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W. J. ASTON

METHANOL DEHYDROGENASE BIOFUEL CELLS AND ENZYME-BASED ELECTRODES

Supervisors: I. J. HIGGINS
A. P. F. TURNER

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To Mum and Dad

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ABSTRACT

This thesis describes the linking of enzymes to electrodes and their application in biofuel cells and as analytical devices.

Methanol dehydrogenase, an NAD independent enzyme was purified by two phase aqueous partition. The enzyme incorporated into a biofuel cell was capable of producing a current in the presence of either a soluble or insoluble mediator. Optimisation of the current was carried out and a variety of alternative membranes, mediators and electrodes were investigated for possible use in the biofuel cell. Although laboratory studies involved the use of platinum electrodes and the soluble mediator N,N,N',N' tetramethyl-p-phenylenediamine, other configurations were investigated, including modified membranes and electrodes. The ability to detect methanol coulometrically in solution led to the construction of a homogeneous poised potential sensor. The device was capable of detecting methanol at concentrations in solution as low as 0.02 uM. The sensor was unaffected by many potential interfering compounds present in water supplies and was used in the analysis of water samples. Retention of the enzyme in the presence of the insoluble mediator 1,1'-dimethylferrocene allowed the construction of a probe. The probe was unaffected by fluctuations in the oxygen tension and was capable of detecting methanol in the range 0.9 uM to 0.1 mM.

Covalent immobilisation of glucose oxidase in the presence of 1,1'-dimethylferrocene enabled the amperometric determination of glucose in the range 0.01 - 30 mM. The device was independent of pH in the normal physiological range with a temperature coefficient of 4.0% $^{o}C^{-1}$. The electrode was used to assay both buffered glucose samples and preliminary work was carried out on untreated samples from diabetic and non-diabetic patients.

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CHAPTER 1

GENERAL INTRODUCTION

1.0 INTRODUCTION

The application of electochemical techniques to biological systems has resulted from a blend of scientific and commercial requirements. The subject of bioelectrochemistry has had a distinguished academic lineage, stemming from the work of Galvani and Volta on animal and fish electricity in the 18th century. The idea that simple unicellular organisms, such as bacteria, or their components were capable of electrical effects seems to have escaped the notice of many scientists. Perhaps one of the most significant papers on the subject was a short work by Cohen (1931) in which he described a bacterial half cell, using mediators to shuttle electrons from the biological catalyst to the electrode. It is this principle which forms the basis of one of the most commercially attractive bioelectrical systems demonstrated to date. The effective coupling of redox proteins to electronic systems has potential applications ranging from bioelectrochemical synthesis to biofuel cells and probably in the short term the most practical proposition, the development of biosensors for both industrial and clinical monitoring.

The work described in this thesis involves two systems, a bio-electrochemical fuel cell and an enzyme-based sensor, both of clinical and industrial use, in which the enzymes methanol dehydrogenase and glucose oxidase are utilised.

1.1 PURIFICATION AND PROPERTIES OF ENZYMES.

1.1.1 Methanol dehydrogenase.

The methanol dehydrogenase present in methylotrophic bacteria is neither a $NAD(P)^+$-dependent nor a flavin containing enzyme (Duine and Frank, 1980, 1981a,c). The enzyme was first described in Pseudomonas M27 (Anthony and Zatman, 1964a,b, 1965, 1967a) and subsequently characterised in a number of other methylotrophic bacteria (Johnson and Quayle, 1964; Patel and Hoare, 1971; Wadzinski and Ribbons, 1975a; Goldberg, 1976; Patel and Felix, 1976; Patel et al., 1972, 1978; Yamanaka and Matsumoto, 1977; Wolf and Hanson, 1978; Bamforth and Quayle, 1978a,b; Duine et al., 1978). Methanol dehydrogenases have been classified into four main groups (Colby et al., 1979) which are shown in Table 1.1. $NAD(P)^+$-independent methanol dehydrogenases linked to artificial electron acceptors in vitro have a requirement for ammonium ions or primary amines as activators. One notable exception to this is the enzyme isolated from Methylobacterium organophilum which, upon storage, developes a dependence for an activator (Wolf and Hanson, 1978). In vivo, methanol dehydrogenase is coupled to cytochrome c, and is located on the periplasmic side of the cytoplasmic membrane in Methylophilus methylotrophus (Jones et al., 1982) and Paracoccus denitrificans (Alefounder and Ferguson, 1981). Evidence for this coupling has been demonstrated by a variety of experiments. It has been shown that methylotrophic bacteria contain more cytochrome c when grown on methanol than when grown on multi-carbon substrates (Tonge et al., 1974; Widdowson and Anthony,

Table 1.1 Classification of methanol dehydrogenases of methylotrophs by Colby *et al* (1979).

Group 1 enzymes are dimeric proteins with molecular weights of 120,000 or 150,000. Enzymes in this group are capable of oxidising primary alcohols and are present in the following organisms: *Pseudomonas* M27, *Methylococcus capsulatus* (Texas), *Ps.* TP1, *Ps.* W1, *Ps.* 2491, *Ps. fluorescens* S25 and S50, *Ps.* RJ1, *Ps.* AM1, *Paracoccus denitrificans*, *Hyphomicrobium* WC and *Hyphomicrobium* X.

Group 2 enzymes are similar to group 1, except they also oxidise some secondary alcohols, although only at low rates. These enzymes have been demonstrated in *Methylobacterium organophilum* and *Pseudomonas* C.

Group 3 enzymes resemble group 1 but are monomeric with molecular weights of 60,000; they are present in *Methylomonas methanica* and *Methylosinus sporium*.

Group 4 enzymes are represented by the alcohol dehydrogenase from *Rhodopseudomonas acidophila* (Bamforth and Quayle, 1978a). This enzyme differs from the group 1 enzymes in that it has a very high K_m for methanol (145 mM) but not for other higher primary alcohols (ethanol 17 μM), secondary alcohols, formaldehyde and acetaldehyde which are oxidised at high rates.

1975; Keevil and Anthony, 1979). Cytochrome c in whole cells of both Pseudomonas AM1 (Anthony, 1975) and Methylophilus methylotrophus (Cross and Anthony, 1980) undergoes rapid reduction upon the addition of methanol. Similarly vesicles prepared from Pseudomonas AM1 were shown to be capable of reducing cytochrome c in the presence of methanol (Netrusov and Anthony, 1979). Mutants of Paracoccus denitrificans (Van Versveld and Stouthamer, 1978), Methylobacterium organophilum (O'Conner and Hanson, 1978) and Pseudomonas AM1 (Anthony, 1975; Widdowson and Anthony, 1975) lacking cytochrome c were incapable of oxidising methanol. Methanol dehydrogenase activity in anaerobically prepared extracts of Hyphomicrobium X is coupled to cytochrome c. The coupling is, however, irreversibly destroyed upon exposure to oxygen; ammonium ions become necessary for activation (Duine et al., 1979a).

The pH optima of methanol dehydrogenases range between 8.5 and 10.5. Most facultative methylotrophic bacteria possess enzymes with low pH optima (8.5 - 9.0) (Anthony and Zatman, 1964b, 1965, 1967a; Metha, 1973; Sperl et al., 1974). Methanol dehydrogenase prepared from Methylococcus capsulatus (Patel et al., 1972; Wadzinski and Ribbons, 1975a) has a pH optimum of 9.5 and that from Pseudomonas C and Methylobacterium organophilum exhibit optima of pH 10.5 (Goldberg, 1976; Wolf and Hanson, 1978).

Formaldehyde is also a substrate for methanol dehydrogenase because it exists in solution as the " hydroxymethanol " hydrate (Sperl et al., 1974). In vivo methanol dehydrogenase may oxidise both methanol and formaldehyde. However mutants of Pseudomonas AM1 lacking

this enzyme were shown to be capable of oxidising methanol at rates similar to the wild type (Heptinstall and Quayle, 1970; Dunstan et al., 1972). This may not be the case in other methylotrophs (Patel et al., 1972; Goldberg, 1976; Yamanaka and Matsumoto, 1977; Wolf and Hanson, 1978).

The stoichiometry of methanol oxidation by Hyphomicrobium X was unaffected by the presence of formaldehyde trapping agents (Duine et al., 1978) suggesting that methanol dehydrogenases oxidise methanol directly to formic acid. It has been reported that Pseudomonas methanica oxidises primary alcohols to the corresponding aliphatic acids (Leadbetter and Foster, 1958) whilst Methylococcus capsulatus (Texas) only produces the corresponding aldehyde (Patel and Hoare, 1971). The substrate range of methanol dehydrogenases has been shown to include benzyl alcohol, 2,2,2 trifluroethanol and acetaldehyde (Sperl et al., 1974) chlorosubstituted alcohols (Patel and Felix, 1976), ethanolamine (Wolf and Hanson, 1978), 1-phenyl ethanol (Sperl et al., 1974; Patel and Felix, 1976), cinnamyl alcohol (Patel and Felix, 1976) and ethylene glycol (Goldberg, 1976). Substitution by methyl groups affects the enzymic activity, the closer to the hydroxyl groups the lower the activity (Sperl et al., 1974).

Inhibitors of methanol dehydrogenases include high phosphate concentration (Higgins and Quayle, 1970; Anthony, 1975; Tonge et al., 1975), EDTA (Patel and Felix, 1976; Ohta et al., 1981), cyanide (Wolf and Hanson, 1978), bipyridyl, manganese and cobalt ions (Ohta et al., 1981).

Since the elucidation of the structure of the prosthetic group

(Fig 1.1) a major advance has been made in the understanding of the mechanistic action of methanol dehydrogenase. Initially, the prosthetic group was proposed to be a pteridine (Anthony and Zatman, 1964b, 1967a,b) due to the release of a fluorescent, green, low molecular weight compound upon denaturation and inactivation by acid, alkaline or heat. It was suggested that the pteridine acted directly as an electron acceptor or was bound to the methanol prior to oxidation. This observation was supported by publications reporting the prosthetic group to be a neopterin cyclic phosphate (Urushibara et al., 1971) and later a lumazine derivative (Sperl et al., 1973). Evidence that the enzyme contained a novel nitrogen containing quinone coenzyme was obtained by electron spin resonance spectroscopy, (Duine et al., 1978; DeBeer et al., 1979; Westerling et al., 1979; Duine and Frank, 1980). Confirmation of the quinone structure was obtained by X-ray crystallography (Salisbury et al., 1979), nuclear magnetic resonance spectroscopy and mass spectrometry of the intact coenzyme (Duine et al., 1980). A semi systematic name, 'pyrrolo-quinoline quinone' (PQQ) was proposed for the quinone containing coenzyme, refering to its systematic name, 2,7,9,tricarboxy-1-H-pyrrolo[2,3-f]quinoline-4,5-dione. The coenzyme has now been synthesised (Corey and Tramantano, 1981; Gainor and Weinreb, 1981) and its properties established (Duine and Frank, 1981a,c). Possible mechanisms have been proposed (Fig 1.2) based on chemical, physical (Salisbury et al., 1979; Forrest et al., 1980) and subsequent electrochemical observations for both PQQ and analogous quinoquinones (Eckert et al., 1982). It has been proposed that during

Figure 1.1 The structure of the prosthetic group of methanol dehydrogenase; pyrroloquinoline quinone.

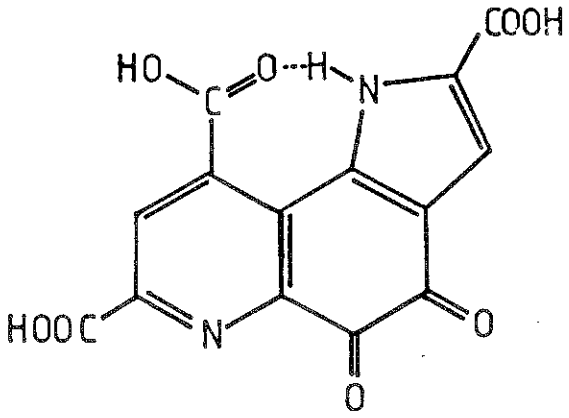
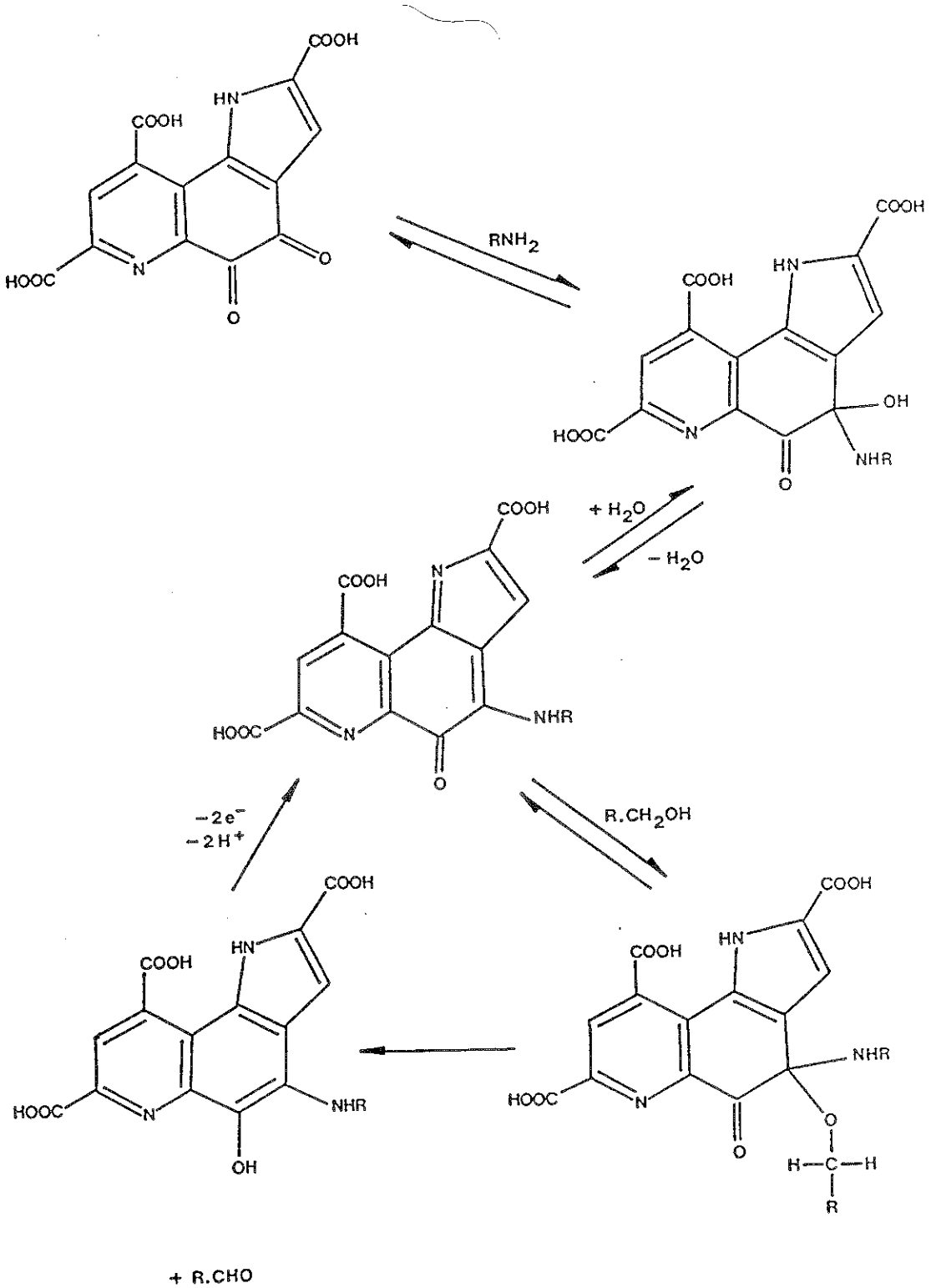

COOH
HO
O
H
N
C
HOOC
N
O
O

Figure 1.2 Proposed mechanism of action of pyrrolo-quinoline quinone oxidation of primary alcohols (from Forrest, Salisbury and Kitty, 1980)


COOH
HN
COOH
HOOC
N
O
O
RNH2
COOH
HN
COOH
OH
HOOC
N
NHR
O
+ H2O
- H2O
COOH
N
COOH
HOOC
N
NHR
O
-2e⁻
-2H⁺
R.CH2OH
COOH
HN
COOH
NHR
HOOC
N
O
O
H—C—H
R
COOH
HN
COOH
NHR
HOOC
N
OH
+ R.CHO

the oxidation of methanol, PQQ(1) forms an addition complex with the amino group of a lysine residue (or ammonia or a primary amine) at the C4 position (II). 1,4- Elimination of water produces the quinone analogue (III) in the active enzyme and 1,4 addition of a primary alcohol allows a cyclic rearrangement with the release of the oxidised product. Duine and Frank (1981a) showed that methanol reacts with PQQ trimethylester forming an adduct at the C5 position. A similar reaction was observed with 4,7-phenanthroline-5,6-dione but not with phenanthrene quinone, suggesting that N6 in PQQ favours methanol addition at C5, although the prosthetic group may not behave the same in vivo. Complex electron spin resonance interpretated as evidence for three electron reduced forms of PQQ (Mincey et al., 1981) has since been shown to be due to the effect of storage (DeBeer et al., 1983).

Although possessing the same prosthetic group the electrophoretic mobilities of methanol dehydrogenases have been shown to vary (Patel et al., 1972; Yamanaka, 1981) due to differences in the amino acid composition between the enzymes (Ohta et al., 1981; Yamanaka, 1981).

The pyrrolo-quinoline quinone prosthetic group has also been shown to be present in methylamine dehydrogenase (DeBeer et al., 1980) and in enzymes from non-methylotrophs such as Acetobacter, Acinetobacter, Pseudomonas and Gluconobacteria species containing the quinoproteins glucose dehydrogenases (Duine et al., 1979b), lactate dehydrogenase, tryptophan dehydrogenase (Duine and Frank, 1981c) and aldehyde dehydrogenase (Ameyama et al., 1981). Removal of the

prosthetic group results in loss of enzymic activity, although reconstitution of a fully active holoenzyme of glucose dehydrogenase from Acinetobacter calcoaceticus has been demonstrated (Duine et al., 1981b; Kilty et al., 1982).

1.1.2 Glucose oxidase.

Glucose oxidase isolated from Aspergillus niger is composed of two subunits, each of 80,000 molecular weight, joined by disulphide bridges. Each subunit contains flavin dinucleotide cofactors (Swoboda, 1969) and a glycoprotein structure (Pazur et al., 1966). The enzyme is composed of 16% carbohydrate (O'Malley and Weaver, 1972), of varying composition (Hayashi and Nakamura, 1981) conferring resistance to inactivation by sodium dodecyl sulphate, urea and heating (Nakamura et al., 1976) but not heavy metals such as silver (Nakamura and Ogura, 1968). Glucose oxidase is also rapidly inactivated by one of its products, hydrogen peroxide, whether the enzyme is in solution (Kleppe, 1966) or immobilised (Greenfield et al., 1975; Krishnawamy and Kittrel, 1978). The properties of the enzyme (Table 1.2), mechanism of action and kinetics have been elucidated (Nakamura and Obura, 1962; Duke et al., 1969). The enzyme catalyses the following reaction:

$$\text{glucose} + O_2 \rightarrow \text{glucono-}\delta\text{-lactone} + H_2O_2$$

$$\text{glucono-}\delta\text{-lactone} + H_2O \rightarrow \text{gluconic acid}$$

Table 1.2 Properties of glucose oxidase

Source	*Aspergillus niger*
Molecular weight	154,000
pH optimum	5.6
Cofactor	2FAD
Co-substrate	Oxygen
K_m for glucose	30 mM

Substrate specificity

Substrate	Relative rate
β-D-glucose	100
2-deoxy-D-glucose	25
6-methyl-D-glucose	2
D-mannose	1
α-D-glucose	0.6

Although glucose oxidase is selective for B-D-glucose, the enzyme from A. niger is capable of oxidising a variety of D-aldohexoses, monodeoxy-D-glucoses and o-methyl-D-glucoses at various rates (Table 1.2); the highest rate being observed with 2-deoxy-D-glucose (Pazur and Kleppe, 1964; Dixon and Webb, 1964).

1.1.3 Enzyme purification.

The commercial development of enzyme-based sensors and fuel-cells for both medical and industrial use requires the economic production of relatively large quantities of enzymes in various degrees of purity. The major processes involved in the extraction and purification of intracellular enzymes include cell separation, disruption, removal of cell debris and finally protein purification.

Normal laboratory scale enzyme production procedures are well advanced and biochemical separation techniques highly adapted. However, scale up of most techniques involved in the purification of enzymes such as methanol dehydrogenase (Anthony and Zatman, 1967a) has not been extensively studied and is severely limited by mechanical properties of resins and materials (Kula et al., 1982). Although preparative chromatography is extensively used in laboratory purification and is readily scaled up to an industrial production; it requires dedicated equipment and components, which are expensive to assemble and operate (Janson, 1977; Curling, 1979; Curling and Cooney, 1982).

Two phase enzyme purification has several advantages over conventional techniques. Although the factors influencing phase

distribution are known (Table 1.3), no mathematical model exists to describe the partition of an enzyme in two phase aqueous systems (Albertsson, 1971). Suitable conditions for extraction therefore have to be found experimentally (Kula et al., 1982).

Two systems commonly employed for two phase extraction are polyethylene glycol/phosphate mixtures and polyethylene glycol/dextran mixtures (Albertsson, 1971). Purified dextan is too expensive to use on an industrial scale (Hedman, 1983), although crude dextran may be used (Kroner et al., 1982a). Polyethylene glycol/phosphate phase systems have been successfully scaled up to an industrial scale (Kula et al., 1977; Hustedt et al., 1980, Kroner et al., 1982b). They are non-toxic (Smyth et al., 1959), biodegradable (Cox, 1978) and may readily be applied to purification procedures used in the food and pharmaceutical industry. Modification of polyethylene glycol may be made by the addition of affinity ligands, resulting in affinity partitioning (Flanagan and Barondes, 1975). The binding capacity per unit volume is considerably higher in aqueous phase systems than in conventional chromatography due to increased ligand density and availability.

Phase separation may be carried out by sedimentation under gravity in settling tanks (Hustedt, 1978). Polyethylene glycol/salt systems settle out faster than polyethylene glycol/dextran systems (Albertsson, 1970; Kula et al., 1982) although conventional centrifuges may be used to reduce phase separation time (Kroner et al., 1978; Hustedt, 1978). Although phase component recovery is not extensively covered in the literature it is a necessary prerequisite

Table 1.3 Parameters affecting partition in two phase aqueous enzyme separation.

1. The composition of the polymer composing the two phase system.
2. Average molecular weight of the polymers.
3. Molecular weight distribution of the polymers.
4. Composition of ions present or added to the system.
5. Ionic strength.
6. pH.
7. Temperature.

for economic scale up. Precipitation by cooling to 6.0°C may be used to recover sodium phosphate from polyethylene glycol/salt systems (Kula et al., 1982). Alternatively, shifting the desired product to the salt phase by adjustment of the PEG phase composition, (Hustedt, 1978) by salting out or by dialysis, may enable the recovery and reuse of the polyethylene glycol fraction.

1.3 FUEL CELLS

Fuel cells may be defined as electrochemical devices in which chemical energy is converted directly into useful electrical energy. Fuel cells and batteries are similar, the major difference being that the latter contain a fixed quantity of fuel or chemical energy, whereas fuel cells require a continuous supply of fuel to enable energy conversion to occur. Normally in fuel cells oxygen, introduced either as a pure gas or air, is reduced at the cathode.

Fuel cells were regarded as only of academic interest for many years after they were first proposed (Grove, 1839), because of problems associated with electrode development, short operational life and high cost. Unlike heat engines, the efficiency of fuel cells is not limited by the Carnot cycle; they therefore offer potential for higher energy conversion (Bockris et al., 1981). The attraction of their thermodynamic efficiency caused rapid development of these devices in the United States with the advent of space exploration (Austin, 1967). Fuel cell

systems may replace both domestic and military power generation enabling electricity to be produced in the area to be served (Huff and Orth, 1960; McBreen, 1980; Anon, 1977; Moss, 1981; Daggitt, 1982). Currently available inorganic fuel cells and those under development are based on the hydrogen-oxygen fuel cell. Various fuel cells are being developed including hydrogen bromide, phosphoric acid and potassium hydroxide. These fuel cells typically require temperatures in the region of 150 - 700^{o}C to operate (Grubb and Michalske, 1964; Wingard et al., 1982). Both the electrolyte composition and elevated operating temperatures impose stringent restrictions on the types of materials and catalysts that can be used for these fuel cells (Wingard et al., 1982).

1.3.1 Biofuel cells

Biofuel cells involve fundamentally the same processes as those which occur in conventional fuel cells, except in the former, the reactions at one or both electrodes are catalysed biologically at ambient temperatures and pressures (Wingard et al., 1982).

The diversity of microbial metabolism allows a large range of fuels to be utilised including many industrial wastes (Suzuki et al., 1978; 1979). The ability of biofuel cells to utilise waste products such as urine, carbon dioxide and faecal materials, led to the proposal that biofuel cells may be developed to operate in confined environments such as those

encountered in space vehicles. Electrical energy, oxygen and food may be produced and waste materials removed (Sisler, 1962; McNeil, 1969). Biofuel cells capable of operating at ambient temperatures, with high efficiency and power density, whilst not emitting traceable products are also important from a military standpoint (Cohen, 1963).

Biofuel cells have generally been classified into one of two types depending on the mode of interaction of the electroactive species with the electrode. Shaw (1963) defined these two classes of biofuel cell. In Type A cells, the fuel is generated biologically in a separate chamber, and electrochemical oxidation occurs either directly or indirectly at the electrode surface. In an indirect fuel cell, the electroactive species such as hydrogen, is oxidised at the anode of a conventional hydrogen-oxygen fuel cell. The overall reaction shown in Fig 1.3. Type B biofuel cells require the direct interaction of the biological catalyst with the electrode, providing a continuous source of electrons. The transfer of electrons to an electrode is often facilitated using a mediator or promotor to effect efficient electron transfer as in the methanol dehydrogenase biofuel cell (Fig 1.4). Classification by other authors has included regenerative, product and depolarizer biofuel cells (Young et al., 1966; Young, 1971; Higgins et al., 1980). Regenerative cells involve the continual transfer of electrons, via a natural or artificial redox compound, to an electrode. A product cell requires the production of electrochemically active

Figure 1.3 The reactions occurring at the electrodes of a conventional hydrogen/oxygen fuel cell.

At the Anode

$$H_2 \rightarrow 2H^+ + 2e^- \qquad E = 0 \text{ Volts}$$

At the Cathode

$$2H^+ + 2e^- + \tfrac{1}{2}O_2 \rightarrow H_2O \qquad E = 1.23 \text{ Volts}$$

Overall

$$H_2 + \tfrac{1}{2}O_2 \rightarrow H_2O \qquad E = 1.23 \text{ Volts}$$

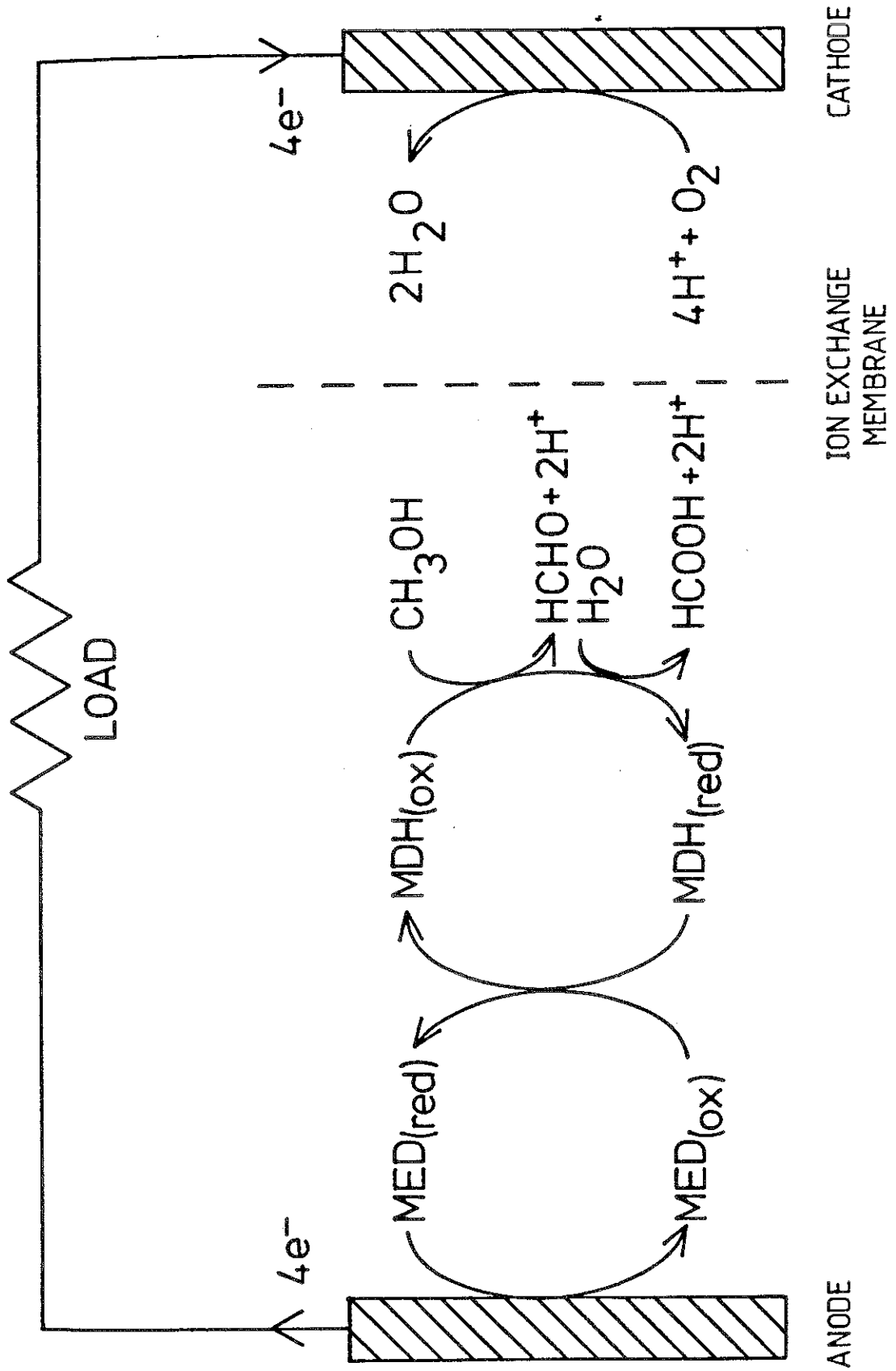
compounds from previously inactive precursors such as hydrogen from glucose (Higgins et al., 1980). In depolarizer cells, biological catalysts act as depolarizers of simple electrochemical reactions, such as oxygen reduction or hydrogen oxidation. Biofuel cells have been classified in this next section into indirect and direct cells, depending upon the interaction of the biological species with the electrode.

1.3.2 Indirect biofuel cells.

Experiments performed in the early part of this century first demonstrated the ability of microorganisms to develope an electrode potential (Potter, 1912). Two half cells containing glucose were connected by a salt bridge; the addition of yeast to one caused a current to be observed. Yudkin (1935) studied the redox potential of washed bacterial suspensions including a facultative anaerobe, a strict aerobe and anaerobe. Although these studies did not involve electron transfer to an external circuit, they did show a good correlation between the redox potentials measured at an electrode and those determined using redox dyes.

The subject was subsequently pursued in the 1960's by NASA in the United States, initially with microbial cells or fermentation broths within the anodic electrode compartment (Austin, 1967). During this period, patent applications were granted for biofuel cells both in the UK and the USA, for processes including alcohol fermentation by yeasts (Hunger and

Figure 1.4 Schematic diagram of a methanol dehydrogenase based biofuel cell where MED (ox/red) are the oxidised and reduced forms of the mediator. MDH (ox/red) are the oxidised and reduced forms of the prosthetic group of the qunioprotein, methanol dehydrogenase.


LOAD
4e⁻
4e⁻
ANODE
CATHODE
ION EXCHANGE MEMBRANE
MED(red)
MED(ox)
MDH(ox)
MDH(red)
CH3OH
HCHO + 2H+
H2O
HCOOH + 2H+
2H2O
4H+ + O2

Perry, 1966), hydrocarbon oxidation by bacteria (Young, 1965) and Actinomycetes (Davis and Yarborough, 1967). An alternative to electrochemical oxidative fuel cells (Grubb and Michalske, 1964) was described by Van Hees (1965). The biofuel cell described by Van Hees (1965) utilised methane oxidation by the methanotroph, Pseudomonas methanica. The cell consisted of two half cells, an air cathode and a methane-sparged anode, the latter containing the obligate methanotroph at a concentration of 0.5×10^9 ml^{-1}. The two compartments were separated by a double membrane arrangement, the middle compartment sparged with nitrogen, thus reducing oxygen transfer from the cathode to the anode. The current density was enhanced by the incorporation of a mediator, but the open circuit potential was unaffected. The fuel cell was capable of developing a maximum power density of 2.8 uW cm^{-2} at 0.35 Volts.

Indirect biofuel cells have been described, based on the generation of ammonia, hydrogen or methanol from high molecular weight materials such as sugars, urea or starches (Lewis, 1966). One of the first systems to be developed incorporated the enzyme urease (Del Duca et al., 1963). Ammonia was supplied to the anodic compartment of an ammonia-oxygen fuel cell containing 3.0 M potassium chloride-tris buffer electrolyte. The biofuel cell was capable of yielding a current density of about 3.0 mA cm^{-1} at 0.4 Volts.

The concept of a "hydrogen economy" has been widely debated (McGown and Bockris, 1980; McAuliff, 1980; Beghi, 1981). Such an

economy is dependent both upon the economic availability of large quantities of hydrogen, with suitable means of handling, storage and transportation. Hydrogen may be either burnt or reacted directly in a fuel cell generating pollution free electricity. Hydrogen gas, even when compressed, however, contains relatively little energy per unit volume. The ideal storage medium would be a comparatively inert liquid which would readily release its energy when required. Two such compounds, methanol and formic acid are suitable substrates for the microbial production of hydrogen at ambient temperatures and pressures (Egorov et al., 1981). Methanol is very attractive as a fuel from an economic and practical viewpiont. It does not pollute the environment, can be produced from fossil fuels as well as other raw materials and can be handled and transported more readily than hydrogen (Egorov et al., 1975).

Hydrogen may be produced by a variety of microorganisms (Gest, 1954) including algae (Gaffron and Rubin, 1949) and purple bacteria (Gest and Kamen, 1949) when exposed to light, this subject has been extensively reviewed (Pfennig, 1967; Gest, 1972; Kesler, 1973; Kondratieva, 1977; Mitsui, 1978).

One of the first bioelectrochemical systems to derive energy from hydrogen was developed by Rourback, Scott and Canfield (1962) in which Clostridium butyricum utilised glucose anaerobically. C. butyricum was capable of yielding large quantities of hydrogen (Brake, 1963a), but the system performed poorly due to the low solubility of hydrogen in the electrolyte

(Brake et al., 1963a,b). The electrode performance was improved by the use of a palladium metal anode, in preference to platinum; this resulted in a current increase from 4.0 mA cm^{-1} to 8.0 mA cm^{-1}. Immobilisation of C. butyricum cells in agar (Suzuki et al., 1980a,b; Karube et al., 1981) and polyacrylamide gel (Karube et al., 1976, 1977b) has resulted in the stabilisation of the enzyme facilitating continuous hydrogen evolution. The rate of hydrogen production by agar gel-entrapped cells was higher than that achieved by cells immobilised in polyacrylamide (Suzuki and Karube, 1978; Suzuki et al., 1978, 1979, 1980b). Such systems resulted in continuous energy production, initially using glucose as substrate (Karube et al., 1977b; Suzuki and Karube, 1978) and subsequently alcohol factory waste (Suzuki et al., 1978, 1979, 1980a,b; Karube et al., 1981). The hydrogen derived from the immobilised cells either reacted directly at the anode surface (Karube et al., 1977b) or indirectly in a conventional hydrogen-oxygen fuel cell (Suzuki et al., 1978, 1980a,b). The current in the direct biofuel cell was shown to be due to a combination of electrochemical reactions involving hydrogen and glucose (Yao et al., 1969; Liu et al., 1978). Microbial-hydrogen has been used to power a 10 W capacity fuel cell (Anon, 1982).

Two theories were proposed to explain the mechanism by which currents were generated in direct fuel cells. The first required the active discharge of organic substances at the electrode irrespective of the presence of bacteria. The second, required the active interaction of the bacteria with the

electrode. It has been demonstrated that bacteria isolated from the electrode were still capable of producing a diminished current (Yudkin, 1935; Videla and Ariva, 1971). While it is not clear exactly what electrochemical reactions occur, it has recently been shown that the kinetics of the bioelectrode reaction are due to two consecutive reactions occuring at the electrode surface (Disalvo et al., 1979). Direct interaction between the electrode and the microorganism was not required but microorganisms have been shown to alter the environment near the electrode (ie. oxygen depletion).

1.3.3 Direct biofuel cells.

An alternative to electrochemical oxidation of microbial products as the basis for a biofuel cell is the development of directly coupled systems thus eliminating intermediates such as hydrogen. The selective permeability of the cell wall and membranes of whole cells limits their use in whole cell biofuel cells (Lewis, 1966; Williams, 1966; Higgins et al., 1980; Wingard et al., 1982). The inclusion of redox mediators capable of shuttling electrons between the microorganism and the electrode provides a mechanism for the operation of efficient whole organism fuel cells (Delany et al., 1983, 1984; Turner et al., 1983). The point of interaction of the mediator with the respiratory chain of such organisms is not clear (Williams 1966; Bennetto et al., 1983), but it has been suggested that it is the NAD^+/NADH couple (Bennetto et al., 1983). The use of either

mediators or redox proteins as intermediates in the electron transfer from the biological catalyst to the electrode, however, reduces the potential difference between the two half cells, since the redox potential exhibited by the electrode is that of the last component of the chain. The redox potential of the mediator should, therefore, be as negative as possible in order to maximise the potential difference between the two electrodes (Bennetto et al., 1983). The coulometric efficiency of mediated biofuel cells has been shown to be high for both whole organism (Delaney et al., 1984; Roller et al., 1983a) and enzyme-based systems (Weibel and Dodge, 1975).

The first clear example of a directly coupled biofuel cell may be attributed to Cohen (1931) who demonstrated that a current of 2.0 mA at a potential of 35 V could be produced by connecting several bioelectrochemical fuel cells in series. Glucose oxidase and microbial fuel cells, both mediated by methylene blue, were investigated by Davis and Yarborough (1962). A range of phenothazine dyes including thionin and methylene blue are reduced by Escherichia coli (Bennetto et al., 1981; Roller et al., 1983b). The incorporaton of these mediators in biofuel cells has resulted in high coulometric yields being attained (Bennetto et al., 1983; Delany et al., 1983; Roller et al., 1983a) a reaction facilitated by the incorporation of chelating compounds (Tanaka et al., 1983).

Methylene blue has been shown to act as a mediator for glucose oxidase (Davis and Yarborough, 1962) in a glucose powered

biofuel cell. The cell consisted of three compartments separated by dialysis membrane. The cathode was sparged with oxygen whilst the anode and inner compartments were maintained anaerobic by sparging with oxygen-free nitrogen. It was demonstrated that in the presence of substrate and methylene blue, glucose oxidase was capable of producing a current. The open circuit potential changed by 100 mV (to 180 mV) and the cell was capable of maintaining 50 to 100 mV across a load of 1000 ohms. Biofuel cells based on glucose oxidase and amino acid oxidase were shown to be capable of producing a current in the absence of mediator (Allen and Yahiro, 1963; Yahiro et al., 1964). No mention was made, however, of the oxygen tension in the anodic compartment. It was latter demonstrated (Yahiro et al., 1964) that alcohol dehydrogenase catalysing an oxygen-independent reaction was incapable of producing a current, even though NADH was produced. NADH is, however, only oxidised electrochemically at high overpotentials (Johanson, 1978) and is not readily oxidised at inert electrodes such as platinum or carbon (Moiroux and Elving, 1978; Jaegfeldt, 1980). The soluble mediator, phenazine methosulphate, has been shown to be capable of rapidly oxidising NADH (Ottaway, 1966) and was subsequently used in a NAD-linked alcohol dehydrogenase fuel cell (Kulis and Malinauskas, 1979). Recent work on modified electrodes (Albery and Hillman, 1981) has enabled electrochemical oxidation of NADH to be performed at relatively low potentials (Tse and Kuwana, 1978; Jaegfeldt et al., 1981; Albery and Bartlett, 1984).

Cofactors such as flavins may be immobilised onto electrode surfaces facilitating electron transfer in biofuel cells and sensors (Wingard and Gurecka, 1978; Wingard, 1978, 1980; Wingard et al., 1982). Implantable biochemical fuel cells using glucose oxidase (Drake et al., 1968) and cofactor requiring enzymes of the tricarboxylic acid cycle (Avampato et al., 1974), linked to mediators such as methylene blue, have been proposed for use in the human body as practical power sources for pacemakers. Incorporation of the hydrogenase enzyme into the anodic compartment of a biofuel cell has been demonstrated, mediated by either methylene blue or methyl viologen (Mizuguchi et al., 1964; Varfolomeev et al., 1977). The coupling using methyl viologen has been developed as a test for hydrogenase activity (Yagi et al., 1975). The enzyme was subsequently immobilised in a polyacrylamide gel and linked to an electrode via the mediator, lithium tetracyano-quinonedimethane (Varfolomeev et al., 1978).

1.3.4 Biocathodes.

Biocathodes of biofuel cells utilise oxygen electrodes or substitutes, such as the ferricyanide-ferrocyanide couple (Allen, 1972) to complete both direct and indirect biofuel cell circuits. The oxygen electrode has the disadvantage of requiring the use of platinum or other catalytic metal electrodes and is not efficient under the mild conditions of pH and temperature normally required for biological systems (Hoare, 1968; Schiffrin, 1983). However, because of the ease of availability of air, it

is considered to be the only practical cathodic electron acceptor (Brake et al., 1963a).

Biofuel cells incorporating either whole cells or enzymes in both electrode compartments have been described (Foremski, 1982; Mizuguchi et al., 1966; Shaw, 1963). As in the previous case of bioanodes, biocathodes may be indirect or direct, incorporating either whole cells or their components. One of the first systems to utilise both a biocathode and a bioanode involved the incorporation of faecal bacteria and algae in the cathode and anode, respectively (Reynolds and Konikoff, 1962). The two compartments, separated by a cation exchange membrane, were capable of developing 0.3 V. Berk and Canfield (1964) developed a fuel cell consisting of two photosynthetic microorganisms; Rhodospirillum rubrum in the anodic compartment produced hydrogen in the presence of malate, whilst algae incorporated in the cathodic compartment completed the circuit by producing oxygen. Although no mediator was used, the biofuel cell was capable producing a short circuit current density of 75 uA cm^{-1} upon illumination. The use of algae in a biofuel cell to supply oxygen to the cathode and fuel to the anode has been described (Sisler, 1964). Immobilised chloroplasts incorporated into the cathodic compartment of a biofuel cell, with Clostridium butyricum and ferredoxin in the anode, showed an enhanced current upon illumination (Suzuki et al., 1980b). Biofuel cells capable of utilising carbon dioxide and urea have been proposed for extended space flights (McNeil, 1969).

Directly coupled cathodic reactions have received some attention. Cytochrome c has been shown to undergo reversible oxidation/reduction at a 4,4'bipyridyl-modified gold electrode (Eddows and Hill, 1979; Albery et al., 1981) and by linkage to an oxidase has been shown to be enable the reduction of molecular oxygen (Hill et al., 1981). Laccase has also been reported to catalyse the reduction of oxygen at an electrode (Mizuguchi et al., 1962, 1966; Berezin et al., 1978), an effect enhanced by the addition of hydroquinone. It was, however, subsequently demonstrated that a non-biological system, consisting of ammonium chloride and copper sulphate was as effective at reducing molecular oxygen as the enzyme (Mizuguchi et al., 1962, 1966). The oxygen reduction capability of an electrode may be biologically enhanced by modification with ferri-ferrocyanide linked, to the amino acid cystine (Mizuguchi et al., 1962, 1966).

1.3.5 Biofuel cell efficiency

1.3.5a Thermodynamic efficiency

Four aspects of fuel cell efficiency must be considered: voltage, current (Faradaic), free energy and comparative thermal efficiencies. The attraction of fuel cells lies in their thermodynamic efficiency with the energy output being dependent upon the difference in the Gibbs free energy (G) between the reactants and the products, with small losses due to entropy

effects. Accepting that the thermodynamic principles applied to bioelectrochemical cells are the same as conventional inorganic cells (Glazebrook and Jones, 1966; Young et al., 1966; Liebhafsky and Cairns, 1968; Bockris and Srinivasan, 1969; Bockris et al., 1981) the net release of Gibbs free energy is related to the reversible potential difference (E) between the two half cells in their standard states and the number of electrons (n) transfered per mole of fuel, according to the equation:

$$G = -n.F.E$$

where F is the Faraday constant (96,490 C mol^{-1}). Thermodynamic data, although incomplete, exists for biological systems (Williams, 1966) enabling e.m.f. values for direct biofuel cells to be predicted from the formal potentials of the substrates. The e.m.f. of a cell may be derived from the standard enthalpy and entropy values:

$$\Delta G = \Delta H - t \Delta S$$

Where G is the Gibbs free energy, H the enthalpy, t, the temperature and S, the entropy. Care should be taken in relating experimental values to E since formal potentials vary with concentration, ionic strength and temperature (Ives, 1971). These equations demonstrate that the work derived from such systems is dependent upon the sign and magnitude of the entropy change.

The thermodynamic efficiency of a fuel cell, defined as the ratio of energy available to do work, to the total energy, may be expressed as a percentage:

$$\%E = (\Delta G/\Delta H).100$$

$$= (1-T\Delta S/\Delta H).100$$

For certain reactions efficiencies greater than 100% are predicted (Bockris and Srinivasan, 1969).

1.3.5b Voltage efficiency

The voltage efficiency is the ratio of the operating voltage to the theoretical voltage. If all the free energy of a hydrogen-oxygen fuel cell was completely converted to electricity then a potential of 1.23 volts would be attained. However, in practice such cells run at a potential of 0.6 - 0.8 volts. Provided the current efficiency is 100% the voltage efficiency has the same value as the free energy efficiency.

1.3.5c Current efficiency

Current efficiency may be defined as:

$$E = \frac{\text{No. of electrons per mole of reactant}}{\text{No. of electrons available per mole of reactant}}$$

or

$$E = \frac{\text{observed current}}{\text{current calculated from rate of consumption of reactants}}$$

or

$$E = \frac{\text{observed charge passed}}{\text{calculated charge available from reactants}}$$

The current efficiency may be less than 100% due either to the occurence of side reactions yielding fewer electrons, a series of consecutive reactions, non-electrochemical decomposition of the reactant or a chemical reaction between the fuel and oxidant or other components present.

1.3.5d Thermal efficiency

In order to facilitate comparison between fuel cells and heat engines the ideal or comparative thermal efficiency may be defined as:

$$E = \frac{\Delta G'}{\Delta H}$$

1.3.5e Electrochemical efficiency

The behaviour of fuel cells under load is dependent upon the kinetics of the processes involved; these have been described by numerous authorities on the subject (Williams, 1966; Bockris and Srinivasan, 1969; Bockris and Reddy, 1970; Bard and Faulker, 1980; Bockris et al., 1981) and have been recently summarised (Wingard et al., 1982). When a current, however small, flows between two electrodes via an external circuit, the equilibrium ceases to be reversible and a net reaction must occur at the

electrodes with unequal forward and reverse reactions. Rapid electrode processes or small currents facilitate minimal departure from equilibrium, resulting in the theoretical and observed cell voltages being similar when the current flows. This ideal situation is frequently not realised and large deviations from the theoretical voltages are often observed. This drop in open circuit potential when a current flows is known as the overvoltage and is associated with resistive polarisation effects and the fall in the theoretical energy transfered to the external ciruit. The main contributers to this are; the activation polarisation arising from the inhibition of charge transfer across the electrode-solution interface, concentration polarisation caused by gradients of components at the electrode interface and ohmic polarisation caused by the resistance of the bulk solution separating the electrodes.

1.3.5f Activation polarisation

The activation overvoltage caused by slow electron transfer has been described in detail by other authors (Bockris and Reddy, 1970; Liebhafsky and Cains, 1968) and may be summed up as follows:

1) A low free energy of activation (or high exchange current) means that the catalyst is efficient and equilibrium rapidly established.
2) The lower the free energy of activation, the lower the polarisation.

3) The larger the electrode the lower the activation energy at a given current.

1.3.5g Concentration polarisation

In order for electrochemical reactions to continue at an electrode surface reactants must arrive and products leave. Interaction between reactants, such as mediators, and electrodes may be by virtue of diffusion or ionic transport. The rate of interaction may be enhanced by agitation, or in the case of directly supplied systems, by external factors. If the rate of transfer is inadequate, serious polarisation effects will occur. At a planar electrode the effects are approximated by linearisation of Ficks first law (Bockris and Reddy, 1970; Bard and Faulkner, 1980; Bockris and Srinavasan, 1969; Bockris et al., 1981) although more complex relations apply at porous electrodes (Bockris and Srinavasan, 1969; Bockris et al., 1981). The limiting current is the current at which a sharp change in potential occurs; in a fuel cell the cell potential will fall to zero.

1.3.5h Ohmic polarisation

Ohmic polarisation arises principally from the solution and membrane resistances separating the two compartments. It causes an appreciable voltage drop at high current densities. Large internal resistances in the cell result in loss of power in the

external circuit (Lewis, 1966; Bockris et al., 1981). These voltage drops may be minimised by careful fuel cell design and construction, such as in thin stacked cells (Eisenberg, 1962).

1.4 BIOSENSORS.

The advantage of enzymes in quantitative assays has been known for some years. The combination of of large number of studies has revealed the elegance of biological catalysts. The most important property is their specificity which, in some cases, enables resolution of stereospecific isomers.

Since the 1970's considerable effort has been focused on improving commercial enzyme purification procedures, which has led to their increasing use for analysis, therapy and as industrial catalysts; the principle usage being in diagnostics and food analysis (for examples see Aston and Turner, 1984). Enzymic methods of analysis have found increasing acceptance and are routinely used in both clinical and quality control laboratories.

Although relatively pure enzymes with high specific activity are now available at reasonable prices, significant economic and practical advantages are offered by enzyme immobilisation. These advantages often include enhanced enzyme stability, both during operation and storage, alongside the ability to perform multiple determinations (Zaborsky, 1973; Pye and Wingard, 1973; Weetal, 1975; Chibata, 1978; Means and Feeney,

1971; Carr and Bowers, 1980).

Immobilisation has in many cases been shown to alter enzyme kinetics from those found in enzymes free in solution (Chibata, 1978). This alteration in the kinetics is due to the formation of heterogeneous systems in which the free passage of molecules to and from the enzyme is limited by either a diffusion or a partioning effect (Wingard et al., 1981; Carr and Bowers, 1980).

Two principal techniques may be used for substrate determination depending on either dynamic or end point measurements. In the former method a physical or chemical change is followed continuously for a short period enabling rapid results to be obtained. The initial velocity of the reaction is dependent on the enzyme concentration, activity, substrate affinity and the concentration of non saturating substrate. In the second method, relatively large amounts of enzyme are used and the reaction is allowed to attain equilibrium thus rendering the technique rather insensitive to physical and chemical conditions affecting enzymic activity. Whereas the latter technique is favoured for substrate determination, the former may be applied to inhibitor and activator assays. Care must be taken, however to control other conditions that may affect the rate of the reaction, especially pH and temperature (Table 1.4).

The straightforward and inexpensive measurement of industrial and clinical samples using a combination of biological material and a secondary detector has been extensively pursued.

Table 1.4 Physical and chemical factors affecting enzyme electrodes.

Physical Factors
Stability of base sensor
Mechanical stability of enzyme layer
Mechanical stability of membrane
Chemical Factors
Enzyme immobilisation techniques
Co-substrate immobilisation technique
Total enzyme activity
Stability of immobilised enzyme
pH
Temperature
Inhibitor concentrations
Storage conditions

Glucose analysis alone includes methods based on spectrophotometric (Dobrick, 1961; Guidotti et al., 1961), potentiometric (Wingard et al., 1983), amperometric (Williams et al., 1970), calorimetric techniques (Mosbach and Danielsson, 1981), and optoelectronic methods (Lowe et al., 1983).

Although the majority of conventional assays involve spectrophotometric analysis, (For examples, see Aston and Turner, 1984) recent reviews on biosensors detail numerous configurations of enzyme-based sensors currently being developed (Carr and Bowers, 1980; Kobos, 1980; Wingard et al., 1981; Guilbault, 1982; Suzuki and Karube, 1981; Suzuki et al., 1982; Lowe et al., 1983; Mosbach, 1983).

One of the most widely known devices, the enzyme electrode, combines the specificity of the biological catalyst with the simplicity of electrochemical techniques. Enzyme electrodes consist of an enzyme retained or immobilised on the surface of an electrode. The enzyme generates or consumes an electrochemically active species in the presence of its substrate. Measurements may be made by operating in either an amperometeric or a potentiometric mode (Wingard, 1983). In addition to the enzyme electrode a reference electrode is required to complete the circuit, enabling current flow or potential changes to be measured. Potentiometric detection is usually applied to enzyme electrodes in which an ionic species is formed or consumed during the reaction (Table 1.5), whereas amperometric devices require one of the species involved in the enzymic reaction to be

Table 1.5 Potentiometric enzyme electrodes

Substrate	Enzyme	Species	Electrode	Reference
L-amino acids	L-amino acid oxidase	NH^{4+}	Mono cation	Guilbault and Hrabenkova (1971)
Creatine	Creatinase	NH^{4+}	Mono cation	Meyerhoff and Rechnitz (1976)
Glucose	Glucose oxidase	I^-	Iodine selective	Nagy *et al* (1973)
Glucose	β-Glucosidase	H^+	pH/glass	Nilsson *et al* (1973)
Penicillin	Penicillinase	H^+	pH/glass	Cullen *et al* (1974)
Urea	Urease	H^+	pH/glass	Nilsson *et al* (1973)

electrochemically consumed (Table 1.6). Enzyme electrodes have many advantages as analytical devices (Table 1.7) enabling particular substrates to be assayed with the minimum of pretreatment. Since these devices are generally non-destructive multiple determinations may be performed. The response is affected by a combination of chemical and physical factors, but is rapid, usually within 30-600 seconds.

1.4.1 Indirect enzyme electrodes.

The term 'enzyme electrode' was first proposed by Clark and Lyons (1962) who determined glucose concentrations using glucose oxidase retained between two cuprophane membranes at an oxygen electrode. A more practical device was demonstrated by Updike and Hicks (1967) in which the enzyme was immobilised in polyacrylamide gel at an oxygen electrode, whilst a second electrode containing heat inactivated enzyme was used to compensate for variation in oxygen tension and interfering substances. Alternative methods for overcoming fluctuations in oxygen tension were to supply sufficient oxygen electrochemically (Romette et al., 1979) or partioning by increasing the oxygen diffusion layer (Bessman and Shultz, 1973, 1974) thereby reducing fluctuations in the hydrostatic pressure caused by loosening of the membrane (Severinghus, 1966).

The detection of hydrogen peroxide, an electrochemically active product (see section 1.2) of the glucose oxidase reaction, has enabled picomolar concentrations of glucose to be determined

Table 1.6 Amperometric enzyme electrodes

Substrate	Enzyme	Species	Electrode	Reference
Alcohols	Alcohol oxidase	O_2	Platinum	Clark (1972)
Alcohols	Alcohol oxidase	O_2	Platinum	Nanjo and Guilbault (1975)
Ethanol	Alcohol dehydrogenase	NADH	Carbon	Suzuki *et al* (1975)
Glucose	Glucose oxidase	O_2	Platinum	Guilbault and Labrano (1973)
Lactate	Lactate dehydrogenase	$Fe(CN_6)^{3-}$	Platinum	Durliat *et al* (1975)
Lactate	Lactate dehydrogenase	NADH	Carbon	Suzuki *et al* (1975)
Monoamine	Monoamine oxidase	O_2	Platinum	Karube *et al* (1980)

Table 1.7 Advantages of enzyme electrodes

Minimal sample pretreatment
Non-destructive towards sample
Small sample volume
Calibration against standard samples
Wide range of sensitivity
Rapid response
Simple to operate
Cheap
Accurate
Specific

(Sittampalam and Wilson, 1982). Electrodes based on this system have been developed for *in vivo* (Shichiri *et al.*, 1982) and *in vitro* (Chua and Tan, 1978) blood glucose analysis. The detection of glucose via hydrogen peroxide production can be achieved using a coupled reaction:

$$2H^+ + H_2O_2 + 2I^- \longrightarrow 2H_2O + I_2$$

The change in iodide concentration can be measured potentiometrically with an iodide selective electrode (Nagy *et al.*, 1973) amperometrically by reduction of iodine (Malstadt and Pardue, 1961; Pardue, 1963) or by coupling to ferricyanide (Blaedal and Olson, 1964). Systems incorporating soluble mediators are, however, not practical for *in vivo* measurements (Thevenot, 1982).

Electrochemical interference from substances such as ascorbic acid, uric acid, tyrosine and certain drugs may occur (Farrance and Aldons, 1981; Lidh *et al.*, 1982). Interference may be compensated for by the use of a second electrode containing inactive enzyme (Updike and Hicks, 1967; Clark, 1972; Thevenot *et al.*, 1978; Thevenot, 1982) or eliminated by the use of a membrane (Lopel and Rishpon, 1981).

The use of ion selective electrodes has increased enormously over the past few years, their mode of operation and basic principles are well documented (Koryta, 1975; Moody and Thomas, 1975; Gamman, 1977; Fricke, 1980; Freiser, 1980). They have been successfully applied to the physiological monitoring of

sodium, potassium, calcium and lithium (Berman, 1974; Meir et al., 1980; Zhukov et al., 1981). Modification of ion selective membrane materials (Rechnitz, 1967) has led to the use of liquid membrane electrodes for in vivo analysis of chloropheniramine (Fukamachi and Ishibashi, 1978), novacine (Negoiu et al., 1981), sulpha drugs (Ionescu et al., 1981), naproxinate (Hogue and Landgraf, 1981), codeine and morphine (Goiha et al., 1978).

Miniaturization of ion selective electrodes has enabled research to be directed towards microscale enzyme electrodes (Brown and Flaming, 1970) which will provide a valuable tool in medicine, biology and physiological reseach (Gamman, 1977). Biosensors based on ion-selective electrodes function by indirect determination of ion formation or utilization (Table 1.6, see Aston and Turner, 1984 for further examples). An example is the urea sensor incorporating the enzyme urease coupled to an ion selective electrode (Katz and Rechnitz, 1963; Guilbault and Montalvo, 1970; Guilbault and Nagy, 1973). Two products are formed, both of which may be assayed using an ion selective electrode:

$$CO(NH_2)_2 + 2H_2O \longrightarrow 2NH_4^+ + CO_3^{2-}$$

Ammonium ions may be measured using an ammonium ion selective liquid membrane electrode (Montalvo and Guilbault, 1969) and carbonate ions determined using a carbonate liquid membrane electrode (Herman and Rechnitz, 1975). Both methods have the disadvantage that they are susceptible to interference

from other ions present in the sample (Katz and Cowans, 1965). Interference may be overcome by the use of gas sensitive electrodes or air gap electrodes, the design and use of which have been reviewed (Bailey and Riley, 1975; Fricke, 1980). Air gap electrodes, introduced in the urease assay were later refined for measurements in both serum and whole blood; (Hansen and Růzuičha, 1974) measuring the ammonia evolved upon exposure to strong alkali. A more rapid and reliable single step assay was developed using immobilised urease (Guilbault and Tarp, 1974). Carbon dioxide, the other product under acidic conditions, may be measured using a gas sensitive electrode (Guilbault and Shu, 1972). These methods are not affected by ions present in the sample and exhibit selectivity, although hydrogen ions may affect the equilibrium (Ross et al., 1974). Whilst enzyme-based sensors generally offer better specificity than whole organism and tissue-based sensors, the latter are capable of mediating complex reaction sequences (Kobos and Pyon, 1981). Workers in Japan, have demonstrated a variety of practical devices based on immobilised whole organims and enzymes (Suzuki and Karube, 1981; Suzuki et al., 1982). Judicious selection of organisms and membrane configurations has yielded devices that are sufficiently selective for use in a variety of industrial processes such as methane determination (Okada et al., 1981; Karube et al., 1982) and biological oxygen demand (Karube et al., 1977a).

1.4.2 Direct amperometric enzyme electrodes.

The potential advantages of simplicity, low cost and accuracy achievable by direct electron transfer between biological and electronic systems, has focused attention on such systems (Fig 1.9). Oxidoreductases may be linked directly to electrodes using either their natural redox couple such as NAD^+/NADH at modified electrodes (Albery and Hillman, 1981) or artificial electron acceptors (Williams et al., 1970).

Electron transfer between glucose oxidase and a platinum electrode has been demonstrated using benzoquinone (Williams et al., 1970). Although the reduced mediator is of industrial use (Vittum, 1951; Alberti and Klibanov, 1982) both forms of the mediator are toxic (Bretherick, 1981) and are therefore unsuitable for in vivo analysis.

Triglycerides may be determined amperometrically in a three enzyme system comprising of lipase, glycerol dehydrogenase and diaphorase; the latter produces NADH which may be determined at an electrode by linking to ferricyanide (Guilbault, 1980). NAD^+-independent yeast lactate dehydrogenase may be linked to ferricyanide (Williams et al., 1970) and incorporated into a lactate sensor (Durliat et al., 1975). The application of the lactate sensor in clinical analysis has been described (Racine et al., 1975). Alternative electron acceptors for enzymes such as glucose oxidase have been proposed including 2,6 dichlorophenol indophenol (Dobrick, 1961). The insoluble mediator, N-methylphenazinium tetracyanoquinodimethanide (NMPTCNQ), has

been successfully coupled to a variety of enzymes both as a pressed pellet and a paste. These include cytochrome $\underline{b}_2$ (Kulis and Svirmickas, 1980; Kulys et al., 1980, 1982), glucose oxidase (Cenas and Kulys, 1981), peroxidase and xanthine oxidase (Kulys et al., 1982) in the determination of L-lactate, D-glucose, hydrogen peroxide, and hypoxanthine as substrates. The results show oxygen interference, presumably due to the reaction of the enzyme with oxygen. The mediator has also been reported to oxidise NADH to NAD^+ (Albery et al., 1984; Albery and Bartlett, 1984).

1.5 OUTLINE OF THESIS.

This thesis describes the purification of methanol dehydrogenase and its incorporation into a biofuel cell. A variety of possible components of the biofuel cell were investigated. The high coulometric efficiency attained using a soluble mediator enabled it to be used as a methanol sensor. Retention of the enzyme by a membrane in the presence of an insoluble mediator allowed methanol to be determined in samples with fluctuating oxygen tensions. Glucose oxidase was immobilised and used as a model amperometric enzyme-based electrode.

AIMS OF THESIS

In recent years the application of enzymes has incresed in an number of fields including analysis, therapy and industrial catalysts. One area of research involves the application of electrochemical techniques to both whole organisms and enzymes. This has resulted in firstly, biofuel cells from which electrical energy may be derived and secondly, sensors capable of the quantitative determinination of specific substrates.

The majority of biofuel cells use whole organisms whose products react either in a conventional fuel cell such as the hydrogen/oxygen fuel cell (Karube et al., 1976; 1977a,b; Suzuki and Karube, 1978) or at electrodes which are in intimate contact with the organisms (Videla and Arvia, 1971; Disalvo et al., 1979). A few incorporate enzymes whose catalytic products react at electrodes within the same compartment (Allen and Yahiro, 1963; Yahiro et al., 1964; Lewis 1966) or in a separate compartment (Del Duca et al., 1963). Of those biofuel cells based on enzymes a smaller number derive energy from the transfer of electrons between the enzyme and electrode via a mediator (Davis and Yarbrough, 1962; Mizuguchi et al., 1966; Weibel and Dodge, 1975).

Sensors also predominantly involve mechanisms in which the electrode measures a substrate or product of the biological reaction, such as oxygen consumption (Clark and Lyons, 1962) or ammonia production (Guilbault and Nagy, 1973). Whilst these systems have been successfully employed, an alternative approach is to exploit the transfer of electrons from the enzyme to an electrode either directly

or via a mediator.

The aim of the research described in this thesis was to investigate the use of a mediator to facilitate the transfer of electrons from an enzyme to an electrode and to develop a biofuel cell and an enzyme-based sensor using this interaction.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Glucose and all inorganic chemicals unless otherwise stated were of analytical grade and supplied by BDH Chemicals Ltd., Poole, Dorset. Agar was otained from Oxoid Ltd., Basingstoke, Hants. Biochemicals were supplied by Sigma Ltd., Poole, Dorset. Platinum and Nickel electrodes were kindly donated by Johnson Matthey Ltd., UK and the International Nickel Corporation Ltd., UK respectively. Formaldehyde releasing biocides and cutting oils were kindly supplied by Dr.E.C.Hill at University College, Cardiff. Dialysis membrane was obtained from Medicell Ltd., London, Nuclepore membrane by Nuclepore Ltd., California, USA. Nafion membranes supplied by Dupont De Nemours and Co., Delaware, USA and ICOM membrane by Dr.H.A.O.Hill. Oxygen and nitrogen (white spot) were obtained from British Oxygen Ltd., London.

2.2 Source of organisms

Pseudomonas AM1 (NCIB 9133), Pseudomonas extorquens (NCIB 9399) and Paracoccus denitrificans (NCIB 8944) were obtained from the National Collection of Industrial Bacteria, Aberdeen, Scotland, UK. Coriolus versicolor, was obtained from the Department of the Environment Research Establishment, Princes Risborough Laboratory, Aylesbury, Bucks.

2.3 Maintenance of cultures.

Stock cultures of Ps. AM1, Ps. extorquens and P. denitrificans were maintained on nutrient agar plates at 4.0°C. They were transfered prior to growth experiments to complete autoclavable minimal media agar plates (see section 2.4) containing methanol, as the carbon source, introduced in the vapour phase as a solution in the lid.

Coriolus versicolor was maintained on malt / yeast / peptone / glucose (MYPG) agar plates (Wickerham, 1951) at 25°C.

2.4 Growth of organisms.

Growth of Ps. AM1, Ps. extorquens and P. denitrificans in liquid culture at pH 7.0 was in 1.0 l conical flasks containing inorganic salts media (250 ml). The medium was either D_2 (Downs and Harrison, 1974) or complete autoclavable salts medium (see section 2.4) with methanol (1.0% V/V unless otherwise stated) as the sole sorce of carbon and energy. The organisms were induced to grow on methanol as their sole source of carbon and energy. This was achieved by placing methanol in the centrewell of an unshaken conical flask containing either of the above mineral salts media maintained at 30°C. Once growth was observed (indicated by an increase in turbidity) these flasks were used to inoculate 1.0 l conical flasks containing the same media (250 ml) as used to induce the organism. The flasks were maintained at 30°C and 150 rpm. Growth was monitored by measuring the optical

density at 600 nm.

Large scale growth of Ps. AM1 on Jayasuriya mineral salts medium (Jayasuriya, 1955) was carried out by Dr.R.C.Hammond, University of Kent, Canterbury, in an electronically controlled 1500 l pilot plant fermenter (New Brunswick, USA) with the assistance of the Fermentation Research Group at Pfizers Research, Sandwich, Kent. The inoculum was also grown on Jayasuriya mineral salts medium, containing methanol (0.4% V/V) as the sole source of carbon and energy, in a 15 l continuous culture (L.H. Engineering Ltd., Stoke Poges, Bucks) at 30°C, dilution rate 0.045 h^{-1}. This was used as the inoculum for a 80 l batch culture on methanol (0.5% V/V) in the same medium as the New Brunswick fermenter at Pfizers, which was subsequently used to inoculate the 1500 l fermenter. Growth was monitored as an increase in the optical density (600 nm). A further addition of methanol (0.5% V/V) was made in the logarithmic phase before harvesting.

2.5 Media.

2.5.1 D_2 Medium. (Downs and Harrison, 1974)

	g l^{-1}
Bulk 1	
Na_2HPO_4	3.0
KH_2PO_4	3.0
$(NH_4)_2SO_4$	3.0
Bulk 2	
$MgSO_4.7H_2O$	0.1

Bulk 3

$FeCl_3.6H_2O$	16.7×10^{-3}

Trace elements

$CaCl_2.2H_2O$	6.0×10^{-4}
$ZnSO_4.7H_2O$	1.8×10^{-4}
$CuSO_4.5H_2O$	1.6×10^{-4}
$MnSO_4.4H_2O$	1.5×10^{-4}
$CoCl_2.6H_2O$	1.8×10^{-4}
H_3BO_3	1.0×10^{-4}
$Na_2MoO_4.2H_2O$	3.0×10^{-4}

The trace elements (10 ml) and bulk 1 (200 ml) were autoclaved together. Bulk 2 (5.0 ml) was autoclaved separately and aseptically added to filter-sterilised bulk 3 (10 ml). Methanol, the source of carbon and energy, was filter sterilised using a 0.22 um type GS filter (Millipore Ltd., Bedford, Massachussets, USA) and added to the medium to give a final concentration of 1.0% (W/V).

2.5.2 Jayasuriya Salts Media (Jayasuriya, 1955)

Salts	$g\ l^{-1}$
$(NH_4)_2SO_4$	0.5
$MgSO_4.7H_2O$	0.2
$CaCl.2H_2O$	10.0×10^{-3}
$FeSO_4.7H_2O$	5.0×10^{-3}
$MnSO_4.4H_2O$	2.5×10^{-3}
$Na_2MoO_4.2H_2O$	2.5×10^{-3}
Phosphates	
K_2HPO_4	1.74
$NaH_2PO_4.2H_2O$	1.56

The phosphates and salts solutions were autoclaved separately

and aseptically added together when cool. Methanol was filter sterilised and aseptically added on cooling.

2.5.3 Complete autoclavable medium. (F.Taylor personal communication)

Component	g l^{-1}
NH_4NO_3	2.0
KH_2PO_4	5.3×10^{-1}
$NaHPO_4$	8.6×10^{-1}
K_2SO_4	1.7×10^{-2}
$MgSO_4.7H_2O$	3.7×10^{-2}
$CaCl.2H_2O$	1.4×10^{-2}
Fe_2+EDTA	8.0×10^{-3}
$ZnSO_4.7H_2O$	$1.15 \times 10_4^{-3}$
EDTA	8.0×10^{-4}
KI	3.3×10^{-4}
$CoCl.6H_2O$	1.9×10^{-4}
$Na_2MoO_4.2H_2O$	1.9×10^{-4}
$CuSO_4.5H_2O$	5.0×10^{-4}
$MnSO_4.4H_2O$	8.9×10^{-5}
H_3BO_3	4.0×10^{-5}
$NiCl_2.6H_2O$	4.0×10^{-5}
$FeSO_4.7H_2O$	4.0×10^{-5}

This medium was developed in our laboratory, having the advantage that it may be autoclaved complete. On cooling, filter sterilised methanol was added.

2.5.4 Medium for the growth of Coriolus versicolor (Fahraeus and Reinhammar, 1962)

Component	g l^{-1}
glucose	20
l-asparagine	2.5
thiamine HCL	5.0×10^{-2}
$MgSO_4.7H_2O$	5.0×10^{-1}
D,L-phenylalanine	1.5×10^{-1}

adenine	2.8×10^{-2}
KH_2PO_4	1.0
$NaHPO_4.2H_2O$	1.0×10^{-1}
$CaCl_2.2H_2O$	1.3×10^{-2}
$FeSO_4.7H_2O$	1.0×10^{-2}
$MnSO_4.4H_2O$	1.0×10^{-3}
$ZnSO_4.7H_2O$	1.0×10^{-3}
$CuSO_4.5H_2O$	2.0×10^{-3}

The medium was made up in distilled water at 30°C and prefiltered through a P25PKX filter bag (GAF Ltd., Wythenshawe, Manchester) prior to filter sterilisation using a millipak 50, 0.22 um disposable filter (Millipore Ltd., Bedford, Massachussets, USA).

2.6 Growth of Ps. extorquens.

Ps. extorquens was grown in fed batch in a 20 l fermenter (L.H. Engineering Ltd., Stoke Poges, Bucks). The basic unit consisted of a fermenter vessel in which a pyrex glass cylinder (QVF Ltd., Stoke on Trent, Staffs) was clamped between two stainless steel plates. Sealing between the glass and steel was achieved by the use of two circular L-shaped neoprene gaskets (Edwards High Vacuum Ltd., Crawley, Sussex). Agitation was maintained at 400 rpm by the use of an eight bladed paddle type impeller attached to the stirrer shaft and driven via a universal coupling. Media feed, effluent gas and control lines were introduced into the fermenter vessel by means of stainless steel tubing set into rubber bungs which were fixed to the top and base plates by screw lock ports. The medium was transfered from a 20 l holding vessel by means of a peristaltic pump (Watson Marlow

Ltd., Marlow, Bucks). To avoid depletion of the volume due to evaporation the exhaust gas outlet pipe was fitted with a stainless steel condenser maintained at $10^{o}C$ (L.H. Engineering Ltd., Stoke Rogers, Bucks). Both the inlet and effluent gasses were passed through bacterial filters (Millipore Ltd., Bedford, Massachussets, USA). Aeration was provided by an air pump (Charles Austin Ltd., Surrey, England) maintained at 0.4 ml min^{-1} for the first 24 hours and then increased to 1.0 l min^{-1}. For fed batch cultures, the outlet pipe consisted of a 1.2 cm stainless steel tube set into the bottom plate of the culture vessel. Culture outflow was conducted; via silicone tubing (Jencons Scientific Ltd., Hemel Hempsted, Herts), to a 20 l sterile receiver vessel.

The fermenter was provided with both pH and temperature monitoring and control systems. The former was monitored by means of an autoclavable glass electrode connected by a liquid salt bridge to a saturated calomel reference electrode (Electronic Instruments Ltd., Chertsey, Surrey). Automatic pH control facilities were used; the pH of the culture was maintained at pH 6.8 by automatic addition of 2.0 M NaOH. The temperature was maintained at $30^{o}C$ with a Churchill laboratory thermocirculator (L.H. Engineering Ltd., Stoke Poges, Bucks) fitted with a platinum resistance thermometer (Sangmo Western Ltd., Enfield, Middlesex). Compensatory cooling of the culture was by a cold water "finger" in the fermenter base plate maintained at $10^{o}C$. Foaming was reduced by the addition of

silicon antifoam (0.1%). Culture sampling and harvesting was by means of a sampling port designed to accept a 1.0 oz Universal glass container (Evans et al., 1970).

The fermenter vessel containing complete autoclavable medium (17.5 l, see section 2.4.3) was autoclaved (121^{o}C, 60 min) and allowed to cool. The medium was aseptically inoculated with a starter culture (500 ml) grown as described in section 2.3. Methanol, was filter sterilised and added either as a 1.0% (V/V) solution for batch growth or continuously, at a rate of 0.14 ml min^{-1}, via the antifoam addition pump. The latter method enabled a 'feed draw' method of growth in which 17.0 l of Ps. extorquens in the log phase of growth was aseptically removed and replaced with 17.0 l of sterile media, and the procedure repeated.

2.7 Growth of Coriolus versicolor.

C. versicolor was grown using medium described by Fahraeus and Reinhammar (1962) in a 20 l fermenter (L.H. Engineering Ltd., Stoke Poges, Bucks). Due to the thermal instability of asparagine and thiamine (Pirt, 1975), the medium was made up in distilled water at 30^{o}C, and prefiltered using a P25PKX filter bag (GAF Ltd., Wythenshawe, Manchester) prior to filter sterilisation using a millipak 50, 0.22 um disposable filter (Millipore Ltd., Bedford, Massachussets, USA) then introducted into a sterile fermenter vessel. The fermenter vessel, maintained at 25^{o}C with an aeration rate of 2.0 l min^{-1} and agitated at 200 rpm, was inoculated with a starter culture of C.

versicolor, previously induced in 2.0 l shake flasks containing 250 ml media maintained at 150 rpm and 25°C inoculated from a single colony of Coriolus versicolor maintained on MYPG plates (see section 2.2).

2.8 Cell harvesting.

Batch cultures of 1.0 l or less were harvested during the exponential phase of growth, by centrifugation (10,000 g, 15 min, 4.0°C) (M.S.E. Mistral 6.0 l refigerated centrifuge M.S.E. Ltd., Crawley, Sussex). The cell pellet was resuspended and washed twice in sodium phosphate buffer (20 mM, pH 7.0).

Larger culture volumes were harvested by passage through a continuous flow separator (Alfa Laval LAB 102B 20, Alfa Laval Ltd., Brentford, Middlesex) at a flow rate of 0.2 - 0.41 l min^{-1}. Cells produced in the pilot plant were harvested using a Westfalia continous-flow centrifuge (Westfalia Ltd., Wolverton, Bucks).

The cells were washed, frozen in liquid nitrogen and stored at -20°C .

