ENZYMATIC DETERMINATION OF UREA AT A POLARIZABLE MEMBRANE-STABILIZED LIQUID-LIQUID INTERFACE

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DEDICATION

TO MY MAM WOROIITUU MIILOO

WHO, ALONE, CATERED ME-AND SENT ME SCHOOL

SAVING NOTHING FOR HER

&

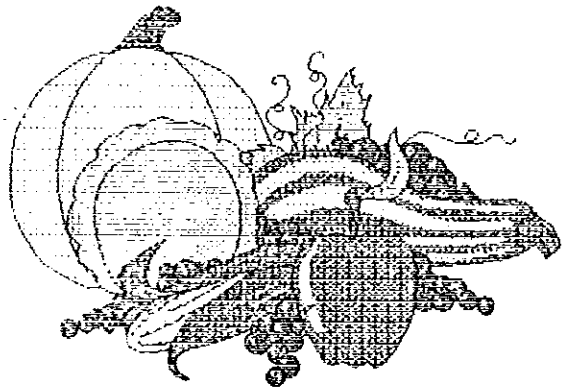
TO ALL THE CHARACTERS WHO BROUGHT TURNING ENCOUNTERS

IN MY LIFE

&

TO ALL OF MY TEACHERS AND MY SCHOOLS

FOR THIS IS THE FRUIT OF THEIR CULTIVATION



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ABSTRACT

The membrane-stabilized water-nitrobenzene interface was used as an amperometric (or voltammetric) ammonium ion sensor for an indirect detection of urea. The ammonium ion was produced by the action of watermelon seeds and soy bean flour on urea in the aqueous phase (5 mmol/L Li_2SO_4). The detection was based on the facilitated transfer of ammonium ions by DB[18]C-6. The organic phase contained 50 mmol/L DB[18]C-6 and 10 mmol/L EthVTPB. The performance of watermelon seeds and soy bean flour as soluble urease reagents is described. The problem caused by potassium indigenous to the tissues is discussed. The sensor was applied to the estimation of potassium in the seeds of some plants.

INTRODUCTION

Electrochemically inactive ions or biological molecules can be analyzed (sensed) electrochemically indirectly in a method which involves the adaptation of principles from biology. Examples are enzymatic and immunological reactions or membrane ion transport mediated by ionophores.

The classical amperometric or voltammetric detection is based on the reduction or oxidation of the species of interest. A new detection principle is based on the electrically induced transfer of ions across the water/ oil interface or more elaborately across the interface between two immiscible electrolyte solutions (ITIES). This can be applied to the determination of ions that are not electro-active in the classical sense. Thus, in addition to the direct determination of ions [1,2], assaying of ionophore [3] and ions whose transfer is ionophore facilitated [4] and neutral bio-molecules giving rise to transferable ions in a biochemical reaction [5-7] have been possible.

Basics of ITIES

The study of the principles making the basis of such analytical application ITIES dates back to 1902 [8]. The works of Ross [9] on liquid-state ion-selective electrodes, Gavach, etal. [10] on polarization phenomena across ITIES, Karpfen and Randles [11] and Hung [12,13] on the thermodynamics of ion transfer across ITIES (where simple, ion association and complex formation were treated) are very fundamental contributions to this branch of electroanalytical chemistry.

The construction of analytically useful ITIES requires the pairing of two mutually insoluble liquid phases. Usually the first is water (w) and the other is an organic liquid (o). The latter must be sufficiently polar (dielectric constant >10) to provide enough conductivity and prevent ion association. The difference in density between the two phases must be high so that a physically stable interface would be formed. For a facile utilization of ITIES in sensors mechanical stability of the electrified interface can be achieved by solidification of the organic phase through gel formation with polyvinylchloride (PVC) [14] or by the use of arrays of micro liquid-liquid interfaces [15].

The analytical application of ITIES is based on the salt partition phenomena across the interface. Some ions are hydrophobic, others hydrophillic and the rest are of intermediate nature. The classification of ions with respect to hydrophillicity has been made simple by the adoption of some extra thermodynamic assumptions which enabled estimation of single ion thermodynamic quantities related to salt partition between two immiscible phases. Usually the "TATB" assumption of Parker is used [16]. Potentiometric, voltammetric and amperometric applications [17] are possible if suitable supporting electrolytes are incorporated in both phases to render them conductive. A hydrophobic salt (e.g. crystalviolet tetraphenylborate, ethylviolet tetraphenylborate) is placed in the organic phase and a hydrophillic salt (e.g. Li_2SO_4, NaF, $MgSO_4$) is placed in the aqueous phase so that the interface becomes polarizable over a range of potentials. The ions of the supporting electrolytes are practically confined to their respective phases. Thus, the interface becomes amenable to the utilization of accepted electrochemical methods so that measurements will be based on currents due to ion transfer across the interface.

The extent of the respective hydrophillicity and hydrophobicity determines the working range (polarization range) of the sensor.

Analysis Based on Ionophore Mediated Transfer of Ions

Since the potential window is usually narrow, many ions, especially very lipophobic ones such as Na^+, K^+, NH_4^+ can not undergo simple ion transfer. It has been shown that the transfer of ions across the interface can be realized by the presence of suitable ionophores such as valinomycin for K^+ [18], nonactin for NH_4^+ [19] via the encapsulation of the ions rendering them lipophilic. These are among the naturally occurring ones. Synthetic ionophores include macrocyclic polyethers such as dibenzo[18]Crown-6. The complex formed are characterized by a polar internal cavity, in which the central cation is situated, and an outer lipophilic envelop, which permits dissolution of the ion in a non-polar medium.

With sensors based on ionophore-facilitated ion transfer, an analyte may be determined either by potentiometry with an ionophore-based ion-selective electrode (ISE), or by electrolysis (voltammetry) at the interface of two immiscible electrolyte solutions [20,21]. In potentiometry, the electrical signal is the electromotive force of the cell consisting of the inner electrode of the ISE, of the membrane and of the reference electrode. In voltammetry, the potential difference at the ITIES is varied in a system of two reference and two auxiliary electrodes by means of a four-electrode potentiostat. The interface is formed of an aqueous solution of a hydrophillic electrolyte and an organic solution of a hydrophobic electrolyte. The resulting potential-current-time characteristics are governed by the same principles

as in the system of an electrode polarized in an electrolyte solution.

The membranes of ionophore-based ISEs are prepared from an inert matrix (eg. PVC) containing an appropriate ion-exchange solution as a plasticizer [22]. This solution contains the salt of the ionophore complex of the determinand cation with strongly hydrophobic anion like tetraphenylborate (TPB^-) or p-chlorotetraphenylborate ($p\text{-ClTPB}^-$). As solvents, o-nitrophenyloctyl ether or phthalic acid ester are used. The ISE of this type include the potassium electrode based on valinomycin, the sodium electrode based on monensin [23] and the ammonium electrodes based on nonactin or monactin.

Voltammetry at the ITIES is particularly suitable for the determination of ionophores. In this case a rather high concentration of the salt of the cation which is complexed by the ionophore is used as the electrolyte of the aqueous phase while the organic phase contains a low concentration of the ionophore. In triangular sweep-voltammetry, the current is controlled either by diffusion of the ionophore or the complex to the interface. This method was applied to the determination of the cocciostat and monensin in cultures of streptomyces cinnamonensis [24]. Similarly the same technique can be adapted for the determination of the cation which is complexed by the ionophore. In this application, the concentration of the cation in the aqueous phase is kept low, while that of the ionophore is kept higher. Under these conditions the current is controlled by the diffusion of the cation to the interface. This method was applied to the determination of K^+ and Na^+ in artificial standard and blood serum samples [25].

reactions of substrates at low concentration is of great importance in chemical analysis. Such determinations of substrates, enzyme activators. enzyme inhibitors and enzymes themselves have been possible.

Four essentially different types of procedures for performing enzyme-involving analysis may be distinguished.

1. Single-stage bulk procedure - the enzyme and the substrate together are allowed either a standard time to react or sufficient time for the reaction to reach completion while a sensor probe is immersed in the solution.

2. Enzyme electrode procedure - enzyme (for substrate analysis) or the substrate (for enzyme analysis) is immobilized in a thin gel, coated onto the surface of the sensor which is immersed in the sample being stirred.

3. Double-stage procedure - similar to procedure 1 except that the reaction and measurement stages are separate. If the optimum pH for enzyme activity is unfavourable for the sensor, both steps will be effected at their suitable pH conditions.

4. Multiple-Stage Bulk Procedure - if the product of an enzyme reaction is not itself detectable by an electrode or probe, additional reaction, enzymatic or otherwise may be performed to generate detectable species (amplification)

Method 1 is highly expensive since it causes high consumption of reagents per analysis. It is also not applicable if there is a discrepancy between the pH optimum of the enzyme and the pH optimum of the sensor. Method 2 is convenient to use since a smaller amount of enzyme is required, the enzyme is reusable and sample pretreatment is minimized.

The concentration of a material participating in an enzymic reaction can be calculated in one of two ways :

1. By measuring the total change that occurs by chemical, physical or enzymatic analysis (the equilibrium method).
2. From the initial rate of the enzyme reaction (the kinetic method)

In the first method, large amounts of enzyme and small amounts of substrate are used to ensure a relatively complete reaction. It is more reliable and can only be used for substrate analysis. In the kinetic method, the initial rate is a function of concentration of the substrate, the enzyme, inhibitor, and activator. This latter method is faster, but pH, temperature and ionic strength must be carefully controlled for maximum sensitivity.

Enzyme sensors or biosensors for urea based on the potentiometric detection have extensively been studied (see section 2). Recently, the possibility of applying ITIES as amperometric sensors in bioelectrodes has been demonstrated in the works of M. Send and Y. Yamamoto [5,7] and T. Bekele [6]. An amperometric urea bioelectrode constructed by immobilizing the enzyme urease (commercial) across

the water-nitrobenzene interface between two dialysis membranes has been described. Urea was determined by the DB[18]C-6-facilitated transfer of NH_4^+ ions (from the urea-urease reaction) to the organic phase. The amperometric sensor has unique characteristics in comparison with the potentiometric one. The amperometric sensor gives a current response, i, which is proportional to the concentration of the analyte, C, that is, $i = K_{amp}C$ where K_{amp} is a constant. The potentiometric sensor gives a potential response, E, which changes linearly with the logarithm of the activity, a (or concentration), of analyte, that is, $E = K_{pot} \ln[a/a_o]$ where K_{pot} is a constant and a_o is a reference activity. Accordingly, the sensitivity (or the change of the response, Δi) of the amperometric sensor is directly proportional to the change of the analyte concentration, ΔC, that is $\Delta i = K_{amp}\Delta C$; whereas that of the potentiometric sensor, ΔE, is directly proportional to the relative change of the analyte concentration, that is, $\Delta E = K^{pot}\Delta C/C$. One of the advantages of the amperometric sensor is that the determination of two or more analytes concentration can be made by a measurement with a single sensor when their characteristic potentials, such as the half-wave potentials or the peak potentials are reasonably separated. Also, the correction for the residual current due to residual components(s) in the test solution can be achieved more easily with the amperometric sensor compared with the potentiometric one.

This thesis is concerned with the use of the membrane-stabilized water-nitrobenzene interface as an amperometric (or voltammetric) ammonium sensor. The ammonium ion is produced by the action of urease (in a solution of tissues rich in this enzyme) on urea. The method is also used for the determination of urea.

2 THEORY

2.1 Thermodynamics of Charge Distribution Across ITIES

For two immiscible solvents such as water (w) and an organic solvent (o) in contact each containing ions of species i of charge z_i under equilibrium distribution between the two phases, the electrochemical potential, μ_i, of each species i in both phases must be equal :

$$\overline{\mu}_{i,w}=\overline{\mu}_{i,o} \qquad 2.1.1$$

The electrochemical potential of an ion in a phase α is given by equation 2.1.2

$$\overline{\mu}_{i,\alpha}=\mu^{\circ}_{i,\alpha}+RT\ln a_{i,\alpha}+Z_iF\varphi_{\alpha} \qquad 2.1.2$$

where $\mu_{i,\alpha}^{\circ}$, $a_{i,\alpha}$ and φ_{α} represent the standard chemical potential, the activity, and the galvani potential, respectively, of the species in the phase in question. R, T and F have their usual significance. As can be derived from equations of the type 2.1.2 for both phases, the galvani potential difference between them, $\Delta_o^w\varphi$, is given by

$$\Delta_o^w\varphi=\varphi_w-\varphi_o=\frac{\mu^{\circ}_{i,w}-\mu^{\circ}_{i,o}}{Z_iF}+\frac{RT}{Z_iF}\ln\frac{a_{i,o}}{a_{i,w}} \qquad 2.1.3$$

$$=\frac{-\Delta^{\circ}_wG^{\circ,w\to o}_{tr,i}}{z_iF}+\frac{RT}{z_iF}\ln\frac{a_{i,o}}{a_{i,w}} \qquad 2.1.4$$

$$=\Delta^w_o\varphi^{\circ}_i+\frac{RT}{z_iF}\ln\frac{a_{i,o}}{a_{i,w}} \qquad 2.1.5$$

where $\Delta G_{tr}^{o,w\rightarrow o}$ is the standard Gibb's free energy of transfer from the aqueous phase to the organic phase and $\Delta_o^w\varphi_i^o$ is the standard galvanic potential difference between two phase with respect to each moiety, i. When a salt AB is partitioned between the phases which are chosen so that the salt is soluble and, at the same time, almost completely dissociated in each, it is possible to derive the following equation for a

$$\Delta_o^w\varphi = \frac{\Delta_o^w\varphi_+^o + \Delta_o^w\varphi_-^o}{2} \qquad 2.1.6$$

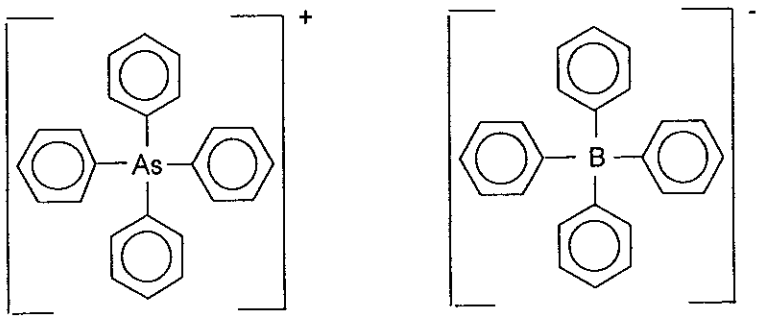
dilute solution so that the activities of the counter ions in the same phase are equal. As it is clear from equation 2.1.6, the galvani potential difference across the interface between immiscible electrolyte solutions is independent of the salt concentration if a single electrolyte is at equilibrium partition [11]. This situation changes if two or more electrolytes are present in the system as it will be seen later, where the hydrophilicity and hydrophobicity of ions comes into consideration. To classify ions between such categories the standard Galvani potentials or the standard Gibb's transfer energies must be known for each of them

The individual ion thermodynamic partition coefficient ($K_{p,i}$), the single ion standard Gibb's energy of transfer / partition ($\Delta G_{p,i}^{o,w\rightarrow o}$) and the standard galvani potential difference $\Delta_o^w\varphi^o$ are related as follows :

$$-\frac{\Delta G^o}{Z_i F} = \Delta_o^w\varphi_i^o = -\frac{RT}{z_i F}\ln K_{p,i}^o \qquad 2.1.7$$

The individual ion activities in a non aqueous solution may be treated in an analogous way as in an aqueous medium using the Deby-Huckel equations for low

concentrations. The influence of the solvent is then reflected only in the difference of standard chemical potentials. The standard Gibb's energy of transfer for an individual ion, in contrast to the electrolyte as whole, is not accessible to a direct measurement and, in order to make possible its quantitative determination, some kind of a non thermodynamic assumption must be made. Most frequently the 'TATB assumption' [16] is made stating that the cation ($TPAs^+$) and the anion (TPB^-) of the salt tetraphenylarsonium tetraphenylborate (TPAsTPB) have equal standard Gibb's transfer energies.



