ADDIS ABABA UNIVERSITY
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ANALYTICAL APPLICATION OF THE MEMBRANE-STABILIZED, ENZYME-MODIFIED INTERFACE BETWEEN TWO IMMISCIBLE ELECTROLYTE SOLUTIONS.

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ABBREVIATIONS

CVTPB :- crystal violet tetraphenyl borate

DB[18]C-6 :- dibenzo-18-crown-6

PNPDCC :- μ-nitrido-bis(triphenylphosphorous)-3,3-como-bis(undecahydro-1,2-dicarba-3-cobalta-closododecarbor)ate

TBATPB :- tetrabutyl ammonium tetraphenyl borate

TOctATPB :- tetraoctyl ammonium tetraphenyl borate

TPAsDCC :- tetraphenyl arsonium-3,3-comobis(undecahydro-1,2-dicarba-3-cobalta-closododecarbor)ate

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ABSTRACT

An amperometric urea biosensor based on facilitated transfer of urease-generated ammonium ion by a neutral carrier (ionophore) dibenzo-18-crown-6 across the polarizable membrane-stabilized interface between water and nitrobenzene is described and discussed. The enzyme urease employed for the investigation was immobilized on the sensor surface by physical entrapment as a gel between dialysis membranes. The current response of the amperometric sensor was found to be directly proportional to the concentration of urea in the sample solution. In addition, correction for residual current due to interfering (residual) substances is relatively easy. The sensor gave a linear response to urea in the concentration range 5×10^{-5} to 5×10^{-3} M. The response time of the sensor was 1 min . The sensitivity appeared to be dependent on the conditions of immobilization of the enzyme.The effect of the thickness of the enzyme layer on the sensor response has been discussed. The life time of the sensor was more than 3 weeks when stored in the buffer solution at room temperature.

1. INTRODUCTION

Ion-transfer across the interface of two immiscible electrolyte solutions (ITIES) has been studied extensively during recent years employing different organic phases and electrochemical techniques [1-3]. Theoretical approaches to the thermodynamics and especially the kinetics of the transfer of an ion and an electron across the interface has been summarized by Samec [4]. The basic principles, concepts and general relations characterizing the transfer of ions across this interface studied by some electroanalytical methods and apparatus, sometimes with application possibilities were described [5]. These studies have shown that such an interface can be electrochemically polarized and thus can serve as an ion-selective electrode for the analytical determination of semihydrophobic ions present in one of the phases. Charge transfer reactions across the ITIES can be studied using the same electrochemical methods employed for the investigation of processes occurring at the metal/electrolyte solution interface. The faradic and non-faradic processes taking place at the interface of two immiscible electrolyte solutions have been compared with analogous processes occurring at the interface metal/electrolyte solution by Koryta [5]. Since the middle of the sixties, a fast growing number of articles have appeared dealing with simple and facilitated ion-transfer as well as the investigation of the double layer structure at the ITIES. It has also been shown that evaluation of the experimental data can be made by using the theoretical frame-work of classical electrochemistry. Studies of such systems help significantly in enlarging the scope of electroanalytical methods and can serve as the simplest model for the charge-transfer reactions taking place in biological systems.

In 1902, Nernst and Riesenfeld treated the problem of single salt transport under constant current across the ITIES [13]. Since the renaissance of the investigation of charge transfer reactions in the sixties the literature has been reviewed by Koryta [5-7]. Chronopotentiometric method was employed in carrying- out the early electrochemical investigations [1,2,8,9]. The introduction of IR-drop compensation by a positive feed-back [10] in cyclic voltammetry [11-14] has proved very useful for the investigation of charge transfer reactions at the ITIES. In addition, other techniques such as current scan polarography and impedance measurements have been employed.

Gaustalla and Gavach [15] pioneered in 1968, the use of electrochemical methodology to show that the water/nitrobenzene interface could be polarized. The same problem was also studied by Blank [16] and Joos [17,18], who used only one electrolyte and attempted

to elucidate the polarization phenomena by different transport numbers of ions transferred in the aqueous phase and in the non-aqueous phase [17]. The basic properties of the ITIES were widely investigated by Koryta, Vanysek and Brezina in 1977 [19]. They indicated the conditions under which the ITIES behaves either as an ideally-polarizable or non-polarizable electrode. The basic equations characterizing the current-potential relationships at the interface have been deduced. Further investigations by other groups working in the field have been made and reported in a number of review articles [5-7,20-25].

The standard Gibbs transfer energy of individual ions between water and organic solvents is the basic quantity characterizing the equilibria at the ITIES. The standard Gibbs transfer energy for an individual ion, in contrast to the electrolyte as a whole, is not accessible to a direct measurement and in order to make its quantitative determination, possible, some kind of a non-thermodynamic assumption must be made. Most frequently the tetraphenylarsoniumtetraphenylborate (TPAsTPB) assumption of Parker [26] and Popovych [27] is made which states that the anion and the cation of tetraphenylarsoniumtetraphenylborate have equal standard Gibbs transfer energies between any pair of solvents. Both ions are quite voluminous that the charge of the central atoms acts only electrostatically on the solvent dipoles. The interaction of the benzene rings with the solvent is the same for both ionic species. On the basis of this assumption a scale for standard Gibbs energies of partition can be calculated for any pair of solvents [28-31]. A summary of standard Gibbs energies of transfer and standard Galvanic potential differences has been given by Koryta [6,19], for various cations and anions in water/nitrobenzene, water/1,1'-dichloroethane and water/1,2-dichloroethane system. An approach for the setting of the Galvanic potential scales and ionic Gibbs energies of transfer between two liquid phases was proposed by Girault and Schiffrin, based on the assumption that the Galvanic potential difference at the potential of zero- charge is zero [32].

The selection of the organic phase to be used for the investigation of the ion-transfer processes at the ITIES is governed by three requirements:-

i- the mutual solubility with water has to be very low, since otherwise the base electrolytes can not be confined to their respective phases and no suitable potential range for polarization of the interphase could be found;

ii- the permittivity ought to be high enough to ensure the dissociation of the supporting electrolytes , so that the organic phase has sufficient conductivity;

iii- the difference in density with respect to water has to be large enough to form

a stable interphase.

Nitrobenzene has been most frequently used, as it meets these requirements. Other organic phases utilized were 1,2-dichloro ethane [33], o-nitrotoluene [34], dichloromethane [29] and acetophenone [35]. In addition to these water-pure organic solvent systems, mixtures of organic solvents have been used. These include nitrobenzene-benzonitrile [36], nitrobenzene-chlorobenzene [37] and nitrobenzene-benzene [38]. Kihara et.al. [21] have investigated systems consisting of a large number of organic solvents.

The supporting electrolytes to be used in the aqueous and organic phases should consist of very hydrophillic ions for the former and very hydrophobic ions for the latter. TBATPB, PNPDCC, TPAsDCC, CVTPB and TBA(p-ClTPB) are commonly used in the organic phase. For the aqueous phase, the sulphates, fluorides, chlorides and bromides of lithium, sodium and magnesium have been widely used. The "potential-window", i.e., the potential range where the transfer of the investigated ions occur without interference from the transfer of the ions of the supporting electrolytes, depends on the hydrophillicity and hydrophobicity of the ions of the supporting electrolytes in the aqueous and organic phases respectively. In practice, the number of ionic species that can be transferred across the water/organic solvent interphase has been limited by the rather narrow range of accessible potential differences between the two phases [7]. This is because the standard potential of some cations, mainly the alkali and the alkaline earth metal ions, are so positive that the anion of the base electrolyte in the organic phase are transferred before these cations or both transfer processes coincide. However, the transfer of such ions can be facilitated by incorporating ionophores (neutral carriers) in the organic phase. The principle of ionophore-facilitated ion transfer was first described by Koryta [5]. An ionophore is a strongly hydrophobic molecule which possess acceptor properties for ions or which is a complex former or a proton acceptor. The resulting ionophore-ion associate must also be hydrophobic. Therefore facilitated transfer of ions such as Na^+[13,38-41,44], K^+[5,41-45], Ca^{2+} [46], H^+ [38,41,47], Li^+ [41,48] and Cd^{2+} [56] can be made possible in the presence of neutral carriers or ionophores which can form relatively stable complexes with the metal ions in the organic phase. The ionophores are macrocyclic uncharged molecules and contain a cavity in which a cation can be embedded. The most known compounds among these are crown ethers which are cyclic polyether compounds with oxyethylene groups and some antibiotics such as valinomycin. Pederson published his first paper [49] dealing with cyclic polyether compounds. This was followed by other reports [50] dealing with crystalline salt

complexes of Na, K, Rb, Cs, Ba, NH_4 salts and some new crown ethers [51]. The effect of ion-association on the distribution of ions and on the Galvanic potential differences across ITIES has been discussed by Hung [52]. Ion-association also influences the transfer of ions which form ion-pairs across ITIES [53].

Determination of the standard Gibbs energy of transfer and the stability constants of the ionophore complexes has been the most important application of the investigation of the ion-transfer across the ITIES [6]. Moreover, such systems have found applications in the elucidation of the response of ion-selective electrodes [49,54-56], the estimation of the rate of the interfacial step of salt extraction [7] and the direct determination of ions in different samples [44,57-60].

The mechanical instability of the liquid/liquid interface and the difficulty in keeping the diffusion layer thickness constant are the major problems associated with the analytical application of the ITIES [60]. The problem of the mechanical stabilization of the electrified liquid/liquid interface has been solved either by solidification of the organic phase through gel formation with polyvinylchloride (PVC) or by insertion of a porous membrane between the phases [60,61]. The thickness of the diffusion layer can be kept constant using a wall-jet arrangement of the sensor in a flow system [62]. The wall-jet cell was first introduced by Glaurt [63] to describe the flow due to a jet of fluid which spreads out over a plane surface , the fluid outside the jet being at rest. The wall-jet arrangement of the sensor ensures the establishment of a hydrodynamic boundary layer within which a hypothetical stagnant diffusion layer adjacent to the hydrophillic membrane is assumed to exist. This simplification of the actual convective diffusion outside the membrane to molecular diffusion enables one to obtain an operational solution for the response dynamics of membrane-stabilized interfaces. In addition, the wall-jet electrode offers high sensitivity and low solution hold-up, and thus appears suited for continuous electrochemical measurements in a flow system. Hundhammer et. al. [60] and Marcek et.al. [68] have used the membrane-stabilized ITIES and the solidified organic phase gel electrode, respectively, for analytical determination, employing a wall-jet cell arrangement in a flow-injection system.

2. THEORY

The electrochemical behaviour of the interface between two immiscible electrolyte solutions (ITIES) is mainly dependent on the ionic composition of the two phases. When one of the phases is water and contains a strongly hydrophillic electrolyte and the other an organic (non-aqueous) solvent containing a strongly hydrophobic electrolyte, then the properties of the ITIES become analogous to those of a polarizable electrode. On the other hand, if there is sufficiently large concentration of ions with high exchange rates present in both phases, then the interface behaves like a non-polarizable electrode. On introduction of a charge to one of the phases from an external source (and, simultaneously, of a charge of opposite sign to the other phase) two processes can take place: the transfer of ions across the phase boundary and the charging of the electrical double-layer formed at the interface.

The ITIES, for example, that formed between water (phase α) and an organic solvent such as nitrobenzene (phase β) has several basic features analogous to the metallic electrode/electrolyte solution interface. Between the two phases there exists an electrical potential difference, the value of which depends on the composition of the two phases.

If both phases contain a common ion B^+ of charge z, which is transferred across them, the condition for equilibrium can be stated as:

$$\overline{\mu_{B^+}}(\alpha) = \overline{\mu_{B^+}}(\beta) \qquad (1)$$

$$\mu^{\circ}_{B+}(\alpha) + RT\ln a_{B+}(\alpha) + zF\varphi(\alpha) = \mu^{\circ}_{B+}(\beta) + RT\ln a_{B+}(\beta) + zF\varphi(\beta)$$

The Galvani potential difference, $\Delta^{\beta}_{\alpha}\varphi$, between the two phases is then given by:

$$\Delta^{\beta}_{\alpha}\varphi = \varphi(\beta) - \varphi(\alpha) = \frac{\mu^{\circ}_{B^+}(\alpha) - \mu^{\circ}_{B^+}(\beta)}{zF} + \frac{RT}{zF}\ln\frac{a_{B^+}(\alpha)}{a_{B^+}(\beta)}$$

$$= -\frac{\Delta^{\beta}_{\alpha}G_{tr,B^+}}{zF} + \frac{RT}{zF}\ln\frac{a_{B^+}(\alpha)}{a_{B^+}(\beta)}$$

$$= \Delta_{\alpha}^{\beta}\varphi_{B^+}^{0} + \frac{RT}{zF}\ln\frac{a_{B^+}(\alpha)}{a_{B}^{+}(\beta)} \qquad (2)$$

Here, $\bar{\mu}_{B+}$ (α), $\bar{\mu}_{B+}$(β) are the electrochemical potentials; $\mu_{B+}{}^{0}(\alpha)$, $\mu_{B+}{}^{0}(\beta)$, the standard chemical potentials; $a_{B+}(\alpha)$, $a_{B+}(\beta)$, the activities of the ion B^+; $\varphi(\alpha)$ $\varphi(\beta)$, the inner (Galvani) potentials; and $\Delta_{\alpha}{}^{\beta}\varphi^{0}{}_{B+}$ the difference in the standard galvani potential; in the phases α and β. The individual ion activities in a non-aqueous solution may be treated in an analogous way as in an aqueous medium using for low concentrations, the Debye-Huckel equations. Then the influence of the solvent is displayed only in the difference of the standard chemical potentials which (after multiplying by -1) is called the standard Gibbs energy of transfer of the ion B^+ from phase α to phase β, $\Delta_{\alpha}{}^{\beta}$ $G_{tr,B+}$. This quantity is, in fact, the difference of standard Gibbs energies of solvation of B^+ in the phases β and α, respectively. The standard Gibbs transfer energy for an individual ion is not accessible to a direct measurement and hence the TPAsTPB assumption is used for its quantitative determination. Since the standard Galvani potential difference of a single ion, B^+, is related to its standard Gibbs transfer energy by:

$$\Delta_{\beta}^{\alpha}\varphi_{B^+}^{0} = \frac{\Delta_{\alpha}^{\beta}G_{tr,B^+}^{0}}{zF} \qquad (3)$$