2.9 Bacterial concentrations.

Bacterial concentrations were determined by both dry weight and optical density measurements. A dry weight versus optical density calibration curve was prepared for Ps. extorquens. Cultures were grown in 1.0 l shake flasks at 30°C on complete

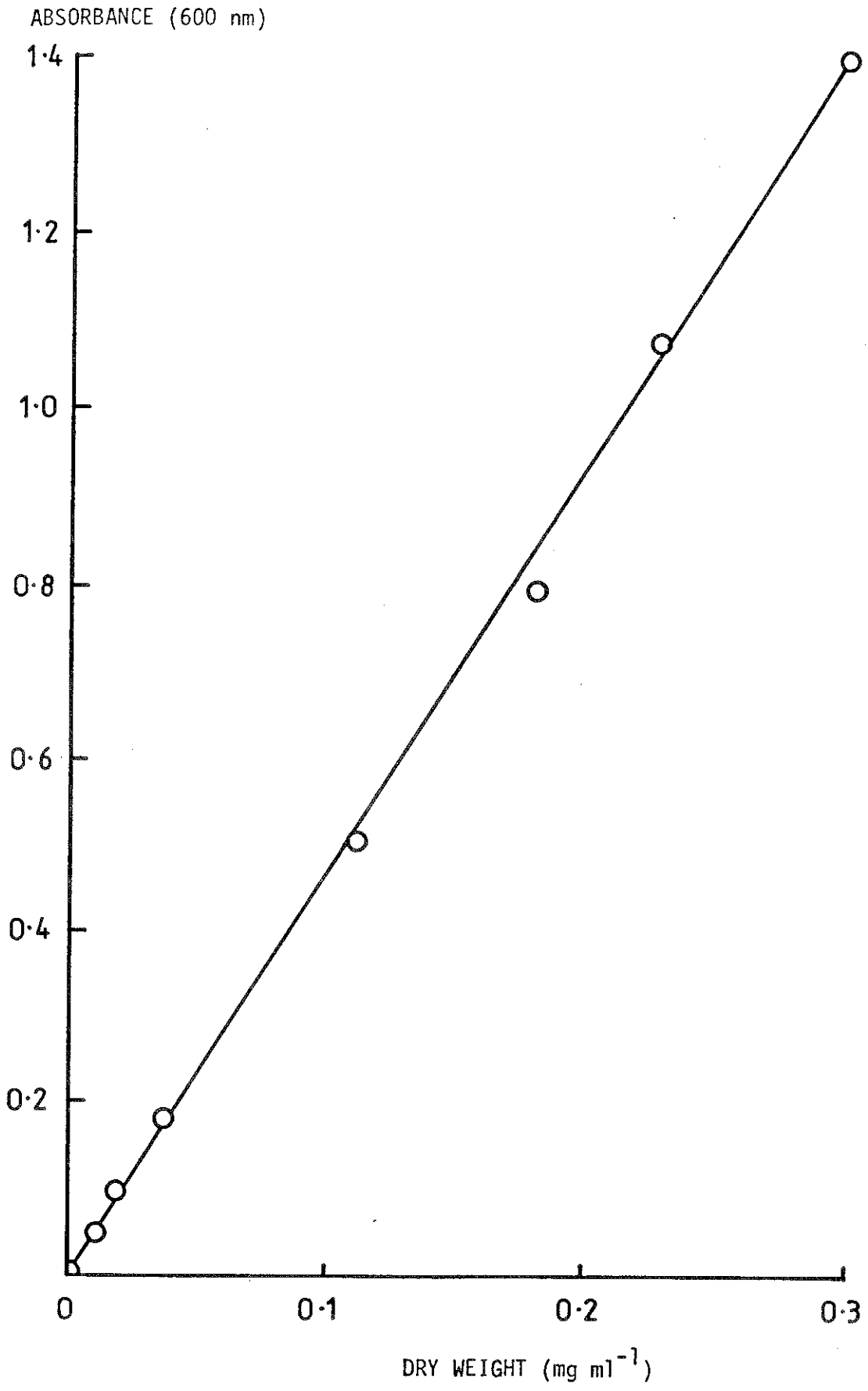
autoclavable media using methanol (1.0%) as the sole source of carbon and energy. Organisms were harvested in the exponential phase of growth by centrifugation at (10,000 g, 10 min, 4.0^{o}C). A thick bacterial cell suspension was made up by resuspending the pellet in distilled water (20 ml); the procedure was carried out rapidly to reduce lysis. Half the suspension was transferred to an equal volume of phosphate buffer (20 mM, pH 7.4). Aliquots of cells suspended in distilled water were pipetted into preweighed glass weighing containers and dried in an air oven at 60^{o}C for 12 hours. Samples were cooled in a desiccator and weighed. Weighings were repeated until a constant result was obtained. Serial dilutions of the bacterial cell suspension in phosphate buffer were prepared, their optical density measured at a wavelength of 600 nm and calibration curves constructed (Fig 2.1).

2.10 Enzyme purification

2.10.1 Conventional purification of methanol dehydrogenase

This method is a modification of that described previously by Anthony and Zatman (1967a). Methanol-grown organisms (200g wet weight) were suspended in 150 ml Tris-HCl buffer (150 ml, 20 mM, pH 8.0) and ruptured by a single passage through a French pressure cell (American Instrument Company, Silver Springs, Maryland, USA) at 135 mPa. Whole cells and cell-debris were

Figure 2.1 Calibration curve of optical density (600 nm) versus dry weight for whole organism suspensions of *Pseudomonas extorquens* (see section 2.9).


ABSORBANCE (600 nm)
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
0·1
0·2
0·3
DRY WEIGHT (mg ml^{-1})

removed by centrifugation (35,000 g, 2.0 h, 4.0°C). The pH of the supernatant was lowered to pH 4.0 with hydrochloric acid (1.0 M) and the resultant pellet removed by centrifugation (35,000 g, 1.0 h, 4.0°C). The pH of the supernatant was raised to 6.0 with sodium hydroxide (1.0 M) before dialysis against Tris-HCl buffer (4.0 l, 20 mM, pH 9.0) and chromatography on DEAE-cellulose, (Whatman Ltd., Maidstone, Kent) previously equilibrated with the same buffer. The void volume, containing cytochrome c and methanol dehydrogenase, was concentrated by ultra-filtration under nitrogen using an Amicon PM10 membrane (Amicon Corporation, High Wycombe, Bucks) followed by gel filtration on a column (60.0 x 5.0 cm) (Parmacia Fine Chemicals Ltd., Uppsala, Sweden) of Sephadex G150 (Parmacia Fine Chemicals Ltd., Uppsala, Sweden) equilibrated with Tris-HCl buffer (100 mM, pH 8.0) containing sodium chloride (200 mM). Methanol dehydrogenase and cytochrome c were resolved into two peaks spectrophotometrically and the fractions containing the former (see section 2.11.3) were pooled and concentrated using an Amicon XM50 ultrafiltration membrane. The purified enzyme was frozen dropwise in liquid nitrogen prior to storage at -20°C.

2.10.2 Two phase enzyme purification of methanol dehydrogenase

Methanol grown cells (200g wet weight) were suspended in 600 ml phosphate buffer (600 ml, 50 mM, pH 7.0), homogenised using a commercially available household blender (Moulinex, France) and ruptured using a Standsted cell disrupter (Standsted

Fluid Power Ltd., Standsted, Essex) at a pressure of 20,000 psi. The disrupted cells (750 ml) were added to a two phase enzyme separation system consisting of; methanol (350 ml, 100 mM), polyethylene glycol 1000 (1.4 l, 50%, W/V) and potassium phosphates (1.05 l, 50% W/V, pH 7.4). The solution was agitated for 15 min in a 5.0 l fermenter vessel at 200 rpm. Initially phase separation was carried out by centrifugation (2,500 g, 5 min, 4.0^{o}C), however, this was subsequently carried out in a settling tank, maintained at 4.0^{o}C. The lower phase containing methanol dehydrogenase, was dialysed against phosphate buffer (10 l, 50 mM, pH 7.0) containing methanol (10 mM), and concentrated using a millipore cassette filter with a 10,000 molecular weight cut-off (Millipore Ltd., Bedford, Massachusettes, USA). DNA was removed by the addition of an equal volume of protamine sulphate (1.0% W/V) made up in the same buffer. After 30 minutes the precipitated DNA was removed by centrifugation (35,000 g, 5 min, 4.0^{o}C). The purified methanol dehydrogenase was frozen by dropwise addition to liquid nitrogen and stored as pellets at -20^{o}C.

2.10.3 Purification of laccase

Coriolus versicolor was chosen as the source of laccase, since it is an extracellular product of this fungus (Fahraeus and Reinhammar, 1962). The organism was grown in a fermenter as previously described (see section 2.7). The production of laccase was induced after 4 days growth by the addition of filter

sterilised 2,5 dimethyl aniline (0.436 g) in ethanol (10 ml, 50% V/V). After a further 7 days, dimethyl aniline (0.218 g) in ethanol (10 ml, 50% V/V) was added with glucose (180 g). The release of laccase was followed by measuring the enzymic oxidation of catechol (see section 2.10.4) and the culture was harvested when this reached a maximum, 8-9 days after the initial induction. The supernatant was pumped through a wine filter (Boots Ltd., UK) containing a saati nylon mesh filter (Sericol group Ltd., London, England) prior to filtering through a GAF filter bag P25PIX (GAF Ltd., Wythenshawe, Manchester). The filtrate was saturated with ammonium sulphate (750 g l^{-1}) in a holding vessel (20 l) and maintained at 4.0°C for 12 hours. The surface precipitate was filtered off and stored at -20°C. A small volume (100 ml) was thawed out, homogenised and dialysed against 10 l phosphate buffer (100 mM, pH 6.0) and stored at -20°C prior to use.

2.11 Enzyme assays

2.11.1 Formate dehydrogenase

The specific activity of formate dehydrogenase was determined spectrophotometrically by the method of Johnson and Quayle (1964). The reaction mixture (2.0 ml) contained the following: sodium phosphate buffer (150 mM, pH 7.0); B NAD (0.4 mM) and formate dehydrogenase. The reaction was initiated by the addition of formate (0.02 mM) and the absorbance monitored at 340 nm using a DU 8 spectrophotometer (Beckman Instruments Ltd.,

California, USA).

2.11.2 Glucose oxidase

The specific activity of glucose oxidase was measured polarographically using a Clark oxygen electrode (Rank Bros, Bottisham, Cambridge) maintained at 30°C. The reaction mixture (3.0 ml) contained phosphate buffer (20 mM, pH 7.4) and enzyme (0.04 ug protein). The reaction was started by the addition of glucose (2.0 ul, 1.0 M).

2.11.3 Methanol dehydrogenase

The specific activity of methanol dehydrogenase was determined polarographically at 30°C by the method of Dunstan et al., (1972). The following additions were made to 2.0 ml Tris-HCl buffer (125 mM, pH 9.0): methanol (150 umol), phenazine ethosulphate (180 umol) and enzyme sample (0.1 - 0.25 mg). The reaction was initiated by the addition of the activator, ammonium chloride (150 u mol).

2.11.4 Laccase

The activity of the extracellular laccase was determined by a modified method described by Fahraeus and Reinhammar (1962). The rate of catechol oxidation (1.0 mM) in acetate buffer (100 mM, pH 5.0) was measured spectrophotometrically at 440 nm. The sample was filtered through a 0.22 um filter to remove fungal

mycelium.

2.12 Chemical assays

2.12.1 Determination of cyanide

Cyanide concentrations were determined by the method of Lambert et al., (1975) which was a modification of the method described by Epstein (1947). Equal volumes of N-chlorosuccinimide - succinimide reagent (succinimide, 100 mM; N-chlorosuccinimde, 7.5×10^{-2} mM) and barbituric acid-pyridine coupling agent (barbituric acid, 500 mM; pyridine, 37 mM) were added to the sample (1.0 ml). The reaction mixture was diluted with distilled water (22 ml) and the absorbance measured at 575 nm after 12 min (Fig 2.2).

2.12.2 Determination of DNA

Two methods were used in the quantitative determination of DNA. The first involved the measurement of absorbance at 260 nm; the second was the indole method based on a colorimetric assay described by Wiener et al., (1976). The latter consisted of the addition of equal volumes (1.0 ml) of both indole (0.08% W/V) and hydrochloric acid (12 M) to a sample (2.0 ml) containing DNA. The sample was incubated for 15 min at $100^{o}C$ and the absorbance measured at 490 nm. Calf thymus DNA type 1 was used for the construction of the standard calibration curve (Fig 2.3).

Figure 2.2 Calibration curve for cyanide determined by the method of Lambert *et al* (1975) (see section 2.12.1).

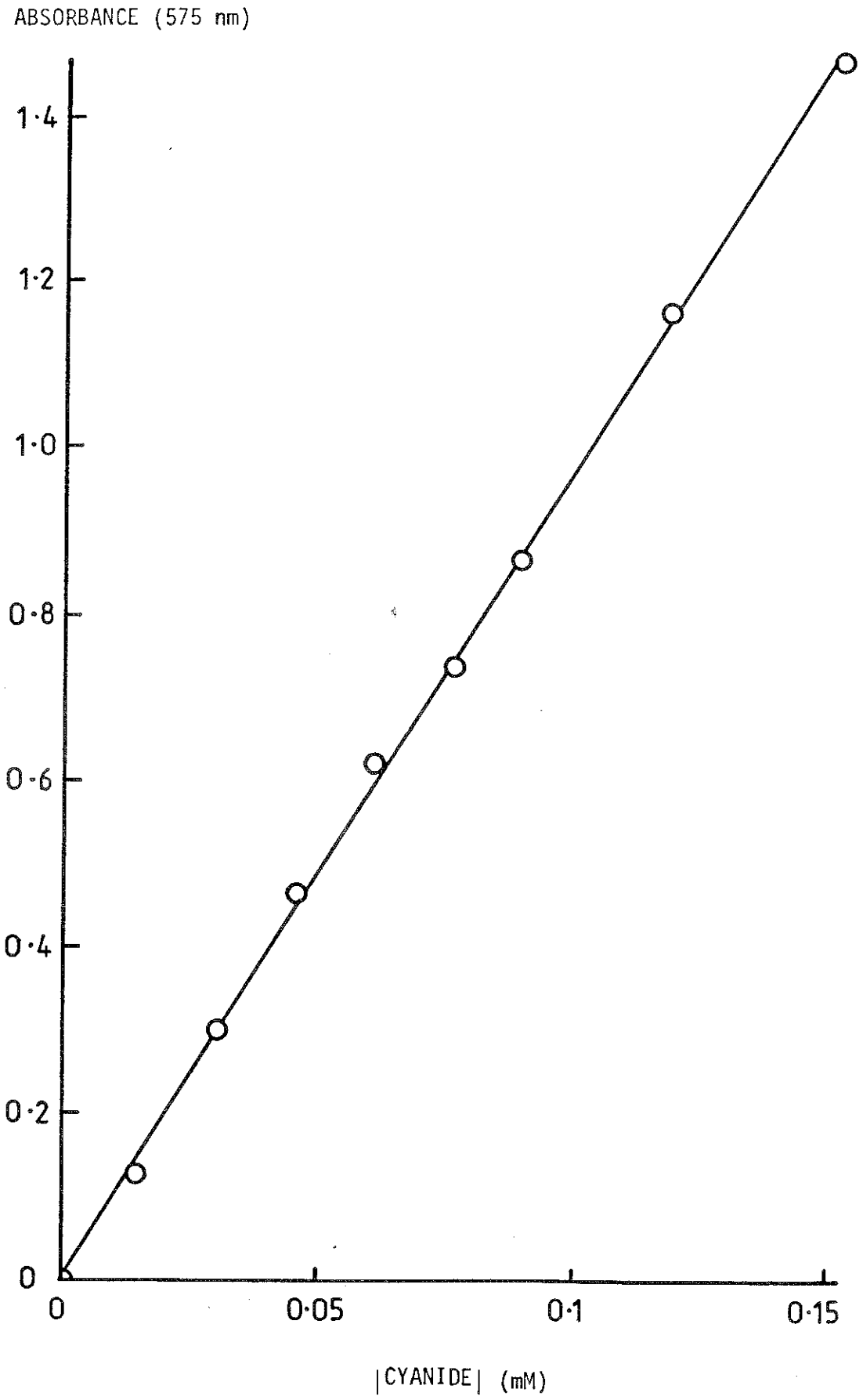
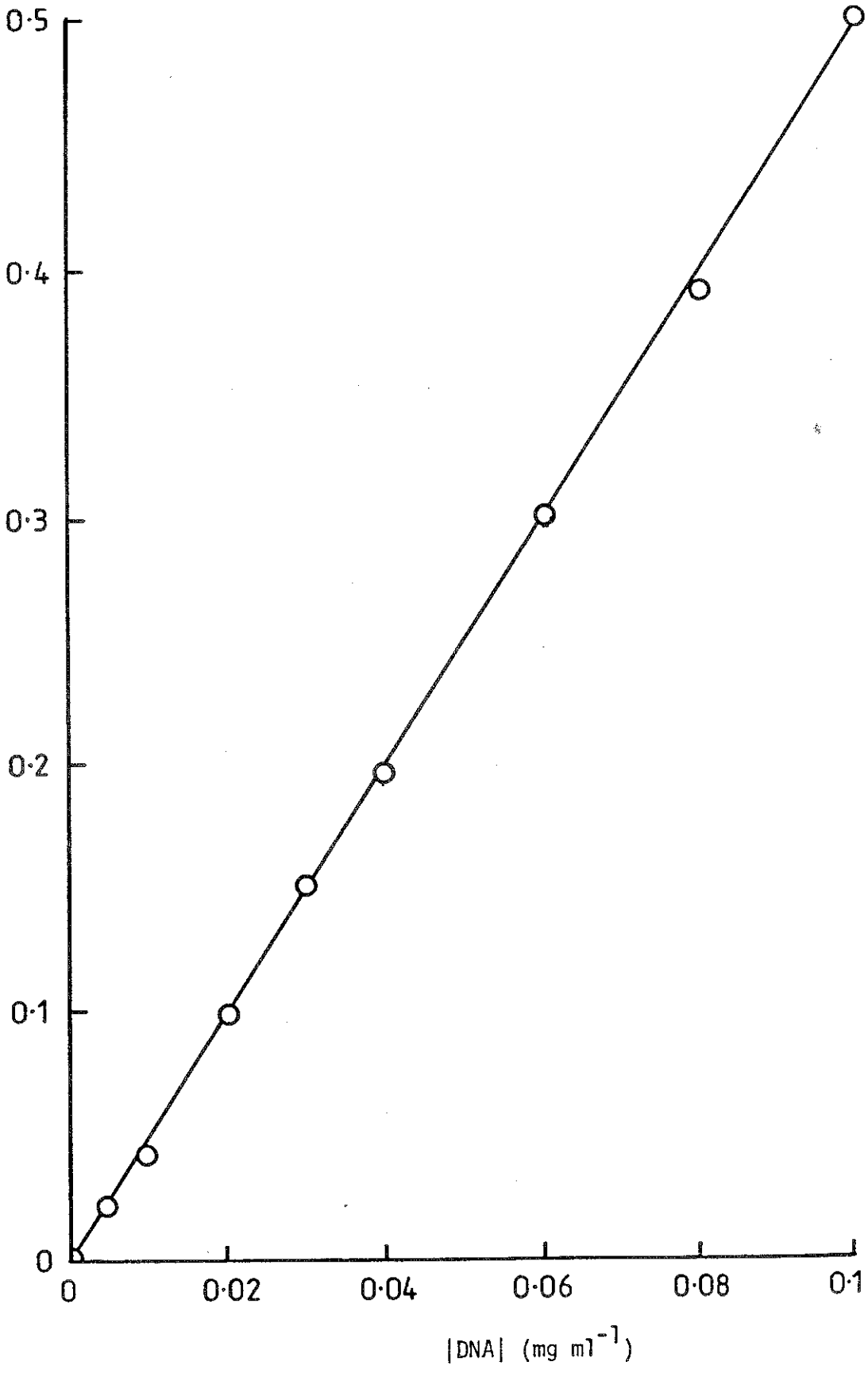

ABSORBANCE (575 nm)
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
0
0·05
0·1
0·15
|CYANIDE| (mM)

Figure 2.3 Calibration curve for DNA, determined by the method of Weiner *et al* (1976) (see section 2.12.2).


ABSORBANCE (490 nm)
0·5
0·4
0·3
0·2
0·1
0
0
0·02
0·04
0·06
0·08
0·1
|DNA| (mg ml⁻¹)

2.12.3 Determination of formaldehyde

Sodium sulphite (25 ml, 0.1 M) was neutralised with hydrochloric acid (0.1 M), using thymolphthalein (0.1% W/V) in ethanol as indicator. A measured volume of formaldehyde was added and the resulting solution titrated (to colorless) with hydrochloric acid (0.1 M). The amount of formaldehyde in the aliquot used was calculated from the equivalence 1.0 ml of 0.1 M hydrochloric acid reacting with 3.003 mg formaldehyde. Formaldehyde was prepared by boiling a solution of paraformaldehyde in a sealed container for 1.0 hour.

2.12.4 Determination of glucose

Glucose was determined colorimetrically either by the use of Diastix and Dextrostix reagent strips (Ames Company Ltd., Stoke Poges, Bucks) or more accurately using a glucose detection kit No. 115 based on hexakinase and glucose dehydrogenase (Sigma Chemical Company Ltd., Missouri, USA).

Amperometric determination was carried out either by measuring hydrogen peroxide production using a glucose analyser (Yellow Springs Instruments, Ohio, USA) or by coupling the enzymic reaction to an electrode via a mediator (see chapter 6).

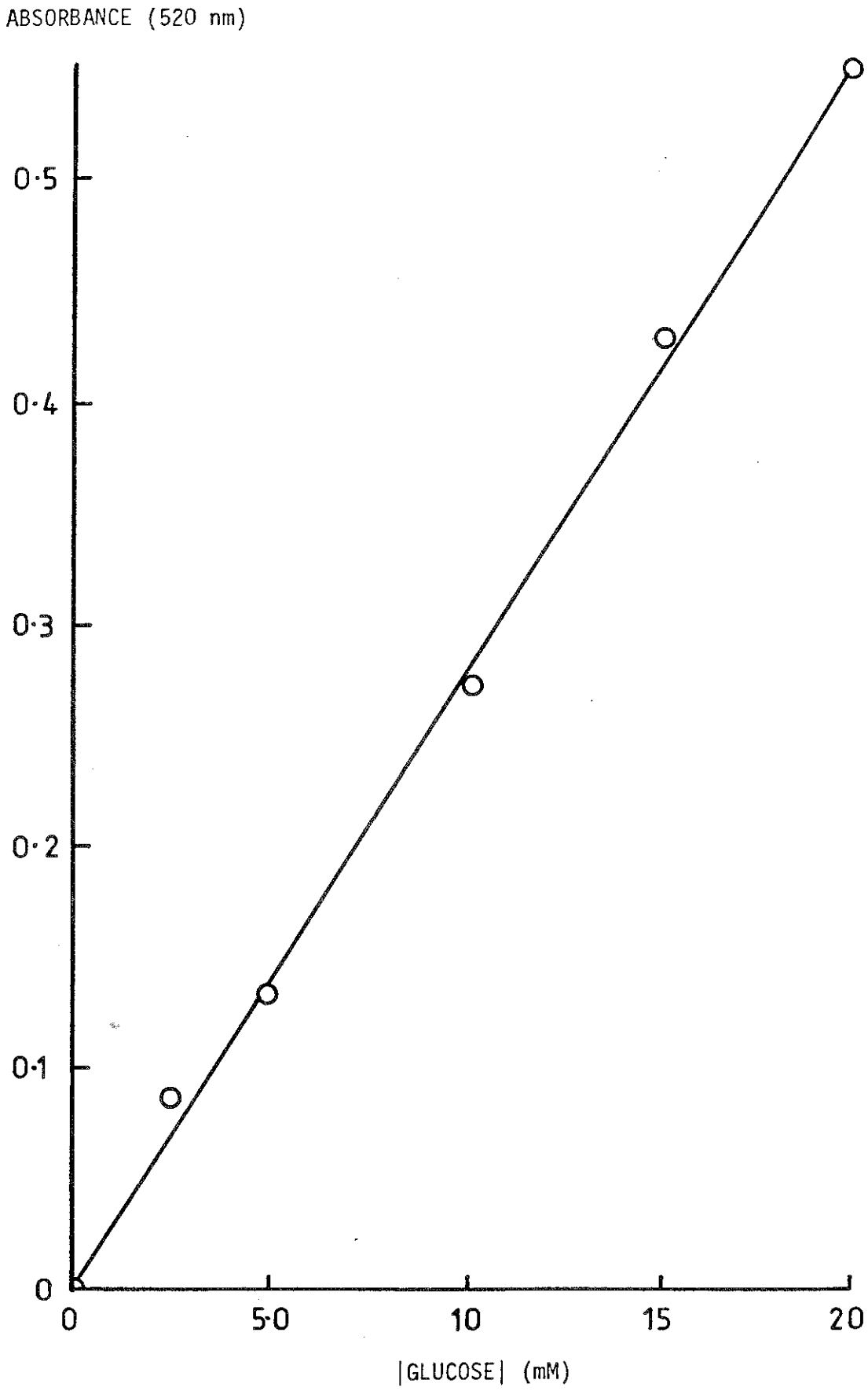
2.12.4a Glucose detection kit (Sigma Chemical Company Ltd., Missouri, USA)

The combined enzyme colour reagent (1.0 ml) was added to the sample (20 ul). The reagent was reconstituted from a vial of glucose enzyme reagent (stock No. 115-20) diluted with distilled water (17 ml) and glucose colour reagent (4.0 ml, stock No. 115-5). Hydrochloric acid was added to the sample (10 ml, 0.1M) and after 5-10 minutes the absorbance was measured at 520 nm. The glucose stock solution provided in the kit (stock No. 635-100) and B-D-glucose were used to construct a standard calibration curve (Fig 2.4).

2.12.4b Dextrostix glucose determination (Miles Laboratories Ltd., Stoke Poges, Slough)

An initial comparison of the reagent area of the Dextrostix with the '0' on the colour chart was carried out to ensure suitablility for use. Sufficient glucose sample (40 ul) was added to the strip to cover the entire reagent surface area. After 60 secs the sample was washed off using distilled water (within 1-2 secs) and the strip blotted dry on a lint-free tissue (Kimberly-Clark Ltd., Maidstone, Kent). Immediately after washing the strip was placed next to the colour chart and a comparative determination of the glucose concentration carried out (Fig 6.22).

Figure 2.4 Calibration curve for glucose determined using a glucose detection kit (Sigma Ltd,, USA) (see section 2.12.4a)


ABSORBANCE (520 nm)
0·5
0·4
0·3
0·2
0·1
0
0
5·0
10
15
20
|GLUCOSE| (mM)

2.12.4c Diastix glucose determination (Miles Laboratories Ltd., Stoke Poges, Slough)

As in the Dextrostix, a comparison was made between the colour of the reagent strip and the '0' of the colour chart to ensure suitability for use. The strip was placed in the sample for 2 seconds after which excess liquid was removed by tapping on the edge of the sample vial. After 30 secs the reagent area was compared with the colour chart to determine the glucose concentration (Fig 6.22).

2.12.4d Yellow Springs Glucose Analyser (Yellow Springs Instruments, Ohio, USA)

Glucose analysis was carried out at Guys Hospital, London, by amperometrically measuring hydrogen peroxide production, using the Yellow Springs glucose analyser. The instrument was initially cleared and calibrated by the injection of a glucose standard (25 ul, 10 mM) and the process repeated for a series of samples.

2.12.5 Determination of hydroxylamine

Hydroxylamine concentrations were determined by the method of Dalton (1977). The sample (250 ul) containing hydroxylamine in the range 20-30 nmol was acidified with hydrochloric acid (50 ul, 1.0 M) and colour reagent added (1.0 ml). The colour reagent contained 8-hydroxyquinoline (1.0% W/V) in ethanol and sodium

carbonate (1.0 M). The absorbance of the reaction mixture was measured at 680 nm after 2 hours (Fig 2.5).

2.12.6 Determination of methanol

Methanol was detected enzymically by means of a biofuel cell and poised potential detector (see section 3.3). Methanol was also determined by gas liquid chromatography using a Pye Unicam 204 gas chromatograph (Pye Unicam Ltd., Cambridge) fitted with a flame ionisation detector. Samples (2.0 ul) were injected directly onto a glass column (2.1 x 4 mm i.d), containing Porapak Q. (J.J's chromatograhy Ltd., Kings Lynn, Norfolk). The column temperature was maintained at 150^{o}C with both the injector and detector at 200^{o}C. The carrier gas (nitrogen) flow rate was maintained at 40 ml min^{-1}.

Methanol standards made up in distilled water were used to calibrate the system with the peak area being measured using a Phillips CDP4 computing integrator (Pye Unicam Ltd., Cambridge).

2.12.7 Determination of polysaccharides

A colorimetric determination of sugars was carried out by the method of Dubois et al., (1956). Aqueous phenol (50 ul, 80% W/V) and sulphuric acid (5.0 ml, conc.) solutions were added to the sample (1.0 ml). After incubation at 25^{o}C for 20 min the optical density was measured at 490 nm. B-D-glucose was used for the construction of the standard calibration curve (Fig 2.6).

Figure 2.5 Calibration curve for hydroxylamine determined by the method of Dalton (1977) (see section 2.12.5).

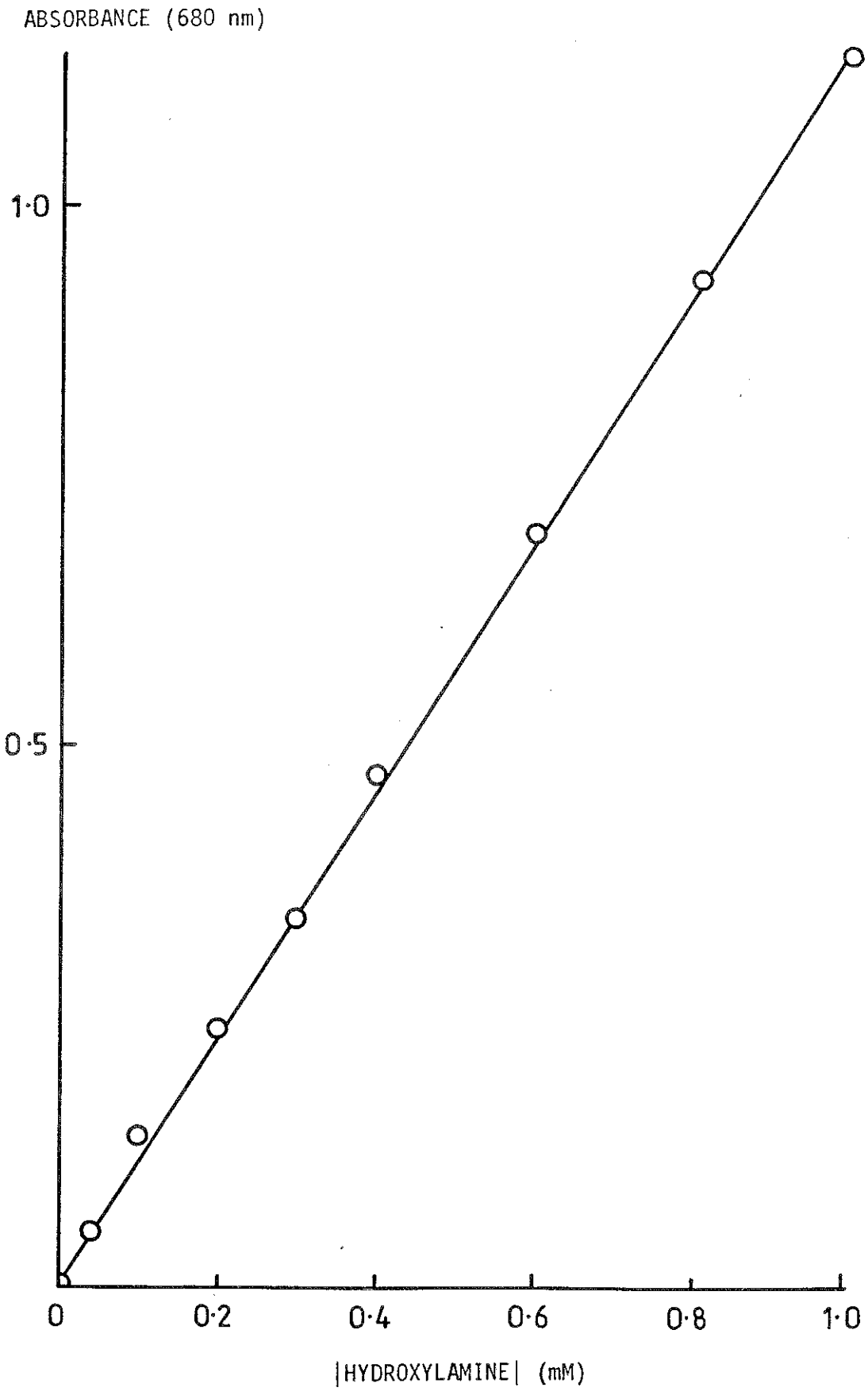
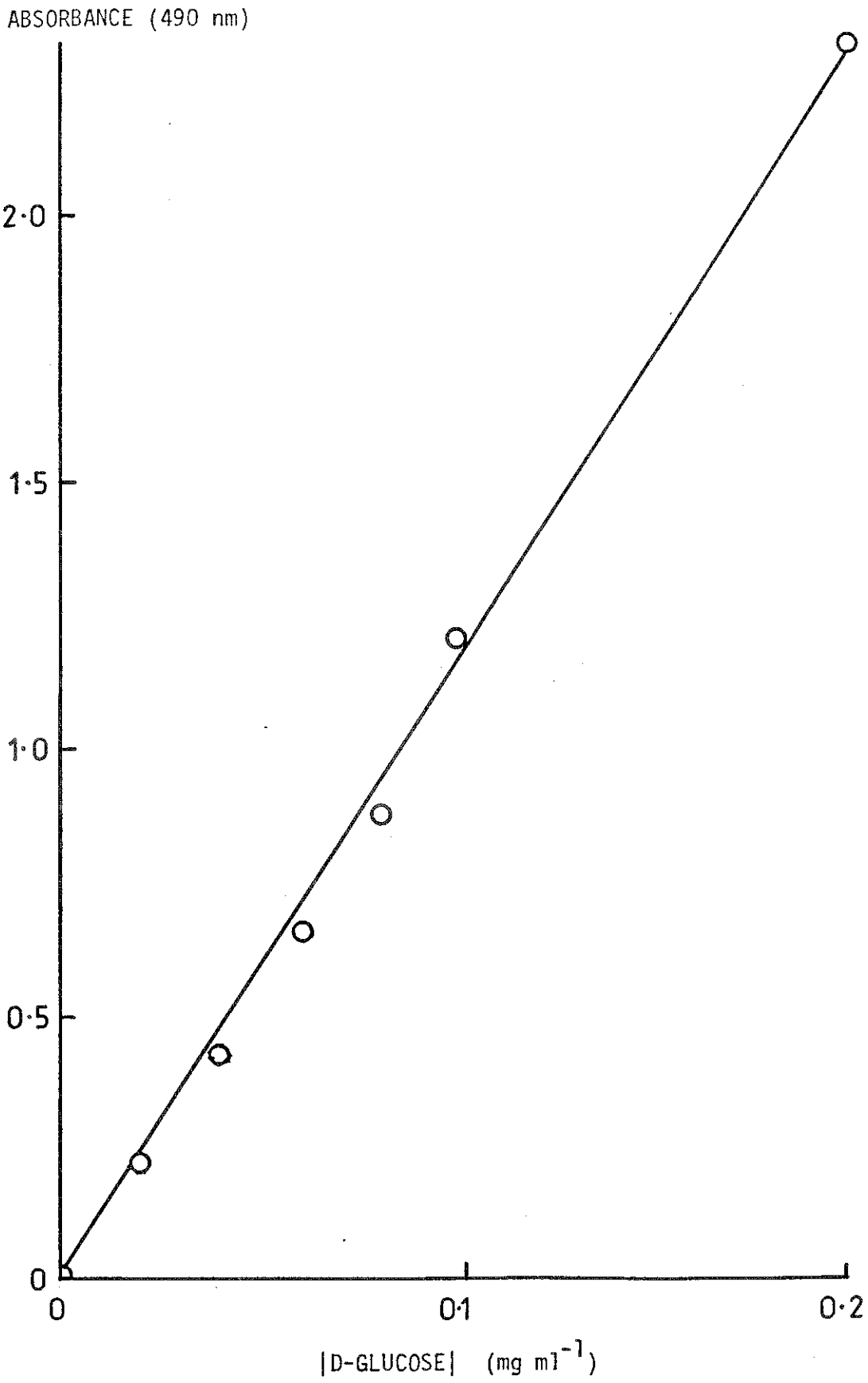

ABSORBANCE (680 nm)
1·0
0·5
0
0·2
0·4
0·6
0·8
1·0
|HYDROXYLAMINE| (mM)

Figure 2.6 Calibration curve for polysaccharides determined by the method of Dubois *et al* (1950) using D-glucose as the standard (see section 2.12.7).


ABSORBANCE (490 nm)
2·0
1·5
1·0
0·5
0
0
0·1
0·2
|D-GLUCOSE| (mg ml⁻¹)

2.12.8 Determination of protein

Soluble protein was measured by the methods of Lowry et al., (1951) and Bradford (1976). Membrane bound protein was determined by the method described by Kennedy and Fewson (1968). In all cases bovine serum albumin (Fraction V) was used for the standard calibration curve.

2.12.8a Lowry et al., (1951)

The following stock solutions were prepared:

A : 2.0 % (W/V) Na_2CO_3 in 0.1 M NaOH

B : 1.0 % (W/V) $CuSO_4.5H_2O$

C : 2.0 % (W/V) sodium potassium tartrate

C*: prepared prior to use A(50 ml): B(0.5 ml): C(0.5 ml)

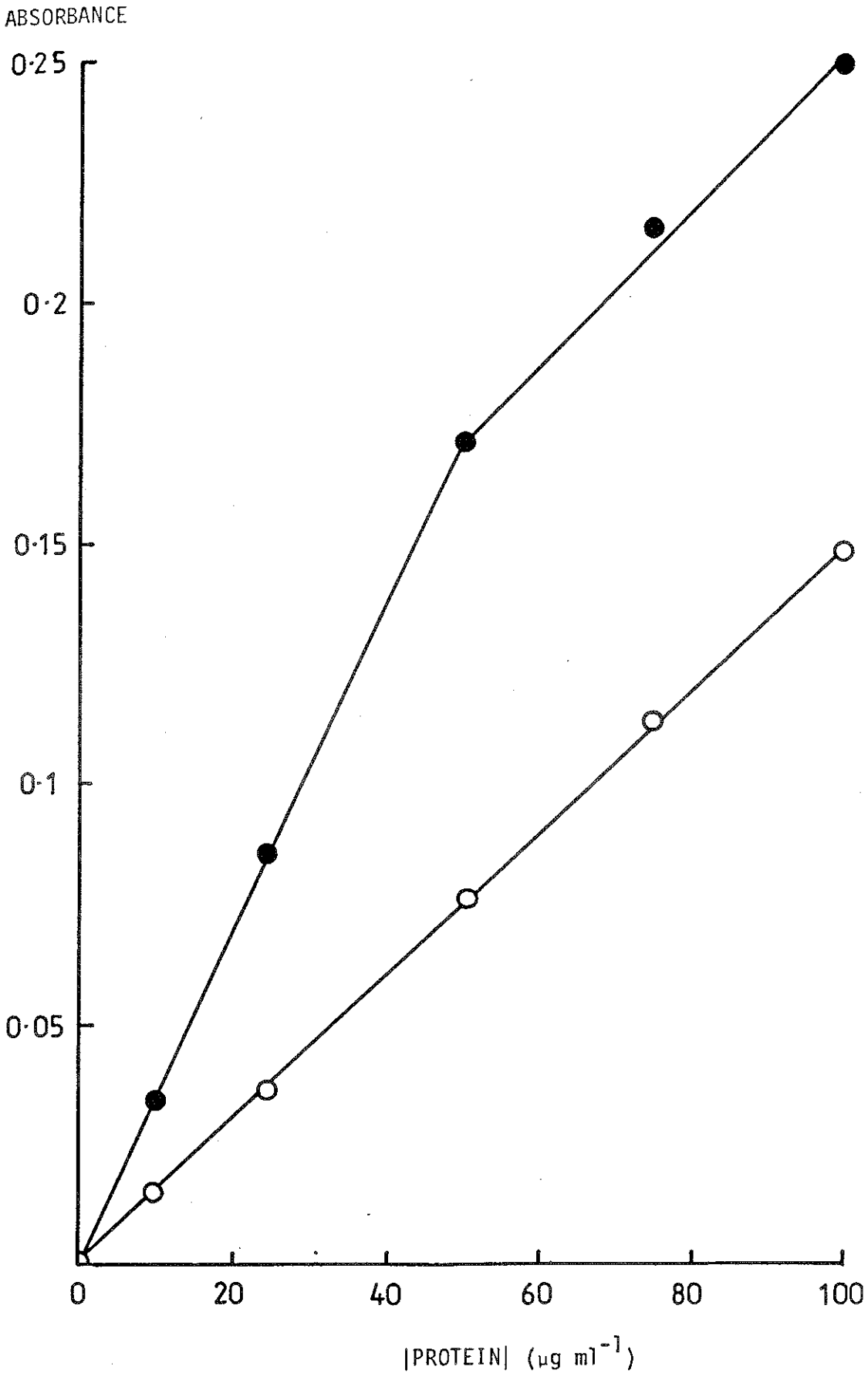
D : Folin-Ciocalteau reagent (5.0 ml) diluted to 15 ml with distilled water, prepared as required.

Protein samples (25-500 ug) were diluted to 1.0 ml with sodium hydroxide (0.1 M) and solution C* was added (5.0 ml) whilst mixing. After 10 minutes solution D (0.5 ml) was added and the solution mixed. The colour was allowed to develop for 30 minutes and the optical density measured at 750 nm (low protein concentration) or 500 nm (high protein concentration) (Fig 2.7).

2.12.8b Bradford (1976)

Coomassie blue G250 (0.1 g) was dissolved in ethanol (50

Figure 2.7 Protein calibration curve determined by the method of Lowry *et al* (1951) using bovine serum albumin (fraction V) as standard (see section 2.12.8a). Determined at 500 nm (○) and 750 nm (●).


ABSORBANCE
0·25
0·2
0·15
0·1
0·05
0
20
40
60
80
100
|PROTEIN| (µg ml⁻¹)

ml), and phosphoric acid (100 ml, 85% W/V). The resulting reagent was diluted to 1.0 l and filtered under reduced pressure prior to use. The protein sample (50 ul) was added to this reagent (2.5 ml) and the mixture shaken. After 2 minutes and the optical density was measured at 595 nm (Fig 2.8).

2.12.8c Kennedy and Fewson (1968)

The following solutions were prepared:

A : 13 % (W/V) Na_2CO_3 in 0.1 M NaOH

B : 2.0% (W/V) $CuSO_4.5H_2O$

C : 4.0% (W/V) sodium potassium tartrate

D : A (100 ml): B (3.0 ml): C (3.0 ml)

An equal volume of reagent D (1.5 ml) was added to the protein sample (10 - 150 ug ml^{-1}) made up in sodium hydroxide (0.66 M). Folin-Ciocalteau reagent (0.5 ml) was added and the solution immediately mixed. The colour was allowed to develop for 30 minutes and the optical density measured at 625 nm (Fig 2.9).

2.13 Preparative isoelectric focusing

Ultrodex (4.0g) was slowly added to an enzyme sample (100 ml), (previously dialysed against glycerol 10 l, 1.0% V/V) and ampholine (2.0% W/V, pH 7.0 - 10.0) using 2.5 ml of ampholine carrier ampholytes

Figure 2.8 Protein calibration curve determined by the method of Bradford (1976) using bovine serum albumin (fraction V) as standard (see section 2.12.8b).

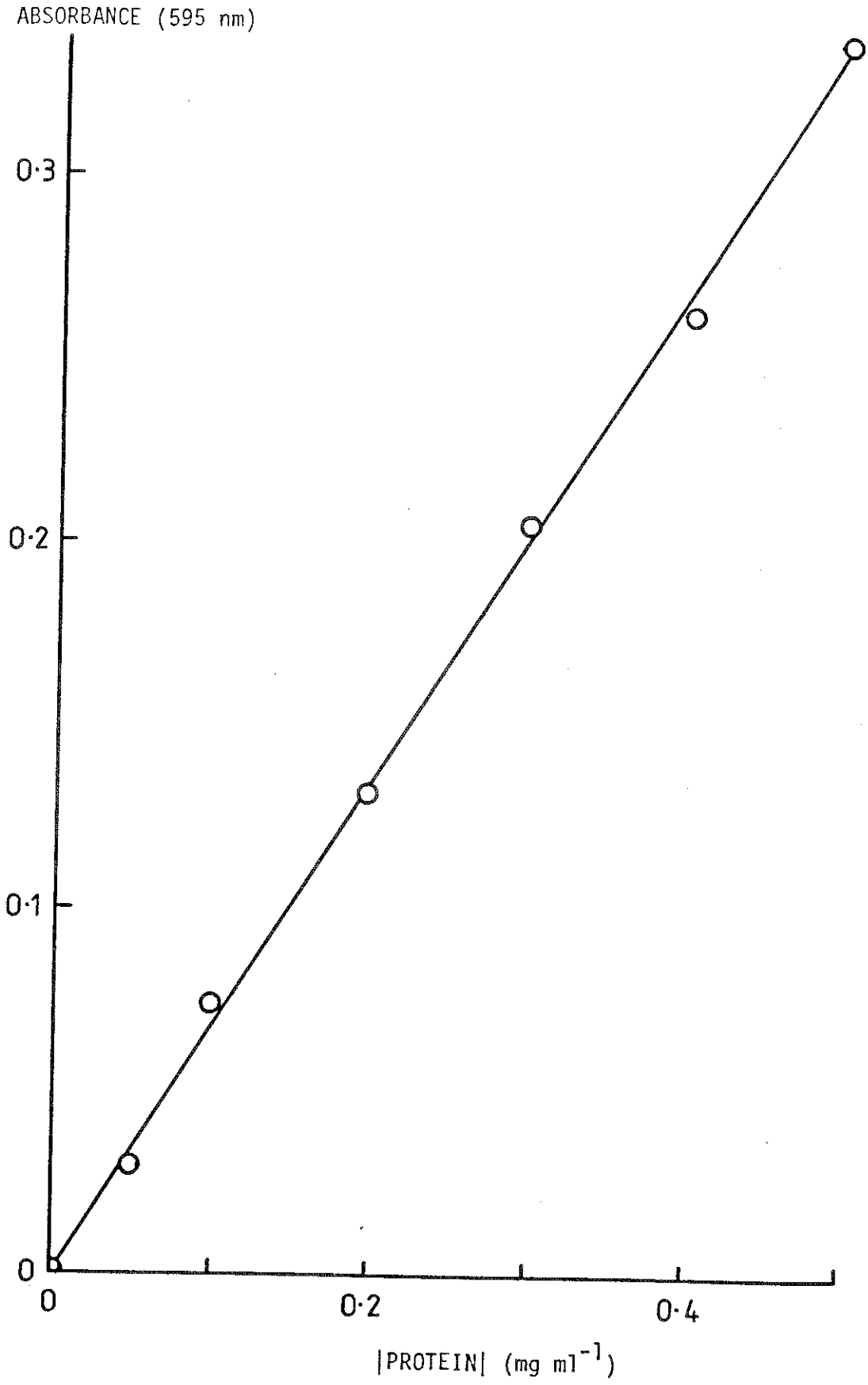
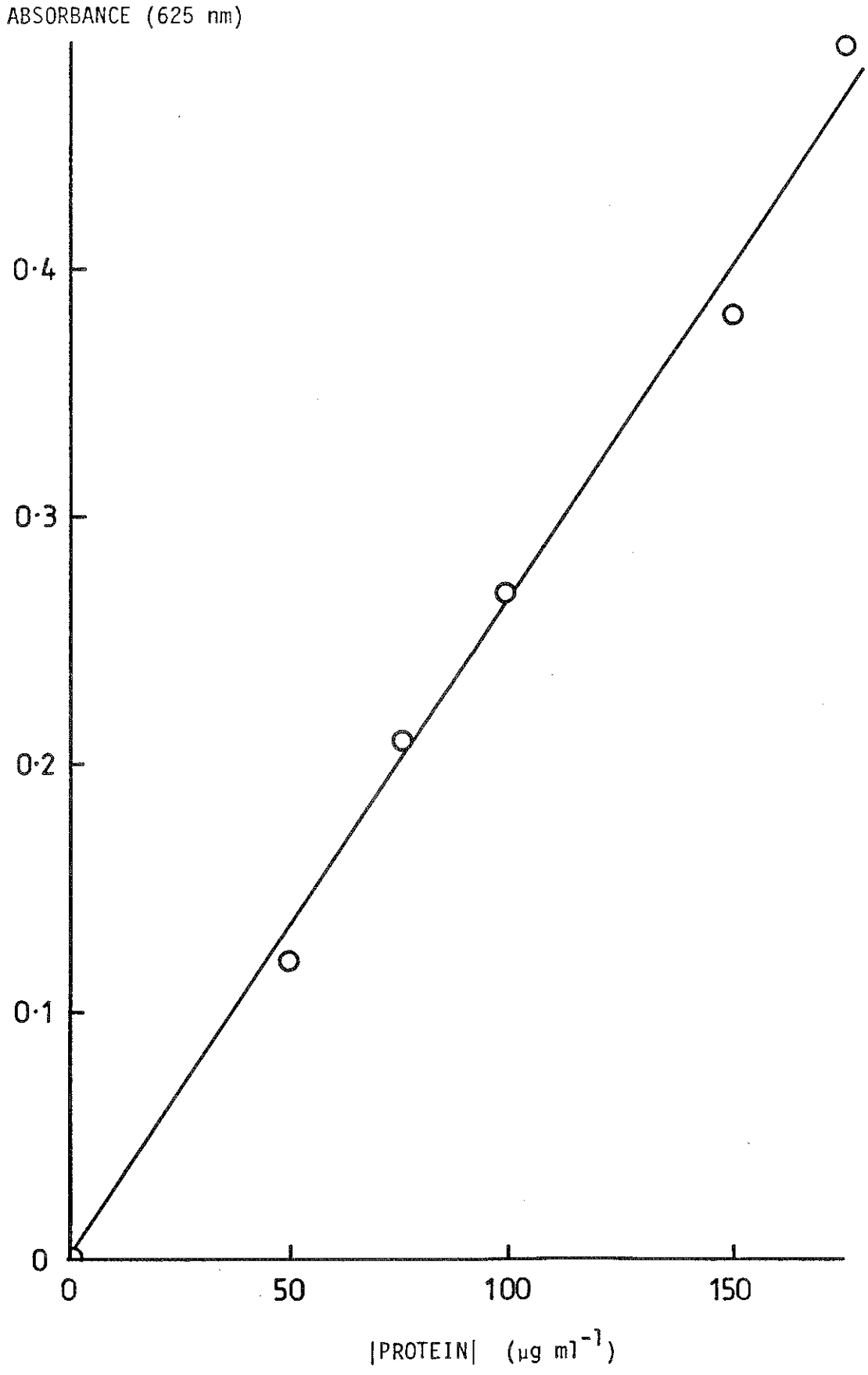

ABSORBANCE (595 nm)
0·3
0·2
0·1
0
0
0·2
0·4
|PROTEIN| (mg ml^{-1})

Figure 2.9 Protein calibration curve for protein determined by the method described by Kennedy and Fewson (1968) using bovine serum albumin (fraction V) as standard (see section 2.12.8c).


ABSORBANCE (625 nm)
0·4
0·3
0·2
0·1
0
0
50
100
150
|PROTEIN| (µg ml⁻¹)

in the pH 7.0 - 9.0 and 9.0 - 11.0 range. The solution formed a slurry which was then weighed. Electrode strips (10.5 cm) soaked in the ampholine solution were placed at each end of the electrofocusing tray (LKB 2117-501) and the tray weighed. The weight of slurry added was determined by reweighing the empty beaker. Evaporation of the slurry was carried out using a hair drier mounted 70 cm above the gel surface until the evaporation limit was reached (60%). The water loss was determined by weighing the tray, the limit being calculated as a percentage of the initial weight of the slurry in the tray. The tray was transfered to a multiphor cooling plate (LKB Ltd., Bromma, Sweden) with a film of Triton X100 (1.0% V/V) on top. An electrode strip soaked in sodium hydroxide (1.0 M) was placed at the cathode and a second strip soaked in phosphoric acid (1.0 M) was placed at the anode. Electrical connection was made to a power supply (LKB 2197) and electrofocusing carried out at 0.39 KV and 20.5 mA for 16 hours at $10^{o}C$. After focusing, a 30 compartment fractionating grid (LKB 2117) was placed over the tray and the pH gradient determined using a pH probe and meter. The bands were scraped off into 5.0 ml plastic columns and the enzyme eluted with borate buffer (200 mM, pH 9.0).

2.14 SDS Polyacrylamide gel electrophoresis

The purity and sub-unit molecular weight of methanol dehydrogenase was determined by SDS polyacrylamide gel electrophoresis (SDS PAGE). Electrophoresis was performed according to the method of Laemmli (1970). Protein samples of known concentration were frozen by dropwise addition to liquid

nitrogen and stored at -80^{o}C until analysed. To prevent proteolysis the samples (1.0 mg ml^{-1} in sample buffer) were solubilised, by heating for 90 sec in boiling water, in sealed polythene tubes. A sucrose crystal was added and the samples were applied to the wells of an acrylamide stacking gel (3.9% W/V) using a Hamilton syringe (Micromeasure Ltd., Bonaduz, Switzerland).

Sample buffer

Tris-HCl (1.0 M, pH 7.0)	6.25	ml
SDS	2.0	g
Glycerol	10.0	ml
Mercaptoethanol	5.0	ml
Bromophenol Blue	1.0 x 10^{-3}	g

make up to 100 ml with glass distilled water. Electrophoresis was carried out at 12 mA for 2 hours in a 1.0 mm thick acrylamide gel (7.5% W/V) with an acrylamide stacking gel (3.0% W/V, Table 2.1). The gels were stained with coomassie brilliant blue R250 in a mixture of distilled water: propan-2-ol: glacial acetic acid (65 : 25 : 10, V/V). The gels were destained in the above solvent mixture and stored in aqueous acetic acid (7.0% V/V). The stained protein bands were scanned in a gel scanner incorporated into a DU8 spectrophotometer (Beckman Instruments, California, USA), at 550 nm.

Table 2.1 Contents of Acrylamide Main and Stacking Gels

Stock solutions for electrophoresis stored in the dark at 4.0°C contained:

A	36.6 g	Tris-HCl	100 ml H_2O
	48.0 ml	HCl (1.0 M)	
C	28.0 g	acrylamide	100 ml H_2O
	0.735g	bisacrylamide	
C_1	24.0 g	acrylamide	100 ml H_2O
	0.6 g	bisacrylamide	
D	19.2 ml	Tris-HCl (1.0 M, pH 7.0)	
	0.8 ml	SDS (20% W/V)	

Stacking gel

	Volume (ml)
Solution D	3.0
Solution C	2.55
Ammonium persulphate (0.28%)	12.0
Distilled water	6.45
TEMED	12.5×10^{-3}

Main gel (7.5% W/V)

	Volume of stock solution(ml)
Solution C_1	6.25
Ammonium persulphate (0.28% W/V)	5.0
SDS (20% W/V)	0.1
Solution A	2.5
Distilled water	5.65
TEMED	11.5×10^{-3}

Electrode buffer (total volume 1.0 l) contained: Tris (3.0 g), glycine (14.4 g) and SDS (1.0 g) in distilled water.

2.15 High pressure liquid chromatography

The purity of methanol dehydrogenase was assayed by high pressure liquid chromatography (HPLC). The enzyme sample (0.4 mg) in phosphate buffer (100ul, 100 mM, pH 7.0) was injected onto a TSK-G-3000 gel filtration column (7.5 x 600 mm) (LKB Ltd., Bromma, Sweden) eluted at a flow rate of 0.5 ml min^{-1} and a pressure of 28 atmospheres using a liquid chromatograph (Varian Ltd., California, USA). The absorbance was measured spectrophotometrically at 280 nm.

2.16 Measurement of absorption spectra

All spectra were recorded using a DU8 single beam spectrophotometer (Beckman Instruments, Irvine, California, USA). Absorption spectra were carried out in matched quartz cuvettes with a light path of 1.0 cm at 21 - 25^{o}C unless otherwise stated. Potassium ferricyanide and sodium dithionite were used as oxidant and reductant respectively.

2.17 Mediator diffusion

The rate of mediator diffusion through various membranes was determined. The membrane was clamped between the two compartments of a biofuel cell containing borate buffer (3.7 ml, anode; 3.0 ml cathode; 50 mM, pH 9.0). The mediator was added to the anode and the cell maintained in the dark. The rate of diffusion through the membrane was determined by absorbance

measurements of the cathodic solution at 563 nm for N,N,N',N' tetramethyl-p-phenylenediamine and 388 nm for phenazine ethosulphate. Mediator diffusion across a cation exchange membrane (BDH Ltd., Poole, Dorset) in borate buffer at various pHs was also determined.

2.18 Proton diffusion

Proton diffusion through a variety of membranes was measured by clamping the membrane between the two compartments of a biofuel cell. Borate buffer (3.7 ml, 50 mM, pH 8.0) was added to the anode and borate buffer (3.0 ml, pH 10.0) added to the cathode. The solution in the anode was stirred and the pH determined using a type CMAWL pH probe (Russel pH Ltd., Auchermuchy, Scotland). Both continuous and discontinuous measurements were carried out by connecting the output of the pH meter to a high-impedance-model chart recorder (JJ Lloyd Instruments Ltd., Warsach, Southampton). The temperature of the cell was maintained at 20^{o}C using a circulating water bath (Gallenkamp Ltd., London).

2.19 Oxygen diffusion

The rate of oxygen diffusion was determined using two methods. Initially the membranes to be tested were clamped between two gas tight half cells and the compartments filled with borate buffer (50 mM, pH 9.0). A miniature oxygen electrode

model 760-45 and meter model 1201 (Transidyne General Corporation, Michigan, USA) was placed in one compartment which was sparged with nitrogen and sealed with silicone rubber sealant. The aerobic compartment was sparged with air at a rate of 100 ml min^{-1} using a peristaltic pump (Watson Marlow Ltd., Marlow, Bucks).

Oxygen diffusion was also measured using an oxygen electrode (Rank Bros Ltd., Bottisham, Cambridge) with the teflon membrane replaced with the membrane to be tested. Measurement of oxygen diffusion rates were recorded whilst sparging with air and nitrogen alternately.

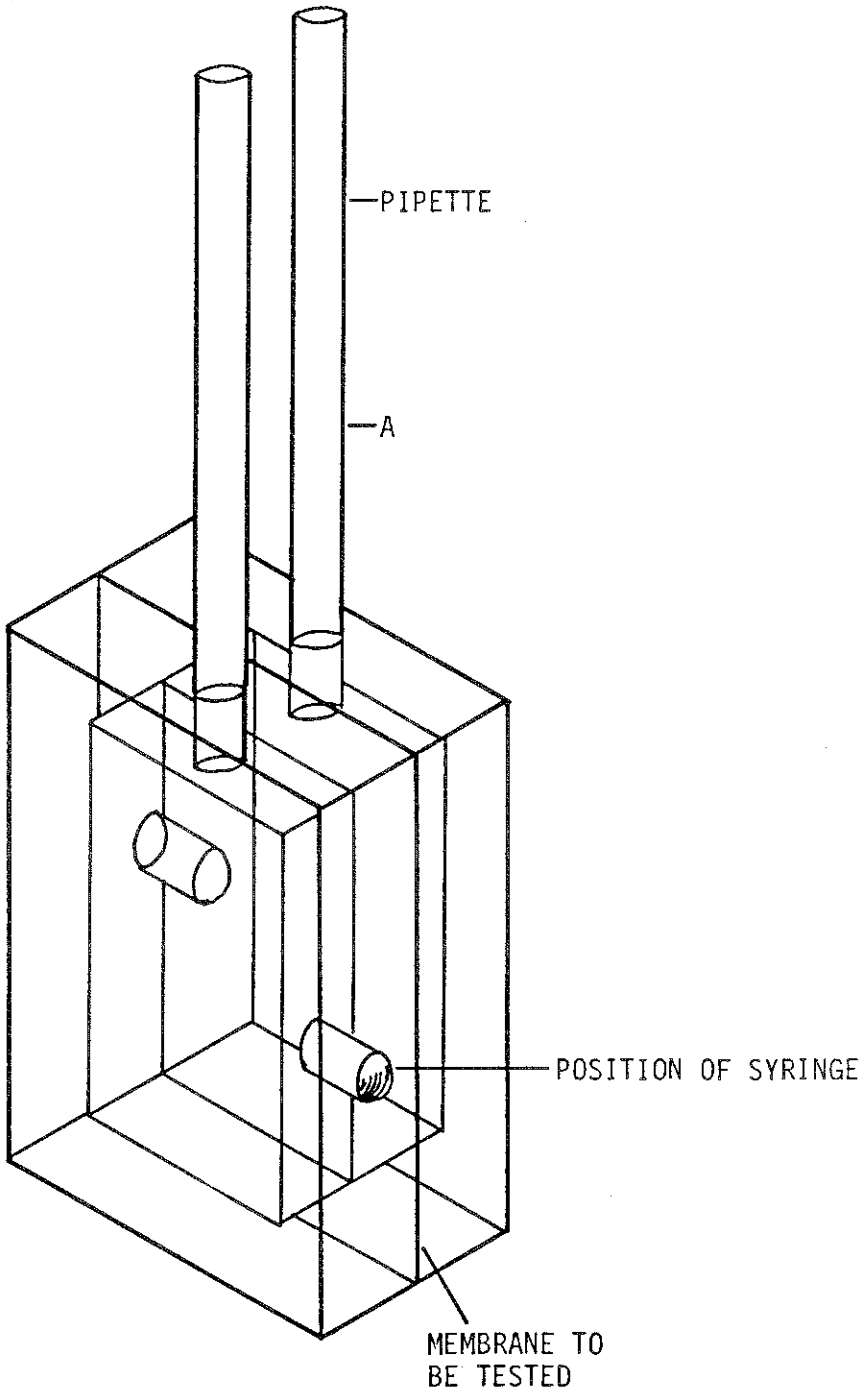
2.20 Enzyme diffusion

The diffusion of methanol dehydrogenase through various membranes was measured by adding borate buffer solution (3.0 ml, 50 mM, pH 9.0) to both compartments of the fuel cell and enzyme to the anodic compartment (0.4 mg). The diffusion of methanol dehydrogenase was measured by removing samples (25 ul) from both compartments and determining the protein concentration by the Bradford method (see section 2.12.8b).

2.21 Osmosis measurements

The measurement of osmosis through various membranes was determined by clamping the membrane to be tested between two halves of a fuel cell as shown in Fig 2.10. Borate buffer (3.0

Figure 2.10 Schematic diagram of a perspex cell used in the determination of osmosis across membranes (see section 2.21).


PIPETTE
A
POSITION OF SYRINGE
MEMBRANE TO
BE TESTED

ml, 50 mM pH 9.0) was added to each of the compartments and sucrose (1.0 M) was added to one compartment. The levels of the liquids in the two compartments were equalised using the two levelling syringes and the levels recorded at predetermined time intervals.

2.22 Stability of mediators to UV light

The stability of phenazine ethosulphate (PES) and N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) to UV light was investigated by exposing a buffered borate solution (50 mM, pH 9.0) containing the mediator to a UV light source 365 nm (20 W) at a distance of 11.0 cm. The absorbance was measured at 563 and 388 nm for TMPD and PES, respectively; the exposure times were recorded. The ability of these redox couples to mediate electron transfer between the enzyme and electrode after exposure to UV was determined by subsequent testing in a methanol dehydrogenase-based biofuel cell.

2.23 Optical screening of mediators

The rapid screening of soluble mediators capable of coupling to the enzyme methanol dehydrogenase was carried out as follows; a solution of the mediator to be tested was made up in borate buffer (50 mM, pH 10.5) containing ammonium chloride (50 mM) and methanol (10 mM). The solution was dispensed into two test tubes maintained at 30^{o}C (Grant Instruments Ltd.,

Barrington, Cambridge). Chemical reduction of one sample was brought about by the addition of a few grains of sodium dithionite. The second sample was reduced by the addition of methanol dehydrogenase (0.2 mg) and the colour change in both tubes was compared.

2.24 Preparation of polyviologen

A solution of 4,4 bipyridyl (0.78 g) and o-dibromoxylene (1.32 g) was made up in acetone (50 ml) in a 100 ml round bottom flask. The solution was stirred and sparged with nitrogen at room temperature for 24 hours. The yellow precipitate of polyviologen (1.5 g, 75% yield) was washed in a small amount of acetonitrile followed by methanol and dried in an air oven at 40^{o}C.

2.25 Preparation of s-1,4-Bis-(dimethylamino)-benzene perchlorate (Wusters blue)

Oxidised N,N,N',N' tetramethyl-p-phenylenediamine was prepared by the method of Michaelis and Granich (1943). N,N,N',N' tetramethyldiamine hydrochloride (4.0 g) was dissolved in a solution of distilled water (70 ml) and methanol (115 ml) containing sodium perchlorate (50 g). The solution was cooled on ice and aqueous bromine added dropwise (0.126 M). The crystals (3.1 g, 77% yield) having a brown metallic luster were washed several times with small portions of ice cold methanol followed

by dry ether and dried in an air oven at 40°C.

2.26 Preparation of 1 methoxy-5-methyl phenazinium methyl sulphate (mPMS)

A sample of 1 Methoxy-5-methylphenazinium methyl sulphate was kindly supplied by Dr.H.A.O.Hill, prepared from 1-methoxyphenazine by the method described by Surry (1955).

2.27 Freeze drying of enzymes and enzyme electrodes

Methanol dehydrogenase was freeze dried using a Edwards freeze drier (Edwards High Vacuum Ltd., Crawley, Sussex). A sample of the enzyme (5.0 ml) was dialysed against distilled water (5.0 l) for 24 hours. The enzyme was frozen dropwise in liquid nitrogen and freeze dried for 24 hours. A second sample was dialysed against phosphate buffer (50 mM, pH 7.0) containing methanol (10 mM) and the procedure repeated.

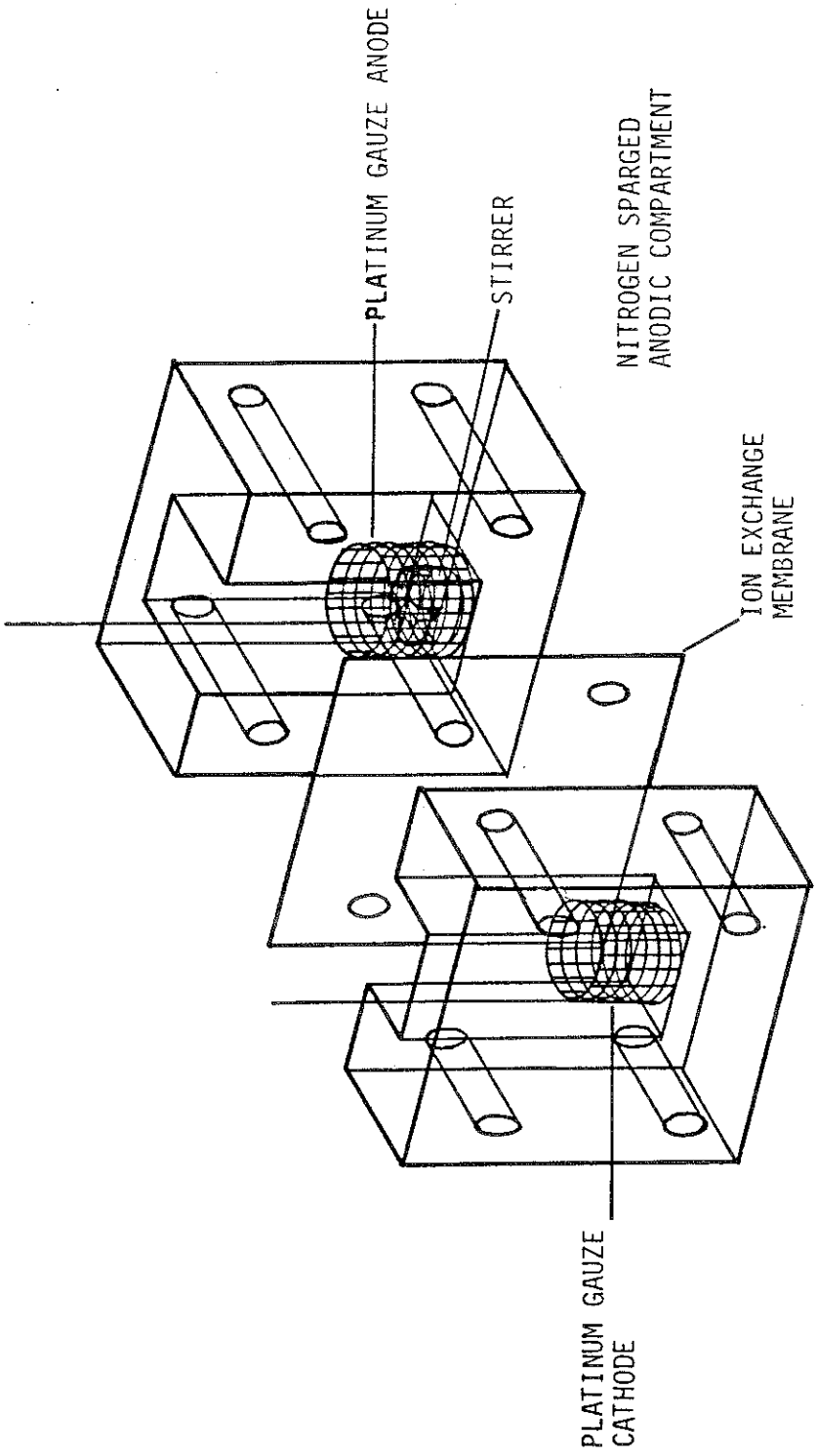
Enzyme-based glucose electrodes (see chapter 6) containing immobilised glucose oxidase were washed in phosphate buffer (50 mM, pH 7.4 or pH 5.0) and frozen in liquid nitrogen prior to freeze drying. In an attempt to improve stability electrodes were also soaked for 1.0 hour in borate buffer (50 mM, pH 7.4) containing D-gluconic acid (100 mM) prior to freeze drying.

2.28 Biofuel cell construction

The biofuel cell consisted of two perspex compartments. The anodic and cathodic compartments had dimensions of 35 x 16 x 15 mm and 35 x 15 x 11 mm, respectively, and were glued using a perspex/chloroform mixture (Fig 2.11). Unless otherwise stated the compartments were separated by a cation exchange membrane (BDH Ltd., Poole, Dorset) and clamped together using 4, 4 BA lengths of studing and nuts. Agitation was achieved by mounting the biofuel cell on a magnetic stirrer (Chem lab Ltd., Hornchurch, Essex). The current was determined by measuring the voltage across a resistance box (Time Electronics Ltd., Tonbridge, Kent) using a high impedance multimeter (Gould Ltd., Hainault, Essex) and chart recorder (JJ Loyd Instruments Ltd., Warsach, Southampton). Electron polarisation curves were determined by placing a standard calomel reference electrode (SCE) (Russel pH Ltd., Auchtermuncy, Scotland) in the electrode compartment being tested and measuring the potential using a high impedance multimeter (Cranwell Ltd., Brentwood, Essex). The fuel cell was maintained at 25^{o}C using a circulating water bath (Grant Instruments Ltd., Barrington, Cambridge). Unless otherwise stated the cathode of the biofuel cell contained borate buffer (3.0 ml, 250 mM, pH 10.5, ammonium chloride, 50 mM) whilst the anode comprised of buffer (3.6 ml) containing TMPD (7.5 mM), methanol (2.5 mM) and methanol dehydrogenase purified from M. methylotrophus (0.1 mg).

Figure 2.11 Exploded view of biofuel cell.

The perspex cell consisted of an anode and cathode (35 x 16 x 15 mm and 35 x 15 x 11 mm) separated by an ion exchange membrane. Typically the anode and cathode were of platinum gauze rolled into cylinders, 50 mesh (1.6 x 4.8 cm) and 80 mesh (1.6 x 2.5 cm) respectively (see section 2.28 and chapter 4).


PLATINUM GAUZE ANODE
STIRRER
NITROGEN SPARGED
ANODIC COMPARTMENT
ION EXCHANGE
MEMBRANE
AIR OR OXYGEN
SPARGED CATHODIC
COMPARTMENT
PLATINUM GAUZE
CATHODE

2.29 Gas lift biofuel cell

The gas lift biofuel cell was constructed of perspex with separate gas lift ports to circulate the solutions over a reticulated carbon anode and platinum gauze cathode (Fig 2.12). The two compartments were separated by a cation exchange membrane (BDH Ltd., Poole, Dorset). The anode was made of reticulated vitreous carbon (Fluorocarbon Co. California, USA), type 2 x 1 x 60s and measured 14 x 29 x 9 mm. It was connected to the external circuit using platinum wire (0.46 mm) and bonded with a silver-based conducting epoxy resin (Johnson Matthey Ltd., UK). Platinum gauze (80 mesh, 33 cm^2) was used as the cathode.

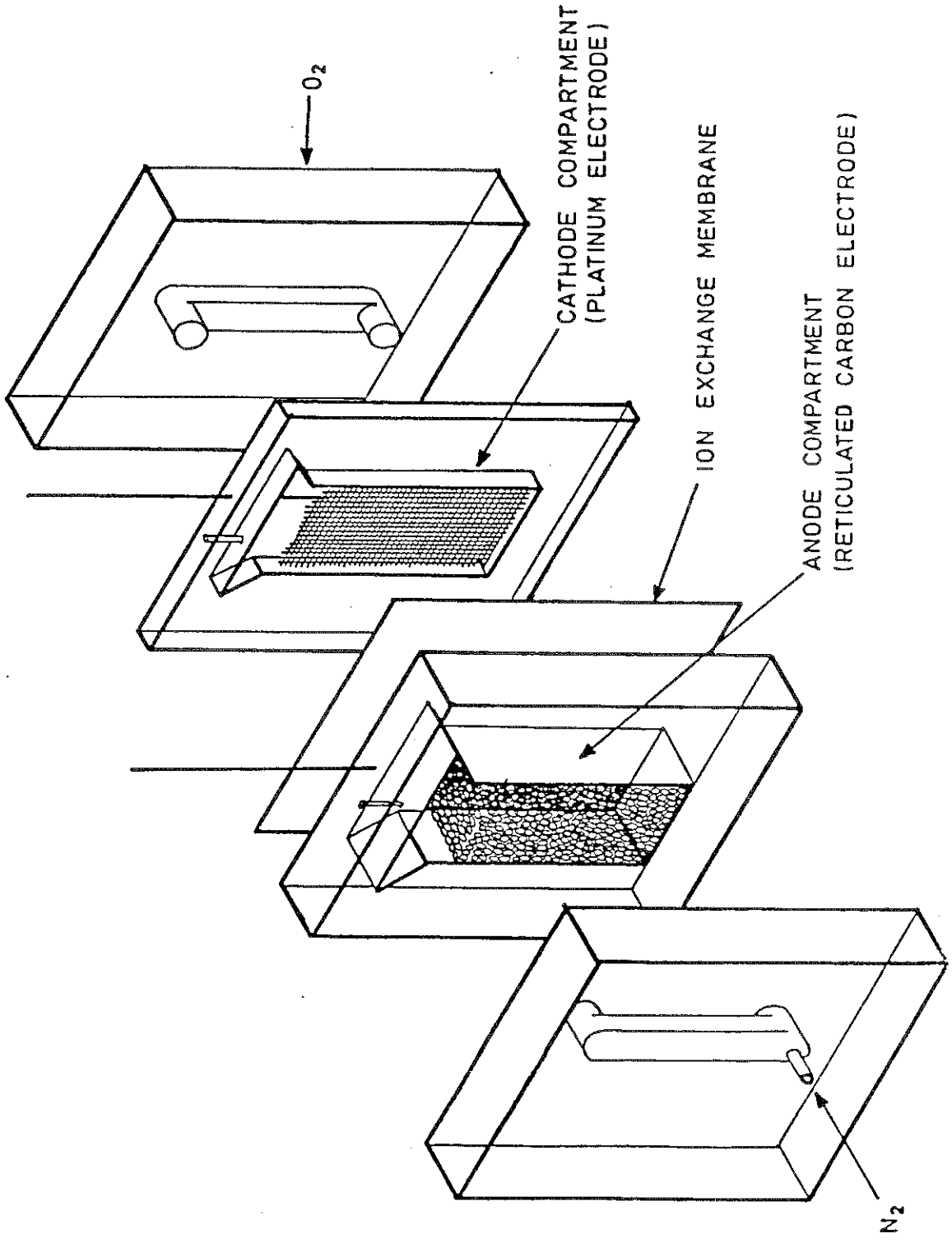
2.30 Temperature variaton during biofuel cell output

The temperature variations of the solutions of the biofuel cell during operation were investigated. The biofuel cell was set up as previously described (section 2.28), but was insulated with expanded polystyrene (5.0 cm). Inlet and outlet tubes were cut through the polystyrene to enable reactants to be added. The temperature was monitored using a thermocouple and digital thermometer (Temcon Instruments Ltd., Arundel, West Sussex).