$TPAs^+$ TPB^-

Fig. 2.1.1 Structures of the tetraphenylarsonium and tetraphenylborate ions.

The application of this assumption is as follows. For the electrolyte AB, in general, the distribution between the two phases is given by the exchange equilibrium below.

$$A^+(w) + B^-(w) \rightleftharpoons A^+(o) + B^-(o) \qquad 2.1.8$$

Then it follows that,

$$\overline{\mu}_{AB,w}=\overline{\mu}_{AB,o} \qquad 2.1.9$$

For the whole salt in phase α we have the following

$$\mu_{AB,\alpha}=\mu^{o}_{+,\alpha}+\mu^{o}_{-,\alpha}+RTlna_{+,\alpha}+RTlna_{-,\alpha} \qquad 2.1.10$$

$$\mu_{AB,\alpha}=\mu^{o}_{+,\alpha}+\mu^{o}_{-,\alpha}+RTln[a_{+,\alpha}a_{-,\alpha}] \qquad 2.1.11$$

When equations of the type 2.1.11 for both phases are equated as in equation 9 it is possible to show that the Gibb's free energy of partition of the salt AB is given by equation 2.1.12.

$$\Delta G^{o}_{p,AB}=-RTln\frac{C^{2}_{AB,o}\gamma^{2}_{\pm,o}}{C^{2}_{AB,w}\gamma^{2}_{\pm,w}} \qquad 2.1.12$$

where $C_{AB,\alpha}$ and $\gamma_{\pm,\alpha}$ are the salt concentration and the mean activity coefficient in the phase indicated. Then, the salt partition coefficient, which is experimentally accessible, is given by

$$K_{p,AB}=\frac{C^{2}_{AB,o}\gamma^{2}_{\pm,o}}{C^{2}_{AB,w}\gamma^{2}_{\pm,w}} \qquad 2.1.13$$

Comparison of equation 2.1.13 with equations 2.1.7 and 2.1.12 makes clear that

$$K_{p,AB} = K_{p,+} \cdot K_{p,-} \qquad 2.1.14$$

In dilute solutions, $\gamma_{\pm}\approx 1$ in both phases so that the salt partition coefficient may be approximated from the distribution constant, $K_{D,AB}$, of the salt using equation 2.1.15.

$$K_{p,AB} = \frac{C^2_{AB,o}}{C^2_{AB,w}} = K^2_{D,AB} \qquad 2.1.15$$

When equation 2.1.14 and 2.1.15 are used for the TATB assumption, we have

$$K_{p,TPAs^+} = K_{p,TPB^-} = \sqrt{K_{p,TPAsTPB}} = K_{D,TPAsTPB} \qquad 2.1.16$$

Thus, once the $K_{p,i}$ values for these ions are known for a pair of immiscible ions those of other ions can be determined by working with their corresponding salts of $TPAs^+$ or TPB^-. A scale for standard values of individual Gibb's free energy of transfer, galvanic potential differences and partition coefficients can therefore be produced as in reference [31 (p.219)].

Ions whose individual partition coefficients lie around unity are designated as semi-hydrophobic, while those with $K_p^{o,w} >> 1$ as hydrophobic. Sets of various salts such as one ion hydrophobic and the other hydrophillic, both ions hydrophobic (hydrophillic) or both of them semi-hydrophobic etc. can be distinguished and studied.

Let us consider a salt of a hydrophobic cation and hydrophillic anion distributed between water and an organic solvent. The cation has the tendency to cross the boundary (ITIES) while the anion behaves quite the opposite way, i.e. the equilibrium illustrated by equation 2.1.17 (below) shifts to the right. These transitions

$$A^+(w) + B^-(o) \rightleftharpoons A^+(o) + B^-(w) \qquad 2.1.17$$

at least to the bulk of the solution, are indeed not possible but the charge distribution occurs at the ITIES by formation of an electric double layer charged positively in the organic solvent and negatively in water. More detailed investigations have shown that it only consists of two diffuse electric double layers [32].

The ITIES has further interesting properties, particularly when the aqueous phase contains a strongly hydrophillic salt A_1B_1 (eg. Li_2SO_4, LiF, LiCl etc.) and the organic phase contains a strongly hydrophobic electrolyte A_2B_2 (like TPAsTPB, EthVTPB etc.). In such cases, the equilibria for the exchange reactions :

$$\begin{aligned} A_1^+(o) + A_2^+(w) &\rightleftharpoons A_1^+(w) + A_2^+(o) \\ B_1^-(o) + B_2^-(o) &\rightleftharpoons B_1^-(w) + B_2^-(o) \end{aligned} \qquad 2.1.18$$

strongly shift to the right. Under these conditions, the determination of the potential difference by the activities of the ions present in the system would be difficult since the activity of an ion in the phase to which it is very phobic is too small. Then $\Delta_o^w\varphi$ is determined by the charge in the double layer, which can be charged from an external source than by equations of the type 2.1.3 - 2.1.4.

An ITIES resulting from such a situation is ideally polarizable, and it is analogous

to that of an ideally polarized metal-electrode/ electrolyte interface as shown elsewhere [21]. The latter involves charging or discharging by supply or removal of electrons, while the former uses ion transport to or from the interface for the same purpose.

It must be stressed that an ITIES for the case being considered behave so only for a limited range of externally applied potential called the potential window. For potentials out of this range, there exists excessive depolarization due to the transfer of ions in question to the phases to which they are phobic.

2.2 Current Across the Interface

Let us consider another system where the lastly discussed ideally polarizable situation is modified by introducing a semi-hydrophobic ion (A_3^+ or B_3^-) at a

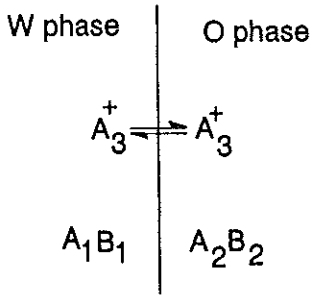


Fig.2.2.1 The salts A_1B_1 and A_2B_2 are practically confined to the phase in which they are indicated. a common semi-hydrophobic ion is depicted to be in an exchange equilibrium between the two immiscible phases.

low concentration (see fig. 2.2.1 above). Now, the electrolytes A_1B_1 and A_2B_2 are in excess over the semi-hydrophobic ions and function as base electrolytes. If a

potential difference (ΔE) in excess of the equilibrium potential $\Delta_o^w\varphi_{eq}$ is imposed

$$\Delta_o^w\varphi = \Delta_o^w\varphi_{eq} + \Delta E \qquad 2.1.19$$

on the system from an external source, the charge introduced is used partly for the transfer of B_3^+ across the interface and partly for charging the double layer. In reference [31] it has been shown how these two effected can be separated. The constant term ΔE in the above equation depends only on the composition of the base electrolytes in (w) and (o), on liquid junction potentials and on the reference electrodes, but it is independent on the nature of the ion B_3^+. The faradaic charge flow across the ITIES is due to the simple charge transfer, which is just analogous to an oxidation reduction reaction at a metallic electrode/ electrolyte interface.

2.3 Analytical Application of ITIES

Before an ITIES is used for analytical purposes, the mechanical stability of the sensor must be ensured either by solidifying the organic phase as PVC gels [1] or by inserting a porous membrane between the two liquid phases [2,33] such as dialysis membranes.

It has been shown that ion-transfer across the ITIES follow formally the same laws as the electron transfer at the metal/ electrolyte solution interface [20,34]. Thus the theory of cyclic voltammetry derived for those electrodes can be adopted to the analytical determination of transferable ions. The transfer of ions

across the interface studied by cyclic voltammetry is diffusion controlled at low sweep rates, hence, the current response of the system to a triangular potential signal can be treated like a reversible electron transfer reaction. From Nicholson and Shain's [35] equation the peak current (i_p) - bulk concentration (C_b), relationship for a system is given by

$$i_P = K_{amp}\, C_i^b \qquad 2.3.1$$

where K_{amp} is a constant varying with square roots of the sweep rate and diffusion coefficients, and the area of the sensor tip. In addition, the Cottrel equation must govern the response in high amplitude potential step (pulse) experiments :

$$i(t) = i_d(t) = \frac{z_i F A D_i^{1/2} C_i^b}{\pi^{1/2} t^{1/2}} \qquad 2.3.2$$

The direct determination of some ions (such as alkaline earth, alkali and ammonium ions) needs the application of very high potentials which are beyond the potential window. But, their transfer can be effected with in the working range by incorporating the so-called neutral carriers (ionophores) in the organic phase. The principle of ionophore-facilitated ion transfer was first described by Koryta [20]. This phenomena occurs due to the complexation of the ions by the ionophores which are hydrophobic, so that the ions will be in a non polar sheath and get their lipophilicity increased. Thus a smaller potential difference (which lies within the potential window) is needed to effect the transfer.

2.4 Enzymatic Electrochemical Determination

In this study three electrochemical methods were used in conjunction with urease catalysed hydrolysis of urea to ammonia and carbon dioxide. The established chemical equation for this reaction is given by equation 2.4.1[36].

$$\text{Urea} + H_2O \xrightleftharpoons{\text{Urease}} 2\,NH_3 + CO_2 \qquad 2.4.1$$

Since determination of urea is based on detection of ammonium ions it is imperative to consider the equilibrium between ammonia and ammonium which is given by equation 2.4.3.

$$NH_3 + H_2O \rightleftharpoons NH_4^+ + OH^- \qquad K_b = 1.66 \times 10^{-5} \qquad 2.4.3$$

Thus, if experiments are performed at around pH ≤ 7.22, more than 99 % of the product is in the form of ammonium ions. The corresponding Michaelis-Menten equation is given by the equation below.

$$\frac{\partial C_{NH_4^+}}{\partial t} = 2\frac{K_{cat}[E_o]\,C_{urea}}{K_M + C_{urea}} \qquad 2.4.4$$

where K_{cat} and K_M are the maximum rate constant and the Michaelis constant of the catalytic reaction and $[E_o]$ is the concentration of the enzyme in the solution. If $K_M >> C_{urea}$, equation 2.4.1.2 simplifies to

$$\frac{\partial C_{NH_4^+}}{\partial t} = 2\,\frac{K_{cat}\,[E_o]\,C_{urea}}{K_M} \qquad 2.4.5$$

Since, the total sum of urea and ammonium species is constant:

$$C_{urea} = C^0_{urea} - \frac{C_{NH_4^+}}{2} \qquad 2.4.6$$

Thus, the instantaneous concentration of ammonium ions is given by

$$C_{NH_4^+}(t) = 2\,[C^0_{urea}]\,(1 - e^{-\frac{K_{cat}[E_0]}{K_M}t}) \qquad 2.4.7$$

At a given time in the course of the enzymatic reaction, the peak currents of voltammograms and the current output in chronoamperometry should reflect the instantaneous concentration of ammonium ions. Thus, peak heights or current versus time curves should correspond to the general equation given below.

Differential Pulse Time Base (DPTB) Technique [37]

This is a chronoamperometric technique. Generally such techniques are used for amperometric titration, amperometric sensors, flow cell, etc. The potential excitation wave form for DPTB technique is illustrated in fig. 2.4.1

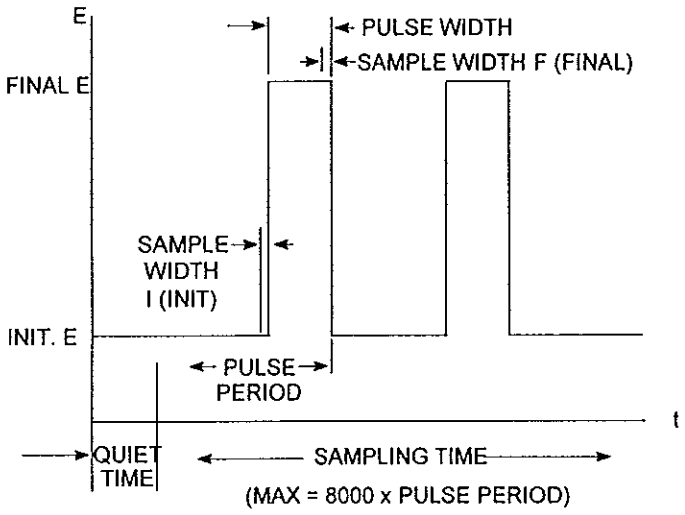


Fig. 2.4.1 Potential excitation wave form for DPTB

In this method a sequence of pulses of constant amplitude (see fig. 2.4.1) are superimposed on the usual time base excitation wave form. As the current is sampled just before the pulse and at the end of the pulse, there is effective discrimination against the background current. As it is the current difference that is displayed, the technique allows examination or use of a defined potential window, which enhances the selectivity of the detection.

3 LITERATURE SURVEY ON THE ESTIMATION OF UREA

3.1 INTRODUCTION

A number of authors have written about the methods utilized for the estimation of urea in clinical and environmental samples. Bailey [28, pp.183], G.G. Guilbault [29], Vadgama [30, pp.26], and Camman [38, pp.98] have discussed about enzyme electrodes used before two decades. Recently, Tailor and Vadgama [39] have produced a generalized analytical review in which spectrometric, electrochemical and some dry methods of clinical urea determination are assessed. A huge number of articles have appeared in the last fifteen years heralding the birth of various urea biosensors with new detection principles and techniques. Among these the innovation of bio-optrodes, utilization of conductimetry, enthalpymetry, coulometry, amperometry and dielectric phenomena as detection principles are to be mentioned. In addition, there have been advances in flow injection analysis, enzyme immobilization techniques, incorporation of conducting polymers and miniaturization of sensors by virtue of thin/ thick film technology, field effect transistors and coated wire electrodes.

The direct methods of urea estimation include the Fearon reaction and the o-phthaldehyde reaction in which quantitation is based on light absorbance by the products, the reaction of p-dimethylaminobenzaldehyde with urea in which manometry of the nitrogen released is used, titration of urea with mercuric salts, and gas chromatography of urethane as a derivative of urea where flame ionization detection is used.

Indirect methods involve the determination of urea by the determination of the products (mostly NH_3) and physico-chemical effects of the urea-urease reaction. Those in common use fall into three main categories : methods based on the Bertholet reaction, those coupled to other enzymes, mainly glutamate dehydrogenase, and conductometric methods. The so called dry chemistry methods also utilize urease. Methods based on amperometric, potentiometric, enthalpymetric, fluorimetric and coulometric detections are in progress.

