ACTA ORTHOPAEDICA SCANDINAVICA
SUPPLEMENTUM NO. 78

From the Orthopaedic Research Laboratories (Head: Göran C. H. Bauer M. D.)
of the Orthopaedic Clinic (Head: Sophus von Rosen M. D.)
Malmö General Hospital, University of Lund, Malmö, Sweden, and
the Hospital for Special Surgery affiliated with the New York Hospital —
Cornell University Medical College, New York City

Stable Calcium Isotopes as Tracers in Studies of Mineral Metabolism

BY G. DUNCAN MCPHERSON

MUNKSGAARD COPENHAGEN 1965

PRINTED IN SWEDEN BY
BERLINGSKA BOKTRYCKERIET
LUND 1965

CONTENTS

General Introduction

The use of stable isotopic tracers in the study of biological systems has several advantages. The system studied is not subjected to any radiation hazard. Stable isotopes do not themselves decay and their distribution in the system studied, and its changes with time may be followed by measurement of the abundance of the isotope in samples which are representative of the system.

The availability of enriched stable calcium isotopes suggested their application as tracers of calcium kinetics. Measurement of abundance of ^{46}Ca and ^{48}Ca is dependent upon precise determination of:

1. the isotopic species, by either mass spectrometry or neutron activation analysis, and
2. the total amount of natural calcium in the sample studied.

This communication describes an investigation of the methods for the use of stable calcium isotopes and the application of this non-radioactive tracer technique to the determination of the exchangeable calcium pool in children.

CHAPTER I

Direct EDTA Titration of Calcium and Magnesium in Biological Samples

I. INTRODUCTION

The refinement of isotope tracer techniques used in the study of calcium metabolism has focussed attention on the need for precise and reliable methods for determination of total calcium in a variety of biological samples. The large numbers of samples which may be accumulated in the course of tracer kinetic studies require as well that such methods be both simple and rapid to avoid overloading laboratory facilities.

Efforts to evaluate the significance of reported differences in specific activity of calcium isotopes between serum and urine led to a study of methods for determination of urine calcium (Medical Uses of ^{47}Ca, Vienna 1962). Dissatisfaction with procedures existing at that time prompted this investigation of a precise method for determination of total calcium which could be applied to urine and solutions of bone, tissue, and feces ash as well as to serum.

The method had to be —

1. accurate over a wide range of concentrations;
2. as precise when applied to samples as to standards;
3. require little or no sample preparation;
4. be applicable to biological samples of varying electrolyte composition without interference from other substances;
5. be free from the subjective influence of the operator.

A. **Determination of Total Calcium**

1. Oxalate Precipitation

Methods involving the quantitative precipitation of calcium (Kramer and Tisdall, 1921; Clark and Collip, 1925; Fiske and Logan, 1931) are subject to a system of errors which have been demonstrated by MacIntyre (1957) using ^{45}Ca tracer techniques.

2. Flame Photometry

Flame photometric methods have proven useful in the routine clinical laboratory estimation of serum calcium but are subject to interference from both sodium and phosphate when used to determine calcium in urine or other solutions of varying electrolyte composition. Most flame photometry procedures involve a prior removal of protein (MacIntyre, 1957) or precipitation of calcium as oxalate, thus in some instances introducing the errors of the oxalate precipitation as well (Toribara *et al.*, 1957; Woollen and Walker, 1959; Brandstein *et al.*, 1963; Loken *et al.*, 1963).

3. Atomic Absorption Spectrophotometry

This new technique has been applied to serum by Zettner and Seligson (1964) and to urine and serum by Sprague (1964) with a high degree of precision. The standard curve is non-linear however, necessitating a series of standards. Deproteinization of serum, and dilution of urine in lanthanum solution are required.

4. Colorimetry and Spectrophotometry

The introduction of metal-indicating dyes and the later application of complexing or chelating agents to the titration of metal ions by Schwarzenbach *et al.* (1946) have revolutionized this field of analytical chemistry. Several reviews summarize progress in the application of these agents (Barnard *et al.*, 1957; Sadek and Reilley, 1959; Reilley and Schmid, 1959; Diehl and Ellingboe, 1960).

Metallochromic indicators have been used in a variety of colorimetric and spectrophometric methods for the direct determination of calcium and calcium plus magnesium (Young *et al.*, 1955; Yanigasawa, 1955; Kingsley and Robnett, 1957; Harper, 1959; Kellerman and Dale, 1960; Brush, 1961). These methods demand strict control of procedure, and usually require a complex series of reference standards to construct a standard absorbance or concentration curve each time the method is used. Colorimetric calcium estimation has also been adapted to the Auto-Analyzer (Boonstra and Jackson, 1962). These methods have not been as widely used for total ion estimation, as they have for the determination of free metal ion concentration in serum (Rose, 1957) and urine (Nordin, 1960). The estimation of total calcium in feces or tissue ash solutions is not feasible because of interference from other electrolytes in samples of such variable composition (Raaflaub, 1956; Rose, 1957).

5. Chelometric Titration of Calcium

The most widely used methods for estimation of total calcium and total calcium plus magnesium employ metal-indicating dyes [murexide, Eriochrome Blue SE, Eriochrome Black T. and many others—see Barnard *et al.* (1957)] as indicators in complexometric titrations. The conditions of such titrations as well as the means for end-point detection have been reviewed by Barnard *et al.* (1957).

The EDTA titration is of potentially great precision provided that the end-point can be accurately interpreted. Titrations dependent upon visual end-points (Greenblatt and Hartman, 1951; Golbyn *et al.*, 1957; Hildebrand and Reilley, 1957; Bachra *et al.*, 1958; Baron and Bell, 1959; Mori, 1959; Lewis and Melnick, 1960; McAllister and Yarbo, 1960; Pappenhagen and Jackson, 1960; Fingerhut and Miller, 1963), and potentially more precise but tedious photometric titrations (Kilbrick *et al.*, 1952; Fales, 1953; Lehmann, 1953; Buckner and Shively, 1955; Eldjarn *et al.*, 1955; Horner, 1955; Van Schouwenberg, 1960) have been followed by other methods of end-point detection. Bett and Fraser (1959) observed rate of change of galvanometer deflection, as did Foss and Andersen (1958). Malmstadt and Hadjioannou (1963) developed an electrical dead-stop titration. In these procedures there is a subjective factor in deciding when the end-point has been reached. This subjective influence is apparent not only in visual titrations and in the graphing of photometric titrations curves, but also in the procedures involving estimation of rate of change of galvanometer deflection at the end-point. The electrical automatic dead-stop titration of Malmstadt and Hadjioannou (1963) may have an over-run (Fales, 1964).

Objective, precise means of detecting the end-point have been described, using a recorder coupled to a photometer or spectrophotometer (Copp, 1963; Jones and McGuckin, 1964). Constant stirring of the titration cell as titrant is added from a constant-rate buret gives a smooth curve of changing transmittance or absorbance on the recorder chart showing a sharp break at the end-point. The precision of such recorded titrations has been demonstrated by Malmstadt and Gohrbandt (1954). Impurities which may be present, if they do not react with the titrant, cause only a shift along the absorbance axis, and do not affect the end-point. If a spectrophotometer is used, there is no need to have a large change in absorbance. Copp (1963) has described a recorded photometric EDTA titration applied to the precise determination of calcium in serum, and in other biological materials after prior removal of phosphate.

B. Determination of Total Magnesium

Interest in methods for determination of magnesium in biological materials has paralleled that of calcium because of the possible relation of these two cations in biological systems.

1. Colorimetry

The classical method for serum magnesium estimation has been the colorimetric procedure using the dye Titan Yellow (Garner, 1946; Heagy, 1948). The method has been extended to estimation of magnesium in urine by Orange and Rhein (1951) and Andreasen (1957). Moscovic (1962) has reviewed the development of this method.

Colorimetric methods with magnesium-specific dyes (Hunter, 1958; Bowen and Martin, 1959; and Bohuon, 1962) have shown good precision but as with Titan Yellow methods also require strict control of procedure. Alcock (1961) has reviewed some of the problems in the estimation of magnesium.

2. Flame Photometry

Van Fossan *et al.* (1959) described a flame photometric method for magnesium in serum, and were followed by Alcock *et al.* (1960) who extended the method to other biological samples as well.

Other flame methods (Montgomery, 1961; Hanna, 1961) share similar difficulties of sample preparation in form of deproteinization, as well as need for a series of prepared standards.

3. Chelometric Titrations

The EDTA titration possesses the same potentially high degree of precision when applied to estimation of calcium plus magnesium as it does with calcium alone. Earlier methods depended upon determination of total calcium plus magnesium, with subtraction of either calcium or magnesium as determined by an independent method (Sobel and Hanok, 1951) or oxalate precipitation of calcium and titration of either calcium or the supernatant magnesium (Friedman and Rubin, 1955; Carr and Frank, 1956).

A stepwise visual titration with adjustment of pH to permit titration of first calcium and then calcium plus magnesium was described by Kovacs and Tarnoky (1960). A similar step-wise titration (Gjessing, 1959) depends upon judgement of the rate of change of galvanometer drift for end-point detection. Methods described by Campbell (1957), Beale and Bostrom (1963) and Bowden and Patston (1963) where calcium and calcium plus magnesium are measured depend also upon estimation of galvanometer drift at the end-point. Other photometric (Wilkinson, 1957) and spectrophotometric (Zak and Hindman, 1956) titrations of calcium and magnesium have only been applied to serum. Malmstadt and Hadjioannou (1959) have developed an automatic titration of calcium and magnesium in serum, as have Jones and McGuckin (1963).

The principle of magnesium determination as the difference between EDTA titration of calcium, and calcium plus magnesium is well established; it remained only to select conditions such that calcium and magnesium could be titrated directly using the precise recorded end-point detection method described for serum calcium by Copp (1963).

C. Principle of Direct EDTA Titration of Calcium and Magnesium in Biological Samples

Ethylenediaminetetraacetic acid (EDTA) like other chelating agents is non-selective, combining with many di, tri and quadrivalent cations (Barnard *et al.*, 1957). Selectivity for specific ion titrations is achieved by the use of masking agents and control of pH.

Above pH 9.0, calcium and magnesium combine with EDTA in a 1 to 1 stoichiometric relationship. At this pH level, other cations such as Fe, Cu, Zn, Mn also combine with EDTA. These are masked from EDTA by addition of cyanide which forms more stable complexes with them than does EDTA.

At pH levels above 12.0, magnesium is removed from solution as the insoluble hydroxide $Mg\ (OH)_2$ which does not react with EDTA. Magnesium may thus be determined by the difference between titration of calcium plus magnesium at pH 10.1 and titration of calcium at pH 12.0 or higher.

Murexide gives a sharp end-point for the calcium titration. Recently, a new indicator Calmagite, has been applied to the calcium plus magnesium titration, giving a sharper end-point than those previously in use.

In samples containing relatively high concentrations of phosphate such as urine, and bone, tissue, or feces ash, interference has been observed in the titration of total calcium (Horner, 1955). At the high pH of the calcium titration, calcium phosphate complexes are formed. While EDTA will eventually remove all calcium from these complexes, the reaction proceeds slowly so that a titration performed too quickly will give a premature or poorly defined end-point, and a low value for total calcium.

In most EDTA titration procedures, this interference has been avoided by prior removal of phosphate from such samples by precipitation (Horner, 1955) and by ion exchange columns (Foss, 1959). Bachra *et al.* (1958) found that citrate delays the formation of such calcium phosphate complexes. Citrate combines with ionic calcium, thus lowering the product of free calcium times phosphate below that critical for complex formation; EDTA however removes calcium readily from the citrate.

The procedure described here extends the semiautomatic spectrophotometric EDTA titration of calcium in serum to the direct titration of calcium and calcium plus magnesium in serum, urine, and tissue, bone, or feces ash solutions. Little sample preparation is required, and the rapid precise quantitation of 0.10 or 0.20 ml aliquots is achieved by a semi-automatic diluting pipet. Addition of citrate to titrated aliquots prevents interference from phosphate.

II. MATERIALS AND METHODS

A. **Apparatus**

The apparatus is shown in a block diagram in Figure 1.

A Beckman B spectrophotometer was fitted with a holder supporting the titration cell, a Pyrex test-tube 15×100 mm, so that the light path was just above the curved bottom of the tube. A small 11/2—3 volt electric motor of the type used in models was incorporated in a light-shielding wooden "mixing" head to spin a soft polyethylene tube with a flattened closed end at a rate adjusted by a rheostat to avoid vortex formation. A constant-speed pump, Harvard Infusion-Withdrawal Pump no. 600-900, with an electro-magnetic clutch supplied titrant at .250 ml/minute through a fine-bore polyethylene tube led into the titrating solution through the mixing head. Mixing and supply tubes ended just above the light path. The output of the Beckman B was recorded, as changing transmittance, on a Sargent SRL recorder, with a 25 MV range plug. The recorder chart drive switch actuated the clutch of the titrant pump. The chart speed was 2 inches per minute.

The titration cell was stirred constantly as titrant was added from the constant flow buret coupled to the recorder drive, and the changing transmittance or absorbance recorded as a line on the chart showed a sharp break at the end-point. The degree of precision was high because the extended intersecting lines drawn at the break were based upon a series of points (i.e. the record) rather than upon a few points as in manual titration methods. Measurement of the distance along the chart from start to the end-point reflected the volume of titrant and hence the Ca or Ca+Mg content of the sample.

