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THE UNIVERSITY OF ALBERTA

MEASUREMENT OF FREE CALCIUM ION CONCENTRATIONS BY ION EXCHANGE/ATOMIC ABSORPTION

by

VASANTHA P. MUTUCUMARANA

A THESIS

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NAME OF AUTHOR VASANTHA P. MUTUCUMARANA

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(Signed) V. P. Mutucumarana

PERMANENT ADDRESS:

10, Bogala Court
Dickman's Road
Havelock Town
Colombo 5
Sri Lanka

DATED January 7th 1988

THE UNIVERSITY OF ALBERTA

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled MEASUREMENT OF FREE CALCIUM ION CONCENTRATIONS BY ION EXCHANGE/ATOMIC ABSORPTION

submitted by VASANTHA P. MUTUCUMARANA

in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE

B. Kratochvil

B. Kratochvil, Supervisor

Frederick J Cantwell

F.F. Cantwell

Allen Hunter

A.D. Hunter

P. Fedorak

P.M. Fedorak, External Reviewer

DATE 7 January 1988

ABSTRACT

A previously developed ion exchange/atomic absorption method for the measurement of free, hydrated metal ion concentrations in solution was employed to measure free calcium. The goal of the work was to measure the unbound calcium ion fraction in urine. Solutions containing different concentrations of calcium ion were equilibrated with a microcolumn of strong acid cation exchange resin under swamping electrolyte concentrations to ensure trace conditions. In this way calibration curves could be obtained for various ranges of free calcium ion levels. Among the conditions studied were the time of equilibration of the ion exchange resin with the sample solution, the concentration and nature of the swamping electrolyte, and the effect of other substances present in urine at significant levels such as magnesium and urea. In addition the presence of complexing ligands normally expected in urine, citrate, tartrate, phosphate, and chloride, was examined. Calcium-citrate systems were investigated in more detail than the others because citrate is the major calcium binding ligand in urine. Calculated and experimental results could not be compared directly for the calcium ligand systems because data for the calculations was available for lower ionic strengths only. Calculated curves trended toward experimental ones

when constants obtained at increasing ionic strengths were used for the calculations. Urine samples were analyzed for free calcium by the ion exchange/atomic absorption method. The total calcium in the samples was measured by titration with EDTA using calcium as the indicator. The results obtained were reasonable.

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CHAPTER 1

INTRODUCTION

1.1 Importance of Measuring Free Calcium Levels in Biological Fluids

Calcium is an important element in biological systems. It is the fifth most abundant element in the human body and it plays a major role in many physiological and biochemical processes. Calcium is involved in muscle contraction, blood clotting, bone formation and homeostasis, regulation of nerve excitability, control of many enzyme reactions, membrane permeability and regulation of many hormone actions [1-4].

It has been known for many years that it is the concentration of ionized or free calcium (coordinated to solvent only) in body fluids that is the most physiologically active form of the element [5-7]. In biological fluids, calcium exists as both the free ion and bound to ligands such as citrate and phosphate [8]. In serum calcium can also be bound to proteins [9]. There has been increasing interest during the last few years in the measurement of ionized calcium in urine, in relation to its importance in pathogenesis of urinary stone

disease, urolithiasis [4,10-12]. Calcium oxalates mixed with calcium phosphates and calcium oxalate alone compose the largest group of urinary stones occurring in humans [13,14]. Since the precipitation of calcium oxalate or calcium phosphate depends on exceeding the solubility product constant of these salts, it has been found useful to determine the concentration of ionized calcium in urine [15]. Analysis for calcium in urine samples is often needed for the diagnosis of patients with kidney stones [16].

The calcium status of a patient can be better examined by determining the concentration of ionized calcium, rather than total calcium, in serum [17,18]. Regular monitoring of ionized calcium in serum has been found to be important in detection of abnormal calcium metabolism [19-21].

There has also been some interest in the measurement of total and ionized calcium in bile in relation to the formation of gallstones. Several calcium salts are known to be able to precipitate from bile either alone or in combination with cholesterol [22]. The concentration of ionized calcium in pancreatic fluid is one of the determinants of the risk of precipitation of calcium carbonate, the main constituent of pancreatic calculi [23].

1.2 Methods Available for the Measurement of Free Calcium

Because of the physiological importance of free calcium in biological systems, several methods have been developed to measure its concentration. Early studies relied on biological assays, such as stimulating an isolated frog cardiac muscle and measuring the magnitude of contraction when the muscle was kept in solutions containing various concentrations of ionized calcium [5,6]. Such methods, however, were impractical for use in routine clinical measurements.

Spectrophotometric methods are widely used to measure ionized calcium concentrations. In these procedures an indicator ligand that forms a weak but highly colored complex with calcium ions is added to the sample under study and the concentration of metal-ligand complex is measured. From this concentration, along with knowledge of the total ligand added and the conditional formation constant of the metal-ligand complex, the free metal ion concentration is calculated. A number of indicators have been investigated for the spectrophotometric measurement of ionized calcium. For example, the dye murexide has been used for many years [24-31]. The main disadvantages of murexide are that its formation constant with calcium

is pH dependent, it forms a weak complex with sodium, and it binds with serum albumin. Some of these problems have been overcome by using tetramethylmurexide, the color of which is independent of pH within the physiological range [32-35]. Arsenazo III is a relatively selective indicator for calcium but commercial samples are impure and difficult to purify [36-39].

A modified spectrophotometric method, called the sample ion increment method, has been developed to measure free metal ion concentrations [40]. This technique permits correction for complexation by the indicator. The method has been applied to the measurement of free calcium in urine samples [41].

In addition to colorimetric indicators, a few fluorimetric indicators have been used for measuring free calcium. Aequorin, a photoprotein isolated from jellyfish, has been used to measure ionic calcium down to 10^{-7} M and lower and is quite selective [42-46]. However, aequorin is difficult to obtain and is unstable on storage. Chlorotetracycline and Quin-2 have been used in the measurement of intracellular ionic calcium concentrations [47,48]. The fluorescence of these calcium-indicator complexes is influenced by variations in pH. The fluorescent indicator Quin-2 AM has been reported to form a calcium complex, the fluorescence of which is not influenced by pH [49].

A number of calcium ion-selective electrodes have now been employed to measure ionized calcium concentrations. These electrodes did not become feasible until the synthesis of suitable organic ion-exchangers about 20 years ago [50]. Since then a number of ion-exchangers with calcium selective properties have been developed, along with a variety of ways of incorporating them into functioning electrodes [18,51-54]. Calcium ion-selective electrodes have been increasingly used for measuring ionized calcium in serum [55-59]. Some of these electrodes have now been incorporated into various commercially available instruments [57,60-62]. However, the use of these ion-selective electrodes in the measurement of ionized calcium in urine is limited as their response is influenced by high sodium ion concentrations and by high concentrations of neutral organic species such as urea.

1.3 Theory of Measurement Based on Ion Exchange

The importance of ionized calcium levels in biological systems was outlined in Section 1.1. Due to limitations in most of the methods discussed in Section 1.2, an alternative method to determine ionized calcium concentrations in biological fluids is needed. In this

work, characterization of the ion exchange column equilibration technique (IEX) to determine free calcium concentrations, and preliminary studies of the application of the method to urine samples, will be discussed. The IEX method has been used to determine free copper ion concentrations in natural waters [63,64]. The following discussion on ion exchange equilibria (Section 1.3.1) and the flow-through column equilibration method (Section 1.3.2) is based on reference 64.

1.3.1 Ion exchange equilibria

In a system containing trace amounts of a divalent metal ion, M^{++}, a high concentration of an inert electrolyte (e.g. $NaNO_3$) and a few milligrams of a strong acid-type cation exchange resin (e.g. Dowex 50W-X8), the following equilibrium will prevail when the pH is low enough that no hydroxide species are formed:

$$M^{++} + 2RNa \underset{K_{IE}^{Th}}{\rightleftharpoons} R_2M + 2Na^+ \qquad (1)$$

where R represents a resin fixed charge site, K_{IE}^{Th} is the thermodynamic ion exchange equilibrium constant for the metal ion distributed between the solution and the resin phases, and is defined as:

$$K_{IE}^{Th} = \frac{a_{R_2M} \cdot a_{Na^+}^2}{a_{M^{++}} \cdot a_{RNa}^2} \qquad (2)$$

Here a_i is the activity of the species i. Equation (2) can be written in terms of species concentrations, [i], and activity coefficients, γ_i:

$$K_{IE}^{Th} = \frac{[R_2M][Na^+]^2}{[M^{++}][RNa]^2} \cdot \frac{\gamma_{R_2M} \cdot \gamma_{Na^+}^2}{\gamma_{M^{++}} \cdot \gamma_{RNa}^2} \qquad (3)$$

where γ_{R_2M} and γ_{RNa} are the respective activity coefficients of R_2M and RNa in the resin phase.

If it is assumed that all test solutions are "swamped" with a high concentration of non-complexing strong electrolyte, it can be safely stated that the ionic strength is constant, and that the activity coefficients are therefore also constant. The presence of a trace amount of metal in any form is not expected to change the total ionic strength of the system, so equation (3) can be written in the form

$$K_{IE} = K_{IE}^{Th} \cdot \frac{\gamma_{M^{2+}} \cdot \gamma_{RNa}^2}{\gamma_{R_2M} \cdot \gamma_{Na^+}^2} = \frac{[R_2M][Na^+]^2}{[M^{2+}][RNa]^2} \qquad (4)$$

where K_{IE} is the concentration equilibrium constant of the metal ion between the aqueous and the resin phases.

A second assumption necessary to the method is that only a small fraction of the exchange sites on the resin will be occupied by the metal, i.e. $R_2M << RNa$. This is controlled by the electrolyte concentration. There must be provided a high enough $[Na^+]$ to guarantee the above condition. The upper limit of the Na^+ concentration is governed by the desire to maintain adequate sensitivity (related to resin uptake) of the metal ion, M^{2+}, and to avoid flow problems associated with increased viscosity.

The two assumptions above provide for "trace" ion exchange conditions [65,66]. Thus, upon changing the metal ion concentration the amount of Na^+ released from or sorbed by the resin is negligible in comparison with the total Na^+ concentration in the system. Under these conditions both $[Na^+]$ and [RNa] may be considered constant. These constants can be incorporated in K_{IE} and, upon rearrangement, equation (4) will take the following form:

$$\frac{[R_2M]}{[M^{++}]} = K_{IE} \cdot \frac{[RNa]^2}{[Na^+]^2} = \lambda_o \qquad (5)$$

where λ_o is the distribution coefficient of the free metal ion between the solution and resin phases. λ_o will be constant as long as trace ion exchange conditions are maintained, and the swamping electrolyte concentration is held constant. If the swamping electrolyte concentration is changed both $[Na^+]$ and K_{IE} will change. The change in K_{IE} is due to the direct effect of ionic strength on activity coefficients in both the solution and resin phases. Thus the value of λ_o will change as a result of both changing K_{IE} and changing $1/[Na^+]^2$.

A third assumption is that the free metal ion is the only calcium containing species sorbed onto the resin. This assumption is not likely to be met since, in principle, any cationic species is likely to undergo ion exchange to some extent. However it is predicted that complexes with lower charge than the free metal will sorb to a lower extent. Uncharged species also may sorb onto ion exchange resins [66].

1.3.2 Flow-through column equilibration method

This method involves passing a test solution, swamped with $NaNO_3$, through a known quantity of a strong cation exchange resin in the sodium form until the total metal

concentration in the effluent is the same as that of the test solution. Thus the resin in the column is brought to equilibrium with the unperturbed test solution. That is, the resin is in equilibrium with a solution from which no metal species in any form have been taken up by the resin. Any further passage of fresh test solution will cause no change in the metal species distribution in either phase.

It is important to recognize that the addition of the swamping electrolyte to the sample solution somewhat perturbs the original sample solution composition because it changes the activity coefficients. This will shift the ion exchange equilibrium system (equation (4)) and thus change K_{IE}. It will also shift the solution equilibria by increasing the acid dissociation constants of organic ligands and decreasing the conditional stability constants of metal complexes, at least at ionic strengths below 1.

Under trace ion exchange conditions, and assuming that M^{2+} is the only species of the metal M that is sorbed onto the resin, the amount of metal sorbed onto the resin is directly proportional to the free metal ion concentration in the solution phase. The value of the distribution coefficient, λ_o (in L/g), can be determined from the slope of a calibration curve obtained in the absence of complexing ligands. The detector signal

measured by an appropriate analytical method upon elution of the equilibrated column with a high concentration of hydrogen ion is proportional to the metal ion concentration in the resin phase, $[R_2M]$.

CHAPTER 2

CHARACTERIZATION OF ION EXCHANGE/ATOMIC ABSORPTION SYSTEM FOR IONIC CALCIUM DETERMINATION

2.1 Introduction

In the ion exchange column equilibration method, the solution of interest is passed through the column of resin, and then to waste, until complete breakthrough has been achieved. At this stage the composition of the column influent and effluent is the same. That is, at complete breakthrough, the resin is in equilibrium with fresh unperturbed test solution. Therefore this method offers a significant advantage of carrying out measurements without perturbation of the existing equilibria in the test solution.

A semiautomated system was developed previously in a study to determine free copper [67]. In this method a miniaturized ion exchange resin column was used and the system was coupled directly to the nebulizer tubing of a flame atomic absorption spectrophotometer. The metal ion was eluted from the resin as a narrow peak, the area of which was proportional to the free metal ion concentration in the initial test solution.

The present study is directed towards determining the concentration of free calcium in urine samples. In addition to the features described above, the system used for this study employs microcomputer-controlled acquisition and processing of data and operation of a series of valves which regulate the flow of solution. This allows the measurement step to be carried out rapidly and efficiently.

2.2 Experimental

2.2.1 Chemicals, resin and stock solutions

The chemicals used were: Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$ (J.T. Baker Chemical Co.); sodium chloride, NaCl (British Drug House); sodium nitrate, $NaNO_3$ (British Drug House); potassium nitrate, KNO_3 (British Drug House); magnesium nitrate, $Mg(NO_3)_2 \cdot 6H_2O$ (Fisher Scientific Co.); urea, NH_2CONH_2 (Fisher Scientific Co.); HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Aldrich Chemical Co.); nitric acid, HNO_3 (Fisher Scientific Ltd.); sodium hydroxide, NaOH (British Drug House), and potassium hydroxide, KOH (American Scientific and Chemical Co.).

The resin used in the column was analytical grade 200-400 mesh Dowex 50W-X8 strong acid type cation exchange resin (J.T. Baker Chemical Co.) with an ion exchange

capacity of 5.1 milliequivalents per gram on a dry basis.

Deionized water was used in preparing all solutions. Distilled water was passed through a column of mixed strong acid and strong base ion exchange resins (Amberlite MB-3, Rohm and Haas Co.) to obtain deionized water. Calcium nitrate stock solution (1000 μg/mL) was prepared by dissolving 11.78 g of the salt in water and diluting to 2 litres. This solution was standardized against EDTA. Sodium chloride solution (1.0 M) was prepared by dissolving 58.44 g of the salt in water and diluting to 1 litre. Sodium nitrate solution (2.0 M) was prepared by dissolving 170.0 g of the salt in water and diluting to 1 litre. Potassium nitrate solution (2.0 M) was prepared by dissolving 202.2 g of the chemical in water and diluting to 1 litre. Magnesium nitrate solution (1000 μg/mL) was prepared by dissolving 10.55 g of the chemical in water and diluting to 1 litre. Urea solution (2.0 M) was prepared by dissolving 60.06 g of urea in water and diluting to 500 mL. HEPES solution (0.01 M) was prepared by dissolving 0.238 g of the chemical in water and diluting to 100 mL. Nitric acid eluent solution (2 M) was prepared by diluting 130 mL of the concentrated acid (70%) to 1 litre with water.

2.2.2 Apparatus

The instrumental system is shown schematically in Figure 1. The variable speed peristaltic pump (Minipuls 2, Gibson, Villiers-le-Bel, France) was fitted with two 0.081-inch i.d. clear standard (PVC) tubes (Technicon Corp.). One of the tubes pumped the sample and the other tube pumped either water or the eluent (2 M HNO_3) depending on the position of valve V_1. Teflon tubing of 0.8-mm i.d. (Mandel Scientific Co., Rockwood, Ontario) connected the remainder of the system. Flow of solution in the system was controlled by a series of solenoid valves (General Valve Corp., Hanover, NJ). Valves V_1 to V_4 are three-way valves. Valves V_5 to V_8 have one common inlet but four separate outlets.

Details of column construction are given in Section 2.2.4. Calcium concentration was monitored with a flame atomic absorption spectrophotometer (AAS) (Perkin-Elmer, Model 290 B). A piece of 0.5-mm i.d. Teflon tubing was connected from valve V_6 to the nebulizer tubing of the AAS. A valve controller unit (built in the electronic workshop, Department of Chemistry, University of Alberta) was used to operate the valves. Both the valve controller unit and the atomic absorption spectrophotometer had been interfaced with an IBM PC microcomputer [41,68]. This enabled microcomputer-controlled operation of valves and processing of data that was acquired from the AAS.