3.2 THE ENZYME UREASE

In light of the fact that most of the methods are indirect, it is imperative to see the literature behind the enzyme urease. Urease (urea amidohydrolase, EC 3.5.1.5) was known in 1876 [39]. It occurs in some 200 species of bacteria, in many moulds and in a large number of higher plants - specially in beans [40, pp.55]. Among the vegetables the ones which contain extractable amounts are jack bean (canavalia ensiformis), squash (curcurbita maxima), watermelon seeds (citrullus vulgaris).

Sumner described the crystallization of jack bean urease in 1926 [41]. It was known to contain free "SH" groups [40]. Its molecular weight has been estimated to be 480,00 - 590,000 [42,43] for jack bean urease and it was 483,000 in one of the earliest reports [44]. Its isoelectronic point is pH 5.0 [40].

Urea is very stable in aqueous media, the half-life of its non enzymatic decomposition to ammonia and cyanic acid is 3.6 years at 38 °C. Urease catalyses the hydrolytic conversion of urea to ammonium and carbamate (fig. 3.2.1a). The

carbamic acid undergoes spontaneous decomposition to carbon dioxide releasing a second ammonia molecule (fig. 3.2.1b). No coenzyme is required [39].

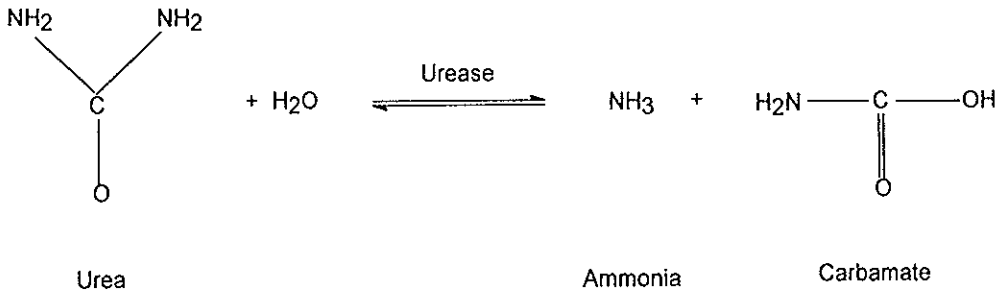


Fig 3.2.1a action of urease on urea

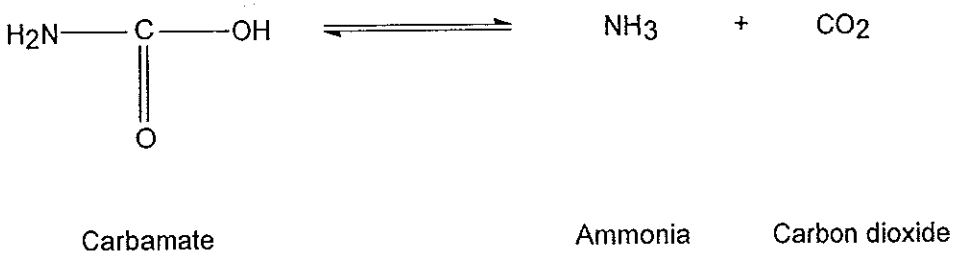


Fig. 3.2.1b consequent decomposition of carbamate to a second NH_3 and CO_2.

Urease has a great specifity for urea, and it is sometimes stated that no other substrate for it is known [36,39]. The K_M for its reaction with urea has been reported to be in the order of 10.5 - 40.0 mmol/L [36] which varies with the type of the buffer used, pH and concentration of the enzyme. In clinical practice the specifity of urease may be regarded as complete [39].

When dealing with enzymes, their efficiency is described by the extent of their (specific) activities. In the literature urease activity is reported in Sumner units per mg protein [36]. One Sumner unit, which is the amount of enzyme required to

produce 1 x 10^{-3} g NH_3 nitrogen in five minutes at 20 °C, is equivalent to 14.3 international units.

Various reaction conditions have been recommended for the urea - urease system. The optimum temperature is 55 °C [45]. In phosphate buffer, for instance, the pH optimum varies significantly with buffer type, ionic strength and urea concentration [46,47]. In various electrochemical urea sensors pH optima of 6.8 - 8.5 have been reported. Many methods use an incubation temperature of 37 °C for EDTA buffers. Ammonia produced in the reaction inhibits it [48], as do sodium and potassium [49], acids, strong alkalis, ultra violet light, heavy metals (Hg, Ag and Cu are especially potent), halogens, quinones (also potent), hydrogen peroxides, borates, fluorides and formaldehyde [40, pp.157-158], Suramin [36] and thiourea [50]. The reaction is reactivated by phosphate, chloride [49], blood serum, proteins, aminoacids, gum arabic, hydrocyanic acid and hydrogen sulphides [40, pp.37].

Impure urease preparations are capable of producing ammonia from other substances. The earliest methods of urease separation and purification from soy bean and jack bean are credited to Van Slyke and Cullen [46] and J.B. Sumner [41,51] and Dounce [52], respectively. Defatted meals, water for extraction and acetone or aqueous acetone for precipitation of the enzyme were used. Recently, conditions for the extraction of urease from citrullus vulgaris (watermelon) were studied [53]. Modification of the older methods has enabled better isolation of the enzyme from soy bean [54].

Methods using urease may be end-point or reaction rate. End-point reactions are actually equilibrium reactions with conditions arranged so that urea is almost completely converted to ammonia in a reasonably short time. These aims may be achieved by removal of the ammonia produced and by ensuring that adequate urease is present in the reaction mixture, even at high initial urea concentrations. In reaction-rate or kinetic methods product formation is monitored over a fixed time to determine the first rate. These methods are more sensitive to reaction conditions than are equilibrium methods, but stable conditions are attainable with automatic analyzers, with frequent monitoring of the rate to ensure first-order kinetics. When enzyme systems are coupled to urease to allow continuous monitoring of the products formed from urea, the urease-urea reaction must be the rate limiting step.

Hypothetically, the urea-urease reaction can be monitored by physico-chemical detection of either of NH_3, CO_2, NH_4^+, and HCO_3^- or their derivatives and by following changes in temperature, pH and conductivity of the reaction system. The potential positive interference with indirect methods is due to ammonia derived from reagents, anticoagulants and the atmosphere [44]. It is possible to include blanks comprising sample plus all reagents except urease to correct this, but in most methods the effect is assumed to be insignificant with short incubation periods.

3.3 POTENTIOMETRIC BIOSENSORS FOR UREA

Both products of the urea-urease reaction may be determined by potentiometry, and urease may be employed as a liquid reagent or immobilized and incorporated in the electrode.

3.3.1 Ammonium Linked Determination

3.3.1.1 Substratum Electrodes with Internal Contact Solution or with Solid Inner Electric Contact

Measurement of NH_4^+ has been the basis of a number of enzyme electrodes for urea which used cation-selective glass electrodes. More than 27 years ago glass membranes with more selectivity towards NH_4^+ ions were designed by using the composition $KABS_{20\text{-}5\text{-}9}$ (20 % K_2O, 5 % Al_2O_3, 9 % B_2O_3 and the remainder SiO_2) [37]. Still the electrode sensed K^+, Rb^+, Li^+ and Cs^+ (potentiometric selectivity coefficient, K^{pot} = 1 - 0.03) in the optimum region of pH 4 - 10 and had a working range of 1 - 5 x 10^{-6} $molL^{-1}$. Later on, the discovery of ionophores was followed by the incorporation of monactin or nonactin in PVC solid matrix to produce a membrane electrode of remarkable selectivity against all ions except K^+ (K^{pot} = 0.12) and little interference from H^+ (K^{pot} = 1 x 10^{-3}). In addition it showed a better lower limit of detection (10^{-6} $molL^{-1}$) and worked in the pH range 4 - 10. In addition to these or their modifications, a number of other potentiometric base sensors such as coated metal electrodes, the ISFETs etc. has been adapted.

Guilbault and Montalvo [55] made the first urea bioelectrodes using the methods of Clark and Lyons [56] and of Hicks and Updike [57]. Urease was immobilized in a polyacrylamide matrix on dacron or nylon nets which covered the active surface of a cation selective glass electrode (responding to ammonium ions) [55]. This procedure suffered serious interference, small sensitivity and short life-time. Later works using cellophane film cover for the enzyme membrane [58], thinner enzyme

membrane [59], differential mode sensing in which a bare glass electrode cell was coupled [60] and antibiotic nonactin [19] could practically improve the mentioned analytical drawbacks. A stability of more than three weeks , a working range of 1.6 x 10^{-1} - 5 x 10^{-5} molL^{-1} and a response time of 25 sec in the pH range 7 - 8.5 were achieved even without resorting to the use of nonactin [61]. Using silicon rubber membrane containing nonactin as the ion-selective electrode proper, with a Ag/ AgCl internal reference electrode in 0.1 molL^{-1} NH_4Cl, determination of 10^{-2} to 10^{-4} molL^{-1} urea in blood, even in the presence of high concentrations of potassium was possible [19].

Since then, similar, modified or new types of urea biosensors have been applied and reported with better or poorer analytical performances. A urea electrode constructed by covering an ammonium-selective electrode with a membrane bearing an immobilized urease was used in a flow system and applied to the analysis of urea in blood [62,63]. Results are claimed to correlate with other methods. Sensors utilizing nonactin-doped PVC-matrix membrane electrodes appear very frequently in the literature. Solid state base sensors with the mentioned membrane modified by further incorporation of a solid carbon-epoxy-resin inner electrical contact [64], epoxy-resin mixed with graphite powder [65-67] and graphite only [68] were reported. It is claimed that immobilization of urease on nylon-net gave faster response (1 min) and long life. In addition, it is said that negligible interference, good precision and good correlation with a spectrophotometric determination were observed on the determination of serum urea [64]. A response time of 10 sec, a sensitivity of 55 mV/ decade of concentration, a life time of ~ 40 days with a linear range of 0.01 - 1 mmolL^{-1} urea are also claimed when urease was immobilized by

aerosol-spraying as an extremely thin layer of reticulate enzyme (1 - 2 μm) on to an ammonium selective PVC membrane which is itself applied directly on to a conductive composite material.

Use of cellulose triacetate as a base membrane matrix was also reported [69]. When urease was immobilized on the surface of hydroxylated asymmetric ammonium-ion-selective cellulose triacetate membranes covering an electrode, use of such ionomer membranes enhanced the selectivity (9 - fold) and accuracy of electrode-based biosensors in a flow injection system where a thin hydrophillic anion exchange membrane was incorporated within a flow-through cell's dialysis unit.

Polypyrol-based potentiometric biosensor for urea has been tried by electropolymerising a solution of pyrol and urease onto metal electrodes [70,71].

3.3.1.2 Substratum Coated-Wire Electrodes

The coated metal wire or metal/ graphite or carbon electrodes as substratum sensors will be considered next. With the technique of metal-connected electrodes, it is possible to eliminate the conventional internal reference system and thus achieve considerable simplification and miniaturization of the ion-selective electrode.

Use of antimony electrodes as base sensors has been reported [72]. When the tip of an antimony electrode was coated with immobilized urease and when the reference electrode was prepared from the same coating mixture but without urease, rectilinear response of 0.1 - 10 $mmolL^{-1}$, with slope 40 - 45 mV per decade of

concentration, response time of 1 min and a minimum three weeks' life time is claimed at an optimum pH of 7.3 in 5 $mmolL^{-1}$ tris buffer. Both coated antimony [73] and coated silanized antimony [70] metals were tried. The former responds to changes in double-layer capacitance and was tested in a flow-cell.

Sensors based on NH_4^+-selective coated-platinum wire electrodes utilizing nonactin incorporated in the coating [74] and the formation of ion permeable channels caused by enzyme-analyte complelxation [75] has been discussed. The latter principle is said to be free from interference by Na^+, K^+, acetate, HPO_4^{2-}, $B_4O_7^{2-}$, Cl^-, and diverse aminoacids. A glassy carbon electrode coated with nonactin-doped and enzyme-modified PVC membranes was reported to be used in a photo switchable mode by incorporation of a dye in the membrane [76]. Detection of 10 $\mu molL^{-1}$ - 1 $mmolL^{-1}$ ammonium is claimed.

3.3.1.3 Ion-Selective Field-Effect Transistors (ISFET) as Substratum Sensors

Such a device was described by Bergveld [as cited in ref. 28, pp.192] in 1972. Moss, Janta and Johnson [77] have studied the construction and performance of potassium ISFET, as the first of its kind. In general, the gates of a conventional metal oxide semiconductor field effect transistor (MOSFET) is replaced with an ion selective membrane. The potential developed across the membrane regulates the potential on the surface of the gate regulator and, hence, the current flowing through the channel between the drain and the source. The current is proportional to the logarithm of the activity of the determinand. The device is sealed in a tube with just the membrane exposed to the solution, and is used in a conventional manner in

become easily available. In addition living tissues are regeneretable and of low cost. The tissue is taken in solution or attached to (immobilized onto) a suitable base sensor. Thus such sensors are viable alternatives to conventional enzyme based electrodes, the major fear being interference from the other enzymes and longer response times probably due to rate limiting diffusion through cell walls.

In line with potentiometric detection of NH_4^+ ions, a biosensor making use of urease-producing bacterium (proteus vulgaris) was described and a Philips NH_4^+-selective electrode gave nerstian response for 2 - 20 $mmolL^{-1}$ urea [80]. Recently, a device consisting of a layer of whole cells of proteus vulgaris applied directly on to an all-solid-state ammonium-selective electrode which has a nonactin-doped PVC membrane was reported [81].

3.3.2 Ammonia Gas Linked Determination of Urea

The design of NH_3-gas electrode [82] in 1973 has enabled total removal of the interference from ions, but the additional gas permeable membrane required leads to slower response. Furthermore, the pH optimum for the urease reaction is inappropriate for detecting ammonia gas produced since the pKa for the NH_3/ NH_4^+ system is 9.0, and a compromise must be made. Yet, for the above mentioned NH_3-gas electrode-based urea sensor, the ammonia produced was enough to give a nice calibration plot due to the sufficiently close proximity of the enzyme layer to the sensing surface.

An alternative ammonia sensitive electrode is the air-gap electrode [83] which

completely avoids protein contamination as the electrode interfaces indirectly with the sample, but requires a separate alkalizing step to liberate gaseous ammonia. A MOS capacitor with a catalytic iridium-platinum surface capable of degrading ammonia was exploited so as to get an ammonia dependent signal. However, this device operates at an elevated temperature and interfaces indirectly with the enzyme reagent.

The construction and use of an ammonium microsensor for urea with urease immobilized at the tip of a rapidly responding NH_3-sensitive microelectrode based on Sb was described. A response time of 30 - 40 seconds was achieved at a pH of 8.5 [84]. Ammonia sensing air-gap microelectrode, with a tip diameter of 2 to 5μm, based on a neutral carrier pH sensing inner electrode gave a 95% response time of 10 - 15 seconds [85].

