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CANADIAN THESES ON MICROFICHE

THÈSES CANADIENNES SUR MICROFICHE

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TITLE OF THESIS/*TITRE DE LA THÈSE* THE DETERMINATION OF CALCIUM AND MAGNESIUM IN BIOLOGICAL FLUIDS.

UNIVERSITY/*UNIVERSITÉ* UNIVERSITY OF ALBERTA

DEGREE FOR WHICH THESIS WAS PRESENTED/ *GRADE POUR LEQUEL CETTE THÈSE FUT PRÉSENTÉE* MSc

YEAR THIS DEGREE CONFERRED/*ANNÉE D'OBTENTION DE CE GRADE* 1975

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THE DETERMINATION OF CALCIUM AND MAGNESIUM IN BIOLOGICAL FLUIDS

by

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A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

DEPARTMENT OF CHEMISTRY

EDMONTON, ALBERTA
FALL 1975

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled The Determination of Calcium and Magnesium in Biological Fluids.

submitted by Peter George Jeremy

in partial fulfilment of the requirements for the degree of Master of Science

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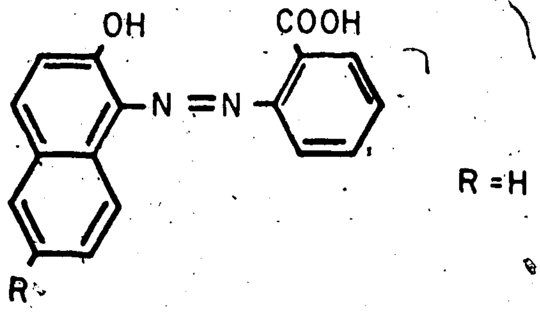
ABSTRACT

A method for the micro-titration of total calcium in urine with EDTA, using calcein as a fluorescent indicator, was developed. Of the major species normally found in urine, only phosphate interferes seriously, and this interference can be overcome by appropriate selection of sample size. A series of urine samples titrated by this method gave values that agreed reasonably well with those obtained by atomic absorption spectroscopy, but averaged slightly higher.

Methods for the determination of ionic calcium in urine were examined. The coated wire calcium ion-selective electrode reported by Freiser and coworkers was prepared and evaluated. It was found unsuitable for the determination of ionic calcium due to erratic and non-reproducible behavior. The spectrophotometric technique of Raaflaub using tetramethylmurexide was found to be more reliable and accurate. A set of urine samples was analyzed for ionic calcium by this method and for total calcium by atomic absorption spectroscopy. An average of 55% ionic calcium was found.

No reliable method has been reported for the determination of ionic magnesium in biological fluids. Work was initiated to find a reagent analogous to tetramethylmurexide for this analysis. The compound 1-(2`-Carboxy-

1'-benzeneazo)-2-hydroxynaphthalene was synthesized and



investigated, but was found to be unsuitable owing to the insolubility of the magnesium complex. A more soluble derivative was prepared by incorporation of a sulphonic acid group in the molecule (R = SO_3H). However, complex formation with magnesium takes place only at pH values above the physiological range, which makes it unsuitable for the analysis of biological fluids.

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CHAPTER I

INTRODUCTION

The analysis of biological fluids such as blood serum and urine has been used for many years as an aid in the diagnosis of certain diseases, in monitoring the progress of patients in hospitals, and in medical research. Calcium is an important element in biological systems and plays a part in many metabolic processes, including conduction of nerve impulses, muscle contraction, and blood clotting. It is also related to kidney stone disease (urolithiasis) since these stones frequently contain calcium.

The measurement of total calcium in biological fluids has been made by a variety of techniques. In hospitals the most commonly used method is a spectrophotometric procedure using cresolphthalein complexone,[1-3] although other reagents, including methylthymol blue and alizarin, have been used.[4-7] Atomic absorption spectrophotometry is now widely accepted for this analysis because it is rapid, accurate and interference effects are generally easily overcome.[8-10] Titrimetric analysis has also been used for the determination of calcium in serum and urine, with ethylenediaminetetraacetic acid (EDTA) as titrant and calcein as indicator.[11-15] However, low results and poor end-points are often encountered when the determination of calcium in untreated urine is attempted by this method.[13-15] Part of the work described in this thesis is concerned with a search for an improved method for the analysis of calcium in urine by titration with EDTA. This would be useful for small laboratories that do not have access to equipment such as atomic absorption spectrophotometers.

The methods outlined above in general measure the total calcium concentration and thus do not distinguish between free and complexed calcium. In blood serum and urine approximately 40% to 60% of the calcium exists as the free ion (co-ordinated to solvent only), the remainder being complexed to ligands such as citrate and phosphate, and in blood serum also to proteins. It has been known for many years that it is the free, or ionic concentration (or more accurately the ionic activity) of calcium that is physiologically active,[16,17] and therefore a measurement of this fraction is often more valuable to the clinical chemist. For example, in the study of urolithiasis it has been suggested that one condition for nucleation of stones is that the urine be supersaturated with respect to two ions which form an insoluble salt, such as calcium and oxalate. Many attempts have been made to correlate ionic concentrations of species such as calcium, oxalate, and phosphate in urine with the formation of kidney stones, but the process is still poorly understood.[18-21]

Various methods have been employed to measure ionic calcium concentrations. These include the use of ion-selective electrodes, and biological, spectrophotometric and calculation techniques. Of these, the greatest advance in recent years has been in the field of ion-selective electrodes. The introduction of commercially available calcium ion-selective electrodes has led to extensive research in clinical chemistry and the biomedical field.[22,23] Most work with the calcium electrode has been done with blood serum, where it has been found to give good results. For urine, the electrode has not been very satisfactory because of difficulties arising from large variations in composition, ionic strength and pH of urine samples.[24] This thesis includes a description of work that was carried out on the preparation and study of a new type of calcium-selective electrode, initially proposed by Freiser and coworkers,[24a] consisting of a liquid ion exchanger in a polyvinyl chloride matrix coated on a platinum wire.

It has been known since 1934 that the amplitude of contraction of the ventricle of an isolated frog heart can be related to ionic calcium concentration,[16,17] and this method has been widely used, although its obvious limitations now render it obsolete.

If information regarding the total concentrations of all major species present in a solution is available, and the equilibrium constants of all the possible complexes are known, then it is possible to calculate the ionic concentration of any of the species. This has been done for calcium in urine by Robertson,[24] using a computer to carry out the calculations by a process of successive approximations. However, the method is tedious since it requires the analysis of the total concentration of each major constituent of the urine sample.

Recent work on the jellyfish protein aequorin has shown that this substance produces a luminescence that is proportional to the ionic calcium concentration,[25,26] and can be used to determine very low concentrations of calcium. However, the method has the disadvantage that the formation constant of the aequorin-calcium complex is sufficiently high that disruption of the equilibrium between bound and free calcium can occur in some systems and thus lead to error.

The dye murexide has been used for many years to determine ionic calcium concentration.[27-29] However, its formation constant with calcium is pH dependant and it forms a weak complex with sodium, which does not make it very satisfactory for analysis of substances such as urine that have a wide variation in pH and sodium concentration. Tetramethylmurexide, reported by Gysling and Schwarzenbach in 1949,[30] also forms a calcium complex that can be used as the basis for the spectrophotometric determination of ionic calcium concentration in blood serum and urine.[31-34] It has the advantage that its formation constant with calcium is independent of pH in the region 4 to 8, where most urines fall. Part of the work in this thesis describes an evaluation of this method for the analysis of ionic calcium in urine.

Magnesium is also important in biological systems, and is known to be related to processes such as protein and nucleic acid synthesis, phosphorylation, and metabolism of carbohydrates.[35,36] As in the case of calcium, the ionic fraction is considered to be the physiologically active species, and thus a method for the analysis of ionic magnesium would be useful for medical and clinical research. A magnesium ion-selective electrode has yet to be developed, and the most commonly employed method is a spectrophotometric procedure using Eriochrome Black T or Eriochrome Blue.[35-37] However, these reagents are far from ideal and the method suffers from interference by calcium and hydrogen ions. The last section of this thesis is concerned with a search for a more suitable reagent for the analysis of ionic magnesium concentration in biological fluids.

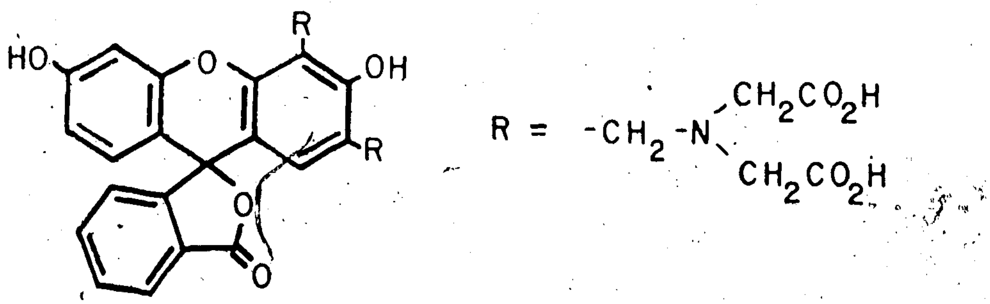
CHAPTER II

DETERMINATION OF TOTAL CALCIUM IN URINE BY TITRATION WITH EDTA

Background

Calcium forms a complex with ethylenediaminetetraacetic acid (EDTA) in alkaline solution ($K_f = 3 \times 10^{10}$) that can be used as the basis for its titrimetric determination. Although EDTA forms complexes with most metal ions, the method can be made essentially specific by choice of optimum pH conditions and by the use of masking agents. In biological fluids such as urine, concentrations of transition metals are usually small and those that are present, principally iron, copper and zinc, can be masked by reagents such as sodium cyanide. Interference from magnesium can be avoided by titration at a pH of 12 or greater, where insoluble magnesium hydroxide is formed.

The principle applied by Schwarzenbach to prepare metallochromic indicators was used by Diehl and Ellingboe[38] in 1956 to prepare a metallofluorescent indicator which they called calcein (also known as fluorexone and fluorescein complexone) which is thought to have the following structure.[39]



Calcein is fluorescent in the pH range of 3 to 10. This green fluorescence is quenched by many metal ions. Above pH 10, calcein does not fluoresce but the complexes with calcium, strontium, barium and magnesium do. This fluorescence can be quenched by the addition of EDTA, which forms stronger complexes with these metal ions than does calcein. It was found that sodium also causes some fluorescence at high pH and thus potassium hydroxide should be used rather than sodium hydroxide to adjust the pH of solutions of calcium prior to titration.[40] The excitation spectrum of the calcium-calcein complex at pH 12 shows a maximum at about 495 nm, and a long wavelength ultraviolet lamp is the best excitation source. The emission (fluorescence) spectrum shows a maximum at about 520 nm, which is visible to the eye as an intense green colour.

The method for calcium as applied to blood serum is usually carried out by placing the sample (containing appropriate masking agents and adjusted to the correct pH) in a darkened enclosure containing an ultraviolet lamp, and titrating with EDTA until the fluorescence disappears.[11-15] As previously mentioned, difficulties are often encountered when this technique is applied to urine samples,[13-15] and this section considers ways of overcoming these problems in an attempt to provide a rapid, simple and accurate method for the determination of total calcium in urine.

It should be noted that rather than using the conventional method of titrating with a buret, all experiments were carried out by gravimetric titration. This was particularly appropriate for this analysis since small volumes of titrant were used.[41]

Experimental

Reagents and Solutions

All chemicals were of reagent grade and used as supplied. Distilled water was used throughout in preparing the solutions.

Standard EDTA Solution. A 1.861 g portion of $Na_2H_2EDTA \cdot 2H_2O$ (Fisher Scientific. Label assay 99.9%) was dissolved in water to give 1 litre of 0.005 *M* solution. A 5-ml portion of this stock solution was diluted to 100 ml with water to give a working solution 0.00025 *M* in EDTA.

Standard Calcium Solution. A stock solution of calcium was prepared by dissolving 2.497 g of $CaCO_3$ (Allied Chemical) in 60 ml of 1 M HCl and diluting to 1 litre with water to give a solution containing 1 mg of calcium per ml. This solution was then diluted by a factor of 100 to give a working solution containing 10 μg of calcium per ml.

Calcein Indicator Solution. Approximately 20 mg of calcein (Fisher Scientific) was dissolved in 25 ml of water and the minimum amount of 0.1 M KOH. The solution was then diluted to 1 litre with water. This solution was found to deteriorate rapidly unless kept frozen. It was convenient to freeze the solution in small sample bottles, so that one bottle could be used each day.

Potassium Hydroxide - Potassium Cyanide Solution. Approximately 1 g of KCN (Matheson, Coleman and Bell) and 5.6 g of KOH pellets (Fisher Scientific) were dissolved in 100 ml of water.

Sodium Chloride Solution. This was prepared by dissolving 4 g of NaCl (Fisher Scientific) in water to give 1 litre of 0.068 M solution.

Urea Solution. An 8 g portion of urea (Fisher Scientific) was dissolved in water to give 1 litre of 0.13 M solution.

Magnesium Solution. This was prepared by dissolving 0.168 g of $MgCl_2.6H_2O$ (Fisher Scientific) in 1 litre of water to give a solution containing 0.02 g Mg per litre. A portion of this solution was then diluted by a factor of two to give a solution containing 0.01 g Mg per litre.

Phosphate Solution. A solution containing 0.2 g P per litre was prepared by dissolving 2.31 g $Na_2HPO_4.12H_2O$ (BDH) in 1 litre of water. A second solution was prepared by dissolving 0.809 g $Na_2HPO_4.12H_2O$ in 1 litre of water to give a solution containing 0.07 g P per litre.

Oxalate Solution. A 0.0122 g portion of $Na_2C_2O_4$ (J.T. Baker Chemical) was dissolved in 1 litre of water to give a solution containing 0.008 g $C_2O_4^{2-}$ per litre.

Citric Acid Solution. This was prepared by dissolving 0.100 g citric acid (Matheson, Coleman and Bell) in water to give 1 litre.

Albumin Solution. Approximately 5 mg of egg albumin (Merck) was dissolved in 1 litre of water.

Simulated Urine Solution. A solution containing some of the major components of normal human urine was prepared by dissolution of 4 g NaCl, 8 g urea, 0.168 g $MgCl_2.6H_2O$, 0.012 g $Na_2C_2O_4$, 0.100 g citric acid, and 5 mg albumin in water to give a final volume of 1 litre.

Sodium Citrate Solution. Approximately 2 g of tri-sodium citrate (J.T. Baker Chemical) was dissolved in 100 ml of water.

Macroreticular Resin. The styrene-divinylbenzene copolymer resin XAD-2 (Rohm and Haas) was used in an attempt to remove fluorescent compounds from the urine samples.

Potassium Dichromate Solution. A 0.1% solution was prepared by dissolving 0.1 g $K_2Cr_2O_7$ (BDH) in 100 ml of water.

Apparatus

A syringe of 5-ml capacity fitted with a needle suitable for addition of small volumes of titrant was used to carry out the gravimetric titrations. The plunger was lightly lubricated with silicone grease to prevent loss of solution and to provide positive action of the plunger. A support for the syringe during weighing on a top-loading balance was made from a 250 ml polyethylene bottle as described by Harris and Kratochvil.[42] The titrations were carried out inside a 'Chromato-Vue' ultraviolet light enclosure (Ultraviolet Light Products Inc.) for illumination of the titration vessel and contents with ultraviolet light, and to exclude other sources of light.