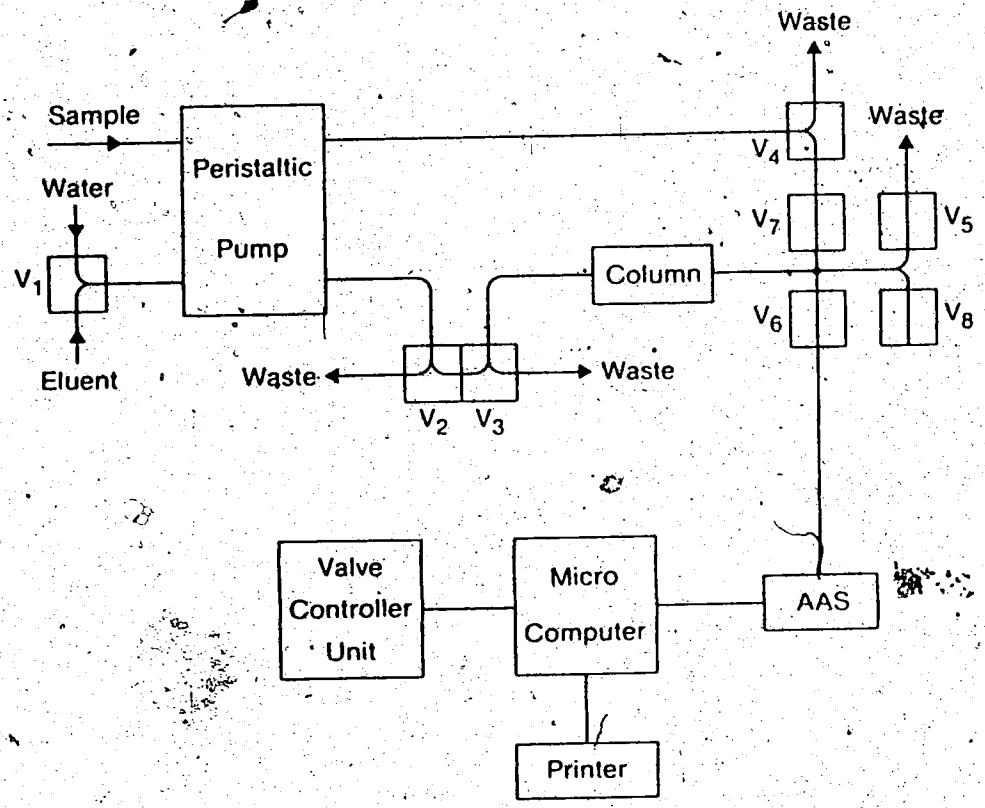


Figure 1. Diagram of semi-automated instrumental system for free calcium determination. V_1 to V_8 denote valves.

The flow of solutions during different cycles in measuring free calcium is shown in Figure 2. A printer model FX-80 (Epson Corp.) was used to provide a printout of the data. A circulating thermostat (Brinkmann Instruments) was used to maintain a constant temperature (±0.5°C) in the water jacket surrounding the column of resin. pH measurements were made with an Orion Research, model 701 pH meter using a microcombination electrode (Cole Parmer Corp.).

2.2.3 Cleaning of equipment

All glassware used for the experiments was first cleaned by washing with liquid detergent and thoroughly rinsing with tap water. This was followed by soaking overnight in 30% (v/v) HNO_3 and then rinsing thoroughly with distilled-deionized water. The flow system and the resin column were cleaned by pumping 2 M HNO_3 acid solution and then rinsing by pumping distilled-deionized water.

2.2.4 Column construction

Analytical grade 200-400 mesh Dowex 50W-X8 cation exchange resin (J.T. Baker Chemical Co.), with an exchange capacity of 5.1 meq/g of dry resin, was used to prepare the column. The resin was pretreated by repeated sedimentation and decantation in water to remove fines and

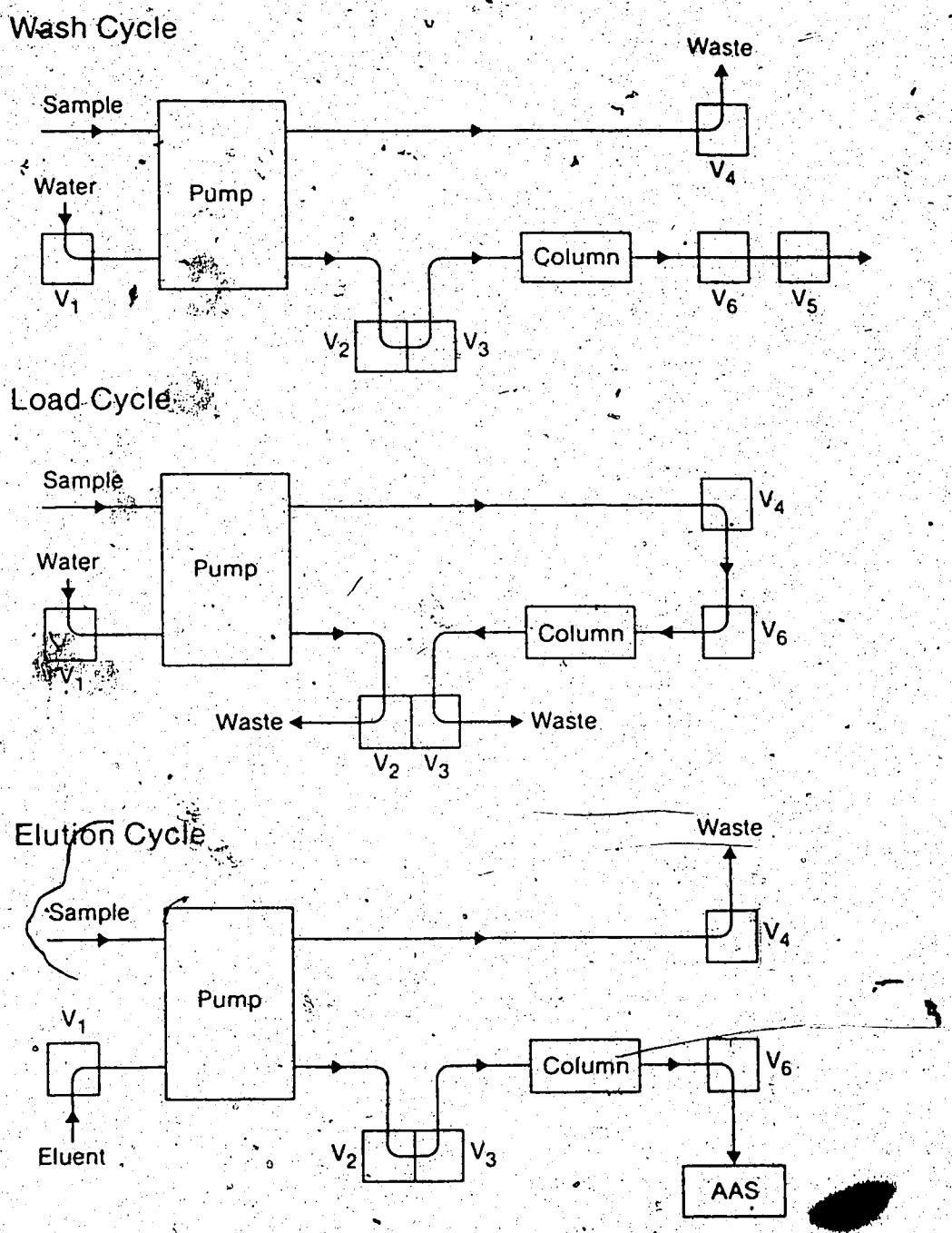


Figure 2. Direction of flow of solution in the different cycles during the determination of free metal using the semi-automated system of Reference 63.

was then washed with HCl, NaOH, ethanol, and water, followed by air drying [65].

The column design was similar to that described in the literature [67]. A 50-mm long Teflon tubing of 1.5-mm i.d. (Mandel Scientific Co., Rockwood, Ontario) was flared at both ends (Figure 3). This length of tubing was required to accommodate the two end fittings (Cheminert, Laboratory Data Control, Riviera Beach, FL). A tightly fitting, small cylindrical glass frit (50-μm porosity, approximately 2 mm long and about 1.5 mm in diameter) was pressed into the Teflon tubing and placed about 20 mm from one end. About 5 mg of the dry resin were packed into the Teflon tube from the opposite end using a fine spatula. The tube was tapped gently during packing to collect most of the resin particles in the center of the tube. A second tightly fitting glass frit was pressed into the tube from the same end through which the resin was packed and placed close to the resin but leaving a space approximately equal in volume to that of the resin. This space was kept to allow for swelling of the resin. The glass frits were made by drilling cores from a sintered glass plate. Finally a tightly fitting Teflon tube of 0.063-inch o.d., 0.02-inch i.d. was pushed through each end of the column to reduce the dead volume. The exact amount of resin in the completed column determined by

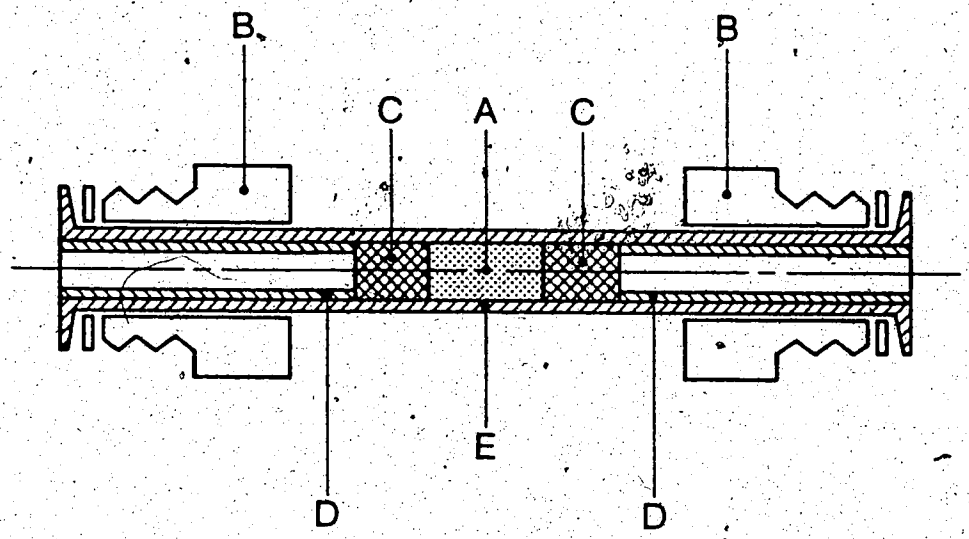


Figure 3. Details of column construction. A, resin sandwiched between two glass frits C; B, end fittings with stainless steel washers; D, Teflon retaining tube; E, Teflon column body.

weighing on a semimicro balance before and after packing, was 4.79 mg.

2.2.5 Instrumental conditions

By adjustment of the peristaltic pump flow control the flow rate in all lines was set to about 4.5 mL/min. This required a setting on the pump of about 666. The atomic absorption spectrophotometer conditions for monitoring the calcium signal are listed in Table 1.

Table 1. Instrumental parameters for monitoring calcium by atomic absorption spectrophotometry [69].

Wavelength, nm	422.7
Lamp current, mA	5
Spectral slit width, Å	7
Mode	Absorbance
Signal	Peak area and peak height
Aspiration rate, mL/min	4.5
Acetylene pressure, psig	8
Air pressure, psig	52

2.2.6 Preparation of test solutions and blanks

An appropriate volume of the electrolyte solution was measured into a clean 250-mL beaker to give the required ionic strength. About 75 mL of deionized water was added to the beaker and the pH adjusted to the desired value

with dilute HNO_3 or dilute NaOH. This was followed by the slow addition of an appropriate volume of a standard calcium solution with magnetic stirring. In some cases a final pH adjustment was required. The solution was then transferred quantitatively to a 200-mL volumetric flask and diluted to the mark with deionized water. The pH was rechecked, and if necessary, adjusted with a very small volume of dilute HNO_3 or dilute NaOH so as to cause negligible change in the final concentration of calcium in the solution.

Blanks were prepared in a similar way but without the addition of the calcium solution.

2.2.7 Procedure for free metal determination

The instrumental setup described in Section 2.2.2 was used to measure free calcium employing a 4.8-mg column of resin. The temperature of the resin column was maintained at 25 ± 0.5°C during all measurements. Flow rate in the system was adjusted to about 4.5 mL/min with the aid of the peristaltic pump. The atomic absorption spectrophotometer conditions used for monitoring the calcium signal are listed in Table 1.

The column was washed by pumping water through it for 1 minute prior to loading with the test solution. The period required to attain complete breakthrough during the

loading cycle was determined by studying the column equilibration curves. After the loading cycle, the column was backwashed with water for 3 minutes to remove any interstitial test solution. During this cycle the flow was diverted to the AAS for 2 minutes. The washing cycle was followed by the elution cycle during which 2 M HNO_3 was passed through the column. Finally the column was washed again with water before introducing the next test solution. The data acquired from the AAS was processed after each measurement to obtain the peak height and peak area.

2.3 Results and Discussion

2.3.1 Column equilibration curves

In this section the time period required to achieve complete breakthrough for different test solutions with the column of resin was examined. Column equilibration curves of the following solutions were studied: 0.5, 1.0 and 5.0 μg/mL Ca, all in 0.1 M NaCl; 25.0 μg/mL Ca in 0.75 M $NaNO_3$ and 25.0 μg/mL Ca in 0.5 M KNO_3. Test solutions were prepared at pH 5.00 ± 0.05 as outlined in Section 2.2.6.

Each test solution was loaded for different periods of time. Flow rate was maintained at 4.5 mL/min. All

experiments were carried out at 25 ± 0.5°C. Similar peak area values were given when 2 M HNO_3 and 3 M HNO_3 were used as the eluents. However 2 M HNO_3 was selected as the eluent, since it had been reported to be more suitable in an earlier study [63].

The results for the loading curves for solutions with 0.5, 1.0 and 5.0 μg/mL Ca in 0.1 M NaCl are presented in Figure 4. When complete breakthrough has been achieved, the calcium content of the resin does not increase with time. Therefore the breakthrough regions are shown as plateaus in these figures. The time required for complete breakthrough does not change as the concentration of calcium is increased at a given electrolyte concentration, in agreement with theory. This is evident by comparing the curves for 1.0 μg/mL and 5.0 μg/mL calcium solutions in 0.1 M NaCl (curves b and c in Figure 4).

The equilibration curves for 25 μg/mL Ca solutions in 0.5 M KNO_3 and 0.75 M $NaNO_3$ are presented in Figures 5 and 6, respectively. These solutions require less than 2 minutes to attain complete breakthrough at a flow rate of 4.5 mL/min. This indicates that the metal ion requires less time for equilibrium to occur between the solution phase and the resin phase when the swamping electrolyte concentration is increased.

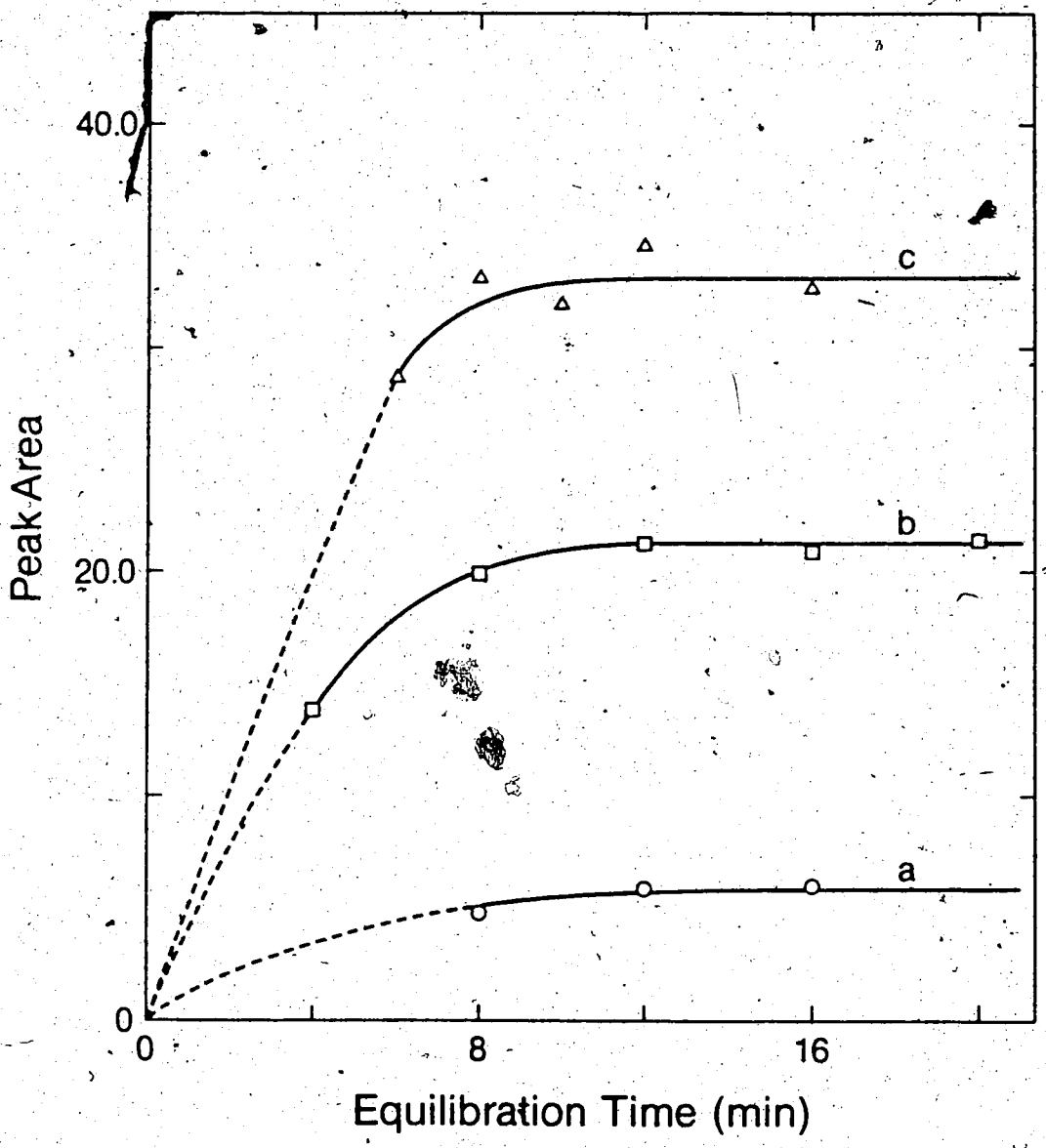


Figure 4. Column equilibration curves. (a) 0.5 µg/mL calcium in 0.1 M NaCl, (b) 1.0 µg/mL calcium in 0.1 M NaCl, (c) 5.0 µg/mL calcium in 0.1 M NaCl. Flow rate, 4.5 mL/min. **Note:** AA sensitivity was changed for the different solutions, so peak areas among plots are not directly comparable.