B. **Reagents**

Reagents were of A.R. quality where possible. Demineralized water was used for making solutions and for diluting samples. Because of the ion exchange properties of glass, reagents were stored in polyethylene bottles. All glassware was acid-washed and rinsed in distilled water.

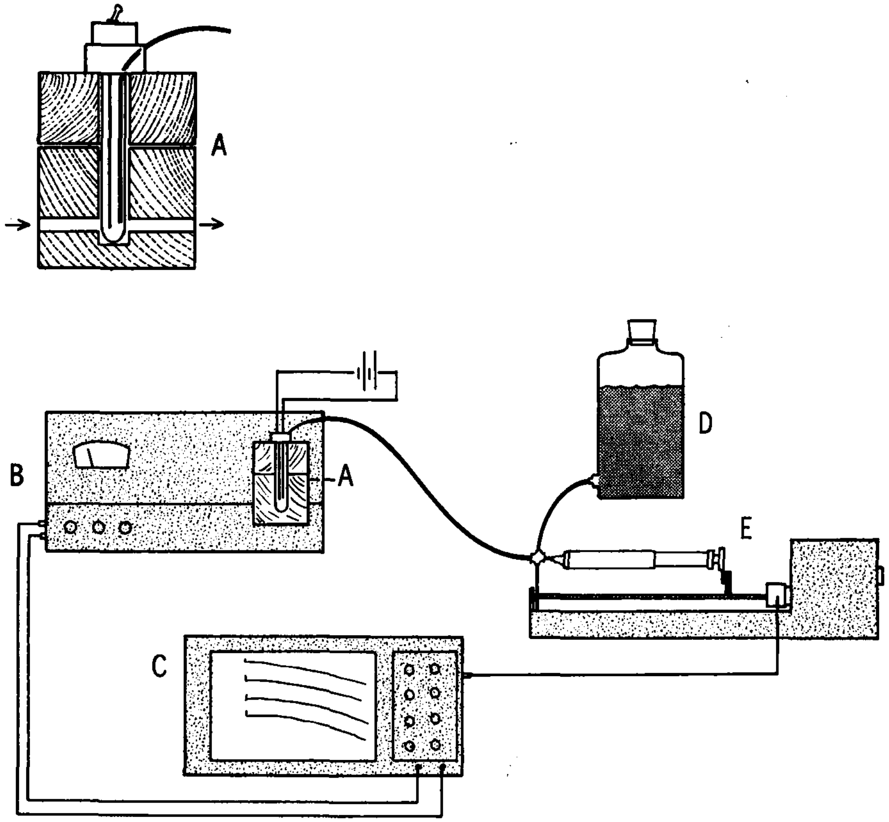


FIGURE 1 Apparatus for recorded titration of calcium and magnesium

A A wooden block holds the sample tube with its lower end in the light path of the spectrophotometer.
A second block supports a small 1 1/2 to 3 volt mixing motor which spins a soft polyethylene tube in the solution during the titration. A second titrant delivery tube of fine bore polyethylene enters the sample tube beside the mixing tube.
B Beckman B. Spectrophotometer: Transmittance is recorded as titration proceeds —Wavelength 480 is used.
C Sargent SRL Recorder: Transmittance on the Beckman B. is recorded at a chart speed of 2 inches per minute.
D EDTA 0.001 M titrant reservoir of polyethylene.
E Harvard No. 600—900 Constant Speed Infusion Pump.
Delivery rate is .250 ml of titrant per minute, using a 30 ml syringe. Two-way stopcock to reservoir is glass. The titrant drive is started by an electromagnetic clutch controlled by the switch of the synchronous motor which drives the chart ensuring simultaneous start and stop of chart drive and titrant delivery.

1. Calcium Standard, 40 mg% and 10 mg%

1.000 Gm. of Ca CO_3 (Mallinckrodt Primary Standard) dried overnight at 105° C, was dissolved in a minimum amount of 5N HCl and diluted to 1 litre water. From this stock solution of 40 mg% Ca^{++} a working standard solution of 10 mg% Ca^{++} was made up as required.

2. Magnesium Standard, 100 mg% and 2 mg%

10.131 Gm. of Magnesium sulfate $MgSO_4 \cdot 7H_2O$ was dissolved in water and diluted to 1 litre. From this 100 mg% stock, a 2 mg% Mg working standard was made up as required.

From chemical equivalence, the amount of EDTA needed to chelate 2.00 mg% Mg is .329 times the amount required to chelate the same volume of 10.0 mg% Ca. Magnesium standard values can thus be determined from the titration of calcium standards alone.

3. EDTA 0.01 and 0.001 M

3.723 Gm. of di-sodium ethylenediaminetetraacetic acid (Mallinckrodt) was dissolved in water and diluted to 1 litre. From this stock 0.01 M EDTA, the titrant solution 0.001 M in water was made up as required.

4. Sodium Citrate 0.1 M

2.490 Gm. of Na Citrate (Mallinckrodt) was dissolved in water and diluted to 1 litre.

5. KCN 1 %

10. Gm. of KCN (Mallinckrodt) was dissolved in water and diluted to 1 litre.

6. NaOH 8.0 N

32.0 Gm. of NaOH (Mallinckrodt) was dissolved in water and made up to 100 ml.

7. NH_4Cl—NH_4OH Buffer

67.5 Gm. NH_4Cl (Mallinckrodt) was dissolved in 570 ml of concentrated NH_4OH (DuPont) and diluted to 1 litre with water. A 10 per cent solution of this stock buffer was made up as required to fill the reservoir of a diluting pipet used for the calcium plus magnesium titration.

C. **Indicators**

1. Calcium Indicator

0.100 Gm. of Murexide (ammonium purpurate acid) (Matheson, Coleman and Bell) was dissolved in water and diluted to 100 ml. The solution was stable for 2—4 weeks at 5° C.

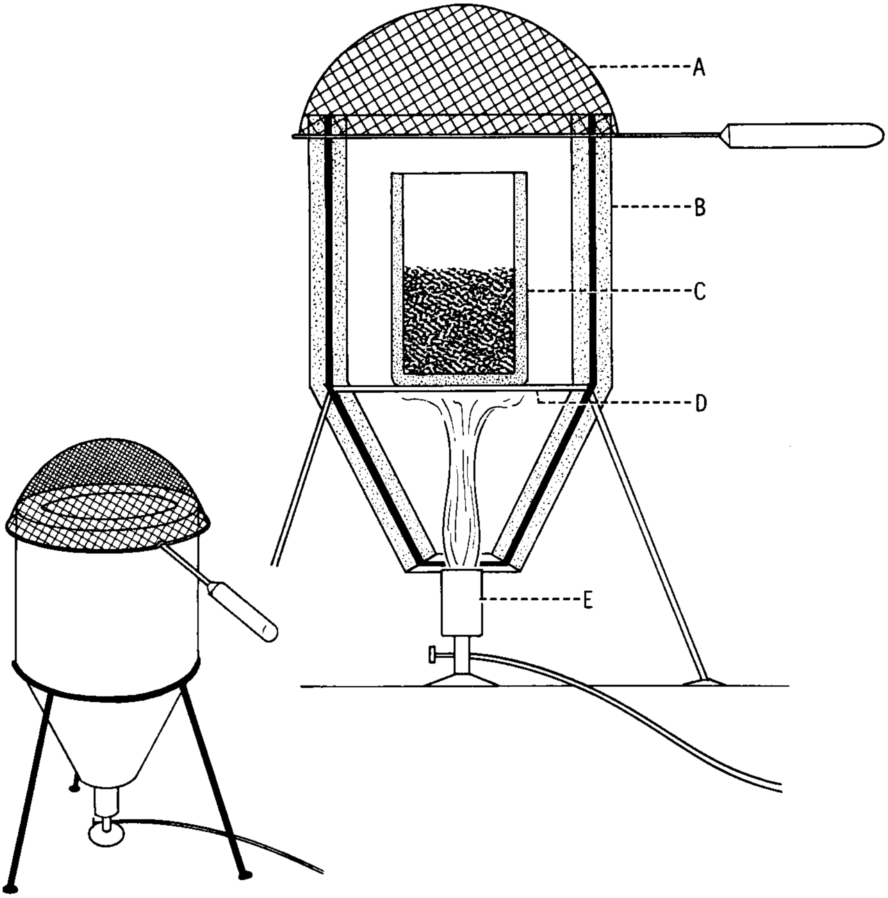


FIGURE 2 Incineration apparatus

A Screen cover of stainless steel (household strainer).
B Insulated chimney of asbestos on a steel cylinder.
C Container for sample—Vycor or glazed porcelain.
D Support rods of steel to hold container over flame.
E Propane burner—industrial type.

2. Calcium plus Magnesium Indicator

0.100 Gm. of Calmagite (Aldrich Chemical Company, Milwaukee, Wisconsin) was dissolved in water and diluted to 100 ml. The solution was stable for 1 month at room temperature.

Na Citrate 0.1 M, KCN 1 %, NaOH 8.0 N and the indicator solutions were kept in polyethylene dropper bottles.

D. **Sample Preparation**

Sample aliquots were taken directly from serum, urine and acid solutions of bone, tissue or feces ash using an automatic diluting pipet (Seligson, 1957).

A 0.20 ml aliquot was drawn up into the pipet and washed into the sample test-tube with 4 ml of the titrating solution using water in the case of calcium, and NH_4Cl—NH_4OH buffer in the case of the calcium plus magnesium titration. Two matched automatic pipets permitted precise quantitation of sample aliquots for both titrations independent of differences in viscosity between samples and standards.

1. Serum, Plasma and Cerebospinal Fluid

Aliquots were drawn directly from fresh samples. If samples had to be kept for some hours they were transferred to polyethylene sample tubes and stored at 5° C.

2. Urine Samples

All samples were acidified to pH 2—3 to dissolve any calcium complexes which might have precipitated. Aliquots were then taken directly.

3. Incineration Procedure for Bone, Tissue and Feces Samples

Feces samples were collected on sheets of thin polyethylene which were then wrapped around the sample for storage in air-tight standard 1 quart paint cans until analysis.

The entire sample and its covering plastic were incinerated in a glazed porcelain or Vycor beaker of 500—800 ml capacity placed over a propane flame in an insulated chimney. See Figure 2. This insulated chimney was modified from a gas and air-fired apparatus described by Buchanan and Sampson (1962), to use a propane heat source. A wide-mouth burner was used at a gas pressure of 10 lbs. per square inch.

Products of combustion were burned completely in passage through the red hot steel mesh covering the chimney. There was no odour, but because of the heat generated, an exhaust hood was used. After burning for 10 to 15 minutes with an orange flame, the sample was reduced to a few ml's of soft grey-white ash. A new sample could be burned to ash on top of the first to combine several days collection. The ash could be further reduced in volume by placing the beaker in a muffle furnace of 500° C for 3 hours.

The small volume of the ash permitted radioactivity measurements to be made upon the ash of an entire period of collection. The ash could then be dissolved in 5 N HCl and diluted appropriately for direct titration of total calcium and calcium plus magnesium in the presence of added citrate.

Bone, tissue, or diet samples were incinerated in the same manner as feces samples.

E. Preparation of Aliquots for Titration

1. Calcium

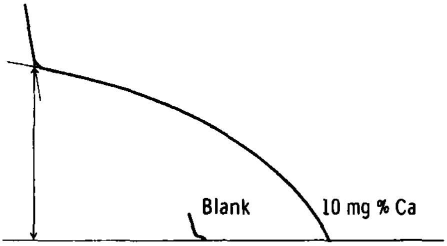
a. Using the automatic pipet, 0.20 ml of sample or standard were washed into a 10×1.5 cm Pyrex test-tube with 4 ml of water.
b. In the case of urine, and bone, tissue or feces ash solution, 3 drops of 0.1 M Na Citrate were then added.
c. 1 drop 1 % KCN was added.
d. 2 drops 8N NaOH were added (4 drops were used if the aliquot had a very low pH).
e. 2 drops indicator solution were added (Murexide 100 mg%).

2. Calcium + Magnesium

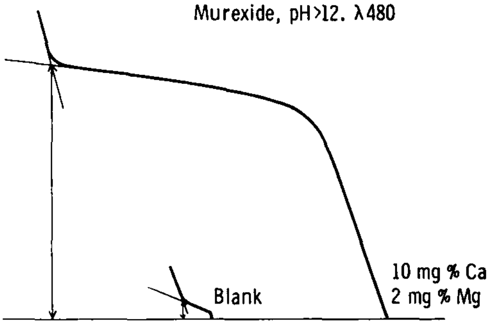
a. Using the automatic pipet 0.20 ml of sample or standard were washed into a test-tube with 4.0 ml of 10 % NH_4Cl—NH_4OH buffer solution.
b. In case of urine, and bone, tissue or feces ash solutions, 3 drops of 0.1 M Na Citrate were added.
c. 1 drop 1 % KCN was added.
d. 2 drops indicator solution were added (Calmagite 100 mg%).

F. Titration Procedure

The procedure was the same for all samples. The test-tube was placed in the holder and covered with the mixing head, ensuring that both mixing tube and titrant delivery tube were in the solution. The mixing motor was started. At wavelength 480 mμ the slit width of the Beckman B was adjusted for 30 % transmittance at sensitivity 3. The recorder chart drive was started, simultaneously actuating the titrant delivery pump. The curve of changing transmittance showed a sharp break



Calcium titration curve
Murexide, pH>12. λ480



Calcium plus magnesium titration curve
Calmagite, pH 10.1. λ480

FIGURE 3
Typical calcium and calcium plus magnesium titration curves recorded as transmittance against time.
Blank titrations are illustrated.

which marked the end-point—see Figure 3. The distance along the chart from the start to the end-point was a measure of the volume of titrant and hence of the metal ion content of the titrated sample. Blank values were determined for both titration and subtracted from both sample and standard values before they were compared.