A number of related tissue bioelectrodes for urea have been reported. The first comprised an ammonia electrode to which was attached a cellulose acetate membrane bearing immobilized mirabilis (a bacterium) as a source of urease [86]. Its precision was comparable to the current spectrophotometric methods applied to biological media. Preparation of proteus vulgaris [87] and sporosarcina [88] urease membranes have been reported. The bacterial electrode comprising the latter one gave a linear response range of 0.1 - 50 $mmolL^{-1}$ urea and functioned for 30 days at room temperature. Urease rich plant tissues such as fresh carnation petals [98], parsley leaves [90], soy bean meal [91] and parsley seeds [92] have also been used either by sandwiching them between the ammonia sensing membrane and a dialysis membrane or immobilized on the electrode's sensitive surface by glutataraldehyde

cross-linking matrix and then dipped in a buffer solution. The sensor with soy bean meal enabled a detection limit of 1 $\mu molL^{-1}$, a response time of 2 - 6 min and a life-time of 60 days. Fresh tissues are not required and so, such sensors are not limited by season.

3.3.3 Carbon Dioxide Gas Linked Determination of Urea

The detection of carbon dioxide linked to reactions of urea with urease in solution [93], with immobilized urease [40] and with urease of P. Vulgaris [94] and P. Mirabilis [95] have been described. Proteus vulgaris was applied as a paste to a dialysis membrane of an Orion 95-02 CO_2-gas sensing electrode and it was held in place with a gas permeable membrane. The electrode was conditioned in a buffer (pH 6.8) solution. Linear performance in the range 0.7 - 30 $mmolL^{-1}$, and a response time of 4 - 5 minutes were observed for the fresh sensor. With a stable response for over a week, the sensors are claimed to be as precise as other methods.

3.3.4 pH Linked Determination of Urea

Potentiometric electrodes may be used to determine pH changes resulting from the urea - urease reaction. Utilization of pH changes linked to this reaction makes a significant part of the literature on urea biosensors. Earlier adaptation of the pH electrode as a substratum entailed interference from acidic and basic components [96]. However, this could minimized by pH adjustments before initiating the enzymatic reaction. A flow-injection method proposed by Ruzicka allowed rapid sample analysis (60/ h) due to accelerated electrode response (30 sec) [97].

Immobilized urease may be integrated with such electrodes by cross-linking with albumin to a palladium-palladium oxide wire [98] or by gel entrapment [99] where an auxiliary pH electrode is used to correct the response. The response time was greater (1 - 3 min) when compared with the one using urease in solution and the method correlated well with a chemical method.

Direct immobilization of urease onto the active surface of a pH electrode may improve the response time when used in a differential measuring system, allowing up to 25 samples per hour to be processed [100]. High storage and operational stability was recorded when a self mounted uniform urease enzyme membrane was formed by dipping glass pH electrode in a solution of the enzyme and a synthetic pre-polymer [101]. The procedure is recommended for the construction of enzyme membranes on other materials such as Carbon and Platinum. A kinetically controlled urease electrode was prepared by wetting a glass pH electrode and dipping it into freeze-dried urease, followed by immersion in a solution of cellulose acetate in CH_2Cl_2 [102]. Equilibrated at pH 7.4 in an electrolyte solution containing NaCl, Na acetate, $CaCl_2$ and $MgCl_2$ before use, the sensor yielded a response time of 30 s and a sampling rate of 20 - 30 h^{-1}. An electrode with a life-time of more than a week and responding in 10 s was prepared in a procedure involving dipping and rotation in the enzyme solution followed by spraying glutaraldehyde [103].

Urea biosensors, with pH detection, based on ISFET coated with cross-linked urease-albumin membrane was described [104]. A pH-ISFET used in conjunction with a photolythically cross-linkable polymer hardened with the use of bovine serum albumin (BSA) and glutaraldehyde has also been reported [105]. A response time

of 20 s and a life-time of 20 days were observed when urease was immobilized onto a 0.1μm thick membrane obtained by vapour-deposition silanization onto the gate insulator of a pH ISFET [106]. A pH static enzyme sensor in which an ISFET is coupled with a noble-metal electrode at which protons are generated to neutralise continuously the OH- produced by the urea-urease reaction has been described [107]. Responses are independent of buffer capacity. The current required to generate the protons so as to keep the pH of the enzyme layer constant is a measure of the enzyme activity.

Placement of two Ta_2O_5-membrane ISFET-MOSFET pair before and after a urease reactor column gave a response time of 30 s [108]. Enzyme based thin metal-film (on silicon wafers) and pH-SFET (pH sensitive FET) sensors using a polyurethane inclusion matrix urease membrane were reported [109]. Good adhesion and retention of the enzyme molecules, good long-term stabilities and good diffusional resistance against electro-active species is claimed. Some Bio-FET transistors, as detectors in FIA, were observed to be stable for 12 weeks and gave results comparable to that of liquid chromatography [110].

A recent innovation is the technique based on enzyme entrapped in a cubic liquid crystalline phase which is coated on to a flat pH electrode [111]. However, slow and non linear response was observed.

3.4 AMPEROMETRIC DETECTION OF UREA

A urea sensor comprising nitrifying bacteria immobilized on an oxygen electrode, a cation exchange membrane, and a urease-collagen membrane has been described [112]. Good agreement with other methods is claimed, along with electrical stability of the sensor for 150 assays. A linear response in the range 2 - 200 $mmolL^{-1}$ also allowed its use in urine assays. The pH dependence of the electrochemical oxidation of hydrazines in the tafel region has been used in conjunction with urease-catalysed splitting of urea to form an amperometric membrane electrode for urea [113]. A commercial oxygen sensor is incorporated in combination with a urease film which is in direct contact with the platinum tip of the electrode filled with an electrolyte. Response time of 20 s, analysis rate of 40 h^{-1}, stability of 2 - 3 weeks and a linear range between 0.8 - 35 $mmolL^{-1}$ urea were observed. Amperometric detection of the ammonia produced via coupled enzyme reactions yielding oxidizable products is possible by detection of the consumption of oxygen. More recently, the same principle, namely the amperometric determination of urea using an NADH-dependent coupled enzyme reaction, was used in conjunction with a vitreous carbon electrode operated at +1V vs Ag/ AgCl [114]. A linear range of 0.01 - 0.2 $mmolL^{-1}$, a detection limit of 5 $\mu molL^{-1}$ and a life-time of 2 week's were reported. Ascorbic acid interfered during measurements. The sensor could be used for $\geq$ 50 assays and it was applied to a fertilizer and clinical assays.

The adaptation of amperometric ammonium ion sensor based on the principle of ion transfer across liquid-liquid interface as a substratum sensor for urea biosensor is one of the many new innovations of the decade [5,7,115]. Nitrobenzene containing

the neutral ionophore DB[18]C-6 and TPATPB , which is the supporting electrolyte, constituted the organic phase which was separated from the aqueous phase by a dialysis membrane. The pulse amperometric technique was used which allows the sensors high reproducibility and long lifetime. A response time of about 300 seconds and a life-time of more than three weeks were observed. It was commented that correction for the residual current was more readily achieved with this sensor than with the potentiopmetric ones. The method was used for the determination of volatile amines in foods and urea in body fluids [5].

3.5 CONDUCTIMETRIC AND COULOMETRIC ASSAYS

Conductimetric Detection

The determination of the increase in electrical conductivity in a reaction mixture due to ammonium ions and carbonate ions produced by the action of urease on urea has been described [116]. Here, the rate of increase in conductivity is related to the urea concentration in the sample. Measurement is commenced a short time after the addition of the sample to the reagent, to allow for changes in conductivity due to the ions in serum.This technique thus avoids the interference by ammonia. An adaptation of conductometric urea determination for flow-injection analysis was described [117], which uses two conductance cells to provide a differential signal and is claimed to compare well with existing methods.

A microelectronic conductimetric biosensor for urea was reported with a response time of 3 min and ± 0.1% reproducibility [118]. Investigation of the use of a.c.

conductometric transducers in urea biosensors resulted in a model sensor demonstrating a detection limit of 1 $\mu molL^{-1}$ and a linear response range of over 2 order of magnitude when urease was covalently bound to collagen membranes coated on either Cu or Pt electrodes [119]. Multi analyte micro-electronic and multi analyte miniature conductance biosensors for a group of analytes including urea have been produced [120,121]. Enzymes are immobilized on metal film electrodes on silicon substratum. In addition to the few seconds' response time achieved, application of these sensors to flow cells enabled continuous determination of urea in a flowing stream. More recently, continuous monitoring of urea in blood during dialysis was facilitated by conductivity measurements by using a separate urease reactor [122]. Calibration graphs were linear from 5 to 50 $mmolL^{-1}$.

Coulometric Detection

It has already been mentioned that coulometric principle was used in connection with pH indication. Recent works based on coulometric titration of NH_3 and CO_2 produced in the urea-urease reaction has been reported [123]. Covalent immobilization of the enzyme on a membrane sandwiched between microporous PTFE membranes, separation of NH_3 and CO_2 at a gas dialysis step, and use of pt detector and generator electrodes were involved. The determination range was 10 $\mu molL^{-1}$ to 1.5 $mmolL^{-1}$, and the results agreed with those of other coulometric methods and no interference was observed except for aliphatic amines and hydrazine.

3.6 METHODS WITH SPECTROPHOTOMETRIC DETECTION

3.6.1 Detection in Soluble Systems [39]

The Bertholet method is based on the reaction of ammonia with phenol to form an indophenol dye on whose light absorbance the quantitation is based. As one of the coupled-enzyme methods, a reaction between ammonia and 2-oxoglutarate, catalysed by glutamate dehdrogenase (EC 1.4.1.3), is used where the associated oxidation of NADH or NADPH is monitored at their characteristic absorbance lines.

Use of ammonia and phthalaldehyde-mercapto ethanol reagent to produce stable fluorescent product has been described. The method is said to be accurate, with linearity in the range 0 - 50 $mmolL^{-1}$, and its sensitivity would allow determination of urea in nano litre samples.

3.6.2 Fluorimetric Detection and Urea Bioptrodes

Works in this decade have contributed new types of urea biosensors called optical-fibre urea biosensors which use optrodes (optodes) as the core detectors. Urea biooptrodes are based on immobilization of urease on the distal tip of fluorescent-based NH_3 optical-fibre sensors [124]. Responses quicker than similar absorbance based sensors or a conventional potentiometric NH_3-gas-sensing electrode were reported. Linear response for 0.25 - 8 $nmolL^{-1}$ urea was achieved using an optrode in which urease was immobilized on a pre-activated immunodyne fixed to the fibre distal tip with the a pH indicator adsorbed on the same membrane. Successful

applications of the sensors to serum [125] and fertilizer [126] are claimed. A typical report reveals a response time of 40 s, selectivity coefficient of 3×10^{-5} and 5×10^{-4} for K^+ and Na^+ ions, respectively, and good agreement with other methods when an ammonium ion selective PVC membrane containing polynonactin was used. The incorporation of nonactin as an NH_4^+ carrier gave better analytical range in a flow system [127].

3.7 OTHER DETECTION METHODS

Uchiyama, et al. [128] have reported the use of immobilized urease in a column; the ammonia generated was measured by ion chromatography. An indirect HPLC method was described by Jansen, et al. [129] using an on line urease-catalysed conversion of urea to ammonia, ion-pair chromatography, and post column derivatization with o-phthalaldehyde for fluorescent detection.

Recently, dielectric-based biosensors have appeared in the literature [130]. A urea sensor in which the dielectric property of immobilized urease are used to monitor the hydrolysis of urea has been prepared.

4. EXPERIMENTAL

4.1 Chemicals and Materials

Commercial nitrobenzene (Fluka or Riedel-deHaen) was purified by consecutively washing three times with 10 % sulphuric acid (BDH) and 6 % sodium hydroxide (BDH) solutions. The resulting alkalinity or acidity was removed by thorough cleansing with distilled water while the pH of the wash was followed. Ethyl violet tetraphenylborate (EthVTPB) was synthesised in the lab from sodium tetraphenylborate (NaTPB) and ethyl violet chloride (s). Dibenzo[18]crown-6 (DB[18]C-6) (Merck), Li_2SO_4 (Aldrich), $Li_2SO_4.2H_2O$ (Merck), cellulose acetate dialysis membrane (PT 600, PT 325), silver wire, a PTFE tube with fitting PTFE-made screw and O-ring were used for the construction of a sensor. NH_4Cl (BDH), KCl (Aldrich), Urea (Analar, Hopkin & Williams Ltd) were used to prepare 'standard' solutions. Petrol-ether, Dowex-cation and anion exchanger (Aldrich), mixed-bed ion exchanger (Aldrich), dustless tissue paper (Cien), methanol (BDH) and acetone (Riedel-deHaen) were used for cleaning purpose.

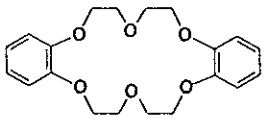
4.2 Instruments

Ac and dc voltammetric techniques were facilitated by a lab-made four-electrode potentiostat which was used in conjunction with other commercial instruments such as function generator (Tektronox), oscilloscope (dual trace, Tektronix), digital multimeter, digitimer (Harris), frequency generator, MPI voltammetry controller, lock-

in analyzer (EG & G Applied Research), integrator, ramp generator, XYt-recorder (PL3-LLOYD Instruments). In addition a pulse amperometric and dc voltammetric techniques were also applied by making use of an electrochemical analyzer with BAS101W electrochemical work station, a personal computer (CTX) with installed BAS programmes.

4.3 Sensor Design and Electrochemical Methods Used

The organic phase was nitrobenzene which was either 10 mmol/L in EthvTPB only or had 50 mmol/L D[18] C-6 additionally. About 1mL of the organic phase was always kept in a PTFE tube whose threaded end was sealed with a hydrophillic dialysis membrane which was fixed using a PTFE O-ring followed by screwing a PTFE-made nut to the tube.The aqueous phase was 5 mmol/L in Li_2SO_4 and 50 mL of it was always taken in a 100 mL beaker so that the membrane was completely inside this phase. As depicted in the diagram below, two reference electrodes (one in the aqueous phase (RE(w)) and the other in the organic phase (RE(o)) and two counter electrodes were used (one in the aqueous phase (CE(w)) and the other in the organic phase (CE(o))). The cell arrangement is shown in fig. 4.2.3.



Dibenzo-18-crown-6

Fig.4.3.1 The structure of dibenzo[18]-crown-6

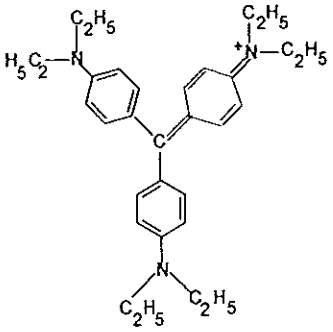


Fig 4.3.2 The structure of ethyl violet

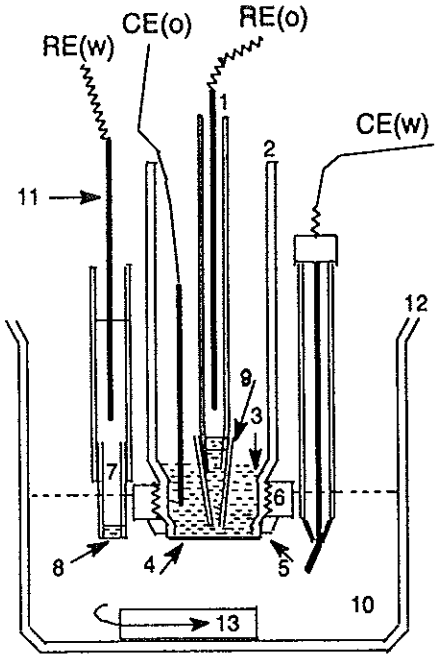


Fig. 4.3.3 diagram of the amperometric sensor based on facilitated ion transfer across the water/ nitrobenzene interface. 1. glass tube containing saturated LiCl, 2. PTFE tube, 3. nitrobenzene (organic phase : 10 mmol/L EthVTPB, 50 mmol/L DB [18] C-6) 4. dialysis membrane, 5. PTFE made O-ring, 6. nut, 7. aqueous phase (5 mmol/L: Li_2SO_4), 8. glass frit, 9. PEP tube adopted from microsyringe tip and filled with the organic phase, 10. aqueous phase, 11. silver wire, 12. beaker, 13. magnetic stirrer.