Procedure

To a small beaker or vial was added 1 ml of the KOH-KCN solution, 1 ml of the working calcium solution (or the sample to be analyzed), 1 ml of water (or the solution to be tested for interference) and 4 drops of calcein indicator solution. A small magnetic stirring bar was added and the vessel placed on a magnetic stirrer inside the ultraviolet light enclosure. The syringe was filled with the EDTA working solution and weighed on a top-loading balance to the nearest milligram. The EDTA solution was then added dropwise from the syringe to the titration vessel until the bright green fluorescence disappeared. The syringe was then reweighed and the weight of titrant required found by difference. Blank titrations were carried out by performing the titration as above but omitting the calcium solution.

Results and Discussion

An initial series of titrations was carried out as described above, using the working calcium solution, in order to assess the accuracy and precision of the method. Analysis of the results for ten titrations gave a relative standard deviation of 0.009, and after subtraction of the blank value an average of 10.0 μg of calcium was found, giving 100% recovery of added calcium.

A study was then made of possible interferences from major components normally present in urine. A table of clinical laboratory values[43] was used to find the major constituents of normal urine, and these were each tested in turn. Titrations were carried out as described in the 'Procedure' section above, using the working calcium solution and adding the substance to be tested in place of the 1 ml of water. The weight of EDTA titrant required in each case was then compared with that used in the titration of pure calcium solutions described above. The results of these tests are summarized in Table I. It can be seen that sodium chloride, urea, oxalate, citric acid and albumin interfere to less than 2% when present at concentrations typically present in urine.

Magnesium was found to cause a reduction in the titre by approximately 2.5% when present in concentrations equivalent to a high (but not abnormal) value for urine. The interference is probably caused by co-precipitation of $Ca(OH)_2$ along with $Mg(OH)_2$. When this concentration of magnesium was combined with sodium chloride, urea, oxalate, citric acid and albumin (the simulated urine solution), a reduction in the titre of less than 1% was observed. This reduction in titration error could be due partly to a neutralization of positive and negative interference effects, or possibly to complexation of the magnesium with oxalate and citrate.

Phosphate was found to interfere more seriously, since it caused a reduction in the titre and gave rise to a 'creeping' end point. This interference is almost certainly caused by formation of calcium phosphate complexes. As EDTA is added to form a stable Ca-EDTA complex, presumably a slow shift in the equilibrium between Ca^{2+} and PO_4^{3-} occurs, liberating more Ca^{2+}, and giving

Table 1. Effects of Selected Constituents of Urine on the EDTA Titration of Calcium

Compound Tested for Interference	Concentration (as added),[a] g per litre	% Recovery of Ca[b]
Sodium chloride	4.0 (NaCl)	101.4
Urea	8.0 ($CO(NH_2)_2$)	101.1
Magnesium chloride	0.02 (Mg)	97.4
" "	0.01 (Mg)	98.5
Sodium oxalate	0.008 ($C_2O_4^{2-}$)	99.2
Citric acid	0.1 ($C_6H_8O_7$)	100.2
Albumin (egg)	0.005 (Albumin)	100.8
Di-sodium hydrogen orthophosphate	0.2 (P)	93[c]
" "	0.07 (P)	97.1[c]
Simulated urine solution	contained all the above except phosphate	99.3

Notes: (a) The bracketed formula following the concentration data indicates the form in which the concentration is expressed. A 1-ml portion of solution was used for each titration.

(b) 10 μg of standard calcium solution was titrated and the results are based on the average of 5 titrations in each case.

(c) Poor end point.

rise to the creeping end point. It has been reported that a back titration will prevent interference from phosphate.[44] This procedure was tested by adding an excess of EDTA to a solution containing calcium and phosphate, and back titrating with standard calcium solution. The results obtained were not satisfactory, since a dragged-out end point was often obtained, particularly when the solution being titrated also contained the simulated urine solution. It has also been reported that by adding citrate the interference from phosphate could be overcome when using cal-red as indicator,[45] presumably because the citrate formed a soluble complex with calcium that prevented precipitation of calcium phosphate. Experiments were carried out to see whether satisfactory results could be obtained with this system. It was found that by adding 10 mg of sodium citrate (0.5 ml of the 2% solution) to the titration solution the creeping end point caused by phosphate no longer occurred. However, the end point was less sharp under these conditions and could not be determined with precision.

The method was then applied to real samples. This was done by diluting the urine by a factor of 10 and titrating a 1-ml aliquot as described under 'Procedure'. A problem became immediately apparent in that the urine samples contained a natural fluorescence under ultraviolet light, that caused difficulty in choosing the end point. This fluorescence was blue and was observed in all samples from both normal individuals and hospital patients. Attempts were made to remove the fluorescence by passing the urine through a column containing styrene-divinylbenzene copolymer macroreticular resin (XAD-2, Rohm and Haas). This treatment was found to remove approximately half of the fluorescence. Destruction of the fluorescent compound by oxidation was also tried. Hydrogen peroxide had no effect but potassium dichromate did remove the fluorescence, probably by a quenching mechanism rather than by oxidation, since reduction of the dichromate by addition of hydrogen peroxide caused return of the fluorescence. The addition of dichromate did not interfere with the EDTA titration and so can be used to eliminate interference from natural fluorescence.

Another problem was that the calcein fluorescence at the end point repeatedly returned for many urine samples. This was almost certainly caused by phosphate interference, since the same effects were observed when phosphate was used in the interference studies.

However, it was found that by using a smaller sample size the problem could be overcome. Use of smaller samples also reduced the natural fluorescence to a point where the use of potassium dichromate was normally not necessary. It was found that good results could usually be obtained when the titre was reduced to 0.5 g or less of 0.00025 M EDTA by appropriate selection of sample size.

The following procedure was finally adopted for the titration of calcium in urine samples:

The urine was diluted by a factor of 20 with distilled water. A 1.00-ml aliquot was then titrated as described in the 'Procedure' section. If the titre was greater than 0.5 g of 0.00025 M EDTA, and if problems arose from a creeping end point or from natural fluorescence, then the titration was repeated using a 0.50 or a 0.25 ml aliquot. If problems still arose from natural fluorescence, 2 to 4 drops of 0.1% potassium dichromate solution were added to quench the interference.

This procedure was applied to a set of urine samples obtained from the University of Alberta Hospital, and the concentration of total calcium in each sample was calculated, correcting for the blank titration and standardizing the EDTA solution against the standard calcium solution. An analysis of the results for ten titrations carried out on one sample gave a value of 21 mg Ca per 100 ml, with a relative standard deviation of 0.041. The results were compared with the values obtained by atomic absorption spectroscopy, carried out by the University of Alberta Hospital clinical laboratory. The results are shown in Table II. From these results, it can be seen that reasonable agreement between the two methods was obtained for most samples. However, in nearly all cases

Table II. Analysis of Urine Samples by Titration with EDTA and Comparison with Atomic Absorption Analysis

Sample No.	Size of Sample Taken for Titration, ml	Calcium Concentration, mg per 100 ml EDTA Titration	Calcium Concentration, mg per 100 ml Atomic Absorption[a]
1	0.05	7.6	6.8
2	0.025	14	12
3	0.0125	21	18
4	0.0125	27	25
5	0.0125	29	23
6	0.05	2.7	3.1
7	0.05	2.6	2.0

[a] Results reported by University of Alberta Hospital clinical laboratory.

the results by titration were higher than those by atomic absorption. No explanation for this could be found.

CHAPTER III

INVESTIGATION OF ION-SELECTIVE ELECTRODES FOR THE DETERMINATION OF IONIC CALCIUM IN URINE

Background

The calcium ion-selective electrode developed by Orion in the nineteen-sixties is based on a liquid ion exchange membrane which is selectively permeable to calcium.[22,46] The liquid ion exchanger, consisting of the calcium salt of didecyl phosphoric acid dissolved in a suitable hydrophobic solvent such as dioctylphenyl phosphonate, is allowed to contact the aqueous sample solution by means of a porous membrane. The inner surface of the ion exchanger-soaked membrane is in contact with an internal reference solution of calcium chloride. When the electrode is placed in a sample solution, calcium ions migrate across the membrane from the solution of higher calcium activity to the one of lower activity until the potential difference set up across the membrane prevents further migration. This potential difference can be measured if the electrical circuit is completed by means of an internal silver-silver chloride reference electrode, brought into contact with the internal calcium chloride solution, and an external reference electrode immersed in the sample solution. This is shown in simplified form in Figure I. The cell potential for calcium is then given by the Nernst equation:

$$E = \text{constant} + \frac{2.303RT}{2F} \log(a_{Ca^{2+}} + \Sigma K_i a_i^{2/Zi})$$

The constant depends largely on the reference electrode used. The term $\Sigma K_i a_i^{2/Zi}$ is the sel~~ec~~tivity factor, where K_i is the selectivity constant between an interfering ion and the calcium ion, a_i is the activity of the interfering ion, and Zi its charge. The Nernst factor, $\frac{2.303RT}{F}$, has a value of 59.16 millivolts at 25°C.

To measure the calcium ion activity of a solution, a calibration graph is prepared by measuring the potentials of reference solutions of various calcium activities, and plotting those potential readings against log $a_{Ca^{2+}}$. The potential given by the sample solution is then read from the calibration plot to give the calcium activity. The lower limit of concentration for this type of electrode is determined by the solubility of the liquid ion exchanger in water, and for this calcium system is in the region 10^{-4} to 10^{-5} M. The most serious interferences likely to be found in biological fluids are hydrogen ions ($K_{H^+} \simeq 10^5$) and magnesium ($K_{Mg^{2+}} = 0.014$). Sodium, potassium and ammonium ions interfere only slightly ($K_{Na^+} = K_{K^+} = K_{NH_4^+} \simeq 10^{-4}$) but can be important in biological fluids where their concentrations may be much larger than that of calcium. As previously mentioned in the intro-

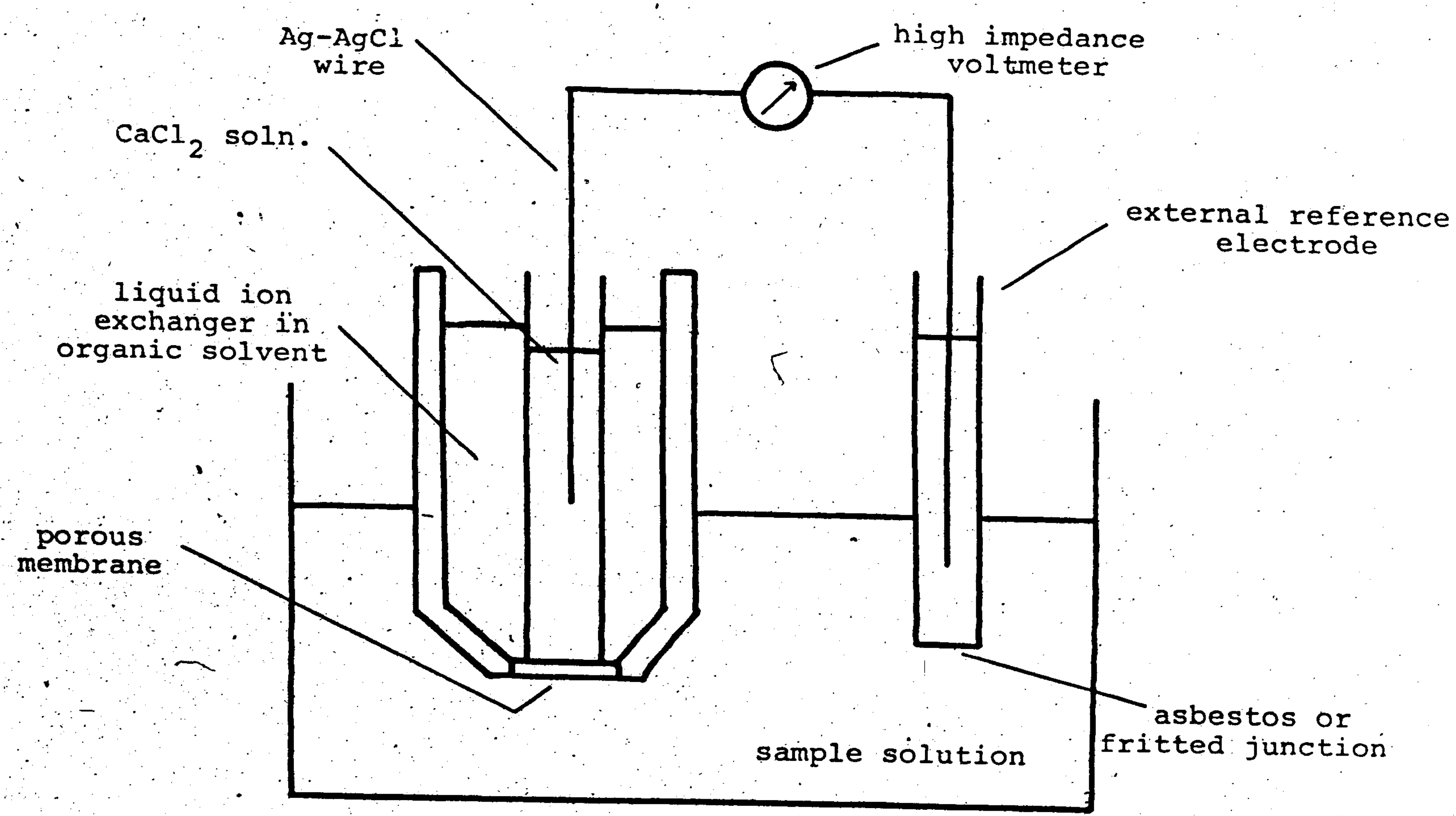


Figure I. Simplified diagram of Orion-type calcium selective electrode.

duction, although these electrodes have been successfully used for the determination of ionic calcium in blood serum, they have not been found to be very satisfactory for use in urine,[47] where the variable ionic strength and range of concentration of molecular species such as urea and uric acid affect the electrode response appreciably.

The promise of this electrode system has led to much research into developing improved types of ion-selective electrodes. Incorporation of the liquid ion exchanger in a solid matrix has been tried by several workers, and appears to be quite promising.[48-50] Other workers have dispensed with the need for an internal reference solution by making direct electrical contact with the solid membrane. Thus Freiser and Cattrall[24a] reported that a calcium selective electrode could be made by incorporating the liquid ion exchanger in a polyvinyl chloride matrix and coating this mixture onto a platinum wire, thus producing an electrode that is compact, inexpensive and simple to make. They reported that this type of electrode was significantly less sensitive to hydrogen and sodium ions than the Orion electrode, and thus would be more satisfactory for use with urine samples. The following section describes the preparation of this type of electrode and its evaluation as a tool in the measurement of ionic calcium concentration.

Experimental

Reagents

The substrate for the electrodes was platinum wire of approximately 0.04 cm diameter. The liquid ion exchanger was obtained from Orion Research (Cambridge, Mass.), code number 92-20-02. Polyvinyl chloride powder was obtained from Magadyne Industries Ltd., Edmonton, Alta. Cyclohexanone, reagent grade, (BDH), was used as a solvent in the preparation of the coating mixture. A stock solution of 0.1 M calcium chloride was prepared by dissolving 10.01 g $CaCO_3$ (Allied Chemical) in the minimum amount of hydrochloric acid, neutralizing the excess acid with sodium hydroxide solution, and diluting with distilled water to give a final volume of 1 litre. Other solutions were prepared from this stock solution by serial dilution to give concentrations of calcium ranging from 10^{-1} to 10^{-5} M.