2.31 Construction of a homogeneous enzyme based methanol sensor

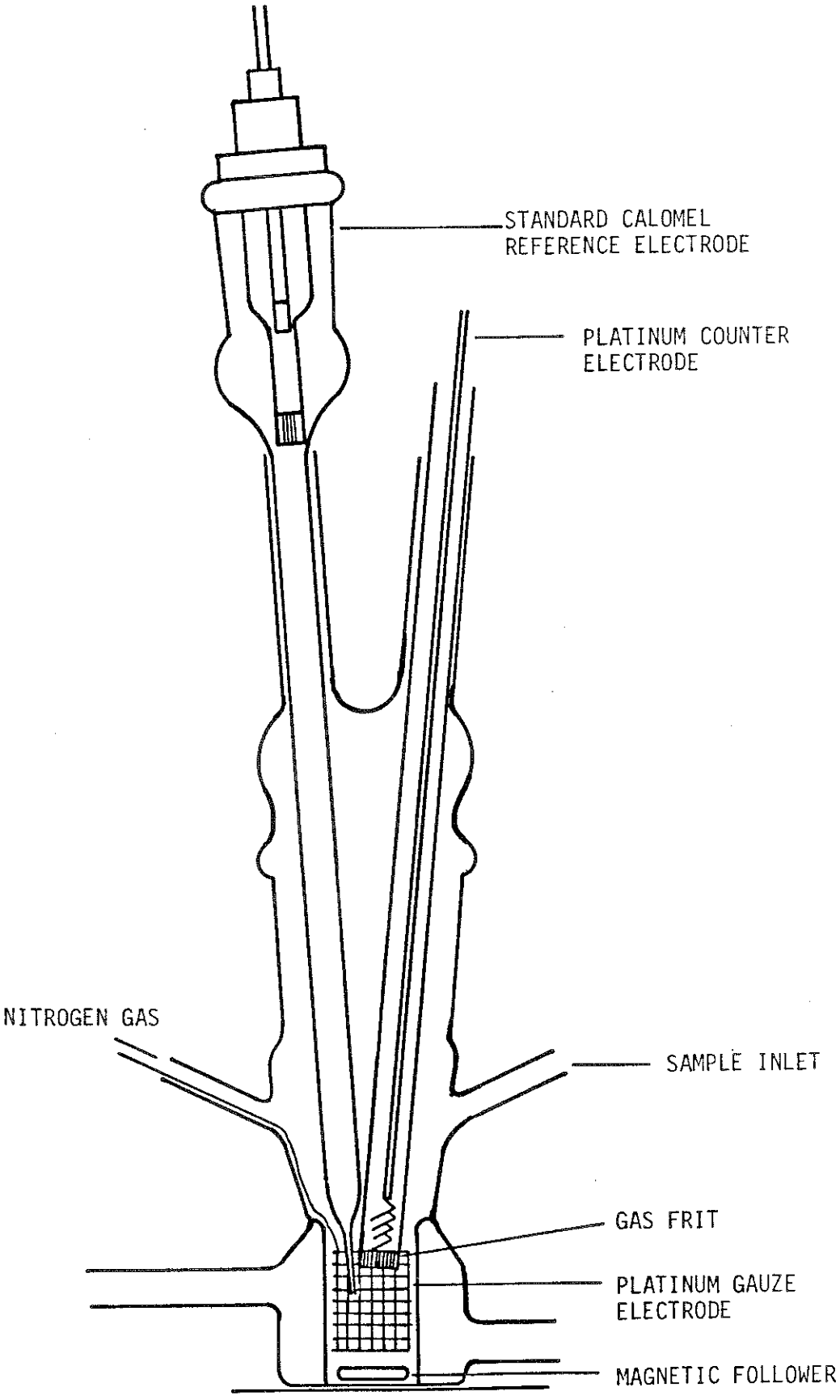
The detector (Fig 2.13) consisted of a jacketed reaction vessel (5.0 ml) (Quickfit, Gallenkamp and Co. Ltd., London) maintained at 30^{o}C, containing borate buffer (3.0 ml, 250 mM, pH

Figure 2.12 Exploded view of gas lift biofuel cell. The reticulated carbon anode (14 x 29 x 9 mm) was bonded to the external circuit using a silver loaded epoxy resin. The sparging gases were introduced as shown and passed up the columns, causing gassing and agitation (see section 2.28 and chapter 4).


O_2
CATHODE COMPARTMENT
(PLATINUM ELECTRODE)
ION EXCHANGE MEMBRANE
ANODE COMPARTMENT
(RETICULATED CARBON ELECTRODE)
N_2

9.0), ammonium chloride (50 mM), phenazine ethosulphate (1.0 mM) and crude extract of Methylosinus trichosporium OB3b (3.0 mg). Methylosinus trichosporium OB3b was grown on methane as the sole sorce of carbon and energy by Dr.D.Scott as previously described (Scott et al., 1981). Organisms were harvested from a steady state culture by centrifugation (10,000 g, 20 min, 4.0^{o}C) and resuspended in sodium phosphate buffer (20 mM, pH 7.0) containing magnesium chloride (5.0 mM). Disruption of the bacteria was carried out by sonication (4 x 45 sec, MSE Type 150W sonicator). Cell debris and unbroken organisms were removed by centrifugation (20,000 g, 1.0h, 4.0^{o}C) and the resulting supernatant used in the sensor. The mixture was continuously stirred using a magnetic stirrer and purged with nitrogen. Prior to use the nitrogen was passed through a Nilox gas purification unit (Jencons Scientific Ltd., Leighton Buzzard, Beds) and water. The working electrode consisting of platinum gauze (50 mesh, 1.0 x 4.8 cm) was soaked in nitric acid and cleaned by cyclic voltammetry in the potential range, -0.26 - +1.3 mV (versus SCE) in sulphuric acid (500 mM) (Sawyer and Roberts, 1974). The working electrode was immersed in the reaction mixture and maintained at +100 mV (verus SCE) using a ministat potentiostat (H.B. Thompson and Associates, Newcastle Upon Tyne). A platinum counter electrode was used; isolated from the reaction mixture by means of a glass frit. Aliquots of nitrogen saturated test solution (100 ul) were added to the system and the amperometric response measured across a 30 ohm resistance. The current/time curve was integrated directly

Figure 2.13 Schematic diagram of a homogeneous enzyme based methanol sensor (see section 2.31 and chapter 5).


STANDARD CALOMEL
REFERENCE ELECTRODE
PLATINUM COUNTER
ELECTRODE
NITROGEN GAS
SAMPLE INLET
GAS FRIT
PLATINUM GAUZE
ELECTRODE
MAGNETIC FOLLOWER

using a CDP4 computing integrator (Pye Unicam Ltd., Cambridge).

2.32 Platinisation of electrodes

Platinisation was carried out by the method described by Feltham and Spiro (1971). The electrodes were cleaned in 50% aqua-regia (HNO_3: HCl: H_2O; 1: 3: 4, V/V) followed by washing in nitric acid and rinsing with water. The electrodes were electrochemically cleaned (see section 2.31). The electrodes were placed in a solution of chloroplatinic acid (3.5% V/V) and lead acetate (5.0×10^{-3}% W/V). The current density was maintained at 30 mA cm^{-1} for a predetermined period of time, typically 10 min.

2.33 Electroless goldplating

Goldplating was carried out by the method described by Feldstein (1974). A copper mesh electrode was cleaned by sonication and placed in a solution containing the following:

$KAu(CN)_2$	3.0×10^{-2} M
KCN	1.0×10^{-1} M
KOH	2.0×10^{-1} M
KBH_4	2.0×10^{-1} M

After 12 hours at 70°C, the electrode was washed in distilled water.

2.34 Production of silver/silver chloride reference electrodes

Silver foil (BDH Chemicals Ltd., Poole, Dorset) was cut (40 x 5.0 x 0.13 mm) and polished using a 0.2 um aluminium-oxide slurry (BDH Chemicals Ltd., Poole, Dorset). A connecting wire was soldered to the electrode and insulated using a non-conductive araldite (Ciba-Geigy, Duxford, Cambridge). The electrode was immersed in a solution of hydrochloric acid (1.0 M) at a potential of +400 mV (versus SCE) for 30 sec and rinsed in distilled water prior to use.

For use on a strip device (see chapter 6), one surface was polished with an aluminium-oxide slurry and bonded to the base electrode using a conductive silver araldite (Johnson Matthey Chemicals Ltd., UK).

2.35 Construction of graphite paste electrodes

Membrane-retained enzyme based sensors were constructed for the determination of methanol. The sensor was constructed as follows; a connecting wire was attached to a platinum disc, (5.0 cm diameter) using a conductive epoxy resin (Johnson Matthey Chemicals Ltd., UK), and placed 1.0 mm inside a length of glass tubing (internal diameter 6.0 mm). The disc was bonded into position using a non-conductive epoxy resin (Ciba-Geigy, Duxford, Cambridge) and allowed to set. Prior to use the platinum working electrode was cleaned and polished using an aluminium-oxide slurry (0.2 um) to remove contaminants.

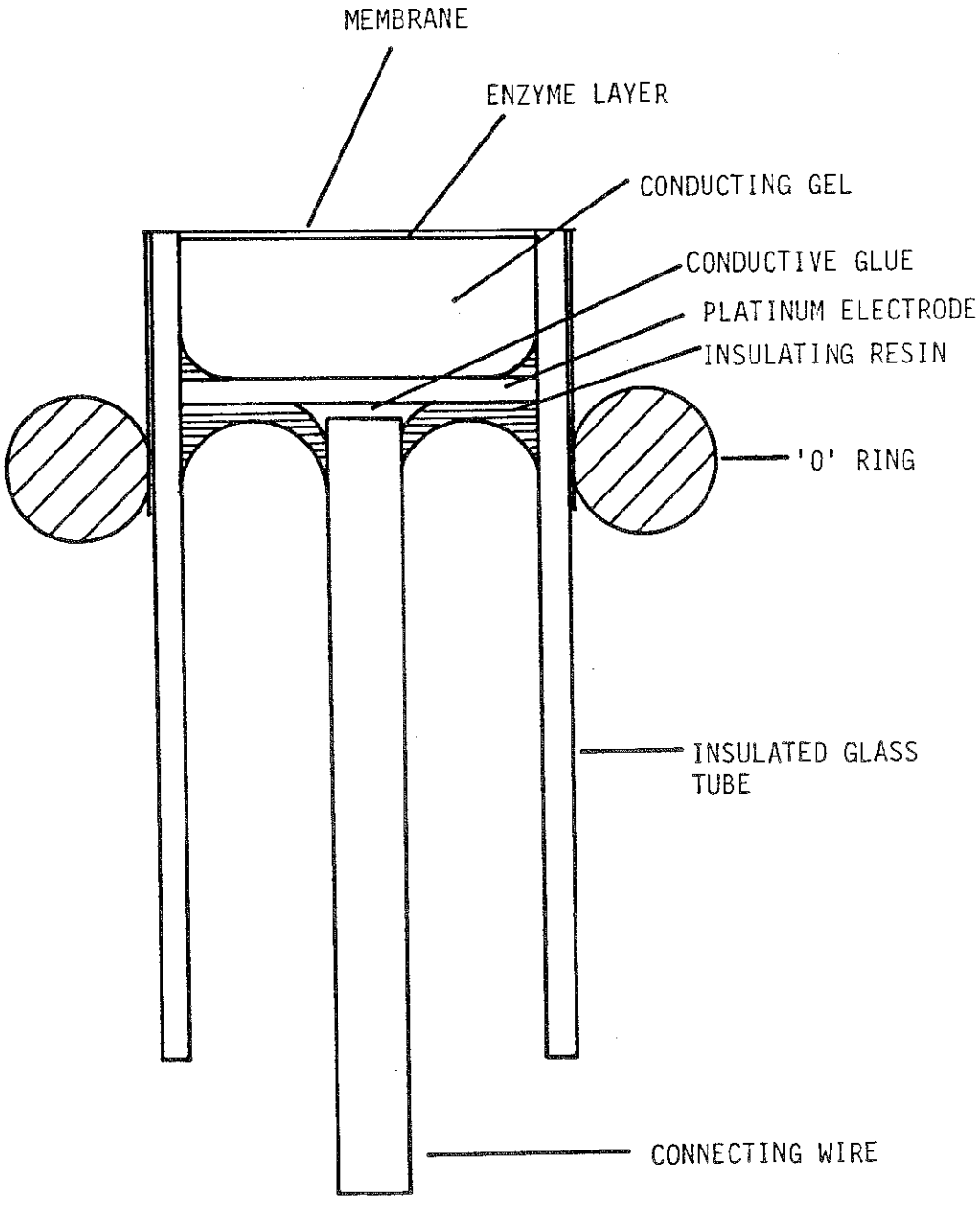
A conductive paste of the following composition was prepared:

Graphite	2.5 g
1,1'-dimethylferrocene	125 mg
liquid paraffin	1.5 ml

The paste was placed in the tip of the working electrode and the surface made smooth. Methanol dehydrogenase (4.0×10^{-2} mg) was placed on the paste and retained on the electrode surface with a membrane (1.2 cm diameter). The membrane, either dialysis or 0.03 um pore size Nuclepore membrane (Nuclepore Corp, California, USA) was held in position with an 'O' ring (Fig 2.14).

The sensor was placed in a reaction vessel maintained at $30^{o}C$ and poised at +150 mV (versus SCE) using a ministat potentiostat and a platinum counter electrode. The solution contained borate buffer (5.0 ml, 250 mM, pH 10.5) and ammonium chloride (50 mM), to which aliquots of methanol were added. Oxygen sensitivity was determined by sparging with nitrogen and subsequently with oxygen. The effect of various membranes on the performance of the enzyme electrode was tested. The membrane to be tested was bonded to a silicone rubber sleeve using an epoxy resin. After drying, the membrane was soaked in buffer for 24 hours prior to testing. A small quantity of buffer was placed on the membrane which was in turn placed over the electrode tip; a needle was introduced between the probe and the sleeve to minimise pressure which was removed when the membrane was in

Figure 2.14 A membrane retained graphite paste enzyme based sensor. The electrode consisted of a platinum disc (6.0 mm id) set into a pasteur pipette using conductive and non-conductive resins as shown (see section 2.35 and chapter 5).


MEMBRANE
ENZYME LAYER
CONDUCTING GEL
CONDUCTIVE GLUE
PLATINUM ELECTRODE
INSULATING RESIN
'O' RING
INSULATED GLASS TUBE
CONNECTING WIRE

place.

2.36 Construction of porous carbon electrodes

Graphite foil, 1.0 mm thick (Union Carbide, Ohio, USA) was cut into discs of 0.6 cm diameter using a standard number 3.0 cork borer. The discs were washed twice in acetone (30 min each wash), once in boiling distilled water (1.0 hour) to remove contaminants and finally dried in an air oven (100^{o}C). The discs were bonded to a length of glass tube (0.6 cm internal diameter) using an epoxy resin and allowed to set at 60^{o}C overnight. Electrical contact was made to the disc by the use of single stranded wire (0.2 mm) (R.S. Components Ltd., London) held in position using a silver loaded conductive resin polymerised at 100^{o}C for 1.0 hour. The resin was insulated by the incorporation of a mixture of epon resin (1.8 g, grade 815) and catalyst (triethylenetetramine 0.23 g) (Polysciences, Inc., Warrington, USA). The probes were allowed to set overnight at 60^{o}C in an air oven.

In the strip device the carbon squares (3.0 x 3.0 mm) were bonded onto the base metal using colloidal carbon after firmly pressing into position any excess carbon was removed (Fig 2.15).

Prior to enzyme immobilisation, the resistance between the carbon electrode and the external contact was tested using a multimeter. Electrodes exhibiting a resistance greater than 3.0 ohms were discarded.

place.

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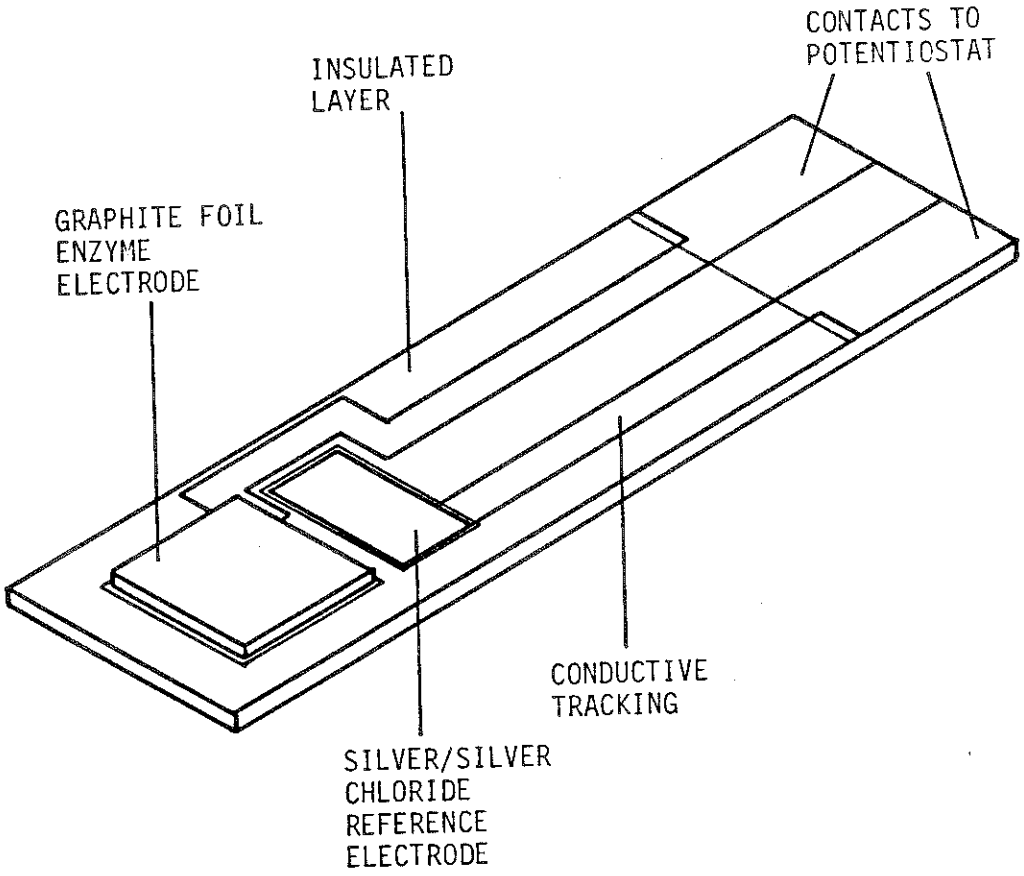
Prior to enzyme immobilisation, the resistance between the carbon electrode and the external contact was tested using a multimeter. Electrodes exhibiting a resistance greater than 3.0 ohms were discarded.

Figure 2.15 Schematic diagram of a glucose oxidase based enzyme electrode. Both the working and silver/silver chloride electrodes were mounted horizontally on a ceramic base. (This configuration was kindly supplied by Genetics International.) (See section 2.36 and chapter 6.)


CONTACTS TO
POTENTIOSTAT
INSULATED
LAYER
GRAPHITE FOIL
ENZYME
ELECTRODE
CONDUCTIVE
TRACKING
SILVER/SILVER
CHLORIDE
REFERENCE
ELECTRODE

2.37 Enzyme immobilisation

The graphite foil electrode surface was doped with 1,1'-dimethylferrocene (5.0 ul, 20 mg ml^{-1} in toluene) and allowed to dry. Immobilisation was carried out by treating the electrode with water soluble-1-cyclohexyl-3-(morpholinoethyl) carbodiimide metho-p-toluenesulfonate (0.15 M) in acetate buffer (100 mM, pH 4.5), for 80 minutes at 25°C. The electrode was washed in distilled water and placed in a solution of glucose oxidase (12.5 mg ml^{-1}, Glucox-ps, John & E. Sturge Ltd., Birmingham), in carbonate buffer (100 mM, pH 9.5), for 60 minutes. Prior to use or freeze drying the electrode was washed in distilled water and frozen at -20°C in phosphate buffer (100 mM, pH 7.4).

When immobilisation was carried out on the strips the silver/silver chloride reference electrode was covered with a water repellant silicone rubber layer. Glucose oxidase (12.5 mg ml^{-1}) was adsorbed into filter paper, frozen in liquid nitrogen and freeze dried. This procedure was repeated with filter paper impregnated with mediator prepared by soaking in a solution of 1,1'-dimethylferrocene in toluene (20 mg ml^{-1}) and allowing to dry.

Once freeze dried the filter paper was cut into discs (0.6 cm diameter) and mounted on the end of a porous carbon electrode impregnated with the same mediator. The disc was retained using a nylon gauze bonded to a silicone sleeve.

Samples of Yellow Springs Glucose Analyser membrane (YSI

2365) were obtained and placed over the end of porous carbon electrode impregnated with mediator and the response of the probe to glucose measured.

2.38 Calibration of stirrer

Stroboscopic calibration of the stirrer speed was carried out in a biofuel cell containing a 0.9 cm stirrer (Radley and Co. Ltd., Sawbridgeworth, Herts). One quarter sector of the magnetic follower was blackened and placed in distilled water within the anodic compartment of a biofuel cell. The stirrer speed was set and the strobes frequency adjusted, until the follower appeared stationary. This procedure was repeated for a series of stirrer setting and the rotation speed determined.

2.39 Membranes metallised by painting

A metallised membrane was made according to the method of Albery and Barron (1982). The membranes to be coated were painted with gold resinate solution (Johnson Matthey Ltd., Cresswell, Staffordshire) to give an electrode of the desired shape. The gold resinate was heated using a hot air gun (Black and Decker Ltd., UK) at a temperature of 600^{o}C for a few minutes until the organic materials had evaporated. Electrical contact was made via a conducting silver epoxy resin (Johnson Matthey Chemicals Ltd., Herts).

2.40 Membranes metallised by sputtering gold palladium

Cation exchange membrane was modified by the deposition of a thin conductive layer of gold palladium on one or both of its surfaces. The membrane was placed within the chamber of a Polaron SEM coating unit E5100 series 11 'Cool' sputter coater (Polaron Equipment Ltd., Watford, England). The chamber was evacuated to 0.02 - 0.03 Torr, using a rotary pump (Edwards High Vacuum Ltd., Crawley, Sussex), with intermediate flushing with argon. The vacuum was then increased to 0.01 Torr. Once this was attained, the ionisation potential was maintained at 2.4 KV and the current held at 20 mA for a maximum sputtering time of 2.0 minutes. The thickness of the deposited layer was determined from the following calculation:

$$th\ (A) = 7.5 \times I \times t$$

where, th (A) is the thickness in Angstroms, t is the time in minutes and I is the current in milliamps. When both surfaces were coated and no masking was used; the electrical contact between the two surfaces was eliminated by trimming the edges of the coated surfaces.

2.41 Membranes metallised by sputtering gold

Gold coating of the membrane was carried out by placing the membrane to be coated in a chamber 10.0 cm from a gold target.

The chamber was evacuated to 0.25 Torr using a rotary pump, type ISC450B (Edwards High Vacuum Ltd., Crawley, Sussex) and subsequently to 10^{-6} Torr using a 6.0 inch water cooled ion diffusion pump (Edwards High Vacuum Ltd., Crawley, Sussex). Fifteen minutes after a full vacuum was achieved; deposition was carried out at 5.0 V, 100 amps for 3.0 minutes.

2.42 Carbon Sputtering

Carbon coating was carried out on both metallised and non-metallised membrane surfaces, using a Coating unit model 12E6/639 (Edwards High Vacuum Ltd., Crawley, Sussex) according to the previously described method for gold metallisation of membranes.

2.43 Satistical analysis

Statistical methods, formulae and tables were those given by Fisher and Yates (1963). The analysis of variance was performed and the detection limits of the homogeneous methanol sensor were calculated according to the Manual on Analytical Quality Control for the Water Industry (WRC Technical Report TR66, 1978). Correlation coefficients were performed using a model TI-51-111 programmable calculator (Texas Instruments, Bedford).

CHAPTER 3

RESULTS AND DISCUSSION: GROWTH OF ORGANISMS AND PURIFICATION OF ENZYMES

3.1 Growth of organisms.

Four methylotrophic bacteria were compared as possible sources of methanol dehydrogenase; these included Ps. extorquens, Ps. AM1, P. denitrificans and M. methylotrophus. P. denitrificans, although containing methanol dehydrogenase (Bamforth and Quayle, 1978b), did not readily adapt to growth on methanol using a simple salts media. In addition, the extracted enzyme lost activity during column chromatography (see section 2.10.1). Ps. extorquens, Ps. AM1 and P. denitrificans were induced to grow on methanol as their sole source of carbon and energy (see section 2.4) using either D_2 medium (see section 2.5.1) or complete autoclavable medium (see section 2.5.3). The specific growth rates of Ps. extorquens and Ps. AM1 on methanol (1.0% V/V) were similar, being 0.105 and 0.108 h^{-1}, with final cell densities of 1.3 and 1.5 mg dry weight ml^{-1}, respectively. The specific activities of methanol dehydrogenase in crude cell free extracts prepared from these cultures were 171.0 and 103.6 nmoles O_2 consumed min^{-1} mg $protein^{-1}$, respectively.

The effect of various methanol concentrations (0.5 - 2.0% V/V) on the growth rate of Ps. AM1 and Ps. extorquens was also investigated. Higher initial methanol concentrations caused a corresponding decrease in growth rate (Table 3.1). Growth of the organisms on 0.5% (V/V) methanol resulted in a decreased cell density. The addition of a further 0.5% (V/V) methanol when the culture reached steady state, however, resulted in a final cell density of 1.1 g dry weight l^{-1} attained for both Ps. AM1 and Ps.

Table 3.1 Comparison of the growth rates and final cell densities of *Pseudomonas* AM1 and *Pseudomonas extorquens* grown in shake flasks at various methanol concentrations.

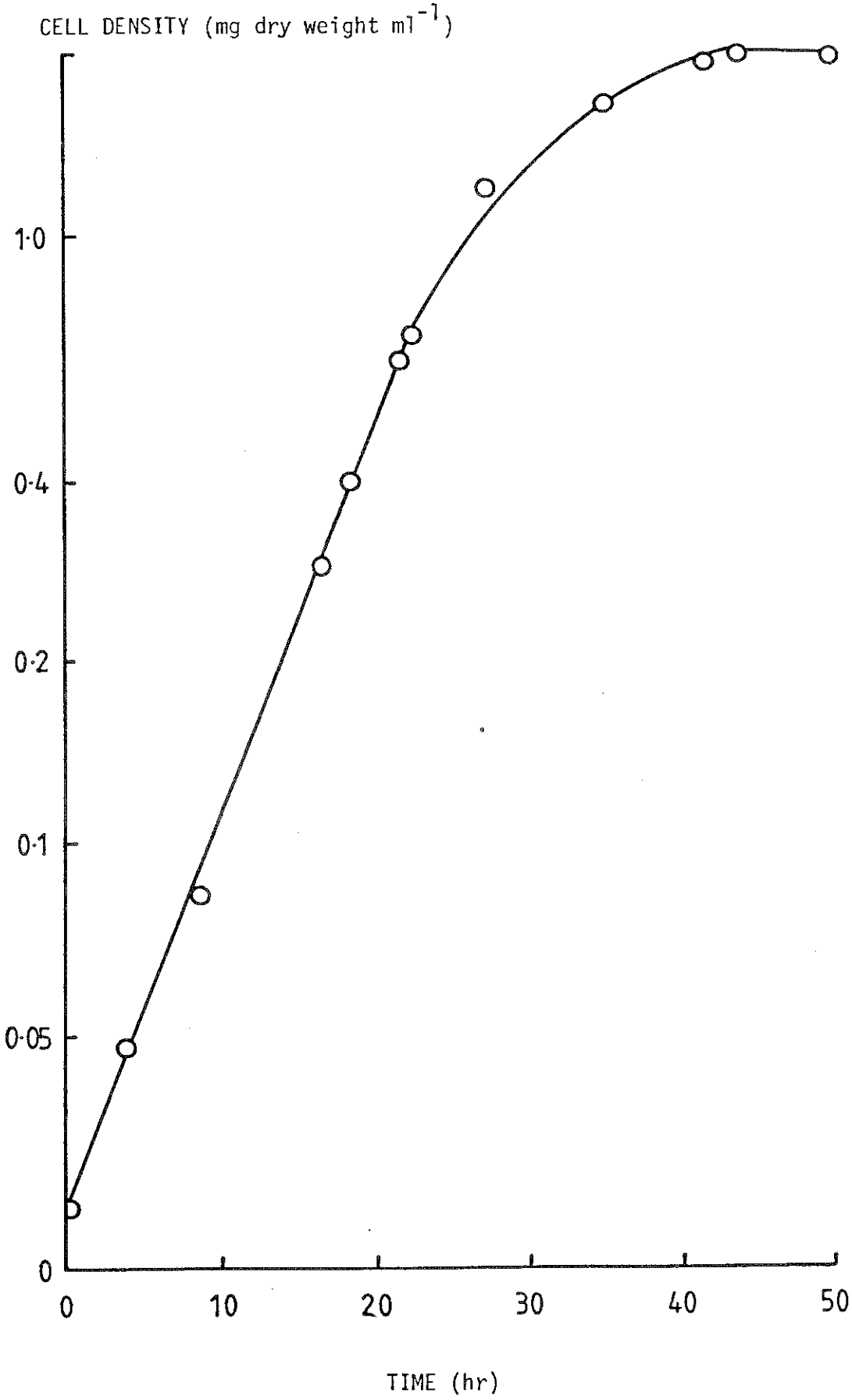
	Pseudomonas AM1			*Pseudomonas extorquens*		
Methanol (%)	Doubling Time (h)	μ (h^{-1})	Final Cell Density (g dry wt l^{-1})	Doubling Time (h)	μ (h^{-1})	Final Cell Density (g dry wt l^{-1})
0.5	5.2	0.13	0.48	4.6	0.15	0.51
1.0	6.4	0.11	1.5	6.6	0.11	1.3
1.5	6.6	0.11	1.0	6.8	0.10	1.0
2.0	8.8	0.08	1.1	8.0	0.09	1.1

extorquens.

Growth of Ps. extorquens in shake flasks on complete autoclavable medium was compared to that on D_2 medium in the presence of methanol (1.0% V/V). When grown on D_2 medium the organisms had a doubling time of 6.5 hours ($u = 0.107\ h^{-1}$). During growth on complete autoclavable medium the doubling time was 6.1 hours ($u = 0.113\ h^{-1}$).

Ps. extorquens was grown in a fermenter (20 l), with methanol as the sole source of carbon and energy, using feed-draw batch growth (see section 2.6). Growth was monitored spectrophotomerically at 600 nm (see section 2.9). Ps. extorquens had a doubling time of 5.2 hours ($u = 0.133\ h^{-1}$) during growth on methanol in the initial batch culture, (Fig 3.1) but when grown in the feed draw mode it exhibited a doubling time of 7.0 hours ($u = 0.088\ h^{-1}$). Organisms were harvested in the mid-exponential growth phase (see section 2.8) since active enzyme was not present in late log or stationary phase of growth (Fig 3.1). The specific activity of crude cell free extracts decreased from 115 nmoles O_2 min^{-1} mg^{-1} protein after 25 hours growth to 70 nmoles O_2 min^{-1} mg^{-1} protein after 50 hours. Initially Ps. extorquens was used as the source of methanol dehydrogenase, although the enzyme was subsequently extracted from M. methylotrophus, the single cell protein organism produced by ICI and donated as a frozen paste.

Figure 3.1 Growth of *Pseudomonas extorquens* grown in batch culture in a 20 l fermenter on methanol (1.0%) as the sole source of carbon and energy.


CELL DENSITY (mg dry weight ml^{-1})
1·0
0·4
0·2
0·1
0·05
0
0
10
20
30
40
50
TIME (hr)

3.2 Purification of methanol dehydrogenase.

The purification of methanol dehydrogenase described by Anthony and Zatman (1967a) does not readily lend itself to laboratory scale up. Summaries of the purification of methanol dehydrogenase from Ps. extorquens by a modified method described by Anthony and Zatman (1967a, see section 2.10.1) and from M. methylotrophus by two phase purification (see section 2.10.2) are given in tables 3.2 and 3.3 respectively.

A two phase aqueous enzyme purification was developed using polyethylene glycol and phosphate systems. Preliminary experiments demonstrated that the ratio of the enzyme in the top phase to that in the bottom was influenced by the molecular weight of the polyethylene glycol used. Methanol dehydrogenase was found to partition entirely into the lower phosphate rich phase when phosphate/polyethylene glycol 1000 was used (see section 2.10.2). The results for the purification of methanol dehydrogenase from M. methylotrophus (Table 3.3) show a 65 fold purification from a disrupted cell suspension and with a 143% yield. Separation of the enzyme rich phosphate phase from the polyethylene glycol was initally carried out by centrifugation (25,000 g, 5.0 min, 4.0^{o}C). Phase separation under gravity was investigated in order to reduce the requirement for centrifugation (Fig 3.2). The enzyme was stable in the phosphate phase for this period of time (48 h).

Fig 3.3 shows SDS polyacrylamide gel electrophoresis (PAGE) (see section 2.14) of crude extracts and purified methanol

Table 3.2 Purification of methanol dehydrogenase from *Pseudomonas extorquens* by a modified method of Anthony and Zatman (1967a).

Fraction	Volume (ml)	Total Protein (mg)	Specific Activity (nmoles O_2 consumed min^{-1} mg^{-1} protein)	Yield (%)	Purification Factor
Crude extract	610	11262	115	100	-
Acid supernatant	1066	4108	300	95	2.6
DEAE cellulose acetate	1500	1500	750	86	6.5
G150-126 eluate	101	292	1140	26	9.9

Table 3.3 Purification of methanol dehydrogenase from *Methylophilus methylotrophus* by two phase aqueous partition

Fraction	Volume (ml)	Protein ($mg.ml^{-1}$)	Total Protein (mg)	Specific Activity (nmoles min^{-1} mg^{-1} protein)	Yield %	Purification (fold)	Nucleic Acid ($mg.ml^{-1}$)	Carbohydrate ($mg.ml^{-1}$)
Disrupted Cells	600	39.53	23,718	93.8	100	0	4.29	0.52
Cell Free Extract	-	6.65	-	668.3	-	7.1	-	-
Diafiltrate/ Concentrate	270	2.09	564.3	5652.1	143	64.8	1.82	0.1
After DNA Precipitation	270	2.09	564.3	5652.1	143	64.8	0.16	-

Figure 3.2 Phase separation of polyethylene glycol 1000 and phosphate under gravity at $4^{o}C$. The phase volumes were determined by measuring the position of the interface in a calibrated settling tank.

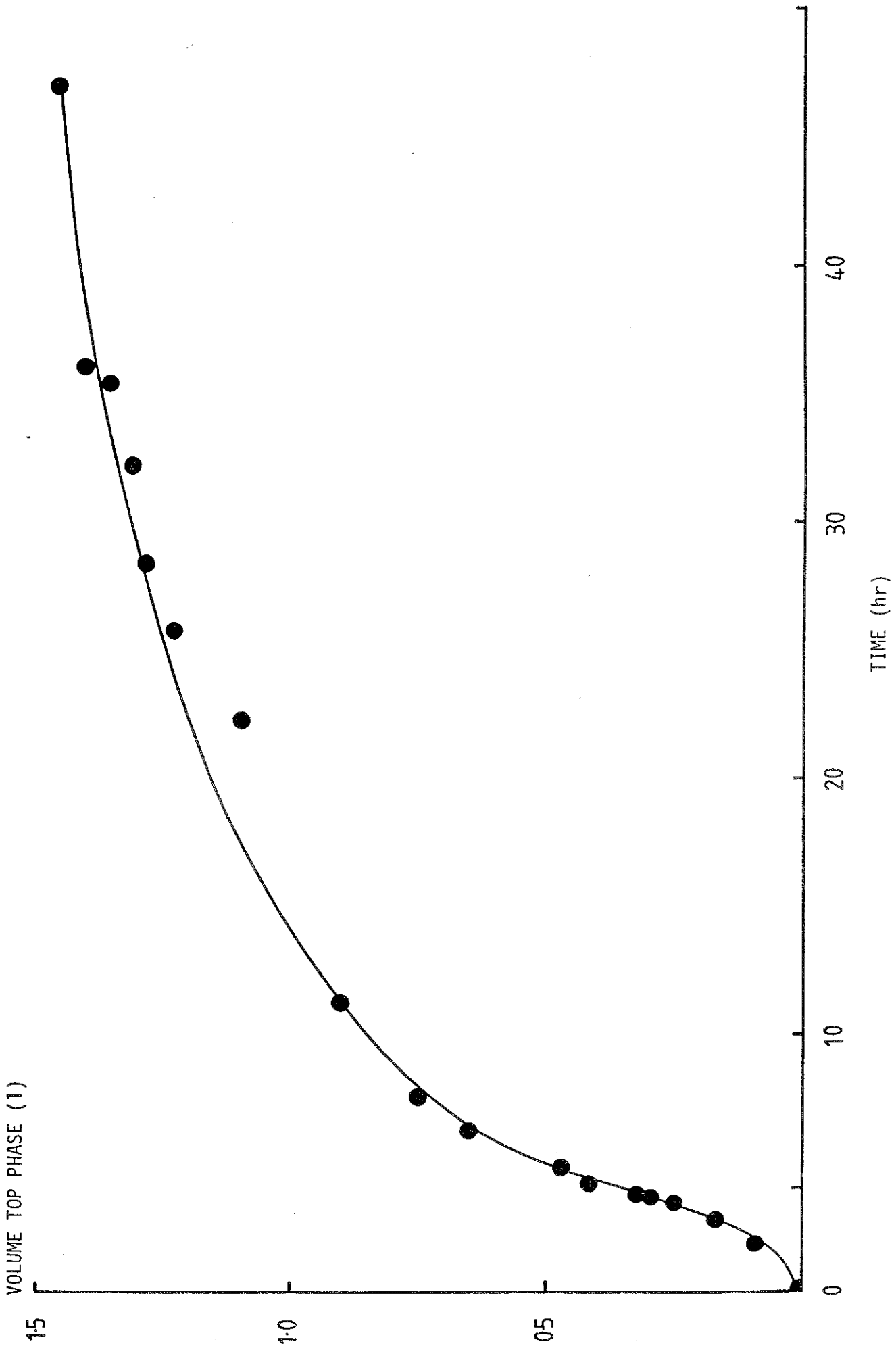
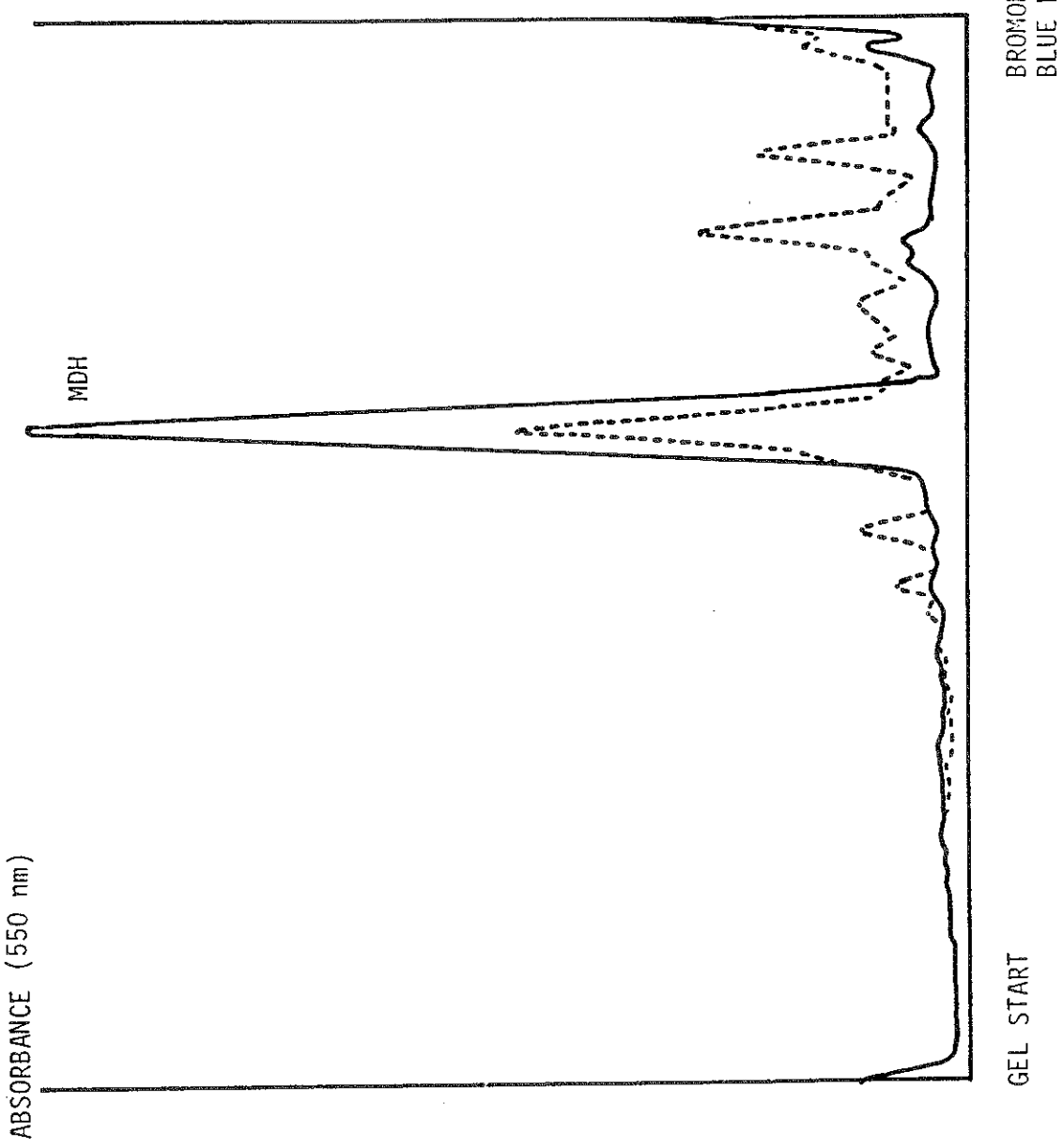

VOLUME TOP PHASE (l)
1·5
1·0
0·5
0
10
20
30
40
TIME (hr)

Figure 3.3 SDS polyacrylamide gel electrophoresis of methanol dehydrogenase obtained from *Methylophilus methylotrophus* by two phase aqueous purification. Prior to (----) and after purification (——).


ABSORBANCE (550 nm)
MDH
GEL START
BROMOPHENOL
BLUE MARKER

dehydrogenase. The purified enzyme was also analysed by gel filtration using HPLC (see section 2.15) (Fig 3.4). Methanol dehydrogenase purified by two phase aqueous partion contained DNA which could be measured by HPLC (Fig 3.4), spectrophotometry (Fig 3.5a,b) and chemical analysis (Table 3.3). This was, however, readily removed by protamine sulphate precipitation (1.0% W/V).

The enzyme constituted about 10% of the soluble potein in crude extracts of Ps. AM1, Ps. extroquens and M. methylotrophus.

The sub-unit molecular weights of the three purified enzymes were shown to be 60,000 when determined by dissociating SDS PAGE (Fig 3.6) and the pH optimum in each case was 10.5 (Fig 3.7). The ability of amino acids and B-cyano-L-alanine to activate the enzyme from Ps. extorquens was tested polarographically (see section 2.11.3). None of those tested, however, resulted in activation of the enzyme which was shown to have an have an absolute requirement for ammonia, methylamine or ethylamine. At 2.5 mM the latter two activators only produced 17% and 9.8%, respectively, of the activity obtained with ammonium chloride (2.5 mM). The specific activity of of all three enzymes doubled for each $10^{o}C$ rise in temperature over the temperature range 40 - $50^{o}C$ (Fig 3.8).

Preparations of methanol dehydrogenase lost about 10% activity after 4 months frozen in liquid nitrogen and stored at $-20^{o}C$. Enzyme from Ps. AM1 and Ps. extorquens lost approximately 30% and 31% activity, respectively after 5 cycles of freezing ($-20^{o}C$) and thawing. Purified enzyme was not stable to freeze

Figure 3.4 Absorption profile of methanol dehydrogenase eluted from a HPLC column. Purified by two phase aqueous separation prior to (----) and after (——) protamine sulphate precipitation to remove DNA.

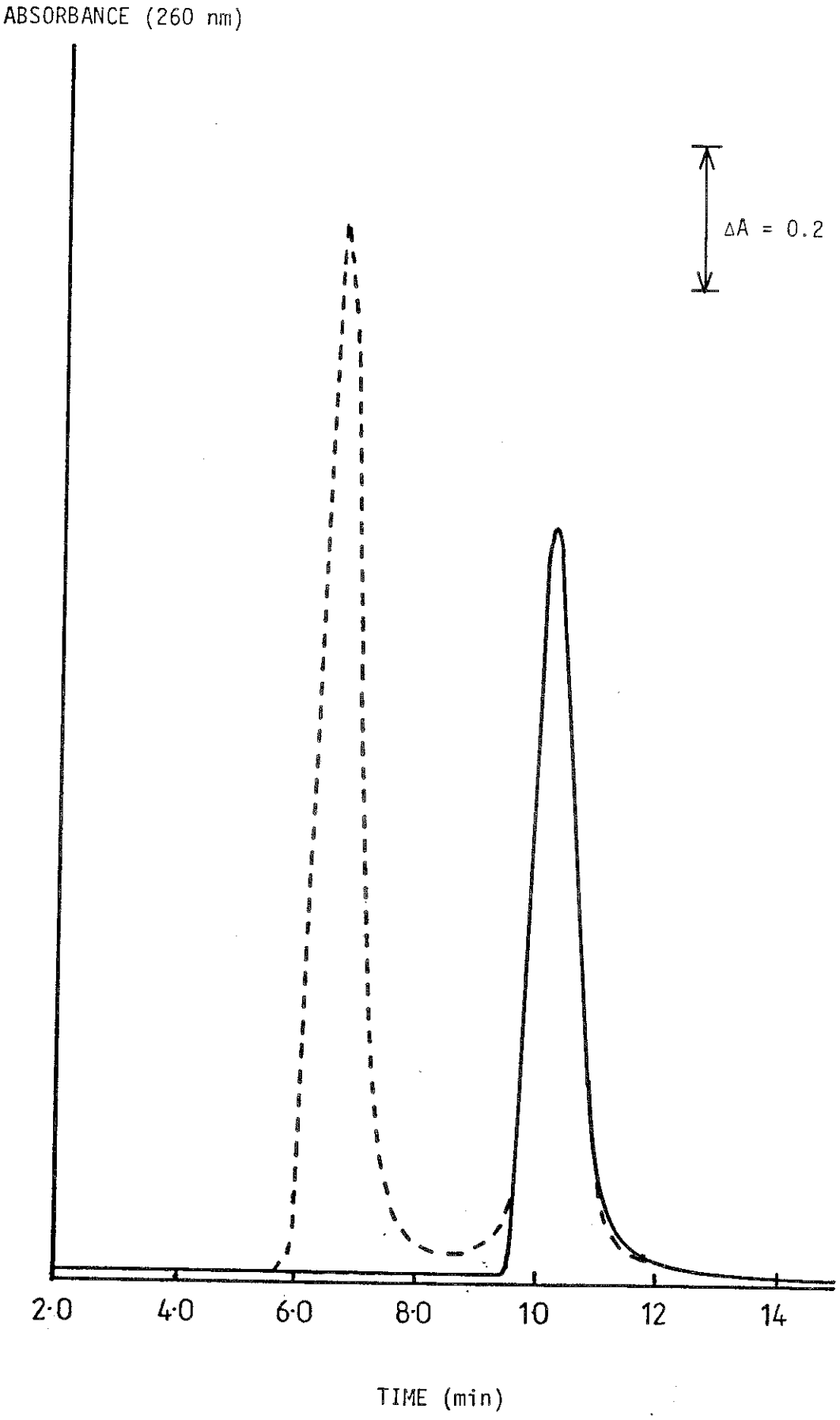

ABSORBANCE (260 nm)
ΔA = 0.2
2·0
4·0
6·0
8·0
10
12
14
TIME (min)

Figure 3.5a Absorption spectrum of methanol dehydrogenase purified from *Methylophilus methylotrophus*. Prior to (----) and after (——) protamine sulphate precipitation.

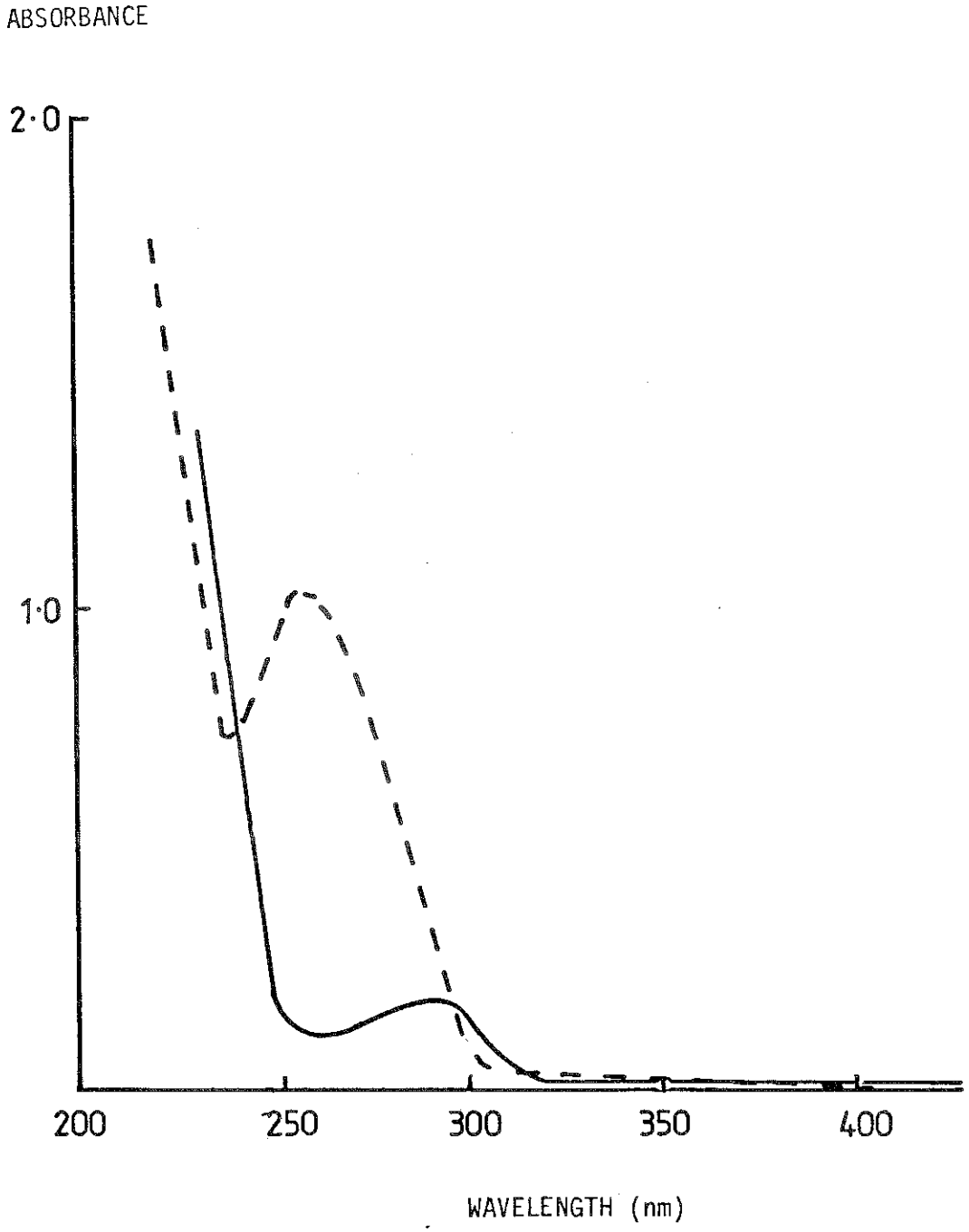

ABSORBANCE
2·0
1·0
200
250
300
350
400
WAVELENGTH (nm)

Figure 3.5b Absorption spectrum of methanol dehydrogenase purified from *Methylophilus methylotrophus* using 0.04 mg ml^{-1} in distilled water. The reference cuvette also contained distilled water.

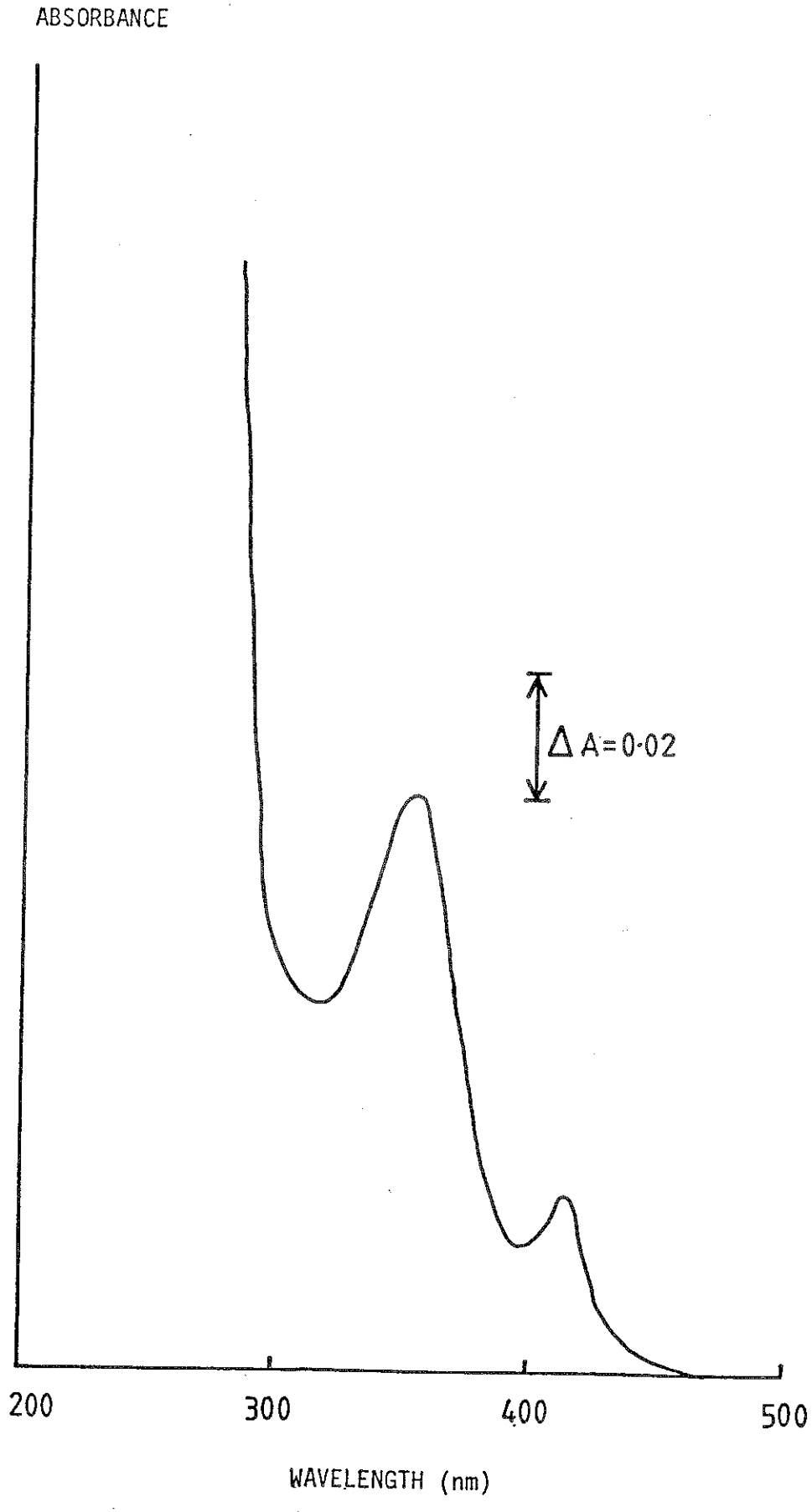

ABSORBANCE
Δ A=0·02
200
300
400
500
WAVELENGTH (nm)

Figure 3.6 Determination of the sub-unit molecular weight of methanol dehydrogenase determined by dissociating SDS polyacrylamide gel electrophoresis. MDH was purified from: *Pseudomonas* AM1; *Pseudomonas extorquens*; *Methylophilus methylotrophus*.

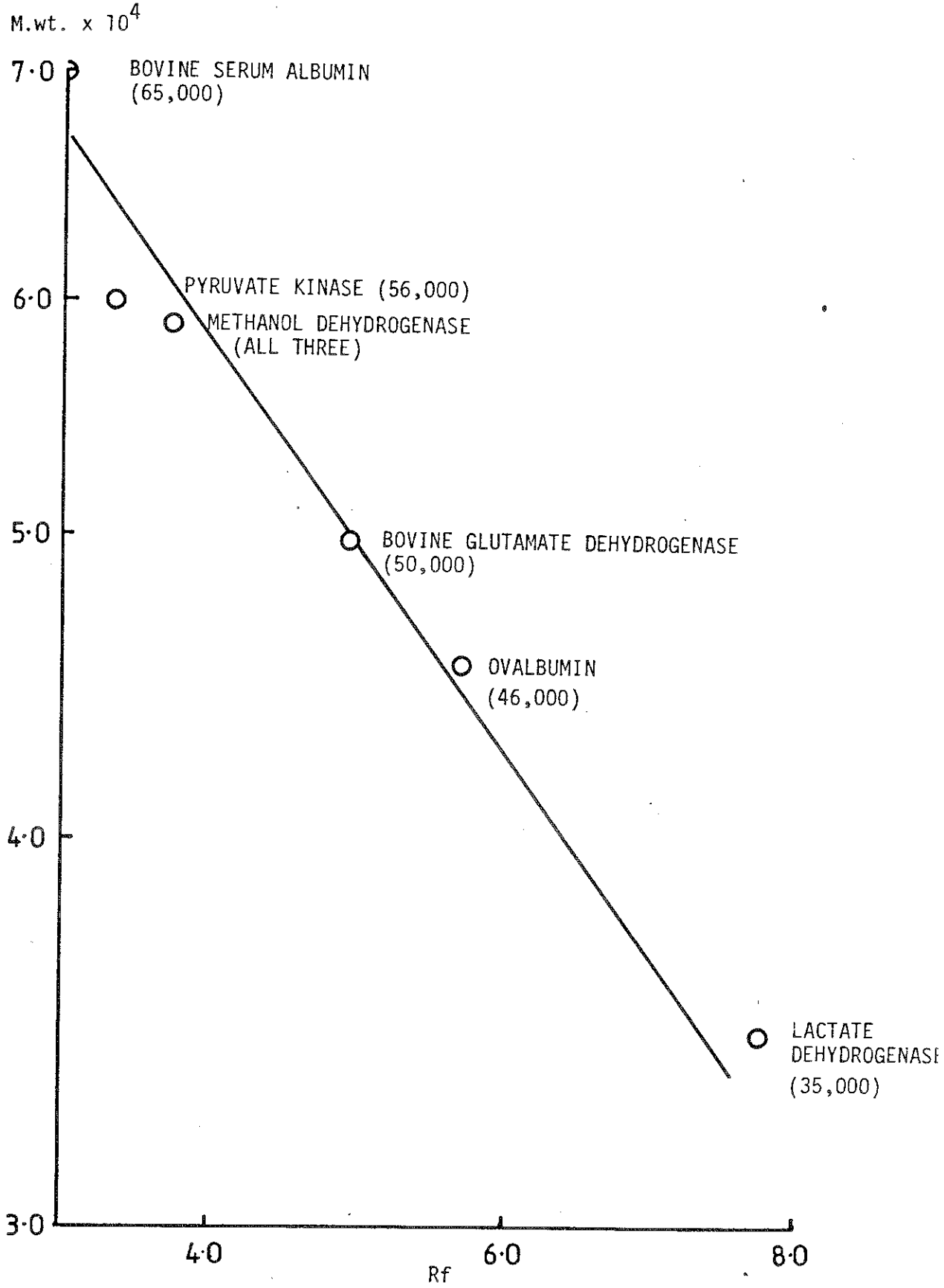

M.wt. x 10^4
7·0
6·0
5·0
4·0
3·0
BOVINE SERUM ALBUMIN
(65,000)
PYRUVATE KINASE (56,000)
METHANOL DEHYDROGENASE
(ALL THREE)
BOVINE GLUTAMATE DEHYDROGENASE
(50,000)
OVALBUMIN
(46,000)
LACTATE
DEHYDROGENASE
(35,000)
4·0
6·0
8·0
Rf

Figure 3.7 pH profile of methanol dehydrogenase purified from *Pseudomonas extorquens*.

o bicarbonate buffer (400 mM)

● borate buffer (200 mM)

□ pyrophosphate buffer (100 mM)

The ionic strength of these buffers were the same (1.0).

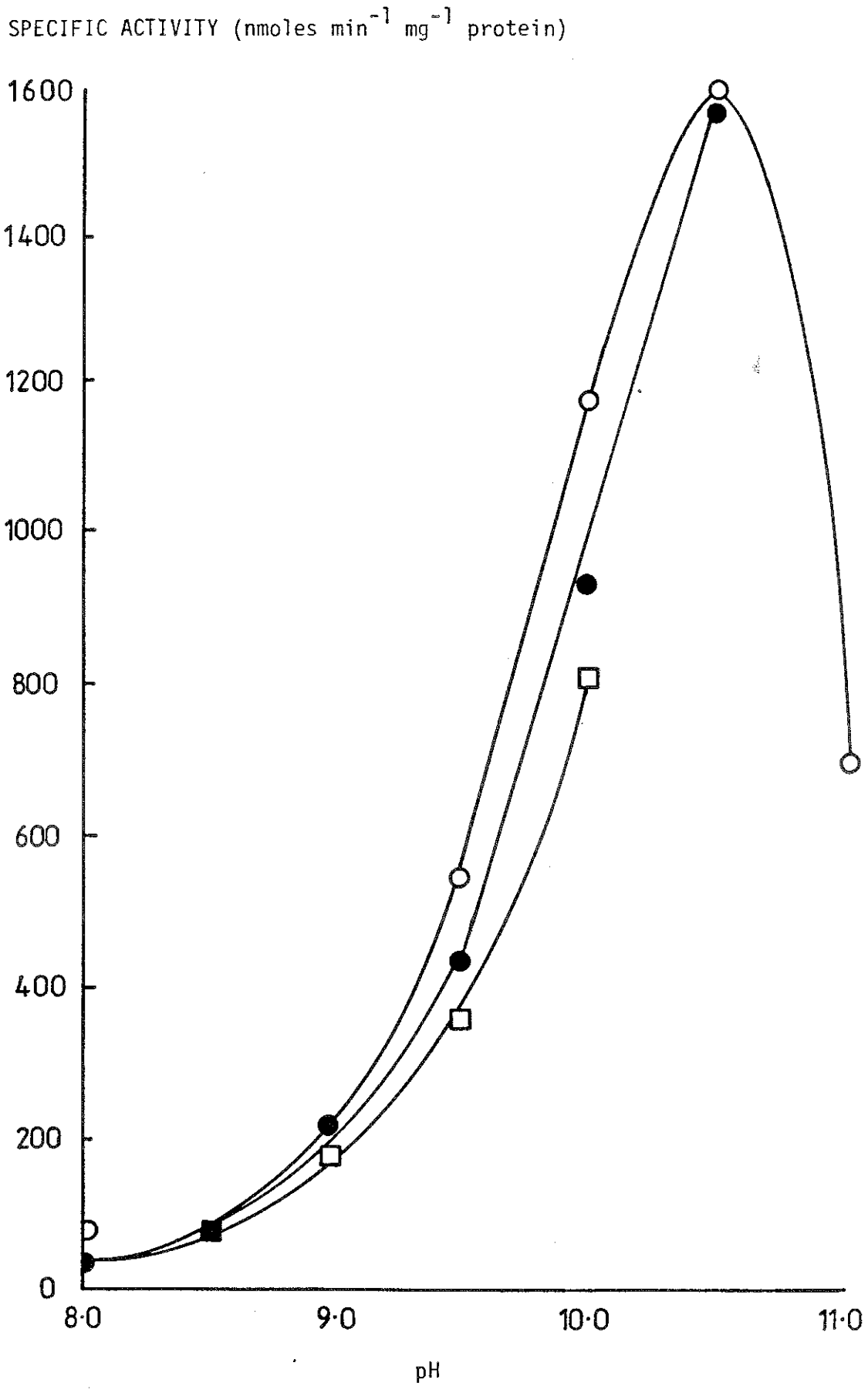
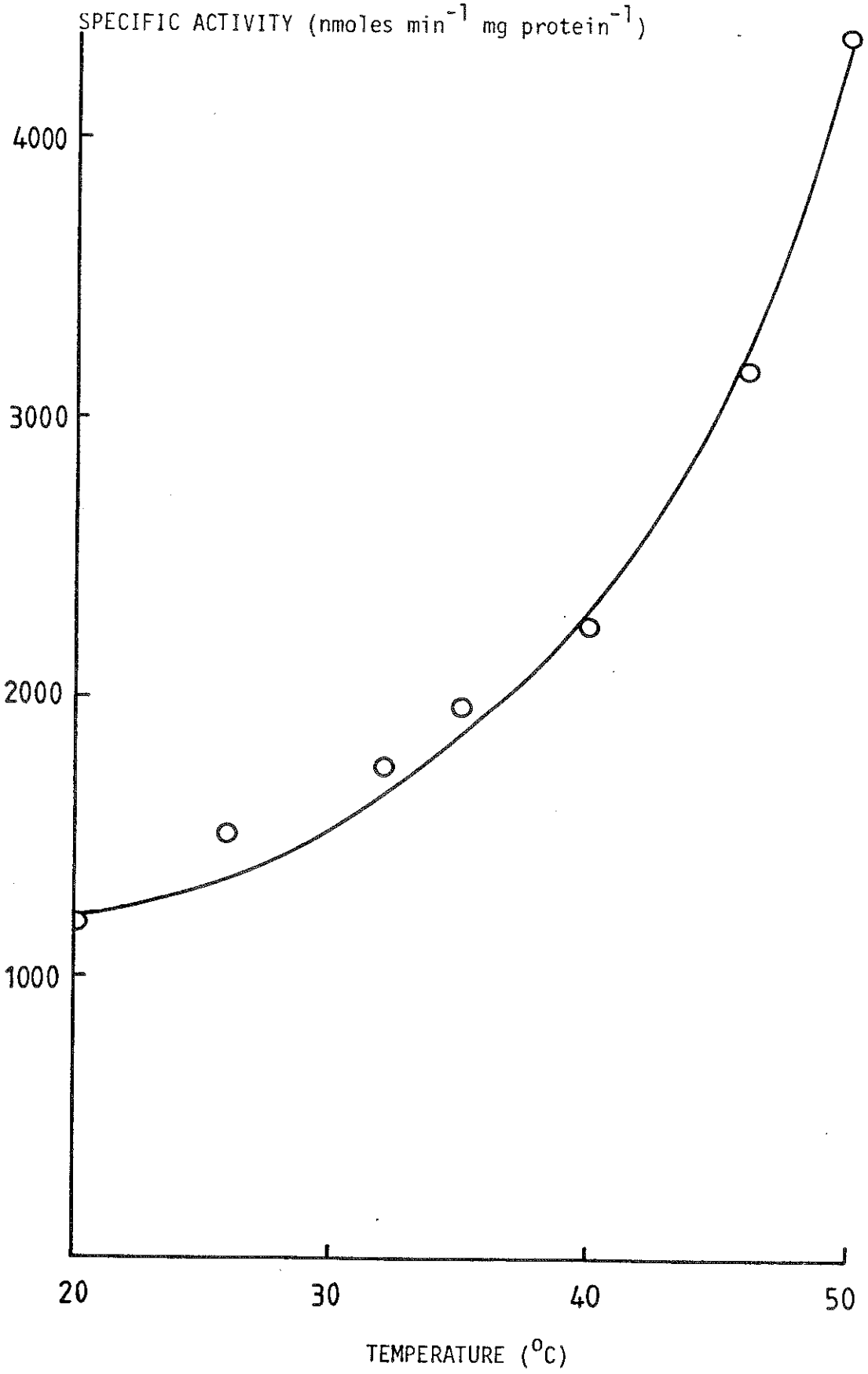

SPECIFIC ACTIVITY (nmoles min^{-1} mg^{-1} protein)
1600
1400
1200
1000
800
600
400
200
0
8·0
9·0
10·0
11·0
pH

Figure 3.8 Effect of temperature on the specific activity of methanol dehydrogenase obtained from *Methylophilus methylotrophus*.


SPECIFIC ACTIVITY (nmoles min^{-1} mg protein^{-1})
4000
3000
2000
1000
20
30
40
50
TEMPERATURE (^{o}C)

drying losing 94% activity (Fig 3.9). Enzyme stability was determined aseptically in autoclaved test tubes plugged with cotton wool. The solutions containing Tris-HCl buffer (100 mM, pH 9.0) and enzyme (0.7 mg) were filter sterilised and maintained at 4.0^{o}C and 30^{o}C. Samples were removed at regular intervals and assayed polarographically (see section 2.11.3). Methanol dehydrogenase purified from Ps. AM1 and Ps. extorquens was stable for 4 weeks at both 4^{o}C and 30^{o}C both with and without added methanol (1.0% V/V).

3.3 Production and purification of laccase.

The growth of Coriolus versicolor and induction of the extracellular enzyme laccase have been described (see sections 2.7 and 2.10.3). The addition of dimethyl aniline to the fermenter at 7.0 days resulted in the rate of catechol oxidation in extracts increasing from 4.0 x 10^{-2} absorbance units min^{-1} ml^{-1} h^{-1} (determined over the first 4 days of growth) to 1.1 x 10^{3} absorbance units min^{-1} $ml^{-1}h^{-1}$. The partially purified enzyme had a pH optimum of 5.0 (see section 2.11.4) when assayed in acetate buffer (Fig 3.10).

3.4 Discussion.

Previously described media for the growth of methylotrophic organisms have ranged from complex medium (Yamanaka and Matsumoto, 1977) to simple salts (Anthony and Zatman, 1964a;

Figure 3.9 The effect of various storage protocols on the activity of methanol dehydrogenase obtained from *Pseudomonas extorquens*.

○ Frozen in liquid nitrogen

● Frozen at -20°C

□ Freeze dried

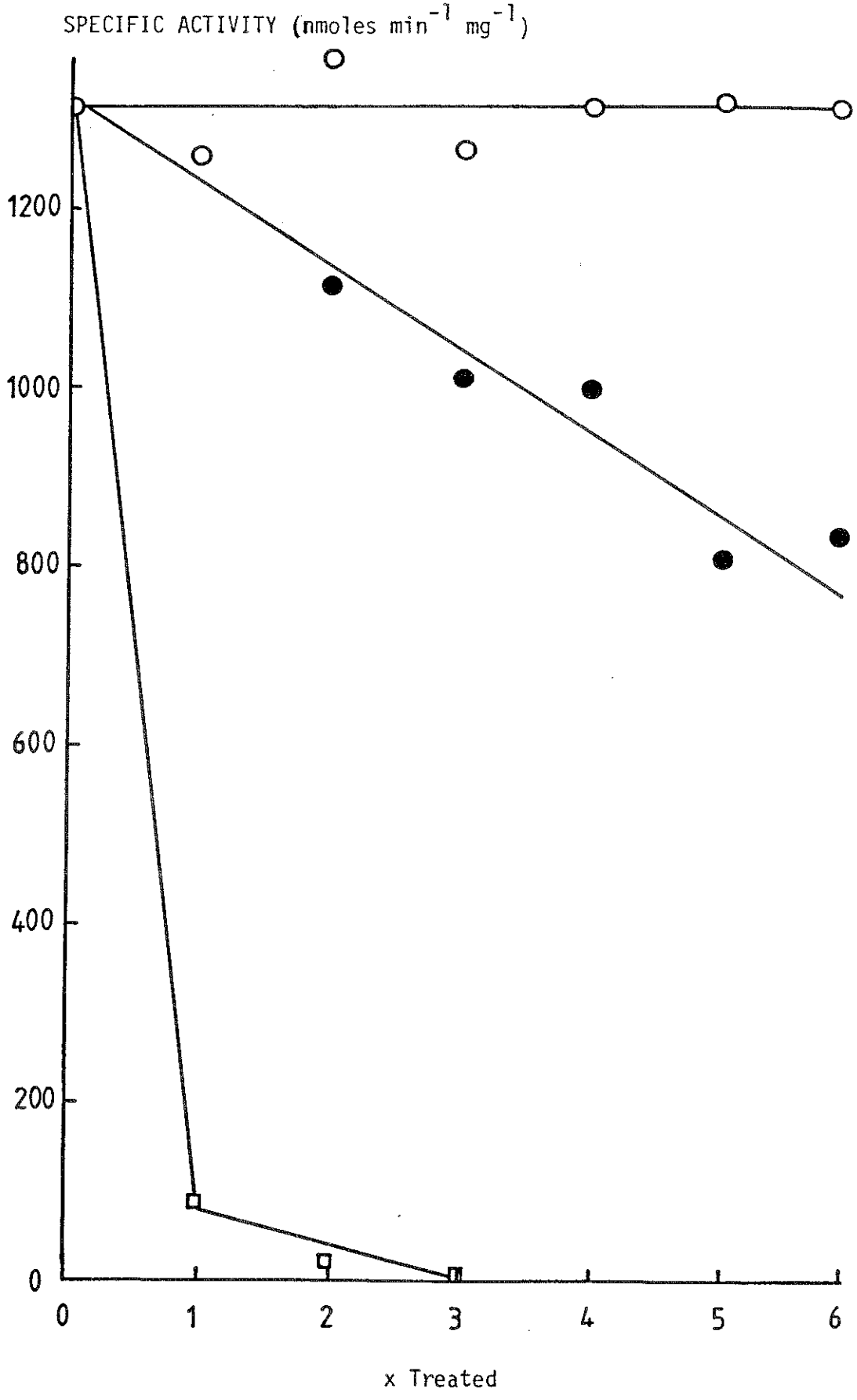
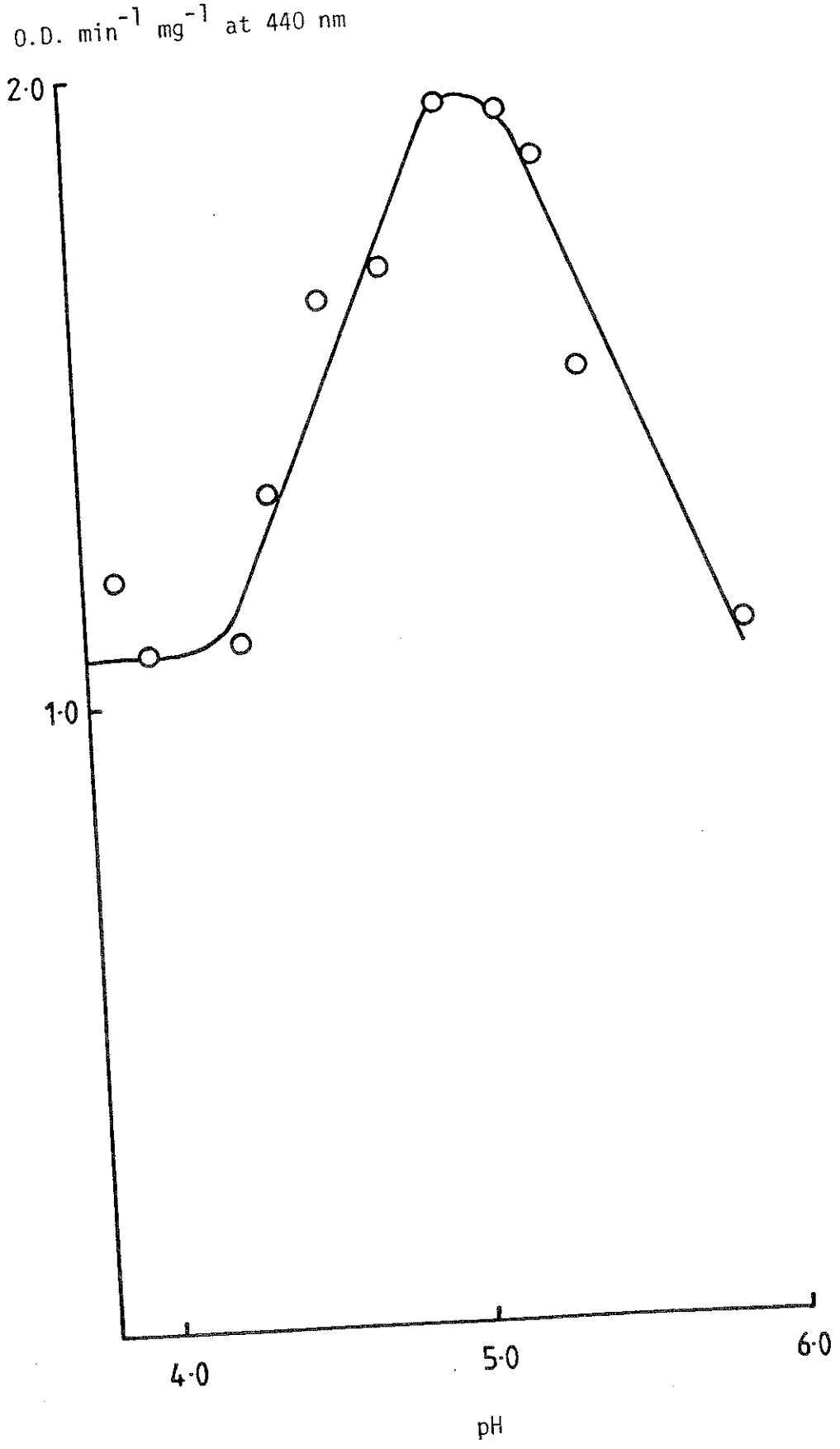

SPECIFIC ACTIVITY (nmoles min^{-1} mg^{-1})
1200
1000
800
600
400
200
0
0
1
2
3
4
5
6
x Treated

Figure 3.10 The pH activity profile of laccase, partially purified from *Coriolus versicolor* in acetate buffer (100 mM) determined spectrophotometrically.


O.D. min^{-1} mg^{-1} at 440 nm
2·0
1·0
4·0
5·0
6·0
pH

Duine et al., 1978). Laboratory scale cultures of Ps. AM1 and Ps. extorquens used D_2 a simple salts media. This medium was, however, unsuitable for large scale industrial growth of organisms and Jayasuriya salts medium was used in preference (Jayasuriya, 1955). This was later replaced with a medium developed in our laboratory (see section 2.5.3) which enabled the constituent salts to be autoclaved complete and methanol added on cooling. Neither the specific growth rate nor the cell density achieved by both Ps. AM1 and Ps. extorquens were affected by changes in the medium (within the range of methanol concentrations tested) although they were affected by the concentration of methanol. Consequently the organisms were grown on 1.0% (V/V) methanol for subsequent experiments although this resulted in a lower growth rate it allowed maximum cell densities to be attained. P. denitrificans did not readily adapt to simple salts media; this may be related to the requirement for yeast extract in the starter culture medium (Cox and Quayle, 1975).