G. Interpretation of Results

$$\text{Calcium in mg\%} = \frac{(\text{Ca sample—Ca blank})\ \text{mm}}{(\text{Ca standard—Ca blank})\ \text{mm}} \times 10$$

Magnesium in mg% =

$$= \frac{[(\text{Ca}+\text{Mg})\ \text{sample—}(\text{Ca}+\text{Mg})\ \text{blank}]\text{—}(\text{Ca sample—Ca blank})\ \text{mm}}{(\text{Ca standard—Ca blank}) \times .329\ \text{mm}} \times 2$$

III. RESULTS

A. Precision and Recovery Studies

1. Titration Conditions

Wavelength 480 mμ provided a sharp break in the transmittance curve for both titrations. Typical curve patterns are shown in Figure 3. The absorption spectra of murexide and Calmagite are shown in Figure 4.

The concentration of the $NH_4OH—NH_4Cl$ pH 10.1 buffer used in the calcium plus magnesium titration was adequate regardless of the initial pH of aliquots. Four drops of 8.0 N NaOH similarly ensured pH above 12.5 when titrating acidified aliquots for calcium. Excess NaOH did not affect the end-point.

The sensitivity of the titration required that blank values be subtracted from observed values. The blank was due to traces of calcium and magnesium in water as well as in reagents used. It was larger in the $NH_4OH—NH_4Cl$ buffer solution. The blank value for the calcium titration corresponded to 0.20 μGm calcium per ml of titration solution.

The mixing lag noted by Copp (1963) appeared actually to be the blank titration value. Blank titration curves are shown in Figure 3.

Using the chart speed, titrant delivery rate and reagent concentration described, the titration of a 0.20 ml aliquot of a 10 mg% solution of calcium required less than one minute. It was quite feasible to run 40 to 50 calcium determinations per hour, preparing aliquots during the time that the apparatus was automatically carrying out titrations.

2. Standard Solutions

There was a linear relation between actual and observed values for calcium solutions from 0.00 to 25.0 mg% and for magnesium solutions from 0.00 to 5.00 mg% as shown in Tables I and II. While the range could be extended with appropriate changes in reagent concentration, in practice it was easier to dilute sample solutions so that they fell within the range used for determination of calcium and magnesium in serum.

3. Precision

a. *Serum Calcium and Magnesium*

18 replicate titrations of calcium and calcium plus magnesium were performed on a single serum sample. The coefficient of variation of the calcium values was .87 %. The coefficient of variation of calcium plus magnesium values was .83 %. The S.E.M. of the Ca titration was .20 % of the mean value and the S.E.M. of the calcium plus magnesium was

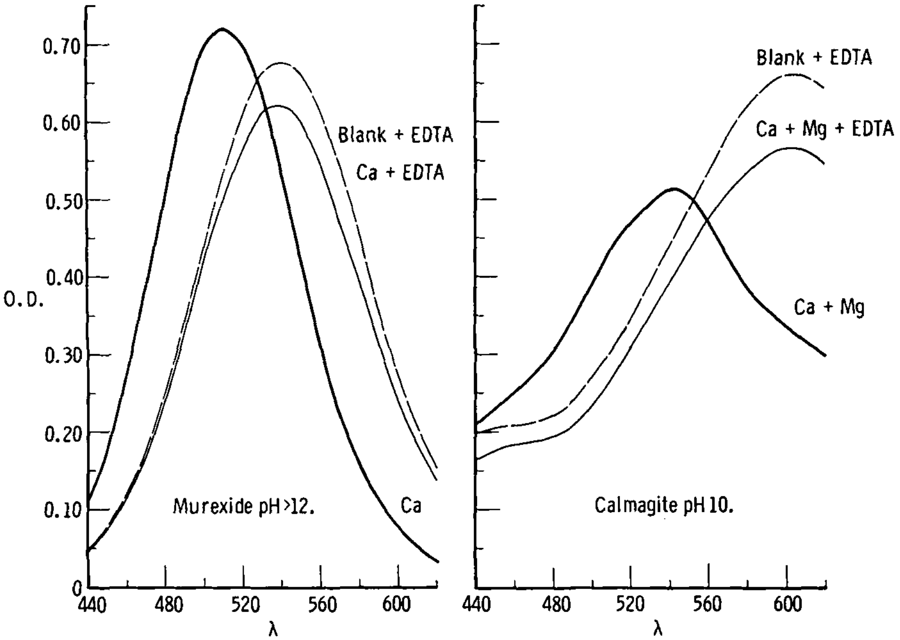


FIGURE 4 Spectra of murexide and calmagite

TABLE I Solution of Calcium Standard 5.00 to 25.0 mg %

Ca std. mg %	5.00	10.0	15.0	20.0	25.0
	5.00	10.00	15.1	20.1	24.9
	4.90	10.00	14.9	19.8	25.0
	5.05	10.10	15.0	20.1	25.2
	5.00	9.90	15.0	20.0	25.0
Mean value mg % ± SD	4.99 ± .06	10.00 ± .08	15.00 ± .08	20.00 ± .14	25.02 ± .13

TABLE II Solutions of Magnesium Standard 1.00 to 5.00 mg %

Mg std. mg %	1.00	2.00	3.00	4.00	5.00
	1.00	1.96	3.05	3.97	5.00
	1.02	1.99	2.95	3.97	5.00
	.97	2.05	3.02	4.02	4.96
	1.00	1.99	2.98	4.05	5.03
Mean value mg % ± SD	1.00 ± .02	2.00 ± .04	3.00 ± .04	3.99 ± .04	5.00 ± .03

TABLE III Recovery of Calcium in Serum

Sample	Ca added mg %	Calculated total Ca mg %	Observed total Ca mg %	$\frac{\text{Observed Value}}{\text{Calculated Value}}$
Serum "A"	5.00	15.00	15.10	100.5
10.00	5.00	15.00	14.98	99.8
(average of 4)	5.00	15.00	15.00	100.
	5.00	15.0	14.95	99.7
	10.0	20.0	20.10	100.5
	10.0	20.0	20.00	100.
	10.0	20.0	19.95	99.7
	10.0	20.0	19.92	99.6

Recovery is 100.00 ± .35 %

TABLE IV Recovery of Calcium in Urine

Sample	Ca added mg %	Calculated total Ca mg %	Observed total Ca mg %	$\frac{\text{Observed Value}}{\text{Calculated Value}}$
Urine "A"	5.00	16.45	16.40	99.5
11.45 mg %	5.00	16.45	16.55	100.5
(average of 4)	5.00	16.45	16.35	99.3
	5.00	16.45	16.45	100.
	10.0	21.45	21.35	99.5
	10.0	21.45	21.50	100.2
	10.0	21.45	21.45	100.
	10.0	21.45	21.50	100.2

Recovery is 99.90 ± .19 %

.19 % of the mean value. The S.E.M. of the magnesium value, which is the difference between these titrations, was .27 % of the mean magnesium value.

b. *Urine Calcium*

50 ml of a urine sample containing 14.2 mg% calcium was mixed with 50 ml of distilled water. 16 replicate titrations of calcium yielded a coefficient of variation of 1.3 % of the mean value of 7.10 mg%. The S.E.M. was .3 % of the mean value.

50 ml of the same urine sample was mixed with 50 ml of a solution of NaH_2PO_4 containing 400 mg% P. 16 replicate titrations of calcium on this sample (containing 7.10 mg% calcium and over 200 mg% P as

TABLE V Recovery of Calcium in Bone Ash

Sample bone ash 60 mg % in HCl	Ca added mg %	Calculated tolal Ca mg %	Observed total Ca mg %	Observed Value / Calculated Value
Bone ash	10.0	30.5	30.60	100.3
60 mg % in HCl	10.0	30.5	30.50	100
Ca content	10.0	30.5	30.6	100.3
20.50 mg %	10.0	30.5	30.5	100

Recovery is 100.1 ± .17 %

TABLE VI Recovery of Calcium in Feces Ash

Feces ash Solution 100 mg % in HCl	Ca added mg %	Calculated total Ca mg %	Observed total Ca mg %	Observed Valne / Calculated Value
Total calcium	10.0	23.1	23.0	99.5
13.10 mg %	10.0	23.1	23.2	100.4
(average of 4)	10.0	23.1	23.15	100.3
	10.0	23.1	23.0	99.5

Recovery is 99.93 ± .49 %

NaH_2PO_4) yielded a coefficient of variation of 1.4 % of the mean value of 7.10 mg%. The S.E.M. was .35 % of the mean values.

4. Recovery of Calcium

Recovery was expressed as percentage of observed over calculated total calcium.

The recovery of 5 and 10 mg% calcium added to a 10. mg% serum ranged from 99.6 to 100.5 % with a mean value of 100.00 ± .35 % (Table III).

The recovery of calcium added to urine ranged from 99.3 to 100.5 % with a mean of 99.9 ± .19 % (Table IV).

The recovery of calcium in bone ash solution ranged from 100 to 100.3 % with a mean value of 100.1 ± .17 % (Table V).

The recovery of calcium in a feces ash solution ranged from 99.5 to 100.4 % with a mean value of 99.93 ± .49 % (Table VI).

5. Recovery of Magnesium

Recovery was expressed as a percentage of observed over calculated total magnesium.

TABLE VII Recovery of Magnesium in Serum

Sample	Mg added mg %	Calculated total Mg mg %	Observed total Mg mg %	Observed Value / Calculated Value
Serum "A"	1.00	2.94	2.96	100.5
1.94 mg %	1.00	2.94	2.92	99.7
(average of 4)	1.00	2.94	2.88	98.0
	1.00	2.94	2.95	100.
	2.00	3.94	3.88	98.5
	2.00	3.94	3.85	98.0
	2.00	3.94	3.98	101.
	2.00	3.94	3.95	100.

Recovery is 99.5 ± 1.3

TABLE VIII Recovery of Magnesium in Urine

Sample	Mg added mg %	Calculated total Mg mg %	Observed total Mg mg %	Observed Value / Calculated Value
Urine "A"	1.00	4.46	4.46	100.
3.46 mg %	1.00	4.46	4.46	100.
(average of 4)	1.00	4.46	4.52	101.
	1.00	4.46	4.39	98.3
	2.00	5.46	5.51	101.
	2.00	5.46	5.38	98.3
	2.00	5.46	5.46	100.
	2.00	5.46	5.51	101.

Recovery is 99.95 ± 1.1 %

The recovery of magnesium in serum ranged from 98.0 to 100.1 % with a mean value of 99.5 ± 1.3 % (Table VII).

The recovery of magnesium in urine ranged from 99.3 to 101 % with a mean value of 99.9 ± 1.1 % (Table VIII).

The recovery of magnesium in bone ash was at least 100 %. The normal magnesium content of bone ash is only about 1 to 2 % of the calcium content, so that it is difficult to quantitate by difference between calcium and calcium plus magnesium titrations. However there was no interference in recovering the added magnesium (Table IX).

The recovery of magnesium in a feces ash solution ranged from 99.2 to 100.8 % with a mean value of 100 ± .66 % (Table X).

TABLE IX Recovery of Magnesium in Bone Ash

Sample bone ash 60 mg % in HCl	Added Mg mg %	Calculated total mg %	Observed total mg %
		(2 to 2.2 mg %)	
Mg content	2.0	2.2	2.1
0.2 mg %	2.0	2.2	2.1
(i.e. 1 % of	2.0	2.2	2.1
Ca content)	2.0	2.2	2.1

TABLE X Recovery of Magnesium in Feces Ash

Feces ash 100 mg % in HCl	Added Mg mg %	Calculated total Mg mg %	Observed total Mg mg %	Observed Value / Total Value
Total Mg	2.00	6.10	6.10	100
is 4.10 mg %	2.00	6.1	6.10	100
(average of 4)	2.00	6.1	6.15	100.8
	2.00	6.1	6.05	99.2

Recovery is 100. ± .66 %

TABLE XI Effect of Phosphate on Titration of Calcium — Magnesium Solution in Presence and Absence of Citrate

Sample Ca 10 mg % — Mg 2 mg %	No added citrate Observed Ca values (mg %)		With Citrate Added Observed Ca values (mg %)				Mean Value — SD
No P	10.0	10.0	10.08	10.0	10.0	9.95	10.01—.05
—50 mg % P	7.25	7.15	10.0	9.88	10.0	10.05	9.98—.07
—100 mg % P	7.50	6.55	9.88	9.95	10.03	10.05	9.97—.08
—150 mg % P	5.90	6.60	10.0	10.0	10.10	10.08	10.05—.05
—200 mg % P	5.60	6.00	10.15	9.75	10.15	10.00	10.01—.18

B. Effect of Citrate upon Phosphate Interference

Table XI demonstrates the decrease of observed calcium with increasing amounts of added phosphorus in a standard calcium plus magnesium solution titrated at pH 13 for calcium. The protective effect of citrate upon the same solution is also shown, with full recovery of calcium in the presence of 200 mg% phosphorus as Na_2HPO_4.

