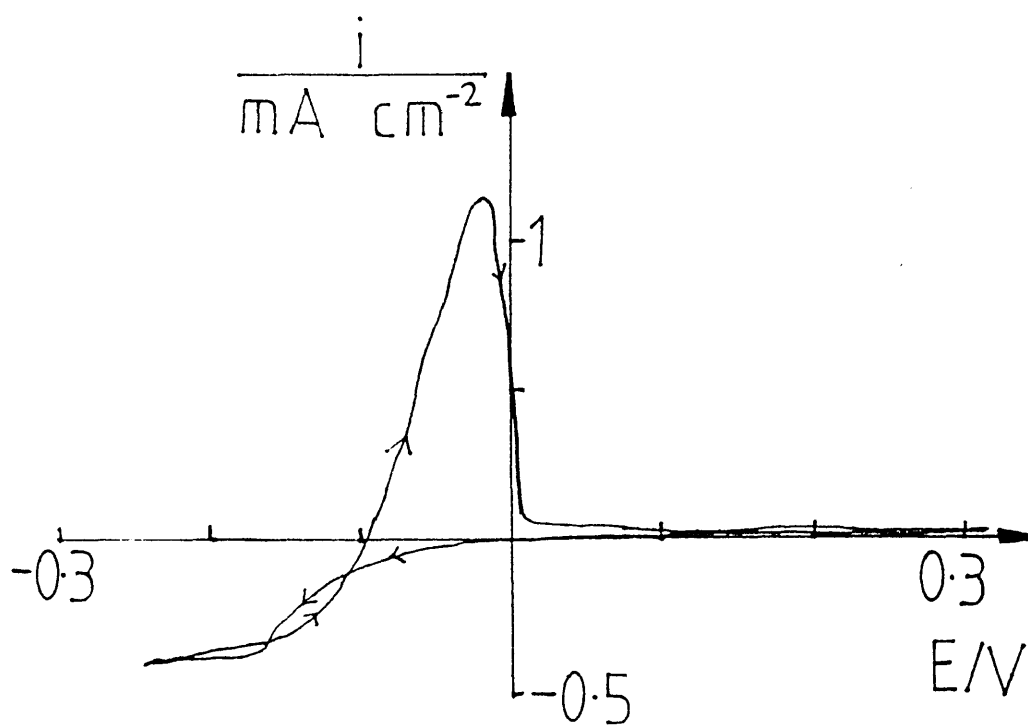


Figure 3.3.17 Rotating disc polarogram of 4.2 mmol dm^{-3} aqueous NMPMeSO_3 (10 mV/s vs SCE).

the synthesis. It is not known whether this species combines with TCNQ^0 to produce the salt. Because of this problem, the second step of the synthesis was carried out by sweeping the TCNQ^0 electrode over a potential range sufficient to reduce the TCNQ^0 to TCNQ^- and to reoxidise " NMP^0 ".

Under these conditions it was hoped that all the TCNQ^0 present should be eventually converted to TCNQ^- which will combine with NMP^+ from the bulk. Any " NMP^0 " which may be made should be reoxidised when the electrode is swept back towards positive potentials and lost into the bulk. The following synthesis was carried out using these principles.

a) Synthesis

A TCNQ^0 electrode was prepared by reducing an aqueous solution of 1 mmol dm^{-3} LiTCNQ and 100 mmol dm^{-3} lithium acetate at +200 mV for 1 minute at a platinum disc electrode (radius 3.5 mm) rotated at 4Hz. At the start of the synthesis the current was 60 μA and at the end it was 56 μA . Altogether 3.4 mC of charge was passed corresponding to the oxidation of 34.2 nmol of TCNQ^- .

The modified electrode was now placed in an aqueous solution of 20 mmol dm^{-3} NMPMeSO_3 , rotated at 4 Hz and swept at 5 mV/s between -70 mV and +55 mV. The disappearance over a number of sweeps of a cathodic peak at -30 mV could clearly be observed. This is attributable to the reduction of TCNQ^0 on the electrode surface. Because the amount of this material on the electrode surface is limited and it can

not be reoxidised at the potentials employed, the decrease in height occurs because it is gradually all converted to TCNQ^- . Once this peak had disappeared, the electrode was taken out of the solution and washed carefully with DDW.

b) Appearance and Electrochemical Properties of the Electrode

The electrode appeared to the naked eye to have an even brownish/black colour with some of the bare platinum metal sometimes showing as a background, depending on its orientation to the viewer. Using scanning electron microscopy, it was possible to see that approximately 30-50% of the electrode surface was covered with microcrystallites. These microcrystallites appeared as irregular polygons, having an average size of about 5 μm across. Due to the depth of these microcrystallites one would therefore expect the appearance of the electrode to change with its orientation. Viewed obliquely, most of the bare platinum would be hidden by the microcrystallites which will extend away from the electrode surface. However, when viewed perpendicularly to the electrode surface, the bare metal would clearly be visible. Thus the appearance of the electrode can be explained by looking at its microstructure.

Initial cyclic voltammetry of the electrode in 100 mmol dm^{-3} KCl shows peaks that are characteristic of the presence of free TCNQ (Figure 3.3.18a). In an effort to remove this from the electrode, a cyclic voltammogram was carried out in 100 mmol dm^{-3} aqueous lithium acetate. It was hoped that TCNQ would be reduced to TCNQ^- , which is soluble in this

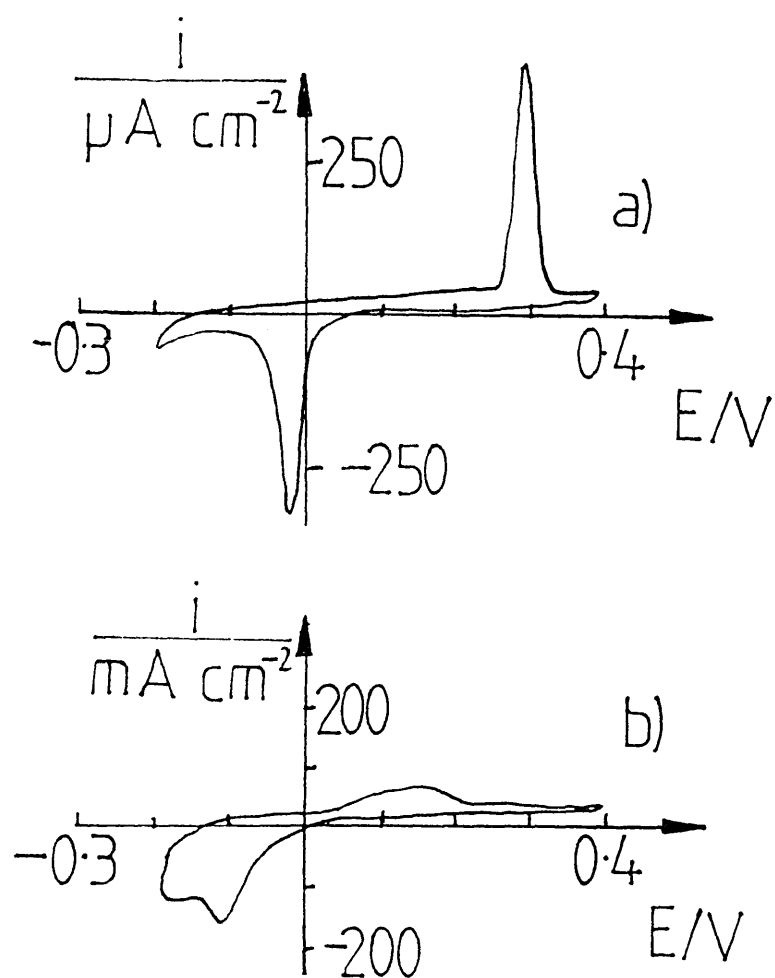


Figure 3.3.18 Cyclic voltammogram of the electrosynthesised NMPTCNQ electrode (20 mV/s vs SCE): a) before reduction, in aqueous KBr; b) during reduction, in lithium acetate.

electrolyte, whilst the NMPTCNQ would not be decomposed because its stable range was not exceeded. However, this process removed all the material from the electrode. Inspection of the cyclic voltammogram of this process (Figure 3.3.18b) shows two cathodic peaks at -100 mV and -170 mV and a broad anodic peak centred at 140 mV. The first could be due to free TCNQ being converted to TCNQ^- . The second peak is more difficult to explain. However, it could possibly be attributable to NMPTCNQ being decomposed to " NMP^0 " and TCNQ^- , or to TCNQ^- being further reduced to TCNQ^{2-} . Its absence in the previous cyclic voltammogram in aqueous KCl may be due to the change in electrolyte. The broad anodic peak may be due to TCNQ^- being reoxidised to TCNQ^0 .

Because of the results from these cyclic voltammograms, this section has been classified as an "attempted synthesis". However, there is a possibility NMPTCNQ was produced, and so the response to GOD and NADH was investigated.

c) Response of the Electrode to GOD and NADH

Electrodes prepared using this method of synthesis tended to have problems with background currents which drifted and were very noisy. Some did not respond to NADH at all, whereas in every case the response deteriorated with time. The best response to NADH was seen when the electrode was used for the first time, when it reached about $2 \mu\text{A cm}^{-2}$ for 1 mmol dm^{-3} NADH (Figure 3.3.19). This value is

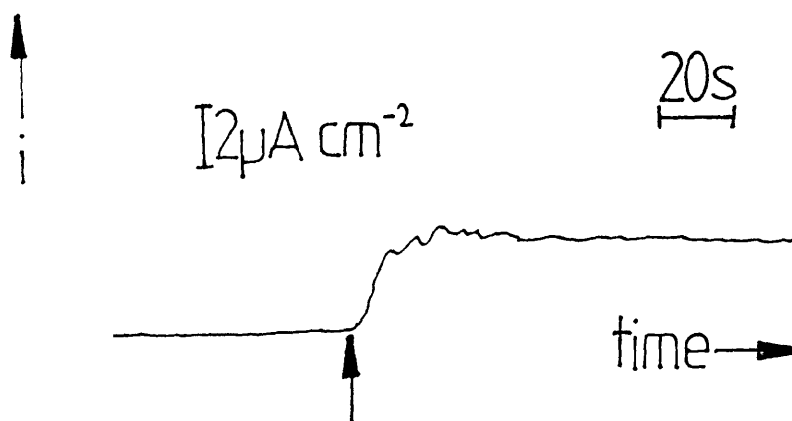


Figure 3.3.19 Response of a fresh electrosynthesised NMPTCNQ electrode to 1 mmol dm^{-3} NADH.

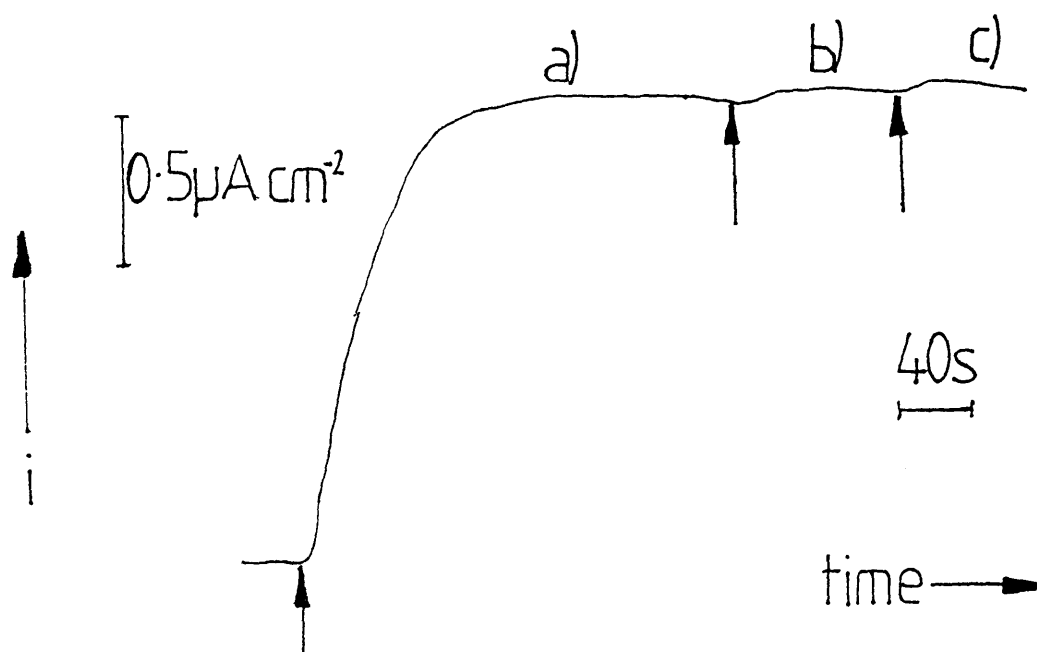


Figure 3.3.20 Response of a fresh electrosynthesised NMPTCNQ/GOD membrane electrode to total glucose concentrations of: a) 1 mmol dm^{-3} , b) 2 mmol dm^{-3} and c) 7 mmol dm^{-3} .

about 25% of that for typical NMPTCNQ polymer paste electrodes, and by a similar argument to that introduced in Section 3.3(1).1, can be explained from the observation that the electrode consists of microcrystallites separated by bare metal.

Turning to GOD, the electrode saturated at 1 mmol dm^{-3} glucose with a saturation current of $1.8 \text{ } \mu\text{A cm}^{-2}$ (Figure 3.3.20). This is around 1% of the typical value for polymer paste electrodes, and again can be expected because the electrode will not be porous and so will have a low actual surface area.

If the electrode was used for prolonged periods of time (such as overnight) the surface of the electrode changed colour from brown/black to purple. This will be discussed in the next section.

d) Surface Coverage of the Electrode

An estimation of the surface coverage of the electrode was difficult because it may have consisted wholly or partially of free TCNQ and because from cyclic voltammetry it was not clear whether NMPTCNQ was actually present. As has already been indicated, some electrodes produced using this method did not respond to NADH. Furthermore, the properties of the electrode seemed to be unrelated to the amount of TCNQ^0 (and hence its thickness) present on the TCNQ^0 modified electrode used. One explanation for the poor performance of the electrodes produced by this method of synthesis is that the resulting modified electrodes consist of an unstable form of NMPTCNQ or a mixture of uncomplexed

NMP and TCNQ. This would explain several of the experimental observations as one could envisage that if either of these hypotheses were true on placing an electrode in a solution containing a potassium or sodium salt as the background electrolyte a soluble form of NMP could be lost from the electrode to leave TCNQ^- on its surface. The latter would give rise to peaks in the cyclic voltammograms and would be observed as a purple insoluble solid on the surface of the electrode. The original brown/black colour could be due to NMPTCNQ or to a combination of the colours of the free donor and acceptor species.

Further investigation of these problems (possibly by spectroscopic techniques) would be interesting, but due to the success of the next synthesis described below were not carried out. One limitation of the above synthesis (and that described in Section 3.3(1).3) could be problems with the electrochemistry the NMP cation. The nature of " NMP^0 " is unknown, and if produced as a byproduct of the synthesis, it could possibly poison an NMPTCNQ electrode, especially if the organic salt only consisted of a thin layer. Alternatively it may form an unstable complex with TCNQ^0 which is non-conducting and/or inactive to NADH and GOD. If either hypothesis were true, then it is difficult to envisage any electrosynthesis for NMPTCNQ succeeding in producing the pure organic salt.

3.3(2).2 PREPARATION OF TTFTCNQ ELECTRODES FROM TTF^0 MODIFIED ELECTRODES

In this synthesis a TTF^0 electrode is oxidised in a solution containing LiTCNQ. The electrochemical behavior of TTF^0 modified electrodes and that of LiTCNQ in solution has already been shown in Figures 3.3.2 and 3.3.15. It can be seen that TCNQ^- can be oxidised at potentials as low as 0 mV, whereas adsorbed TTF^0 shows a peak at 130 mV. The approach of sweeping the potential of the electrode around this peak would therefore result in very large amounts of insoluble TCNQ^0 being produced, especially because the concentration of TCNQ^- will be very high due to its use as the background electrolyte. Whilst it may be possible to remove this electrochemically, the more circumspect approach of galvanostatically controlling the oxidation process was adopted. Knowing the amount of TTF^0 bound to the electrode, the charge passed could be controlled to oxidise the same number of moles of TCNQ^- , so optimising the chances of producing the maximum amount of the organic salt with the least amount of impurities.

a) Synthesis

A TTF^0 modified electrode was prepared as follows. A gold disc electrode (radius 3.5mm) was rotated at 16 Hz in an aqueous solution containing 1 mmol dm^{-3} TTFC1 and 100 mmol dm^{-3} lithium acetate. The electrode was poised at 0 mV and electrolysis was carried for 422 seconds, after which 10 mC of charge had been passed at the electrode (this was monitored using a current integrator). At the start of the

synthesis the current was 32 μA . This decreased to 24 μA by the end of the synthesis. The electrode was then carefully washed in DDW.

The TTF^0 modified electrode was then placed in an aqueous solution containing 25 mmol dm^{-3} LiTCNQ , rotated at 16 Hz and an oxidising current of 100 μA was passed for 100 s (ie total charge passed 10 mC). The electrode was taken out of solution and washed with DDW.

b) Appearance and Electrochemical Behavior of the Electrode

The electrode was evenly covered in a thin layer of a dark grey/black material. No bare metal was visible and an oblique view of the electrode showed the layer of organic salt clearly standing out on the surface with a thickness that was approximately uniform over the entire edge of the electrode.

Figure 3.3.21a shows a cyclic voltammogram of the electrode in a pH7.4 buffer containing 150 mmol dm^{-3} NaCl and 100 mmol dm^{-3} sodium phosphate. No significant peaks are apparent. A cyclic voltammogram for the same electrode is shown in Figure 3.3.21b after being reduced in aqueous KCl . New peaks have appeared which are characteristic of TTFTCNQ . We can therefore conclude that this synthesis has been successful in producing TTFTCNQ .

c) Response of the Electrode to NADH and GOD

An unusual property of the TTFTCNQ electrode prepared by this synthesis was that transients for potential steps were

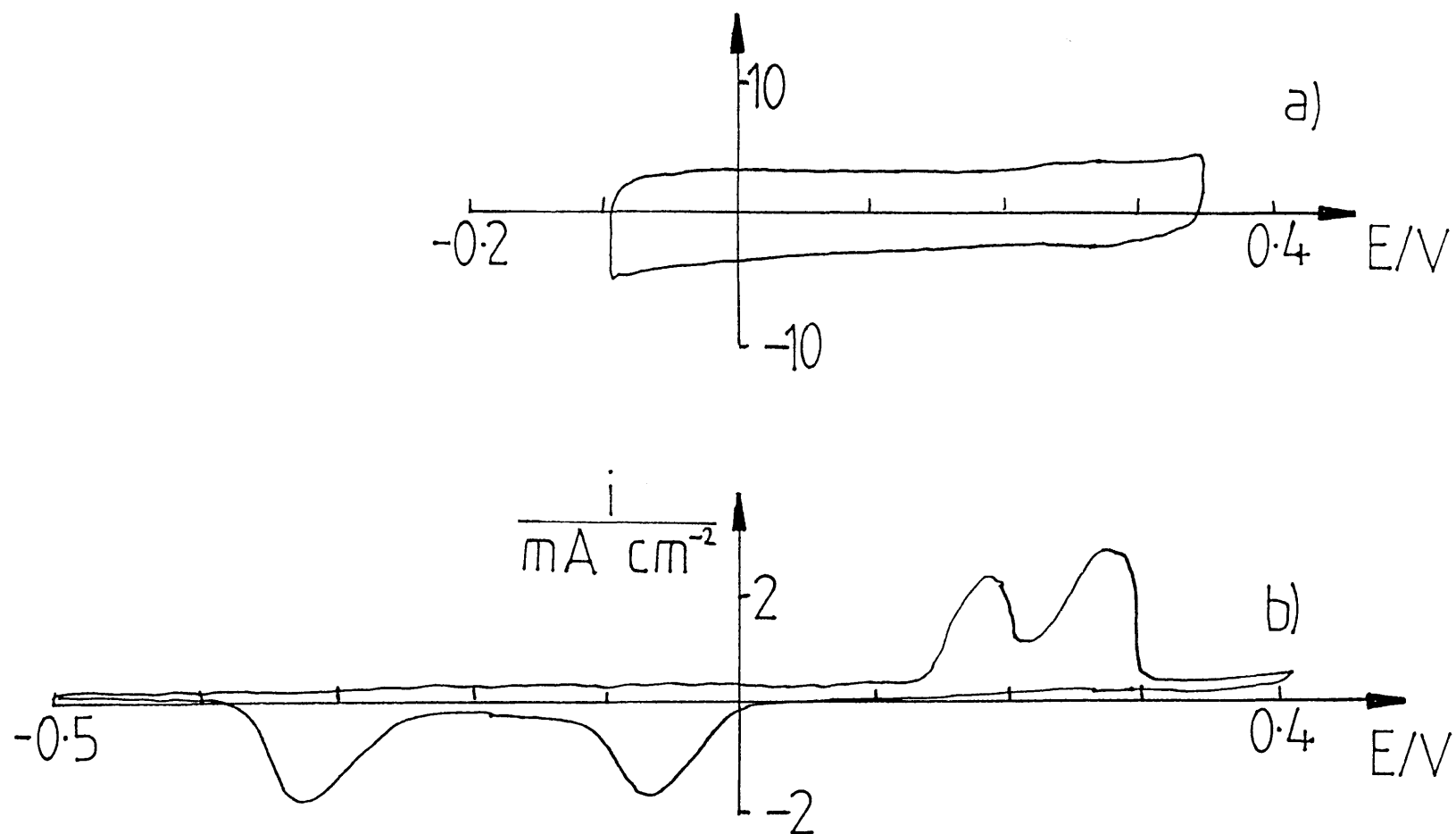


Figure 3.3.21 Cyclic voltammogram of the electrosynthesised TTFTCNQ electrode (vs SCE): a) before reduction, 100 mV/s; b) after reduction, 50 mV/s.

much faster than those experienced with polymer paste and silicone oil electrodes. Because of this, a rotating disc polarogram of NADH could be carried out. To minimise capacitive and other effects a very slow sweep rate was adopted. Figure 3.3.22 shows such a polarogram (taken in an aqueous solution of pH7.4 buffer containing 1 mmol dm^{-3} NADH, 100 mmol dm^{-3} sodium phosphate and 150 mmol dm^{-3} NaCl at a rotation speed of 4 Hz and sweep rate of 0.2 mV s^{-1}), after allowance had been made for the background current. Unlike NMPTCNQ electrodes, the rate of oxidation of NADH is potential dependent in this region and furthermore the currents obtained are comparatively small. This will be discussed in Section 3.4.

This data can also be used to find an apparent transfer coefficient α , for NADH at a TTFTCNQ electrode. This assumes that the electrochemical rate constant for NADH k' , is given by the following equation

$$k' = k^* \exp\{\alpha n F (E - E^*) / RT\} \quad (3.4)$$

The current i , is then given by

$$nFA/i = 1/(k'[NADH]) + nFA/i_{\text{lim}} \quad (3.5)$$

where i_{lim} is the limiting current for a process such as mass transport or adsorption. Substitution of equation 3.4 into 3.5, and taking logarithms gives:

$$\ln\{(i_{\text{lim}}/i) - 1\} = -(\alpha n F / RT)E + \text{constant} \quad (3.6)$$

This is, in fact, a modified form of the Tafel plot^[86], and such a plot is shown for the NADH/TTFTCNQ system in Figure 3.3.23. The gradient of this plot gives a value for α of 0.88. The significance of this result will be discussed in

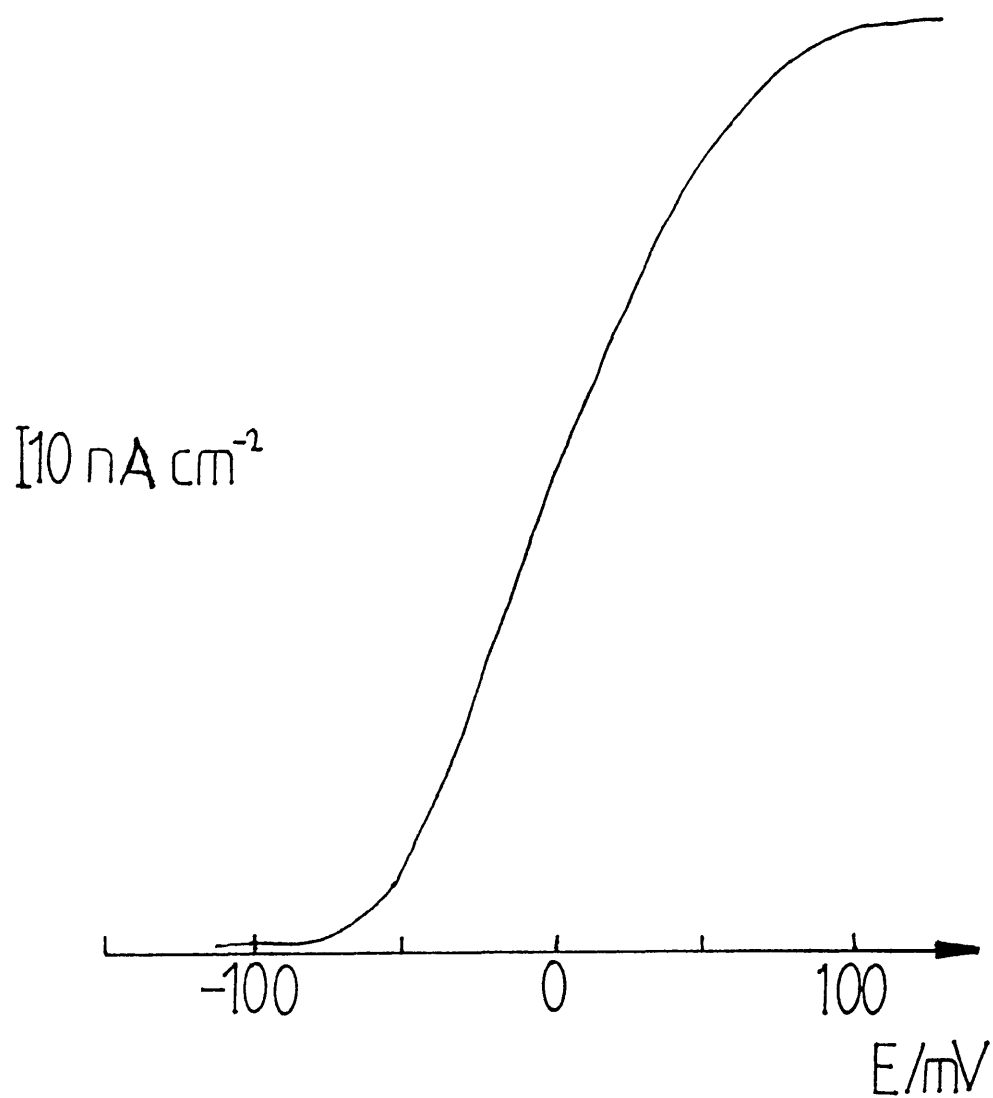


Figure 3.3.22 Rotating disc polarogram of 1.0 mmol dm^{-3} NADH at the electrosynthesised TTFTCNQ electrode (0.2 mV/s vs SCE).