Organisms were harvested in mid-exponential phase of growth. The yield of methanol dehydrogenase (27%) and purification attained (9.9 fold) using conventional techniques was similar to that achieved by previous authors (Patel et al., 1972; Mehta, 1973; Sperl et al., 1974; Anthony and Zatman, 1967a,b; Patel and Felix, 1976; Bellion and Wu, 1978; Ohta et al., 1981). The yield achieved using a single step two phase purification system (143%) was far greater than that previously published (13%) for the same organism (Ghosh and Quayle, 1981)

and therefore offers a superior technique for the extraction and purification of this enzyme. The high yield may be due the removal of inhibitory components present in the crude extract. The availability of large quantaties of commercially produced cell paste of M. methylotrophus from ICI and the development of the two phase technique enabled large quantities of the enzyme to be purified with relative ease and low cost. This technique is readily amenable to scale up, decreasing process time and saving both manpower and energy input (Kroner et al., 1982b).

The enzyme purified from Ps. AM1 had similar properties to that purified from Ps. M27 (Anthony and Zatman, 1967a,b) including the same subunit molecular weight determined by SDS polyacrylamide gel electrophoresis, absorption spectrum as Pseudomonas M27 and isoelectric point of 8.8. The enzyme had a pH optimum of 10.5 which was similar to some other methanol dehydrogenases (see section 1.2.1) such as that isolated from Pseudomonas C (Goldberg, 1976), but different from the optimum pH of 9.0 reported for Ps. M27 (Anthony and Zatman, 1967a,b).

Partially purified laccase was capable of oxidising catechol with a pH optimum of pH 5.0 as previously described (Fahraeus and Reinhammar, 1962). Its incorporation into a biofuel cell to enhance the cathodic reaction is discussed in sections 1.3.4 and 4.4.4.

CHAPTER 4

RESULTS AND DISCUSSION: METHANOL DEHYDROGENASE -BASED BIOFUEL CELLS

4.1 Mediators

The ability of methylotrophic bacteria to derive energy from the oxidation of methanol using methanol dehydrogenase has been investigated by many authors (see section 1.2.1). The enzymic oxidation of methanol is an especially interesting redox reaction for the development of an enzyme-based biofuel cell, since the substrate is oxidised to formate via formaldehyde with the release of four electrons (Wingard et al., 1982).

The efficient conversion of substrates in biofuel cells is facilitated by the presence of mediators capable of rapid electron transfer between the enzyme and an electrode. Previous biofuel cell studies using methanol dehydrogenase have utilised phenazine (m)ethosulphate as mediators (PMS; PES) (Plotkin et al., 1981). These mediators, however, decompose both photochemically and at the optimum pH of the enzyme (Ghosh and Quayle, 1979; see section 3.2). Whilst work using PES was pursued other mediators, membranes and electrodes were also investigated. An alternative mediator, TMPD, was shown to be a stable substitute to PES and PMS.

The use of artificial redox couples in the study of biological systems has been well documented (Hewlitt, 1950; Fultz and Durst, 1982; Johnson et al., 1983) and their application in biofuel cells has been recently reviewed (Wingard et al., 1982).

Mediators replace the natural redox partners in biological systems. They should be capable of rapid electron transfer between the electron source and the electron acceptor. They should be stable under the conditions used, non-toxic, not substrates for the enzyme

and have redox couples close to that of the biological system in order to derive the maximum energy.

Phenazine methosulphate (PMS) and phenazine ethosulphate (PES) are frequently used in polarographic and colorimetric enzyme assays (Dickens, 1936; Peel, 1972; Dunstan et al., 1972) and were initially used in the methanol dehydrogenase-based biofuel cell (Plotkin et al., 1981). Both of these mediators, however, possess low solubility in the reduced form (Kulys and Malinauskas, 1979) and undergo UV inactivation (McIlwain, 1937; Ghosh and Quayle, 1979). It was therefore necessary to screen a variety of mediators exhibiting improved properties for use in a biofuel cell.

4.1.1 UV Stability of mediators.

The rate of UV inactivation of PES was measured spectrophotometrically and its ability to mediate in a methanol dehydrogenase-based biofuel cell tested (see section 2.22). To determine the wavelength of maximum absorption (see section 2.16) an absorbance spectrum of a freshly prepared solution of PES was compared to one exposed to ultraviolet light (Fig 4.1.1). UV inactivation was determined by exposing a solution of PES (0.03 mM) in borate buffer (50 mM, pH 9.0), containing ammonium chloride (50 mM), to a UV light source (365 nm) maintained at a distance of 11.0 cm (see section 2.22). Samples were removed at various time intervals and the absorbance measured at 388 nm. The absorbance decreased as shown (Fig 4.1.2). The rate at which the spectral change occurred was not affected by sparging the solution with nitrogen or air during exposure

Figure 4.1.1 Absorption spectrum of phenazine ethosulphate (0.05 mM) in borate buffer (50 mM, pH 9.0). The absorption spectrum was measured prior to (——) and after (---) 6 minutes exposure to UV light (see section 4.1.1).

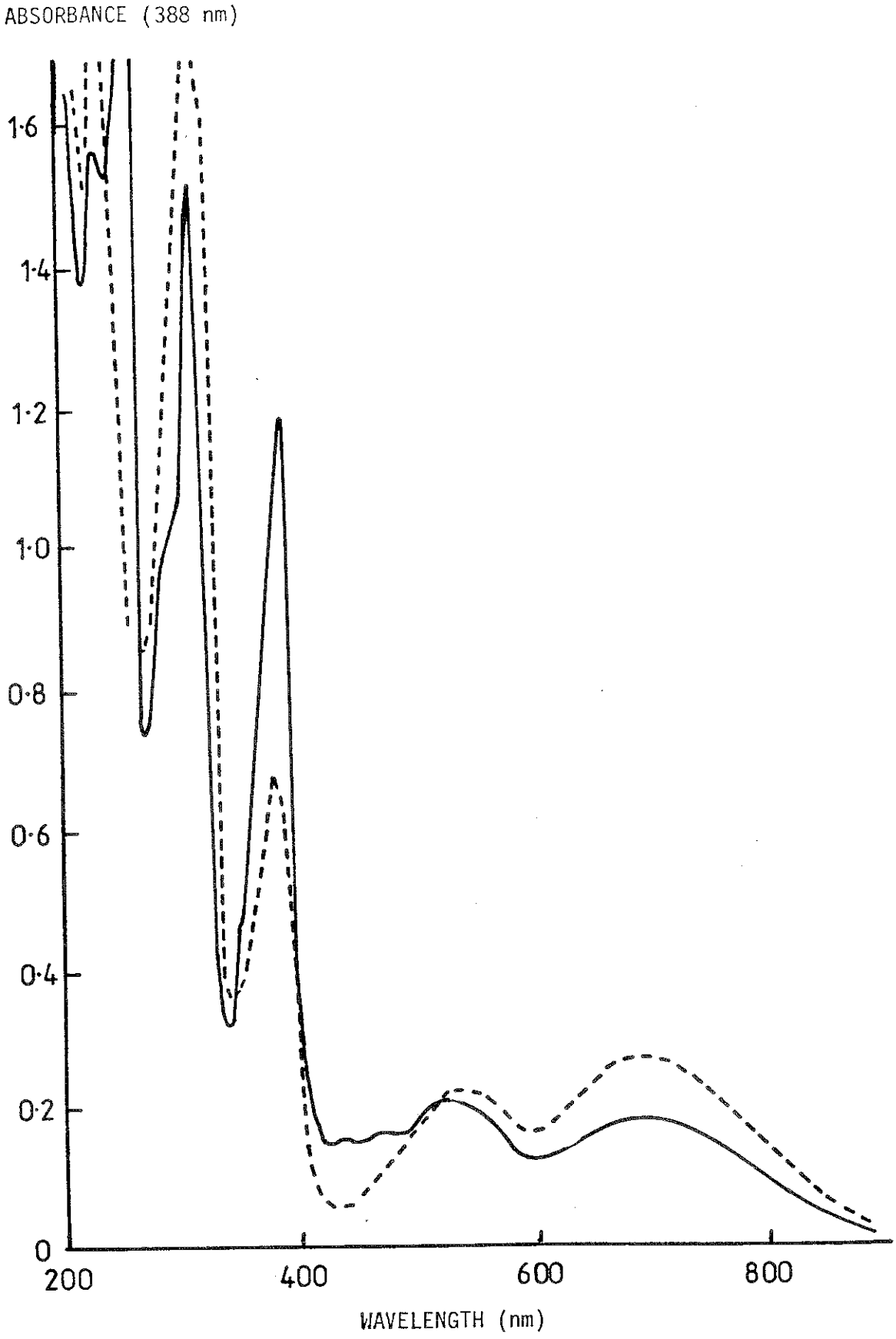
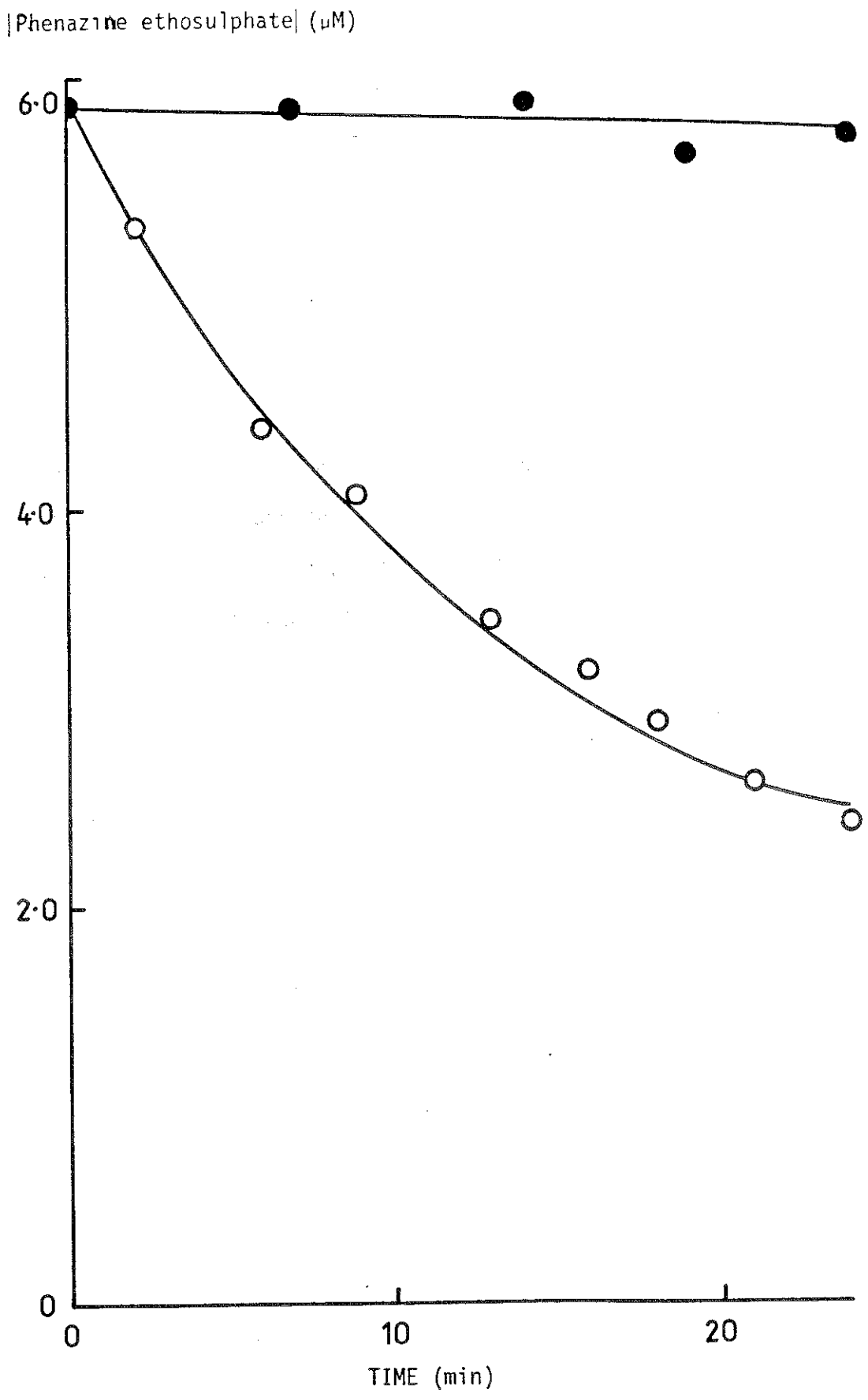

ABSORBANCE (388 nm)
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
200
400
600
800
WAVELENGTH (nm)

Figure 4.1.2 The decrease in absorbance (388 nm) of PES (0.03 mM) in borate buffer (50 mM, pH 9.0) exposed to UV light (O) and maintained in the dark (●) (see section 4.1.1).


|Phenazine ethosulphate| (μM)
6·0
4·0
2·0
0
0
10
20
TIME (min)

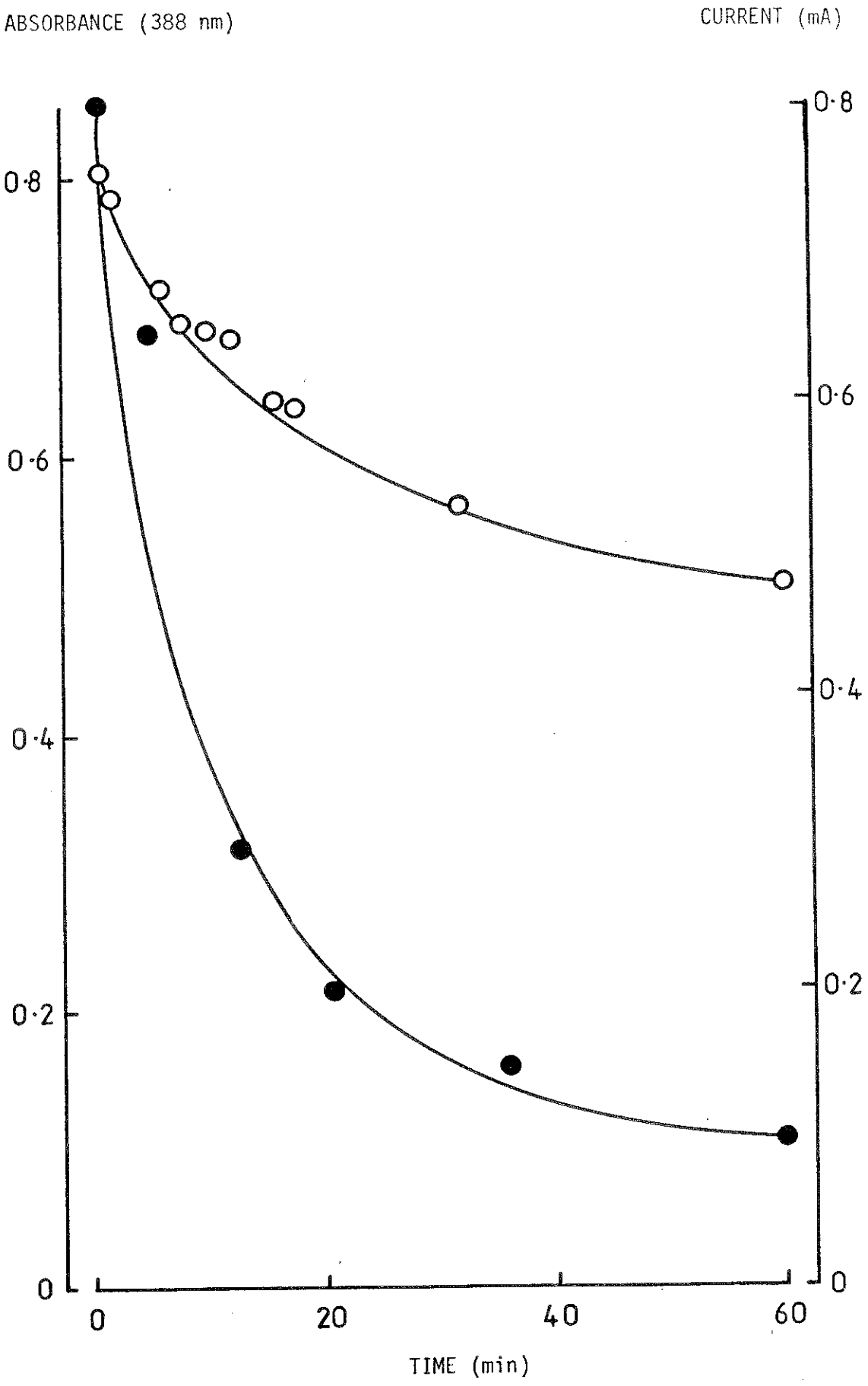
to the light. Buffered solutions of PES in the pH range 8.0 to 11.0 exposed to UV light underwent the same absorbance changes when measured at 388 nm.

A solution of PES (0.03 mM) maintained in the dark in borate buffer (250 mM) at pH 9.0 or above (pH 11.0) became red in colour after 12 hours. When maintained at pH 8.0 - 8.5 the solution remained yellow for longer than 24 hours. The colour changes were unaffected by the presence of ammonium chloride (50 mM).

The ability of PES (3.0 mM) exposed to ultraviolet light to transfer electrons from methanol dehydrogenase to the electrode was investigated. Samples of the mediator exposed to UV light were removed and the absorbance determined (388 nm). The current produced by adding mediator (0.01 mM) to a biofuel cell containing enzyme (0.4 mg), methanol (2.5 mM), borate buffer (250 mM, pH 9.0) and ammonium chloride (50 mM) was measured. The procedure was repeated for a range of exposure times. Exposure of PES to UV light resulted in its inability to mediate in a methanol dehydrogenase biofuel cell (Fig 4.1.3).

The experiment was repeated with N,N,N',N'tetramethyl-p-phenylenediamine (TMPD). The absorption spectrum and ability to mediate electron transfer between the enzyme and electrode was unaffected by exposure to UV light when tested at pH 9.0 and 10.5 over a 1.0 hour period. TMPD was stable at the pH optimum of the enzyme, 10.5 (see section 3.2) whilst PES became rapidly hydrolysed to form a red solution of 1-hydroxyphenazine (Haas and Zumbrunnen, 1981) incapable of mediating in the biofuel cell.

Figure 4.1.3 The ability of PES exposed to UV light to mediate in a biofuel cell. The absorbance of a solution of PES exposed to UV light was measured at 388 nm (O), an aliquot was added to a biofuel cell and the maximum current measured (●) (see section 4.1.1).


ABSORBANCE (388 nm)
CURRENT (mA)
0·8
0·6
0·4
0·2
0
0·8
0·6
0·4
0·2
0
0
20
40
60
TIME (min)

4.1.2 Soluble mediators.

The ability of methanol dehydrogenase to reduce mediators was investigated colorimetrically, polarographically and electrochemically both in the biofuel cell and sensor configurations.

Although the polarographic analysis enabled possible mediators to be screened (Table 4.1.1) it was replaced with a colorimetric assay in which changes of the oxidised and reduced forms of the mediator were visually observed.

Colorimetric analysis of soluble redox couples (see section 2.23) involved a visual observation and comparison of the colour changes upon both chemical and enzymic reduction. Soluble mediators were made up in borate buffer (50 mM, pH 10.5) containing ammonium chloride (50 mM) and methanol (1.0 mM) incubated at 30^{o}C. The solution containing the mediator (0.1 mM) was divided equally between two tubes, both sparged with nitrogen. A crystal of sodium dithionite was added to one and the colour change observed. The ability to produce the oxidised form was demonstrated by vigorous shaking and sparging of the tube with air. Methanol dehydrogenase (0.2 mg) was added to the second tube and the colour change compared to that obtained by the chemical reduction. This technique, although allowing rapid screening of possible mediators such as TMPD and thionin, which undergo colour changes upon reduction (Table 4.1.2), was not suitable for insoluble mediators or those which do not produce observable spectral changes.

The ability of mediators to facilitate electron transfer was tested in the biofuel cell configuration (see section 2.28). The

Table 4.1.1 The relative rates of oxygen consumption by methanol dehydrogenase in the presence of substrate, activator and a variety of potential mediators determined using a Clark oxygen electrode (see section 2.19).

Polarographic measurements

Mediator	Rate relative to PES (%)
PES	100.0
mPMS	20.0
Thionin	9.7
Brilliant blue R	7.3
Meldolablau	6.3
Resorufin	0.0
Toluidine blue O	0.0
Brilliant cresol blue	0.0
Chlorophenol	0.0
Crystal violet	0.0
2,3 Dichloro-1,4 naphthoquinone	0.0
Gallocyanin	0.0
1,2 Naphthoquinone	0.0

4.1.2 Colorimetric analysis of possible mediators. A visual comparison of colour changes exhibited by various possible mediators upon chemical and enzymic reduction (see sections 2.23 and 4.1.2).

)R	OXIDISED	REDUCED	REVERSIBLE	ENZYMIC REDUCTION
ıe Orange (1,2)	Yellow	Clear	+	-
no-di(3 ethyl azoline sulphonic liammonium salt	Green	Clear	+	+
:resol green	Blue	Red	+	-
ıymol blue	Blue	Blue	-	-
ıinone	Blue	Clear	+	+
ıethy acridine				
: (2)	Green	Yellow	-	
ınt blue G	Blue	Blue	-	-
ınt blue R	Blue	Clear	+	-
ınt cresol blue	Blue	Clear	+	-
fuschin	Purple	Clear	+	-
ıhenol	Yellow	Clear	+	-
ıhenol red	Blue	Clear	+	-
red	Red	Red	-	-
violet	Purple	Clear	+	-
:hlorophenol- :nol	Blue	Clear	+	-
ıydroxynaphthalene	Clear	Clear	-	-
:hylamineo-2,3- ıenocazine	Purple	Clear	+	+
'anin	Purple	Clear	+	-
te green (2)	Green	Clear	+	-
green	Blue	Clear	-	-
red	Yellow	Yellow	-	-
viologen	Clear	Purple	-	-
violet	Purple	Clear	+	-
:ne blue	Green	Yellow	+	-
:hol	Clear	Clear	-	-
red	Yellow	Yellow	-	-
ue (2)	Blue	Clear	+	-
ne ethosulphate (1,3)	Yellow	Brown	+	+
red	Red	Red	-	-
franin	Red	Clear	+	-
ı-quinoline quinone	Yellow	Yellow	-	-
'in	Purple	Clear	+	-
n O	Red	Clear	+	-
ne	Red	Yellow	+	-
:-tetracyanoquino- ne (1)	Green	Orange	-	-
N'-tetramethyl-*p*- :nediamine	Blue	Clear	+	+
: (2)	Purple	Clear	+	+
ne blue O	Blue	Clear	+	+

:ic; (2) Precipitated out of solution; (3) Became red in colour

biofuel cell was set up (see section 2.28) containg the mediator to be tested (1.0 mM). The open circuit potential was measured prior to and after the addition of enzyme (0.1 mg). If a change in the open cicuit potential was observed, the voltage was measured across a series of known resistances and the maximum power output determined (Table 4.1.3).

Of the 57 mediators tested colorimetrically and in the biofuel cell 20 caused a change in the potential; of these thionin and TMPD, produced the largest output. However, thionin, which had been previously used in whole cell biofuel cells (Bennetto et al., 1980), readily precipitated out of solution at pH 10.5 the optimum pH of the enzyme (see section 3.2).

4.1.3 Insoluble mediators.

Insoluble mediators were tested by their incorporation into a carbon paste in the presence of methanol dehydrogenase (see section 2.35). The probe was placed in nitrogen sparged borate buffer (250 mM, pH 10.5) containing ammonium chloride (50 mM). The potential of the probe was poised at +200 mV (versus SCE). After allowing the background current to decay, aliquots of methanol were added and the steady state current measured. It was possible to demonstrate that insoluble mediators such as ferrocene, 1,1'-dimethylferrocene and N-methylphenazinium tetracyanoquinodimethanide (NMPTCNQ) were capable of mediating between methanol dehydrogenase (see chapter 5) and an electrode, whilst p-chloranil, polyvinyl ferrocene and polyviologen were not.

Table 4.1.3 The maximum power output using possible mediators in a methanol dehydrogenase based biofuel cell (see sections 2.28 and 4.1.2).

Mediator	Maximum Power (μW)
TMPD	30.55
Thionin	30.3
Methylene blue	5.3
Orange H-ER	2.5
Phenol red	1.0
Amino ferrocene	9.4×10^{-1}
Malachite green	6.6×10^{-1}
Dimethyl ferrocene	6.0×10^{-1}
Dichlorophenolindophenol	5.7×10^{-1}
Dicarboxyferrocene	4.3×10^{-1}
Red HE-3B	3.3×10^{-1}
Monocarboxy ferrocene	3.2×10^{-1}
Resofurin	2.1×10^{-1}
Bromophenol red	1.0×10^{-1}
Bromophenol blue	4.2×10^{-2}
Cresol red	4.2×10^{-2}
Brilliant blue C	6.3×10^{-2}
Brilliant blue G	6.0×10^{-2}
Methyl green	2.6×10^{-2}
Blue HE-RD	9.9×10^{-3}

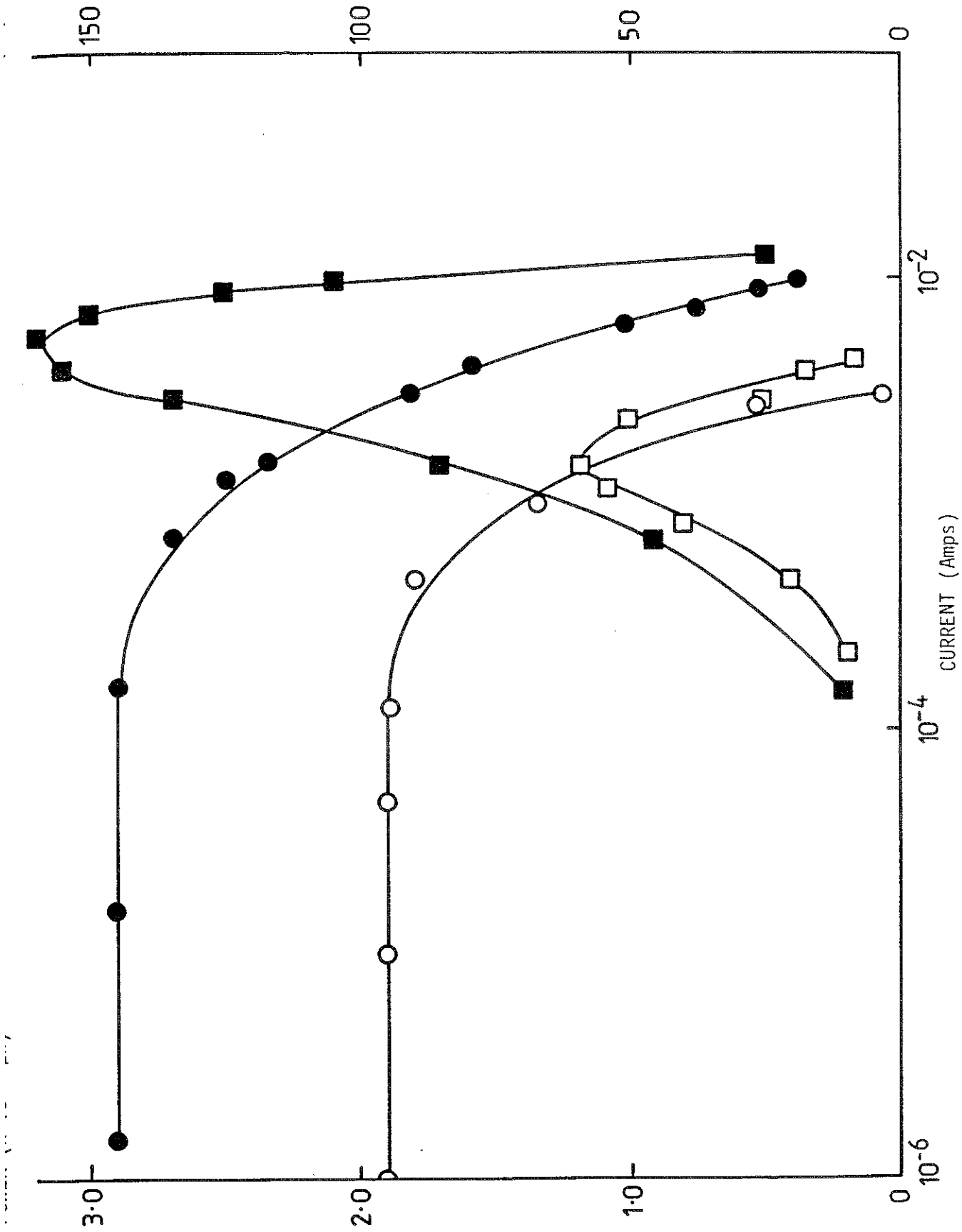
It was possible to coat a platinum electrode with 1,1'-dimethylferrocene and use this modified electrode in the biofuel cell in the presence of methanol dehydrogenase. The platinum gauze electrode was dipped in a solution of the mediator dissolved in toluene (20 mg ml^{-1}), removed and allowed to dry at $50^{o}C$ prior to use. The modified electrode was incorporated into the anodic compartment of a biofuel cell (see section 2.28) containing methanol (5.0 mM) and enzyme (0.4 mg) in the absence of TMPD. The platinum gauze cathode was sparged with air, whilst the anode was sparged with nitrogen. The current produced was measured using a high impedance chart recorder by determining the voltage across a known resistance. By measuring the current at various resistances it was possible to show that the biofuel cell had an internal resistance of 1.0×10^{4} ohms (see section 4.3) and was capable of producing 3.2 uW with an open circuit potential of 148 mV upon the addition of enzyme (Fig 4.1.4).

4.2 Membranes.

Membranes for use in biofuel cells should facilitate the rapid passage of protons whilst retaining mediator, substrate and oxygen (Turner et al., 1982). Early investigators used dialysis membranes (Davis and Yarbough, 1962; Van-Hees, 1965) now membranes exhibiting properties better suited to applications in biofuel cells are commercially available and have been used in such cells (Bennetto et al., 1982, 1983; Turner et al., 1982). Diffusion of components such as oxygen, mediator, enzyme or substrate cause a reduction in the coulometric efficiency of the cell. The membrane used in a sensor

Figure 4.1.4 Changes in voltage and power output with variation in current for a biofuel cell mediated by an insoluble mediator, 1,1'-dimethylferrocene. The biofuel cell was set up as described in section 2.28, but with the platinum gauze anode doped with 1,1'-dimethylferrocene.

	No methanol dehydrogenase	Methanol dehydrogenase
Voltage	○	●
Power	□	■


150
100
50
0
3·0
2·0
1·0
0
10^{-2}
10^{-4}
10^{-6}
CURRENT (Amps)

should facilitate the diffusion of substrate whilst retarding the free passage of interfering substances. Modification of the diffusion properties of membranes, such as allowing the preferential passage of oxygen has enabled in vivo blood glucose analysis to be carried out in fluctuating oxygen tensions (Shichiri et al., 1982).

4.2.1 Proton diffusion.

Membranes used in both biofuel cell and sensor configurations, should facilitate proton diffusion. In the methanol dehydrogenase biofuel cell the protons produced by the enzymic reaction diffuse across the ion exchange membrane to the cathode, where they are oxidised to complete the cell. A change in pH occuring because of poor proton diffusion would affect the enzymic activity in both biofuel cells and sensors (see sections 3.2 and 4.4).

Several membranes were tested for suitability in the biofuel cell. A pH gradient was created across the membrane between pH 10.0 and pH 8.0 using borate buffer (250 mM). The rate of proton diffusion was determined by measuring the pH change using a micro pH probe (Russel pH Ltd., Auchtermuchy, Fife, Scotland) (see section 2.18). Although some of the membranes tested did not allow any detectable diffusion to occur, others were freely permeable to proton diffusion. Membranes were grouped into 6 sections depending on the rate of proton diffusion (Fig 4.2.1).

It was also shown that the membrane used in biofuel cell studies (BDH cation exchange membrane) was sufficiently permeable to protons so as not to cause a deleterious change in pH. Fig 4.2.2 shows that

Figure 4.2.1 Proton diffusion through various membranes. The pH was measured using a micro pH probe (Russel pH Ltd., Auchtermuchy, Scotland), across an initial pH gradient of borate buffer (250 mM) pH 10.0 and pH 8.0.

I	PTFE
	POLYETHYLENE
	CELLULOSE
	TEFLON 'C' 3/16 ml
II	NAFION 125
	BDH ANION
	NAFION 117
III	BDH CATION
IV	DIALYSIS TUBING
	ICOM
V	OSMOSIS MEMBRANE
VI	POLYACRYLAMIDE

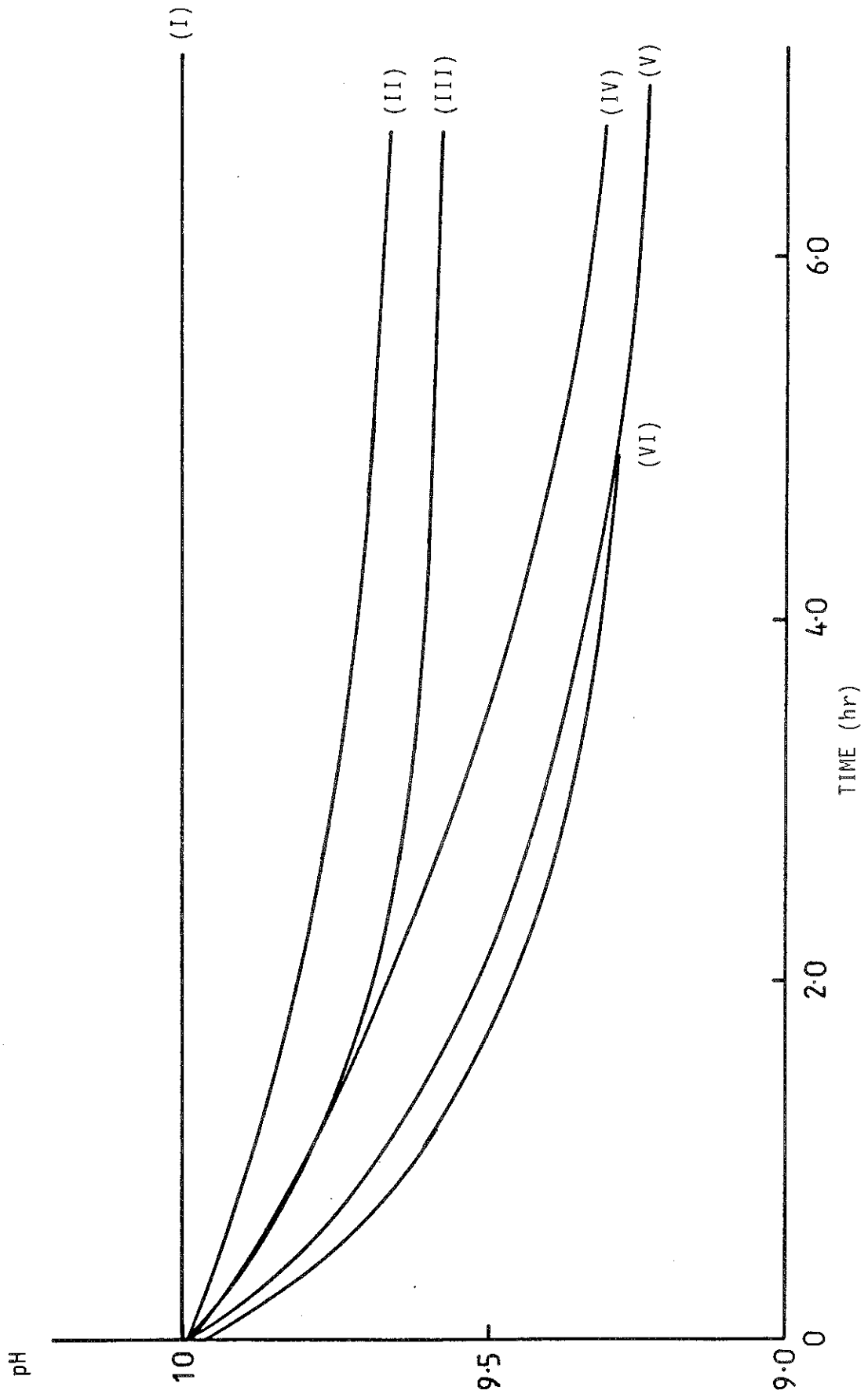
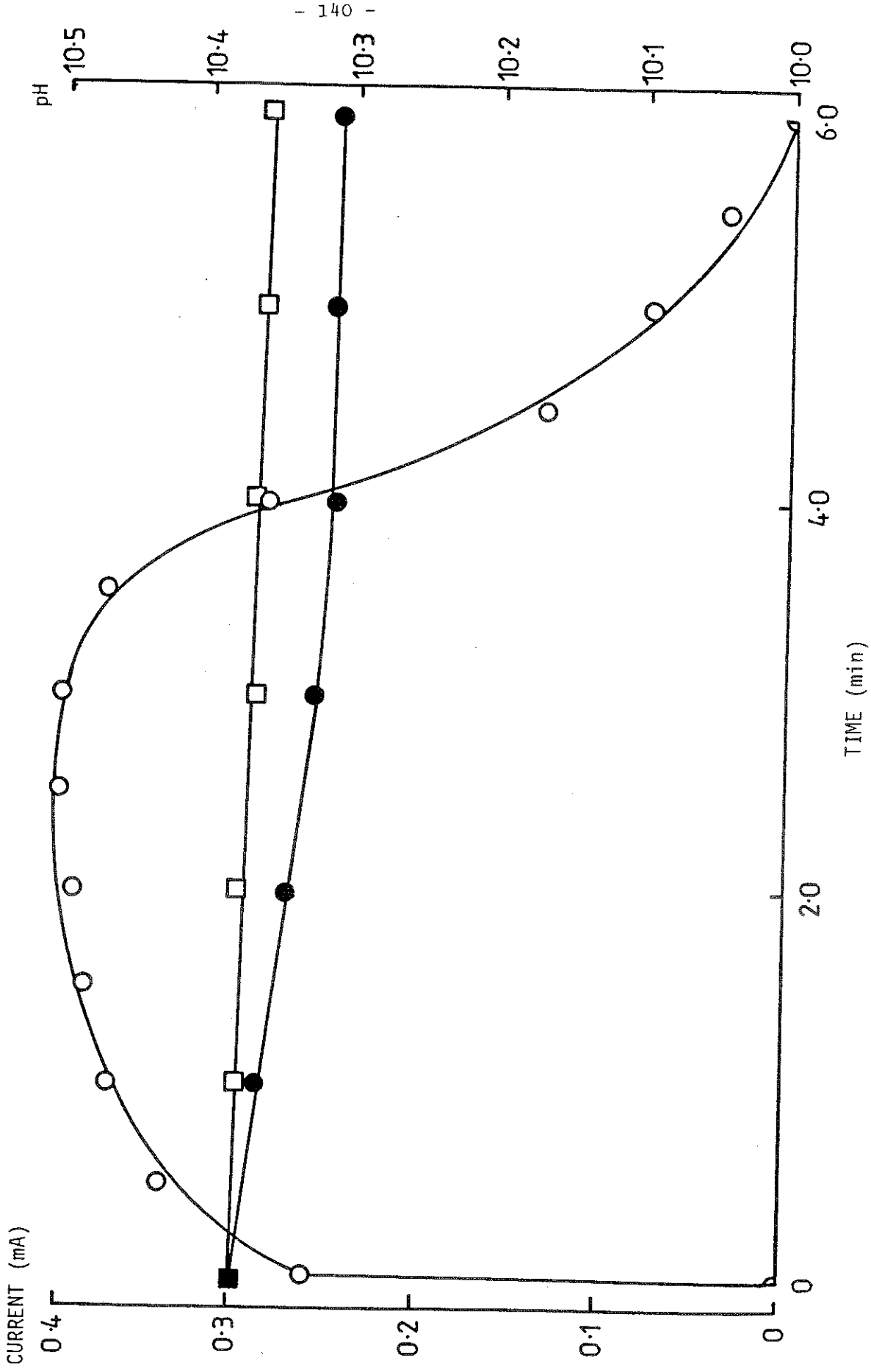

pH
10
9·5
9·0
0
2·0
4·0
6·0
TIME (hr)
(I)
(II)
(III)
(IV)
(V)
(VI)

Figure 4.2.2 The effect of current output (○) on the pH of the anodic (●) and cathodic (□) compartments (see sections 2.27 and 4.2.1).


pH
10·5
10·4
10·3
10·2
10·1
10·0
6·0
4·0
2·0
0
TIME (min)
CURRENT (mA)
0·4
0·3
0·2
0·1
0

the pH of the anodic compartment in the biofuel cell during 6.0 min of operation changed by only 0.02 pH units.

4.2.2 Oxygen diffusion.

The diffusion of oxygen across the membrane will reduce the coulometric efficiency of a fuel cell due to preferential oxidation of the mediator. The presence of oxygen in the anodic compartment of a biofuel cell (see section 4.4) or a homogeneous methanol sensor (see chapter 5) utilising an autooxidisable mediator resulted in no detectable current upon the addition of substrate. This was demonstrated by sparging each of the two configurations alternately, with oxygen and nitrogen and measuring the charge passed upon the addition of methanol (5.0 ul, 0.1 mM) to a biofuel cell (see section 2.28) containing methanol dehydrogenase (0.4 mg). No detectable response was recorded upon the addition of an aliquot of methanol although the coulometric efficiency was calculated to be 100% when sparged with nitrogen.

Oxygen diffusion through membranes was measured by both incorporating a micro oxygen probe in a modified biofuel cell and using a Clark oxygen electrode. The micro oxygen electrode was incorporated into one of the compartments of a gas tight biofuel cell. Both fuel cell compartments contained magnetic followers to allow stirring. The membrane was placed between the two compartments and sealed using silicon rubber (see section 2.19). After initial calibration of the oxygen electrode, the electrode containing compartment was sparged with nitrogen for a few minutes and sealed.

The adjoining compartment was sparged with air, at a rate of 50.0 ml min^{-1} and the oxygen tension in the sealed compartment measured. All the membranes tested including dialysis, BDH cation, BDH anion, Nuclepore, Nafion 117 and Nafion 125 membranes allowed the passage of oxygen (Table 4.2.1). The results are similar to those obtained using a Clark oxygen electrode. In the Clark oxygen electrode the membrane to be tested was placed over a electrode, replacing the teflon membrane and the compartment sparged alternately with air and nitrogen. The time taken to reach equilibrium was measured (Table 4.2.1).

4.2.3 Mediator diffusion.

The lag time and the rate of diffusion of PES and TMPD through various membranes was studied spectrophotometrically. Initially, an absorption spectrum was carried out to determine the wavelength of maximum absorption of the mediator. These were 388 nm for PES (Fig 4.1.1) and 563 nm for TMPD. The mediator (30 mM PES or 7.14 mM TMPD) was made up in borate buffer (250 mM, pH 9.0) and placed in the anodic compartment of a biofuel cell. The diffusion of the mediator was measured by assaying samples from the cathodic compartment at regular time intervals. The rate of diffusion and the lag time prior to diffusion commencing for both PES (Table 4.2.2) and TMPD (Table 4.2.3) was shown to be dependent on the membrane. The procedure was repeated with BDH cation exchange membrane for both mediators at pH values in the range, pH 7.5 - 11.0. Whilst it was not possible to monitor PES diffusion above pH 9.0, due to absorbance changes (see section 4.1.1),

Table 4.2.1 Rates of oxygen diffusion through various membranes. Two methods to determine the rate of oxygen diffusion were used; a Clark oxygen electrode (see sections 2.19 and 4.2.3) and a Transdyne microelectrode, incorporated into a gas tight biofuel cell (see sections 2.19 and 4.2.3).

Membrane	Equilibrium time (Clark oxygen electrode)	% O_2 Saturation min^{-1} using a Transdyne O_2 electrode
TEFLON	1.1	1.55
ICOM	1.2	0.83
NAFION	2.4	0.12
NAFION	2.4	-
BDH Cation Exchange	3.5	0.08
BDH Anion Exchange	3.8	0.08

Table 4.2.2 Variation in the lag time prior to diffusion and the rate of diffusion of PES through various membranes in borate buffer (100 mM, pH 9.0) (see sections 2.17 and 4.2.3).

Membrane	Lag Time (h)	Rate ($mMh^{-1}cm^{-2}$)
NAFION 117	>24.0	7×10^{-4}
NAFION 125	18.3	7×10^{-4}
BDH Cation	3.3	6.8×10^{-4}
BDH Anion	-	6.8×10^{-4}

Table 4.2.3 The lag time and initial diffusion rates of TMPD through various membranes in borate buffer (100 mM, pH 9.0) (see sections 2.17 and 4.2.3).

Membrane	Time prior to diffusion (h)	Diffusion rate (A_{563} $h^{-1}cm^{-2}$)
Cellophane	2.0	6.3×10^{-4}
Polyethylene	<0.1	2.6×10^{-4}
BDH cation exchange	2.0	7.9×10^{-3}
BDH anion exchange	9.0	5.7×10^{-3}
Nafion 125	<0.1	2.9×10^{-3}
Millipore filter (0.22 μm)	<0.1	9.9×10^{-2}
Osmosis membrane	<0.1	9.6×10^{-2}
Chicken's egg amniotic membrane	<0.1	5.0×10^{-1}
ICOM membrane	2.5	2.9×10^{-1}
Agar	<0.1	rapid
Polyacrylamide	<0.1	rapid
Gold coated PVC	<0.1	rapid
Dialysis (10,000 mw cut off)	<0.1	rapid

the rate of diffusion of both mediators and the lag time prior to diffusion were shown to be influenced by the pH of the solution (Figs 4.2.3 and 4.2.4).

Cation exchange membranes used in biofuel cell experiments were washed with borate buffer (250 mM, pH 9.0 or 10.5) between experiments in preference to water. Washing with water reduced the lag time before diffusion started, but did not affect the rate at which diffusion occured.

4.2.4 Enzyme diffusion

The diffusion of enzyme in both the biofuel cell and sensor configurations should be minimized. Loss of enzyme would ultimately cause a reduction in the current. Methanol dehydrogenase (1.0 mg ml^{-1}) was purified from M. methylotrophus (see chapter 3) and placed in the anodic compartment of a biofuel cell containing borate buffer (3.6 ml, 250 mM, pH 9.0). Enzyme diffusion across the membrane (see section 2.20), was determined colorimetrically using the Bradford reagent (see section 2.12.8b). To ensure that the enzyme remained dimeric (see section 1.2.1) the activity of the enzyme was measured both before and after the experiment. The membranes tested included ICOM, dialysis, BDH cation and anion exchange membrane. In all cases the specific activity remained at 1.1 u moles O_2 consumed min^{-1} mg $protein^{-1}$. Diffusion only occured through ICOM membrane the rate being 2.4×10^{-3} mg ml^{-1} h^{-1} cm^{-2}.

Figure 4.2.3 The effect of pH on the lag time prior to diffusion (O) and the rate of diffusion (●) of PES through cation exchange membrane (BDH Ltd., Poole, Dorset) (see sections 2.17 and 4.2.3).

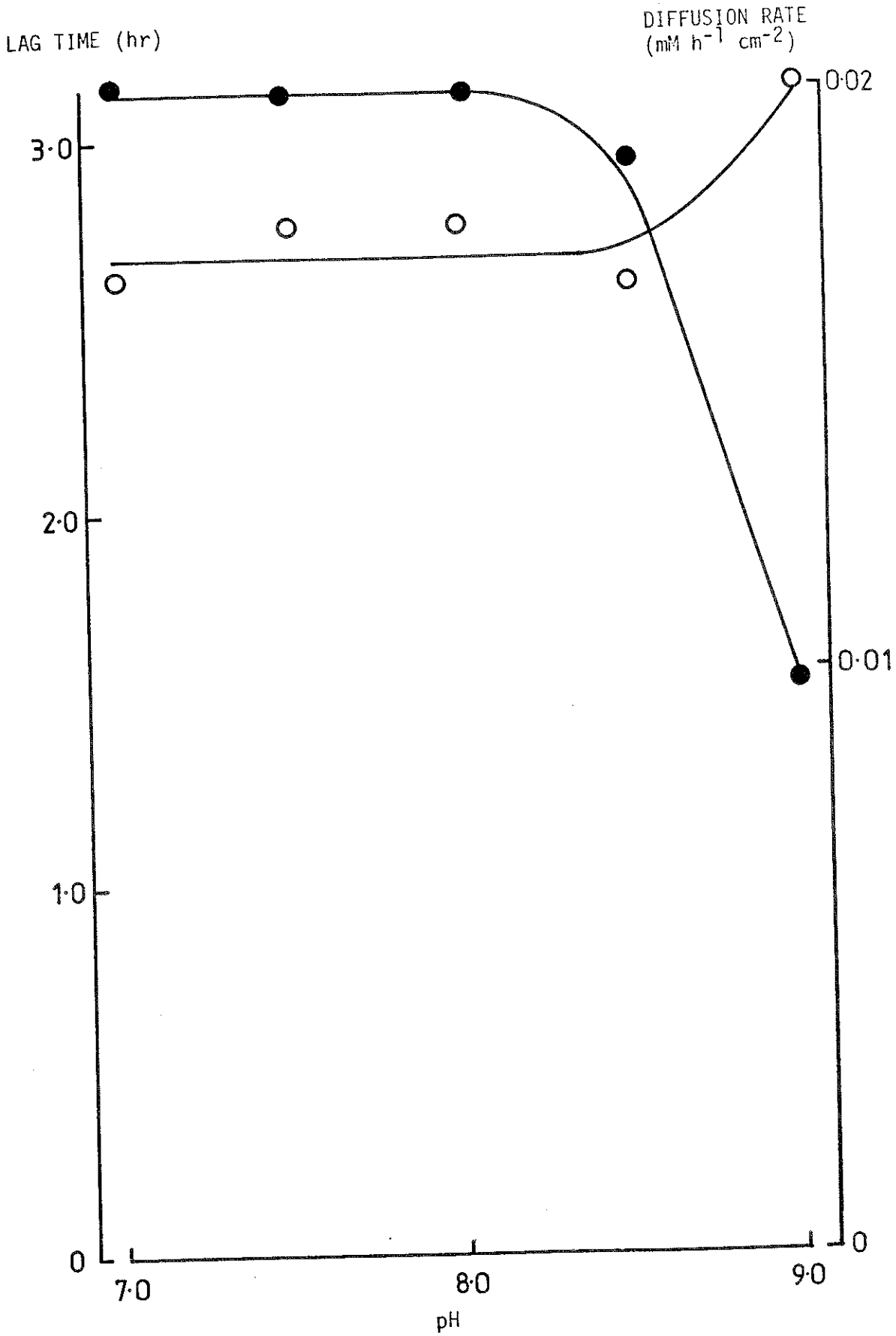
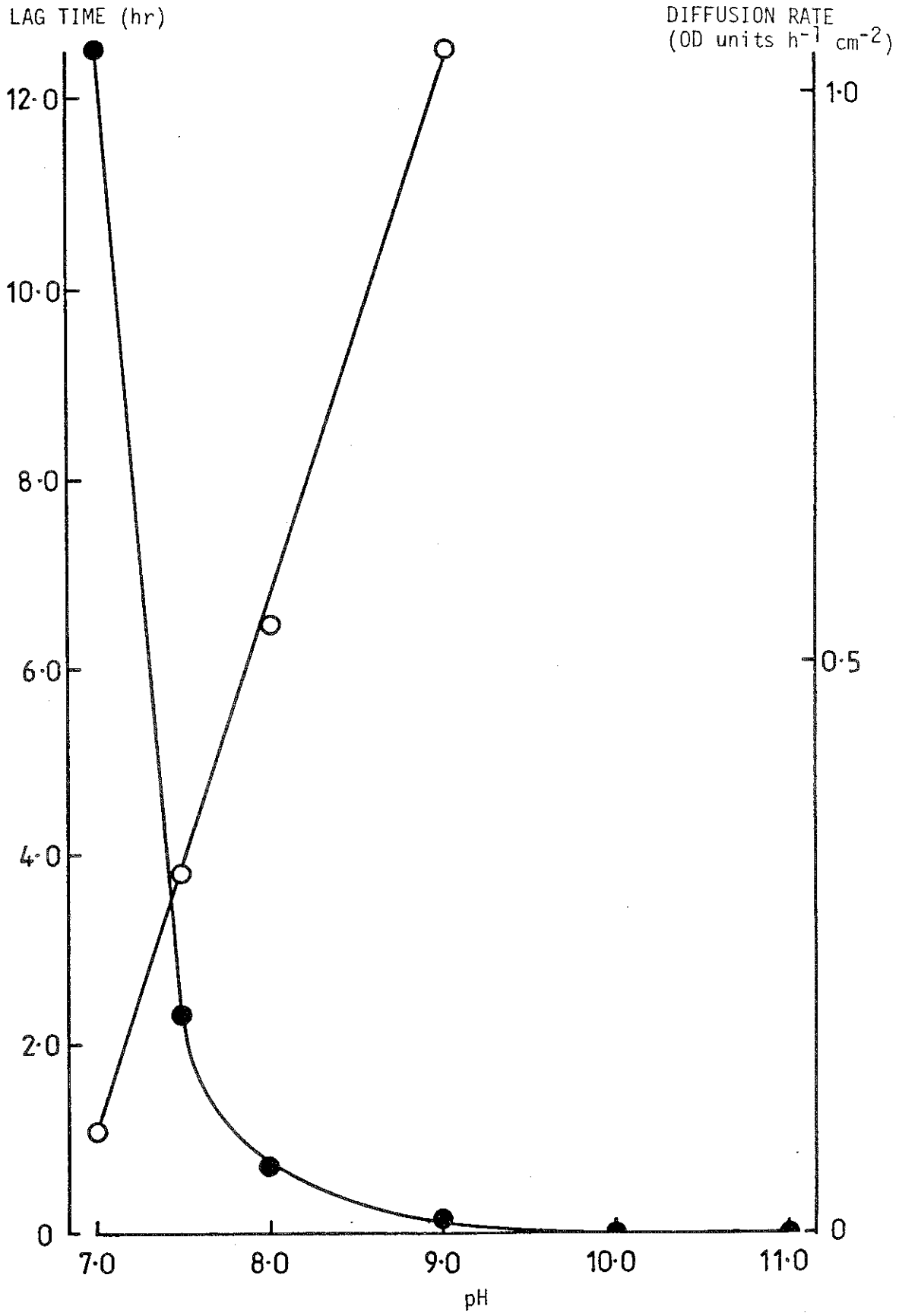

LAG TIME (hr)
DIFFUSION RATE
(mM h⁻¹ cm⁻²)
3·0
2·0
1·0
0
0·02
0·01
0
7·0
8·0
9·0
pH

Figure 4.2.4 The effect of pH on the lag time (O) and the rate of diffusion (●) of TMPD through cation exchange membrane (BDH Ltd., Poole, Dorset (see sections 2.17 and 4.2.3).


LAG TIME (hr)
DIFFUSION RATE
(OD units h-1 cm-2)
12·0
10·0
8·0
6·0
4·0
2·0
0
1·0
0·5
0
7·0
8·0
9·0
10·0
11·0
pH

4.2.5 Substrate diffusion.

Substrate diffusion, whilst required in a sensor configuration, needs to be minimised in the biofuel cell. Methanol diffusion across membranes was measured by gas chromatography (see section 2.12.6) and by using the enzyme-based methanol sensor (see section 2.31 and chapter 5).

A biofuel cell was set up containing borate buffer (250 mM, pH 9.0) separated by the membrane to be tested. Methanol (75 mM) was placed in the anodic compartment and diffusion measured by removing samples (10 ul) from the adjoining compartment at regular time intervals. Nuclepore and dialysis membrane used in the sensor configuration readily allowed the diffusion of methanol (Table 4.2.4) whilst BDH cation exchange membrane used in the biofuel cell allowed a slower rate of diffusion.

4.2.6 Osmosis.

The unequal distribution of solute molecules across a membrane would be expected to cause osmosis. Osmosis was measured by placing dialysis membrane in the configuration previously described (see section 2.21). Borate buffer (250 mM, pH 9.0) was added to one compartment and buffer containing methanol dehydrogenase (0.2 mg ml^{-1}) added to the other. The levels of the solutions in the two compartments were equalised using leveling syringes and recorded at regular time intervals. No change in volumes of the solutions in each of the two compartments was observed over a 6.0 hour period. The

Table 4.2.4 Rates of methanol diffusion through various membranes, determined both by gas-liquid chromatography and the homogeneous enzyme-based methanol sensor (see sections 2.12.6, 2.31, 4.2.5 and chapter 5).

Membrane	Detection Method (Diffusion rate μmoles min^{-1} cm^{-1})	
	Gas Chromatography	Methanol Sensor
Nucleopore	1.7×10^{-6}	1.6×10^{-6}
Dialysis	1.3×10^{-6}	1.5×10^{-6}
Nafion 117	1.8×10^{-6}	-
Nafion 125	1.4×10^{-6}	1.0×10^{-6}
BDH Anion	8.0×10^{-7}	-
BDH Cation	5.0×10^{-7}	7.0×10^{-7}

procedure was repeated with sucrose (1.0 M) for various membranes. The rate at which osmosis occurred (Table 4.2.5) was shown to be influenced by the membrane separating the compartments.

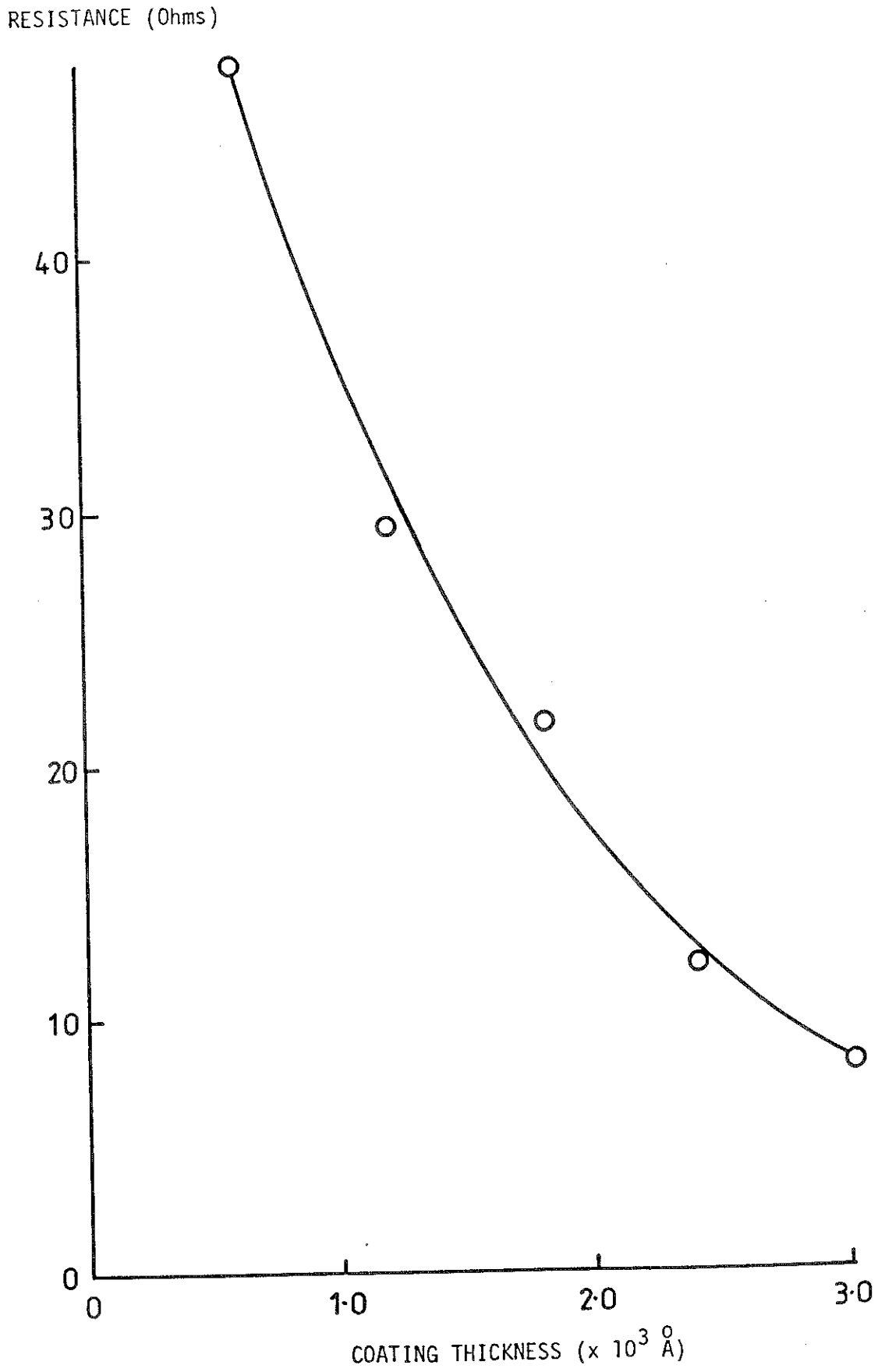
4.2.7 Modified membranes.

Coating an electrode material directly onto a membrane should enable the construction of electrodes which are both thin and proton permeable. A cation exchange membrane (BDH Ltd., Poole, Dorset), which was previously shown to be resistant to toluene was painted with gold resinate in toluene (12% W/V) (see section 2.39). The resistance of the membrane painted with gold resinate was, however, too high to enable it to function as an electrode (> 999 K ohms cm^{-1}). Heating the coated membrane to 600^{o}C, using an hot air gun evaporated off the organic residues (see section 2.39) and reduced the resistance, but at this temperature the membrane became brittle and prone to cracking. It was possible to coat a gold surface onto PTFE to give an electrode with a resistance of 0.2 ohms cm^{-1}, but this was not proton permeable (see section 4.2.1). Gold and gold palladium could be deposited onto temperature sensitive materials, including ion exchange membranes and nylon gauze, by sputter coating (see sections 2.40 and 2.41). The deposition of metal on each of the surfaces of the membrane enabled them to function as an electrode whilst being electrically isolated. Increasing the coating time increased the thickness of the deposited layer (see section 2.40) and reduced the sheet resistance (Fig 4.2.5). Membranes coated with gold palladium were as permeable to protons as untreated membranes; the initial pH in tests changed by 7 x 10^{-3} pH

Table 4.2.5 Rates of osmosis through various membranes. The membrane to be tested was placed between two half cells (see section 2.21), one containing sucrose (1.0 M) in borate buffer (250 mM, pH 9.0) (see sections 2.21 and 4.2.6).

Membrane	ml h^{-1}
Dialysis	1.3×10^{-1}
ICOM	5.2×10^{-2}
Nafion 117	7.5×10^{-3}
BDH Cation	7.5×10^{-3}
Nafion 125	4.0×10^{-3}

Figure 4.2.5 Change in resistance with variation in the thickness of gold palladium deposited (see sections 2.40 and 4.2.7).


RESISTANCE (Ohms)
40
30
20
10
0
0
1·0
2·0
3·0
COATING THICKNESS (x 10³ Å)

min^{-1} with a pH gradient between 10.5 and 8.0 (see sections 2.18 and 4.2.1). The rate of mediator diffusion (PES, 20mM) in borate buffer (250 mM, pH 9.0) measured at pH 9.0 was unaffected by the thickness of the deposited layer, although the lag time increased (Fig 4.2.6).

A gold palladium electrode (1.0 cm^2) was deposited onto one surface of a cation exchange membrane by masking an area of cation exchange membrane (BDH Ltd., Poole, Dorset) with a card. The electrode was connected to the external circuit via an gold wire (0.25 mm) using silver loaded epoxy resin and insulated from the solution with a non-conductive paint. The modified membrane was soaked in borate buffer (250 mM, pH 10.5) for 24 hours and mounted in a biofuel cell (see section 2.28) with a platinum gauze cathode. Power curves were obtained for the biofuel cell by measuring the voltage across a variable resistance, prior to and after the addition of enzyme (0.1 mg ml^{-1}) in the presence of TMPD (18 mM). The biofuel cell developed a current, albeit at low density, in the presence of enzyme (Fig 4.2.7).

Carbon was deposited on the gold palladium (see section 2.42) by the same process. Glucose oxidase was immobilised onto the carbon in the presence of 1,1'-dimethylferrocene (see section 2.37). Power curves were obtained in the presence of phosphate buffer (100 mM, pH 7.4) containing sodium chloride (150 mM) prior to and after the addition of glucose (32 mM), but it was not possible to measure any glucose dependent current. The electrode was, however, capable of responding to glucose, when the potential of the membrane electrode was poised at +150 mV (versus SCE). The current increased from 7.5

Figure 4.2.6 The effect of coating gold palladium on cation exchange membrane on the lag time (O) and the rate of PES diffusion (●) (see sections 2.17 and 4.2.7).

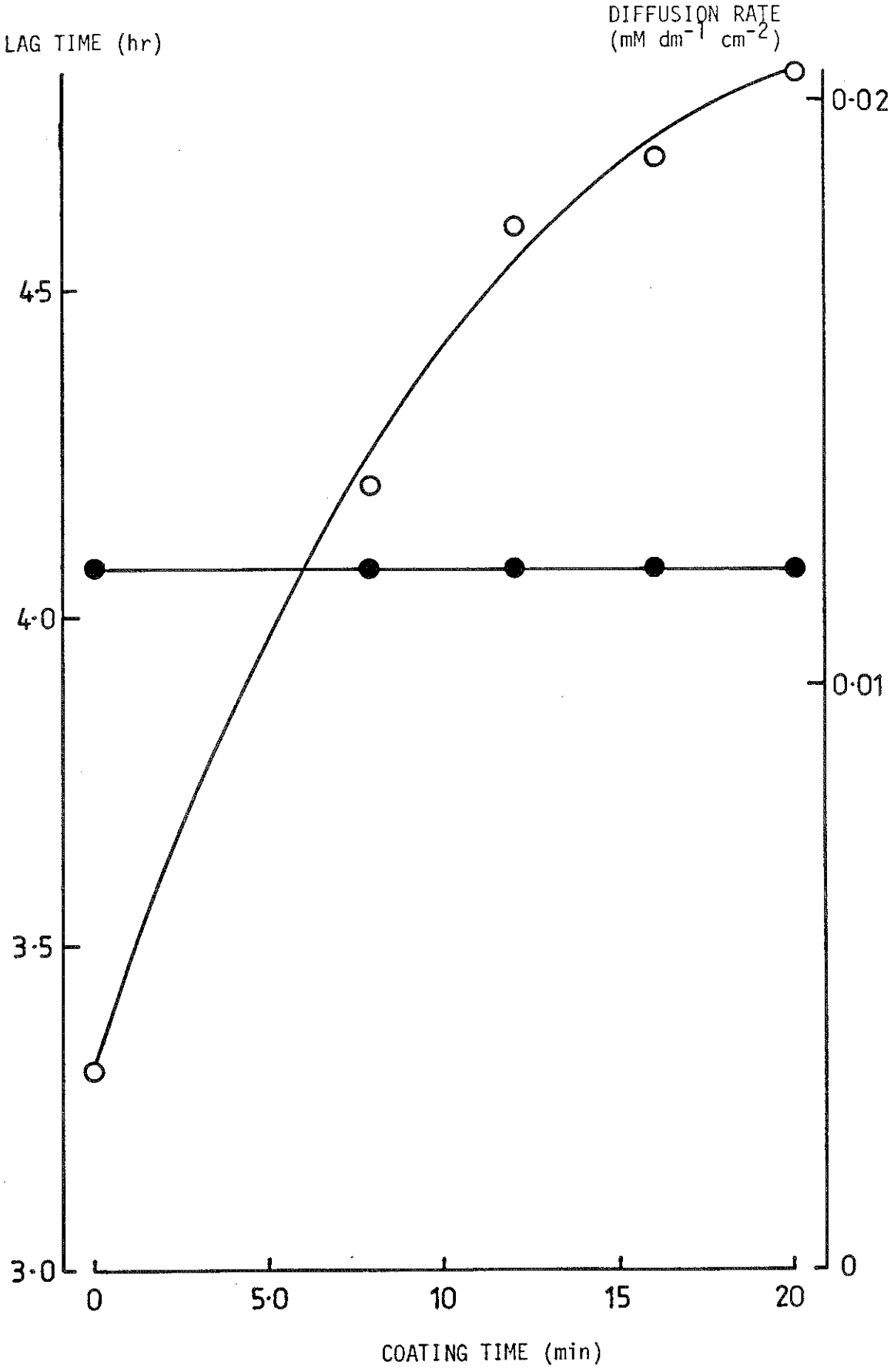
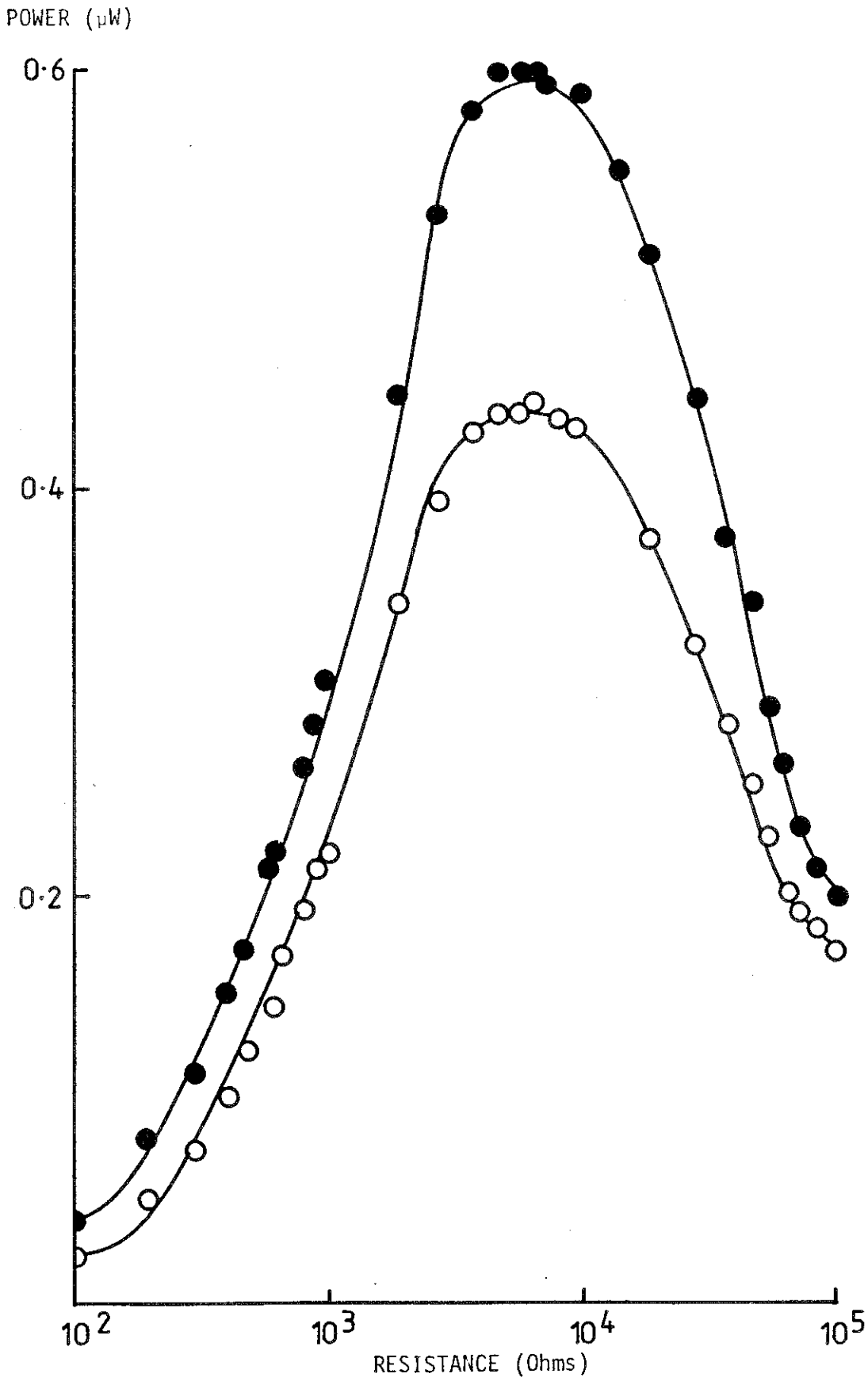

LAG TIME (hr)
DIFFUSION RATE
(mM dm^{-1} cm^{-2})
4·5
4·0
3·5
3·0
0·02
0·01
0
0
5·0
10
15
20
COATING TIME (min)

Figure 4.2.7 The variation in power output for a gold palladium modified membrane electrode, in the absence (O) and presence of methanol dehydrogenase (●) (see section 4.2.7).


POWER (μW)
0·6
0·4
0·2
10^2
10^3
10^4
10^5
RESISTANCE (Ohms)

uAmps to 13.0 uAmps on addition of glucose (32 mM).

4.3 Electrodes.

Electrodes for use in biofuel cells should be inert, non-toxic to the biological catalyst, economic, readily available and facilitate the rapid transfer of electrons to the mediator. Various materials, ranging from carbon to the noble metals were investigated to determine their feasibility as electrodes in the biofuel cell.

4.3.1 Potential electrodes.

Carbon is commercially available in several forms including powders, cloths and glassy carbons. It is cheaper than the noble metals and frequently used in the immobilisation of enzymes (Cho and Bailey, 1979; Osborn et al., 1982). Carbon cloth was not suitable since its fibrous nature caused it to disintegrate during use. Reticulated carbon used in whole cell and enzyme-based biofuel cell studies (Bennetto et al., 1980; Turner et al., 1982) whilst having the advantage of being inert and possessing a high surface area, was considered unsuitable for use in the laboratory biofuel cell. Reticulated carbon is brittle and difficult to maintain electrical contact to it. The electrochemical coating of reticulated carbon with platinum (see section 2.32) did not result in an even deposition or allow any enhanced currents above those obtained with an untreated carbon

of the same dimensions (see section 4.4.5).

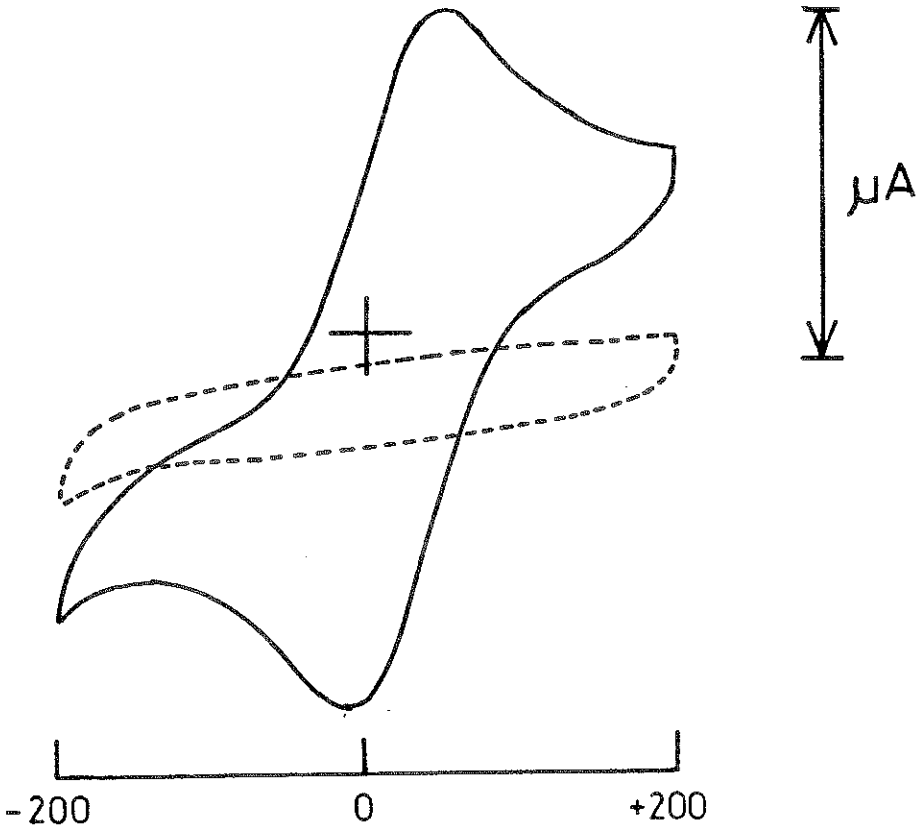
The sheet resistance of electrode materials available was determined by measuring the average resistance (10 measurements) between two points 1.0 cm apart on the electrode surface. The points consisted of two pointed steel pins, fixed at a distance of 1.0 cm, protruding 0.5 mm from the surface of a perspex holder. The device enabled materials to be divided into two types: the first had high conductivity included the noble metals, nickel and pyrolytic carbon. The second group, with resistances greater than 1.0 K ohms comprised of charcoal cloth, indium tin oxide, ruthium oxide and conducting foam (Table 4.3.1).

The reversible transfer of electrons between the electrode and the mediator was investigated electrochemically. D.C. cyclic voltammetry of the electrodes (1.0 cm^2) was carried out in borate buffer (250 mM, pH 10.5) containing ammonium chloride (50 mM) and TMPD (0.5 mM). The electrode was placed in solution and the potential cycled between -200 mV and +200 mV (versus SCE) at a rate of 10 mV sec^{-1} using a sweep generator (Oxford Electrodes Ltd., Garsington, Oxford). The peak catalytic current of TMPD at +50 mV was measured using a X-Y plotter (Bryans Ltd., Mitcham, Surrey) and used as an indication of the ability of the mediator to exchange electrons with the electrode (Fig 4.3.1). Of the materials available, nickel, platinum, gold, silver and glassy carbon enabled oxidation and reduction of the mediator, whilst aluminium foil, ruthenium oxide, charcoal cloth, indium tin oxide, gold coated PVC and conducting foam did not show any detectable oxidation or

Table 4.3.1 Properties of possible electrode materials and their ability to couple to a mediator (see section 4.3.1).