Apparatus

An Orion Model 701 pH meter, equipped with a commercial saturated calomel reference electrode was used for all potential measurements.

Preparation of Electrodes

A solution containing 0.5 g of polyvinyl chloride (PVC) in 10 ml of cyclohexanone was prepared, and 6 ml of this solution was mixed with 1 ml of the liquid ion exchanger. The platinum wire was then coated by dipping it into the mixture and allowing it to air dry for several hours. The thickness of the coating could be increased by repeating the immersion. The wire was coated to within about one inch of one end so as to prevent contact of the bare wire with the test solution. The uncoated end of the wire was then pushed through a small cork to provide a support that would fit the electrode holder.

Procedure

The coated platinum wire was placed in an electrode holder and connected to the pH meter along with the calomel reference electrode. The electrodes were then immersed in the calcium solutions and millivolt readings taken, normally 10 and 60 sec after immersion. All solutions were stirred magnetically during the measurements. The millivolt readings were plotted against the negative logarithm of the molar calcium concentration to give calibration graphs over the concentration range studied.

Results and Discussion

It was found to be difficult to obtain reproducible results using the coated wire electrodes. This appears to be the result of a number of variables in preparation and use, some of which could not be controlled. These variables included thickness of coating, previous conditioning of the electrode surface, and time of immersion in the test solution.

Effect of Coating Thickness

Electrodes were prepared as described above, using 1 to 5 immersions in the coating mixture to vary the thickness of the coating. Potential measurements were then made as described in the 'Procedure' section above, and calibration graphs plotted for each electrode. The results for several of these electrodes are shown in Figure II. It can be seen that the more thickly coated electrodes gave better linearity and slopes close to the theoretical 29.5 mV per concentration decade (at 25°C) as calculated by the Nernst equation. With 4 coatings, reasonable linearity down to 10^{-4} M Ca was usually obtained along with slopes on the order of 25 mV/decade. No further improvement in linearity was found by increasing the coating thickness beyond 5 layers.

Time Dependency of the Electrode Potential

The electrode response was found to be highly time dependent. In the study of short term time dependency, readings were taken at fixed periods after immersion of the electrode in the test solution. Very large changes in the potential, up to 30 mV, usually occurred in the first minute, but generally a steady potential would be reached after about 3 minutes. After a period of 24 hours or more, an electrode usually gave a totally different

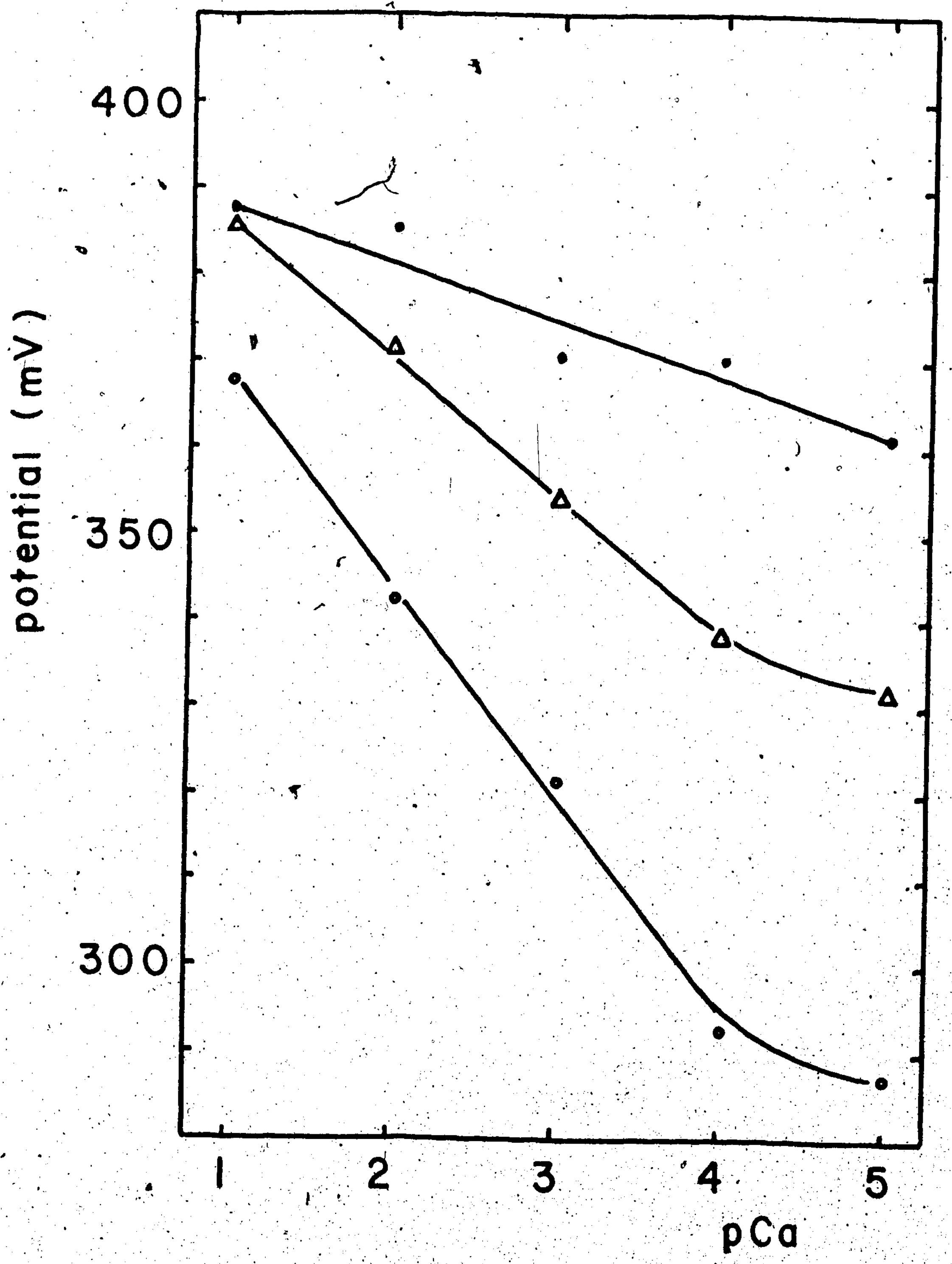


Figure II. Effect of coating thickness on electrode behavior.

- • 1 coating
- Δ 2 coatings
- O 4 coatings

response, and calibration plots often showed a marked decrease in slope. Some of these effects are shown in Figure III and Figure IV. The reason for this behavior is not clear, but may be partly due to a tendency of the coating to absorb or desorb both water and calcium.[51]

Conditioning of Electrodes

On the basis of these results, it was thought that an improvement in reproducibility might be achieved by conditioning the electrodes prior to use. Experiments were carried out in which electrodes were immersed in solutions of different calcium concentrations for various lengths of time before measurements were taken. No significant improvement was found, although it appeared that by soaking the electrodes in 10^{-1} M calcium for 15 minutes prior to use the reproducibility from hour to hour was improved slightly, and the electrodes still gave reasonable linearity after a day or more.

Composition of Coating Mixture

Initially a 5% solution of PVC in cyclohexanone was used in a 6:1 ratio with the liquid ion exchanger. The effect of changing the composition of the coating mixture was studied. Thus a 10% solution of PVC in cyclohexanone was mixed in a 6:1 ratio with the liquid ion exchanger, and electrodes made with this mixture. Also, a 10% solution of PVC in cyclohexane was mixed in a 6:2

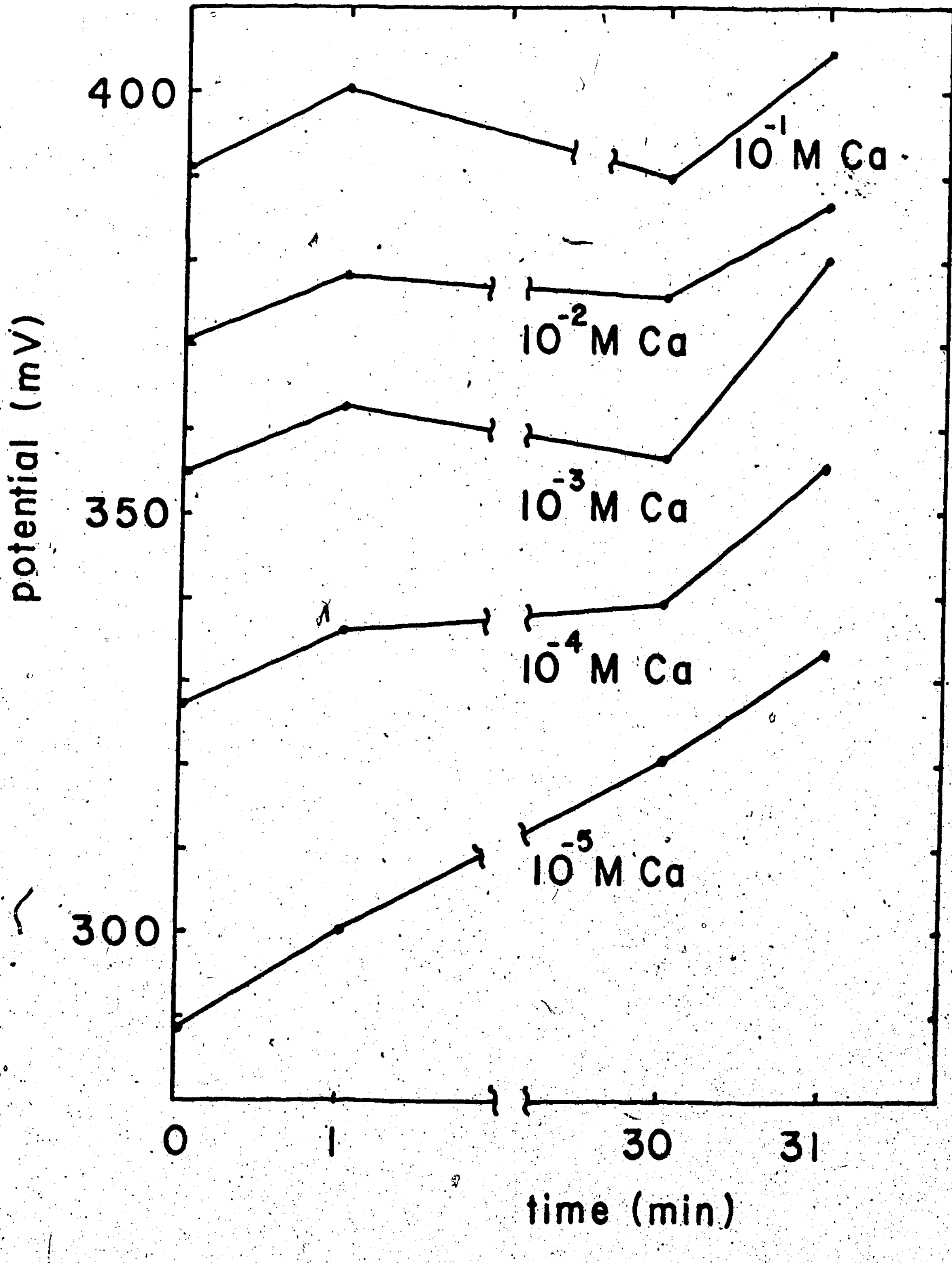


Figure III. Short-term time effects on electrode behavior. Electrode removed from solution after 1 minute, re-immersed after 30 minutes.

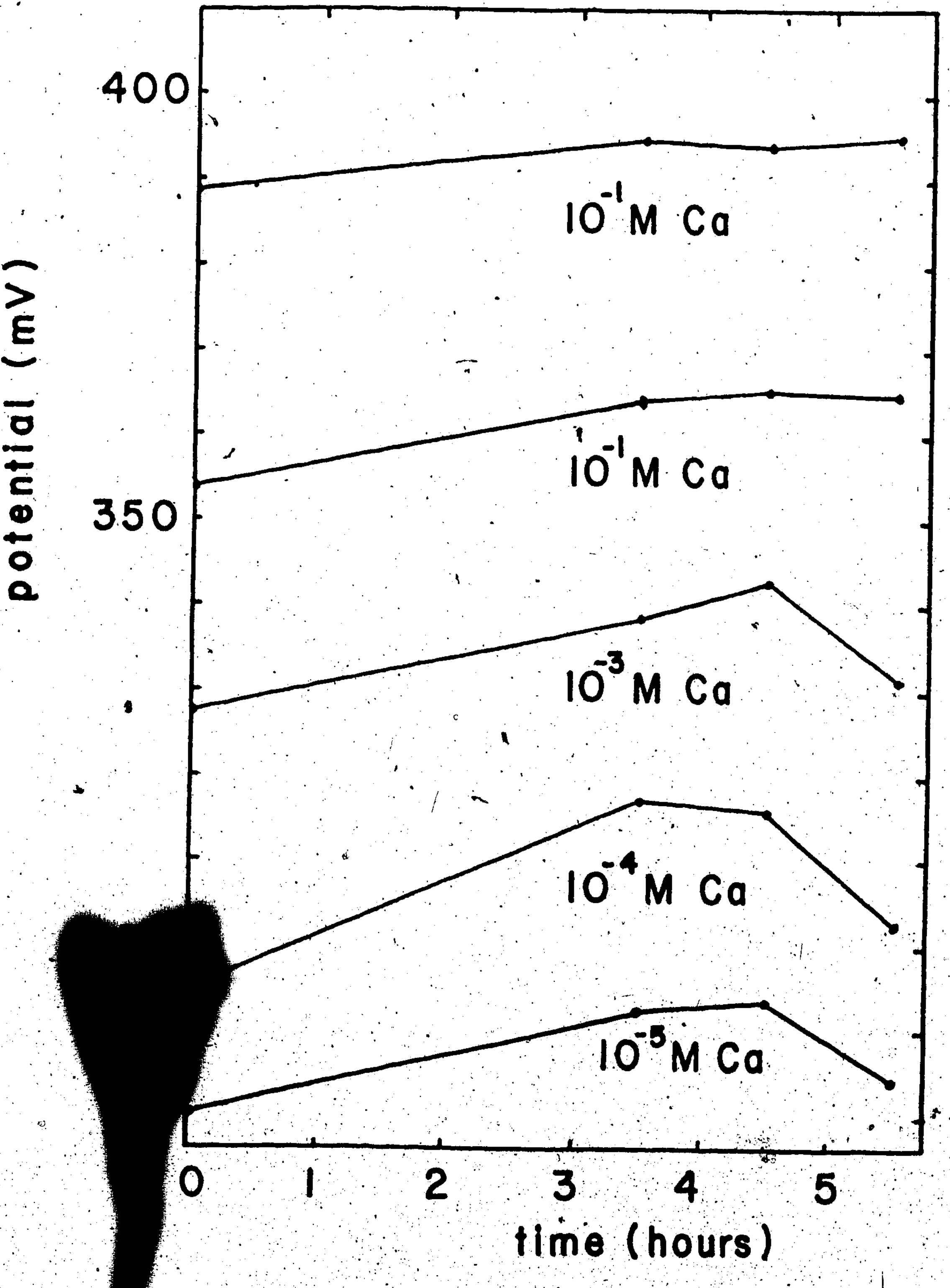


Figure [illegible]. Long-term time effects on electrode behavior. Electrodes stored in air between readings and conditioned by soaking in 10^{-1} M Ca solution for 15 minutes prior to readings.

ratio with the liquid ion exchanger, and electrodes prepared with this mixture. No significant change in the performance of the electrodes was detected in either case.