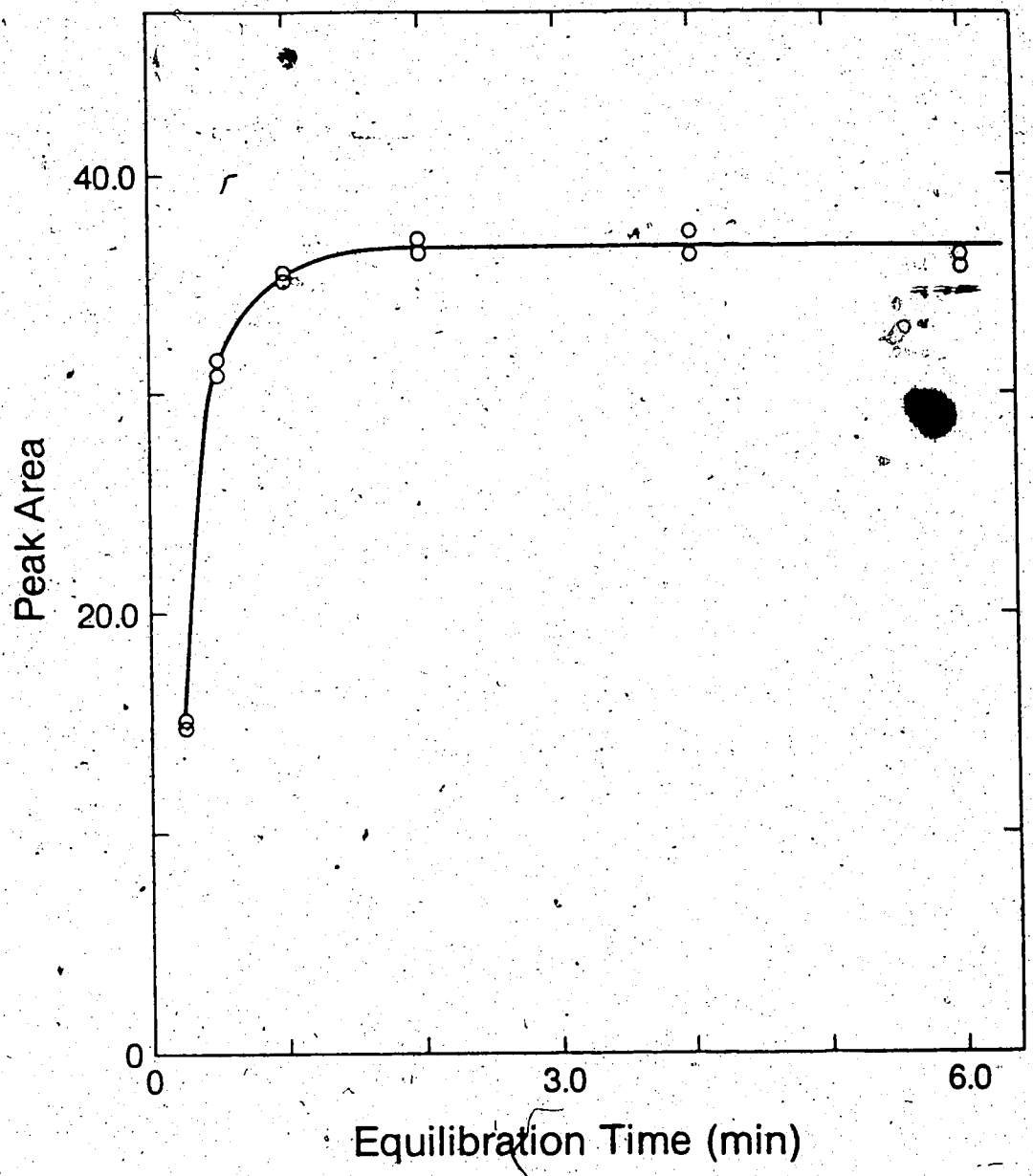


Figure 5. Column equilibration curve for 25 μg/mL calcium solution in 0.5 M KNO_3, Flow rate, 4.5 mL/min.

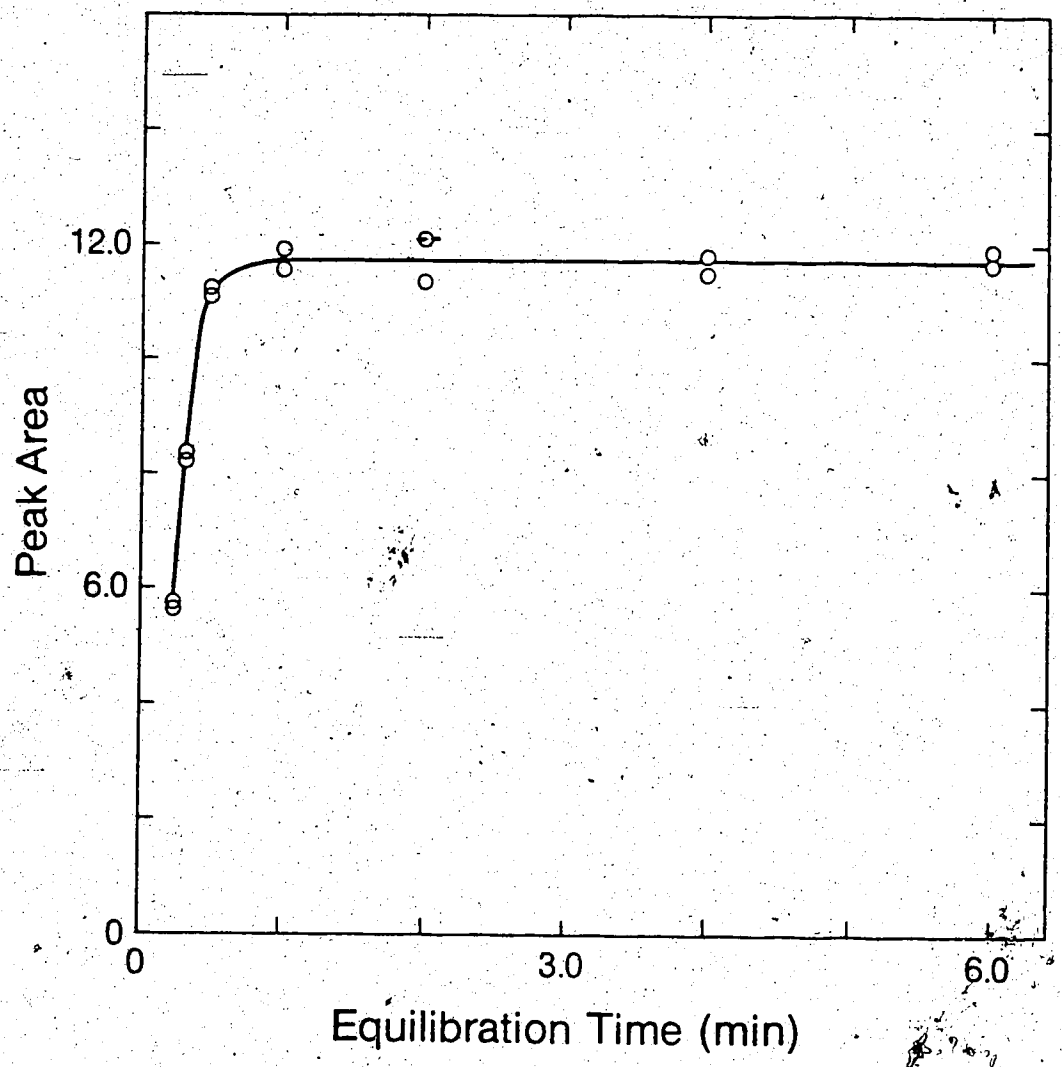


Figure 6. Column equilibration curve for 25 µg/mL calcium solution in 0.75 M $NaNO_3$. Flow rate, 4.5 mL/min.

2.3.2 Calibration curves with 0.1 M NaCl as electrolyte

Standard calcium solutions ranging from 25 μg/mL to 125 μg/mL were prepared at pH 5 as outlined in Section 2.2.6. The above range was selected in order to match the concentration of calcium that was usually present in urine [70]. Each solution was passed through the column of resin for a period of 10 minutes and the free metal was determined as described in Section 2.2.7. The calibration curve for the standard solutions prepared in 0.1 M NaCl is shown in Figure 7. Peak area values were used in the preparation of all calibration curves. The curve does not show linearity, even at the lowest calcium concentration studied, 25 μg/mL. This indicates that the column is not operating under trace conditions at higher concentrations when the ionic strength is 0.1.

This result was further examined by carrying out the following experiment. Each solution was passed through the column for a period of 10 minutes as before but in the elution cycle the effluent from the column was collected in a 50-mL volumetric flask instead of passing it directly to the nebulizer tubing of the AAS. The contents of the flask were diluted to the mark with 2 M HNO_3 and the resulting solution was aspirated into the AAS. The concentration of calcium in these solutions was determined by referring to a calibration plot made by aspirating a

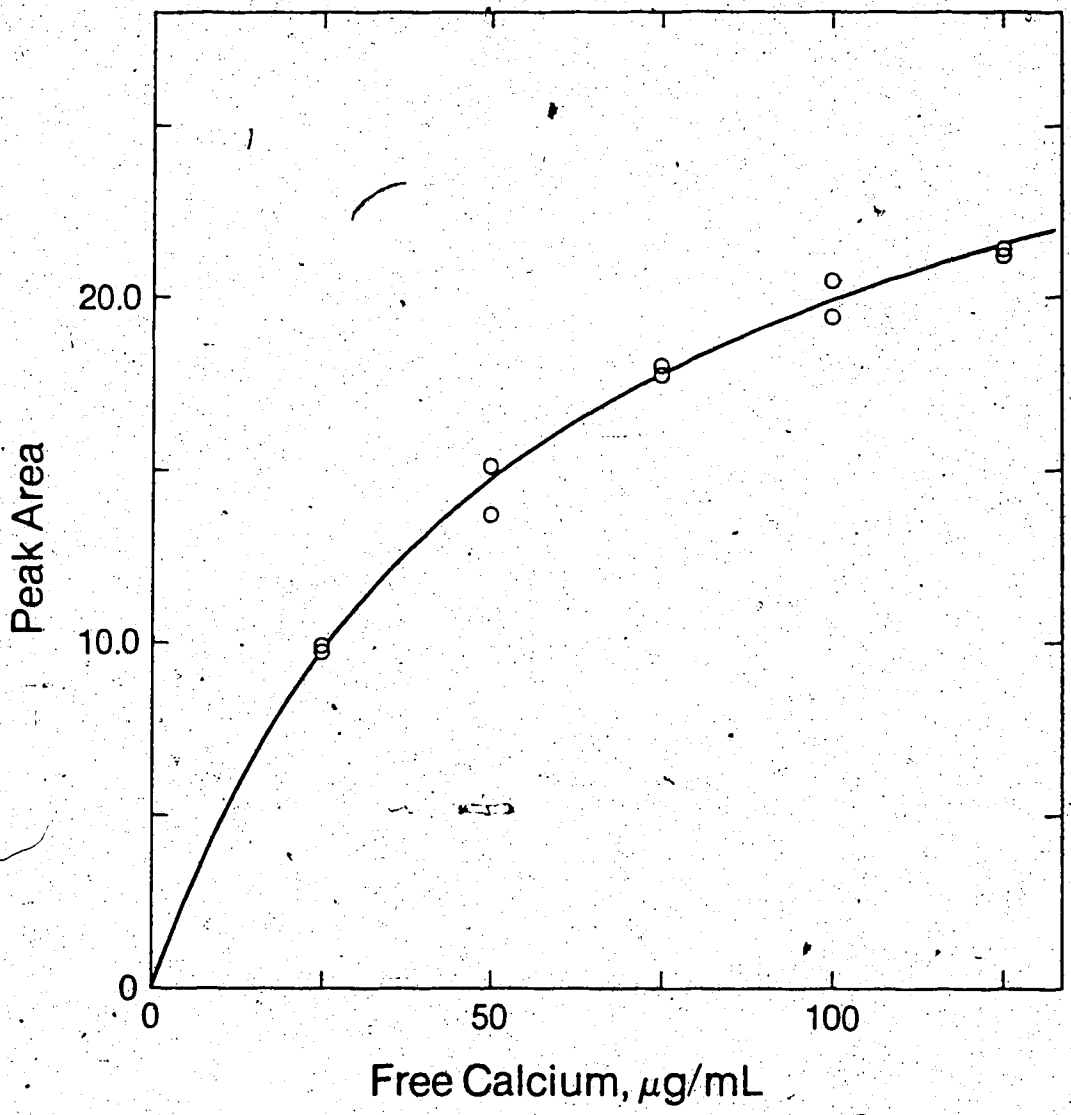


Figure 7. Calibration curve for standard calcium solutions in 0.1 M NaCl at pH 5.

set of standard calcium solutions, ranging from 5 μg/mL to 25 μg/mL, prepared using 2 M HNO_3 as the diluent. These standards were prepared in 2 M HNO_3 in order to match the matrix of the effluent from the column.

The fraction of resin in the calcium form was calculated as follows:

If the concentration of calcium in the effluent (diluted to 50 mL) is defined as x mg/L, then the number of millimoles of calcium present in that volume is given by

$$\frac{x}{1000} \times 50 \times \frac{1}{40.08}$$

where 40.08 is the atomic weight of calcium. The number of milliequivalents of calcium on the resin is then

$$\frac{x}{1000} \times 50 \times \frac{1}{40.08} \times 2$$

Since the exchange capacity of the resin used in this study is 5.1 meq/g, and the weight of resin in the column is 0.00479 g, then the total number of equivalent sites on the resin may be written as

$$5.1 \times 0.00479 \text{ meq}$$

Thus the fraction of resin in the calcium form is expressed by

$$\frac{x \times 50 \times 2}{\frac{1000 \times 40.08}{5.1 \times 0.00479}} = 0.1021\ x$$

Table 2. Fraction of resin in the calcium form at breakthrough conditions after equilibrating the column with standard calcium solutions in 0.1 M NaCl.

Concentration of calcium standard solution (μg/mL)	Fraction of resin in the calcium form
25	0.10
50	0.18
75	0.29
100	0.35
125	0.41

The results (Table 2) clearly show that the column is not operating under trace conditions with the calcium solutions considered above in 0.1 M NaCl.

Free metal determinations carried out on a set of solutions (25 and 50 μg calcium/mL) in 0.2 M NaCl also exhibited non-trace conditions of calcium on the resin. Therefore it was necessary to examine solutions prepared in higher electrolyte concentrations in order to achieve suitable calibration curves.

2.3.3 Calibration curves at higher electrolyte concentrations

2.3.3.1 With sodium nitrate as electrolyte

At higher electrolyte concentrations, $NaNO_3$ was used instead of NaCl to minimize any complexation of free calcium by the electrolyte anion. Solutions were prepared as described in Section 2.2.6 with the addition of an appropriate volume of a 2.0 M sodium nitrate stock solution and adjustment of pH to 5.00 ± 0.05 with dilute HNO_3. Calibration curves were examined at three different electrolyte concentrations - 0.5 M, 0.75 M and 1.0 M $NaNO_3$. All the solutions were equilibrated with the column of resin for a period of 5 minutes and free calcium determined by the method described in Section 2.2.7. A study of the column equilibration curves (Section 2.3.1) showed that this loading period was sufficient to achieve complete breakthrough conditions. Results are presented in Figures 8 to 10 for the different sodium nitrate electrolyte concentrations.