The calculation of $\Delta G^{o}_{tr,B+}$ becomes possible if the standard Galvani potential difference can be obtained experimentally. It has been shown that the ion-transfer across the interface of immiscible electrolytes follow formally the same laws as those describing the electron transfer at the metal/electrolyte solution interface [4,5]. Thus, the theory of cyclic voltammetry derived for those electrodes can be adopted to describe the voltammetric behaviour of the ITIES. 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. It has been shown by Nicholson and Shain [64] that the current-potential relationship for such a system is given by:

$$\mathbf{i = z^{3/2}\, FAC^{b}_{i}(D_i\, \nu\Pi)^{1/2}\; X(at)} \qquad (4)$$

where the area A of the interface is expressed in cm^2, the bulk concentration, C^b_i, in mol dm^{-3} and the sweep rate υ in Vs^{-1}. The values for $\Pi^{1/2}X(at)$ vs $(E - E_{1/2})$ [or in this particular case, vs $(\Delta^{\alpha}_{\beta}\varphi - \Delta^{\alpha}_{\beta}\varphi_{1/2})$] are tabulated by Nicholson & Shain [64]. Following this concept the evaluation of $\Delta^{\alpha}_{\beta}\varphi_i$ from voltammetric data is straightforward since the relationships

$$\Delta^{\alpha}_{\beta}\varphi_P = \Delta^{\alpha}_{\beta}\varphi_{1/2} - \frac{1.109RT}{zF} \qquad (5)$$

$$\Delta^{\alpha}_{\beta}\varphi^0 = \Delta^{\alpha}_{\beta}\varphi_{1/2} - \frac{RT}{2zF}\ln\frac{D^{\alpha}_i}{D^{\beta}_i} - \frac{RT}{zF}\ln\frac{\gamma^{\beta}}{\gamma^{\alpha}} \qquad (6)$$

can be employed provided that the experimental $\Delta_{\beta}{}^{\alpha}E$ scale with an arbitrary reference point, dependent upon the experimental condition can be transformed into a potential scale with $\Delta_{\beta}{}^{\alpha}\varphi = 0$ as zero point. In the above equations, $\Delta_{\beta}{}^{\alpha}\varphi_p$ is the Galvani potential difference at the current maximum and $\Delta_{\beta}{}^{\alpha}\varphi_{1/2}$, the Galvani potential difference at the half-wave potential. The transformation of the zero point is possible if the TPAsTPB is used as the supporting electrolyte in the organic phase and Li_2SO_4 or LiF in the aqueous phase. The voltammogram will be limited at negative potentials by the transfer of $TPAs^-$ from the organic phase to water, and at the positive side by the transfer of TPB^- from the organic phase to water. Here, $\Delta_{\beta}{}^{\alpha}E_{1/2}$ for $TPAs^+$ and TPB^- can be estimated by means of eqn.(4) from the initial part of the current-potential curve related to the transfer of these two ions. Since TPAsTPB is the supporting electrolyte in the organic phase, an error is introduced due to the contribution of the ion migration to the observed current. Inspite of this, no error will arise in fixing the $\Delta_{\beta}{}^{\alpha}E'$ value where $\Delta_{\beta}{}^{\alpha}\varphi$ is zero by

$$\frac{\Delta^{\alpha}_{\beta}E_{1/2,\,TPAs^+} + \Delta^{\alpha}_{\beta}E_{1/2,\,TPB^-}}{2} = \Delta^{\alpha}_{\beta}E' \qquad (7)$$

Since the assumption is justified that the diffusion coefficients and the absolute mobilities of the two ions are equal.

In order to avoid errors in solvents with a low permittivity due to ion association,

eqn. (6) should be replaced by:

$$\Delta_{\beta}^{\alpha}\varphi_i^0=\Delta_{\beta}^{\alpha}\varphi_{1/2}-\frac{RT}{zF}\ln\frac{D_i^{(\alpha)1/2}}{D_i^{(\beta)1/2}+a_c^{\beta}K_aD_{ic}^{(\beta)1/2}}-\frac{RT}{zF}\ln\frac{\gamma_i^{\beta}+\gamma_{ic}^{\beta}}{\gamma_i^{\alpha}} \quad (8)$$

where $a_c{}^{\beta}$ is the activity of the anion or the cation of the supporting electrolyte in the organic phase; K_a, the ion association constant of the transferred ion with the respective ion of the supporting electrolyte, and the subscript 'ic' indicates the ion associate.

Similarly for a system consisting of a uni-univalent electrolyte, A_1B_1, (Fig. 1) consisting of the ions A_1^- and B_1^+,

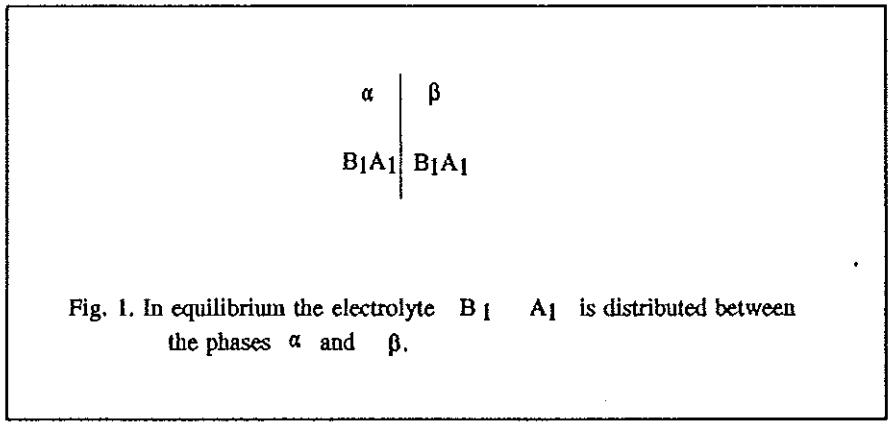


Fig. 1. In equilibrium the electrolyte B_1 A_1 is distributed between the phases α and β.

the expression for the inner (galvani) potential difference is given by:

$$\Delta_{\alpha}^{\beta}\varphi=\frac{\Delta_{\alpha}^{\beta}\varphi_{B_1^+}{}^0+\Delta_{\alpha}^{\beta}\varphi_{A_1^-}{}^0}{2}=\frac{\Delta_{\alpha}^{\beta}G_{tr,A_1^-}^0-\Delta_{\alpha}^{\beta}G_{tr,B_1^+}^0}{2} \quad (9)$$

In this case, the potential difference depends entirely on the ability of the ions A_1^- and B_1^+ to be transferred from the phase α to β expressed by standard Gibbs transfer energies. If $\Delta^{\beta}{}_{\alpha}G^0{}_{tr,A1^-} < \Delta_{\alpha}{}^{\beta}G^0{}_{tr,B1^+}$, i.e., the transfer of the anion to β is easier than that of the cation, the phase β is negatively charged with respect to α ($\Delta_{\alpha}{}^{\beta}\varphi < 0$) and an electrical double

layer is formed at the interface with a negative charge prevailing in its part situated in β. In the case shown in Fig.1, the potential difference effected by different abilities of ion for transfer from one phase to the other one is independent of their overall concentration.

In another case shown in Fig.2, the transfer of A_1^- from α to β and that of A_2^- from β to α is difficult compared with the transfer of the common cation B_3^+

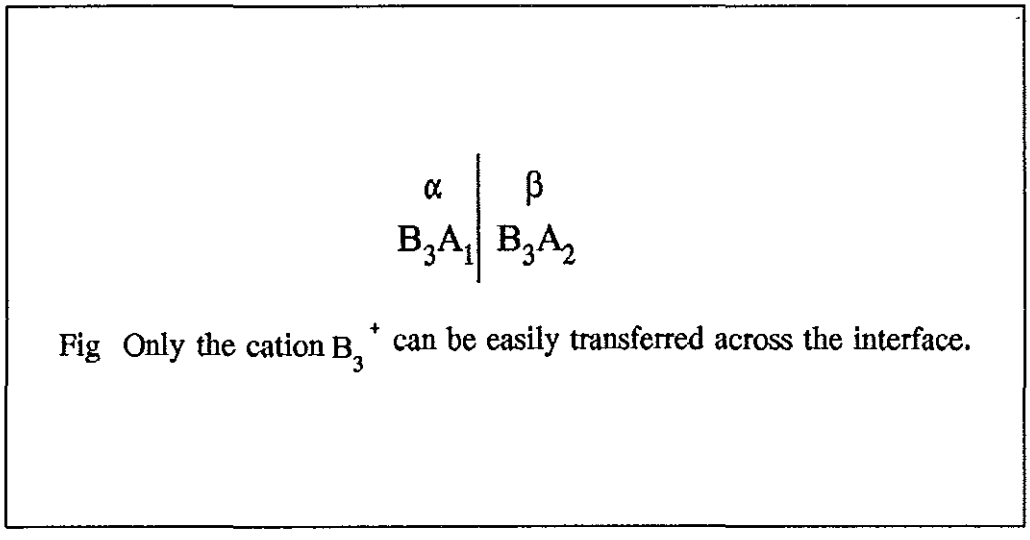


Fig Only the cation B_3^+ can be easily transferred across the interface.

from this it follows that,

$$\Delta_{\alpha}^{\beta}G_{tr,A_1^-}^{0} \gg 0$$

$$\Delta_{\alpha}^{\beta}G_{tr,A_2^-}^{0} \ll 0$$

$$\Delta_{\alpha}^{\beta}G_{tr,A_2^-}^{0} \ll \Delta_{\alpha}^{\beta}G_{tr,B_3^+}^{0} \ll \Delta_{\alpha}^{\beta}G_{tr,A_1^-}^{0}$$

$$/\Delta_{\alpha}^{\beta}G_{tr,B_3^+}^{0}/ \ll \Delta_{\alpha}^{\beta}G_{tr,A_1^-}^{0}\,;\; /\Delta_{\alpha}^{\beta}G_{tr,B_3^+}^{0}/ \ll -\Delta_{\alpha}^{\beta}G_{tr,A_2^-}^{0} \qquad (10)$$

Under these conditions the electrical potential difference between the two phases will be determined practically only by the activities of B_3^- in both phases according to eqn.(2) if the concentrations of the salts in both phases have comparable values. The Galvani potential difference across the interface in terms of the anions will be given by

$$\Delta_{\alpha}^{\beta}\varphi = \Delta_{\alpha}^{\beta}\varphi_{A_1^-}{}^{0} + \frac{RT}{F}\ln\frac{a_{A_1^-}(\beta)}{a_{A_1^-}(\alpha)} \qquad (11)$$

$$= \Delta_{\alpha}^{\beta}\varphi_{A_2^-}{}^{0} + \frac{RT}{F}\ln\frac{a_{A_2^-}(\beta)}{a_{A_2^-}(\alpha)}$$

However from the conditions stated in eqn.(10), $\Delta_{\alpha}{}^{\beta}\varphi^{0}{}_{A1}$ has a large negative value that are, in absolute values, considerably larger than $\Delta_{\alpha}{}^{\beta}\varphi$. Therefore $a_{A1}(\beta)$ and $aA_2(\alpha)$ must be very small. They will attain their equilibrium values by the transfer of a small quantity of the salt B_3A_1 to the phase β and of B_3A_2 to α (assuming both phases have comparable volumes) so that practically no change occurs in the concentrations of B_3A_1 in α and B_3A_2 in β. Under these conditions the phase α, for example, plays a completely analogous role towards phase β as a cationic electrode towards the electrolyte solution containing the corresponding cations. The structure of the electrical double layer at the interface depicted in Fig.2, depends on the sign of $\Delta_{\alpha}{}^{\beta}\varphi$.

The third case is a system consisting of a very hydrophillic salt A_1B_1, that is practically confined only to aqueous phase (phase α) and a very hydrophobic salt A_2B_2, confined in the non-aqueous phase (phase β) as shown in Fig.3.

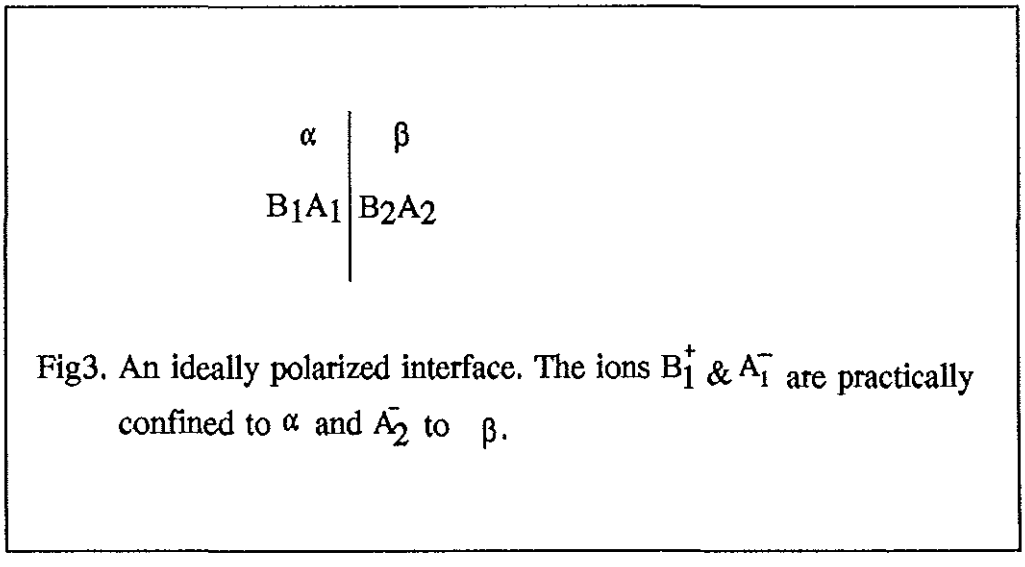


Fig3. An ideally polarized interface. The ions B_1^+ & A_1^- are practically confined to α and A_2^- to β.