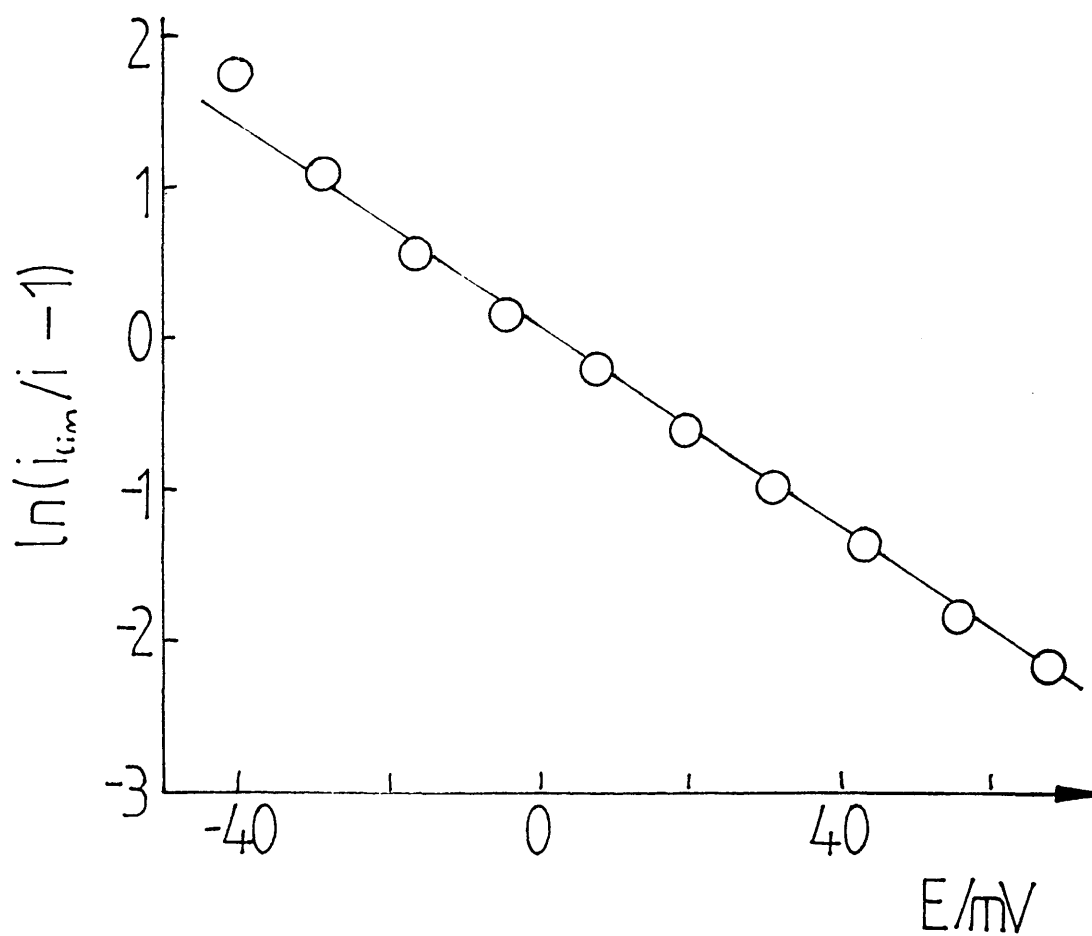


Figure 3.3.23 Modified Tafel plot of data shown in Figure 3.3.22

the next chapter.

The response of the electrode to GOD was tested at 8 potentials between -50 and +300 mV. Most of these results are shown in the next chapter. For comparison with the other electrosynthesised organic salt electrodes the response to GOD at 50 mV is shown in Figure 3.3.24. The electrode begins to saturate at about 2 mmol dm^{-3} glucose with a final saturation current of $3.6 \mu\text{A cm}^{-2}$. This is substantially below that for a polymer paste electrode, but this would be expected if the electrode was not porous or had a low roughness factor (ie smoother surface). A fuller discussion of these results, and a comparison of results from this electrode and electrodes made from a silicone oil paste, will be given in the next chapter.

d) Surface Coverage of the Electrode

Using the formula given in Section 3.3.1, and from the cyclic voltammogram shown in Figure 3.3.21b, the amount of TTFTCNQ reduced during the experiment was about 19 nmol cm^{-2} , corresponding to about 150 monolayers. As this is close to the limit to the number of monolayers which can be reduced before the electrode becomes too resistive, this value must represent a lower limit to the amount of TTFTCNQ in the modified electrode. This gives an electrosynthetic yield of greater than 15%.

The reproducibility of this synthesis seemed to depend greatly on the freshness of the solutions of the donor and acceptor species used. It was found that poor electrodes

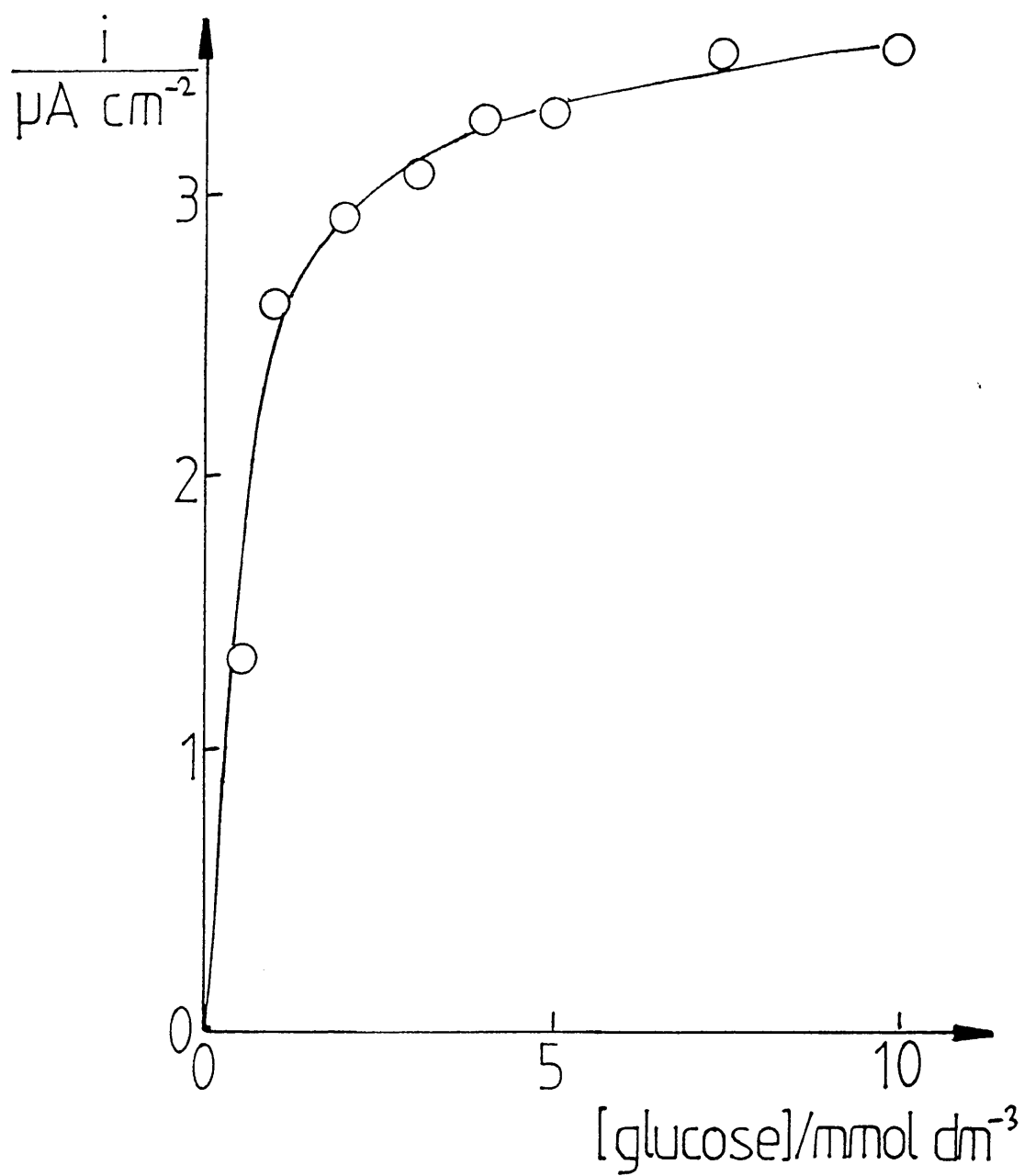


Figure 3.3.24 Response of the electrosynthesised TTFTCNQ/GOD membrane electrode to glucose.

were generally the result of employing solutions that were not properly degassed or where the substrates had been allowed to decompose in solution. Otherwise the success rate of this synthesis in producing thin-layer TTFTCNQ electrodes seemed to be quite good.

3.4 DISCUSSION

In this chapter, five syntheses for the production of organic salt electrodes are described based on two different synthetic routes. The first route consisted of reducing or oxidising a solution of both the donor and acceptor in the appropriate oxidation states. In the second route, a modified donor or acceptor modified electrode was produced in the first step, and then reduced or oxidised in a solution containing the corresponding ionic acceptor or donor.

Two syntheses for NMPTCNQ are described, based on the two different methods. Using the first technique, the modified electrode that resulted from the synthesis was patchy and probably consisted of separate microcrystallites of NMPTCNQ. This hypothesis is supported by its appearance, activity towards NADH and GOD, and cyclic voltammetry. Employing the second method, the electrode again consisted of separate microcrystallites (seen quite clearly by scanning electron microscopy) and was active towards NADH and GOD, although its cyclic voltammetry gave inconclusive results.

TTFTCNQ was produced by three syntheses. In the first synthesis described, a solution of TTF^0 and LiTCNQ was oxidised to produce a TTFTCNQ modified electrode, although the yield was low. Identification of the electrode was confirmed by its cyclic voltammetry, and supported by its activity to GOD and appearance. The second synthesis for TTFTCNQ consisted of reducing a solution of TTF^+ and

TCNQ⁰ in acetonitrile. Black, needle-like, crystals of TTFTCNQ were produced in relatively large amounts. Again, cyclic voltammetry confirmed these crystals as the desired organic salt. Finally, a thin-layer TTFTCNQ electrode was produced in the last synthesis described in this chapter by oxidation of a TTF⁰ electrode in the presence of TCNQ⁻. This electrode was active towards GOD and NADH, and cyclic voltammetry showed it to be the desired product.

Three major questions of scientific interest arise when we compare the results of these syntheses:

- i) why did only the final synthesis for TTFTCNQ result in a thin-layer electrode;
- ii) why did the second synthesis for TTFTCNQ produce large amounts of this material, whereas the yields for the other syntheses based on the same route were low?
- iii) why did the activity of the TTFTCNQ electrodes towards NADH vary so widely?

Addressing the first question, as already mentioned this problem could be due to the number of sites available for nucleation on the electrode surface. Results from the second synthesis for TTFTCNQ suggest that once nucleated, the crystals grow longitudinally away from the electrode to form needles. A plausible explanation for this observation would be that growth is much faster along the conducting axis of the crystal than in the other two directions. Turning to the third synthesis for TTFTCNQ, effectively we have created all the nucleation sites we require because we

have modified the whole surface of the electrode with TTF^0 . TCNQ can therefore lock onto all these sites to produce the salt in a thin, even layer. The reason why a thin layer NMPTCNQ electrode could not be produced in a similar way remains to be found. However, the synthesis could have failed because " NMP^0 " was made during the final step which may complex with TCNQ and/or poison NMPTCNQ.

An answer to question ii) is not readily apparent. It is interesting that this was the only synthesis where the donor and acceptor species were both in solution at concentrations higher than 1 mmol dm^{-3} . It may be that in the other two syntheses based on the same method the organic salt was precipitated in solution and not on the electrode itself. The factors governing crystallisation are not very well understood at present^[91], and so an answer to this question probably must wait for further advances in this field of science.

The last question is again difficult to explain. Variable results have been reported for the oxidation of NADH at TTFTCNQ polymer paste electrodes. The reason for this irreproducibility is unknown. However, a clue may be found from the polarogram of NADH on the TTFTCNQ electrode presented in Figure 3.3.22. It shows that there is a high overpotential for this species on this organic salt compared to NMPTCNQ, which oxidises NADH satisfactorily throughout its stable range. Furthermore the limiting current is very small compared to those seen on NMPTCNQ electrodes. One possible explanation could be that the observed

electrochemical rate constant is very dependent on the nature of the surface of the electrode. If this were true, the limiting current may be determined by absorption kinetics, not diffusion of the substrate from the bulk. The high currents for this process seen on the TTFTCNQ electrode produced by the modified El Kacemi/Lamache synthesis may therefore be due to its high surface area compared to a thin-layer electrode, or to differences in its surface. We may also envisage that the small amount of uncomplexed TTF present in the first TTFTCNQ electrode synthesised may be responsible for the inactivity of this electrode towards NADH, especially if it was concentrated on the surface.

After answering these questions, we must finally note that the aim of this work was to fabricate thin-layer organic salt modified electrodes and all the syntheses described in this chapter must be evaluated with this in mind. Although NMPTCNQ was made in at least one synthesis, it formed as separate microcrystallites and not as a thin-layer. Of the three syntheses described for TTFTCNQ, that using a modified TTF⁰ electrode succeeded in producing a thin-layer of the organic salt. The next chapter utilises such an electrode in some mechanistic studies of the GOD/TTFTCNQ system.

CHAPTER 4

STUDIES ON THE MECHANISM OF THE OXIDATION OF GOD AT TTFTCNQ ELECTRODES

4.1 INTRODUCTION

This chapter is made up of two main parts. The first part is concerned with the elucidation of the mechanism by which GOD is oxidised at a TTFTCNQ electrode made using a silicone oil paste. This particular type of organic salt electrode was employed because it is easy to fabricate and can be polished to give a smooth reproducible surface. The validity of two kinetic models for this process is tested by means of rotating disc studies with GOD in solution.

In the second part of this chapter the potential dependence of a membrane electrode for glucose is examined with reference to the kinetic model presented for the "one-substrate system", introduced in Chapter 2. This was facilitated with the use of an electrosynthesised TTFTCNQ electrode, so reducing problems due to adsorbed enzyme and from the slow transients seen at polymer paste electrodes after a potential step. The latter point is important, as it allows one to make measurements at a range of different substrate concentrations and potentials in one day, using the same electrode. By comparing the response at each potential and with reference to the kinetic model, the rate limiting processes that govern the response of the electrode are determined. Results using the membrane electrode are

also compared with the rotating disc results and the theoretical models with a view to further elucidation of the mechanism by which GOD is oxidised at these electrodes.

In the next part of this introductory section a discussion of the qualitative aspects and the evidence for and against each model will be discussed. A more rigorous mathematical analysis of the two models will be presented later.

4.1.1 PRESENT MODELS OF THE GOD/TTFTCNQ SYSTEM

The first experiments using conducting organic salts as electrodes for GOD were made by the school of Kulys^[43]. They postulated a mechanism based on homogeneous mediation of the enzyme. A scheme of his model is shown in Figure 4.1.1. Here, some kind of mediator M, dissolves from the electrode into the bulk. Reduced enzyme GODH_2 reacts with this mediator to form GOD and a reduced form of the mediator M^- . M^- can then diffuse into the bulk (and hence effectively is lost into solution) or back to the electrode, where it is reoxidised to M. In this model, the response of the electrode depends crucially on the concentration of dissolved mediator at the electrode surface and the second order rate constant for the reaction between M and GODH_2 . Kulys measured this rate constant (for TCNQ^0 as M) to be $3 \times 10^6 \text{ mol}^{-1} \text{ dm}^3 \text{ s}^{-1}$ [92]. Other mediators gave lower values of this parameter. The currents obtained could then be as expected if the mediator concentration was $13.5 \mu\text{mol dm}^{-3}$ (again assuming TCNQ^0 was M). It was also stated that if

electrode

solution

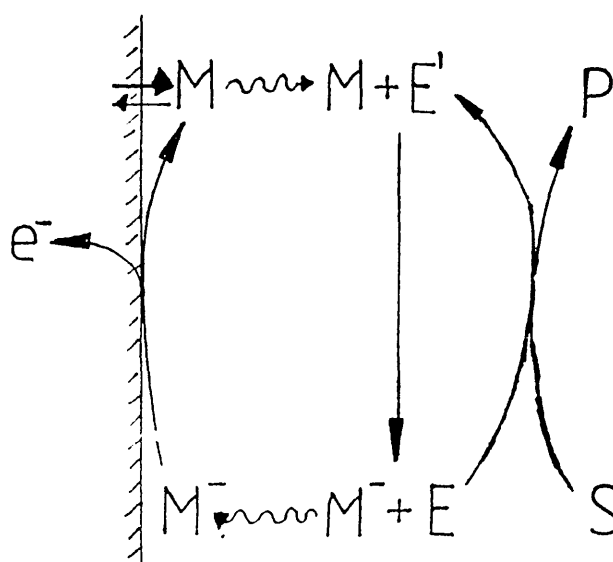


Figure 4.1.1 Reaction scheme for the homogeneous mediation model.

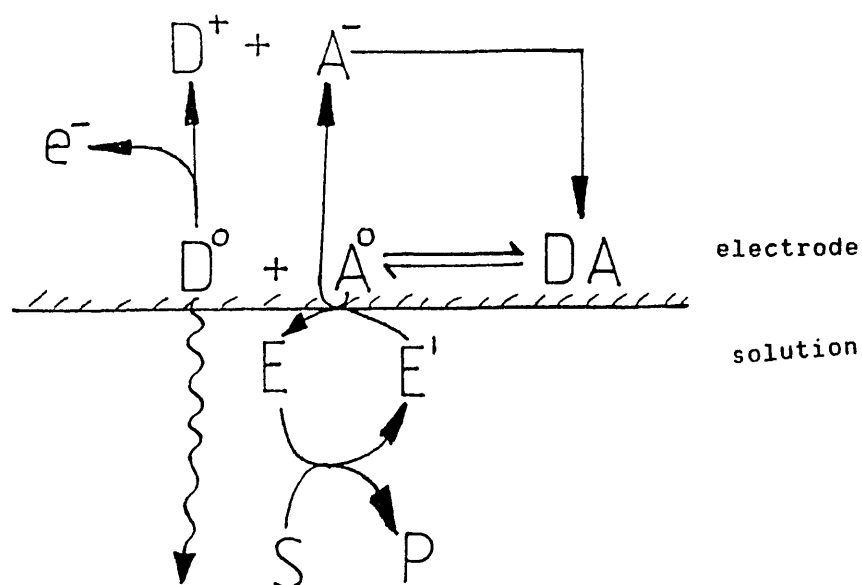


Figure 4.1.2 Reaction scheme for the heterogeneous redox catalysis model.

the electrode was rough this concentration could be much lower, as the electrode surface area would be relatively large.

In 1985, Albery et al carried out experiments using a rotating platinum ring/TTFTCNQ disc electrode^[40]. They failed to detect significant amounts of any mediator at the ring. It was therefore concluded that the mediator concentration at the surface was μ molar or less. Furthermore, scanning electrode microscopy showed that the scale of roughness was not enough to justify the differences in currents observed^[41]. They concluded that GODH₂ was therefore involved in a direct electron transfer at the electrode surface.

However, further investigation of the system by Bartlett using single crystal electrodes revealed that the current was proportional to the square root of the enzyme concentration^[41]. This result was incompatible with the direct electron transfer theory but could be explained by the Kulys model. The original objections to this model remained however, and so another mechanism was proposed which involved heterogeneous redox catalysis (Figure 4.1.2). In this model, there is an equilibrium at the surface of the electrode between the organic salt DA, and free D^0 and A^0 adsorbed on its surface. The enzyme reacts with A^0 to form A^- . D^0 is oxidised electrochemically to D^+ (hence producing a current), and these two products recombine to form DA. Under certain circumstances, the observed relationship

between current and enzyme concentration is then expected. In this thesis, results are presented which show that there is an inverse relationship between the current at a rotating disc electrode and the rotation speed. The model was therefore further refined to allow D^0 to dissolve into solution. Here, the loss of D^0 will increase with rotation speed. Less D^0 will therefore be available for electrochemical oxidation and so the current will decrease.

It would of course be desirable to use independent methods to detect intermediates and measure individual rate constants as well as theoretical analysis to differentiate between the possible models for this system. However, up to the present time this has proved difficult because of the low solubilities of the various species of interest and because organic salt electrodes tend to have high background currents and do not seem amenable to non-steady state measurements. For the moment, we must therefore rely on using analytical models of each mechanism to predict the experimental rate laws. This process forms the basis of the first part of this chapter.

4.2 EXPERIMENTAL

4.2.1 CHEMICALS AND SOLUTIONS

All chemicals were either commercially obtained of AnalaR grade, or synthesised as given in Section 2.2.1, Chapter 2 and Section 3.2.1, Chapter 3.

4.2.2 MEMBRANES

Membranes were treated prior to use as described in Section 2.2.2, Chapter 2.

4.2.3 ELECTRODES

Rotating gold disc and cavity electrodes were made by Mr M Pritchard. Details of reference and counter electrodes are found in Section 2.2.3, Chapter 2. All potentials are quoted against the saturated calomel electrode.

4.2.4 APPARATUS

Details of all the apparatus employed are given in sections 2.2.4 (Chapter 2), and 3.2.4 (Chapter 3).

4.2.5 EXPERIMENTAL PROCEDURES

GOD membrane electrodes were made as described in Section 3.2.5, Chapter 3. Their response to glucose was monitored as described for choline electrodes in Section 2.2.5, Chapter 2, except that initially the electrode was

poised at -50 mV, and at each substrate concentration the potential was stepped to +300 mV in 50 mV increments. After each step the current was allowed to reach a steady state. When the steady state current at +300 mV had been measured the potential was stepped back to -50 mV, and the next aliquot of glucose solution added.

For rotating disc experiments, the electrode was poised at the desired potential in a 20 ml solution containing 150 mmol dm⁻³ glucose in pH7.4 buffer. The steady state background current was measured at 1 Hz, 4 Hz and 9 Hz, and then aliquots of glucose oxidase solution were added using a micro pipette. After each aliquot was added, the steady current was measured at 4 Hz, and additionally at 1 Hz and 9 Hz for selected concentrations of enzyme.

4.3 THEORY

In this section two kinetic models are analysed for the oxidation of glucose oxidase at an organic salt electrode. Before we examine each model in turn we will ask ourselves if we can predict what the likely value of each of the various kinetic and experimental parameters are that appear in each theory.

First, we will look at the diffusion of species to and from the electrode. The important parameters here are the diffusion coefficient D_i (where i is the species of interest) and the diffusion layer thickness Z_D .

The diffusion layer thickness can quite simply be calculated for the rotating disc electrode, and is inversely proportional to the square root of the rotation speed. It is given by the following equation^[86]:

$$Z_D = 0.642\nu^{1/6}(D_i)^{1/3}\omega^{-1/2} \quad (4.1)$$

where ν is the kinematic viscosity and ω the rotation speed in Hz. A typical value of ν is $0.01 \text{ cm}^2 \text{ s}^{-1}$ [93] (although this is an approximation, the very low power to which it is raised means Z_D is very insensitive to changes in this parameter) and so

$$Z_D \approx 0.298(D_i)^{1/3}\omega^{-1/2} \quad (4.2)$$

For this system, several of the diffusion coefficients for the species of interest have been measured. A list of these together with the corresponding diffusion layer thicknesses at 1 Hz ($Z_{D,1}$) appears in Table 4.3.1. The diffusion coefficients of TTF^0 , TCNQ^0 and TCNQ^- do not

TABLE 4.3.1

Diffusion parameters for species involved in the TTFTCNQ/GOD system.

Species	$10^7 D / \text{cm}^2 \text{ s}^{-1}$	$z_{D,1} / \mu\text{m}$
GOD	4.1 ^[11]	22
Glucose	67 ^[94]	56
TTF ⁺	60 ^[95]	~54

TABLE 4.3.2

Second-order rate constants for reaction of reduced GOD with various mediators.

Mediator	$10^4 k_M / \text{mol}^{-1} \text{ dm}^3 \text{ s}^{-1}$
TTF ⁺	"efficient" ^[96]
TCNQ ⁰	3.3 ^[92]
TCNQ ⁻	300 ^[92]

appear to have been determined. However, they are likely to be close to the value for TTF^+ , considering that they are similar in size to TTF^+ and the latter is not highly charged.

We must now turn to the kinetic rate constants for the reactions in solution. The rate at which glucose reduces the enzyme may, of course depend on its concentration, being determined by particular kinetics of the enzyme. However at the glucose concentration employed in the work presented in this thesis the enzyme will be approaching saturation (the K_m for glucose is about 33 mmol dm^{-3} [11]). The rate of turnover for enzyme will therefore be close to its k_{cat} of 3400 s^{-1} [15]. The rate at which GOD reacts with the possible mediators has been more difficult to determine, due to the low solubilities of TCNQ^0 and TTF^0 and problems due to the synthesis of water soluble TTF^+ salts. However reported values for these constants are shown in Table 4.3.2.

As already stated, the surface concentration of mediator at the electrode surface is important. However, this parameter is unknown, as is the solubility product of TTFTCNQ . The only work reported in this field appears to be the rotating disc measurements of Craston who concluded that there was no mediator present in concentrations above $1\text{--}2 \text{ } \mu\text{mol dm}^{-3}$ [39].

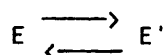
Finally, in the heterogeneous redox catalysis theory rate constants are introduced for the reaction of enzyme at the electrode surface and for the electrochemical oxidation

of adsorbed species, as well as a constant to describe the equilibrium between the organic salt and TTF and TCNQ adsorbed to its surface. None of these parameters have been determined at the present time.

4.3.1 THE HOMOGENEOUS MEDIATION THEORY

The kinetic scheme of this model was shown in Figure 4.1.1. A complete analysis of this theory would take into account the diffusion of M and M^- , GOD and $GODH_2$, and glucose and its product between the electrode and the bulk, as well as all the reactions between them. However, this would be too difficult to analyse mathematically and so we must make some simplifying assumptions.

First, we will ignore product inhibition of the enzyme. The concentration of product will be too small compared to the concentration of glucose, as the latter is present at 150 mmol dm^{-3} , and anyway glucose oxidase does not seem to show strong product inhibition^[14]. Second, the observed fluxes are so low, and the glucose concentration so high, that we can assume glucose is present at constant concentration throughout the layer. Third, we can look at the ratio of oxidised to reduced enzyme in the reaction layer. This can be calculated by looking at the local equilibrium between the oxidised enzyme E, and the reduced enzyme E' at the electrode surface:



Equating the fluxes, we have (where e and e' denote the

concentrations of E and E' respectively, m_0 is the concentration of M at the electrode surface, k_M represents the second order rate constant between E' and M, and k_E is the observed first order rate constant for reaction of E and substrate)

$$e/e' = (k_M m_0)/k_E \quad (4.3)$$

We shall call this ratio γ . For the work presented in this thesis $k_E \sim k_{cat} \sim 10^3 \text{ s}^{-1}$. The value of m_0 is unlikely to be significantly greater than $10^{-6} \text{ mol dm}^{-3}$. Hence, using a value for k_M of $10^6 \text{ mol}^{-1} \text{ dm}^3 \text{ s}^{-1}$, we find $\gamma \sim 10^{-3}$. This means that even if k_M and m_0 were both ten times as large as estimated, almost all the enzyme will be in the reduced form and e' can be assumed to be equal to the total enzyme concentration e_T , throughout the reaction layer. It should be noted that this situation exists because for the work presented in this thesis the substrate concentration is very high. When the latter is small we may find that $\gamma > 1$. This case is being investigated in the laboratories of Bartlett and Albery and will be discussed in the thesis of Miss L J Murphy^[97].