A comparison of the calibration curves shows that the linear region of the plots extends up to a higher concentration of calcium as the electrolyte concentration is increased. For example, the peak area observed for the solution with 125 μg/mL calcium in 1.0 M $NaNO_3$ falls within the linear region whereas this is not the case with

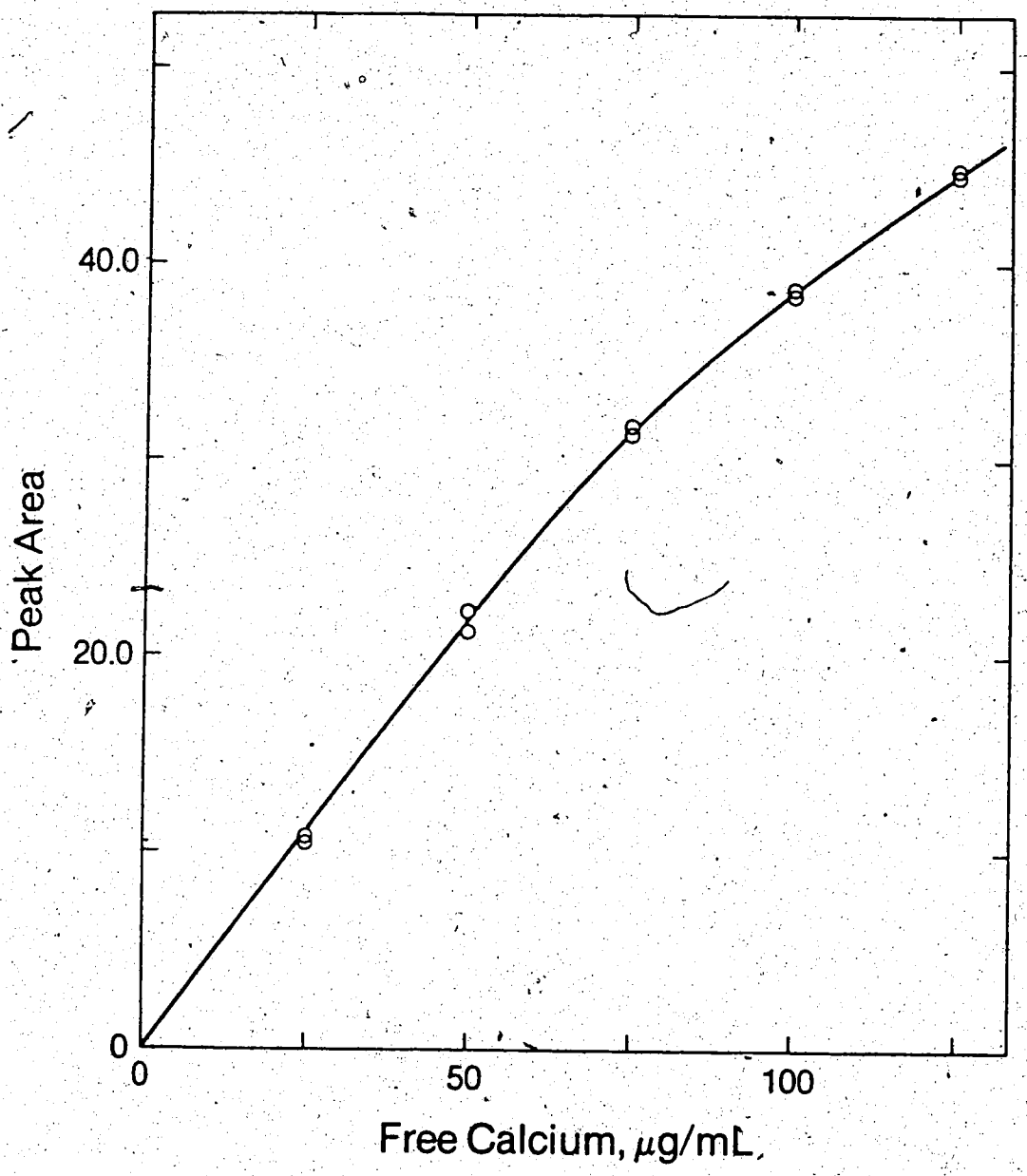


Figure 8. Calibration curve for calcium standards in 0.5 M $NaNO_3$ at pH 5.

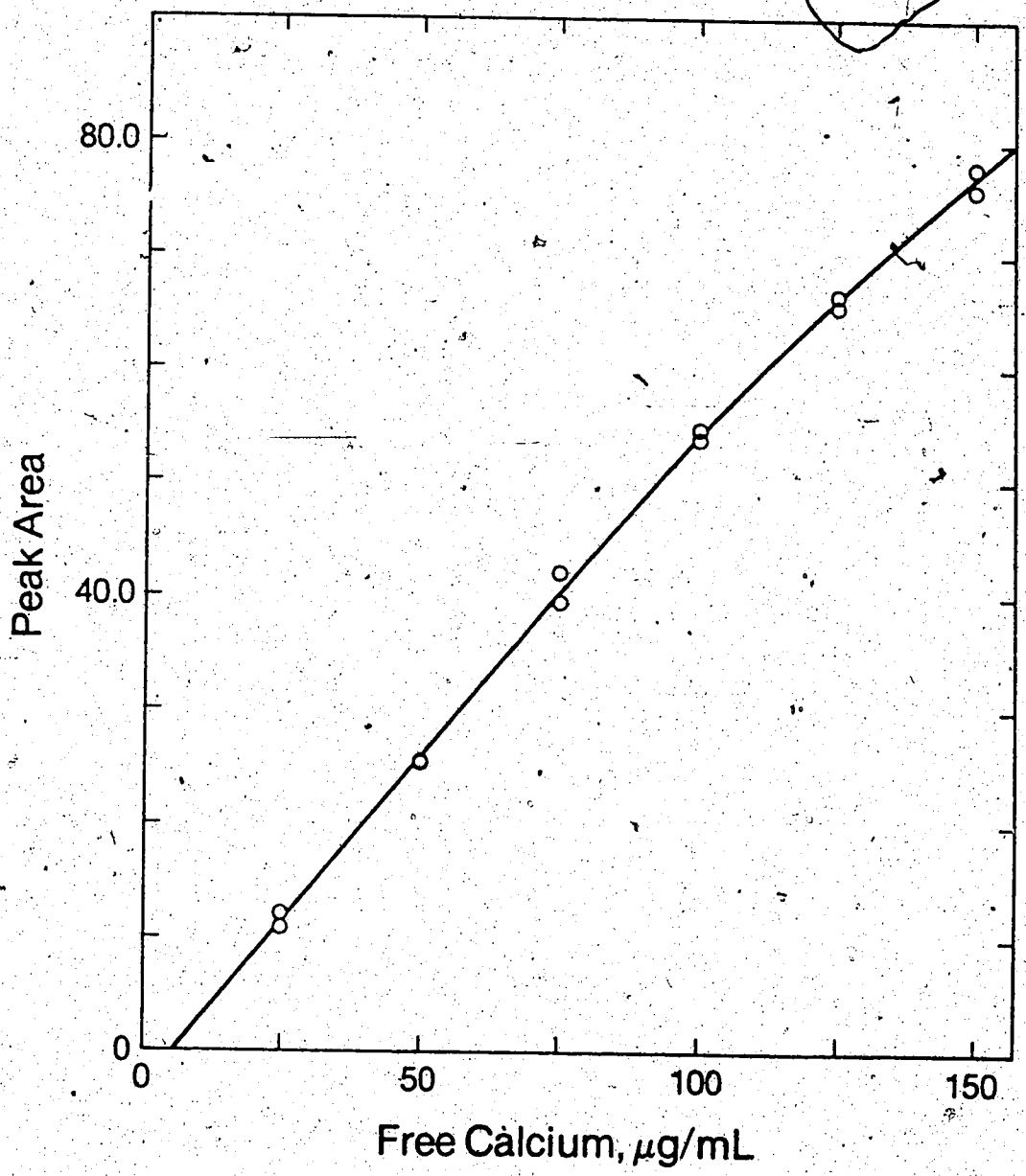


Figure 9. Calibration curve for calcium standards in 0.75 M $NaNO_3$ at pH 5.

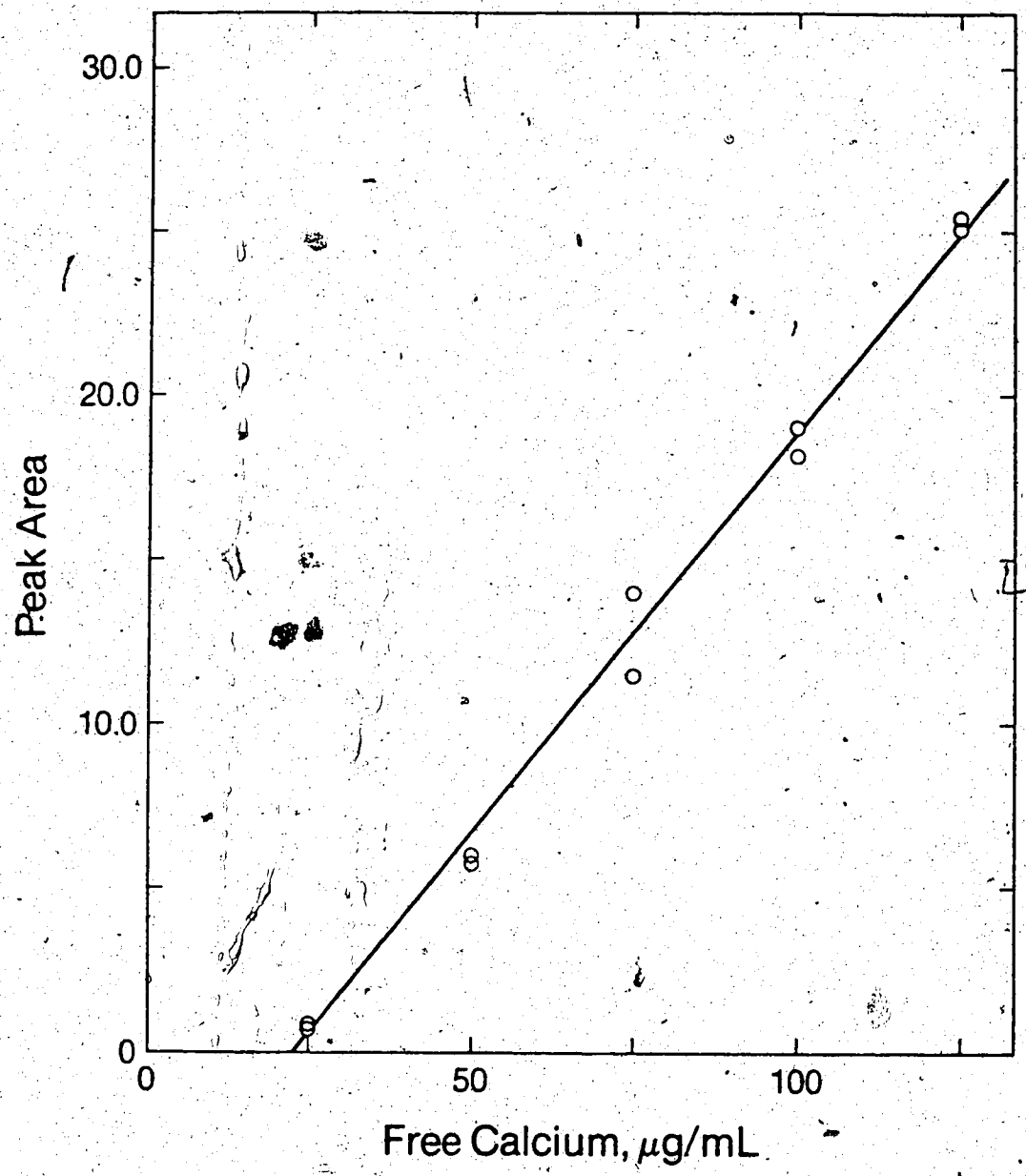


Figure 10. Calibration curve for calcium standards in 1.0 M $NaNO_3$ at pH 5.

the other electrolyte concentrations considered. However, the non-linear region observed with the plot having 0.75 M $NaNO_3$ shows a reasonable change in peak area with concentration so that it is still useful as a calibration curve. The figures show that the calibration plots with 0.75 M $NaNO_3$ and 1.0 M $NaNO_3$ have a negative intercept while the plot with 0.5 M $NaNO_3$ passes through the origin, the reason for which is not known. Blanks consisting of only supporting electrolyte were run for each calibration curve, and peak areas were always negligible.

If activity coefficients are shifted by increasing ionic strength, a change in slope would be predicted, but the intercept would still be expected to be zero. At high ionic strengths the resin behaves as though calcium ions are excluded until they attain some threshold level, after which they are able to force their way into the highly charged solution within the resin pores. Among the experiments that might be conducted to investigate this behavior are the replacement of calcium with other divalent or trivalent ions, both organic ions and metal ions, replacement of the sodium or potassium with other monovalent cations such as lithium, ammonium, or alkyl ammonium species, and use of a calcium ion buffer solution in place of calcium alone in preparation of the calibration curves.

The non-linear nature at higher calcium concentrations for curves with 0.5 M $NaNO_3$ and 0.75 M $NaNO_3$ can be explained as follows: By examining the equation:

$$\frac{[R_2M]}{[M^{++}]} = K_{IE} \cdot \frac{[RNa]^2}{[Na^+]^2}$$

it is clear that $[RNa]/[Na^+]$ has to be constant in order to have a linear relationship between $[R_2M]$ and $[M^{++}]$. Upon changing the calcium ion concentration, changes in the amount of Na^+ released from or sorbed by the resin would be negligible in comparison with the total Na^+ in the system only if there is a high enough concentration of sodium in the solution. Under such conditions both $[Na^+]$ and [RNa] can be considered to be constant, thus giving a linear relationship between $[R_2Ca]$ and $[Ca^{++}]$.

2.3.3.2 With potassium nitrate as electrolyte

Examination of calibration curves at KNO_3 concentrations of 0.5 M and 1.0 M is described in this section. Solution preparation and free calcium determination were carried out as detailed in Section 2.3.3.1 but with the addition of an appropriate volume from a 2.0 M potassium nitrate solution as the electrolyte. Results are presented in Figures 11 and 12.

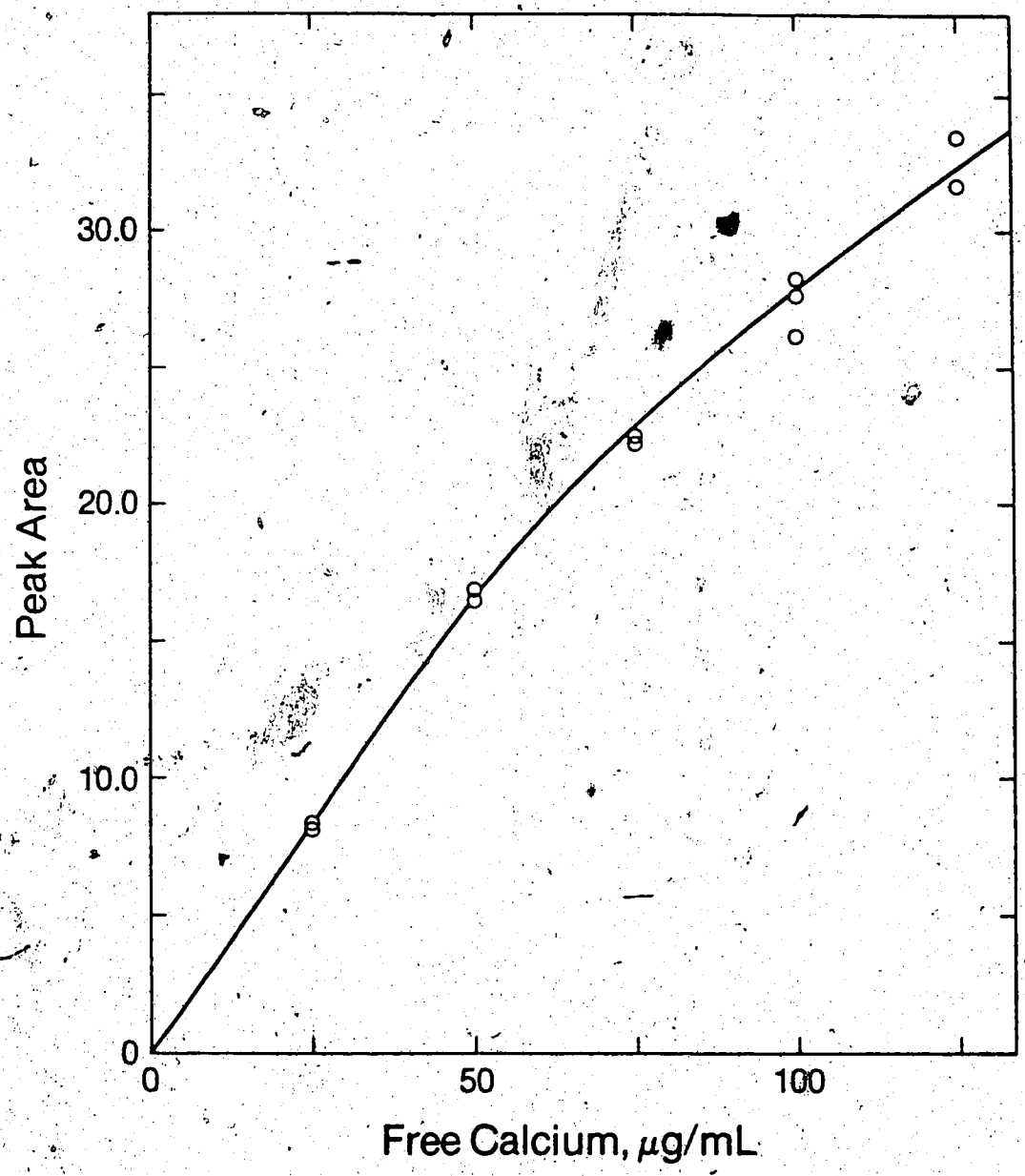


Figure 11. Calibration curve for calcium standards in 0.5 M KNO_3 at pH 5.