Effect of Coating Part of the Wire with Paraffin Wax

Freiser and Cattrall[24a] coated only part of the wire, and wrapped the remainder of the bare wire with paraffin film to prevent contact of the metal surface with the solution. In the experiments outlined here, exposure of the bare wire to the test solution was prevented since the coating reached close to the top of the wire. In order to find out whether these two methods gave different electrode characteristics, electrodes were prepared in a way similar to those of Freiser and Cattrall. The electrodes were first coated in the normal manner but when dry, part of the coating was cut through and removed so as to leave approximately 0.75 inches length of coating at one end of the wire. The exposed part of the wire was then repeatedly dipped into melted paraffin wax until a suitable layer of wax had built up along the length of the wire. Measurements were then made in the normal way, using both the paraffin coated and the previously described types of electrodes. No significant difference in the characteristics of the two types of electrode could be observed.

Other Factors Involved and General Conclusions

Several other factors were checked and found to give small effects on the potential readings. The speed of magnetic stirring influenced the readings by ± 5 mV, so constant stirring speeds were maintained for a particular experiment. The depth of immersion of the electrode affected the readings to a similar extent, deeper immersion giving lower millivolt readings. Therefore a constant immersion depth was maintained for a particular experiment; this problem also can be overcome by using the paraffin-coated type of electrode. Good electrical contact between the platinum wire and lead was vital for reproducible results; ideally a soldered joint should be used. In general, newly prepared electrodes give the best results with regard to linearity, low scatter and close to theoretical slopes of the calibration graphs. It was also noticed that linearity was frequently achieved down to calcium concentration of 10^{-4} M, but at lower concentrations the behavior of the electrodes was erratic and unpredictable. Due to the general lack of reproducibility, no further work was carried out with these electrodes.

CHAPTER IV

AN EVALUATION OF TETRAMETHYLMUREXIDE AS A REAGENT FOR THE DETERMINATION OF IONIC CALCIUM IN URINE

Background

In 1949, Gysling and Schwarzenbach described the preparation of the dye tetramethylmurexide from tetramethylalloxantine, and commented on the spectral properties of the free dye and its calcium complex.[30] In 1962, Raaflaub introduced tetramethylmurexide as a reagent for measuring concentrations of ionic calcium in urine and other biological fluids.[31] He described the advantage of tetramethylmurexide over murexide for this purpose - its pH independence over the physiological pH range. However, he found it necessary to prepare standard solutions which matched the sample with respect to sodium concentration and ionic strength. Ionic strength effects occur because tetramethylmurexide responds to changes in calcium activity which in turn is related to ionic strength.

The ionic strength of urine varies widely, and at the present time no accurate and convenient method is available for its determination. Hunt and King estimated the ionic strength of urine samples by comparing their conductance with standards prepared from potassium chloride.[52] However, the relationship between conductance and ionic strength of urine has been shown to be only an approximation.[53] Other workers have estimated the ionic strength from measurements of the major cations present in urine,[54] but this is tedious and again gives only approximate results. The tetramethylmurexide method for measurement of ionic calcium concentration in urine is therefore inherently subject to some error. This is discussed further in the 'Results and Discussion' section.

The interference from sodium arises because it forms a complex with tetramethylmurexide with an absorption maximum in the same region as the calcium complex. The formation constant of the sodium complex is much smaller than that of the calcium complex ($K'_{fNa} = 1.6$, $K_{fCa} = 300$). However, because of the high concentration of sodium normally present in urine it is necessary to make a correction for this interference. This is relatively easy to carry out since the sodium concentration in the urine samples can be rapidly and accurately determined by flame spectroscopy. Various methods can then be used to apply a correction. Hunt and King[52] and Raaflaub[31] prepared calibration graphs for each urine sample using matched sodium concentrations. Nordin and Smith[55] and Robertson, Peacock and Nordin[56] used empirically obtained correction factors for sodium. French, Cham and Cross[33] and Cham[57] prepared sodium calibration graphs to make the correction~~. This~~ is the method that was used in these experiments, and is described in detail in the 'Procedure' section.

The free dye and its calcium complex have overlapping spectra, and a solution containing both forms must be treated as a two-component system. Various theoretical bases for this type of analysis have been used. Raaflaub[58] derived an equation relating the absorbance ratio of the complex to free dye, E_x/E_o, at a given wavelength to the ionic calcium concentration.

$$\frac{E_x}{E_o} = 1 + \frac{a_x - a_o}{a_o} \cdot \frac{K_f[Ca^{2+}]}{1 + K_f[Ca^{2+}]}$$

where E_x = absorbance of the complex
E_o = absorbance of the free dye
a_o = molar absorptivity for the free dye
a_x = molar absorptivity for the complex
K_f = formation constant of the complex

Ettori and Scoggan[29] derived equations for this type of system which involved measurements at two wavelengths, λ' and λ".

$$[Ca^{2+}] = \frac{a'_o - a''_o\phi}{K_f(a''_x\phi - a'_x)}$$

where a'_o = molar absorptivity of the free dye at λ'
a''_o = molar absorptivity of the free dye at λ"
a'_x = molar absorptivity of the complex at λ'

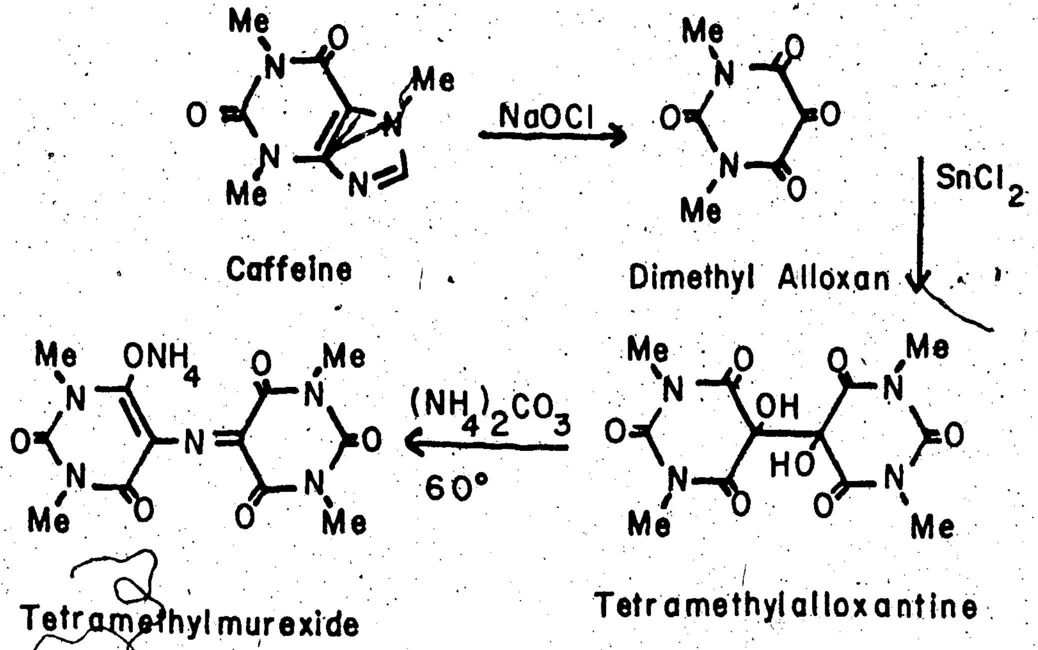
a''_x = molar absorptivity of the complex at λ''

ϕ = $\frac{A'}{A''}$, where A is the measured absorbance.

This two wavelength method has been used by several authors for the determination of ionic calcium in biological fluids with tetramethylmurexide.[56,32,33]

In the work described here, the method of Nordin and Smith,[55] adapted from Raaflaub, was used with some modification. Thus standards were prepared at a fixed ionic strength corresponding to typical urine samples, and no further correction was made for ionic strength effects. Measurements were made at a single wavelength and calibration graphs prepared by plotting absorbance vs. ionic calcium concentration. A set of urine samples was analyzed for ionic calcium by this method, and for total calcium by atomic absorption spectroscopy. The percentage of ionized calcium was then calculated.

Tetramethylmurexide was prepared from tetramethylalloxantine by the method of French, Cham and Cross[59] with some modification in the isolation procedure. Tetramethylalloxantine was prepared from caffeine, as described by Cope, Heyl, Peck, Eide and Arroyo.[60] The synthetic pathway is shown below.



Experimental

Reagents

Preparation of Tetramethylalloxantine. Into a 3-litre, 3-necked flask was placed 100 g of caffeine (J.T. Baker). A mixture of 120 ml of hydrochloric acid and 50 ml of water was then added, with mechanical stirring. A further 200 ml of water was added and the mixture stirred until the caffeine had dissolved. The flask was then immersed in an ice bath and 1125 ml of sodium hypochlorite solution (commercial laundry bleach containing 5% available chlorine) added slowly with stirring, the temperature being held between 35°-40°. A precipitate formed initially then slowly went back into solution; the addition of bleach was stopped when solution was complete. A solution of 50 g of stannous chloride di-hydrate (Shawinigan) in 37 ml of hydrochloric acid and 37 ml of water was then added rapidly with stirring. After five minutes, the stirrer was stopped and the mixture allowed to stand for two hours. The precipitate of tetramethylalloxantine was then filtered off using a suction flask and Whatman No. 1 filter paper. The damp precipitate was then boiled with 125 ml of water, cooled, and again filtered, with suction continued for one hour. The material was then dried in an oven at 60° for four hours, leaving 64 g of an off-white powder of melting point 205°-220°.

Preparation of Tetramethylmurexide. The method of French, Cham and Cross was followed with some modification. Approximately 25 g of the dry tetramethylalloxantine was mixed with 490 g of powdered ammonium carbonate (BDH) in a large crystallizing dish. This was then heated at 60° for 9 hours in an oven, equipped with an air vent and located in a fume hood (to allow escape of ammonia). After this time, the mixture occupied about one third of its original volume, and had turned pink. The isolation procedure of French et al., was tried on a test portion of the product, but was not successful. This method involved adding small amounts of water and decanting off the saturated tetramethylmurexide solution, followed by a salting out step using ammonium chloride. No precipitate formed on addition of the ammonium chloride, and since it was suspected that the product contained a large amount of unreacted or unvolatilized ammonium carbonate which prevented the tetramethylmurexide from salting out, the following method was devised. A 10-g portion of the product was heated with 50 ml of water in a beaker at about 60°. Large amounts of ammonia were evolved, confirming the above suspicions. Heating was continued until evolution of ammonia had ceased. The solution was then cooled to room temperature, filtered, and ammonium chloride (Fisher Scientific) added to the filtrate with stirring, until no more would dissolve (about 15 g was needed). The red precipitate that formed was collected by centrifugation, and the salting out step repeated by redissolution in a minimum amount of water, and addition of ammonium chloride to re-precipitate the tetramethylmurexide. After centrifugation, the precipitate was transformed to a filter paper in a filter funnel and allowed to air dry. The remainder of the impure product was treated in the same way, giving a total yield of 0.21 g.

A portion of the material was submitted for elemental analysis to the microanalytical laboratory, Department of Chemistry. The results were: carbon, theor. 42.35%, found 33.3%. Hydrogen, theor. 4.74%, found 5.27%. Nitrogen, theor. 24.70%, found 24.4%. The spectral properties of the material were investigated by dissolution of 25 mg in 50 ml of distilled water to give a 1.47 x 10^{-3} M solution. A 0.1 ml portion of this solution was diluted with 5 ml of distilled water and the spectrum obtained over the visible region using a Unicam SP800 spectrophotometer with 1-cm cells and distilled water as a reference. A single broad peak was observed with a maximum absorbance of 0.41 at 530 nm (lit.[30] λ_{max} = 530 nm). Another 0.1 ml of the original solution was then mixed with 4.5 ml of water and 0.5 ml of standard calcium solution (1 ml = 1 mg Ca). The spectrum of this solution showed that the absorption band was now shifted, with a maximum absorbance of 0.47 at 495 nm (lit.[30] λ_{max} = 492 nm in the presence of excess Ca). The spectra are shown in Figure V.

For the calcium analyses, this 0.05% solution of tetramethylmurexide was used and was kept refrigerated and protected from light as much as possible. Under these conditions, it was found to undergo slow decomposition as shown by a decrease in absorbance of about 0.5% per day, and so it was normally discarded after about two weeks and a fresh solution prepared.

Other Reagents. A standard calcium solution was prepared by dissolving 2.497 g of calcium carbonate (Allied Chemical) in 50 ml of 1 M hydrochloric acid and diluting to 1 litre with distilled water, to give a solu-

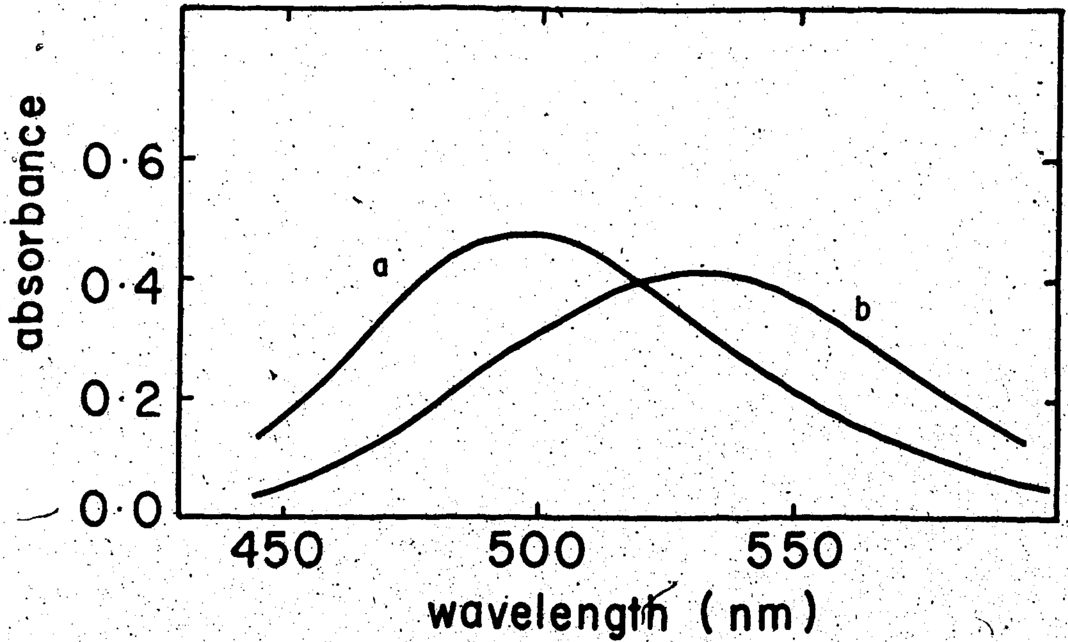


Figure V. Visible spectrum of tetramethylmurexide and calcium complex. (a) 0.1 ml of 0.05% tetramethylmurexide + 5 ml of calcium solution (0.1 mg Ca per ml); (b) 0.1 ml of 0.05% tetramethylmurexide + 5 ml of distilled water.

tion containing 1 mg Ca per ml.

A buffer solution of pH 8 was prepared by mixing 175 ml of 1 M hydrochloric acid with 300 ml of 1 M tris-(hydroxymethyl)aminomethane (Fisher Scientific).

A 5% lanthanum solution was prepared by dissolving 12.7 g of $LaCl_3 \cdot 6H_2O$ (Fisher Scientific, low-in-calcium grade) in 25 ml of hydrochloric acid and 25 ml of distilled water, and diluting to 100 ml with distilled water.

