Characterisation of Electrodes for Analytical Potentiometry

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I hereby certify that the material, which I now submit for assessment on the programme of study leading to the award of Master's of Science is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed : Éamon Mc Evoy Date : 17/12/93

Date : _____

Abstract

This thesis reports on 4 projects undertaken as part of the experimental work for the degree. Participation in a study to determine the feasibility of a new calcium cell, the Covington reference cell or CRM, as the basis for a reference method for ionised calcium measurement in blood products, was carried out. This involved the testing of "blind" samples, both aqueous and protein containing, to investigate the precision and accuracy of the method. In our laboratory, the cell was found to have comparable precision with commercial analysers used in clinical laboratories and results tallied well with the assigned values of protein solutions. This cell was used to investigate the effect of increasing protein on measured ionised calcium and showed that there was an apparent rise in measured ionised calcium with increasing protein levels. Use of an isotonic salt bridge in the reference electrode cell resulted in reduced measurements of ionised calcium compared that using the normal hypertonic salt bridge junction.

A novel KCl-doped resin, RepHex, was evaluated as a reference electrode material. When used as the reference electrode with a pH electrode, it was found to give comparable pH measurements in unstirred solution but much improved stability and precision in stirred solutions compared to a conventional frit-restricted calomel electrode. The leakage from this junction was found to be much less than that of the conventional electrode, particularly when surface areas were normalised.

Preliminary studies on the ionophoric capabilities of five novel calixarene compound for potassium ions were carried out. Compounds Ia and Ib were found to have a linear range of 10^{-3} to 10^{-1}M K^+ and Compounds II,III and IV had a linear range of 10^{-4} to 10^{-1} M K^+. Limited lifetime studies were carried out and compounds Ib and II were still functioning after 10 days. The selectivities for these compounds were such that it is unlikely that they could be incorporated into electrodes for general use.

1 Introduction

1.1. Introduction to Potentiometry

Potentiometry is one of the most popular electroanalytical techniques currently in use to-day. The simplicity of potentiometry, a two electrode system, has been exploited in a wide range of applications. Initial applications were in industry and environmental monitoring but in the last 25 years, its use in in clinical chemistry has expanded rapidly, so that a wide range of biologically important ions, both cations and anions, may now be measured with ion-selective electrodes (ISEs) [1].

The technique is based on the selective exchange of ions at a membrane-sample boundary. The mathematical principles governing these exchange processes were first described by Nernst in 1888 [2,3] for ideal (specific) membranes. Cremer then discovered that certain glass membranes exhibited some pH sensitivity [4]. Over the next 30 years, much work was performed to develop and refine the technique, with ion-selective electrodes for ions other than the hydrogen ion being developed. In 1936, Beckman marketed the first pH meter and so brought the technique to a wider audience. Pioneering work by Nikolskii led to further understanding of the processes at work when he first postulated the effects of other ions on the response function of the glass electrode and designed equations to quantify their effect on the signal generated [5,6].

The development of electrodes in potentiometry took its next leap forward in 1962 with the development of the first non-glass ion-selective electrode [7]. The next major step was the development of a liquid membrane electrode which comprised of an electroactive agent, dispersed in

a water immiscible solvent [8]. The inconvenience of this configuration lead to the development of other ionophore support matrices (e.g. silicone rubber). However membranes based on PVC have become the most popular [9]. Initially liquid membranes employed ion-exchange type electroactive agents but work in the late 1960's and early 1970's led to neutral carriers such as antibiotics and their derivatives being used as ionophores in ISEs the most famous of which is the valinomycin based potassium ISE. Work on the development of new ionophores has lead to the use of poly- and crown ethers [10] and recently calixarene compounds [11,12] as ionophores in PVC membrane electrodes.

The other component of the potentiometric system, the reference electrode, has in contrast changed little since the development of the technique. The main types of reference electrodes are the silver/silver chloride and calomel electrodes with the fluoride electrode being sometimes used as a pseudo- reference electrode [13].

In using potentiometry as an analytical technique, careful consideration must be given to all aspects of the measuring system. The electrode must be adequate for its intended use in terms of characteristics such as:

(1) **Sensitivity** i.e. the reponse of the electrode to changes in activity of the primary ion. Ideal electrodes will give a Nernstian response, i.e. 59.16/z mV/decade, where z = charge of the primary ion

(2) **Selectivity** i.e. the response of the electrode to ions, other than the primary ion, in the sample matrix. Ideal electrodes will show no reponse to other ions in the sample matrix.

(3) **Speed of Response** i.e. how quickly the electrode responds to

changes in activity. Ideally response time should be of the order of a few seconds or less.

(4) **Stability of the signal** i.e. the change in measured signal in solution of constant activity as a function of time. Ideal electrodes will demonstrate no change in measured signal with time.

These characteristics should be established when choosing a measuring system or when evaluating a new ionophore.

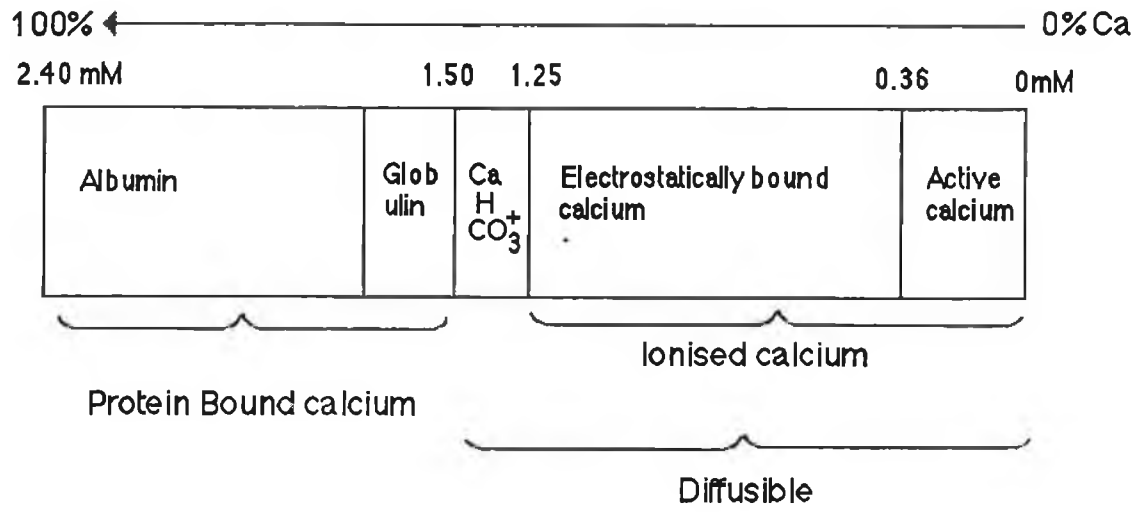
However in reality, the response of the electrode can be expected to be affected by the sample matrix and so the composition of the sample should be known. This will be important in clinical applications where the sample may contain proteins, lipids, blood cells, and high proportions of ionic constituents e.g. sodium, bicarbonate or in industrial applications where the sample of interest may contain high levels of extraneous contaminants or even, in some cases, very low ionic strength. Thus the choice of ISE and reference electrode will be of great importance in order to achieve precise and accurate measurement of the ion of interest.

As approximately 50% of this thesis deals with the measurement of ionised calcium in blood products, a guide to calcium homeostasis will be given.

1.2. Calcium Homeostasis

Calcium in the bone acts as a reservoir for calcium in extracellular fluid. Calcium in plasma circulates in the body in three distinct states; bound to proteins, complexed to inorganic anions and as free calcium ions hereafter referred to as ionised calcium, Ca^{2+} (cf Figure 1.1).

Figure 1.1 Various calcium fractions in serum



The protein bound calcium which makes up approximately 50% of the total serum calcium is called the non-diffusible fraction and the remainder is known as the diffusible fraction. The ionised fraction is the physiologically active portion of the extracellular calcium as was demonstrated by a series of historic experiments carried out by McLean and Hastings [14]. The level of ionised calcium is maintained by the body within very narrow limits e.g. 1.19--1.33 mM. The two most important hormones responsible for the regulation of this balance are the parathyroid hormone, PTH, and a metabolite of Vitamin D, 1,25 dihydroxyvitamin D or 1,25 $(OH)_2$ Vit.D. PTH acts through its mobilisation of calcium from the bone and also by causing increased resorption of calcium in the kidney tubules. 1,25 $(OH)_2$ Vit.D on the other hand increases absorption of calcium from the intestine and affects mobilisation of calcium from the bone. Also important in proper calcium homeostasis is the hormone calcitonin, which prevents calcium reabsorbtion from bone and hence lowers plasma calcium. The roles of these hormones in calcium regulation are represented in Figure 1.2.

When blood calcium falls

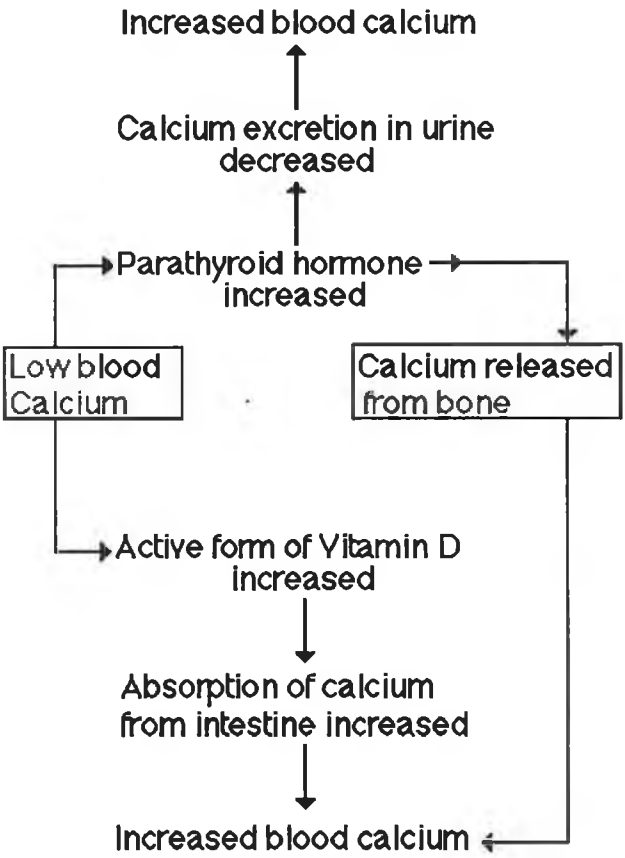


Figure 1.2. Graphical representation of the pathways involved in calcium homeostasis in the body [15]

1.2.1. Protein bound calcium

The binding of calcium to serum protein is thought to be purely to facilitate the transport of calcium throughout the body in a manner comparable to Iron bound to transferrin [16]. Alternatively, it may be a method of modulating calcium activity, as ionised calcium is the physiologically active portion. 90% of all protein bound calcium is bound to albumin and experiments have suggested that there are approximately 30 binding sites, classified on the basis of the magnitude of the binding constants, available at a physiological pH of 7.40 [17]. Each of these binding sites have different binding or association constants. A more simplistic model has been suggested and this states that there are 12 ± 1 binding sites all with an apparent association K of 95L/mol at pH = 7.40 and at normal physiological levels, only 10% of the available binding sites are saturated [18].

There are many factors other than protein concentration or available calcium which influence the binding of the calcium to proteins. These are

(1) pH [19];

(2) temperature [19];

(3) interfering alkali metal ions [20];

(4) ionic strength [20];

1.2.1.1. Effect of pH on protein bound calcium

There is an inverse relationship between the binding of calcium to albumin and pH i.e. as pH decreases, the ionised calcium concentration increases. This happens because the increasing H_3O^+ concentration leads to competition with calcium ions for binding sites on the albumin molecule.

Therefore as pH decreases, the apparent binding constant also decreases and so bound calcium is released after competition with H_3O^+ ions at the binding sites. The pH effect on ionised calcium also depends on the factor causing the shift in pH. If pH is lowered using HCl, the increase in ionised calcium concentration is greater than if the pH drop is caused by pCO_2 changes [19]. If the pH drop is caused by a rise in pCO_2 , there will be a simultaneous rise in bicarbonate concentration. Bicarbonate binds the calcium ions and thus the rise in ionised calcium is less than if the bicarbonate concentration had remained constant.

1.2.1.2. Effect of temperature on protein bound calcium

The protein binding of calcium is temperature dependent, with binding increasing slightly with increasing temperature [19]. The magnitude of these changes are quite small but measurements should be performed on serum samples at 37°C. This should be done so as to prevent errors due to the effect of temperature on the measuring system as temperature fluctuations can cause significant error in potentiometric measurements.

1.2.1.3. Effect of interfering ions on protein bound calcium

It has been shown that there is some binding of sodium to albumin [21] but this is thought to be very small and not significant. However, due to the divalent nature of magnesium and its closeness in size to calcium, competitive binding between magnesium and calcium is very important [20]. Both ions are said to bind with equal strength and to the same binding sites on the albumin molecule.

1.2.1.4. Effect of ionic strength on protein bound calcium

An inverse relationship exists between calcium binding to albumin and the ionic strength of the medium [20]. This effect is not dependent on the electrolyte composition of the medium being identical in both KCl and NaCl solutions and this would suggest that this relationship is not due to competitive binding from potassium or sodium but rather some other process, probably via conformational changes in the albumin molecule.

1.2.2. Diffusible calcium

That calcium not bound to serum proteins is termed the diffusible portion i.e. complexed and uncomplexed calcium. The complexed portion consists mainly of the calcium-bicarbonate [$CaHCO_3^+$] ion-pair with small amounts of calcium-lactate ion-pair and calcium citrate complex. The calcium citrate complex is of great importance in organ transplantation where massive transfusions of blood are required [22]. The importance of the complexed calcium lies in the fact that it reduces the concentration of free ionised calcium.

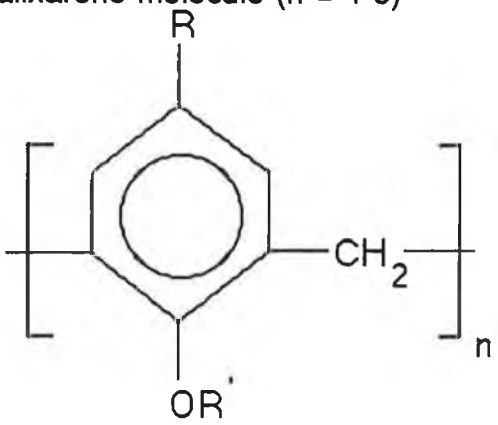
1.2.3. Ionised calcium

The remainder of serum calcium is present as free calcium ions. This is slightly misleading as only a portion of this, 15%, is biologically active. The rest is inactivated by electrostatic forces due to the other ionic constituents of the plasma.

1.3. Calixarenes

Calixarene is a term introduced by Gutsche to describe a homologous series of macrocyclic phenol-formaldehyde condensates [23]. The basic calixarene molecule is represented in Figure 1.3.

Figure 1.3 Basic Calixarene molecule (n = 4-8)



where R = H, Alkyl group e.g. *t*-Butyl

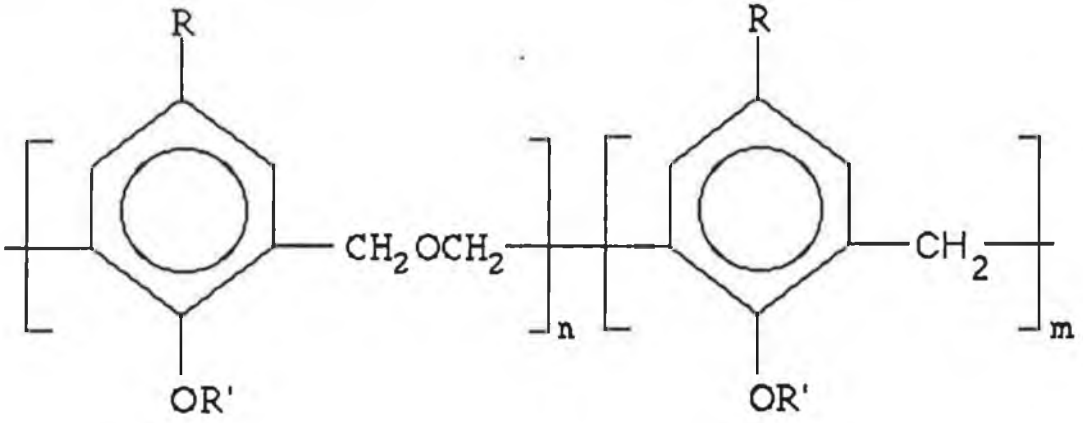
R' = H, Ester, Ether, Thiol etc.

They have recently become the focus of much work in the area of host-guest chemistry and have been exploited for a variety of applications, notably in heavy metal absorbtion [24], catalysis [25], and alkali metal complexation and transport [26]. Most recently, they have been exploited as active agents in potentiometric sensors for sodium, and caesium [11,12].

Calixarenes behave as neutral carriers in potentiometric sensors. The possess the main features of neutral carriers i.e. a well defined cavity size and the presence of inwardly facing polar groups which define a polar cavity. The cavity size depends on the number of repeating units in the molecule and the nature of the bridging groups between the units. Tetrameric calixarenes (n=4) have been shown to preferentially sequester sodium [11] and the hexameric calixarenes (n=6) have demonstrated caesium selectivity [12]. The potassium ion is intermediate in size to sodium and caesium and

so a calixarene compound with a cavity size appropriate for potassium was sought. Efforts to synthesize a calixarene with the optimum cavity size resulted in the formation of a new series of calixarenes, the oxacalixarenes. These tetrameric calixarenes have larger $-CH_2O-$ moieties inserted into the methylene bridge structure of the basic calixarene molecule, Figure 1.4 [27] and this results in larger cavities than the conventional tetrameric calixarene. The evaluation of some of these compounds are described.

Figure 1.4. Basic structure of the new oxacalixarene molecules



where m = 4 - n

n = 1, monoxacalixarene

n = 2, dioxacalixarene

R = H, Alkyl group e.g. *t*-Butyl

R' = H, Ester, Ether, Thiol etc.

An alternative route to achieve potassium selectivity was also tried. Tetrameric calixarenes with a partial cone configuration (cf Section 6.1., p 109) were synthesized. It was hoped that the more open nature of the cavity might result in a potassium selective sensor. Two of these compounds were also evaluated as potassium sensors.

1.4. Summary of Thesis

In this thesis, the work carried out in the participation in a European study aimed at the development of a reference method for the measurement of ionised calcium in blood products is described. Investigation of the effect of sample constituents on measured calcium as measured by the prototype calcium cell and two commercial analysers was also studied. Evaluation of a new type of ionophore as a potential potassium sensor, and the use of a new reference electrode material in the measurement of pH of high purity water are other studies reported.

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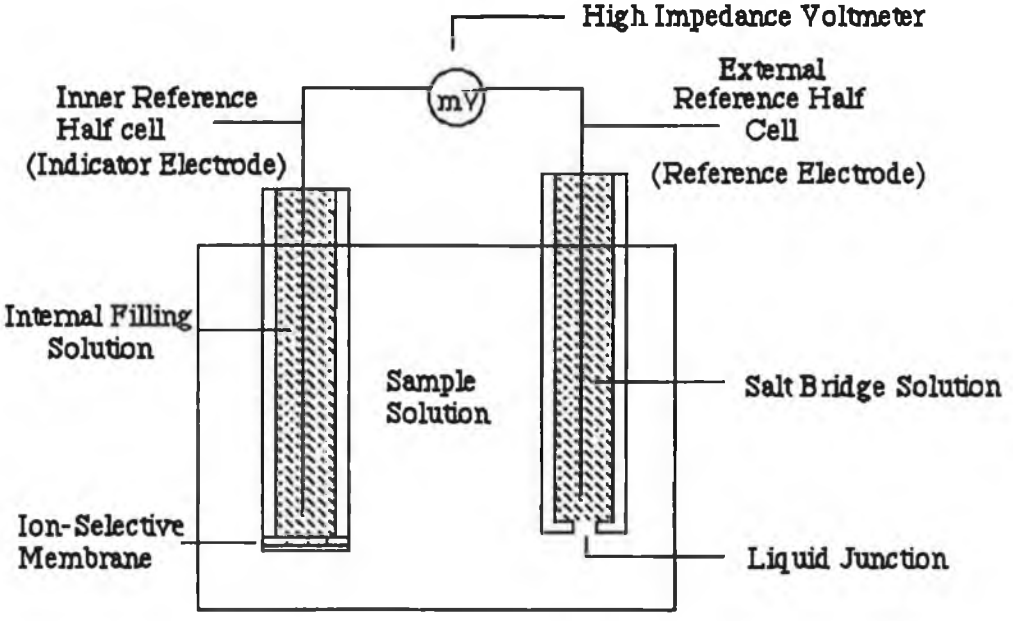
2 Theory

2.1. Introduction

Potentiometric techniques involve the measurement of the EMF generated in a galvanic cell under zero current conditions. Such a galvanic cell consists of two half-cells in electrical contact by means of a salt bridge. The half-cells in this system are known as the indicator or ion-selective electrode (ISE) and reference electrode half cells respectively. The circuit is completed by a high impedance voltmeter. This high impedance is required to prevent any current drainage and hence current flow which would disturb the various equilibria which generate the cell potential. The indicator and reference electrode will be considered separately. A typical ISE based potentiometric cell is graphically represented in Figure 2.1.

Figure 2.1 A Typical Potentiometric system, comprised of the indicator electrode and an external reference electrode.



This electrochemical system may also be represented by the following cell diagram:

Ag | AgCl | KCl (XM) || sample | membrane| A^+(const. M) |AgCl | Ag.

External Reference electrode — Indicator Electrode or Ion-Selective Electrode

where XM is the molarity of the salt bridge

| represents a change in phase or liquid junction

The potential of the cell, E_{cell} (in ideal circumstances) is comprised of two potentials

$$E_{cell} = E_{ind} - E_{Ref} \quad (1)$$

where E_{ind} is the potential of the indicator electrode

E_{Ref} is the potential of the external reference electrode

The potential of the indicator electrode, E_{ind}, is composed of contributions from an internal reference electrode, $E_{int(1)}$, and the membrane, E_m, responsive for the primary ion.

$$E_{ind} = E_{int(1)} + E_m \quad (2)$$

$$= \text{Constant} + Em$$

where $E_{int(1)}$ = potential of the inner reference electrode = constant

E_m = potential of membrane which varies as a function of the primary ion activity.

The potential of the external reference electrode is composed of contributions from an internal reference electrode, $E_{int(2)}$ and a potential which arises at the interface of the electrode and the sample, E_{jn} .

$$E_{Ref} = E_{jn} + E_{int(2)} \quad (3)$$
$$= \text{Constant} + E_{jn}$$

where E_{Ref} = Potential of the external reference electrode

E_{jn} = potential at the sample interface

$E_{int(2)}$= constant potential

Combining equations (2) and (3) and providing E_{jn} is constant,

$$E_{cell} = (\text{Constant} + E_m) - (\text{Constant} + E_{jn})$$
$$= \text{Constant} + E_m - E_{jn}$$
$$= E^o + E_m \quad (4)$$

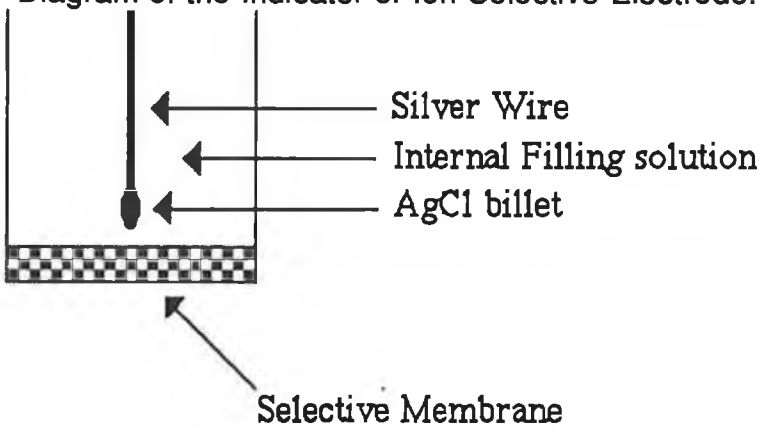
where E^o = standard cell potential i.e. cell potential measured under standard conditions of temperature (298K), pressure (1 atm.) and unity activity of the primary ion.

The processes involved for $E_{int(1)}$, E_m and E_{jn} are discussed in subsequent sections.

2.2. The Ion-Selective Electrode or ISE

The ISE is comprised of a selective membrane, an internal filling solution and an internal reference electrode. Thus the potential of the ISE is composed of two different potentials, the potential of the internal reference electrode, $E_{int(1)}$, and the potential generated in the membrane of the ISE, E_m. The inner reference electrode is usually a silver/silver chloride wire and the internal filling solution is a chloride salt of the primary ion. The potential of this reference electrode,$E_{Ag/AgCl}$, is constant provided that the chloride activity is constant. The ISE may be represented as in Figure 2.2.

Figure 2.2 Diagram of the Indicator or Ion-Selective Electrode.



Therefore, the potential of the indicator electrode is given by;

$$E_{ind} = E_{Ag/AgCl} + E_m \text{ or } E_{ind} = E_{int(1)} + E_m \qquad (2)$$

2.2.1. The Membrane Potential

The potential of the membrane is induced by a charge separation of the primary ion A^+, and its anion, X^- at the membrane interface.

When the membrane, selective to A^+ ions is dipped into a salt solution of A^+X^- ions, there will be a carrier mediated flux of A^+ ions through the membrane from the solution of higher activity towards the solution of lower activity. The anions will remain in situ as they are unable to enter the membrane and the two populations will be separated by the membrane boundary, hence the charge separation. At equilibrium, the migration of the counter-ions through the membrane will cease, opposed by a potential set up between the membrane and the sample solution in order to maintain electrical neutrality. This potential will be related to the differences in activity of A^+ on each side of the membrane. There will be no further net movement of the A^+ ions from each side of the membrane to the other. The potential of the membrane cannot be measured directly but may be measured by

immersing two reference electrodes into the solutions on either side of the membrane. For a membrane that is exclusively selective towards the primary ion, A^+ , the zero current potential is a directly related to the activities of the contacting solutions on either side of the membrane and the magnitude of this potential varies in a way predicted by the Nernst equation, equation (4).

$$E_m = RT/nF \ln a'/a'' \qquad (4)$$

or $= S \ln a'/a''$

where $S = RT/nF$

a' refers to the activity of A^+ in the external solution

a" refers to the activity of A^+ in the internal solution

R is the gas constant

T is the absolute temperature

n refers to the charge of the primary ion

F is the Faraday constant.

2.2.2. Membrane Boundary and Diffusion Potentials

The potential of the membrane may be resolved into three components, two boundary potentials $E_{b'}$ and $E_{b''}$ and an internal potential, called the diffusion potential E_d where

$$E_m = E_{b'} + E_{b''} + E_d \qquad (5)$$

In an ideal electrode, E_d will be constant and so the membrane potential may be related only to the boundary potentials. The boundary potentials are a measure of the ion-exchange processes that take place at the membrane surfaces and thus there are two boundary potentials. These potentials are dependent on the primary ion activity at both interfaces of the membrane. Therefore,

$$E_{b'} = RT/nF \ln a'/a_m \quad (6)$$

and $$E_{b''} = RT/nF \ln a''/a_m \quad (7)$$

where $E_{b'}$ is the boundary potential at the sample interface and a' is the activity of the primary ion

$E_{b''}$ is the boundary potential at the internal interface and a'' is the activity of the primary ion.

a_m is the activity of the primary ion in the membrane

Thus the potential of the membrane,

$$E_m = E_{b'} - E_{b''} + E_d$$

$$= RT/nF \ln (a'a_m/a''a_m) + E_d$$

$$= RT/nF \ln a'/a'' + E_d \text{ and as a'' and Ed are constant}$$

$$E_m = \text{constant} + S \ln a'.$$

2.3. The External Reference Electrode

The basic requirement of a reference electrode is that it provides a stable potential, independent of sample matrix effects, against which the potential of the indicator electrode may be measured. The two most common types in general applications are the calomel and the silver/silver chloride electrodes:

(1) The calomel electrode which may be represented as

KCl (satd.) | $Hg_2Cl_{2(s)}$(satd.)| $Hg_{(l)}$.

and the half-cell reaction is $Hg_2Cl_{2(s)} + 2e \rightleftharpoons 2Hg_{(l)} + 2Cl^-_{(aq)}$. The electrode potential for this cell is + 0.2420V against a standard hydrogen electrode (SHE) at 25°C.

(2) The silver/silver chloride which may be represented by

KCl (satd.)|$AgCl_{(s)}$(satd.)| $Ag_{(s)}$

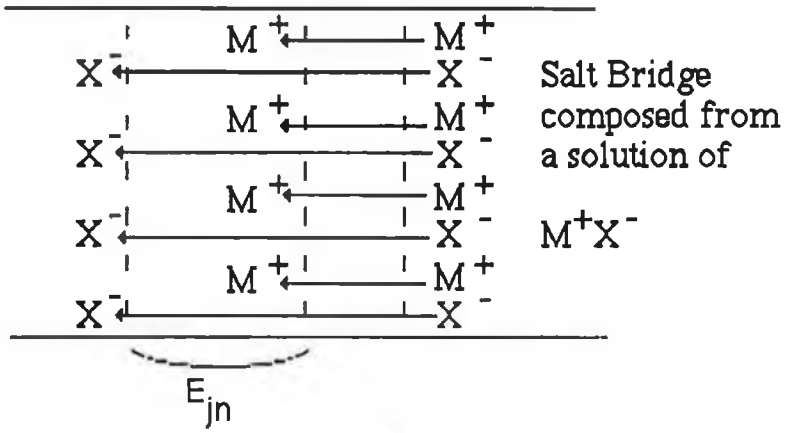
and the half-cell reaction is $AgCl_{(s)} + e \rightleftharpoons Ag_{(s)} + Cl^-_{(aq)}$ The electrode potential for this cell is + 0.2046V against the SHE at 25°C.

The reference electrode is constructed using an internal electrode system as shown above. This internal electrode is responsive to Cl^- ions and is bathed in a solution of constant Cl^- concentration (KCl satd.). As the Cl^- concentration remains constant, the potential will be constant. The overall potential of the reference electrode is also composed of a potential which is generated at the interface between the salt bridge solution and the sample. This potential is known as the liquid junction potential.

2.3.1. Generation of a Junction potential

At the junction of two dissimilar solutions, an unequal diffusion of the ions in the solutions will occur, due to the differing mobilities of the ions in the solutions. This results in a charge separation at the junction which in turn gives rise to a potential (Figure 2.3).