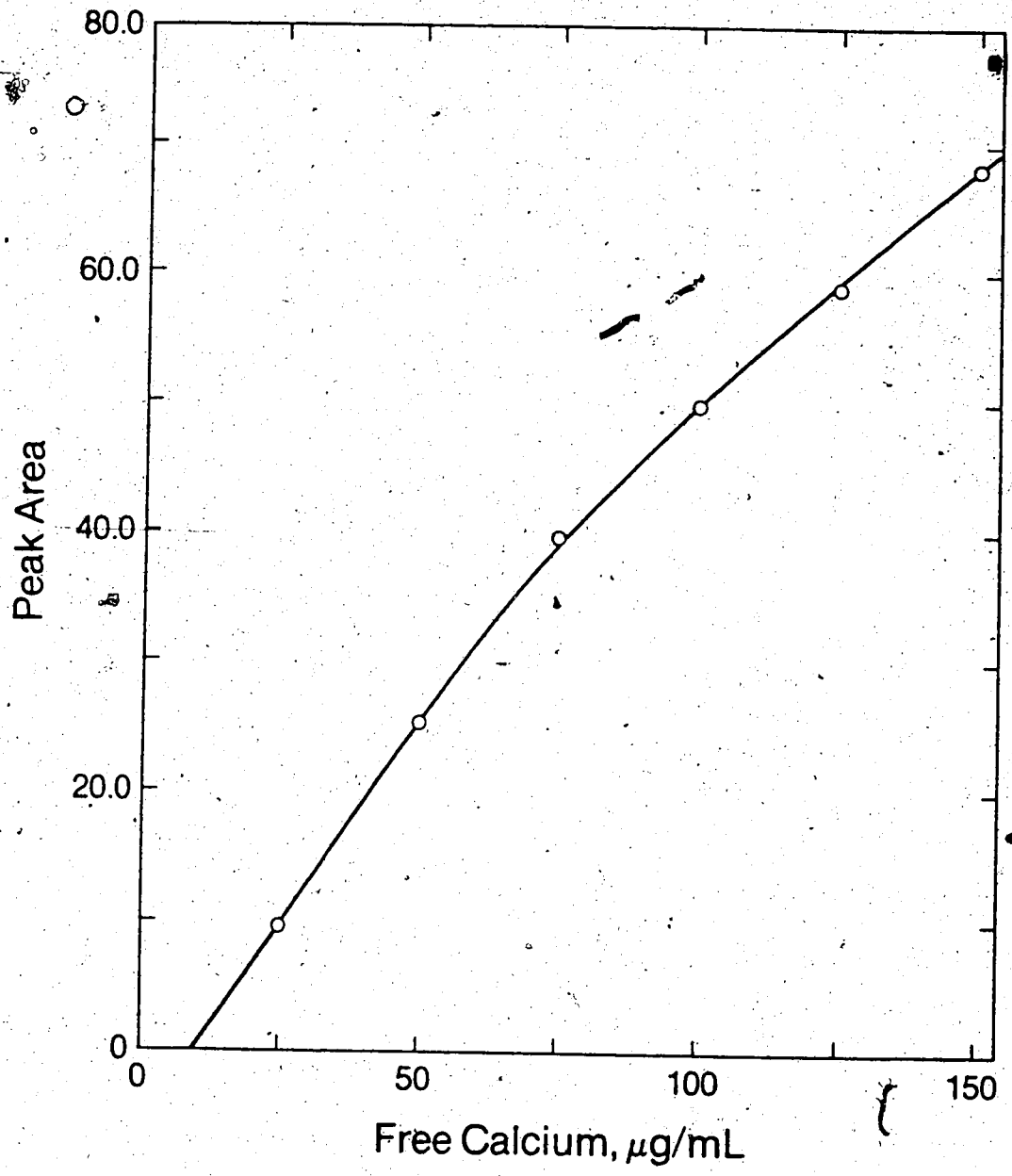


Figure 12. Calibration curve for calcium standards in 1.0 M KNO_3 at

The plots with 0.5 M KNO_3 show a similar trend to those obtained with 0.5 M $NaNO_3$ as the electrolyte (Section 2.3.3.1). However, the calibration curves for the two electrolytes at 1.0 M concentrations are different. The plot relating to 1.0 M KNO_3 begins to curve off around a calcium concentration of 60 μg/mL whereas that relating to 1.0 M $NaNO_3$ gives a linear region even up to a concentration of 125 μg/mL in calcium.

A possible explanation for the above experimental observation could be that at higher ionic strengths more calcium ions in a given solution are sorbed onto the resin when KNO_3 is used as the electrolyte instead of $NaNO_3$. The sorption of more calcium onto the resin would cause the system to operate under non-trace conditions for calcium. In dilute solutions, potassium shows a greater affinity than sodium towards the strongly acidic cation exchange resins due to its smaller hydrated ionic size [66]. On this basis it might be expected that at a given electrolyte concentration less calcium would be sorbed onto the resin when KNO_3 is used as the swamping electrolyte than when $NaNO_3$ is used with the same solution of calcium. However, a reversed selectivity order of metal ions from the expected trend towards ion exchange resins at high ionic strengths has been demonstrated previously by several studies [71-73].

2.3.4 Effect of magnesium on free calcium determination

The effect of magnesium, at the levels usually present in urine, on the determination of free calcium was examined. Three test solutions, each containing a total calcium concentration of 75 μg/mL and an electrolyte concentration of 0.5 M KNO_3, were prepared. An appropriate volume from a magnesium nitrate solution was added to two of these solutions to bring the final magnesium concentration to 50 μg/mL and 100 μg/mL respectively. The normal range of magnesium in urine is about 7-109 μg/mL. The third solution contained no magnesium. The pH of the solutions was adjusted to 5.00 ± 0.05 with dilute HNO_3. Each solution was equilibrated with the column for 5 minutes and the elution peaks were compared. The peak area for the solution without magnesium was 21.90 ± 0.69 (n = 2) while that for the solutions containing 50 μg/mL and 100 μg/mL magnesium were 22.24 ± 0.35 (n = 2) and 21.61 ± 0.38 (n = 2), respectively. Therefore it is clear that magnesium, at the levels usually present in urine, does not interfere with the measurement of free calcium. So long as the equilibrium between the swamping electrolyte and magnesium is such that magnesium uptake by the resin is small (less than 1%), magnesium would not be expected to interfere. Under these conditions the equilibration of calcium and

magnesium with the resin can be considered to be independent of each other. Interference by a third cation with the equilibrium between calcium and the swamping electrolyte would only be expected to occur when the third cation is so strongly taken up by the resin that the number of exchange sites available for the calcium equilibrium is significantly reduced. A major reduction in the number of exchange sites available to the calcium swamping electrolyte equilibrium would have the effect of a reduction in the amount of resin available in the column, and would reduce the amount of calcium eluted from the column during the measurement step. This would lead to low results.

2.3.5 Effect of urea on free calcium determination

Two test solutions, each containing a total calcium concentration of 75 μg/mL and an electrolyte concentration of 0.5 M KNO_3, were prepared. Urea was added to one of these solutions to give a final concentration of 0.57 M. This corresponds to the upper limit of urea usually present in urine, since the normal range of concentration of urea in urine is 10 to 35 g/24 h [74], and the normal volume of urine voided is 1 to 1.5 L/24 h. The pH of the solutions was adjusted to 5.00 ± 0.05 with dilute HNO_3. Each solution was equilibrated with the column for 5

minutes and the elution peaks were compared. The solution without urea gave a peak area value of 22.98 ± 1.13 for 3 replicates, while that for the solution with urea (0.57 M) was 22.04 ± 0.53 for 4 replicates. This indicates that there is no interference from urea, at levels usually present in urine, in the determination of free calcium by this method.

2.3.6 Effect of pH on free calcium determination

Determination of free calcium on two test solutions, at pH 5 and 7 respectively, is described in this section. The pH of urine normally falls within a range of 5 to 7. The total calcium concentration of each solution was 75 μg/mL and electrolyte (KNO_3) concentration was 0.5 M. The pH of one of the solutions was adjusted to 5.00 ± 0.05 with dilute HNO_3. HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer was added to the other solution and the pH adjusted to 7.00 ± 0.05 with dilute KOH. The final buffer concentration of this solution was kept at 10^{-3} M. It has been reported that the complexing capacity of HEPES with calcium is negligible [75]. The addition of a buffer was necessary since the pH of the solution was not stable around 7 without it.

Each solution was equilibrated with the resin column for 5 minutes. The elution peaks gave values of 24.61 ±

0.20 (n = 4) and 24.70 ± 0.10 (n = 3) respectively for the solut[illegible] at pH 5 and 7. These results demonstrate that the amount of free calcium determined in the standard solutions is not affected by pH within the range of 5 to 7. It agrees with the fact that the ion exchange capacity of strongly acidic cation exchange resins is not affected by pH [66].

Furthermore these results show that there may not be any sorption of calcium containing colloidal particulates onto the resin around pH 7. Such a process would have increased the peak area value at pH 7. Presence of filterable particulate copper-containing species at pH 8 has been reported in a study carried out to determine free copper using the ion exchange equilibration method [63]. Also formation of species such as $CaOH^+$ around pH 7 may not be taking place. If such species had formed, the fraction of free calcium would have decreased which would have reduced the peak area at pH 7.

2.4 Conclusions

Results indicate that the electrolyte concentration of the standard solutions needs to be adjusted to 0.75 M with $NaNO_3$ in order to achieve suitable calibration curves to measure free calcium in the range of about 25 to 125 µg/mL. A higher electrolyte concentration would enable

the upper limit of this range to be raised. However, at higher ionic strengths activity coefficients and equilibria may shift significantly.

Determination of free calcium at lower ionic strengths, which would have been desirable, is not possible due to curvature beginning at low concentrations in the calibration plots. The calibration curves with 1.0 M KNO_3 as the electrolyte showed deviation from the expected trend. Therefore $NaNO_3$ was used as the swamping electrolyte in most of the experiments, especially at high ionic strengths.

Urea at a concentration of 0.57 M does not affect the measurement of free calcium. This is in contrast to its effect on calcium ion measurements with ion selective electrodes, which are perturbed by urea.

Free calcium measurements on standard solutions are not affected by pH in the range of 5 to 7. Therefore, it is not critical that the pH of the standard solutions be matched with that of urine samples as long as the pH of the samples falls between 5 and 7. In fact, the pH of normal urine falls within this range. Dependence of free calcium determination on pH at higher pH values was not examined.

CHAPTER 3

DETERMINATION OF FREE CALCIUM BY THE ION EXCHANGE/ATOMIC ABSORPTION SYSTEM IN THE PRESENCE OF COMPLEXING LIGANDS

3.1 Introduction

In this chapter, the effect of some of the important complexing ligands present in urine on the determination of free calcium is examined. Presence of complexing ligands result in the binding of some of the metal ions and thereby lower the free metal ion content. The extent of such binding depends on the stability constants of the complexes of the metal ion with the ligand species, the concentration of the ligand, and the pH of the medium. Depending on the ligand charge, the metal-ligand complex can be either cationic, anionic or neutral. In the ion exchange column equilibration method, it is assumed that only the free metal is sorbed onto the resin. However, sorption of cationic complexes by similar mechanisms to those of free metal ions and sorption of neutral complexes by physical interactions can also take place [66]. Such processes can give rise to falsely high values for the amount of free calcium determined. Formation of anionic complexes would not interfere to a significant extent as

they bear the same sign of charge as the fixed resin sites.

The fraction of free calcium available in synthetic solutions with various ligand concentrations was examined in order to study the effect of ligands on metal speciation. The ligands considered were citrate, tartrate, phosphate, and chloride, all of which are usually present in urine. Citrate was studied most extensively as it appears to be the ligand that binds more calcium than the others at the normal concentrations present in urine. The fraction of free metal ion can also be determined theoretically when the stability constants of metal-ligand complexes and the acid dissociation constants of the ligand are known at a given ionic strength.

3.2 Experimental

3.2.1 Chemicals and solutions

The chemicals used were: Sodium citrate, $HOC(COONa)-(CH_2COONa)_2 \cdot 2H_2O$ (J.T. Baker Chemical Co.); potassium citrate, $HOC(COOK)(CH_2COOK)_2 \cdot H_2O$ (British Drug House); tartaric acid, $HOOC(CHOH)_2COOH$ (Fisher Scientific Co.); disodium hydrogen orthophosphate, $Na_2HPO_4 \cdot 7H_2O$ (Matheson, Coleman and Bell); sodium chloride, NaCl (British Drug

House).

Sodium citrate solution (0.20 M) was prepared by dissolving 5.882 g of sodium citrate in water and diluting to 100 mL. Potassium citrate solution (0.40 M) was prepared by dissolving 12.97 g of potassium citrate in water and diluting to 100 mL. Tartaric acid solution (0.50 M) was prepared by dissolving 7.506 g of tartaric acid in water and diluting to 100 mL. Disodium hydrogen orthophosphate solution (0.40 M) was prepared by dissolving 53.61 g of the chemical in water and diluting to 500 mL. Sodium chloride solution (2.0 M) was prepared by dissolving 58.44 g of the salt in water and diluting to 500 mL. The other chemicals and reagents used were described in Chapter 2.

3.2.2 Apparatus and procedure for the determination of free calcium

The instrumental setup described in Section 2.2.2 was used for the determination of free calcium. The procedure described in Section 2.2.7 was used to measure free calcium in the solutions containing complexing ligands. The free calcium content was determined by referring to a calibration plot made by measuring a series of standard solutions prepared at the corresponding ionic strength. Flow rates in the system were maintained at about 4.5

mL/min. Each solution was equilibrated with the column for a period of 5 minutes, except in the case relating to Section 3.3.1.4. The temperature of the resin column was kept at 25 ± 0.5°C during all measurements.

3.2.3 Calculation of free calcium at different ligand concentrations

The general method used will be discussed by considering the complexes formed between a ligand such as citric acid (H_3L) and a divalent metal ion (M^{++}) such as Ca^{++}.

$$M^{2+} + L^{3-} \rightleftarrows ML^{-} \qquad \beta'_{ML^-} = \frac{[ML^-]}{[M^{2+}][L^{3-}]}$$

$$M^{2+} + HL^{2-} \rightleftarrows MHL \qquad \beta'_{MHL} = \frac{[MHL]}{[M^{2+}][HL^{2-}]}$$

$$M^{2+} + H_2L^{-} \rightleftarrows MH_2L^{+} \qquad \beta'_{MH_2L^+} = \frac{[MH_2L^+]}{[M^{2+}][H_2L^-]}$$

The total metal concentration M_T, is given by

$$M_T = [M^{2+}] + [ML^-] + [MHL] + [MH_2L^+] + [MOH^+]$$

$$+ [M(OH)_2] + \ldots [M(OH)_n^{-(n-2)}]$$

By substituting the corresponding conditional

stability constants (β') and concentrations,

$$M_T = [M^{2+}] + \beta'_{ML}[M^{2+}][L^{3-}] + \beta'_{MHL}[M^{2+}][HL^{2-}]$$

$$+ \beta'_{MH_2L^+}[M^{2+}][H_2L^-] + \beta'_{MOH}[M^{2+}][OH^-]$$

$$+ \beta'_{M(OH)_2}[M^{2+}][OH^-]^2 + \cdots \beta'_{M(OH)_n-(n-2)}[M^{2+}][OH^-]^n$$

$[OH^-] = \frac{k_w}{[H^+]}$ where k_w is the autoprotolysis constant of water.

$$M_T = [M^{2+}] + \beta'_{ML}[M^{2+}][L^{3-}] + \beta'_{MHL}[M^{2+}][HL^{2-}]$$

$$+ \beta'_{MH_2L^+}[M^{2+}][H_2L^-] + \beta'_{MOH}[M^{2+}] \frac{k_w}{[H^+]}$$

$$+ \beta'_{M(OH)_2}[M^{2+}] \frac{k_w^2}{[H^+]^2} + \cdots \beta'_{M(OH)_n-(n-2)}[M^{2+}] \frac{k_w^n}{[H^+]^n}$$

Fraction of free metal, $\alpha_{M^{2+}} = \frac{[M^{2+}]}{M_T}$

$$\alpha_{M^{2+}} = 1/(1 + \beta'_{ML}[L^{3-}] + \beta'_{MHL}[HL^{2-}] + \beta'_{MH_2L^+}[H_2L^-]$$

$$+ \beta'_{MOH} \frac{k_w}{[H^+]} + \beta'_{M(OH)_2} \frac{k_w^2}{[H^+]^2} + \cdots \beta'_{M(OH)_n-(n-2)} \frac{k_w^n}{[H^+]^n})$$

When the total ligand concentration (C_L) is much greater

compared to the amount of ligand bound to the metal,

$$C_L = [H_3L] + [H_2L^-] + [HL^{2-}] + [L^{3-}]$$

The concentration of each ligand species can be calculated from the acid dissociation constants and the pH value of the solution [97].

$$\alpha_{L^{-3}} = \frac{[L^{3-}]}{C_L} = \frac{K_1K_2K_3}{[H^+]^3 + K_1[H^+]^2 + K_1K_2[H^+] + K_1K_2K_3}$$

where $\alpha_{L^{3-}}$ is the fraction of the ligand species L^{3-} and K_1, K_2 and K_3 refer to the acid dissociation constants of the ligand.

$$\alpha_{HL^{2-}} = \frac{[HL^{2-}]}{C_L} = \frac{K_1K_2[H^+]}{[H^+]^3 + K_1[H^+]^2 + K_1K_2[H^+] + K_1K_2K_3}$$

$$\alpha_{H_2L^-} = \frac{[H_2L^-]}{C_L} = \frac{K_1[H^+]^2}{[H^+]^3 + K_1[H^+]^2 + K_1K_2[H^+] + K_1K_2K_3}$$

The fraction of free metal can be determined by substituting the values of $[L^{3-}]$, $[HL^{2-}]$ and $[H_2L^-]$ in the expression for $\alpha_{M^{2+}}$. If more than about 5% of the ligand is bound by the metal, then a new value of C_L is calculated and the expression reiterated to constant C_L.