Apparatus

A Unicam SP800B spectrophotometer, equipped with a constant wavelength time scanning device, was used for the absorbance measurements, and a piston type 100 μl micropipet (Eppendorf) was used for addition of the tetramethylmurexide solution. A Perkin-Elmer 290B atomic absorption spectrophotometer was used for the total calcium determinations in the urine samples, and a Heath modular spectrophotometer consisting of a Heath flame module, Heath 703 monochromator, Heath EU 701-30 photomultiplier module, and a Heath EU 20-28 log/linear current module recorder, was used for the sodium analyses of the urine samples.

Procedure

Ionic Calcium Analysis. For the preparation of the calibration curve, standard calcium solutions were prepared using 1.964 g of potassium chloride (Shawinigan) plus 0, 2, 5, 10, 15 and 20 ml of the standard calcium solution, and diluted to 100 ml with distilled water. For the measurement step, 5 ml of each of these solutions in turn were then mixed with 0.1 ml of 0.05% tetramethylmurexide, immediately transferred to a 1-cm glass cell, and the absorbance at 480 nm recorded as a function of time. The absorbance value obtained 0.80 minutes after the time of mixing was then used to construct a calibration graph of calcium concentration against absorbance. A typical calibration plot is shown in Figure VI.

For the analysis of urine, a fresh sample should be used if possible. Otherwise, it should be kept refrigerated without the addition of any preservative that could upset the ionic calcium equilibrium. The samples were centrifuged if any suspended particles or turbidity was present, and 5 ml were mixed with 0.1 ml of 0.05% tetramethylmurexide solution and the absorbance measured as for the standards, except that a portion of the urine sample was used in the reference cell instead of distilled water. The ionic calcium concentration was then found by reading the absorbance (corrected for sodium) from the calibration graph.

To correct for interference from sodium, solutions were prepared containing sodium chloride in the concentration range of 0.1 to 0.4 M. These solutions were then used to prepare a calibration graph in the same way as for

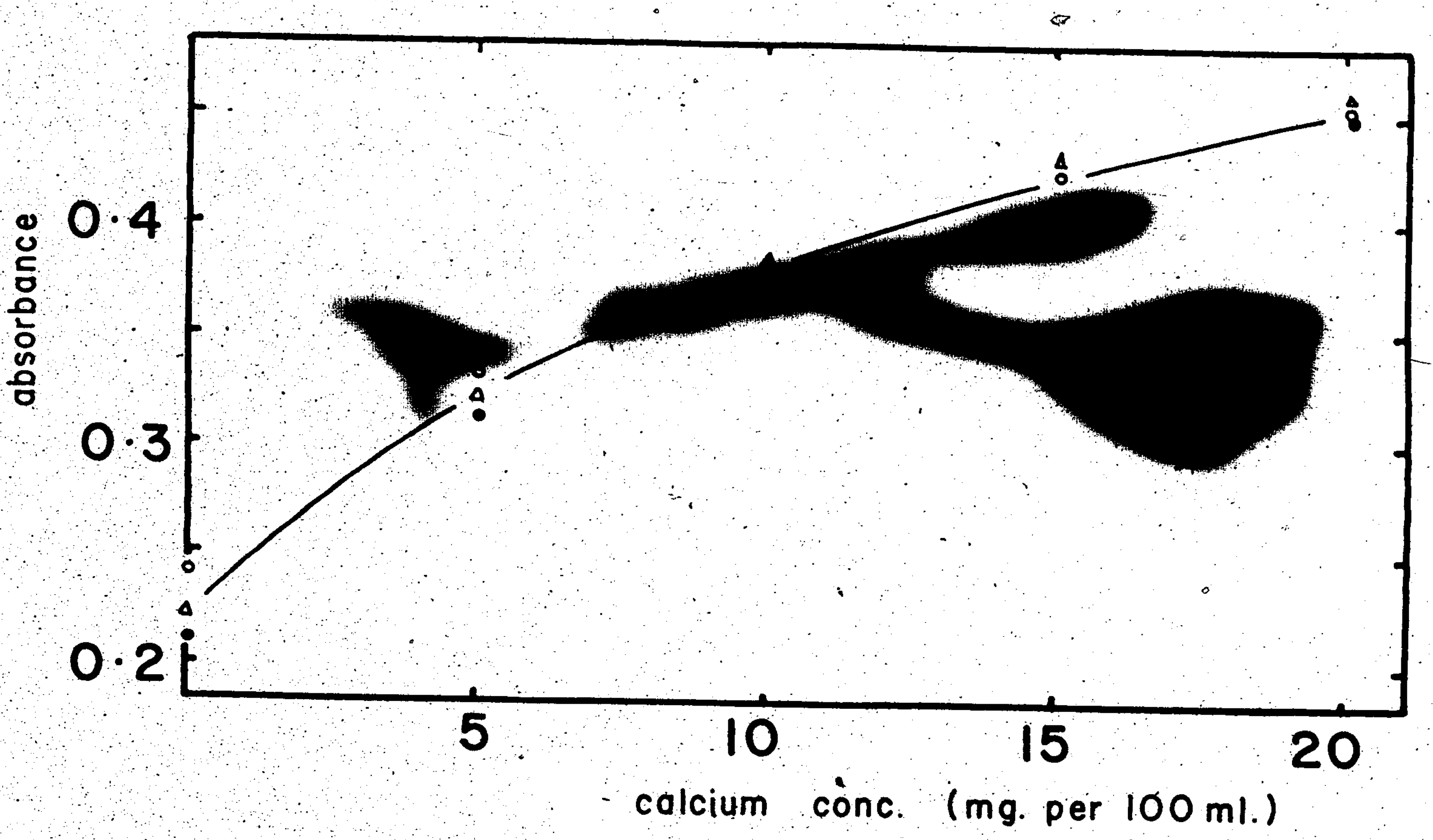


Figure VI. Calibration curves for calcium with tetramethylmurexide in the presence of varying amounts of sodium.

● 0.25 M KCl
Δ 0.12 M KCl + 0.13 M NaCl
O 0.25 M NaCl

calcium. The sodium concentration of the urine sample was then determined, and the absorbance produced by this concentration of sodium read off from the calibration graph. This absorbance value was then subtracted from that obtained for the urine sample, and this corrected absorbance read off on the calcium calibration graph to give the true calcium concentration of the sample. A typical sodium calibration graph is shown in Figure VII.

The sodium concentrations of the urine samples were measured by flame emission spectroscopy. Standards were prepared from sodium chloride to give solutions containing 5 to 100 ppm of sodium in distilled water. The Heath modular spectrophotometer was set up using a hydrogen-oxygen flame, a slit width of 22 μm, and a wavelength of 589 nm. The average chart readings, obtained in the %T mode, were plotted against concentration of sodium to give a calibration graph. The urine samples were then diluted by a factor of 50 with distilled water, and these solutions aspirated under the same conditions as for the standards. The sodium concentrations in the samples were then read from the calibration graph.

Total Calcium Analysis. This was carried out by atomic absorption spectroscopy.[61,62] Standards were prepared by first diluting the standard calcium solution (1 ml = 1 mg Ca) to give a solution containing 20 mg of calcium per litre. Then 0, 5, 10, 15, 20, 25 and 30 ml

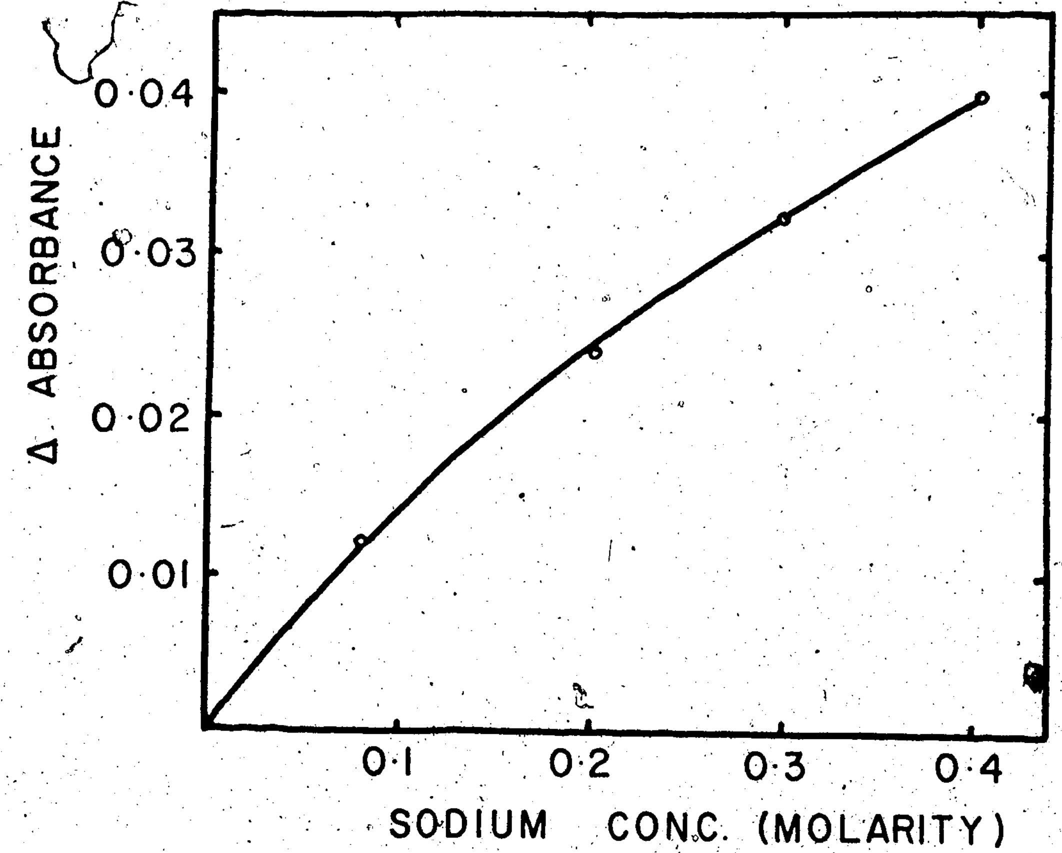


Figure VII. Calibration curve for tetramethylmurexide with sodium. Δ Absorbance values were obtained by subtracting the blank value at 480 nm from the measured absorbance values at this wavelength.

of this solution was mixed with 10 ml of 5% lanthanum chloride solution and diluted to 100 ml with distilled water. These solutions were then aspirated into an air-acetylene flame in the atomic absorption spectrophotometer, and the absorbance recorded. A calibration curve was then plotted of the meter reading vs. calcium concentration. Sample solutions were prepared by mixing 1 ml of urine with 5 ml of lanthanum solution and diluting to 50 ml with distilled water. These solutions were then aspirated under the same conditions as for the standards. The concentration of total calcium in each sample was then read from the calibration plot.

Results and Discussion

Stability of the Calcium-Tetramethylmurexide Complex.

The absorbances of the calcium-tetramethylmurexide solutions were found to decrease slowly with time. This is in agreement with the findings of Hunt and King[52] but is contrary to those of Pedersen.[32] The rate of decomposition appears to be approximately proportional to the amount of calcium present; the blank, which contained no calcium, did not show a decrease in absorbance. A typical rate of decrease in absorbance for the standard containing 15 ml of calcium solution was about 4% per minute. Thus it was necessary to measure the absorbance of the solutions soon after addition of the tetramethylmurexide. It was found that about 0.80 minute was sufficient time to fill the cell, place it in the cell holder and start the scan, so this time was arbitrarily chosen as the time of measurement.

Accuracy of the Tetramethylmurexide Method for the Determination of Ionic Calcium.

An experiment was carried out to verify the accuracy of the method by measuring the absorbance of a solution containing a known amount of calcium and a known amount of citrate which complexed with part of the calcium and thus reduced its ionic concentration. The ionic calcium concentration was then read from a calibration graph, the dissociation constant of the calcium citrate complex was calculated, and the value compared with the literature data. The experiment was carried out at pH 8 so that the citrate would be essentially in the form cit^{3-}. The fraction of citrate in this form was calculated using acid dissociation constants[63] of $Ka_1 = 1.15 \times 10^{-3}$, $Ka_2 = 4.6 \times 10^{-5}$ and $Ka_3 = 2.4 \times 10^{-6}$ in the expression:

$$\alpha_3 = \frac{[cit^{3-}]}{Ccit} = \frac{Ka_1Ka_2Ka_3}{[H^+]^3 + Ka_1[H^+]^2 + Ka_1Ka_2[H^+] + Ka_1Ka_2Ka_3}$$

At pH 8, $\alpha_3 = 0.996$, so 99.6% of the total citrate is present as cit^{3-} at pH 8.

Since ionic concentrations were used rather than activities, a conditional dissociation constant was determined rather than a thermodynamic constant. Thus it was important to know the ionic strength of the solution used in the experiment. Most literature values for the dissociation constant of calcium citrate were measured at an ionic strength of 0.16, so this value was used in order to be able to make a comparison more readily. Contributions to the ionic strength in this experiment originated from the buffer solution, the calcium ions, the chloride ions present as counter-ions to the standard calcium, the calcium citrate complex, free citrate ions and sodium counter-ions. These contributions were calculated as follows.

For the buffer solution, representing tris(hydroxymethyl)aminomethane (THAM) as $T\text{-}NH_2$, reaction with hydrochloric acid gives the protonated form $T\text{-}NH_3^+$. Protonation of the excess $T\text{-}NH_2$ can also occur by hydrolysis.

$$T\text{-}NH_2 + H_2O \rightleftarrows T\text{-}NH_3^+ + OH^-$$

as given by $K_b^{63a} = 1.4 \times 10^{-6} = \dfrac{[T\text{-}NH_3^+][OH^-]}{[T\text{-}NH_2]}$

The total concentration of THAM in the stock buffer solution was 0.632 M, and in the experiment a ten-fold dilution was used, so the final concentration of THAM was 0.0632 M.

Therefore, $[\text{T-NH}_2] + [\text{T-NH}_3^+] = 0.632$

From above, at pH 8, $\dfrac{[\text{T-NH}_3^+]}{(0.0632-[\text{T-NH}_3^+])} = \dfrac{1.4 \times 10^{-6}}{10^{-6}}$

and $[\text{T-NH}_3^+] = 0.036$ M.

For electroneutrality in the buffer solution, the chloride ion concentration must be the same, so $[\text{Cl}^-] = 0.036$ M. The total concentration of calcium in the solution under study was 2.50×10^{-3} M, and the total concentration of citrate was 2.27×10^{-3} M.

$$C_{ca} = 2.50 \times 10^{-3} = [\text{Ca}^{2+}] + [\text{CaCit}^-]$$

$$C_{cit} = 2.27 \times 10^{-3} = [\text{Cit}^{3-}] + [\text{CaCit}^-]$$

$$K' = \frac{[\text{Ca}^{2+}][\text{Cit}^{3-}]}{[\text{CaCit}^-]} = 7.0 \times 10^{-4} \text{ at } 25°,\ \mu = 0.16^{64}$$

$$\therefore\ 7.0 \times 10^{-4} = \frac{[\text{Ca}^{2+}]\{C_{cit} - (C_{ca} - [\text{Ca}^{2+}])\}}{C_{ca} - [\text{Ca}^{2+}]} \quad (1)$$

and $[\text{Ca}^{2+}] = 1.58 \times 10^{-3}$ M. Then $[\text{CaCit}^-] = 9.2 \times 10^{-4}$ M, and $[\text{Cit}^{3-}] = 1.35 \times 10^{-3}$ M. The citrate was added as trisodium citrate dihydrate, thus the concentration of sodium $= 3C_{cit} = 3 \times 2.27 \times 10^{-3}$, or 6.81×10^{-3} M.