Using the above assumptions we have now arrived at a stage where we can begin to analyse the problem. We are now following the original treatment by Kulys^[92]. Mathematical analysis now shows (see Appendix 2) that the flux j , is given by:

$$j = k_D m_0 [(K_M)^{1/2} \coth(K_M)^{1/2} - 1] \quad (4.4)$$

where k_D is the mass transport rate constant for M given by $k_D = D_M/Z_D$ and $K_M = k_M(Z_D)^2 e_T/D_M$. The value of m_0 may be

governed by thermodynamics or its dissolution kinetics, and in Appendix 2 a simple model is introduced which shows that:

$$m_o = m_{o,T} / (1 + \theta_M) \quad (4.5)$$

where $m_{o,T}$ is the thermodynamic solubility of M and θ_M is given by $\theta_M = k_D/k_b$, k_b being a first order rate constant for the reincorporation of M onto the electrode after it has dissolved. Thus substitution for m_o in equation 4.4 gives

$$j = k_D m_{o,T} [(K_M)^{1/2} \coth(K_M)^{1/2} - 1] / (1 + \theta_M) \quad (4.6)$$

This equation splits up into two cases, depending on the value of K_M . Case I is where $K_M \gg 1$. The flux is then given by:

$$j = n(D_M k_M e_\Gamma)^{1/2} / (1 + \theta_M) \quad (4.7)$$

Here M reacts in a thin reaction layer and does not escape into the bulk.

However if $K_M \ll 1$, then most M is lost to the bulk and its reaction with E' is relatively slow. This is Case II and now the flux is given by:

$$j = (1/3) n k_M m_{o,T} e_\Gamma Z_D / (1 + \theta_M) \quad (4.8)$$

Each of these cases can be subdivided into sub-cases depending on whether θ_M is greater than or less than unity. If $\theta_M \ll 1$ (sub-cases I_T and II_T) then m_o will be thermodynamically controlled and equal to $m_{o,T}$. On the other hand, if $\theta_M \gg 1$ (sub-cases I_K and II_K) then $m_o \ll m_{o,T}$ and will be inversely proportional to k_D . Table 4.3.3 summarises the expression for the flux for each of the four sub-cases and the case diagram is shown in Figure 4.3.1.

In the results presented in this work the current is

TABLE 4.3.3

Approximate equations for flux for each case of the homogeneous mediation theory.

Case	K_M	Control of m_o	Flux Given By
I_T	>1	Thermodynamic	$n(D_M k_M e_I)^{1/2} m_{o,T}$
I_K	>1	Kinetic	$n[(k_M e_I D_M)^{1/2} m_{o,T}]/\theta_M$
II_T	<1	Thermodynamic	$n(1/3) k_M e_I Z m_{o,T}$
II_K	<1	Kinetic	$n[(1/3) k_M e_I Z m_{o,T}]/\theta_M$

TABLE 4.3.4

Order of flux with respect to enzyme concentration and rotation speed for each case of the homogeneous mediation model.

Case	Order w.r.t. e_I	Order w.r.t. ω
I_T	1/2	0
I_K	1/2	-1/2
II_T	1	-1/2
II_K	1	-1

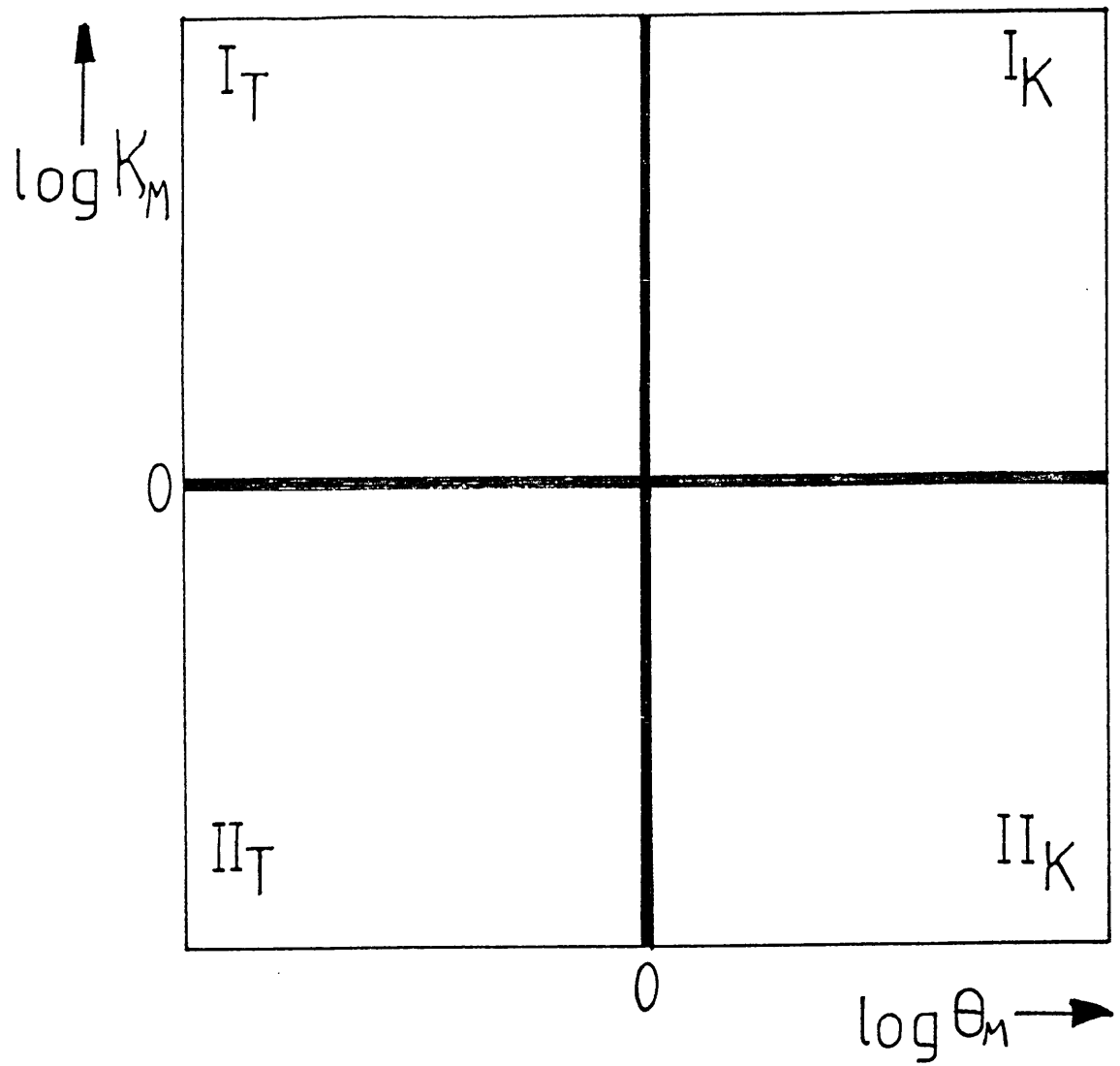


Figure 4.3.1 Case diagram for the homogeneous mediation model.

determined at different values of e_L and W . The order of the flux with respect to these two variables for the four sub-cases is shown in Table 4.3.4. Results are also presented at different potentials. Alteration of this latter variable could change $m_{O,T}$ and k_b and will be discussed in Section 4.4(1).5.

When confronted with experimental results, the first stage of the analysis is to determine the order of current with respect to e_L and W . Comparing these values to those shown in Table 4.3.4, we can see which sub-case (if any) of the theory is applicable. The treatment of the experimental data will then depend on these initial conclusions, and will be discussed in more detail in Section 4.4(1).3, which contains the experimental results.

4.3.2 THE HETEROGENEOUS REDOX CATALYSIS THEORY

A scheme of reactions for this model has been shown in Figure 4.1.2. On the surface of the organic salt DA there is supposed to be a labile equilibrium involving the salt and species D^0 and A^0 . D^0 is assumed to be oxidised to D^+ on the electrode surface with a first order rate constant k_2 . The enzyme is assumed to react with A^0 with a second order rate constant k_3 to produce A^- . D^+ is then supposed to combine rapidly with A^- to reform DA, the organic salt. A further possibility included in the model is that D^0 can be lost from the electrode by dissolving into solution and

then diffusing out into the bulk. This process can be described by the partition coefficient K_S and a mass transfer rate constant k_D :

$$K_S = d_{\text{sol}}/d \quad (4.9)$$

$$k_D = D_D/Z_D \quad (4.10)$$

where d_{sol} and d are the concentration of D in solution at the electrode surface (mol cm^{-3}) and adsorbed to the electrode surface (mol cm^{-2}) respectively, D_D is the diffusion coefficient of D and Z_D is the length of the diffusion layer. In the steady state the diffusion of D^0 from the surface must be matched by an equal flux of A^- leaving the surface.

In solution the enzyme reacts with substrate with a first order rate constant k_E . In a similar argument to that presented in section 4.3.1, we can presume $k_E \sim k_{\text{cat}}$ and there is no concentration polarisation of glucose throughout the reaction layer.

A full mathematical treatment of this system is presented in Appendix 3. Here we will summarise the results. We will first introduce the dimensionless parameters $\theta_D = K_S k_D / k_2$, $K_E = k_E (Z_D)^2 / D_E$, $B = (n e_I / K_1 k_2 k_3) (D_E / Z_D)^2$ and f , where f is given by:

$$j_e = f n D_E e_I / Z_D \quad (4.11)$$

where j_e is the flux of electrons and equal to i/AF . The system is then described by the following equation:

$$B f^2 + f \tanh[(K_E)^{1/2}] / (K_E)^{1/2} - (1 + \theta_D)^{-1} = 0 \quad (4.12)$$

K_E describes the chances that E will escape into the

bulk before it reacts with substrate. If $K_E \gg 1$ then E is more likely to react than be lost to the bulk, whereas the converse is true if $K_E \ll 1$. B describes the balance between the surface kinetics and mass transport. If $B \gg 1$ then the surface kinetics are relatively rapid and the flux will tend to be controlled by the regeneration of enzyme or mass transport. On the other hand if $B \ll 1$ then mass transport is rapid and the flux will tend to be controlled by the surface kinetics or enzyme regeneration. θ_D describes the chances of D being electrochemically oxidised before dissolving into solution. It will be greater than unity if D is more likely to escape into solution than be oxidised, and less than one if the converse is true.

We are now in a position to derive three approximate solutions for f which correspond to three simple cases. We will first consider the case where

$$B/(1+\theta_D) \gg \tanh^2[(K_E)^{1/2}]/K_E \quad (4.13)$$

then we find equation 4.12 reduces to:

$$f \approx \{B(1+\theta_D)\}^{-1/2} \quad (4.14)$$

and so

$$j_e = \{nK_1k_2k_3e/(1+\theta_D)\}^{1/2} \quad (4.15)$$

In this case (Case S) the surface kinetics are rate limiting.

Reversing the inequality of equation 4.13 we find:

$$f \approx \{(K_E)^{1/2} \coth[(K_E)^{1/2}]\}/(1+\theta_D) \quad (4.16)$$

If $K_E \ll 1$ we find

$$f \approx 1/(1+\theta_D) \quad (4.17)$$

hence we obtain Case T:

$$j_e = nD_E e_L / \{Z_D (1 + \theta_D)\} \quad (4.18)$$

The rate limiting step is now mass transport of E' across the diffusion layer.

Finally we will consider the final case where $K_E \gg 1$.

Enzyme regeneration is now rapid and we find

$$f \approx [(K_E)^{1/2}] / (1 + \theta_D) \quad (4.19)$$

giving

$$j_e = n(D_E k_E)^{1/2} e_L / (1 + \theta_D) \quad (4.20)$$

This is Case E. E is now consumed in a reaction layer close to the electrode given by $(D_E/k_E)^{1/2}$.

We have now found three simple cases as solutions to our original problem. These cases can be further subdivided, depending on the value of θ_D . They are shown on the case diagrams displayed in Figures 4.3.2 (for $\theta_D \ll 1$, sub-cases E, S and T) and 4.3.3 (for $\theta_D \gg 1$, sub-cases E_θ , S_θ and T_θ). Table 4.3.5 summarises the approximate expression for the flux in each sub-case.

The analysis of experimental data will follow the same procedure as for the homogeneous mediation theory. We first determine the experimental orders of the current with respect to e_L and W and compare them with those predicted by the theory, shown in Table 4.3.6. Further analysis then depends as to which case we believe is applicable, and will be discussed further in Section 4.4(1).4 with the experimental results.

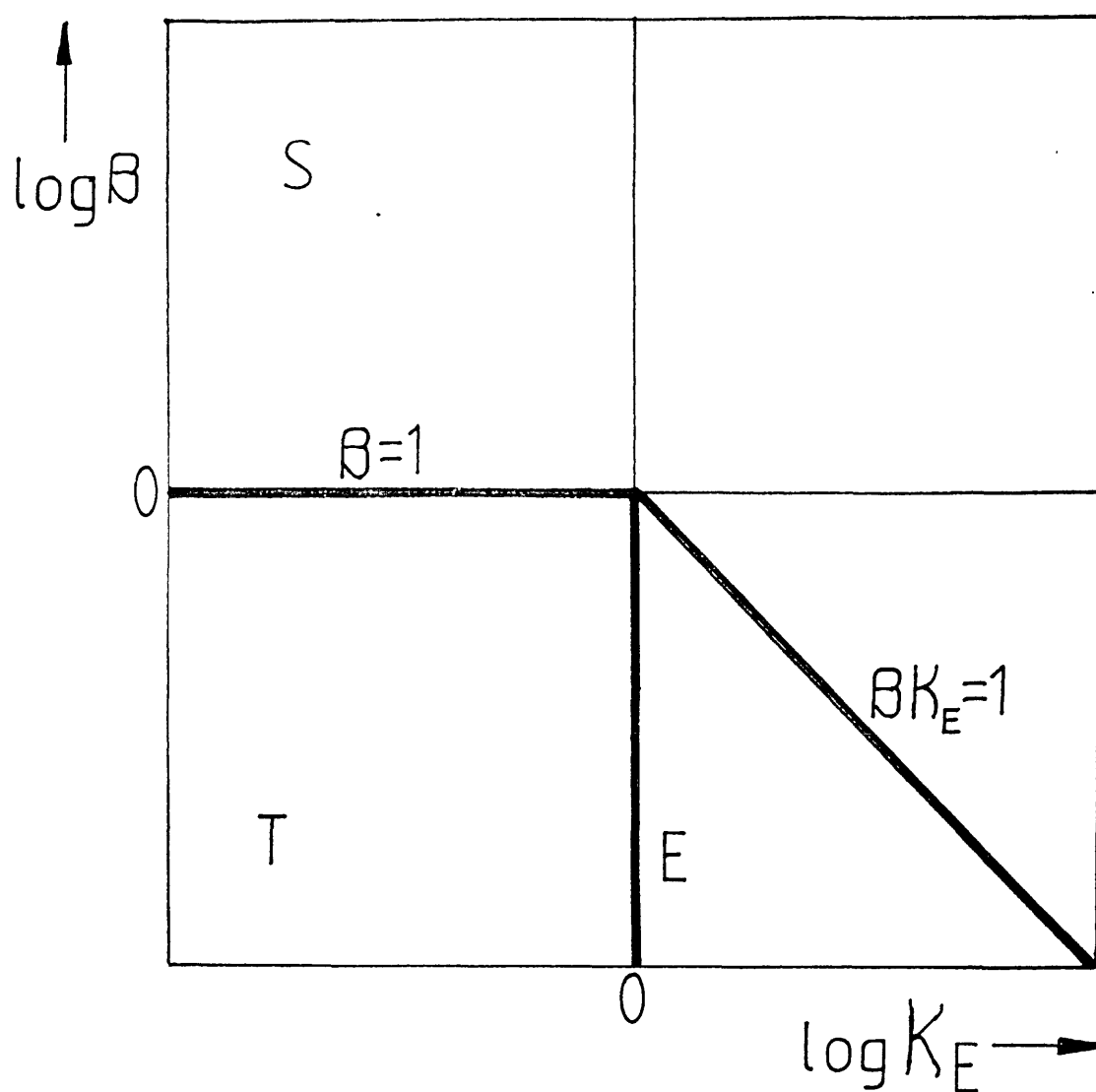


Figure 4.3.2 Case diagram for the heterogeneous redox catalysis model ($\theta_D \ll 1$).

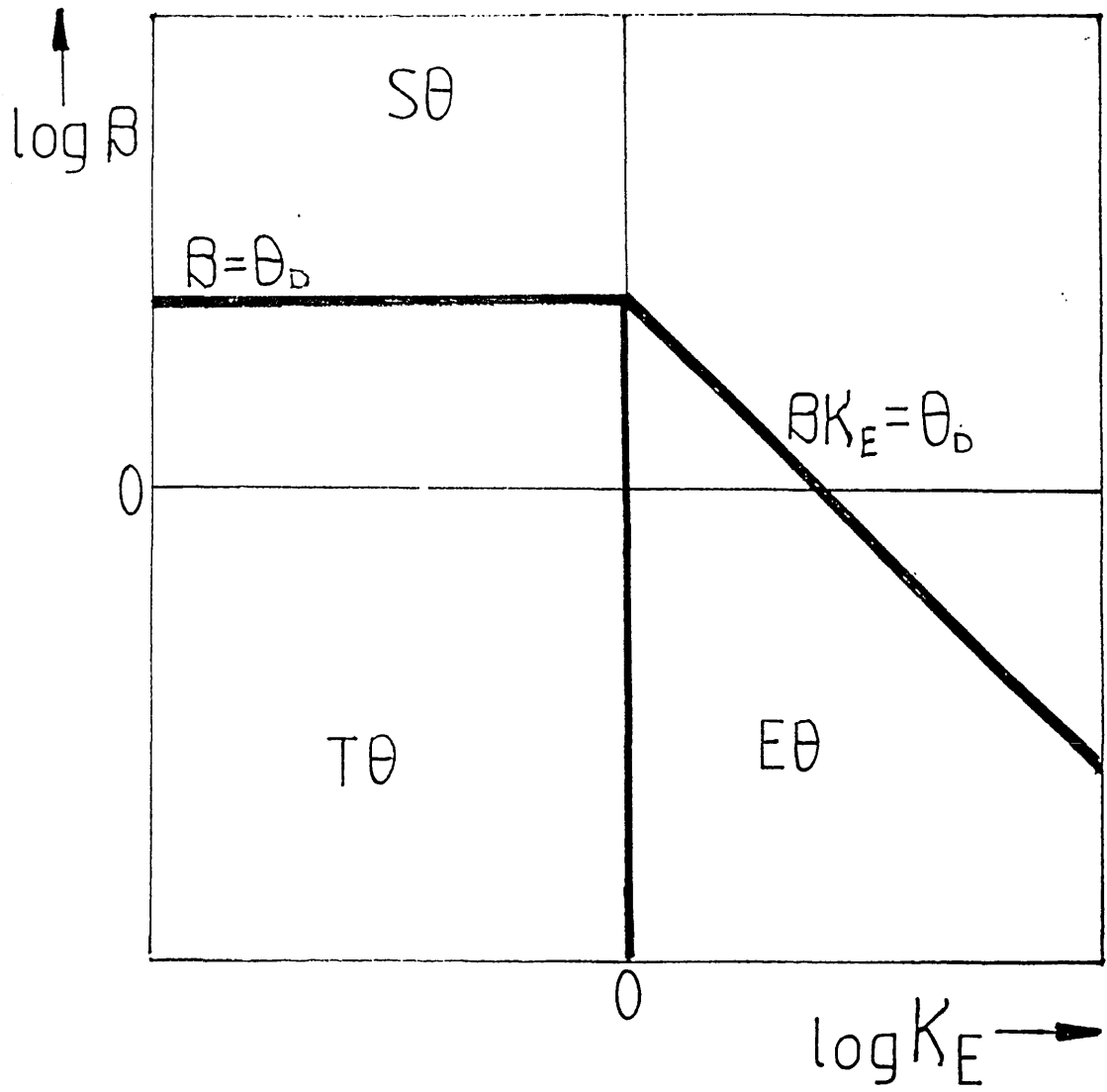


Figure 4.3.3 Case diagram for the heterogeneous redox catalysis model ($\theta_D \gg 1$).

TABLE 4.3.5

Approximate equations for flux for each case of the heterogeneous redox catalysis model.

Case	θ_D	Flux Given By
E	<1	$n(D_E k_E)^{1/2} e_\Sigma$
S	<1	$(nK_1 k_2 k_3 e_\Sigma)^{1/2}$
T	<1	$nD_E e_\Sigma / Z_D$
E_θ	>1	$n[(D_E k_E)^{1/2} e_\Sigma] / \theta_D$
S_θ	>1	$[(nK_1 k_2 k_3 e_\Sigma)^{1/2}] / \theta_D$
T_θ	>1	$n[(D_E e_\Sigma) / Z_D] / \theta_D$

TABLE 4.3.6

Order of flux with respect to enzyme concentration for each case of the heterogeneous redox catalysis model.

Case	Order w.r.t. e_L	Order w.r.t. W
E	1	0
S	1/2	0
T	1	+1/2
E_θ	1	-1/2
S_θ	1/2	-1/2
T_θ	1	0

Correlation coefficients for data shown in Figures 4.4.1a
and b:

Potential /mV	Correlation Coefficient	Points Used for Calculation *
300	0.9976	3-10
250	0.9988	4-10
200	0.98934	1-10
150	0.99583	2-10
100	0.99763	3-10
50	0.9981	1-10

Correlation coefficients for data shown in Figures 4.4.2a
and b:

Potential /mV	Correlation Coefficient	Points Used for Calculation *
300	0.99985	3-10
250	0.99976	4-10
200	0.99947	1-10
150	0.99977	2-10
100	0.99984	3-10
50	0.99388	1-10

* points are taken to be numbered sequentially in order of
increasing enzyme concentration from 1 (lowest
concentration) to 10 (highest concentration)

4.4 RESULTS

4.4(1) ROTATING DISC STUDIES

This section contains results from rotating disc studies of GOD at substrate saturation using a TTFTCNQ/silicone oil paste electrode. The current was measured at 10 enzyme concentrations between 10 and 90.9 units/ml for six potentials between 50 and 300 mV. Additionally the dependence of the current with rotation speed was investigated for three enzyme concentrations.

4.4(1).1 DEPENDENCE OF CURRENT ON ENZYME CONCENTRATION

Raw data showing the dependence of current i , with enzyme concentration e_L , at 4 Hz is shown in Figures 4.4.1a and b for the six potentials employed. It can be seen that the current is not linear with e_L at any potential and that it does not appear to change significantly with potential above +150 mV. Replotting the current against $(e_L)^{1/2}$ (Figures 4.4.2a and b), we find that we obtain more linear plots* (except for the lowest concentrations of enzyme) and we can therefore conclude that the current is approximately proportional to $(e_L)^{1/2}$. This means that in terms of the two proposed mechanisms we must be substantially in Case I (for homogeneous mediation) or Case S (for heterogeneous redox catalysis) perhaps approaching the boundary of another case at low enzyme concentrations.

*See table (opposite) for correlation coefficients

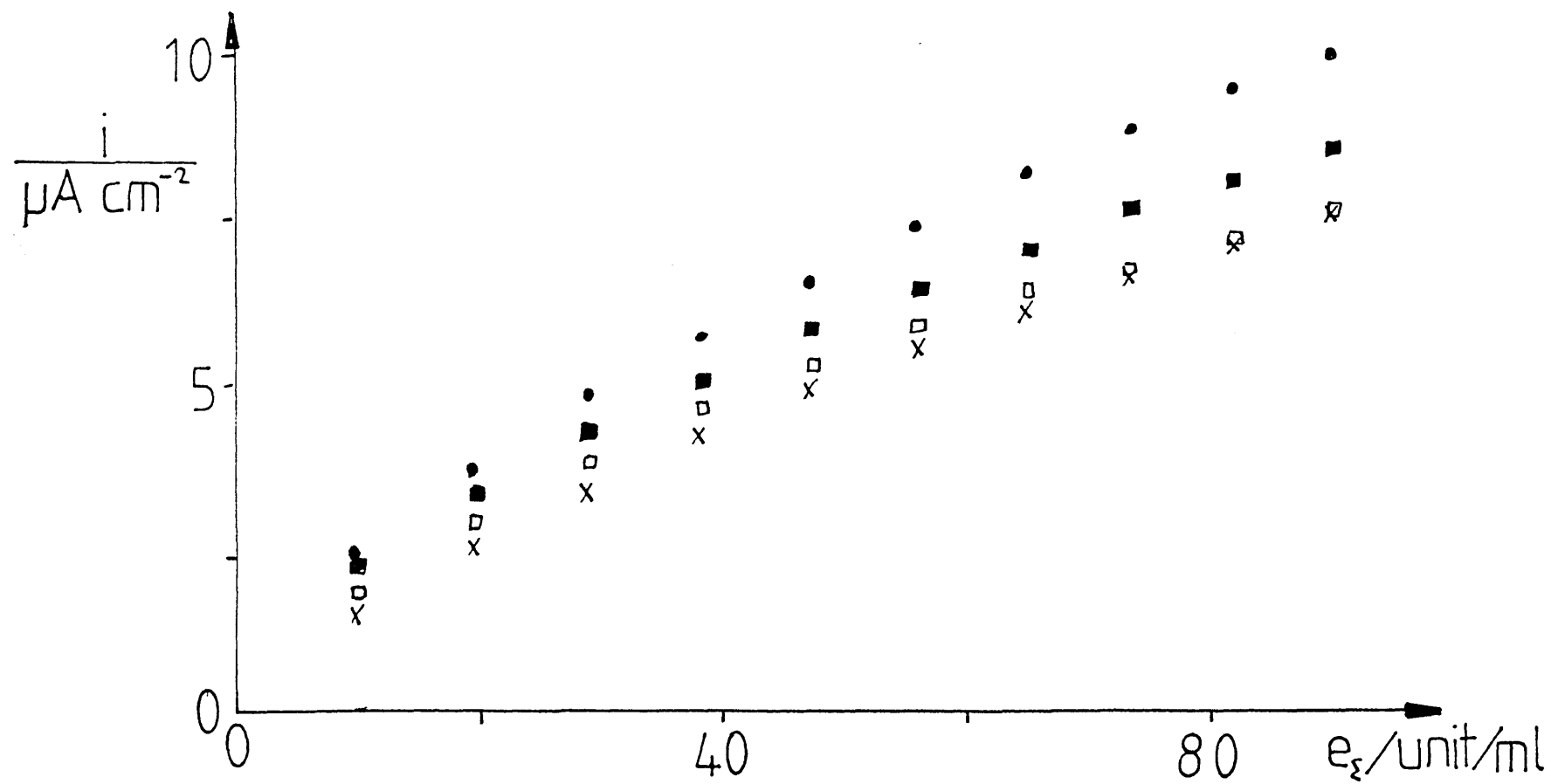


Figure 4.4.1 a) Response of the electrode to different concentrations of enzyme: (■) 150 mV, (□) 200 mV, (●) 250 mV and (×) 300 mV.