3.3.1.4 Preparation of test solutions and blanks

An appropriate volume of the electrolyte solution was placed in a clean 250-mL beaker. This was followed by the addition of the corresponding volume of the ligand solution (often added as the sodium or potassium form) that was being tested. The amount of sodium or potassium introduced from the ligand was also taken into consideration in calculating the volume of electrolyte solution that was required to give the final ionic strength. About 75 mL of deionized water was added to the beaker and the pH adjusted to 5.00 ± 0.05 with dilute HNO_3 (except when tartaric acid was used as the ligand where NaOH was added to adjust the pH). This was followed by the slow addition, with magnetic stirring, of an appropriate volume of the standard calcium solution to give a final total calcium concentration of 100 µg/mL in each solution (except in Section 3.3.1.4). A pH readjustment was necessary in some cases. The solution was transferred quantitatively into a 200-mL volumetric flask and diluted to the mark with deionized water. These test solutions contained an equal amount of total calcium with varying ligand concentrations.

Blanks were prepared in the same manner as described above but without the addition of the calcium solution.

3.3 Results and Discussion

This section lists and discusses the results obtained when the ligands citrate, tartrate, phosphate, and chloride are added singly and together to a solution of calcium that is equilibrated with the micro ion exchange column. The effect of citrate was studied at pH 5 and 7, using both $NaNO_3$ and KNO_3 at different concentrations as the electrolyte.

3.3.1 Effect of citrate on free calcium determination

3.3.1.1 With $NaNO_3$ as electrolyte (0.75 M) at pH 5

Four test solutions were prepared as described in Section 3.2.4. The ligand was added as sodium citrate to give final citrate concentrations of 1.0×10^{-3}, 2.0×10^{-3}, 4.0×10^{-3}, and 6.0×10^{-3} M. The amount of free calcium in each solution was determined as described in Section 3.2.2. Relative fraction of free calcium at different total citrate concentrations is shown in Figure 13.

Citric acid, $HOC(CH_2CO_2H)_2CO_2H$, forms CaL^-, CaHL and CaH_2L^+ complexes with Ca^{2+} where L^{3-} denotes the $HOC(CH_2CO_2^-)_2CO_2^-$ anion. Stability constants for the calcium-citrate complexes and protonation constants for citric acid at different ionic strengths are given in Table 3 [76]. Values of these constants at an ionic

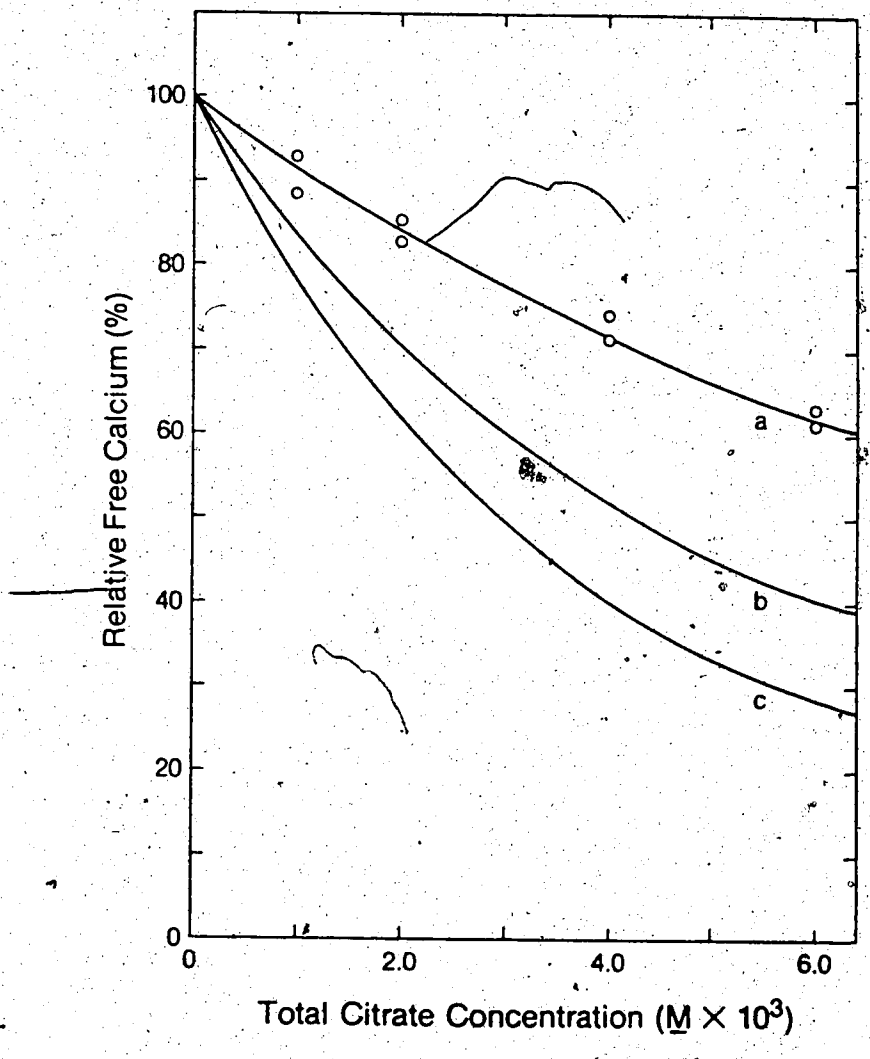


Figure 13. Variation in relative free calcium as a function of total citrate concentration. (a) At pH 5 in 0.75 M $NaNO_3$ electrolyte as measured by the ion-exchange equilibration method. (b) Theoretical curve at pH 5 using β_{CaL^-} at μ = 0.16, and β_{CaHL}, $\beta_{CaH_2L^+}$ and ionization constants (K_1, K_2, K_3) of citric acid at μ = 0.1 to calculate the fraction of free calcium. (c) As in (b), but with β_{CaL^-} at μ = 0.1.

Table 3. Stability constants for calcium-citrate complexes and protonation constants for citric acid at different values of ionic strength μ. Data from Ref. 76.

	log K (25°C, μ=0)	log K (25°C, μ=0.1)	log K (25°C, μ=1.0)
$Ca^{2+} + L^{3-} \rightleftarrows CaL^-$	4.68	3.50, 3.55,[a] 3.18[b]	-
$Ca^{2+} + HL^{2-} \rightleftarrows CaHL$	3.09	2.10[a]	-
$Ca^{2+} + H_2L^- \rightleftarrows CaH_2L^+$	1.10	1.05[a]	-
$H^+ + L^{3-} \rightleftarrows HL^{2-}$	6.40	5.69	5.33
$H^+ + HL^{2-} \rightleftarrows H_2L^-$	4.76	4.35	4.08
$H^+ + H_2L^- \rightleftarrows H_3L$	3.13	2.87	2.80

[a] 20°C, μ=0.1

[b] 25°C, μ=0.16

strength of 0.75 are not available in the literature. Therefore it is not possible to compare the experimental results directly with calculated values for free calcium at this ionic strength. However, the experimental curve (a) shows the expected trend, the fraction of free calcium decreasing with an increase in citrate concentration.

Curves b and c in Figure 13 were derived from calculations employing the stability constants and ionization constants under the given set of conditions. In curve b, the stability constant for the CaL^- species at μ=0.16 was used whereas all the other constants were at at μ=0.1. All constants were at μ = 0.1 in curves b and c. These curves illustrate that the fraction of free calcium is raised with an increase in ionic strength due to the lowering of the stability constants. However the extent of such an increase in free calcium cannot be estimated without knowledge of the values of the related stability constants and ionization constants at that ionic strength.

3.3.1.2 With $NaNO_3$ as electrolyte (0.75 M) at pH 7

Test solutions were prepared as outlined in Section 3.2.4 but the pH was adjusted to 7.00 ± 0.05 with dilute NaOH. The ligand was introduced as sodium citrate to give a final concentration of 1.0×10^{-3} M, 2.0×10^{-3} M, 4.0×10^{-3} M, 6.0×10^{-3} M, and 8.0×10^{-3} M in the

solutions. A solution of HEPES buffer was added prior to the addition of NaOH in order to keep the pH stable at 7. The buffer concentration in each solution was 10^{-3} M. Free calcium in each solution was determined as described in Section 3.2.2. The variation of the relative fraction of free calcium with total citrate concentration at pH 7 is shown in Figure 14.

Examination of the experimental curves in Figures 13 and 14 indicates that the fraction of free calcium measured in the solution at a given citrate concentration is approximately the same at pH 5 and pH 7, even though the calculated curves indicate that it should be lower at pH 7. This unexpected result could not be explained; further work in this area was not attempted owing to lack of time. A comparison of the corresponding values is also given in Table 4.

3.3.1.3 With KNO_3 as electrolyte (0.5 M) at pH 5

Solutions were prepared as described in Section 3.2.4 with KNO_3 as the electrolyte. The final citrate concentrations in the solutions were adjusted to 1.0×10^{-3} M, 2.0×10^{-3} M, 4.0×10^{-3} M, 6.0×10^{-3} M and 8.0×10^{-3} M by adding potassium citrate as the ligand. The amount of free calcium in each solution was determined as outlined in Section 3.2.2. Figure 15 illustrates the variation of the fraction of free calcium with total

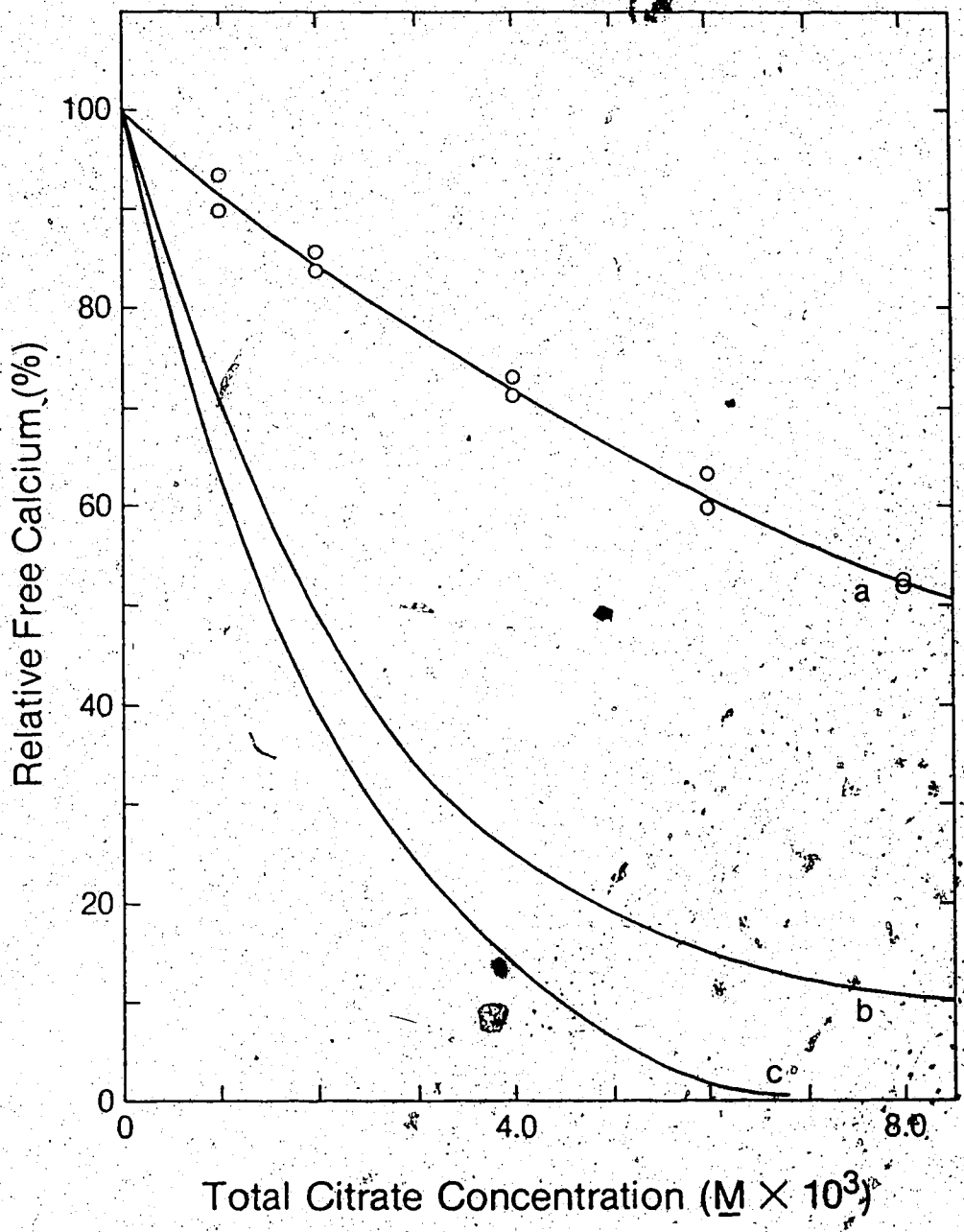


Figure 14. Variation in relative free calcium with total citrate concentration at pH 7 in 0.75 M $NaNO_3$ electrolyte. (a) experimental; (b) calculated using β_{CaL^-} at $\mu = 0.16$, all other constants at $\mu = 0.1$; (c) calculated using all constants at $\mu = 0.1$.

Table 4. Comparison of the fraction of free calcium, as determined experimentally, at pH 5 and 7 with citrate as the ligand, in 0.75 M $NaNO_3$. (Two runs made at each pH and citrate concentration.)

Citrate concentration (M)	Relative fraction of free calcium (%) pH 5	pH 7
1.0×10^{-3}	88, 93	90, 93
2.0×10^{-3}	83, 85	84, 86
4.0×10^{-3}	71, 74	71, 73
6.0×10^{-3}	61, 63	60, 63

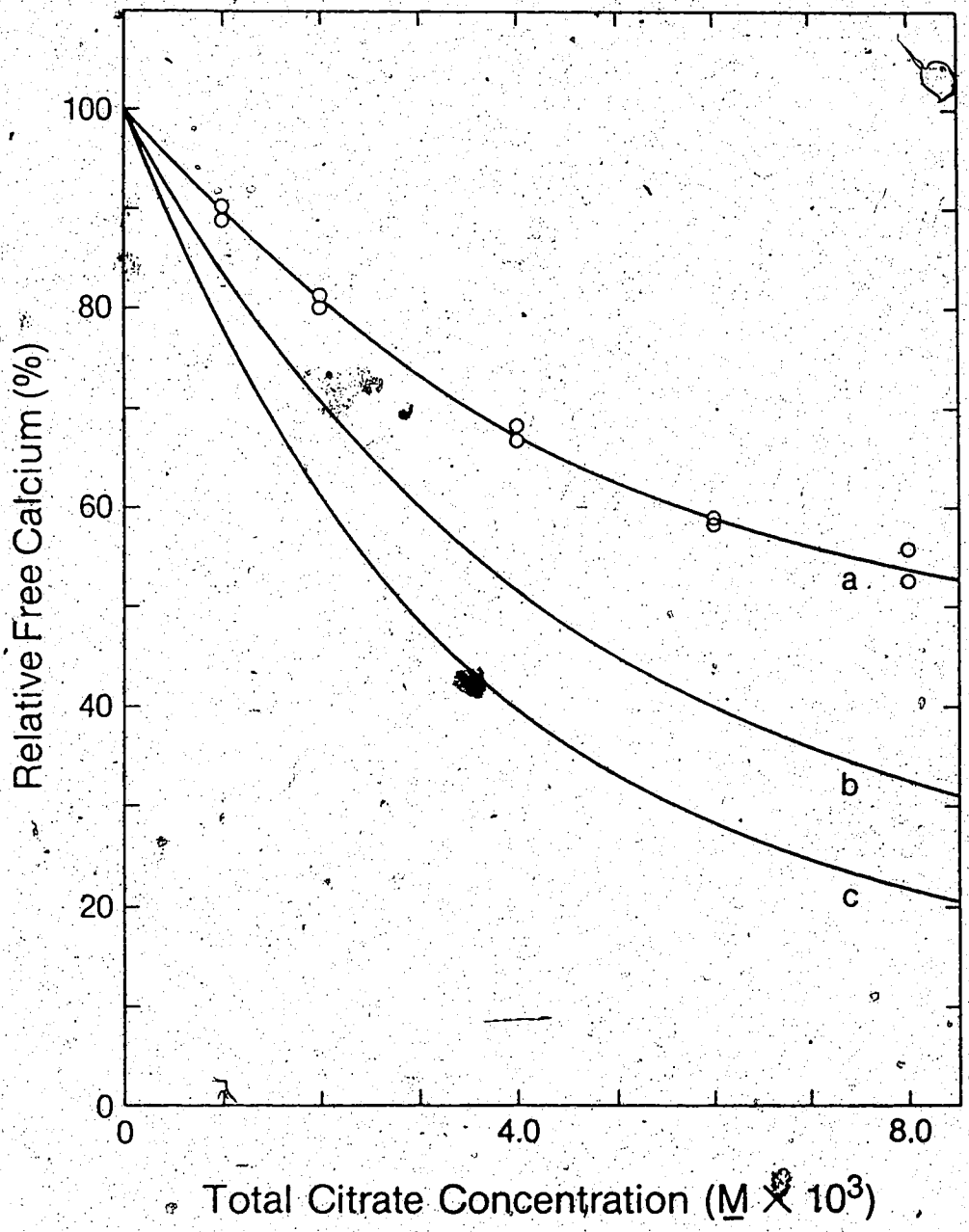


Figure 15. Variation in relative free calcium with total citrate concentration at pH 5 in 0.5 M KNO_3 electrolyte. (a) experimental; (b) and (c) as in Figure 13.

citrate concentration when KNO_3 (0.5 M) is used as the electrolyte, at pH 5. The trend of the curve is similar to that obtained with 0.75 M $NaNO_3$ at pH 5 and 7 although there is a slight reduction in the amount of free calcium at the given citrate concentrations. This could possibly be due to the higher stability constant values for the calcium-citrate complexes at a lower ionic strength which decreases the amount of free metal ion.