Electrode Material	Resistance (ohms cm^{-1})	Observable Changes	Anodic Peak (μA)
Nickel	0.1	Support Rusted	20.0
Gold	0.1	-	17.5
Glassy carbon	4.0	-	17.5
Platinum	0.1	-	17.5
Silver	0.1	White Surface	17.5
Aluminium foil	0.1	Grey Surface	0
Charcoal cloth	0.99K	-	0
Conducting foam	>99.0K	-	0
Gold coated PVC	10.0	-	0
Indium tin oxide	1.1K	-	0
Platinum coated PVC	1.4	-	0
Reticulated carbon	2.0	-	-

Figure 4.3.1 D.C. cyclic voltammetry of TMPD (1.0 mM), in borate buffer (250 mM, pH 9.0) containing ammonium chloride (50 mM) (see section 4.3.1). In the presence (——) and absence of mediator (----)

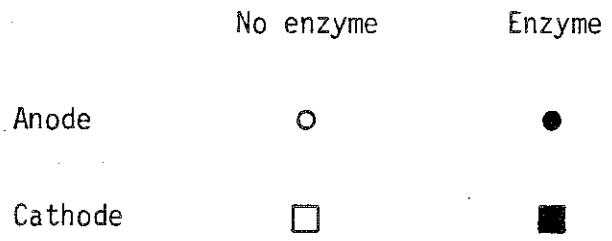

μA
-200
0
+200
Potential (mV versus SCE)

reduction of the mediator (Table 4.3.1).

The variation in the potentials of the anode and cathode were measured at different currents in a biofuel cell (see section 2.28). The potential of the two electrodes was monitored using a standard calomel reference electrode and high impedance multimeter. The cathode was made of platinum gauze and the anode of platinum foil (1.0 cm^2). The potential of both electrodes was determined for a range of currents in the presence and absence of enzyme (Fig 4.3.2). The biofuel cell was washed out and the procedure repeated for a range of electrodes, including silver, gold, nickel, glassy carbon and carbon cloth. In all cases the potential of the cathode was unaffected by the addition of enzyme, whilst the potential of the anode increased. The maximum currents produced by a variety of electrodes (1.0 cm^2) was determined (Table 4.3.2).

The conductivity of various borate buffers was measured using either a commercially available conductivity meter (Gallenkamp and Co. Ltd., London) or a meter based on a 555 timer (Mann and Jamieson, 1981) with the electrodes maintained at a preset distance (1.0 cm, unless otherwise stated). The results show that the conductivity of these buffers was dependent on molarity (Fig 4.3.3), pH (Fig 4.3.4), concentration of ammonium chloride (Fig 4.3.5) and the distance between the electrodes (Fig 4.3.6). The redox potential of a platinum electrode in borate buffer was measured with respect to a standard calomel reference electrode. The potential was shown to be dependent on the pH of

Figure 4.3.2 Variation in the potential of a platinum anode and cathode with change in current output, in the presence and absence of enzyme (see section 4.3.1).



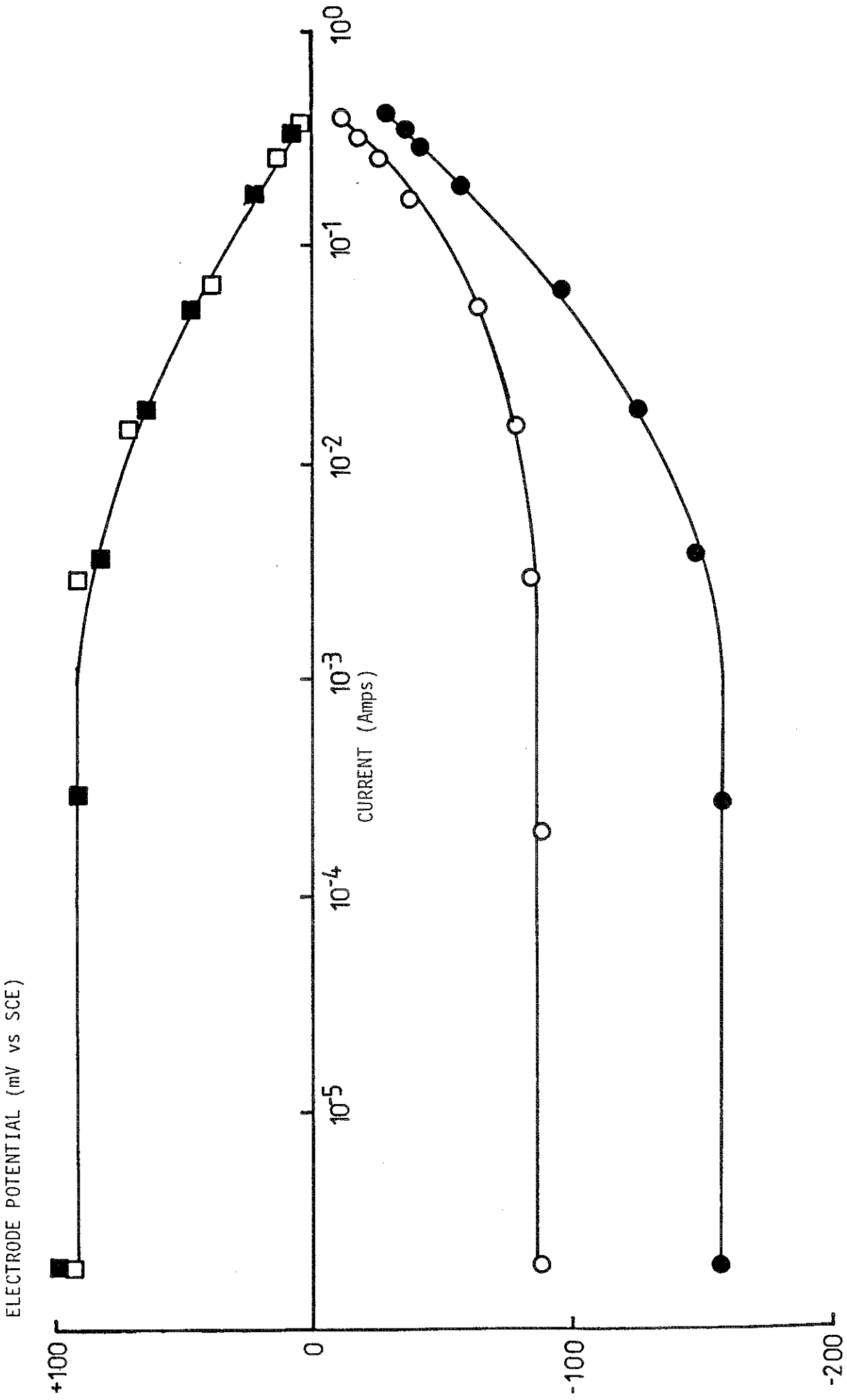

ELECTRODE POTENTIAL (mV vs SCE)
+100
0
-100
-200
CURRENT (Amps)
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}

Table 4.3.2 The variation in potential and current output of various anodes in biofuel cells with platinum cathodes measured across 10 and 9.9×10^{-7} ohms (see section 4.3.3).

	Resistance (ohms)					
	10 ohms			9.9×10^{7} ohms		
Electrode Material	Current (mA)	Anodic Potential (mV/SCE)	Cathodic Potential (mV/SCE)	Current (mA)	Anodic Potential (mV/SCE)	Cathodic Potential (mV/SCE)
Carbon	0.68	-72	-48	1.9×10^{-6}	-140	+53
Copper	0.68	-74	-48	4.2×10^{-6}	-364	+52
Gold	0.32	-42	-30	2.4×10^{-6}	-191	+49
Platinum	0.1	-12	-14	1.8×10^{-6}	-136	+49
Silver	0.2	-30	-19	3.4×10^{-6}	-286	+47

Figure 4.3.3 The effect of molarity on the conductivity of borate buffer (pH 10.5, containing ammonium chloride, 50 mM) (see section 4.3.1).

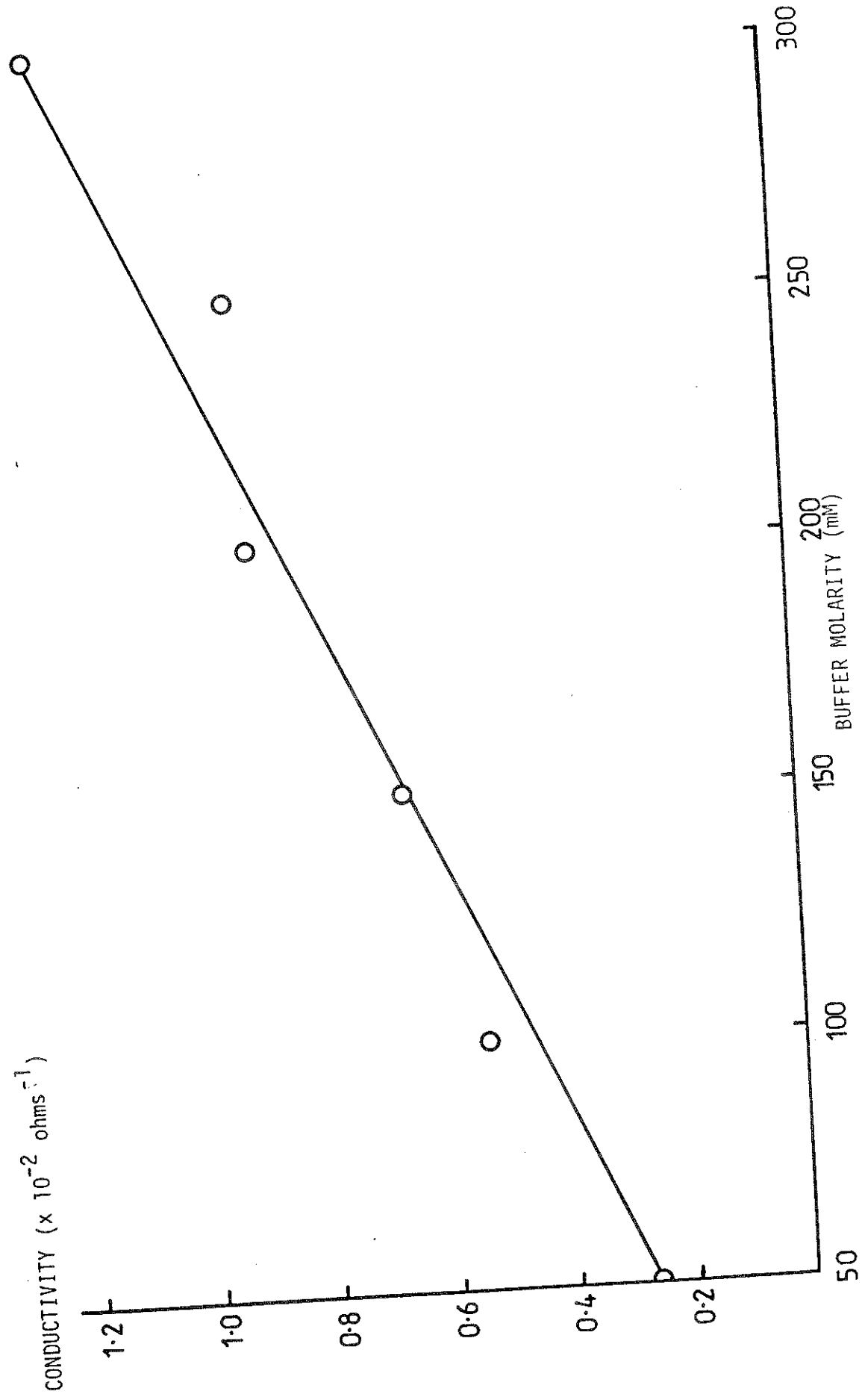
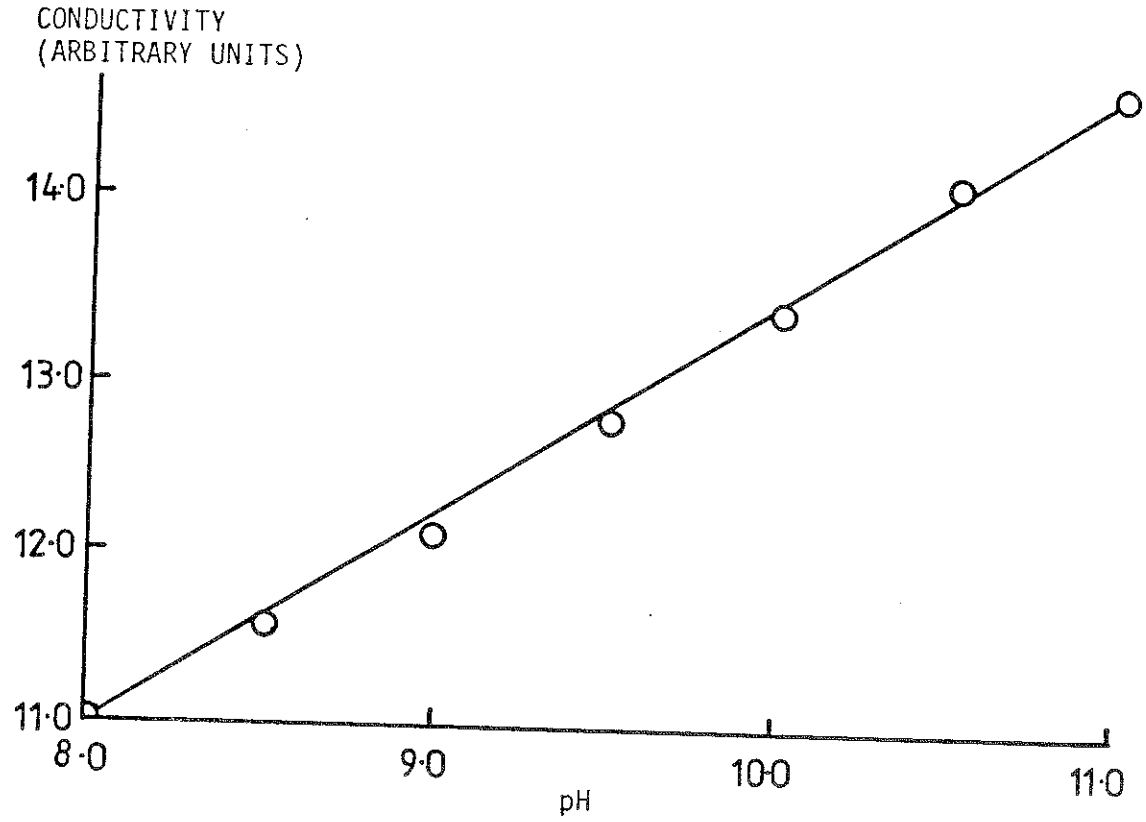

CONDUCTIVITY (x 10^{-2} ohms^{-1})
1·2
1·0
0·8
0·6
0·4
0·2
50
100
150
200
250
300
BUFFER MOLARITY (mM)

Figure 4.3.4 Effect of pH on the conductivity of borate buffer (250 mM) containing ammonium chloride (50 mM) (see section 4.3.1).

Figure 4.3.5 Effect of ammonium chloride on the conductivity of a borate buffer (250 mM, pH 10.5) (see section 4.3.1).


CONDUCTIVITY
(ARBITRARY UNITS)
14·0
13·0
12·0
11·0
8·0
9·0
10·0
11·0
pH

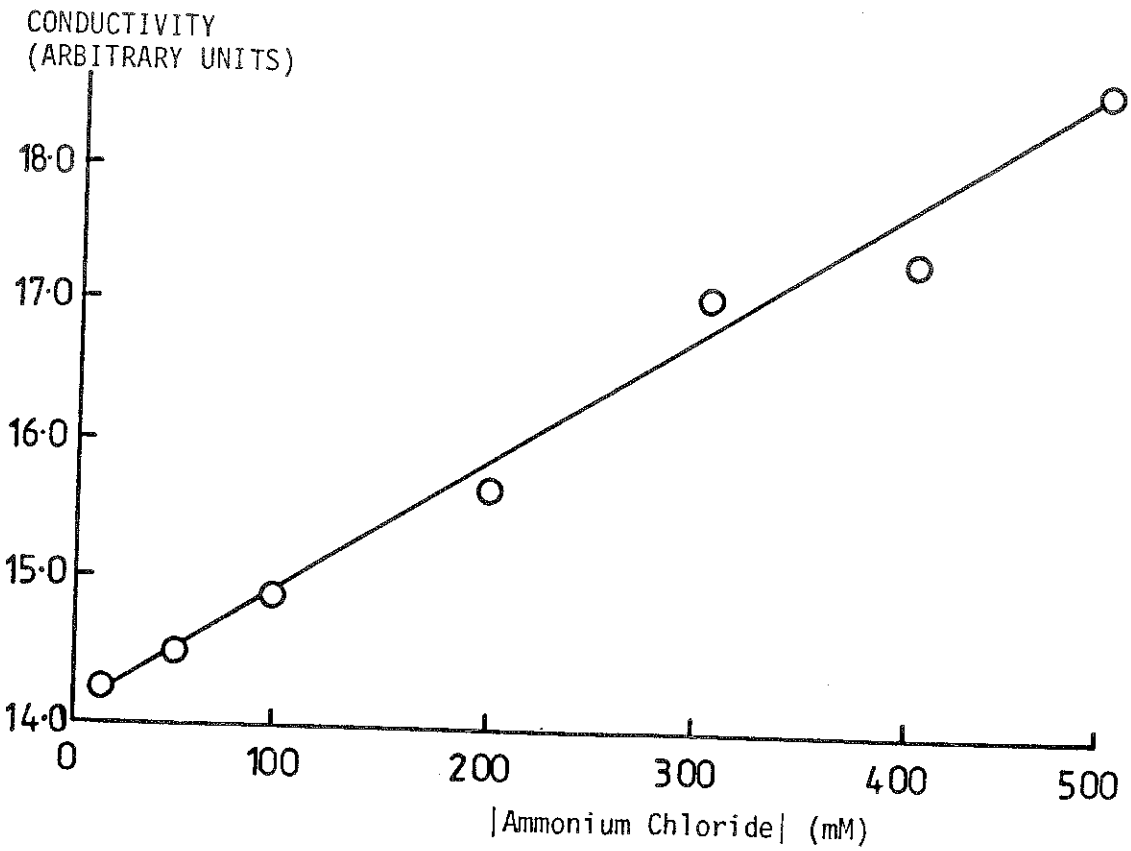
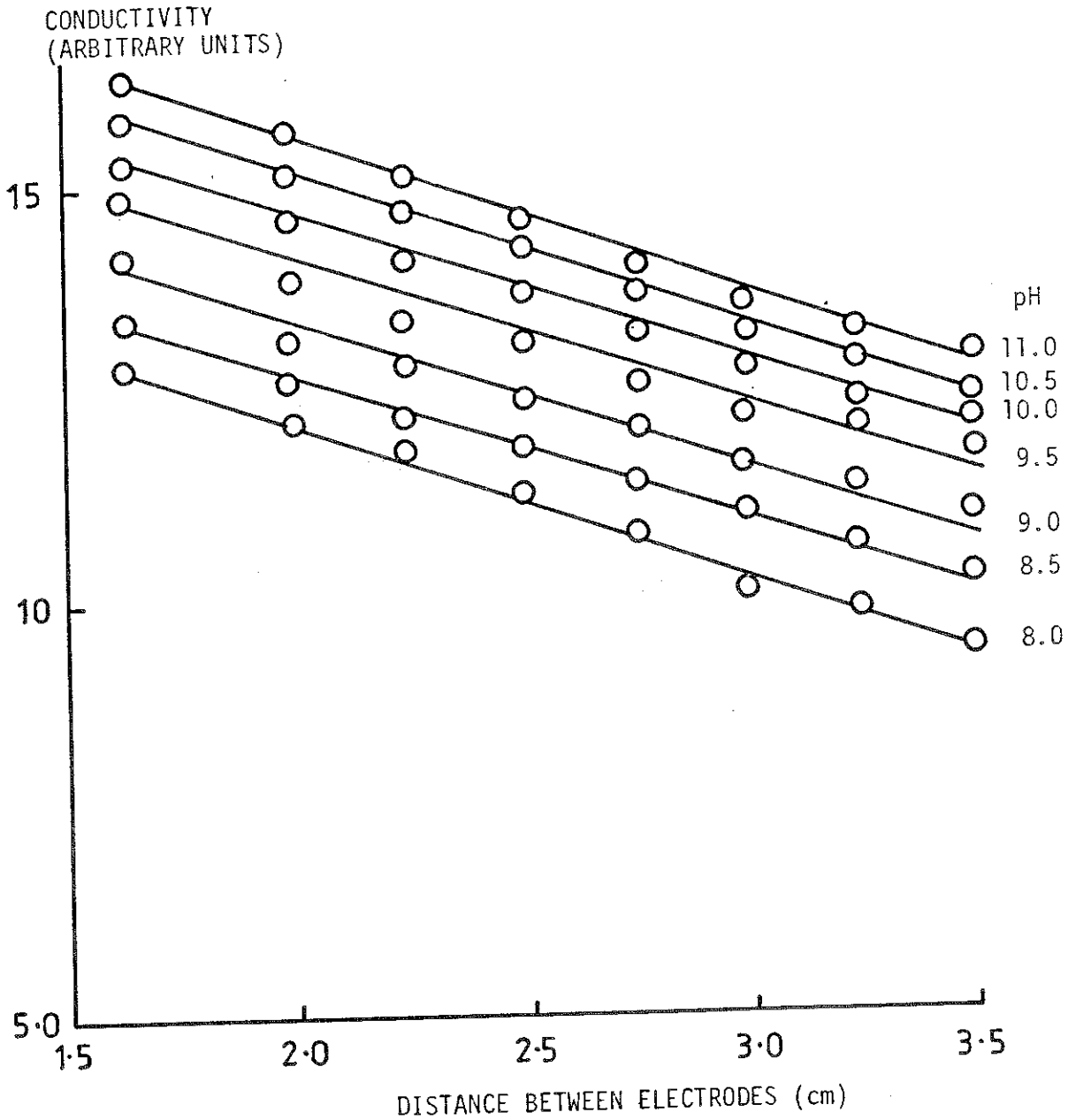

CONDUCTIVITY
(ARBITRARY UNITS)
18·0
17·0
16·0
15·0
14·0
0
100
200
300
400
500
|Ammonium Chloride| (mM)

Figure 4.3.6 The effect of variation in the distance between two electrodes and pH on the conductivity of borate buffer (100 mM containing 50 mM ammonium chloride) (see section 4.3.1).


CONDUCTIVITY
(ARBITRARY UNITS)
15
10
5·0
1·5
2·0
2·5
3·0
3·5
DISTANCE BETWEEN ELECTRODES (cm)
pH
11.0
10.5
10.0
9.5
9.0
8.5
8.0

the buffer, decreasing at a rate of -51.14 mV per pH unit when measured between pH 8.0 and 11.0. Increasing the molarity caused an increase in the potential, with a slope of 1.0×10^{-2} mV mM^{-1} when measured in borate buffer (pH 10.5) between 50 mM and 300 mM.

The addition of TMPD (7.5 mM) to borate buffer (250 mM, pH 10.0) containing ammonium chloride (50 mM) caused the conductivity to change from 1.3×10^{-2} ohm^{-1} to $1.26 \times 10^{-2} ohm^{-1}$ and the pH to 9.89. The addition of methanol (2.5 mM) did not cause a change in either the conductivity or the pH. The addition of enzyme (0.04 mg ml^{-1}) in phosphate buffer (20 mM, pH 7.0) resulted in the conductivity changing to 1.24×10^{-2} ohm^{-1} and the pH decreasing to 9.81.

4.3.2 Electrode stability and toxicity.

The stability of the materials available and their possible toxicity to methanol dehydrogenase was investigated. Electrodes were exposed to borate buffer (250 mM, pH 9.0) containing ammonium chloride (50 mM) to ensure that they were stable in the alkaline conditions present in the biofuel cell and that chemical reactions did not occur. The electrodes (5 at 25 mm^2) were examined visually, weighed and placed in borate buffer (10 ml, 250 mM, pH 10.5). They were removed at regular time intervals over several months, assessed for tarnishing then dried, weighed and replaced in the buffer. Carbon and the noble metals appeared to be stable under these conditions with no change in appearence

or weight. However, aluminium foil became tarnished while high porosity nickel, although appearing stable, was supported on a metal gauze base which reacted with the solution. Copper electrodes reacted with the buffer resulting in a blue solution. Gold coated copper gauze electrodes (see section 2.33) were not suitable due to an uneven surface coating exposing small areas of copper which reacted with the buffer (Table 4.3.1).

In the alkaline conditions the ions of metals such as copper, cobalt, manganese, nickel and iron may be toxic to the enzyme and cause inactivation. The effect of the metal ions on the specific activity of methanol dehydrogenase was studied polarographically (see section 2.11.3) using soluble salts of the metals. The enzyme was incubated in the presence of the appropriate metal ions in borate buffer (250 mM, pH 9.0), PES (3.0 mM) and methanol (7.5 mM) for 2.0 minutes at various ionic concentrations (0 - 30 mM) and the reaction started by the addition of ammonium chloride (22.5 mM). All the salts tested caused inactivation of the enzyme (Table 4.3.3) at the relatively high concentrations used (see also chapter 5).

4.4 TMPD Mediated biofuel cell studies.

4.4.1 Charateristics of the biofuel cell.

Platinum gauze was used as the electrode material for both the anode and cathode of the biofuel cell. No current was produced by the biofuel cell in the absence of enzyme, substrate,

Table 4.3.3 Toxicity of metal ions in solution to methanol dehydrogenase purified from *Pseudomonas extorquens*. The ID_{50} is the concentration of the ion (mM) which caused 50% inactivation of the enzyme assayed polarographically (see section 2.11.3 and 4.3.2).

Metal Ion	Salt	ID_{50} (mM)
Nickel	Chloride	6.5
	Sulphate	7.0
Iron	Chloride	5.5
	Sulphate	7.0
Copper	Chloride	6.5
	Sulphate	6.5
Cobalt	Chloride	5.5
	Sulphate	2.3
Manganese	Chloride	2.4
Lead	Acetate	2.3

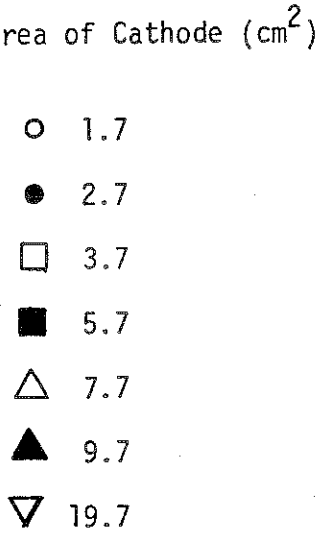
mediator or activator. It was demonstrated that the reaction at the cathode was not limiting the current using a biofuel cell (see section 2.28) containing TMPD (19.0 mM). The cathode and anode (10 cm^2) were sparged with air and nitrogen, respectively. A platinum cathode of large surface area in relation to the anode was used. The enzyme was added in aliquots and the current recorded. The biofuel cell was washed and the experiment repeated for a variety of cathode areas. A ratio of cathode to anode area of 1:1 or greater removed any limitations due to the cathodic side (Figs 4.4.1 and 4.4.2).

The effect of sparging the cathode with oxygen and air (40 mls min^{-1}) was investigated with respect to the size of cathode. Sparging the cathode with oxygen resulted in outputs greater than those produced with air, irrespective of the ratio of anode to cathode area (Fig 4.4.3). This difference decreased with increased external loading (Table 4.4.1)

The effect of variation in the rate of sparging the cathodic compartment with air and oxygen at different external loads was studied. The biofuel cell (see section 2.28) containing methanol dehydrogenase (0.4 mg) was washed out and the experiment repeated at various resistances. At high resistances the current produced was less dependent upon the rate of air sparged into the cathodic compartment (Fig 4.4.4).

The optimum pH for the current production was determined. The biofuel cell was set up (see section 2.28) containing TMPD (4.5 mM) and enzyme (0.04 mg) at various pH values between pH 8.0

Figure 4.4.1 Effect of variation in the size of the cathode on the current output of the biofuel cell (see sections 2.28 and 4.4.1).



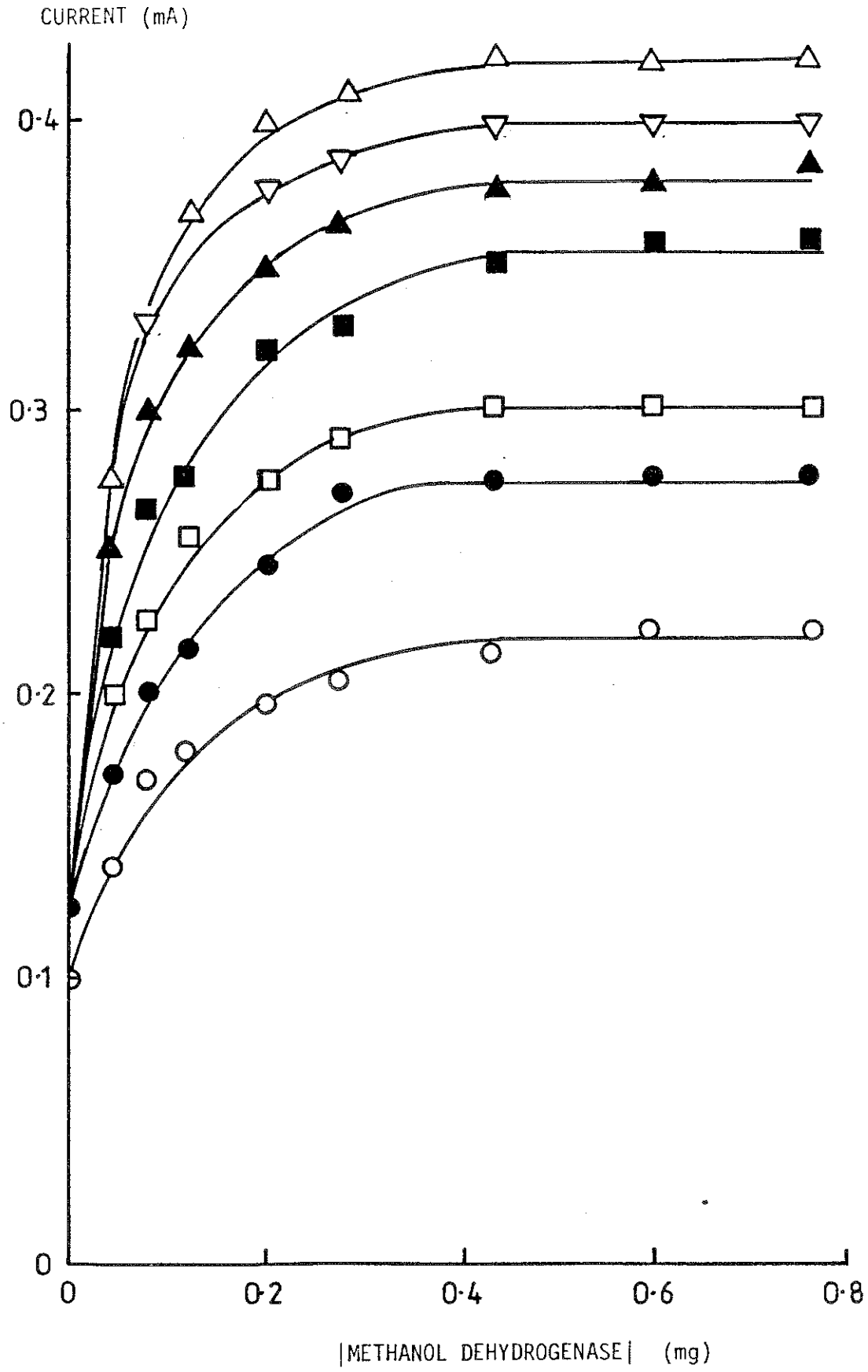

CURRENT (mA)
0·4
0·3
0·2
0·1
0
0
0·2
0·4
0·6
0·8
|METHANOL DEHYDROGENASE| (mg)

Figure 4.4.2 The effect of variation in the area of the cathode on the maximum current produced by a biofuel cell (see section 4.4.1).

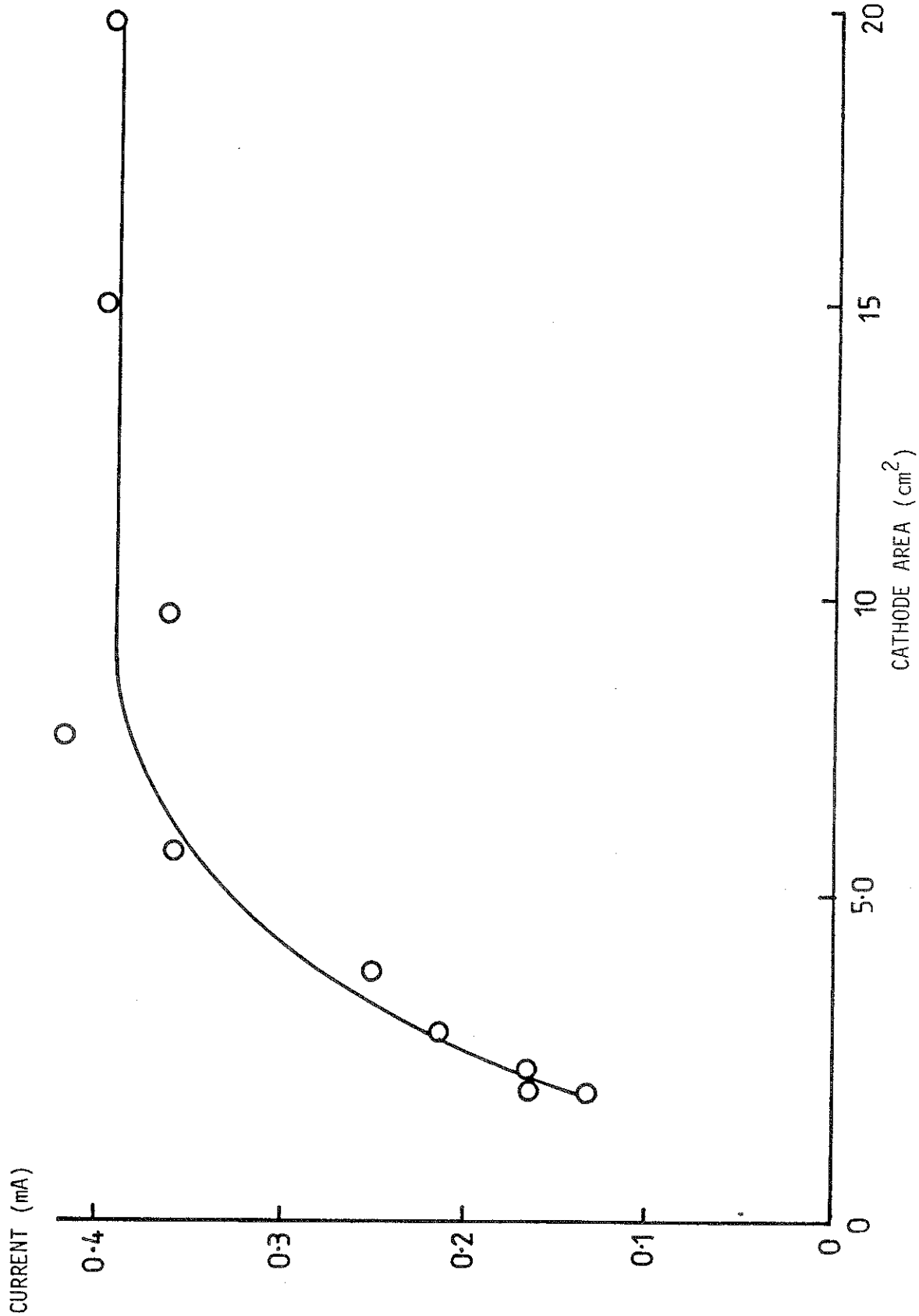

CURRENT (mA)
0·4
0·3
0·2
0·1
0
5·0
10
15
20
CATHODE AREA (cm²)

Figure 4.4.3 The effect of sparging the cathodic compartment with air (O) and oxygen (●) at various ratios of anode to cathode (see section 4.4.1). The area of the cathode was 10 cm^2.

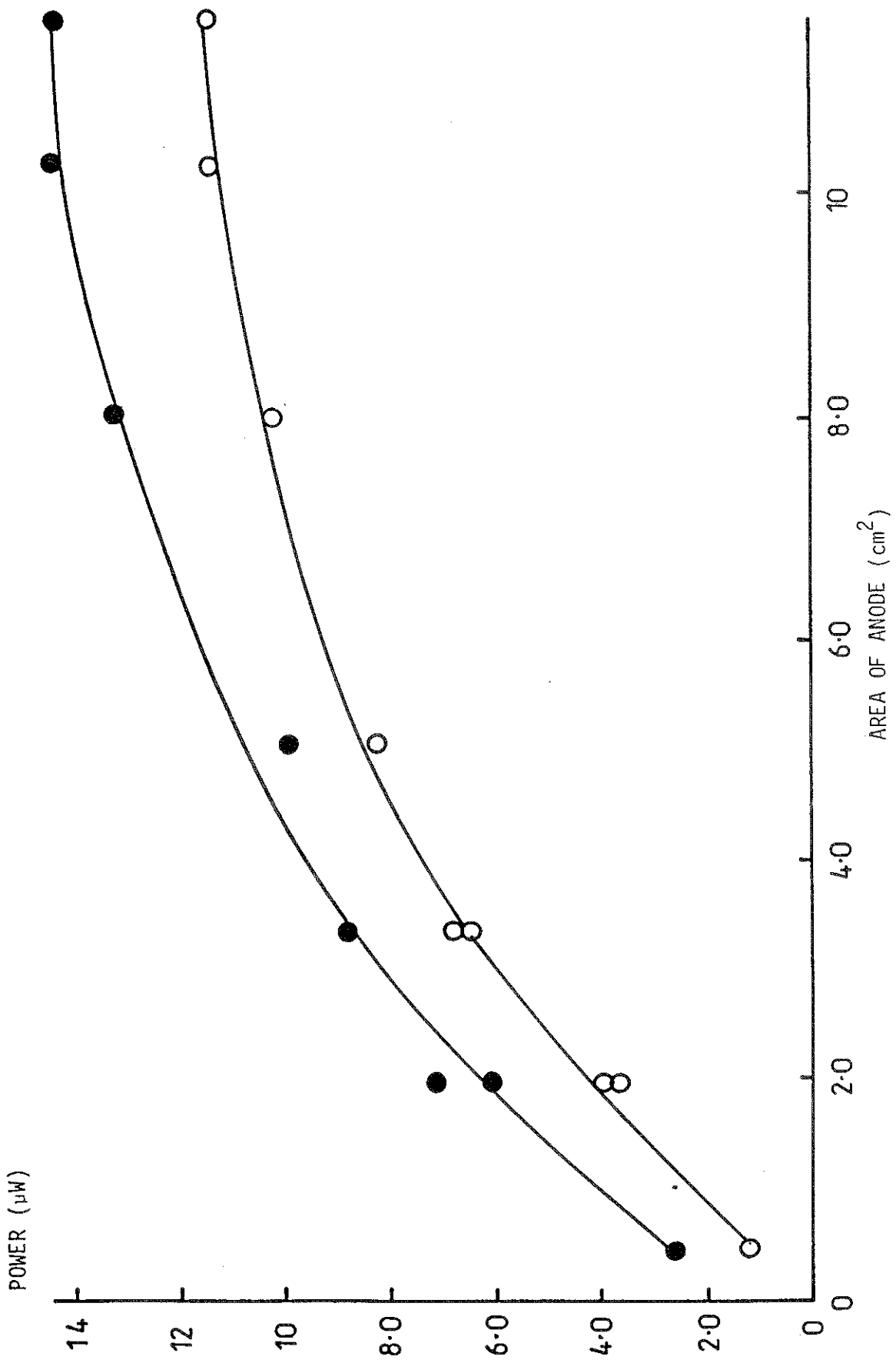
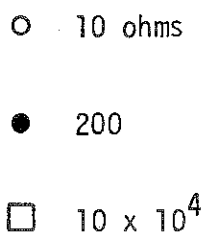
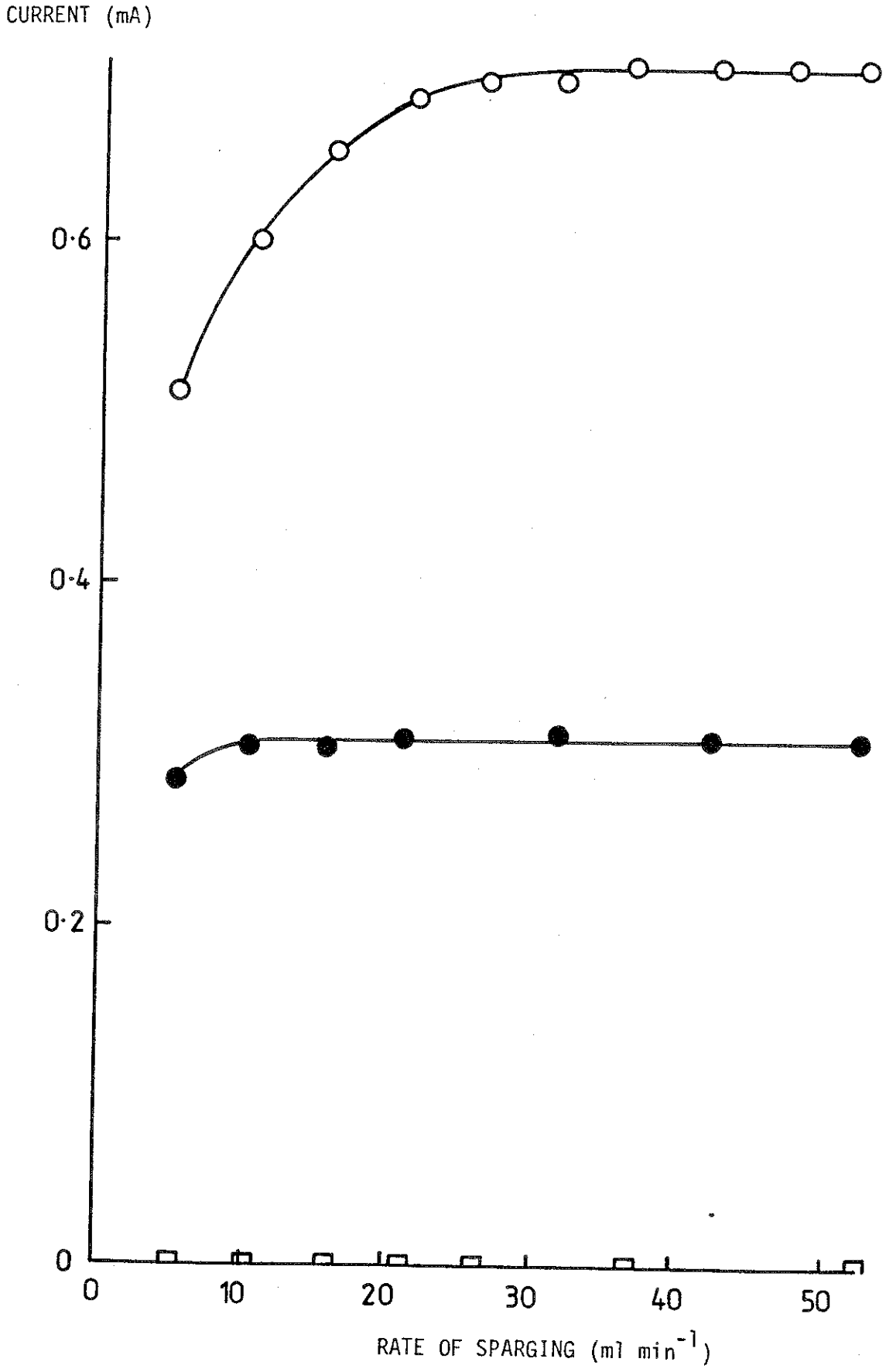

POWER (μW)
14
12
10
8·0
6·0
4·0
2·0
0
2·0
4·0
6·0
8·0
10
AREA OF ANODE (cm²)

Table 4.4.1 The effect of sparging the cathode with air and oxygen (40 ml min^{-1}) at various resistances on the current output of a biofuel cell (see section 4.4.1).

	Air Sparged Cathode			Oxygen Sparged Cathode			
External Resistance (Ohms)	Background Current (mA)	Current in the presence of enzyme (mA)	Difference (mA)	Background Current (mA)	Current in the presence of enzyme (mA)	Difference (mA)	Difference in the enzyme dependent currents produced by oxygen and air (mA)
10	0.128	0.43	0.302	0.22	0.68	0.46	0.158
200	0.085	0.24	0.16	0.123	0.32	0.20	0.04
1×10^4	0.008	0.018	0.01	0.009	0.018	0.01	0.0

Figure 4.4.4 The effect of the rate of sparging the cathodic compartment at various resistances (see section 4.4.1).




CURRENT (mA)
0·6
0·4
0·2
0
0
10
20
30
40
50
RATE OF SPARGING (ml min^{-1})

and 12.0. The pH optimum for maximum current output was 10.5 (Fig 4.4.5; Table 4.4.2). The current output increased up to pH 10.5 above which rapid reduction in current and enzyme stability occurred (see section 4.4.3). Whilst PES was unstable at pH values greater than 9.0, it was possible to use TMPD at the optimum pH of the enzyme (see section 4.1). Increasing both the enzyme (Fig 4.4.6) and mediator (Fig 4.4.7) concentrations resulted initially in an increase in the current output proportional to the amount added, until a maximum current was attained.

The effect of agitation of the anodic solution was investigated. This was demonstrated by varying the rate of rotation of a magnetic follower incorporated in the anodic compartment. The magnetic stirrer was calibrated stroboscopically (see section 2.38). Although a current is produced in the absence of stirring, increased agitation caused an increase in the steady state current produced until a maximum current of 0.39 mA was attained (Fig 4.4.8).

The variation in the temperature of the biofuel cell during operation was investigated. A biofuel cell was set up (see section 2.28) insulated with 50 cm of expanded polystyrene through which inlet and outlet tubes were cut allowing reactants to be added (see section 2.30). During a 60 minute period the temperature was monitored using a glass thermometer and thermocouple. The procedure was repeated with the solution agitated by means of a magnetic stirrer at 2100 rpm and

Figure 4.4.5 The effect of pH on the rate of attainment of maximum current produced by a TMPD mediated biofuel cell (see section 4.4.1 for details).

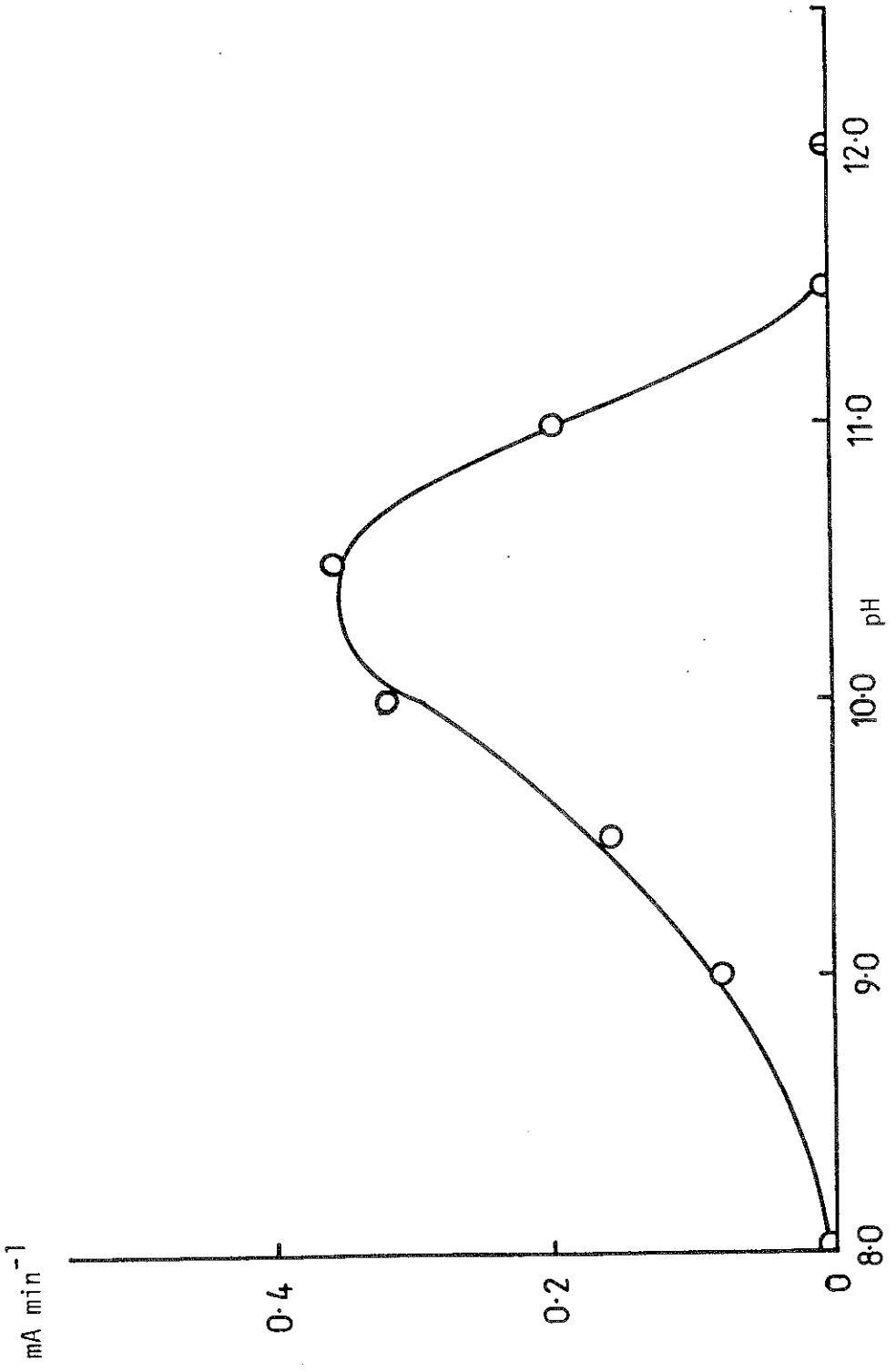

mA min^{-1}
0·4
0·2
0
8·0
9·0
10·0
11·0
12·0
pH

Table 4.4.2 The change in conductivity, current output and open circuit potential with change in pH for a methanol dehydrogenase based biofuel cell (see section 4.4.1).

pH	Conductivity prior to the addition of medium ($Ohms^{-1}x10^{-2}$)	pH on addition of mediator	Conductivity after addition of mediator ($Ohms^{-1}x10^{-2}$)	Maximum current (mA)	Initial current (mA)	Current (mA)	Open Circuit Potential (mV)
8.0	6.2	7.80	3.0	0.28	0.28	0	152.3
9.0	7.9	8.71	7.9	0.3	0.2	0.1	159.0
9.5	8.9	9.24	5.88	0.3	0.185	0.115	110.4
10.0	9.9	9.82	9.7	0.3	0.15	0.15	-
10.5	10.0	10.44	8.8	0.3	0.12	0.18	114.0
11.0	11.0	10.83	11.37	0.25	0.135	0.115	-
11.5	-	-	-	0.23	0.13	0.101	98.9
12.0	-	-	-	-	-	-	-
12.5	12.7	12.50	12.7	0.075	0.075	0	95.4

Figure 4.4.6 The effect of enzyme concentration on the current output of a methanol dehydrogenase based biofuel cell, measured across a 10 ohm resistance (see section 4.4.1).

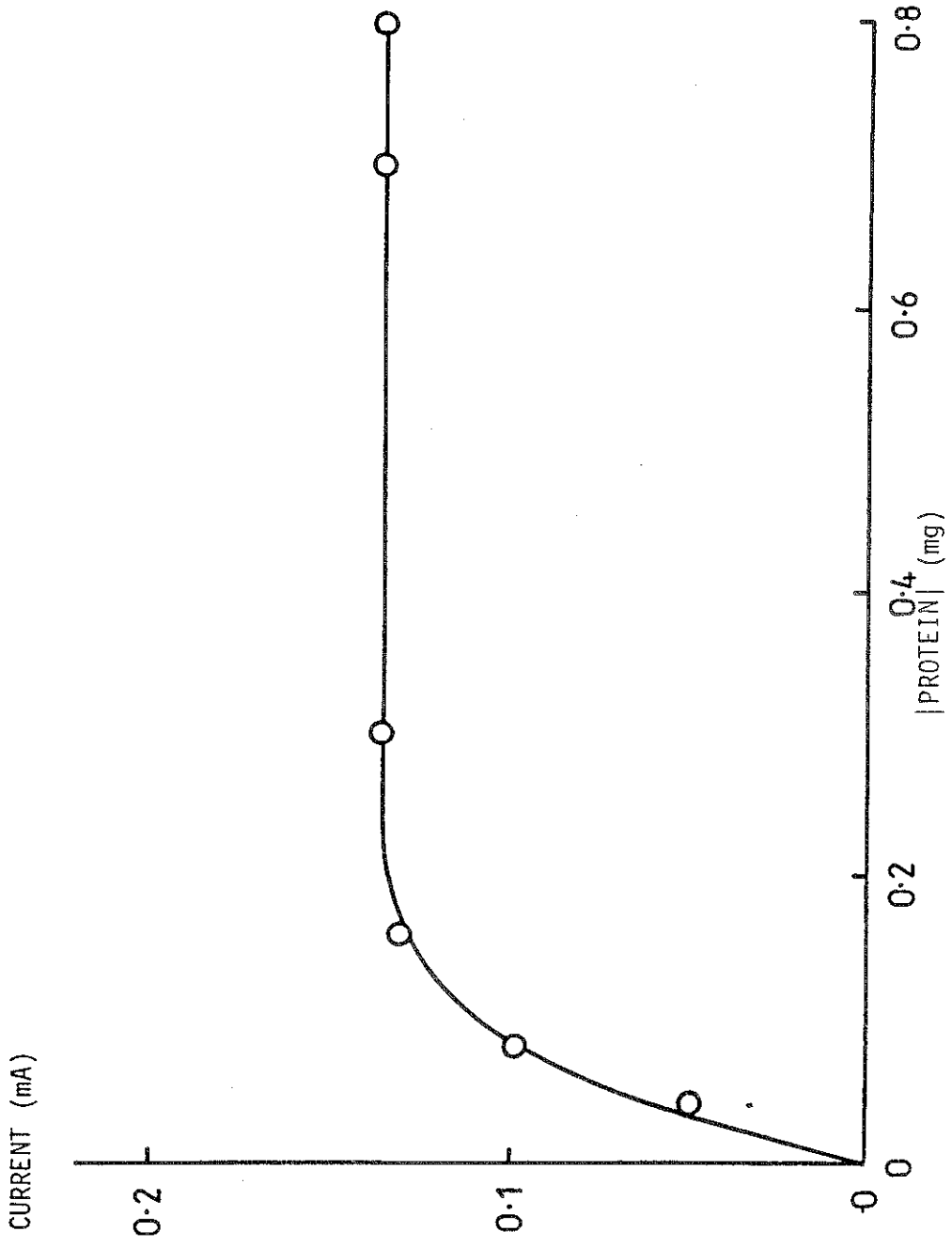

CURRENT (mA)
0·2
0·1
0
0
0·2
0·4
0·6
0·8
PROTEIN (mg)

Figure 4.4.7 The effect of mediator concentration on the current output of a methanol dehydrogenase based biofuel cell (see section 4.4.1).

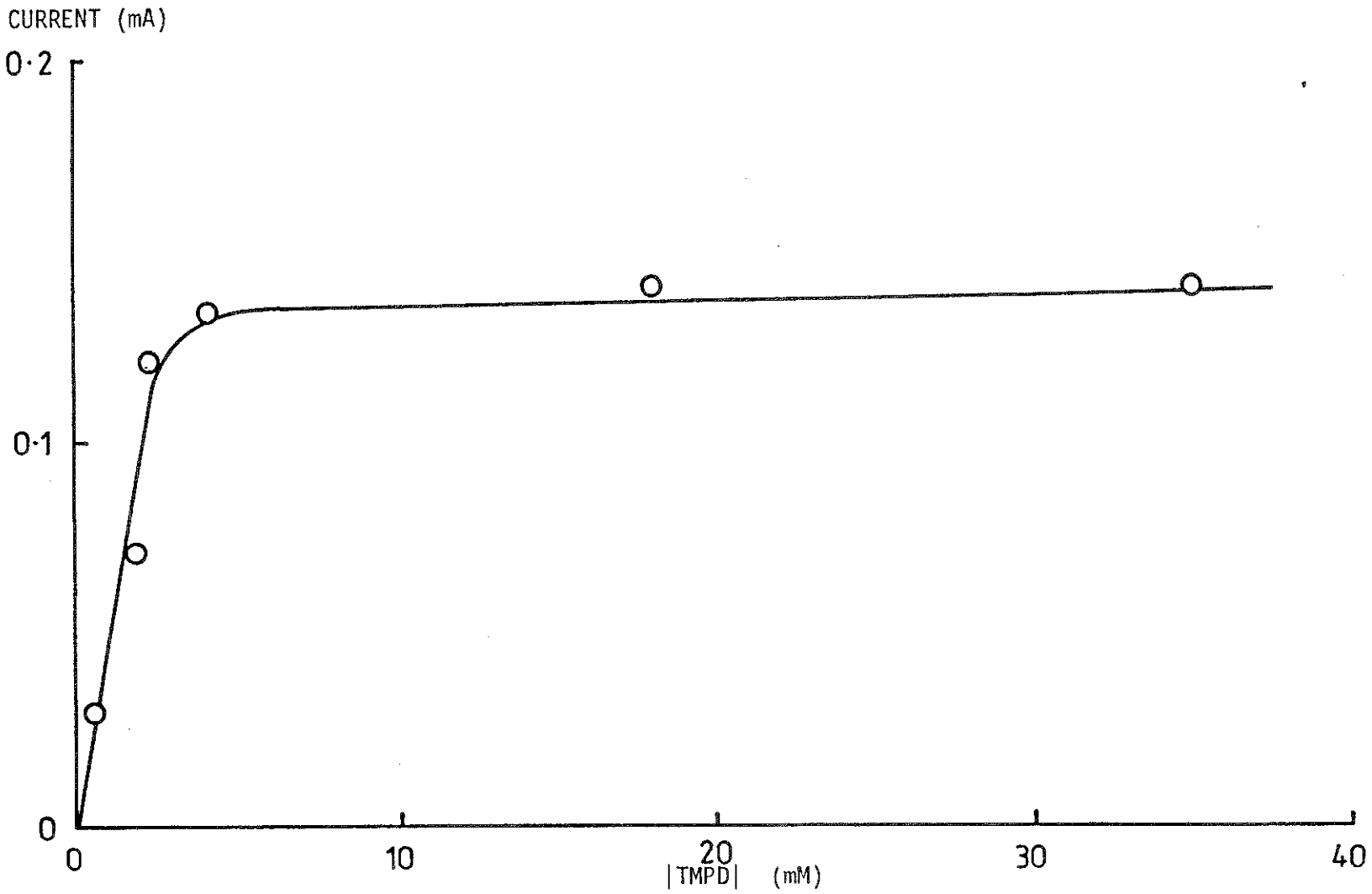
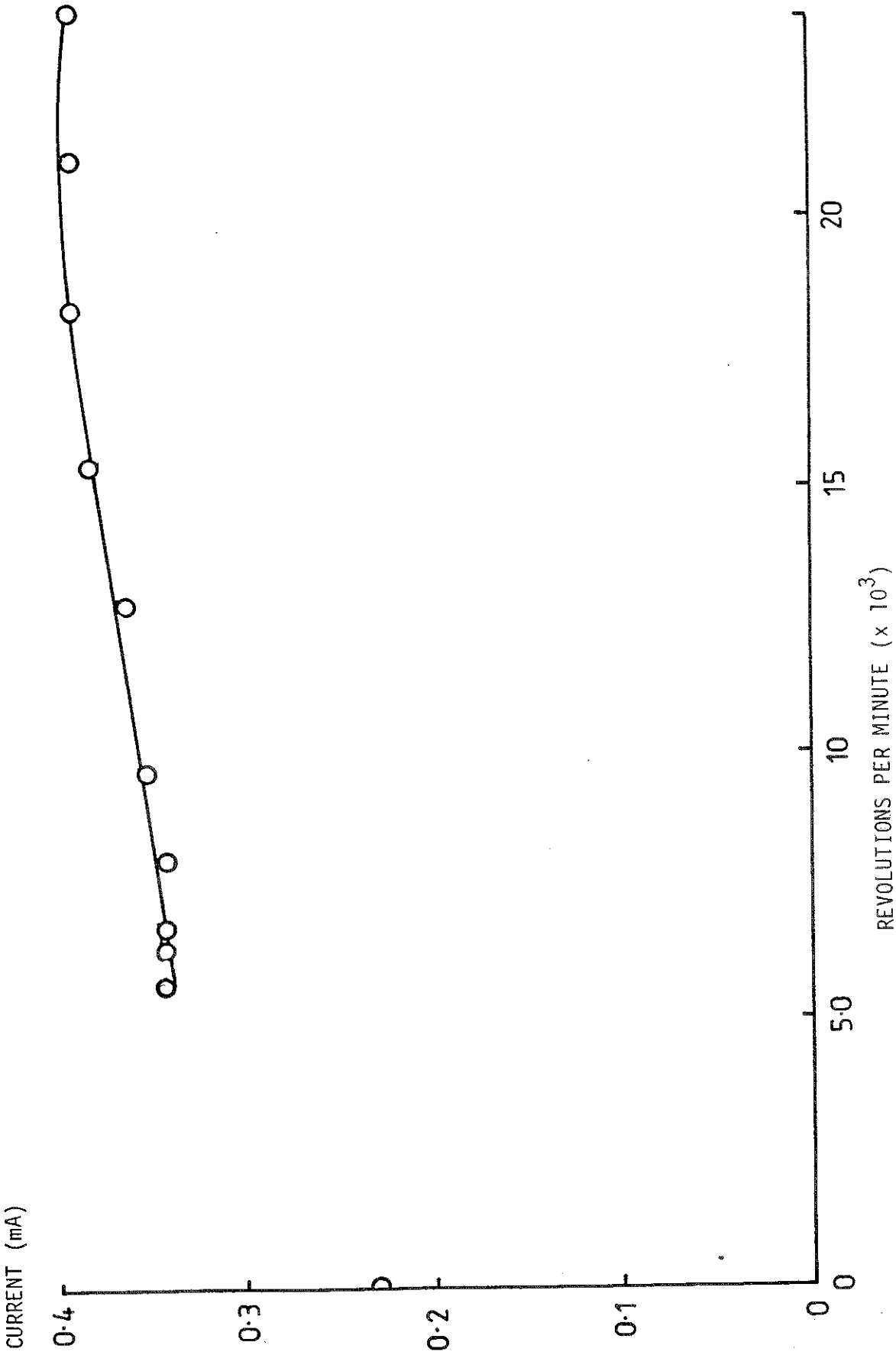

CURRENT (mA)
0·2
0·1
0
0
10
20
30
40
|TMPD| (mM)

Figure 4.4.8 The effect of stirring rate on the current output of a methanol dehydrogenase based biofuel cell (see section 4.4.1).

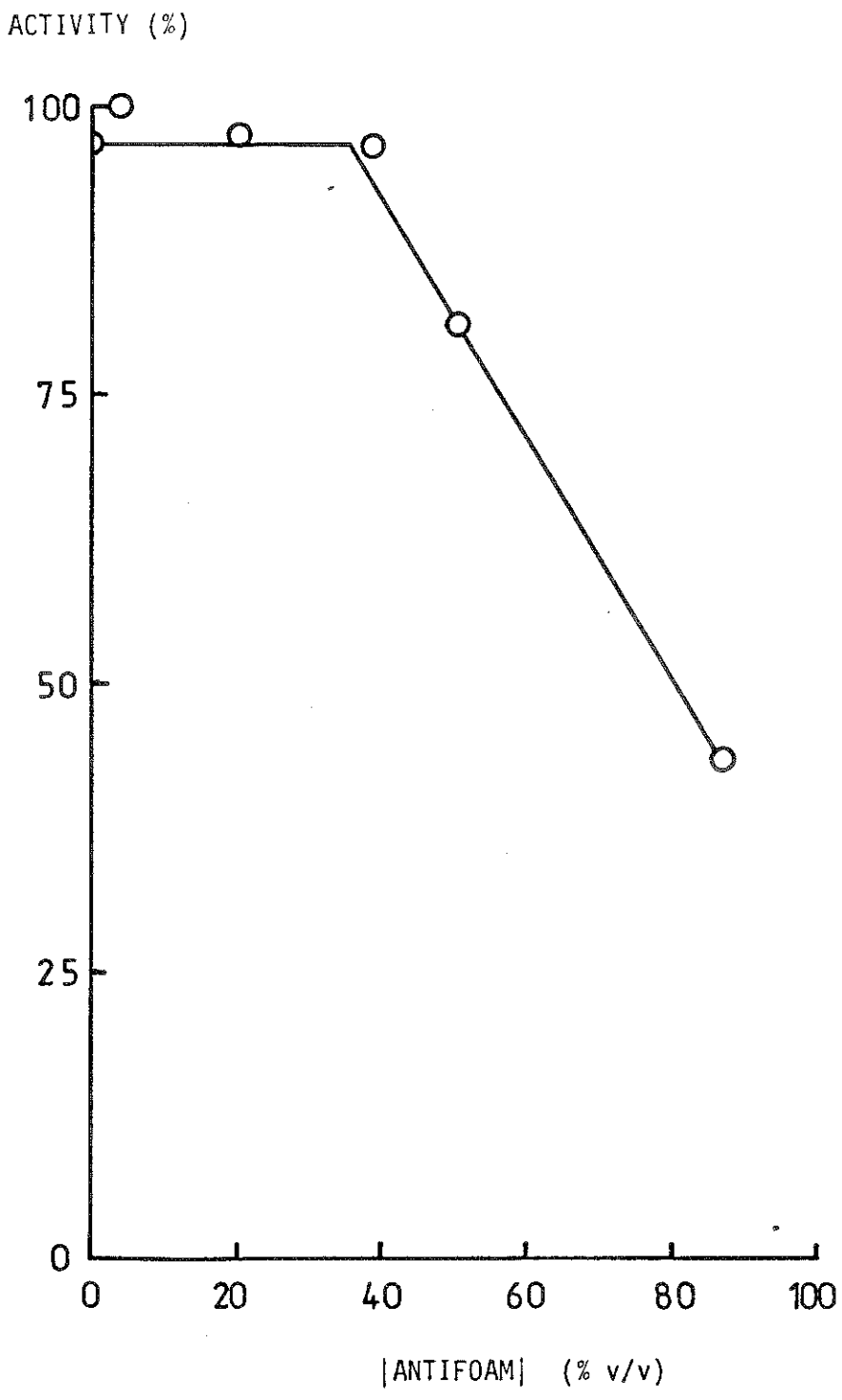

CURRENT (mA)
0·4
0·3
0·2
0·1
0
0
5·0
10
15
20
REVOLUTIONS PER MINUTE (x 10^3)

subsequently in the presence of enzyme. The temperature recorded by both devices did not alter, remaining at 21.5^{o}C.

Sparging nitrogen gas through the anodic compartment caused foaming of the solution. This was eliminated by the addition of silicone antifoam or by passing the gas over the surface. Whilst in this work nitrogen gas was passed over the surface of the anodic chamber, the effect of antifoam on the enzyme was investigated both polarographically and in a biofuel cell. The enzyme was incubated in the presence of various concentrations of antifoam for 5 minutes prior to the addition of ammonium chloride. The concentration of antifoam added to prevent foaming (0.01-0.1% V/V) did not affect the specific activity of the enzyme (Fig 4.4.9). The effect of antifoam on the methanol-based biofuel was determined by setting up a biofuel cell (see section 2.28) containing methanol dehydrogenase (0.2 mg). Once a steady state current was attained, antifoam was added (2.5×10^{-3}% V/V) and the steady state current measured. No detectable variation in the current was observed.

The effect of the distance between the electrodes on the current output of the biofuel cell was investigated. Two platinum electrodes (1.0 cm^2) were mounted on a stand enabling the distance between them to be altered. The electrodes were placed in the anode and cathode compartments of a biofuel cell, (see section 2.28) separated by an ion exchange membrane and sparged with nitrogen and air, respectively. The current was measured at various resistances and electrode distances.

Figure 4.4.9 The effect of silicone antifoam on the activity of methanol dehydrogenase measured polarographically (see sections 2.11.3 and 4.4.1).


ACTIVITY (%)
100
75
50
25
0
0
20
40
60
80
100
[ANTIFOAM] (% v/v)

Decreasing the distance between the two electrodes resulted in an increased current (Table 4.4.3).

The change in internal resistance and the maximum power produced on the addition of enzyme was investigated. The biofuel cell was set up (see section 2.28). The voltage was measured across a series of known resistances and the current determined. The procedure was repeated for various concentrations of enzyme. Although the method is crude it can be seen that the internal resistance decreases with increasing enzyme concentration until maximum power is achieved (Fig 4.4.10).

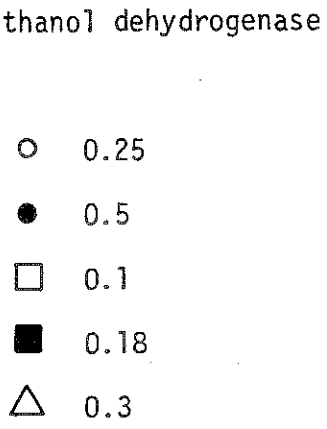
The internal resistance of a biofuel cell is composed primarily of the bulk solution resistance and the charge transfer resistance (see section 1.3.5). Determination of the total internal resistance of the biofuel cell is possible by measuring the resistance at which the maximum current is produced, although this does not allow the contribution of each component to be determined. The solution resistance and the charge transfer resistance may be determined by setting up a biofuel cell and applying a sinusoidal frequency between the electrodes (Armstrong et al., 1976; Britz, 1980; Hladky et al., 1980).

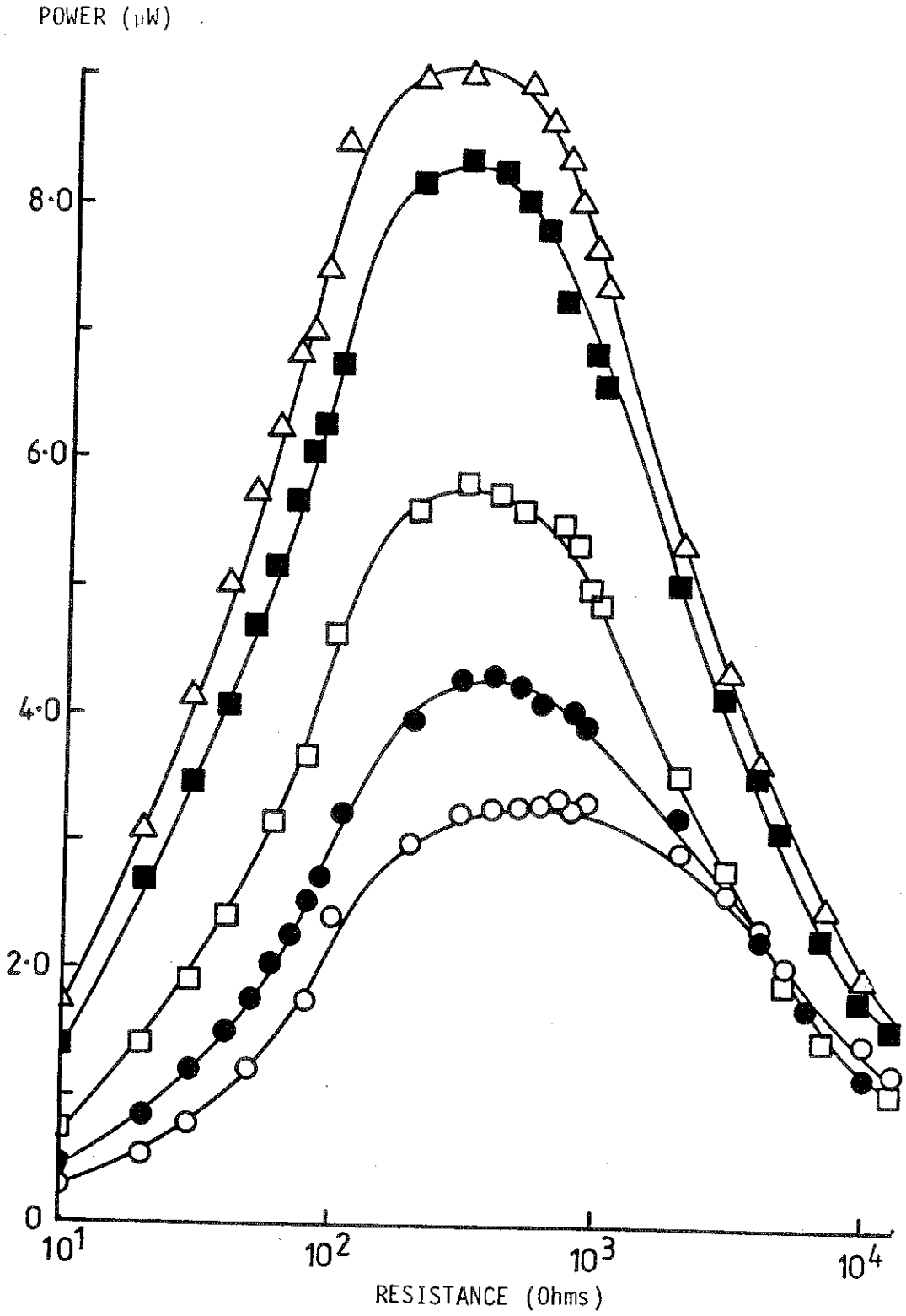
A biofuel cell was set up as described previously (see section 2.28). The potential of the working electrode was maintained at +70 mV (versus SCE) and the frequency swept between 9.9×10^{4} - 3×10^{-2} H_z using a frequency analyser (Solarton Instruments Ltd., Slough, Berks) coupled to a microcomputer (Commodore Ltd., Slough, Berks). Values for the solution

Table 4.4.3 The effect of varying the distance between two platinum electrodes on the current output of a methanol dehydrogenase based biofuel cell (see section 4.4.1).

	Distance between electrodes (cm)			
	0.3		1.5	
Resistance (ohms)	Current with no enzyme	Current in the presence of enzyme	Current with no enzyme	Current in the presence of enzyme
10	8.75×10^{-2}	1.35×10^{-1}	8.75×10^{-2}	9.5×10^{-2}
200	7.5×10^{-3}	1.0×10^{-2}	7.0×10^{-3}	8.0×10^{-3}
10×10^{6}	1.05×10^{-6}	1.4×10^{-6}	1.05×10^{-6}	1.28×10^{-6}

Figure 4.4.10 The effect of the concentration of methanol dehydrogenase on the maximum power produced, with change in resistance, by a methanol dehydrogenase based biofuel cell (see section 4.4.1).




POWER (μW)
8·0
6·0
4·0
2·0
0
10^1
10^2
10^3
10^4
RESISTANCE (Ohms)

resistance and the charge transfer resistance were calculated to be approximately 12.0 ohms and 500 ohms respectively (Fig 4.3.11). The resistances determined by this method are similar to those previously determined using a conductivity meter and those calculated from power curves. The largest component of the biofuel cell resistance is the charge transfer resistance, which decreases on addition of enzyme.

4.4.2 Substrate specificity of methanol dehydrogenase.

The biofuel cell functioned as a coulometric sensor for the analysis of primary alcohols. Integration of the current time curves on the addition of aliquots of substrate enabled the charge passed to be determined (Fig 4.4.12). This procedure, carried out for alcohols of known concentrations, enabled the coulometric efficiency and the concentration of substrates to be determined (see chapter 5). The biofuel cell was capable of determining low concentrations of straight chain primary alcohols up to decan-1-ol; formaldehyde and diols including 1,3-propanediol and 1,4-butanediol (Table 4.4.4). However, the addition of 1,2-ethanediol, secondary and tertiary alcohols did not result in production of a current. Determination of the charge passed and the gradient of the slopes enabled the charge passed per mole to be calculated. Oxidation of methanol and the diols is associated with 4.0 Faradays $mole^{-1}$ of charge whereas formaldehyde and the other primary alcohols give values of 2.0

Figure 4.4.11 Frequency analysis of a biofuel cell in the absence (O) and presence (●) of enzyme (see section 4.4.1).