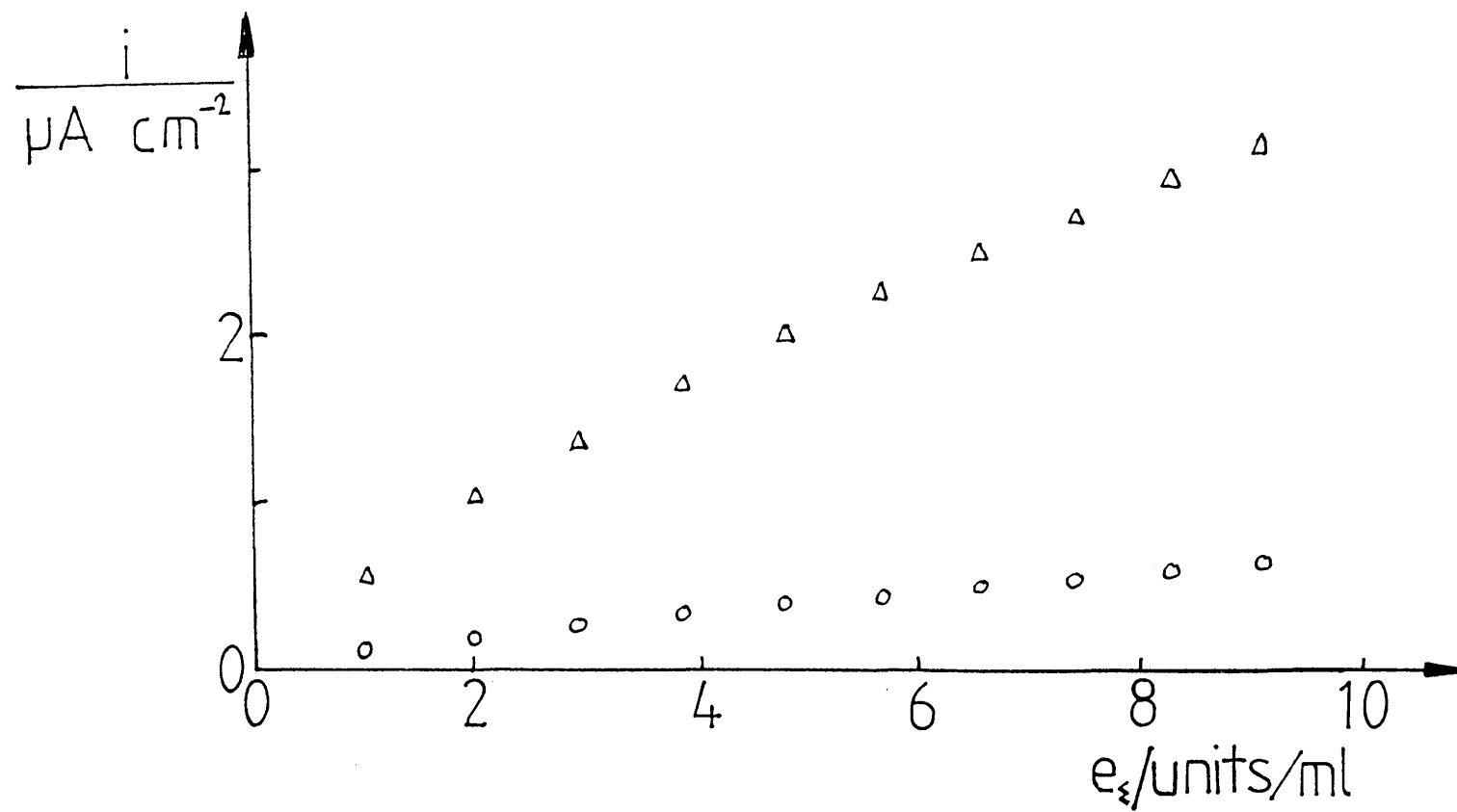


Figure 4.4.1 b) Response of the electrode to different concentrations of enzyme: (O) 50 mV and (Δ) 100 mV.

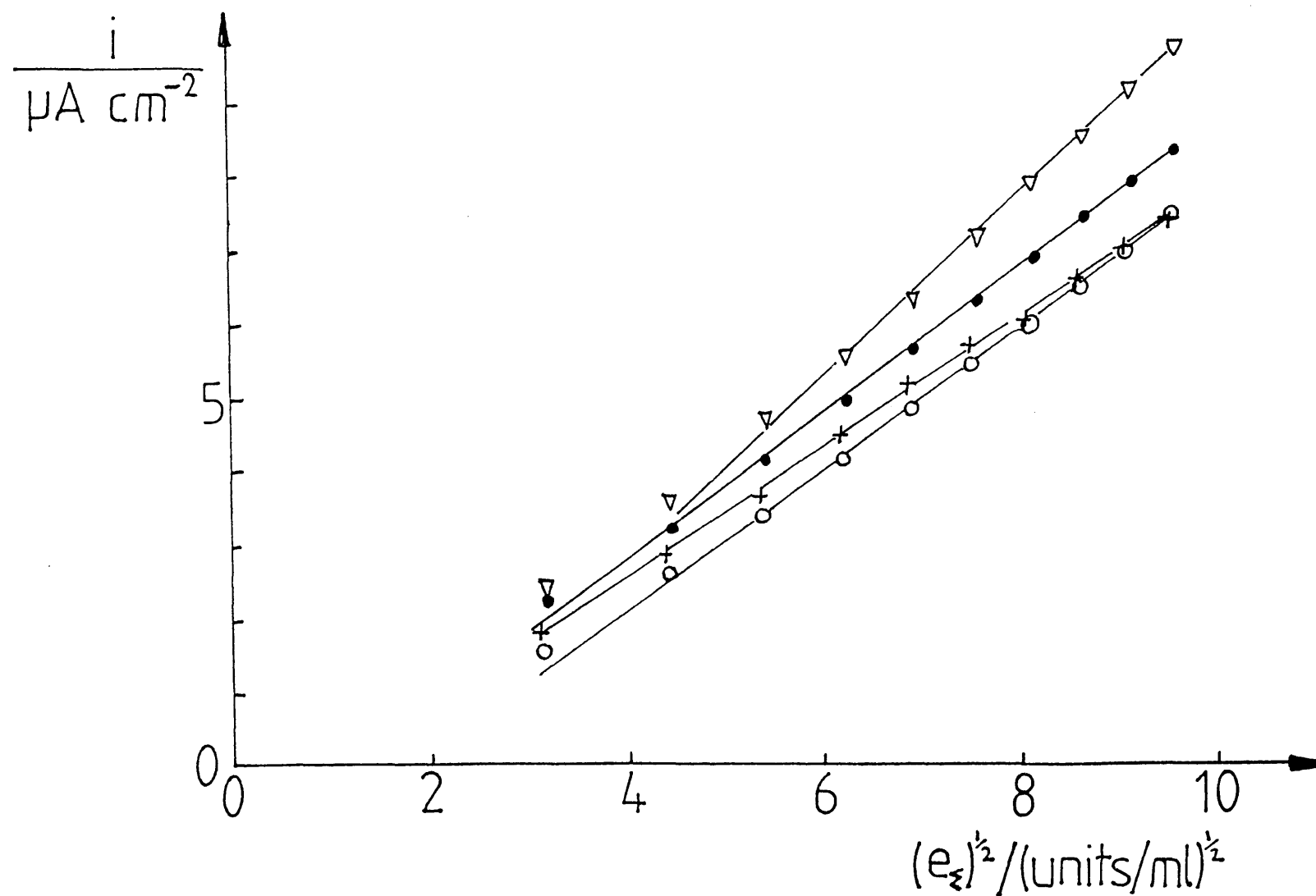


Figure 4.4.2 a) Data from Figure 4.4.1 replotted against square root of enzyme concentration: (●) 150 mV, (×) 200 mV, (▽) 250 mV and (○) 300 mV.

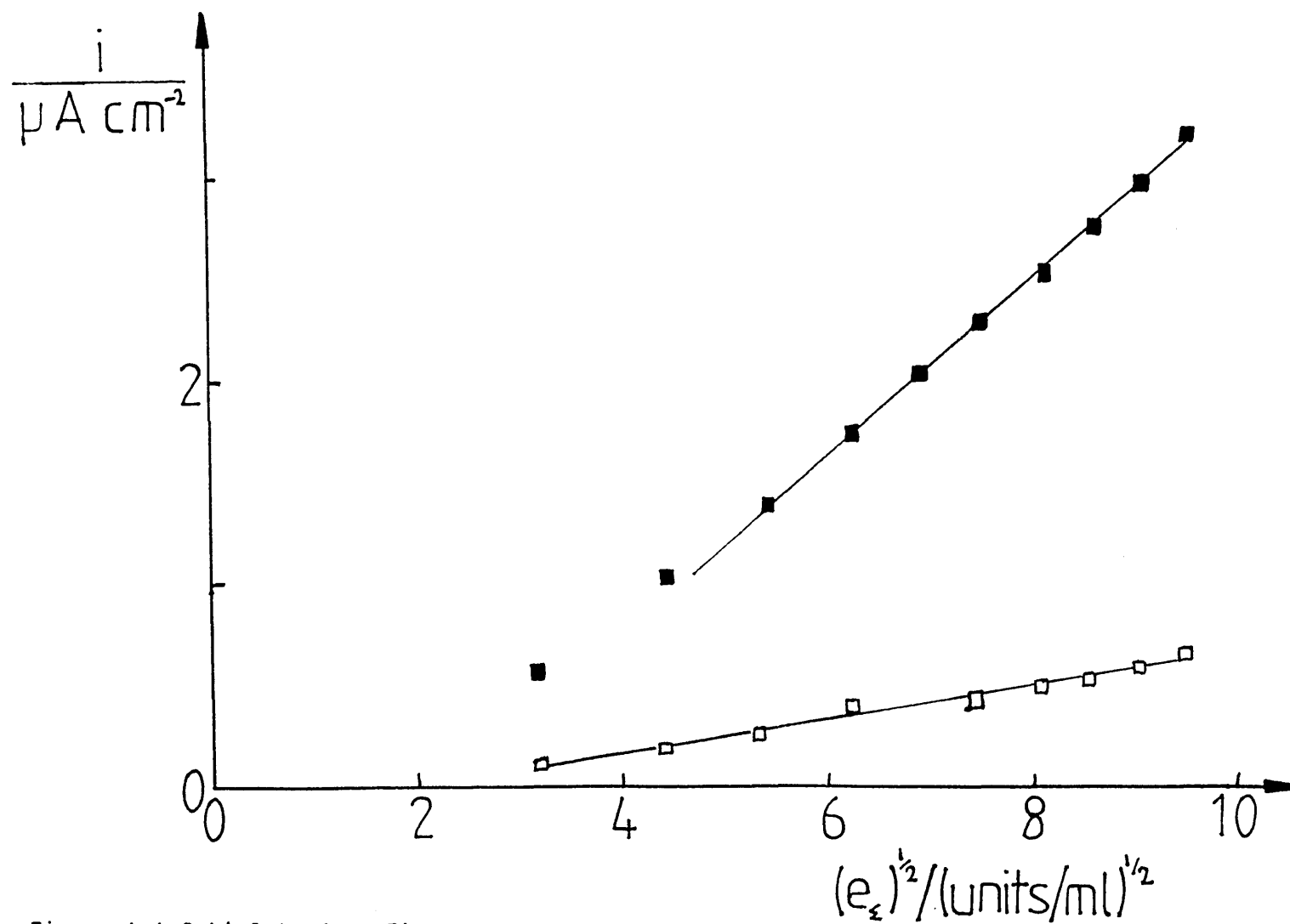


Figure 4.4.2 b) Data from Figure 4.4.1 replotted against square root of enzyme concentration: (□) 50 mV and (■) 100 mV.

4.4(1).2 DEPENDENCE OF CURRENT ON ROTATION SPEED

Figures 4.4.3a, b and c show how the current changes with rotation speed for enzyme concentrations of 29.1, 56.6 and 90.9 units/ml respectively. There is definitely an inverse relationship between current and rotation speed, except at +50 mV, where the current does not seem to change significantly with rotation speed. However it is obvious that i is not proportional to $W^{-1/2}$ because if so the current at 1 Hz would be thrice that at 9 Hz. This inverse relationship would seem to eliminate Case T of the heterogeneous redox theory as being important. This was expected as we can calculate that $K_E \gg 1$ (see Appendix 3) Furthermore, we cannot be solely in Case II of the homogeneous mediation theory because otherwise the current would depend substantially more on rotation speed.

As it is difficult to assess a priori the effect of changing the potential, for the moment we will treat the data at each potential individually. Comparing our conclusions from the previous section, we can predict that for the homogeneous mediation theory we are either working solely in Case I_K (with θ_M being comparable to unity, because of the relationship between current and rotation speed) or moving away from the I/II boundary into Case I with increasing enzyme concentration. In a similar way, for the heterogeneous redox catalysis theory, we must be in Case S_θ , or moving away from the E_θ/S_θ boundary with increasing enzyme concentration. We will examine these two alternatives in the next two sections, and finally we will

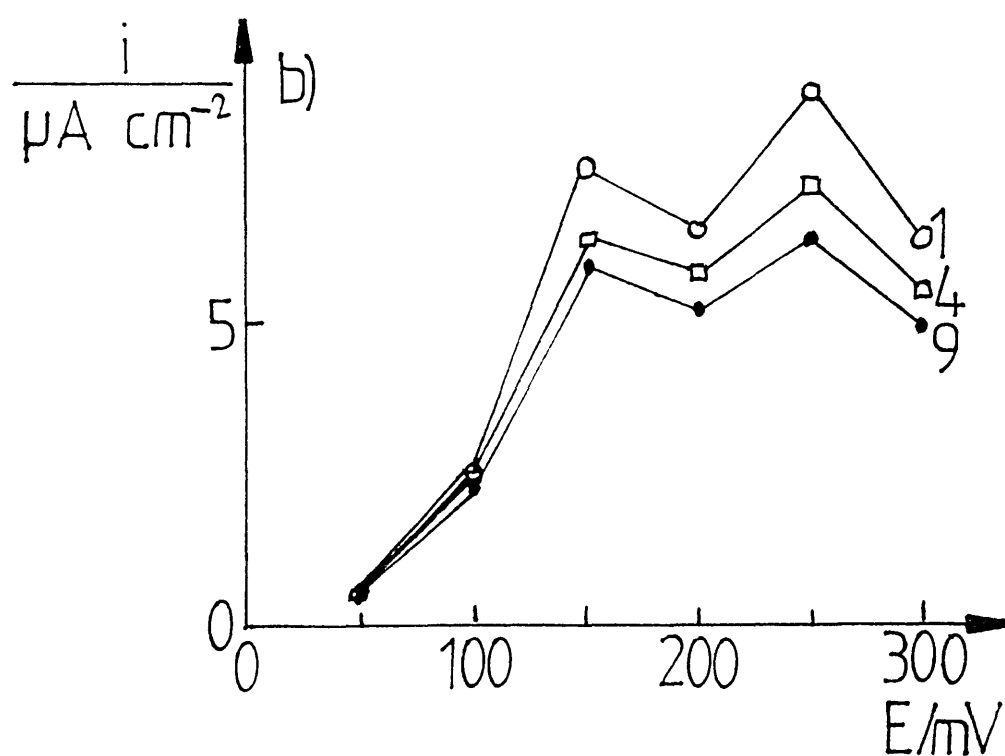
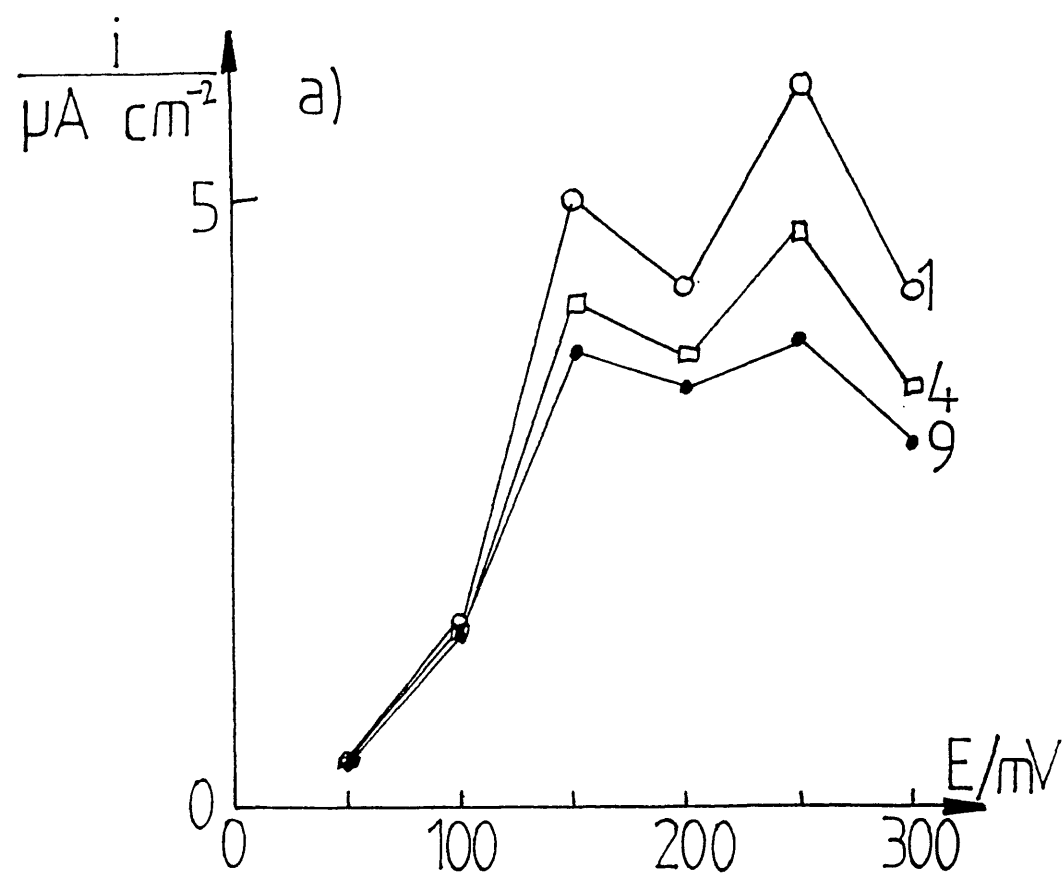


Figure 4.4.3 Variation of current with rotation speed for an enzyme concentration of a) 29.1 units/ml and b) 56.6 units/ml. Numbers 1, 4 and 9 on figures refer to rotation speed in Hz.

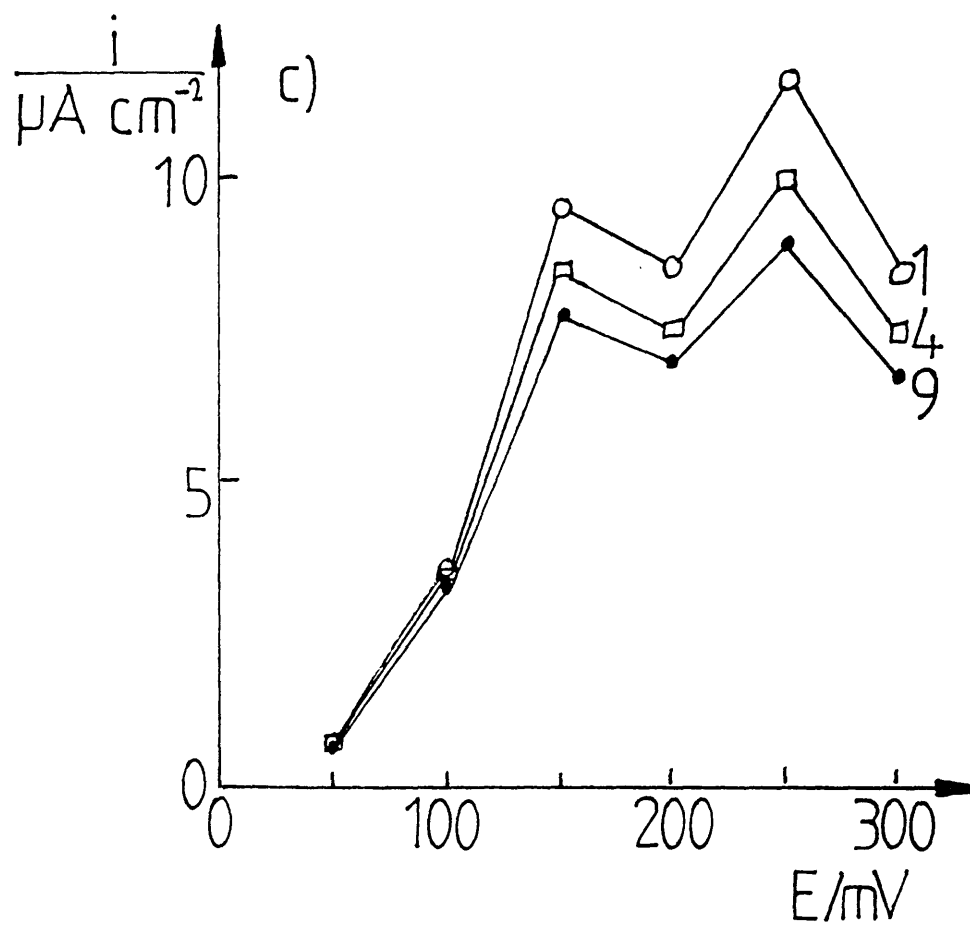


Figure 4.4.3 c) Variation of current with rotation speed for an enzyme concentration of 90.9 units/ml. Numbers 1, 4 and 9 on figure refers to rotation speed in Hz.

examine if it is possible to explain the potential dependence of the current.

4.4(1).3 FIT OF THE EXPERIMENTAL DATA TO THE HOMOGENEOUS MEDIATION MODEL

The master equation describing this system is as follows:

$$j = k_D m_{O,T} [(K_M)^{1/2} \coth(K_M)^{1/2} - 1] / (1 + \theta_M) \quad (4.21)$$

Analysis of the data can be made much easier if we can make some kind of simplifying assumption. Rotation speed dependence arises in the terms for K_M , k_D and θ_M . It is possible to show that, whatever the values of θ_M and k_D , $K_M > 4$ because otherwise we would observe much more rotation speed dependence. Thus we can assume that $\coth(K_M)^{1/2} \sim 1$ and so substituting for K_M , k_D and θ_M the expression simplifies to:

$$j = (C_1 (e_\Sigma)^{1/2} - C_2 W^{1/2}) / (1 + C_3 W^{1/2}) \quad (4.22)$$

where $C_1 = (D_M k_M)^{1/2} m_{O,T}$, $C_2 = (D_M / Z_{D,1}) m_{O,T}$ and $C_3 = D_M / (Z_{D,1} k_b)$.

It should be noted that because we have assumed that $K_M > 4$, j will always be positive in this expression (as we would expect from physical intuition). We can now rearrange this equation to give:

$$(j/W^{1/2}) = C_2 [(C_1/C_2) (e_\Sigma/W^{1/2}) - 1] / (1 + C_3 W^{1/2}) \quad (4.23)$$

If the homogeneous mediation model is correct, then experimental plots of $j/W^{1/2}$ against $(e_\Sigma/W^{1/2})$ must

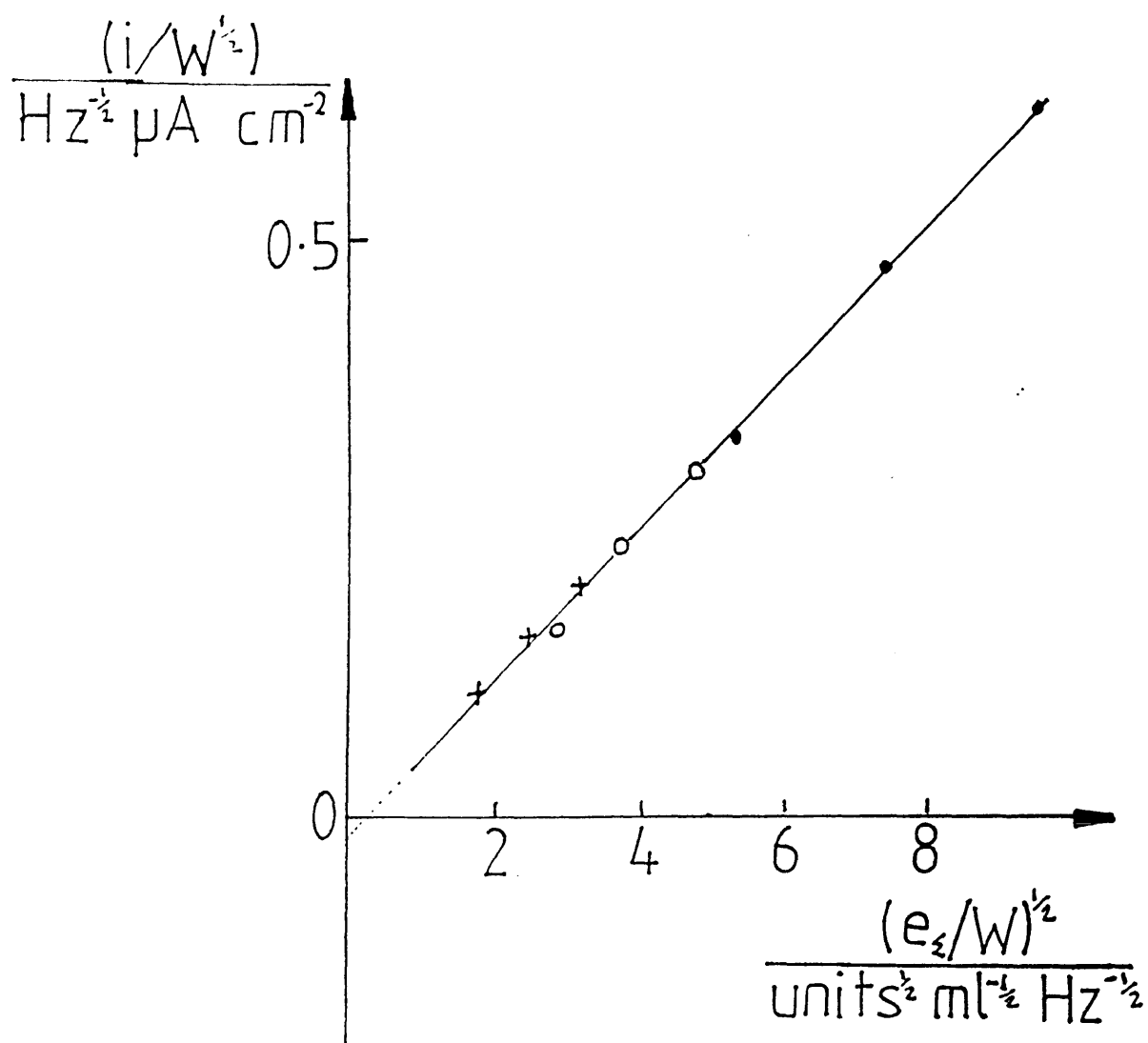
therefore satisfy the following conditions:

- i) at each rotation speed, we must obtain a straight line;
- ii) these straight lines should pass through a common intercept on the abscissa (we shall call this point I);
- iii) the gradients G , of each line should either remain constant or decrease with rotation speed and furthermore in the latter case a plot of $1/G$ against $W^{1/2}$ should be linear.