The tip of RE(o) was placed as close as possible to the membrane. Experiments were done under stirring

Dc/ Ac-Sweep Voltammetry

A four electrode potentiostat was connected to the sensor through the corresponding electrodes in such a way that the reference electrode in the organic phase (RE(o)) was kept at virtual ground and the reference in the aqueous phase (RE(o)) was kept

at desired potentials. Both linear and cyclic sweeps were used at sweep rate of 25 mVs^{-1}. The iR drop could be compensated manually by using a positive feed back loop. Overcompensation was detected on an oscilloscope and the compensation resistance was set at a value just before the onset of oscillation.

In the ac voltammetric technique a voltage of frequency 35 Hz and amplitude 5 mV was superimposed on to a triangular dc excitation wave. Current outputs were taken directly from a lock in analyzer. Amplification and recording of sensor outputs were facilitated by use of an XYt recorder with adjustable sensitivity.

Differential Pulse Amperometry

The pulse amperometric method with the potential excitation wave form presented in section 2.4 was applied using an electrochemical analyzer (Bioanalytical Systems-BAS 100A) which was connected to an on-line personal computer equipped with BAS programmes. Current responses were easily followed versus time.

Before and after each experiment, the sensor was always washed with distilled water and electrochemically cleansed in the supporting electrolyte until a voltammogram characteristic of the supporting electrolyte was recovered.

4.4 Standards Used and Sample Handling

0.1 mol/L aqueous solutions of NH_4Cl, Urea and KCl were prepared and used. Plant samples which were believed to be rich in the enzyme urease [40] were collected

and studied. The list includes some beans, watermelon seeds, squash seeds and soy bean flower as presented in the table below.

Table 4.1 list of plant tissues

Sample code/ common name	Sientific name	place of sampling	other
Bean 1 (Java bean) seeds	*Phaseolus lunatus*	Zuway town	large , kidney shaped, Red + white with red mosaic
Bean 2 seeds	*Phaseolus vulgaris*	Merkato/ Gambela	medium sized, light brown with brown mosaic
Bean 3 seeds	same	Merkato	small, white all in all
Bean 4 seeds	same	Merkato	violet + Black with black mosaic
Squash seeds	*Curcurbita maxima*		
watermelon 1	*Citrullus Vulgaris*		from fruits with light green and green stripes
watermelon 2	same		from green fruits
Soy bean flour	*Soja hispida*		

The samples were dried, their hulls removed and the cotyledons ground in an agate mortar. Portions from watermelon 1 and soy bean flour were defatted using petroleum ether. The defatting process involved extraction and decantation followed by drying in the open air until the powder has lost the smell of the solvent. Mostly, 50 mg of a tissue was taken in the aqueous phase.

4.5 Preliminary Investigation

Voltammograms were recorded for the supporting electrolytes and ammonium ions. Each of the collected plant tissue was tested for urease activities. Responses in the presence and the absence of DB[18]C-6 were checked.

4.6 The Potassium Problem

Since the preliminary experiments indicated disturbing amounts of endogenous K^+ ions in the collected samples, three methods were tried to check if it could be possible to remove these ions. Watermelon seeds and soy bean seeds were chosen for this purpose and treated as follows :

1 A 40% acetone/ water mixture was prepared and used to wash a portion of soy bean flour. Centrifugation and decantation were used for separation. The wet powder was left in open air to dry before it was tested.

2 Portions of the same tissue and a mixed-bed ion exchanger were shaken together in distilled water. The same separation methods as in 1 were used before the tissue was reexamined.

3 Two columns were prepared from ordinary plastic syringes. One was packed with a cation exchanger (Dowex 50Wx8 treated with sufficient 10% HCl and washed with distilled water up to neutrality). The other was packed with an anion exchanger (Dowex 1x2 treated with 10 % NaOH until no Cl^- was detected in the eluate and finally it was washed with distilled water up to neutrality). A portion of watermelon 1 was mixed with water and was stirred well. The mixture was centrifuged and supernatant was passed through the columns which were placed one over the other. Aliquots from the collected solution was tested for enzymatic activity.

4.7 Calibration Experiments

The sensor was calibrated with respect to NH_4^+ and K^+ ions at different ages of the sensor. Peaks height increments of linear sweep dc-voltammograms and current rises in time-based differential pulse amperometric responses were measured for each aliquots of 'standard' NH_4Cl and KCl added to 50 mL of the aqueous phase.

4.8 Estimation of Potassium Content of some Plant Tissues.

The amount of endogenous K^+ was estimated for each of the collected tissues because potassium is reported to inhibit urease and it interfered with experiments by causing high background current. It was also felt necessary to evaluate the performance of the sensor for a direct analytical determination of potassium in untreated plant samples. Dc- linear sweep voltammetry was used and steady state current peak heights were measured both before and after the addition of aliquots

of standard 0.1 M KCl to the mixture containing 1mg of sample per ml of the aqueous phase.

4.9 Watermelon Seeds and Soy Bean Flower as Soluble Enzymatic Reagents For the Estimation of Urea

Sets of experiments were done on three urease active tissues with the following goals.

- to compare their relative activities
- to see the effect of defatting
- to see the influence of NH_4^+ ions on the enzymatic activities
- to see the effect of the tissues on the sensitivity of the sensor

During the study the differential pulse time base technique was used to follow current vs time when standards were added and enzymatic reaction were going on. Before the start of an experiment iR drop was undercompensated by 10 KΩ.

5. RESULTS AND DISCUSSION

5.1 Preliminary Investigation

5.1.1 The Working Range

Fig 5.1.1 shows the dc-cyclic voltammogram of the supporting electrolytes that were used. Ac- and dc-linear sweep voltammograms of the same electrolytes are shown in fig. 5.1.1.2. The limit of the potential window at positive potentials (indicated by straight arrow) is either due to the transfer of Li^+ or TPB^- which usually occurs around 650 mV (see equations 5.1.1.1 below). The lowest positive limit of the

$$Li^+(w) \longrightarrow Li^+(o)$$
$$TPB^-(o) \longrightarrow TPB^-(w) \qquad 5.1.1.1$$

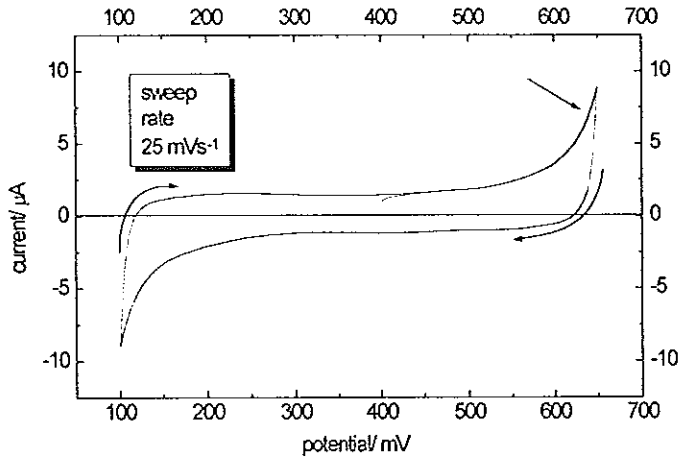
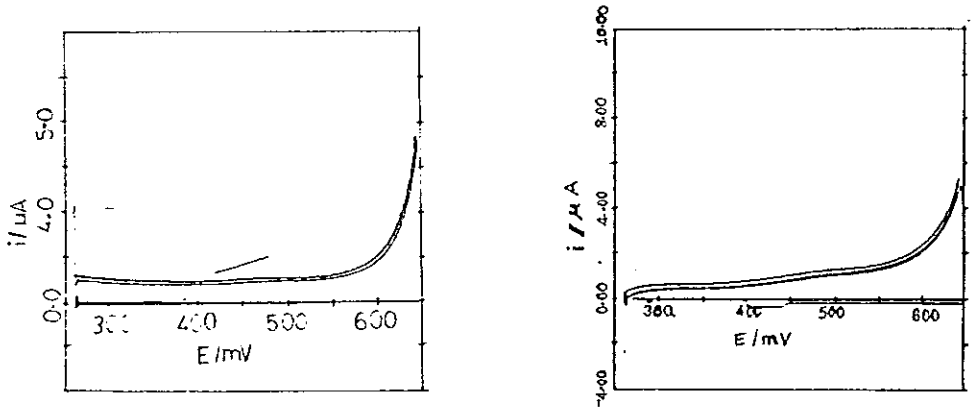


Fig 5.1.1.1 Dc-cyclic voltammogram of the supporting electrolytes used at the membrane-stabilized water-nitrobenzene interface. (Water: 5 mmol/L Li_2SO_4; nitrobenzene: 10 mmol/L EthVTPB + 50 mmol/L DB [18] C-6, sweep rate = 25 mV/s, stirring)

potential, which was not important to deal with, may be attributed to the transfer of either of sulphate or ethylviolet ions. In the presence of K^+ ions in the aqueous phase, the transfer of TPB^- from the organic to the aqueous phase leads to the



(a) (b)

Fig 5.1.1.2 Ac- (a) and dc- (b) linear sweep voltammograms of the supporting electrolytes at the membrane stabilized water-nitrobenzene interface (Water: 5 mmol/L Li_2SO_4; nitrobenzene: 10 mmol/L EthVTPB + 50 mmol/L DB [18] C-6, sweep rate = 25 mV/s). multiple sweep curves are seen since it was always repeated to make sure the constancy of background currents.

the pores of the membrane. The use of another more hydrophobic anion such as tetra (p-chlorophenyl) borate (fig. 5.1.1.3) may help to extend the working range

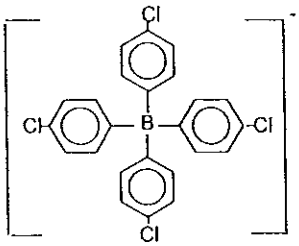


Fig 5.1.1.3 The structure of p-chloro tetraphenyl borate. More hydrophobic ions such as this one may widen the working range.

of the interface to more positive potentials. That would be the case during the study of some ions such as ammonium ions whose transfer occurs at potentials around 600 mV. Since urea determination was based on the amperometric/ voltammetric detection of ammonium ions at the membrane-stabilized water-nitrobenzene interface, the transfer of this ions was studied. Its cyclic sweep wave appears as a shoulder to the cathodic limit of the window as depicted in the figure below.

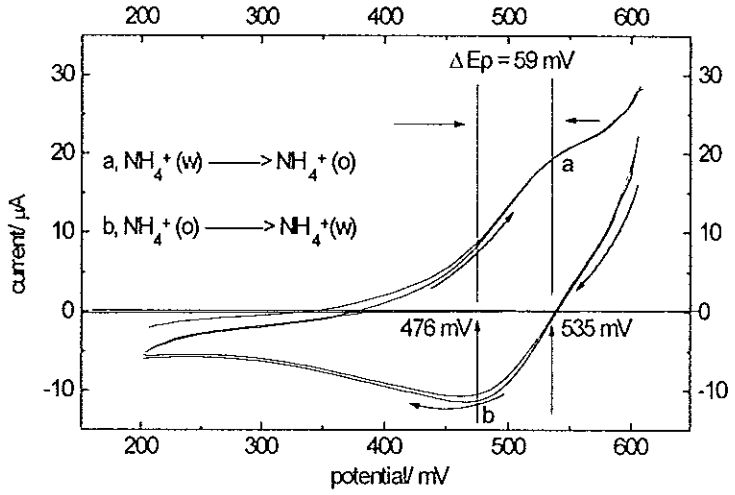


Fig. 5.1.1.4 Dc-cyclic voltammogramic wave for the facilitated transfer of ammonium ions across the membrane-stabilized water-nitrobenzene interface (Water: 5 mmol/L Li_2SO_4; nitrobenzene: 10 mmol/L EthVTPB + 50 mmol/L DB [18] C-6, sweep rate = 25 mV/s). The peak potential separation of 59 mV indicates a reversible and diffusion controlled transfer of the ions in question.

In addition, previous studies as well as the preliminary studies have shown that the ionophore DB[18]C-6 also facilitates the transfer of potassium and sodium ions within the potential window that is being discussed. The transfer of both ions occur at less positive potentials than that of the ammonium ion in the order $NH_4^+ > Na^+ > K^+$. Thus, urea determination based on the ionophore-facilitated transfer of ammonium is in danger of interference from these ions.

5.1.2 Preliminary Experiments with the Tissues

When 50 mg of watermelon meal was added to 50 mL of 5 mmol/L Li_2SO_4 the voltamogram shown in fig. 5.1.2.1. was obtained. The peak current was increasing with time and became constant after 15 min. From the peak potential and in comparison with the voltammogram obtained in the presence of potassium ion this peak was identified to be due to the transfer of potassium ion present in the watermelon. All other investigated seeds have given similar voltammograms indicating the presence of potassium in the plant tissues.

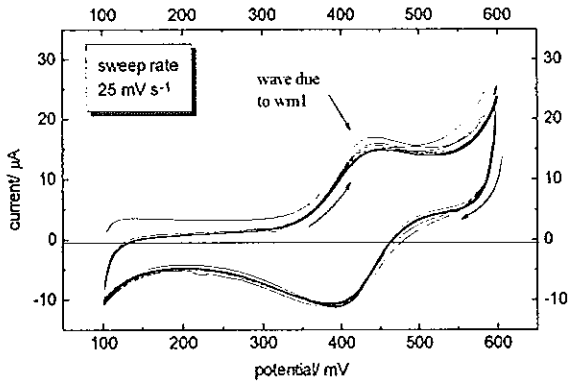


Fig 5.1.2.1 Cyclic-voltammogram of an aqueous suspension of watermelon 1 at the membrane-stabilized-water-nitrobenzene interface (Water: 5 mmol/L Li_2SO_4; nitrobenzene: 10 mmol/L EthVTPB + 50 mmol/L DB [18] C-6, sweep rate = 25 mV/s). Such peaks were detected for each of the samples.