Figure 2.3 Representation of a liquid junction of a solution of M^+X^- with dilute sample. The Anion X^- diffuses more quickly than M^+ and so a charge separation occurs and hence a potential is developed.



2.3.2. Estimation of this potential

The magnitude of this junction potential may be estimated by use of the Henderson equation. In order to use this equation, the concentrations of the salt bridge and the sample must be known and the mobilities of the ions in each of these solutions is also required.

$$E_d = \frac{-\sum_n Z_n U_n (C_n' - C_n'')}{\sum_n Z_n^2 U_n (C_n' - C_n'')} \times \frac{RT}{F} \times \ln \frac{\sum_n Z_n^2 U_n C_n'}{\sum_n Z_n^2 U_n C_n''} \qquad (8)$$

where

Σ is the sum of all the charged species

z_n is the charge number of the n^{th} species

u_n is the absolute mobility of the n^{th} species

$c_{n'}$ is the concentration (or corresponding activity a_n') of the nth species in the sample solution

$c_{n''}$ is the concentration (or corresponding activity a_n'') of the nth species in the salt bridge of the reference electrode.

Under optimal conditions, the first factor of equation (8) is dominated by the term due to the bridge electrolyte and thus $u_n c''$ should be chosen as large as practicable in order to achieve a buffering of E_D against changes in the composition of the sample.

2.3.3. Minimising this potential

The size of this junction potential may be minimised by the use of a highly concentrated solution of a 1:1 equitransferent electrolyte e.g. KCl or CsCl. If these electrolytes might lead to contamination of the sample, other electrolytes such as NH_4Cl, KNO_3, or NH_4NO_3 may be used. The use of highly concentrated salt bridges present problems when used in blood products. The hypertonic salt bridge usually employed in clinical analysers has been shown to denature the protein in serum samples [1] and this is thought to give rise to elevated results as the protein concentration rises [2]. This rise occurs due to the formation of immobile poly-anions at or near the junction which alter the local potential and thus lead to errors [3]. In whole blood measurements, the effect of the haemocrit is also of importance as the sedimentation of the red blood cells causes a suspension effect which in turn alters the junction potential. Studies have shown that altering the composition of the salt bridge can help minimise this effect [3].

2.3.4. Residual Liquid Junction Potentials

The Junction potential contributes to the total cell potential by contributing to the potential of the external reference electrode, E_{Ref}. Differences in composition between the calibrating solutions and sample solution will result in different liquid junction potentials. This difference is

known as the residual liquid junction potential or RLJP. This potential may be considerable and to minimise errors in the final analysis, the composition of the calibration solutions should be closely matched to the composition of the sample being measured. An alternative method is to add a concentrated solution of an ion which does not interfere with the measuring electrode i.e. a total ionic strength adjustment buffer or TISAB to the calibrating and sample solution. Thus as the ionic strength of the solutions measured are equal, the activity coefficient of the ion of interest will be constant .

2.3.5. Features of a good reference electrode junction

The requirements of a good Reference Electrode junction are as follows:

(1) it must be reproducible

(2) it should not be affected by stirring or streaming of the solution being measured

(3) it should not be affected by particulate matter in the sample

(4) there should be no memory affects due to trapped solutions in the junction and carried over to the next

In order to achieve these requirements, the electrode must possess a constant positive outflow of the salt bridge solution so that the flow from the junction will be sufficient to overcome the backflow of sample ions into the junction. There are many different junction configurations to achieve this e.g. ceramic frit, fibre wick, ground glass sleeve but the best junction configuration is a constant flowing electrode or one where the junction is renewed for each measurement [4]. The usual choice for the bridge solution of a reference

electrode is a concentrated or saturated KCl solution. This solution is chosen as the K^+ and Cl^- ions are equitransferent. This bridge solutions may cause interference in some analyses e.g measurement of K^+ or the measurement of Ag^+ or Pb^{2+} solutions (as Cl^- will react with these species and they will precipitate out at the junction). In analyses such as these, a double junction electrode is used. In this electrode, the conventional reference electrode is separated from the sample by a solution containing ions which are compatible with the reference electrode yet do not affect the analytical measurement.

2.4. Concentration versus Activity

A distinction must be made between concentration and activity. The preceding discussions have used the term activity. This should not be confused with concentration. There is a relationship between the activity measured by an ISE and concentration. The activity measured may be related to the concentration by its activity coefficient;

$$a_i = f_i . c_i \qquad (9)$$

where f_i = activity coefficient of the ion i

c_i = concentration of ion i

In dilute solutions, the activity coefficient approaches unity. The activity of an ion depends on its environment and thus the presence of other ions will affect this coefficient. The activity coefficient will therefore be a function of the ionic strength. Using the Davies expression, in dilute solutions

$$\log f_i = A.z_i^2 \left[I^{0.5}/(1+I^{0.5}) - 0.2I \right] \qquad (10)$$

where A is a function of the temperature and the dielectric constant of the solvent (i.e. 0.512 in water at 25 C)

I = Ionic strength = $0.5 \Sigma c_i z_i^2$

z_i = charge of any ion i

It is important therefore in clinical applications that calibrating solutions have ionic strengths of close approximation to that of blood plasma.

2.5. Classification of Ion-Selective Electrodes

Ion-Selective electrodes may be classified according to the membrane type used.

1) Solid-State membrane electrodes based on crystalline materials
2) Glass Membrane electrodes
3) Electrodes which have a charged carrier as the electroactive agent dispersed in a water-immisible solvent or held in an inert matrix
4) Electrodes which have a neutral carrier as the electroactive agent dispersed in a water-immisible solvent or held in an inert matrix
5) Special electrodes such as enzyme electrodes, gas electrodes [5]

As neutral carrier ionophores were used mainly in this work, most discussion will focus on these compounds.

2.6. Liquid Membrane electrodes

Liquid membrane sensors are widely used in potentiometric arrangements. Initial membranes consisted of the ion sensing material or ionophore, dissolved in a suitable liquid phase, which was held in place by means of a ceramic frit or a filter paper soaked in the organic phase. With the development of poly(vinyl chloride) (PVC) membranes [6], this unsatisfactory arrangement was discontinued.

A typical PVC membrane may be made by dissolving the ionophore in a suitable plasticiser or mediator. The PVC is then added and stirred to give a slurry and a volatile solvent e.g. tetrahydrafuran (THF) is used to dissolve the mixture. The membrane cocktail is poured into a suitable mould and the solvent, usually tetrahydrafuran is allowed to evaporate, forming a self-supporting, flexible and mechanically strong membrane. Details of this method are available in reference [7]. The composition of such a membrane is usually

1% Ionophore

66% Plasticiser

33% PVC

An additive such as potassium tetra-kis(p-chlorophenyl) borate is commonly added to improve the characteristics of the membrane such as stability, resistance to anion interference, and lowering membrane impedance [8]. Care should be exercised when using this additive as its presence is known to affect the selectivity, with larger cations such as caesium being preferentially exchanged. The function of the membrane may also be influenced by the choice of solvent used. An electrode using the ion-exchanger, di-n-octylphenylphosphonate, may be converted to a "water hardness" electrode by using decanol as the solvent [9]. The above composition is typical of a membrane using a neutral carrier ionophore.

2.6.1. Neutral Carriers

A neutral carrier ionophore is an electrically neutral molecule that will form a reversible complex within the membrane with the ion of interest .

$$A^+(aq.) + X^-(aq) + L\ (Mem.) \rightleftharpoons LA^+(Mem.) + X^-(aq.) \qquad (11)$$

where A+ is the primary ion

L represents the ionophore

X^- is the anion (counter-ion)

When such a compound is contained in a membrane, it will facilitate the entry of certain cations into the membrane and the resulting complex will be mobile within the membrane. The selective complexing of cations by naturally occuring neutral carrier antibiotics was first noted in 1964 [10] and their excellent alkali ion complexing properties were recognised by Stefanac and Simon in 1966 [11]. The potential for neutral carriers to be used as active agents in liquid membrane electrodes was subsequently exploited and neutral carrier electrodes were introduced first in the late 1960's [12]. The use of naturally occurring neutral carriers such as Valinomycin and Nonactin has been complemented by the use of synthetic neutral carriers such as ETH 1001 for Ca^{2+} [13], and other macrocyclic ligands such as crown ethers [14] and calixarenes [15,16]. Neutral carrier ionophores are selective for their target ion, not because of a binding action but rather because they provide an environment of conformation and atomic interaction into which the ion fits.

2.6.2. Features of Neutral Carrier Compounds

The structural requirements for a neutral carrier to behave as an ionophore are summarised below:

1) The carrier should be composed of polar and non-polar groups and should have as high a lipophilicity as possible.

2) The carrier should be multidentate and able to assume a stable conformation that provides a cavity, surrounded by polar groups,

suitable for the uptake of a cation, while the non-polar groups form a lipophilic shell around the coordination sphere.

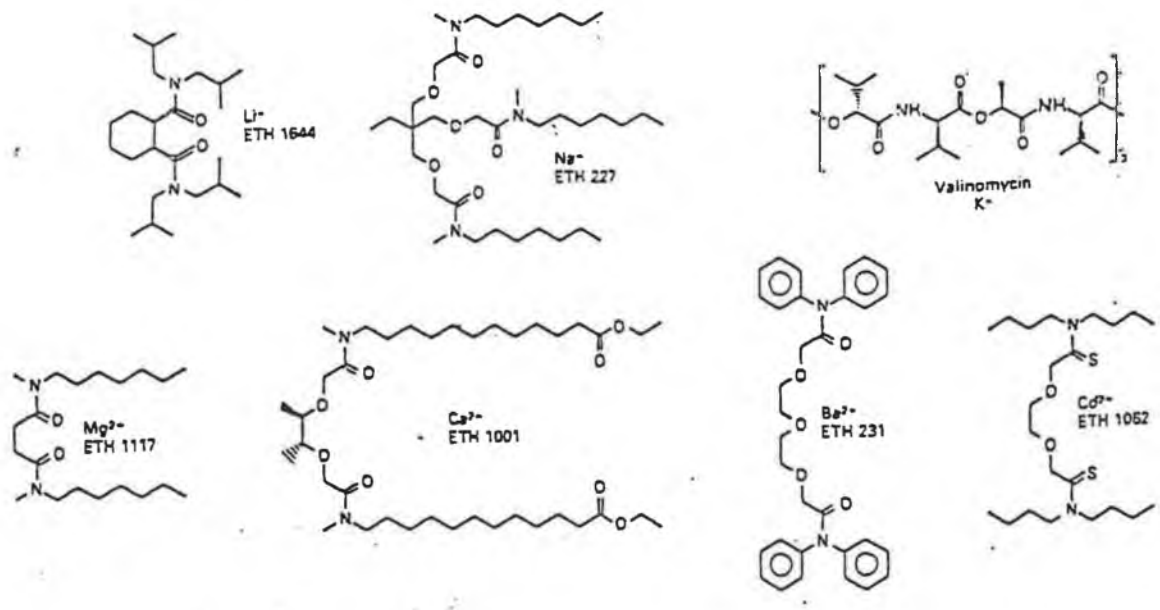
3 There should be 5-8 , but not more than 12, coordination sites preferably containing carbonyl or amide oxygen atoms which can provide strong ion-dipole interaction.

4) The coordination sites should form a rigid arrangement around the cavity. This rigidity may be enhanced by the presence of bridged structured e.g. hydrogen bonds. Within a group, the ion that fits the cavity best will be preferred.

5) The carrier should be flexible enough to allow a fast exchange of the primary ion between the complexed and uncomplexed state.

6) The carrier should be sufficiently small to allow mobility through the membrane [17]

Figure 2.4 Some cation selective ionophores which possess the characteristics described above [18]



The ionophore also has to induce semipermeability in the membrane and the selectivity depends on the Gibb's free energy change of transfer of ions from the aqueous phase to the membrane. The selectivity will depend on the selectivity of the carrier for the ion and also it will depend on the extraction properties of the plasticiser for the ion.

2.7. Characterisation of an Ion-Selective Electrode

The previous discussion has been about an ideal ion-selective membrane. i.e. a membrane with response characteristics which obey the Nernst equation. The membrane may be permeable to other interfering ions of similar charge to the primary ion which will contribute to the potential of the membrane. The effect of these interfering ions, j, on the potential of the membrane may be described by the Nikolskii/Eisenmann equation;

$$E_{cell} = E^{o} + S \ln [a_i + \Sigma (K_{ij}^{pot} a_j^{zi/zj})] \qquad (12)$$

where

K_{ij}^{pot} = the selectivity of the membrane for the primary ion, i, over the interfering ion j.

a_i= the activity of the primary ion i

a_j= the activity of the interfering ion j

z_i= charge of the primary ion i

z_j= charge of the interfering ion j

2.7.1. Selectivity

The selectivity coefficient is a measure of the preference by the membrane for the primary ion over the interfering ion. An ideally selective electrode would show selectivity coefficients for all interfering ions

approaching zero and the above relationship simplifies to the Nernst equation. Thus if the selectivity coefficient is known, the activity of the primary ion may be determined even in the presence of interfering ions.

The selectivity coefficient of the electrode to the various interfering ions may be experimentally determined by use of two main methods, the separate solution method and the mixed solution method.

2.7.1.1. Separate Solution (S.S.) Method

The potential of a fixed concentration of the primary ion i and the same concentration of the interfering ion j is measured and the selectivity coefficient may be calculated by the equation

$$K_{ij}^{pot} = 10^{\Delta E/S} \qquad (13)$$

where ΔE = difference between the potential of the interfering ion and the potential of the primary ion.

S = the slope of the electrode

This method is not particularly valid as it does not represent a realistic situation but its simplicity and ease of use means that it is usually employed when initial characteristic studies on a new membrane are performed. It is also only applicable for ions of the same charge.

2.7.1.2. Mixed Solution (M.S.) Method

This method is that recommended by the International Union of Pure and Applied Chemistry (I.U.P.A.C.) for the determination of selectivity coefficients. There are two variations on this technique;

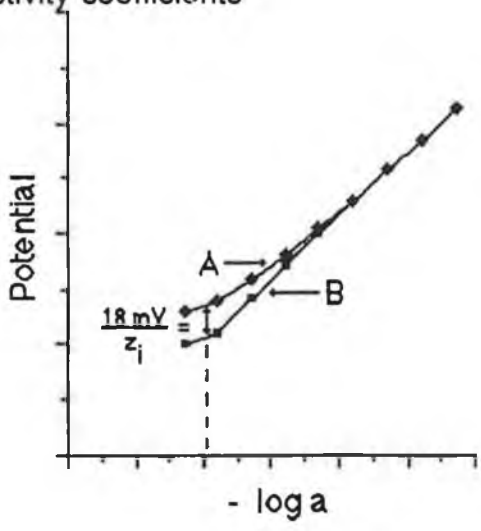
(a) a fixed concentration of the interfering ion, j, is contained in varying primary ion concentrations (usually between 10^{-6}M and 10^{-1}M). The

potential of these solutions are measured and plotted (curve A). Extrapolation of the straight line of the curve as shown in Figure 2.5 gives the activity of the primary ion producing the same potential as the interfering ion (curve B). The selectivity is given by

$$a_i = K_{ij}^{pot} a_j^{zi/zj} \qquad (14)$$

a_i may be determined by finding where curves A and B differ by $18mV/z_i$ and then using the equation above.

Figure 2.5 Graphical representation of Mixed Solution method for determination of selectivity coefficients



(b) In this case, the activity of the primary ion i, is kept constant, and the activity of the interfering ion j, is varied.

Selectivity coefficients quoted must be treated with caution and determined experimentally in accordance with its intended use as the selectivity coefficient depends on many parameters such as ionic strength, stirring rate, slope of the electrode, solution composition, and membrane composition.

2.7.2. Response Time

The reponse time may be defined as the time taken for the EMF to change from its initial value to a given limit of the final value e.g. 90% of its final value, t_{90}. The response time of the electrode should be as fast as possible, in the order of 30 seconds or less for commercial analysers.

Two methods can be used to determine the reponse time:

(a) The immersion method; In this case the electrode is simply immersed in a solution of the primary ion and the transient reponse monitored.

(b) The Injection Method; A small volume of a solution of the primary ion is made into a dilute solution which is being stirred rapidly. The response of the electrode is monitored as a function of time from the injection to the final steady-state potential using a chart recorder.

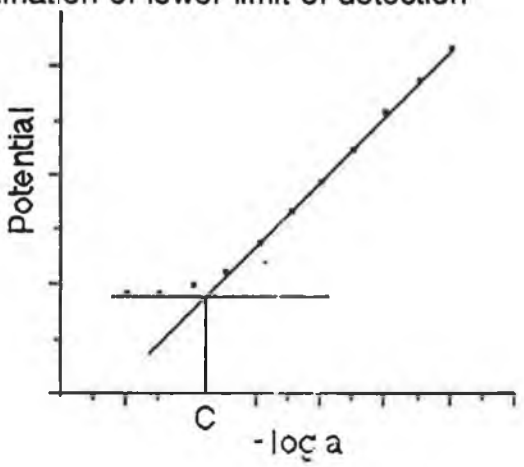
The processes that contribute to the response time of the membrane are many fold e.g. membrane type e.g. glass, solid-state, liquid membrane, the direction of the concentration change, speed of stirring, the free energy of activation of the carrier reaction with the primary ion, ionophore concentration.

2.7.3. Limits of Detection

The upper limit of detection for liquid membrane electrodes is approximately 0.1M of the primary ion. Immersion in solutions above this concentration result in the membrane being saturated with primary ion. The lower limit of detection is usually the more important quantity that restricts the use of ISEs for analysis. The IUPAC definition of the lower limit of detection states "The lower limit of detection is the concentration of the ion under investigation at which the extrapolated linear portion of the calibration graph

at extreme dilution of that ion intersects the extrapolated Nernstian portion of the graph". This is indicated by point C (Figure 2.6).

Figure 2.6 Determination of lower limit of detection



2.8. Desired Characteristics of Membranes for Clinical analysis

The discussion that follows is an attempt to specify the characteristics of the membrane that are required if the electrode is to be used in clinical samples e.g. whole blood, serum and plasma. The electrode should be made up from a well balanced optimisation of the following properties: selectivity, useful lifetime, stability and response time.

2.8.1. Selectivity

Clinical samples are complex with contributions from a range of different electrolytes and proteins whose normal concentrations in serum will be known. By using the Nicolskii/Eisenman equation, the required selectivity factor may be calculated by

$$K_{ij,max}^{pot} = \frac{(ai,min)}{(aj,max)^{z_i/z_j}} \times \frac{P_{ij}}{100} \qquad (15)$$

where $K_{ij,max}^{pot}$ = the highest tolerable value of the selectivity coefficient

$a_{i,min}$ = the lowest expected activity of the measuring ion i

$a_{j,max}$ = the highest expected activity of the interfering ion j

P_{ij} = the highest % tolerable error in the determined activity of the primary ion a_i due to interferences of a_j [19].

If this factor is achieved, it means that the interference due to interfering ions in the sample will be less than 1%.

2.8.2. Lifetime

The major source of membrane failure apart from mechanical failure e.g. contamination, tearing, is the loss of membrane components (ionophore, additives, plasticiser) into the sample. Such losses affect the selectivity and membrane resistance and they eventually result in membrane failure. To ensure a lifetime of at least one year, the partition coefficient, K, of the ionophore between the aqueous sample solution and the membrane phase should be larger than $10^{5.5}$ [19]. The lipophilic nature of the lipids and proteins in serum favours the extraction of these membrane components and to minimise this extraction, the ionophore should be as lipophilic as possible. It has also been shown that resistance measurements of the membrane may also be useful in giving some indication as to whether the membrane is nearing the end of its lifetime [20]

2.8.3. Stability

For clinical analysers, the stability of an electrode may be examined by the following criteria:

reproducibility

drift

shift in standard potential

As calibrations are performed during the run, the reproducibility of the potential of the standard and the sample will be of the prime importance. In in-vivo or process applications, where periodic calibration is not very feasible, the drift of electrode will be the important criteria. A shift in the standard potential will occur in serum samples due to the deposition of protein on the membrane surface but this may be minimised by the incorporation of an -OH group in the PVC used in the membrane or by the use of a highly lipophilic plasticiser [21]. The stability of the measurement will also be a function of the whole electrical circuitry and the stability of the reference electrode.

2.9. Accuracy of measurement

The electrode resistance plays an important role in the accuracy of potentiometric measurements. The definition of potentiometry specifies that it is a measurement of the potential at zero current. However, in order to measure the signal a finite current, i, has to flow in the circuit.

$$i=E/(R+r) \qquad (16)$$

where E = potential of the cell

R = input resistance of the meter

r = resistance of the measuring cell (usually dominated by the ISE membrane)

The current causes a potential drop, ΔE, across the cell and this will be the error of the reading. If the maximum error tolerable is 0.1%, then $\Delta E/E < 0.001$. To fulfil this requirement , R must be greater than 999r. Therefore the meter input resistance must be at least 1000 times greater than the resistance of the electrode membrane.

The discrimination of the meter will be important. Evident from the Nernst equation is the fundamental fact that an error of 0.1 mV is equivalent to an error of 0.4% for monovalent ions and 0.8% for divalent ions at all measured concentrations.

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3 Reference Method for the Measurement of Ionised Calcium in Human Blood, Serum and Plasma

3.1. History of ionised calcium measurement

The majority of calcium found in the body is found in the bone. It exists largely in the form of Hydroxyapatite, a crystalline structure composed of calcium, phosphate and hydroxyl ions. Bone acts as the reservoir for calcium and is involved in a continuous process of resorption and renewal. The most important fraction of calcium is found in the extracellular fluid and is one of the most important ions circulating in the body as it plays important roles in many life sustaining processes e.g. neuromuscular and cardiac activity, blood clotting, and in bone and teeth formation. Calcium is present in blood in three distinct forms

(1) Nondiffusible (protein bound)

(2) Diffusible nonionised (as complexes or chelates)

(3) Ionised calcium

The importance of calcium in the body was first demonstrated by Ringer in 1883 [1]. Serum calcium was found by dialysis experiments to be present in two forms, protein bound or nondiffusible and diffusible [2] and McLean and Hastings then showed that the diffusible fraction could be further divided and showed that this extra fraction, the ionised or free fraction was the biologically active fraction [3]. They also noted a relationship between the protein and pH of the serum and ionised calcium and so developed the first nomogram or algorithm for the estimation of ionised calcium using total calcium determinations [4]. Over the next two decades, further refinements to

the nomogram took place but always a direct method of measurement was sought. Many different methods were tried such as bio-assay and photometric techniques but these were labour intensive and prone to interference and so never replaced the measurement of total calcium as the method of choice.

The development of a direct method of analysis for ionised calcium came with the rapid development in potentiometry that took place in the 1960s and 1970's. Ross developed the first calcium ion-selective electrode [5]. Improvements in ionised calcium measurement have come hand in hand with developments in potentiometric techniques. The development of PVC membrane technology [6], i.e. immobilisation of an active agent in a poly (vinyl chloride) matrix, allowed electrodes with longer lifetimes to be manufactured and made clinical analysers feasible. Simon et. al. developed the neutral carrier ionophore, ETH 1001, in 1972 [7] and this has since become the most popular ionophore for calcium measurement [8]. Further improvements in electronics and microprocessor technology has resulted in analysers that can measure pH and ionised calcium simultaneously and so calculate the ionised calcium level corrected to a pH of 7.40. This value of pH is taken to represent the "normal" blood pH in human subjects.

3.2. Reason for Study

While ionised calcium measurements have been possible for the past 20 years, there has been considerable reluctance to favour the use of ionised calcium measurements. This reluctance to change may be due to the earlier unreliability of ionised calcium measurement or the absence of a reference method. Since the potentiometric determination of ionised calcium is a

comparative one, the analyser (ISE) needs to be calibrated. A group of like-minded scientists and clinicians met in Copenhagen in May of 1982 to address the issue of a reference method for ionised calcium. This was important as the standardisation of measurement and equipment was clearly needed. The International Federation of Clinical Chemistry (IFCC) laid down the specifications for primary standards [9] as they worked towards their stated aim of developing a reference method for ionised calcium.

A subsequent EC project, RM 380, was established, the aims of which were to "work out through collaborative studies, the details and specifications of the IFCC measurement procedure". Bowers [10] first outlined the steps taken in the development of a reference method for total serum calcium and then using a similar rationale, stated the requirements for a reference method for ionised calcium. The sequence of events needed to develop a reference method can be summarised as follows:

(1) The measuring units must be decided upon (mM)

(2) A definitive method must be proposed (None Available)

(3) A primary reference material must be established ($CaCO_3$)

(4) A reference method must be decided on if no definitive method available. (The method of choice was Potentiometry)

(5) Secondary reference materials must be created (in order to validate the method)

(6) The method must be validated in expert laboratories

(7) Finally, the method must be introduced to a field application

The ultimate aim of this endeavour was to establish some criteria for the measurement of ionised calcium i.e. units to use, calibration, quality control standards etc.. The details of the proposed reference method were defined

[11,12] and when the method had been shown to be practical, the study protocol was established.

Figure 3.1 Steps taken in the development of the proposed reference method for the measurement of ionised calcium.

Reference Method for the Determination of Blood ionised calcium

International Federation of Clinical Chemistry = IFCC

(defined the units of measurement, primary standards)

↓

European Working Group on Ion-Selective Electrodes = EWGISE

(designed the cell to be used in the development of the reference method and assessed its capabilities)

↓

Commission of the European Communities BCR programme for applied Metrology and Chemical Analysis

(provided the financial backing to allow the European-wide collaboration study)

↓

EC Project RM 380

"Ionised Calcium in Human blood serum and plasma"

3.2.1. Study Protocol

Twelve Covington reference cells (hereafter referred to as the CRM) were distributed to selected laboratories throughout Europe. It was decided that parallel studies with commercial analysers should be performed with the same transfer standards as used with the reference method [13]. This would give an indication of the variability of ionised calcium measurement in expert laboratories.

3.2.2. Aims of the Study

1 Establish the reference cell for ionised calcium measurement

2 Test the reference cell in different laboratories.

3 Establish and test Quality Control protein-based standards

4 Determine the between- and within- laboratory precision for both reference cell and commercial instruments.

5 Establish the relative importance of factors affecting variability such as : carbon dioxide, carryover, protein build up, choice of primary, secondary calibration solutions versus quality control solutions [14].

3.3. Experimental procedure

3.3.1. Reagents and Materials

The reagents for the study consisted of three calibrating solutions:

Solution 1 1.25 mM Ca^{2+} (Primary Calibrating solution)

Solution 2 0.40 mM Ca^{2+}

Solution 3 2.50 mM Ca^{2+}

Each solution was adjusted to an ionic strength of 0.16 M by the addition of NaCl. The ionised calcium concentration of five aqueous samples, H1 to H5, six protein containing samples, B1-B3, D1-D3, and six Human serum samples, HS1 to HS6, was measured using the CRM. The solutions were contained in air-tight ampoules to prevent pH changes due to the atmosphere.

The flush solution with which the CRM was cleaned was a 1.25 mM $CaCl_2$ solution with an ionic strength of 0.16M and was prepared using a 1.0M stock solution of $CaCl_2$ and analar grade sodium chloride. The internal filling solution for the external reference electrode was a saturated potassium chloride solution at 37°C and was prepared using analar grade KCl. The internal filling solution for the calcium electrode was prepared by saturating the 1.25 mM primary standard, Solution 1, with AgCl. Distilled deionised water was used in the preparation of all solutions.

3.3.1.1. Preparation of solutions used in the study

The chemicals used in the preparation of solutions and samples H,B,D, and HS were as follow: analar grade 1M $CaCl_2$ (BDH), NaCl, KCl, $MgCl_2.6H_20$, $LiCl.H_20$, and $NaHCO_3$ (all Riedel-de Haen), and NaHEPES,HEPES, Bovine albumin (all Sigma) were used. Distilled, carbon dioxide-free water was used as the diluent.

The primary calibration solutions were prepared by weighing appropriate amounts of NaCl, 1M $CaCl_2$ and water.

The secondary calibration solutions, H1-H5 were prepared by weighing appropriate amounts of NaCl, 1M $CaCl_2$, NaHEPES, HEPES and water (cf Section 3.4.3., p 56 for details)

The Protein containing samples, B, were prepared by using concentrated bovine albumin solution (170 gL^{-1}). To this, appropriate amounts of NaCl, 1M $CaCl_2$, $MgCl_2.6H_20$, $LiCl.H_20$, NaHEPES, HEPES and water were added. The final albumin concentration was approximately 70gL^{-1}. This solution was then filtered through sterile 0.22mm filters.

Table 3.1 Composition of the protein solutions , B and D, used in the evaluation of the precision of the CRM. Solutions D were tonometered with CO_2 to a preset pH. pH given was measured by the ICA2 at our laboratory.

Sample	Composition	pH
B1	Normal pH, Na^+, K^+, Li^+, Ca^{2+}, Mg^{2+}, TCa, Cl^-	7.40
B2	Low pH, Na^+, K^+, Li^+, Ca^{2+}, Mg^{2+}, TCa, Cl^-	7.21
B3	High pH, Na^+, K^+, Li^+, Ca^{2+}, Mg^{2+}, TCa, Cl^-	7.59
D1	Normal pH, pO_2, pCO_2, Na^+, K^+, Li^+, Ca^{2+}, Mg^{2+}, TCa, Cl^-	7.40
D2	High pH, Na^+, K^+, Li^+, Mg^{2+}, TCa, Cl^- Low pO_2, pCO_2, Ca^{2+}	7.58
D3	Low pH, Na^+, K^+, Li^+, Mg^{2+}, TCa, Cl^- High pO_2, pCO_2, Ca^{2+}	7.21

The Protein containing samples, D, were prepared as above except that $NaHCO_3$ was added and the solution was equilibrated with CO_2/N_2 gas mixtures to achieve the desired pH values. A guide to the composition of samples B and D is given in Table 3.1. The exact composition of these samples were not known as part of the "blind" testing protocol.

Human sera samples were prepared from a serum pool by addition of appropriate amounts of NaCl, 1M $CaCl_2$, $MgCl_2.6H_20$, and $LiCl.H_20$. Different salt concentrations provided six distinct calcium levels for ionised calcium determination and all were at physiological pH. Each sterile sample pool was tonometered with an appropriate CO_2/N_2 gas mixture used for tonometry. The samples simulated the clinically significant range for ionised calcium. The composition of the sample are given in Table 3.2

The ionised calcium concentration of the protein containing samples B, D, and HS, was assigned by measurements using four Radiometer ICA2 analysers at the coordinators laboratories at Newcastle-upon-Tyne and Utrecht.