It will be seen that when we attempt to fit the experimental data to the heterogeneous redox catalysis model the conditions that the plots need to satisfy are practically the same, thus making comparison between the two models fair.

Figures 4.4.4a to f show such plots for the experimental data. For three potentials (namely +150, +200 and +250 mV) the data seems to satisfy the above conditions reasonably well. There is a common intercept on the abscissa and the gradients tend to decrease with W . The difference in the gradients is very small, however, showing that C_3 and hence θ_M are not very significant. For the other three potentials the data do not fit as well. However, from the previous three plots, we can assume that in these cases θ_M is quite small, and thus force a straight line through all the data. This should give us a rough idea of the values of $m_{O,T}$ and k_M at these potentials.

From the gradients and the intercept we therefore find for $m_{O,T}$ and k_M as shown in Table 4.4.1. The values for



• 1 Hz

○ 4 Hz

+ 9 Hz

Figure 4.4.4 a) Diagnostic plot for homogeneous mediation model for data at 50 mV.

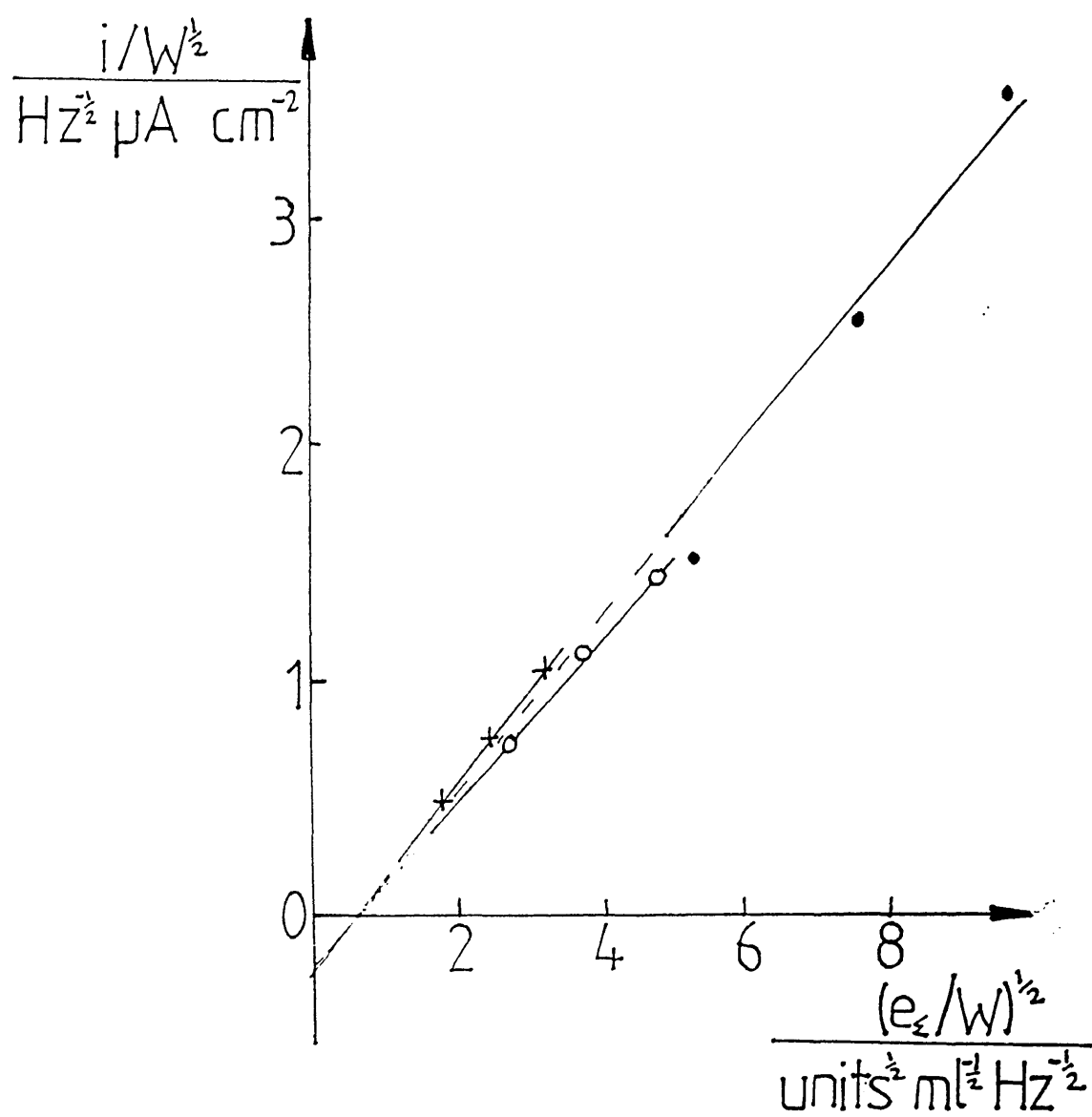
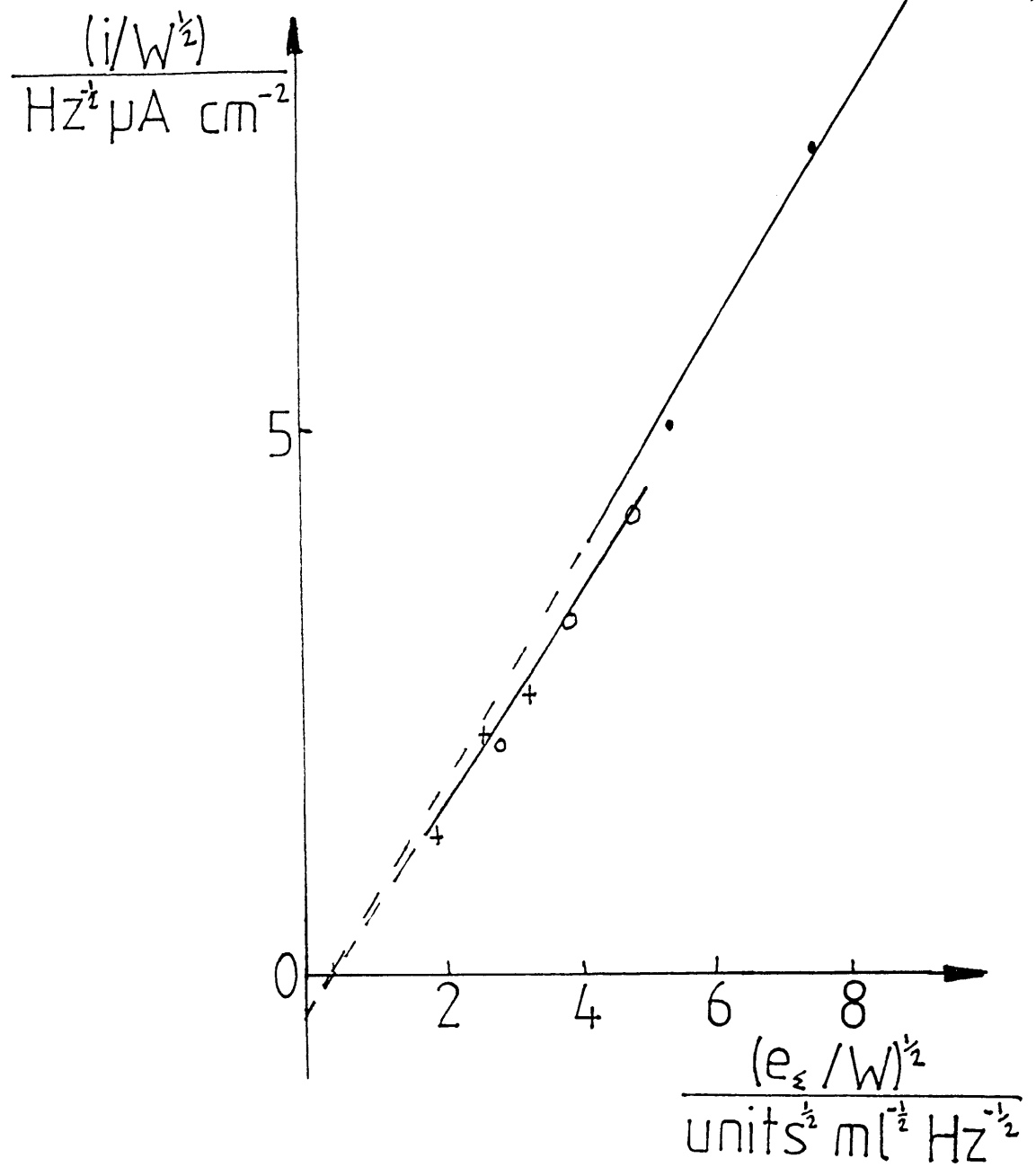


Figure 4.4.4 b) Diagnostic plot for homogeneous mediation model for data at 100 mV.



• 1 Hz
 ○ 4 Hz
 + 9 Hz

Figure 4.4.4 c) Diagnostic plot for homogeneous mediation model for data at 150 mV.

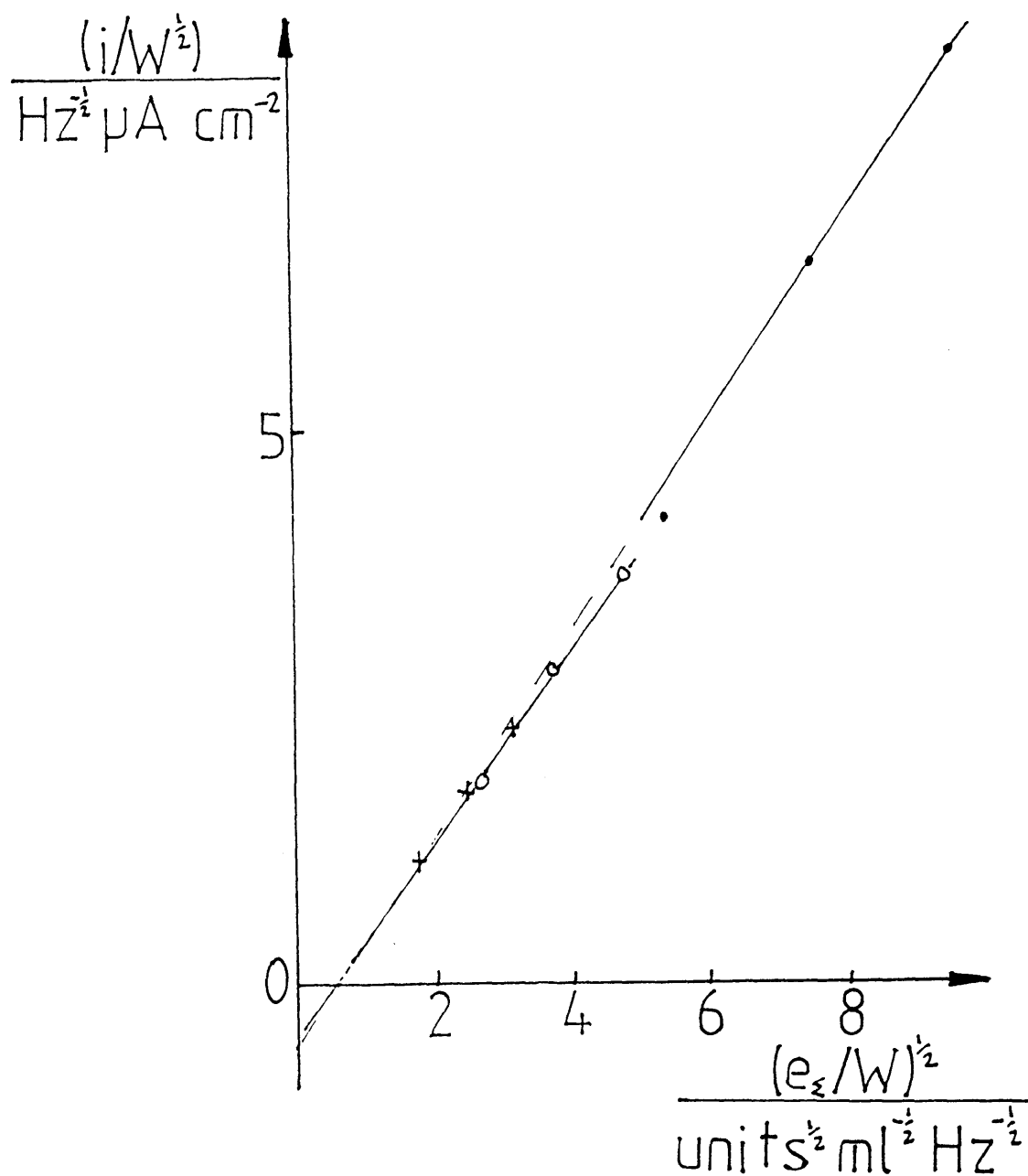


Figure 4.4.4 d) Diagnostic plot for homogeneous mediation model for data at 200 mV.

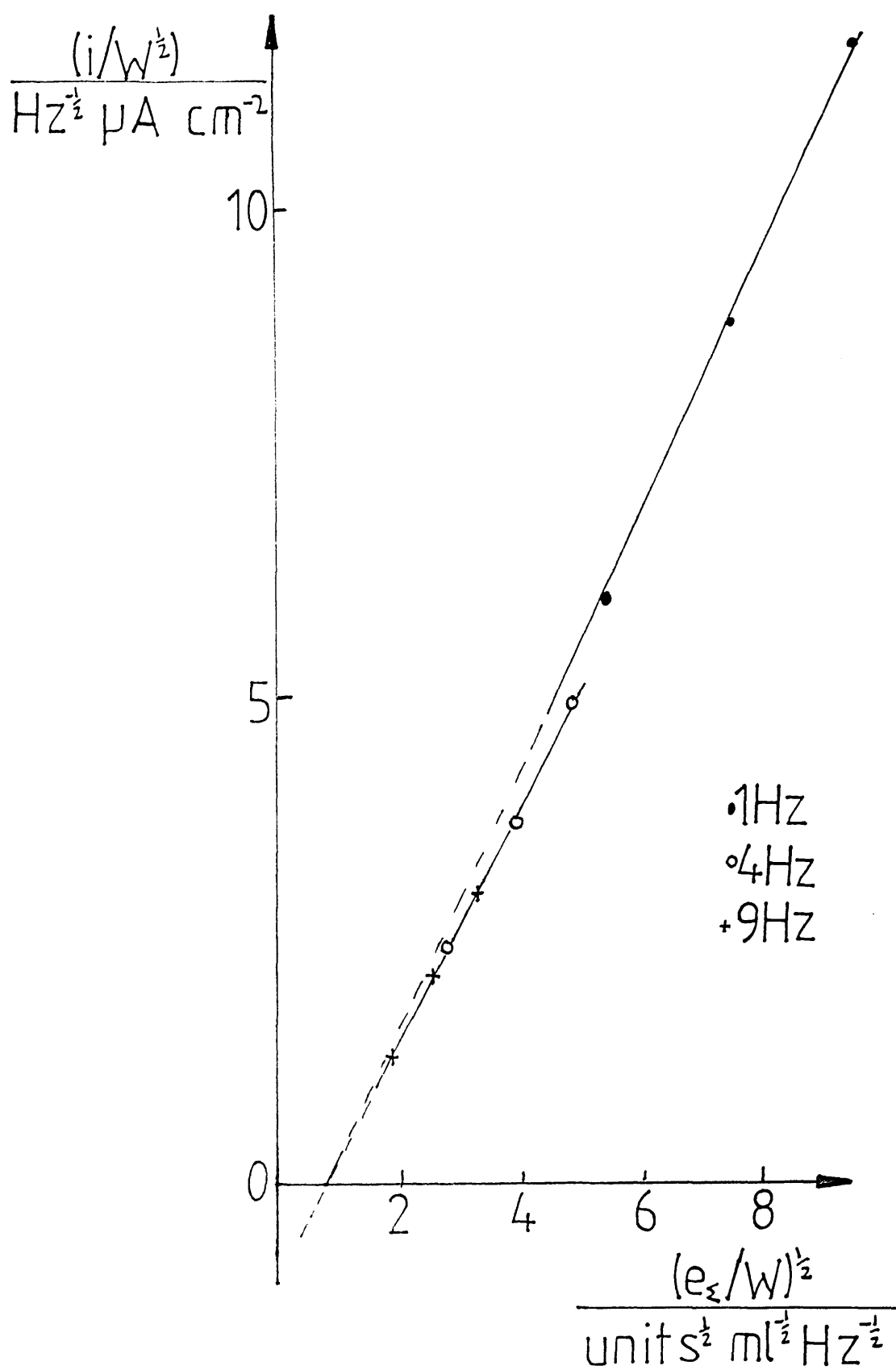
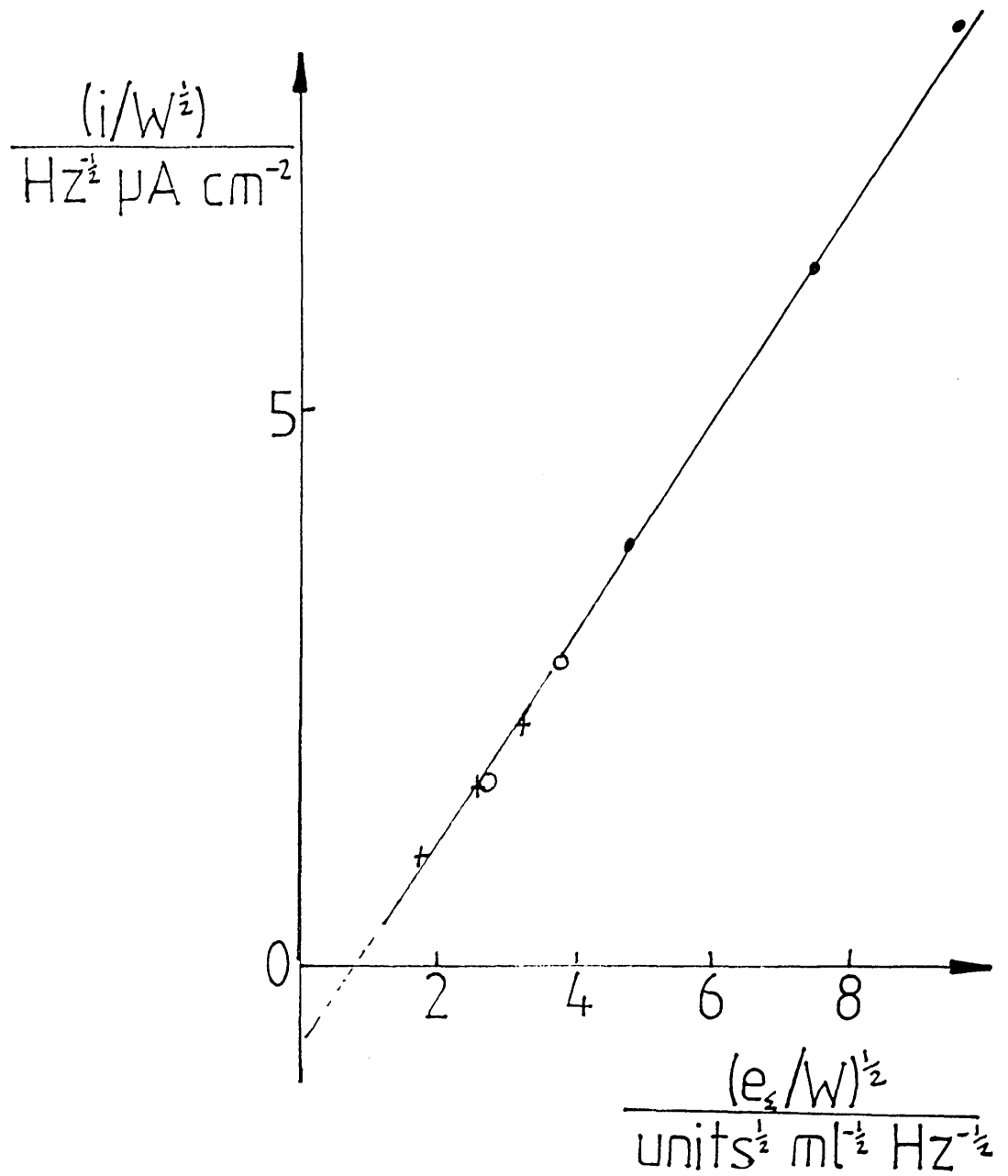


Figure 4.4.4 e) Diagnostic plot for homogeneous mediation model for data at 250 mV.



• 1 Hz
 ○ 4 Hz
 + 9 Hz

Figure 4.4.4 f) Diagnostic plot for homogeneous mediation model for data at 300 mV.

TABLE 4.4.1

Kinetic parameters determined experimentally from the diagnostic plots.

HOMOGENEOUS MEDIATION MODEL

Potential	$m_{o,T}$	$10^{-6} k_H$
/ mV	/ $\mu\text{mol dm}^{-3}$	/ $\text{mol}^{-1} \text{dm}^3 \text{s}^{-1}$
50 [*]	0.06	140
100 [*]	1.4	8
150	2.4	23
200	2.2	22
250	4.3	11
300 [*]	4.0	6

HETEROGENEOUS REDOX CATALYSIS MODEL

Potential	$(D_E k_E)^{1/2}$	$10^{-15} K_1 k_2 k_3$	K_S/k_2
/ mV	/ cm s^{-1}	/ $\text{mol cm}^{-1} \text{s}^{-1}$	/ $\text{cm}^{-1} \text{s}$
50 [*]	0.006	8.5	-
100 [*]	0.015	460	118
150	0.082	4500	391
200	0.067	2200	200
250 [*]	0.11	5500	450
300	0.055	3600	333

* values of parameters at these potentials were obtained from poorer quality plots and therefore will have a greater error.

$m_{O,T}$ all lie in the μ molar range or below, which would be expected given previous experimental evidence. However the values of k_M obtained seem much higher than those measured for reaction of GOD with either $TCNQ^0$ or $TCNQ^-$ in solution, and if this model is correct we must therefore rule out these two species as candidate mediators. This leaves TTF^+ (or perhaps TTF^{2+}) as the only alternatives. As the value for k_M has not been definitively measured for either of these two cations, their determination should be an important part of future work.

The diagnostic plots presented above therefore seem clearly to support the homogeneous mediation model as the true mechanism for this system. Whilst three potentials gave plots that were not perfect, they still yielded reasonable values for $m_{O,T}$ and k_M . It is also interesting that the values for k_M are all relatively constant with potential (the value obtained at 50 mV is much higher than the rest, but this may be because of the higher experimental error for this data). This is just as expected and is further evidence in support of this model. The plots confirm our preliminary conclusions that we are working in Case I_T. As we have shown that θ_M is not very significant the mediator concentration must be controlled mainly by the thermodynamics of the system and not its dissolution kinetics. At low enzyme concentrations we are quite close to Case II_T and this is why there is some dependence of current on rotation speed. We will now see how well the experimental results fit the heterogeneous redox catalysis

model.

4.4(1).4 FIT OF THE DATA TO THE HETEROGENEOUS REDOX

CATALYSIS THEORY

The master equation for this theory is:

$$Bf^2 + f \tanh[(K_E)^{1/2}] / (K_E)^{1/2} - (1 + \theta_D)^{-1} = 0. \quad (4.24)$$

Because we know that $K_E \gg 1$, we can assume that

$\tanh(K_E) \sim 1$. Hence substituting for K_E , f , B and θ_D after rearrangement we find

$$j = (e_L/j)F_1 / (1 + F_2 W^{1/2}) - F_3 \quad (4.25)$$

where $F_1 = nK_1 k_2 k_3$, $F_2 = (K_S D_D) / (Z_{D,1} k_2)$ and

$$F_3 = (K_1 k_2 k_3) / (D_E K_E)^{1/2}.$$

If this mechanism is correct, then a plot of i against i/e_L should give a straight line at constant rotation speed. The gradient should either decrease or remain constant with rotation speed depending on the value of F_2 , and the intercept on the ordinate should always be the same at any rotation speed. Furthermore, a plot of the reciprocal of the gradient against rotation speed should be a straight line. As stated in the preceding section, these conditions are almost the same as the fit for the homogeneous mediation theory, except that the intercept must be constant on the ordinate in this case and not the abscissa. Figures 4.4.5a to f show such plots for the experimental data.

For two of the potentials (namely +200 and +300 mV) the

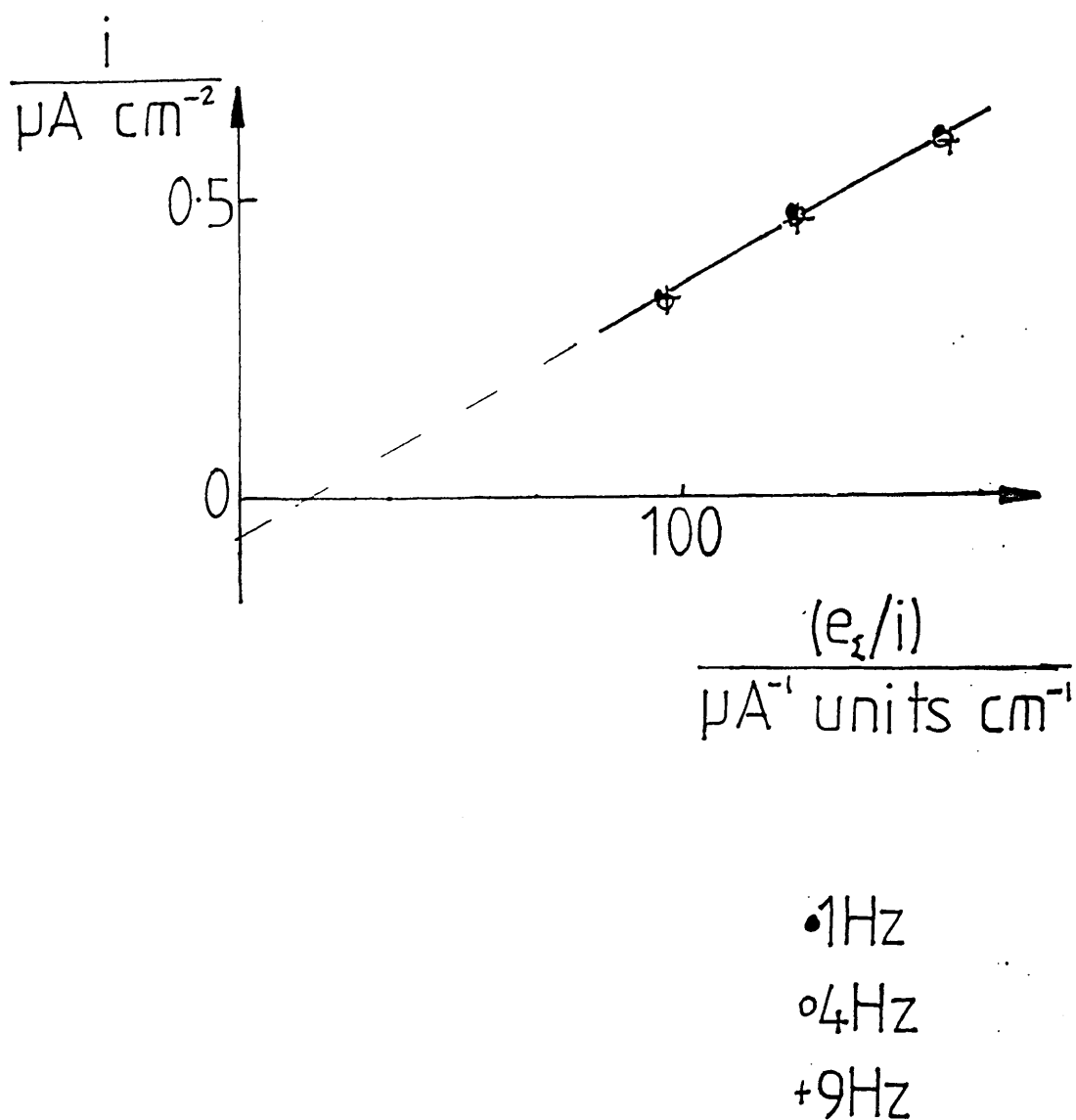


Figure 4.4.5 a) Diagnostic plot for heterogeneous redox catalysis model for data at 50 mV.