The chloride counter-ion in the standard calcium solution was equal to twice the total calcium concentration, or 5.0×10^{-3} $\underline{M}$.

The total ionic strength, then, given by $\mu = \frac{1}{2}\Sigma\, C_iZ_i^2$, is

$$\mu = \frac{1}{2}\{0.036 + 0.036 + 1.58 \times 10^{-3} \times 4 + 9.2 \times 10^{-4} + 1.35 \times 10^{-3} \times 9 + 6.81 \times 10^{-3} + 5.0 \times 10^{-3}\}$$

$$\mu = 0.051$$

To provide a final ionic strength of 0.16, potassium chloride was added (0.812 g per 100 ml).

The procedure for estimation of the conditional formation constant of calcium citrate was as follows. A mixture of 0.812 g KCl, 10 ml of pH 8 THAM buffer solution, 0.0668 g of tri-sodium citrate dihydrate and 10 ml of standard calcium solution (1 ml = 1 mg) was diluted with distilled water to 100 ml in a volumetric flask. The ionic calcium concentration was then determined as described in the 'Procedure' section. The standard calcium solutions used in the preparation of the calibration graph were also adjusted to an ionic strength of 0.16 using potassium chloride.

Results

An ionic calcium concentration of 1.10×10^{-3} $\underline{M}$ was found. Thus $[Ca^{2+}] = 1.10 \times 10^{-3}$, $C_{ca} = 2.50 \times 10^{-3}$

and $C_{cit} = 2.27 \times 10^{-3}$. Substituting these values into Eq. 1 gives a value for $K_{dissoc.} = 6.8 \times 10^{-4}$ at 25° and an ionic strength of 0.16. This compares well with the literature values of 6.8×10^{-4} (25°, $\mu = 0.15$)[65], 7.0×10^{-4} (25°, $\mu = 0.16$)[64], and 6.0×10^{-4} (23°, $\mu = 0.16$)[66] and thus demonstrates that the tetramethylmurexide method does accurately measure ionic calcium concentration.

Effect of Sodium

Sodium forms a complex with tetramethylmurexide that absorbs in the same region as the calcium complex. It thus causes an increase in the apparent calcium concentration. However, in complexing with the tetramethylmurexide it removes some of the free dye that would otherwise be available for complexation with calcium, with the result that the apparent calcium concentration decreases. The first of these two opposing effects predominates in the concentration range used here, as shown in Figure VI. The sodium correction method of French, Cham and Cross,[33] and Cham,[57] which was used here, does not take into account the second effect. When the highest accuracy is desired calibration graphs should be prepared for each sample, using matched sodium concentrations. The calculation method (see Appendix) also corrects for both sodium effects.

Effect of Ionic Strength

As mentioned in the background section, errors due to ionic strength effects will invariably occur when the tetramethylmurexide method is applied to biological fluids of unknown ionic strength. The following calculations were carried out to estimate the error that can occur when analyzing urine samples for ionic calcium concentration by this method. The ionic strength of urine was calculated using literature values for the most abundant ionic species normally present, which were usually reported as a range of values. The maximum and minimum individual ionic strength contributions were summed to give the total maximum and minimum ionic strength. The activity coefficients for the calcium ion were then calculated using the extended Debye-Huckel equation, and compared with the value obtained for the standards. The results for the urinary values are summarized in Table III, which shows that the minimum ionic strength for normal urine is in the region of 0.16 and the maximum value is around 0.45. However, it should be realized that pathological samples could lie outside this range, for example when a hospital patient is on a diet that includes a large intake of water. The standards were prepared at an ionic strength of 0.25, which is considered to be the average value for urine. The effect of these ionic strength values on the activity coefficient of the calcium is shown in the following calculations.

$$-\log f_{ca^{2+}} = \frac{0.\text{[illegible]}i^{2}\sqrt{\mu}}{1 + \sqrt{\mu}} \quad \text{(in water at room temperature)}$$

Table III. Ionic Strength Contributions in Urine

Ion	mEq/day[43] low	mEq/day[43] high	Ionic Strength Contribution* low	Ionic Strength Contribution* high
Ca^{2+}	1.3	12.5	0.002	0.025
Mg^{2+}	4	16	0.008	0.032
K^{+}	20	123	0.010	0.061
Na^{+}	40	200	0.02	0.10
NH_4^{+}	29	35	0.015	0.018
$H_2PO_4^{-}$	6.6	19.2	0.003	0.010
HPO_4^{2-}	4.4	12.8	0.009	0.026
SO_4^{2-}	0.4	10.4	0.001	0.021
Cl^{-}	160	260	0.08	0.13
citrate^{3-}	2	5	0.009	0.023
uric acid anion	3	6	0.002	0.003
		Total	0.156	0.446

* Assuming a daily volume of one litre.

For $\mu = 0.16$: $fca^{2+} = 0.27$

For $\mu = 0.25$: $fca^{2+} = 0.22$

For $\mu = 0.45$: $fca^{2+} = 0.16$

Since concentration is related to activity by $a = c \times f$, the relative error introduced when a urine sample has $\mu = 0.16$ will be 19% and for a sample with $\mu = 0.45$, the error will be 27%. This demonstrates that unless the ionic strength of the sample can be determined with reasonable accuracy and a correction carried out, large errors can be introduced.

Analysis of Urine Samples

Four urine samples (obtained from patients at the University of Alberta Hospital) were analyzed for total calcium and sodium by flame spectroscopy, and for ionic calcium, as described in the 'Procedure' section. A comparison between ionic and total calcium was made, and the percentage of ionic calcium was calculated. The results are shown in Tables IV and V. It is seen that the percentage of ionic calcium varied from 40 to 65%, with a mean of 52%. This is in reasonable agreement with the findings of Cham[57], who found values ranging from 41-60% with a mean of 50%, and with Robertson[24], who found an average of 50% ionized calcium.

Table IV. Analysis of Urine Samples for Ionic Calcium.

Sample No.	Sodium Concentration, M	Absorbance Correction For Sodium	Original Absorbance	Corrected Absorbance	Corrected Ionic Calcium Concentration, mg per 100 ml
1	0.020	0.003	0.205	0.202	0.4 ± 0.3
2	0.035	0.004	0.250	0.246	1.5 ± 0.2
3	0.257	0.029	0.415	0.386	13.6 ± 0.7
4	0.218	0.025	0.400	0.375	13.7 ± 0.6

Table V. Fraction of Ionized Calcium in Urine Samples.

Sample No.	pH	Total Ca Concentration (Atomic Absorption), mg per 100 ml	Ionic Ca Concentration, mg per 100 ml	% Ionic Ca
1	8.5	1.0	0.4	40 ± 30
2	7.0	2.3	1.5	65 ± 10
3	6.0	31	13.6	44 ± 2
4	4.5	23	13.7	60 ± 2

Uncertainty in figures is based on reading error of 0.005 absorbance units.

CHAPTER V

THE SPECTROPHOTOMETRIC DETERMINATION OF IONIC MAGNESIUM CONCENTRATION IN BIOLOGICAL FLUIDS

Background

The spectrophotometric determination of metal ion concentrations in biological fluids by the approach described in the previous chapter requires a reagent that has properties of a very stringent nature. It must be sufficiently water soluble that a small volume of the reagent solution in high concentration can be added to the sample, to minimize dilution effects. It must form a complex with the metal ion that has a high molar absorptivity in the visible region, preferably with an absorbance maximum at a wavelength well separated from that of the free reagent. For the analysis of urine samples a wavelength away from the yellow region (380 - 460 nm) is advantageous. The complex should have a sufficiently small formation constant that the equilibrium between the bound and free metal ion is disturbed as little as possible. However, the formation constant must not be so small that little or no complexation takes place under the conditions of the analysis. The reagent should be specific for the metal ion to be determined, and free from interference by other substances. For urine samples the complex formation should be unaffected by variation in pH over the range of about 4 to 8.

The reagent must of course disturb the metal ion equilibrium to some extent if it is to form a complex. For a maximum error of 1%, [M] > 100[ML], where M represents the metal ion to be analyzed, and L the reagent ligand. The charges on M, L and ML have been omitted for simplicity.

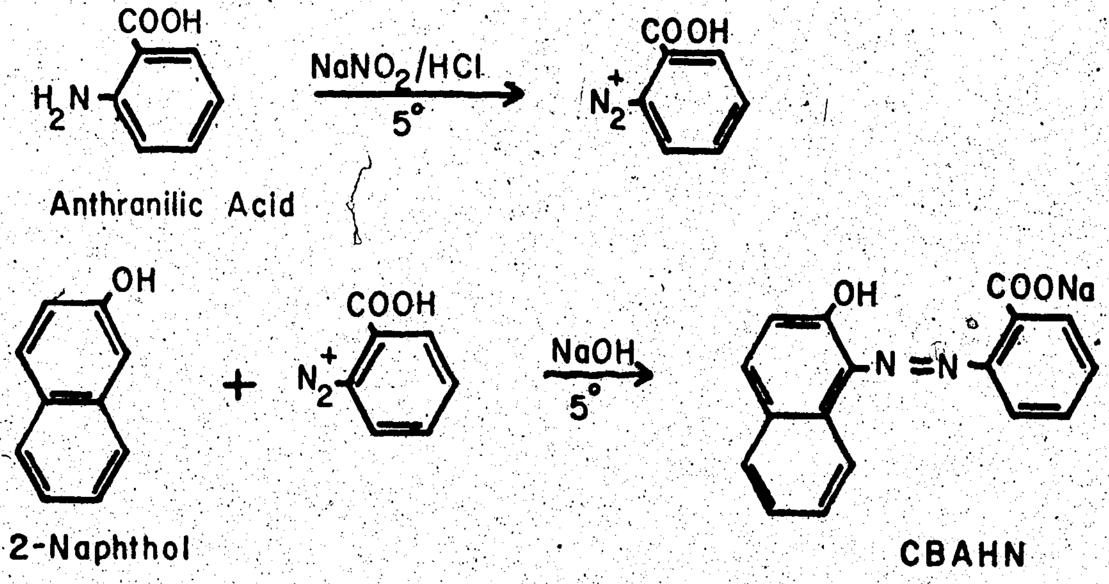
$$K_f = \frac{[ML]}{[M][L]}$$

$$\therefore \; K_f < \frac{1}{100[L]} \quad \text{for a maximum error of 1\%.}$$

The proportion of the metal ion that is bound to the reagent can thus be controlled by the concentration of reagent. However, the concentration of reagent to be used depends also on the molar absorptivity of the free reagent and its complex with the metal ion, since absorbance readings above 0.1 are normally desired for adequate accuracy.

A reagent has not yet been reported that fulfils all of the above requirements for the determination of magnesium in biological fluids. Both Eriochrome Black T and Eriochrome Blue SE have been proposed,$^{35-37}$ but these reagents are pH sensitive and are subject to interference from calcium. Eriochrome Black T is also sensitive to trace amounts of cobalt, nickel, copper and iron.

A literature search was carried out to find whether a reagent was available that possessed all or most of the required properties listed above. The work of Diehl and Ellingboe[67] was useful since they had investigated the complex-forming properties of a large number of azo compounds with calcium and magnesium. One of these compounds, 1-(2'-carboxy-1'-benzeneazo)-2-hydroxynaphthalene, was reported to give a weak complex with magnesium (log K_f = 2.1) but no complex with calcium. Its acid dissociation constants were reported to be favourable in that neglible ionization would be expected in the pH range 4 to 8. The compound was therefore synthesized and its properties investigated. The synthesis was carried out by diazotization of anthranilic acid and coupling with 2-naphthol, as shown below.



For convenience, this compound is referred to here as CBAHN. A more soluble derivative of this dye was also prepared and investigated, by using 2-naphthol-6-sulphonic acid in place of 2-naphthol. This derivative is referred to as CBAHNS.

Experimental

Reagents

Synthesis of CBAHN. The method used was adapted from those for similar dyes[68-70] and from the work of Diehl and Ellingboe on the same dye.[67] About 50 g of anthranilic acid (BDH) was ground with dilute hydrochloric acid (92 ml of concentrated HCl and 150 ml of water), the slurry cooled to 0-5°, and placed in a 3-litre, 3-necked flask fitted with a mechanical stirrer and thermometer. A cooled solution of 26 g of sodium nitrite in 350 ml of water was added slowly over a period of about 40 minutes until a test portion of the reaction mixture gave a positive reaction with starch-iodide paper, indicating that excess nitrite was present. During the addition, the mixture was stirred continuously and kept below 5° by immersion in an ice bath. The resulting turbid yellow diazonium solution was filtered and kept below 5° while the next stage was set up.

About 52 g of 2-naphthol (BDH) was dissolved in a solution of 80 g of sodium hydroxide in 430 ml of water.

Approximately 300 g of ice was added to the warm solution to cool it to about 5°; care was taken to avoid precipitation of naphthol. The cooled solution was transferred to a 3-litre, 3-necked flask equipped with a mechanical stirrer and thermometer, and the previously prepared diazonium solution was added slowly over about 45 minutes, with constant stirring and with the temperature held at about 5°. It was necessary to add water (cooled to 5°) to dilute the slurry of azo compound that formed, otherwise stirring became inefficient. After the diazonium solution had been added, the mixture was stirred for a further 30 minutes, then held at 5-10° for 3 hours before filtering off the red precipitate by suction. The product was allowed to air dry and then was recrystallized from acetic acid as red, needle shaped crystals with a melting point of 275-277° (lit.[71] mp 278°). Elemental analysis gave the following: carbon, theor. 69.9%, found 69.3%; hydrogen, theor. 4.15%, found 4.27%; nitrogen, theor. 9.59%, found 8.54%. A mass spectrum showed a high intensity peak at 292, consistent with the calculated molecular weight for the protonated compound of 292.3. Isotope peaks of smaller intensity were obtained at 293 and 294.