3.3.1.4 With $NaNO_3$ as electrolyte (0.1 M) at pH 5

The possibility of measuring free calcium in diluted solutions in order to avoid high ionic strengths is examined in this section. Test solutions were prepared by a method similar to that described in Section 3.2.4 except that each solution contained 10 µg/mL of total calcium and the electrolyte ($NaNO_3$) concentration was adjusted to 0.1 M. The ligand was added as sodium citrate and its concentrations in the solutions were 1.0×10^{-4} M, 2.0×10^{-4} M, 4.0×10^{-4} M, 6.0×10^{-4} M, and 8.0×10^{-4} M. Each solution was equilibrated with the column for a period of 12 minutes and free calcium determined by the method described in Section 2.2.7. The calibration plot was made by measuring a series of standard solutions with calcium concentrations ranging from 2.5 µg/mL to 12.5 µg/mL.

The time period required for complete breakthrough to be achieved was determined by studying a column equilibration curve with a 1.0 μg/mL calcium solution in 0.1 M $NaNO_3$ at pH 5. The equilibration curve obtained was similar to that obtained with 0.1 M NaCl as electrolyte at pH 5 (Figure 4) where a time period of 12 minutes was adequate to attain complete breakthrough conditions.

Variation of the fraction of free calcium with total citrate concentration is shown in Figure 16. Comparison of these results with those discussed in Sections 3.3.1.1 to 3.3.1.3 indicates that the fraction of free calcium is raised with the decrease in ligand concentration.

3.3.2 Effect of tartrate on free calcium determination

Test solutions were prepared as described in Section 3.2.4 with tartaric acid concentrations of 0.5×10^{-2} M, 1.0×10^{-2} M, 2.0×10^{-2} M, and 4.0×10^{-2} M. The pH of the solutions was adjusted to 5.00 ± 0.05 with dilute NaOH. The amount of sodium introduced by the addition of NaOH was taken into consideration in the adjustment of the electrolyte ($NaNO_3$) concentration to 0.75 M. Free calcium of each solution was determined by the method described in Section 3.2.2.

The change in the fraction of free calcium with total tartrate concentration is illustrated in Figure 17.

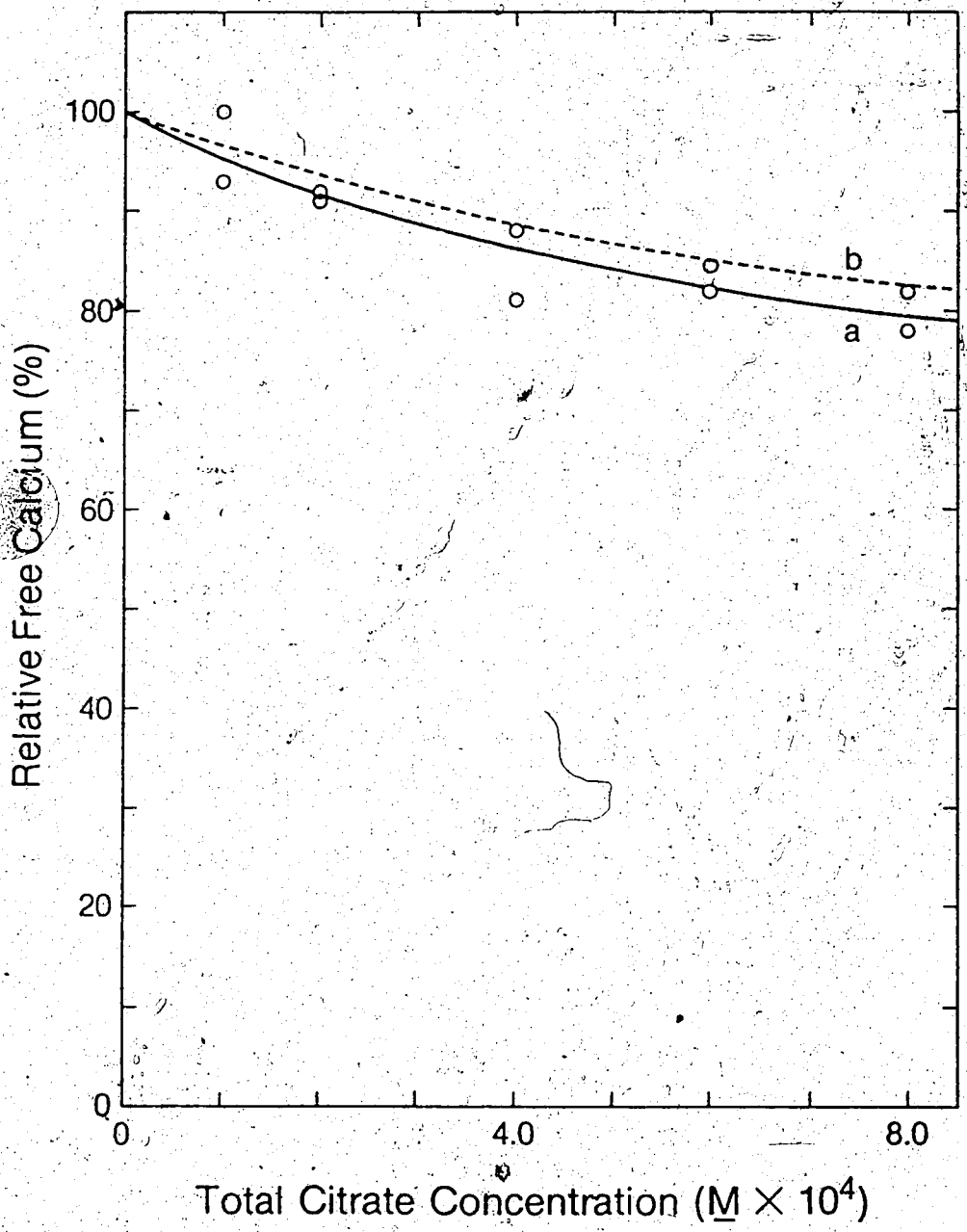


Figure 16. Variation in relative free calcium with total citrate concentration at pH 5 in 0.1 M $NaNO_3$ electrolyte.

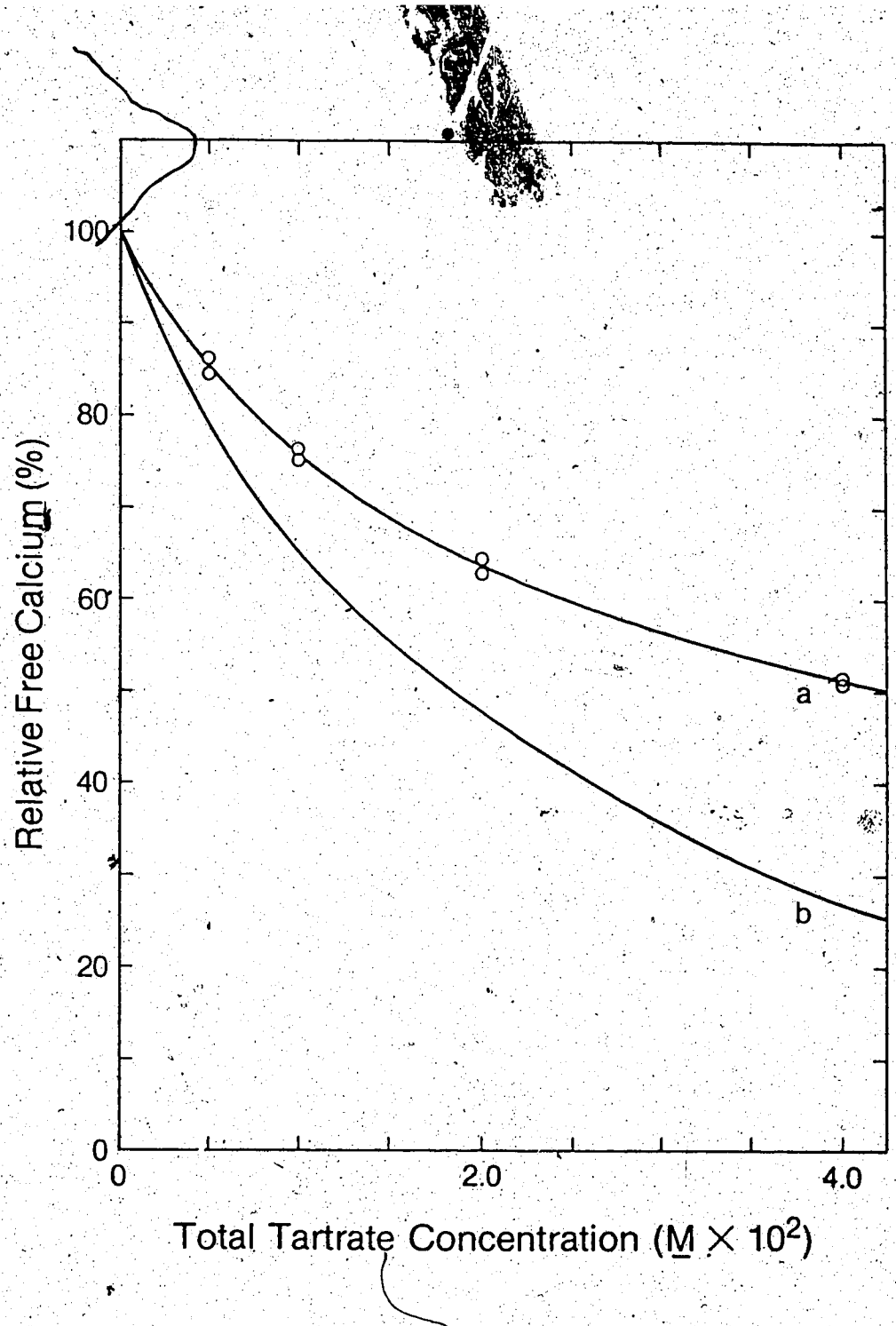


Figure 17. Variation in relative free calcium with total tartrate concentration at pH 5 in 0.75 M $NaNO_3$ electrolyte.

Tartaric acid, $HO_2CCH(OH)CH(OH)CO_2H$, forms CaL and $CaHL^+$ complexes with calcium where L^{2-} denotes the $^-O_2CCH(OH)CH(OH)CO_2^-$ anion. Log β (stability constant) values for CaL and $CaHL^+$ are given as 1.80 and 1.11 at an ionic strength of 0.2 [76]. CaL has a log β value of 2.80 at a zero ionic strength [76].

3.3.3 Effect of phosphate on free calcium determination

Calcium forms the complexes $CaPO_4^-$, $CaHPO_4$ and $CaH_2PO_4^+$ with phosphate. The log β values for the above complexes at 25°C and at zero ionic strength are given as 6.46, 2.74 and 1.4 to 0.8, respectively [77]. Another set of values for log β of $CaHPO_4$ and $CaH_2PO_4^+$ under the same conditions are given as 2.41 and 0.6, respectively [78,79]. The $CaHPO_4$ complex has a log β value of 1.50 at 25°C and an ionic strength of 0.2 [14].

Test solutions were prepared as described in Section 3.2.4, introducing the ligand as Na_2HPO_4. The ligand concentrations in the solutions were 0.07 M, 0.14 M, 0.21 M and 0.28 M. The amount of free calcium in each solution was determined as described in Section 3.2.2. The change in the fraction of free calcium with total phosphate concentration is represented in Figure 18. A comparison of the calcium-phosphate system with that of the calcium-

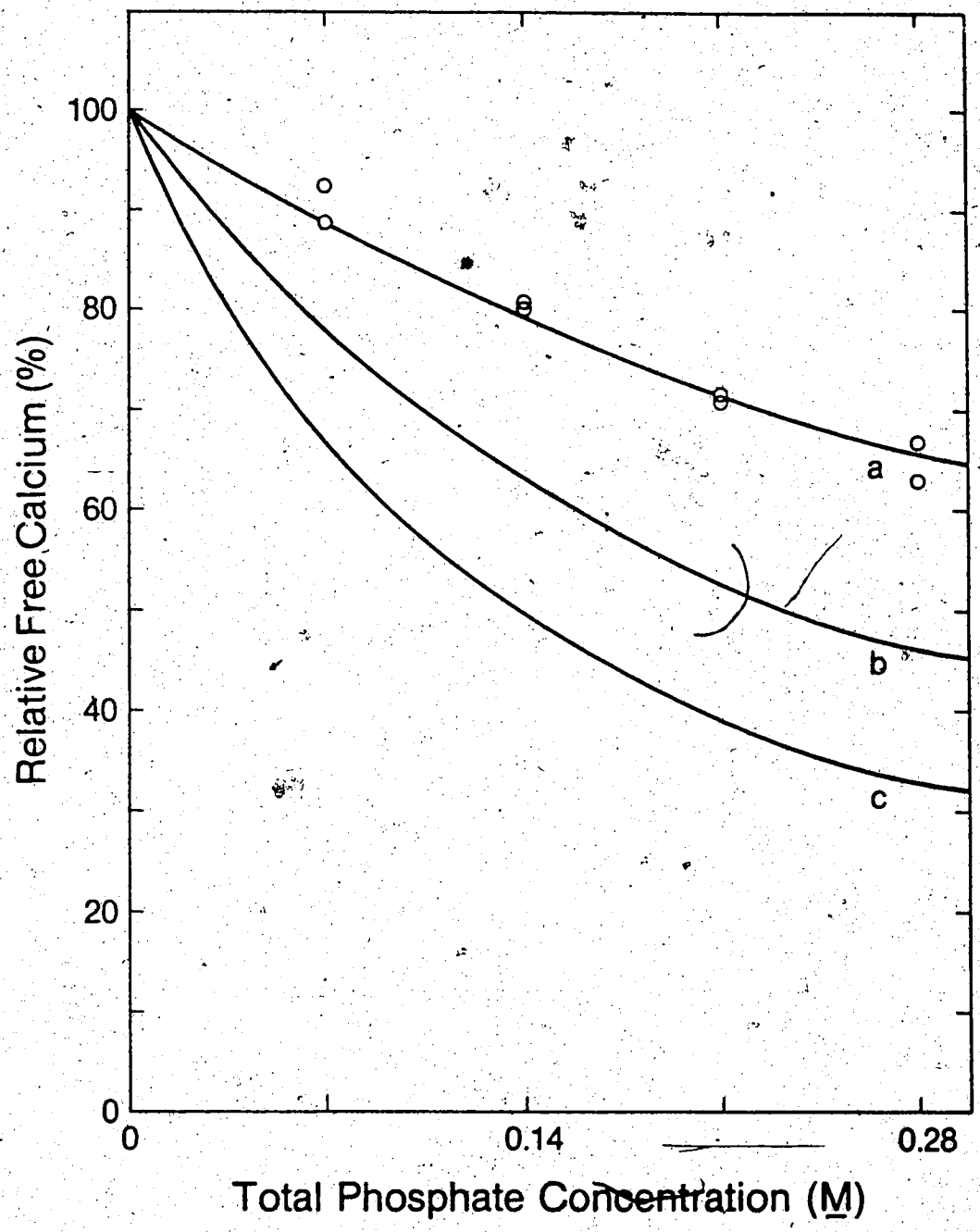


Figure 18. Variation in relative free calcium with total phosphate concentration at pH 5 in 0.75 M $NaNO_3$ electrolyte.

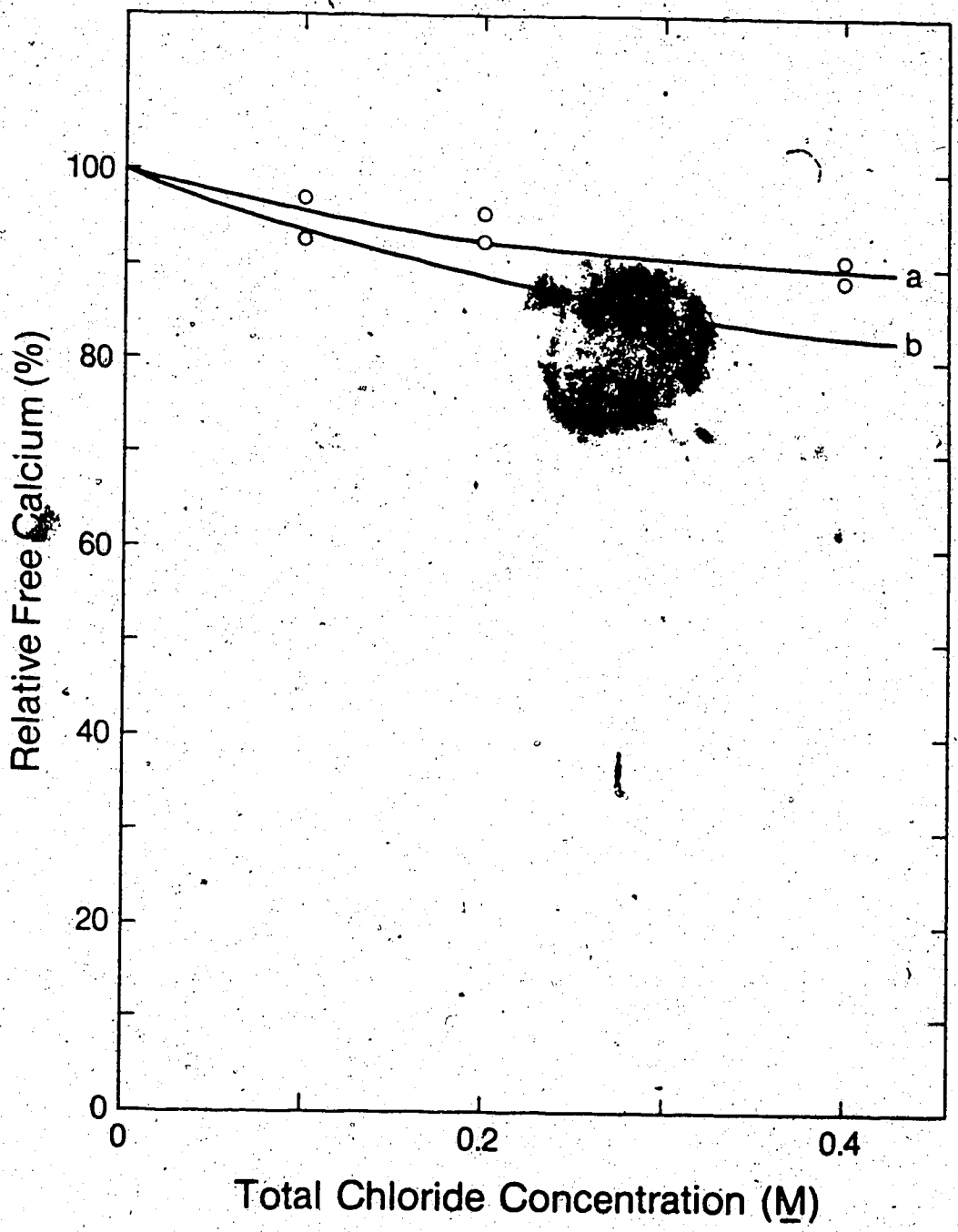
higher concentration of phosphate is required to reduce the free metal ion concentration by a given amount.