If a charge is injected to one of the phases from an external source (and, simultaneously, a charge of opposite sign to the other phase), then there exists a potential range ("potential-window") where ITIES behaves like an ideally polarized electrode, i.e., the injected charge is used only for double-layer charging. The equilibria of the exchange reactions:

$$A_1^-(\alpha) + A_2^-(\beta) = A_1^-(\beta) + A_2^-(\alpha) \quad (12)$$

$$B_1^+(\alpha) + B_2^+(\beta) = B_1^+(\beta) + B_2^+(\alpha) \quad (13)$$

are strongly shifted to the left-hand side. The standard Gibbs transfer free energies for the ionic species in the system follows :

$$\Delta_\alpha^\beta G^0_{tr,B_1^+} + \Delta_\alpha^\beta G^0_{tr,A_1^-} \gg 0$$

and,

$$\Delta_\alpha^\beta G^0_{tr,B_2^+} + \Delta_\alpha^\beta G^0_{tr,A_2^-} \ll 0 \quad (14)$$

From these expressions, it can be seen that the equilibrium distribution of both electrolytes between the two phases is such that the electrolyte A_1B_1 is present almost completely in phase α while A_2B_2 is present almost completely in phase β. Under these conditions there exists a range of $\Delta_\alpha^\beta\varphi$ where the determination of the potential difference by the activities of the ions present in the system is impossible since $a_{A1^-}(\beta)$, $a_{A2^-}(\alpha)$, $a_{B1^+}(\beta)$ and $a_{B2^+}(\alpha)$ are very small. Then $\Delta_\alpha^\beta\varphi$ is determined mainly by the charge in the electrical double layer, which can be changed by charging the phases from an external source. This situation is completely similar to that of an ideally polarizable electrode as illustrated in Fig.4. In both cases, the phases are charged by a supply of charge to or a removal from the interface. In the case of the metal electrode/electrolyte solution system, the charge supplied to or removed from the metallic side are electrons while in the case of the ITIES only ion transport to and from the interface takes place.

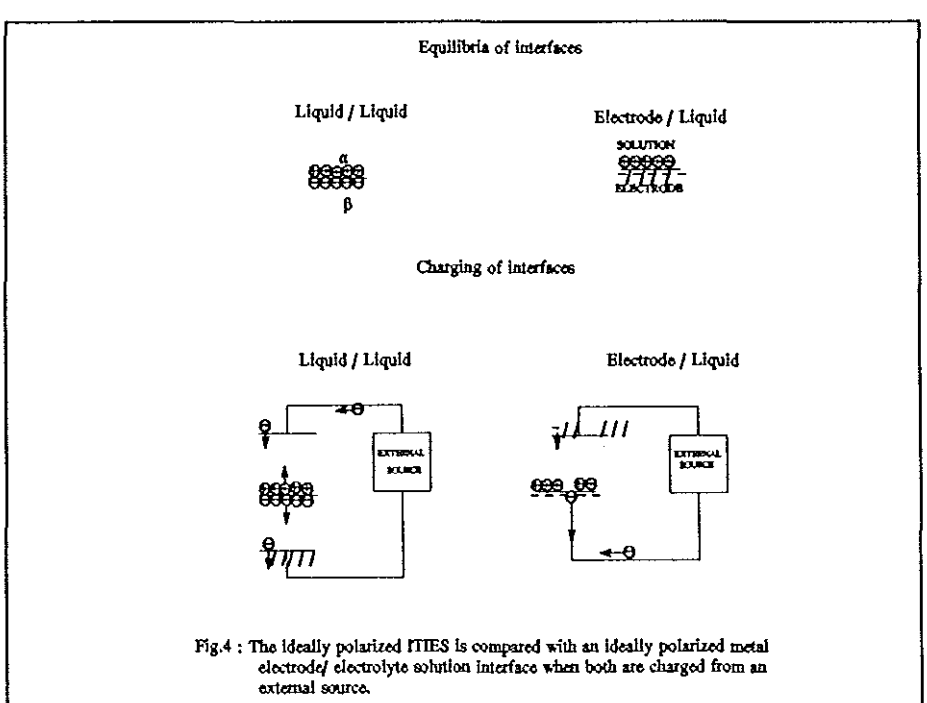


Fig.4 : The ideally polarized ITIES is compared with an ideally polarized metal electrode/ electrolyte solution interface when both are charged from an external source.

2.1. Current flow across the interface

Let us consider a case where semi-hydrophobic ion (B^+_3) is introduced in the previous system at a low concentration as shown in Fig.5.

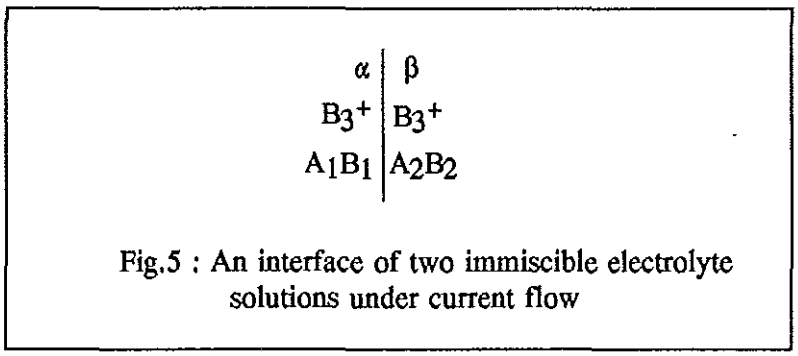


Fig.5 : An interface of two immiscible electrolyte solutions under current flow

The electrolytes A_1B_1 and A_2B_2 are in excess over the introduced ion and function as base electrolytes used in polarography and other methods of electrochemical kinetics. The cation B^+_3 has similar characteristics as B^+_3 in Fig.2. In the absence of current flow an equilibrium is established in the system, the equilibrium potential difference, $\Delta\varphi_{eq}$, being given by eqn.(1). If a potential difference, $\Delta V = \Delta\varphi + \eta$, in excess of the equilibrium potential is imposed 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 electrical double layer formed at the interface (Fig.6). The constant term, η, depends only on the composition of the solution of the base electrolytes in phases α and β, on liquid junction potentials and on the reference electrodes used; but is independent of the nature of the ion B^+_3. If the contribution of the charging current can be neglected, the remaining current is due to a simple charge transfer across the interface. This is analogous to an oxidation-reduction reaction at a metallic electrode/electrolyte solution interface as shown in Fig.6.

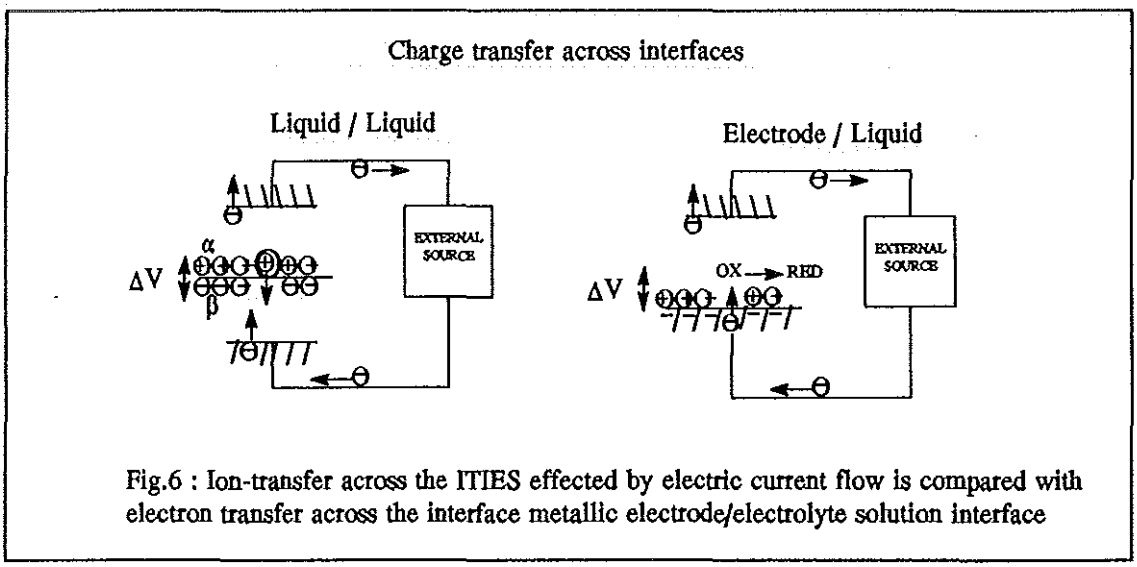


Fig.6 : Ion-transfer across the ITIES effected by electric current flow is compared with electron transfer across the interface metallic electrode/electrolyte solution interface

2.2. Facilitated ion transfer

The standard potential of some ions are so positive or negative that their transfer can't be studied because it occurs out of the range of the 'potential window' limited by the transfer of the ions of the supporting electrolytes. To shift the transfer of cations to more negative potential values, a suitable reagent, L, called an ionophore, is incorporated in the organic phase, which will then react with the ion under investigation.

For ion-transfer facilitated by such reagents, the transfer reaction is followed by a chemical reaction according to :

$$M^+(W) \leftrightarrows M^+(O) \qquad (15)$$

$$M^+(O) + L(O) \leftrightarrows ML^+(O) \qquad (16)$$

where $M^+(W)$, $M^+(O)$ refer to the metal ion in aqueous and organic phase; L(O) and $ML^+(O)$, to the ionophore and the metal-ionophore complex in the organic phase respectively.

The stability constant, K, of the complex formed is given by:

$$K = \frac{[ML^+]_0}{[M^+]_0 [L]_0} \qquad (17)$$

From this the concentration of the transferred ion can be obtained, i.e.,

$$[M]_0 = \frac{[ML^+]_0}{[L]_0 K} \qquad (18)$$

substitution of this expression into that for the galvani potential difference across the interface gives:

$$\Delta_\beta^\alpha \varphi = \Delta_\beta^\alpha \varphi_{M^+}^0 + \frac{RT}{zF} \ln \frac{1}{K} + \frac{RT}{zF} \ln \frac{[ML^+]_0}{[L]_0 [M^+]_w} \qquad (19)$$

The theoretical current-potential relationship for a reversible case, assuming that both reactions are reversible has been derived by Nicholson and Shain [64]. This treatment can be adopted when experimental conditions are chosen so that either of eqn.(20) or (21) holds:

$$[L]_o >> [M^+]_w \qquad (20)$$

$$[M^+]_w >> [L]_o \qquad (21)$$

The current-potential dependence of facilitated ion transfer is then given by eqn.(4) into which the diffusion coefficients and the concentrations of species with the lower concentrations in the respective phase has to be inserted.

The flux balance at the half-wave potential is either

$$D^{1/2}_{ML+}[ML^+]_{o,x=0} = D^{1/2}_{M+}\,[M^+]_{w,x=0} \qquad (22)$$

for the condition expressed in eqn. (20), or

$$D^{1/2}_{ML+}[ML^+]_{o,x=0} = D^{1/2}_{L}[L]_{o,x=0} \qquad (23)$$

when condition in eqn.(21) is used.

The respective Expressions for the half-wave potentials can be obtained by substituting eqn. (22) or eqn. (23) into eqn. (19),

$$\Delta_{\beta}^{\alpha}\varphi_{\frac{1}{2}} = \Delta_{\beta}^{\alpha}\varphi_{M^+}^{o} + \frac{RT}{zF}\ln\frac{1}{K} + \frac{RT}{zF}\ln\frac{1}{[L]_o} + \frac{RT}{2zF}\ln\frac{D_{M^+}(w)}{D_{ML^+}(o)} \qquad (24)$$

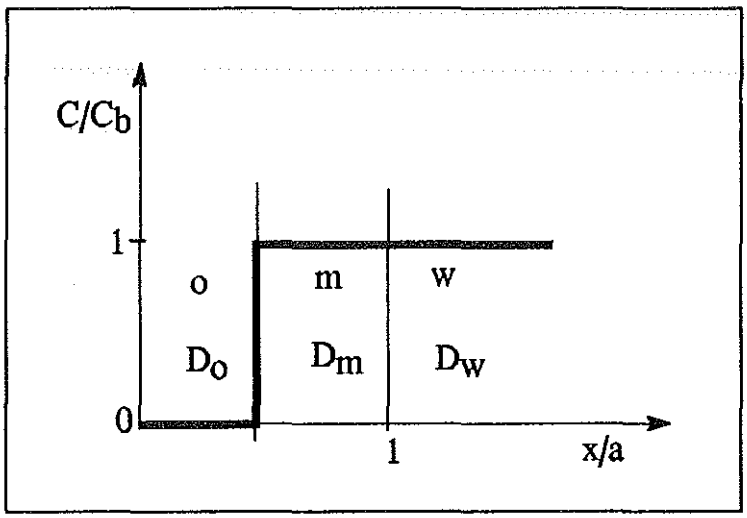
$$\Delta_{\beta}^{\alpha}\varphi_{1/2} = \Delta_{\beta}^{\alpha}\varphi_{M^+}^{o} + \frac{RT}{zF}\ln\frac{1}{K} + \frac{RT}{zF}\ln\frac{1}{[M^+(w)]} + \frac{RT}{2zF}\ln\frac{D_L(o)}{D_{ML^+}(o)} \qquad (25)$$

The above two equations have been verified experimentally and have been employed for the determination of the stability constants and for the analytical determination of concentration of either the ion transferred or of the ionophore [8,41,43].