Since the aim of the research was to study the enzymatic activity of the samples by following up the amount of ammonia formed in the enzymatic hydrolysis of urea the presence of potassium ion in the seeds was a serious drawback for the investigation. In order to observe the peak due to NH_4^+ transfer, one had to ensure that preceding K^+ peak was constant. Hence the system had to be allowed sufficient time for the K^+ peak to get constant. In the experiments carried out only watermelon

seeds and soy bean flour were found to exhibit enzymatic activity. When an aliquot of urea was added to an aqueous suspensions of a tissue, a peak evolves through

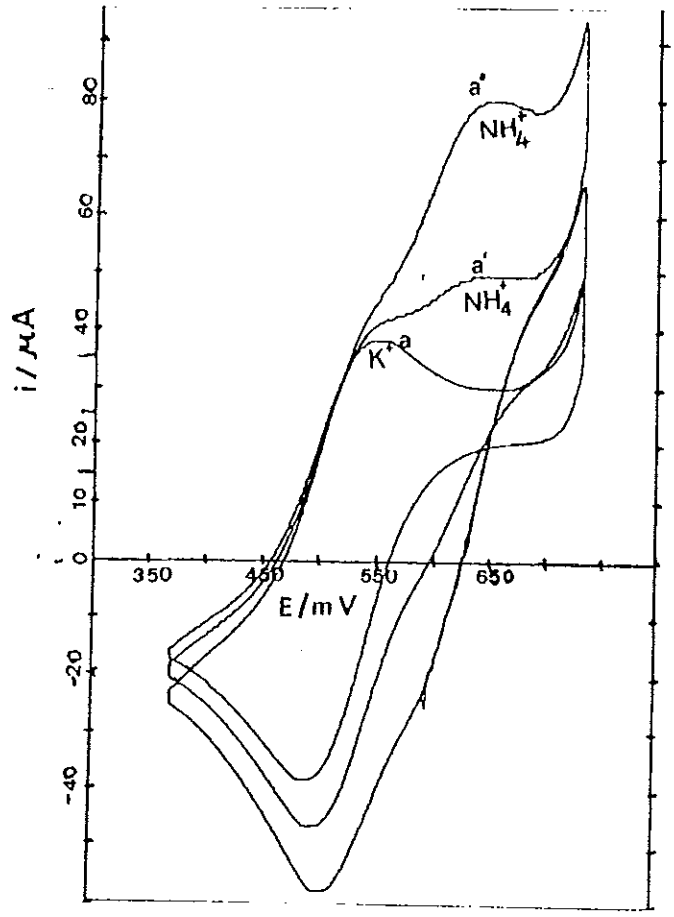


Fig 5.1.2.2 Dc-cyclic voltammogram of facilitated ion transfer at the membrane-stabilized water-nitrobenzene interface (Water: 5 mmol/L Li_2SO_4; nitrobenzene: 10 mmol/L EthVTPB + 50 mmol/L DB[18]C-6, sweep rate = 25 mV/s, stirring) showing the action of soy bean flour on urea through the detection of generated ammonium ions whose transfer was facilitated by DB[18]C-6. (a) shows a peak due to potassium when 120 mg of the tissue was dissolved in the aqueous phase. (a') and (a'') appeared after 3 min and 10 min, respectively, on addition of 0.5 mL of 0.1 mol/L urea.

time near the positive potential limit of the window. It was enhanced when standard ammonium chloride was added. Thus, it has been attributed to the facilitated transfer of NH_4^+ ions produced by the urease catalysed hydrolysis of urea. Fig. 5.1.2.2 (above) depicts a typical voltammogram showing the action of soy-bean-flour urease on urea as peak height growth through time.

5.2 Trials to Remove Potassium ions From the Tissues

When one tries to remove the indigenous potassium from a tissue, one has to make sure that the method does not hurt or destroy the enzyme for which the tissue is important. It is known that urease has been recrystallized from its aqueous solutions by addition of 38 % acetone/ water as a precipitant. It is clear that this combination of water and acetone should have an ability to solvate potassium ions. It is a known fact that ion exchangers help to remove charged impurities. Based on this understanding some experiments were carried out as already described in the experimental section. The results are discussed hence forth.

When soy bean flour was washed with acetone/ water mixture, the height of the potassium peak slightly decreased and a decline in urease activity was observed as compared to the voltammogram of the suspension of the untreated tissue. The evolution of ammonia reached its steady state after about 65 min.Usually not more than 30 minutes were required. Shaking with or passing through ion exchangers totally removed K^+ ions. However, this was accompanied by total loss of enzymatic activity (compare fig. 5.2.1 and fig. 5.2.2). A weak activity was observed when the tissue/ exchanger contact time was reduced to about 10 minutes (the previous being more than 30 min) for the batch treatment on watermelon 1 (see fig. 5.2.3). Steady state was reached after three hours.

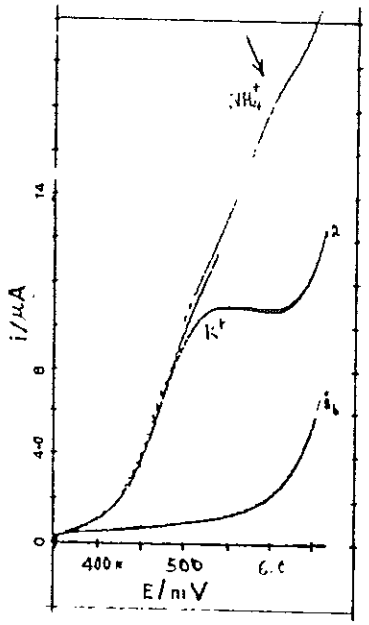


Fig. 5.2.1 Dc-linear sweep voltammogram at the membrane-stabilized water-nitrobenzene interface (Water: 5 mmol/L Li_2SO_4; nitrobenzene: 10 mmol/L EthVTPB + 50 mmol/L DB[18]C-6, sweep rate = 25 mV/s, stirring) showing the action of untreated watermelon seeds on urea. Curve (2) was obtained on addition of 2.5 mL of an aqueous extract containing 20 mg of the tissue per mL. Curve (3) was obtained on addition of 50 μL of 0.1 mol/L urea.

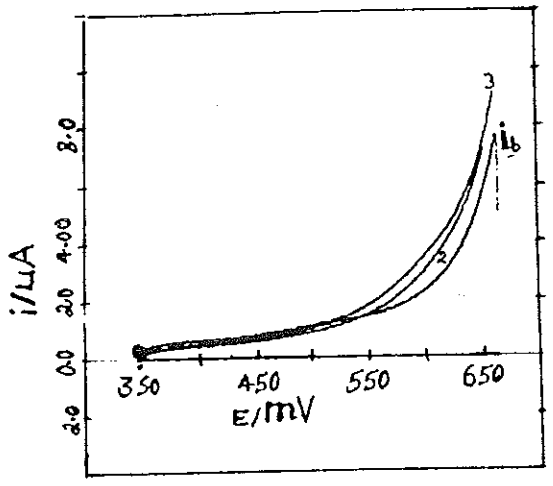


Fig. 5.2.2 Dc-linear sweep voltammogram at the membrane-stabilized water-nitrobenzene interface (Water: 5 mmol/L Li_2SO_4; nitrobenzene: 10 mmol/L EthVTPB + 50 mmol/L DB[18]C-6, sweep rate = 25 mV/s, stirring) at a sweep rate of 25 mVs^{-1}. No potassium peak (curve 2) and no urease activity (curve 3) is shown when the suspension of watermelon seeds described in fig. 5.2.2 was shaken with mixed-bed ion exchanger for more than 30 minutes.

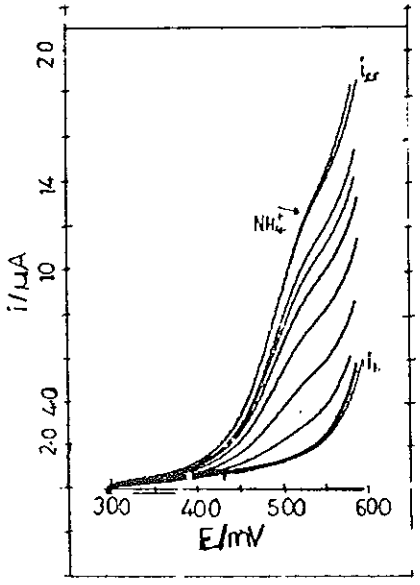


Fig.5.2.3 Dc-linear sweep voltammogram at the membrane-stabilized water-nitro benzene interface (Water: 5 mmol/L Li_2SO_4; nitrobenzene: 10 mmol/L EthVTPB + 50 mmol/L DB[18]C-6, sweep rate = 25 mV/s. stirring) at a sweep rate of 25 mVs^{-1}. When the contact time between the exchanger and the aqueous tissue was decreased to about 10 min, a weak activity was observed. 3 hrs were required to reach the steady state (i_{ss})

5.3 Calibration Experiments

The sensor was calibrated by applying dc-linear sweep voltammetry and the differential pulse time base amperometric methods. Ac-linear sweep voltammetry for the same purpose could not be used due to non-linear responses (see fig. 5.3.1 and the accompanying calibration graph).

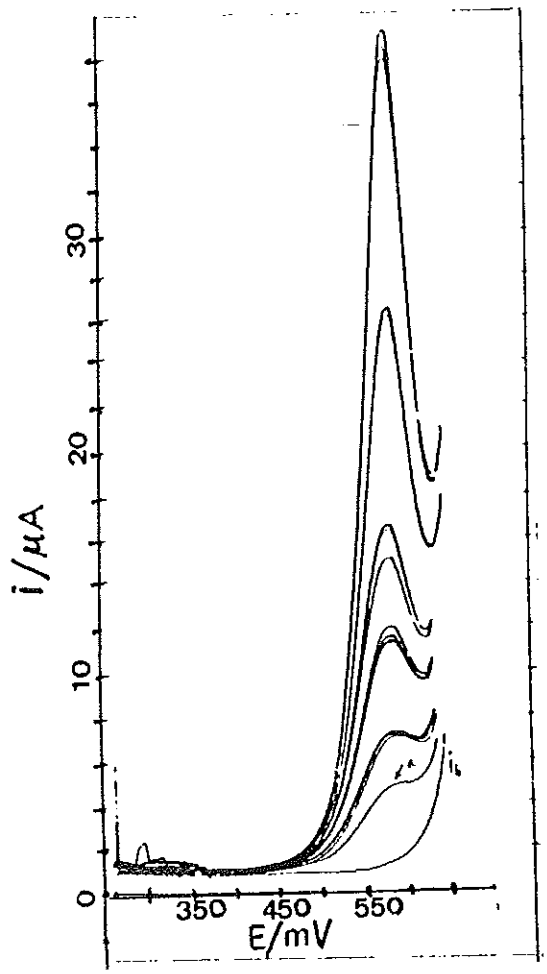


Fig. 5.3.1 Ac-linear sweep voltammogram at the membrane-stabilized water-nitrobenzene interface (Water: 5 mmol/L Li_2SO_4; nitrobenzene: 10 mmol/L EthVTPB + 50 mmol/L DB[18]C-6, sweep rate = 25 mV/s, stirring) showing an apparent non linear growth of peak height on successive 25 μL additions of 0.1 mol/L NH_4Cl. Curve (a) was obtained after addition of 50 μL of the same standard.

Table 5.3.1 Dependence of ac-voltammetric peak height on slight changes in iR drop

iR compensation/ KΩ	current/ μA	change in iR compensation/ KΩ
2.40 (initial)	5.3 (initial)	-----
2.42	6.5	0.02
2.44	6.65	0.04
2.46	7.3	0.06
2.48	7.9	0.08
2.50	8.6	0.10

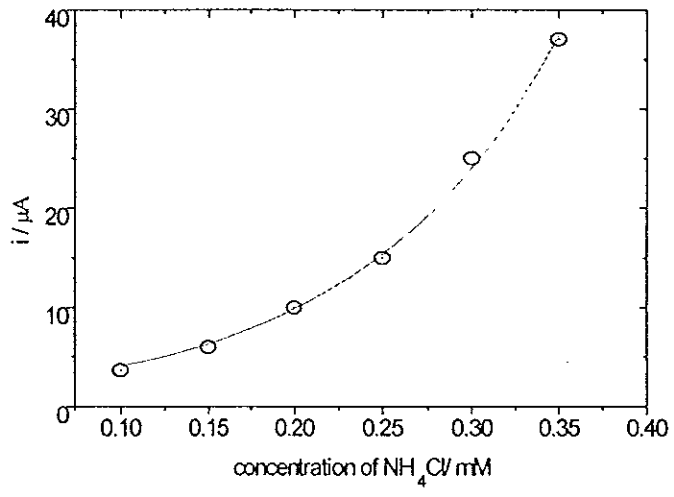


Fig. 5.3.2 Calibration curve following from the experiment presented in fig. 5.3.1. The curve clearly shows the apparent exponential increase in current with increasing ammonium concentration in the aqueous phase.

The cause of this behaviour is not clear. The phenomenon requires further theoretical works. Ac-peak currents are also dependent on iR drop. Table 5.3.1 demonstrates the susceptibility of ac peak heights by starting at arbitrary sets of iR compensation and current, and then followed by slight increments in iR compensation. Figures 5.3.3 and 5.3.4 (below) show some experiments done to calibrate the sensor with respect to NH_4^+ and K^+.