In Table XII titration of calcium in urine in the presence of added phosphorus showed the same incomplete recovery, which in the pres-

TABLE XII Effect of Added Phosphate on Titration of Urine Calcium in Presence and Absence of Added Citrate

Sample Urine "B',	Observed total Ca -- No citrate mg %	Observed total Ca with citrate mg %
Total Calcium		
10.45 mg %	9.50	10.45
(average of 4)		
50 mg % P added	8.90	10.50
+100 mg % P	7.88	10.55
+150 mg % P	5.90	10.50
+200 mg % P	7.30	10.50

TABLE XIII IAEA Comparison of Urine Calcium Methods

Urine Sample	IAEA value	EDTA titration	Atomic Absorption*
A	22.8	22.81	22.6
B	1.8	1.41	1.4
C	10.7	10.9	10.9
D	8.35	8.61	8.4
E	9.5	9.65	9.9
F	13.7	14.0	14.3

Values are mg% total calcium. Urine D contained added NaCl. Urine E contained added $NaCl+MgSO_4 \cdot 7H_2O$.

* Courtesy of S. Sprague, Perkin-Elmer Corporation.

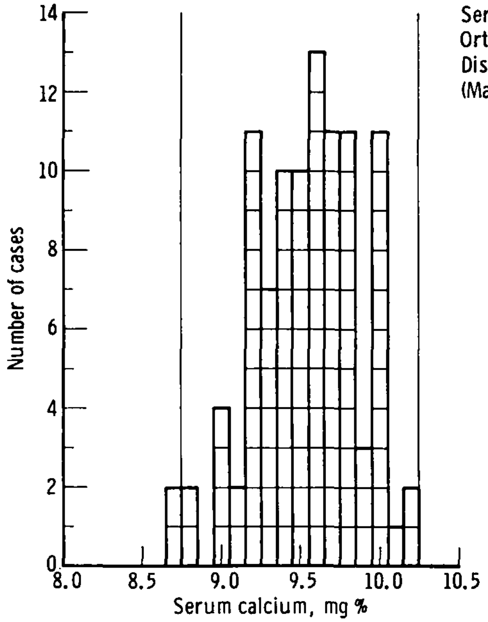
ence of citrate, full recovery was achieved with up to 200 mg% phosphorus added as Na_2HPO_4.

The protective effect of citrate allowed titrations to be done without interference from high phosphate levels up to 15 minutes after the addition of NaOH to the sample.

C. Interlaboratory Comparison of Urine Calcium Methods

As part of a continued examination of calcium analysis procedures under the auspices of the International Atomic Energy Agency, Veall (^{47}Ca Panel 1964) conducted an interlaboratory comparison with 6 unknown samples of urine.

The results by this method, as well as those contributed through the courtesy of Miss S. Sprague of the Perkin Elmer Corporation using



Serum calcium values
Orthopedic Clinic outpatients
Distribution of 100 cases
(Malmö General Hospital)

FIGURE 5

atomic absorption spectrophotometry, are shown in Table XIII. Although urine B was said to contain 1.8 mg% Ca, it apparently contained 0.7 mg% calcium so firmly bound that it was not exchangeable with ^{47}Ca in vitro even after incubation for 48 hours (Veall, 1964). If then this sample, actually contained only 1.1 mg% calcium, the present method had a consistent overestimate of 2 % suggesting a difference in standard solutions, but a high degree of internal consistency particularly in the presence of added NaCl and $MgSO_4 \cdot 7H_2O$ in samples D and E respectively (^{47}Ca Panel Report 1964).

D. Serum Calcium—Normal Values

All values are averages of duplicates agreeing within 1.5 per cent.

To establish the normal distribution of serum calcium values in an Orthopaedic Department, blood was drawn from 100 non-fasting patients presenting at the Orthopaedic Outpatient Clinic of the Malmö General Hospital, who did not have evidence of bone disease or bone injury.

The 53 male patients had serum calcium of 9.54 ± .34 mg% and the serum calcium of the 47 female patients was 9.52 ± .35 mg%. There was no relation between calcium concentration and age or sex. The distribution of the calcium value is shown in Figure 5. Only 2 of the 100 cases fell outside of the range 8.80 to 10.20 mg%.

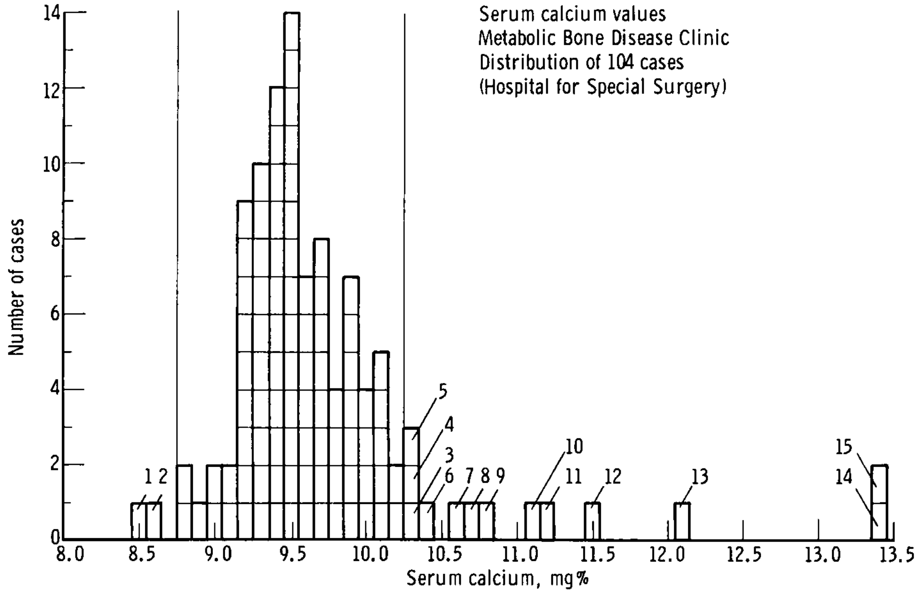


FIGURE 6

In 104 patients of both sexes drawn from the Metabolic Bone Disease Clinic at the Hospital for Special Surgery the serum calcium was 9.77 ± .84 mg%. The distribution of the calcium values in this series is shown in Figure 6.

Taking the normal range of serum calcium as 8.80 to 10.20 mg% (approximating ± 2 S.D. from the mean) the values from the Metabolic Bone Disease Clinic were recalculated on the 89 values falling within this range; this resulted in a mean of 9.53 ± .33 mg%. The 15 cases which fell above or below this narrow range of normal are shown in Table XIV. While it is apparent that within the range 8.8 to 10.2 there are cases with metabolic bone disease in the form of Paget's disease, multiple myeloma, and other conditions, the 15 cases with values outside these limits all have an explanation for their hypercalcemic or hypocalcemic state. The two proven cases of parathyroid adenoma were detected entirely on the basis of the serum calcium done routinely in the Metabolic Bone Clinic.

A further study to assess the range of plasma calcium in man was performed on blood from 227 healthy male blood donors at the Malmö General Hospital. In these the plasma calcium was 9.25 ± .35 mg%. The standard deviation remained the same as in the non-fasting group of OPD patients. The lower mean value may have been related to the

TABLE XIV Serum Calcium Values—Metabolic Bone Disease Clinic—Hospital for Special Surgery

Patient		Sex	Age	Serum Calcium (mg %)	Diagnosis
1	C. H.	M	68	8.45	Gastrectomy, cancer of the prostate, large doses of estrogens.
2	M.A.	F	80	8.60	Osteoporosis; poor calcium absorption by ^{47}Ca study.
3	I. R.	F	63	10.30	Post-menopausal osteoporosis.
4	H. M.	F	61	10.30	Paget's disease; tuberculosis of the hip.
5	M. W.	F	58	10.30	Osteoporosis, healing fractures; alcoholism.
6	R. M.	F	32	10.40	Idiopathic osteoporosis; myasthenia gravis; high calcium diet for 3 years.
7	P. M.	F	15	10.6	Vitamin D resistant rickets on 75,000 units daily for one year.
8	V. F.	F	49	10.65	Hypercalcuria, low phosphorus, probable parathyroid disease.
9	J. S.	F	49	10.80	Paget's disease.
10	J. F.	M	65	11.10	Gastrectomy, suspected parathyroid disease.
11	A. D.	F	64	11.18	Proven parathyroid adenoma.
12	R. R.	F	12	11.50	Osteogenesis imperfecta, acutely decalcifying spine with fractures.
13	I. F.	F	68	12.1	Proven parathyroid adenoma.
14	M. W.	F	74	13.35	Multiple myeloma.
15	P. B.	M	17	13.40	Osteogenic sarcoma with metastases.

fasting state of the blood donors. Another factor may have been slight hemodilution, as the blood was from the end of the donation period.

In a study of 102 sera taken for routine latex fixation tests at the Hospital for Special Surgery, the mean serum calcium was 9.44±.39 mg%.

E. Serum Magnesium—Normal Value

All values given are averages of duplicates. In the same 100 outpatients at the Malmö General Hospital—free of any evidence of bone disease, the serum magnesium of 53 males was 2.24±.25 mg% and of 47 females 2.23±.21 mg%.

In the same group of 227 fasting male blood donors, plasma magnesium was 2.17±.21 mg%.

IV. DISCUSSION

A. Direct Titration of Calcium and Magnesium

The advantages of the method described for determination of calcium and magnesium in biological samples are specificity, precision, simplicity, and freedom from the operator's influence.

Precision in the measurement of aliquots is assured by the automatic pipet calibrated to contain, which is washed by the diluting solution for complete delivery independent of differences in viscosity between samples and standards.

The simple dropwise addition of reagents to the titrating solution is not a critical step since doubling any of these has no measurable effect upon the end-point. Impurities leading to colour changes can only affect the absorbance axis of the record and not the titration end-point. Similarly the colour of the serum or urine aliquot itself has no effect on the titration end-point.

The interferences recognized in other methods do not affect this procedure as described. Excesses of Na or K which affect flame methods have no influence on the EDTA titration. Magnesium does not react at the pH at which calcium is measured, yet is titrated quantitatively at the lower pH of the calcium plus magnesium titration.

Phosphate interference which in other methods necessitates prior sample treatment is eliminated by the simple addition of citrate in excess. No prior treatment aside from acidification is required for the determination of calcium or calcium plus magnesium in urine.

Freedom from interference permits precise measurements of calcium and calcium plus magnesium directly on aliquots of any biological material in solution with total calcium content of the aliquot as low as 10—20 μGm.

The incineration procedure removes much of the difficulty of assaying stool and tissue calcium content.

B. Serum Calcium and Magnesium in Man

Advances in the precision of analytical methods have led to a new concept of the normal range of serum calcium and magnesium in man.

The results reported here for serum calcium are in agreement with those reported by Copp (1963) who found the mean value for 54 normal adults to be $9.40 \pm .32$ mg% Ca and Jones and McGuckin (1964) who found serum calcium in 40 normal adults to be $9.47 \pm .29$. The range of normal is remarkably low attesting to the precision of the homeostatic mechanisms controlling the concentration of this electrolyte in body fluids.

If 95 % of all serum calcium values in a normal population fall within the range 8.80 to 10.20 mg% (mean ± 2 S.D.), it is apparent that values outside this narrow range take on a new meaning when compared to the previously accepted much wider range of serum calcium. The 15 cases from the Metabolic Bone Clinic series which were outside this normal range supported this view. See Table XIV.

Continued reliance in the clinical laboratory upon serum calcium methods with a precision of ± 5 % can not be justified when the range of normal is so narrow.

Serum magnesium also falls within a narrow range. The values reported here agreed well with the 2.23 ± .27 mg% serum Mg reported in 18 cases by Malmstadt (1959), using an EDTA titration method.

C. Determination of Abundance Levels of Calcium Isotopes

Estimation of the relative abundance levels of the calcium isotopes has depended until recently upon mass spectrometric analysis. With the development of neutron sources and improved measurement techniques, activation analysis has been used to study the abundance of calcium isotopes in the natural environment (Corless, 1964). The precision of the estimation of abundance of calcium isotopes with this technique is dependent upon the precision of total calcium estimation by EDTA titration.

The production of enriched ^{48}Ca and ^{46}Ca has made possible this use as stable tracers of calcium kinetics (McPherson, 1964). The amount of ^{46}Ca or ^{48}Ca in a calcium sample is determined by activation analysis, counting the ^{47}Ca or ^{49}Ca produced in samples and standards. The abundance level of stable isotopes can then be determined if the total calcium of the activated sample and standard can be determined accurately. The EDTA titration described here has been used for the determination of total calcium in such activated samples. Abundance levels of ^{46}Ca and ^{48}Ca in samples expressed as per cent dose per gram of total calcium permit construction of specific activity curves for stable isotopes used as tracers.

V. CONCLUSIONS

A direct recorded EDTA titration procedure permits precise measurement of total calcium and total calcium plus magnesium, giving magnesium by difference, in a variety of biological materials.

Phosphate interference during titration is prevented by the addition of citrate in excess.

The normal range of serum calcium and serum magnesium is very narrow (9.50 ± .35 mg% and 2.24 ± .21 mg% respectively). Deviations from this narrow range observed in a sample of patients from a Metabolic Bone Clinic have been important in the diagnosis of a variety of conditions.

Precision in estimation of total calcium in small samples has permitted the use of this method in the determination of the abundance of stable calcium isotopes used as tracers of mineral metabolism, using neutron activation analysis.