2.3. The diffusion problem at the membrane-stabilized interface between two immiscible electrolyte solutions

Before using the membrane-stabilized ITIES as the base sensing element for the construction of the enzyme-based urea sensor, theoretical solutions for the diffusion problem at the interface were obtained inorder to get a clear picture about the diffusion of the substrate into the membrane of the transducer to be used. To check whether the theoretical predictions agree with that of the experiment, preliminary investigation was undertaken. The analysis starts with the theoretical descriptions of the concentration profiles, which is a function of both time and the distance from the electrode surface, and the response of the system to a potential-step excitation in chronoamperometric and chronocoulometric modes.

The figure below shows the supposed, normalized distance-concentration profile prior to the potential-step excitation ($t \leq 0$) where o, m, and w denote the space occupied by the organic phase, the membrane and the aqueous phase respectively. $D_{(o,m,w)}$ are the diffusion coefficients in the respective phases. The membrane inserted has a thickness of a. The potential difference, $\Delta_m{}^o\varphi$, is acting at $x = 0$. Semi-infinite diffusion is assumed in the aqueous phase and in the organic phase.



The calculation of the diffusion-limited current, $i_d(t)$, and the concentration profiles $C_{(o,m,w)}(x,t)$ in the three regions requires the solution of the linear diffusion equation:

$$\frac{\partial C_{(o,m,w)}(x,t)}{\partial t} = D_{(o,m,w)} \frac{\partial^2 C_{(o,m,w)}(x,t)}{\partial x^2} \qquad (31)$$

in the respective region. The initial and boundary conditions are as follows:

t = 0

$$-\infty < x < 0 \; ; \; C = C_0 = 0 \qquad (32)$$

$$0 < x < +\infty \; ; \; C = C_m = C_w = C_b \qquad (33)$$

t > 0

$$C_o = 0 \quad ; \quad x \rightarrow -\infty \qquad (34)$$

$$C_w = C_b \quad ; \quad x \rightarrow \infty \qquad (35)$$

$$Cw = Cm \quad ; \quad x = a \qquad (36)$$

$$x=0 \; ; \; \Theta = \left(\frac{C_o}{C_m}\right)_{x=0} = \exp\frac{[z_i F(\Delta_m^o\varphi - \Delta_m^o\varphi^o)}{RT}] \qquad (37)$$

The flux balance is given by

x = a ;

$$D_w\left(\frac{\partial C_w}{\partial x}\right)_{x=a} = D_m\left(\frac{\partial C_m}{\partial x}\right)_{x=a} \qquad (38)$$

x = 0 ;

$$D_o\left(\frac{\partial C_m}{\partial x}\right)_{x=0} = D_m\left(\frac{\partial C_m}{\partial x}\right)_{x=0} \qquad (39)$$

Laplace transformation yields for the concentration profile in the three regions,

$$-\infty < x < 0 \; ; \; \overline{C_o}(x, s) = A\exp[q_o x] \qquad (40)$$

$$0 < x < a \; ; \; \overline{C_m}(x, s) = \frac{C_b}{s} + B\exp[q_m x] + D\exp[-q_m x] \qquad (41)$$

and

$$a < x < \infty \; ; \; \overline{C_w}(x, s) = \frac{C_b}{s} = E\exp[-q_w x] \qquad (42)$$

$$where, \; q_{(o,m,w)} = \sqrt{\frac{s}{D_{(o,m,w)}}} \qquad (33)$$

The constants A, B, D and E have to be evaluated according to the boundary conditions and finally we obtain for the concentration profile within the membrane in the Laplace domain

$$\overline{C_m}(x,s) = \frac{C_b}{s} - C_b \exp\frac{[-q_m x]}{s(\lambda+1)} + \frac{C_b \sum_{n=0}^{n=\infty} (-1)^n \Gamma^n \left(\frac{1-\sigma'}{1+\sigma'}\right)^{n+1} \exp[-q_m\{(n+1)2a - x\}]}{s(\lambda+1)} + \frac{C_b \sum_{n=0}^{n=\infty} (-1)^n \Gamma^{n+1} \left(\frac{1-\sigma'}{1+\sigma'}\right)^{n+1} \exp[-q_m\{(n+1)2a + x\}]}{s(\lambda+1)} \qquad (43)$$

where

$$\Gamma=\frac{\sigma-\Theta}{\sigma+\Theta};with\sigma=\sqrt{(\frac{D_m}{D_o})};\lambda=\frac{\sigma}{\Theta};\sigma^*=\sqrt{(\frac{D_m}{D_w})} \qquad (35)$$

The concentration profile in the time domain can be obtained by table transformation and is

$$C_m(x,t)=C_b-\frac{C_b erfc[\frac{x}{(4D_m t)^{1/2}}]}{\lambda+1}+\frac{C_b\sum_{n=0}^{n=\infty}(-1)^n\Gamma^n(\frac{1-\sigma^*}{1+\sigma^*})^{n+1}erfc[\frac{(n+1)2a-x}{(4D_m t)^{1/2}}]}{\lambda+1}+\frac{C_b\sum_{n=0}^{n=\infty}(-1)^n\Gamma^{n+1}(\frac{1-\sigma^*}{1+\sigma^*})^{n+1}erfc[\frac{(n+1)2a+x}{(4D_m t)^{1/2}}]}{\lambda+1}$$

with

$$j(0,t)=-D_m(\frac{\partial C_m}{\partial x})_{x=0}; \qquad (37)$$

we obtain,

$$j(0,t)=-\frac{C_b D_m^{1/2}}{(\pi t)^{1/2}(\lambda+1)}-\frac{C_b D_m^{1/2}\sum_{n=0}^{n=\infty}(-1)^n\Gamma^n(\frac{1-\sigma^*}{1+\sigma^*})^{n+1}\exp[-\frac{(n+1)^2 2a^2}{D_m t}]}{(\pi t)^{1/2}(\lambda+1)}+\frac{C_b D_m^{1/2}\sum_{n=0}^{n=\infty}(-1)^n\Gamma^{n+1}(\frac{1-\sigma^*}{1+\sigma^*})^{n+1}\exp[-\frac{(n+1)^2 2a^2}{D_m t}]}{(\pi t)^{1/2}(\lambda+1)}$$

The time dependence of the limiting current [C_m (0,t)→ 0]; i_{lim}(t)= - j_{lim}(0,t)z_iFA is given by

$$i_{\lim}(t)=\frac{z_i FAD_m^{1/2}C_b}{(\pi t)^{1/2}}\{1+2\sum_{n=0}^{n=\infty}(\frac{1-\sigma^*}{1+\sigma^*})^{(n+1)}[\exp[-\frac{(n+1)^2 2a^2}{D_m t}]\} \qquad (46)$$

Taking the integral from t=0 to t gives the chronocoulometric response of the membrane-stabilized interface , i.e. ,

$$Q_{\text{lim}}(t)=\frac{2z_i FAD_m^{1/2}C_\delta t^{1/2}}{\pi^{1/2}}\{1+2\sum_{n=0}^{n=\infty}\left(\frac{1-\sigma^*}{1+\sigma^*}\right)^{(n+1)}[\exp[-\frac{(n+1)^2 2a^2}{D_m t}]-\frac{(2\pi)^{1/2}a}{(Dt)^{1/2}}erfc(\frac{(n+1)2^{1/2}a}{(D_m t)^{1/2}})]\} \qquad (47)$$

The experimentally obtained response characteristics of this interface is given in the first part of the results and discussion section.

2.4. Electrochemical sensors based on biological principles

Application of principles taken from biology to electroanalytical chemistry dates from the beginning of this century. In the search for a suitable biological membrane model, Cremer [65] used thin glass membranes and found that the membrane potential depends on hydrogen ion concentration in the bathing solution. This finding led Haber and Klemensiewicz [65] to the construction of the glass electrode.

A biosensor is a device incorporating a biological sensing element either intimately connected to or integrated within a transducer. Usually the aim is to exploit the molecular recognition capabilities of proteins and biological substances to provide the selectivity normally lacking in purely chemical devices, so as to produce a signal reflecting the concentration of a substance of biological interest. The signal-transducing technology is then more-or-less conventional.

The processes from which electrochemical biosensors originated include:

i- membrane ion transport mediated by ionophores,

ii- enzymatic reactions resulting in formation of electrode or membrane active products or in consumption of similar substances,

iii- immunochemical reactions by effect of which concentrations of electrode active substances are changed.

Ionophore mediated ion transport

Since 1951 when Berger et.al. [66] described solubilization of sodium and barium ions in low permittivity solvents by a metabolite later identified as the antibiotic nigericin, a number of analogous natural and synthetic substances were described. They are either

macrocyclic or acyclic but form cyclic structures when entering into complexes and possess the following common properties:

i- they form relatively stable complexes with alkali metal (in some cases with alkaline earth metal) ions while the stability of the complexes follows ion-selective series,

ii- they enable transfer of hydrophillic ions across the lipid membranes of the cells and cell membranes, across artificial lipid bilayer membranes (BLM) and across relatively thick membranes of organic solvents, .

iii- they ion-selectively uncouple oxidative phosphorylation in mitochondria,

iv- their action results in formation of a defined membrane potential at BLM and thick membranes forming a basis for their use in ion-selective electrodes.

These substances were called ionophores by Pressman [65] while the term 'ion-carrier' is also used. Their basic structure can be exemplified by the typical member of this group, valinomycin. The polar carbonyl groups of the carboxyls stretch into the internal cavity of the molecule while the external 'envelope' of the structure is made hydrophobic by the isopropyl groups. This enables complex formation with alkali metal ions, particularly with potassium ion , which fits well into the internal cavity while the hydrophobic outer surface causes dissolution of the complex in solvents of low polarity as well as in the interior of BLM and biological membranes.

Enzymatic reactions

The vast majority of biochemical pathways are catalyzed by specific proteins , the enzymes. They act according to the scheme:

$$\text{substrate} + \text{cofactor} \overset{\text{enzyme}}{\rightarrow} \text{products} \qquad (26)$$

The utilization of enzyme catalyzed reactions for electrochemical sensors requires that either the cofactor or the products be electrode- or membrane-active . An example of the first group of sensors is the oxidation of β-glucose by oxygen which is catalysed by the enzyme β-glucose oxidase:

$$\beta\text{-glucose} + O_2 \overset{\beta\text{-glucoseoxidase}}{\rightarrow} \text{gluconic acid} + H_2O_2 \qquad (27)$$

Both oxygen and hydrogen peroxide are electroactive.The second group is exemplified by the hydrolysis of urea catalysed by urease:

$$CO(NH_2)_2 \xrightarrow{\text{urease}} CO_2 + NH_3 (\text{or } NH_4^+) \quad (28)$$

Ammonia or ammonium ions influence membrane potentials of various ion-selective electrodes.

2.4.1. Principles of measurement

Generation of the electrical signal in the sensor will be dealt with, i.e., the manner by which the extent of biochemical transformation of the analyte is converted to an electrical quantity (in the biochemical way of speaking, the purely electrochemical part of the sensor acts as a transducer of energy) that is related to its concentration.

With sensors based on ionophore-facilitated ion transfer two alternatives will be considered. In the most frequent version, i.e., in potentiometry with an ionophore-based ion-selective electrode ,the electrical signal is the e.m.f. of the cell consisting of the inner electrode of the ion-selective electrode, of the membrane and of the reference electrodes:

reference electrode 1 /analyte/membrane/inner electrolyte/reference electrode 2 (29)

The e.m.f., E, is given by the equation (when the liquid junction potentials between the reference electrodes and the analyte and the inner electrolyte are neglected) :

$$\mathbf{E = E_2 + \Delta\phi_m - E_1} \quad (30)$$

where E_2 and E_1 are the potentials of the reference electrodes and $\Delta\phi_m$ is the membrane potential.

The second alternative is the voltammetric version of potentiometry with ion-selective electrode, electrolysis at the interface of two immiscible electrolyte solutions (ITIES) [5,6]. Here 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 solvent solution of a hydrophobic electrolyte. The resulting E-I-T characteristics are governed by the same principles as in the system of an electrode polarized in an electrolyte solution.

With enzyme electrodes, the usual method is to determine the cofactor or the product amperometrically. Very often the Clark oxygen electrode is used to monitor oxygen consumption in oxidase catalized reactions. A particular version of the amperometric method is based on a chemically modified electrode with the cofactor bound to the

electrode. The second mode of measurement is potentiometry with ion-selective electrode by which the concentrations of the product is measured. Finally, the differential capacity changes due to reversible adsorption of the substrate of an enzymatic reaction is measured [65].

2.4.2. Structure and function of electrochemical biosensors

The membranes of ionophore-based ion-selective electrodes are prepared from poly(vinylchloride) matrix containing an appropriate ion-exchanging solution as plasticizer [67]. This solution contains the salt of the ionophore complex of the determinand cation with strongly hydrophobic anion like tetraphenylborate or p-chlorotetraphenylborate. As solvents, o-nitrophenyloctyl ether or phthalic acid esters are used. The ion-selective electrodes of this type include the potassium electrode based on valinomycin, and the sodium electrode based on monensin [65].

Voltammetry at the ITIES is particularly suitable for 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. With the mode of triangular-sweep voltammetry, the current is controlled either by diffusion of the ionophore or of the complex formed to ITIES. This method was applied to the determination of the coccidiostat, monensin, in cultures of *streptomyces cinnamonensis* [38]. Similarly, the same technique can be applied 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 ITIES. This method was applied to the analytical determination of K^+ and Na^+ in artificial standard and blood serum samples [44].

Typical enzyme electrodes consist of an immobilized enzyme layer attached to the internal sensor. The immobilization of the enzyme is carried out either by the physical adsorption or, preferably, by chemical binding to a polymer layer. Enzyme electrodes have the advantage of being simple, reliable, essentially reagentless, and sensitive besides being inherently selective. The most important electrode with amperometric indication is the glucose sensor which is based on reaction expressed in eq. (27).

The typical potentiometric enzyme sensor, the urea electrode, is based on reaction given by eq.(28). The product, ammonia or ammonium ion, is then determined either by

the ammonia gas probe or by the ammonium ion-selective electrode based on the ionophore nonactin [68].