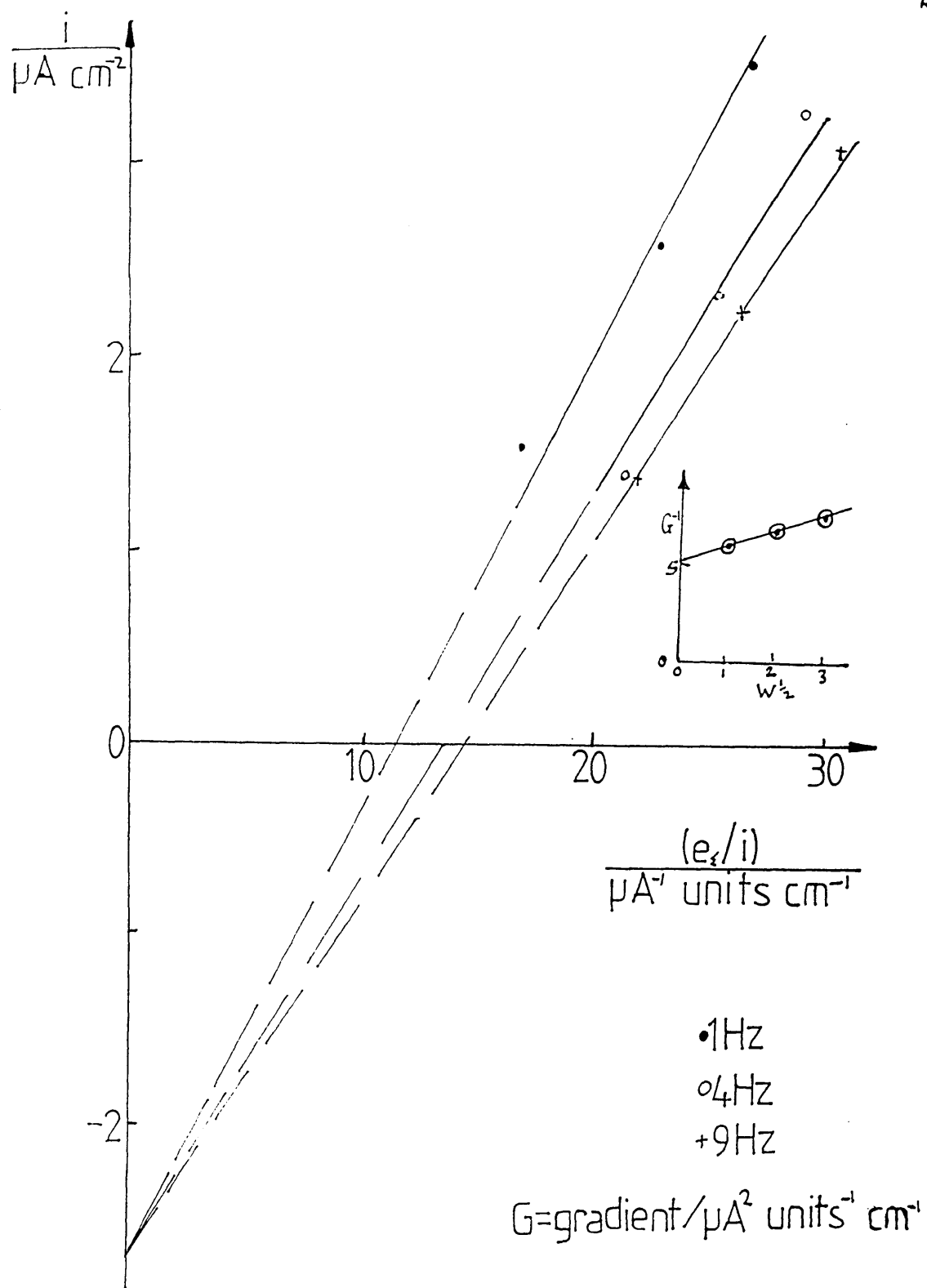


Figure 4.4.5 b) Diagnostic plot for heterogeneous redox catalysis model for data at 100 mV.

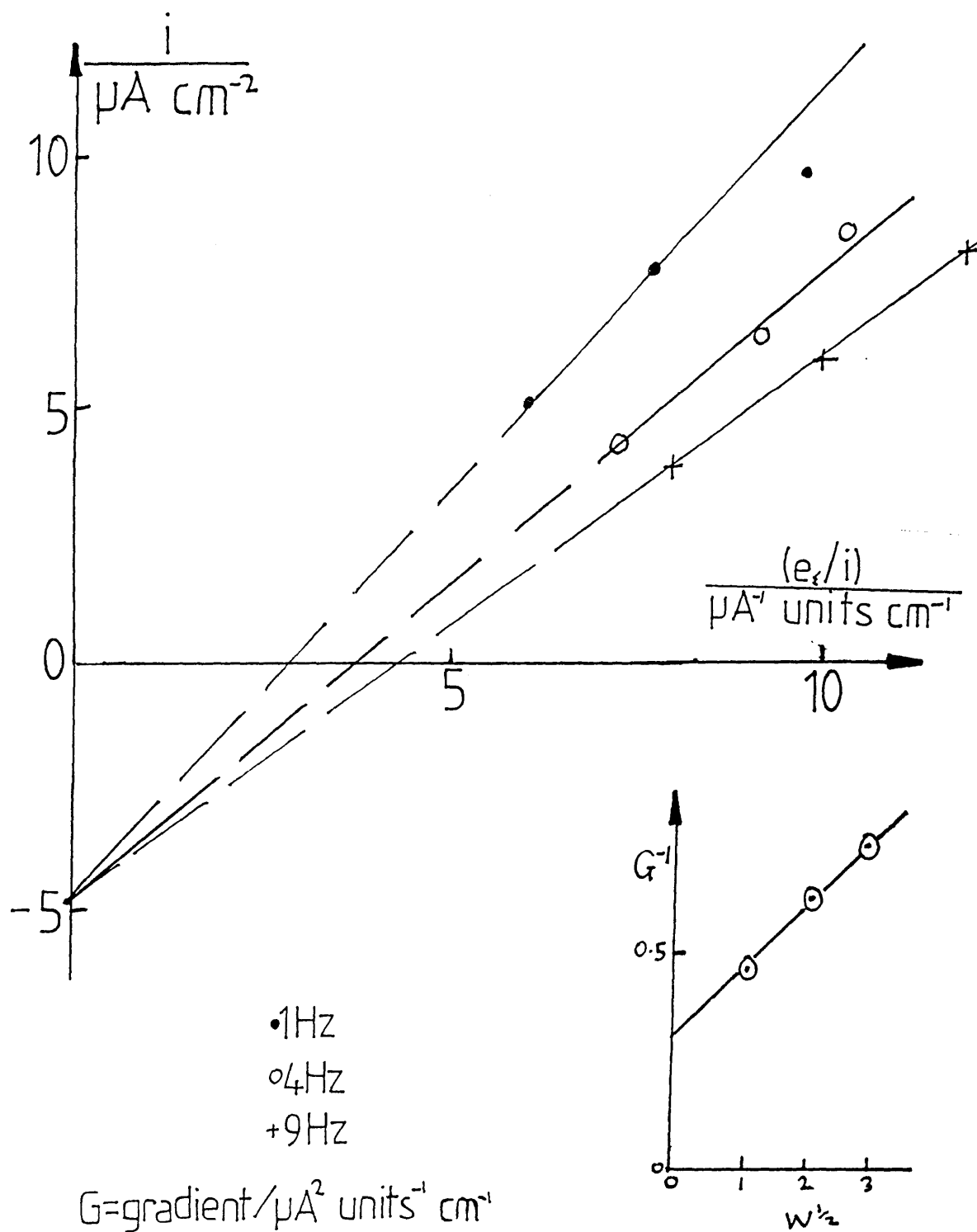


Figure 4.4.5 c) Diagnostic plot for heterogeneous redox catalysis model for data at 150 mV.

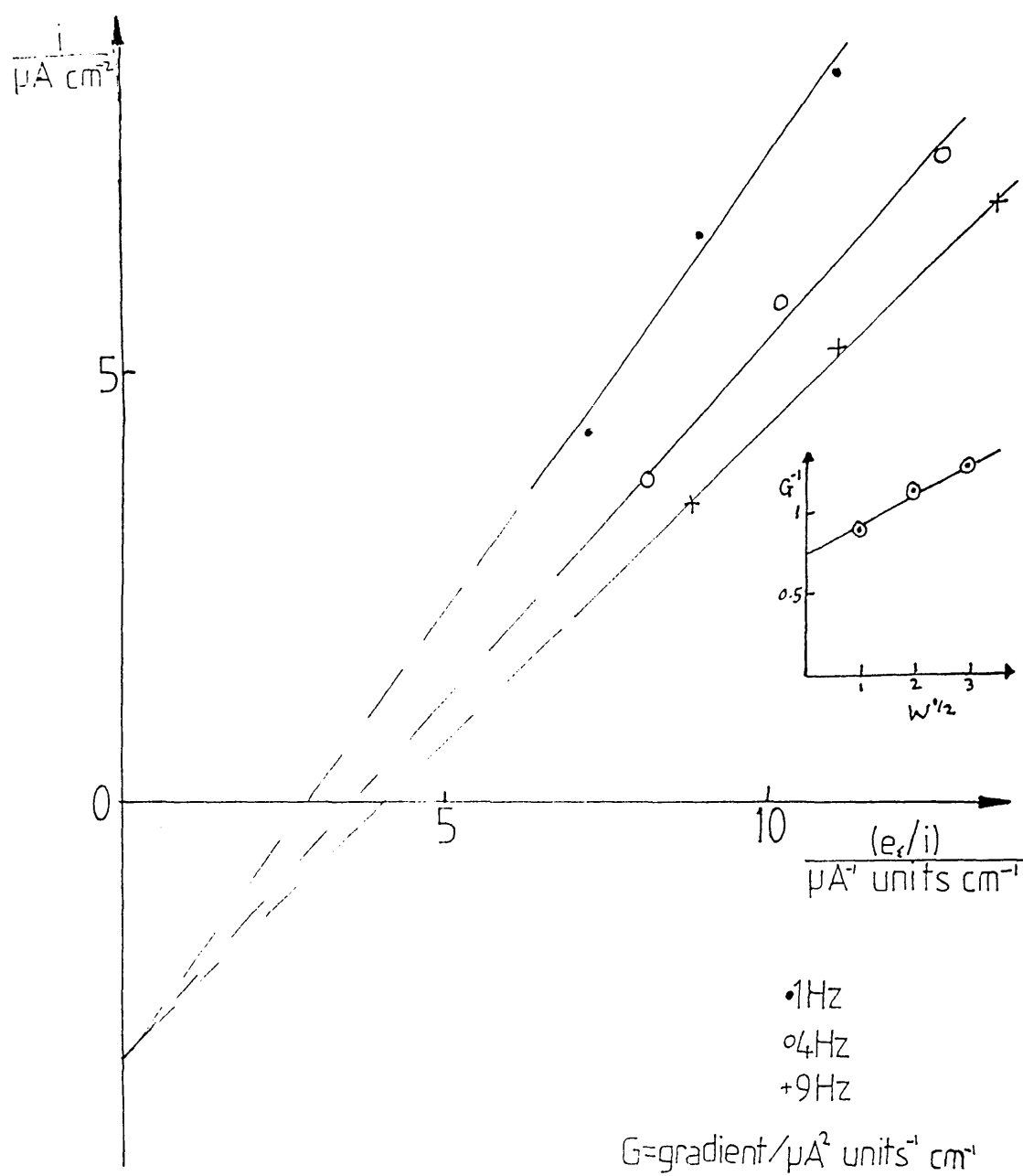


Figure 4.4.5 d) Diagnostic plot for heterogeneous redox catalysis model for data at 200 mV.

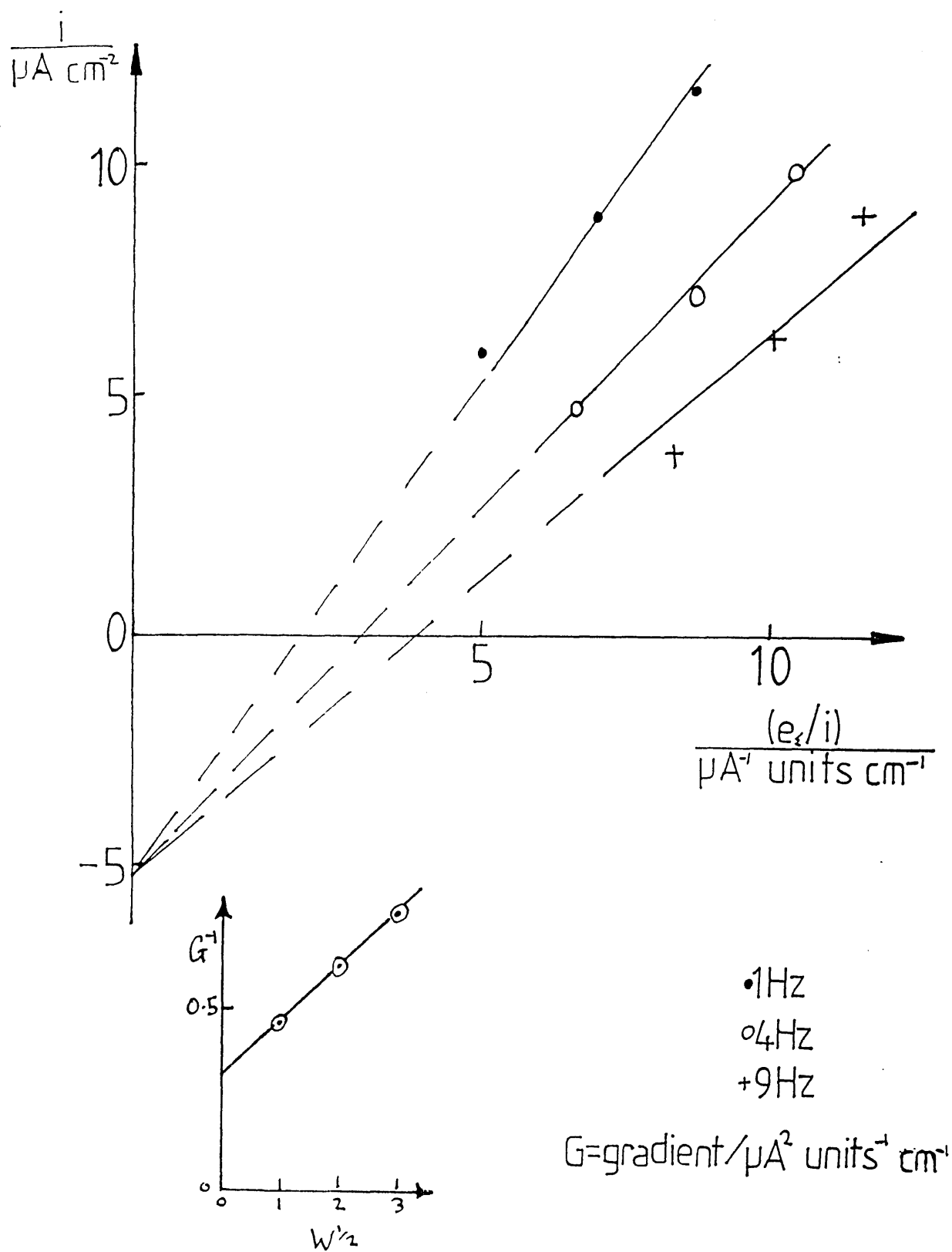


Figure 4.4.5 e) Diagnostic plot for heterogeneous redox catalysis model for data at 250 mV.

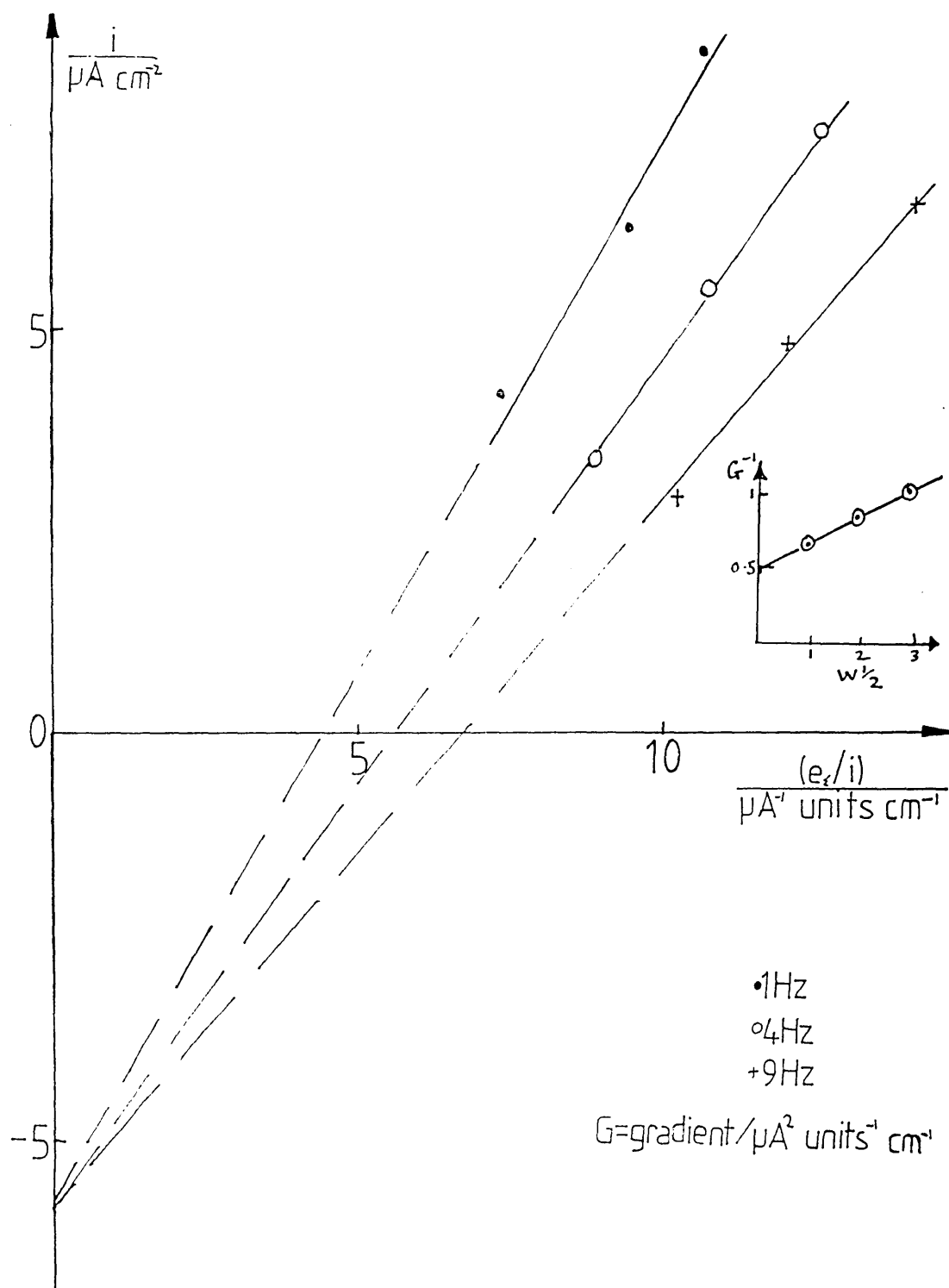


Figure 4.4.5 f) Diagnostic plot for heterogeneous redox catalysis model for data at 300 mV.

data does indeed behave as this theory would predict. At +150 mV the data could also fit the theory reasonably well. However, the plots at the other potentials do not appear to fit the theory quite so well. As in the treatment for the homogeneous mediation model, we can attempt to force the data to fit the theory, and this is shown in the plots.

From all the plots we can therefore find values for F_1 , F_2 , and F_3 and thus obtain estimates for the rate constants shown in Table 4.4.1. It is difficult to estimate values independently for K_1 , k_2 , k_3 and K_S but we can estimate from published data (see Section 4.3) that the value of $(D_E k_E)^{1/2}$ should be $\sim 0.04 \text{ cm s}^{-1}$. Except for the value obtained at 50 mV, this agrees quite well with those determined experimentally. The discrepancy seen for the 50 mV data may again be due to experimental error.

On the basis of these diagnostic plots we can not eliminate this model as a possible mechanism to describe this system. Two, and possibly three, potentials fit the theory quite well, but the other potentials gave poor plots although within experimental error yielded reasonable values for $(D_E k_E)^{1/2}$. It is however satisfactory that $K_1 k_2 k_3$ seems to be smaller at low potentials, which would be expected because of the smaller currents under these conditions. K_S/k_2 also seems to decrease at low potentials, which again is to be expected because of the loss of rotation speed dependence. The reason why some of the plots were so unsatisfactory may not be because this model is wrong but because of unknown experimental factors (such as adsorbed

enzyme or drifting background currents) which may be affecting the data. Further work now being carried out in these laboratories should resolve this question^[97].

4.4(1).5 DEPENDENCE OF CURRENT WITH POTENTIAL

Qualitative analysis of the data shows that the current increases with potential and that at low potentials it becomes less dependent on rotation speed. For the homogeneous mediation theory θ_M and $m_{O,T}$ could both change with potential. The master equation is of the form:

$$j = \{(m_{O,T}/(1 + \theta_M))\} \cdot f(e_{\Sigma}, W^{1/2}) \quad (4.26)$$

We have already shown that at high potentials θ_M is quite small and that the rotation speed dependence comes from the function $f(e_{\Sigma}, W^{1/2})$. This function should not change with potential. We must therefore explain the decrease in current by assuming that $m_{O,T}$ increases with potential and/or that θ_M becomes larger as the potential decreases. Inspection of Table 4.4.1 shows that indeed $m_{O,T}$ does increase with potential and so this appears to explain the smaller currents at low potentials.

Unfortunately for this model we now arrive at a contradiction when we also consider the loss of rotation speed dependence at low potentials. Inspection of equation 4.26 shows that changes in $m_{O,T}$ would not affect the rotation speed dependence. θ_M cannot be increased at low potentials because this would give rise to more rotation

speed dependence. Thus it is impossible to explain the experimental results by this model in its present form.

Turning to the heterogeneous redox catalysis theory, potential dependence arises out of the terms involving θ_D and $K_1 k_2 k_3$ whilst rotation speed dependence is determined by the magnitude of θ_D . A decrease in current can be explained by an increase in θ_D or a decrease in $K_1 k_2 k_3$. Table 4.4.1 confirms that the latter parameter does decrease considerably with potential. K_S/k_2 and hence θ_D also decrease with potential, thus explaining the loss of rotation speed dependence at low potentials. This model therefore accounts for the changes in the system with potentials in a very satisfactory manner.

4.4(1).6 COMPARISON OF RESULTS FOR THE HOMOGENEOUS MEDIATION AND HETEROGENEOUS REDOX CATALYSIS MODELS

In order to evaluate both models it is necessary to look at the results regarding the current's dependence on rotation speed, enzyme concentration and potential and the quality of the experimental plots obtained for each mechanism. Qualitatively the overall results can not be explained by the homogeneous mediation theory because it can not explain the observed decrease in rotation speed dependence at low potentials. However the fit of the data to the homogeneous mediation model appears to be better than that for the heterogeneous catalysis model. Using either model, we derive values for parameters such as k_M and $(D_E k_E)^{1/2}$ and $m_{O,T}$ which appear credible.

Of course we must not exclude the possibility that there may be a feature of either model that we have ignored. However the results presented here seem to be rather inconclusive. Work now being carried out in these laboratories involving further ring/disc studies and investigation of the system at low substrate concentrations should give us a clearer picture in the near future^[97].

4.4(2) THE GLUCOSE MEMBRANE ELECTRODE

In this section results are presented which show how the response of a glucose membrane electrode changes with potential over a range of glucose concentrations which encompasses both saturated and unsaturated conditions. The organic salt electrode employed was electrochemically synthesised exactly as described in Section 3.3(2).2 of Chapter 3.

The response of the electrode to glucose at each potential is shown in Figures 4.4.6a and b. Looking at these curves it can be seen that $i_{\max}$ increases with potential and that the dynamic range of the sensor is also increased. If we now turn to the Hanes plots of the data (Figure 4.4.7) it can also be seen that at low concentrations the response of the electrode becomes more linear as the potential becomes larger and the curves at each potential seem to reach a common intercept (the response is linear when the gradient of the line is zero).