CHAPTER II

Heavy Calcium Isotopes as Tracers of Calcium

I. INTRODUCTION

A. Reported Ca* Specific Activity Differences

Unexplained differences in specific activity of heavy calcium isotopes between blood and urine have been reported by several investigators in man and in experimental animals. The apparent differences observed have been attributed in some cases to binding of these isotopes by blood proteins as evidence of a calcium pool within plasma (Wiester *et al.*, 1963; Veall and Parsons, 1964). Others have reported variation in S.A. ratios of blood and urine but have been unable to state whether these were real or due to systematic analytic errors (Dow and Stanbury, 1960). Profound variations in blood/urine Ca* specific activity ratios have been related to starvation in both rats (Likins and Craven, 1960) and sheep (Giese and Comar, 1964).

B. Isotope Effects

Isotopes of an element do possess different chemical characteristics and some degree of fractionation has been shown to occur in both geological and biological processes. These isotope mass effects range all the way from the profound disturbances produced in biological systems by Deuterium (Kritchevsky, 1960) to the slight variation in O_2, C, and S isotope abundance ratios which are important in geological studies (McDowell, 1964).

While Deuterium and Tritium are particular examples of the importance of isotope mass effects, the mass differences between ^{40}Ca and its heavy isotopes radioactive ^{45}Ca and ^{47}Ca, and stable ^{46}Ca and ^{48}Ca are relatively greater than those that occur in other elements commonly used as tracers in biological systems, and because of this one might expect some fractionation to occur. Differences in abundance of isotopes in the natural environment have usually been observed when the element has been taken through complex systems, such as carbon in photosynthesis, sulfur in bacterial decay processes in petroleum deposits, and carbon in precipitation of calcite (McDowell, 1964). Corless has suggested that $^{48/40}$ calcium isotopic abundance variations may occur in calcified tissues (IAEA 1964).

Processes which do discriminate tend to conserve isotopes of lower mass, so that if fractionation of calcium isotopes did occur, one would expect that heavier ^{45}Ca and ^{47}Ca would be discriminated against by active transport mechanisms such as occur in the renal tubules. Reabsorption processes absorb relatively more easily the isotope requiring the least energy i.e. ^{40}Ca. Differences on this basis due to biological fractionation would thus tend to result in higher Ca* S.A. in the urine than in the serum.

That some biological fractionation of calcium isotopes may exist is not seriously questioned. However, such differences are probably far less than those of biological variation inherent in experiments undertaken in the complex milieu of the intact animal. Such factors as renal blood flow, delay of urine in transit related to its flow rate etc. will of course affect short term studies, but will be less significant in experiments over several hours, days or weeks as is the usual case in calcium tracer studies.

The purpose of the investigation reported here was to evaluate reported Ca* S.A. differences between serum and urine in human subjects. Special attention was given to the possibility that heavy Ca isotopes are fractionated as compared to lighter Ca isotopes.

II. MATERIALS AND METHODS

A. Specific Activity Studies with ^{47}Ca

1. Subjects

Case 1, R.M. A 77 year old male with diabetes, and a vertebral compression fracture secondary to post-gastrectomy osteoporosis. The patient was confined to bed during most of the study period.

Case 2, W.J. A 40 year old male with back pain secondary to rheumatoid spondylitis and associated severe osteoporosis. The patient was ambulatory during the study.

Case 3, E.G. A 48 year old female with severe back pain related to osteoporosis and compression fractures of vertebrae, secondary to corticosteroid therapy of dermatomyositis. The patient was confined to bed and wheel chair during study.

All cases received a normal hospital diet.

2. Procedure

After voiding, each subject received 50—75 μc ^{47}Ca intravenously as CaCl$_2$ solution containing a negligible amount of carrier calcium.

Blood was drawn from the antecubital veins. Serum was separated and stored in the cold.

Patients voided at their usual times using a clean acid-washed glass bottle for each voiding. The time period of each collection was recorded.

Collections continued over a 7 to 9 day period.

3. ^{47}Ca Specific Activity Determination

a. *^{47}Ca Assay*

3.0 ml aliquots of serum and of each urine sample were placed in glass sample tubes and counted in an automatic well-counter (Nuclear Chicago) against a ^{47}Ca standard solution representing .17 % of the dose administered.

Counting accuracy was kept to at least 2 per cent.

b. *Total Calcium Assay*

Total calcium concentration in the serum and in urine samples was measured using direct EDTA titration.

Specific activity was expressed as % dose ^{47}Ca per gram total calcium.

B. **^{48}Calcium Abundance Studies**

Calcium isolated as calcium carbonate from samples of human and rat bone and human urine was analyzed for its ^{48}Ca abundance in relation to standard $CaCO_3$ samples by neutron activation analysis. ^{49}Ca produced from the ^{48}Ca by n, γ reaction under neutron bombardment was counted in relation to standard samples. Total calcium estimation on the activated standard and samples gave abundance ratios of ^{48}Ca in the samples in relation to the reference standard employed.

III. RESULTS

A. **Radioactive Studies Using ^{47}Ca**

Figures 7, 8 and 9, show the change with time of serum and urine calcium in the three cases studied with ^{47}Ca.

The urine specific activity is plotted at the mid points of collection periods. While this does tend to a slight over-estimation this is not significant after the initial rapid drop of ^{47}Ca S.A. in the first day.

It is apparent that urine and serum ^{47}Ca specific activity fall on the same curve in the three cases.

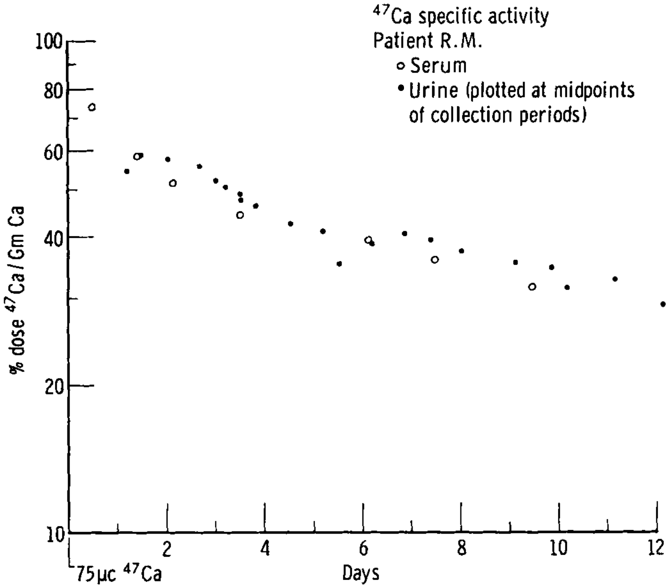


FIGURE 7

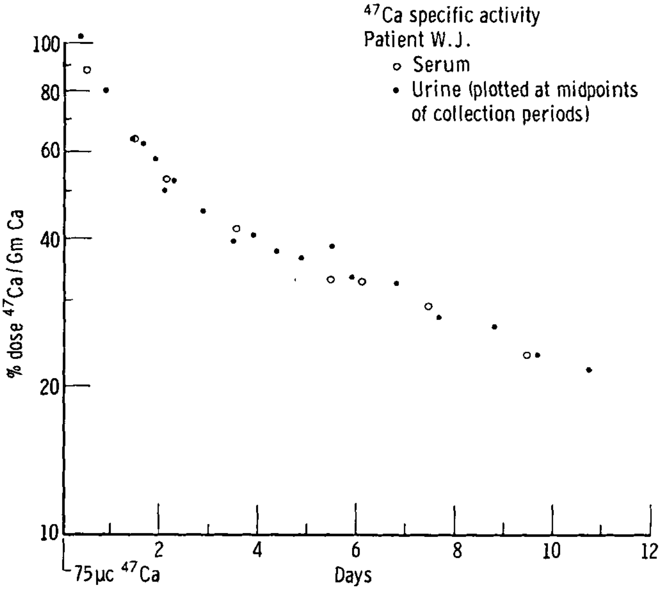


FIGURE 8

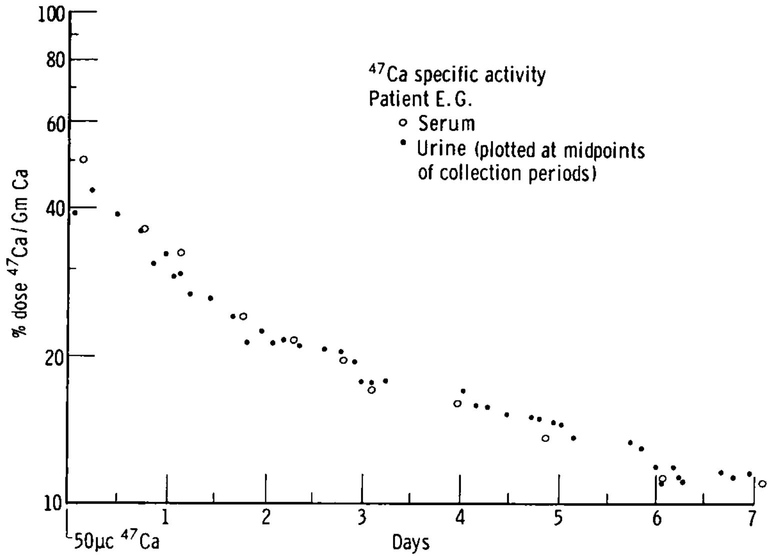


FIGURE 9

TABLE XV

Calcium Source	Total Calcium Activated (mg)	^{48}Ca Abundance	$\frac{^{48}\text{Ca Abundance Sample}}{^{48}\text{Ca Abundance Standard}} \times 100$
CaCo$_3$	6.50	.182 %	100.
CaCO$_3$	5.58	.182	100.
Human Urine A (2 precipitations)	7.51	.182	100.
Human Urine A (3 precipitations)	7.91	.173	95.
Rat Bone Ash R	8.02	.174	95.6
Rat Bone Ash R	5.92	.178	97.8
Human Bone Ash H	8.21	.181	99.4
Human Bone Ash H	7.14	.181	99.4

* Primary Calcium Standard, Mallinckrodt 4071.

B. ^{48}Ca Abundance Studies

^{48}Ca abundance values in samples of rat and human bone, and urine from several patients as a ratio of standard calcium samples are shown in Table XV. The isotopic ratios in the samples are similar. The control and post-study ^{48}Ca abundance levels in a series of patients injected with ^{48}Ca as tracer showed abundance levels similar to standard calcium samples (Table XV).

IV. DISCUSSION

A. Previous Studies

Considerable evidence is available to support the contention that serum Ca*S.A. is reflected by the urine Ca*S.A. in experiments where reasonable time has been allowed to elapse for equilibration in body pools before sampling.

Govaerts (1949) studied the excretion of ^{45}Ca infused in dogs and reported the same S.A. in serum and urine. In 1945, Blau *et al.* gave ^{45}Ca orally to two patients and found the S.A. curves of plasma and urine to be the same over a 9-day period of study. After the second hour following ^{45}Ca given orally, urine S.A. was representative of serum S.A. They confirmed these observations in other human cases in which the tracer was given intravenously.

Bronner *et al.* (1956) injected ^{45}Ca intravenously in 9 adolescent mentally defective boys and found that serum and urine S.A. fell in the same curve. In one young adult this was true over a longer period of 30 days.

Krane *et al.* (1956) also followed the serum and urine S.A. of 15 cases of thyroid disease given ^{45}Ca intravenously and found S.A. to be the same.

Heaney and Whedon (1958) also showed similar urine and serum S.A. in 4 representative cases of 12 studies with intravenous ^{45}Ca for periods up to 10 days.

The subject of Ca* S.A. was considered at the Second Panel on Medical Uses of ^{47}Ca (Vienna, 1964). Corey *et al.* (1964) stated that "fractionated urine can't be obtained early in the study and the S.A. of urine calcium is often different from that in serum"; however they had no difficulty in the use of Ca* S.A. in saliva as representative of S.A. in blood. There seems little reason to expect basic differences in S.A. to be produced by the filtration and reabsorption processes in renal tissue that are not produced by the secretion processes of the salivary glands.

At the same Panel on ^{47}Ca Soto *et al.* (1964) reported that in a variety of disease states as well as in normals, the ^{47}Ca S.A. of serum and urine did not differ significantly after the first day of 7 to 8 day studies. In their series were included 10 normals, 11 hyperthyroids, 9 hypothyroids, 6 cured cases of thyrotoxicosis, 4 hypoparathyroids and 5 cases of osteoporosis. If differences were to appear, then this series should certainly have presented opportunity with its variety of metabolic disorders. Soto *et al.* (1964) reported the same findings in 8 cases given ^{47}Ca orally.

Szymendera (1964) presented data on three patients given ^{47}Ca intravenously showing excellent agreement of S.A. in plasma and urine and employing EDTA titration to assay total calcium in the samples. The ease of sample preparation in ^{47}Ca studies compared to the relative complication of precipitation procedures needed to prepare ^{45}Ca for isotope assay suggests that ^{47}Ca S.A. measurements on serum and urine are more likely to be true values.

Animal experiments on normal dogs by Miller *et al.* [1964 (a)] and by Giese and Comar (1964) on normal sheep showed the same S.A. of serum and urine ^{45}Ca.

Investigations noting a difference in Ca* S.A. between blood and urine have been reported by a number of authors. In the light of evidence provided by the studies discussed above, as well as the Ca* S.A. relationship observed in the three cases studied with ^{47}Ca described here, these reports have been examined.

The controversy appears to date from a report by Dow and Stanbury (1960) describing serum to urine ^{45}Ca S.A. ratios of from 1.18 to 3.86 in human subjects.