Table 3.2 Assigned ionised calcium concentration and ionic composition of the Human sera samples ,HS1-HS6, used in the evaluation of the CRM. (The ionised calcium concentration was measured by four ICA2 analysers in co-ordinators laboratories and Na^+ and K^+ concentration was measured by FAAS)

Sample	Assigned Ca^{2+} mM	Na^+ mM	K^+ mM
HS1	1.76	122.8	8.78
HS2	1.53	130.3	7.33
HS3	1.22	140.8	5.68
HS4	1.11	145.4	5.02
HS5	0.88	153.4	3.85
HS6	0.69	157.1	2.81

The compositions of the calibration solutions of the commercial analysers are given in the Appendix A.

3.3.2. Apparatus

3.3.2.1. The Covington Reference Method apparatus (CRM)

The cell design used in the study was that cell which had been proposed by Covington as part of the reference method (CRM) at the IFCC meeting in Stressa in 1988 [12] and is shown in Figure 3.2. The ionophore used in the selective membrane of the CRM was the ETH 1001 neutral carrier and the composition of the membrane may be found in [12]. The membranes used in the study were prepared in Professor Covington's laboratory in accordance with recognised practices. The reference electrode salt bridge solution of the CRM was a KCl solution which had been saturated at 37°C. The temperature of the CRM was maintained at 37°C by means of a water jacket, fed from a thermostated water bath via a circulating pump. The temperature was measured using the temperature probe of the Jenway 3040 ion-analyser, which has a 0.1°C discrimination. The reference half-cell consisted of a commercial calomel electrode immersed in a saturated KCl solution in the reference vessel, (Figure 3.2). An open liquid junction between the sample solutions and the salt bridge was formed by suspending a capillary contact into the KCl reservoir as shown in Figure 3.2. Electrical contact with the sample is maintained via the opening between the two compartments of the reference vessel. The potentials generated were measured by a Jenway 3040 ion-analyser which had a 0.1 mV discrimination as laid down in the specifications [12].

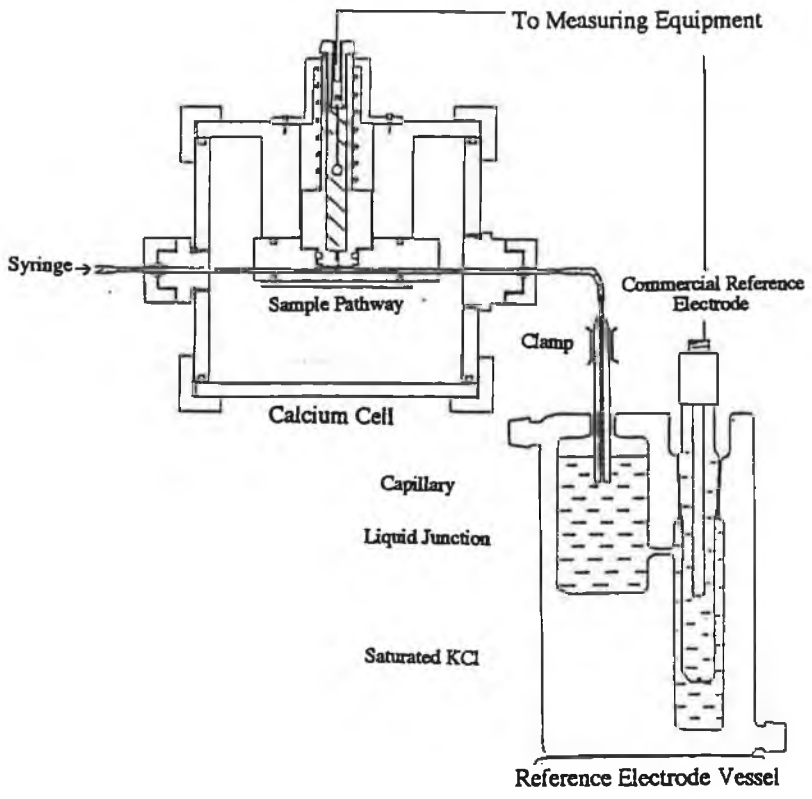
The initial step was to assemble the CRM and details of the construction are given in Appendix B. The performance had then to be established and shown to confirm to the specifications laid down in references [11,12].

Table 3.3. Required performance criteria for the CRM [12]

Criteria	Specifications
Relative Sensitivity*	1.00, +0.02, -0.05
Response Time	< 30 seconds
Stability	< 0.13 mV hr^{-1}

* Relative Sensitivity = Measured slope/Theoretical slope

Figure 3.2 Experimental set-up of the CRM in measurement mode.



3.3.2.2. The commercial calcium analysers

The ionised calcium concentration of the samples were measured using two commercial analysers: the Radiometer ICA2 analyser (ICA2), and the Baker Analyte+2 (Baker). The calcium sensing agent used in the commercial analysers was calcium (di-n-octylphenyl) phosphate ion-exchanger. The reference electrode salt bridge solution in the ICA2 was a 4.6 molal sodium formate solution while the Baker used a 1.5 M KCl solution. Both analysers utilised an open static liquid junction and gave simultaneous pH measurement. The recommended calibration solutions and aspiration techniques were used when measuring the sample and each of the solutions were measured in triplicate.

3.3.3. CRM sample introduction

The sample was introduced by means of aspiration. The following procedure was performed.

All samples measured were placed in the water bath so that they would be in thermal equilibrium before being aspirated into the CRM. This was done as it has been shown that it take approximately 4-5 minutes for the sample to reach 37°C in the sample pathway when the sample is at room-temperature, compared to approx. 2 minutes when it has been at 37±3°C [15].

1 A syringe with 3 mL of flush solution was attached to the cell by means of a short length of tubing.

2 This solution was pushed slowly through the sample pathway and during flushing, air was allowed to pass through the cell at least three times .

This was achieved by withdrawing the syringe from the sample pathway. The solution would then drain under gravity.

3 The sample to be measured was then drawn carefully into a syringe from the ampoule.

4 The syringe was inverted and any trapped air removed. This was then attached to the cell and the sample passed through the cell until seven drops had escaped from the capillary tip.

5 The syringe was removed and the sample allowed to drain. This allowed air to enter the system and helped minimise carry-over from previous samples. This was twice repeated and finally the sample was passed through the cell until ten drops escaped from the capillary tip.

6 The excess sample was carefully removed from the capillary tip before immersing in the reference vessel as shown in figure 3.2.

3.3.4. Measurement Protocol

7 After placing the capillary in the KCl, the potential of the cell was recorded every 30 seconds and a final reading was taken after 3 minutes.

8 The capillary was removed from the KCl, the syringe removed and the sample allowed to flow to waste.

Steps **1** to **8** were carried out for all aqueous solutions, both calibrating and samples. The procedure was modified slightly when protein containing samples were measured in that in step 1, 5 mL of flush solution rather than 3 mL was used to clean the sample pathway between samples. Each sample was measured in quintuplicate by the CRM. Carryover between solutions was not found to be a problem.

3.3.5. Calibration and Calculation of Results

The results for the samples measured were calculated by a mathematical interpolation using the potentials measured for the calibration solutions (1 to 9) and the potentials for the sample in question, measured in accordance with the following sequence.

Number	Solution Type	Potential
1	Solution 1	$E_{1,1}$
2	Solution 2	$E_{2,1}$
3	Solution 1	$E_{1,2}$
4	Solution 3	$E_{3,1}$
5	Solution 1	$E_{1,3}$
6	Solution 2	$E_{2,2}$
7	Solution 1	$E_{1,4}$
8	Solution 3	$E_{3,2}$
9	Solution 1	$E_{1,5}$
10	Sample X	$E_{x,1}$
11	Solution 1	$E_{1,6}$
12	Sample X	$E_{x,2}$
13	Solution 1	$E_{1,7}$

The concentration c_x of the ionised calcium in the samples were calculated as follows.

$$c_x = c_1 . 10^{y}$$

and

$$y = \frac{\Delta E_x}{\Delta E_s} \log \frac{c_s}{c_1}$$

where c_X = the sample concentration of ionised calcium in mM

c_1 = the midpoint calibration solution (solution 1)

c_S = the second calibration solution of ionised calcium which should be solution 2 when $c_X < 1.25$ mM or solution 3 when $c_X > 1.25$ mM.

ΔE_X = the average potential difference between the midpoint solution and the sample

ΔE_S = the average difference between the midpoint solution and the second calibration solution.

$$\Delta E_X = 1/4 \{ (E1,5 - Ex,1) + (E1,6 - Ex,1) + (E1,6 - Ex,2) + (E1,7 - Ex,2)\}$$

$$\Delta E_S = 1/4 \{ (E1,1 - E1,2) + (E1,2 - E2,1) + (E1,3 - E2,2) + (E1,4 - E2,2)\}$$

or depending on the concentration

$$\Delta E_S = 1/4 \{ (E1,2 - E3,1) + (E1,3 - E3,1) + (E1,4 - E3,2) + (E1,5 - E3,2)\}$$

Relative Sensitivity

The relative sensitivity S of the electrode is defined as:

$$S = \frac{g}{g^o}$$

where g = sensitivity of the electrode

g^o = theoretical Nernstian sensitivity

and

$$g_2 = \frac{\Delta E_S}{\log c_1 - \log c_2}$$

$$g_3 = \frac{\Delta E_S}{\log c_1 \quad - \quad \log c_3}$$

g_2 = sensitivity of the electrode in the range 0.4 mM to 1.25 mM ionised calcium

g_3 = sensitivity of the electrode in the range 1.25 mM to 2.50 mM ionised calcium.

The relative sensitivity $S_2(g2/g^o)$ and $S_3(g_3/g^o)$ of the CRM should not deviate more than +0.02 to -0.05 from the theoretical value (1.00) [11].

The calculation of sample concentration and sensitivity was performed using a rudimentary computer program written in GWBASIC. A printout of this program may be viewed in the appendix B.

The measurement and calculation of the concentration of ionised calcium in both commercial analysers was under microprocessor control.

3.4. Results

The results obtained in the study using the CRM and commercial analysers are summarised in tables 3.5, 3.6, and 3.7. The solutions were analysed in the following sequence: H1 - H5, B1, B2, D1, D2, HS1, HS3, HS2, HS5, HS6, HS4, B3, D3.

3.4.1. Performance of the CRM

3.4.1.1. Sensitivity

The relative sensitivity, S, of the CRM, S_2 and S_3, was determined before each sample measurement. A summary of the relative sensitivities as measured before each analysis is given in Table 3.4. The relative

sensitivities were within the specifications outlined in Table 3.1 on all but 3 occasions, H1 and S_2 of B2.

Table 3.4 A summary of the relative sensitivities, S_2 and S_3, measured during the analysis of the European standards. Values given are the average of 5 measurements.

Solution	S_2 ± s.d.	S_3 ± s.d.
H1	0.9047 ± 0.0050	0.9468 ± 0.0044
H2	0.9585 ± 0.0057	0.9878 ± 0.0038
H3	0.9520 ± 0.0057	0.9813 ± 0.0059
H4	0.9549 ± 0.0062	0.9824 ± 0.0095
H5	0.9552 ± 0.0027	0.9846 ± 0.0048
B1	0.9572 ± 0.0028	0.9873 ± 0.0052
B2	0.9458 ± 0.0222	0.9710 ± 0.0161
B3	0.9598 ± 0.0126	1.0029 ± 0.0287
D1	0.9625 ± 0.0042	0.9916 ± 0.0045
D2	0.9628 ± 0.0060	0.9894 ± 0.0062
D3	0.9648 ± 0.0032	0.9905± 0.0054
HS1	0.9700 ± 0.0039	0.9829 ± 0.0098
HS2	0.9690 ± 0.0050	0.9932 ± 0.0054
HS3	0.9680 ± 0.0048	0.9910 ± 0.0040
HS4	0.9666 ± 0.0121	0.9837 ± 0.0145
HS5	0.9668 ± 0.0030	1.0018 ± 0.0195
HS6	0.9680 ± 0.0030	0.9975 ± 0.0243

3.4.1.2. Speed of Response

The response time may be defined as the time taken for the EMF to change from its initial value to a given limit of the final value e.g. 90% of its final value, t_{90}. The response time of the electrode should be as fast as possible, in the order of 30 seconds or less for commercial analysers. The potential of the CRM was found to become stable and steady after 30 seconds and typical response data are given in Table 3.2. These data are for solution D3 and it is obvious that the t_{90} value was reached within 30 seconds.

Table 3.5. Typical response data from the measurement of a D3 solution

Potential	Time (seconds)					
(mV)	30	60	90	120	150	180
$E_{1,1}$	25.9	25.8	25.8	25.9	25.9	26.0
$E_{2,1}$	11.3	11.3	11.1	11.2	11.3	11.3
$E_{1,2}$	26.0	25.9	25.9	25.9	25.9	26.0
$E_{3,1}$	35.3	35.3	35.2	35.3	35.3	35.3
$E_{1,3}$	26.0	26.1	26.0	26.0	26.0	26.0
$E_{2,2}$	11.2	11.3	11.4	11.3	11.3	11.3
$E_{1,4}$	25.9	25.0	25.9	25.9	25.9	25.9
$E_{3,2}$	35.1	35.1	35.1	35.1	35.1	35.1
$E_{1,5}$	26.0	25.9	26.0	25.9	25.9	26.0
$E_{x,1}$	30.7	30.5	30.5	30.4	30.2	30.3
$E_{1,6}$	25.9	25.8	25.8	25.8	25.9	25.8
$E_{x,2}$	30.5	30.4	30.5	30.3	30.3	30.2
$E_{1,7}$	25.6	25.6	25.7	25.7	25.8	25.7

3.4.2. The European Average or Euro. Ave.

The CRM was used in 10 expert laboratories throughout Europe and the data collected at the laboratories incorporated as the "Euro. Ave." or averaged results of the laboratories involved. The data given [14] includes the data from 8 of these expert laboratories, 2 laboratories being excluded as outliers. Our laboratory is included in this Euro. Ave.

3.4.3. Aqueous samples, H

These were solutions whose ionised calcium concentration are well defined. They were composed of varying concentrations of calcium, in the form of $CaCl_2$, and contained 4.06 mM N-2-Hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES), 5.00 mM NaHEPES and NaCl to give a final ionic strength of 0.16M. The results (Table 3.6) show an obvious negative bias between the assigned result and the obtained result using the CRM.

Table 3.6 A summary of the concentrations of the solutions H1-H5 as measured by the three analysers and the grand average for all CRMs used (including our CRM). Also given is the % difference or bias between the actual concentration and the measured concentration.(n = 5 for CRM, n = 3 for ICA2 and Baker and n = 40 for Euro. Ave.)

Sample	Analyser	Exact Ca^{2+} mM	Measured Ca^{2+} mM	s.d. mM	% Bias	%CV
H1	CRM	0.10	0.095	0.0049	- 5.00	5.16
	ICA2	0.10	0.160	0	+60.00	0
	Baker	0.10	0.137	0.0058	+37.00	4.23
	Euro. Ave.	0.10	0.104	0.0164	+4.00	15.76

Sample	Analyser	Exact Ca^{2+} mM	Measured Ca^{2+} mM	s.d. mM	% Bias	%CV
H2	CRM	7.00	6.722	0.0608	- 4.00	0.90
	ICA2	7.00	6.857	0.0058	- 2.04	0.08
	Baker	7.00	7.263	0.0416	+3.76	0.57
	Euro. Ave.	7.00	6.832	0.2831	- 2.40	4.14
H3	CRM	2.00	1.918	0.0045	- 4.10	0.23
	ICA2	2.00	1.963	0.0058	- 1.85	0.29
	Baker	2.00	2.013	0.0058	+0.65	0.23
	Euro. Ave.	2.00	1.946	0.0304	-2.70	1.56
H4	CRM	0.75	0.714	0.0055	- 4.80	0.77
	ICA2	0.75	0.767	0.0058	+2.27	0.76
	Baker	0.75	0.767	0.0058	+2.27	0.76
	Euro. Ave.	0.75	0.733	0.0312	- 2.67	4.26
H5	CRM	1.25	1.200	0.0158	- 4.00	1.32
	ICA2	1.25	1.243	0.0058	- 0.56	0.47
	Baker	1.25	1.267	0.0058	+1.36	0.46
	Euro. Ave.	1.25	1.226	0.0224	- 1.92	1.83

A negative bias of approximately 4% was observed when the aqueous samples were measured using the CRM. This negative bias was also

experienced by the other CRMs in the study, Euro. Ave., but not to the same extent. The precision of the measurements with the CRM were good in all cases except solution H1. The %CVs within the physiological range were 0.23% for H3, 0.77% for H4 and 1.32% for H5.

The commercial analysers gave results comparable to the exact concentration at all measurements except the lowest concentration standard, H1, where a large bias of 60% for the ICA2 and 37 % for the Baker was recorded. There appeared to be no systematic bias with the commercial analysers as was experienced by the CRM. As expected, the precision of the commercial analysers was excellent.

3.4.4. Protein containing samples, B and D

There was no systematic bias obvious in the measured ionised calcium in the protein containing solutions using the CRM.

Table 3.7 A summary of the assigned concentration of protein containing samples B and D and the concentrations as measured with the three analysers (n = 5 for CRM, n = 3 for ICA2 and Baker and n = 40 for Euro. Ave.)

Sample	Analyser	Assigned Ca^{2+} mM*	Measured Ca^{2+} mM	s.d. mM	% Bias	%CV
B1	CRM	1.25	1.292	0.0192	+ 3.36	1.49
	ICA2	1.25	1.223	0.0058	- 2.16	0.47
	Baker	1.25	1.227	0.0058	- 1.84	0
	Euro. Ave.	1.25	1.277	0.0564	+ 2.16	4.42

Sample	Analyser	Assigned Ca^{2+} mM*	Measured Ca^{2+} mM	s.d. mM	% Bias	%CV
B2	CRM	0.76	0.750	0.0122	- 1.31	1.63
	ICA2	0.76	0.730	0	- 3.95	0
	Baker	0.76	0.730	0.0100	- 3.95	1.37
	Euro. Ave.	0.76	0.726	0.0280	- 4.47	3.86
B3	CRM	1.76	1.758	0.0268	- 0.11	1.52
	ICA2	1.76	1.750	0	+ 1.14	0
	Baker	1.76	1.780	0	+ 1.14	0
	Euro. Ave.	1.76	1.793	0.0564	+ 1.87	3.15
D1	CRM	1.25	1.238	0.0130	- 1.20	1.05
	ICA2	1.25	1.230	0	- 1.60	0
	Baker	1.25	1.267	0.0058	+ 1.36	0.46
	Euro. Ave.	1.25	1.252	0.0603	+ 0.16	4.81
D2	CRM	0.75	0.664	0.0055	- 11.47	0.83
	ICA2	0.75	0.690	0	- 8.00	0
	Baker	0.75	0.677	0.0058	- 9.73	0.85
	Euro. Ave.	0.75	0.669	0.0375	- 10.80	5.60

Sample	Analyser	Assigned Ca^{2+} mM*	Measured Ca^{2+} mM	s.d. mM	% Bias	%CV
D3	CRM	1.73	1.738	0.0045	+ 0.46	0.26
	ICA2	1.73	1.710	0.0265	- 1.16	1.54
	Baker	1.73	1.760	0.0100	+ 1.73	0.57
	Euro. Ave.	1.73	1.756	0.0733	+ 1.48	4.17

* Assigned values as described p 46

The CRM gave results which closely matched the assigned concentrations for the B and D solutions with one exception, that of solution D2. This was also noted with the two commercial analysers. The precision of the D solutions were better than that of the B solutions (cf Table 3.6) . Overall the precision of measurements with the CRM was excellent with %CVs <1.63% in our laboratory. This is in marked contrast to the inter-laboratory imprecision as shown by the Euro. Ave.

3.4.5. Human sera samples, HS

Measurements were made with Human serum to test the CRM with realistic samples. The CRM performed well and the precision was excellent with CVs in the range 0.37% to 1.52%. The concentrations measured by our CRM were consistently lower than those measured by the two other analysers (Table 3.8). At higher concentrations, the CRM gave values which were close to the assigned value but as the samples became less

concentrated, the measured value diverged from the assigned value (% Bias HS1= -2.27%, % Bias HS6 = -13.91).

Table 3.8 A summary of the concentrations of the Human sera samples as measured by the three analysers (n = 5 for CRM, n = 3 for ICA2 and Baker and n = 40 for Euro. Ave.).

Sample	Analyser	Assigned Ca^{2+} mM*	Measured Ca^{2+} mM	s.d. mM	% Bias	% CV
HS1	CRM	1.76	1.720	0.0122	- 2.27	0.71
	ICA2	1.76	1.727	0.0058	- 1.87	0.33
	Baker	1.76	1.700	0.0300	- 3.40	1.76
	Euro. Ave.	1.76	1.758	0.1144	- 0.11	6.51
HS2	CRM	1.53	1.476	0.0055	- 3.53	0.37
	ICA2	1.53	1.513	0.0058	- 1.11	0.38
	Baker	1.53	1.487	0.0058	- 2.81	0.39
	Euro. Ave.	1.53	1.513	0.0871	- 1.11	5.76
HS3	CRM	1.22	1.146	0.0114	- 6.06	0.93
	ICA2	1.22	1.190	0	- 2.46	0
	Baker	1.22	1.170	0	- 4.10	0
	Euro. Ave.	1.22	1.175	0.0634	- 3.69	5.39

Sample	Analyser	Assigned Ca^{2+} mM*	Measured Ca^{2+} mM	s.d. mM	% Bias	%CV
HS4	CRM	1.11	1.028	0.0084	- 7.39	0.82
	ICA2	1.11	1.073	0.0058	- 3.33	0.54
	Baker	1.11	1.050	0	- 5.40	0
	Euro. Ave.	1.11	1.046	0.0617	- 5.76	5.89
HS5	CRM	0.88	0.800	0.0122	- 9.09	1.52
	ICA2	0.88	0.857	0.0058	- 2.61	0.67
	Baker	0.88	0.837	0.0058	- 4.88	0.69
	Euro. Ave.	0.88	0.816	0.0352	- 7.27	4.31
HS6	CRM	0.69	0.594	0.0055	- 13.91	0.92
	ICA2	0.69	0.647	0.0058	- 6.23	0.89
	Baker	0.69	0.623	0.0058	- 9.71	0.93
	Euro. Ave.	0.69	0.597	0.0331	- 13.48	5.54

* Assigned values as described in p 46

The commercial analysers gave results which were closer to the assigned results. As before, the precision of these analysers were better than that of the CRM.

The results for ail solutions are graphically represented in Figures 3.3.

Figure 3.3 Graphical comparison of results from the analysers in our laboratory and the grand European Average, Euro. Ave., for aqueous H1-H5 solutions, protein containing solutions B1-B3,D1-D3, and Human sera HS1-HS6. (Results for H2 has been removed for the sake of clarity).

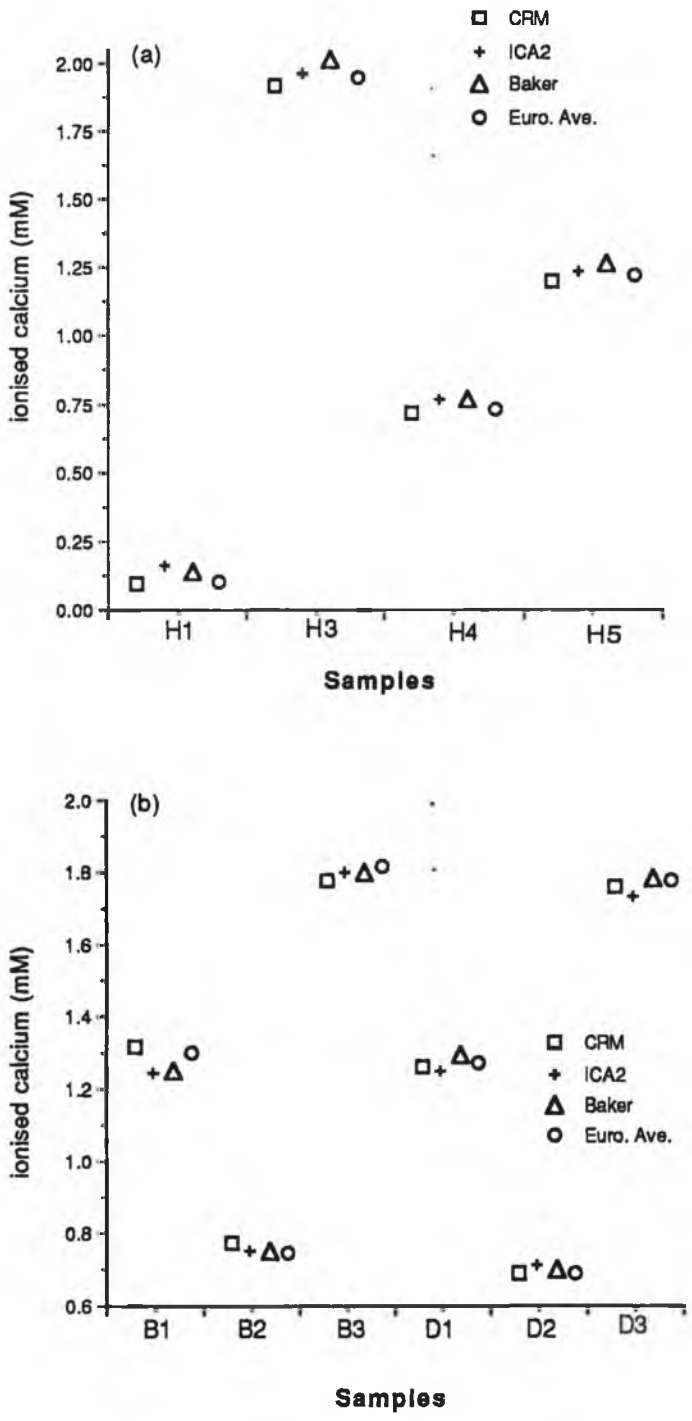


Figure 3.3 (Cont.) Graphical comparison of results from the analysers in our laboratory and the grand European Average, Euro. Ave., for the Human sera samples, HS1-HS6.

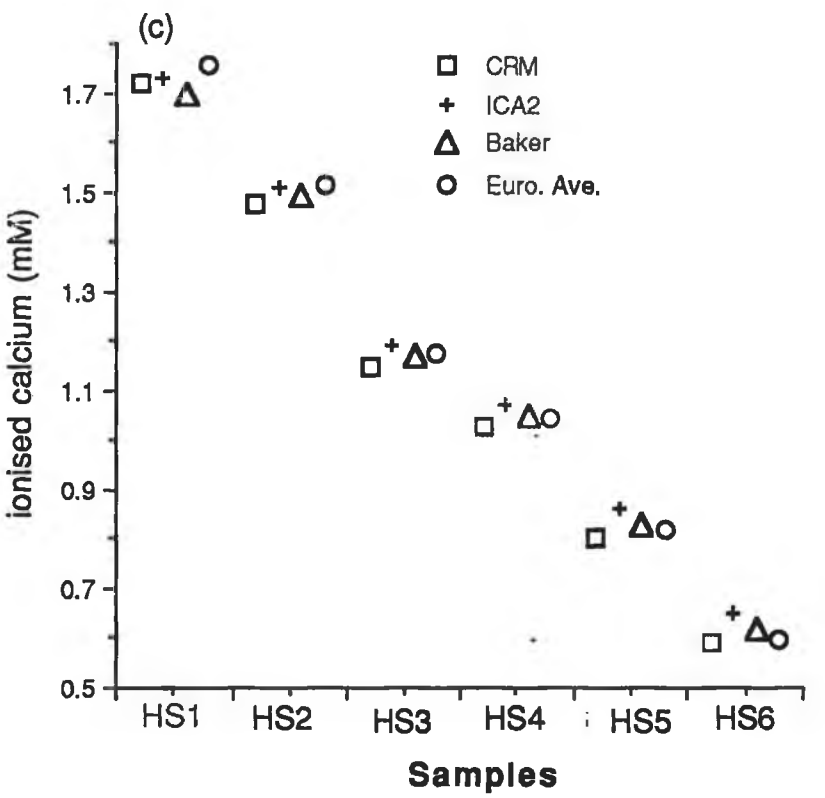


Figure 3.3 (Cont.) Comparison of measure ionised calcium versus assigned values for the analysers used in the study and the European Average, Euro. Ave..