We can look at the results in a different way by examining how the current changes with potential at a given substrate concentration. This is shown in Figure 4.4.8, where $\ln(i)$ is plotted against potential E , for several different glucose concentrations. At high potentials and low glucose concentrations, the response of the electrode remains approximately constant. This is why the Hanes plots at each potential have a common intercept. However, at high substrate concentrations the plot almost becomes a straight

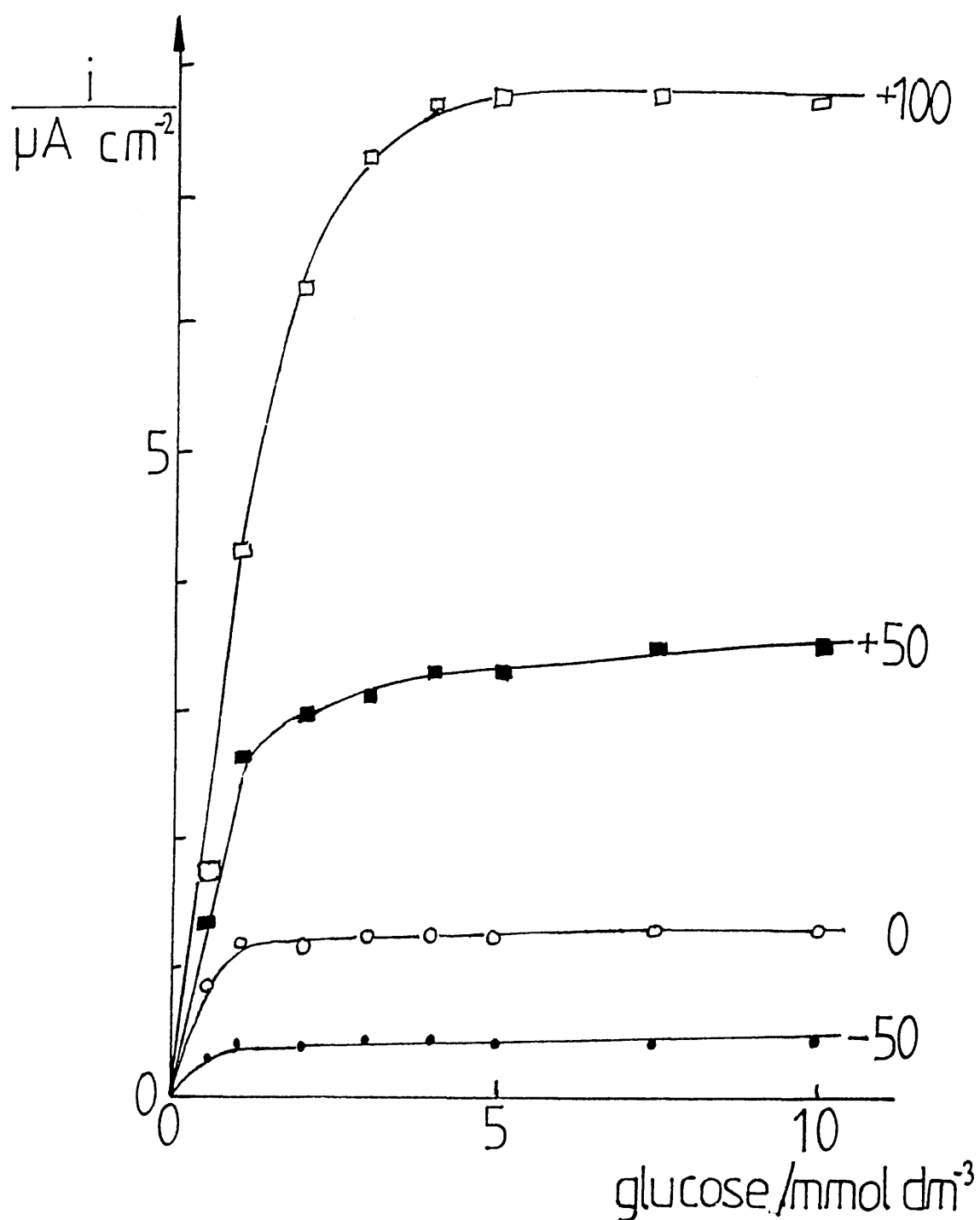


Figure 4.4.6 a) Response of the membrane electrode to glucose at different potentials. Numbers -50, 0, +50 and +100 on figure refer to potential in mV.

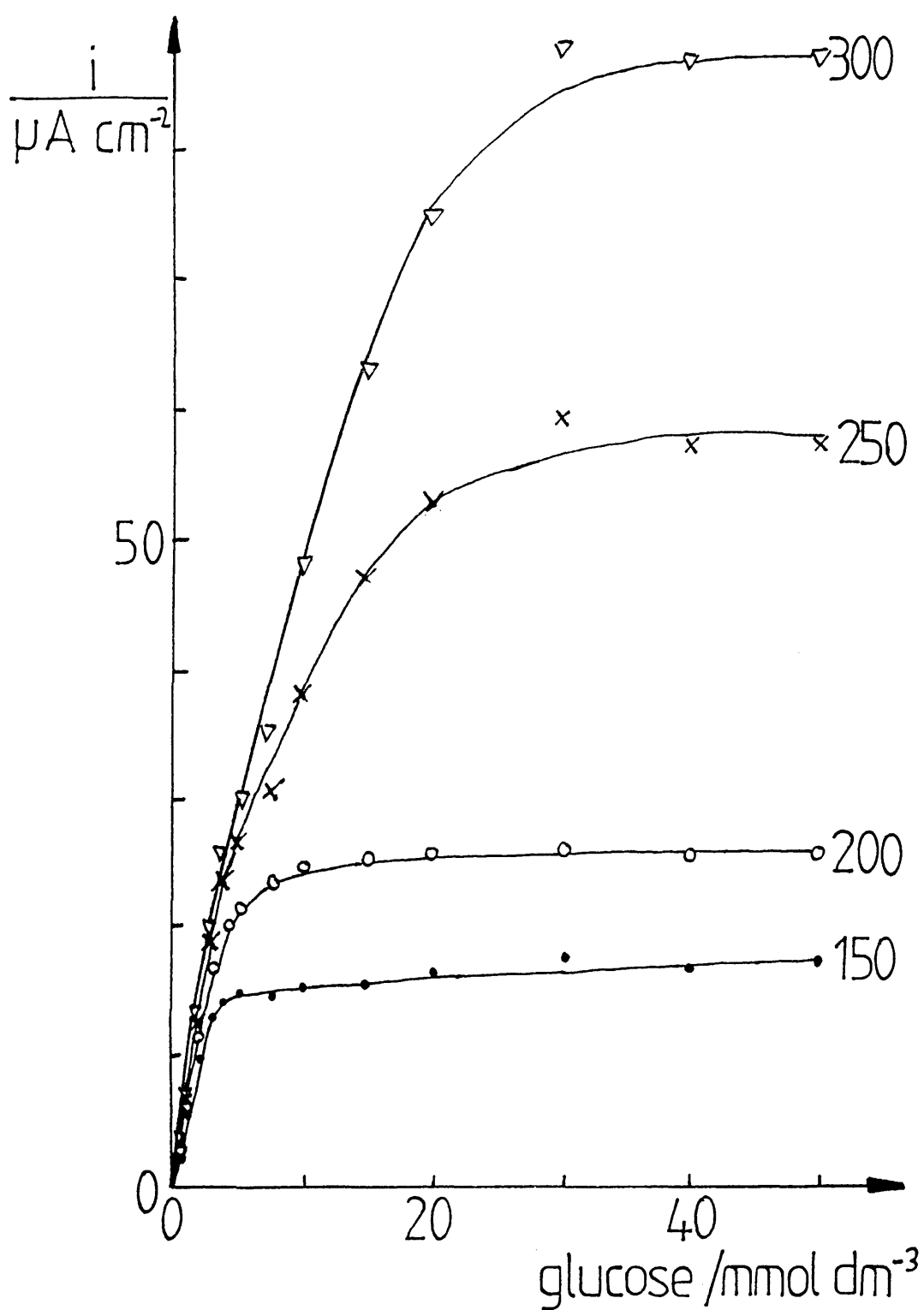


Figure 4.4.6 b) Response of the membrane electrode to glucose at different potentials. Numbers 150, 200, 250 and 300 on figure refer to potential in mV.

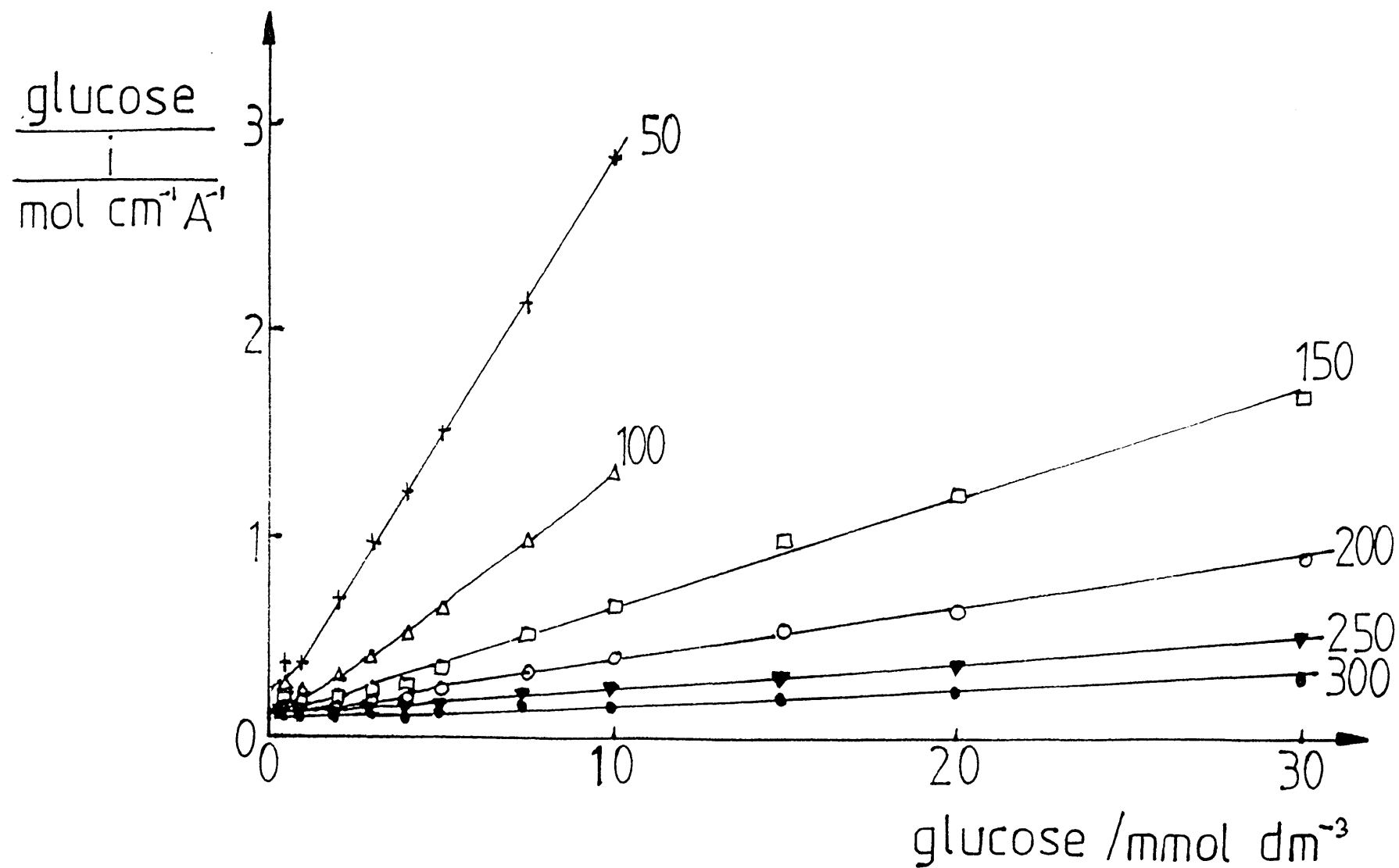


Figure 4.4.7 Hanes plots of data shown in Figures 4.4.6a and b. Numbers 50, 100, 150, 200, 250 and 300 on figure refer to potential in mV.

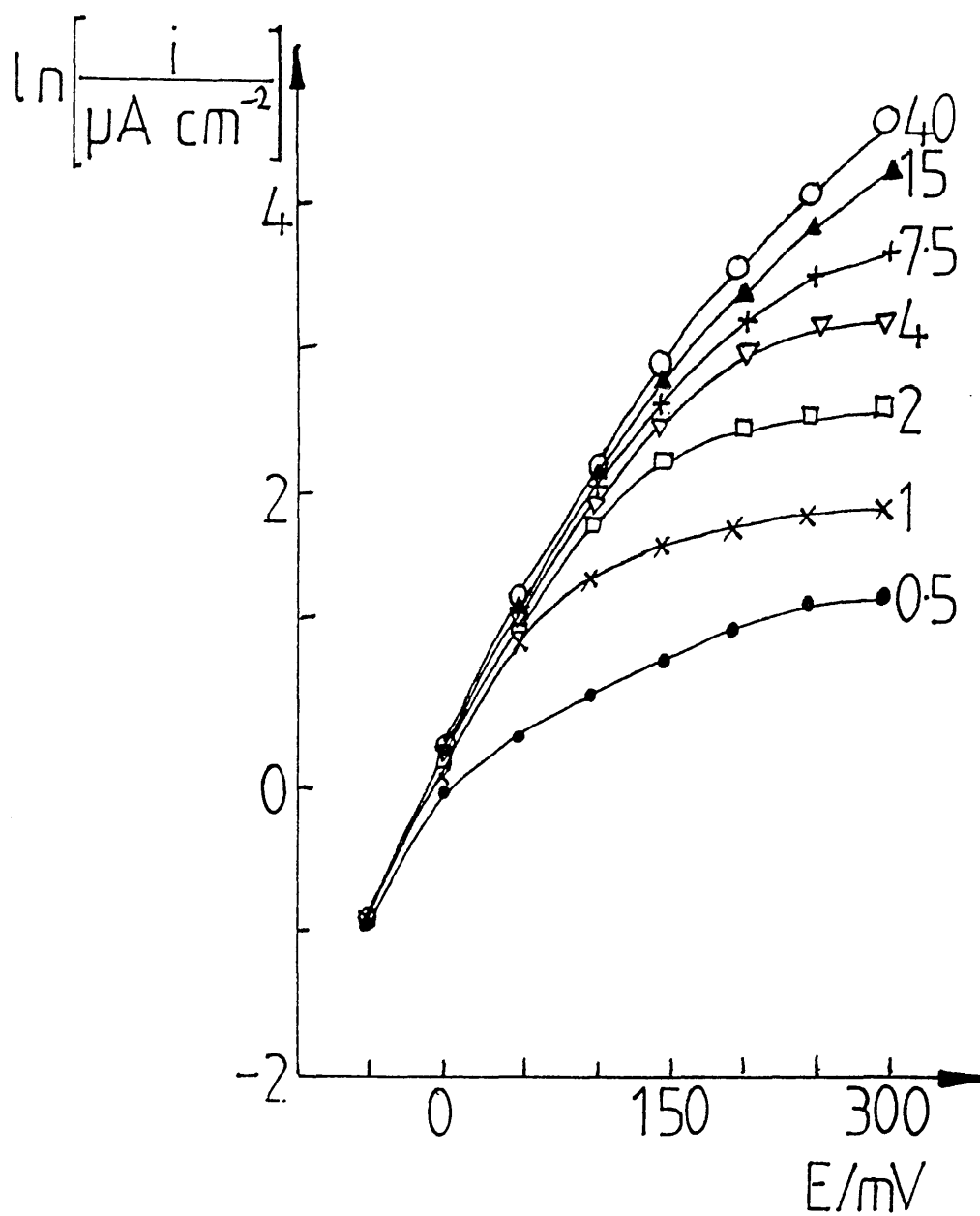


Figure 4.4.8 Variation of the current from the membrane electrode at different potentials. Numbers 0.5, 1, 2, 4, 7.5, 15 and 40 on figure refer to glucose concentration in mmol dm^{-3} .

line, with a positive gradient. Furthermore we can see that each individual curve tends to approach this straight line at lower substrate concentrations for lower potentials, again showing that the dynamic range of the electrode is larger at higher potentials.

We can explain all these observations using the theory introduced in section 2.3.1, Chapter 2. The complete equation describing this system is:

$$\frac{[S]_{\infty}}{j} = \frac{1}{k_{me}} + [S]_{\infty} \left[\frac{1}{Lk_{cat}[E]_t} + \frac{j}{(k')^2[E]_t} \right] \left[1 - \frac{j}{k_s[S]_{\infty}} \right] \quad (4.27)$$

First, we can see that the Hanes plots come to a common intercept because at low values of $[S]_{\infty}$, $[S]_{\infty}/j$ tends to $1/k_{me}$. If we now turn to the situation at high substrate concentrations, it has already been shown that

$$[E]_t/j_{max} = 1/(Lk_{cat}) + j_{max}/(k')^2 \quad (4.28)$$

Thus the increase in i_{max} with potential must be explained by an increase in k' (as k_{cat} is potential independent). However, the plot of $\ln(i)$ against E is not quite linear even at the highest substrate concentrations, indicating that some other rate limiting process is becoming important at high potentials. This will be discussed more fully later.

The dynamic range of the sensor can be roughly estimated by the value of $K_{me} = j_{max}/k_{me}$. This can be thought of as analogous to the Michaelis constant of an enzyme (K_m). The dynamic range will be larger as the value K_{me} increases. At each potential, k_{me} is constant but j_{max} increases. This

leads to an increase in K_{me} and so an increase in the dynamic range, as observed.

Further analysis of the data can be conveniently separated into examination of the response at low, high and intermediate values of glucose concentration. We will look at each of these regions in turn.

4.4(2).1 RESPONSE AT LOW GLUCOSE CONCENTRATIONS

In this region the flux is limited by the membrane or the enzyme kinetics. As a qualitative guide, we know that at high potentials the current is linear with substrate concentration. This points to membrane control.

Furthermore k_{me} is defined by the equation:

$$1/k_{me} = 1/k_s + K_M/Lk_{cat} [E]_t \quad (4.29)$$

The experimental value of k_{me} is $3.6 \times 10^{-5} \text{ cm s}^{-1}$. This compares with a value of k_s measured as 4.7×10^{-5} by Alberly and Craston^[40] and estimated value of $K_M/Lk_{cat} [E]_t$ of $>1 \times 10^{-4} \text{ cm s}^{-1}$ (using a low value for L of $1 \mu\text{m}$). This again points to membrane control. Confirmation of this conclusion would usually be made by employing the ρ or modified ρ plot. However, these plots do not give satisfactory results. The reason for this lies in the rate determining processes at high and intermediate concentrations, discussed below.

4.4(2).2 RESPONSE AT HIGH GLUCOSE CONCENTRATIONS

At high concentrations of glucose the response is obviously governed by some potential dependent process. In

terms of homogeneous mediation theory this must be due to changes in the concentration of mediator, whilst in terms of the heterogeneous redox catalysis model it must be due to changes in K_1 , k_2 , k_3 or K_S . It must be remembered that we can not however apply exactly the same mathematical analysis to this system as for the rotating disc electrode, because the membrane alters the boundary conditions of the problem. The simplest view to take would be that because of the membrane k_D will be effectively smaller for membrane electrodes than that for the rotating disc system. However the effect of the membrane may also be to allow a build-up of any species dissolving from the electrode into the enzyme/electrolyte layer. For both mechanisms, this would tend to increase the observed current if θ_M or θ_D were important. However, we can use both models as a guide for interpretation of the data where the current is not limited by membrane transport.

The analysis is easier for the heterogeneous redox catalysis model and we will start with this. At the highest concentrations of glucose the current is potential dependent. This means we are in Case S and the flux is given by

$$j = (nK_1k_2k_3e_I)^{1/2} \quad (4.30)$$

We have here assumed that the decrease in k_D will make θ_D negligible. At high potentials some other process is involved in determining the flux, as $\ln(i_{\max})$ is not quite linear with potential. We can gain some idea of the

magnitude of this second process by assuming that if it was entirely rate-determining it would give rise to some limiting current, i_{lim} . Using a modified Tafel plot (cf Section 3.3(2).2, Chapter 3) we find the best straight line is obtained when $i_{lim} \sim 119 \mu A cm^{-2}$ (see Figure 4.4.9). It is interesting that if we were in Case E and the rate-determining process was the reaction of E with S in a thin reaction layer we can calculate that the current would be about $112 \mu A cm^{-2}$. Inspection of the case diagram for this mechanism (Figure 4.3.1) shows that as we increase the value of $K_1 k_2 k_3$ we eventually move from Case S to Case E. The potential dependence of i_{max} thus apparently seems to be explicable with this model.

We shall now move on to the homogeneous mediation theory. We have already seen that although i_{max} is potential dependent, a Tafel plot of $\ln(i_{max})$ against potential is not a straight line. Using the modified Tafel plot we obtained a value for i_{lim} of $119 \mu A cm^{-2}$. As has already been stated this value of i_{lim} is close to that expected if the rate-limiting process was reaction of enzyme and substrate in a thin reaction layer. We also know that the potential dependent contribution must come from changes in $m_{o,T}$. If $m_{o,T}$ became large, then we may find that we reach a situation where $\gamma > 1$. In this case a possible consequence is that the rate-limiting step can change from reaction of mediator with E' in a thin reaction layer to reaction of E with S in a thin reaction layer^[97]. The data therefore seems to be explicable also in terms of this

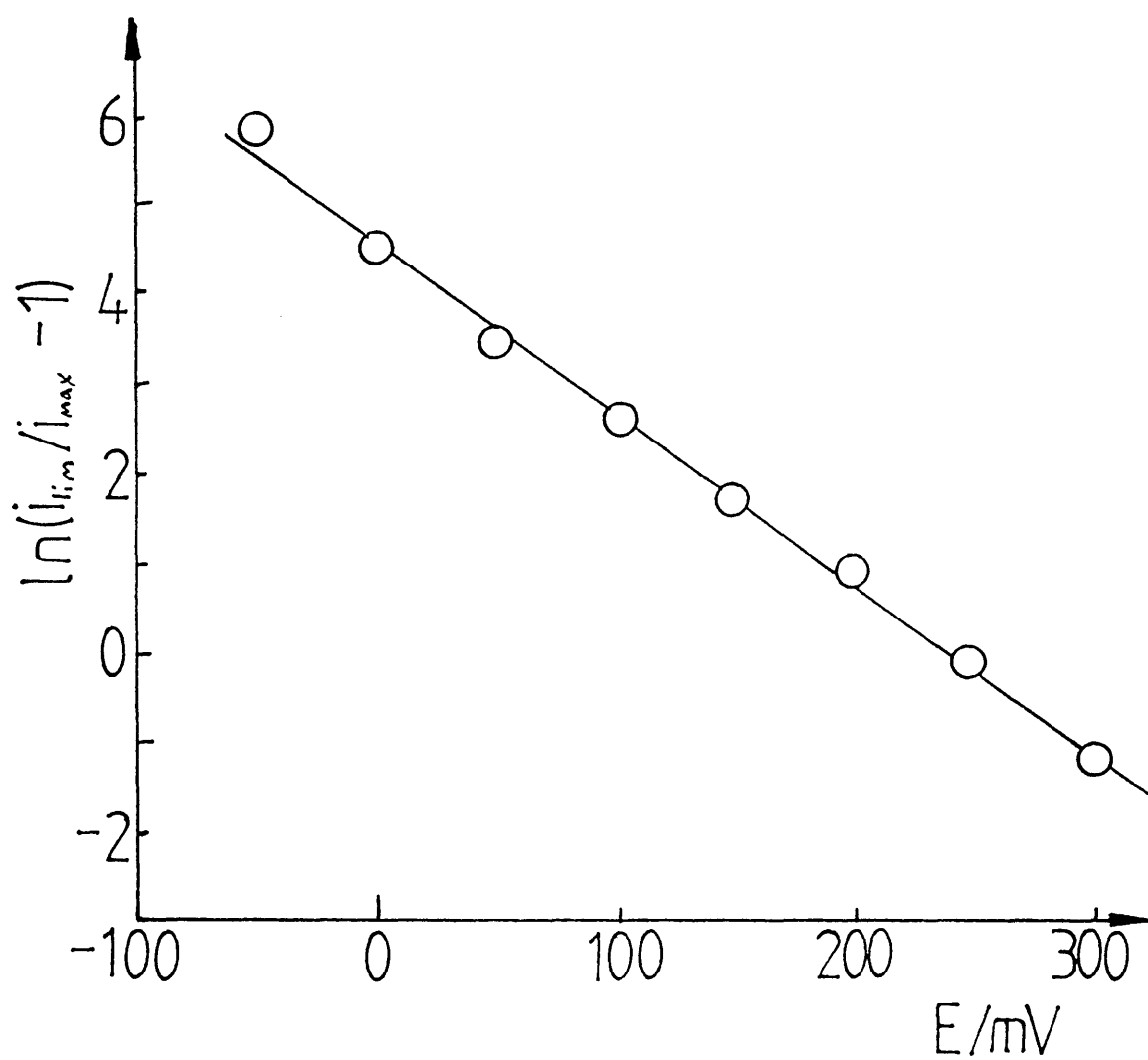


Figure 4.4.9 Modified Tafel plot for i_{max} at each potential using a value for i_{lim} of $119 \mu\text{A cm}^{-2}$.

mechanism.

Unfortunately, both analyses seem contradictory to the rotating disc results, which show little potential dependence at the higher potentials. One could argue that changes in the value of θ_M or θ_D because of the differences in k_D between the membrane and the rotating disc electrodes may affect the potential dependence. We have already shown that for the homogeneous mediation theory θ_M is unimportant and so for this model this possibility can be discounted. However, in the case of the heterogeneous redox theory θ_D is significant and so this explanation must be considered, although one must point out that the rotating disc results seem to indicate that $K_1 k_2 k_3$ is approximately constant at the higher potentials (see Table 4.4.1). Thus it is difficult to explain this paradox. Could there be a difference in the surfaces of a silicone oil paste electrode and an electrosynthesised electrode which produces changes in potential dependence?

Purely physical differences between the two surfaces such as surface roughness would probably lead to increased currents at all potentials but the same potential dependence and can therefore be ruled out. The difference in potential dependence would therefore probably have to be ascribed to the chemical rather than physical differences in the surfaces. Investigation of this would require the application of other techniques. Perhaps the thin-layer TTFTCNQ has a different microstructure than that found in

the silicone oil electrode. Another possibility may be that the silicone oil forms a thin-film on the surface of the electrode and electron transfer or dissolution of the mediator is limited in some way by this film. However, it is obvious in the latter case that if the effective solubility of M is larger at "pure" TTFTCNQ electrodes than those made of silicone oil, its true solubility must be in the range of 10's of μmolar . This should almost certainly have been detected in ring/disc studies as the disc electrode in that case was a pressed pellet of TTFTCNQ. The answer to this question must await further work.

4.4(2).3 RESPONSE AT INTERMEDIATE GLUCOSE CONCENTRATIONS

At intermediate concentrations of substrate the following processes could be rate limiting:- mass transport across the membrane, reaction of the enzyme with substrate or mediator behind the membrane either in the whole of the enzyme/electrolyte layer or in a reaction layer.

The first process can be eliminated where the response is not linear with substrate. Dealing with the heterogeneous redox catalysis model first, we must now consider whether the enzyme is concentration polarised. It has already been stated that the reaction layer thickness L_R is given by $(D_E/k_E)^{1/2}$. Under unsaturated conditions k_E is given by multiplying the apparent second order rate constant for reaction of E and S with the substrate concentration in the enzyme electrolyte layer, [S]. For GOD, this rate constant has a value of $10,000 \text{ (mol dm}^{-3}\text{) s}^{-1}$ [14]. In

Chapter 2, it has already been shown in the case of choline that we probably have enzyme concentration polarisation at saturation. In that case the value of k_E was 10 s^{-1} . For the glucose system $k_E > 10 \text{ s}^{-1}$ when $[S] > 1 \text{ mmol dm}^{-3}$. From the value of k_s we can calculate $[S]$ at any given current and bulk glucose concentration $[S]_\infty$. Taking the data at 300 mV as an example we find that $[S] > 1 \text{ mmol dm}^{-3}$ when $[S]_\infty > 10 \text{ mmol dm}^{-3}$. It therefore seems likely that at intermediate glucose concentrations the enzyme is concentration polarised and so the q -plot may not be linear.