The significance of this finding in relation to the 15 cases described in 1956 by Krane *et al.* in which Stanbury took part is not explained. Dow and Stanbury (1960) were unable to state whether their observations were real or were related to analytical errors in the precipitation procedure used for both serum and urine calcium. Veall (1962) in studying such procedures checked with ^{47}Ca has found from 62 to 75 % recovery of total calcium from urine with oxalate precipitation methods. The co-precipitation of magnesium in urine under certain conditions can give a false high estimate of urine total calcium and account for a low S.A. in urine relative to plasma (Fiske and Logan, 1931).

Following Dow and Stanbury's (1960) report, other authors have commented upon the possibility of multiple calcium pools in blood which do not exchange as rapidly as expected and could thus account for S.A. differences.

Veall and Parsons (1964) proposed that the complexed diffusible forms of calcium in plasma i.e. calcium bound to organic acids, sulfate and phosphate, were not available for rapid exchange with calcium tracers. These forms of calcium were supposed to be preferentially excreted since the ionic form is more available for tubular reabsorption. Although Veall stated that this complexed pool of calcium should be considered in models of calcium kinetics he did not present evidence to support this view.

Further support for this hypothesis came from Wiester *et al.* (1963) based upon 4 blood samples drawn from a parathyroidectomized dog given an infusion of ^{45}Ca and ^{40}Ca over a period of three hours. Their observation that urine S.A. was only 55—70 % that of plasma ultrafiltrate depended upon oxalate precipitation of calcium—with possible errors in over-estimation of total calcium in urine due to magnesium interference.

Giese and Comar (1964) reported similar Ca* S.A. in the serum and urine of 2 normal sheep, but found lower urine Ca* S.A. in the same 2 animals when starved 48 hours despite the fact that total plasma calcium changed only slightly. The lower S.A. was associated with increased ^{45}Ca in the urine in one sheep, and increased ^{40}Ca in the other when compared with the values in the fed control experiment. An in vitro equilibration of ^{45}Ca with plasma of normal and starved sheep showed no difference. This did not support the idea that complexed, non-exchangeable pools of calcium in plasma were responsible for the Ca* S.A. difference in the starved sheep.

Similar apparent differences in ^{45}Ca S.A. in starved rats were noted by Likins and Craven (1960). In both the rats studied by Likins and Craven (1960) and the sheep studied by Giese and Comar (1964) the total calcium of urine was determined by oxalate precipitation. Since magnesium may interfere in these methods, and since starved animals may excrete many times as much magnesium as calcium in urine (Fiske and Logan, 1931), these two factors may explain these results in starved animals.

Studies of the S.A. of blood and urine of dogs by Miller (1964) and Miller and Neuman (1964) leave little doubt that in the normal state, Ca* S.A. is the same in blood and urine. They employed EDTA titration of the counted samples and so avoided the possibility of magnesium interference. The differences observed in their experimental preparations are related to marked disturbances in renal function performed to study calcium exchange in kidney tissue.

Table XVI summarizes the evidence which has been presented concerning the serum urine Ca* S.A. ratio. The 3 cases reported here studied with ^{47}Ca showed that urine Ca* S.A. was representative of serum Ca* S.A. and are in agreement with the findings reported by Soto *et al.*, 1964, Szymendera, 1964, Heaney and Whedon, 1958, Krane *et al.*, 1956, Blau *et al.*, 1954, and Bronner, 1956 in a total of 86 human subjects, including normal cases and cases suffering from various metabolic bone diseases. Dow and Stanbury's (1960) report on 10 cases is the only one in which S.A. differences were observed in man. They were unable to rule out systematic error in the urine calcium method employed (Stanbury, 1964).

In normal animals, the Ca* S.A. of urine and serum was the same in rats (Likins and Craven, 1960) dogs (Govaerts, 1949; Miller, 1964; Miller *et al.*, 1964) and sheep (Giese and Comar, 1964). Ca* S.A. differences

TABLE XVI Serum — Urine Calcium* Specific Activity

Author	Cases	Subjects	Isotope	Urine Calcium method	Relation of serum to urine S.A.
Blau et al. 1954	2	man	^{45}Ca	precipitation	same
Krane et al. 1956	15	man	^{45}Ca	precipitation	same
Bronner et al. 1956	9	man	^{45}Ca	precipitation	same
Heaney and Whedon 1958	12	man	^{45}Ca	precipitation	same
Dow and Stanbury 1960	10	man	^{45}Ca	precipitation	urine S.A. lower than serum in all cases
Szymendera 1964	3	man	^{47}Ca	EDTA titration	same
Soto et al. 1964	45	man	^{47}Ca	EDTA titration	same
Govaerts 1949		dogs	^{45}Ca	precipitation	same
Likins and Craven 1960	6	rats	^{45}Ca	precipitation	same in normals
Likins and Craven 1960	8	rats	^{45}Ca	precipitation	urine S.A. lower in starved animals
Wiester et al. 1963	1	dog	^{45}Ca	precipitation	urine S.A. lower
Giese and Comar 1964	2	sheep	^{45}Ca	precipitation	same in normals, urine S.A. lower in starved animals
Miller et al. 1964		dogs	^{45}Ca	EDTA titration	same

found in animals have been in 4 blood samples from 1 parathyroidectomized dog (Wiester *et al.*, 1963), starved rats (Likins and Craven, 1960) and starved sheep (Giese and Comar, 1964) as well as in the specialized stop-flow studies of renal calcium exchange reported by Miller *et al.*, 1964. Magnesium interference in the methods used for urine calcium in the starved animals may account for the apparent lower Ca* S.A. in these studies.

B. **Present Study**

The close agreement of urine and serum ^{47}Ca S.A. in the three cases reported here did not support the existence of significant isotopic fractionation processes due to either complexed calcium pools in plasma or to active transport mechanisms in the kidney.

The constancy of $^{48/40}Ca$ abundance ratios in a variety of biological samples including urine and bone did not suggest that there was a significant degree of biological fractionation which would discriminate against the heavy calcium isotopes, otherwise there would have been differences in ^{48}Ca abundance not only between bone samples and urine samples but also between different urine samples. These findings do not support the observations of Corless and Winchester (1964) who found differences of $^{48/40}$Ca abundance ratios between human teeth and geological calcium samples. They acknowledged however, that the method employed required further refinement.

The use of urine Ca*SA as a measure of plasma Ca*SA is of considerable importance. First, it is possible to extend the time of a tracer study by concentration of urine calcium by various means, and also to decrease the dose of tracer according to the concentration factor. Secondly, it is possible to employ the isotope abundance pattern of urine Ca as a measure of the isotope abundance pattern of plasma calcium. In studies of calcium kinetics using stable isotopes of calcium, the 10 mg samples needed for activation analysis can be isolated from timed urine collections, thus avoiding the need for large volumes of plasma. This method has been adopted as a means of following the plasma S.A. of stable ^{46}Ca and ^{48}Ca used as tracers of calcium kinetics in man.

V. CONCLUSIONS

Studies of ^{47}Ca specific activities in serum and urin in three human subjects did not reveal evidence of fractionation of heavy calcium isotopes sufficient to invalidate kinetic studies using that tracer.

^{48}Ca abundance in a variety of samples of biological origin was constant in relation to standard calcium samples. The abundance of ^{48}Ca is apparently constant in nature.

Urine calcium specific activity is representative of serum calcium specific activity. Studies of calcium kinetics using stable isotopes may be performed using activation analysis of 10 mg samples of calcium isolated from urine.

Kinetic studies with calcium isotopes can be extended in time by concentrating urine.

CHAPTER III

^{46}Ca and ^{48}Ca as Tracers in Studies of Mineral Metabolism

I. INTRODUCTION

Tracer studies using radioisotopes have led to a dramatic increase in knowledge not only of the mechanisms but also of the kinetics of physiological processes. At the present time more than two dozen radioisotopes possess properties which make them of actual or potential value in the study of skeletal tissues alone (McLean and Budy, 1964).

A. **Stable Isotopes of Calcium**

Recently, the development of more efficient separation procedures in an effort to secure high specific activity radioactive tracers has led to the production of stable isotopes in highly enriched forms. Table XVII lists the stable isotopes of calcium and their enrichments available at the present time (Beauchamp, 1964).

Stable isotopes are suitable for tracer studies provided that:

1. normal abundance of the isotope is low in nature,
2. highly enriched forms of the isotope are available,
3. increased abundance of the isotope in sample materials can be accurately measured.

This report describes the use of two stable isotopes of calcium, ^{46}Ca and ^{48}Ca as tracers in studies of calcium metabolism in man and animals.

B. **Abundance Measurement**

1. Mass Spectrometry

The abundances of the isotopes of calcium determined by mass spectrometry are shown in Table XVIII. These values compare very favourably with those given by Nier (1938) who discovered ^{46}Ca and ^{48}Ca.

Mass spectrometry provides the most precise means of estimating the abundance of isotopic species provided that a pure sample of the element can be obtained. In the case of calcium, as little as 100 μGm of

TABLE XVII

Isotope	Percentage enrichment
^{42}Ca	25 to >85
^{43}Ca	20 to >65
^{44}Ca	75 to >98
^{46}Ca	0.5 to 40
^{48}Ca	5 to >95

Stable Calcium Isotope Enrichment Levels Available April, 1964.

TABLE XVIII Abundance of the Isotopes of Calcium in Nature

Mass	40	42	43	44	46	48
Mole per cent	96.92	0.64	0.132	2.13	0.0032	0.179

From White and Cameron, Physical Reviews 74: 991, 1948.

the element is sufficient for analysis of abundance by this method. As the result is a complete pattern of the abundances of the isotopes in relation to each other, the total weight of sample is not critical. This method is readily applied to determination of the abundance of ^{42}Ca, ^{43}Ca, ^{44}Ca and ^{48}Ca. It has been used to study the isotopic composition of the calcium of meteorites (Hirt and Epstein, 1964), and is used routinely to accurately determine enrichment levels of isotopes prior to the production of radioactive isotopes by neutron bombardment.

2. Neutron Activation Analysis

The demonstration that radioactive isotopes could be produced artificially led Hevesy as early as 1936 to suggest that the technique we now know as neutron activation analysis could be used to quantitate elements at tracer levels. The wider application of this analytical method awaited the later development of better neutron sources with higher fluxes, and more sensitive counting apparatus employing multichannel analyzers and gamma ray spectrometry.

Activation analysis is a two-stage process. A small amount of the sample to be analyzed is exposed for a suitable period of time to a neutron flux. Many of the stable isotopes in the sample will be activated—depending upon the flux, the cross-section for neutron capture of the isotopes present, and their abundance in the sample.

In the second stage the induced radionuclides are identified by their characteristic γ ray spectra and half-lives. Comparison of the activity in the sample with that of a standard radiated under the same conditions permits quantitation of the induced radioisotopes and thus the amount of their stable precursors in the sample.

The relation between radioactivity induced in an isotopic species by a constant flux, and the amount of the isotope present is given by the equation.

$A_x = F\sigma_a N_x (1 - e^{-\lambda t})$

where N_x = no. of target atoms of type X
σ_a = activation cross-section of type X atoms
A_x = disintegration rate of the radioisotope produced from X as result of neutron bombardment
λ = disintegration constant
t = time of bombardment
F = flux of neutrons

Disintegration rate is proportional to mass and the sensitivity is proportional to both the cross-section for neutron capture and the flux employed. Theoretically this relationship permits absolute quantitation of the isotopic species present, but in practice standard samples are usually used for reference. Repeated analyses of the same sample are possible as the method is non-destructive.

Neutron activation analysis has been used to determine trace elements in tissue and in serum (Bethard *et al.*, 1963; Koch *et al.*, 1958; and Rieffel *et al.*, 1957).

For example, the exposure of 10 ml of human serum to a neutron flux of 2.5×10^{12} n/cm^2/sec for one hour produces the following range of isotopes with over 30 disintegrations/min. ^{24}Na, ^{38}Cl, ^{80m}Br, ^{80}Br, ^{82}Br, ^{128}I, ^{42}K, ^{49}Ca, ^{27}Mg, ^{60}Co, ^{56}Mn (Spencer *et al.*, 1957). Unless a separation procedure is performed before or after the activation step and before counting, the high background activity of the other isotopes may interfere with measurement of a particular nuclide.

Because of both its high cross-section for neutron capture and its generally high concentration in biological samples, sodium in the form of ^{24}Na ($^1/_2$ 15 hrs) is responsible for most of the activity induced in serum and other biological samples. At 12 hours, other isotopes with shorter half-lives have decayed to the point where 99·6 % of the gamma activity is due to ^{24}Na (Spencer *et al.*, 1957).

The short-lived γ emitting isotope ^{45}Ca ($^1/_2$ 8.7 min) produced by n, γ reaction from ^{48}Ca under neutron bombardment must be counted soon after activation. Although ^{24}Na does not interfere with counting of the

TABLE XIX

	^{46}Ca	^{48}Ca
Abundance in natural calcium	.0032 %	.179 %
Cross-section for neutron capture	.3	1.1
Induced isotope (nγ reaction)	^{47}Ca	^{49}Ca
Half-life of induced isotope	4.9 days	8.7 minutes

high energy peak of ^{49}Ca at 3.1 MeV, it is desirable to reduce the Na content of such samples by prior treatment to produce as pure a sample of calcium as possible.

The longer-lived isotope ^{47}Ca ($^{1}/_{2}$ 4.9 days) produced by n, γ reaction from ^{46}Ca may be counted after 7 days, at which time ^{24}Na has decayed sufficiently that it does not interfere significantly.

A consideration of the relative cross-sections and abundances of ^{48}Ca and ^{46}Ca shows that a much longer period of neutron bombardment (days) is needed to activate ^{46}Ca, than the short period (minutes) needed to activate ^{48}Ca. See Table XIX.