In some cases coupled enzyme reactions are used for preconditioning of the analyte by an enzymatic reaction for elimination of the interferents and for recycling the substrate to amplify product formation [69].

On the whole, the enzyme electrodes with an amperometric indication have definite advantage against the potentiometric sensor, in the first place because of consumption of product of the enzymatic reaction in the electrode reaction which decreases the response time of the sensor.

The enzymatic processes resulting in concentration change of electroactive cofactors or products can also be substantiated by direct application of biological systems, i.e., by tissues, microorganisms and cell organelles. In the tissue electrodes a thin layer of a tissue is fixed to the internal indicating system which senses the products of enzymatic reactions of the substrate taking place in the tissue layer.

Table 1 shows some commercially produced enzyme electrodes based on amperometric indication.

Table1. Commercially Produced Enzyme Electrodes (Amperometric)[65]

ANALYTE	ENZYME
Glucose	β-glucose oxidase
Sucrose	Invertase+β-glucose oxidase
Lactose , galactose	Galactose oxidase
Lactose, galactose	Uricase
D-Lactate	D-lactate oxidase
L-Lactate	L-lactate oxidase cytochrome b_2
Choline	Choline oxidase
L-Lysine	L-lysine oxidase
Alcohol	Alcohol oxidase
α-Amylase	β-glucose oxidase + maltase

An important step forward would be achieved if some of these biosensors could be miniaturized as it is usual with ion-selective microelectrodes. An ion-selective field-effect transistor (ISFET) could serve as a suitable internal probe [70]. However, a major construction breakthrough will be needed before practical biochips will appear in analytical and clinical laboratories.

The practical applications of electrochemical biosensors of the type of enzyme electrodes is still rather small. The most elaborate and mainly used enzyme electrode, the glucose sensor, can only be found in a fraction of clinical laboratories where spectrophotometric methods are mostly used. The situation can be compared with the penetration of the glass electrode into analytical laboratories where it took about 30 years from Cremer's and Haber and Klemensiewicz's discovery to the marketing of the first pH-meter by the Beckman company. A more recent example is the history of sodium and potassium ion-selective glass electrodes. The sodium glass electrode was known since the 1930's but as there was no suitable electrochemical sensor for potassium the analysis of these ions was made exclusively by means of flame photometry in clinical laboratories. However, when the valinomycin-based potassium electrode was discovered by simon in 1969 [65] the automatic potentiometric method for Na^+/K^+ determination found a widespread use so that after 15 years, more than half of the assays are done by ion-selective electrodes than by flame photometry [71].

Thus, the big effort devoted to the study of biosensors will doubtless not be wasted. The increase of the stability of these sensors as well as an important exemplary success like a glucose sensor will open their way to practical applications.

2.4.3. Fundamental aspects of biosensor operation

Though a wide variety and ever increasing number of sensing methods have been employed in biosensors, some fundamental principles are generally common to all. First, the biological components, either enzymes, antibodies or receptors, are employed to provide specifity. This specifity derives from biological recognition based on the binding of the target analyte by the biological component employed and consequently a reaction sequence involving such a binding step will generally be encountered. Second, sensing will generally take place at a surface and transport to this surface prior to reaction at it will frequently be an important factor. Third, pseudo-steady-state conditions, in which the sensor signal is

dependent on a reaction proceeding at a constant rate, will often be encountered, for example, in enzyme-based and amperometric systems. The kinetics of transport coupled with surface reaction(s) will therefore be important in such cases. Even where a true equilibrium measurement is to be made, the rate of approach to equilibrium will be important in determining the response-time. In this case the kinetics of the process will again require consideration.

Finally, as most enzymatic reactions used for enzyme electrodes are irreversible, these biosensors deplete the substrate at their surface. Thus supply of substrate to the sensor surface will be affected by hydrodynamic conditions in its vicinity. It is therefore important to control solution flow by stirring (probe sensor) or by circulation of the sample solution (flow-through sensor). Alternatively, The sensor may be rotated in the test solution.

2.4.4. Urea biosensors -a survey

Guilbault and Montalvo [72,73] prepared several electrodes for urea by physically entrapping urease in a polyacrylamide gel held over the surface of a monovalent cation electrode by cellophane film. The urea diffuses into the urease layer, where it is converted to ammonium ions, which are sensed by the cation electrode. The electrode could be used up to 3 weeks with no loss of activity and responded to urea in the concentration range 5×10^{-5} to 1.6×10^{-1} M with a response time of about 35 sec.

Because sodium and potassium ions interfered in the measurement, Guilbault and Hrabankova [74] used an uncoated NH_4^+ ion electrode as reference electrode to the urease-coated NH_4^+ electrode, and added ion-exchange resin in attempts to develop an urea electrode useful for assay of blood and urine. Good precision and accuracy were obtained.

In attempts to improve the selectivity of the urea determination, Guilbault and Nagy [68] used a silicon rubber-based nonactin ammonium ion-selective electrode as the sensor for the NH_4^+ ions liberated in the urease reaction. The selectivity coefficients of this electrode were 6.5 for NH_4^+/K^+, 7.50×10^2 for NH_4^+/Na^+, and much higher for other cations. The reaction layer of the electrode was made of urease enzyme chemically immobilized on polyacrylamide gel. A still further improvement was described by Guilbault, Nagy, and Kuan [75] using a three electrode system, which allowed dilution to a constant interference level. Analysis of blood serum showed good agreement with spectrophotometric methods, and the enzyme electrode was stable for 4 months at 4^0 C.

Still further improvement in the selectivity of this type of electrode was obtained by Anafalt, Granelli, and Jagner [76], who crosslinked urease directly onto the surface of

an Orion ammonia gas electrode probe by means of glutaraldehyde. Sufficient ammonia was produced in the enzyme reaction layer even at pH's as low as 7 - 8 to allow direct assay of urea in the presence of large amounts of Na^+ and K^+. A response time of 2 - 4 min was observed.

Guilbault and Tarp [77], described a still better, total interference-free, direct-reading electrode for urea, using the air-gap electrode of Ruzicka and Hansen [78]. A thin layer of urease chemically bound to polyacrylic acid was used, at a solution pH of 8.5, where good enzyme activity was still obtained, yet where sufficient NH_3 is liberated to yield a sensitive measurement with the air-gap NH_3 electrode. The urea diffuses into the gel; the NH_3 produced diffuses out of solution to the surface of the air-gap electrode, where it is measured. A linear range of 3×10^{-2} to 5×10^{-5}M was obtained with a slope of 0.75 pH unit per decade. The electrode could be used continuously for blood serum analysis for up to 1 month (at least 500 samples) with a precision and accuracy of less than 2%. The response time is 2-4 min at pH 8.5, and the electrode is washed under a water tap for 5-10 sec after each measurement. Absolutely no interference from any levels of substances commonly present in blood was obtained (Na^+, K^+, ascorbic acid, etc).

A urea electrode using physically entrapped urease and a glass electrode to measure pH change in solution were described by Mosbach et.al. [79]. The response time of the electrode to urea was about 7 - 10 min and had a linear range from 5×10^{-5} to 1×10^{-2} with a change of about 0.8 pH unit per decade. The electrode could be kept at room temperature for about 2 - 3 weeks . The ionic strength and pH were controlled using a weak (10^{-3}M) Tris buffer and 0.1 M NaCl.

Still another possibility for urea electrode is the use of a CO_2 sensor to measure the second product of the urea-urease reaction. Guilbault and Shu [80] evaluated the use of the CO_2 sensor and found that a urea electrode, prepared by coupling a layer of urease covered with a dialysis net with a CO_2 electrode, had a linear range of 1×10^{-4} to 1×10^{-1} M, a response time of about 1 - 3 min and a slight response to only acetic acid. Na^+ and K^+ ions had no interference.

Canh Tran-Minh and Didier Vallin [81] described the method for the construction of urease-bound thermistor to be used for the determination of urea. The sensitive part of a thermistor was coated with an artificial enzyme membrane obtained by the cross-linking of urease with an inert protein using glutaraldehyde as a bifunctional reagent. The pH and temperature dependence of the hydrolysis of urea by urease given by the sensor were

compared with the results previously obtained by other methods. O'Keeffe and his coworker [82] compared urea levels in compound feed determined by three methods: the phenol-hypochlorite measurement of ammonium ions released by urease; the p-dimethyl aminobenzaldehyde (DMAB) color reaction with urea; and the conway microdiffusion measurement of ammonia released by urease action. The phenol-hypochlorite method had comparable accuracy and precision to the official DMAB method and was an improvement on the microdiffusion method. The enzyme-based method was found to be simple, rapid, inexpensive and suited for automation. Yasuda et. al. [83] described a urea electrode using an ammonium ion-selective electrode with an immobilized urease membrane. The measurement of blood urea was carried out accurately without any pretreatment of the samples. A conversion equation related to haematocrit was derived, which made it possible to determine plasma urea concentration from whole blood urea concentration. Rapid determinations of plasma urea concentration were also made possible.

Christian et. al. [84] applied reflectance spectrophotometry to the flow-injection measurement of pH in order to perform an assay for urea in water and serum samples via the enzymatic (urease) degradation of urea to ammonia and hydrogen carbonate. Detection was based on commercial non-bleeding acid-base indicator papers situated in the flow stream of an integrated micro-conduit at the tip of a fibre-optic bundle. The sample and reagent were injected via a split-loop injection technique. The serum was analysed by a stopped-flow kinetic measurement to avoid errors in measurements due to the variability in the background sample pH. Another flow-injection system was described for the assay of urea in undiluted whole blood by Petersson [85]. Urea was quantified by means of an ammonium ion-selective electrode covered with a membrane covered with covalently immobilized urease. Interference from potassium ion was reduced by adjusting the potassium ion concentration in the carrier stream and in the aqueous calibration solution to 4.0 mM; it can be eliminated by measuring the potassium ion concentration in the sample separately and applying a mathmatical correction for the potassium ion contribution to the signal. The linear measuring range was 1-40 mM urea, with an injection frequency of $40h^{-1}$ and a standard deviation of 1 % for whole blood samples.

A microprocessor for urea was described by Paney and his coworker [86] which was based on immobilized urease on the tip of a 10 μm diameter ammonia gas electrode made from a polypyrrole film coated onto a platinum wire. The sensor responded rapidly reaching a steady-state within 20 - 40s when the concentration of urea in solution is changed from

1×10^{-4} to 5×10^{-2} M. The enzymatic response was current limiting. Responses in a wide range of substrate concentrations were achieved (1×10^{-3} to 5×10^{-2} M). High storage and operational stability (more than 32 days) have been recorded for the urea microprocessor.

The objective of the present research is to construct a urea biosensor which is based on the facilitated transfer of the urease-generated ammonium ions employing the neutral carrier dibenzo-18-crown-6, across the membrane-stabilized interface between two immiscible electrolyte solutions.

3. EXPERIMENTAL

3.1. Reagents and chemicals

Organic phase

Nitrobenzene was used as the organic phase after purification. It was purified by washing three times with 10% (v/v) H_2SO_4, then three times with 10% (w/v) KOH and finally with distilled water until it became neutral. 0.01 M solutions of tetraoctylammoniumtetraphenylborate (TOctATPB) and crystalviolettetraphenylborate (CVTPB) in nitrobenzene were used independently as the supporting electrolytes in the organic phase. CVTPB wasprepared by mixing equimolar amounts of crystalviolet chloride (BDH) and sodium tetraphenylborate (MERCK), both dissolved in methanol. The product was filtered and the violet precipitate was washed with redistilled water until the test for chloride was negative. TOctATPB was prepared from equimolar amounts of tetraoctylammonium chloride (BDH) and sodium tetraphenyl borate following the same procedure. The neutral carrier dibenzo-18-crown-6 (DB[18]c-6) (MERCK), 0.01M in nitrobenzene, was used as the ionophore in the organic phase.

Aqueous phase

0.01M $MgSO_4$ was used as the supporting electrolyte in this phase. The buffer used in this work is 0.01M (pH 8.6, I=0.03M) solution of the Lithium salt of ethylenediamine tetraacetic acid (Li_2EDTA) which was prepared from EDTA (BDH) and LiOH (BDH) solutions mixed in stoichiometric ratios. This solution was also used as the supporting electrolyte in this phase. It was proved by separate experiment that the use of Li_2EDTA solution as the base electrolyte in the aqueous phase permits voltammetric measurements.

0.01M $LiClO_4$ was also used in the preliminary investigations of the membrane-stabilized ITIES.

Enzyme-gelatine solution

Two tablets of Urease (EC 3.5.1.5, Jack-bean meal, BDH, 400 E.U. per tablet) were dissolved in 5% gelatine solution(in the buffer) to make 5ml solution.

Substrate standard solution

A stock 0.01M urea solution dissolved in the buffer was prepared. For calibration, aliquots of this solution were added into the beaker containing the buffer to generate a series of concentration steps.

All chemicals, except nitrobenzene, were of analytical grade and were used without further purification. Stock solutions were prepared at a concentration of 0.1M for all studied electrolytes, and were diluted with double-distilled water as required.

Following the usual sign convention, the transfer of cations from the organic phase to water is indicated by a negative current and that for the transfer of anions from the organic phase to water is shown by a positive current.

All measurements were made at a laboratory temperature of 22 ^{0}C.

3.2. Cell arrangement

Construction of the electrochemical cell

Hydrophillic dialysis membranes (PT 600 and PT 325) were used to stabilize the interface. The geometrical area of the interface was 0.25 cm^2. The membranes were soaked in the buffer/supporting electrolyte for an hour before use. The thickness of the swollen membranes were 41μ and 70μ respectively. A disk of the membrane, 1.5 cm in diameter, was fit onto the tapered end of a teflon tube by the help of a plastic 'O'-ring. The inner compartment of the tube was filled with the solution of the organic phase (0.01M solutions of TOctATPB or CVTPB and DB[18]C-6 in nitrobenzene). The resulting cell was then dipped into the aqueous solution for equilibration or pending the enzyme immobilization.