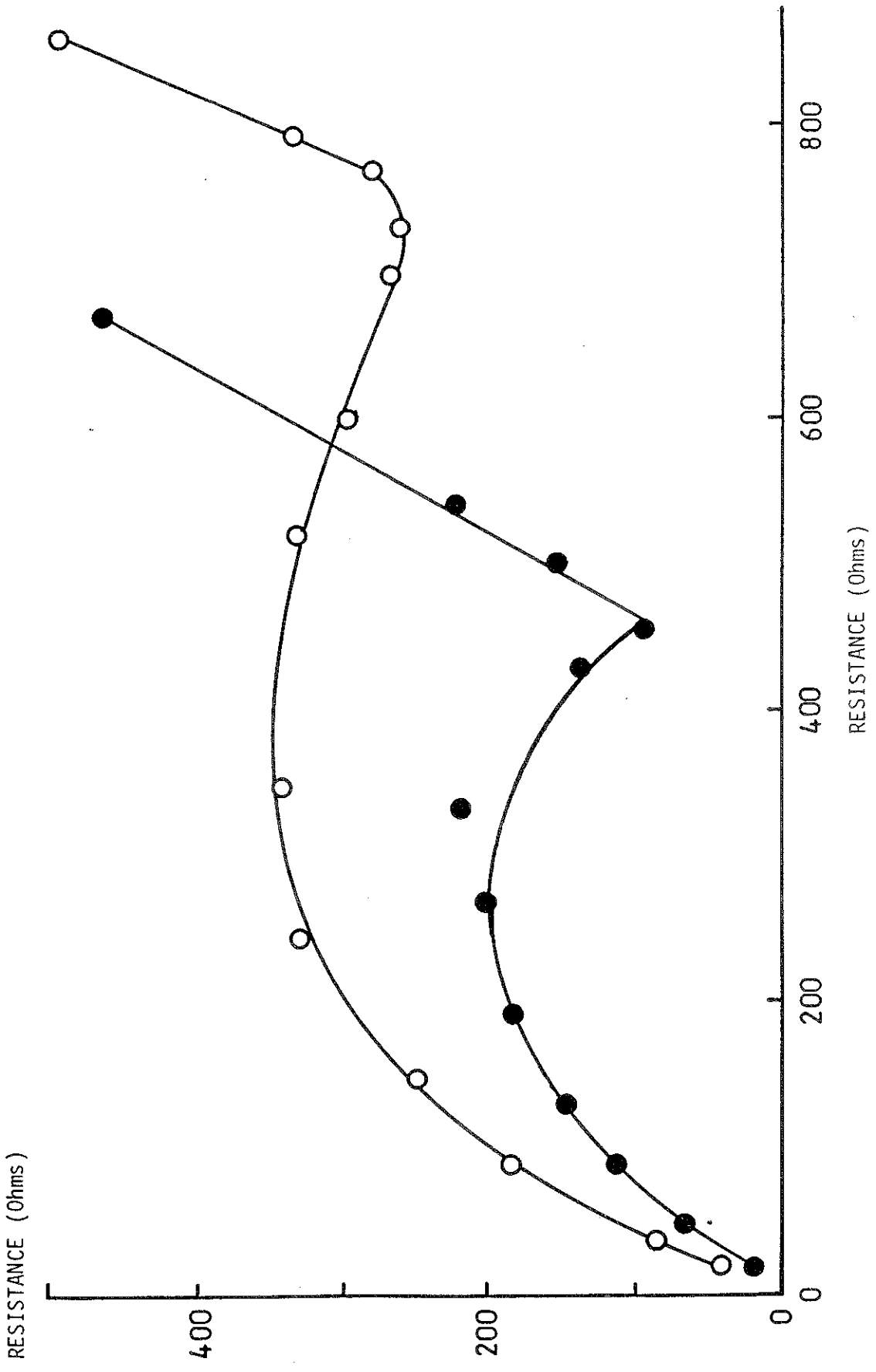
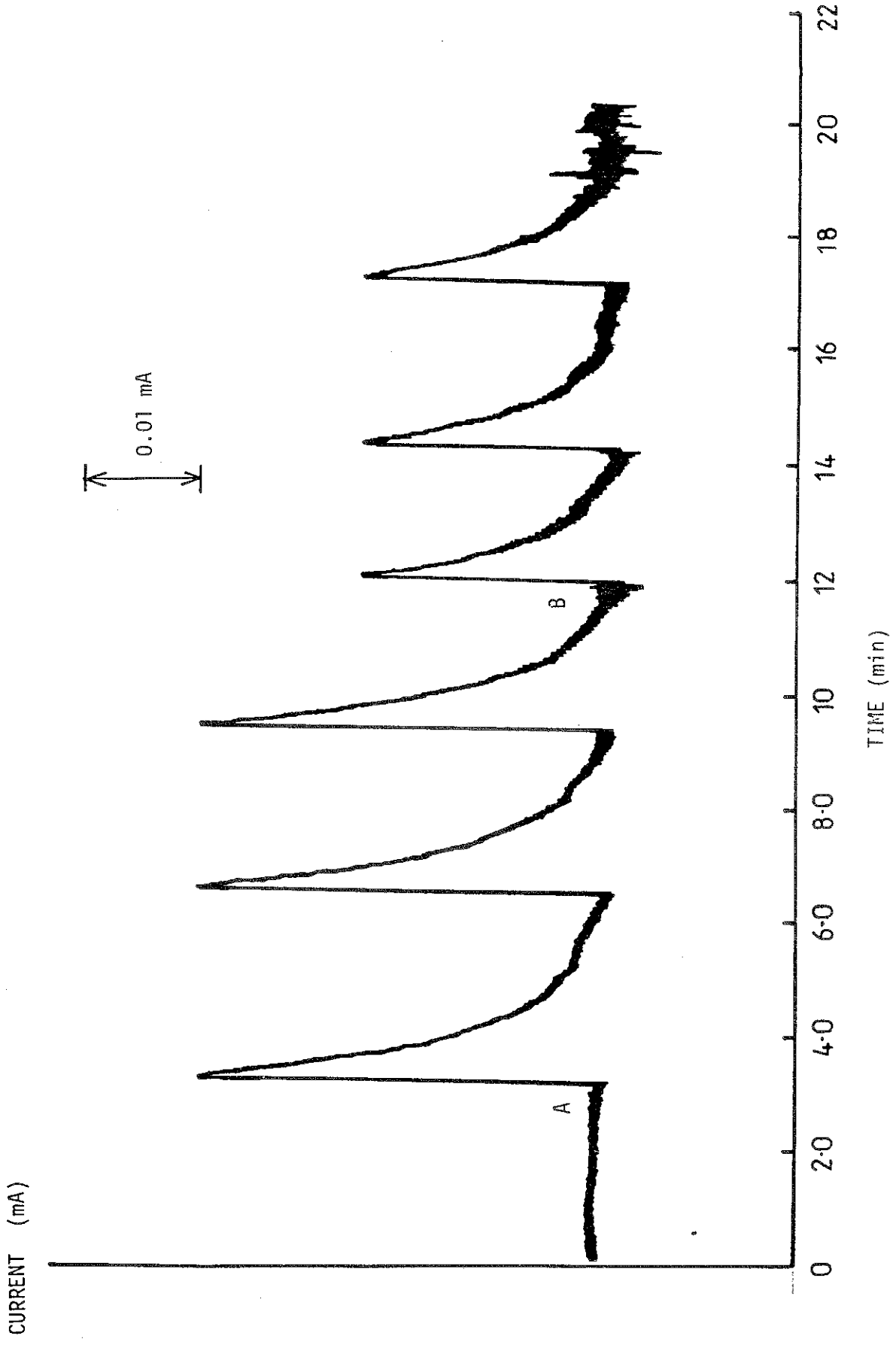

RESISTANCE (Ohms)
400
200
0
0
200
400
600
800
RESISTANCE (Ohms)

Figure 4.4.12 The current response on triplicate additions of methanol at (A) 3×10^{-10} μmoles and (B) 5×10^{-10} μmoles to the biofuel cell (see section 4.4.2).


CURRENT (mA)
0.01 mA
A
B
0
2·0
4·0
6·0
8·0
10
12
14
16
18
20
22
TIME (min)

Faradays $mole^{-1}$ (Table 4.4.4). The results for methanol support the proposal that methanol dehydrogenase coverts methanol to formate via the intermediate production of formaldehyde (see section 4.4.4).

4.4.3 Stabilisation of methanol dehydrogenase.

Methanol dehydrogenase has been shown to be irreversibly inactivated in the presence of mediator (Duine and Frank, 1980; Anthony, 1982). Loss of the enzymic activity was investigated with respect to the concentration of ammonium chloride, pH, molarity and mediator.

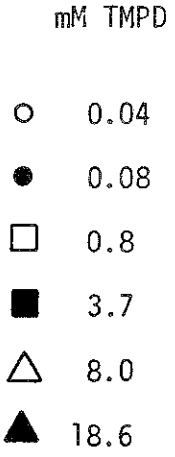
The stability of the enzyme in the biofuel cell (see section 2.28) in the presence of various concentrations of mediator (TMPD) was tested. The peak current on the addition of methanol (0.025 mM) was used as a measure of the amount of active enzyme present. Increasing the concentration of mediator caused a decrease in the stability of the enzyme and its ability to produce a current (Fig 4.4.13). The incorporation of oxidised TMPD (3.7 mM) (see section 2.25) did not affect the maximum current attained or the rate of loss (0.22 mA h^{-1}).

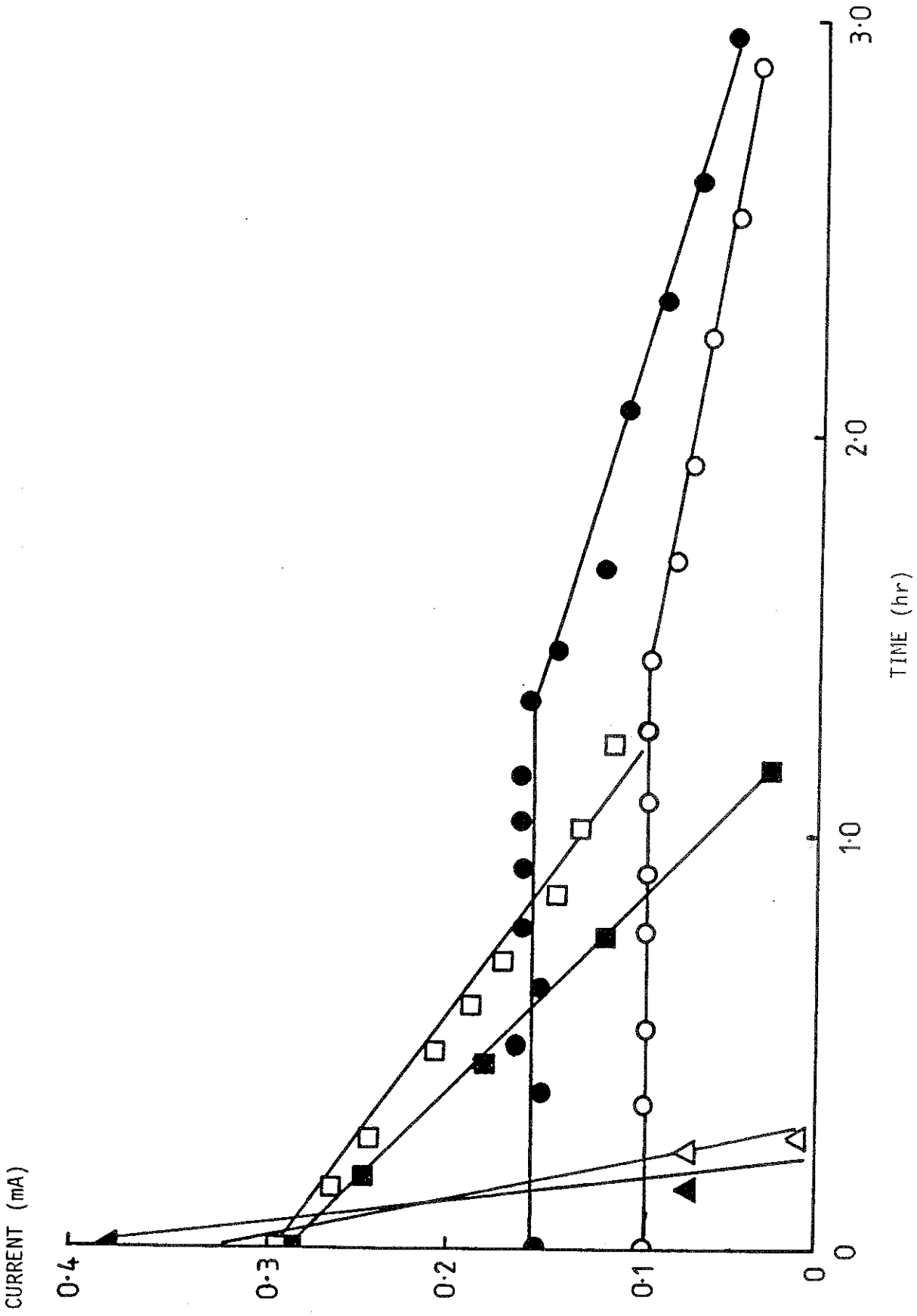
The coulometric efficiency of the biofuel cell was determined for two concentrations of methanol at various concentrations of mediator. The biofuel cell, mediated by TMPD (18.6 mM), was set up as described previously (see section 2.28) and the total charge passed on the addition of methanol measured. A high coulometric efficiency was achieved at a methanol

Table 4.4.4 The biofuel cell used as a coulometric sensor, with the lowest concentrations detected for a range of substrates tested (see section 4.4.2).

Alcohol	Lowest limit detected (moles)	Charge passed ($c\ mole^{-1}$)
Methanol	5×10^{-10}	3.8×10^{5}
Ethanol	5×10^{-10}	1.7×10^{5}
Propanol	1×10^{-9}	1.7×10^{5}
Butanol	3×10^{-9}	1.1×10^{5}
Pentanol	5×10^{-10}	1.0×10^{5}
Hexanol	5×10^{-10}	1.25×10^{5}
Heptanol	5×10^{-10}	1.45×10^{5}
Octanol	1×10^{-9}	1.7×10^{5}
Nonanol	3×10^{-9}	1.7×10^{5}
Decanol	1×10^{-9}	2.1×10^{5}
Formaldehyde	-	1.7×10^{5}
Acetaldehyde	-	0
Isopropanol	-	0
Isobutanol	-	0
1,2-Ethanediol	-	0

Figure 4.4.13 The effect of mediator concentration on the ability of a methanol dehydrogenase biofuel cell to produce a current (see section 4.4.3). Each point is the maximum current attained on the addition of an aliquot of methanol (0.025 mM).




CURRENT (mA)
0·4
0·3
0·2
0·1
0
0
1·0
2·0
3·0
TIME (hr)

concentration of 0.025 mM, whilst in the presence of 2.5 mM methanol and high concentrations of mediator a reduction in the coulometric efficiency was observed (Fig 4.4.14).

The effect of buffer molarity on the enzymic activity was investigated in a biofuel cell (see section 2.28) containing TMPD (0.75 mM). The maximum current attained, measured with a 10 ohm load, was unaffected by the molarity in the pH range tested. The stability of the enzyme at three molarities was tested (50, 150 and 300 mM). At all three concentrations the rate of decrease of the peak current on the addition of aliquots of methanol was similar (Fig 4.4.15), losing all activity after 4 hours.

The loss of activity of the enzyme was investigated with respect to pH. The initial rate at which the current was produced and the maximum current developed was measured on the addition of aliquots of methanol (0.025 mM). The rate of increase in current at pH 9.5 was half that obtained at pH 10.5 (Fig 4.4.5) although in both cases the maximum current attained was 0.25 mA. The rate of inactivation was determined by measuring the peak current on the addition of aliquots of methanol over a 35 minute period. At pH 10.5 and pH 9.0 the peak current decreased at rates of 3.5×10^{-3} mA min^{-1} and 3.1×10^{-3} mA min^{-1}, respectively. When repeated at pH 11.0 inactivation of the enzyme occured within 10 minutes. The maximum current attained at pH 10.5 and pH 8.5 was the same, although the background currents were 0.15 and 0.24 mA, respectively.

The effect of ammonium chloride on the enzyme was

Figure 4.4.14 The effect of mediator concentration on the maximum current output (O) and the coulometric efficiency of a biofuel cell mediated by TMPD (see section 4.4.3). The coulometric efficiency was measured at:

- ● 2.5 mM methanol
- □ 0.025 mM methanol

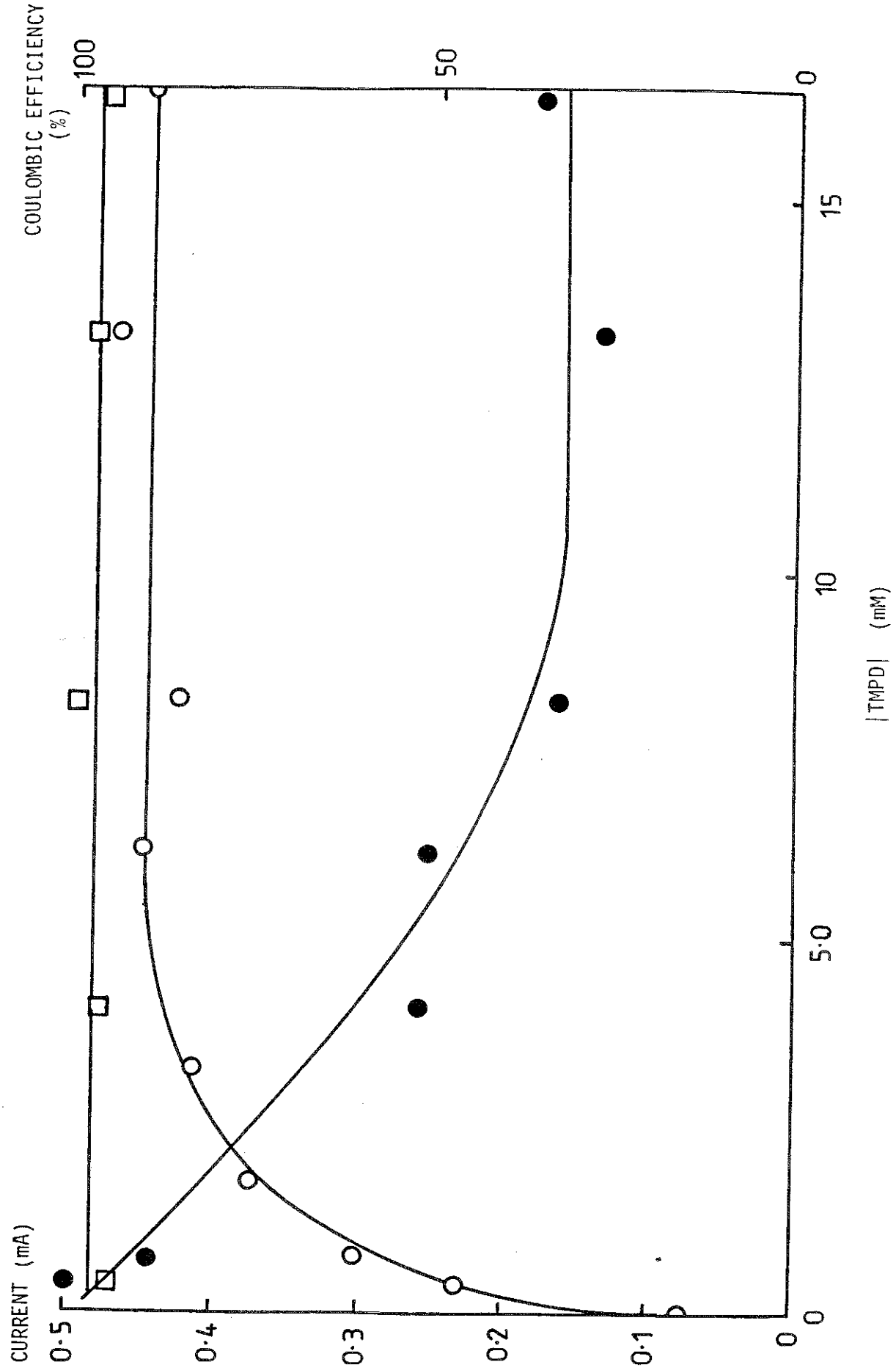
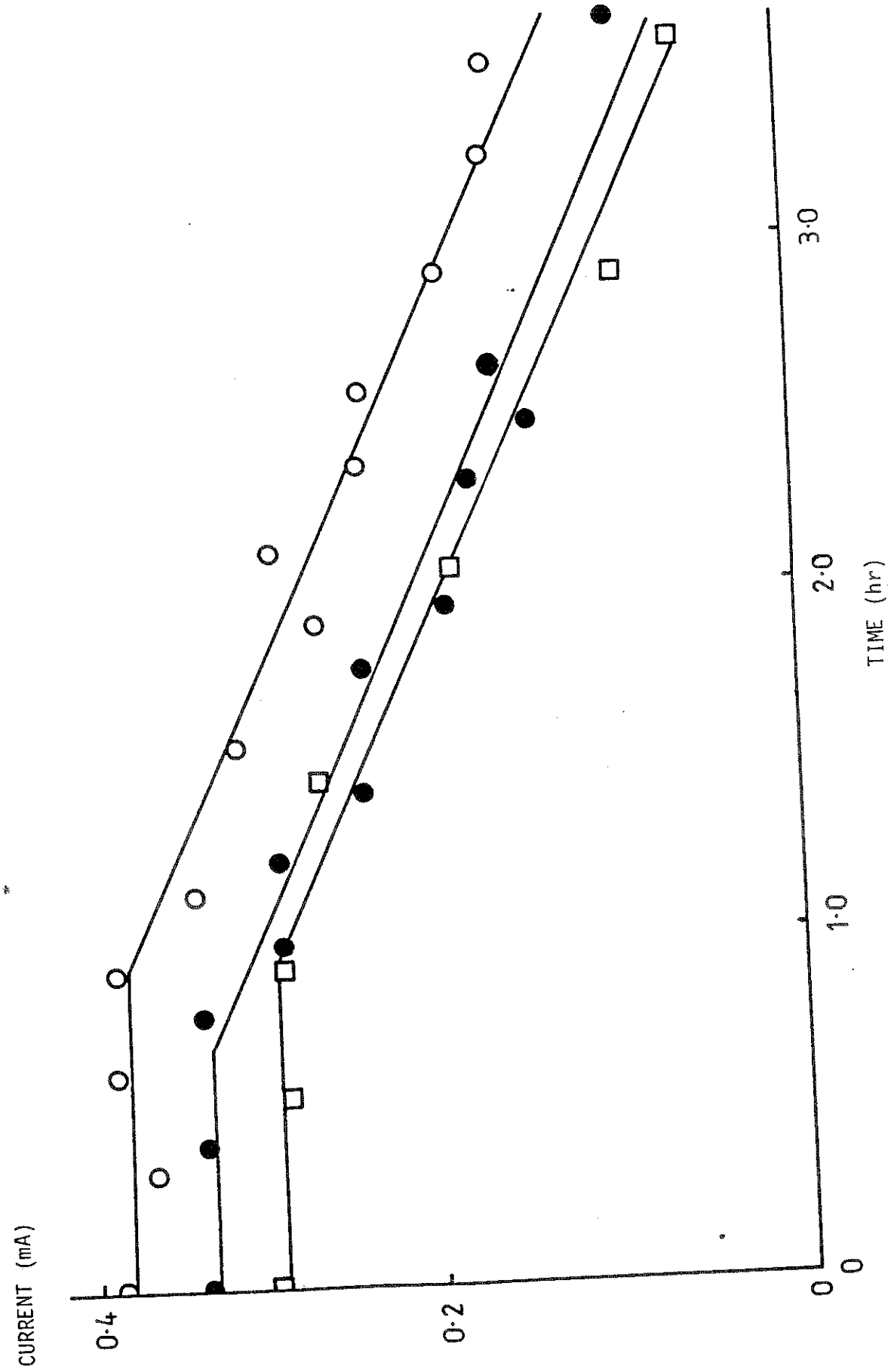

COULOMBIC EFFICIENCY (%)
100
50
0
CURRENT (mA)
0·5
0·4
0·3
0·2
0·1
0
0
5·0
10
15
|TMPD| (mM)

Figure 4.4.15 The effect of buffer (pH 10.5) concentration on the response of a methanol dehydrogenase biofuel cell (see section 4.4.3). The peak current was measured on the addition of methanol (0.025 mM). Buffer strengths were:

○ 50 mM borate buffer

● 150 mM borate buffer

□ 300 mM borate buffer


CURRENT (mA)
0·4
0·2
0
0
1·0
2·0
3·0
TIME (hr)

investigated both polarographically and in the biofuel cell (see section 2.28). Active enzyme was determined by measuring the peak current on the addition of aliquots of methanol (0.025 mM) at various times. The rate at which the maximum current was attained was unaffected by the concentration of ammonium chloride (50 mM and 500 mM) tested, but in all cases the enzyme lost activity within 3.0 hours. When tested polarographically, the addition of ammonium chloride between the concentrations 6.0 and 15 mM did not affect the specific activity of the enzyme.

Due to the alkaline conditions in the biofuel cell, ammonia is released. The concentration of ammonia was monitored using an ammonia sensing probe (Russel pH Ltd., Auchtermuchy, Fife, Scotland). The probe was calibrated by measuring the ammonia released from a solution of ammonium chloride on the addition of sodium hydroxide (91 mM) (Fig 4.4.16). The release of ammonia was measured in borate buffer (250 mM, pH 10.5) containing ammonium chloride (50 mM). Initially, a rapid release of ammonia occured (Fig 4.4.17) although this decreased with time. It was thought that loss of ammonium chloride from a biofuel cell might account for loss in current output. However, when ammonium chloride (25 mM) was added to a biofuel cell that had been operating for 3.0 hrs in the presence of excess methanol it did not restore activity. The subsequent addition of enzyme resulted in a current.

Two other compounds, methylamine and ethylamine have been demonstrated to be activators of methanol dehydrogenase (see

Figure 4.4.16 Standard calibration curve for ammonia using an ammonia selective electrode (see section 4.4.3). Calibration was carried out by the addition of sodium hydroxide (5 ml, 1.0 mM) to a solution of ammonium chloride (50 ml) and measuring the steady state potential.

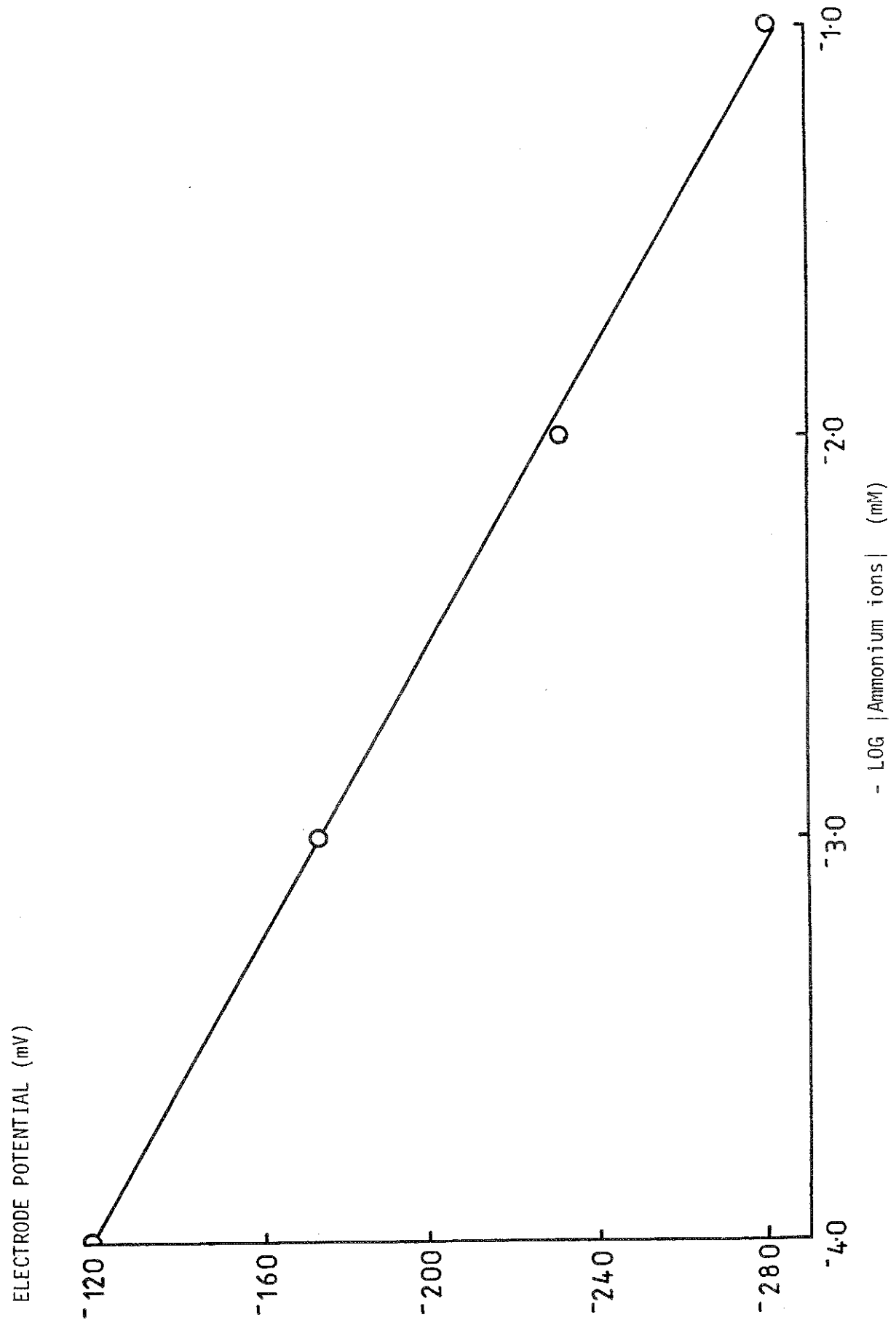
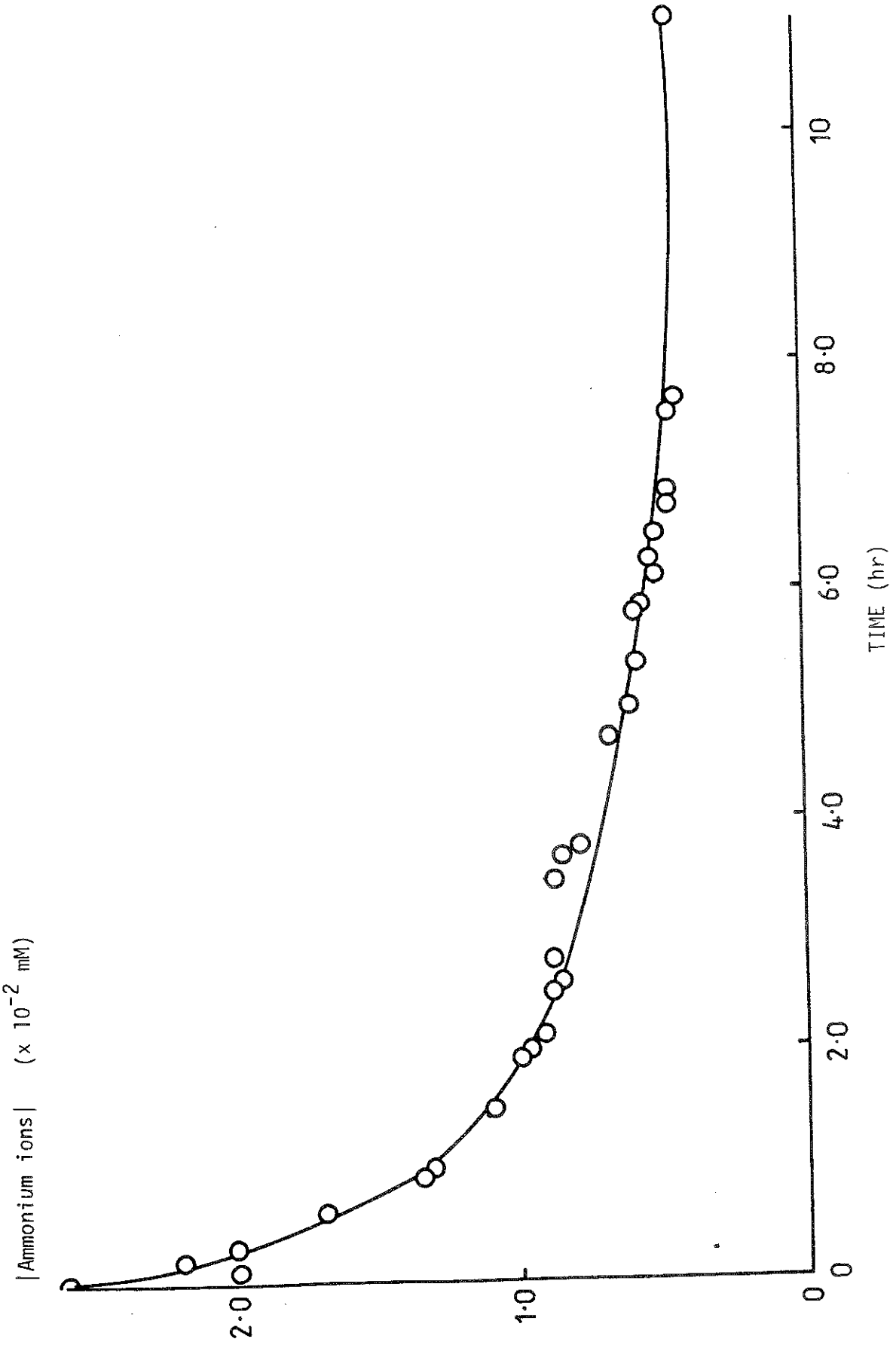

ELECTRODE POTENTIAL (mV)
-120
-160
-200
-240
-280
-1·0
-2·0
-3·0
-4·0
- LOG |Ammonium ions| (mM)

Figure 4.4.17 The variation in concentration of ammonia present in a freshly prepared solution of borate buffer (250 mM, pH 9.0) containing ammonium chloride (50 mM).


|Ammonium ions| (x 10⁻² mM)
2·0
1·0
0
0
2·0
4·0
6·0
8·0
10
TIME (hr)

section 2.28) containing enzyme (0.4 mg) was tested with each of the activators (50 mM). In all three cases the addition of methanol (0.25 mM) resulted in a maximum current of 0.25 mA which was lost at a rate of 1.2×10^{-5} mA min^{-1}.

The effect of various stabilising agents on methanol dehydrogenase were tested polarographically in an oxygen electrode. The presence of methanol in enzyme preparations prevented analysis of the effect of stabilising agents on enzyme in the absense of methanol. Hence crude methanol dehydrogenase was prepared from methane-grown Methylosinus trichosporium OB3b (Scott et al., 1981) supplied by Dr.D.Scott. Enzyme preparations from organisms grown under these conditions contain only bound methanol. Cell-free extract (0.03 mg) was incubated in an oxygen electrode in borate buffer (25 mM, pH 9.0) containing ammonium chloride (22.5 mM) and PES (3.0 mM) at $30^{o}C$ in the absence of methanol. Once the endogenous methanol was utilised methanol (7.5 mM) was added after a predetermined time and the specfic activity determined. The oxygen electrode was washed out and the procedure repeated for a range of times in the presence and absence of the stabilising agent under test. The variation in the specific activity of the enzyme in the absence of the stabilising agent was compared to those obtained in their presence (10 mM). The stabilising agents tested included azide, glycerol, dithiothreitol, cysteine, mercaptoethanl, boiled crude extract (0.75 mg ml^{-1} protein), bovine serum albumin (3.0%), sucrose (3.0%), menthol, polyethylene glycol 8000,

phenylmethylsulfonyl fluoride, thioglycollate and B-cyano-L-alanine; none exhibited any stabilising influence on the enzyme.

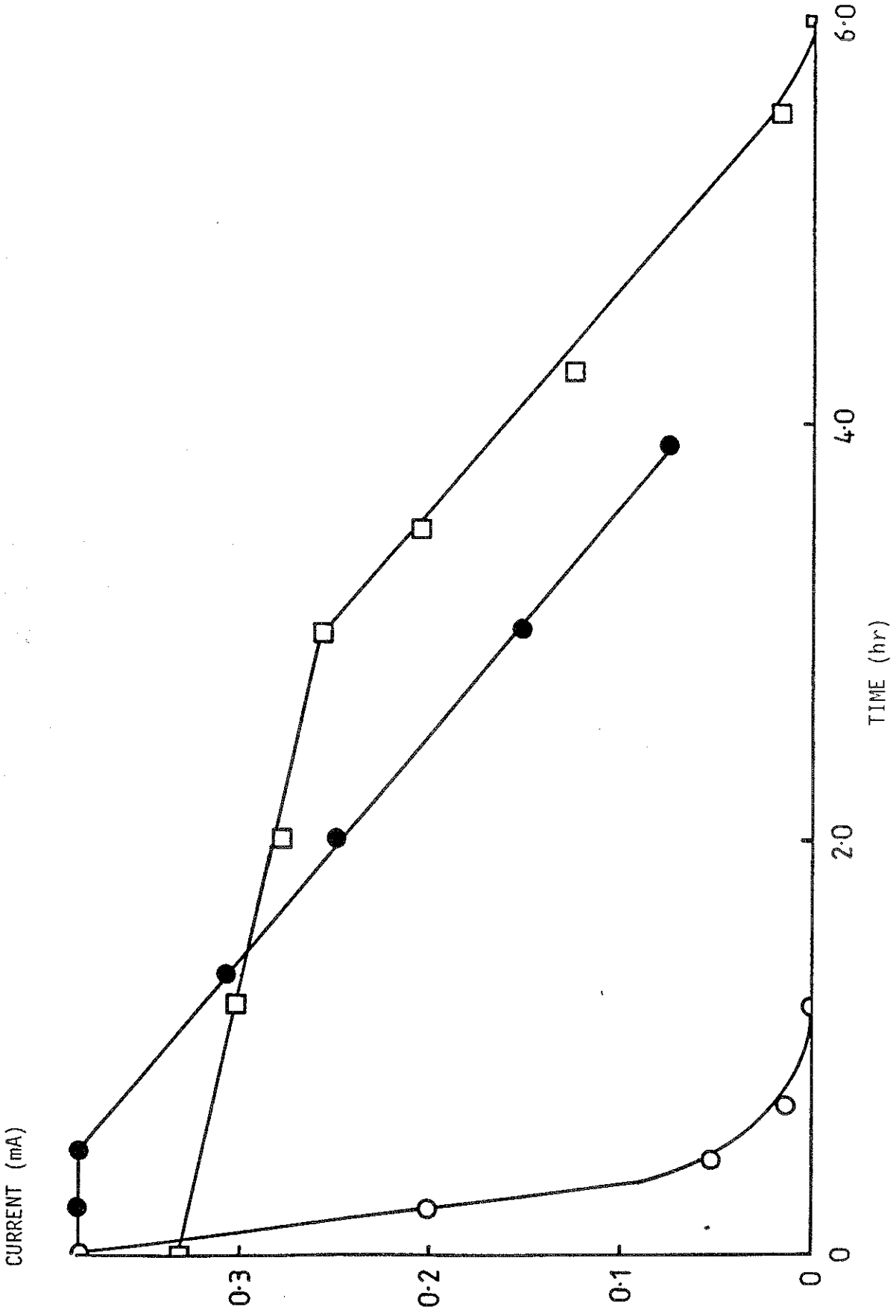
Two compounds, hydroxylamine and cyanide, have been shown to be competitive inhibitors of methanol dehydrogenase and capable of maintaining activity of the enzyme in the absence of substrate (Diune and Frank, 1980, 1981c). The stability of the enzyme in a biofuel cell was determined in the absence of methanol. The biofuel cell (see section 2.28) contained TMPD (4.3 mM) and methanol dehydrogenase (0.05 mg) obtained from Ps. AM1. Once utilisation of the endogenous methanol was completed, methanol was added (24 mM) and the current measured. The biofuel cell was washed, cleaned, the membrane replaced and the procedure repeated for a range of times as above. The experiment was repeated in the presence of hydroxylamine (0.25 mM). After 2.0 hours, 74% of the initial activity remained in the presence of hydroxylamine compared to 10% in its absence. The experiment was repeated with methanol dehydrogenase obtained from M. methylotrophus (0.03 mg) in the presence of TMPD (18.0 mM) at two concentrations of hydroxylamine 0.25 mM and 2.5 mM. Although the stability of the enzyme was enhanced in the presence of hydroxylamine (Fig 4.4.18) this effect was not maintained. The effect of oxygen on the stabilising influence of the hydroxylamine was determined. A solution of borate buffer (250 mM, pH 10.5) containing ammonium chloride (50 mM) was degassed by sonication (4 x 45 sec) and boiling (100^{o}C, 10 mins). The buffer

Figure 4.4.18 The effect of hydroxylamine on the stability of methanol dehydrogenase (see section 4.4.3). Each point represents the maximum current attained with a freshly prepared biofuel cell.

○ no hydroxylamine

● 0.25 mM hydroxylamine

□ 2.5 mM hydroxylamine


CURRENT (mA)
0·3
0·2
0·1
0
0
2·0
4·0
6·0
TIME (hr)

was dispensed into three containers to each of which was added hydroxylamine (2.5 mM). One container was sealed; the second sparged with nitrogen (containing 0.18 ppm O_2) and the third with oxygen-free nitrogen (0.05 ppm O_2). Samples were removed at predetermined times and the concentration of hydroxylamine determined (see section 2.12.5). Whilst hydroxylamine is stable when maintained anaerobic, the presence of oxygen even in trace amounts (0.05 ppm O_2), caused rapid oxidation to occur (Fig 4.4.19).

Cyanide is also a competitive inhibitor of methanol dehydrogenase (Duine and Frank, 1980, 1981c) and was investigated as a possible stabilising agent. Cyanide (2.5 mM) was added to the anodic compartment of a biofuel cell (see section 2.28). In the presence of cyanide the maximum current was not rapidly attained; an initial current (0.1 mA) was observed for 10 minutes after which a maximum current (0.25 mA) was attained which was equal to that obtained in the absence of cyanide.

The loss of cyanide from solution was demonstrated by making up a solution of cyanide (1.0 mM) in borate buffer (250 mM, pH 10.5). The solution was stirred using a magnetic stirrer and the concentration of cyanide in solution determined (see section 2.12.1). The loss of cyanide from solution was enhanced by stirring, but could be reduced by sealing the container (Fig 4.4.20).

The release of cyanide from solution may therefore be reduced by sealing the anodic compartment. However, cyanide may

Figure 4.4.19 The stability of hydroxylamine in various borate buffer solutions (250 mM, pH 10.5) (see section 4.4.3).

- ○ sparged with nitrogen
- ● sparged with oxygen-free nitrogen
- □ degassed and maintained in a sealed container

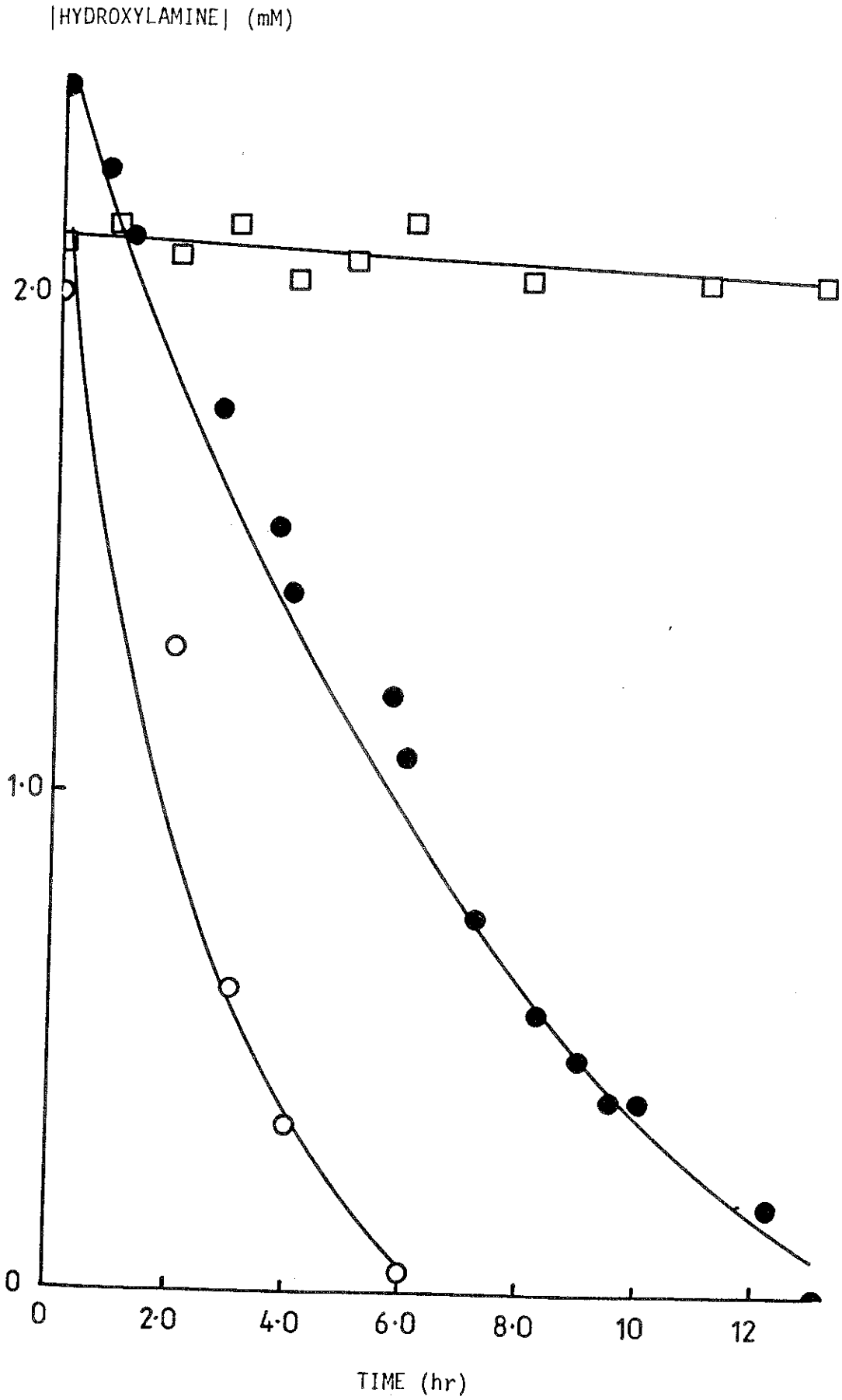
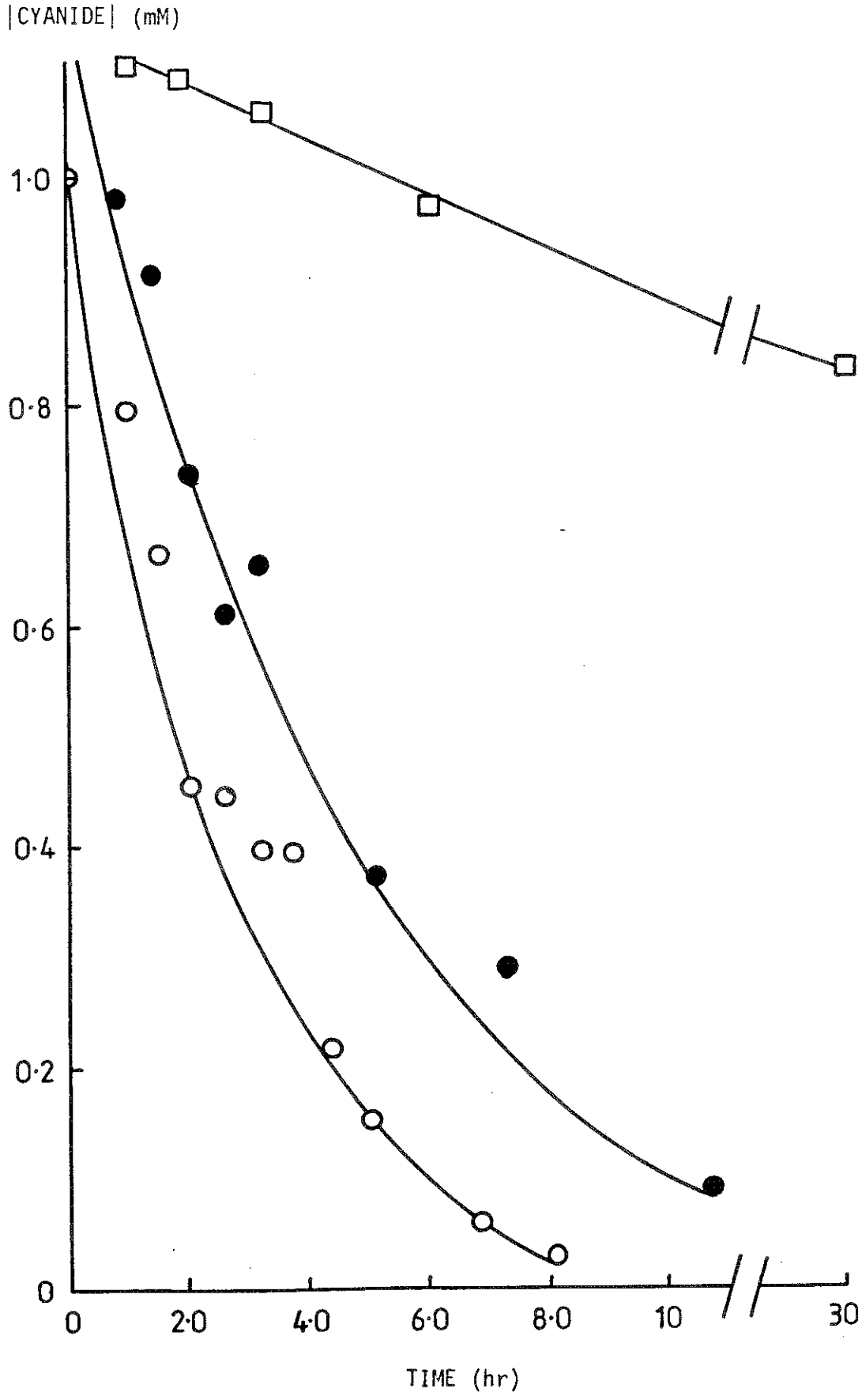

|HYDROXYLAMINE| (mM)
2·0
1·0
0
0
2·0
4·0
6·0
8·0
10
12
TIME (hr)

Figure 4.4.20 The stability of cyanide in various borate buffer solutions (250 mM, pH 10.5) (see section 4.4.3).

- ○ stirred and open to the atmosphere
- ● unstirred but open to the atmosphere
- □ sealed container

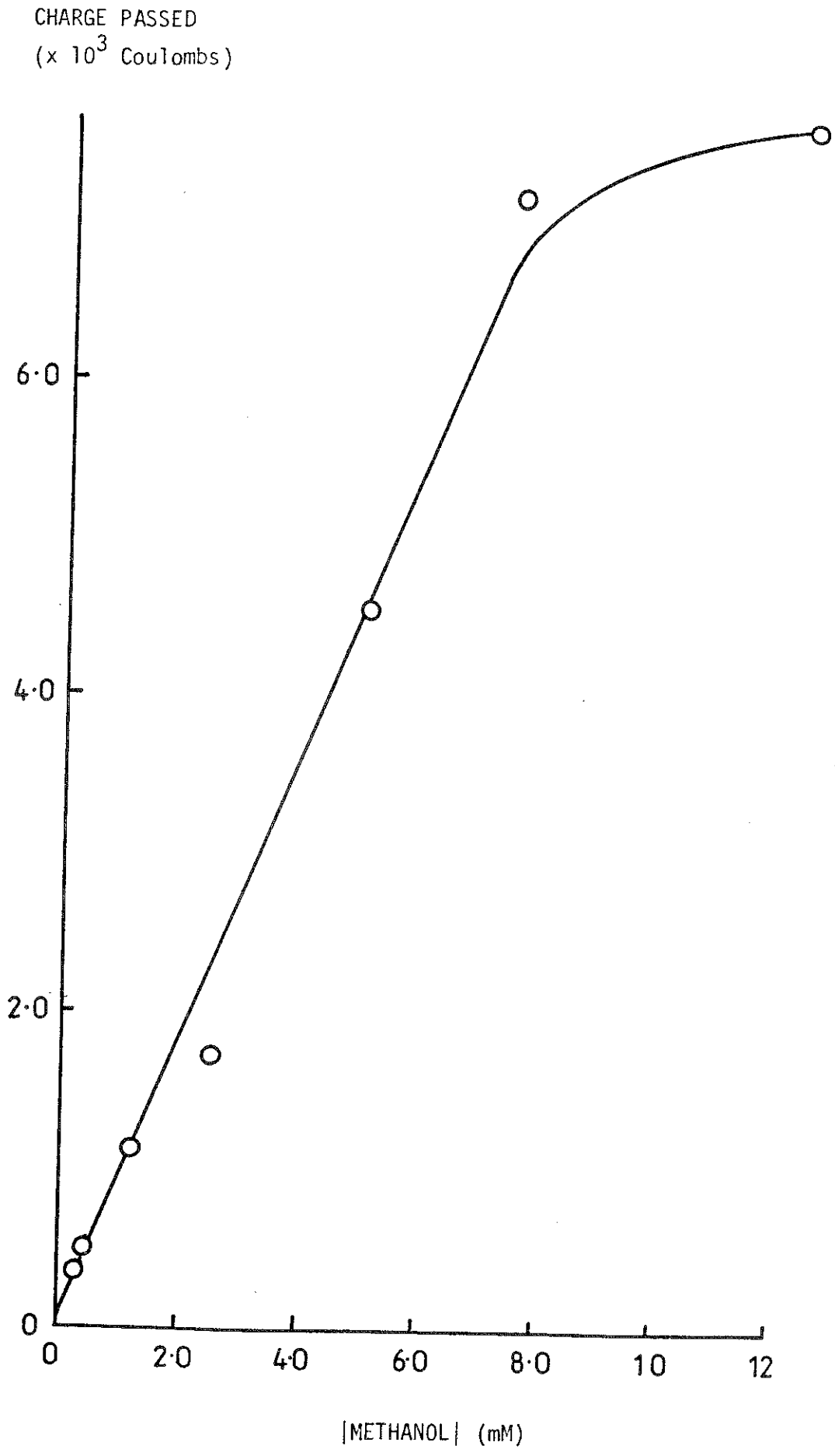

|CYANIDE| (mM)
1·0
0·8
0·6
0·4
0·2
0
0
2·0
4·0
6·0
8·0
10
30
TIME (hr)

also diffuse into the cathodic compartment via the cation exchange membrane. A biofuel cell was set up containing borate buffer (250 mM, pH 10.5) and ammonium chloride (50 mM). Cyanide (10 mM) was added to the anodic compartment and its diffusion through the membrane measured colorimetrically by the removal of samples from the cathode at regular time intervals (see section 2.12.1). Over the three hour period tested, cyanide was shown to diffuse through the membrane, the concentraton in the cathodic compartment increasing at a rate of 1.6×10^{-5} mM min^{-1} cm^{-1}.

4.4.4 Biofuel cell efficiency.

Determination of the charge passed enabled the coulometric efficiency of the biofuel cell to be determined. This was shown to be high for primary alcohols up to decan-1-ol, formaldehyde and diols. The affinity of the enzyme for its substrate enabled the device to be used a sensor (see chapter 5). High coulometric efficiency at various concentrations of substrate is desirable. The effect of increasing the substrate concentration on the enzyme was investigated both polarographically and in a biofuel cell. When tested polarographically, methanol did not affect the specific activity of the enzyme up to 30 mM methanol. The effect of increasing methanol concentrations on the biofuel cell (see section 2.28) was tested. The maximum current attained and the charge passed were measured at various concentrations of methanol. Increasing the concentration of substrate resulted in a decrease in the coulometric efficiency (Fig 4.4.21); however,

Figure 4.4.21 The effect of methanol concentration on the coulometric output of the biofuel cell (see section 4.4.4).


CHARGE PASSED
(x 10^3 Coulombs)
6·0
4·0
2·0
0
0
2·0
4·0
6·0
8·0
10
12
|METHANOL| (mM)

the maximum current attained (0.25 mA) and the rate at which it was attained (0.3 mA min^{-1}) were unaffected by the concentration of methanol up to 12 mM.

The effect of variation in the external load was investigated. A biofuel cell was set up as described previously (see section 2.28). The charge passed was measured as previously described and the procedure repeated for a range of external resistances (Fig 4.4.22). Increasing the external resistance caused a decrease in the coulometric efficiency (Fig 4.4.23). The current efficiency of the biofuel cell was determined with TMPD at pH 10.5 when the enzyme was current limiting. The theoretical efficiency was determined from:

$$E = \frac{n.\ F.\ \text{Specific activity}}{60}$$

where n is the number of electrons transfered and F is the Faraday constant (96,487 coulombs per mole). A theoretical efficiency of 1.7 mA mg $protein^{-1}$ was predicted with TMPD. The observed current was, however, 1.25 mA mg $protein^{-1}$; representing 73% of the enzyme activity released as current at $20^{o}C$. The maximum power derived from the cell was 12 uW, eqivalent to 14 KW mol^{-1} catalyst, with a power density of 20 mW m^{2}.

The effect of formate, the product of the enzymic reaction, was investigated both polarographically and in the biofuel cell. No effect was observed polarographically in an oxygen electrode

Figure 4.4.22 The power and resistance characteristics of a TMPD mediated biofuel cell (see section 4.4.4).

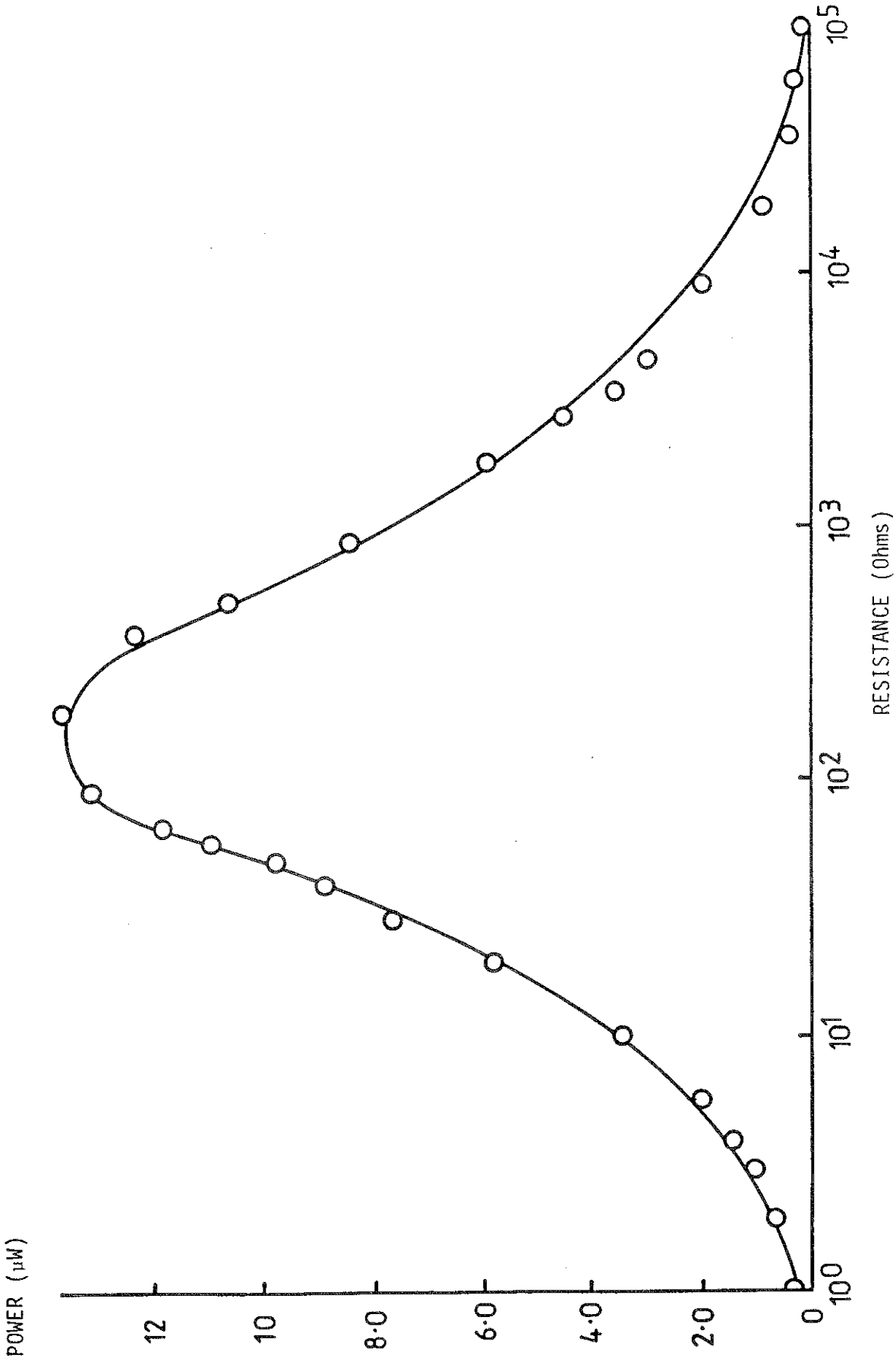
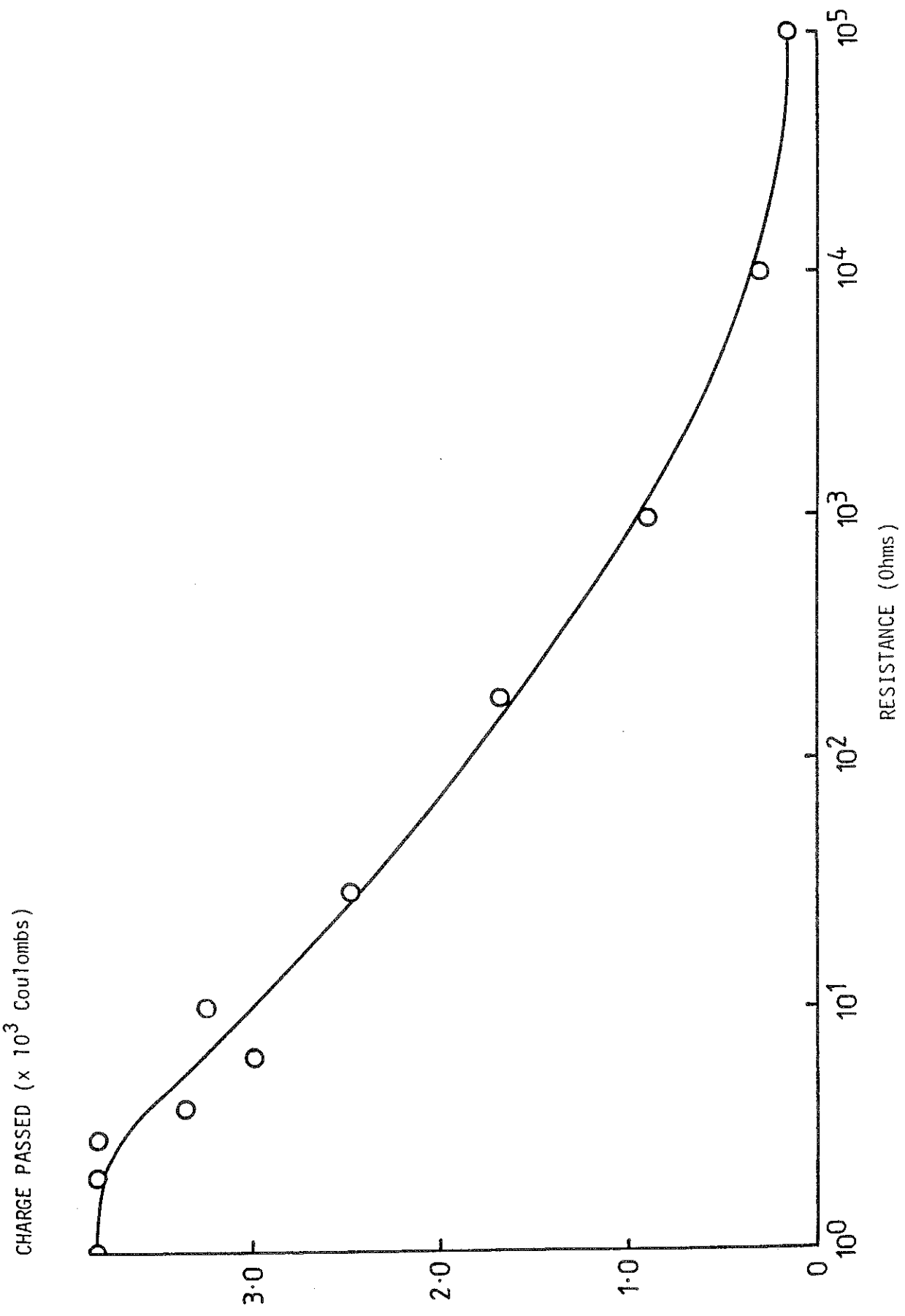

POWER (μW)
12
10
8·0
6·0
4·0
2·0
0
10^0
10^1
10^2
10^3
10^4
10^5
RESISTANCE (Ohms)

Figure 4.4.23 The effect of the external load on the coulometric output of a biofuel cell (see section 4.4.4).


CHARGE PASSED (x 10^3 Coulombs)
3·0
2·0
1·0
0
10^0
10^1
10^2
10^3
10^4
10^5
RESISTANCE (Ohms)

when tested up to 25 mM. A biofuel cell (see section 2.28) containing TMPD (0.8 mM) was set up. The rate of current increase (0.2 mA min^{-1}), the maximum current attained (0.21 mA) and the coulometric efficiency (100%) were unaffected by the presence of formate (2.5 mM).

In order to increase the coulometric efficiency of the biofuel cell the incorporation of formate dehydrogenase was considered. This enzyme brings about the conversion of formate to carbon dioxide and water, with the corresponding release of a further two electrons. The enzyme was shown spectrophotometrically to have a pH optimum of 7.0. The incorporation of formate dehydrogenase (1.0 mg) in a biofuel cell in the presence of NAD+ (1.4 mM) did not result in an increase in either current or coulometric yield.

The incorporation of a biological catalyst in the cathodic compartment has been previously described (see section 1.3.4). One system utilises laccase; an extracellular enzyme produced by Coriolus versicolor. The enzyme was prepared (see sections 2.7 and 2.10.3) and added to the cathodic compartment of a biofuel cell (see section 2.28) containing methanol dehydrogenase (0.5 mg). The addition of laccase (2.2 mg in phosphate buffer 0.2 ml, 100 mM, pH 6.0) did not affect the current produced by the biofuel cell.

4.4.5 Gas lift biofuel cell.

The laboratory studies described above used a biofuel cell

which was stirred with a magnetic stirrer and sparged with nitrogen. This would not be appropriate for practical devices, which would be expected to operate for prolonged periods of time with the minimum of attention. Electrodes should possess the maximum surface area whilst being both inert and economical. One possible electrode material is reticulated carbon. In laboratory studies involving this material, however, difficulty was encountered in maintaining electrical contact with the external circuit (see section 4.3.1). A device utilising reticulated carbon was constructed (see section 2.29) in which nitrogen gas was used to both mix and sparge the solution components. Preliminary experiments involved sparging nitrogen gas through the solution, but this caused bubbles to become entrapped within the electrode material. A system was constructed in which the nitrogen was passed along a tubular glass system; gas introduced at the bottom of the tube formed bubbles causing the solution to be lifted and hence circulated. Increasing the gas flow rate, however, caused the gas to flow out of the lower of the two tubes, past a constriction, causing the system to stop. A second configuration was adopted in which perspex sheets were cut and assembled as shown in Fig 2.12. This system was based on the same principle, but had the advantage that the flow of gas up the tube was maintained at high flow rates. In addition, the system was easily constructed and modified. A biofuel cell incorporating reticulated carbon was set up containing borate buffer (100 mM, pH 9.0), ammonium chloride (25 mM), methanol (100

mM) and phenazine ethosulphate (3.5 mM). The current increased with increasing concentration of enzyme until a maximum current of 0.5 mA was attained (Fig 4.4.24). The biofuel cell was washed out and the procedure repeated (Fig 4.4.24). Washing the electrode with distilled water and immersion in nitric acid caused a red colouration of the solution due to adsorbed mediator. Platinisation of reticulated carbon (see section 2.32) of the same dimensions and its use in this configuration did not result in currents greater than those obtained with non-platinised carbon.

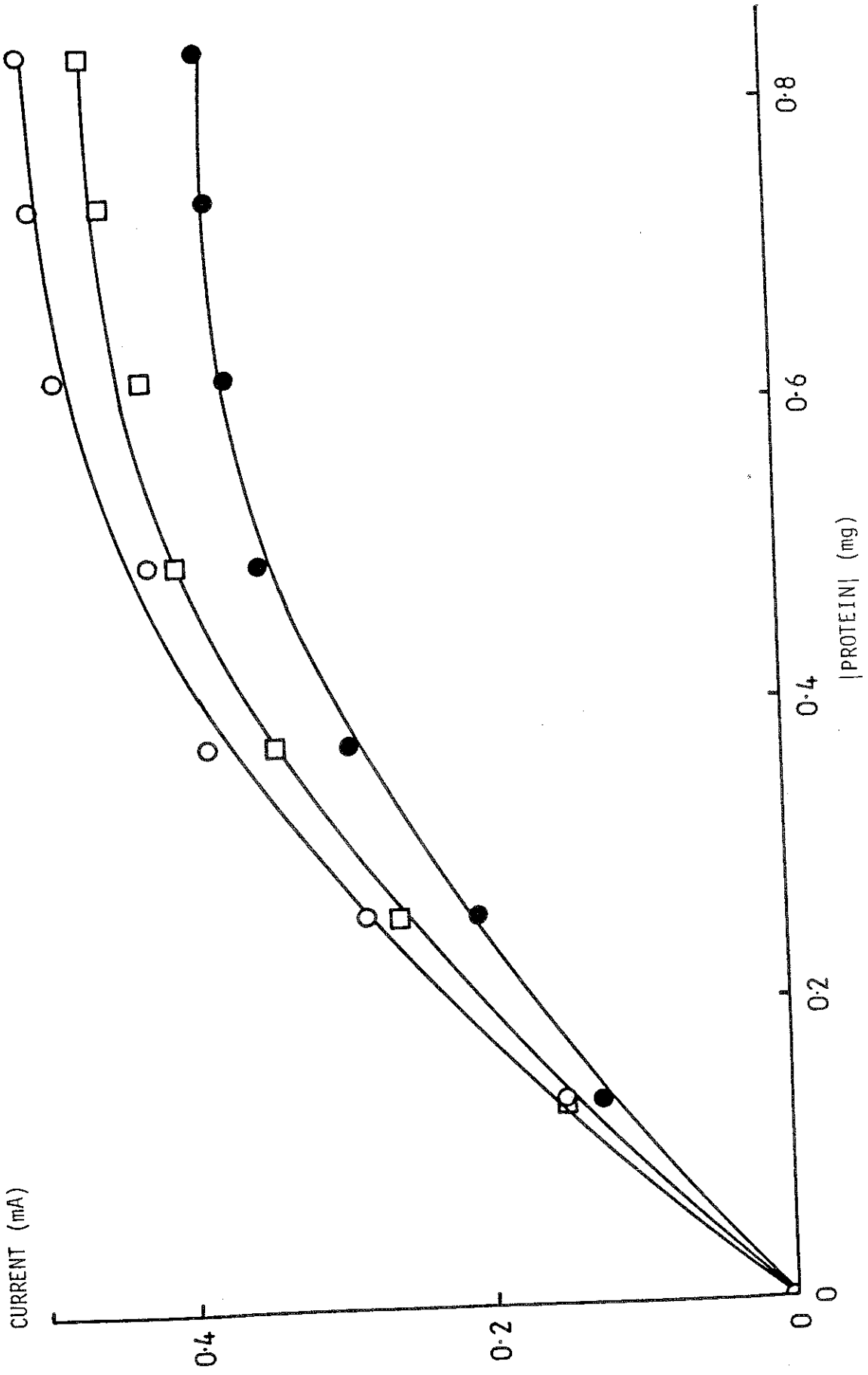
4.5 Discussion.

Efficient enzyme-based biofuel cells require the interaction of the biological catalyst with an electrochemically active mediator. The production of a current required the transfer of electrons from the substrate, via the enzyme, to the electrode; this process was facilitated by the use of mediators (Fig 1.4). Electrons pass around the external circuit to the cathode, where in the presence of protons, oxygen is reduced enabling electrical energy to be produced and hence work to be performed. Components of the methanol dehydrogenase-based biofuel cell, including mediators, membranes and electrodes were investigated.

A variety of membranes were examined with respect to their ability to retain the diffusable components of the biofuel cell,

Figure 4.4.24 The current response of a biofuel cell incorporating a reticulated carbon anode. The cell was degassed and agitated by the passage of gas bubbles around an isolated loop (see sections 2.29 and 4.4.5).

- ○ 1st run
- ● Repeat of the 1st run, after washing the cell with borate buffer
- □ Repeat of previous run, after dismantling the cell and rinsing the reticulated carbon anode in nitric acid


CURRENT (mA)
0·4
0·2
0
0
0·2
0·4
0·6
0·8
|PROTEIN| (mg)

such as oxygen, protons, substrate and mediator. Dialysis membrane and Nuclepore were shown to be freely permeable to oxygen, mediator and substrate. Cation exchange membrane (BDH Ltd., Poole, Dorset) allowed oxygen, substrate and mediator to diffuse across the membrane although at a lower rate. The diffusion of mediator across the membrane was shown to be influenced by the pH of the buffer and the mediator tested. Whilst none of the membranes tested possessed ideal properties, BDH cation exchange membrane was the best compromise, reducing the diffusion of oxygen, enzyme and mediator whilst allowing the diffusion of protons.

Although it was not possible to metallise this membrane by heating in the presence of gold resinate, it was possible to deposit gold and gold palladium by sputtering. The deposition of gold by this technique resulted in the deposited material cracking off the surface of the membrane when it was soaked in borate buffer. In the second method a thinner layer of gold palladium was deposited whilst maintaining the temperature of the membrane at approximately 25^{o}C. The modified membrane was capable of functioning as an electrode whilst retaining properties of the membrane such as proton diffusion. The subsequent deposition of carbon onto this metallised membrane enabled the immobilisation of an enzyme, glucose oxidase, in the presence of 1,1'-dimethylferrocene. The modified membrane did not produce a substrate-dependent current in a fuel cell, but did respond to glucose when poised in an amperometric configuration.

The properties of several electrode materials ranging from carbon to the noble metals were investigated for possible incorporation into the biofuel cell. These tests included measuring the resistance of the material, the ability to oxidise and reduce a mediator (TMPD), the chemical and physical stability in buffered solutions and the toxicity of the metal ions to methanol dehydrogenase. Of the materials tested, two were found to be suitable for incorporation into the cell: reticulated carbon and platinum. Reticulated carbon was, however, brittle and unable to withstand the repeated handling during experiments although it was successfully incorporated in a biofuel cell. This cell did not use a magnetic stirrer and both agitation and sparging were achieved by the passage of gas bubbles in a separate column connected to the cell. The use of commercially available reticulated carbon sheets would have advantages in scale up as it would enable electrodes of large surface area to be constructed. By placing these sheets close together it would eliminate the requirement for stirring at the same time as reducing the internal resistance of the biofuel cell. Recent work involving whole organism biofuel cells has also utilised this principle (Delany et al., 1983, 1984).

In order to overcome some of the disadvantages of a cell mediated by PES (Plotkin et al., 1981) a range of possible mediators were investigated by a variety of polarographic, colorimetric and electrochemical techniques. Of those tested, methoxy phenazine methosulphate (mPMS), thionin and TMPD were

demonstrated to be capable of coupling to methanol dehydrogenase. mPMS was photochemically stable but was not stable at the optimum pH of the enzyme. Thionin, used in previous whole organism biofuel cell studies (Bennetto et al., 1983) precipitated out of alkaline solutions in the methanol dehydrogenase based biofuel cell. TMPD was photochemically stable, capable of reversible electron transfer and stable at pH 10.5. A biofuel cell utilising methanol dehydrogenase, coupled to a platinum electrode via TMPD, produced a current at ambient temperatures and pressures. The device utilised a range of primary alcohols up to decanol, certain diols and formaldehyde with high current and coulometric efficiency.

At low substrate concentrations, the high coulometric efficiency of the cell enabled it to be used in the quantitative determination of primary alcohols. Increasing the substrate concentration and the external resistance caused a decrease in the coulometric efficiency; an effect also observed in methanol based inorganic fuel cells. The problems associated with such systems are mainly due to (1) slowness of the electrode reaction of methanol and its intermediates, (2) poisoning of the electrode by the intermediates, (3) formation of formaldehyde and formic acid by products on a number of electrocatalysts (Bockris and Srinivasan, 1969).

NAD^+-linked formate dehydrogenase from Pseudomonas oxalaticus was incorporated into the biofuel cell in the presence of NAD in order to bring about the complete oxidation of methanol

to carbon dioxide. This linkage did not, however, result in an enhancement of either current or charge passed. Formate dehydrogenase was shown to have a pH optimum of 7.0 whilst the corresponding optimum of methanol dehydrogenase was 10.5.

Whilst methanol dehydrogenase was shown to be stable in solution, the presence of mediator (TMPD) in the biofuel cell caused inactivation of the enzyme. Stabilisation of methanol dehydrogenase in the absence of substrate was investigated using several potential stabilising agents. It was proposed that addition of substrate would cause the displacement of the inhibitor and production of a current, utilisation of the substrate would result in rebinding and hence stabilisation. Hydroxylamine, a competitive inhibitor of methanol dehydrogenase enhanced the stability of methanol dehydrogenase, however, it readily undergoes oxidation (Morrison and Boyd, 1972; Windholz et al., 1976). In biofuel cells the cathode is aerated (9.0 ppm O_2) and oxygen has been shown to diffuse across the cation exchange membrane (see section 4.2.2), facilitating the oxidation of the hydroxylamine.

The biofuel cell could be operated for periods of up to 12 hours. Some advance towards continuous operation was achieved by linking the enzyme to an insoluble mediator (1,1'-dimethylferrocene) which was coated onto the surface of a carbon electrode deposited on a cation exchange membrane. The immobilisation of methanol dehydrogenase onto such a modified membrane would enable electrodes to be constructed which would be

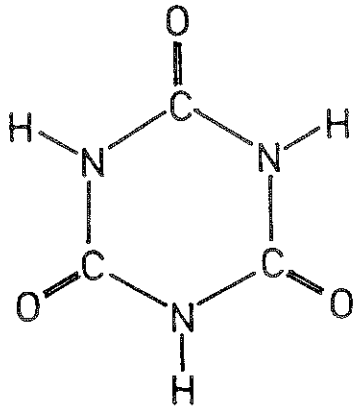
thin whilst possessing large surface areas. Substrate could be pumped though the cell facilitating continuous operation.

Loss in efficiency of the biofuel cell may be due to side reactions or short circuits within the cell. This may involve either reactions of the substrate or intermediates with other components present in solution or reoxidation of the mediator, due to oxygen or mediator diffusion through the membrane.