Measurement of ^{47}Ca or ^{49}Ca induced in a sample relative to that induced in a standard, combined with precise determination of the mass of total calcium in the sample and standard, results in a value for the abundance of the stable isotope in the sample in relation to its abundance in the standard. Abundance changes in samples following administration of enriched stable isotopes may thus be measured by neutron activation analysis combined with total calcium estimation to give data in terms of specific activity (% dose/Gm element) of the stable isotope administered.

In this investigation the methods used in determining ^{46}Ca and ^{48}Ca abundance levels in biological samples were evaluated, employing these stable isotopes as tracers of calcium.

Parallel kinetic studies in man and animals were performed to assess the precision of the stable isotopic tracer technique compared with a radioactive tracer method.

II. MATERIALS AND METHODS

A. Determination of ^{46}Ca and ^{48}Ca Abundance

1. Isolation of Calcium from Biological Samples

A double oxalate precipitation was modified from methods described by Clark and Collip (1925) and Kolthoff and Sandell (1960). The hot precipitation of calcium oxalate at pH 4.0 results in a coarse, easily washed precipitate with low levels of contamination by sodium or magnesium (Kolthoff and Sandell, 1960).

a. *Apparatus*

All glass-ware was acid-washed and rinsed in distilled water. Distilled water was used for all solutions and for all washing procedures.

b. *Reagents*

1. HCl (Dupont) 10 N was used to ensure pH of less than 3.0 in urine samples to dissolve any calcium complexes.
2. Ammonium oxalate 4.0 %. 40 Gm. of ammonium oxalate (Mallinckrodt) was dissolved in 1 litre of water making a nearly saturated solution.
3. Ammonium hydroxide 20 %. 200 ml of ammonium hydroxide (Dupont) was diluted to 1 litre with water.

c. *Sample Preparation*

(i) Urine

Urine collections of 300 to 500 ml volume were acidified to pH 3.0 with concentrated HCl. A 20 ml aliquot was kept for EDTA titration of the total calcium content of the sample.

(ii) Bone

After ashing at 500° C for 3 to 4 hours bone ash was dissolved in 5 N HCl and diluted appropriately with water to give a calcium concentration approximating that of the urine samples.

A quantitative precipitation was not necessary, as the object was only to secure a minimum of 5 to 10 mg of pure calcium as carbonate from each sample precipitated.

d. *Precipitation and Ashing of Calcium Oxalate*

Acidified urine and bone ash solutions of 500 to 800 ml volume in 1500 ml Pyrex beakers were heated in a fume hood to 80° to 90° C. At this temperature 50 ml of 4.0 % ammonium oxalate was added to each sample with stirring. 20 % ammonium hydroxide was then added dropwise with stirring until a cloudy white precipitate formed. pH was adjusted to 4.0 and the sample set aside to cool for $^1/_2$ hour.

The contents of each beaker were filtered on Munktell's 007 ash-free paper in Buchner funnels using suction. An additional 500 ml of water was used to wash the filtered precipitate. After drying, the filter paper containing precipitated calcium oxalate was ashed in a muffle furnace at 500° C for 2 hours.

The calcium carbonate resulting from the first precipitation and ashing procedure was then dissolved in 2N HCl and washed into a Pyrex beaker, and after dilution to 500 ml, the entire hot precipitation, filtration and ashing procedure was repeated.

The calcium carbonate from the second precipitate was transferred to Pasteur pipettes the ends of which were sealed with Parafilm.

The small polyethylene capsules used to contain 25 mg of calcium carbonate for activation analysis were easily filled by placing the narrow end of the Pasteur pipet inside the capsule and tapping the pipet gently while the capsule rested on a balance.

e. *Removal of Sodium During Isolation of Calcium*

To determine the extent to which the precipitation and washing procedures removed sodium, tracer ^{22}Na was added to a series of urine samples and the ^{22}Na in the first and second precipitate was measured in relation to the recovery of calcium.

2. Measurement of Stable Isotopes

a. *Mass Spectrometry Analysis of Abundance*

Analyses were performed upon 100 μGm samples of calcium at the OakRidge National Laboratory.

b. *Neutron Activation Analysis of ^{46}Ca and ^{48}Ca*

An early coded series of samples in which the total calcium content was measured by weight of $CaCO_3$ in the sample activated, was analysed for ^{48}Ca content in three activation analysis laboratories, Sweden (Hägermark, 1963), France (Laverlochere, 1963) and the United States (Rupp, 1963). At the three laboratories, weighed samples of 5 to 10 mg calcium as calcium carbonate in polyethylene containers were activated in a neutron flux from 10^{12} to 6.5×10^{13} n/cm^2/sec for periods of one to 10 minutes depending on the neutron flux of the reactor concerned. The ^{49}Ca produced was counted for 3 to 5 minutes using gamma scintillation spectrometry.

In each case the abundance of ^{48}Ca in the samples was expressed as a ratio relative to a standard with normal abundance.

In all later series ^{48}Ca samples activated at the Union Carbide Research Reactor, Tuxedo Park, N.Y., the calcium content of the activated samples was determined by direct EDTA titration. The Union Carbide Facility is a 5 megawatt swimming pool reactor with a flux of 3×10^{13} n/cm^2/sec. Activation time for 10 mg samples of calcium as carbonate was 1 or 2 minutes. After a 5 minutes delay period to allow decay of very short-lived nuclides, the ^{49}Ca in the samples was measured by gamma scintillation spectrometry. Counting times of 1 to 5 minutes for each sample at 50,000 c/m were employed. Data were collected on punch tape and computed automatically to measure ^{49}Ca by its 3.1 MeV peak.

Typical γ ray spectra recorded during counting periods on a sample and a standard of calcium carbonate illustrate the peaks due to other nuclides.

Samples containing increased abundance levels of ^{46}Ca were prepared in the same manner used for ^{48}Ca. Twenty-five mg of $CaCO_3$ was placed in a quartz container with a capacity of 1 ml. Each container was then tightly wrapped in aluminium foil. All the samples and standards from two human studies were placed in an aluminium cylinder 20×6 cm and sent to the Brookhaven National Laboratory Upton, N.Y. for activation. The container was placed in a flux of 1.4×10^{13}/cm^2/sec for 7 days.

For counting of the ^{47}Ca in the activated samples a 2 1/2″ well crystal and a multi-channel analyser were used. One week following removal from the reactor the shortlived contaminating isotopes had decayed to a negligible level. The ^{47}Ca activity was now estimated by counting the 1.3 MeV photo-peak. There was reason to believe that activity from other activation products interfered only slightly at this point in time. Three weeks later however (more than 4 ^{47}Ca half-lives) more long-lived contaminations began to predominate; the most important of these seemed to be ^{59}Fe.

For the effective use of ^{46}Ca as a tracer more elaborate counting procedures will probably be required. Stability of the equipment is of great importance; computer analysis of spectra, or radiochemical separations will be needed to confirm the observations made during the decay of these activated samples.

3. Determination of Total Calcium

Calcium content of activated samples and of urine and bone ash solutions was determined using a direct recorded EDTA titration procedure.

4. Calculation of Stable Isotope Dosage

Addition of an enriched stable isotope to a quantity of calcium e.g. the exchangeable body pool, increases the abundance in that pool in proportion to the mass of the isotope added. The abundance increase above the normal level may then be expressed in terms of per cent dose stable isotope per gram natural calcium. In the case of ^{46}Ca (abundance 0.0032 %) one gram of natural calcium contains .032 mg of ^{46}Ca. Addition of as little as .32 mg of 100 % abundance ^{46}Ca would raise the abundance level in one gram of calcium ten times *above* the normal level. As the isotope is diluted by exchange and removal processes acting upon this theoretical one gram pool, the increase of abundance level *above normal* at any point in time is proportional to the specific activity of the isotope (% dose ^{46}Ca/Gm calcium).

^{48}Ca has a normal abundance of .179 % so that 1 gram of natural calcium contains 1.79 mg ^{48}Ca. Increasing the abundance level in a 1 gram pool by ten times above normal requires a dose of 17.9 mg of 100 % enriched ^{48}Ca.

The choice of isotope and enrichment used, is thus governed by expected pool size, natural abundance, and the available enrichment of the isotope in relation to the total mass of natural calcium in the dose, an important consideration when low enrichments are used.

B. Kinetic Studies with ^{46}Ca and ^{48}Ca

1. Parallel Kinetic Studies with ^{47}Ca and ^{48}Ca in Man

Urine was collected from two human subjects following intravenous injection of a tracer dose of ^{47}Ca and ^{48}Ca as its 10.4 % enrichment in the form of $CaCl_2$ in sterile water. The dose administered was checked by counting the ^{47}Ca activity in the syringe before and after injection. Each patient received 20 μc ^{47}Ca combined with 4.0 mg of ^{48}Ca as its 10.4 % enrichment. Subject H 1 was a 20 year old normal male with a knee injury, and Subject H 2 was a 48 year old woman with osteoporosis. There were no restrictions of diet or activity.

The ^{47}Ca specific activity of the urine samples was determined by counting ^{47}Ca activity of the entire sample in a large well counter followed by determination of total calcium in the sample by an EDTA titration method.

^{48}Ca abundance in the samples was determined using both mass spectrometry and neutron activation analysis of pure calcium isolated as $CaCO_3$. Total calcium of the activated samples was determined by weighing the 25.0 mg of $CaCO_3$ submitted for activation.

The specific activity of both ^{47}Ca and ^{48}Ca was expressed as % dose per gram calcium.

2. Parallel Kinetic Studies with ^{47}Ca and ^{48}Ca in Rats

Five female Wistar rats fed a normal diet were each injected intraperitoneally with 25 μc ^{47}Ca combined with 2.0 mg ^{48}Ca as its 39.7 % enrichment in the form of $CaCl_2$ in 2 ml sterile water. The dose given each animal was determined by weighing the syringe used.

At 7 days the animals were killed with ether, and the incisors, femora, and tibiae were removed. The 4 incisors, 4 shafts of the 4 bones, and the 8 ends of the long leg bones, as well as the remainder of the rat were ashed at 500° C for 12 hours.

The resulting ash was dissolved in 2 N HCl and diluted to an appropriate volume with water. The total calcium and the ^{47}Ca activity of an aliquot of each ash solution was measured to give specific activity of ^{47}Ca in each sample as % dose ^{47}Ca/Gm calcium.

Calcium as calcium carbonate was isolated from each solution by double oxalate precipitation and 25 mg aliquots of $CaCO_3$ were placed in sealed polyethylene capsules for activation analysis of ^{48}Ca content at the Union Carbide Reactor.

Total calcium in the activated samples was measured by direct EDTA titration of the contents dissolved in HCl.

3. Kinetic Studies with ^{48}Ca in Man

Two human subjects were injected intravenously with tracer doses of ^{48}Ca. Subjects B.N. and D.M. were 32 year old physicians in good health who continued normal activities and consumed a normal diet during the study.

^{48}Ca was given as the 7 % enrichment in solution as $CaCl_2$. B.N. received 2.94 mg calcium containing .206 mg ^{48}Ca. D.M. received 2.64 mg calcium containing .185 mg ^{48}Ca.

Calcium was isolated by oxalate precipitation from timed urine collections during the week following injection. Samples of 25.0 mg $CaCO_3$ were then activated by neutron bombardment for one week at the Brookhaven National Laboratory, and the ^{48}Ca abundance was then determined as described above.

C. **Normal Abundance of ^{48}Ca**

The abundance of ^{48}Ca in samples of human bone, rat bone, human urine, and a Primary Calcium Standard (Mallinckrodt 4071) was determined by neutron activation analysis of calcium isolated as $CaCO_3$. The total calcium of the activated samples was determined by direct EDTA titration to study the range of ^{48}Ca abundance in such diverse samples.

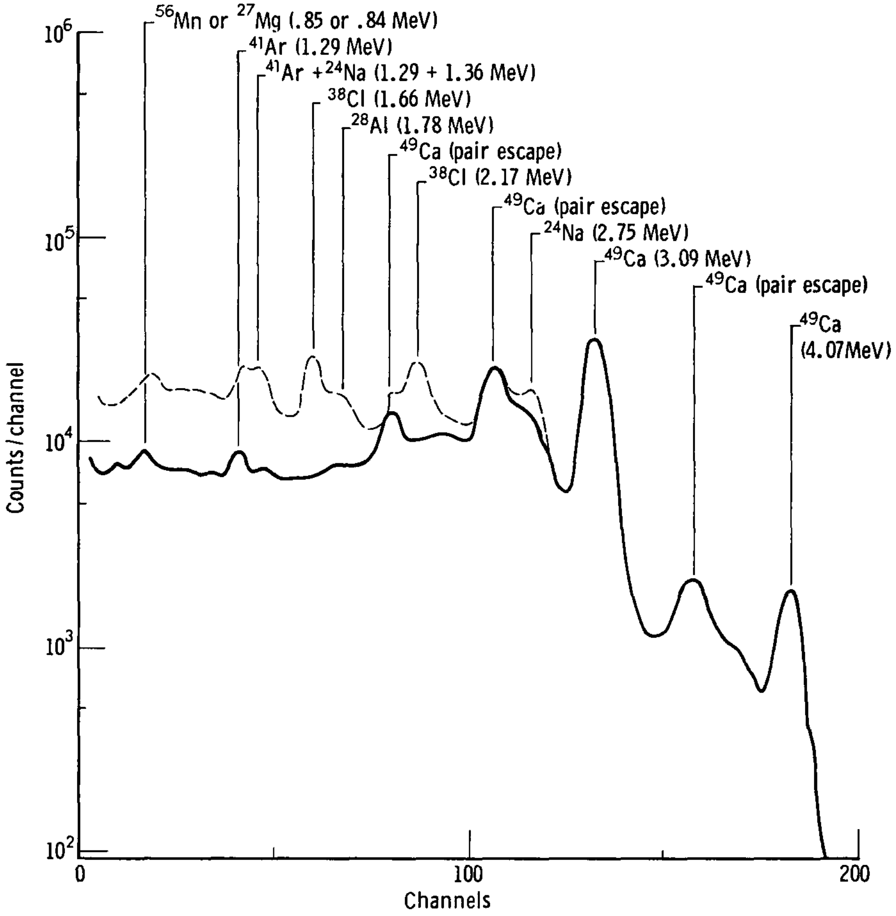


FIGURE 10 Gamma ray spectrum of 10 mg sample of calcium isolated from human urine (normal abundance) shown by thin line, compared to spectrum of 10 mg of standard calcium (Primary Standard Calcium Carbonate, Mallinckrodt 4071) shown by the dark line.