In the homogeneous mediation theory, by altering substrate concentration we are changing k_E and hence γ . At low and intermediate substrate concentrations we may find that $\gamma > 1$. The analysis would become much more difficult in this case. However we do know that γ fixes the concentration of E' at the electrode surface at any substrate concentration (cf equation 4.3). This concentration would be unlikely to be the concentration of enzyme close to the membrane and so the enzyme will be concentration polarised and the q -plot again may not give satisfactory results, especially as we may be changing from one case to another when increasing the substrate concentration results in changing the value of γ to less than unity.

4.4(2).4 THE APPARENT TRANSFER COEFFICIENT, α

From the gradient of the modified Tafel plot shown in Figure 4.4.9 we can calculate a value for the apparent

transfer coefficient, α . This is 0.49. It is interesting that this is almost half the value of that for NADH. If both oxidations were mediated in solution, then we would expect the same value of α , because α would be a parameter representing the solubility of M. It could be suggested for this mechanism that α represents the potential dependence for the reoxidation of M^- . Again, in this case we would expect the same value of α and furthermore it would imply that except at the highest potentials $[M^-]$ at the electrode surface was non-zero. This is unlikely because in that case the current would be more dependent on W for the rotating disc studies as more M^- would be lost to the bulk when the thickness of the diffusion layer decreased, especially at low potentials.

On the other hand, if we consider the heterogeneous redox catalysis model, it is easy to see that if k_3 was potential dependent, α could change with the nature of the species being reduced. Care should be taken with this conclusion however, because it should be noted that as long as the electron transfer occurred at the surface other mechanisms may predict different values of α for different materials. The possibility that there are different mechanisms for the oxidation of different substrates should also not be eliminated.

4.5 DISCUSSION

In this chapter studies were made on the GOD/TTFTCNQ system using both membrane and rotating disc electrodes. The main thrust of these studies was to see how well the two current models for the oxidation of GOD on TTFTCNQ could predict the experimental behavior of the system. The dependence of current with enzyme concentration, rotation speed and potential was measured and diagnostic plots were made on the data to test each theory.

Both models could adequately explain the dependence of the current on each of these variables taken in isolation. The diagnostic plots seem to favour the homogeneous mediation model, although the poor quality of some of the plots for the heterogeneous redox catalysis model may be due to experimental factors. Values obtained from these plots for parameters such as $(D_E k_E)^{1/2}$, $m_{O,T}$ and k_M seemed reasonable, although the latter seems to eliminate $TCNQ^0$ and $TCNQ^-$ as candidates for the mediator, M. The heterogeneous redox model could convincingly explain the dependence of the current with potential which was not possible using the homogeneous mediation theory in a consistent manner.

Results for the membrane electrode could support either theory, although it is difficult to reconcile these results with the evidence from rotating disc experiments. Again, the heterogeneous redox catalysis model can qualitatively explain this dichotomy, but fails once the experimentally determined rate constants are taken into

account. Comparison of the values for α for GOD and NADH for oxidation at TTFTCNQ seems to favour the heterogeneous redox theory, as the homogeneous mediation theory would predict similar values for this parameter for any substrate, unless they are oxidised using different mechanisms.

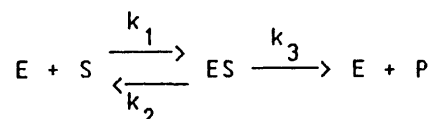
The experimental evidence presented in this chapter therefore seems to favour neither model conclusively. The fit of the data to the heterogeneous redox catalysis model seems poor, but in order to explain the difference in the behavior of the system between low and high potentials the homogeneous mediation theory would have to be modified in some way. It is, however, difficult to imagine what form this modification would take as it would not only have to explain the loss of rotation speed dependence at low potentials and the differences in α for GOD and NADH, but also still allow for the current be proportional to $(e_{\text{r}})^{1/2}$ with an inverse rotation speed dependence at low potentials.

Much more work needs to be carried out before a definitive conclusion can be made. This may not be surprising, because the models are quite similar and we do not have accurate, independently determined, values for several important parameters for both models. However, this study has served to show the importance of examining the effect of all of the different experimental variables when attempting to elucidate the mechanism of this system and of applying the data to theoretical analysis. Only by using all the evidence at our disposal can we hope to find a solution to this problem.

APPENDIX 1

DERIVATION OF THE MICHAELIS MENTEN EQUATION

This appendix gives the Briggs-Haldane derivation^[98] of the Michaelis Menten equation for an enzyme, E that reacts with substrate, S to give an enzyme/substrate intermediate, ES. This intermediate breaks down to give a product, P and the free enzyme, E. In this model, product inhibition will be ignored and the concentration of S is assumed to be much greater than the total enzyme concentration, $[E]_t$.



Using the steady-state approximation:

$$d[ES]/dt = 0 = k_1[E][S] - (k_2 + k_3)[ES] \quad (A1.1)$$

The rate, v is obtained by considering the breakdown of ES:

$$v = k_3[ES] \quad (A1.2)$$

Finally, the total concentration of enzyme is given by:

$$[E]_t = [E] + [ES] \quad (A1.3)$$

We can find [ES] from equation A1.2 and substitute this result into equation A1.1 to obtain [E]. Substitution for [E] and [ES] into equation A1.3 and rearrangement then gives:

$$v = k_{cat} [E]_t [S] / (K_m + [S]) \quad (A1.4)$$

where $k_{cat} = k_3$ and $K_m = (k_2 + k_3)/k_1$

APPENDIX 2

THE HOMOGENEOUS MEDIATION MODEL

This appendix contains the mathematical analysis for the homogeneous mediation model for the oxidation of GOD at TTFTCNQ electrodes. The underlying assumptions and kinetic scheme for the model were discussed in Section 4.3.1 of Chapter 4. Using these assumptions, we are following analysis first carried out by Kulys in 1981^[92].

The differential equations describing the diffusion and kinetics of the two species M and M⁻ are as follows

$$D_M \partial^2 m / \partial z^2 = k_M e_L m \quad (A2.1)$$

$$D_M \partial^2 m' / \partial z^2 = -k_M e_L m \quad (A2.2)$$

where m and m' denote the concentrations of M and M⁻ respectively, the total concentration of E is e_L, and z is the distance perpendicular from the electrode. The diffusion coefficients of M and M' are presumed to be the same.

The boundary conditions are as follows:

At the electrode surface (z=0):

$$m = m_0 \text{ and } m' = 0$$

At the edge of the diffusion layer (z=z_D):

$$m = m' = 0$$

The flux of electrons j, from the conversion of M' to M at the electrode surface is given by

$$j = nD_M (\partial m' / \partial z)_0 \quad (A2.3)$$

Addition of equations A2.1 and A2.2 followed by integration

gives after application of the boundary conditions:

$$m + m' = m_0(1 - z/Z_D) \quad (A2.4)$$

We can now introduce the dimensionless variables $X=z/Z_D$, $u=m/m_0$, $u'=m'/m_0$, and $K_M=k_M(Z_D)^2 e_L/D_M$. This latter parameter describes the chances of the mediator M escaping into the bulk before it reacts with enzyme. If $K_M < 1$ then M is more likely to escape than react with E'. If $K_M > 1$ then most of the M will react rather than escape. Substitution into equations A2.1 and A2.4 then gives

$$\partial^2 u / \partial X^2 = K_M u \quad (A2.5)$$

$$u + u' = 1 - X \quad (A2.6)$$

and the boundary conditions are

at $X=0$, $u=1$ and $u'=0$

at $X=1$, $u=u'=0$

The flux of electrons is given by

$$j = (nD_M m_0 / Z_D) (\partial u' / \partial X)_0 \quad (A2.7)$$

We can easily solve equation A2.5, and use of the boundary conditions gives

$$u = \cosh\{(K_M)^{1/2} X\} - \coth\{(K_M)^{1/2}\} \sinh\{(K_M)^{1/2} X\} \quad (A2.8)$$

Using equation A2.6, with differentiation we find

$$(\partial u' / \partial X)_0 = (K_M)^{1/2} \coth\{(K_M)^{1/2}\} - 1 \quad (A2.9)$$

Hence the flux is given by

$$j = n(D_M / Z_D) m_0 \{(K_M)^{1/2} \coth\{(K_M)^{1/2}\} - 1\} \quad (A2.10)$$

We will now deal with simple kinetic scheme for the value of m_0 .

The Value of m_0

An important question is whether m_0 is determined

thermodynamically or kinetically. To illustrate how important this is we will take a simple kinetic scheme for the dissolution of M from the electrode. This is shown in Figure A2.1. M dissolves from the organic salt with a zero-order rate constant k_f . It can then diffuse into solution, react with the enzyme or reprecipitate at the electrode surface with a first-order rate constant k_b . However, it is also formed at the electrode surface by the oxidation of M^- . In the steady state we can equate the flux of M away from the electrode with its net rate of formation:

$$k_f - k_b m_o + D_M (\partial m' / \partial z)_o = -D_M (\partial m / \partial z)_o \quad (A2.11)$$

Hence,

$$k_f - k_b m_o = -D_M \{ (\partial m' / \partial z)_o + (\partial m / \partial z)_o \} \quad (A2.12)$$

But differentiation of equation A2.4 yields

$$(\partial m' / \partial z)_o + (\partial m / \partial z)_o = -m_o / Z_D \quad (A2.13)$$

Comparison of the above two equations, gives after rearrangement:

$$m_o = k_f / (k_b + k_D) \quad (A2.14)$$

where $k_D = D_M / Z_D$.

The value of m_o therefore depends on the ratio of k_b to k_D . If $k_b \gg k_D$ then the value of m_o can be considered to be the thermodynamically controlled value of k_f / k_b . On the other hand, if $k_b \ll k_D$ then m_o can be considered to be kinetically controlled with a value of k_f / k_D . In this latter case, m_o will be sensitive to rotation speed as $m_o \propto (1/k_D) \propto Z_D \propto \omega^{-1/2}$.

Finally, if we assume the thermodynamic solubility of M

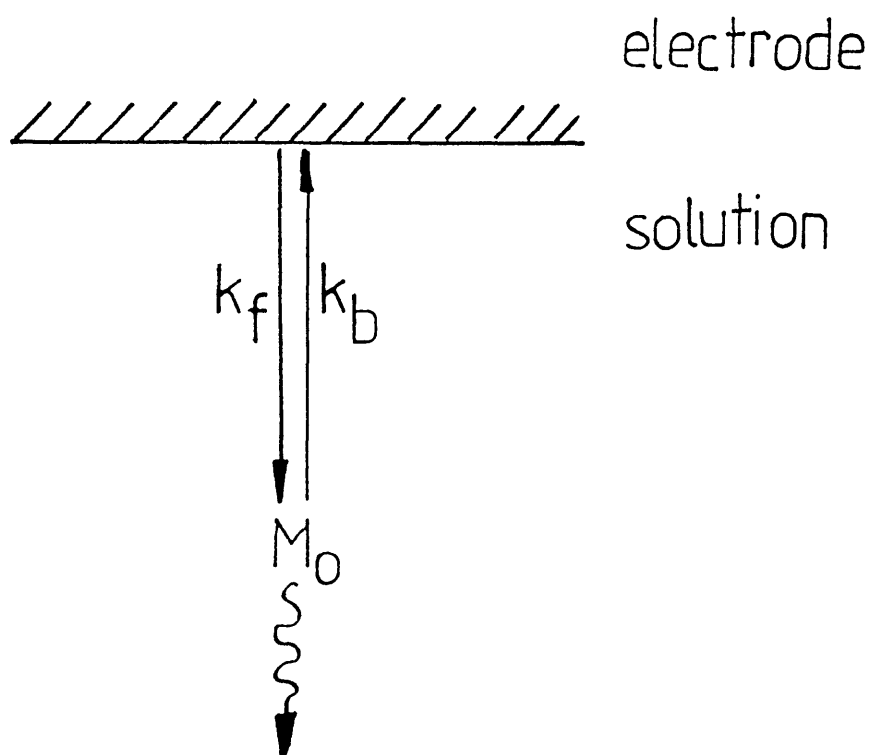


Figure A2.1 Kinetic scheme for the dissolution of M .

to be $m_{o,T} = k_f/k_b$ then we can rearrange equation A2.14 to give:

$$m_o = m_{o,T} (1 + \theta_M)^{-1} \quad (\text{A2.15})$$

where $\theta_M = k_D/k_b$. Substitution for m_o into equation A2.10 then yields:

$$j = n(D_M/Z_D) m_{o,T} \{ (K_M)^{1/2} \coth[(K_M)^{1/2}] - 1 \} / (1 + \theta_M) \quad (\text{Equation A2.16})$$

This expression is discussed in Section 4.3.1 (Chapter 4), where approximate expressions for the flux are derived.

APPENDIX 3

THE HETEROGENEOUS REDOX CATALYSIS MODEL

The basis of this model was discussed in Section 4.3.2 of Chapter 4. It was first proposed by Alberty et al in 1987^[41]. The analysis involves finding an expression for the flux of electrons in terms of the concentration of E' at the electrode surface and then examining what this concentration will be using Fick's second law.

Because D^0 may be lost from the electrode surface the flux of electrons j_e will not be equal to the flux of enzyme j_E . If small letters denote the concentrations of D and A on the electrode surface we have the following

$$K_1 = da \quad (A3.1)$$

$$j_e = k_2 d \quad (A3.2)$$

$$j_E = k_3 a e'_0 \quad (A3.3)$$

In the steady state the fluxes of D^0 and A^0 must match:

$$(k_2 + K_S k_D) d = n k_3 e'_0 a \quad (A3.4)$$

where e'_0 is the concentration of E' at the electrode surface. Elimination using the above equations gives

$$j_e = n j_E / (1 + \theta_D) \quad (A3.5)$$

Here θ_D is a dimensionless parameter given by

$$\theta_D = K_S k_D / k_2 \quad (A3.6)$$

where $k_D = D^0 / Z_D$. θ_D describes the likelihood of D^0 being electrochemically oxidised before it is lost into solution. If $\theta_D \gg 1$ then most D^0 will dissolve rather than be oxidised. On the other hand if $\theta_D \ll 1$ then most D^0 will tend to be oxidised rather than escape to the bulk. Further

rearrangement and elimination between the above equations yields:

$$j_E = [K_1 k_2 k_3 (1 + \theta_D) e'_0 / n]^{1/2} \quad (\text{A3.7})$$

We must now look at the differential equation describing the diffusion and kinetics of the enzyme in a direction z normal to the electrode:

$$D_E \partial^2 e' / \partial z^2 = -k_E e \quad (\text{A3.8})$$

where e denotes the concentration of E . If we assume E and E' have the same diffusion coefficient then

$$e + e' = e_\Sigma \quad (\text{A3.9})$$

We assume all the enzyme in the bulk of solution is in the reduced form. Hence at $z = Z_D$, $e' = e_\Sigma$ and $e = 0$.

Secondly at the electrode surface ($z = 0$):-

$$D_E (\partial e' / \partial z)_0 = j_E \quad (\text{A3.10})$$

Introducing the dimensionless parameters $v = e' / e_\Sigma$,

$K_E = k_E (Z_D)^2 / D_E$ and $X = z / Z_D$ we find

$$\partial^2 v / \partial X^2 = -K_E (1 - v) \quad (\text{A3.11})$$

with the boundary conditions that at $X = 1$, $v = 1$ and that at $X = 0$

$$j_E = (D_E e_\Sigma / Z_D) (\partial v / \partial X)_0 \quad (\text{A3.12})$$

Integration of equation A3.11, followed by application of the boundary conditions and differentiation gives

$$(\partial v / \partial X)_0 = (1 - v_0) (K_E)^{1/2} \coth\{(K_E)^{1/2}\} \quad (\text{A3.13})$$

We now define the dimensionless parameter, f , to describe the flux of electrons

$$j_e = f n D_E e_\Sigma / Z_D \quad (\text{A3.14})$$

From equations A3.5, A3.12 and A3.14 we then find

$$(\partial v / \partial X)_0 = f(1 + \theta_D) \quad (\text{A3.15})$$

Hence from equations A3.7, A3.10, A3.14 and A3.15 we obtain

$$v_0 = (1 + \theta_D) B f^2 \quad (\text{A3.16})$$

$$\text{where } B = (n e_{\Sigma} / K_1 k_2 k_3) (D_E / Z_D)^2$$

Substitution of the above two equations into equation A3.13

then gives

$$B f^2 + f \tanh[(K_E)^{1/2}] / (K_E)^{1/2} - (1 + \theta_D)^{-1} = 0 \quad (\text{A3.17})$$

Approximate solutions for the flux using this equation, are given in Chapter 4, where the significance of K_E and B is also discussed. At this point we can estimate that $K_E \sim 10^3$ from the values for Z_D , D_E and k_{cat} given in Section 4.3 in Chapter 4. This will make application of equation A3.17 to experimental results much easier as we can then assume that $\tanh(K_E) \sim 1$. This procedure is carried out in Section 4.4(1).4 of the same chapter.

An Alternative Mechanism

In the above analysis it has been assumed that E^+ reacts with A^0 and that D^0 is electrochemically oxidised to produce the current. However an alternative mechanism could be proposed in which the salt dissociates giving D^+ and A^- . The enzyme then reacts on the surface with D^+ to produce D^0 and A^- is electrochemically oxidised to give A^0 , which recombines with D^0 to give back DA . In this case, the analysis would be equivalent, and we would obtain the same equations for the flux.

REFERENCES

1. A R Fersht, "Enzyme Structure and Mechanism", 2nd Ed, W H Freeman, New York (1984);
2. N C Price & L Stevens, "Fundamentals of Enzymology", Oxford University Press, Oxford (1982);
3. S A Bernhard, "The Structure and Function of Enzymes", W A Benjamin, New York (1968);
4. P F Leadlay, "An Introduction to Enzyme Chemistry", The Chemical Society, London (1978);
5. M Dixon & E C Webb, "Enzymes", 3rd Ed, Longman, London (1982);
6. L Michaelis & M L Menten, Biochem Z 49 33 (1913);
7. C S Hanes, Biochem J 26 1406 (1932);
8. H J Bright & D J Porter, p421 in "The Enzymes", (P D Boyer, ed), Vol XIIB, 3rd ed, Academic Press, New York;
9. G Palmer & V Massey, p263 in "Biological Oxidations", (T P Singer, ed), Interscience, New York (1968);
10. G Dryhurst, "Biological Electrochemistry", Vol 1, p456, Academic Press, New York (1982);
11. B E P Swoboda & V Massey, J Biol Chem 240 2209 (1965);
12. D Keilin & E F Hartree, Biochem J 42 221 (1948);
13. B E P Swoboda, Biochim Biophys Acta 175 365 (1965);
14. Q H Gibson, B E P Swoboda & V Massey, J Biol Chem 239 3927 (1964);
15. H J Bright & M Appleby, J Biol Chem 244 3625 (1969);
16. F Scheller, G Strnad, B Neumann, M Kuhn & W Ostrowski, J Electroanal Chem 104 117 (1979);
17. R M Ianiello, J J Lindsay & A M Yacynych, Anal Chem 54 1098 (1982);
18. R M Ianiello, J J Lindsay & A M Yacynych, Anal Chim Acta 141 23 (1982);
19. K Narashimham & L B Wingard Jr, Anal Chem 58 2984 (1986);

20. C Bourdillon, J P Bourgeois & D Thomas, J Am Chem Soc 102 4231 (1980);
21. H Durliat & M Comtat, Anal Chem 56 148 (1984);
22. F R Duke, R N Kust & L A King, J Electrochem Soc 116 32 (1969);
23. S Ikuta, S Imamura, H Misali & Y Horuti, J Biochem 82, 1741 (1977);
24. M Ohta-Fukuyama, Y Miyake, S Emi & T Yamana, J Biochem 88 197 (1980);
25. Y Tani, N Mori, H Yameda & R Hayashi, Agric Biol Chem 45 539 (1981);
26. H Yameda, N Mori & Y Tani, Agric Biol Chem 43 2173 (1979);
27. N Mori, Y Tani, H Yameda & R Hayashi, Agric Biol Chem 43 815 (1979);
28. N Ohishi & K Yagi, Biochem Biophys Res Comm 86 1084 (1979);
29. J H Perlstein, Angew Chem Int Ed Engl 16 519 (1977)
30. D O Cowan & F M Wiygul, Chem Eng News (July 21st) 28 (1986);
31. G Wegner, Angew Chem Int Ed Engl 20 261 (1981);
32. D S Acher, R J Harder, W R Hertler, W Mabiler, L R Melby, R E Benson & W E Mochel, J Am Chem Soc 82 6408 (1960);
33. L R Melby, R J Harder, W R Hertler, W Mahler, R E Benson & W E Mochel, J Am Chem Soc 84 3374 (1962);
34. J B Torrance, Acc Chem Res 12 79 (1979);
35. J B Torrance, Ann New York Acad Sci 313 210 (1978);
36. M R Bryce, Chem Br 24 781 (1988);
37. R C Wheland & J C Gillson, J Am Chem Soc 98 3916 (1976);
38. C D Jaegar & A J Bard, J Am Chem Soc 102 5435 (1980);
39. D H Craston, PhD Thesis, Univ London (1986);
40. W J Albery, P N Bartlett & D H Craston, J Electroanal Chem 194 223 (1985);

41. W J Albery, P N Bartlett & A E G Cass, Phil Trans R Soc London B316 107 (1987);
42. C D Jaegar and A J Bard, J Am Chem Soc 101 1690 (1979);
43. J J Kulys, A S Samalius & G-J S Svirmikas, FEBS Letts 114 7 (1980);
44. W J Albery, P N Bartlett, M Bycroft, D H Craston & B J Driscoll, J Electroanal Chem 218 119 (1987);
45. W J Albery & P N Bartlett, J Chem Soc Chem Comm 234 (1984);
46. W J Albery, P N Bartlett, A E G Cass & K W Sim, J Electroanal Chem 218 127 (1987);
47. M Sharp, Anal Chim Acta 59 137 (1972);
48. J C Crawhall & W C Purdy, Trends Anal Chem 1 181 (1982);
49. M Thompson & U J Krull, Trends Anal Chem 3 173 (1984);
50. "Biosensors: Fundamentals and Applications" (A P F Turner, I Karube & G S Wilson, eds), Oxford University Press, Oxford (1987);
51. "Biosensors" (M Akhtar, C R Lowe & I J Higgins, eds), Royal Society London (1987); also contained in Phil Trans Roy Soc Lond B316 pp1-181 (1987);
52. A E G Cass, Life Chemistry Reports 1 1 (1983);
53. I J Higgins & H A O Hill, Essays in Biochemistry 21 119 (1985);
54. W J Albery, P N Bartlett, A E G Cass, D H Craston & B G D Haggett, J Chem Soc Faraday Trans (1) 82 1033 (1986);
55. G G Guilbault & J G Montalvo, J Am Chem Soc 91 2164 (1969);
56. I Karube, p471 in ref 50;
57. J Koryta, Electrochim Acta 31 515 (1986);
58. G S Wilson, p165 in ref 50;
59. L Clark & C Lyons, Ann New York Acad Sci 102 29 (1962);
60. L Clark, BRD Patent K1 421 3104 Nr 1598 285 (Feb 4, 1971);

61. G Davies, *Biosensors* 1 161 (1985);
62. D Williams, A Doig & A Korosi, *Anal Chem* 42 118 (1970);
63. G Jonsson & L Gorton, *Biosensors* 1 355 (1985);
64. W Mindt, P Racine and P Schlaepfer, *Ber Bunsenges Phys Chem* 77 804 (1973);
65. A E G Cass, D G Francis, H A O Hill, W J Aston, I J Higgins, E V Plotkin, L D L Scott & A P F Turner, *Anal Chem* 56 667 (1984);
66. D R Matthews, E Brown, A Watson, R R Holman, T Steemson, S Hughes & D Scott, *Lancet* 1(8537) 778 (1987);
67. M Boutelle, C Stanford, M Fillenz, W J Albery & P N Bartlett, *Neurosci Letts* 72 283 (1986);
68. Y Degani & A Heller, *J Phys Chem* 91 1286 (1987);
69. P N Bartlett, R G Wittaker, M J Green & J Frew, *J Chem Soc Chem Comm* 1603 (1987);
70. "Choline and Acetylcholine: Handbook of Chemical Assay Methods" (I Hanin, ed), Raven Press, New York (1974);
71. T Uwajima & O Terada, *Trends Anal Chem* 1 55 (1981);
72. K Honda, K Miyaguchi, H Nishino, H Tanaka, T Yao, & K Imai, *Anal Biochem* 153 50 (1986);
73. C Eva, M Hadjiconstantinou, N H Neff & J L Meek, *Anal Biochem* 143 320 (1984);
74. J Ferraris, D O Cowan, V Walatka Jr & J R Perlstein, *J Am Chem Soc* 95 948 (1973);
75. P N Bartlett, PhD Thesis, Univ London (1981);
76. W J Albery & P N Bartlett, *J Electroanal Chem* 194 211 (1985);
77. S L T Durrant, PhD Thesis, Univ London (1987);
78. K D Zigel, H K Frensdorff & D E Blair, *J Polymer Sci (A-2)* 7 809 (1969);
79. G Kneebone, unpublished results;
80. R B Lennox, personal communication;

81. P Kathirgamanathan & D R Rosseinsky, J Chem Soc Chem Comm 86 (1979);
82. P Kathirgamanathan & D R Rosseinsky, J Chem Soc Chem Comm 356 (1980);
83. K El Kacemi & L Lamache, Electrochimica Acta 31 1197 (1986);
84. J Przeworski, PhD Thesis, Univ London (1984);
85. K W Sim, personal communication;
86. A J Bard & L R Faulkner, "Electrochemical Methods", Wiley, New York (1980);
87. K El Kacemi & M Lamache, Electrochim Acta 31 1197 (1986);
88. G Rao, personal communication;
89. W J Albery, "Electrode Kinetics", Clarendon Press, Oxford (1975);
90. A R Mount, personal communication;
91. "Instrumental Methods in Electrochemistry", Southampton Electrochemistry Group, Ellis Horwood, Chichester (1985);
92. N K Cenas & J J Kulys, J Electroanal Chem 128 103 (1981);
93. "Handbook of Chemistry and Physics", 59th ed, CRC Press, Florida;
94. R A Kamin & G S Wilson, Anal Chem 52 1198 (1980);
95. M Eddowes & M Gratzel, J Electroanal Chem 152 143 (1983);
96. M F Cardosi & A P Turner, p272 in ref 50;
97. L J Murphy, PhD Thesis, Univ London, to be submitted (1989);
98. G E Briggs & J B S Haldane, Biochem J 19 338 (1925).