Recent uncompleted preliminary work carried out in collaboration with Dr.J.K.M. Sanders (Cambridge University) using 13C-nuclear magnetic resonance, has shown that methanol dehydrogenase produces free formaldehyde in the presence of methanol. The enzymic product of the biofuel cell reaction, formate, did not inactivate the enzyme at the concentration tested (2.5 mM) but formaldehyde may inactivate the enzyme. Interpretation of the 13C-nmr spectra suggested that free formaldehyde reacted with ammonium ions in the buffer, producing hexamine (Fig 4.4.25), which may reduce the coulometric efficiency and have a deleteroius effect on the enzyme.

This work has demonstrated the coupling of methanol dehydrogenase to an electrode using both soluble and insoluble mediators. The crude configuration described may have applications in the study of enzymes and enzymic reactions by enabling the stoichiometry and kinetics of reactions to be determined whilst also allowing product analysis. Development of mediated enzyme and whole cell biofuel cells may have both industrial and clinical applications. Indirect biofuel cells are

Figure 4.4.25 HEXAMINE, the proposed structure of the reaction between formaldehyde and ammonia (see section 4.5).



currently being developed to enable the utilisation of waste water (Suzuki et al., 1978, 1979). Biofuel cells may also be used in the detoxification of water supplies. The application of biofuel cells in clinical analysis has centred on heart pacemakers, the configurations described typically utilise soluble mediators (Drake et al., 1968; Avampato et al., 1974). Ferrocene and its derivatives posses properties which offer alternative mediated systems, which have to compete with non biological fuel cells and batteries. However considerably more work is required to produce a practical configuration but the application of enzyme linked systems has led to the development of enzyme based sensors. These have generated much interest for both industrial and clinical applications. Preliminary results for two configurations are described, including a homogeneous methanol sensor utilising soluble components (chapter 5) and an immobilised glucose sensor (chapter 6).

CHAPTER 5

RESULTS AND DISCUSSION: DEVELOPMENT OF AN ENZYME -BASED METHANOL SENSOR

5.1 Limits of detection of the methanol sensor.

Methanol was determined using either pure or crude extracts of methanol dehydrogenase in a biofuel cell (see section 4.4). The total charge passed or the current was measured (Figs 4.4.12). Problems associated with the use of a biofuel cell as an analytical instrument included the tendency for the substrate to diffuse out of the anode and the necessity for an air cathode (see sections 4.1 and 4.4).

A more practical configuration was adopted, as described in section 2.31. The sensor consisted of a coulometric device in which the potential of a platinum gauze was poised at +100 mV with respect to a standard calomel reference electrode (SCE) (Fig 5.1). By measuring the maximum current or the charge passed (Fig 5.2), the sensor was shown to be capable of detecting methanol in solution at concentrations as low as 0.025 mg l^{-1}, lower than can be attained using conventional gas chromatography (Fig 5.3). The criteria for testing the methanol sensor were those laid down by the Water Research Centre (WRC) for a feasibility study carried out by members of staff of CIT.

The limits of detection of the enzyme based methanol sensor were determined using methanol standards made up in distilled water. The total charge passed was measured after the addition of aliquots of sample (100 ul). The lowest limit of detection was calculated as 0.03 mg l^{-1} (0.94 uM).

The reproducibility of the system was determined in the range, 0.025 - 1.0 mg l^{-1}. Five batches of methanol, consisting

Figure 5.1 The effect of the applied potential on the amperometric response of a homogeneous enzyme based methanol sensor.

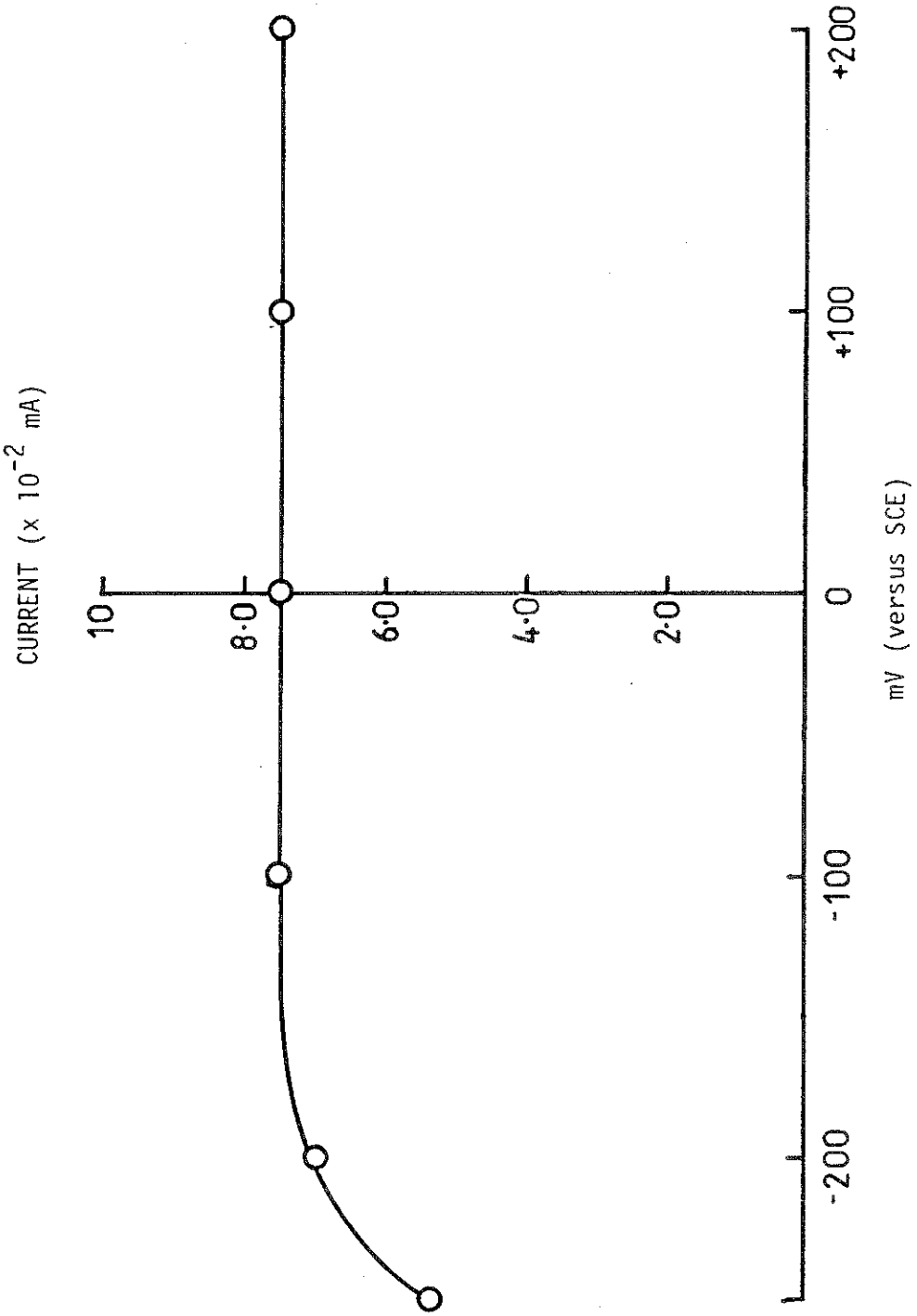

CURRENT (x 10^{-2} mA)
10
8·0
6·0
4·0
2·0
0
-100
-200
+100
+200
mV (versus SCE)

Figure 5.2 Calibration curve for methanol using a homogeneous methanol sensor. Determined by measuring the peak height (O) or the area under the curve (●).

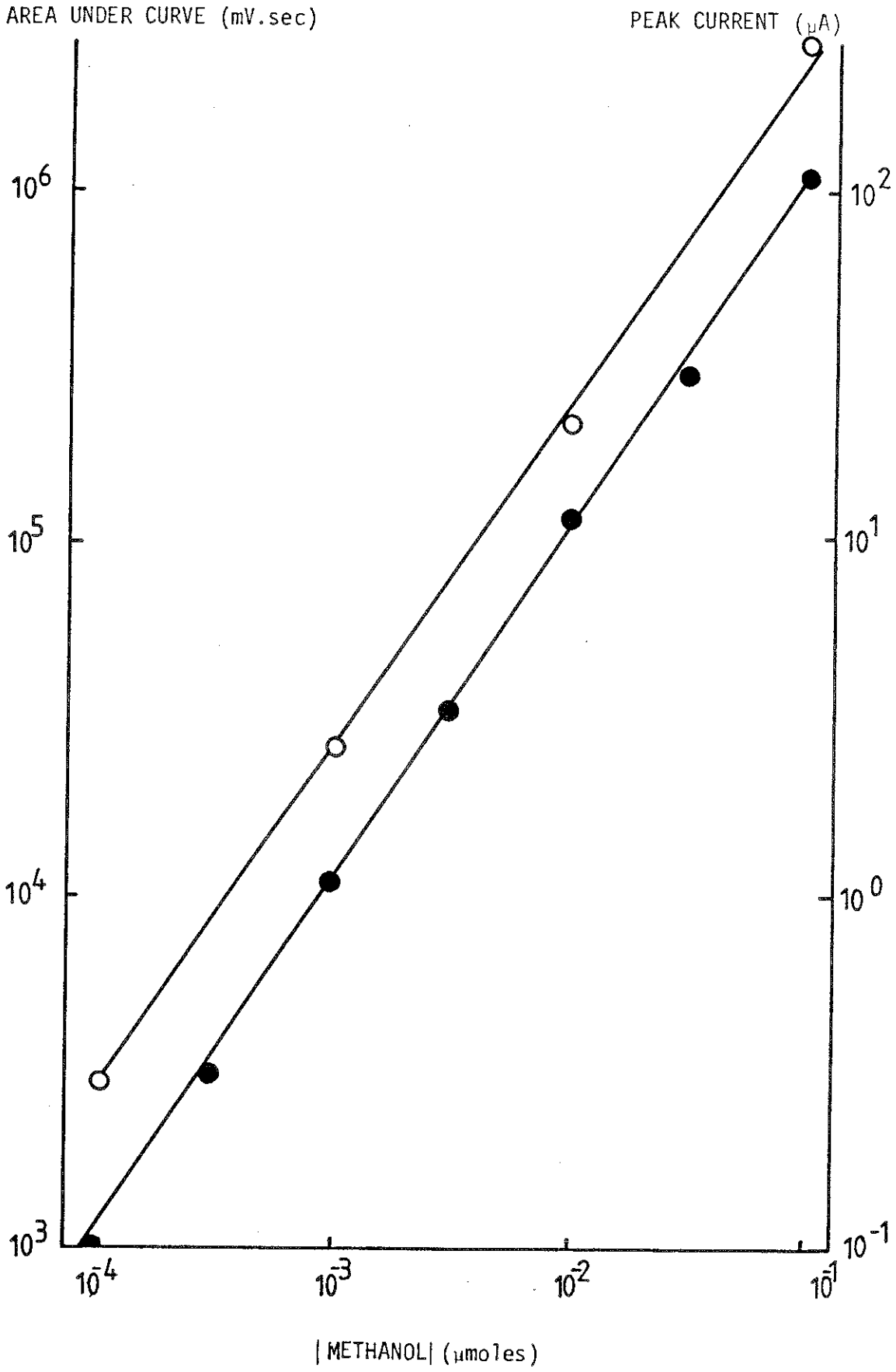
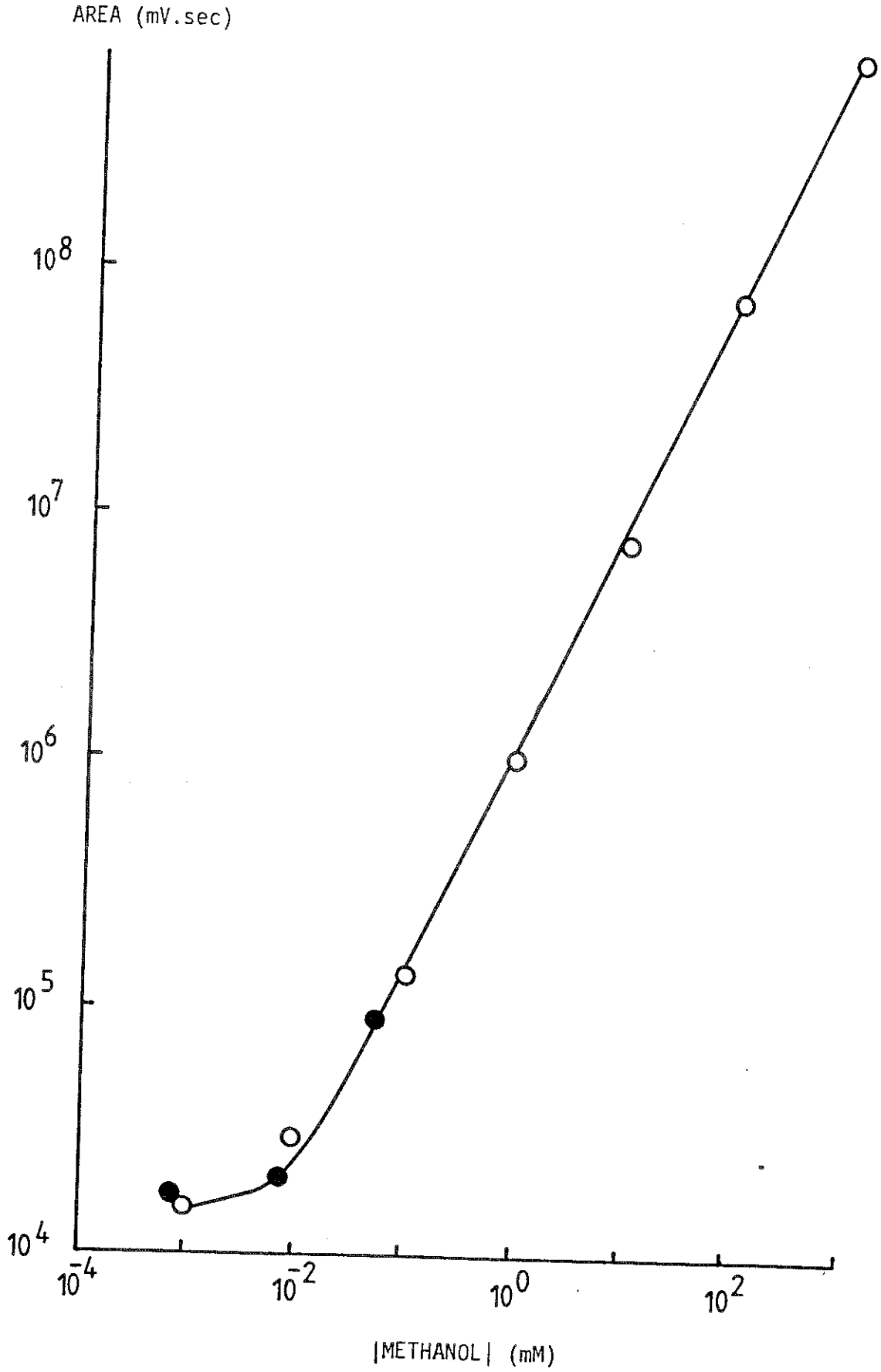

AREA UNDER CURVE (mV.sec)
PEAK CURRENT (μA)
10^6
10^5
10^4
10^3
10^2
10^1
10^0
10^-1
10^-4
10^-3
10^-2
10^-1
| METHANOL | (μmoles)

Figure 5.3 A calibration curve for methanol, determined using gas chromatography. Using a Porapak Q column at a column temperature of 150^{o}C and nitrogen at a flow rate of 40 ml min^{-1} (O). (●) are the concentrations of methanol in solution determined by the homogeneous enzyme based methanol sensor.


AREA (mV.sec)
10^8
10^7
10^6
10^5
10^4
10^{-4}
10^{-2}
10^0
10^2
|METHANOL| (mM)

of four replicates at 0.025, 0.25 and 1.0 mg l^{-1} were analysed. The order of measurement being randomised with respect to the methanol concentrations. Each batch was analysed using a freshly prepared cell on different days and was divided into two runs of two replicates, each pair of replicates was preceeded and followed by five calibration standards. The calibration preceeding each run was analysed by simple linear regression and used to convert the sample reading to mg l^{-1} methanol. Analysis of variance was performed (Table 5.1) and the detection limit (for $\alpha = \beta = 0.05$) of the sensor calculated according to the following equation:

$$2t_{0.1}\sqrt{2.S_w}.$$

Where t is the student t and S_w the within batch standard deviation. The results (Table 5.1) show that even a crude enzyme electrode configuration may be used in analysis at concentrations as low as 0.25 mg l^{-1}. Whilst the concentrations of methanol in the standard solutions were 0.025, 0.25 and 1.0 mg l^{-1}, due to dilution the actual concentrations in the detector were less than 7.0×10^{-4}, 7.0×10^{-3} and 3.1×10^{-2} mg l^{-1} respectively.

The similarity of between batch and within batch standard deviations (Table 5.1) implies that errors were due to the technique and not interference due to contaminants. The confidence limits for the mean determination of methanol (0.25 mg l^{-1}) at a 95% probability are +- 0.07 mg l^{-1} representing a reasonable accuracy of estimation of methanol concentrations in

Table 5.1 Determination of the precision of methanol detection using the homogeneous enzyme based methanol sensor. The means and standard deviations were calculated from 20 determinations (5 batches x 4 replicates).

		Degrees of Freedom	Concentration of Methanol in Distilled Water mg l^{-1}		
Prepared concentration		-	0.025	0.25	1.0
Mean of determinations	$\bar{x}$	-	0.0312	0.2506	1.0085
Standard deviation	s	19	0.0120	0.0379	0.0905
Standard deviation between batches	s_b	4	0.0094	0.0238	0.0655
Standard deviation within batches	s_w	15	0.0073	0.0295	0.0624

water.

5.2 Interference tests

The response of the detector was tested with river water obtained from the Great Ouse to which methanol had been added at 0.25 mg l^{-1}. The means and standard deviations were each calculated from 20 determinations, consisting of 5 batches of 4 replicates. The response of the sensor to river water alone (Table 5.2) although below the estimated limit of detection may be an indication of a low concentration of enzymically or electrochemically oxidisable substrates. A sample of denitrified river water (supplied by the WRC) was divided into two portions: to one, methanol was added, to give a final concentration of 1.0 mg l^{-1}. The samples were filtered, through a 0.22 um filter, to clarify the solutions and duplicate determinations of methanol concentration made against known methanol standards made up in distilled water. The means and standard deviations of the determinations of added methanol were, at 0 and 1.0 mg l^{-1} in denitrified river water, 0 and 0.9684 +- 0.0651 mg l^{-1} respectively.

The response of the detector to formaldehyde was also investigated. Formaldehyde was prepared by boiling paraformaldehyde in a sealed container (see section 2.12.3) then known aliquots were added to the detector. The charge passed was approximately half (48%) that produced by an equal concentration of methanol. The ability of the methanol dehydrogenase based

Table 5.2 The effect of the addition of methanol to river water samples obtained from the Great Ouse, on the response on the homogeneous enzyme based methanol sensor. Means and standard deviations were calculated from 20 determinations (5 batches x 4 replicates).

Concentration added ($mg\,l^{-1}$)		0	0.25
Mean of determination	$\bar{x}$	0.0281	0.2437
Standard deviation	s	0.12	0.0350
Standard deviation between batches	s_b	0.0104	0.0163
Standard deviation within batches	s_w	0.006	0.0310

sensor to detect formaldehyde in solution allowed the analysis of formaldehyde in cutting oils and biocides (see section 5.4).

Interference tests were performed for chemicals present in drinking water, at concentrations greater than the upper limits normally encountered. The interference chemicals were made up in distilled water and added to the cell to give the required final concentration shown (Table 5.3). Triplicate additions of methanol (1.0 mg l^{-1}) were made to the reaction mixture and the charge passed was determined. The interfering chemical to be tested was added followed by a second set of triplicate standards. The cell was cleaned and reassembled for each of the seven interference compounds tested, a total of 42 results were obtained.

5.3 Operational stability

The effect of temperature on the stability of the sensor was investigated, in relation to both the peak current and charge passed in the temperature range 20 - 50^{o}C. The peak current increased with increased temperature (Fig 5.4), as observed previously with soluble enzyme (Fig 3.8), whilst the charge passed remained constant. Increasing the temperature, however, decreased the stability of the sensor eg. at 50^{o}C the sensor became inactivated within an hour. At 30^{o}C, although the peak height decreased with time, measurements of the charge passed, enabled the sensor to be operated continuously for over 12 hours, whilst at 20^{o}C continuous operation was maintained for 24 hours.

Table 5.3 Interference tests of possible contaminants present in water supplies tested at concentrations greater than normally encountered.

Compound	Concentration l^{-1}	Result $mg.l^{-1}$
Sodium hypochloride	2.0 mg Cl_2	1.07
Sodium sulphite	2.0 mg SO_2^-	0.995
Ammonium sulphate	10.0 mg NH_4^+	0.920
Chloramine	2.0 mg NH_2Cl	1.115
Ferric chloride	2.0 mg Fe^{3+}	0.955
Aluminium oxide	1.0 mg Al^{3+}	0.910
Lead acetate	50 µg Pb^{2+}	1.02*
Copper sulphate	50 µg Cu^{2+}	
Zinc sulphate	1000 µg Zn^{2+}	
Nickel chloride	50 µg Ni^{2+}	
Mercuric chloride	1.0 µg Hg^{2+}	
Cadmium sulphate	5.0 µg Cd^{2+}	

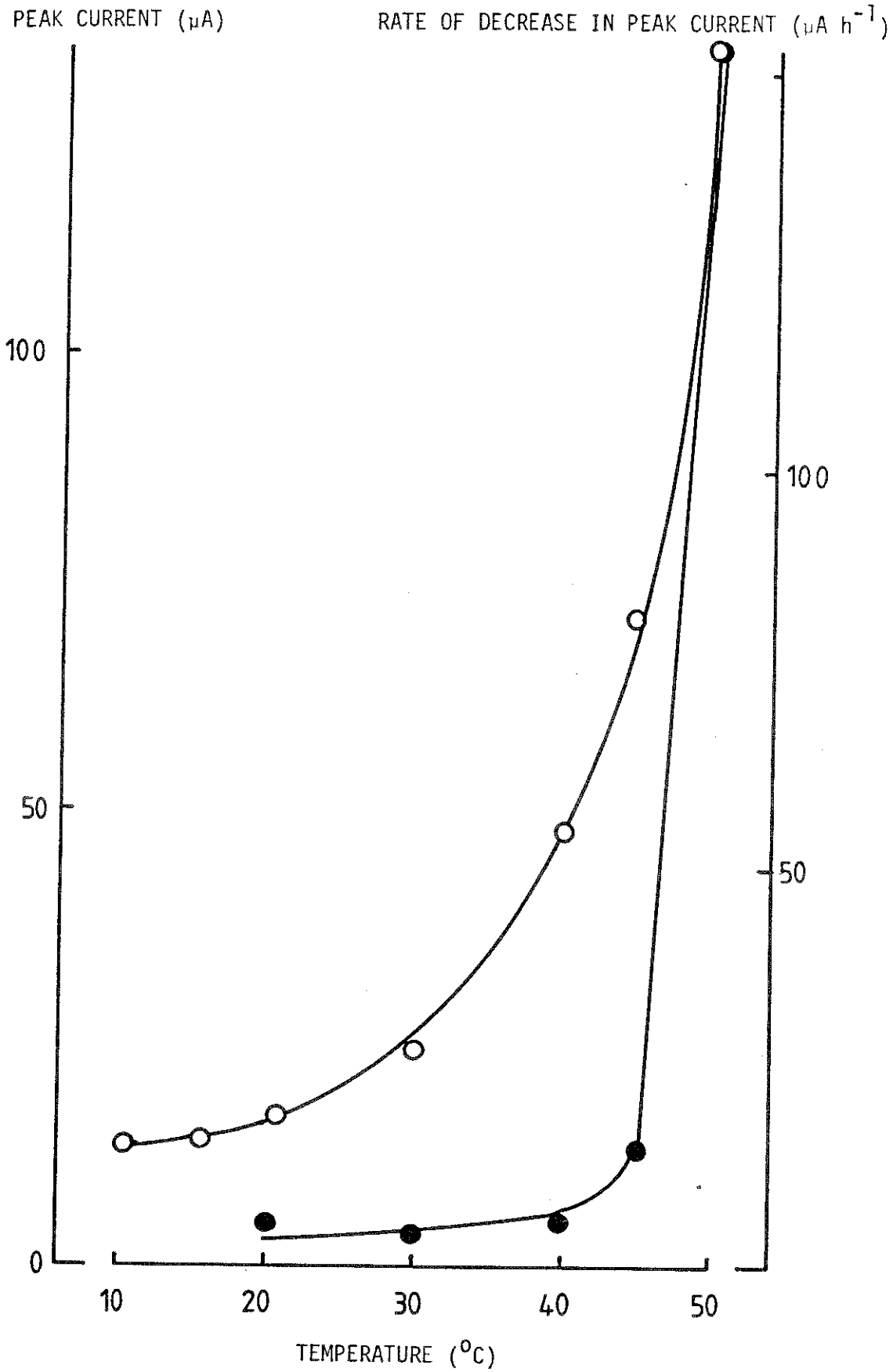
	Sum of Squares	Difference	Mean of Squares
Treatments	0.988	13	0.076
Error	9.7522	70	0.139
Total	10.74	83	0.129

$$LSD = t_{(0.5)}\frac{\sqrt{2s^2}}{n}$$

$= 0.15\ mg.l^{-1}$ for 13 degrees of freedom

* Heavy metals were added as a single solution

Figure 5.4 The effect of temperature on the peak current (O) and stability (●) of the homogeneous methanol sensor.


PEAK CURRENT (μA)
RATE OF DECREASE IN PEAK CURRENT (μA h⁻¹)
100
50
0
100
50
10
20
30
40
50
TEMPERATURE (°C)

4 Analysis of formaldehyde releasing biocides and cutting ls

The ability of methanol dehydroganse to oxidise ›rmaldehyde enabled the homogeneous methanol sensor to be used ı the analysis of formaldehyde in commercially available ocides and cutting oils. Samples of three commercially ⁄ailable biocides and three formaldehyde releasing cutting oils ›re kindly supplied by Dr.E.C.Hill, University College, Cardiff, ıd the published properties of these compounds are shown (Table .4). The methanol sensor was set up as described previously and ıe charge passed measured on the addition of triplicate aliquots f methanol standards (10 ul). Methanol standards were used ›cause of their availability and it had been shown previously ıat the charge passed upon the addition of methanol was ›proximately twice that of formaldehyde. The formaldehyde ›leasing biocides were prepared at the concentrations recomended ›r use in industrial situations (Table 5.4). Triplicate ddition of the samples (10 ul) were added in a random order, ollowed by further methanol standards. The charge passed was sed to convert directly the readings into the concentration of ormaldehyde. Although the limits of formaldehyde detection were ot determined, the results show that the sensor was capable of etecting an enzymically oxidisable substrate in some of the olutions tested and at the concentrations used in industrial ituations (Table 5.4). No detectable response was, however, bserved in two of the cutting oils tested, even with the sample

Table 5.4 Properties and analysis of formaldehyde releasing biocides and cutting oils (see section 5.4).

Chemical Name FORMALDEHYDE releasing Biocides	Commercial Trade Name	Acute Toxicity	Concentration tested	Coulometric response (mM)
Hexahydro-1,3,5 Tris (2-Hydroxyethyl) striazine	GROTAN BK	0.678-0.944 ml kg	0.1% v/v	7.8
Tris (hydroxy methyl) nitromethane	TRIS NITRO	0.5-1.0 g kg^{-1}	0.1% w/v	0.1
Hexahydro-1,3,5 Triethyl-5-triazine	VANICIDE	0.316 ml kg^{-1}	0.1% w/v	7.9
FORMALDEHYDE releasing Cutting Oils				
No information available	DROMAS B	No information available	1.0% v/v	0
No information available	DELAPENA S750	"	1.0% v/v	7.0×10^{-2}
No information available	KUTWELL 40	"	2.0% v/v	0

size increased to 100 ul.

5.5 Enzyme based methanol probe

A soluble derivative of ferrocene, amino ferrocene, was shown to be capable of mediating in a homogeneous sensor configuration irrespective of the oxygen tension. This was demonstrated by the addition of methanol (10 ul, 1.0 mM) to a homogeneous methanol sensor containing amino ferrocene (1.0 mM) ammonium chloride (50 mM) and methanol dehydrogenase (0.27 mg) in borate buffer (250 mM, pH 10.5). The peak current (6×10^{-5} Amps) and the area under the curves upon the addition of methanol were unaffected when sparged alternately with oxygen and nitrogen. When operated over a two hour period the peak current initially decreased at a rate of 3.75×10^{-7} Amps min^{-1}, irrespective of the gas sparged.

The coupling of the enzyme to the insoluble mediator 1,1'-dimethylferrocene enabled the construction of a enzyme-based methanol probe. The sensor was prepared as previously described (see section 2.35) with the enzyme retained behind either dialysis or Nuclepore membrane. The electrode was placed in borate buffer (250 mM, pH 10.5) maintained at $25^{o}C$ and sparged with nitrogen. The potential of the electrode was poised at +150 mV (versus SCE) with a platinum auxillary electrode. Aliquots of methanol (10 ul) were added and the steady state current measured. The sensor detected methanol at concentrations as low as 0.03 mg l^{-1}, exhibited a linear response up to 3.2 mg l^{-1} and

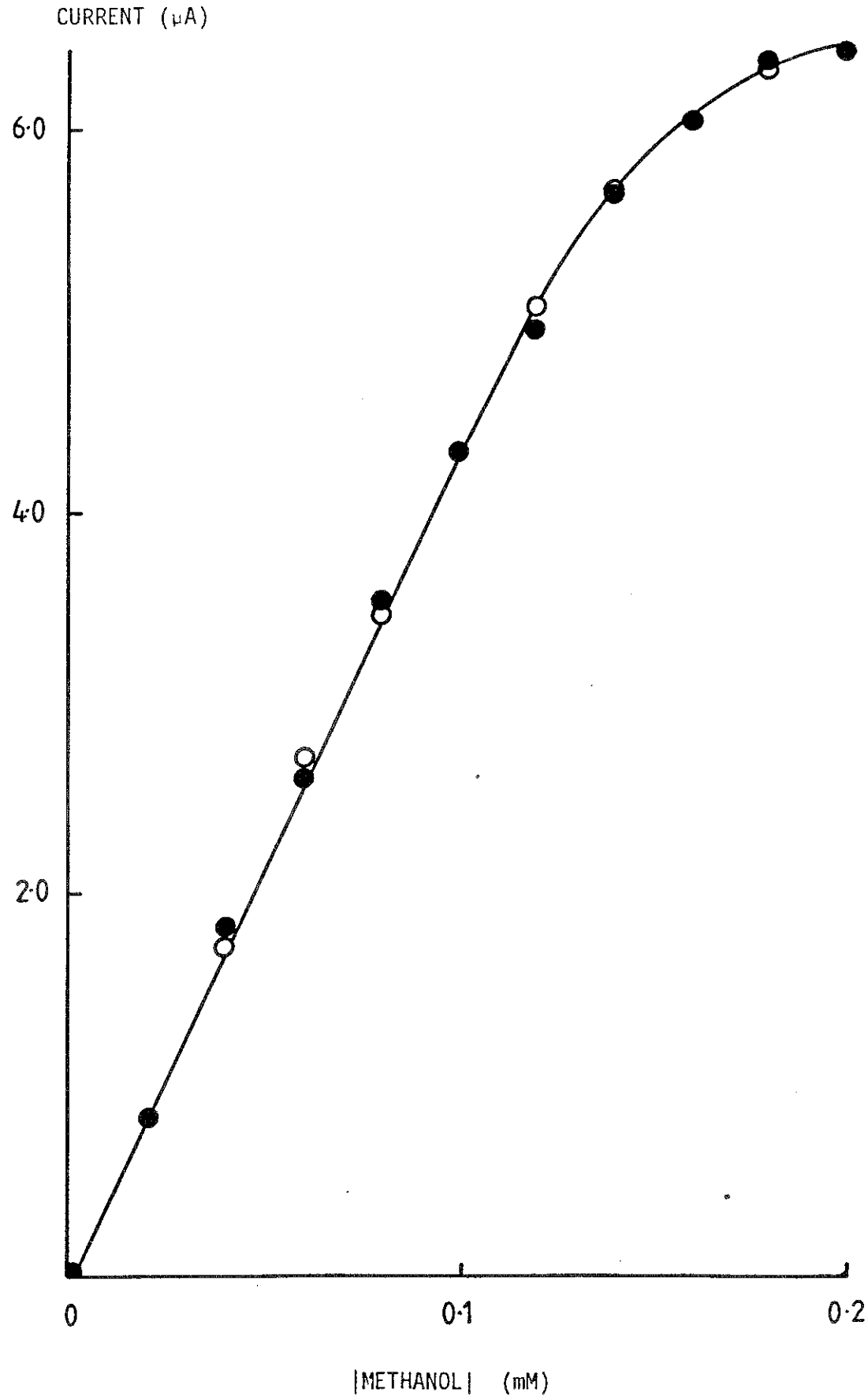
attained steady state currents within 20 seconds of the addition of a sample. Fluctuations in oxygen tension did not affect the response of the probe; this was demonstrated by alternate sparging of the buffer reaction mixture with oxygen and nitrogen (Fig 5.5). Whilst the homogeneous sensor was stable for 12 hours at 30^{o}C, the enzyme based probe lost 90% activity within 3.0 hours.

Interference due to compounds and ions in solution may be reduced or eliminated by the use of ion exchange membranes. Either cation or anion exchange membranes were mounted in a silicon rubber sleeve, soaked for 24 hours in borate buffer (250 mM, pH 10.5) and placed over the end of the enzyme probe. These modified sensors, however, did not elicit a detectable response in the range tested 0 - 0.2 mM. This may be due to the reduced rate at which methanol diffuses through ion-exchange membranes (see section 4.2.5).

5.6 Other configurations tested

N-methylphenazinium tetracyanoquinodimethanide (NMPTCNQ) prepared by the method of Melby (1965) was obtained from Imperial College, London. The mediator was made up in a graphite paste as previously described for the 1,1'-dimethylferrocene paste electrode, (see section 2.35) but containing NMPTCNQ (125 mg) as mediator. The enzyme (4 x 10^{-2} mg) was retained at the electrode surface by dialysis membrane. The probe was placed in buffer (250 mM, pH 10.5) containing ammonium chloride (50 mM) and

Figure 5.5 The steady state amperometric response of an enzyme based methanol sensor. The enzyme methanol dehydrogenase was retained at the electrode surface using dialysis membrane and linked to the electrode via an insoluble, non-autooxidisable mediator, 1,1'-dimethylferrocene. Sparged with oxygen (O) and nitrogen (●).


CURRENT (μA)
6·0
4·0
2·0
0
0·1
0·2
|METHANOL| (mM)

maintained at 25^{o}C. The potential of the electrode was poised at +200 mV (versus SCE) with a platinum counter electrode. Aliquots of methanol were added and the steady state current measured. NMPTCNQ was capable of acting as a mediator between methanol dehydrogenase and an electrode. The electrode responded rapidly upon the addition of methanol (less than 20 seconds to steady state). In the presence of 1.0 mM methanol the response was unaffected by sparging with either air or nitrogen and in both cases 90% of the initial activity of the probe was lost after 12 minutes. Polyviologen incorporated in the graphite paste did not mediate in the range 0 - 0.2 mM methanol.

5.7 Discussion

The ability of the methanol sensor to detect methanol in solution was demonstrated, enabling quantitative determinations to be made in the range 0.25 - 1.0 mg l^{-1}. The feasibility of using a poised potential detector for industrial water analysis was examined.

Due to intensive farming techniques the water industry has become increasingly concerned over the past few years with rising nitrate levels in water supplies. High nitate levels have been shown to cause secondary (toxic) methaemoglobinaemia. In this condition haemoglobin is converted to methaemoglobin which is unable to bind oxygen; this is often fatal for young infants (Darling and Roughton, 1942; Cornblath and Hartman, 1948; Ross and Deforges, 1959; Jaffer and Heller, 1964).

A maximum acceptable concentration of nitrate (NO_3, 50 mg l^{-1}) has been set by the EEC to be implemented by 1985. One technique currently being investigated for the removal of nitrate involves the use of the natural methylotrophic flora. These organisms when supplied with a source of energy (methanol) are capable of carrying out denitrification (Gauntlett and Zabel, 1982). A temporary limit of 0.25 mg l^{-1} methanol has been set by the committee on New Chemicals and Materials of Construction for use in Public Water Supply and Swimming Pools, while the denitrification process is evaluated.

The method presently used to assess the quality of waste water discharge involves monitoring the ventilatory behavior of fish (Gruber and Cains, 1981). An alternatve method involves monitoring the light output of luminescent bacteria (Bulich, 1982). The use of an indirect enzyme electrode for alcohol monitoring has been described based on glutaraldehyde immobilised alcohol oxidase (Verduyn et al., 1983). By measuring oxygen consumption the electrode was capable of responding to alcohols within 1.0 - 2.0 minutes, was linear in the range 1 - 25 mg l^{-1} and was stable for over 2 weeks. Alcohol electrodes based on the consumption of oxygen or the production of hydrogen peroxide (Clark, 1972; Mason, 1983), are affected by fluctuations in oxygen tensions and require the removal of catalase (see section 1.4).

These results show that an enzyme based sensor utilising crude cell free extracts of methanol dehydrogenase may be used in

the analysis of water samples. The standard deviations, although somewhat high for an analytical technique enabled the determination of methanol at concentrations as low as 0.25 mg l^{-1}. The means and standard deviations of the determinations of methanol at 0 and 1.0 mg l^{-1} were comparable to the experiments involving common pollutants. The differences caused by the addition of the test substances were all less than the least significant difference (LSD) of 0.15 mg l^{-1}, calculated in Table 5.3. These are comparable to the earlier experiments involving the response of the detector to river water containing added methanol, implying that the addition of the pollutants at the concentrations used did not cause interference. Formaldehyde elicited half the response obtained with the same concentration of methanol, enabling the sensor to function as a formaldehyde detector. Although the concentration of free formaldehyde in the cutting oils and biocides tested was not confirmed by a second method; the sensor was capable of detecting an enzymically oxidisable substrate in the formaldehyde releasing biocides.

Coupling the enzyme to an insoluble mediator demonstrated that it was possible to construct a probe which enabled methanol concentrations to be determined rapidly under various oxygen tensions. The immobilisation of this enzyme in a configuration similar to that described for glucose analysis (see chapter 6) would be more practical and have applications both in clinical (Clark, 1972) and industrial analysis (Verduyn et al., 1983; Mason, 1983).

CHAPTER 6

RESULTS AND DISCUSSION: DEVELOPMENT OF AN ENZYME -BASED GLUCOSE SENSOR

This work was done in collaboration with Dr.H.A.O.Hill at the Department of Inorganic Chemistry, Oxford University. The work described in this section was carried out by myself as part of a multidisciplinary group at Cranfield Institute of technology and Oxford University.

6.1 Effect of physical and chemical factors on the enzyme-based glucose sensor.

The immobilisation of glucose oxidase to graphite foil in the presence of the mediator 1,1'-dimethylferrocene (see sections 2.36 and 2.37) enabled glucose concentrations to be determined amperometrically. The steady state current was shown to be dependent on the potential applied to the enzyme electrode. Increasing the potential caused a corresponding increase in the current until a maximum was achieved (Fig 6.1). Poised at +150 mV (versus SCE) the enzyme electrode gave a linear response to glucose at concentrations between 0 - 30 mM. Steady state currents were rapidly attained enabling glucose concentrations to be determined within 1.0 minute. More rapid determinations were possible by making measurements prior to the attainment of steady state (Fig 6.2). Rapid stirring (2500 rpm) in the presence of 8.0 mM glucose caused a 10% increase in the steady state amperometric response of the electrode when compared to measurements in an unstirred solution.

The effect of pH in the range pH 5.5 to 9.0, on the

Figure 6.1 The effect of the applied potential (mV versus SCE) on the amperometric response of a glucose oxidase based enzyme electrode, in phosphate buffer (50 mM, pH 7.4) (O) and in the presence of glucose (30 mM) (●).

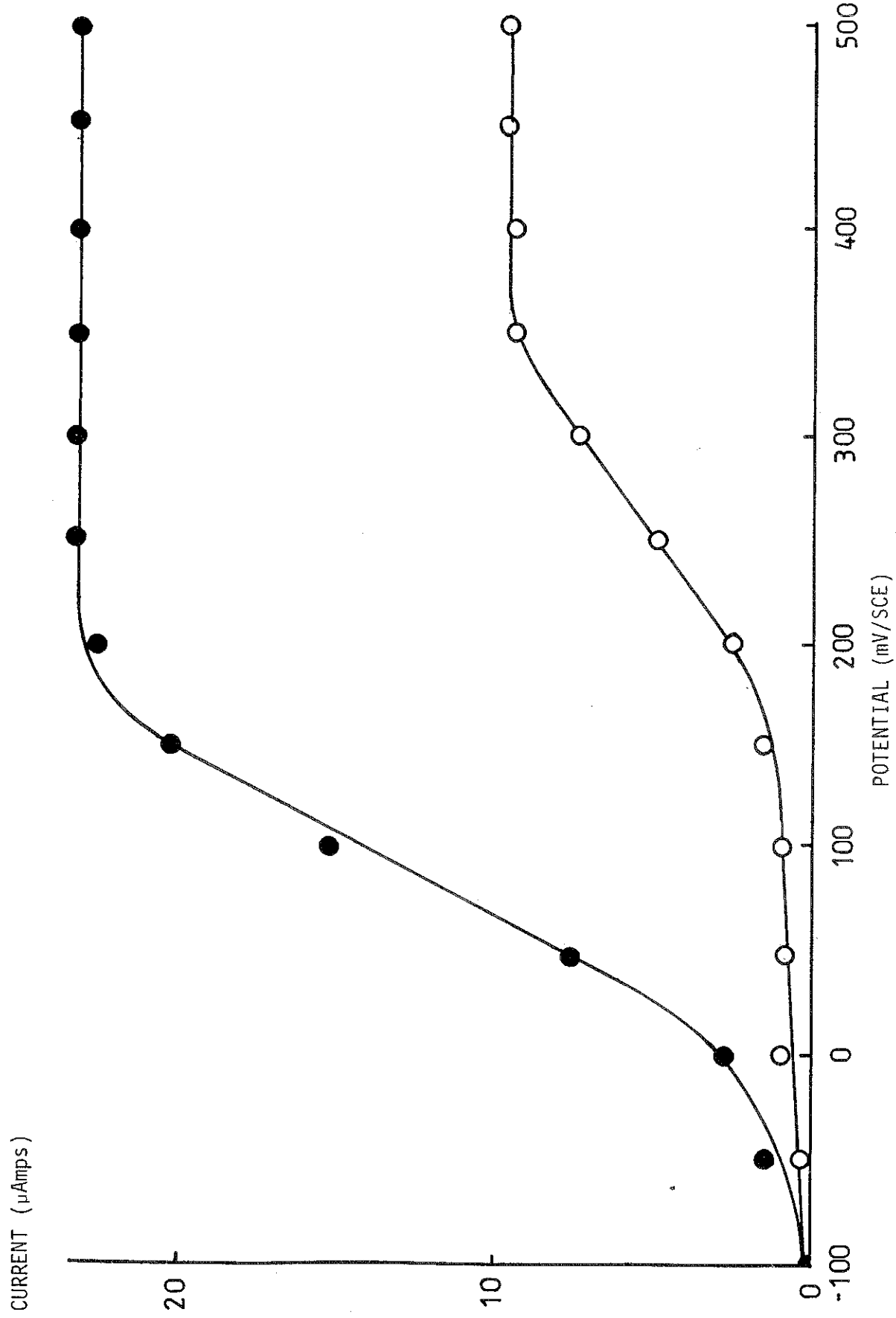
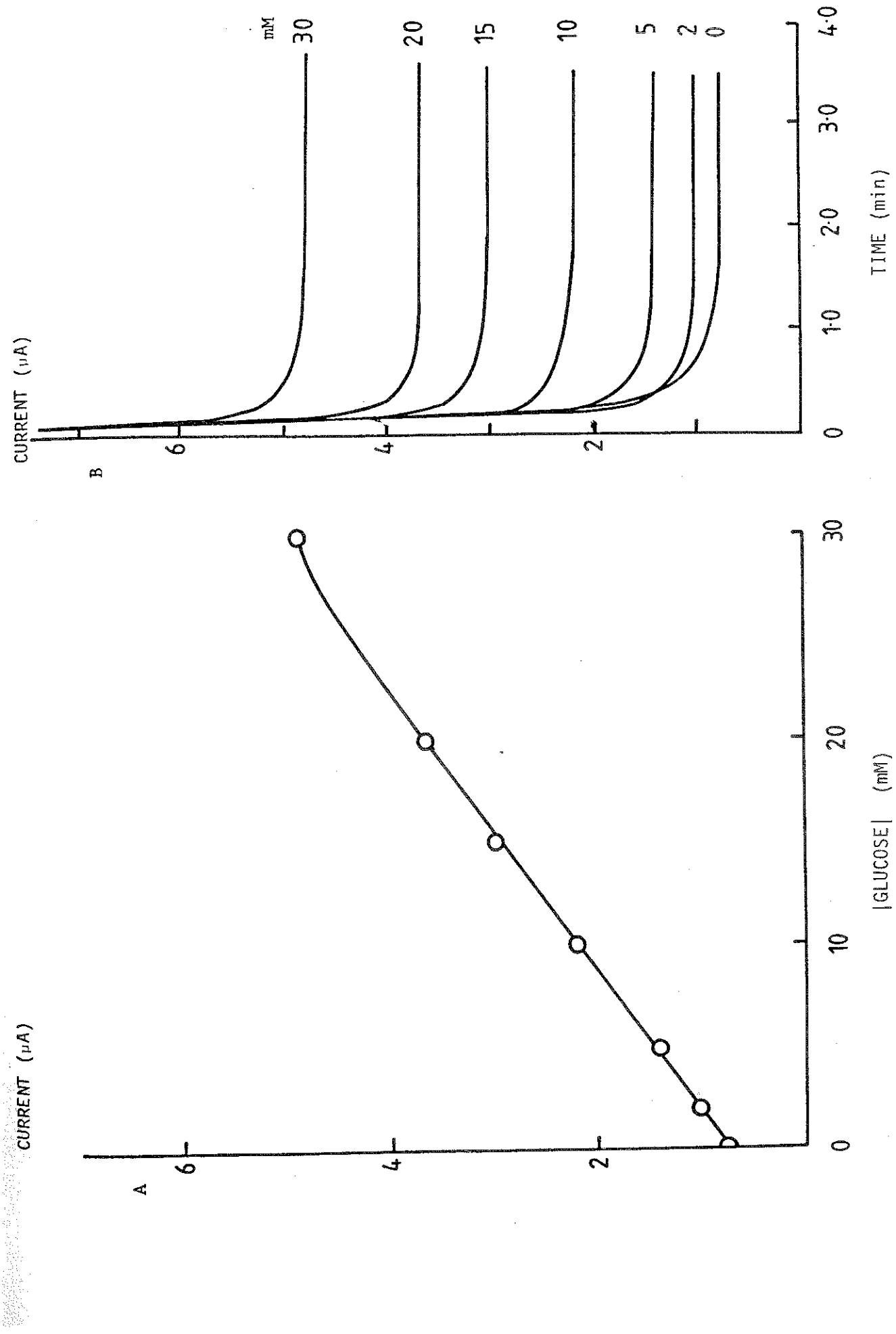

CURRENT (μAmps)
20
10
0
-100
0
100
200
300
400
500
POTENTIAL (mV/SCE)

Figure 6.2 The amperometric response of a glucose oxidase based sensor to glucose (Graph A) and the time taken to attain these values (Graph B).

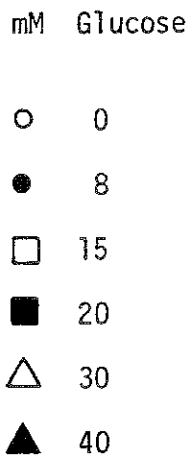

A
CURRENT (μA)
6
4
2
0
10
20
30
|GLUCOSE| (mM)
B
CURRENT (μA)
6
4
2
0
1·0
2·0
3·0
4·0
TIME (min)
mM
30
20
15
10
5
2
0

activity of the probe was investigated in both phosphate and borate buffers (100 mM) containing sodium chloride (150 mM). In the normal physiological range of glucose concentrations (2 - 10 mM) the probe was unaffected by changes in pH between 7.0 and 9.0 (Fig 6.3).

The effect of temperature on the sensor's response time and on the steady state current on addition of glucose was investigated in the range 25 - 65^{o}C. The steady state current, measured at 8.0 mM glucose, increased by 0.2 uA $^{o}C^{-1}$ within the range 25 - 45^{o}C, (Figs 6.4 and 6.5) the rate of attainment decreasing from 3.0 min to 0.8 min. Above 45^{o}C inactivation of the enzyme occured (Figs 6.6 and 6.7). Above 40^{o}C, at low mediator concentrations, the background current increased at a rate of 0.33 mA $^{o}C^{-1}$ (Fig 6.6) whilst electrodes prepared with higher mediator concentrations did not show this effect (Fig 6.7).

The response of the probe at various dissolved oxygen tensions was determined by sparging phosphate buffer (100 mM, pH 7.4 containing 150 mM sodium chloride) with nitrogen, air or oxygen. Air and oxygen elicited responses of 81% and 61% respectively (Fig 6.8) when compared to nitrogen (100%). Increasing the potential of the electrode to +600 mV (versus SCE) enabled glucose analysis to be performed at various oxygen tensions, the response being unaffected by sparging with either nitrogen, air or oxygen. Increasing the potential to +500 mV did, however, cause an increase in the time taken to attain

Figure 6.3 The effect of pH on the steady state current response of a glucose oxidase based electrode at various concentrations of glucose.



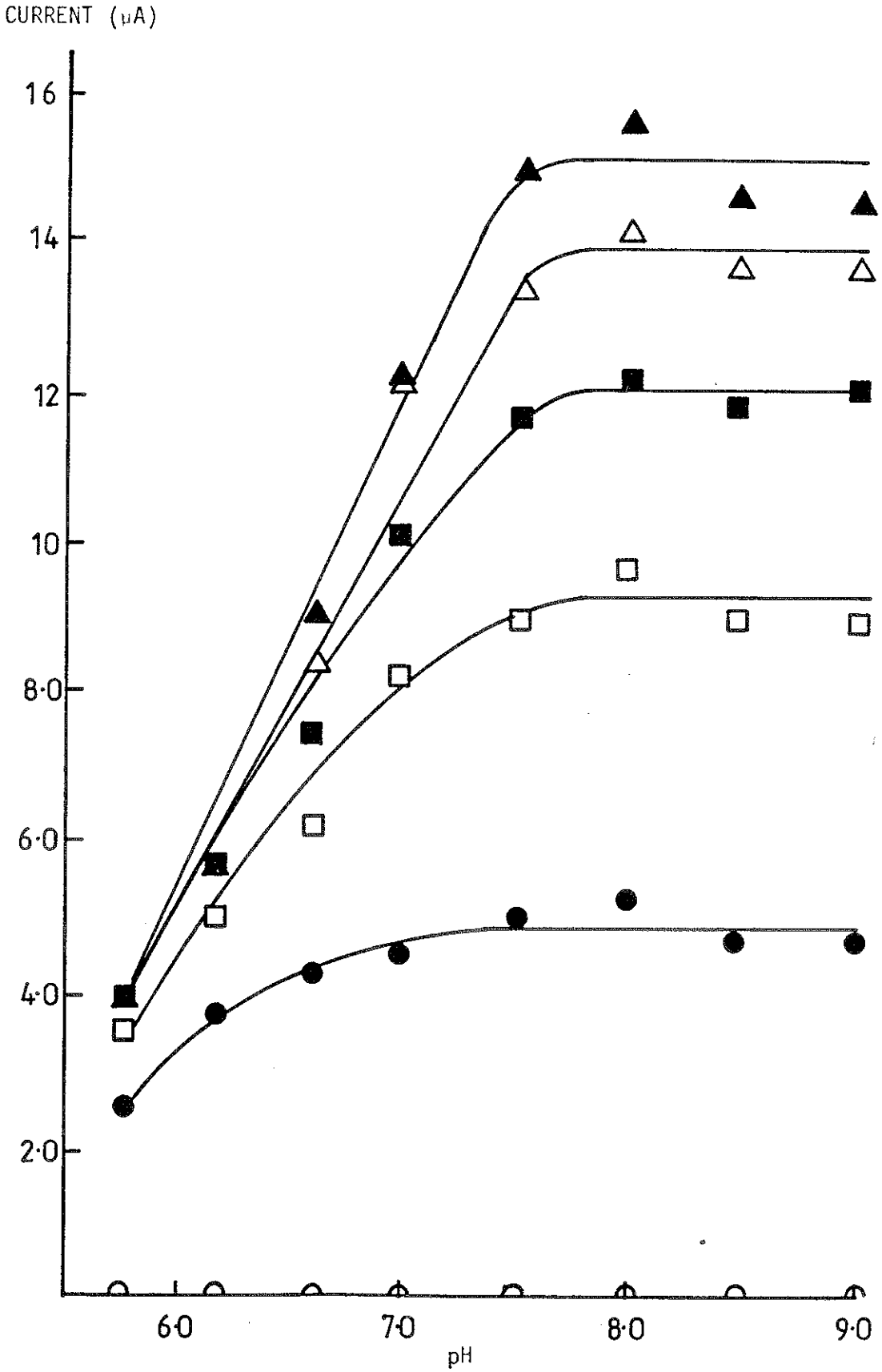

CURRENT (μA)
16
14
12
10
8·0
6·0
4·0
2·0
6·0
7·0
8·0
9·0
pH

Figure 6.4 The effect of temperature on the steady state response of the glucose oxidase based enzyme electrode at various concentrations of glucose.

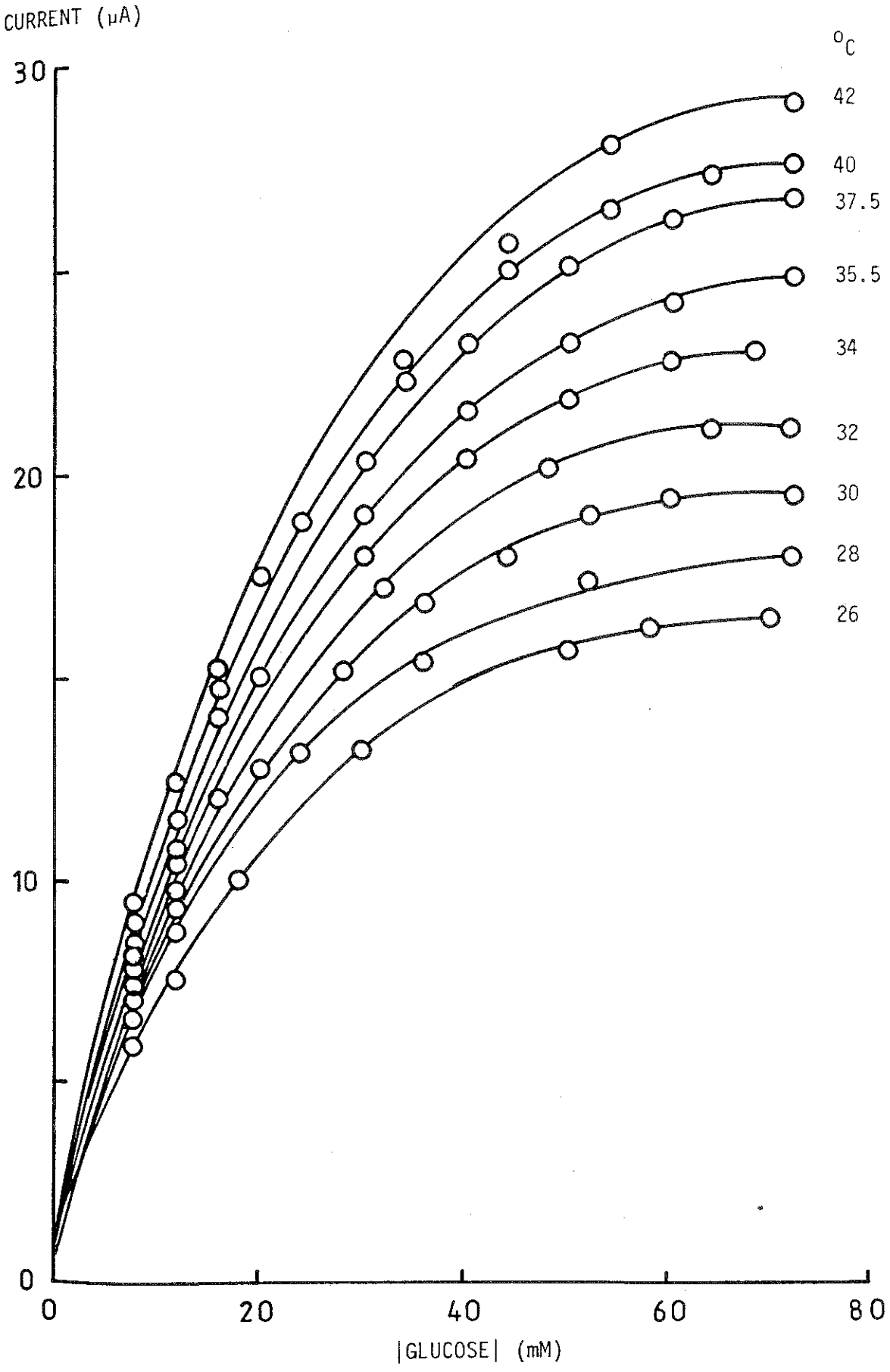

CURRENT (μA)
°C
42
40
37.5
35.5
34
32
30
28
26
30
20
10
0
0
20
40
60
80
|GLUCOSE| (mM)

Figure 6.5 The effect of temperature on the amperometric response of a glucose oxidase based enzyme electrode at 8.0 mM glucose.

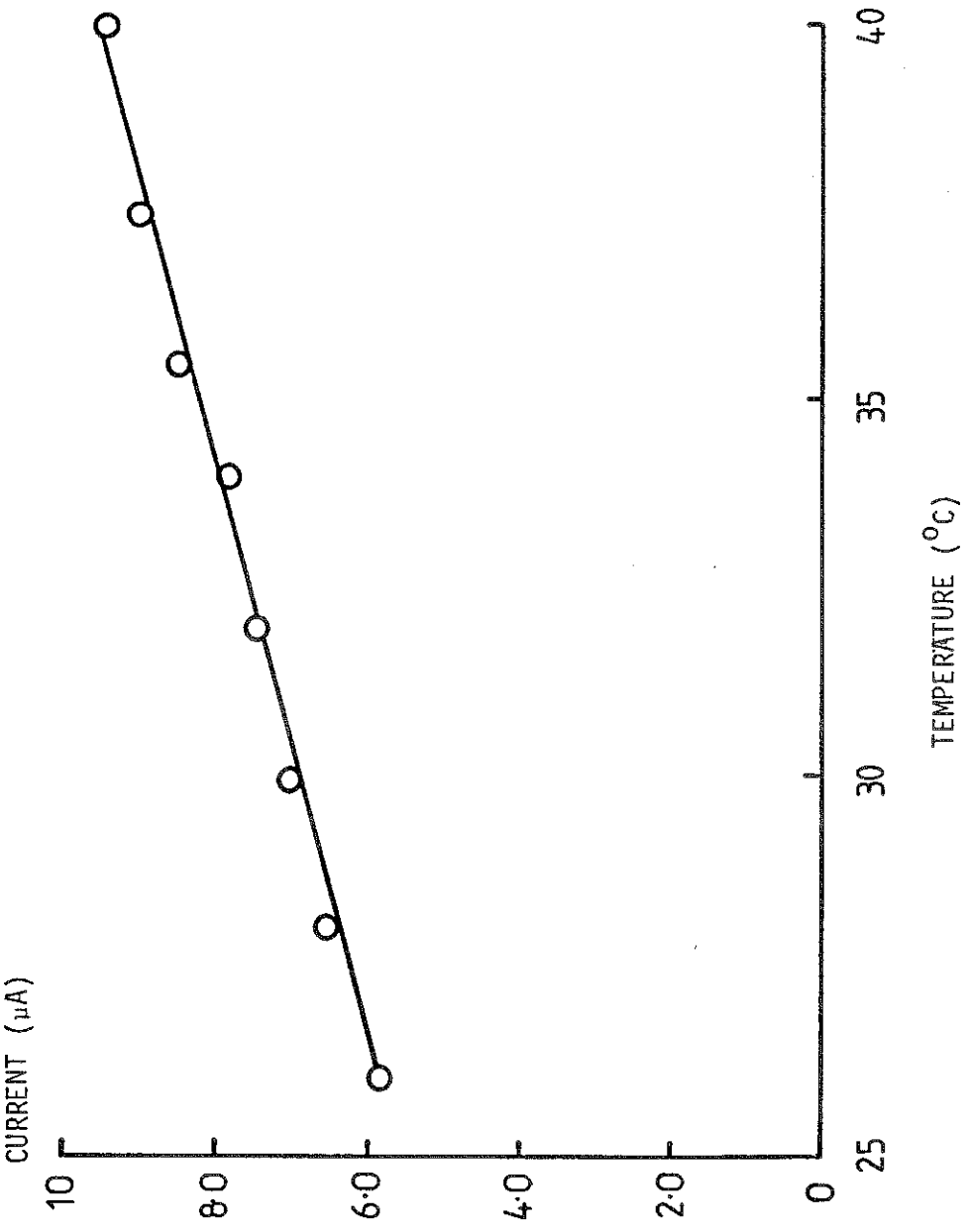

CURRENT (μA)
10
8·0
6·0
4·0
2·0
0
25
30
35
40
TEMPERATURE (°C)

Figure 6.6 The effect of temperature on the amperometric response of a glucose oxidase based enzyme electrode exhibiting low background currents. In the absence (○) and presence (●) of 30 mM glucose. (□) is the enzymically produced current.

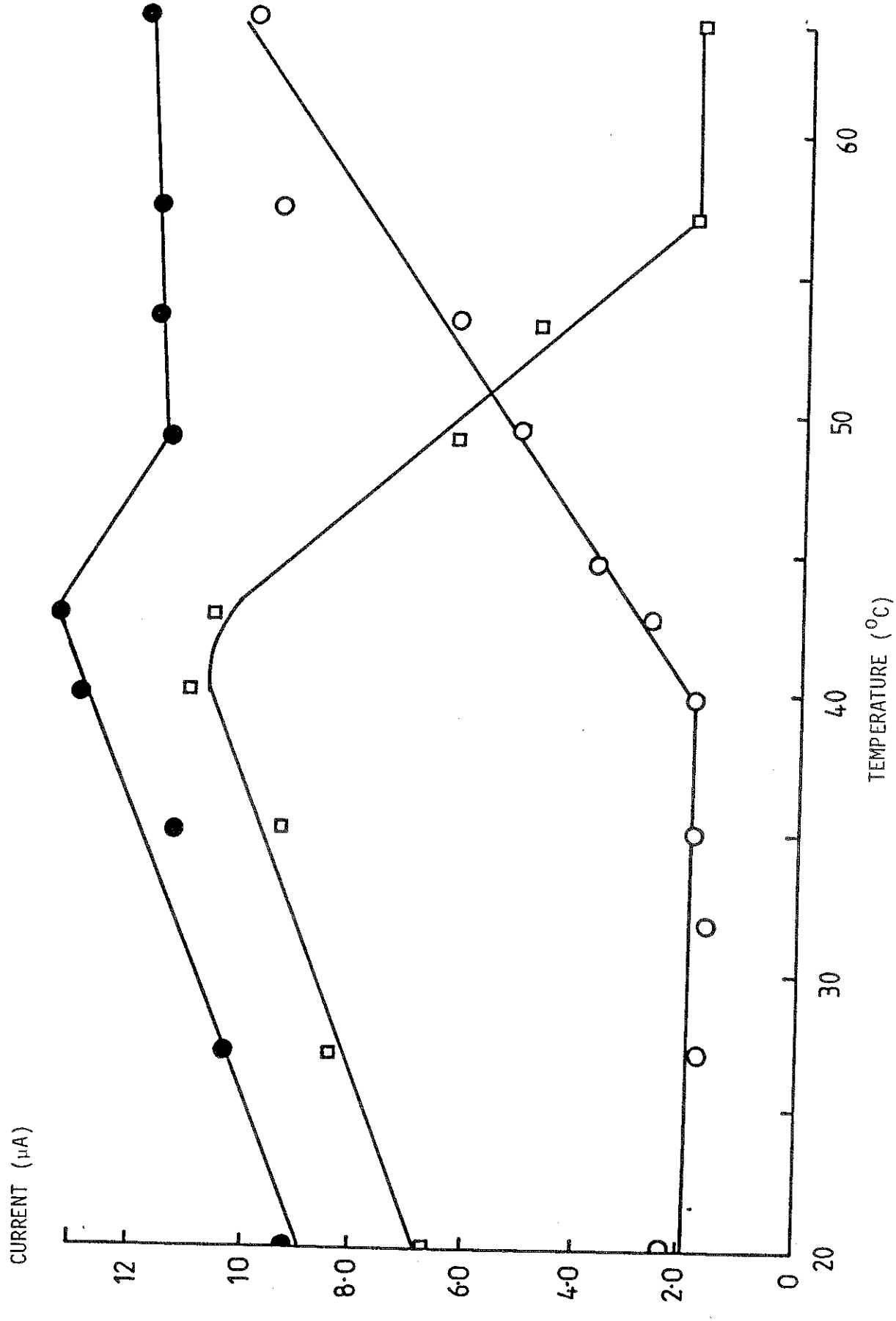

CURRENT (μA)
12
10
8·0
6·0
4·0
2·0
0
20
30
40
50
60
TEMPERATURE (°C)

Figure 6.7 The effect of temperature on the amperometric response of a glucose oxidase based enzyme electrode exhibiting high background currents. In the absence (O) and presence (●) of 30 mM glucose.

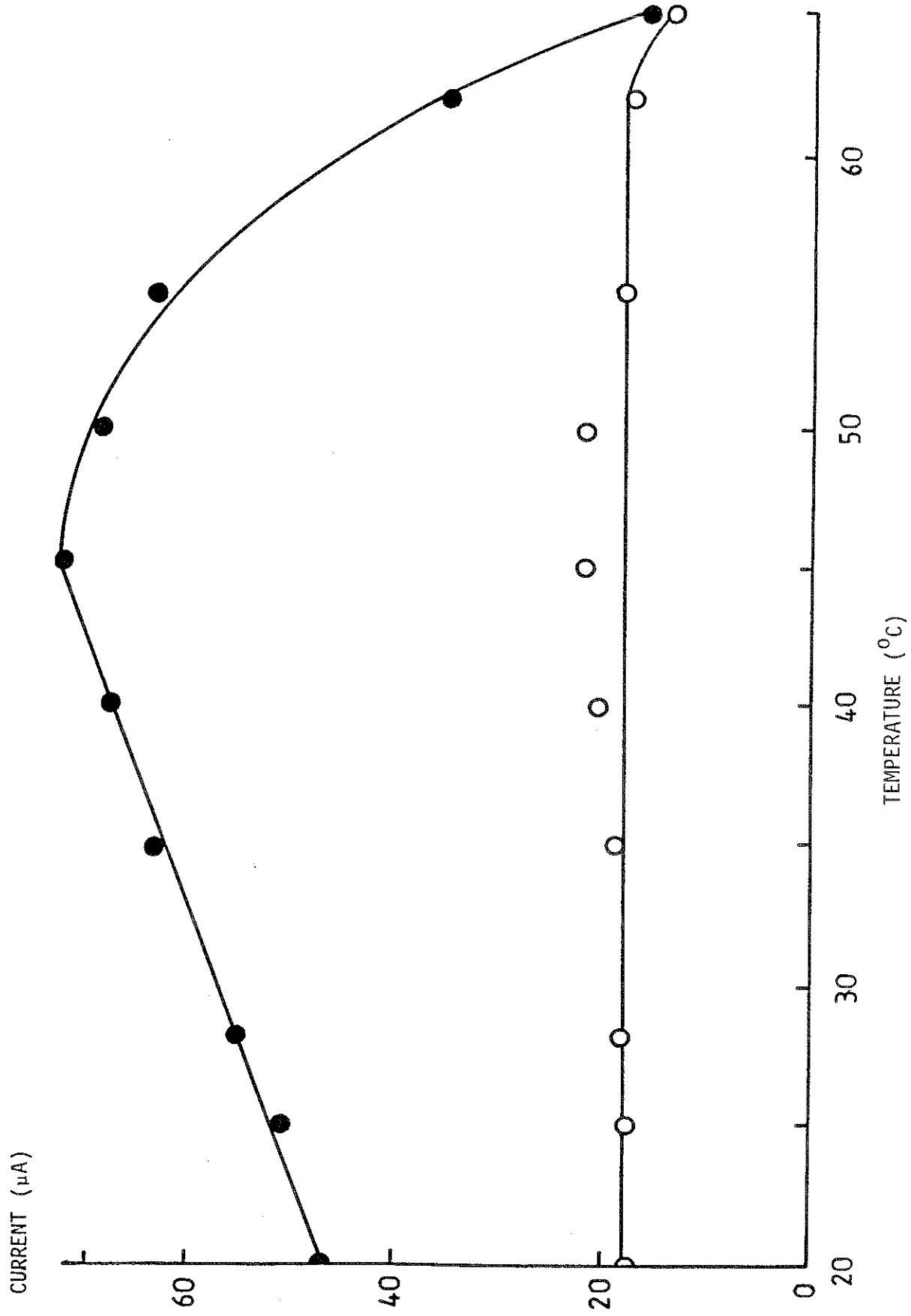
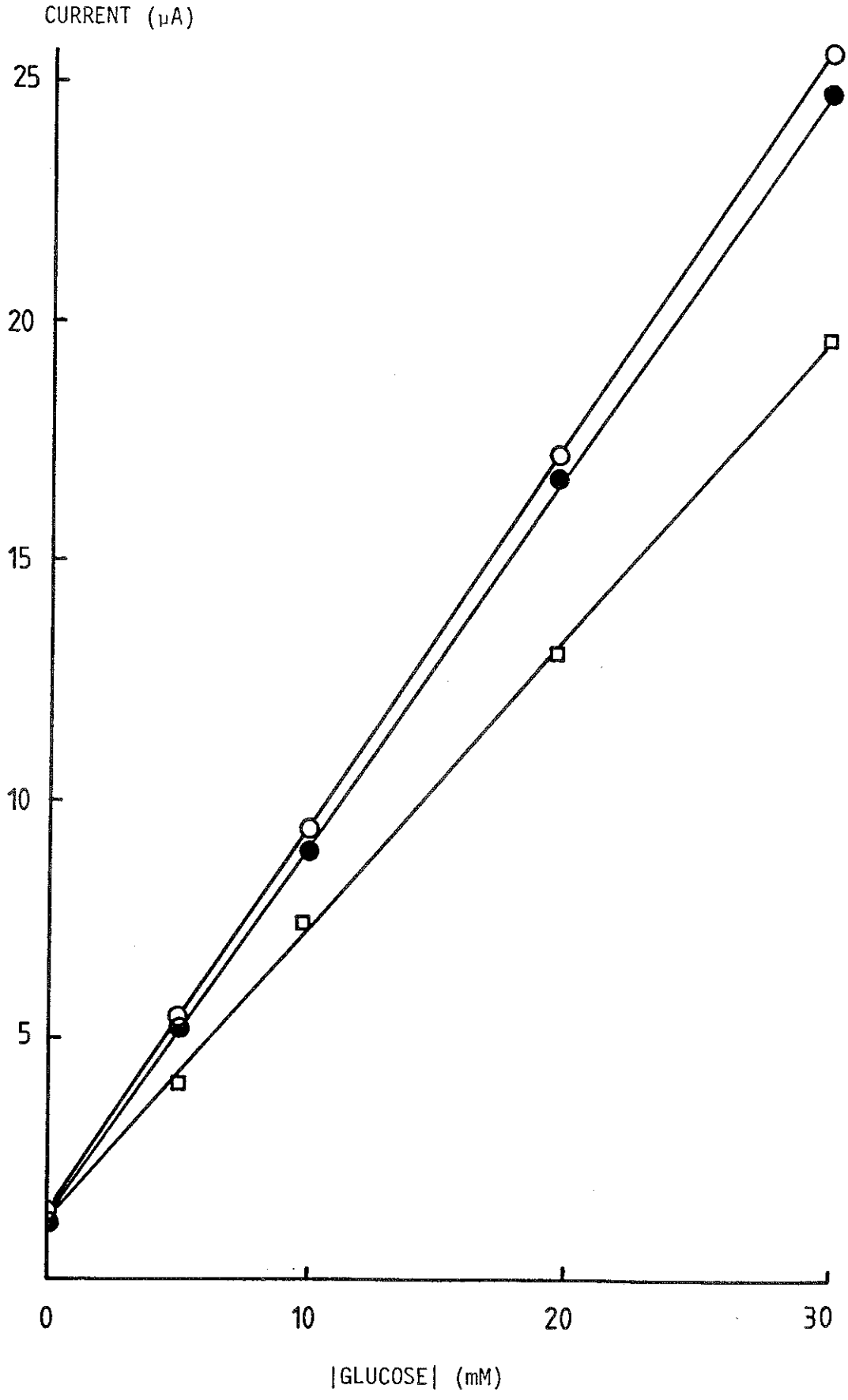

CURRENT (μA)
60
40
20
0
20
30
40
50
60
TEMPERATURE (°C)

Figure 6.8 The effect of oxygen tension on the steady state response of a glucose oxidase enzyme based electrode. The probe was sparged with either nitrogen (○), air (●) or oxygen (□).


CURRENT (μA)
25
20
15
10
5
0
10
20
30
|GLUCOSE| (mM)

steady state currents, when measured in the presence of 30 mM glucose (Fig 6.9).

The effect of covering the enzyme-based glucose sensor with either dialysis or Nuclepore polycarbonate membrane was investigated. The membrane to be tested was placed over the end of a glucose electrode and retained using an 'O' ring. The response (Fig 6.10) of the electrode was unaffected by Nuclepore membrane. Dialysis membrane, however, caused a change in the apparent K_m from 24mM to 73mM.

6.2 Immobilisation procedures

The effect of enzyme loading was determined by varying the concentration of glucose oxidase used in the immobilisation procedure between 0.01 and 100 mg ml^{-1}(see section 2.37). The response of the sensor was subsequently determined by measuring the steady state currents on the addition of glucose (Fig 6.11). Although it was not possible to determine the concentration of protein immobilised, increasing the enzyme concentration caused an increase in the steady state amperometric response, whilst at low enzyme concentrations (0.01 mg ml^{-1}) the sensor did not show a linear response over the range 0 - 30 mM glucose.

The effect of variation in the mediator concentration applied to the sensor was investigated by preparing sensors using 1,1'-dimethylferrocene solutions (5.0 ul in toluene) in the range 1.0×10^{-3} to 1.0×10^{2} mg ml^{-1}. The background current was shown to increase with increasing mediator concentrations (Fig

Figure 6.9 The effect of the applied potential (versus SCE) on the amperometric response of a glucose oxidase enzyme based electrode in the presence of glucose (30 mM).

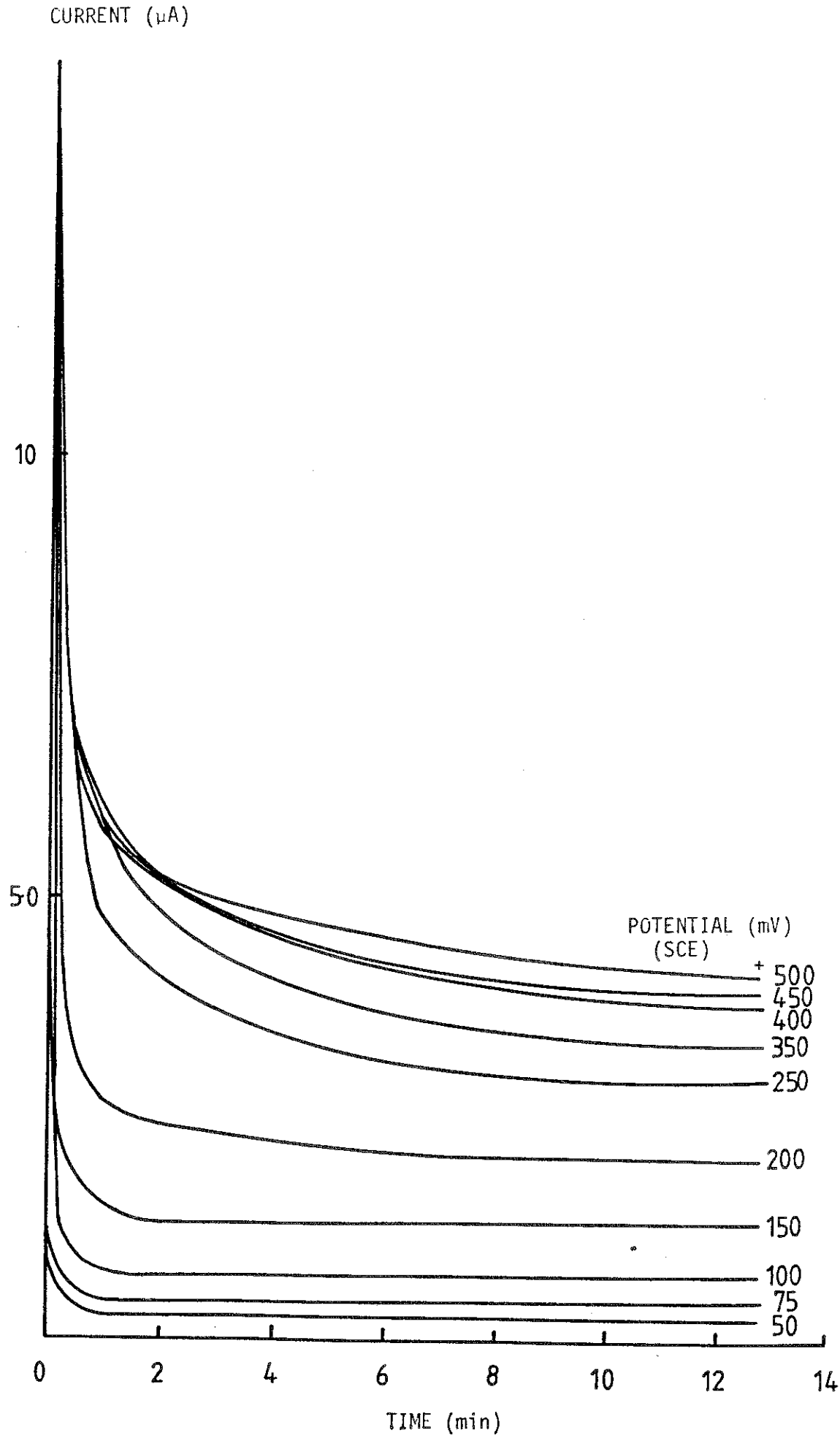

CURRENT (μA)
10
50
POTENTIAL (mV)
(SCE)
+
500
450
400
350
250
200
150
100
75
50
0
2
4
6
8
10
12
14
TIME (min)

Figure 6.10 The effect of dialysis membrane (○) and Nuclepore membrane (●) on the amperometric response of a glucose oxidase based electrode, in comparison to that with no membrane (□).