3.3.4 Effect of chloride on free calcium determination

Calcium forms $CaCl^+$ with chloride ion which has a stability constant value of 0.6 at an ionic strength of 1.0 M [78]. Test solutions, prepared by the method given in Section 3.2.4, contained 0.1 M, 0.2 M and 0.4 M chloride ion concentrations. The ligand was added as NaCl. The amount of free calcium in the solutions was determined as described in Section 3.2.2. The variation of the fraction of free calcium with total chloride concentration is represented in Figure 19. The results show that the reduction in free calcium concentration by chloride ion takes place only to a slight extent, particularly when compared with the other ligands that were studied. A chloride ion concentration of 0.4 M decreases the free calcium amount only by about 10%.

3.3.5 Effect of a mixture of citrate, phosphate and chloride on free calcium determination

A set of solutions containing citrate, phosphate and chloride were prepared in order to examine the effect of a mixture of ligands on the amount of free calcium. Each solution contained 100 µg/mL of total calcium and was


100
80
60
40
20
0
0
0.2
0.4
a
b
Relative Free Calcium (%)
Total Chloride Concentration (M)

3.2.4. The ligand concentrations were adjusted to 2.0×10^{-3} M citrate, 2.0×10^{-2} M phosphate and 0.1 M chloride in one of the solutions and 3.5×10^{-3} M citrate, 3.0×10^{-2} M phosphate and 0.2 M chloride in the other solution. The above values were selected so that they fall within the normal levels of these ligands present in urine [70]. The ligands were added as sodium citrate, disodium hydrogen phosphate and sodium chloride. The electrolyte concentration in each solution was adjusted to 0.75 M with $NaNO_3$, taking into consideration the amount of sodium introduced by the ligands. The amount of free calcium in each solution was determined as described in Section 3.2.2.

The percentage of free calcium in the solution with lower concentrations of ligands was 77 while that in the other solution was 67. As expected, an increase in the concentration of the ligands has decreased the amount of free calcium in the solution.

3.4 Conclusions

Among the calcium-ligand systems investigated, citrate appears to be the most important ligand with regard to binding with calcium and lowering the amount of free calcium available in the solutions. Citrate reduces

the free calcium content to a greater extent than the other ligands examined at a given concentration. For example, with a total calcium concentration of 2.5×10^{-3} M, a citrate concentration of 5×10^{-3} M reduces the fraction of free calcium to about 66% while the same concentration of tartrate and phosphate lowers the fraction only to about 85% and 99%, respectively. The decrease in the amount of free calcium with a chloride concentration of 5×10^{-3} M is negligible.

Results obtained with the ligands examined were in agreement with the expected trend, the free calcium level decreasing with increasing concentration of ligand. However, it was not possible to compare the experimentally observed values for free calcium with any predicted values as the stability constants of the calcium-ligand complexes and ionization constants of ligands are not known at the ionic strengths considered.

CHAPTER 4

DETERMINATION OF IONIC CALCIUM IN URINE

4.1 Introduction

The importance of measuring ionized calcium levels in urine, with regard to urinary stone disease, was outlined in Section 1.1. Several techniques involving spectrophotometry using murexide [80-82] or tetramethylmurexide [83,84] and ion selective electrodes [83,85,86] have been employed to measure free calcium levels in urine. However, as discussed in Section 1.2, these methods are influenced by differences in sodium concentration and ionic strength so that there are difficulties in matching standards to samples. When murexide is used as the ionized calcium sensitive dye, variations in urinary pH must also be taken into account. The ion exchange column equilibration method was used in the present study to measure free calcium concentrations in urine samples.

The measurement of the total calcium concentration in urine is also required in order to determine the fraction of the unbound metal. Total calcium in serum and urine has been determined by atomic absorption [87-89] and by spectrophotometry with o-cresolphthalein complexone

[90-92]. Titrimetric analysis with ethylenediaminetetra-acetic acid (EDTA) as the titrant and calcein as the indicator has also been used for measuring total calcium in serum and urine [93-96]. The total calcium measurements discussed in this chapter were based on the microtitrimetric method with EDTA [93].

4.2 Determination of Total Calcium in Urine by Microtitration

4.2.1 Introduction

Calcium forms a stable complex with EDTA in alkaline solution that can be used as the basis for its titrimetric determination. The method can be made specific by choosing optimum pH conditions and by use of masking agents. It has been reported that direct manual titration without preliminary treatment is unsatisfactory for the determination of total calcium in urine [94-96]. This analysis has been improved by the development of a simple and rapid microtitrimetric method with EDTA and calcein as the fluorescent indicator [93]. Interference from phosphate and natural fluorescence is minimized by the use of small sample volumes. The low concentrations of transition metals present in urine are masked by reagents such as potassium cyanide. Interference from magnesium is

avoided by titration at a pH of 12 or greater, where insoluble magnesium hydroxide is formed.

The indicator calcein is fluorescent in the pH range of 3 to 10. Above pH 10, calcein does not fluoresce whereas the calcein-calcium complex does. This fluorescence is quenched by the addition of EDTA, which forms a stronger complex with calcium than does calcein. Since sodium also causes some fluorescence at high pH, potassium hydroxide is used rather than sodium hydroxide to adjust the pH of solutions of calcium prior to titration [98]. The procedure is carried out by a weight titration technique which is suitable for the analysis since small volumes of the titrant are used.

4.2.2 Experimental

4.2.2.1 Chemicals and solutions

The chemicals used were: Disodium ethylenediaminetetraacetate, $Na_2H_2EDTA \cdot 2H_2O$ (Fisher Scientific Co.); calcium carbonate, $CaCO_3$ (J.T. Baker Chemical Co.); potassium hydroxide, KOH (J.T. Baker Chemical Co.); potassium cyanide, KCN (British Drug House); potassium dichromate, $K_2Cr_2O_7$ (British Drug House); and calcein, $C_{30}H_{26}N_2O_{13}$ (Fisher Scientific Co.).

Deionized water was used in preparing all solutions. Standard EDTA solution (0.005 M) was prepared

by dissolving 1.861 g of the chemical in water and diluting to 1 litre. A 5-mL portion of this stock solution was diluted to 100 mL with water to give a 0.00025 M [illegible] solution. Standard calcium solution (1.0 mg/mL) was prepared by dissolving 2.497 g of calcium carbonate in 60 mL of 1 M hydrochloric acid and diluting to 1 litre with water. 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 was prepared by dissolving approximately 20 mg of the indicator in 25 mL of water and the minimum amount of 0.1 M potassium hydroxide and then diluting to 1 litre with water. This solution was kept frozen in order to prevent deterioration. Potassium hydroxide (1 M)-potassium cyanide (0.15 M) solution was prepared by dissolving approximately 5.6 g of potassium hydroxide pellets and 1 g of potassium cyanide in 100 mL of water. Potassium dichromate solution (0.1%) was prepared by dissolving 0.1 g of the chemical in 100 mL of water.

4.2.2.2. Apparatus

A syringe of 5-mL capacity fitted with a 26-gauge stainless-steel needle was used as the buret to add small volumes of the titrant during the weight titrations [99]. The plunger was lightly lubricated with silicone grease to prevent loss of solution and to provide positive

plunger action. A support for the syringe during weighing on a top-loading balance was made from a 250-mL polyethylene bottle, as described in Ref. 100. All titration end points were observed under ultraviolet radiation from a Chromatovue light enclosure (Ultraviolet Light Products Inc.).

4.2.2.3 Procedure

A 2.5-mL portion of the urine sample to be analyzed was diluted to 50 mL with water and mixed. To a 10-mL beaker were added 1 mL of the potassium hydroxide-potassium cyanide solution, 1 mL of water, a 0.125-mL aliquot of the diluted urine sample, 4 drops of potassium dichromate solution (0.1%) 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, filled with the EDTA working solution, was weighed on a top-loading balance to the nearest mg. The EDTA solution was then added dropwise from the syringe to the titration vessel until the bright green fluorescence disappeared. The syringe was reweighed and the weight of titrant used was found by difference. Titrations with the working calcium solution (10 μg/mL) were carried out to standardize the EDTA solution. Blank titrations were carried out with the omission of the calcium solution.

4.3 Determination of Free Calcium in Urine by the Ion Exchange Column Equilibration/Atomic Absorption Spectrophotometry Method

Urine samples, collected from three individuals, were used for the determination of total and free calcium concentrations. The total sodium and potassium levels of each sample were measured by Norine Motkosky using flame emission spectroscopy. A Varian Spectra AA-10 atomic absorption spectrophotometer was used for the measurements and the procedures given in Ref. 101 were followed for this analysis. Urine samples were diluted 400-fold for both the sodium and potassium determinations. The samples were refrigerated upon collection and were analyzed within one to two days. In some cases second analyses were performed one to two weeks later on the same samples.

On the basis of the sodium and potassium analyses, sufficient solid sodium nitrate was added to each urine sample to bring the total concentration of sodium and potassium to 0.75 M. Each sample was equilibrated with the column of resin for a period of 5 minutes and peak areas measured by the method described in Section 2.2.7. The free calcium levels were determined by referring to a calibration plot made with standard calcium solutions in 0.75 M $NaNO_3$ (Section 2.3.3.1).

4.4 Results and Discussion

Five urine samples (A1, A2, B1, C1, and C2) were analyzed for total and free calcium by the methods described in Sections 4.2 and 4.3. The symbols A, B, and C were used to denote samples collected from different persons and the numbers 1 and 2 were used to denote samples collected from the same person but on two different days. The total calcium and free calcium levels in the samples are given in Table 5. The total calcium values of 84 and 312 were near the lower and upper normal limits for a healthy population.

The free calcium levels in samples C1 and C2 were too high to be determined from the calibration plots, which were useful up to about 150 μg/mL calcium. The free metal ion concentrations in C1 and C2 can be estimated to be 225 ± 10 and 186 ± 5 μg/mL of calcium. This gives an approximate fraction for the free metal ion of 72% and 76% in these samples.

Samples A1 and A2, which were collected from the same person but on different days, gave almost the same fraction of free calcium even though they contained varying amounts of total and free calcium. The fraction of free calcium in sample B1 was high even though the total calcium concentration was low.

Table 5. Total calcium and free calcium levels in urine samples. Total calcium was determined by microtitration with EDTA [93] and free calcium was determined by the ion exchange column equilibration method. Sodium and potassium were determined by atomic absorption spectroscopy. The letter n refers to the number of replicates on a given solution.

Sample	Total sodium concentration (M)	Total potassium concentration (M)	Total calcium concentration (μg/mL)	Free calcium concentration (μg/mL)	Fraction of free calcium (%)
A1	0.157	0.061	112 ± 5 (n=3)	63 ± 1 (n=4)	56
A2	0.090	0.078	163 ± 4 (n=3)	92 ± 1 (n=3)	56
B1	0.036	0.015	84 ± 4 (n=4)	78 ± 1 (n=3)	93
C1	0.151	0.071	312 ± 6 (n=4)	-	-
C2	0.114	0.032	244 ± 4 (n=4)	-	-

Sodium and potassium are usually present in relatively high concentrations in urine [70]. Therefore an estimate of these ion concentrations in a urine sample is required prior to the measurement of free calcium in order to bring the electrolyte concentration of the sample to that of the standards used to prepare the calibration curve. The electrolyte concentration in the sample was closely adjusted to that of the standard by adding an appropriate amount of solid sodium nitrate.

As observed with samples C1 and C2, high concentrations of free calcium could not be measured using the above procedure. It may be possible to measure such high levels of the free metal ion by raising the electrolyte concentration of standards (Section 2.3.3) and samples above 0.75 M. However, the changes in activity coefficients of the species present in the medium with ionic strength also need to be considered before raising the electrolyte concentration any further.

An alternative would be to dilute the urine sample to lower the free calcium level to a measurable value. This would also alter all equilibria between calcium and ligands present in solution since it would lower all species concentrations, leading to greater dissociation of the calcium complexes.

The urine samples were not filtered before passing them through the column of resin. A possibly high value for free calcium could result if calcium containing particulates were trapped in the resin phase and not removed during the wash cycle. These particles would dissolve in the HNO_3 eluent and be detected in the elution cycle. This can be a problem for samples that are stored for any length of time, since crystallization or precipitation can occur.

4.5 Conclusions

The results of the preliminary investigations reported here show that the ion exchange column equilibration method (IEX) can be used to determine the concentration of free calcium in urine. As outlined in Section 1.3.1, the IEX method requires the addition of a swamping electrolyte in order to allow only a small fraction of exchange sites on the resin to be occupied by the metal to be determined. It is recognized that the addition of a high concentration of electrolyte may perturb the equilibrium in the system to some extent. It was shown in Section 2.3.3 that an electrolyte ($NaNO_3$) concentration of about 0.75 M is required in order to measure free calcium levels comparable to those present in normal urine using this method. However, the

determination of free calcium concentrations in urine samples with relatively high levels of the free metal ion necessitates raising the electrolyte concentration further in the sample and the standards. An alternative method for the analysis of such samples could be to dilute them and then measure the free metal ion concentration in the diluted sample. But here again, dilution will perturb the equilibrium in the system and raise the free calcium level unless calcium ion buffering by ligands in the solution is very large.

CHAPTER 5

FUTURE WORK

An investigation of the possibility of carrying out the measurements at lower ionic strengths is recommended. This would minimize changes in the activity coefficients due to ionic strength effects. Such a study would require that a swamping electrolyte, the cation of which is held more strongly onto the resin, be added to the solutions. Examples of such electrolytes would be [illegible] and quaternary ammonium salts. The expense of using such salts in routine analysis might need consideration.

Further study of the non zero intercept observed for calcium calibration curves at concentrations of swamping electrolyte above about 0.5 M should be done. Among the variables that could be studied are nature of the ion being determined, nature of the swamping electrolyte cation and counterion, and the effect of metal ion buffering. The cross-linking of the resin is another variable that might be altered to see whether it affects the shift in intercept.

It would be useful to determine stability constants at high ionic strength for one or more of the calcium-ligand systems studied in this work. Knowledge of these constants would allow direct comparison of experimental and calculated values for free calcium concentrations, and thereby permit a definitive test of the validity of the method.

Free calcium measurements carried out in the presence of citrate ligand in 0.75 M $NaNO_3$ gave experimentally similar values at pH 5 and 7. However, calculated curves at these pH values differed (see Figures 13 and 14), free calcium levels at pH 5 being higher than those at pH 7 for a given citrate concentration. The factors relating to this difference in trends between the experimentally and theoretically observed results should be examined.

The preliminary studies discussed in this work suggest that the ion exchange column equilibration method can be applied to measure free calcium in urine. The importance of free calcium levels, not only in urine but also in other biological fluids, was outlined in the introductory chapter. The possibility of applying the method to measure free calcium levels in serum and other biological fluids would be a study of importance. Considering the normal levels of calcium in biological fluids, a reasonably low volume of the sample is required.

to attain equilibrium with the column of resin, which makes such a study possible. The method would not have been feasible if a large volume of biological fluid, such as serum, was required for the analysis.

Only a few of the constituents present in urine were tested for their effect on the determination of free calcium. These included magnesium and urea. It would be useful to examine whether the presence of albumin has an effect on the measurements. Also, possible interferences on the measured values from calcium-containing particulates in urine should be investigated. This requires that the urine samples be filtered to remove such particulates before passage through the column of resin.

The ion exchange column equilibration method can also be applied to determine free magnesium concentrations in urine. Magnesium is also an important element in biological systems and determination of its free metal ion levels would be clinically useful. It could be possible to measure the ionic levels of both calcium and magnesium in urine and other biological fluids by coupling the ion exchange flow system to an inductively coupled plasma atomic emission spectrophotometric detector. These measurements may also be carried out using neutron activation analysis. Such techniques would provide procedures for the rapid determination of multielement free ion concentrations in biological fluids.

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