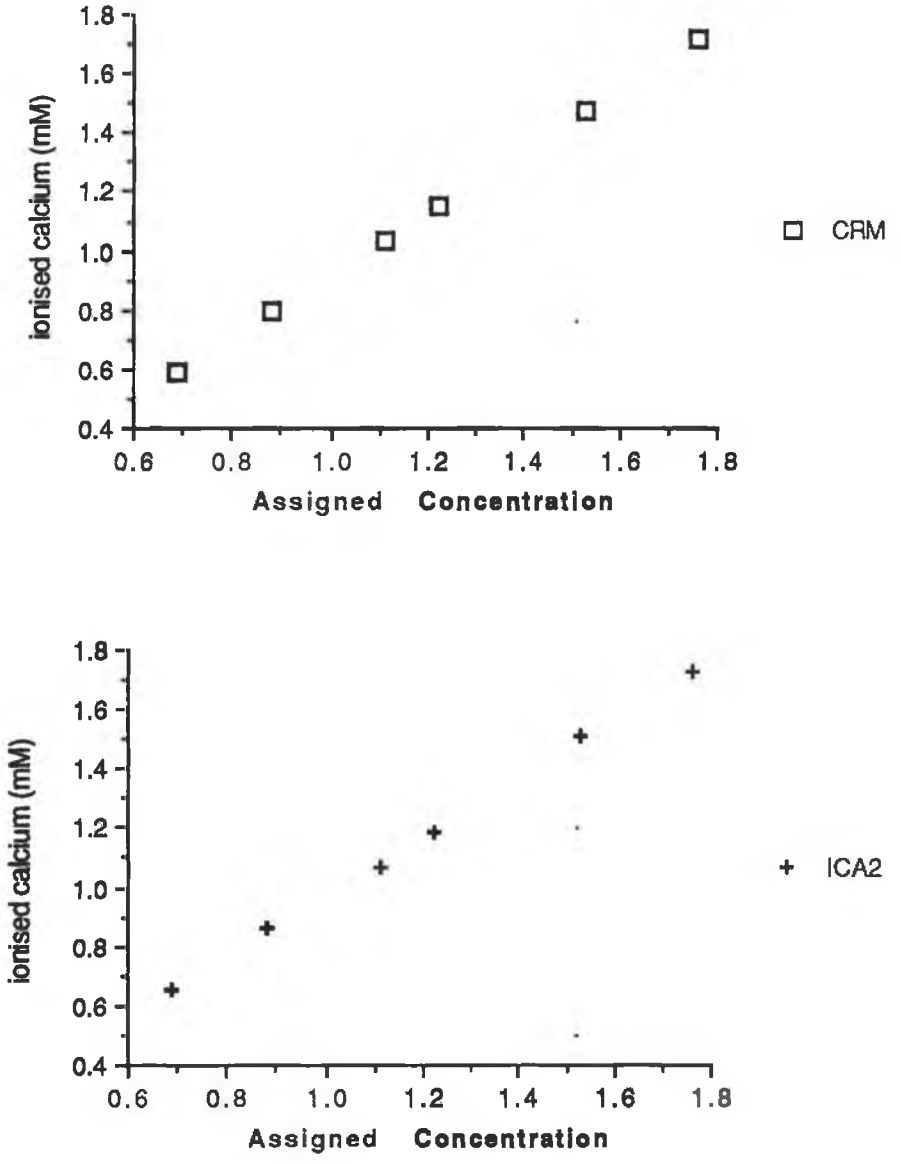
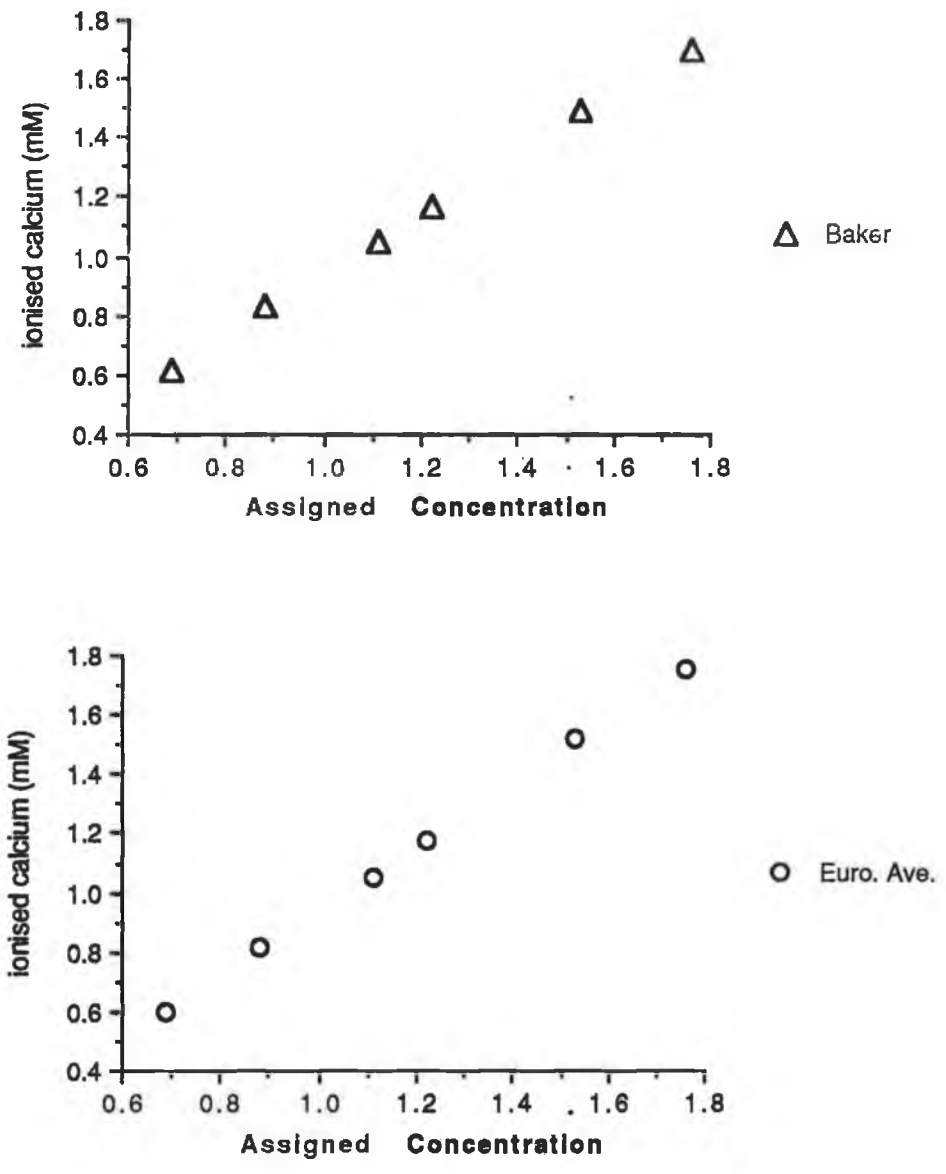


Figure 3.3 (Cont.) Comparison of measure ionised calcium versus assigned values for the analysers used in the study and the European Average, Euro. Ave..



3.4.6. Using the CRM

Shifts of 0.2-0.3 mV in the standard potential were observed after the first contact with a protein sample but after this initial rise, the signal became stable. There was a slow monotonic drift in the standard potential with continuous use of the CRM. Periodically, during the analysis of the Human sera, the potential of the cell would sometimes suddenly fall by 1-1.5 mV. After this, the cell would stabilise to this new potential and sensitivity was not affected

3.5. Discussion

The aim of this study was to develop a reference method for ionised calcium and to assess the feasibility of this method. The end use of such a method would be to create a method by which the accuracy and precision of commercial analysers might be assessed and defined. By definition, a reference method must have precision and accuracy commensurate with its intended use [10].

The accuracy of the analyser may be established using aqueous standards. Protein containing standards are difficult to formulate because of the binding of calcium to protein and the debate over the actual ionic strength of serum samples, 0.16M or 0.30M, if the effect of albumin is taken into account [16]. The comparative results obtained with the protein containing standards, H,D, and HS, give a measure of the relative precision of the CRM but cannot give a measure of the accuracy of the method, due to the reasons outlined above. Protein standards may be made in two ways, using pooled Human sera samples or made using a bovine serum albumin, BSA, solution and adding salts to mimic the ionic composition of serum. As pH plays an

important role in the calcium binding of protein, these BSA samples must be buffered. The samples analysed were carefully created to investigate the effect of buffers, electrolytes and protein type in order that quality control standard might be created.

The accuracy of the measuring cell was assessed by use of the aqueous solutions, H1-H5. The CRM was found to have an approximate 4% negative bias between the measured concentrations and the exact concentrations at all concentrations. The only difference between these solutions and the primary calibration solutions was the presence of HEPES/NaHEPES buffer, the ionic strength being constant at 0.16M. Therefore this consistent bias may be assumed to be due to the presence of this HEPES/NaHEPES. HEPES may affect the measurement in two ways. It may bind to the calcium in the solution and thus lower the actual presence of the calcium. Alternatively, it may result in a residual liquid junction potential (RLJP) due to the different compositions of the calibration solutions and the H standards. For concentrations HEPES/NaHEPES up to 50 mM, the effect of ion association may be excluded [17]. Therefore, the bias is most likely caused by a RLJP established between the primary calibration solutions and the H standards. The commercial analysers demonstrated greater accuracy in the aqueous solution than the CRM. This better accuracy is perhaps due to the calibration solutions, which contained buffers (cf Appendix A), used with these analysers which more closely matched the H1-H5 solutions than the primary calibrating solutions of the CRM.

As outlined in section 3.2.4., one of the aims was to test BSA vs Human serum based quality controls. The BSA standards also contained HEPES buffer which may lead to RLPJ errors but as the assigned

concentrations are already biased because they were measured using 4 Radiometer ICA2 analysers, the magnitude of this error may not be established. The CRM had precision comparable with the commercial analysers and as experience grew, this precision approached parity with the commercial analysers (Tables 3.6, 3.7 and 3.8). The precision of the commercial analysers should be inherently better then that of the CRM as they have a strict automated sampling and measurement regime. The concentration of the BSA protein solutions, B and D, as measured by the CRM were close to the assigned concentrations with one exception, that of solution D2 (Table 3.7). This was found to be markedly different from the assigned value using all analysers in our laboratory. Analysis of data from Europe showed that the majority of CRMs also experienced this deviation but it was not as evident with the other commercial analysers in the European study (cf Figure C4 in appendix C). This deviation must be due to the solution itself as it was measured using the same technique as the other BSA samples.

The concentration of the Human serum samples, HS, were found to diverge from the assigned values as the assigned ionised calcium concentration decreased. While this % bias increased as ionised calcium decreased (Table 3.8), there was a consistent difference of approximately 0.08 mM between the assigned and measured ionised calcium concentration for samples HS3,4,5,and 6 as measured by the CRM. The basis for this difference is not due to a RLJP due to HEPES as the Human serum samples did not contain HEPES. There was remarkable consistency in the results as evident in Figure 3.3 (Graphs c and d). It must be noted that results in graph c have been offset for the sake of clarity and it can be seen that each method

has a different slope. Again it must be noted that analysis is made difficult because of the inherent bias associated with the assigned ionised calcium concentrations.

The choice of quality control standard for protein samples was one of the aims of this project. From the results obtained, the D solutions (based on BSA and tonometered to desired pH) would offer the best option as a quality control standard. In an ideal situation, Human serum would be best but would be problematic as the samples would be susceptible to CO_2-based pH changes. The BSA solutions on the other hand, contain HEPES buffer which would minimise this risk. Precision of measurements on solutions D and HS were better than those of solution B. Both D and HS had been tonometered to obtain the desired pH and so any protein containing quality control standard must be tonometered rather than adjusted by use of buffers.

An asymmetry potential when samples containing protein are measured with an ISE, which has been noted by another researcher [18], was also observed during the analysis of protein samples. Shifts of approximately 0.2-0.3 mV were noted but after this initial rise, the potential remained steady. In the analysis of the protein samples, once this shift had occured, the sensitivity of the ISE was not affected (Table 3.4) and the stability of the potential reading was also excellent (Table 3.5). Ideally, this asymmetry potential should not arise. It has been attributed to a coating of serum components on the selective membrane [18]. Therefore the use of a protective cellulose acetate membrane may alleviate this. On a practical level, a protective membrane is not feasible with the CRM as there are already difficulties in getting the selective membrane seated in the CRM without having another membrane present. Alternatively, the use of an -OH

modified PVC membrane which has been shown to minimise the hystersis due to protein contamination [18] may be another alternative.

During analysis of serum samples, there were sudden potential drops. This may have been due to an earthing effect. With the continuous use of the calibrating solutions, a static build up would occur and when earthed, the potential would suddenly drop. It may also have been due to a shedding of a protein layer from the membrane surface. The use of a protective membrane over the selective membrane may reduce this but would present difficulties as already stated. Further studies are needed to investigate the reason for this effect but with better earthing, one might be able to greatly reduce it.

An initial protocol for measurement was given (Appendix B) but each laboratory was allowed to develop their own. The precision achieved at our laboratory was amongst the best in the Europe-wide study (cf Figures in appendix C). The imprecision of the method in other laboratories (Figures C.1, C.2, C.3, C.4, and C.5 in appendix C), may be judged to be due to non-standardised methods of sample introduction, i.e. human error or due to careless sample introduction. The precision of the Euro. Ave. was much less than that of the CRM in our laboratory. This is not surprising as the s.d. of the Euro. Ave. is based on the inter-laboratory variations whereas the s.d. of our laboratory is an intra-laboratory variation. The commercial analysers which had a strict sampling regime demonstrated the greatest precision. Therefore the need for automated sample introduction is an absolute requirement if the Reference method is to achieve its aims.

The Reference Method is intended as a method against which a commercial analyser's performance is evaluated and to create secondary reference standards. The need for this was demonstrated by the study but

from a hands-on experience, the feasibility of the method must be drawn into question. The long analysis time required for just one sample (approximately 80-90 minutes) is too long. The use of a shorter calibration sequence and shorter analysis time i.e. potential measurement taken after 90 seconds rather than the recommended 180 seconds, was found not to affect the precision of measurements of serum samples (cf Section 4.5). Therefore a shorter calibration sequence might be introduced.

3.6. Conclusions to European Study

The study has shown the need for standardisation of calibration solutions. The introduction of HEPES buffer gave a negative bias of 4% in the aqueous solutions measured. The nature of the solutions B, D and Human serum samples measured does not allow a definitive analysis to be made as the processes that may take place in the solutions are not easily modelled. The CRM in our laboratory showed precision comparable to that of the commercial analysers and its accuracy, i.e. closeness to the assigned value, is commensurate with the commercial analysers. The time of analysis is too long and so the calibration sequence must be shortened. The use of a shorter calibration sequence and shorter time before potential measurement has been shown not to affect the precision of the CRM in serum samples (Section 4.5). A strict protocol of sample introduction must be introduced if further progress is to be made and the use of an automated sampling procedure may also be of benefit.

3.7. References

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4 Study on the Effect of Protein/Albumin on the Measurement of Ionised Calcium

4.1. Introduction

Since Ferreiria and Bold [1] first noted that the ionised calcium determined in serum was higher than that of its ultrafiltrate, much work has been done on the effects of protein on the performance of calcium analysers. This has become increasingly important now that the Bureau Communautaire de Reference (BCR) is addressing the problem of defining conditions for a reference method for the measurement of ionised calcium. There have been conflicting reports on the effect of protein on ionised calcium determinations. Thode [2] used in-vitro equilibrium dialysis activity measurements to support his theory that protein does not directly influence calcium activity measurements in serum. In addition, his in-vivo data, in contrast to that of Butler [3] failed to show any significant correlation between serum albumin levels and ionised calcium measured. In-vitro studies by Payne and our group [4,5] have shown a positive bias due to increasing albumin concentration and we also demonstrated an in-vivo effect [5]. Payne's most recent publications [6-8] suggest that the composition of the salt bridge electrolyte of the reference electrode is the most critical factor in determining the level of protein interference in ionised calcium measurements.

4.2. Rationale of Study

As there is great debate surrounding the effect of albumin on ionised calcium measurements, we wished to examine the effect of albumin changes on ionised calcium measurements made with the CRM. Changes in protein levels in serum samples were induced by venostasis in healthy human volunteer subjects. Venostasis was used as the increase in albumin/protein is due to a natural pooling effect . In this way, the effect of natural albumin and protein changes on ionised calcium measurement could be evaluated. Also, because of the recommendations of Payne [6] that the bridge solution should be an isotonic solution, we wished to examine the effect of variation in the salt bridge electrolyte concentration on the determination of ionised calcium.

4.3. Experimental procedure

4.3.1. Study Design

Blood was taken from 17 healthy volunteers, aged 22-36 who were not on medication. The protocol was approved by the ethics committee at St. Michael's Hospital Dublin and informed consent was obtained from all volunteers. An intravenous cannula was inserted without venostasis and the first blood was drawn. A sphyogomanometer cuff was applied to the arm and inflated to 90mm/Hg. Blood was taken, without fist clenching, at 2.5, 5.0,7.5,10.0 and 15.0 minutes after application of the cuff. The blood was collected in vacutainers and allowed to clot naturally on standing. It was then centrifuged and the serum transferred anaerobically to syringes. The samples were then analysed with the smallest possible delay. Total calcium, ionised calcium, albumin, total protein and pH were measured.

4.3.2. Measurement protocol

4.3.2.1 The commercial calcium analysers

Details of the measurement protocol of the commercial analysers may be found in Section 3.3.2.2.

4.3.2.2. Measurement with the CRM

The measurement protocol was as described in section 3.4.3. and 3.4.4. with the following changes: The calibration sequence was a shortened version of the one laid down by EWGISE [9] . The potentials of the calibrants and serum samples were measured after 90 seconds in order to maintain similar analysis times to the commercial analysers. Serum samples were measured twice and a one point calibration was performed before and after each serum sample to check for drift. If the potential between each one-point calibration differed by more than 0.1 mV, a two-point calibration was carried out. Two point calibrations were carried out after every fourth serum sample. The cell was flushed out with 5 mL of flush solution between each serum sample, as described in section 3.3.5..

4.3.3. Effect of isotonic salt bridge

A second experiment assessed the effect of altering the salt bridge electrolyte concentration on measured ionised calcium. The saturated KCl in the CRM was replaced with isotonic (0.150M) KCl and 21 paired samples from hypocalcaemic, normocalcaemic and hypercalcaemic patients were analysed by the CRM in the hypertonic and isotonic configurations. Paired samples were used so that the protein and electrolyte composition would be identical. Thus, any differences in the ionised calcium measurement

observed would be due to the salt bridge solution alone, not ionic strength or protein alterations. The measurement protocol was as in 4.3.2..

4.3.4. Analyses of composition of sera analysed

Total serum calcium was determined by atomic absorption spectroscopy. In the analysis of protein and albumin, pre-and post-venostasis samples were analysed in the same batch. The protein concentration was determined using the Biuret method. The precision of the method was 1.09% within batch and 1.64% between batch. Albumin was determined by the Bromocresol Green technique. Precision was 0.58% within batch and 2.62% between batch.

4.3.5. Statistical analysis

Percentage changes of albumin, total protein and total and ionised calcium from basal levels were calculated and subjects divided into two groups based on these changes. An arbitrary albumin rise of 15% was chosen. Group 1 contained 10 subjects whose serum albumin level increased greater than 15% over the basal level. Group 2 contained the remaining 7 subjects whose maximum change in serum albumin during the same period was only 5%. Group 2 served as a control group. A paired student's *t* test was performed to compare basal values with those at each of the five time intervals; a Bonferroni correction of the level of significance was calculated in view of the multiple comparisons [10]. The effect of changes in albumin and total protein concentrations on ionised calcium was examined by linear regression analysis using the least squares method. The p value refers to a two-tailed test .

4.4. Results

4.4.1. Precision of the CRM with Human Sera

The precision of the CRM in human sera samples had been shown to be excellent with the samples HS1-HS6 (c.f. Section 3.6 and Table 3.7). As the calibration and measurement protocol had been changed for this study, the precision had to be re-established for this measurement protocol. This was achieved by the measurement of a pooled serum sample 10 times, in both the hypertonic and isotonic configurations.

Table 4.1 Precision study for the CRM using the modified measurement protocol

	Serum	Hypertonic ionised calcium (mM)	Isotonic
	1	1.289	0.977
	2	1.289	0.986
	3	1.289	0.990
	4	1.310	0.994
	5	1.310	0.990
	6	1.310	0.982
	7	1.310	1.000
	8	1.299	0.993
	9	1.305	0.985
	10	1.310	0.981
Hypertonic	Mean = 1.302	s.d. = 0.0097	%CV = 0.74
Isotonic	Mean = 0.988	s.d. = 0.0069	%CV = 0.70

The precision of the CRM in both modes of operation was found to be excellent, with %CV < 1% (cf Table 4.1)

4.4.2. Venostatsis Study

The CRM was used in the hypertonic mode only for the venostasis study. All subjects participating in the study showed an increase in serum total protein and albumin concentrations following venostasis and the magnitude of the increases varied significantly between subjects (Tables 4.1 and 4.2). Serum pH did not alter significantly in individual subjects during the period of venostasis (Tables 4.1 and 4.2). The subjects were therefore divided into two categories as previously stated in 4.3.5.

Table 4.2 Effect of venostasis on total calcium, serum albumin, total protein and ionised calcium measured by the three analysers in group 1 subjects (n=10,in all cases except the CRM ionised calcium results, n = 6)

Variables	Basal Value	Mean Values post-venostasis ± s.d.				
Time (minutes)	0.0	2.5	5.0	7.5	10.0	15.0
Total Ca (mM).	2.392 ±0.063	2.414* ±0.072	2.503* ±0.060	2.545* ±0.080	2.558* ±0.127	2.597* ±0.109
albumin (g/L)	45.3 ±2.7	46.9* ±2.4	50.6* ±4.1	52.3* ±4.7	53.4* ±3.7	56.2* ±4.3
Protein (g/L).	76.1 ±3.6	78.4* ±3.6	86.7* ±7.4	88.8* ±7.7	89.9* ±8.6	95.2* ±6.3
Ca(ICA2) (mM).	1.263 ±0.042	1.274* ±0.041	1.288* ±0.042	1.293* ±0.048	1.293* ±0.050	1.301* ±0.052
Ca(Baker) (mM).	1.250 ±0.037	1.255* ±0.028	1.266* ±0.030	1.274* ±0.042	1.270* ±0.042	1.287* ±0.043
Ca(CRM) (mM).	1.278 ±0.019	1.290* ±0.025	1.308* ±0.023	1.317* ±0.019	1.317* ±0.026	1.317* ±0.036
pH.	7.373	7.361	7.361	7.361	7.361	7.358

where * denotes significant p value at 0.01 according to the Bonferonni correction of 0.05/n, where n=5

Table 4.3. Effect of venostasis on total serum calcium, serum albumin, total protein and ionised calcium measured by the three analysers in group 2 subjects (n=7, in all cases except the CRM ionised calcium results, n = 5)

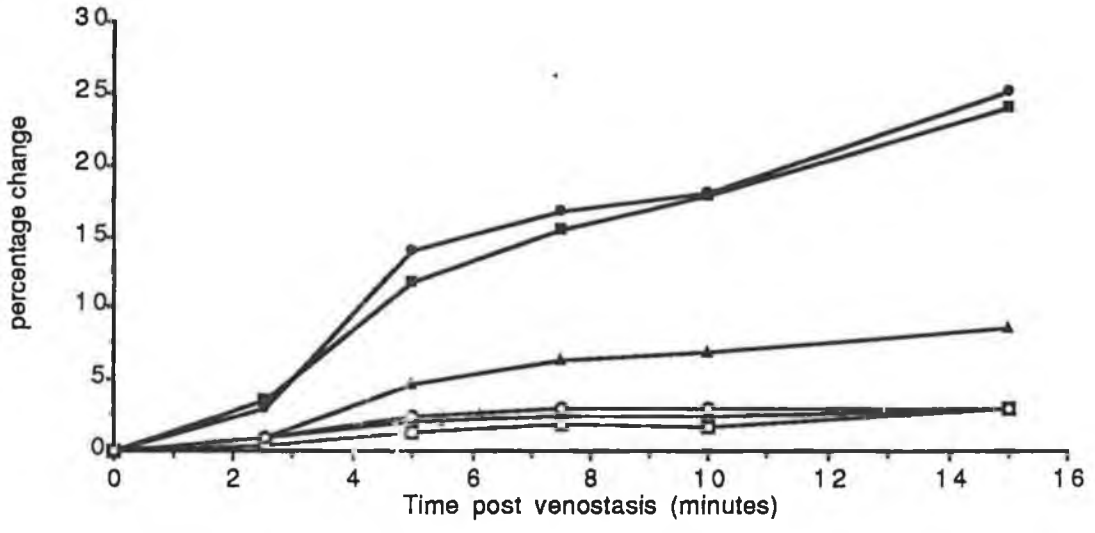
Variables	Basal Value	Mean Values post-venostasis ± s.d.				
Time (minutes)	0.0	2.5	5.0	7.5	10.0	15.0
Total Ca (mM).	2.384 ±0.080	2.374 ±0.100	2.404 ±0.099	2.430* ±0.078	2.453 0.059	2.463 ±0.090
Albumin (g/L).	46.4 ±2.8	45.6 ±4.3	45.7 ±2.8	47.9* ±2.6	48.3 ±3.3	48.6 ±3.4
Protein (g/L)	77.6 ±4.6	77.6 ±7.3	77.7 ±4.7	81.8 ±5.5	83.5 ±9.5	83.6 ±7.9
Ca(ICA2) (mM)	1.259 ±0.031	1.256 ±0.035	1.264 ±0.034	1.267 ±0.033	1.258 ±0.039	1.266 ±0.030
Ca(Baker) (mM).	1.240 ±0.035	1.241 ±0.045	1.244 ±0.040	1.244 ±0.039	1.240 ±0.040	1.250 ±0.031
Ca(CRM) (mM)	1.204 ±0.050	1.200 ±0.042	1.202 ±0.040	1.214 ±0.034	1.208 ±0.047	1.208 ±0.062
pH	7.380	7.389	7.387	7.390	7.395	7.387

where * denotes significant p value at 0.01 according to the Bonferonni correction of 0.05/n, where n=5

4.4.3. Group comparisons

Group 1 subjects showed marked increases in total calcium, albumin and protein levels with a significant increase in measured ionised calcium levels following venostasis (Figure 4.1).

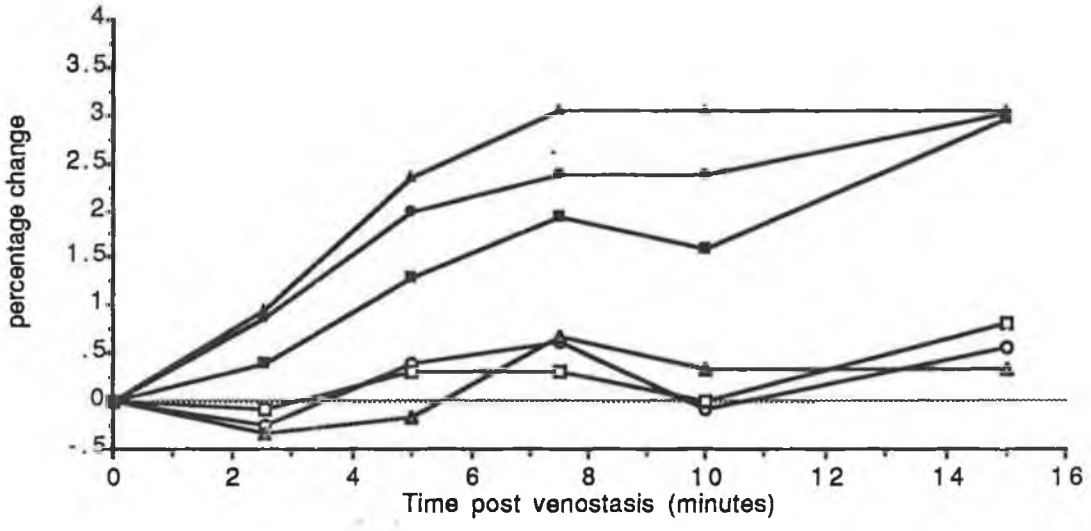
Figure 4.1 Effect of venostasis on total serum calcium (●), serum albumin (■), total protein (▲), and ionised calcium as measured by the three analysers, ICA2 (○), Baker (□), and the CRM (△) in group 1. The symbols represent mean percentage changes.



There were significant increases from basal in all parameters after 2.5 minutes venostasis ($p < 0.01$) in group 1 (Table 4.2.).

In group two, there were significant increases in total calcium and protein after 7.5 minutes venostasis but there was no significant rise in ionised calcium (Table 4.3). The relative increases in ionised calcium measured by all three ion-selective electrodes in groups 1 and 2 are graphically represented in Figure 4.2.

Figure 4.2 Effect of venostasis on ionised calcium measured by the three analysers in group 1 and group 2 subjects. In group 2: ICA2 (o), Baker (□) and the CRM(Δ). In group 1: ICA2 (●), Baker (■) and the CRM (▲). The symbols represent mean percentage changes.



Correlations between measured ionised calcium and albumin/total protein concentrations in the serum samples during venostasis are shown in Table 4.3.

Table 4.3 Regression equations of albumin and total protein on ionised calcium measured by the three analysers

Instrument	Regression Equation	r values	p values
Radiometer	Y=0.00269x + 0.00570 (Albumin)	0.62	<0.001
ICA2	Y= 0.00213x - 0.00396 (Protein)	0.76	<0.001
Baker	Y= 0.00197x + 0.00115(Albumin)	0.50	<0.001
Analyte+2	Y= 0.00160x +0.00389 (Protein)	0.62	<0.001
CRM	Y= 0.00312x + 0.00035(Albumin)	0.53	<0.001
	Y= 0.00168x - 0.00071 (Protein)	0.50	<0.001

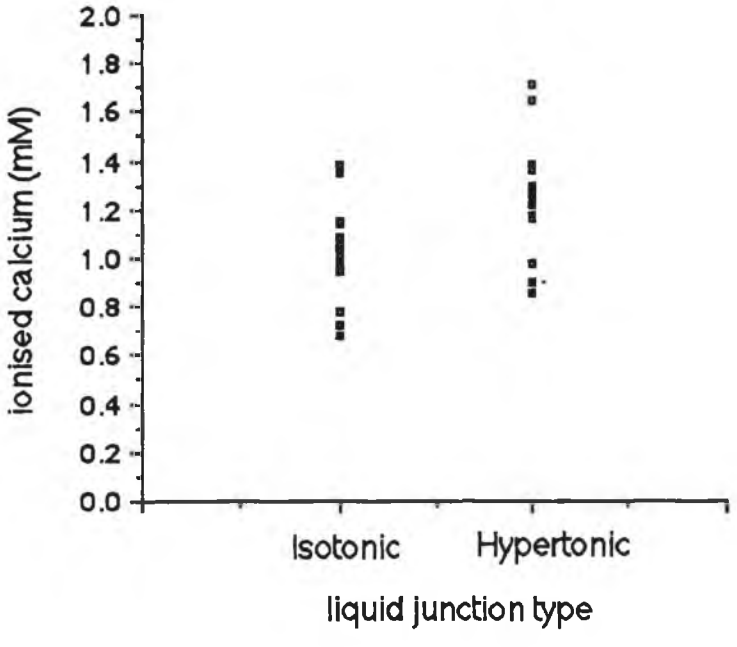
The results suggest that an increase of 10 g/L of albumin would result in an increase in ionised calcium of 0.0269mM/L measured by ICA2, 0.0197

mM/L measured by the Baker and 0.0312 mM/L measured by the CRM. An increase of 10 g/L in protein would result in an increase in ionised calcium of 0.0213 mM/L measured by ICA2, 0.0160 mM/L measured by the Baker and 0.0168 mM/L measured by the CRM.

4.4.4. Analysis using the CRM with isotonic salt bridge

Analysis of paired serum samples (n=21) showed that the ionised calcium measured using the isotonic configuration was greatly reduced ($p<0.001$) compared to the results generated in the saturated KCl mode. The mean ionised calcium ± s.d. measured with the isotonic system was 1.026 ± 0.170 (range 0.68-1.39) compared to 1.255 ± 0.197 (range 0.86-1.71) measured with saturated KCl. These results are graphically represented in

Figure 4.3. Serum ionised calcium concentration in 21 paired serum samples measured by CRM using both isotonic and hypertonic KCl as the salt bridge electrolyte.



4.4.5. Increases in serum calcium

During the venostasis study, 3 of the 17 subjects had serum total calcium which exceeded the reference range for healthy subjects. In one of the subjects, ionised calcium was abnormally high when measured with the Baker analyser. Ionised calcium measured by the ICA2 was also raised in one subject post-venostasis. The increase in ionised calcium occured only after significant albumin/protein increases.