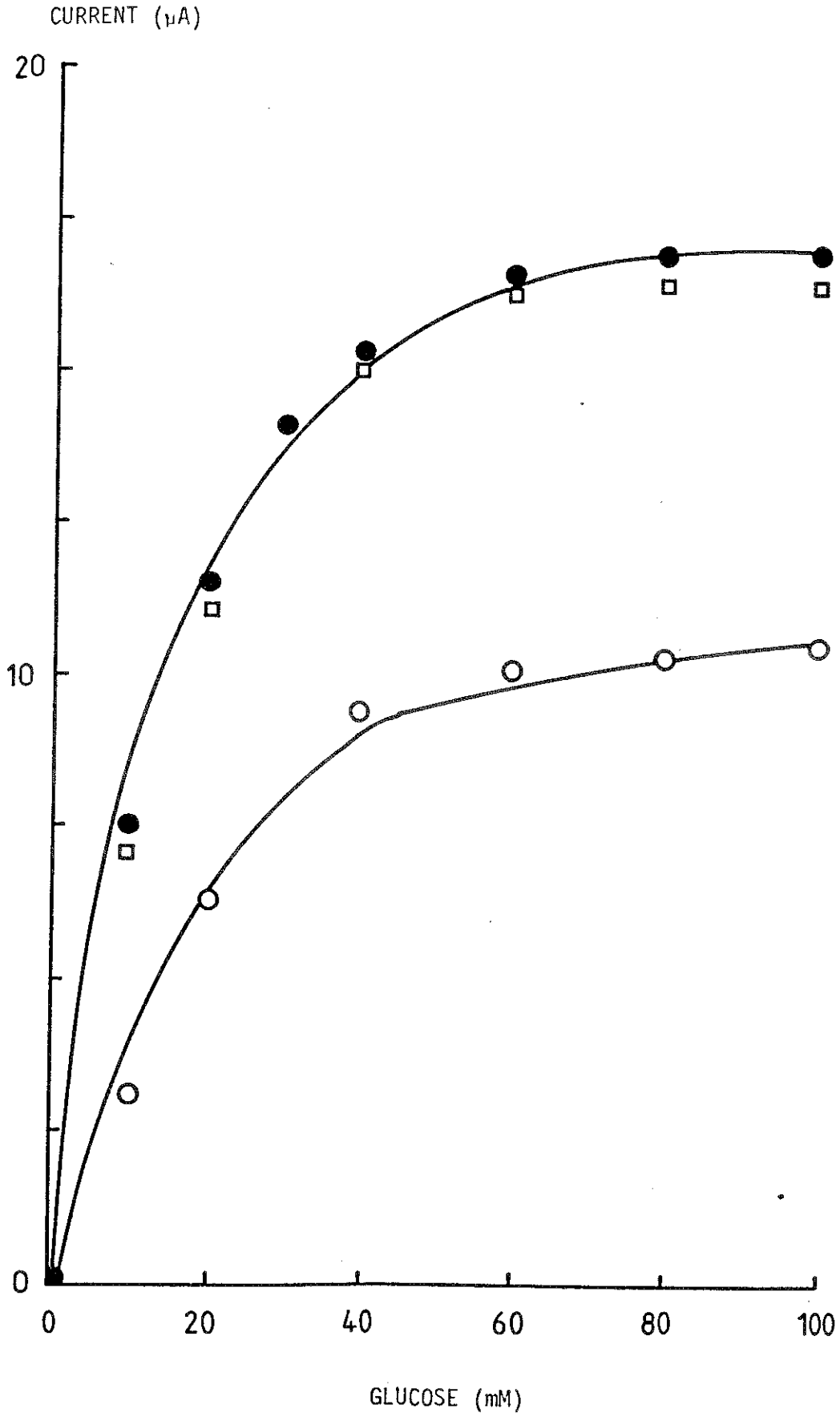
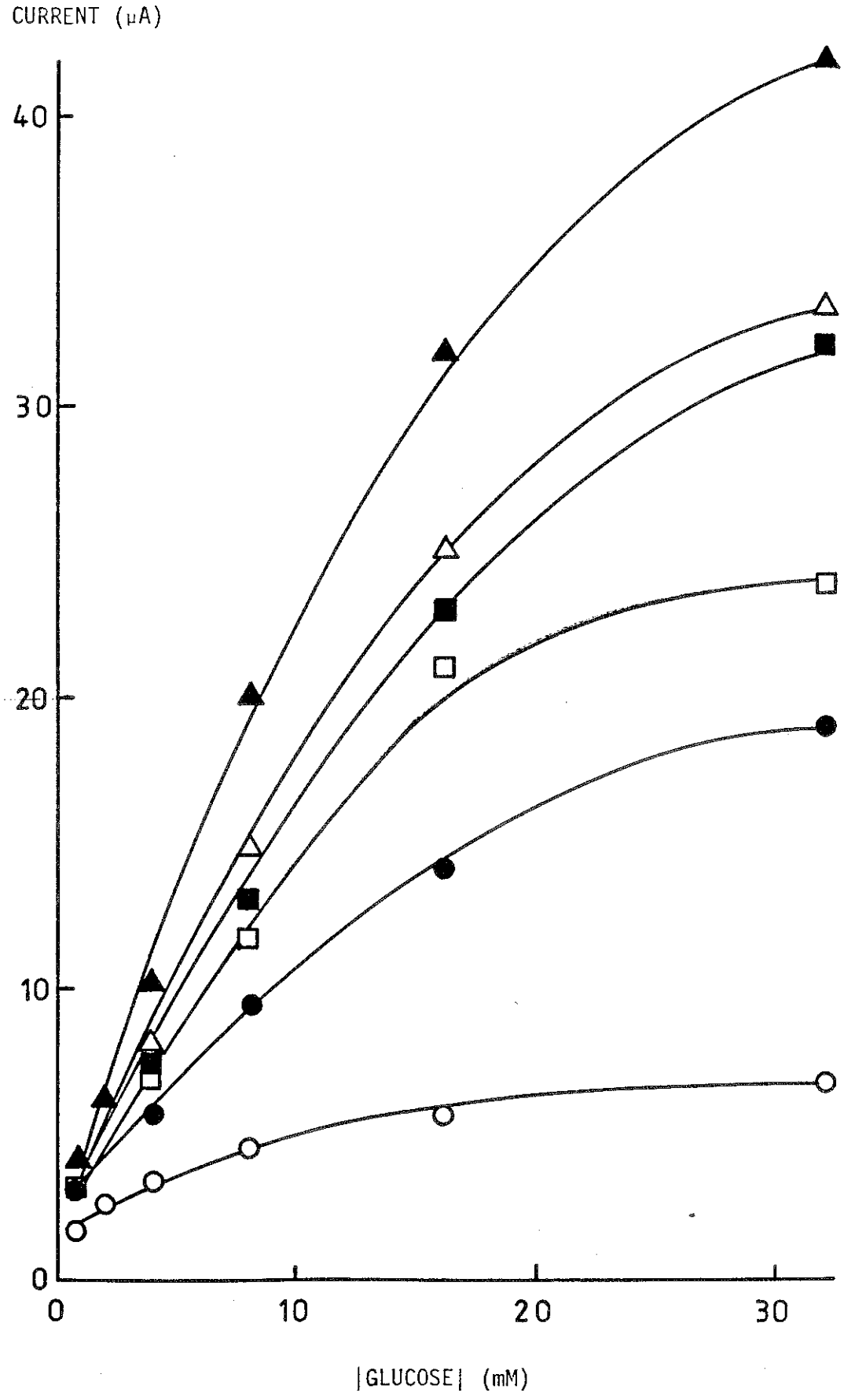

CURRENT (μA)
20
10
0
0
20
40
60
80
100
GLUCOSE (mM)

Figure 6.11 The effect of the concentration of enzyme used in the immobilisation procedure on the amperometric response of the glucose oxidase based enzyme electrode to glucose.

mg ml^{-1} Glucose oxidase	
○	0.01
●	0.04
□	0.1
■	1.0
△	10.0
▲	100.0


CURRENT (μA)
40
30
20
10
0
0
10
20
30
|GLUCOSE| (mM)

6.12) whilst the linearity and the response time of the probe were not affected.

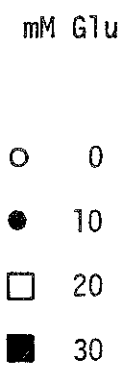
The distribution of mediator and enzyme through the graphite foil was examined by sequential removal of carbon layers and subsequent analysis of the electrode response to glucose. The response was unimpaired by removal of half the layers of electrode material (Fig 6.13) but removal of subsequent layers caused a decrease in the current.

Prior to the sensor being used for a practical application, it was neccessary to demonstrate the reproducibility of response. This was achieved by assembling 50 electrodes and testing them in buffered glucose solutions as described previously (see section 2.36). All the sensors constructed exhibited a linear response up to 30 mM glucose, with an average slope of 0.339 $^{+}$- 0.083 uA mM^{-1} and an intercept of 1.903 $^{+}$- 0.453 uA. All but one exhibited a correlation coefficient of better than 0.99 (n=50).

6.3 Operational stability of the enzyme electrode

The sensor did not exhibit hysteresis (Fig 6.14) in the range of glucose concentrations tested, (0 - 30 mM) and was capable of both discontinuous (Fig 6.13, 6.14) and continuous operation (Fig 6.15). During discontinuous operation the electrode response decayed with time at a rate of 3.0% per assay at 30 mM. The response of the electrode was unaffected by freezing at -20^{o}C. The behaviour of the probe on continuous operation for a seven day period was tested (Fig 6.15). The

Figure 6.12 The effect of the concentration of 1,1'-dimethylferrocene applied to the glucose oxidase enzyme electrode on the amperometric response to glucose.



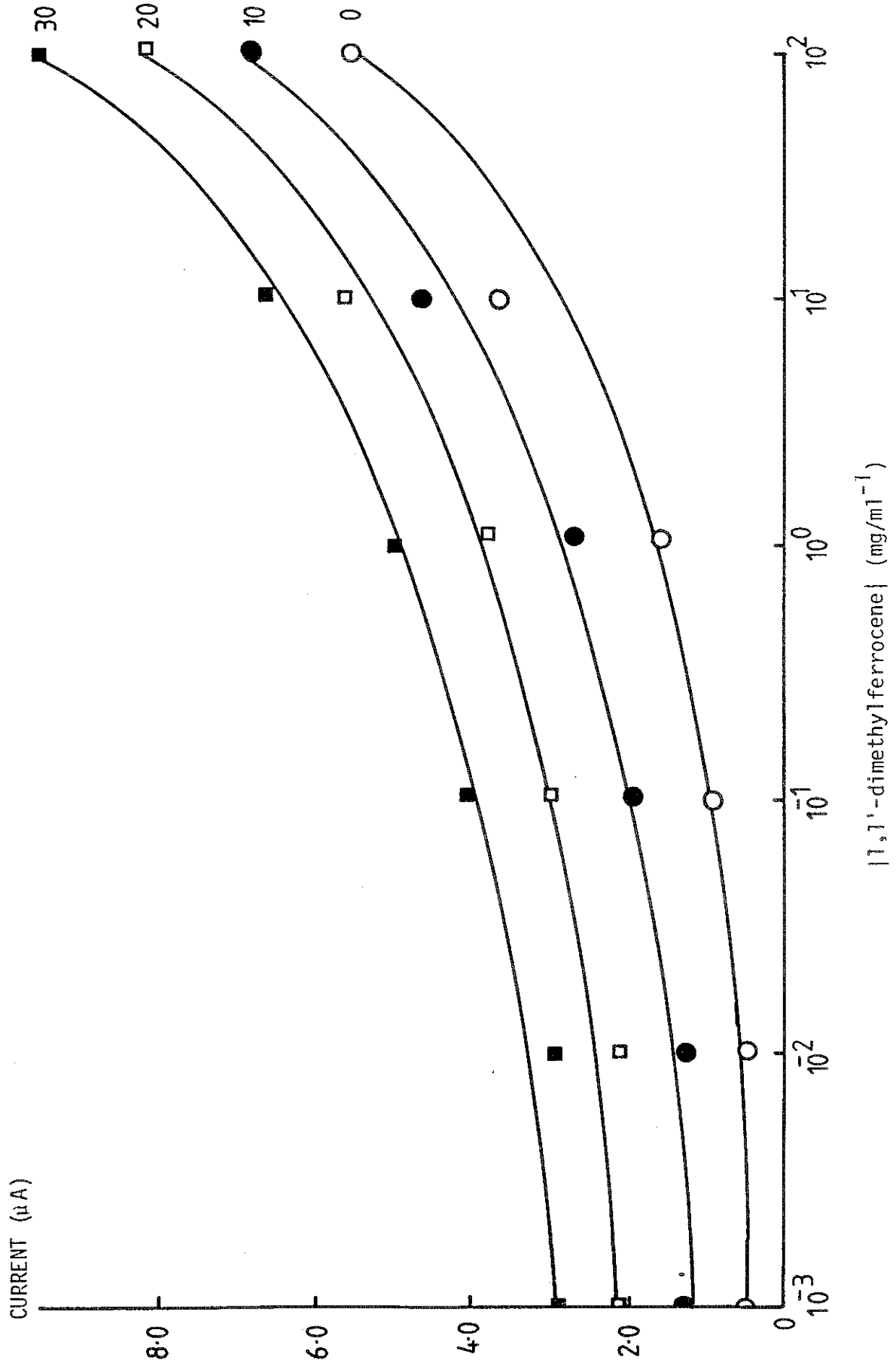
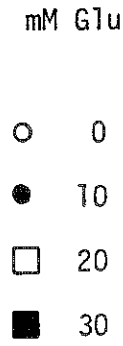

30
20
10
0
CURRENT (μA)
8·0
6·0
4·0
2·0
0
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
[1,1'-dimethylferrocene] (mg/ml^{-1})

Figure 6.13 The effect of the removal of layers of graphite foil on the amperometric response of the glucose oxidase based enzyme electrode to glucose.



The broken lines are the amperometric response with no layers removed.

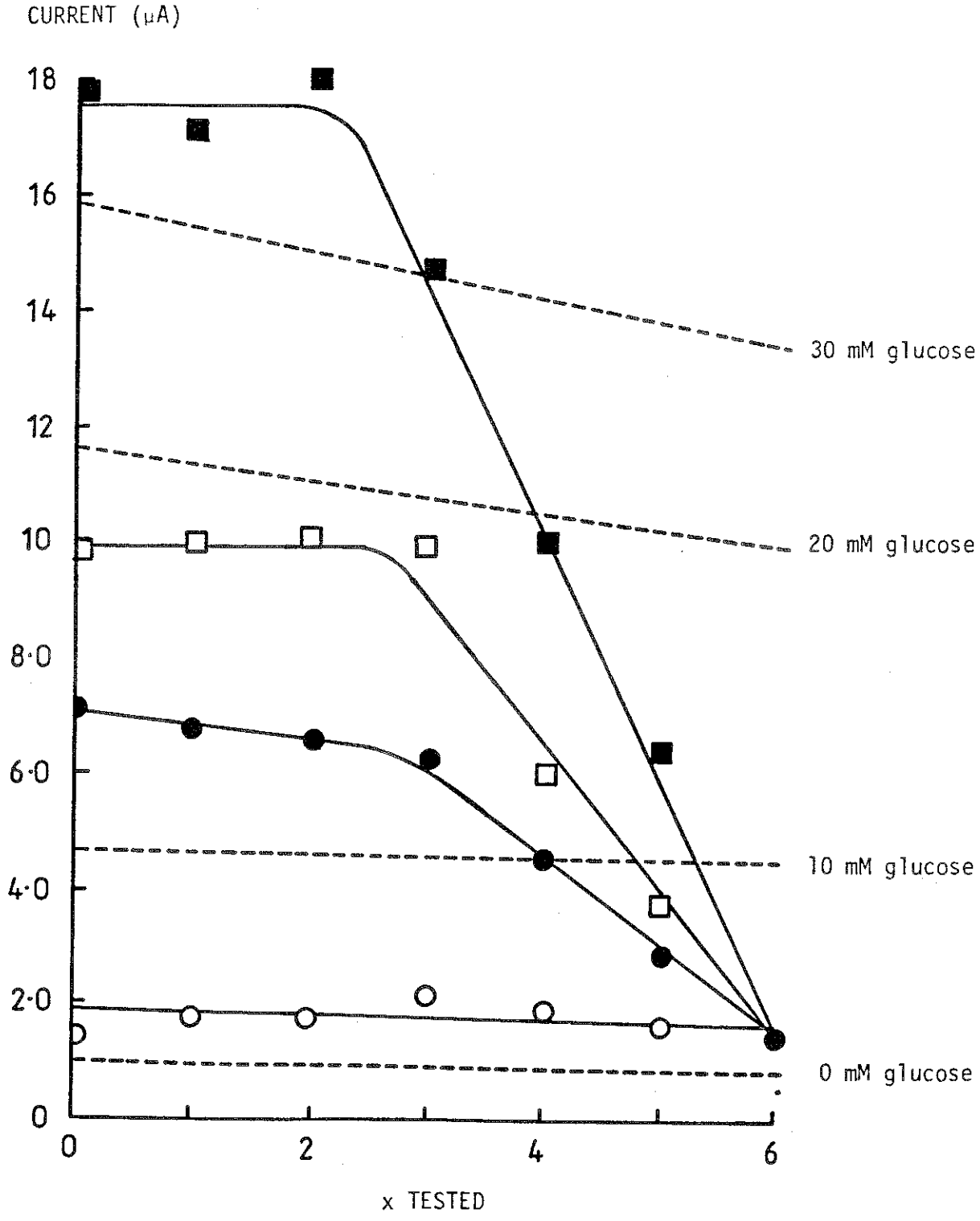

CURRENT (μA)
18
16
14
12
10
8·0
6·0
4·0
2·0
0
0
2
4
6
x TESTED
30 mM glucose
20 mM glucose
10 mM glucose
0 mM glucose

Figure 6.14 The amperometric response of a glucose oxidase based enzyme electrode to increasing (O) and decreasing (●) concentrations of glucose.

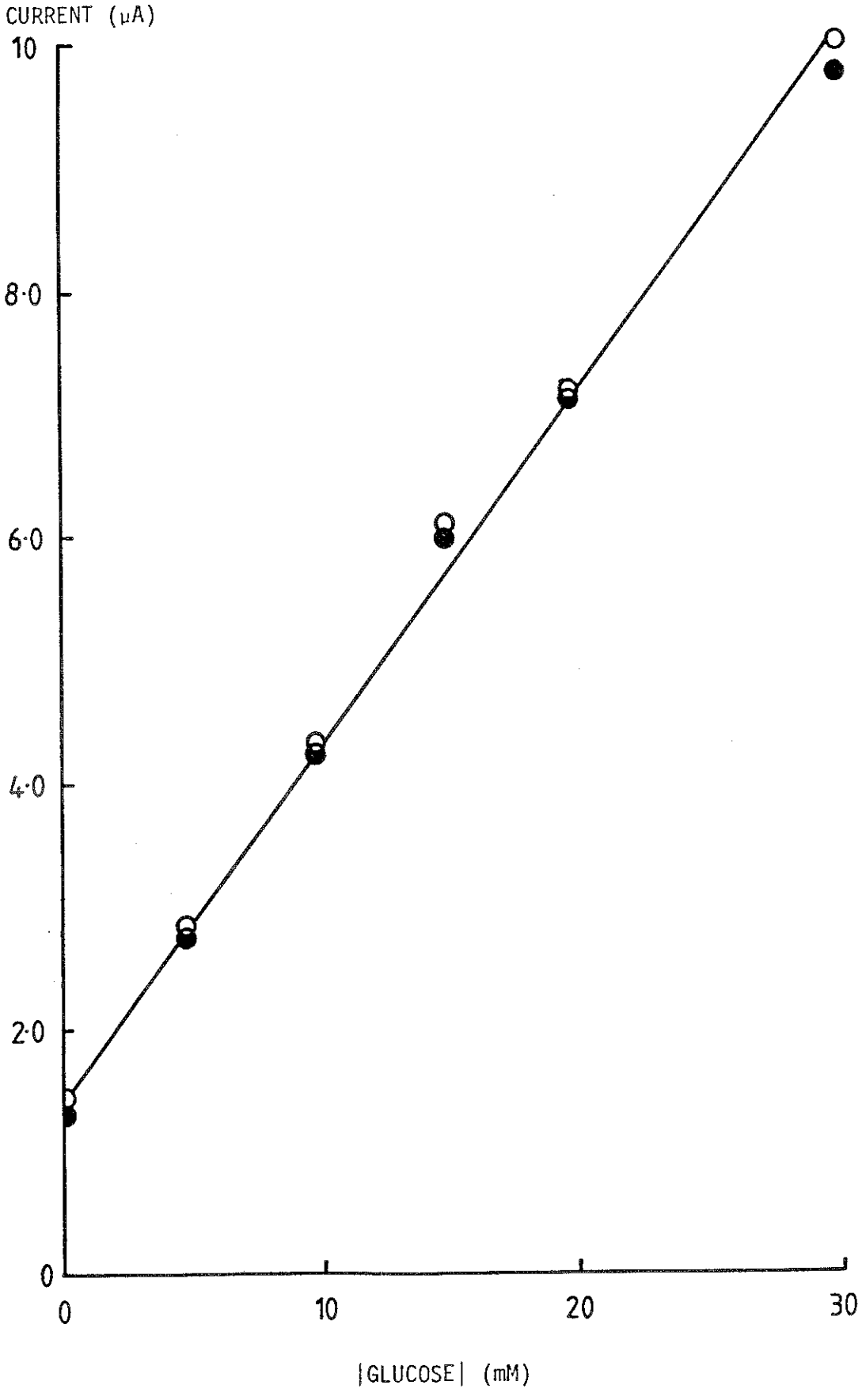

CURRENT (μA)
10
8·0
6·0
4·0
2·0
0
0
10
20
30
|GLUCOSE| (mM)

Figure 6.15 The effect of continuous operation on the amperometric response of a glucose oxidase enzyme based electrode to glucose (30 mM). Tested intermittently with buffer and glucose at:

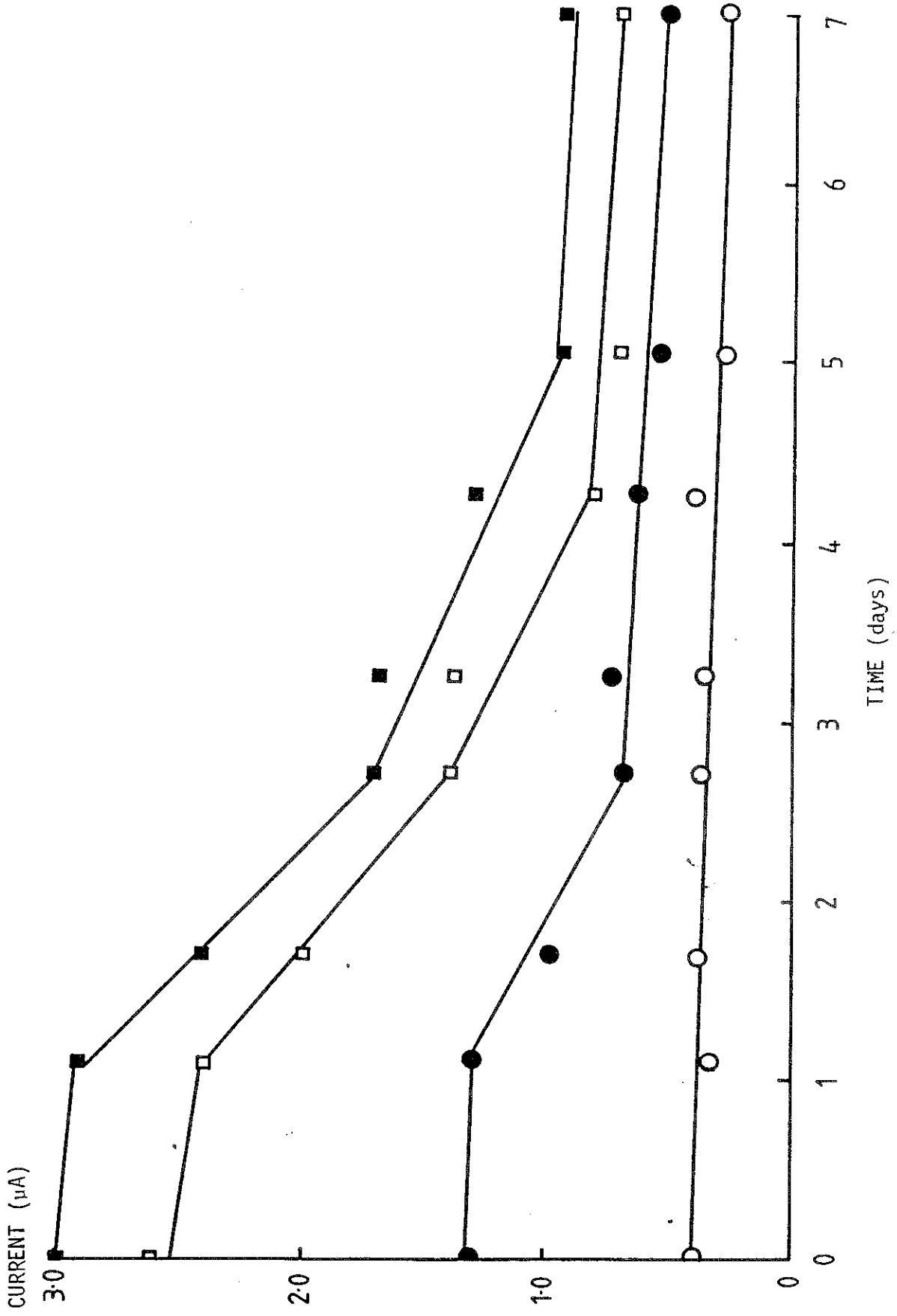
mM Glucose

○ 0

● 10

□ 20

■ 30


CURRENT (μA)
3·0
2·0
1·0
0
0
1
2
3
4
5
6
7
TIME (days)

nitrogen sparged phosphate buffer (100 mM, pH 7.4) containing sodium chloride (150 mM), glucose (30 mM) and penicillin (0.01 mM) was replaced every 24 hours to minimise possible bacterial contamination. During the seven day period, the response of the electrode to various glucose concentrations was determined. The amperometric response of the electrode, although initially remaining steady was shown to decrease rapidly after one day and subsequently attain a lower steady state output. The time taken to attain steady state currents increased from an initial response time of 1.0 minute to 15 minutes after 7 days. The sensor was stored frozen at -20^{o}C in phosphate buffer (100 mM, pH 7.4) containing sodium chloride (150 mM) for a further seven days, after which the test procedure was repeated. The response of the probe to 30 mM glucose underwent a steady decrease of 2.5 x 10^{-4} uA h^{-1} and did not undergo the initial rapid decrease, as in the previous run.

6.4 Storage stability of the enzyme based electrode.

It was possible to store the enzyme electrodes frozen at -20^{o}C in phosphate buffer (100 mM, pH 7.4 containing 150 mM sodium chloride) without loss in activity for at least six months. Glucose oxidase is stable to freeze drying and is commercially available in this form. The freeze drying of glucose electrodes (see section 2.7) resulted in 83% loss in activity, although this was reduced to 65%, by soaking the electrodes in borate buffer (50 mM, pH 7.4) containing gluconic

acid (150 mM) for 30 minutes prior to freeze drying (Fig 6.16).

Glucose oxidase based sensors lost activity on storage desiccated at 25^{o}C after freeze drying (see section 2.27), whether subjected to gluconic acid or not. The inability of the electrodes to respond to glucose after prolonged storage may be due to loss of either mediator or enzyme activity.

The melting point of 1,1'-dimethylferrocene was determined as 37^{o}C using a melting point apparatus (Gallenkamp Ltd., London).

The volatility of the mediator was demonstrated by exposing discs of graphite foil (0.6 cm diameter) previously impregnated with 1,1'-dimethylferrocene (20 mg ml^{-1} in toluene), to a constant stream of nitrogen gas (0.1 l min^{-1}) maintained at 30^{o}C. At various time intervals, five impregnated discs were removed and individually weighed. Each disc was placed in toluene (1.0 ml) for 24 hours and the optical density of the mediator in the solvent determined at 440 nm (Fig 6.17). The absorption maximum was determined by dissolving a sample of the mediator in toluene and carrying out a wavelength scan. The concentration of 1,1'-dimethylferrocene on graphite foil discs subjected to a nitrogen stream was shown to have decreased after 5 weeks to 27% of its initial concentration.

Loss in enzyme activity during storage desiccated at 25^{o}C was demonstrated by immobilising glucose oxidase on a probe in the absence of mediator (see section 2.36). The response of the probe was tested in phosphate buffer (100 mM, pH 7.4) containing

Figure 6.16 The effect of freeze drying on the amperometric response of a glucose oxidase based enzyme electrode. An average of five electrodes were examined prior to (○) and after freeze drying in the absence (●) and presence (□) of gluconic acid.

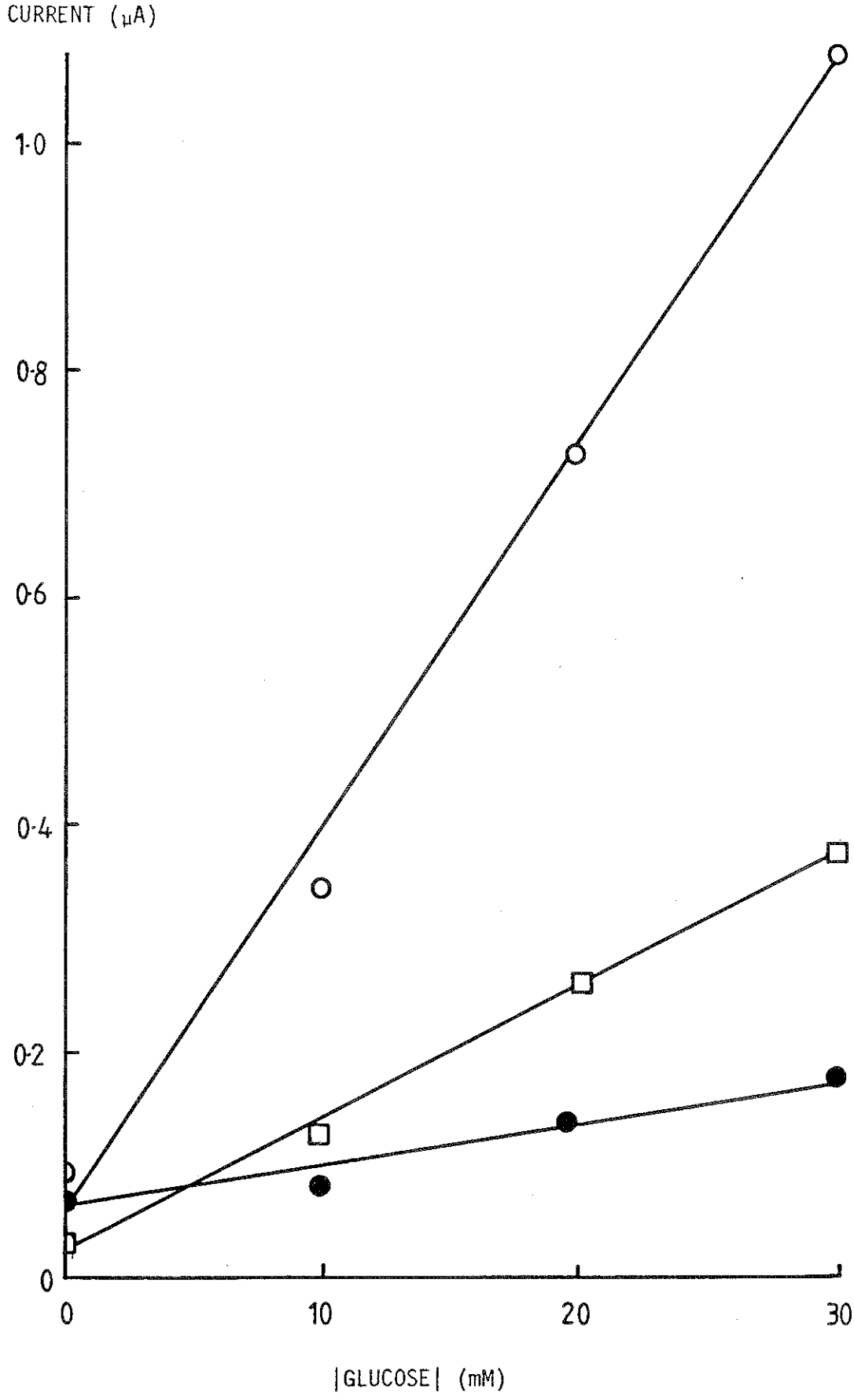
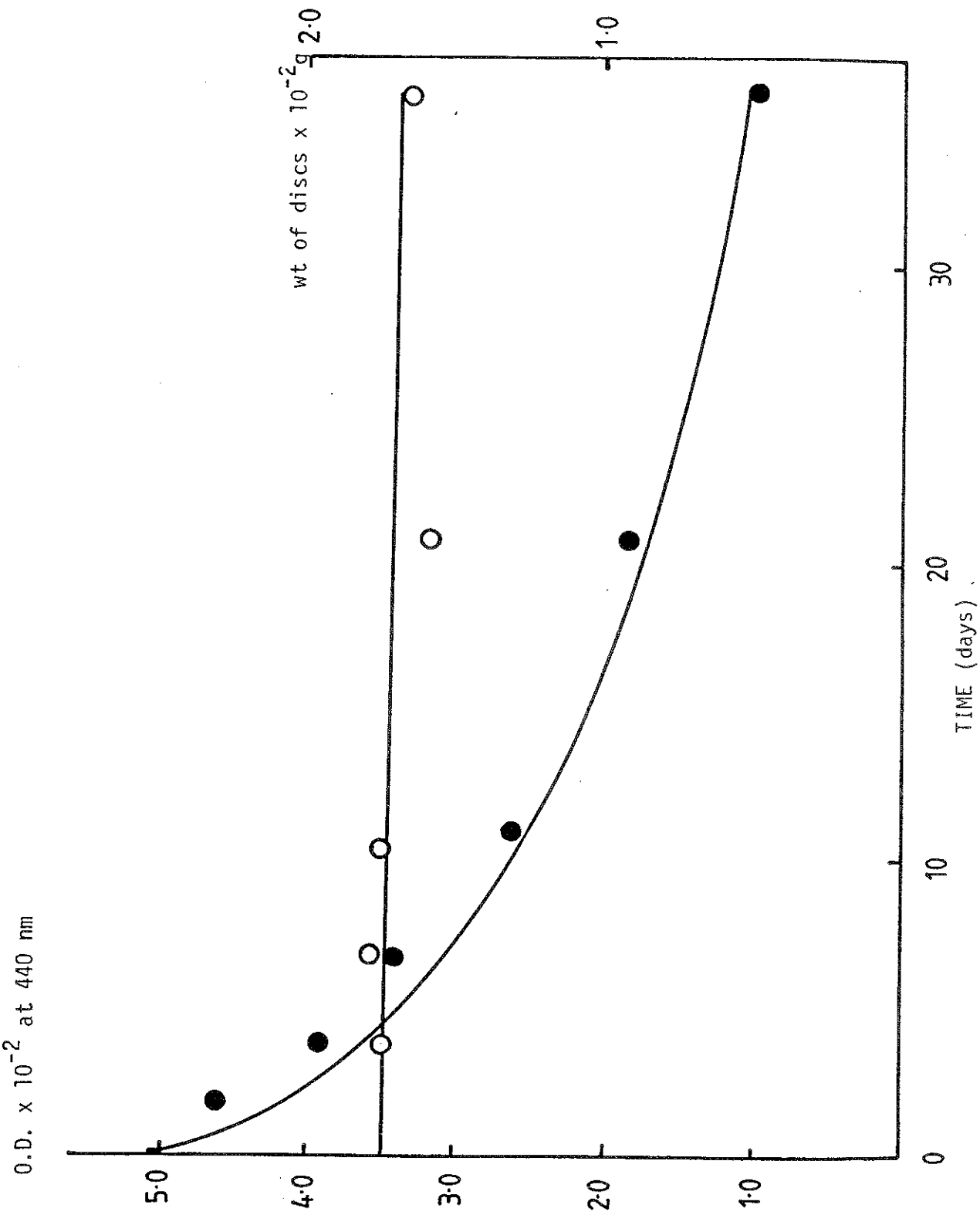

CURRENT (μA)
1·0
0·8
0·6
0·4
0·2
0
0
10
20
30
|GLUCOSE| (mM)

Figure 6.17 The loss of 1,1'-dimethylferrocene from graphite foil discs maintained at 30°C sparged with nitrogen at a rate of 0.1 ml min^{-1}. The discs were weighed (○) and 1,1'-dimethylferrocene was extracted in toluene and determined spectrophotometrically at 440 nm (●).


O.D. x 10^{-2} at 440 nm
wt of discs x 10^{-2} g
TIME (days)
5·0
4·0
3·0
2·0
1·0
2·0
1·0
0
10
20
30

sodium chloride (150 mM) and glucose, in the range 0 - 30 mM (Fig 6.18). After excess solution had been removed, 1,1'-dimethylferrocene (5.0 ul, 20 mg ml^{-1} in toluene) was added and the procedure repeated. Only in the presence of both mediator and glucose oxidase did the probe respond to glucose (Fig 6.18). A probe previously stored desiccated at 25°C for 2 months exhibited low current responses when tested in buffered glucose solutions. To determine if the response of the probe was limited due to loss of mediator, 1,1'-dimethylferrocene was added as above. The addition of the mediator did not, however, enhance the response of the sensor to glucose, although the background current was increased (Fig 6.19).

6.5 Clinical analysis.

Whilst a three electrode sensor configuration was suitable for studying the characteristics of the enzyme electrode, it was considered unsuitable for practical applications where small samples are available. One such application is the analysis of blood glucose, in which samples of the order of 0.1 ml are obtained from patients.

Due to the small currents produced by the enzyme electrode it was possible to use a silver/silver chloride reference electrode in place of the standard calomel reference and platinum counter electrodes. A more practical device was constructed by mounting the enzyme based glucose electrode and reference electrodes horizontally on a ceramic base (Fig 2.15). The

Figure 6.18 The current response of an enzyme based glucose electrode in the presence of enzyme only (○), mediator only (●) and in the presence of both components (□).

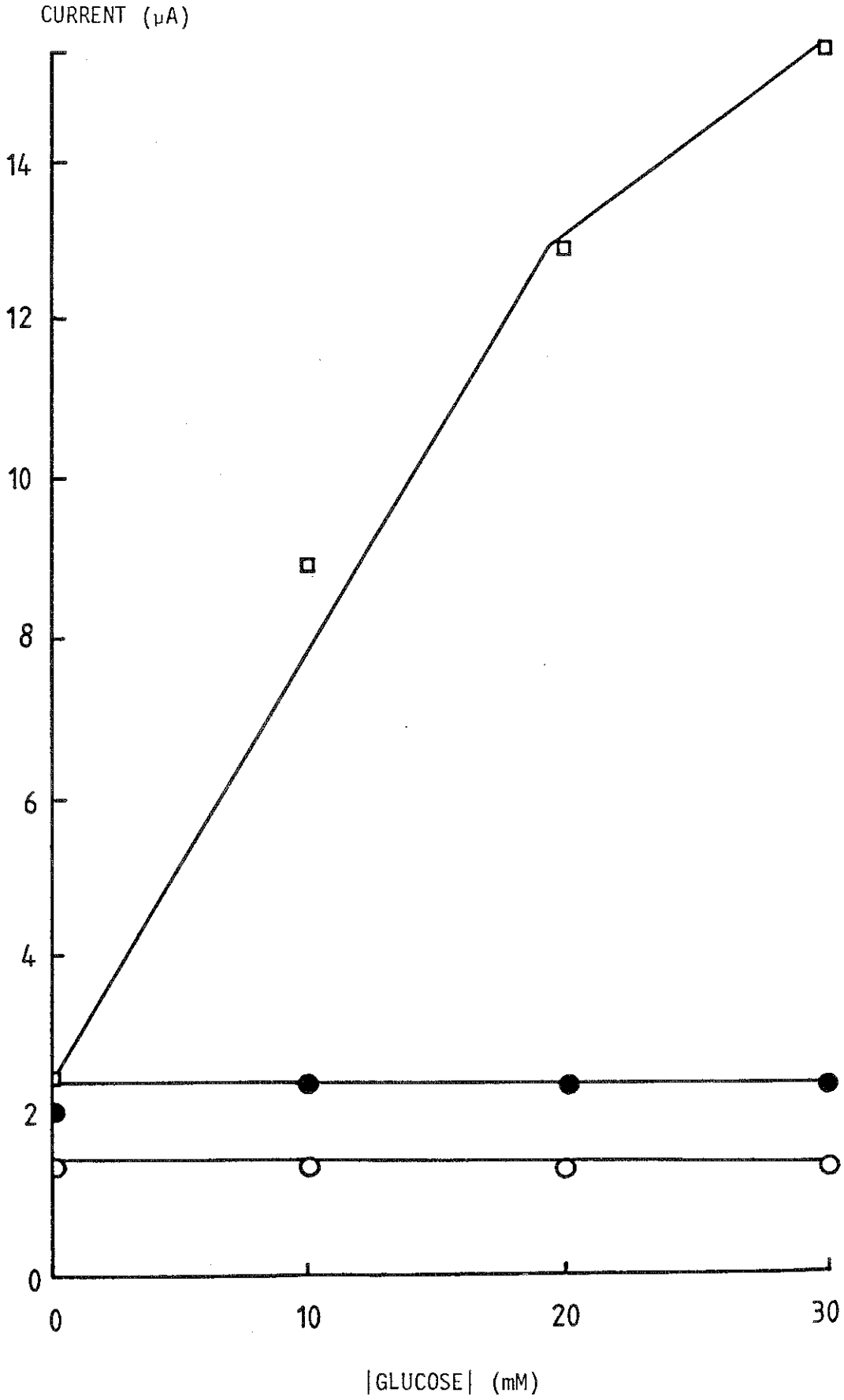
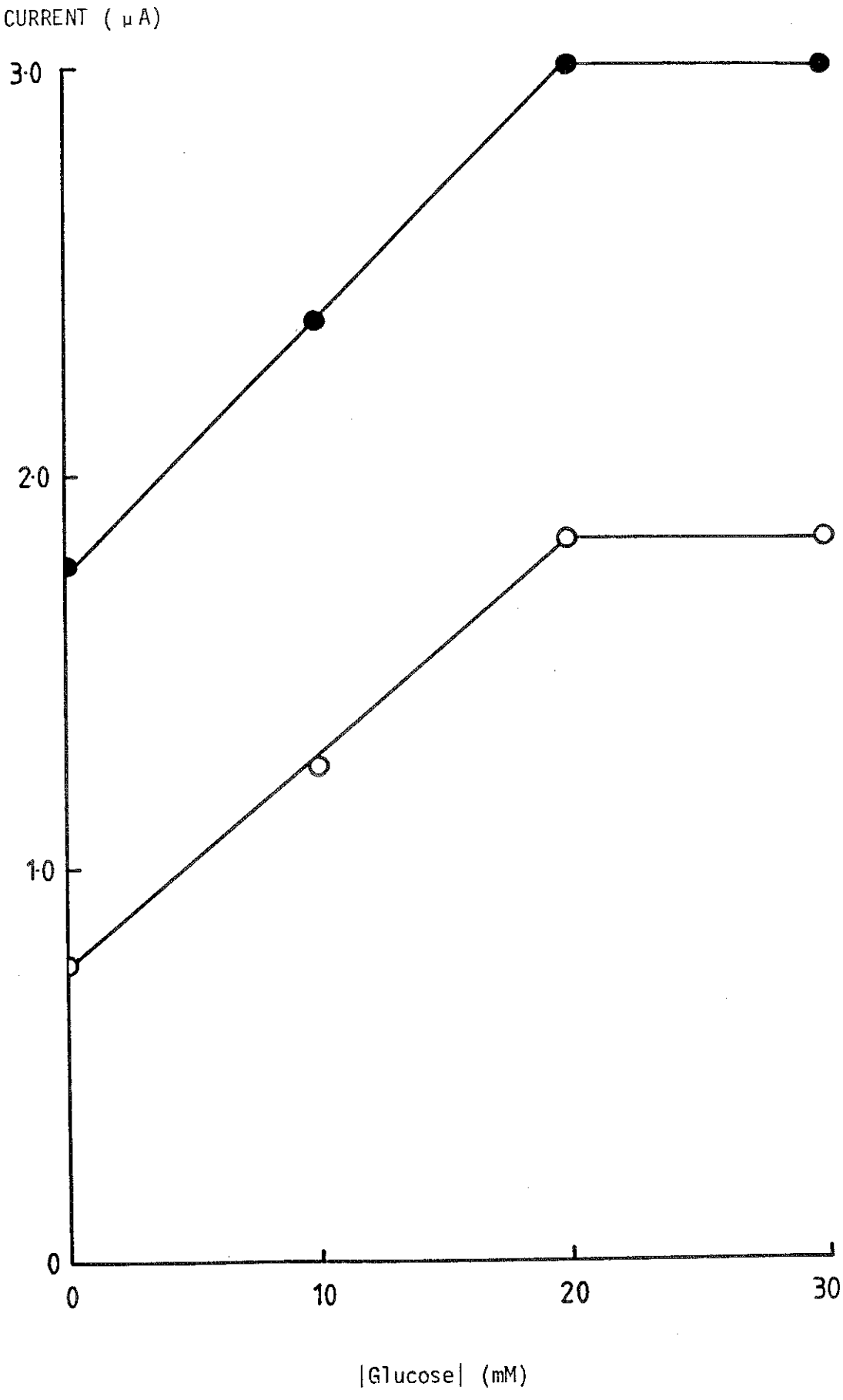

CURRENT (μA)
14
12
10
8
6
4
2
0
0
10
20
30
|GLUCOSE| (mM)

Figure 6.19 The effect of doping a glucose oxidase enzyme electrode, stored desiccated at $25^{o}C$ for one month, with additional 1,1'-dimethylferrocene (50 µl, 20 mg ml^{-1} in toluene). Prior to (O) and after (●) the addition of mediator.


CURRENT (μA)
3·0
2·0
1·0
0
0
10
20
30
|Glucose| (mM)

electronics were minaturised and incorporated into a hand held device. A further modification of this system involved the incorporation of a timer, enabling currents to be determined prior to the attainment of steady state values. These devices, provided by Genetics International Inc. (Boston, USA) produced a linear response up to 30 mM (Fig 6.20), with the lowest concentration determined being 0.1 mM (Fig 6.21). The electrode attained steady state within 1.0 minute, but responses could be measured within 35 seconds (Fig 6.22) using the timer. Glucose determinations were possible in both buffered solutions and authentic whole blood samples (40 ul), obtained from either normal or diabetic patients, without any pretreatment. Preliminary results were similar to those obtained by currently used clinical techniques including the Yellow Springs Instrument (YSI) and Diastrix methods of glucose analysis (Table 6.1).

6.6 Other configurations tested.

A probe constructed of a filter paper disc (see section 2.36) retained on the end of a graphite carbon electrode impregnated by 1,1'-dimethylferrocene also responded to glucose. The electrode poised at +150mV (verses SCE) exhibitied a linear response to glucose up to 30mM. However the probe did not elicit the rapid response of the previously described system but took 20 mins to reach steady state.

It was possible to place a Yellow Springs membrane over the end of a graphite carbon electrode (see section 2.27). The

Figure 6.20 The amperometric response of a glucose oxidase strip electrode to glucose in the range 0-30 mM using a two electrode configuration (see text for details).

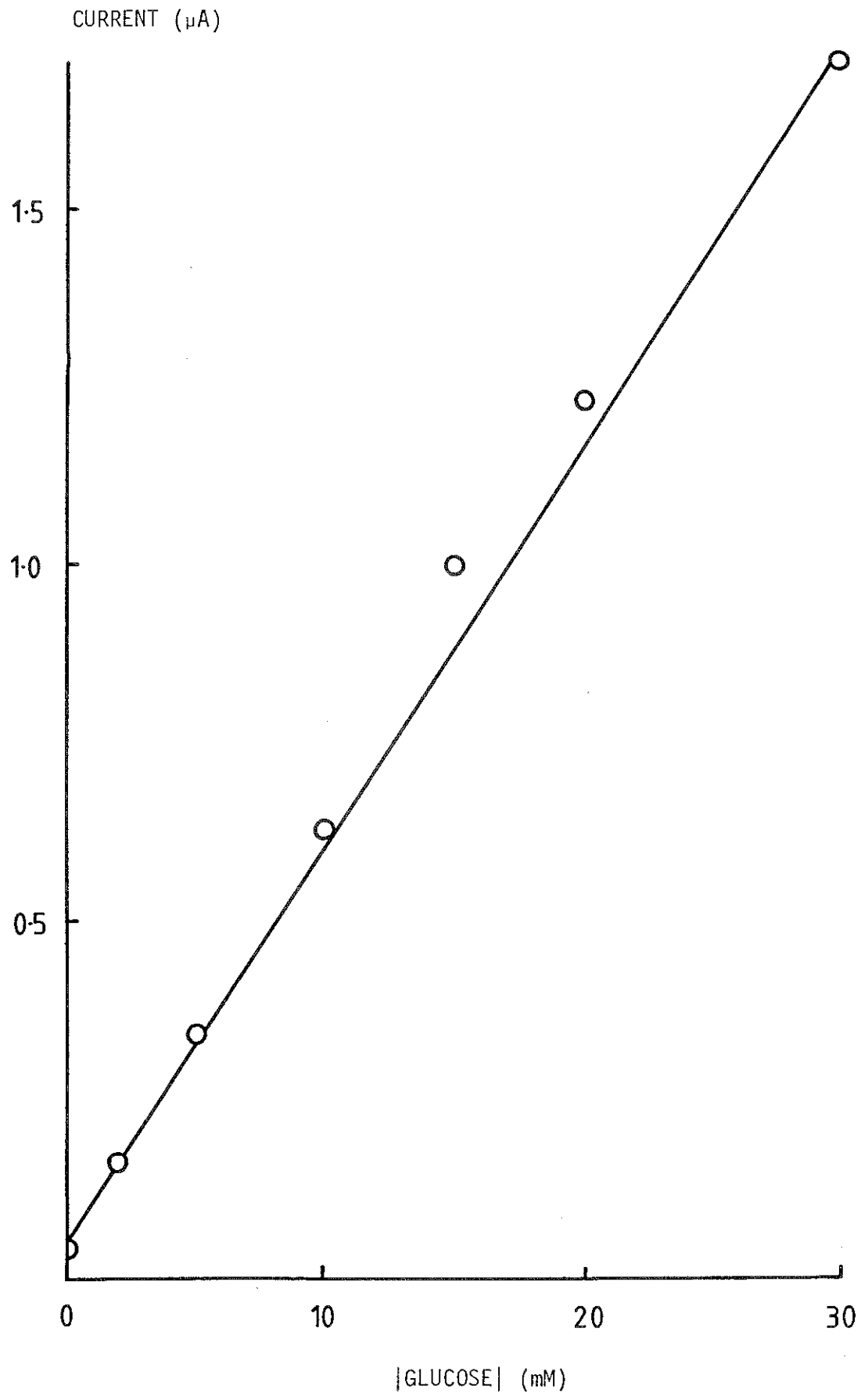

CURRENT (μA)
1·5
1·0
0·5
0
10
20
30
|GLUCOSE| (mM)

Figure 6.21 The amperometric response of a glucose oxidase based strip electrode to glucose in the range 0-5 mM using a two electrode configuration (see text for details).

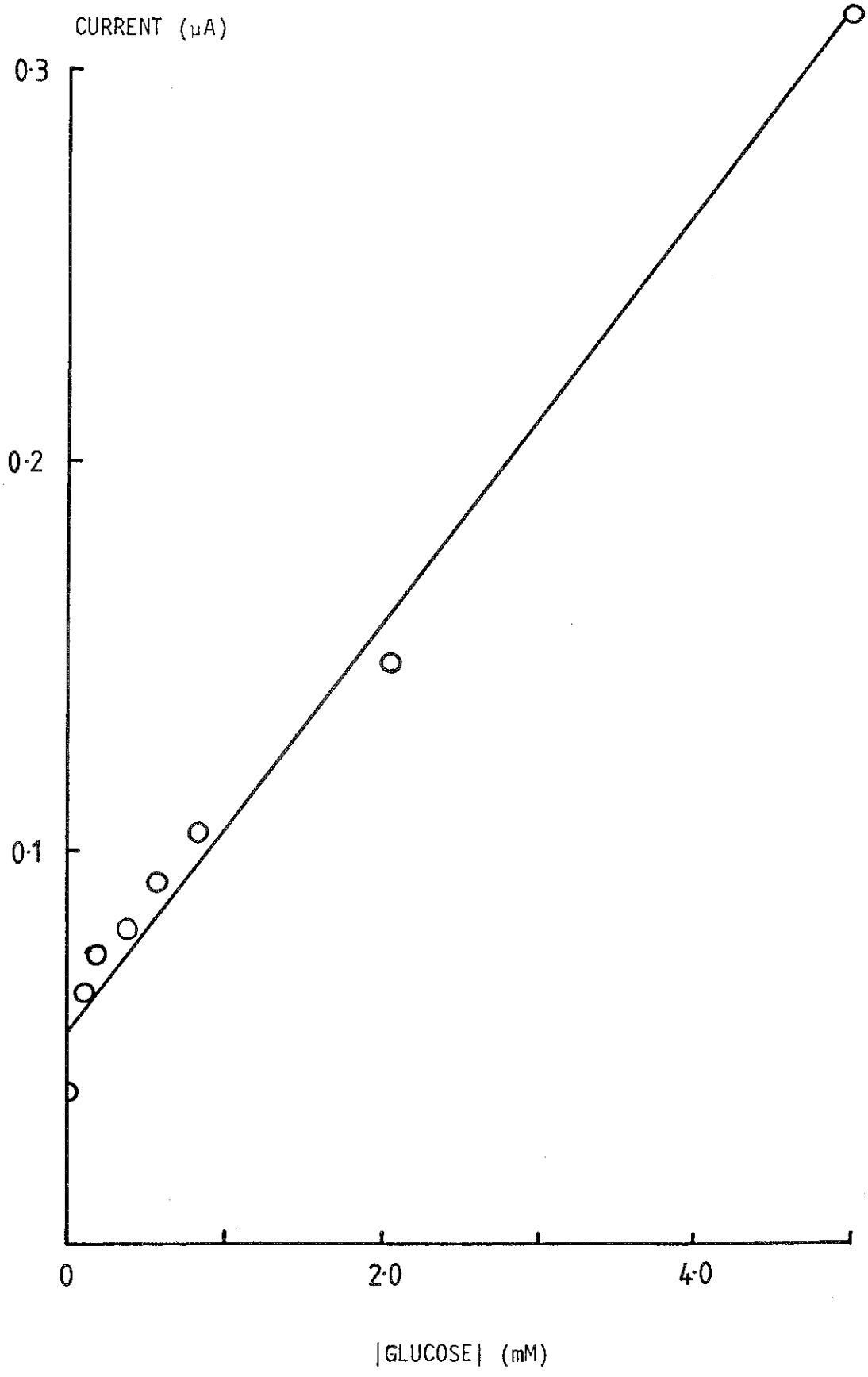

CURRENT (μA)
0·3
0·2
0·1
0
2·0
4·0
|GLUCOSE| (mM)

Figure 6.22 The amperometric response of a glucose oxidase based strip electrode to glucose recorded after 35 seconds, compared to two commercially available colorimetric strip devices, DIASTIX (A) and DEXTROSTIX (B) (see section 2.12.4).

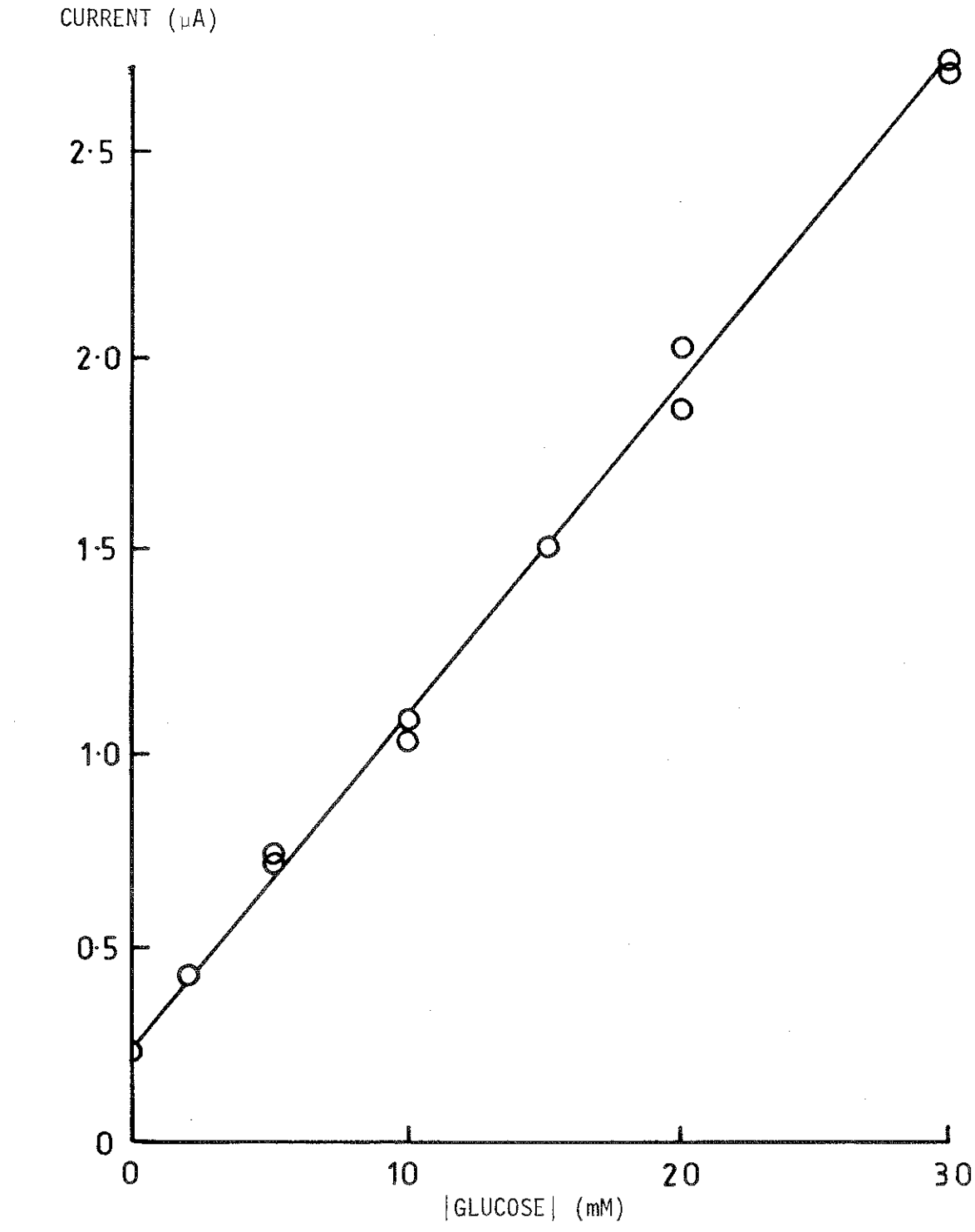

CURRENT (μA)
2·5
2·0
1·5
1·0
0·5
0
0
10
20
30
|GLUCOSE| (mM)
A
B

Table 6.1 Preliminary data on the analysis of blood glucose obtained from a non-diabetic (1) and a diabetic (2) patient. Samples were obtained from the index finger using an 'Autolet' lance (Genetics International Inc.).

Analytical Method	Glucose Concentration (mM) Patient	
	1	2
Glucometer visual	4.7	9.3
Glucometer meter	5.0	9.1
Yellow Springs Instrument	4.9	10.3
Glucose oxidase based enzyme electrode	4.9	9.2

electrode tested as previously described produced a steady state current to glucose up to 30 mM attaining steady state currents within 30 seconds.

It was possible to replace 1,1'-dimethylferrocene with NMPTCNQ in the immobilised glucose electrode (Fig 6.23). The probe, poised at +200 mV (versus SCE), exhibited a linear response to glucose up to 20 mM. Oxygen tensions affected the response; at 30 mM glucose the steady state current for nitrogen, air and oxygen saturated buffer were 5.75 mA (100%); 5.63 mA (98%) and 3.0 mA (52%), respectively.

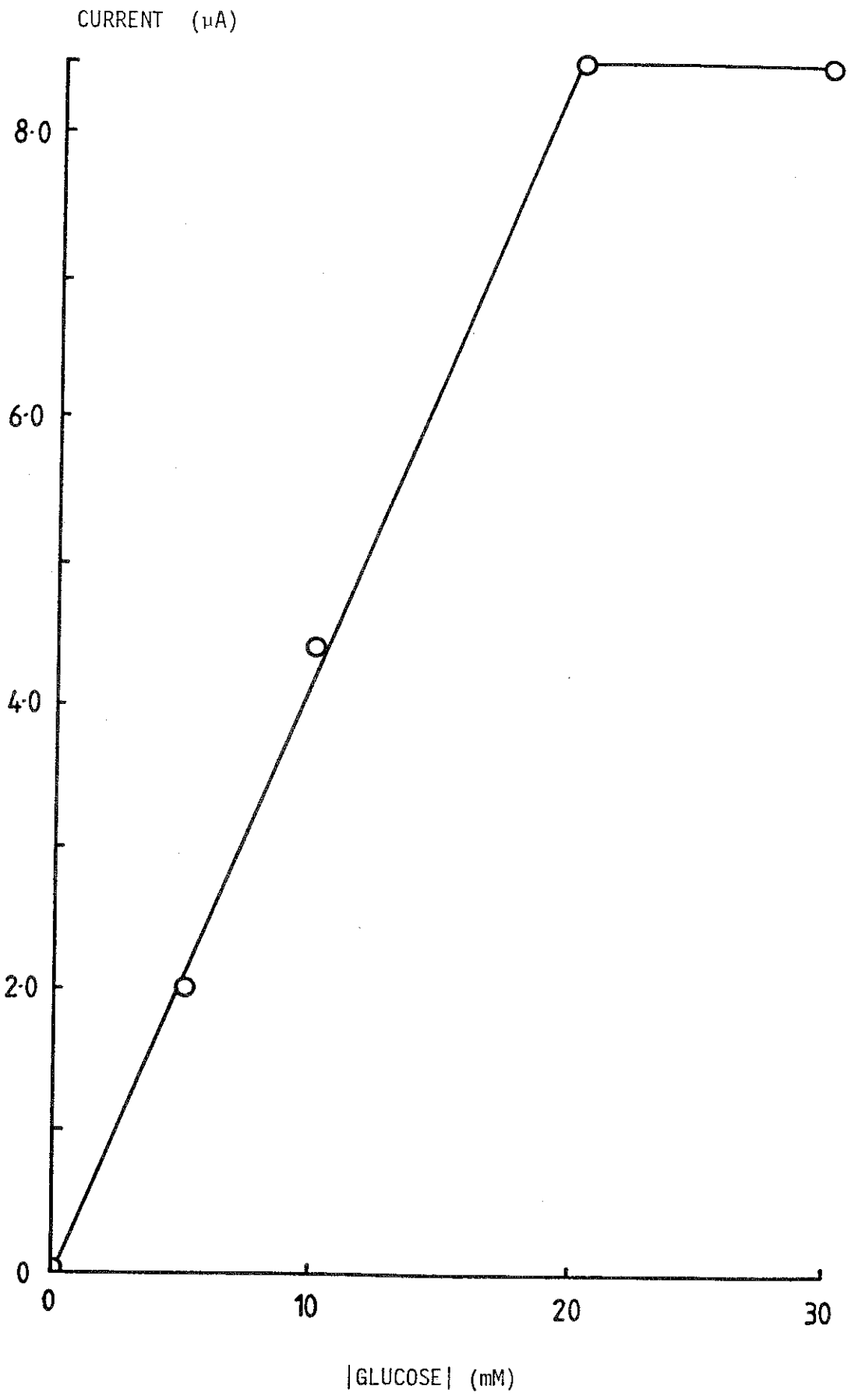
6.7 Discussion.

The construction of the enzyme electrode described, enabled glucose concentrations to be determined both in laboratory and clinical samples. The processes occuring within the electrode are not fully understood, and are complicated by the porous nature of the electrode and are influenced by a number of chemical and physical factors.

The response of the detector to buffered glucose solutions was affected by the concentration of enzyme and mediator used in the immobilisation procedure.

The glucose oxidase electrode responded rapidly to glucose enabling steady state readings to be made within 1.0 minute. The response was linear up to 30 mM with the lowest limit detected 0.1 mM. The electrode response was linear with respect to the glucose concentration normally found in diabetic patients

Figure 6.23 The amperometric response of a glucose oxidase based enzyme electrode, mediated by NMPTCNQ, to glucose.


CURRENT (μA)
8·0
6·0
4·0
2·0
0
0
10
20
30
|GLUCOSE| (mM)

(Turner and Pickup, 1984) and was independent of pH over the range 7.0 - 9.0. The electrode had a temperature coefficient of ca. 4.0% $^{o}C^{-1}$ between 20^{o}C and 45^{o}C and above this temperature inactivation was observed. At high mediator concentrations the background current was unaffected by increasing the temperature above 40^{o}C, however, at low concentrations of 1,1'-dimethylferrocene there was an increase in the background current corresponding to the melting point of 1,1'-dimethylferrocene. The probe was unaffected by variation in oxygen tension when poised at +600 mV (versus SCE), although at this potential interference from other electrochemically active substances, such as ascorbate, present in the blood would occur (Scott, personal communication). This interference may however may be reduced or eliminated by the use of membranes described by other authors (Lobel and Rishpon, 1981). The loss in ability to respond to glucose on storage is presumably due to loss of enzymic activity and not loss of mediator. Preliminary work suggested that the device gave comparable results to other techniques used in glucose analysis. Comparison to the Yellow Springs glucose analyser resulted in a correlation coefficient of 0.98 (n=23) to be attained (Davis, personal communicaton).

Development of the glucose sensor should lead to applications in industrial and clinical monitoring. The feasibility of the latter has been demonstrated, but the sensor requires further development to enable reproducible in vitro and in vivo glucose analysis.

The graphite foil used in this study was not ideal for mass production of electrodes. It did, however, allow an electrode to be constructed which consisted of multiple layers of carbon between which mediator and enzyme are retained.

Ferrocene and its derivatives may be suitable mediators for in vivo use since they are relatively non-toxic. They are used in the administration of iron (Dahl, 1979; Dahl and Briner, 1980; Yarrington et al., 1983) have antibiotic properties (Edward et al., 1975, 1976) and do not undergo autoxidation (Szentrimay et al., 1977).

Oxygen insensitivity of the glucose electrode offers advantages over other oxygen-dependent enzyme-based glucose electrodes. In previous configurations it was necessary to make sufficient oxygen available by either electrochemical generation (Enfors, 1981; Cleland and Enfors, 1982) the use of membranes (Suzuki et al., 1982) or by incorporating sufficient oxygen in the electrode (Rossette et al., 1979). Oxygen tensions in the human body are normally maintained at 30 - 40 mm Hg, but have been shown to decrease upon implantation of a sensor (Hunt et al., 1969) to as low as 6.0 mm Hg after 14 days (Niinikoski et al., 1972). In addition the oxygen independence of the ferrocene-based system relieves the requirement for the removal of catalase, an impurity which interferes with oxygen dependent systems. Removal of catalase is both expensive and difficult.

Many of the previously described amperometric glucose electrodes elicited responses within a few minutes, but utilised

mediators which were either soluble (Nagy et al., 1973) or toxic (Williams et al., 1970) and therefore unsuited to development for in vivo analysis.

Glucose oxidase-based enzyme electrodes suffer from oxygen interference due to it being a co-substrate (see section 1.2.2). The use of an oxygen independent enzyme, such as glucose dehydrogenase (Duine et al., 1979b; D'Costa et al., 1984) should enable the quantitative determination of glucose to be made in samples exhibiting varying oxygen tensions.

Whilst disposable enzyme-based glucose test strips have been developed for home analysis (see sections 2.11.4b and 2.11.4c) difficulty may be encountered in their use (Fig 6.22). The application of glucose based enzyme electrodes should benefit many sufferers of diabetes (Albisser and Spencer, 1982). Diabetes is one of the commonest chronic diseases and affects about 6.0% of the adult population of the western world (WHO, 1980). Associated complications include heart disease, strokes, amputations and blindness (Turner and Pickup, 1984). These should be reduced by improved electronic control of blood glucose levels by enabling a relatively constant glucose concentration to be maintained (Albisser and Spencer, 1982). Several devices may be envisaged that would improve general diabetes management such as convenient subcutaneous sensors that may ultimately to be linked to an automated feed back loop coupled to a portable insulin pump.

CHAPTER 7

GENERAL SUMMARY

7.1 Summary of work presented in this thesis.

Initial purification of methanol dehydrogenase was based on the published procedures of Anthony and Zatman (1967a). Using this conventional technique only limited quantities could be purified predominantly due to the maximum loading the equipment. An alternative purification procedure involving aqueous two phase enzyme separation was developed. The method had the advantage that once optimum conditions for enzyme extraction were determined it was possible to increase the volumes enabling large quantities to be purified. The yield and specific activity of the purified enzyme attained by this method was higher than that obtained previously using conventional techniques (Ghosh and Quayle, 1981). Whilst the reasons for the higher yield and enzyme activity were not fully investigated this may be due to the milder conditions employed and the speed with which the process is completed, an effect observed by other authors using similar techniques on other enzymes (Kroner et al., 1982b). The enzyme purified by two phase separation technique exhibited properties similar to that purified by conventional techniques, such as subunit molecular weight and absorption spectrum. The pH optimum was higher than that published previously (Anthony and Zatman, 1967a,b) although these authors did not test methanol dehydrogenase and phenazine ethosulphate above pH 9.0.

Initial work utilised phenazine ethosulphate as the mediator (Plotkin et al., 1981) although it possessed several disadvantages. These include photoinactivation and instability at the pH optimum of the enzyme (Ghosh and Quayle, 1979). To overcome these problems a

range of alternative mediators were screened. N,N,N',N' tetramethyl-p-phenylenediamine (TMPD) was shown to be the most suitable alternative. It was readily soluble, capable of reversible, single electron transfer, stable at pH 10.5 and did not undergo photochemical inactivation. In the biofuel cell, TMPD mediated electron transfer between methanol dehydrogenase and the electrode although, as with other fuel cells, the coulometric efficiency was reduced with increasing external resistance (Liebhafsky and Cairns, 1968). It was also observed that increasing both mediator and substrate concentrations caused a reduction in the coulometric efficiency. This reduction in efficiency is probably due to diffusion through the membrane by methanol, oxygen and mediator or side reactions such as that postulated between formaldehyde, the intermediate product formed, and ammonium ions which are required for activation of the enzyme. In an attempt to produce a more efficient biofuel cell and overcome some of these problems, the components of the biofuel cell were investigated. To reduce the effect of oxygen a non-autooxidisable mediator, 1,1' dimethylferrocene, was utilised. A platinum electrode was succesfully doped with 1,1'-dimethylferrocene which, although capable of mediating, produced lower currents in comparison to those obtained with TMPD, due to the ferrocene derivative having an increased redox potential and possibly the reduced interaction of the soluble enzyme with the modified electrode.

Reticulated carbon was investigated as an electrode to reduce the internal resistance of the biofuel cell. Whilst this material has been successfully used by other authors (Bennetto et al., 1980) it

was brittle and could be not easily handled. A variety of techniques were investigated to deposit the electrode material directly onto the membrane in an attempt to reduce the internal resistance and the diffusion of mediator, oxygen and substrate. Sputtering provided an electrode on both surfaces, which was capable of functioning either as an anode or a cathode without destroying the properties of the membrane. Although the use of the modified electrode as a cathode was not pursued, it was demonstrated that it was capable of electron transfer from both soluble and insoluble mediators. A biofuel cell was constructed which incorporated both immobilised enzyme and mediator. Carbon was sputtered onto gold palladium deposited previously onto the membrane, and glucose oxidase was immobilised onto the modified surface. Using this configuration, the cell produced small currents and exhibited a high electrode resistance and an enzyme-dependent current could not be measured. However, by poising the potential of the electrode an enzyme-dependent current was observed on the addition of substrate.

The majority of enzyme-based sensors utilise indirect systems (Carr and Bowers, 1980) which possess several disadvantages (see section 1.4). Methanol oxidase, for example, enables alcohol concentrations to be determined by measuring either oxygen consumption (Verduyn et al., 1983) or hydrogen peroxide production (Mason, 1983). Whilst these configurations can function as sensors, they are of limited application since they require oxygen to be in excess. The product, hydrogen peroxide, inactivates the enzyme and sensors based on this configuration require the removal of catalase, a frequent

impurity of methanol oxidase enzyme preparations. The quinoproteins such as methanol dehydrogenase are not limited by such restraints and when linked to non-autooxidisable mediators could be used in solutions with a range of oxygen tensions.

Two amperometric enzyme electrode configurations based on a mediated system were investigated. The first utilised the soluble mediator phenazine ethosulphate and methanol dehydrogenase for the analysis of methanol. The device was unaffected by potential contaminants of water supplies enabling methanol to be determined coulometrically at concentrations at the limit of those detected by gas liquid chromatography. This configuration, although not requiring sophisticated and expensive equipment, may not be considered practical because of the requirement for ammonium ions and anaerobic conditions due to the use of an autooxidisable mediator.

To overcome some of these restraints the enzyme was retained in a probe sensor mediated by a non-autooxidisable mediator, 1,1' dimethylferrocene. This configuration still required the presence of ammonium chloride but was unaffected by variations in oxygen tension, a property not exhibited by previous alcohol sensors (Mason, 1983; Verduyn et al., 1983).

Interest in sensors has focused attention on the measurement of glucose due to its practical application, especially in the field of medicine. The majority of enzyme-based glucose sensors measure oxygen consumption (Guilbault and Labrano, 1973), hydrogen peroxide production (Shichiri et al., 1982) or utilise toxic mediators (Williams et al., 1970). Although these have in some cases been

succesfully developed into commercial systems, such as the Yellow Springs glucose analyser (YSI), they are of limited application especially were implantation is considered. A range of techniques have been used in an attempt to overcome the limitations of oxygen based systems ranging from a requirement for oxygen in the sample (YSI), the use of membranes to cause preferential diffusion of oxygen (Schichiri et al., 1982) and electrochemical oxygen production (Romette et al., 1979). Although these sytems are being pursued their practical long term application may be considered doubtful.

Ferrocene and its derivatives are non-toxic and couple to the flavoprotein glucose oxidase. Immobilisation of glucose oxidase onto a carbon electrode with 1,1'-dimethyferrocene as a mediator in a probe exhibited many of the features required for the analysis of clinical samples (Thevenot, 1982). These include response within the normal physiological pH range, independence of oxygen and volume of the sample. Due to the properties of the graphite used the substrate was diffusion controlled thereby reducing the temperature dependence of the electrode. Non-toxicity is especially important if in vivo applications are to be developed. Although the solubility of the ferricinium ion may cause problems during continuous operation, this may be overcome by the use of a suitable membrane (eg. a positively charged membrane). Based on the results obtained in this thesis it should be possible to develop a range of enzyme-based sensors utilisising those enzymes which may be linked to ferrocene and its derivatives mediate electron transfer to an electrode eg. amino acid oxidase, xanthine oxidase, carbon monoxide oxidoreductase, glucose dehydrogenase and lactate dehydrogenase.

7.1 Future work.

The development of enzyme-based analytical techniques such as enzyme electrodes requires economic production of enzymes. Two phase aqueous enzyme purification of methanol dehydrogenase has been demonstrated and investigation of the factors affecting enzyme distribution should enable its application to other enzymes such as glucose dehydrogenase.

Directly coupled enzyme-based biofuel cells may be considered to be in their infancy; possessing many of the problems which were initially encountered with other fuel cells. Indeed, many of the lessons learnt in the development of conventional fuel cells may be applied to biofuel cells, which range from systems based on whole organisms to purified enzymes. The immobilisation of the biological catalyst, such as methanol dehydrogenase, would facilitate continuous operation allowing the continuous addition of substrates and removal of products. The immobilisation and coupling of methanol dehydrogenase to its natural redox partner may enable the development of more direct electron transfer.

Analytical applications of enzyme-based systems are in the short term of the greatest commercial interest especially in view of the increasing number of suitable enzymes available. Whilst a greater understanding of the mechanism of action of these coupled systems is required, the enzyme-based electrodes should be developed rapidly and adapted to the analysis of industrial samples.

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