<u>Synthesis of CBAHNS</u>. The first stage of the synthesis, diazotization of anthranilic acid, was carried out as described above in the synthesis of CBAHN. For the

second stage, 105 g of 2-naphthol-6-sulphonic acid, sodium salt (Eastman Kodak) was mixed with a solution of 80 g of sodium hydroxide in 430 ml of water; additional water was added, with stirring, until dissolution was complete. The solution was then cooled to about 5° and placed in a 3-litre, 3-necked flask equipped with a mechanical stirrer and thermometer. The cold diazonium solution was then added slowly to the naphthol solution over a period of one hour with continuous stirring, and with the temperature held at about 5°. A deep red solution was produced which was stirred for a further 30 minutes to ensure complete reaction. The product was isolated by the addition of 5 M hydrochloric acid to the solution, with stirring, until a dark red precipitate formed (presumably the protonated form of CBAHNS). This was filtered by gravity, washed with 1 M hydrochloric acid and again filtered and allowed to air dry. Attempts to recrystallize the product from ethanol, acetic acid and butanol were unsuccessful. An adaptation of the purification method for Calmagite used by Lindstrom and Diehl[72] was then tried. The partially dry solid was repeatedly extracted into hot acetone by stirring while heating on a steam bath, then decanting off the mother liquor and repeating the extraction using fresh portions of acetone until the acetone extract was almost colourless. The combined acetone extracts were filtered and evaporated to dryness on a steam bath, leaving a shiny black solid. The non-extractable solid was allowed to air dry yielding a brick red powder. The non-extractable fraction was designated CBAHNS-1 and the extracted fraction CBAHNS-2. Both fractions had melting points above 360°, although CBAHNS-1 darkened above 300°. Sodium was determined by ignition of CBAHNS moistened with sulphuric acid and weighing the residue as Na_2SO_4. Found, for CBAHNS-1, 12.9%; for CBAHNS-2, 0.31%; theor. for the monosodium salt, 5.8%. Chloride was an expected impurity, and was determined by turbidimetry.[73] Found, for CBAHNS-1, 15.2%; for CBAHNS-2, 0.46%. For CBAHNS-1, this result was confirmed by gravimetric analysis as AgCl, which gave a value of 15.1% Cl. Potentiometric titrations with sodium hydroxide were carried out on both fractions. Plots of pH against volume of base added are shown in Figure VIII. For CBAHNS-1, only one potential break was observed, but for CBAHNS-2, two potential breaks were seen. This suggests that CBAHNS-1 was the monosodium salt and that CBAHNS-2 was the protonated compound, which is in reasonable agreement with the above results for the sodium analysis. On this basis, the percentage purity was calculated from the potentiometric results, which showed CBAHNS-1 to be 83.2% and CBAHNS-2 to be 99.6% pure. This is in good agreement with the results for sodium and chloride, since

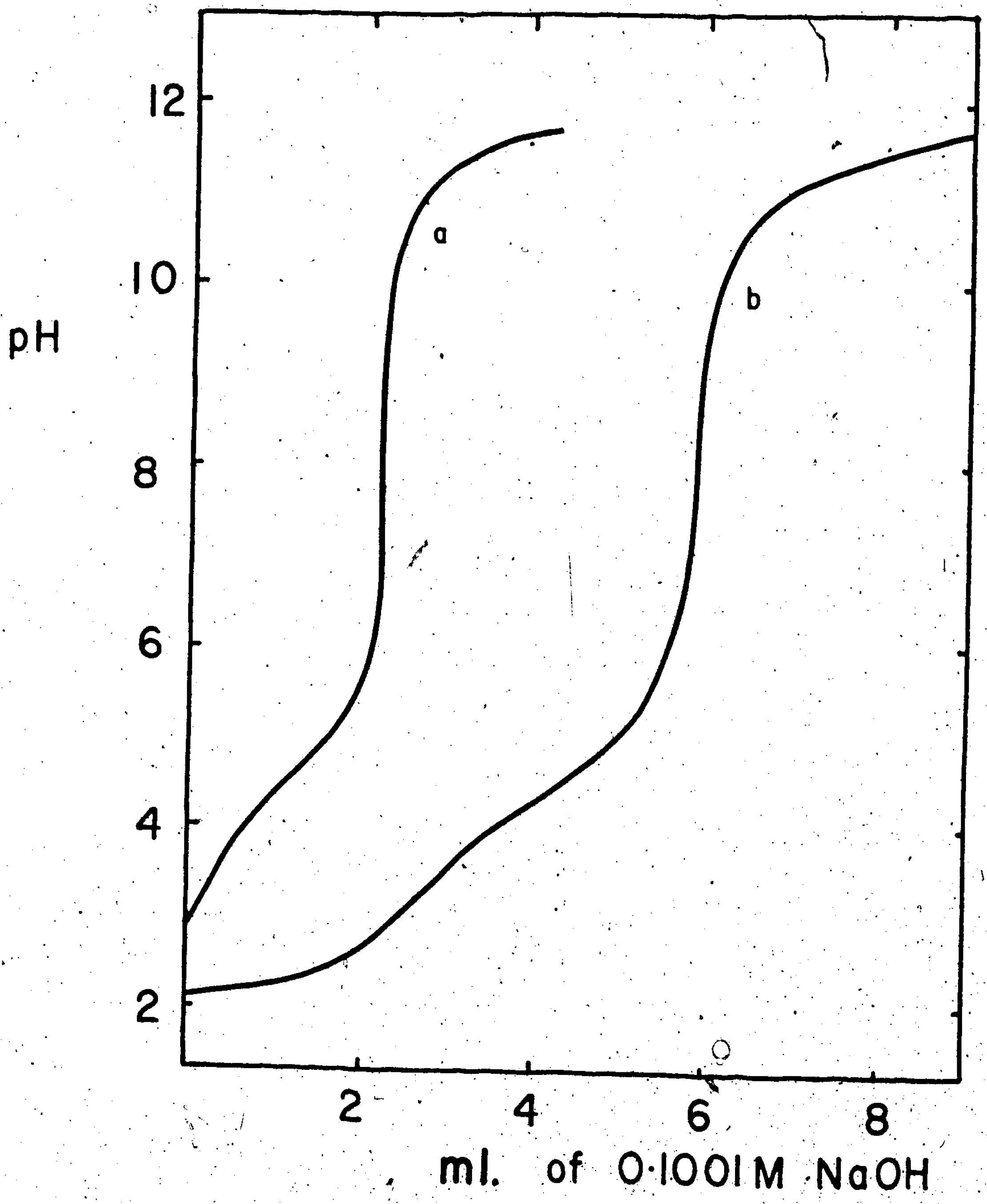


Figure VIII. Potentiometric titration curves for CBAHNS.

(a) CBAHNS-1 (0.1062 g)
(b) CBAHNS-2 (0.1085 g)

the monosodium salt has a theoretical sodium content of 5.8% and this leaves 7.1% unaccounted for. If this excess sodium is combined with the chloride then this gives 17.6% NaCl present as impurity in the CBAHNS-1, which is in accord with the 83.2% assay. Elemental analysis, carried out by the microanalytical laboratory, Department of Chemistry, gave the following results. For CBAHNS-1: carbon, theor. 51.8%, found 36.2%; hydrogen, theor. 2.82%, found 3.3%; nitrogen, theor. 7.11%, found 4.2%, sulphur, theor. 8.13%, found 5.8%. For CBAHNS-2: carbon, theor. 54.8%, found 55.0%; hydrogen, theor. 3.25%, found 3.9%; nitrogen, theor. 7.52%, found 2.8%; sulphur, theor. 8.61%, found 6.6%. The low results obtained for CBAHNS-1 can be attributed in part to sodium chloride impurity. After correction for 17.6% NaCl, the theoretical values become: carbon, 43.2%; hydrogen, 2.35%; nitrogen, 5.93%, and sulphur, 6.78%. The poor results for CBAHNS-2 may have been caused by difficulties in the analysis, since potentiometric titration gives an equivalent weight within 0.4% of the theoretical.

Other Reagents. Standard magnesium solution was prepared by dissolving 0.100 g of magnesium sulphate (Fisher Scientific) in 100 ml of distilled water to give a solution containing 0.02 g Mg/100 ml. The following buffer solutions were prepared. For a buffer of pH 4, 50 ml of 0.1 M potassium hydrogen phthalate (Fisher Scientific) was mixed with 0.1 ml of 0.1 M hydrochloric acid and diluted to 100 ml with distilled water. For a buffer of pH 6, 0.1 M hydrochloric acid was added to 20 ml of 0.1 M tris-(hydroxymethyl)aminomethane (THAM, Fisher Scientific) until the pH reached the required value as measured by a standardized pH meter. For a buffer of pH 7, 50 ml of 0.1 M THAM was mixed with 46.6 ml of 0.1 M hydrochloric acid and diluted to 100 ml with distilled water. For a buffer of pH 8, 50 ml of 0.1 M THAM was mixed with 29.2 ml of 0.1 M hydrochloric acid and diluted to 100 ml with distilled water. For a buffer of pH 10, 50 ml of 0.05 M sodium bicarbonate (Allied Chemical) was mixed with 10.7 ml of 0.1 M sodium hydroxide and diluted to 100 ml with distilled water.

A 0.1 M solution of silver nitrate (Johnson, Matthey and Mallory) was used for the chloride analysis. Sodium chloride (Fisher Scientific) was used to prepare standards for the turbidimetric chloride analysis. Sulfamic acid (high purity grade, Matheson, Coleman and Bell) was used to standardize the sodium hydroxide solution used in the potentiometric titration of CBAHNS.

Apparatus

A Unicam SP 800B spectrophotometer was used to obtain all spectra. A Coleman 28C pH meter (Coleman Instruments, Perkin Elmer Corp.) equipped with a glass electrode and a calomel reference electrode, was used for the potentiometric titrations of CBAHNS with standard sodium hydroxide.

Procedure

CBAHN. This was found to have a very low solubility in water, although with increasing pH the solubility increased. A saturated solution was prepared by dissolving CBAHN in distilled water adjusted to pH 11 with sodium hydroxide solution. This solution was diluted by a factor of ten with distilled water in order to bring the absorbance down to a reasonable level. A 5-ml portion of this solution was mixed with 2 ml of buffer solution pH7 and 2 ml of distilled water, and the spectrum recorded over the visible region in 1-cm glass cells. The measurements were repeated using buffer solutions of pH 6 and pH 8, and finally the whole procedure was repeated using 2 ml of standard magnesium solution (0.02 g Mg per 100 ml) in place of the 2 ml of distilled water.

CBAHNS. A 25 mg portion of each fraction was dissolved in distilled water to a final volume of 50 ml. This was then diluted by a factor of four, and to 1 ml of this solution was added 2 ml of buffer solution (pH 4, 6, 8 or 10), 2 ml of distilled water, and the spectra recorded over the visible region using 1-cm cells. The measurements were then repeated using 2 ml of standard magnesium solution (0.02 g Mg per 100 ml) in place of the 2 ml of distilled water.

Results and Discussion

CBAHN. The spectra obtained when no magnesium was present were independent of pH over the range studied, with absorbance maxima at 488 nm, as shown in Figure IX. With magnesium present, no change in the wavelength of maximum absorbance occurred, but a reduction in the absorbance values was observed, and the absorbance found to decrease with time. After about 30 minutes, the absorbance had decreased to about 25% of its original value and a faint red precipitate was observed on the sides and bottom of the cell, which suggested that the magnesium complex was slowly coming out of solution. This was substantiated by the fact that the baseline between 550 and 650 nm shifted from zero to a small positive value as the absorbance at 488 nm decreased. Thus, both the reagent and its magnesium complex are too insoluble to be suitable for the determination of ionic magnesium. At this point, synthesis of a more soluble derivative of the dye by incorporating a sulphonic acid group into the molecule was studied.

CBAHNS. The spectra for CBAHNS-1 and CBAHNS-2 were similar, except that CBAHNS-1 gave greater absorbance readings in each case. The shape of the spectral curves resembled those of CBAHN, CBAHNS-1 having an absorbance maximum of 0.96 at 487 nm and CBAHNS-2 having an absorbance maximum of 0.68 at 488 nm. When no magnesium was present, no change in the spectra was observed over the pH range

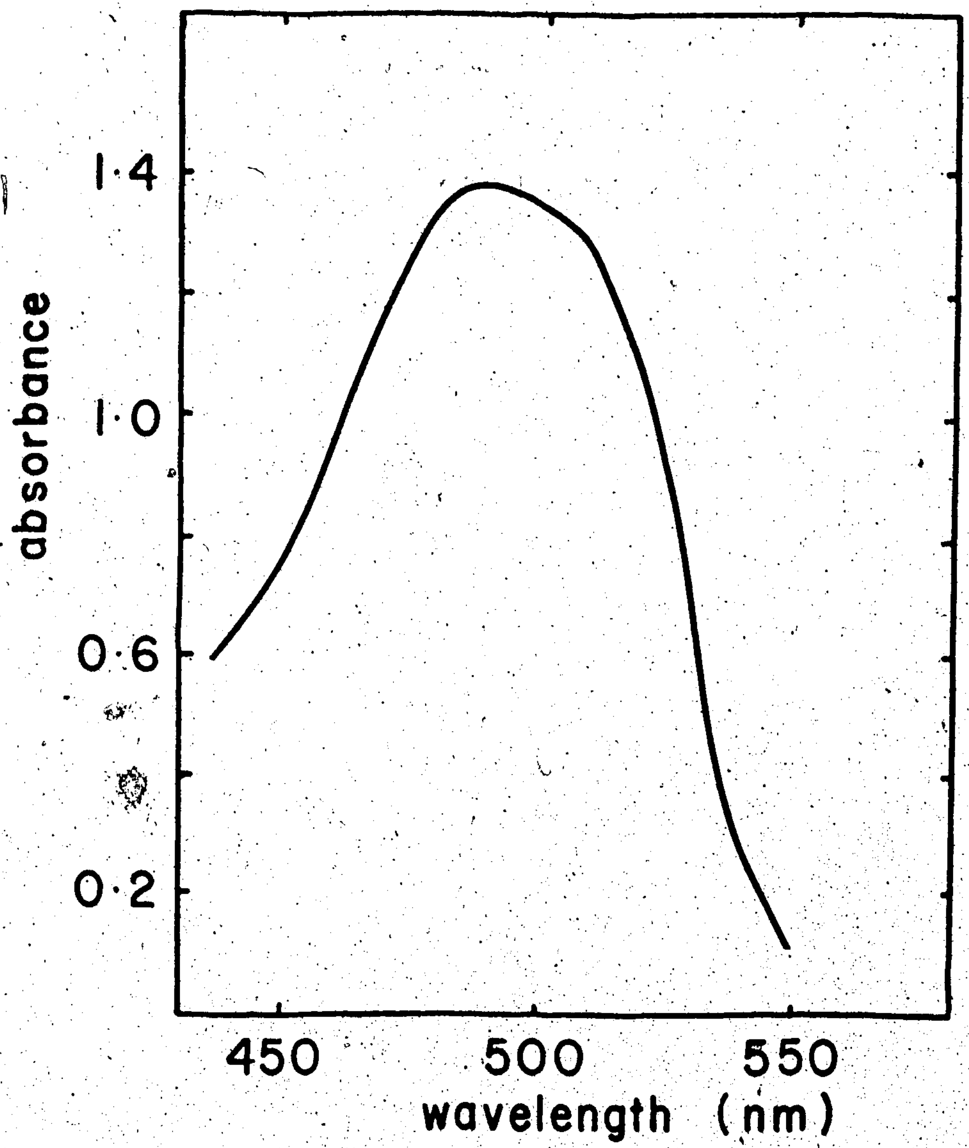


Figure IX. Visible spectrum of CBAHN at pH 7.

4-10. With magnesium present, no change was observed over the pH range 4-8, but at pH 10 a difference was observed. Thus for CBAHNS-1, the wavelength of maximum absorbance was shifted to 484 nm and the absorbance valve decreased to 0.79 at this wavelength. Complex formation with magnesium therefore appears analytically useful only above pH 8, which is outside the pH range required. The reagent is therefore not suitable for the determination of ionic magnesium concentration in biological fluids. It may be possible to find a derivative of this compound that forms an analytically useful complex with magnesium for clinical application.

SUMMARY

A method for the determination of total calcium in urine was developed by titration with EDTA, using the fluorescent indicator calcein. The method was tested for interference from compounds normally found in urine. Only phosphate was found to interfere seriously. The titration of urine samples was hindered by interference from phosphate and by a naturally occurring fluorescence which obscurred the end point. Both these problems could be overcome by the use of smaller samples. Comparison of the results obtained for a set of urine samples by this method and by atomic absorption spectroscopy gave reasonable agreement for most samples although the EDTA titration gave slightly higher results.

The coated wire type of calcium electrode was evaluated for use in the determination of ionic calcium. It was found to be unsuitable since non-reproducible results were obtained. Factors affecting the reproducibility included the thickness of the coating, time of immersion in the sample solution and age of the electrode.