Enzyme immobilization

The enzyme was 'immobilized' by physical entrapment, in a gel, between two dialysis membranes. 0.1ml of the enzyme-gel solution was placed on the membrane surface of the sensor and allowed to form a gel. It was then covered with an outer protective membrane and held in place with an 'O'-ring. Before measurements are made, the biosensor was immersed in the buffer solution for equilibration.

Fig.7 shows the sensor used for the investigation. Two auxiliary electrodes, A1 and A2 (platinum wires) served as the source and drain of current while the potential difference across the interface was controlled by means of two reference electrodes, R1 and R2 (Ag/AgCl and silver wire); in the aqueous and organic phases respectively.

3.3. Electronic set-up

The measurements were carried out with a four electrode potentiostat with automatic IR compensation by means of positive feed-back. The IR compensation was set to the nearest point before the potentiostat starts oscillating. The potentiostat was set up from operational amplifier modules (McKee-Pedersen Instruments) and a differential amplifier (AM 502, Tektronix). The voltammograms were recorded with an x-y / x-t recorder (PH 8041, Phillips). The triangular voltage pulse was generated by a ramp generator (MP 1502).

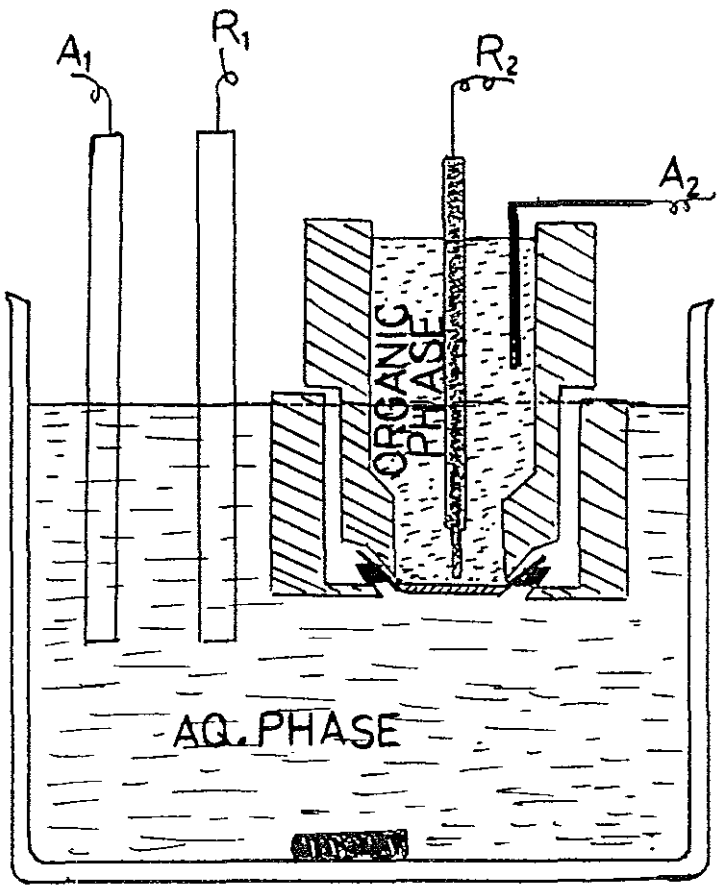


Fig.7. The electrochemical cell used for the investigation. R_1 and R_2 are the reference electrodes while A_1 and A_2 are the counter electrodes in the aqueous and organic phases respectively.

4. RESULTS AND DISCUSSION

The following figures show the experimentally obtained response of the membrane-stabilized interface to a potential step excitation in the chronoamperometric (Fig. 8. a, b) and chronocoulometric (Fig. 9) modes. As can be seen, the experimentally observed response characteristics are in accordance with those predicted by the theoretical expressions derived.

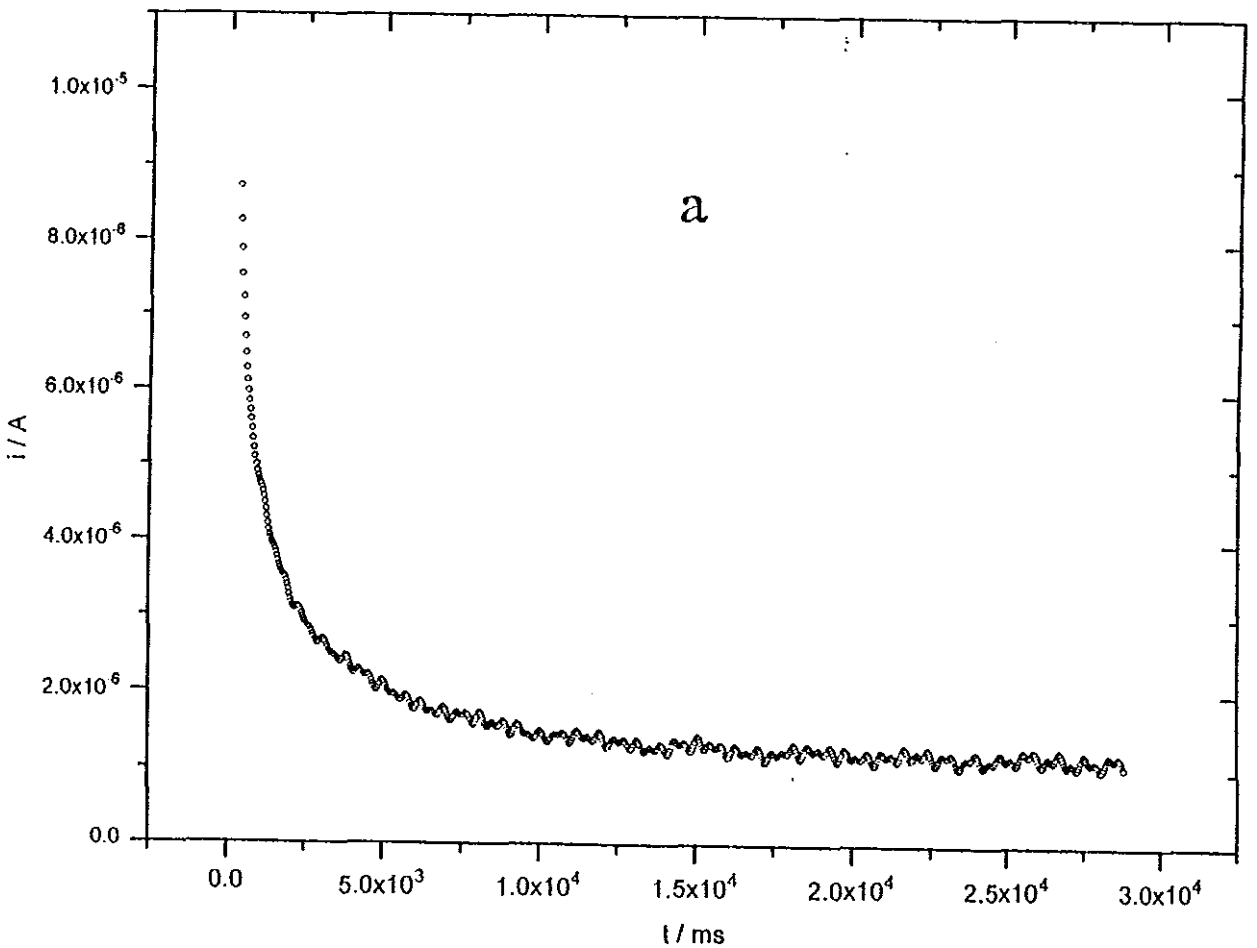


Fig.8a. Experimental chronoamperometric response of the membrane-stabilized ITIES to a potential step excitation(current as a function of time).

Membrane:PT 600

Aqueous phase: $5x10^{-3}$ M $MgSO_4$ + $5x10^{-4}$ M $LiClO_4$

Organic phase: $1x10^{-2}$ M CVTPB in nitrobenzene

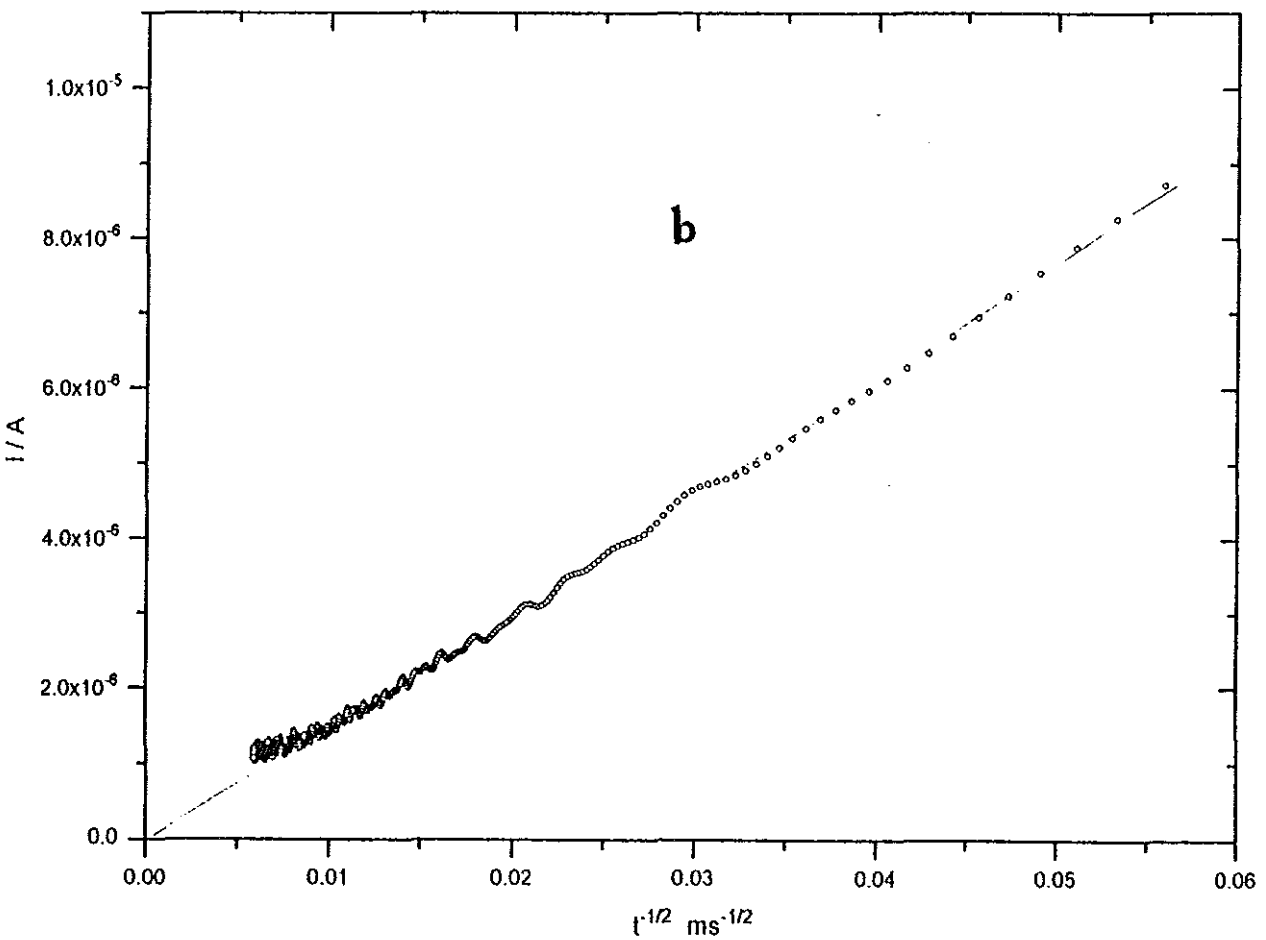


Fig.8b. Experimental chronoamperometric response of the membrane-stabilized ITIES to a potential step excitation (current as a function of inverse of square root of time).