4.5. Discussion

Venostasis which results in a steady rise in albumin/protein levels over time, allows the investigation of the effect of increasing albumin levels on measured ionised calcium in human subjects. The variation of serum albumin in response to venostasis within the test subjects was co-incidental rather than a feature of study design. It did however allow comparison of the effect of marked increases in albumin/protein concentrations in one group of human subjects compared with a second group showing lesser changes over the 15 minute venostasis period.

It was shown that the apparent rise in ionised calcium with increasing albumin and protein levels is independent of the instrumentation design and configuration. There was a statistically significant rise in ionised calcium levels as measured by all three analysers following venostasis and albumin/protein increases in group 1. (Table 4.2). Subjects in group 2 whose mean increase was less than 5% did not show these significant alterations in ionised calcium (Table 4.3). On a purely empirical basis, the percentage change, in group 1 subjects, in ionised calcium from basal is greater, by a factor of at least 3, than the method error associated with the instruments and

so this rise is unlikely to be a random occurrence. In group 2 the increase was less than 1% while in group 1 it rose by 3% from basal levels (Figure 4.1.). The precision of the measuring equipment used with the CRM was 0.1 mV. From the Nernst Equation it is clear that an error of 0.13 mV is equivalent to an error of 1% at all measured concentrations. A variation of 0.1 mV will correspond to an error in ionised calcium of approximately 0.78% and at typical physiological concentrations e.g. 1.25 mM, this would correspond to a change of 0.01 mM in measured concentration. The magnitude of the change in mV over the period of venostasis was approximately 0.4 mV or 0.04 mM. In healthy people, ionised calcium is maintained within very narrow limits. The reference range spans approximately 0.14 mM. This corresponds to a change in potential of 1.4 mV. Therefore the change of 0.4 mV observed in group 1 subjects, while small, is substantial in terms of the reference range.

While the design of the experiment allowed us to assess the magnitude of ionised calcium rise, it did not allow us to us to investigate the cause. A real increase may have occured if the pH of the sera had fallen but there were no significant changes in the pH of the sera of either group (cf Tables 4.2 and 4.3)

The use of the term "apparent" with respect to the protein induced rise in ionised calcium is important as different views are held as to whether the increase in ionised calcium is a direct affect of protein on the calcium electrode system or a predictable consequence of the Donnan equilibrium at the selective membrane. A previous study has listed evidence for both of these theories [5]. However the differences in the designs of the analysers does allow us speculate about events. There are two main areas where a

direct albumin/protein effect might occur; at the selective membrane or at the reference electrode liquid junction. The increase is unlikely to be a direct effect of the protein binding on the selective membrane as the ICA2 analyser, which has a cellulose acetate protective membrane covering the selective membrane, experiences the same magnitude of change as the other two unprotected analysers. The suspicion that the rise is due to protein interference at the reference electrode liquid junction, as Payne suggests, is borne out by the fact that all analysers, which use hypertonic salt bridge solutions, experienced an increase in measured ionised calcium. In this study, the Baker, which uses the least hypertonic bridge solution showed less pronounced rises in ionised calcium until gross changes (>20% rise) in albumin occured (Figures 4.1 and 4.2).

Venostasis was chosen as the method by which the albumin/protein of serum may be increased. The albumin/protein would rise as the sphyogomanometer would close the vein and the albumin/protein in the blood would naturally pool at this point. There would no pooling of the electrolytes in the blood, including ionised calcium, as these are diffusible and so would diffuse through the venous walls and enter the extracellular fluid. As there would be no pooling of other electrolytes e.g. sodium or potassium, there would be no ionic strength effects which would cause dissociation of calcium ions from the albumin molecule. The apparent association, K_A, constant for the equilibrium, $Ca^{2+} + alb^{2-} \rightleftharpoons Caalb$ or

$$K_A = \frac{[Caalb]}{[Ca^{2+}]\ [alb^{2-}]} \qquad (1)$$

is 95L/mol [11] and so the tendency to dissociate will not be favoured. Also the concentration of albumin [alb^{2-}] also rises and will negate the tendency for dissociation. The only other means by which ionised calcium would actually rise is if there was a decrease in the pH of the serum. There was no significant increase in the pH of the sera (Table 4.2) and so the increase in ionised calcium measured is an apparent rise, not an actual rise.

At hypertonic liquid junctions, protein is denatured [12] and forms immobile poly-anions at the junction between the protein and KCl which gives rise to a positive junction potential [13]. Payne has carried out many elegant experiments to support the hypothesis than the interference occurs at the liquid junction. In one such experiment, he found that as the bridge solution in the salt bridge went from hypertonic to isotonic, protein interference becomes negligible [6].

A further study comparing ionised calcium in a small number (n=21) of paired serum samples tended to support the idea of salt bridge dependence. Paired samples would of course have the same electrolyte and protein composition and thus any changes that result are due to the effect at the liquid junction alone. The CRM was used to measure serum ionised calcium levels in the paired samples. The CRM used a hypertonic and an isotonic salt bridge solution in the reference vessel (cf Figure 3.2) for the measurement of the paired samples. .The ionised calcium levels measured using the isotonic salt bridge in the CRM were greatly reduced compared to those measured using a hypertonic bridge solution. While the ionised calcium measured by the isotonic configuration is significantly lower, there is a good correlation between results generated by both configurations $r=0.98$, $p<0.001$. In a recent study, Payne used a Ciba-Corning 634 analyser with an

isotonic NaCl salt bridge electrolyte [14] to investigate his ealier premise that an analyser with an isotonic bridge did not suffer from protein interference [6]. He found that, as with our investigation, ionised calcium measurements made using the isotonic configuration were greatly reduced and that the precision was not unduely affected [14]. Unfortunately he found a significant positive correlation with serum chloride and so concluded that an isotonic NaCl reference electrode offered no major clinical advantage for the measurement of ionised calcium .

That these large changes in measured ionised calcium occur supports the premise of the liquid junction as the major site where protein influences ionised calcium measurement, rather than by activity coefficient effects. Other researchers have reported that protein interference is not observed using ion-selective electrode measurement of ionised calcium or sodium when cells without liquid junctions are used [15]. If the suggestion that isotonic KCl shows no protein interference is true [6], it is possible that these figures reflect the true ionised calcium level in blood. These findings are especially important now that a reference method for ionised calcium is being developed. The implications for the BCR study is that potential calibrating solutions must contain protein in order to reduce the positive bias, caused by aqueous calibrants, when measuring protein samples. Also the use of a saturated salt bridge solution in the CRM must be examined.

4.6. Conclusions of Study

A small but significant increase, (3%), in ionised calcium measured, which occurs with increasing serum albumin/protein levels, is evident with 2

commercial analysers and a calcium reference half-cell. This rise was independent of the membrane type used and was present when a hypertonic salt bridge electrolyte was used. Further work on ionised calcium measurement by ion-selective electrodes must focus on the salt bridge dependence noted and encompass both effects of protein and electrolyte changes. The use of a solid-state reference electrode or one with no liquid junction would perhaps allow us to elucidate the cause for this apparent rise in ionised calcium.

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5 Evaluation of RepHex as a Reference Electrode Junction Material

5.1. Introduction

An often neglected consideration when choosing an Ion-Selective Electrode (ISE) system is that of a suitable reference electrode. Indeed, recent extensive reviews cite only 12 papers devoted to reference electrode considerations in potentiometric analyses for the period 1988-92 [1,2]. Conventional calomel and Ag/AgCl electrodes are suitable for general applications but in specialised areas such as measurement of pH in low ionic strength water, the characteristics of these electrodes may not be suitable.

The fundamental consideration for a reference electrode is that it provides a stable junction potential. The maintenance of this potential is probably one factor which causes most difficulty in potentiometric measurements. In potentiometry, one monitors the cell potential (E_{CELL}), which includes a contribution from the reference electrode junction potential (E_{jn}), the reference electrode half-cell potential (E_{REF}) and the ISE potential (E_{ISE}). On transferring between solutions, the change in cell potential (ΔE_{CELL}) is given by;

Potential in solution 1 $E_{CELL(1)} = E_{ISE(1)} - E_{REF(1)} + E_{jn(1)}$ (1)

Potential in solution 2 $E_{CELL(2)} = E_{ISE(2)} - E_{REF(2)} + E_{jn(2)}$ (2)

The change in potential $\Delta E_{CELL} = E_{CELL(2)} - E_{CELL(1)}$ (3)

The potential of the cell will be wholly determined by the the ISE if there is no difference in the potentials of the reference electrode due to the sample i.e.

$E_{REF(1)} = E_{REF(2)}$ and $E_{jn(1)} = E_{jn(2)}$

The reference half-cell, e.g. Ag/AgCl or calomel, is not affected on transfering between solutions but the maintanence of stable junction potentials can be a problem.

This problem arises from the need to provide an electronically conducting pathway between the ISE and the reference half-cell which passes through the sample but which does not allow bulk mixing of the reference electrode salt-bridge and the sample solution. Commercial electrodes incorporate a porous ceramic frit, fibre wick, micro capillary or ground sleeve to enable the salt bridge ions to diffuse into the sample.

For high precision pH work, a renewable liquid junction as in a free diffusion junction is recommended [3]. In process applications this is not preferred as it may lead to contamination of the sample and will also increase maintenance needs of the system. Frits may experience residual memory effects from buffer solutions which could give rise to errors [4]. For pure water applications, it has been recommended that the porosity of the junction may be increased by reducing the length of the ceramic frit to decrease junction potentials [5]. In many situations, the reference electrode junction potential can become unstable e.g. due to clogging or poisoning (in hostile industrial environments), dilution of the salt bridge (in pure waters) or precipitation of protein (in clinical samples) [6]. KCl is normally used as the bridge electrolyte as the almost equitransferrent ions minimise the magnitude of the junction potential.

The performance of potentiometric systems for the measurement of pH in water with low ionic strength is one of the more difficult applications. This may be encountered in natural water samples and in boiler feedwater at power stations, where the pH must be carefully monitored in order to

minimise corrosion [7]. This application was therefore chosen in order to compare the performance of reference electrodes incorporating a RepHex junction to conventional reference electrodes with a ceramic frit junction.

5.2. Experimental procedures

5.2.1. pH measurements

5.2.1.1. Materials and Reagents

Measurements were made on deionised water that had a base conductivity of 1.5-2.0 μS. Buffers conforming to DIN 19267 standard at pH 7.00 and 4.01 (Reagecon Diagnostics Ltd.) were used to calibrate the electrodes.

5.2.1.2. Apparatus

The following potentiometric cells were used in the investigations;

(1) RepHex Cell:

Ag | AgCl | KCl (2.8M)| RepHex | sample | pH membrane| pH buffer | AgCl |Ag

(2) Ceramic Frit Cell:

Hg, Hg_2Cl_2 | KCl (2.8M) | ceramic frit | sample | pH membrane | pH buffer | AgCl| Ag

where | represents a solid/liquid interface

The potentials of these cells were measured using a Jenway 3040 ion-analyser which had a 0.1 mV discrimination.

5.2.2. Leakage studies

5.2.2.1. Materials and Reagents

Leakage measurements were made on deionised water that had a base conductivity of 1.5-2.0 µS. A 25 mM HCl/ 0.25 mM DAP-HCl (DL-diaminopropionic monohydrochloride) solution was prepared as the mobile phase and a 100 mM TBAOH (Tetrabutylammonium hydroxide) solution was used as the column regenerant.

5.2.2.2. Apparatus

A Jenway 3070 conductivity meter was used to measure the conductivity of water in which the reference electrodes were immersed. A Dionex Ion-Chromatography system Ion-Pac CS3 column with 25 mM HCl/ 0.25 mM DAP-HCl (DL-diaminopropionic monohydrochloride) as the mobile phase was used measure the K^+ ions leaked from the electrodes. The flow rate was 1.5 ml/min and the chromatogram was measured over 7 minutes. A 100 mM TBAOH (Tetrabutylammonium hydroxide) solution was used as the column regenerant.

5.3. Fabrication of RepHex reference Junctions

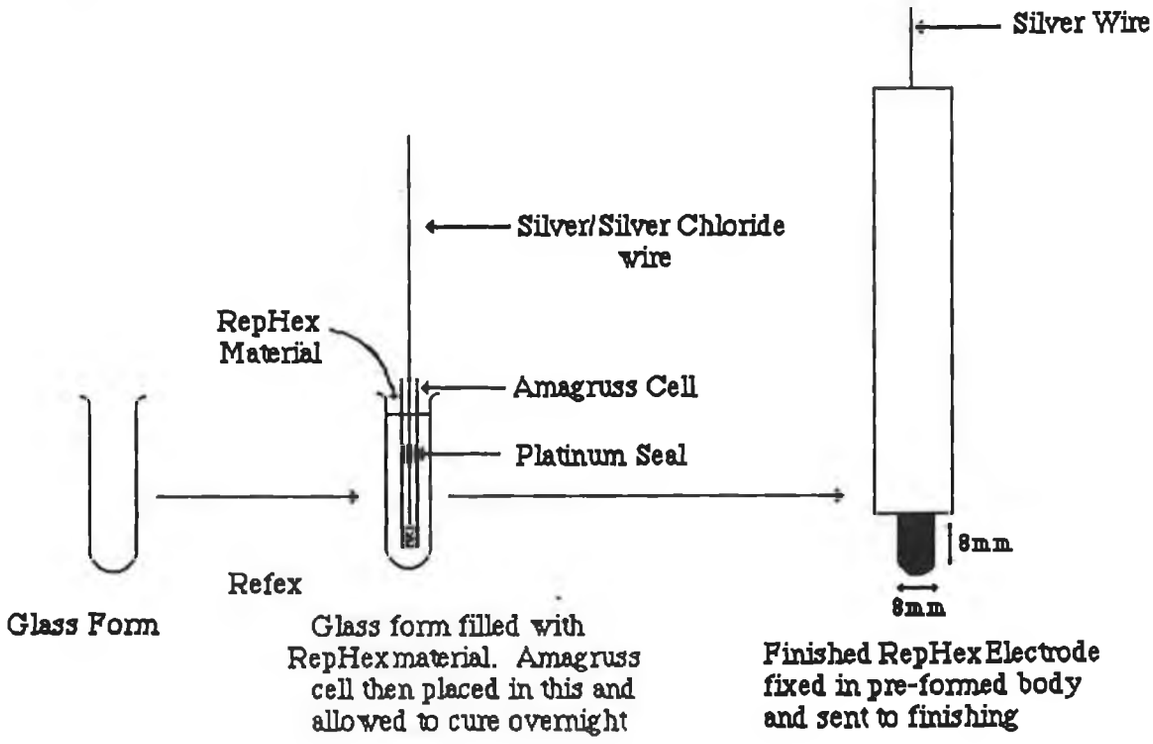
To 100g of a polyvinyl ester resin (Derakane 470-36), an appropriate amount of initiator was added. 100g of KCl and 8g LiCl were added to this resin as well as small amounts of quartz and graphite. The mixture was stirred thoroughly to ensure even dispersion of the components. Initially, the mixture was quite fluid and could be spun or moulded into the desired form. After curing overnight, it was a hard crystalline material which may be machined, although it was quite brittle. The exact details of this procedure

are described in a patent, European Patent No. 0247535 [8]. Two types of junction design were investigated.

5.3.1. Type A RepHex Electrodes

The resin/KCl mixture was poured into a glass form with an external diameter of 10 mm. An Ag/AgCl half-cell, with a filling solution of 2.8M KCl, was then placed in this mixture and positioned as close to the edge of the form as possible. The RepHex material was then allowed to cure overnight. The cast was then removed from the glass form and fixed into an epoxy body so that 12 mm of the RepHex protruded (Figure 5.2).

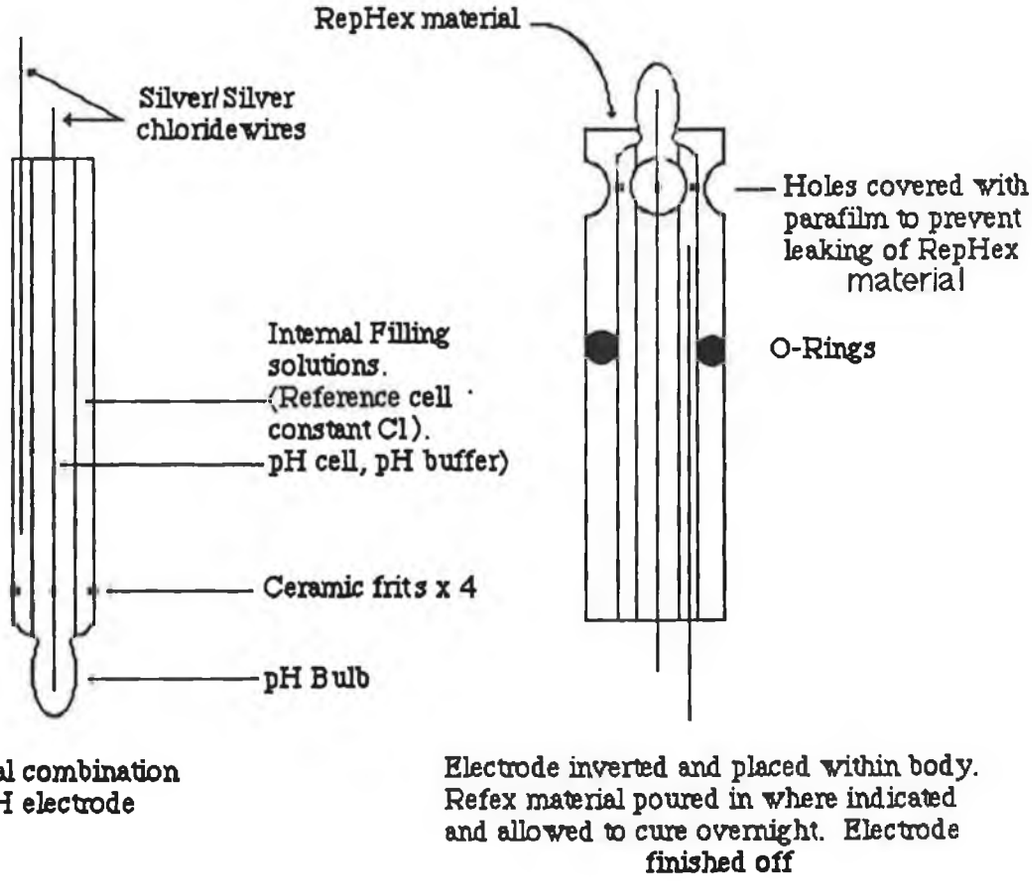
Figure 5.2 Graphical representation of the manufacture of the type A electrodes



5.3.2. Type B RepHex Electrodes

An internal pH combination electrode with 4 frit restricted liquid junctions was positioned inside an epoxy body with four windows by means of O-Rings so that the four ceramic frits were opposite the windows in the body. These windows and the pH membrane were then protected with parafilm. The body of the electrode was then inverted and filled with the RepHex material. This was allowed to cure overnight (Figure 5.3).

Figure 5.3 Graphical representation of the manufacture of RepHex type B electrodes



5.4. Methods

5.4.1. pH measurements in deionised water:

Measurements were taken in both static and slowly stirred solutions. The electrodes were initially calibrated with buffers of pH 7.00 and 4.01. pH measurements in pure water were taken at 30 second intervals up to 10 minutes. This procedure was repeated ten times.

5.4.2. Leakage monitoring using conductivity

The RepHex electrodes were soaked in deionised water for 2/3 days to remove any KCl which may have built up on the outer surface. 100 ml of the pure water was placed in a polycarbonate bottle and the conductivity of the water was measured prior to insertion of the electrodes. The bottle was then sealed with parafilm to prevent any particulate contamination. When measurements were taken, the electrode was removed, the water stirred for one minute, the conductivity probe placed in the water and the conductivity measured after 1 minute. The electrode was then replaced in the water and resealed until the next measurement. The leakage from the electrodes was monitored over a period of 5 days.

5.4.3. Leakage monitoring using K^+ concentration measurements

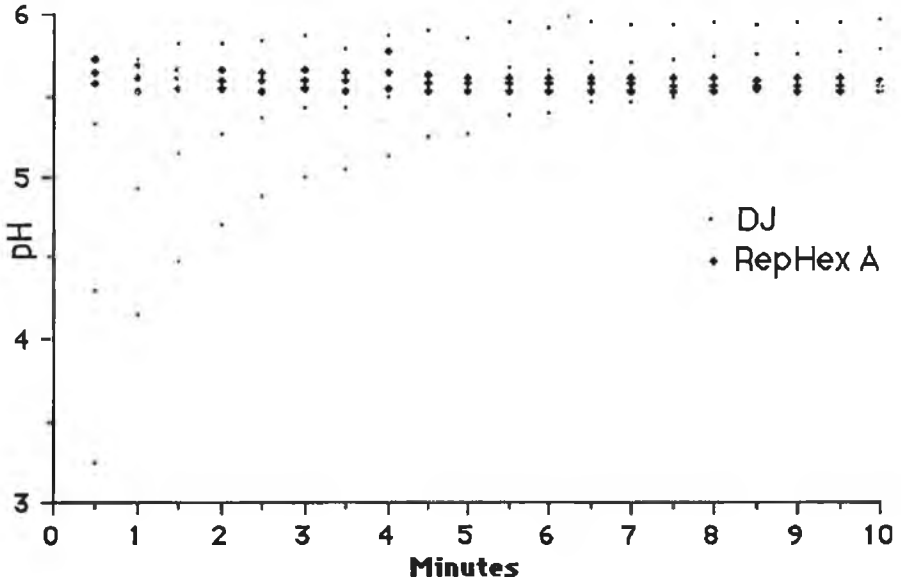
The electrodes used in this study were a Type A RepHex (Figure 5.1), a Type B RepHex electrode (Figure 5.2) and a conventional frit restricted electrode. Samples for analysis were gathered using the same procedure as above except that samples were taken over 4 days.

5.5. Results

5.5.1. pH determinations

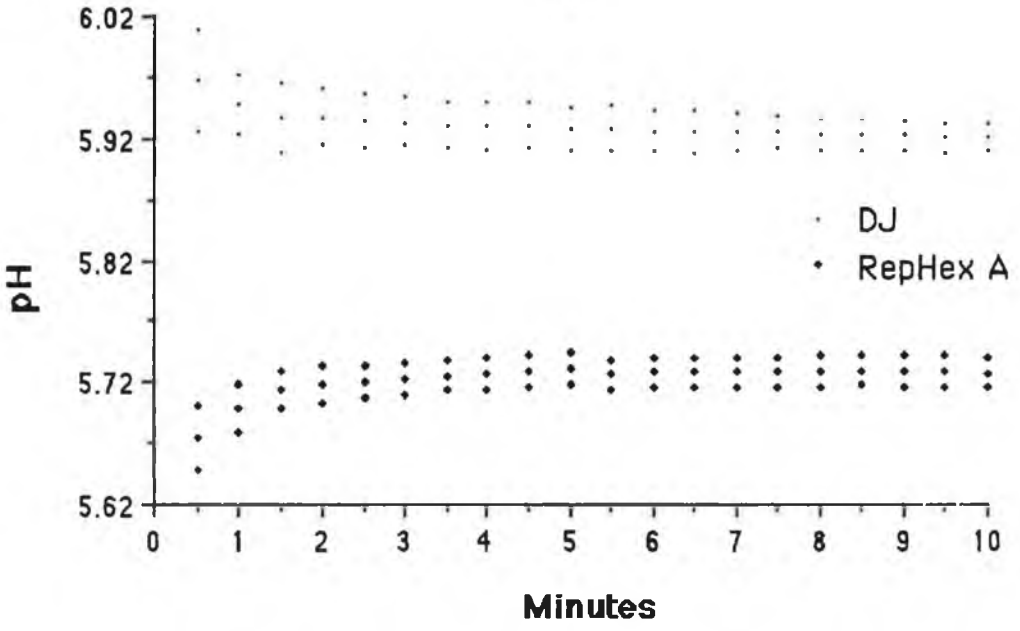
The results obtained with the RepHex A and conventional ceramic frit double junction (DJ) reference electrodes are shown in Figure 5.4 and Figure 5.5 for slowly stirred and unstirred solutions, respectively.

Figure 5.4 Graphical representation of pH results ± s.d. as measured using a type A RepHex, "+", and ceramic frit double junction electrode, ".", as the reference electrodes in slowly stirred water. Note the large instability associated with the ceramic frit double junction electrode compared to the RepHex A electrode.



In unstirred solutions, there was little difference between the ceramic frit junction and the RepHex junctions in terms of precision (Figure 5.5) but the precision of measurements made in stirred solutions was markedly better for the type A RepHex than the ceramic frit double junction electrode (Figure 5.4).

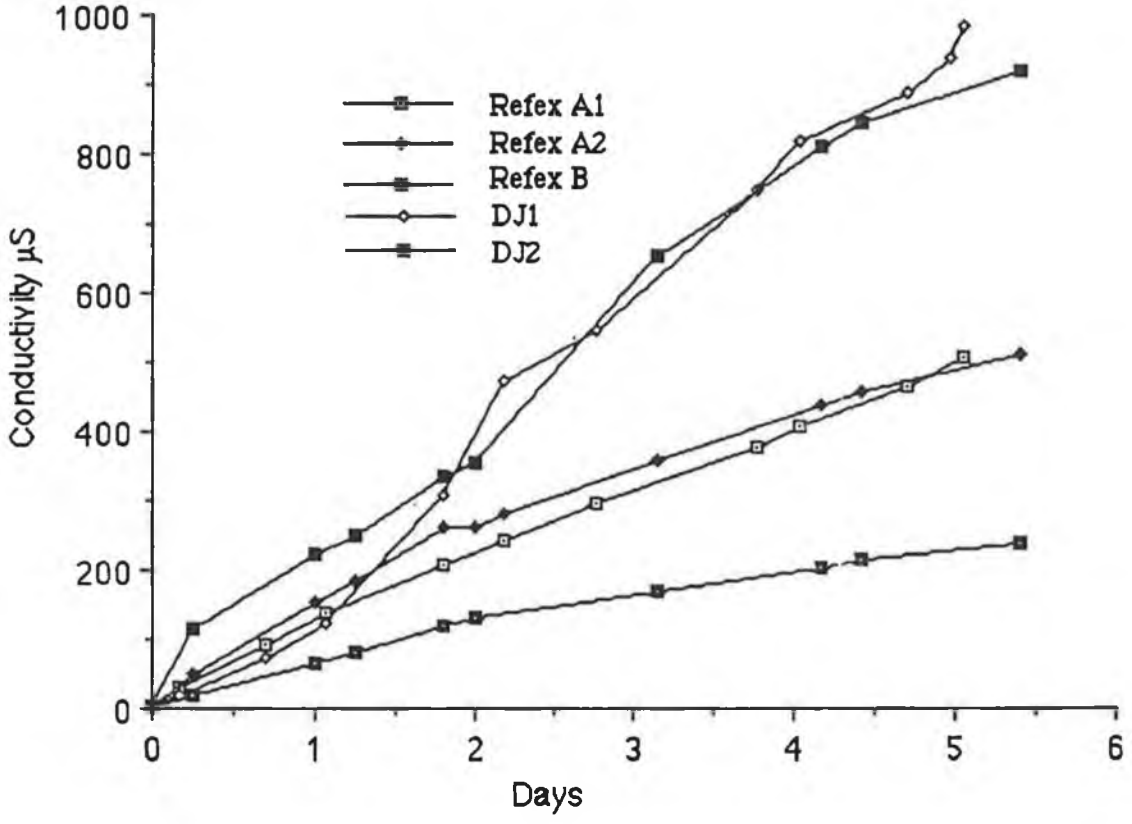
Figures 5.5 Graphical representation of pH results ± s.d. as measured using a type A RepHex, "+", and ceramic frit double junction electrode, ".", as the reference electrodes in unstirred water. Note the similar stability associated with the ceramic frit double junction electrode and the RepHex A electrode.



5.5.2. Leakage studies

The results of the conductivity studies are graphically represented in Figure 5.6 for five reference electrodes, two with type A RepHex junctions (RepHex A1 and RepHex A2), two double junction reference electrodes with ceramic frit junctions (DJ 1 and DJ 2), and one combined glass electrode with the RepHex B type junction (RepHex B). Increases in conductivity brought about by leakage of KCl from the reference electrode salt bridge through the junction, was greatest with the ceramic frit electrodes. These trends were confirmed by monitoring the increase in K^+ in the storage water using ion-chromatography.

Figure 5.6 Graph following the leakage from the type A and type B RepHex electrodes and a ceramic frit electrode as monitored using conductivity measurements.



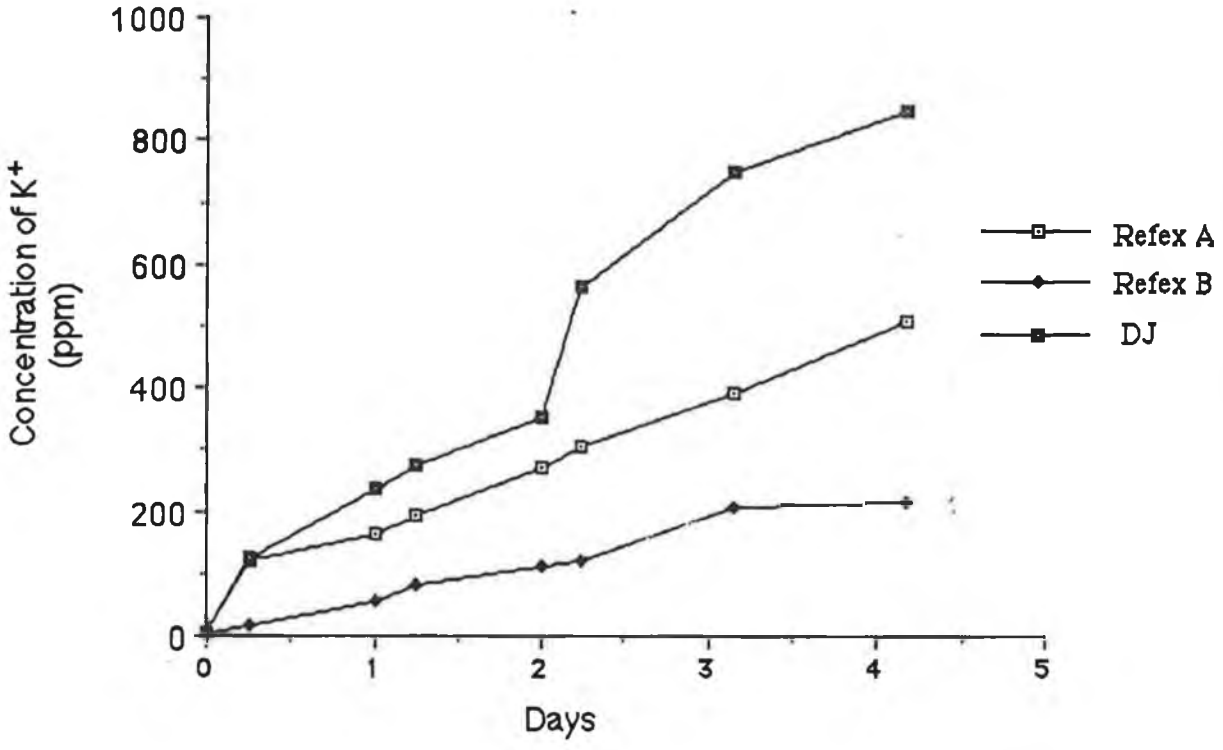
The leakage from the reference electrodes was also measured quantitatively by ion-chromatography. In this analysis the leakage from a type A electrode, a type B electrode and a ceramic frit junction was quantified. The results are summarised in Table 5.1.