The use of tetramethylmurexide as a reagent for the determination of ionic calcium concentration in urine was evaluated. Tests were carried out that verified that the method would accurately measure the ionic concentration of calcium in a solution of known ionic strength. In solutions of unknown ionic strength, such as urine, it was

demonstrated that errors of up to 30% could occur if no correction is made for ionic strength effects. A set of urine samples was analyzed for ionic calcium by this method and by atomic absorption spectroscopy for total calcium. An average value of 52% ionic calcium was found.

An attempt was made to find a reagent analogous to tetramethylmurexide for the determination of ionic magnesium concentration in biological fluids. The compound 1-(2`-carboxy-1`-benzeneazo)-2-hydroxynaphthalene was synthesized and its properties investigated. The magnesium complex is too insoluble to be useful for the determination of ionic magnesium concentration, so a more soluble derivative was prepared by incorporating a sulphonic acid group in the molecule. This compound was found to have sufficient solubility, but the magnesium complex formed only above pH 8. Since this is outside the physiological range, the reagent is not suitable for the determination of ionic magnesium concentration in biological fluids.

BIBLIOGRAPHY

1. Gitelman, H.J., Anal. Biochem., 18, 521 (1967).
2. Connerty, H.V. and Briggs, A.R., Am. J. Clin. Path., 45, 290 (1966).
3. Kessler, G. and Wolfman, M., Clin. Chem., 10, 686 (1964).
4. Frings, C.S., Cohen, P.S. and Foster, L.B., Clin. Chem., 16, 816 (1970).
5. Connerty, H.V. and Briggs, A.R., Clin. Chem., 11, 716 (1965).
6. Gindler, E.M. and King, J.D., Am. J. Clin. Path., 58, 376 (1972).
7. Mager, M. and Farese, G., Clin. Chem., 12, 236 (1966).
8. Trudeau, D.L. and Freier, E.F., Clin. Chem., 13, 101 (1967).
9. Willis, J.B., Anal. Chem., 33, 556 (1961).
10. Grunbaum, B.W. and Pace, N., Microchem. Journal., 15, 666 (1970).
11. Wallach, D.F.H. and Steck, T.L., Anal. Biochem., 6, 176 (1963).
12. Rudolf, G.G., Holler, J.J. and Ford, W.J., Clin. Chim. Acta, 18, 187 (1967).
13. Toribara, T.Y. and Koval, L., Talanta, 7, 248 (1961).
14. Slanina, J., Vermeer, P., Mook, G., Reinders, H.F.R. and Agterdenbos, J., Z. Anal. Chem., 260, 354 (1972).

15. Raman, A. and Chong, Y.K., Clin. Biochem., 7, 106 (1974).

16. McLean, F.C. and Hastings, A.B., J. Biol. Chem., 107, 337 (1934).

17. McLean, F.C. and Hastings, A.B., J. Biol. Chem., 108, 285, (1935).

18. Modlin, M., Journal of Urology, 97, 567 (1967).

19. Robertson, W.G. and Morgan, D.B., Clin. Chim. Acta, 37, 503 (1972).

20. Robertson, W.G., Peacock, M. and Nordin, B.E.C., Clin. Sci., 34, 579 (1968).

21. Hodgkinson, A., Marshall, R.W. and Cochra[illegible], M., Israel J. Med. Sci., 7, 1230 (1971).

22. Moore, E.W. Chapter 7 in "Ion-Selective Electrodes", Durst, R.A., Ed., NBS Special Publication 314, Washington, D.C., 1969.

23. Hattner, R.S., Johnson, J.W., Bernstein, D.S., Wachman, A. and Brackman, J., Clin. Chim. Acta, 28, 67 (1970).

24. Robertson, W.G., Clin. Chim. Acta, 24, 149 (1969).

24a. Freiser, H. and Cattrall, R.W., Anal. Chem., 43, 1905 (1971).

25. Shimomura, [illegible], Johnson, F.H. and Saiga, Y., Science, 140, 1339 (1963).

26. Izutsu, K.T., Felton, S.P., Seigel, I.A., Nicholls, J.I., Crawford, J., McGough, J. and Yoda, W.T., Anal. Biochem., 58, 479 (1974).

27. Raaflaub, J., Z. Physiol. Chem., 288, 228 (1951).

28. Rose, G.A., Clin. Chim. Acta, 2, 227 (1957).

29. Ettori, J. and Scoggan, S.M., Arch. Biochem. and Biophys., 78, 213 (1958).

30. Gysling, H. and Schwarzenbach, G., Helv. Chim. Acta, 32, 1484 (1949).

31. Raaflaub, J., Z. Physiol. Chem., 328, 198 (1962).

32. Pedersen, K.O., Scand. J. Clin. Lab. Invest., 25, 199 (1970).

33. French, T.J., Cham, B.E. and Cross, R.B., Lab. Practise, 21, 333 (1972).

34. Leme, C.E., Coslovsky, R. and Wajchenberg, B.L., Biomedicine, 19, 431 (1973).

35. Berlin, R.D., Anal. Biochem., 14, 135 (1966).

36. Scarpa, A., Biochem., 13, 2789 (1974).

37. Walser, M., Anal. Chem., 32, 711 (1960).

38. Diehl, H. and Ellingboe, J.L., Anal. Chem., 28, 882 (1956).

39. Diehl, H. "Calcein, Calmagite and *O,O*'-Dihydroxyazobenzene - Titrimetric, Colourimetric and Fluorometric Reagents for Calcium and Magnesium", G. Frederick Smith Chemical Co., Columbus, Ohio, 1964.

40. Korbl, J., Vydra, F. and Pribil, R., Talanta, 1, 281 (1958).

41. "Gravimetric Titrimetry". Technical Information Bulletin 1014, Mettler Instrument Corporation, Princeton, N.J., 1967.

42. Harris, W.E. and Kratochvil, B., "Chemical Separations and Measurements: Background and Procedures for Modern Analysis", W.B. Saunders Company, 1974, p. 160.

43. Handbook of Clinical Laboratory Data, 2nd Ed., 1968. Chemical Rubber Company.

44. Yalman, R.G., Bruegemann, W., Baker, P.T. and Garn, S.M., Anal. Chem., 31, 1230 (1959).

45. Bachra, B.N., Dauer, A. and Sobel, A.E., Clin. Chem., 4, 107 (1958).

46. Ross, J.W., Science, 156, 1378 (1967).

47. Private Communication with Orion Research Inc.

48. Rechnitz, G.A. and Hseu, T.M., Anal. Chem., 41, 111 (1969).

49. Moody, G.J., Oke, R.B. and Thomas, J.D.R., Analyst, 95, 910 (1970).

50. Ansaldi, A. and Epstein, S.E., Anal. Chem., 45, 595 (1973).

51. Bagg, J. and Vinen, R., Anal. Chem., 44, 1773 (1972

52. Hunt, L.D. and King, J.S., Invest. Urol., 1, 83 (1963).

53. Isaacson, L.C., Invest. Urol., 5, 406 (1968).

54. Pak, C.Y.C., Diller, E.C., Smith, G.W. and Howe, E.S., Soc. Exp. Biol. and Med. Proc., 130, 753 (1969).

55. Nordin, B.E.C. and Smith, D.A., in "Diagnostic Procedures in Disorders of Calcium Metabolism", Churchill, London, 1965, p. 181.

56. Robertson, W.G., Peacock, M. and Nordin, B.E.C., Clin. Sci. 34, 579 (1968).

57. Cham, B.E., Clin. Chim. Acta, 37, 5 (1972).

58. Raaflaub, J., in "Methods of Biochemical Analysis", Vol. III, Wiley, New York, 1956, p. 301.

59. French, T.J., Cham, B.E. and Cross, R.B., Lab Practise, 20, 424 (1971).

60. Cope, A.C., Heyl, D., Peck, D., Eide, C. and Arroyo, A., J. Amer. Chem. Soc., 63, 356 (1941).

61. Willis, J.B., Anal. Chem., 33, 556 (1961).

62. Trudeau, D.L. and Freier, E.F., Clin. Chem., 13, 101 (1967).

63. Li, N.C., Lindenbaum, A. and White, J.M., J. Inorg. Nucl. Chem., 12, 122 (1959).

64. Schubert, J. and Lindenbaum, A., J. Amer. Chem. Soc., 74, 3529 (1952).

65. Joseph, N.R., J. Biol. Chem., 164, 529 (1946).

66. Hastings, A.B., McLean, F.C., Eichelberger, L., Hall, J.L. and DaCosta, E.J., J. Biol Chem., 107, 351 (1934).

67. Diehl, H. and Ellingboe, J., Anal. Chem., 32, 1120 (1960).

68. Clarke, H.T. and Kirner, W.R. in "Organic Syntheses", Coll. Vol. I. Gilman, H., Ed., John Wiley and Sons Inc., New York, 1941, p. 374.

69. Atkinson, E.R. and Lawler, H.J. in "Organic Syntheses", Coll. Vol. I. Gilman, H., Ed., John Wiley and Sons Inc., New York, 1941, p. 222.

70. Conant, J.B. and Corson, B.B. in "Organic Syntheses", Coll. Vol. II. Blatt, A.H., Ed., John Wiley and Sons Inc., New York, 1943, p. 36.

71. Schetty, G. and Kusler, W., Helv. Chim. Acta, <u>44</u>, 2193 (1961).

72. Lindstrom, F. and Diehl, H., Anal. Chem., <u>32</u>, 1123 (1960).

73. Rosin, J., "Reagent Chemicals and Standards", D. Van Nostrand Co. Inc., 1961.

APPENDIX

DETERMINATION OF IONIC CALCIUM CONCENTRATION IN THE PRESENCE OF SODIUM USING TETRAMETHYLMUREXIDE - CALCULATION METHOD

The ionic calcium concentration can be calculated using the equation derived below. Using this method, no calibration graphs are required, and both effects of sodium complexation with tetramethylmurexide described on p. 52 are taken into account.

$$\text{Total absorbance, } A_T = A_{CaD} + A_{NaD} + A_D \qquad (1)$$

where D represents tetramethylmurexide. Assuming a path length of 1 cm throughout:

$$A_{CaD} = a_{CaD}C_{CaD} \qquad (1a)$$

$$A_{NaD} = a_{NaD}C_{NaD} \qquad (1b)$$

$$A_D = a_D C_D \qquad (1c)$$

where a = molar absorptivity

C = molar concentration

$$(C_D)_T = C_D + C_{CaD} + C_{NaD} \qquad (2)$$

where $(C_D)_T$ = total concentration of tetramethylmurexide

$$K_f = \frac{C_{NaD}}{C_{Na^+}C_D} = 1.6$$

$$C_{NaD} = 1.6\ C_{Na^+}C_D \qquad (3)$$

Similarly, $$C_{CaD} = 300\ C_{Ca^{2+}}C_D \qquad (4)$$

Substituting (1a), (1b) and (1c) into (1), and (3) and (4) into the resulting equation and into (2) gives:

$$A_T = a_{CaD}C_{CaD} + a_{NaD}C_{NaD} + a_DC_D$$

$$= a_{CaD}300C_{Ca^{2+}}C_D + a_{NaD}1.6C_{Na^+}C_D + a_DC_D \qquad (5)$$

$$(C_D)_T = C_D(1 + 300C_{Ca^{2+}} + 1.6C_{Na^+}) \qquad (6)$$

Rearranging (6) to solve for C_D, and substituting into (5) gives

$$A_T = \frac{(a_{CaD}300C_{Ca^{2+}} + a_{NaD}1.6C_{Na^+} + a_D)(C_D)_T}{(1 + 300C_{Ca^{2+}} + 1.6C_{Na^+})} \qquad (7)$$

Solving (7) for $C_{Ca^{2+}}$ yields

$$C_{Ca^{2+}} = \frac{(C_D)_T(a_D + 1.6a_{NaD}C_{Na^+}) - A_T(1 + 1.6C_{Na^+})}{300(A_T - a_{CaD}(C_D)_T)} \qquad (8)$$

CALCULATION OF MOLAR ABSORPTIVITIES OF TETRAMETHYLMUREXIDE AND ITS CALCIUM AND SODIUM COMPLEXES (AT 480 NM)

The concentrations of free tetramethylmurexide [D], calcium-tetramethylmurexide [CaD] and sodium-tetramethylmurexide [NaD] were calculated by the same approach used on p. 50.

For $(C_D)_T = 2.88 \times 10^{-5}$ and total calcium concentration of 2.45×10^{-3},

$$[D] = 1.67 \times 10^{-5} \text{ and } [CaD] = 1.2 \times 10^{-5}$$

For $(C_D)_T = 2.88 \times 10^{-5}$ and total sodium concentration of 0.200,

$$[D] = 2.13 \times 10^{-5} \text{ and } [NaD] = 0.75 \times 10^{-5}$$

These values were then used to calculate the molar absorptivities using the following experimental data:

For the blank, $A = 0.214$

$$\therefore\ a_D = \frac{0.214}{2.88 \times 10^{-5}}$$

$$= \underline{7.43 \times 10^3}$$

At a total calcium concentration of 2.45×10^{-3}, $A = 0.387$

$$A_T = a_{CaD}C_{CaD} + a_DC_D$$

$$\therefore \quad 0.387 = a_{CaD} \times 1.21 \times 10^{-5} + 7.43 \times 10^{3} \times 1.67 \times 10^{-5}$$

$$a_{CaD} = \underline{2.17 \times 10^{4}}$$

At a total sodium concentration of 0.200, A = 0.242

$$A_T = a_{NaD}C_{NaD} + a_D C_D$$

$$0.242 = a_{NaD} 0.75 \times 10^{-5} + 7.43 \times 10^{3} \times 2.13 \times 10^{-5}$$

$$a_{NaD} = \underline{1.12 \times 10^{4}}$$

SAMPLE CALCULATIONS USING EXPERIMENTAL DATA

The calculation method was tested using experimental data from known mixtures of calcium and sodium, and using the above values for the molar absorptivities.

1. For $C_{Ca^{2+}} = 2.45 \times 10^{-3}$ and $C_{Na^+} = 0.250$, $A_T = 0.382$ at 480 nm. Substituting the values into equation (8) gives $C_{Ca^{2+}} = 2.63 \times 10^{-3}$. By the graphical method described on p. 43 $C_{Ca^{2+}} = 1.94 \times 10^{-3}$.
2. For $C_{Ca^{2+}} = 1.225 \times 10^{-3}$ and $C_{Na^+} = 0.200$, $A_T = 0.333$ at 480 nm. Substituting the values into equation (8) gives $C_{Ca^{2+}} = 1.40 \times 10^{-3}$. By the graphical method described on p. 43 $C_{Ca^{2+}} = 0.88 \times 10^{-3}$.

3. For the analysis of the urine samples shown in Table IV:

Sample No. 2, by the calculation method gives

$C_{Ca^{2+}} = 2.84 \times 10^{-4}$

and by the graphical method gives

$C_{Ca^{2+}} = 3.75 \times 10^{-4}$

Sample No. 3, by the calculation method gives

$C_{Ca^{2+}} = 3.79 \times 10^{-3}$

and by the graphical method gives

$C_{Ca^{2+}} = 3.40 \times 10^{-3}$

Sample No. 4, by the calculation method gives

$C_{Ca^{2+}} = 3.16 \times 10^{-3}$

and by the graphical method gives

$C_{Ca^{2+}} = 3.43 \times 10^{-3}$

Note: The values for the formation constants of the calcium and sodium complexes of tetramethylmurexide obtained from Ref. 32 have not been verified by other workers. The rather high results obtained by the calculation method for the known mixtures may be due to inaccuracy in these formation constants.