Membrane: PT 600

Aqueous phase : $5x10^{-3}$ M MgSO4 + $5x10^{-4}$ M $LiClO_4$

Organic phase : $1x10^{-2}$ M CVTPB in nitrobenzene

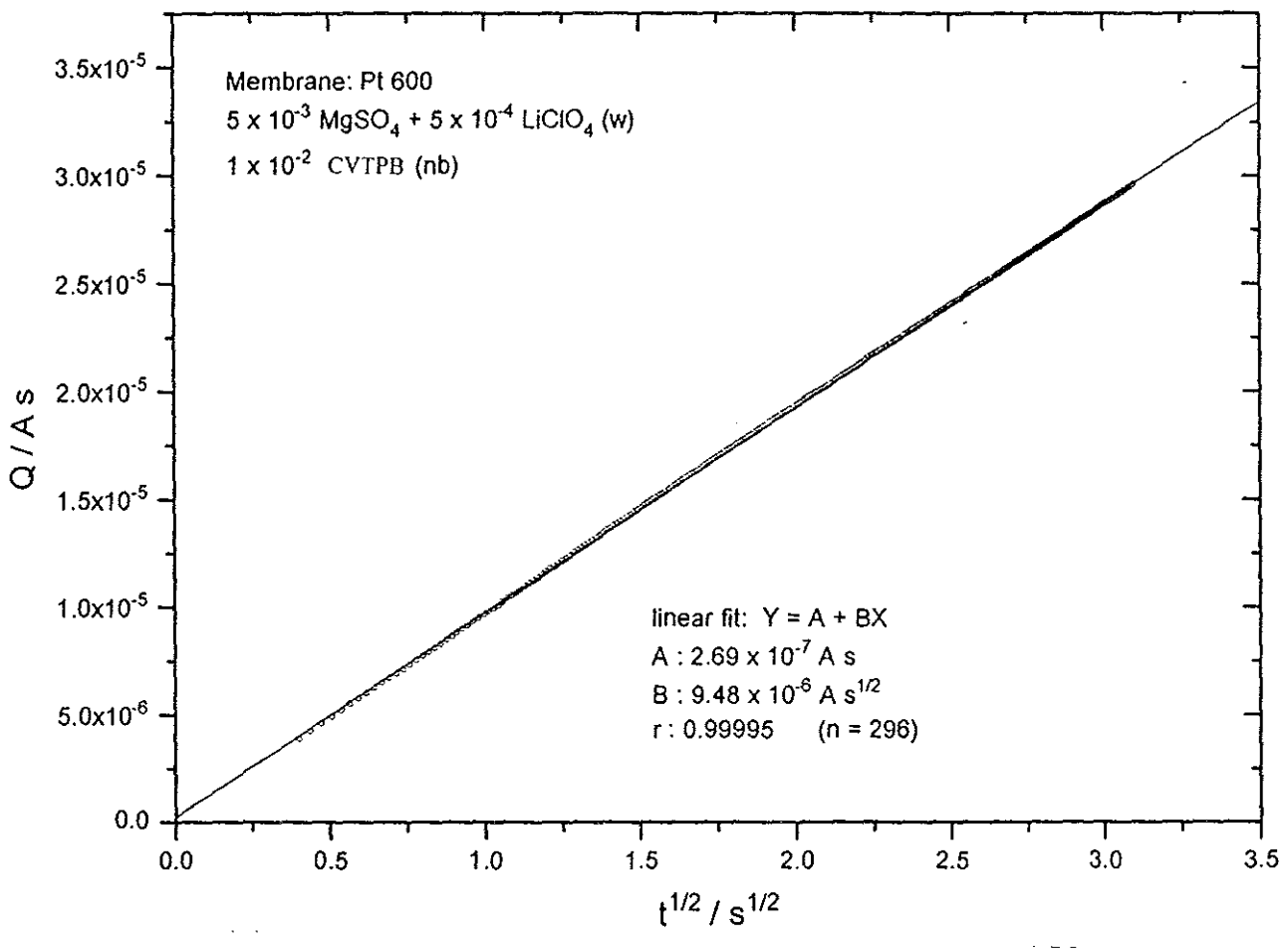


Fig. 9. Experimental chronocoulometric response of the membrane-stabilized ITIES to a potential step excitation.

A schematic diagram of the enzyme electrode is shown in fig.7. The electrode sensing element is the membrane-stabilized ITIES. Immediately adjacent to the membrane of the cell is the enzyme layer which formed by the entrapment of the enzyme within a gel. The performance of the sensor depends on formation of a layer of enzyme which is as thin as possible but with enzyme immobilized at the highest possible specific activity. Failure to do this results in sensors with poor sensitivity and long response times. The final component in the sensor is the outer protective membrane. This membrane serves several important functions. First, it is a protective barrier which prevents large molecules such as proteins from entering the enzyme layer. The membrane layer will also prevent the leakage of the enzyme into the sample solution. A properly chosen membrane exhibits permselective properties which are additionally beneficial to the function of the sensor. Finally the membrane can serve as a diffusional barrier for the substrate itself. Most enzymes follow some form Michaelis-Menten kinetic which leads to enzyme reaction rates largely non-linear with substrate concentrations. As a result, sample dilution is usually necessary for clinical assays. The laboratory made, Enzyme-based sensor, however, was capable of a linear dynamic ranges of several orders of magnitude because the response is controlled by diffusion through the membrane and not by enzyme kinetics. If the enzyme layer activity is low, then a thick membrane will be required to achieve good linear response. This will also lead to slow response. On the contrary, if the enzyme layer activity is high, a thin outer membrane is sufficient and a rapid response may be obtained.

As pointed out earlier, the immobilization method used is physical entrapment. Unless one is using a very labile enzyme that can be stabilized by chemical immobilization, it is better to use physical entrapment as a gel between dialysis membrane and avoid the time, chemical and enzyme consuming process of optimizing the cross-linking (chemical binding) procedure. In addition the amount of enzyme that can be immobilized onto the same surface area is much greater than that obtained using the chemical methods.

Before resorting to the use of the commercially obtained dialysis membrane, the possibility of casting cellulose acetate membranes over the sensor body (drop coating) have been investigated. During the experiments, two major problems were encountered; making such membranes unsuitable for the purpose. One of the problems was the difficulty of producing a film of reproducible thickness which was found to affect the response characteristics of the sensor. The other problem was mechanical, i.e., the membrane cannot be manipulated without tearing. It was found to peel off from the sensor surface when the

'O'-ring was put in place, releasing the enzyme into the aqueous solution.

The presence of the gelatine in the enzyme layer has several functions. It has high degree of swelling, i.e., it has high water sorption capacity. This provides an aqueous environment for the enzyme and minimizes intracarrier transport resistances to the diffusion of the substrate and products of enzyme catalysis. The hydrophillic nature of the gel also tends to stabilize the enzyme.

The effect of the type of enzyme used on the sensor response has also been investigated. Two types of enzyme preparations, supplied by different companies were employed:

i- urease powder (Ames Division, MILES Ltd, 1500 E. U. per vial)

ii- urease tablets (Jack bean meal, BDH, 400 E. U. per tablet).

Both preparations were dissolved in the gelatine-buffer solution followed by immobilization according to the procedure previously described. After equilibration time, voltammograms of the buffer solution were run using the newly prepared electrodes.

The use of the powder form of the enzyme was found to interfere with the measurements. It gave peaks within the 'potential-window' of the system which was interpreted to be due to transfer of sodium and nitroprusside ions blended with the preparation by the supplier. This preparation was intended for use in the colorimetric determination of urea based on the Berthelot's indophenol method. Subsequent runs using the tablet form of the enzyme gave no peak within the usable potential range. Hence this latter preparation was chosen for the investigation.

4.1. Determination of Urea by the facilitated transfer of urease-generated ammonium ions with dibenzo-18-crown-6

The cyclic polyether dibenzo-18-crown-6, has six oxygen atoms in the polyether ring, that are the bond places of the ionophore, and two benzene rings as depicted below.

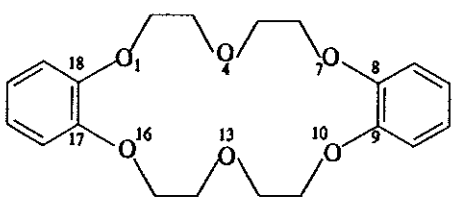


Fig. 10. Dibenzo-18-crown-6.

It forms a stable complex with ammonium ions released from the urease action on urea present in the test solution according to the reaction:

$$(NH_2)_2CO + 2H_2O \rightarrow 2NH_4^+ + CO_3^{2-}$$

The complex is formed by ion-dipole interaction between the ammonium ion and the oxygen atoms symmetrically placed in the polyether ring.

The transfer of ammonium ions across the ITIES was found to be facilitated through complexation with DB[18]C-6. Fig.11 shows the experimentally obtained voltammograms for 1 x 10^{-3}M urea in the test solution in the presence (1) and absence (2) of the ionophore in the organic phase. As can be seen, there is no peak within the 'potential-window' of the system in the absence of the ionophore whereas its presence gave a well-defined peak within the range of the potential used. The positive peak current obtained can be ascribed to the transfer of ammonium ions from water to nitrobenzene induced by the complex formation at the interface.

For the quantitative determination of urea, the amperometric mode of measurement was employed. The sensor gave a current response, I, which was proportional to the

concentration of urea in the sample solution. The current response is defined by the applied potential, E_{app}(which is equal to the polarographic half-wave potential, $E_{1/2}$)

$$I = K_{amp}\, C$$

where $K_{amp}$is a constant and depends on the voltammetric technique used as well as the other features of the experiment (such as cell and electrode design, stirring rate etc..). The relationship describing the variation of the response with the variation of the analyte concentration is given by:

$$\Delta I = K_{amp}\Delta C$$

Namely, the current response variation of the amperometric sensor indicates directly the change of concentration.

4.2. Calibration and characterization of the sensor response

Calibration of the sensor was made by adding standard solutions of the substrate and was carried out by measuring the steady-state response of the sensor. Aliquots of the standard urea solution were added into a beaker containing the buffer solution , under constant stirring, to generate a series of concentration steps.

The steady-state response was defined as the plateau reached in monitoring the changes in current as a function of time It was calculated by comparing the steady-state current either to the background current (I_b) in the absence of substrate or to the steady-state current corresponding to the previous addition. Fig.12 shows the experimentally obtained curves in which (a)- ($I - I_b$) vs concentration, C; (b)- $\Delta I/\Delta C$ vs C and (c)- $\Delta I/\Delta C$ vs logC were plotted. It was possible to measure the steady-state response over a large range of analyte concentration and successive substrate determinations were possible every 1 - 2 min by washing the sensor and rinsing the cell. After washing, it was found to be necessary to wait for some minutes for the current response to return to the back ground levels.

The linear range of the calibration curve was determined by plotting $\Delta I/\Delta C$ vs C (as in Fig.12b) or by comparing $\Delta I/\Delta C$ values for successive substrate additions. These method is much more definitive than plotting ($I - I_b$) vs C.

The response time of the sensor was determined for each substrate pulse into the cell

and are measured to 90 or 95 % of the steady-state response under constant stirring. Fig.13 shows the current-time response of the biosensor to a concentration step from zero to 5 x 10^{-4}M followed by washing and immersing in the buffer solution. The concentration step was generated by adding 0.25 ml of 1 x 10^{-2}M urea standard solution into the beaker containing 49.75 ml of the buffer under constant stirring. The time required to reach 95 % of the steady-state value, i.e., the response time, was 1 min. When the sensor was re-immersed back into a new buffer solution, the current decayed reaching the baseline value within 1-2 min.

Fig.14 shows the sensor response to subsequent additions of urea solutions in the concentration ranges 5 x 10^{-4} to 1.5 x 10^{-3}M. As can be seen the noise level of the measurement increases at higher concentrations because of increased back ground current corresponding to previous additions. In addition, the sensitivity of the sensor decreases with increasing concentration because of possible depletion of the enzyme. Therefore, to get a wide linear range of the measurement it was found to be preferable to measure steady-state signals corresponding to separate additions; rinsing the sensor with the buffer and allowing the current to return to the back ground level between each run.

4.3. Assessment of specifity and interferences

Selectivity depends first upon the enzyme chosen. The enzyme used in this work was urease- an absolute-specific enzyme. Hence no other substrate can be acted upon by the enzyme. There is also no interference from electroactive species which can diffuse to the sensor surface to be oxidized since the potential imposed on the system is low.

On the other hand, the sensor responds to residual (preformed) ammonium ion and monovalent cations such as sodium and potassium that are invariably present in clinical or soil samples. However, correction for the residual current due to these species in the test solution can be easily made. For this purpose a sensor of the same structure as the urea sensor but urease being removed from the enzyme layer was fabricated. This urease removed urea sensor gave the current response proportional to the concentration of these species but no response to urea. Since the normal sensor (with urease) responds to all species, the concentration of urea corrected for the residual interferents was obtained by taking the difference of the two signals. To account for possible differences in geometry, the observed signals in both sensors was divided by the geometrical area of the sensors.

Last but not least is the possible poisoning of the enzyme by heavy metal ions present in analyte solutions. If not removed, these ions will inactivate the enzyme and prevent a response to the analyte, resulting in negative interference. This interference is usually removed by addition of complexing agents such as EDTA. The use of EDTA as a buffer thus plays dual role : in addition to maintaining the pH of the system at the desired level, it will also complex these metal ions which may otherwise inhibit the enzyme.

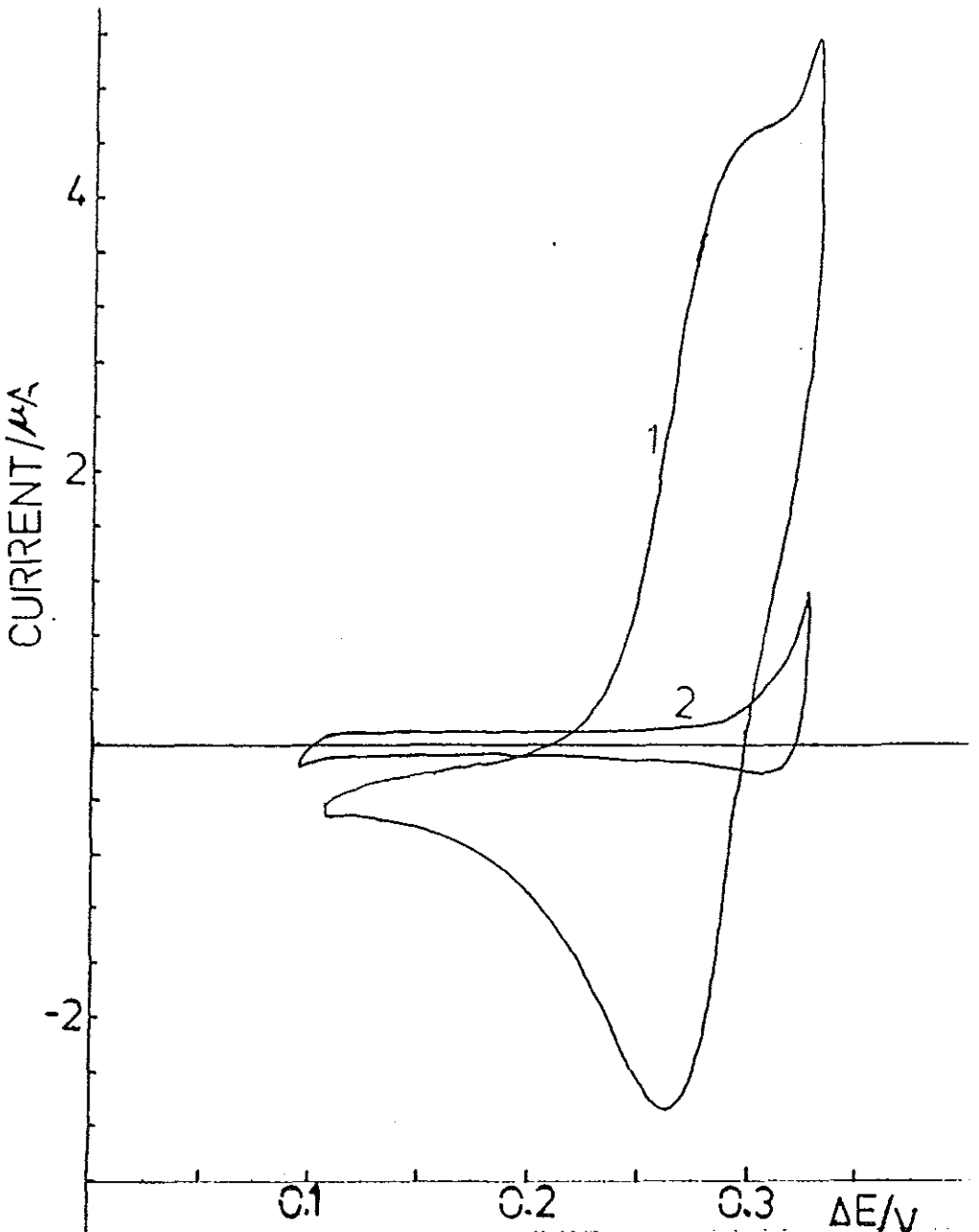
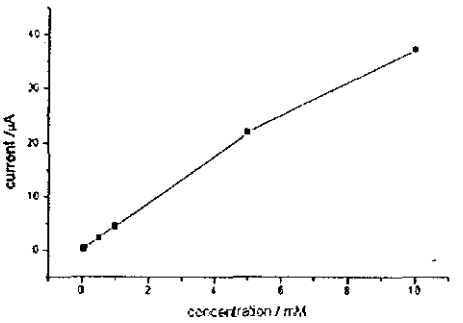
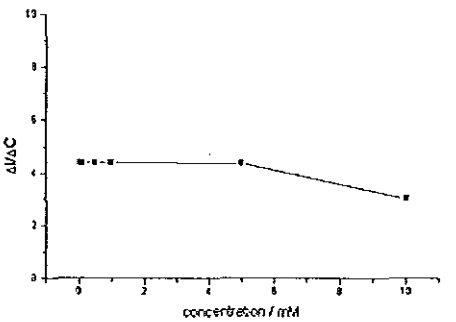