Table 5.1 Leakage from the reference electrodes, type A and type B RepHex and ceramic frit double junction as measured with the Dionex.

	Concentration K^+ (ppm)		
Time (h)	Type A RepHex	Type B RepHex	Ceramic frit Jn.
0	3	3	3
6.2	121	17	125
24.1	165	57	235
30.1	195	84	275
48.1	272	112	354
53.6	307	119	563
75.6	392	207	748
100.1	507	215	850

These results are graphically represented in Figure 5.7. Once again, the leakage of K^+ is greatest with the ceramic frit. When the leakage rates are normalised for junction area, the rate of K^+ leakage with RepHex A and RepHex B junctions are almost the same at around 6.0×10^{-8} mol/h/mm^2 whereas the frit junction is almost three orders of magnitude higher at 2.67×10^{-5} mol/h/mm^2.

Figure 5.7 Graph showing the leakage from the type A and B RepHex and ceramic frit electrodes as monitored by measurement of the potassium concentration. Graph generated using data from Table 5.1.



In terms of volume of KCl leaked through the junctions, this is difficult to quantify for the RepHex junctions as the KCl is released from the doped resin rather than from the internal filling solution. The ceramic frit electrode loses KCl at around 4.81 x 10-5 mol/hr (10 µl/h), which is typical for junctions of this type [9,10].

5.6. Discussion

Although the pH of water is assumed to be 7, the pH of the water measured in the study was found be in the range of pH 5.5-6.0. This does not invalidate any of the findings as this is typical for water in equilibrium with CO_2. In unstirred solutions, the pH value reached a steady value within three

minutes but the ceramic frit electrode exhibited better precision than the RepHex junction (Figure 5.5).

In stirred solutions, the RepHex electrodes gave much more precise results over the entire 10 minute measurement period compared to the ceramic frit electrode (Figure 5.4). Even after 10 minutes, the standard deviation of results obtained with the ceramic frit junction was approximately 0.18 pH. Furthermore, the mean value did not stabilise for around six minutes. In contrast, the cell incorporating a RepHex junction gave almost instantaneously stable results with much better precision (standard deviation 0.07 pH after 0.5 minutes, decreasing to 0.035 pH after 10 minutes). On moving from unstirred to stirred solutions, a decrease of around 0.2 pH was obtained with both cells (Figure 5.4 and 5.5) an effect which has been noted by other investigators [11] and results obtained with the RepHex electrode were approximately 0.2 pH lower than those obtained with the ceramic frit electrode.

The leakage from the RepHex electrodes was significantly less than that from the ceramic frit electrode. This is surprising, considering the much greater junction area (see Table 5.2) and heavy KCl loading of the RepHex electrodes.

Table 5.2 Characteristics and summary of leakage data from the three different junctions used.

Junction Type	Dimensions/mm	Area/mm^2	Absolute Leakage rate mol K^+/h	Leakage rate normalised for area of contact mol $K^+/h/mm^2$
RepHex A	hemisphere; Radius = 4.0 cylinder; height = 8.0 circumference = 25.1	301.6	1.92×10^{-5}	6.38×10^{-8}
RepHex B	circular windows x 4 Redius = 4.0	201.1	1.18×10^{-5}	5.84×10^{-8}
Frit	radius = 0.75	1.8	4.81×10^{-5}	2.67×10^{-5}

The lower leakage rate obtained with the RepHex B to RepHex A electrode is predictable from the smaller junction area. The leakage was found to be extremely slow compared to other junction designs [9,10] when normalised in terms of junction area. From the leakage data (Figure 5.6 and 5.7, Table 5.1), the release of KCl occured in a consistent and controlled manner and so we would expect stable and reproducible junction potentials. As the KCl loading of the RepHex electrode is very large (1:1 w/w KCl : Resin), there is a huge reservoir of KCl within the electrode and so the lifetime of these electrodes will be much greater than conventional electrodes. The robust nature of the resin in which the KCl is entrapped means that the RepHex junction would be ideal in process applications. The precision and stability of pH measurements in stirred pure water solutions (Figure 5.4) demonstrates the stability of the junction potential and so this electrode, due to its stability and low leakage of KCl compared to conventional electrodes (Table 5.2), would be useful in measurements made at power stations [7]. The stability is further enhanced by the ability to use

large junction areas compared to other designs based on diffusion of KCl from an internal bridge solution which is restricted by means of a narrow capillary, a ceramic frit or a fibre wick. Hence clogging, coating or blockage can be expected to be much less problematic with RepHex junctions.

Fundamental studies on the mechanism of functioning of the RepHex junction were performed in Finland and are described in appendix D. The following conclusions were drawn from these results;

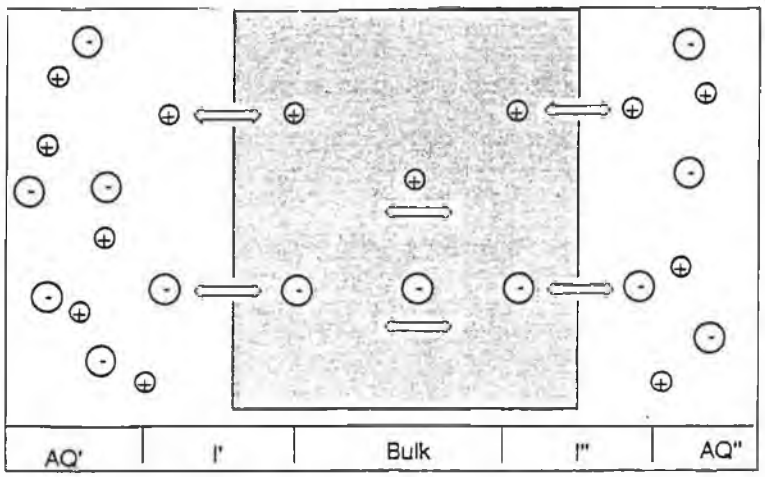
a) The incorporation of the KCl salt into the polymer matrix is crucial for the RepHex junction to demonstrate its excellent electronic properties.

b) The charge transfer mechanism occurring at the RepHex junction is ionic in nature.

c) RepHex has very low electrical resistance at low frequencies (i.e. essentially zero Hz or d.c.) at which potentiometric measurements are made.

d) RepHex can be expected to have a low impedance pathway at high frequencies and this suggests that the electrode may be used for a.c. measurements. The charge transfer through the RepHex material may be described by consideration of the five regions shown in Figure 5.8.

Figure 5.8 Transport processes involved at the RepHex junctions



AQ' and AQ" represent the bulk aqueous solutions on the inside (') and outside (") of the electrode, I' and I" represent the interfaces of the RepHex with the internal and external solutions respectively and Bulk represents the RepHex material itself.

In the regions AQ' and AQ", the mechanism of charge transport is dominated by diffusion of the ions present in the solutions. In the regions I' and I", evidence from studies in Finland and the present study show that the charge transport is via the KCl at both surfaces but there is no evidence to suggest whether the K^+ or Cl^- dominates this process or whether each is involved equally. Leakage studies confirmed that KCl is transferred into the sample solution. In the bulk region, the mechanism of charge transport has been shown to be ionic in nature and of low impedance (<10kΩ) but whether some or all of the ions take part in this movement is not known. The movement of these ions may be due to two reasons:

<1> In a manner similar to that ascribed to positive holes or electrons in a classical semiconductor. The large negative lattice enthalpies of KCl and LiCl means that the solid salts are not involved but lattice defects may account for some ability to hop from position to position.

<2> Small amounts of water may be trapped along with the salts during manufacture or water may be drawn into the material due to the presence of the diliquescent LiCl in the material. The water molecules would solvate the ions in the material and they might be able to migrate from one ionic region to another within the resin, in the direction of the potential gradient driving the process.

The nature of the RepHex junction results in a pressure insensitive junction [12]. This is a great improvement compared to conventional reference electrodes in that no special holders are needed when they are used under pressure. Recently, a pressure insensitive electrode for voltammetric measurements has been developed [13]. While this has shown good stability, lifetimes are very short (14 days). The heavy loading of the RepHex junction results in long lifetimes and thus may provide an excellent replacement for the electrode described by Jermann et. al. [13].

5.7. Conclusions

This study shows that despite the heavy salt loading and large areas of contact of the RepHex junctions investigated, leakage of KCl into sample solutions can be expected to be less than that occurring with conventional ceramic frit junctions. Furthermore, pH measurements in deionised water suggest that the RepHex junctions provide a stable junction potential which is quick to stabilise and relatively constant with time and between stirred/unstirred solutions. Future work will centre on optimisation of loading of the resin and the geometry of the junction, and investigating its performance as a conducting substrate for solid-state sensors. Also its use in voltammetric analysis may yield further areas of application.

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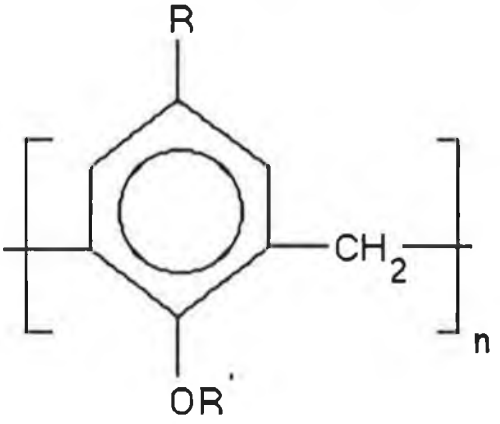
6 Novel calixarenes as Potassium Ionophores

6.1. Introduction

An area of rapid development has been the inclusion of calixarene compounds into PVC membranes as ionophores. Calixarene molecules act as neutral carriers when included in such membranes. Certain calix(4)arenes or tetramers which are selective for sodium have been incorporated into PVC membranes for ISEs and been shown to have similar precision to commercially available analysers for the measurement of sodium in blood [1]. Calix(6)arenes or hexamers have been successfully used to make caesium selective electrodes [2]. Calixarenes possess the main characteristic of a neutral carrier ionophore which is a well defined polar cavity attached to a non-polar macrocyclic backbone. The ion complexing properties of the basic calixarene, Figure 6.1, have been improved by the addition of esters, ketones, amides and other such functional groups via the phenolic oxygen.

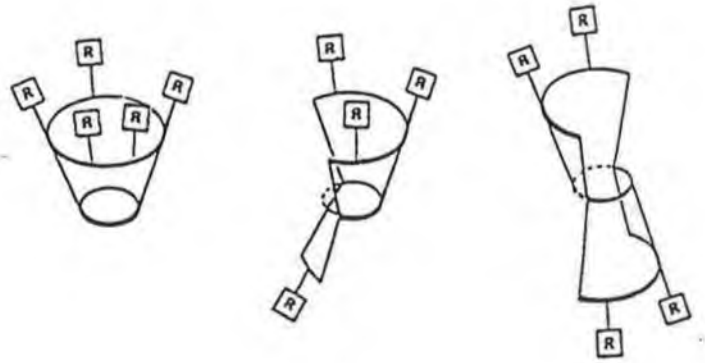
Figure 6.1 Structure of the basic calixarene molecule (n=4-8)



where R and R' = H

The size of the cavity is of fundamental importance in determining which ions are optimally held within the cavity. The movement of the ion into the cavity is a best-fit process. The tetramer and the hexamer have cavities of an optimum size for the sodium and caesium ions respectfully. The incorporation of a $-CH_2O-$ in the methylene bridge of the calix(4)arene macromolecule [3] increases the cavity size and therefore, compounds of this type might prove to be selective for potassium (ionic radius = 1.38A). Another type of calixarene compound which may be suitable as a potassium ionophore are the partial cone calix(4)arenes. The more open nature of the cavity of these compounds may preferentially sequester potassium rather than sodium.

Figure 6.2. Representation of the types of cone conformation of calix(4)arenes (a = normal cone conformation, b = partial cone conformation and c = 1,2 alternate conformation).



6.2. Experimental procedure

Initial performance characteristics of 5 calixarene compounds, 3 oxacalixarenes and 2 partial cone calix(4)arenes, were assessed. Their

slope, linearity and selectivity coefficients against a range of interfering ions were measured. Some lifetimes were also measured.

6.2.1. Materials

6.2.1.1. Calixarene compounds used

Oxacalixarenes

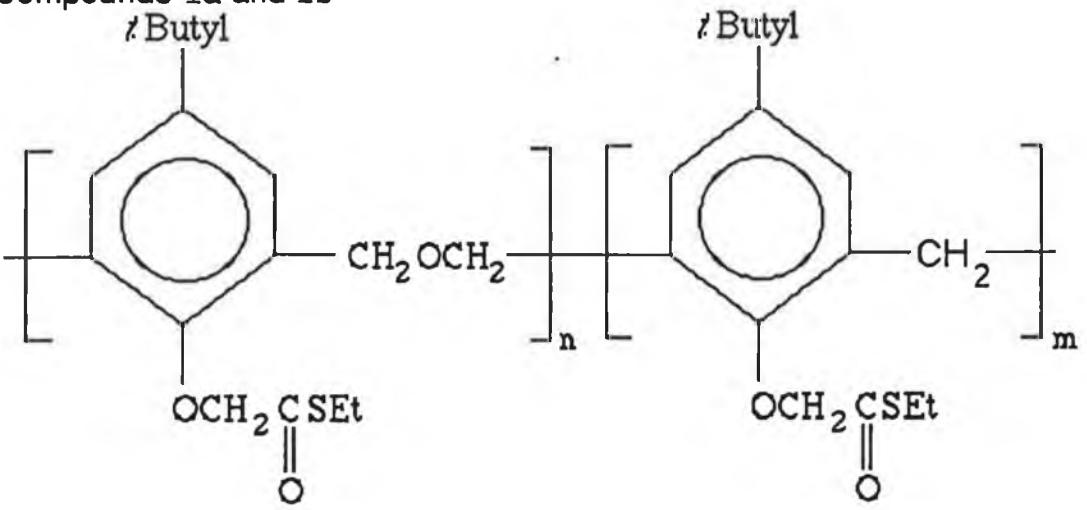
1) Thiol derivative of a *p*-*t*-butyl monooxacalix(4)arene Ia
2) Thiol derivative of a *p*-*t*-butyl dioxacalix(4)arene Ib
3) Methyl Ketone derivative of a *p*-*t*-butyl dioxacalix(4)arene II

Partial Cone Calixarenes

1) Butyl ester calix(4)arene III
2) Methoxy ester calix(4)arene IV

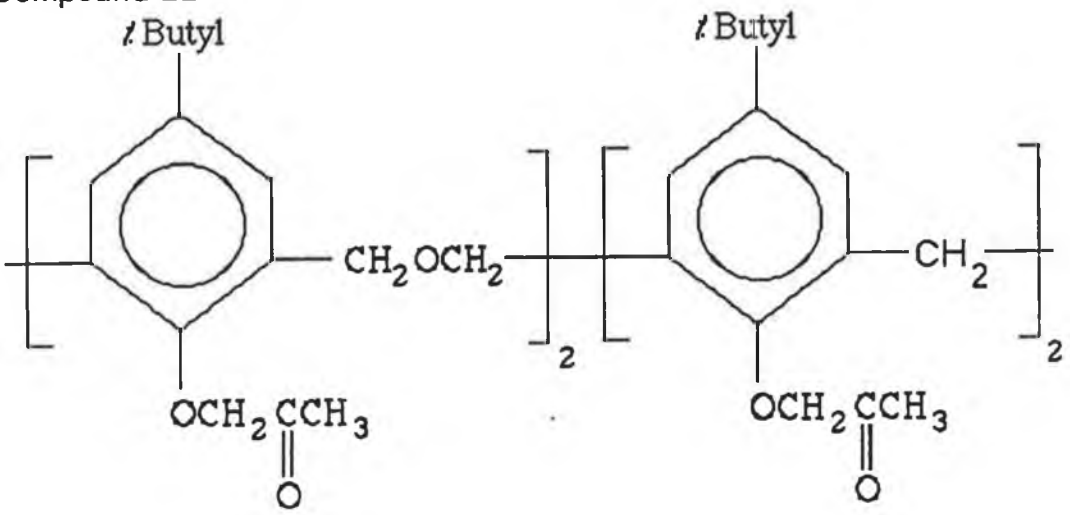
These compounds are represented in Figure 6.3.

Figure 6.3 Representations of the calixarene compounds used in this study, .
Compounds Ia and Ib

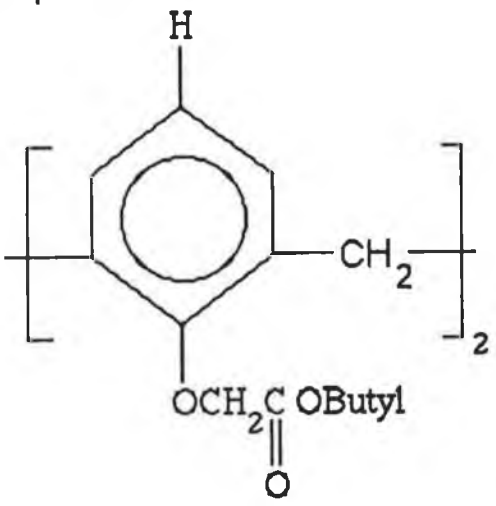


where m = 4 - n
n = 1, Compound Ia
n = 2, Compound Ib

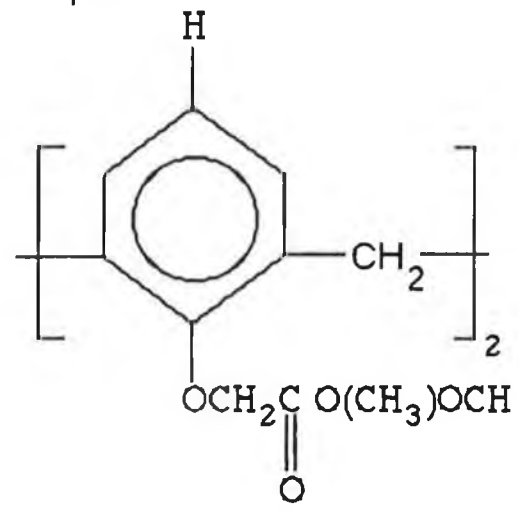
Compound II



Compound III



Compound IV



6.2.1.2. Reagents

All solutions were prepared in distilled deionised water (Millipore grade). Analar grade chlorides of lithium, sodium, potassium, ammonium, rubidium, caesium, calcium, and magnesium were obtained from Riedal-de-Haen. The calibration solutions and solutions used to assess selectivity coefficients were prepared by serial dilution of stock 1M solutions of the appropriate ion. The membrane materials were as follows and were obtained from Fluka (Buchs, Switzerland): Poly(vinyl chloride) (PVC), 2-nitrophenyl octyl ether (o-NPOE) and potassium tetra-kis (p-chlorophenyl) borate (KTpClPB).

The calixarene compounds used in the study were synthesised by Dr. Stephen Harris at Dublin City University.

6.2.1.3. Fabrication of electrodes

Membranes were fabricated in the manner described by Moody and Thomas [4]. In brief, the calixarene, plasticiser and PVC were mixed and dissolved in THF. Where at all possible, membranes were first made without

the use of the ion-exchanger, potassium tetra-kis (p-chlorophenyl) borate (KTpClPB). For membranes that contained the ion-exchanger, a 4:1 mole ratio in favour of the ionophore was used. THF was used as the solvent and was added until a fluid mixture resulted. The cocktail was poured into a mould and a tissue was placed over the mould to prevent contamination by dust etc. and the THF was allowed to evaporate off overnight. This resulted in a flexible membrane and a 9mm disc was cut from this and inserted into the cap of a Russell gas sensing electrode (model ISE 97-7809). The electrode was filled with 10^{-1}M KCl as the internal reference solution and was allowed to condition overnight before measurements were taken. The electrode membranes were stored in a 0.1 M K^+ solution during periods between calibration and while they were not in use.

6.2.1.4. Measuring apparatus

Potentiometric measurements were made relative to a saturated calomel electrode using a high impedence Russell 660 pH/millivolt meter. Potential measurements were taken after 1 minute at room temperature. The selectivity coefficients (Log K_{ij}^{pot}) were determined by the separate solutions (S.S) method using 10^{-1}M solutions of the primary and interfering ions. EMF measurements were carried out on cells of the type:

Hg, Hg_2Cl_2 / KCl (Satd) / sample / PVC membrane / 0.1 M KCl (aq) / AgCl / Ag

6.3. Results

6.3.1. Linearity

All compounds, Ia and Ib, II, III, and IV demonstrated a linear response to potassium in the range 10^{-3} to 10^{-1} M . Compound II, III, and IV demonstrated linear responses to potassium in the range 10^{-4} to 10^{-1} M. Typical calibration curves are shown in Figures 6.3, 6.4, and 6.5. Figure 6.4 represents the calibration curves of compound Ib when electrodes were fabricated with and without the ion-exchanger, KTpClPB. There was no deterioration in the linearity when the electrode was fabricated without the presence of the ion-exchanger, but the response at lower concentrations was more variable (Figure 6.4).

Figure 6.3 Typical calibration curves for electrodes based on compounds Ia, Ib, and II

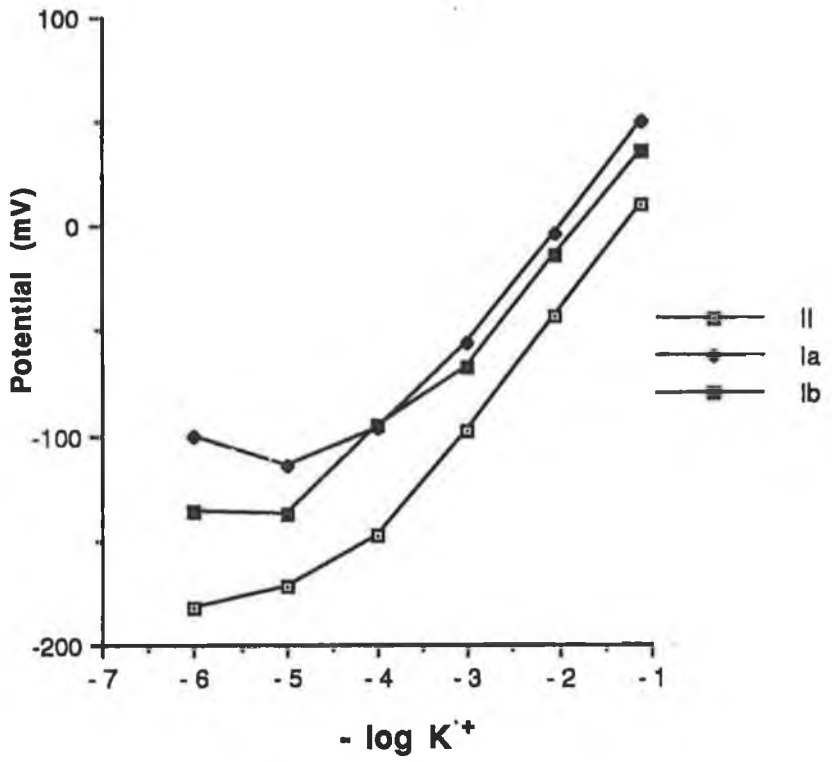


Figure 6.4 **Comparison of calibration curve of electrode based on compound Ib with and without the presence of KTpClPB in the membrane.**

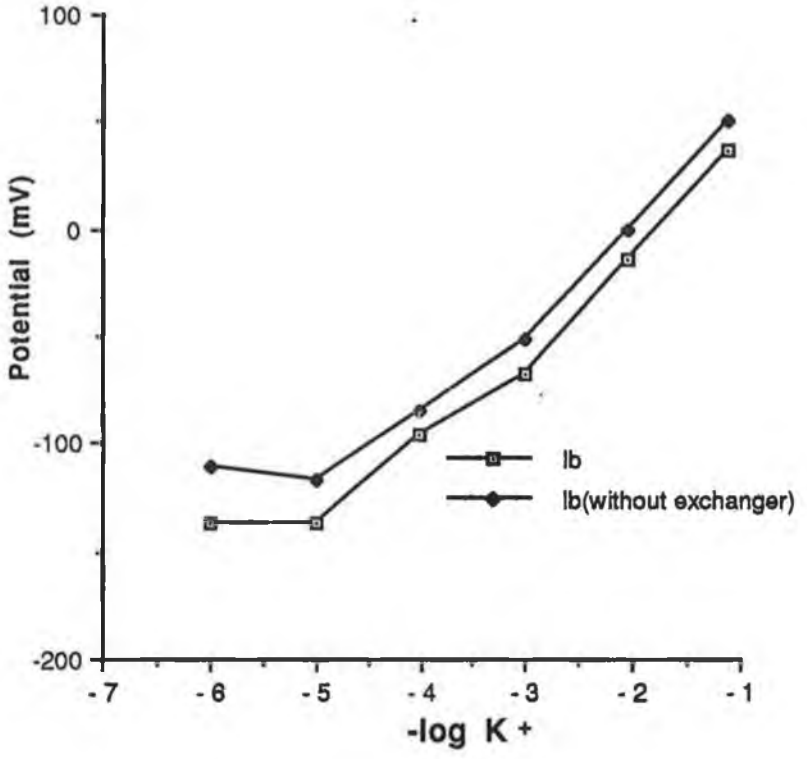
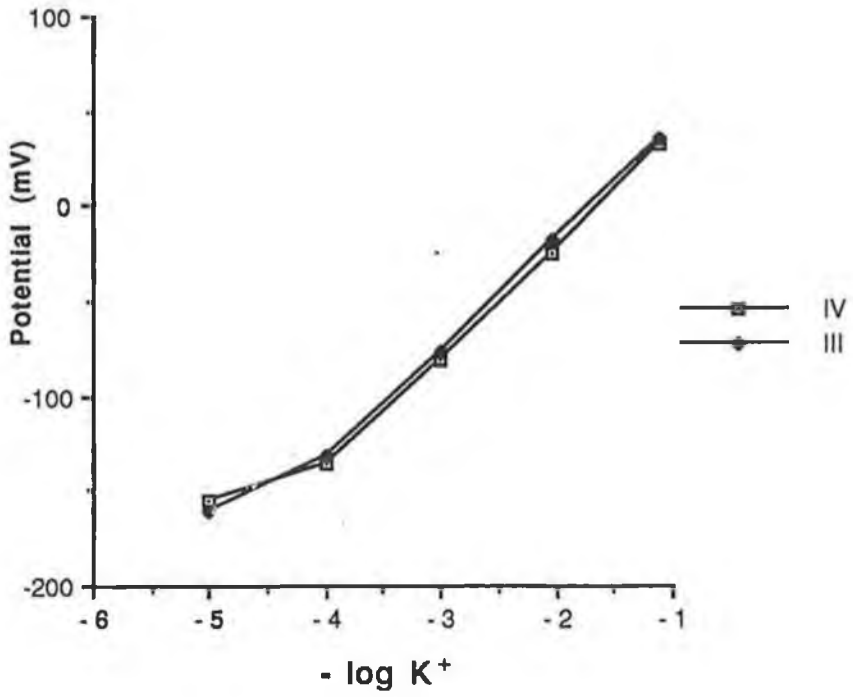


Figure 6.5 Typical calibration curves for electrode based on compound III and IV.



6.3.2. Sensitivity

The electrode based on compound Ia initially demonstrated a slightly sub Nernstian response to potassium, 55.65 mV/decade, but after storage in 0.1 M K^+ for 5 days, the response dropped to 50.38 mV/decade (Table 6.1).

Table 6.1 Summary of response characteristics of electrode based on compound Ia

Slope (mV/decade)	55.65	50.38	50.74
		Selectivity Log K_{ij}^{pot}	
Interfering ion	Day 1	Day 6	Day 15
Na^+	-1.42	-1.25	-1.23
Li^+	-2.21	-2.08	-1.84
Rb^+	+0.34	+0.27	+0.12
Cs^+	+0.69	+0.40	+0.41
H^+	-1.55	-0.89	-0.91
NH_4^+	-0.64	-0.72	-0.66
Mg^{++}	-1.88	N/A	-1.82
Ca^{++}	-2.25	N/A	-1.91

On storage for a further 9 days, no further deterioration was observed with the electrode having a response of 50.74 mV/decade.

The electrode based on compound Ib had a Nernstian response to potassium throughout the analysis period of fourteen days, with a slope of 54.86 mV/decade measured on day 1, 55.20 mV/decade measured on day 5 and 56.74 mV/decade measured on day 14 (Table 6.2). The electrode based on compound Ib, without the ion-exchanger, had a lower Nernstian slope factor, slope = 51.23 mV/decade and was stable for one day only (Table 6.2).

Table 6.2 Summary of response characteristics of electrode based on compound Ib

Slope (mV/decade)	54.86	55.20	56.74	51.23*
Selectivity Log K_{ij}^{pot}				
Interfering ion	Day 1	Day 5	Day 14	Day 1*
Na^+	-1.42	-1.60	-1.55	-1.62
Li^+	-2.27	-2.17	-2.12	-2.25
Rb^+	+0.41	+0.27	+0.19	+0.11
Cs^+	+0.77	+0.64	+0.48	+0.41
H^+	-1.47	-2.17	-1.91	-1.23
NH_4^+	-0.67	-0.84	-0.91	-1.04
Mg^{++}	-1.90	-1.96	-1.93	-1.88
Ca^{++}	-2.77	-2.32	-2.12	-2.45

* Electrode based on compound Ib which did not have KTpClPB in the membrane

The electrode which was made without the presence of the ion-exchanger, KTpClPB, exhibited a Nernstian slope of 51.23 mV/decade but this response was not as stable as slope decreased to 43.14 mV/decade after storage in 0.1M K^+ for 5 days.

The electrode based on compound II showed a slope of 56.03 mV/decade on day 1, 55.41 mV/decade on day 3, 58.46 mV/decade on day 4 and 57.22 mV/decade on day 10 (Table 6.3).