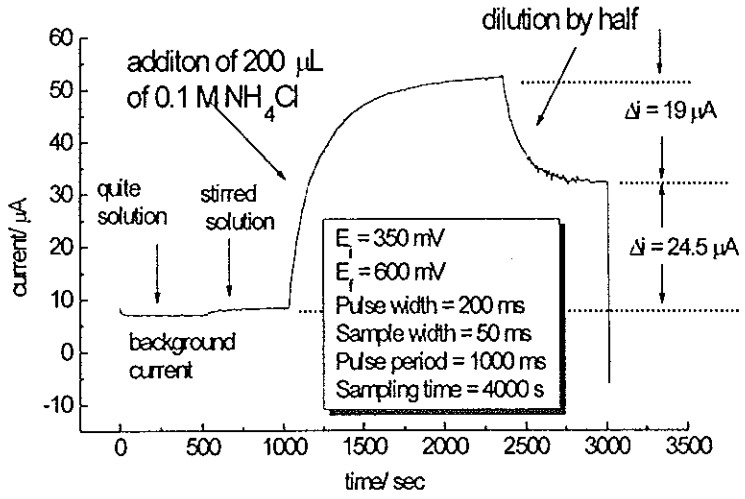


Fig. 5.3.3 a

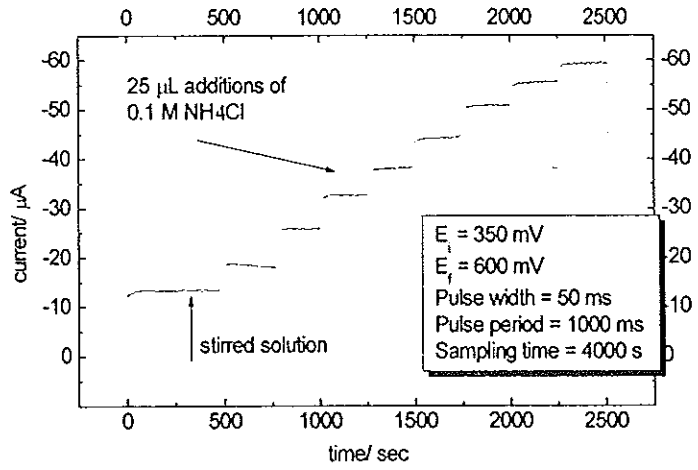
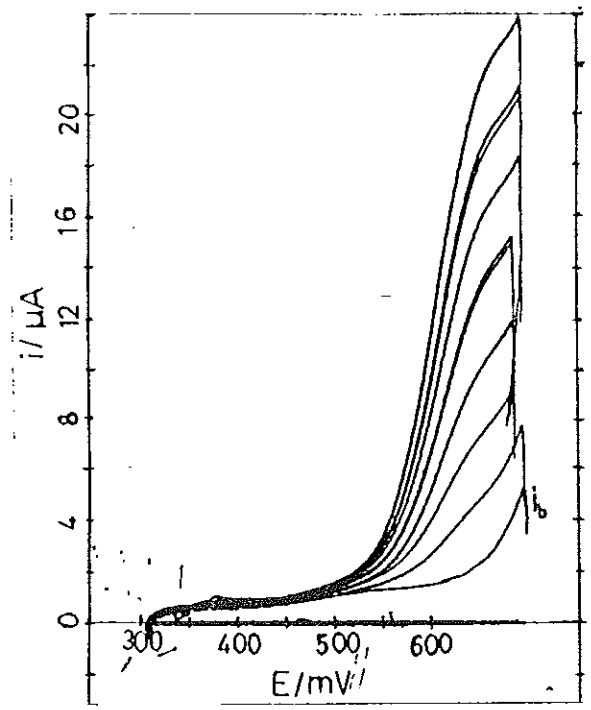
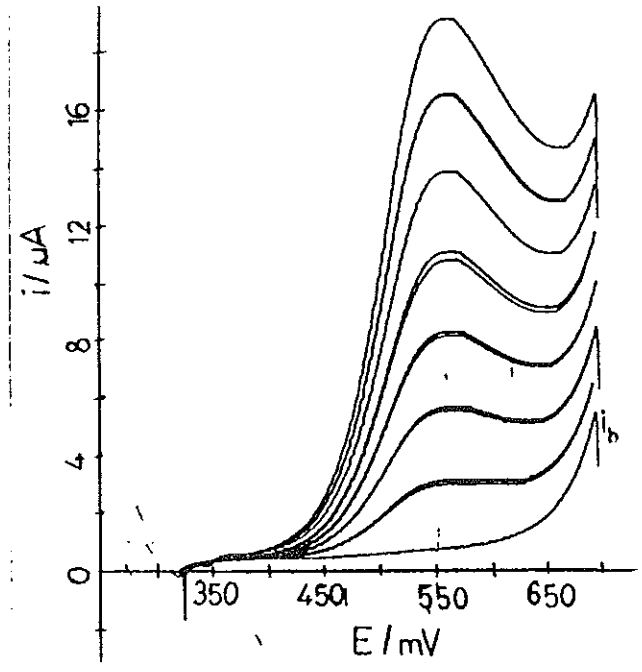


fig. 5.3.3 b

Fig 5.3.3 Calibration experiment on the sensor with respect to NH_4^+ ions where the dptb amperometry at the membrane-stabilized water-ntrobenzene interface (Water: 5 mmol/L Li_2SO_4; nitrobenzene: 10 mmol/L EthVTPB + 50 mmol/L DB[18]C-6, stirring) was used. Fig 5.3.3 a shows a simple calibration test where addition of 200µL of 0.1mol/L NH_4Cl followed by dilution by doubling the volume of the aqueous phase. Fig 5.3.3b shows a calibration experiment where 25 µL aliquots of 0.1mol/L NH_4Cl were added consecutively.



(b)



(a)

Fig. 5.3.4 Dc-linear sweep voltammogram obtained by using the membrane-stabilized water-nitrobenzene interface (Water: 5 mmol/L Li_2SO_4 (50 mL); nitrobenzene: 10 mmol/L EthVTPB + 50 mmol/L DB[18]C-6, sweep rate=25mV/s, stirring) as a voltammetric sensor. Curve i_b is the background current due to the supporting electrolytes. The consecutively growing peaks at around 650 mV were due to facilitated NH_4^+ transfer (fig 5.3.4 a) and the one that occurs at around 550 mV was that of K^+ (fig 5.3.4 b). The figures show calibration experiments where 25 µL aliquots of 0.1 mol/L NH_4Cl and KCl were added to the aqueous phase.

Fig. 5.3.5, 5.3.6 and 5.3.7 show calibration curves that follow from the above discussed experiments, i.e. from fig. 5.3.3 b, 5.3.4 a and 5.3.4 b, respectively.

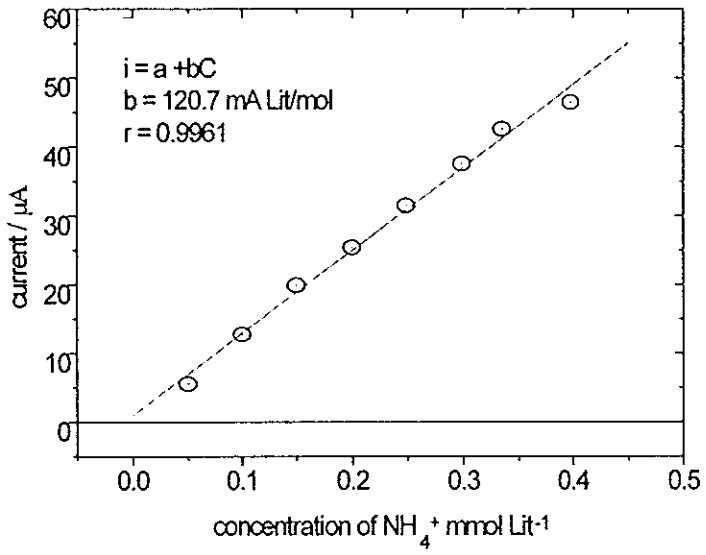
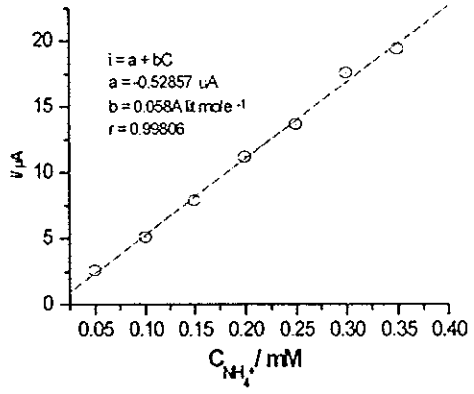


Fig. 5.3.5 Calibration curve of the sensor with respect to ammonium ions using data taken from the dptb curve shown in fig.3.3.3b



Fg. 5.3.6 Calibration graph for the sensor with respect to ammonium ions. Data were taken from peak height measurements from the experiment described in fig. 5.3.4 a.

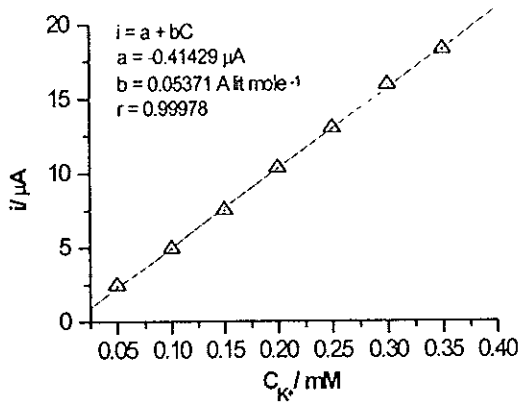


Fig. 5.3.7 calibration curve of the sensor with respect potassium ions. Data were taken from the calibration experiment described in fig 5.3.4(b).

The amperometric constant or the sensitivity, K_{amp}, was observed to vary even when two consecutive experiments were compared (Table 5.3.1). The high correlation coefficients exhibited enable quantitation of an analyte by standard addition method. The dptb technique was observed to render much higher sensitivity which was more than twice those found by dc-voltammetry.

Table 5.3.2 Variation of sensitivity

analyte	K_{amp}/ mAM^{-1}	correlation coefficient (r)	method
	51.07	0.99995	dc-linear sweep
NH_4Cl	47.70	0.99964	same
	58.00	0.99806	same
	120.53	0.9977	dptb
KCl	53.71	0.99978	dc-linear sweep
	56.5	0.99978	same
	68.00	0.99958	same

5.4 Estimation of Potassium in Some Plant Tissues

The sensor was used to determine the potassium content of the plant materials collected. Peak currents of dc-voltammograms were measured before (i_x) and after (i_{x+s}) addition of 2 µL (V_s) of 0.1 mol/L (C_s) KCl to 50 mL (V_x) of aq. tissue

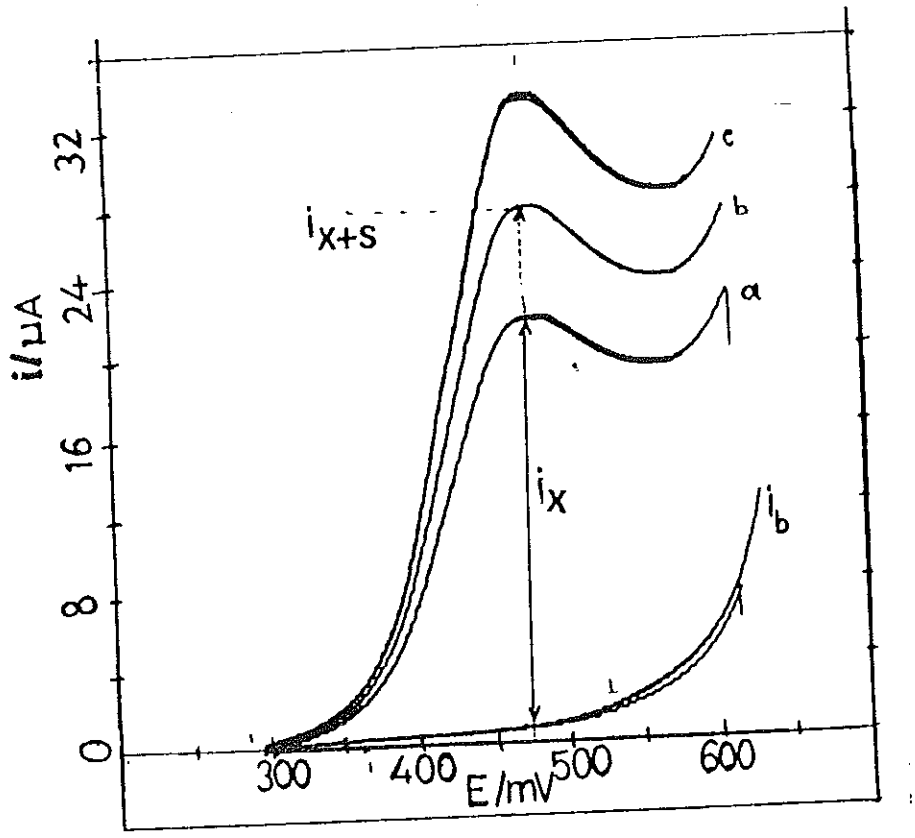


Fig. 5.4.1 Dc-LV voltammogram obtained at the membrane-stabilized water-nitrobenzene interface (Water: 5 mmol/L Li_2SO_4; nitrobenzene: 10 mmol/L EthVTPB + 50 mmol/L DB[18]C-6, sweep rate = 25 mV/s, stirring) for determination of potassium in an aqueous suspension of bean 2 (a variety of phaseolus vulgaris seeds). Analysis by standard addition method is described. Peak currents were measured both before and after the addition of a standard to an aqueous suspension of the tissue. Curves i_b, a, b, and c correspond to the supporting electrolyte, addition of 50 mg seed powder, addition of 50 µl standard (0.1 mol/L KCl) and addition of another 50 µl of the standard.

suspension which contained unknown concentration (C_x) of potassium. Fig 5.4.1 (above) shows a typical voltammogram used to estimate potassium in bean 1 seed (*phaseolus vulgaris*). Calculations were based on equation 5.4.1 (below) after background currents were subtracted.

$$i = K_{amp} C \qquad \text{.....5.4.1}$$

Since $C_s << C_x$, (assuming negligible increase in volume) the following equation was used to calculate the concentration of potassium in the aqueous phase.

$$C_x = \frac{V_s C_s}{V_x} \left(\frac{i_x}{i_{x+s} - i_x} \right) \qquad \text{.....5.4.2}$$

Percent of the analyte was then estimated using the equation given below,

$$\%K = \frac{3900 C_x V_x}{50} \qquad \text{..... 5.4.3}$$

The percent compositions of K^+ in the samples collected are presented in the table 5.4.1.The precision observed were very promising. The percent standard deviation varied from 2 to 3 only except for three of the eight samples.

Table 5.4.1 Values of estimated potassium content of some plants (%)

sample code	average $\%K^+$ n=4	standard deviation (S_{n-1})	$\%S_{n-1}$
squash seed	0.459 (n=6)	0.01	2.18
bean 1	1.86	0.14	**7.53**
bean 2	1.45	0.04	2.76
watermelon 1	0.752	0.056	7.45
soy bean flower	1.572 (n=6)	0.061	3.88
bean 3	1.504	0.041	2.73
bean 4	2.482	0.075	3.02
watermelon 2	0.722	0.014	1.94

5.5 Watermelon Seeds and Soy bean Flour as Soluble Reagents for Amperometric Urea Determination. Performance Evaluation of the Tissues.

Differential pulse amperometric studies on urease activities of watermelon 1 (wm1), watermelon 2 (wm2) and soy bean flour (sbf) are presented in fig. 5.5.1, 5.5.2 and 5.5.3, respectively. From these figures and the accompanying table (table 5.5.1), it can be inferred that watermelon 1 seeds gave the highest increase in current per µL additions of 0.1 mol/L standard urea. This table compares current changes at steady state (Δi_{ss}), time required to 95% of steady current changes ($t_{95\%\Delta iss}$) initial urease-urea reaction rates ($rate_o$), ratios of total current change to changes in concentration due to additions of ammonium chloride ($\Delta i_{eq}/\Delta C_{amml} \cong K'_{amp}$, an estimation of the amperometric constant), and estimated values of urease activities in Sumner Units (SU).

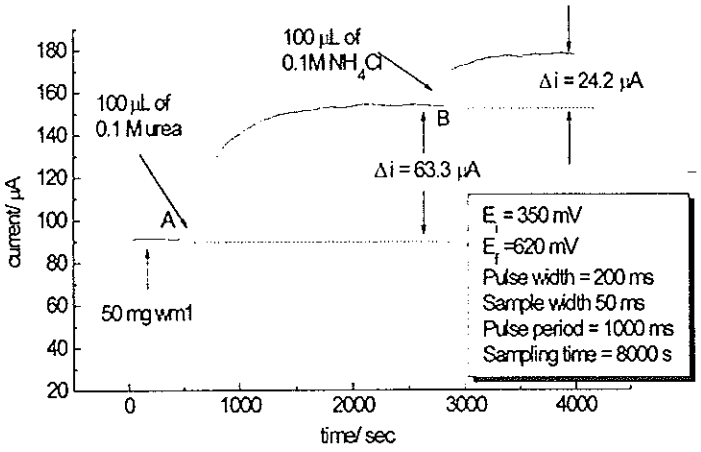


Fig. 5.5.1 Differential pulse amperometry at the membrane stabilized water-nitrobenzene interface. The curve before point (A) was a sum due to supporting electrolytes (Nitrobenzene: 10 mmol/L EthVTPB, 50 mmol/L DB[18]C-6; Water: 5 mmol/L Li_2SO_4) and 50 mg wm1 dissolved in the aqueous phase. At point (A) 100 µL of 0.1 mol/L urea was added and, the extension of the curve up to point (A) shows the current increase due the generation of ammonium ions by wm1-urease-urea reaction. At point (B), where the enzymatic reaction was in a steady state, 100 µL of 0.1 mol/L NH_4Cl was added. A fresh sensor was used for this experiment.