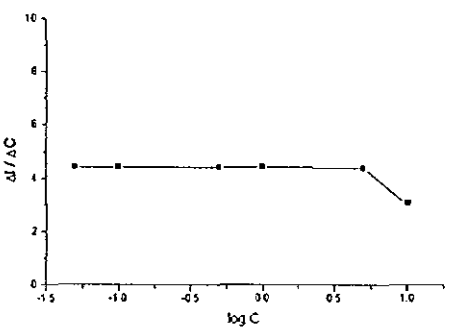
Fig.11.Cyclic voltammogram of the response of the biosensor to $1x10^{-3}$M urea with (curve 1) and without (curve 2) the ionophore DB[18]C-6 in the organic phase. The supporting electrolytes are 10^{-2}M TOctATPB and 10^{-2}M Li_2EDTA (pH 8.6) in nitrobenzene and water respectively. Polarization rate: 0.25 mVS^{-1}.



(a)



(b)



(c)

Fig. 12.Calibration graphs for peak currents (steady-state) corresponding to a series of concentration steps;

(a)- $I-I_b$ (b)- $\Delta I/\Delta C$ vs C (c)- $\Delta I/\Delta C$ vs logC.

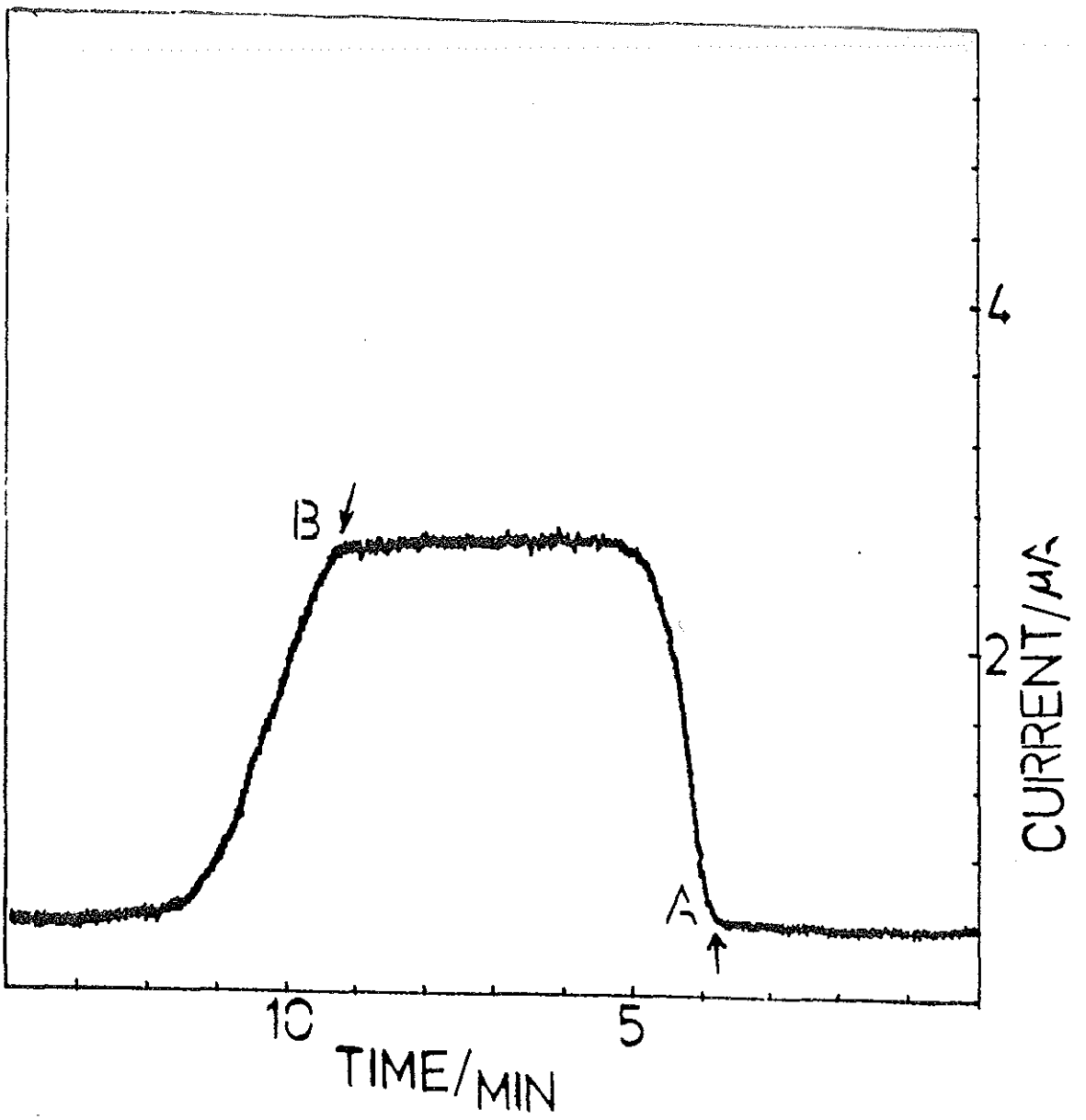


Fig.13. Typical current-time response of the biosensor to a concentration step from 0 to $5x10^{-4}$M(A) followed by washing and immersing in the buffer solution(B).

aqueous phase: 10^{-2} M Li_2EDTA, pH 8.6

organic phase: 10^{-2}M TOctATPB + 10^{-2}M DB[18]C-6 in nitrobenzene.

polarization rate: 0.25 mVS^{-1}

E_{app}= 270 mV

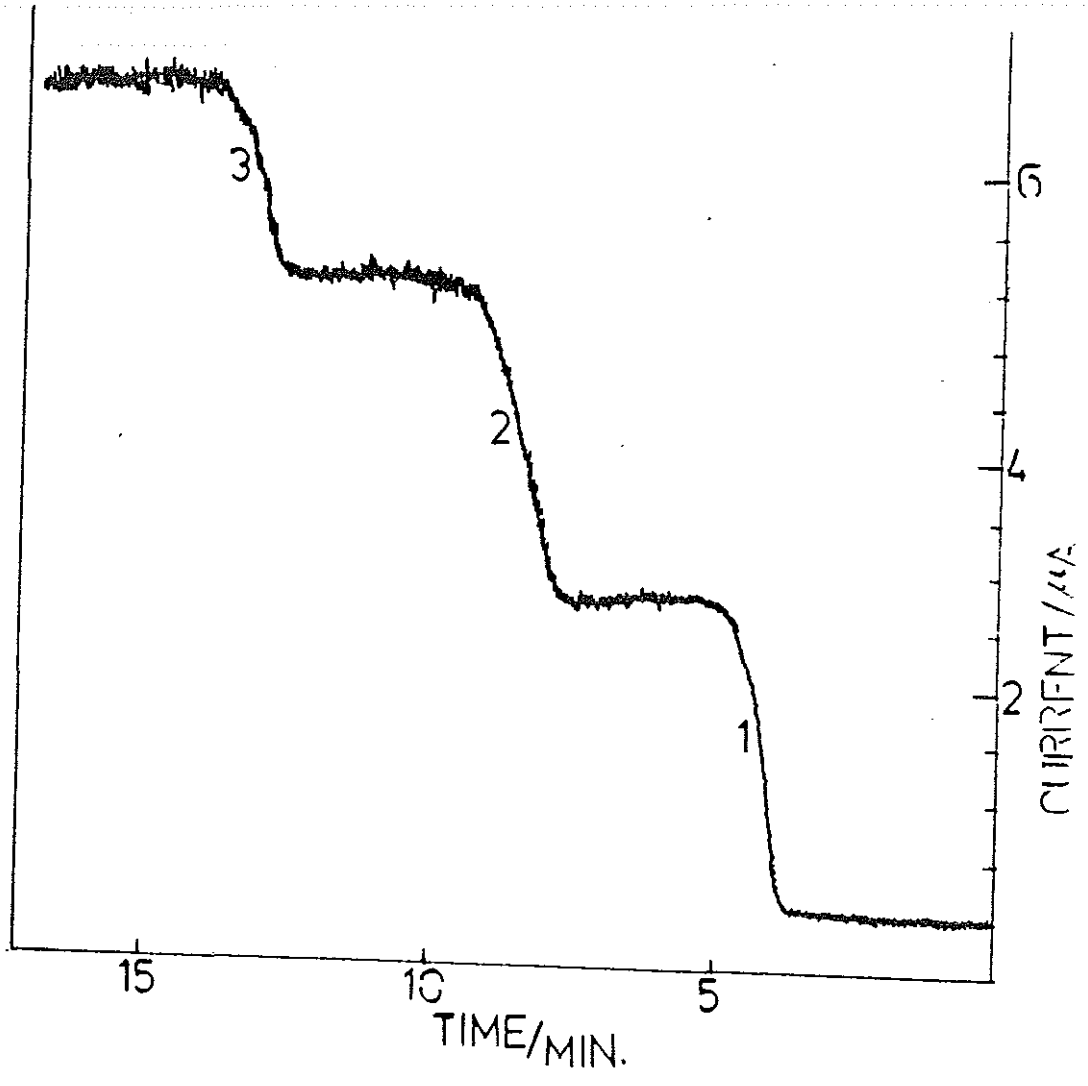


Fig.14.. Current-time profile of the biosensor to subsequent concentration steps:-

1. $5x10^{-4}$M 2. $5x10^{-4}$ M & 3. $3x10^{-4}$M urea standard solutions.

Aqueous phase: 10-2M Li_2EDTA

Organic phase: 10^{-2}M TOctATPB + 10^{-2}M DB[18]C-6 in nitrobenzene.

Sweep rate: 0.25 mVS^{-1} ; E_{app}= 270 mV

5. CONCLUSION

The coupling of enzymatic reactions with electrochemical monitoring is expanding rapidly. A large number of enzyme electrodes had been fabricated, resulting in relatively selective and convenient sensor for a variety of substrates. Generally, the coupling of biochemical and electrochemical reactions appears to have a great analytical promise. These devices combine convenient electrochemical principles of operation with the selectivity of enzymes. Much attention has recently been given to the development of specific biosensors for various substrates of biomedical and biochemical interest by using immobilized enzymes in conjunction with amperometric and/or potentiometric ion sensing electrodes.

The present work dealt with carrying out preliminary experiments in order to test the possibility whether the modification of the membrane-stabilized ITIES by enzymes can enhance the analytical performance of enzyme catalyzed reactions in comparison with the classical methods. The analytical application of the amperometric sensor constructed in this work is based on the transfer of ammonium ion, released in the urea-urease reaction, across the polarizable membrane-stabilized water/nitrobenzene interface employing its facilitated transfer by the neutral carrier DB[18]C-6 in the organic phase. The course of the reaction is followed by monitoring the accompanying current as a function of time and/or concentration. Ideas about the kinetics of the reaction can easily be obtained from the change in the current (substrate concentration) as a function of time. The quantitative determination was based on measurement of the steady state current, which was found to be directly proportional to the urea concentration in the sample solution. The results obtained were satisfactory. We have tried to extend the method for use in a flow-injection system. But the sensitivity obtained was very low. This was because of the short contact/residence time of the flowing substrate solution with the enzyme-layer of small surface area. However, there is a possibility of adopting it to a flow-injection analysis if the enzyme is immobilized on packed-bed reactors of relatively larger surface area (continuous-flow immobilized enzyme reactors). It was expected that this system would provide continuous and reproducible method for assay of urea in various samples without interferences.

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DECLARATION

I, the undersigned, declare that this thesis is my original work and has not been presented for a degree in any other university.

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Place and date of submission

Chemistry Department, Addis Ababa University, June, 1995.

This thesis has been submitted for examination with my approval as a university advisor.

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