Table 6.3 Summary of response characteristics of electrode based on compound II

Slope (mV/decade)	56.03	55.41	58.46	57.22
Selectivity Log K_{ij}^{pot}				
Interfering ion	Day 1	Day 3	Day 4	Day 10
Na^+	-1.65	-1.53	-1.58	-1.34
Li^+	-3.16	-2.80	-2.38	-2.33
Rb^+	+0.08	+0.09	+0.11	+0.16
Cs^+	-0.19	-0.14	-0.06	0
H^+	-2.05	-2.08	-2.40	-1.44
NH_4^+	-1.08	-1.10	-1.08	-0.77
Mg^{++}	-2.10	-2.10	-2.07	-2.04
Ca^{++}	-2.65	-3.20	-2.56	-2.93

The slope of electrodes based on compounds III and IV were measured on day 1 only. The response of these electrodes on day 1 were excellent with the electrode based compound III having a measured slope of 58.64 mV/decade and compound IV having a slope of 58.01 mV/decade (Table 6.4).

Table 6.4 Summary of response characteristics of electrode based on compounds III and IV

Slope (mV/decade)	58.64	58.01
Selectivity Log K_{ij}^{pot}		
Interfering ion	Compound III	Compound IV
Na^+	-0.17	-0.71
Li^+	-1.94	-2.03
Rb^+	-0.81	-0.63
Cs^+	-1.27	-0.59
H^+	-2.71	-2.58
NH_4^+	-1.72	-1.48
Mg^{++}	-1.79	-1.77
Ca^{++}	-2.63	-2.57

6.3.3. Lifetime

Only day 1 measurements were taken with electrodes based on compounds III and IV. The electrode based on compound Ia suffered a response deterioration after storage in a 0.1 M K^+ for 5 days. On further storage the response remained constant at its reduced level. Electrodes based on compounds Ib and II demonstrated stable lifetimes up to at least 10 days. Time did not allow any further lifetime studies to be undertaken. An electrode based on compound Ib but without the addition of the ion-exchanger KTpClPB had a lifetime of only 1 day as the slope of the electrode decreased to 43.14 mV/decade after storage for 5 days.

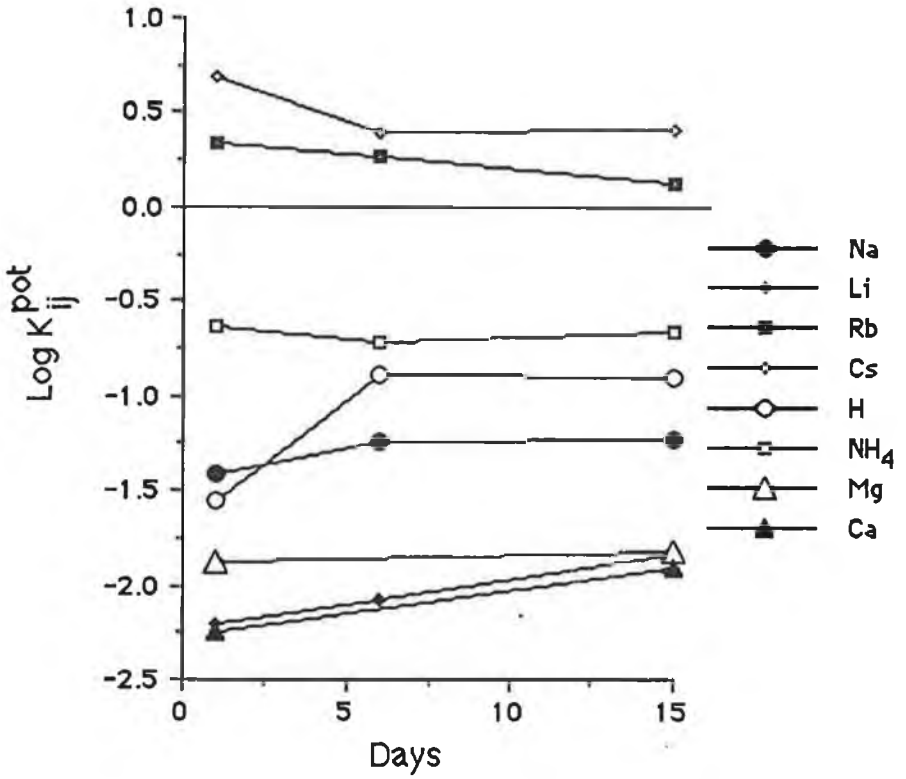
6.3.4. Selectivity

The following selectivity trends were observed for the compounds tested and are summarised in Table 6.5 and are graphically represented in Figures 6.6, 6.7, and 6.8. The selectivity coefficients, Log K_{ij}^{pot}, with the exception of $Cs^+$and H^+,calculated for the electrode based on compound Ia were found to tend towards zero on storage in 0.1 M K^+ solution. After an initial decrease, the selectivity of the electrode against Cs^+ and H^+ stabilised, Table 6.1 and Figure 6.6. The selectivity coefficients, Log K_{ij}^{pot} for the alkali ions, with the exception of sodium were also found to tend towards zero on storage and use. The selectivity coefficients against H^+ and Ca^{++} was not consistent, unlike those calculated for NH_4^+ and Mg^{++}. With the exception of Ca^{++} and Li^+ ,the selectivity of the electrode based on compound II showed good consistency. Unlike electrodes based on Ia or Ib, there was no appreciable response to Cs^+ or Rb^+, compared to K^+.

Table 6.5 Selectivity patterns for the electrodes tested. Data available in Tables 6.1, 6.2, 6.3, and 6.4.

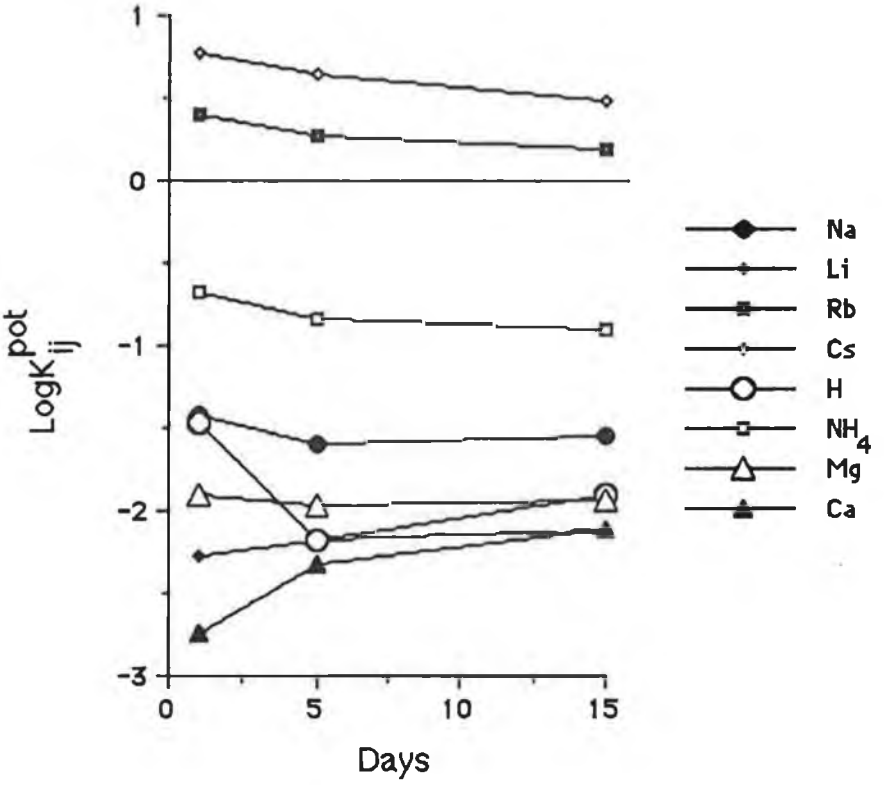
Compound	Selectivity Pattern
Ia	Cs > Rb > K > NH_4 > Na > H> Mg > Li > Ca
Ib	Cs > Rb > K > NH_4 > Na > Mg > Li =H > Ca.
II	Rb > K > Cs > NH_4 > Na Mg > H > Ca=Li
III	K > Na >Rb > Cs > NH_4 > Mg > Li > Ca=H
IV	K > Cs=Rb > Na > NH_4 > Mg > Ca=H

Figure 6.6 Selectivity of electrode based on Ia as a function of time



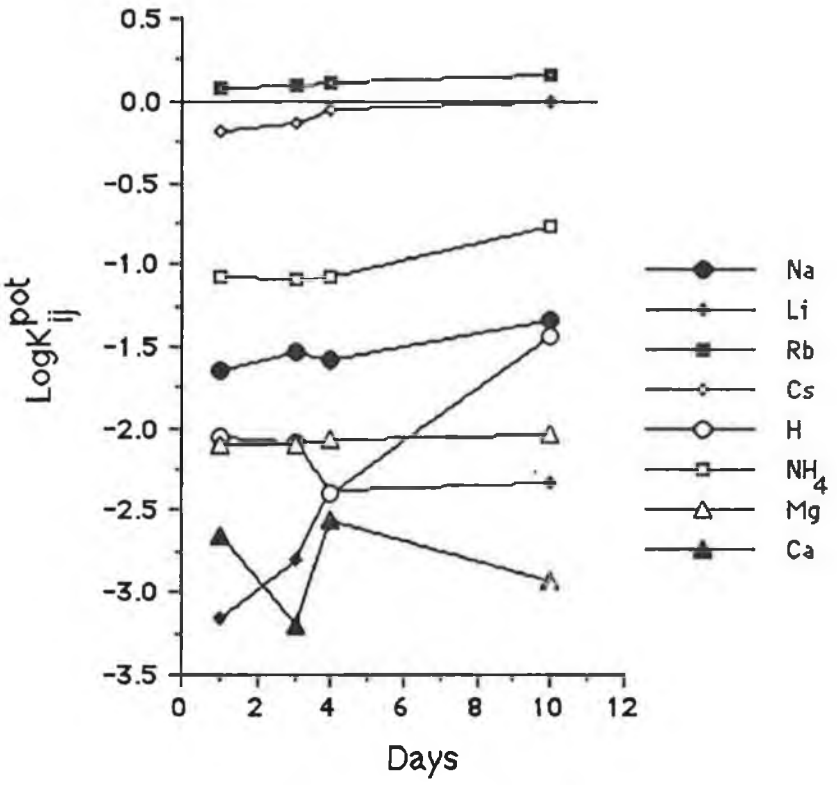
Compounds, Ia and Ib, exhibited a unique selectivity pattern, being more responsive to caesium and rubidium than potassium. The selectivity of the dioxa- thiol derivative, compound Ib, over sodium was better than that of the monoxa- thiol derivative, Ia (Tables 6.1 and 6.2). Compound Ib also exhibited less interference from H^+ and NH_4^+ ions. Both compounds had similar selectivity coefficients for the divalent ions.

Figure 6.7 Selectivity of Electrode based on Ib as a function of time



Compound II was found to have similar selectivity coefficients for sodium as compound Ib (Tables 6.2 and 6.3). There was little discrimination in response between the larger alkali ions, Rb^+, Cs^+ and K^+. It demonstrated excellent preference for K^+ over the other interfering ions with Log K_{ij}^{pot} values < -2 except for H^+ and NH_4^+ ions , Table 6.3.

Figure 6.8 Selectivity of electrode based on II as a function of time



The partial cone compounds were found not to have good selectivity for potassium over sodium. The preference for potassium over the larger alkali metal ions was much better than the oxacalixarenes. They showed excellent selectivity over the other interfering ions. Improvements in selectivity against sodium were found to result in deterioration of selectivity against caesium and rubidium (Table 6.4).

6.4. Discussion

The linear range of the electrodes was found to be 10^{-4}-10^{-3} to 10^{-1} M K^+. This linear range may have been increased if an alternative reference electrode was used since the calomel electrode will introduce K^+ ions to the

sample which effect the membrane response especially at the lower concentrations.

The lifetimes of the electrodes were not assessed fully but two of the electrodes based on compounds Ib and II were still functioning satisfactorily after 10 days. The benefit of the addition of the ion-exchanger, KTpClPB, to the membrane was demonstrated in the membranes made using compound Ib. An electrode based on this compound, without the ion-exchanger, failed after storage for 5 days while the electrode with the ion-exchanger was still functioning after 10 days.

The selectivities of these membrane electrodes based on the oxacalixarenes, were found to decrease on storage. This may be due to leaching of the ionophore or ion-exchanger into the storage solution. With proper attention to the plasticiser used, this decrease in selectivity may have been combatted.

Electrodes based on compounds Ib and II were found to have a greater preference for potassium over sodium than the electrode based on monooxacalixarene, compound Ia. Both these compounds were dioxacalixarenes and thus the presence of the extra spacer unit $-CH_2O-$ must result in a cavity size of better fit for the larger alkali metal ions rather than sodium or lithium (Tables 6.1, 6.2, and 6.3).

Compounds, Ia and Ib, exhibited a unique selectivity pattern, being more responsive to caesium and rubidium than potassium. Compound II did not demonstrate this phenomenon. This would suggest that compound II possessed a cavity size which was close to the optimum for potassium. In the absence of an optimum fit, cations are drawn into the membrane in reverse order to their hydration enthalphies. In the case of the alkali ions, this means

that ions of the largest radius and hence the lowest hydration enthalphy will be preferentially drawn into the membrane. Caesium (1.70A) and rubidium (1.52A) have larger radii than potassium (1.38A) and so will be drawn into the cavity in the following order, Cs>Rb>K. This trend was observed for electrodes based on compound Ia and Ib, Table 6.5 but not for compound II. The ion-exchange KTpClPB generally favours large cations [5] and PVC membranes with KTpClPB only, can demonstrate caesium selectivity [6]. Electrodes based on compounds Ia and Ib demonstrated a greater preference for Rb^+ and Cs^+ than for K^+. This preference for Rb^+ and Cs^+ would not appear to be due to the presence of KTpClPB as an electrode made with compound Ib, without KTpClPB, was found to have similar responses to both rubidium and caesium as the electrode with KTpClPB (Table 6.2).

The selectivity of the electrodes based on the partial cone compounds against sodium, was not very good, with electrode III having almost similar responses to potassium and sodium. The introduction of the longer derivative chain into the ligand resulted in a better discrimination between potassium and sodium but this discrimination was less than 10-fold. These electrodes exhibited much better selectivity against Rb^+ and Cs^+ than the oxacalixarenes. The lack of preference for potassium over sodium, with the excellent selectivity against Rb^+ and Cs^+, would suggest that these compounds exhibit much of the features of an ordinary calix(4)arene.

The ultimate aim was to determine if any of these new compounds were suitable as a potassium ionophore. To date, the best potassium ionophore has been found to be the valinomycin ionophore. Electrodes based on this compound have been shown to have excellent preference for

potassium over sodium. Other compounds have been tried as ionophores for potassium such as the crown ethers [7] but these have been unable to meet the characteristics of the valinomycin electrode. A main area of use for a potassium ionophore would be in the analysis of potassium in serum. Ion-selective electrodes are becoming more popular in clinical analyses due to the ease of use and speed of response. In serum samples, the main interfering ion in such samples would be sodium which is present in serum at a concentration of 135-150 mM. In order that the compound would be useful as a potassium ionophore, the required selectivity coefficient (Log K_{ij}^{pot}) for sodium should be of the order of -3.6 [8]. None of the compounds tested approached this requirement. Only one mediator, o-NPOE, was used. The nature of the mediator is known to affect the response of the membrane. Therefore, other mediators should be used to investigate whether the selectivity for potassium over sodium and the lifetime of the electrodes may be increased.

6.5. Conclusions

All compounds tested exhibited Nernstian responses to potassium. The linear range was 10^{-3} to 10^{-1} M K^+ for electrodes based on compounds Ia and Ib and 10^{-4} to 10-1 M K^+ for electrodes based on compound s II,III and IV. The selectivities calculated for the compounds used in this study were such that it is unlikely that any of these compounds would be incorporated into ion-selective electrodes for general use. Compound II would probably be the most suitable as a potassium ionophore as it would appear to have a cavity size closer to the size of the potassium ion than compound Ia or Ib. Structural modifications to the oxacalixarenes should be pursued to try and

produce a compound with better selectivity against sodium. Other plasticisers should be used to investigate whether the selectivity for potassium over sodium may be increased and the lifetime of the electrodes may be increased.

6.6. References

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Appendix A

Composition of Calibration solutions for the Commercial analysers.

Radiometer ICA2 analyser

Substance	Normal (mmol/ Kg H_2O)	High (mmol/ Kg H_2O)
Calcium Chloride	1.26	2.80
Sodium Chloride	154	101
HEPES Buffer		103
Tris Buffer	4.60	
Sodium Hydrogen Carbonate	0.40	0.40

Baker Analyte+2 Analyser

Substance	Low (mM)	High (mM)
Calcium Chloride	1.20	2.55
Sodium Chloide	120	200
Potassium Chloride	4	7.1
Hydrogen Chloride	37.7	46.6
Tris Buffer	50	50

Both Baker calibration solutions contained Triton X-100 as a preservative.

Appendix B
Details of assembly for the CRM as given to each of the participating Laboratories

CHECKLIST OF APPARATUS PROVIDED

The cell includes:

1	water jacket body	(a)
2	large 'O' rings, BS/USA size No. 139	(b)
2	grey screw-on caps	(c)
1	inner section	(d)
1	base plate	(e)
1	sample flow section	(f)
2	'O' rings, BS/USA size No. 012	
1	large screw-in side plug	(g)
1	'O' ring, BS/USA size No. 016	(h)
1	small screw-in side plug	(i)
1	'O' ring, BS/USA size No. 012	(j)
2	end caps	(k)
4	PTFE olives (2 spare)	(l)
1	electrode body + porous ceramic frit	(m)
1	'O' ring, BS/USA size No. 010	(n)
1	top-cap clamp	(o)
1	stainless steel spring	(p)
1	internal electrode (Ag/AgCl)	(q)

Other apparatus included:

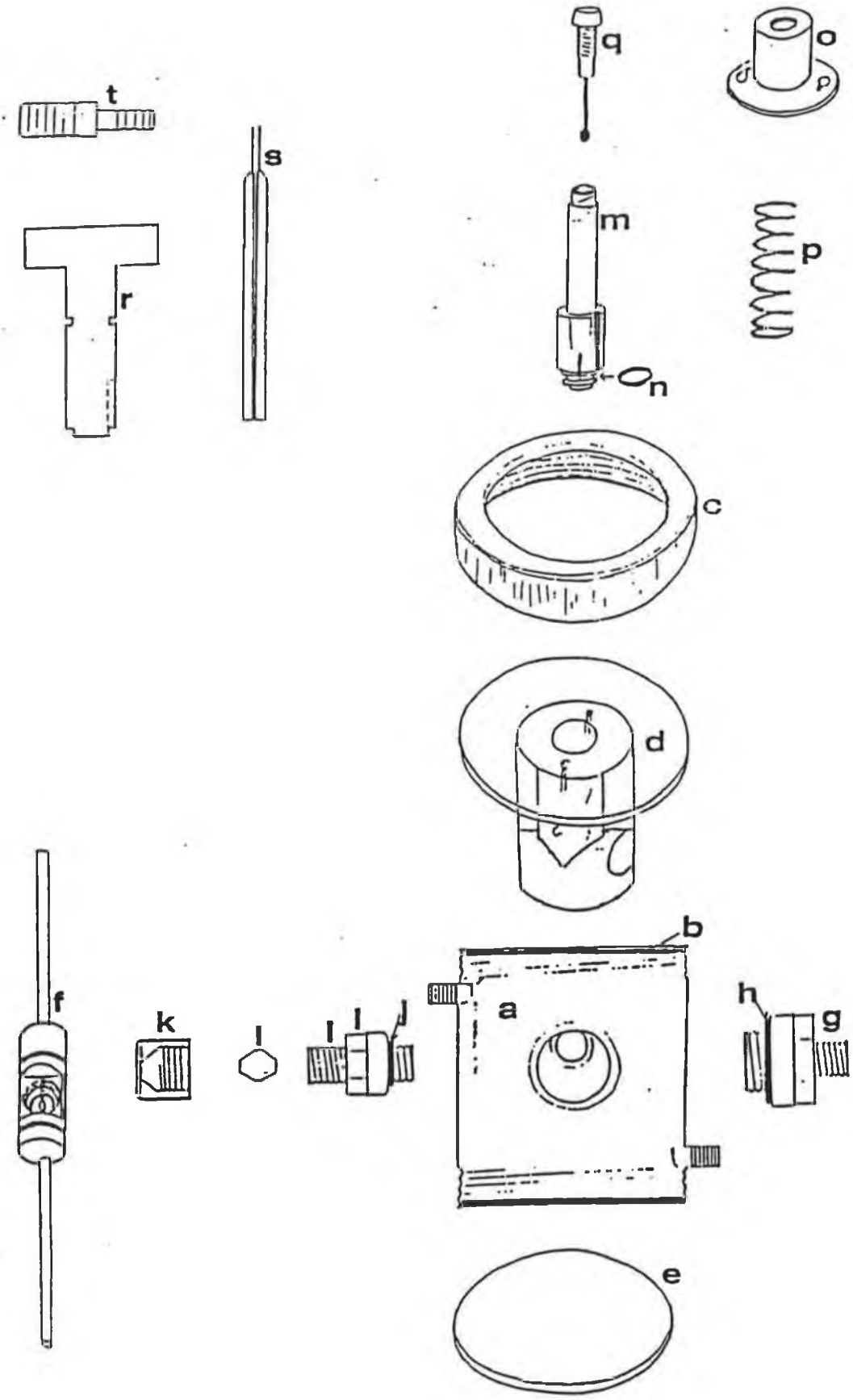
1	key	(r)
1	0.5 mm glass capillary	(s)
2	adaptors for water jacket tubing	(t)
1	length of tubing to fit water jacket	

1 2 mm plug
1 glass reference electrode vessel
1 calomel electrode
membranes
silver chloride

EXTRA APPARATUS REQUIRED

- Plastic syringes (5 ml) and adaptors for sample insertion
- Water bath (37° C)
- Temperature control and water circulation unit + tubing
- High input impedance buffer amplifier + digital voltmeter, or equivalent, eg. research pH meter with 0.1 mV discrimination
- Shielded lead + connector to meter terminating in a 2 mm-plug
- Solutions (1), (2) and (3), as specified by the IFCC reference document [1] (see Appendix 1) (Table 1). Do not use the EUROTROL solutions until the system is working satisfactorily. The solutions can be made up by volume and weight in plastic bottles using the quantities shown in Table 3.2. 1 mol dm^{-3} calcium chloride volumetric solution from BDH is suitable for making up the stock Ca^{2+} solution.
- Solution (1), to act as flush solution
- Saturated potassium chloride solution
- Solution (1) saturated with silver chloride, for use as the internal filling solution of the electrode.

Reference cell parts and accessories



ASSEMBLY OF THE CELL

1. If the side plugs are attached to the cell, remove them (Fig. 3.2a).

2. Secure the inner section in place by screwing on a grey ring (Fig. 3.2b).

3. Screw base plate into place with the other grey ring.

4. Moisten the 'O' rings on the sample flow section with distilled water(Fig. 3.2c).

5. Keeping the sample flow section upright, push it into the cell body through the larger hole (Fig. 3.2d).

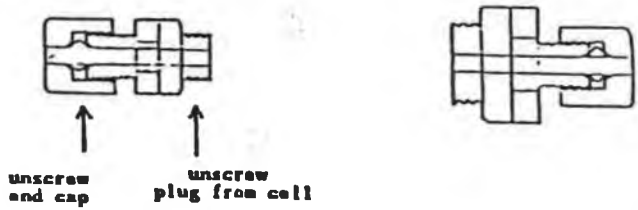
6. Once the sample flow section is in position, it should be aligned properly using the grey key (Fig. 3.2e). Do not pull the tubing - push from either side. If necessary, pressure can be applied to the body of the sample flow section using an appropriate object, eg. thin metal rod. The angle of the flow section can be altered by removing the base plate and applying a spanner to the end with flat sides.

7. Replace the key by the electrode body, and clamp the electrode body in position using the spring and top clamp.

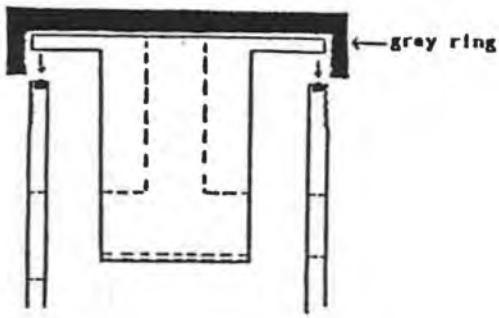
8. Slide the plugs over the tubing and screw them into position in the cell walls. Although a spanner grip is provided on the plugs, finger tightness is normally adequate.

9. Slide the PTFE olives and the outer caps along the tubing and tighten the caps up to FINGER TIGHTNESS ONLY (not much pressure is necessary to make a seal, and over tightening will damage the olives) (Fig. 3.2f).

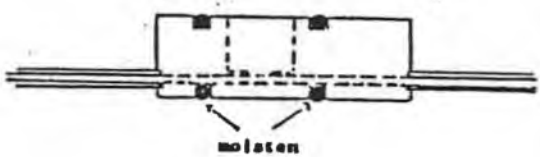
a



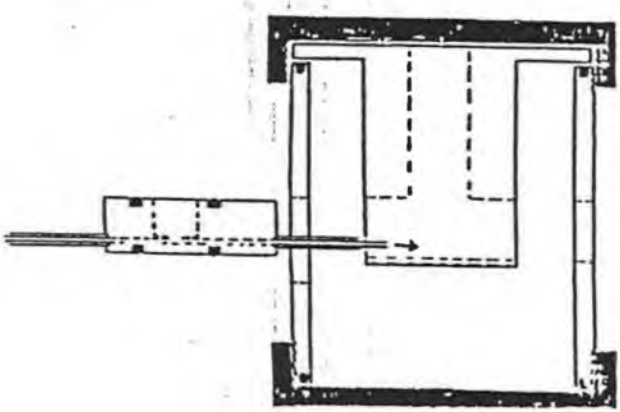
b



c

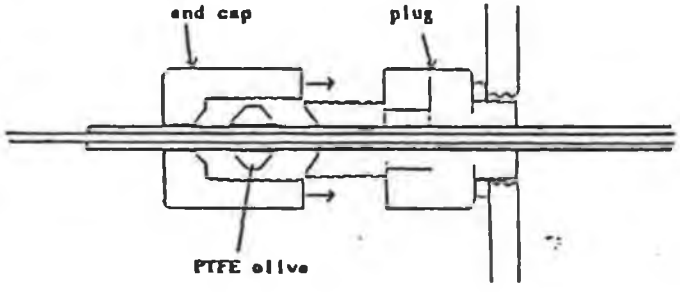


d



e

f



Assembly of the reference cell

USE OF THE CELL

1. Attach the cell to a water temperature and circulation unit and warm to 37 °C.

2. Pre-warm some internal filling solution (solution 1 saturated with silver chloride) to 37 °C.

3. Fill the electrode body with warmed internal filling solution.

4. Place a membrane disc (5 mm diameter) in position in the cell, over the sample flow area.

5. Put a small drop of internal filling solution either in the centre of the membrane, or on the end of the frit at the base of the electrode body.

6. Push the electrode body into place, aligning the slot in its side with the pin inside the cell. Push down firmly and clamp into place with the spring and top clamp.

7. Screw the internal electrode into the electrode into the electrode body, mopping up excess internal solution with a tissue as it is forced out.

8. Seal the internal electrode/electrode body join with sealing tape* to prevent evaporation from the internal solution.

9. Flush the membrane repeatedly with the mid-range calibration solution (solution (1)). Leaving this solution in the sample path, allow the membrane to condition for several hours, flushing occasionally, if possible.

*e.g. Nescofilm, from Nippon Shoji Kaisha Ltd., Osaka, Japan

Taking measurements

Solution pre-warmed to 37 ^{o}C is pushed through the cell, slowly, using a syringe, the syringe should be removed and replaced three or four times during this process to allow small air segments to pass through the cell. The syringe should be emptied of air bubbles after filling with solution as relaxation of air compressed while pushing the solution through the cell can cause solution to be drawn up the capillary and affect the liquid junction.

The syringe is left in place for the measurement, to hold the solution in the capillary. The liquid junction is formed by wiping off excess solution from the capillary and then bringing the reference electrode vessel up under the capillary, as shown in Fig. 3.3.

The emf should be read after 3 minutes.

After a sample solution has been measured, the cell should be flushed with the flush solution (equivalent to IFCC solution 1 - but do not use the EUROTROL solution) before the next calibration solution is put through.

The emf measured for solution 1 (1.25 mmol dm^{-3} Ca^{2+}) should be around 20 - 50 mV. If it is large and changing rapidly, leakage may be occurring around the sides of the membrane.

If no reading is obtained

(ie large voltage, wandering erratically)

This implies a break in circuit, possible causes:

Not plugged in somewhere
Liquid junction not formed
Large bubble in sample solution
No solution between membrane and frit in electrode body
Internal electrode not covered by solution
Solution in external electrode not making contact
Faulty reference electrode
Faulty wiring

Causes of noise

Usually owing to a bad connection somewhere in the circuit:

Bubbles formed in the sample
- at the membrane
- in the connecting tubing
- at the liquid junction

Air trapped between the frit in the electrode body and the membrane

Bubbles formed in the electrode body between the internal electrode and the frit

CHECK:

Sample solution - look for bubbles
- push more solution through and measure again

The internal reference solution - look for bubbles
- top up or replace solution

The membrane/frit interface - remove the electrode body and place a drop of solution on the base of the frit. Replace the electrode body and check the response.