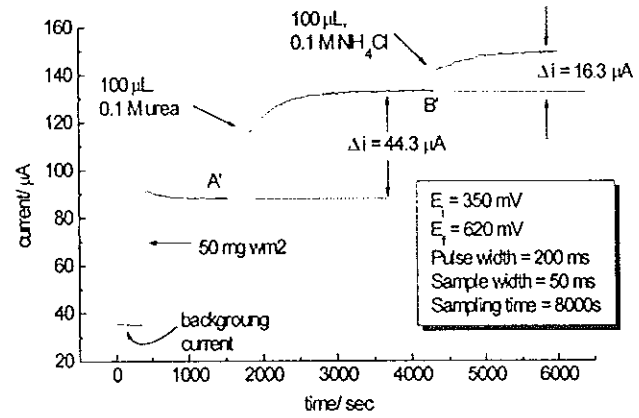


Fig. 5.5.2 Differential pulse amperometry at the membrane stabilized water-nitrobenzene interface. The curve before point (A') a sum due to supporting electrolytes (Nitrobenzene: 10 mmol/L EthVTPB, 50 mmol/L DB[18]C-6; Water: 5 mmol/L Li_2SO_4) and 50 mg wm2 dissolved in the aqueous phase. Here, both of them are shown. At point (A') 100 µL of 0.1 mol/L urea was added and, the extension of the curve up to point (B') shows the current increase due the generation of ammonium ions by wm2-urease-urea reaction. At point (B'), where the enzymatic reaction was in a steady state, 100 µL of 0.1 mol/L NH_4Cl was added. This experiment was done next to the one presented in fig. 5.5.1.

Urease activities were estimated from the figures in question by reading the current at 300 s (i_{300}) after the addition of urea. One sumner unit is the amount of enzyme required to produce 10^{-3} g or 5.88 x 10^{-5} moles of NH_3 in five minutes [40]. Urease activity per mg of tissue in solution was estimated by the following equation.

$$activity = \frac{1}{50mg}\frac{n_{NH_3}}{5.88X10^{-5}} = \frac{V_x}{50mg}\frac{C_{NH_3,300}}{5.88X10^{-5}} = \frac{V_x}{50mg}\frac{i_{300}}{5.88X10^{-5}K'_{amp}} \qquad 5.5.1$$

where $n_{NH3,300}$ is the number of moles of ammonia generated in five minutes and V_x is the volume of the aqueous phase.

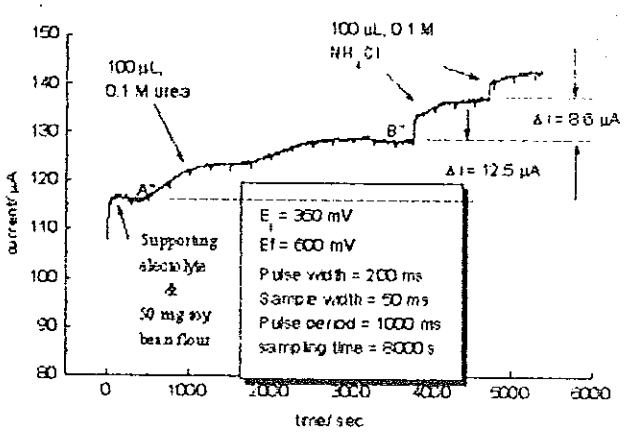


Fig. 5.5.3 Differential pulse time base amperometry at the membrane stabilized water-nitrobenzene interface. The curve before point A is a sum due to the supporting electrolytes (Nitrobenzene: 10 mmol/L EthVTPB, 50 mmol/L DB[18]C-6; Water: 5 mmol/L Li_2SO_4 , stirring) and 50 mg soy bean flour dissolved in the aqueous phase. At point (A") 100 µL of 0.1 mol/L urea was added and, the extension of the curve up to point (A')' shows the current increase due the generation of ammonium ions by soy bean-urease-urea reaction. At point (B"), where the enzymatic reaction was in a steady state, 100 µL of 0.1 mol/L NH_4Cl was added. This experiment was done next to the one presented in fig. 5.5.2.

Watermelon 2 was relatively much more active than soy bean flour. Steady states were reached in 17, 17 and 33 minutes for wm1, watermelon 2 and undefatted soy bean flour, respectively. Both varieties of watermelon seeds showed initial reactions of equal rate. The poor urease activity of, and high back ground current caused by soy bean flour (defatted) made it unsuitable as a reagent. An additional experiment

Table 5.5.1 Summary of fig. 5.5.1, 5.5.2 and 5.5.3

tissue	addition of	i_{ss} / µA	$t_{95\%\Delta ieq}$ in sec	$rate_0$/ µA/ sec	K_{amp}/ mAM^{-1}	activity/ S.U. mg^{-1}
wm1 seeds	50 mg wm1	58.62		0.1254	120	0.00506
	100 µL urea	63.3	17.1 min			
	100 µL NH_4Cl	24.2				
wm2 seeds	50 mg wm2	54.3		0.1254	81.5	0.00524
	100 µL urea	44.9	17 min			
	100 µL NH_4Cl	16.3				
sbf	50 mg sbf	96.03		0.0123		0.00208
	100 µL urea	12.5	33 min			
	100 µL NH_4Cl	8.6	4.3 sec		43	
	100 µL NH_4Cl	5.9			29.5	

on the same tissue using a freshly prepared sensor gave the same results, i.e. low sensitivity and very slow initial reaction rate. In addition, its solution was observed to foam on stirring, causing noisy current output (see fig. 5.5.3).

Other consecutive experiments were done with undeffated (fig. 5.5.4) and defatted (fig. 5.5.6) portions of watermelon 1. Aliquots of standard ammonium chloride were added before the tissues were dissolved in the aqueous phase. After adding 100 µL of 0.1 mol/L urea, the same volume of 0.1 mol/L ammonium was added again at the steady state. This procedure was used so that one could compare the sensitivity of the sensor before and after the addition of a tissue. Table 5.5.2 and 5.5.3 present tabular summary of these experiments. Here, defatted watermelon better enzymatic

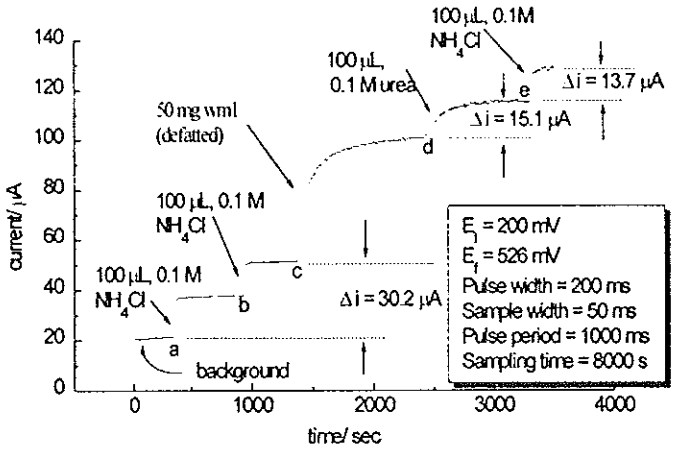


Fig. 5.5.4 Differential pulse time base amperometric study on the urease activity of watermelon seeds on urea when the addition of the former is preceded by the other. The sensor was the membrane-stabilized water-nitrobenzene interface (Nitrobenzene: 10 mmol/L EthVTPB, 50 mmol/L DB[18]C-6; Water: 5 mmol/L Li_2SO_4 , stirring)

activity than the untreated portion. The sensitivity of the sensor had decreased when the undefatted portion was examined. This is seed showed discernible from the

Table 5.5.2 Performance of defatted watermelon seed powder when its addition was preceded by addition of ammonium solution (summarised from fig. 5.5.4).

point	addition of	i_{ss}/ µA	$t_{95\%\ iss}$	K_{amp} µA/ mM
a	100 µL NH_4^+	16.43	17.1 sec	82.15
b	100 µL NH_4^+	13.77	.....	68.85
c	50 mg powder	49.33	8 min	-------
d	100 µL urea	15.1	8.7 min	75.5
e	100 µL NH_4^+	13.7	.....	68.5

curves that equal additions of ammonium at points a and a' did not give the same increase in current. Compare points e and e' where 100 µL of the standard was added after completion of the urease-urea reaction. The sensitivity of the sensor had

already been reduced by 50 % at point a' during the second experiment.

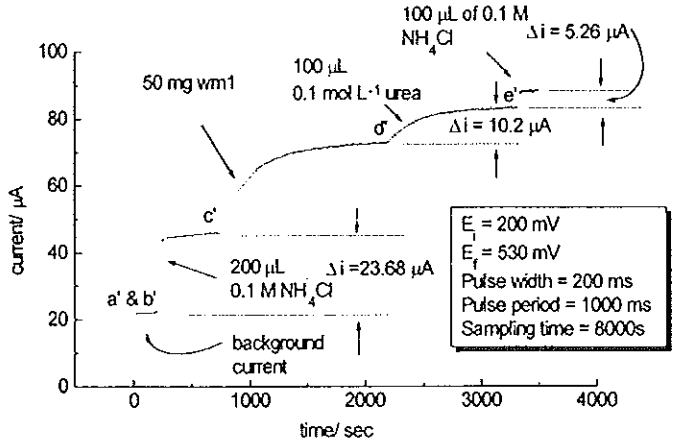


Fig. 5.5.5 Differential pulse time base amperometric study on the urease activity of watermelon seeds on urea when the addition of the former is preceded by the other.The sensor was the membrane-stabilized water-nitrobenzene interface (Nitrobenzene: 10 mmol/L EthVTPB, 50 mmol/L DB[18]C-6; Water: 5 mmol/L Li_2SO_4 , stirring)

Table 5.5.3 Performance of undefatted watermelon seed powder when its addition was preceded by addition of ammonium solution (summary of fig. 5.5.5).

point	addition of	Δi_{ss}/ µA	$t_{95\%\ iss}$	K_{amp} / µA/ mM	$rate_0$
A'	200 µL NH_4^+	23.68	1.5 min	59.2	
B'	50 mg powder	27.3	15 min	-------	
C'	100 µL urea	10.2	10 min	51	0.03129
D'	100 µL NH_4^+	5.26	----------	26.3	

Both the amount of dissolved potassium and its rate of dissolution seem to increase by 100%. The evaluated initial urease-urea reaction rates are very much lower than the one reported in table 5.5.1. This may be attributed to the effect of the already added ammonium chloride.

6. CONCLUSIONS

The present work dealt with the utilization of urease-rich plant tissues as soluble reagents for the enzymatic determination of urea. Ammonium ions produced by the catalytic hydrolysis of urea were amperometrically detected at the membrane-stabilized water-nitrobenzene interface. The ionophore DB[18]C-6 was dissolved in the organic phase which contained EthvTPB as the supporting electrolyte. Li_2SO_4 was the aqueous supporting electrolyte. In addition the sensor was applied to the direct determination of potassium in some plant tissues without any pretreatment.

Voltammetric studies showed that ammonium ion transfer peak occurred as a shoulder to the cathodic limit of the potential window. The amperometric sensor responded to K^+ and Na^+ ions, too. Calibration curves for NH_4^+ and K^+ ions showed very high correlation coefficients (> 0.998). However, the sensitivity of the sensor (under the conditions of the study) decreased even when calibration curves from two consecutive experiments were compared. Differential pulse amperometry rendered the sensor much more sensitive as compared to dc-voltammetry. The sensor did not stay more than 10 days.

A finite portion of dry and powdered tissue was kept in the aqueous phase to which aliquots of standard urea were added. Reaction progress versus time could be easily followed by a pulse amperometric technique in which current versus time output was displayed on a computer's screen. Dc-linear sweep voltammetry, although less sensitive, could also be used in preliminary investigation and the determination of potassium.

Enzymatic activities could easily be estimated. Watermelon seeds and defatted soy bean flour showed urease activities. The former ones gave higher initial urease-urea reaction rate and high increase in current per unit addition of standard urea when compared with defatted soy bean flower.

Watermelon seeds were found to contain very smaller amount of indigenous potassium ions when compared to soy bean flour. potassium ions whose dissolution step further lengthened the response time. Washing the meals with acetone/ water mixture or use of ion exchangers to remove indigenous potassium ions have been found to weaken or destroy the enzyme activity. The former treatment partially removed the ions, while ion exchangers totally did so. Study must be made to adopt these methods for the non-destructive removal of K^+ ions. The effect of defatting with petrol-ether on urease activity is not clear. However, it increased the dissolution rate of potassium from the tissue ($\geq$100%).

To experiment with the tissues, one had to wait until all the potassium had gone into solution before urea was added as an analyte or a standard. Response times were very long (10 - 15 min). If potassium dissolution step was considered, the response time would double for the undefatted watermelon. The tissue may be utilized for determination of urea by the reaction-rate (kinetic method) method provided that conditions are controlled by buffering, thermostating, etc. The suspensions of the tissues were found to be acidic (between pH 6 and 6.5). This might be due to the fact that the aqueous phase itself was acidic (less than pH 6). Future works must manage to utilize the enzyme as much as possible at a pH close to that of the tissue.

The presence of the tissues in the aqueous phase entailed increased rate of deterioration of sensitivity. However, it can wisely be used for the standard addition method. This approach proved promising for the utilization of watermelon seeds for enzymatic estimation of urea and the direct determination of potassium in plant materials.

Recommendations for Future Work

Based on the out comes of the works discussed some recommendations may be worth mentioning. If the positive limit of the working potential range was caused by the transfer of TPB^- ions to the aqueous phase, the limit may be pushed to more positive potentials if p-Cl or p-F tetraphenylborate are utilized instead of tetraphenylborate. Since 5 mmol/L Li_2SO_4 is acidic (pH < 6), a buffer (such as that of Li_2EDTA) must be used so as to work at a neutral pH or at a pH closer to that of the tissues. Since the discussed dptb experiments involved iR undercompensation by 10 KΩ through out an experiment, results may have been distorted by changes in iR drop. Thus, such experiments must be designed in such a way that iR drop can be compensated instantaneously.

The methods for defatting and for removal of potassium should be redesigned so that the urease activity is not harmed. Already prepared tissue suspensions may be used to alleviate the time required for the complete dissolution of potassium. Since it seems that particles of the powdered tissues blocked the pores of the membrane, a water-gap ammonium sensor may be proposed. Finally, immobilization of watermelon meal across the interface is expected to yield better performances.

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DECLARATION

I, the undersigned, declare that this thesis is my original work, has not been presented for a degree in any other university and that all sources of material used for the thesis have been duly acknowledged.

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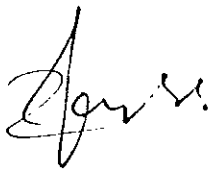
Signature 

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