The computer printout of the programme in GWBASIC used to calculate the concentration of the samples measured in the study from the potential readings of the calibration solutions and the sample solutions.

```
10 REM  A computer programme to calculate concentration of samples
50PRINT "input  the variables"
60 REM The following lines input the potential readings from the calibrating
70 REM SOLUTIONS 1,2, and 3. Also inputed are the potential readings for
the unknowm samples.
80 INPUT "E1,1";A
90 INPUT "E1,2";B
100 INPUT "E1,2";C
110 INPUT "E3,1";D
120 INPUT "E1,3";E
130 INPUT "E2,2";F
140 INPUT "E1,4";G
150 INPUT "E3.2";H
160 INPUT "E1,5;I
170 INPUT "Ex,1";J
180 INPUT "E1,6";K
190 INPUT "Ex,2";L
200 INPUT "E1,7";M
210 REM The average potential difference between the unknown sample and
the mid-point calibrating solution, solution 1 is calculated. This will be later
used to determine the calcium concentration in the unknown sample.
220 LET P=((I-J)+(K-J)+(K-L)+(M-L)
230 REM
240 LET T=P/4
250 PRINT "Ex=";T
260 REM
270 REM
280 REM The average potential difference between the mid-point and low
concentration calibrating solutions (i.e. between solution 1 and 2 is
calculated).
```

```
290 REM This is used to calculate the sensitivity of the electrodes between these concentrations (i.e how closely to the theoretical response the electrode is functioning)
300 LET Q=((A-B)+(C-B)+(E-F)+(G-F))/4
310 PRINT "delta Es";Q
320 REM This is used to calculate the sensitivity of the electrode between these concentrations (i.e how closely to the theoretical response the electrode is functioning)
330 LET W=Q/.4948
340 PRINT "SENSITIVITY g2";W
350 REM The average potential difference between the mid-point and high
360 REM concentration calibrating solutions (i.e sloution 1 and 3) is calculated.
370 LET R= ((C-D)+(E-D)+(G-H)+(I-H)/4
380 PRINT "delta ES";r
390 REM The sensitivity of the electrode between the mid-point and high concentration calibrating solutions (solution 1 and 3) is calculated.
400 LET Y= (R/(-.30103))
410 PRINT  "SENSITIVITY G3";Y
420 REM The following two lines take the percentage sensitivities calculated and convert them to millivolt readings
430 LET X=W/30.77
440 LET V=Y/30.77
450 REM The sensitivity of the electrodes as measured using the calibrating solutions are printed on the screen for recording
460 REM
470 REM
480 PRINT "The sensitivity Sii is...";X
490 PRINT "The sensitivity Siii is...";V
500 REM
510 REM
520 REM Depending on whether the unknown solution is less or more concentrated than the mid-point calibrating sloution, the concentration of the unknown is calculated in separate portions of the program
530 IF T>O THEN GOSUB 590
```

```
540 IF T<0 THEN GOSUB 650
550 Following lines make it possible to repeat the program or abort
560 PRINT "DO YOU WANT TO USE IT AGAIN?Y/N"
570 INPUT X$
580 IF X$="Y" THEN GOTO 10 ELSE END
590 REM Calculation of unkown concentration when the unknown is of lower
concentration than the mid-point calibrating solution
600 LET AA=((T/Q)*-.49485
610 REM
620 LET CX=(1.25 * (10^AA))
630 PRINT "THE CONC. IS";CX"mM"
640 return
650 REM Calculation of unknown concentration when the unknown is of
higher concentration than the mid-point calibrating solution (solution 1)
660 LET BB=((T/R)*.30103)
670 REM
680 LET DX=(1.25 *(10^BB))
690 PRINT "THE CONC IS ";DX"mM"
700 RETURN
```

Appendix C

Comparison of Results obtained at our laboratory and those obtained at other participating laboratories. In the figures below, our results are denoted by an asterisk below the number of the laboratory for the CRM results and the letter of the analyser used.

Figure C.1 Aqueous sample H5. Actual concentration=1.25 mM

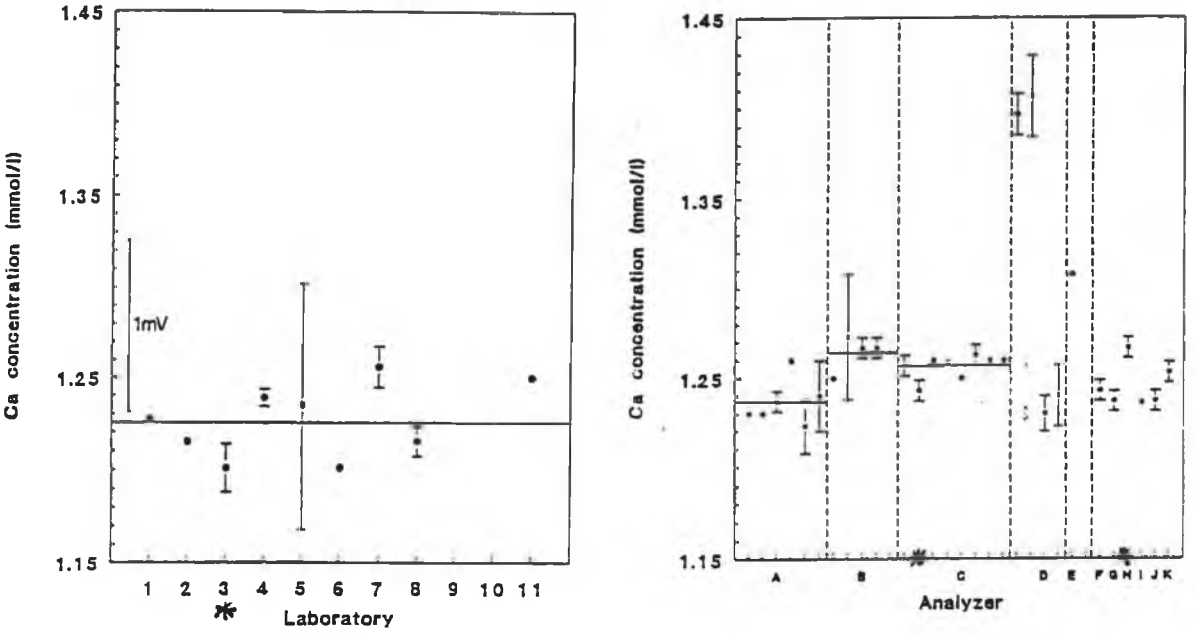


Figure C.2 Protein containing sample B1. Assigned value = 1.25 mM

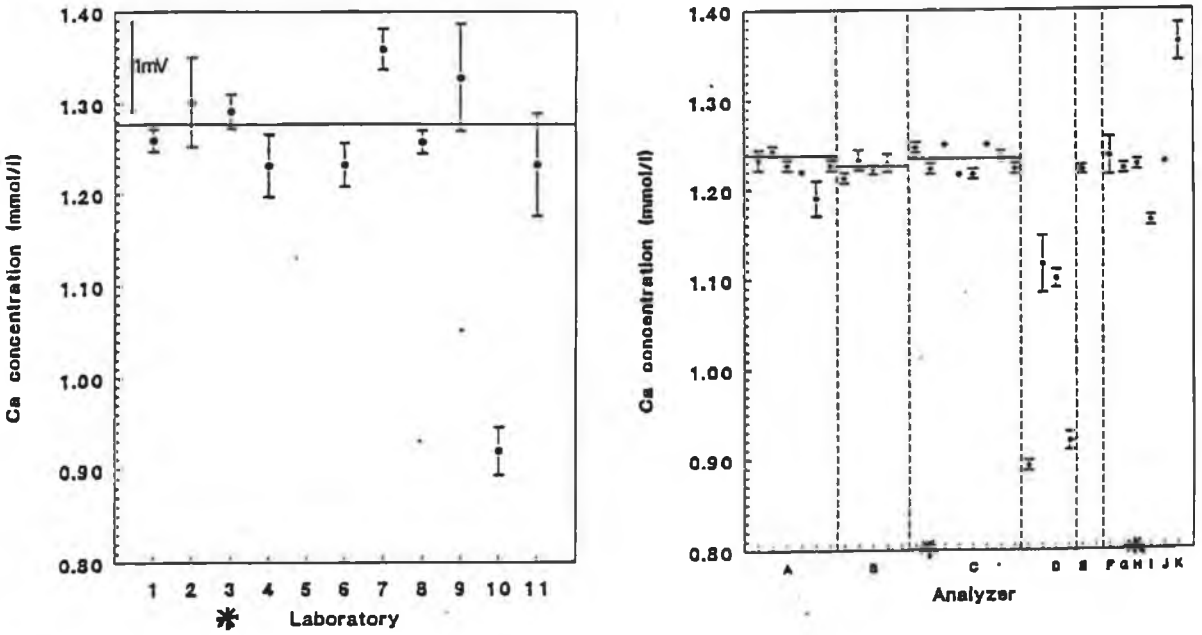


Figure C.3 Protein containing sample D1. Assigned value = 1.25 mM.

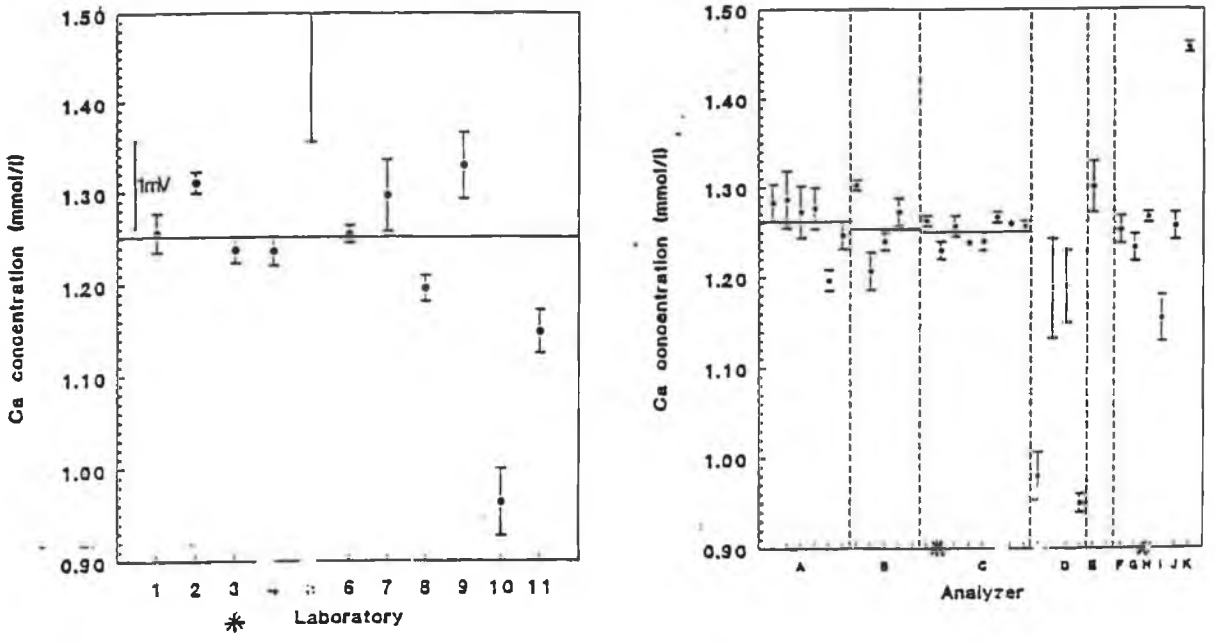


Figure C.4 Protein containing sample D2. Assigned value = 0.75 mM.

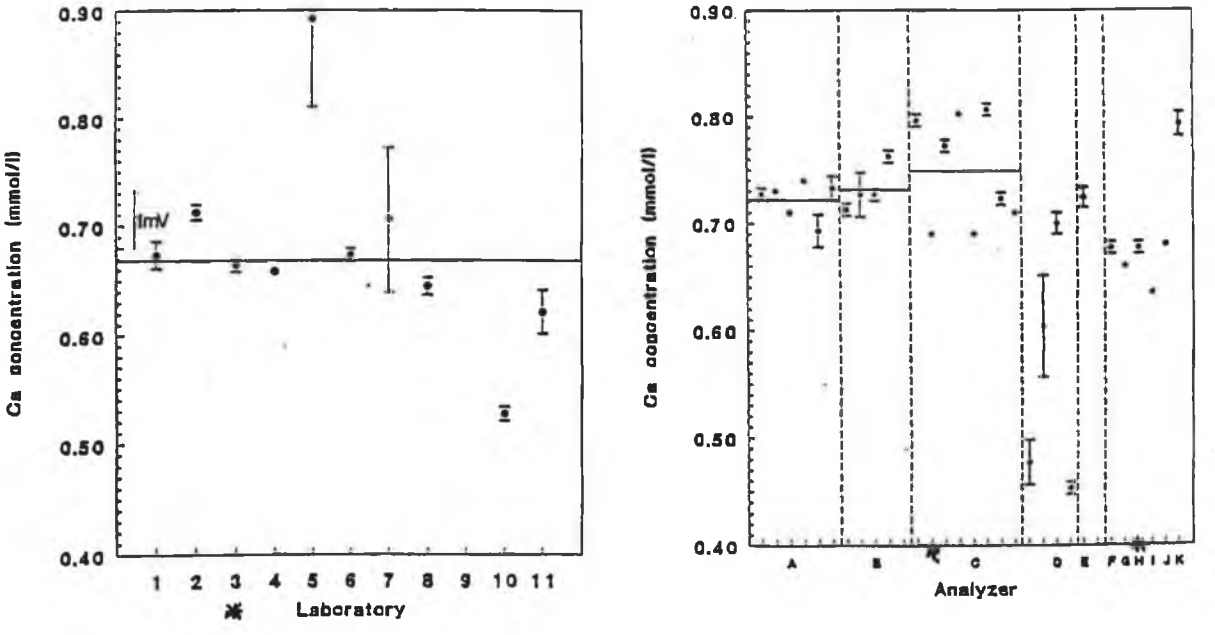
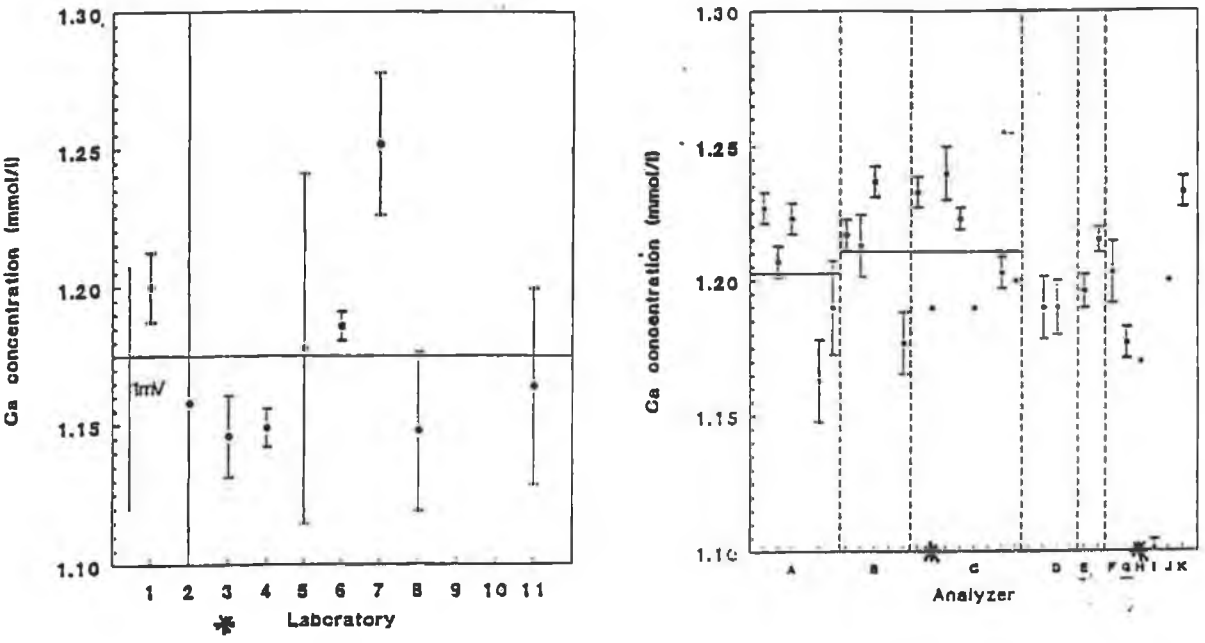


Figure C.5 Human Serum sample HS3. Assigned Value = 1.22 mM



Appendix D

D.1. Introduction

Fundamental research into the processes occuring in the RepHex junction were carried out at the Laboratory for Analytical Chemistry, Abo Akademi, SF-20500 Turku, Finland. The stability of the junction was assessed in various buffers and the characteristics of the material were investigated using impedence measurements.

D.2. Stability Measurements

The stability of the RepHex electrode was assessed by titration of NaCl in Buffer solutions using commercially available Orion double junction Ag/AgCl, NEK and Ag/AgCl disc electrodes. The NaCl was added incrementally, to a final concentration of 0.016M , to see if the addition would perturb the junction potential

D.3. Impedence Measurements

Impedence measurements were carried out on two electrodes, a standard RepHex junction and an undoped (i.e. no salts added) RepHex junction. Impedence measurements were made using a potentiostat/galvanostat with a GPIB interface (NF Circuit Design Block Co. Ltd., Japan). The typical amplitude of the sinusoidal signal was 100 and 200 mV.

Table D.1

Supporting Electrolyte	0.1M acetic acid buffer pH 5
Initial Volume	50 mL
Titrant	1.0 mol dm^{-3} NaCl
Indicator Electrode	Ag/AgCl disk electrode
Reference Electrode	RepHex (active)

V/μL	C/mol dm^{-3}	E/mV (after 1 minute)		
0	0.000	290.2	289.7	289.6
50	0.001	197.4	197.1	
100	0.002	181.2	181.1	
200	0.004	164.7	164.7	
400	0.008	148.4	148.3	
800	0.016	133.0	132.0	131.9

Table D.2

Supporting Electrolyte	0.1M acetic acid buffer pH 5
Initial Volume	50 mL
Titrant	1.0 mol dm^{-3} NaCl
Indicator Electrode	Ag/AgCl disk electrode
Reference Electrode	RepHexTM (inactive)

V/μL	C/mol dm^{-3}	E/mV (after 1 minute)		
0	0.000	unstable	unstable	unstable
50	0.001	unstable	unstable	unstable
100	0.002	unstable	unstable	unstable
200	0.004	unstable	unstable	unstable
400	0.008	unstable	unstable	unstable
800	0.016	unstable	unstable	unstable

Table D.3

Supporting Electrolyte	0.1M acetic acid buffer pH 5
Initial Volume	50 mL
Titrant	1.0 mol dm^{-3} NaCl
Indicator Electrode	RepHex (active)
Reference Electrode	Orion D/J Ag/AgCl

V/μL	C/mol dm^{-3}	E/mV (after 1 minute)	
0	0.000	2.3	2.3
50	0.001	2.3	2.3
100	0.002	2.4	2.4
200	0.004	2.4	2.4
400	0.008	2.4	2.4
800	0.016	2.4	2.4

Table D.4

Supporting Electrolyte	0.1M acetic acid buffer pH 5
Initial Volume	50 mL
Titrant	1.0 mol dm^{-3} NaCl
Indicator Electrode	RepHex active
Reference Electrode	NEK

V/μL	C/mol dm^{-3}	E/mV (after 1 minute)	
0	0.000	-45.9	-45.9
50	0.001	-45.8	-45.9
100	0.002	-45.8	-45.8
200	0.004	-45.8	-45.8
400	0.008	-45.8	-45.8
800	0.016	-45.8	-45.8

Table D.5

Supporting Electrolyte	0.1M phosphate buffer pH 7.1
Initial Volume	50 mL
Titrant	1.0 mol dm^{-3} NaCl
Indicator Electrode	Ag/AgCl disk electrode
Reference Electrode	RepHex (active)

V/μL	C/mol dm^{-3}	E/mV (after 1 minute)	
0	0.000	2.3	2.4
50	0.001	2.4	2.4
100	0.002	2.3	2.4
200	0.004	2.4	2.5
400	0.008	2.4	2.4
800	0.016	2.4	2.4

Table D.6

Supporting Electrolyte	0.1M boric acid buffer pH 9.2
Initial Volume	50 mL
Titrant	1.0 mol dm^{-3} NaCl
Indicator Electrode	RepHex (active)
Reference Electrode	NEK

V/μL	C/mol dm^{-3}	E/mV (after 1 minute)	
0	0.000	-43.9	-43.8
50	0.001	-43.8	-43.9
100	0.002	-43.8	-43.7
200	0.004	-43.8	-43.7
400	0.008	-43.7	-43.6
800	0.016	-43.7	-45.6

Table D.7

Supporting Electrolyte	0.1M boric acid buffer pH 9.2
Initial Volume	50 mL
Titrant	1.0 mol dm^{-3} NaCl
Indicator Electrode	RepHex (active)
Reference Electrode	Orion D/J Ag/AgCl

V/μL	C/mol dm^{-3}	E/mV (after 1 minute)
0	0.000	
50	0.001	
100	0.002	
200	0.004	
400	0.008	
800	0.016	

D.4 Stability in Buffer solutions

The results obtained from seven studies are summarised in Tables D.1- D.7 and in Figure D.1., (traces 1-7). The importance of KCl doping for the proper functioning of the RepHex junction was demonstrated in Table D.2 where no stable measurements were possible with the undoped inactive RepHex electrode. The stability of the RepHex junction compared to commercially available reference electrodes was demonstrated by titration of NaCl into buffer solutions at pH 5.0, 7.1 and 9.1 (Tables D.3-D.7 and Figure D.1). The juncion potential was not perturbed above the resolution of the meter used (0.1 mV) except in boric acid buffer where a maximum of 0.3 mV was obtained.

D.5. Impedence Studies

The impedence spectra for the undoped (inactive) and doped (active) RepHex type A junctions are shown in Figures D.2 and D.3 respectively, together with equivalent circuits for each. The almost vertical line in Figure D.2 is typical of a blocked interface [1] with no d.c. resistance or d.c. current.

The equivalent circuit shows a double layer capacitance (C_{dl}) in series with a bulk resistance and capacitance (R_m and C_m), which gives rise to the very high impedence at low frequencies. In contrast, the impedence spectrum shown in Figure D.3 is typical of an unblocked interface [1]. It shows two adjacent semicircles reflecting two relaxational processes with time constants (τ' and τ'') given by

$$\tau' = R_m C_m = 1/\omega' = 0.03\text{ms} \quad (1)$$

$$\tau'' = R_{ct} C_{dl} = 1/\omega'' = 0.4\text{ms} \quad (2)$$

This indicates that the ion transfer at the solution/RepHex interface is dictating the kinetics of the electrode response.

Figure D.1 Stability of RepHex type A electrode in various buffer solutions (Details given in Tables D.1 to D7).

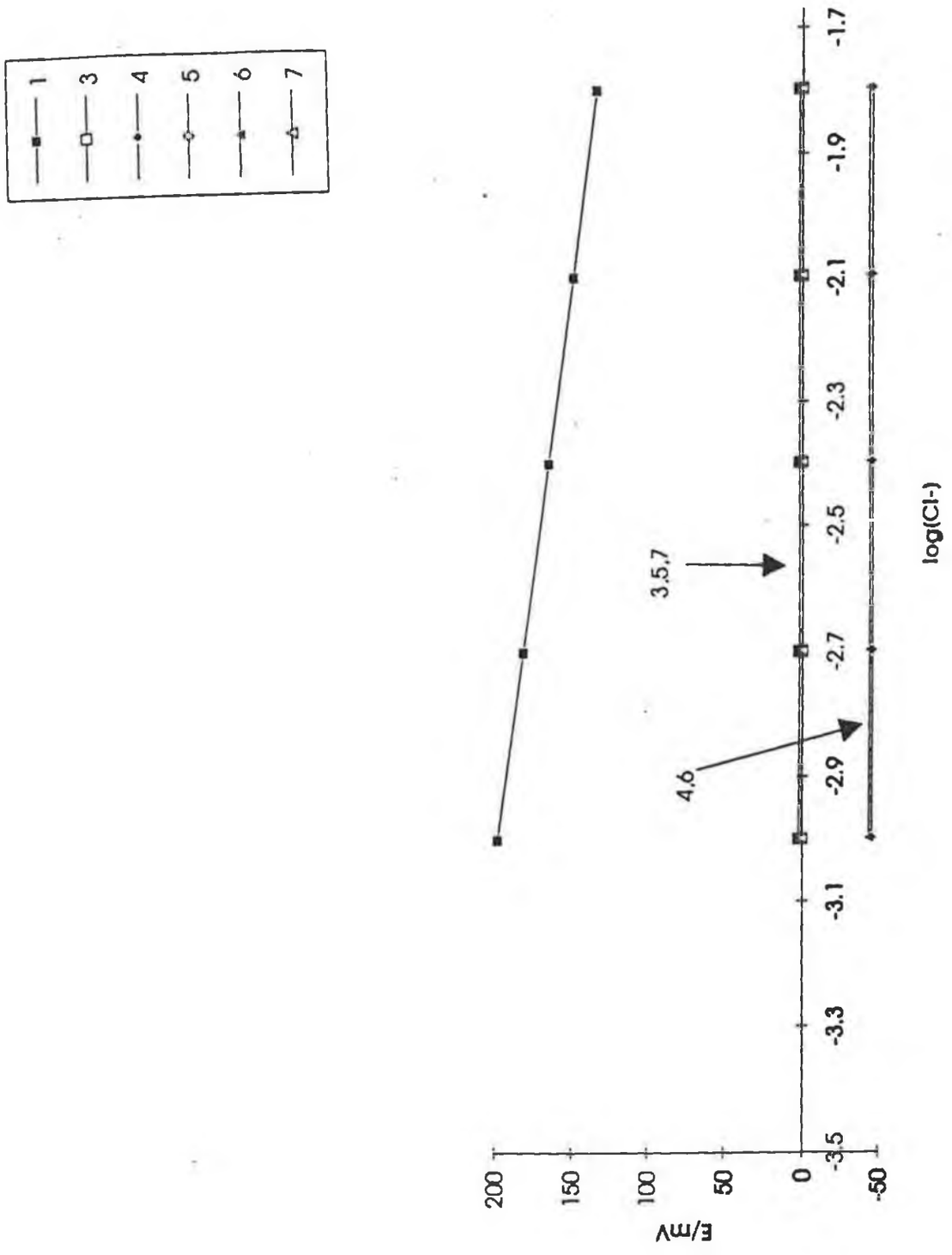
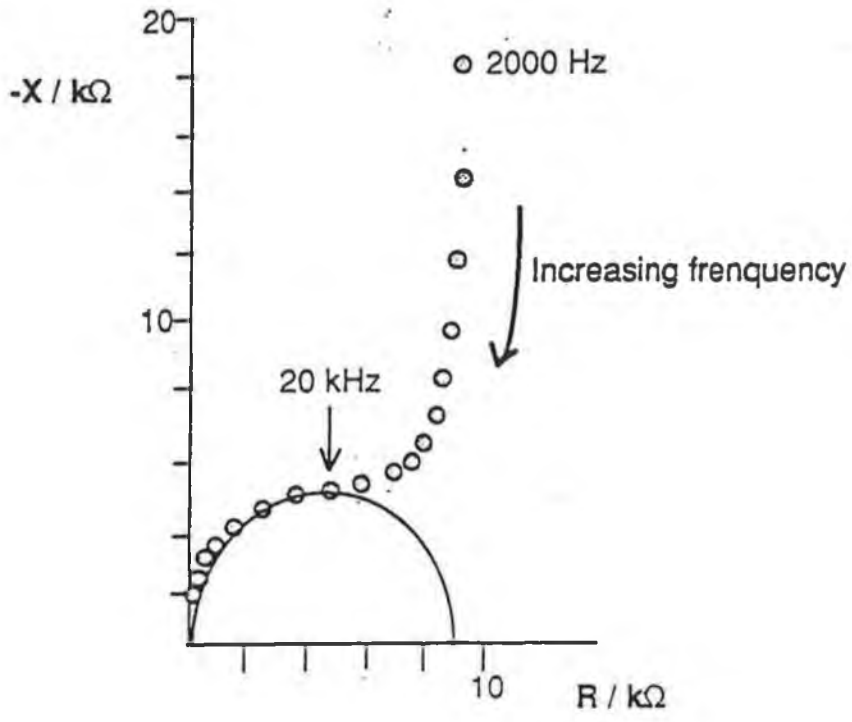
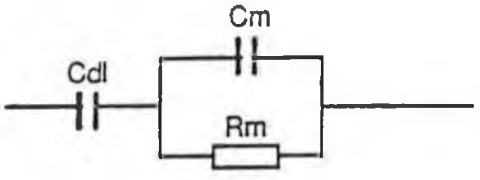


Figure D.2 Impedance spectrum (a) and equivaent circuit (b) for inactive RepHex type A electrode.

(a) Impedance Spectrum



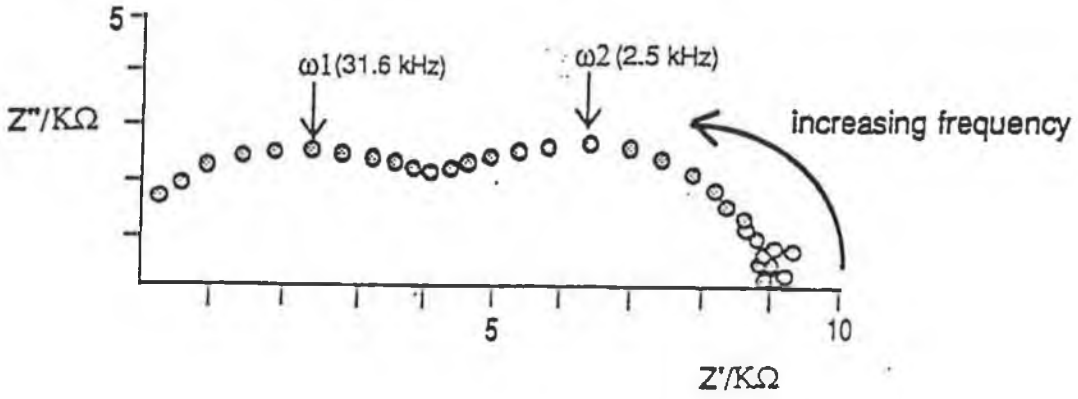
(b) Equivalent Circuit



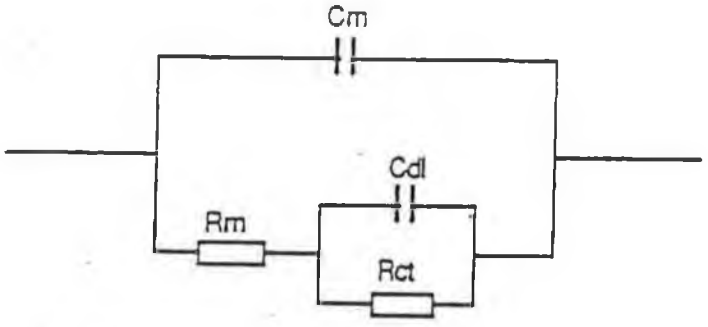
Rm = 9.2 kΩ
Cm = 1 x 10^{-9} F
Cdl = 4 x 10^{-9} F

Figure D.3 Impedance spectrum (a) and equivalent circuit (b) for active RepHex type A electrode.

(a) Impedance Spectrum



(b) Equivalent Circuit



R_m = 4.5 kΩ
C_m = 7.03 x 10^{-9}F
R_{ct} = 4.5 kΩ
C_{dl} = 8.89 x 10^{-8} F

1 Buck R. P., Ion-Selective Electrode Rev., 1982, 4, 3.