III. RESULTS

A. Determination of Abundance Levels of ^{46}Ca and ^{48}Ca

1. Isolation of Calcium from Biological Samples

The double oxalate precipitation and ashing procedure isolated calcium from urine and bone samples as calcium carbonate of sufficient purity that no significant interferences from other elements were encountered in mass spectrometric analysis or neutron activation analysis in any of the four laboratories concerned. Comparison of the gamma ray spectra

TABLE XX **Removal of Sodium during Oxalate Precipitation of Calcium from Urine**

Per cent Sodium Remaining in Sample					Per cent Total Calcium Remaining in Sample			
Sample	A	B	C	D	A	B	C	D
First Precipitation	0.79	0.84	1.84	1.32	87	94	85	95
Second Precipitation	0.004	0.003	0.004	0.005	81	84	81	85

of a standard and a sample of calcium isolated from urine demonstrates the relative purity of the samples (Fig. 10).

Table XX shows calcium and ^{22}Na recovery during a typical double precipitation. The hot precipitation and washing was very effective in removing sodium which is the most important source of interference (as ^{24}Na) in the activation analysis of biological samples.

Although ^{24}Na did not interfere with the counting of ^{49}Ca due to the differences in their gamma ray energies, the removal of sodium before activation markedly lessened the over-all activity of the samples and simplified the postradiation counting and handling procedures.

2. Abundance Measurement of ^{48}Ca by Mass Spectrometry and Neutron Activation Analysis

The abundance of ^{48}Ca in calcium samples from two patients injected with ^{47}Ca and ^{48}Ca was determined by neutron activation analysis in three laboratories. Mass spectrometry was also used to estimate abundance in some of the samples (Table XXI). The values are expressed as a ratio (per cent) of the ^{48}Ca abundance of enriched samples to the ^{48}Ca abundance of calcium isolated from pre-injection urine samples.

The discrepancies noted in these results could have been due to the fact that total calcium values were derived from the weight of $CaCO_3$ in the activated samples. Differences in the true value of calcium mass by this means related to temperature variations during ashing could thus affect the true value for ^{48}Ca abundance.

In all later studies performed, total calcium was determined directly by EDTA titration of activated samples to improve the precision of total calcium and thus the estimation of abundance levels of both ^{46}Ca and ^{48}Ca.

TABLE XXI ^{48}Ca Abundance in Urine Samples (per cent of normal ^{48}Ca Abundance)
Patient H-1 (L. H.)

	Neutron activation analysis			Mass spectrometry
Sample	Sweden	France	United States	United States
I	176	163	177	180
II	157	136	137	148
III	113	121	123	120
Patient H-2 (S.A.)				
SA 1*	97	100.		
SA 2*	101	100.	100.	100.
SA 3	148	151	156	144
SA 4	178	186	204	179
SA 5	132	136		
SA 6	124.5	129		
SA 7	124.5	125		
SA 8	122.5	120	105	120

* SA 1 and SA 2* — Pre-injection urine calcium.

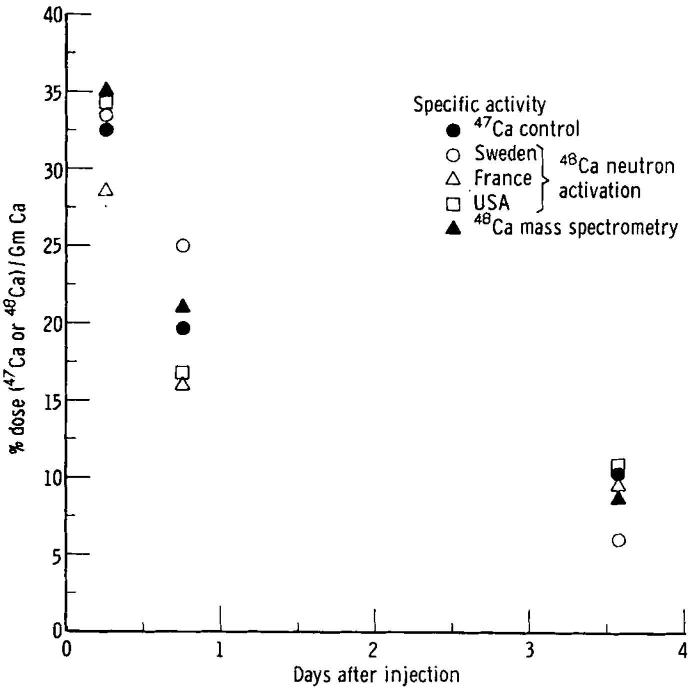


FIGURE 11

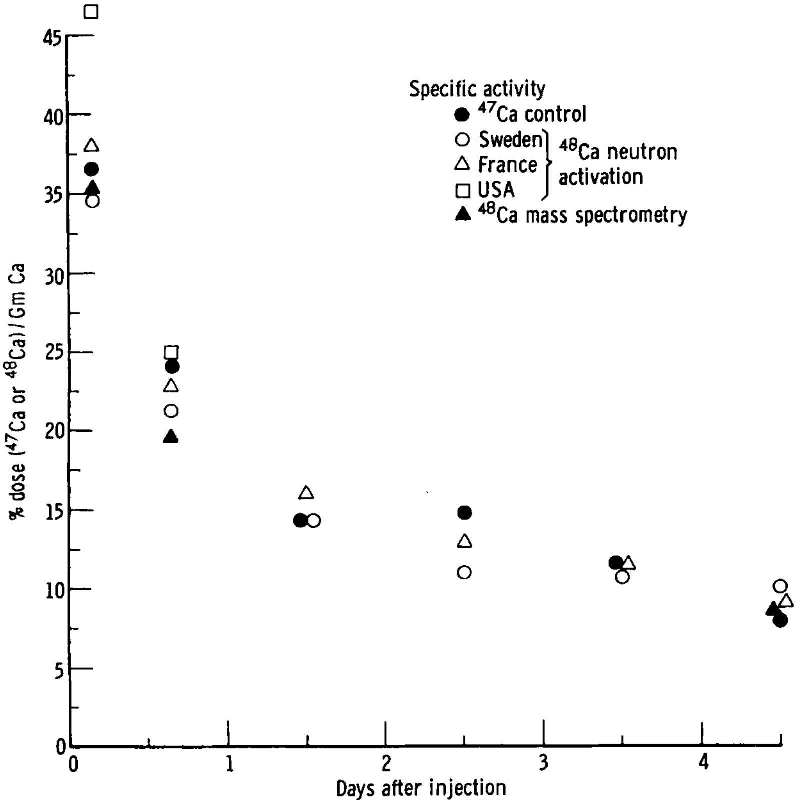


FIGURE 12

B. Kinetic Studies with ^{46}Ca and ^{48}Ca

1. Parallel Kinetic Studies with ^{47}Ca and ^{48}Ca in Man

The results of parallel kinetic studies using ^{47}Ca and ^{48}Ca in 2 human subjects are shown in Figures 11 and 12. The agreement between specific activity values derived from the two isotopes was satisfactory. Errors related to weighing the 25.0 mg samples of $CaCO_3$ which were activated in these 2 cases could account for some of the discrepancies noted, but the validity of the use of stable calcium isotopes as tracers is apparent.

2. Parallel Kinetic Studies with ^{47}Ca and ^{48}Ca in Rats

The relation between specific activity of ^{47}Ca and ^{48}Ca in the samples of calcium isolated from the teeth, shafts, and ends of long bones, as well

TABLE XXII Relation between ^{47}Ca and ^{48}Ca Specific Activity in Calcium isolated from Rat Bones and Teeth, and Whole Body

Whole Body	^{47}Ca Specific Activity	^{48}Ca Specific Activity
No. 16	42.8	41.5
17	44.3	40.3
18	—	—
19	—	—
20	42.0	39.0
4 Teeth		
16	—	—
17	45	42.2
18	47.5	41.6
19	44.5	48.5
20	—	—
4 shafts of long bone (2 femurs+2 tibias)		
16	22.4	24.2
17	26.8	26.3
18	26.2	25.3
19	19.4	24.0
20	20.6	19.
8 ends of long bones (2 femurs and 2 tibias)		
16	55.5	58.5
17	53.5	51.0
18	58.2	59.5
19	49.0	53.0
20	55.2	56.0

as the whole rat one week after injection is shown in Table XXII. The two independent methods of determining bone specific activity show good agreement.

3. Kinetic Studies with ^{46}Ca in Man

The results of one of the dilution studies are shown in Figure 13 where the curve represents the ^{46}Ca abundance in urine calcium relative to ^{46}Ca abundance in 4 standard samples. Normal abundance of 0.0032 % ^{46}Ca is taken as 100 %. For comparison the per cent dose ^{46}Ca administered per Gm calcium in the samples is shown in the right of the figure.

The presence of many other nuclides in samples activated for such a long period of time complicated the measurement of ^{47}Ca in these

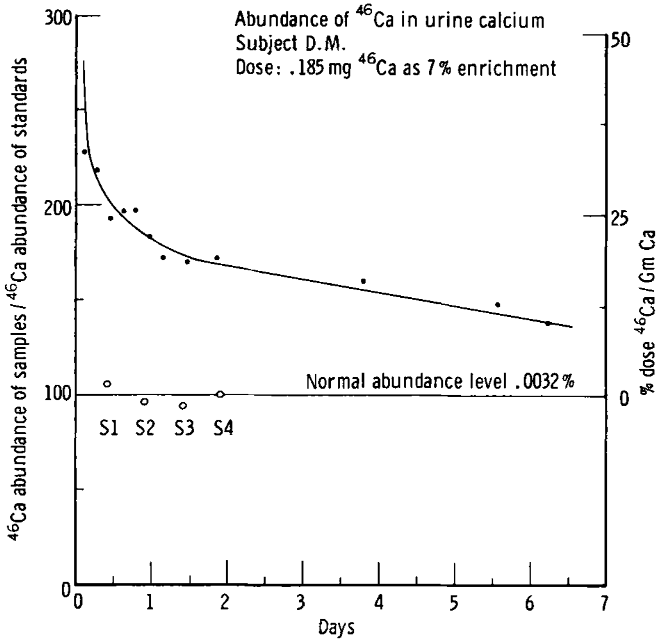


FIGURE 13

samples. A simple post-radiation radiochemical separation using a ^{47}Ca spike would simplify the counting procedure and improve precision.

Despite these counting difficulties the pattern of the curve in the case shown demonstrates that ^{46}Ca may also be used as a stable tracer of calcium.

C. Normal Abundance of ^{48}Ca

Table XV shows the ^{48}Ca abundance level of samples isolated from bone, and urine, expressed as a ratio of the abundance of ^{48}Ca in samples of Primary Standard Reagent Grade $CaCO_3$ (Mallinckrodt 4071).

Little variation in abundance of ^{48}Ca is apparent in these biological samples.

IV. DISCUSSION

A. Determination of Abundance Levels of ^{46}Ca and ^{48}Ca

Stable isotopes of calcium can be used as tracers in vivo. Mass spectrometry provides a precise method for determination of abundance levels of ^{46}Ca, and ^{48}Ca in samples of calcium which can be easily isolated from biological materials by oxalate precipitation. Samples of 1 mg are sufficient for such analysis.

^{46}Ca and ^{48}Ca may be activated to produce radioactive ^{47}Ca and ^{49}Ca by neutron bombardment of samples from 5 to 10 mg in mass. ^{48}Ca has a large cross-section for neutron capture so that very short (1 minute) exposure to a neutron flux activates sufficient ^{49}Ca so that precise counting can be performed in a short period (1—2 minutes). The short half life (8 · 7 minutes) of ^{49}Ca is not a problem as the sample can be counted immediately upon ejection from the reactor tube. The high gamma ray energy (3.1 MeV) of ^{49}Ca removes the problem of interference from other isotopic species which may contaminate the sample.

Precision in ^{49}Ca estimation in the contents of sealed polyethylene capsules, plus precision in the estimation of total calcium in the same samples by EDTA titration make possible accurate abundance level determinations in samples collected following injection of enriched stable ^{48}Ca.

^{46}Ca has a much lower abundance, and a much smaller cross-section for neutron capture than ^{48}Ca, so that samples of 5—10 mg calcium must be exposed to a neutron flux for a period of several days to activate enough ^{47}Ca for precise counting. This long period of activation produces other isotopes which make counting of the ^{47}Ca more difficult. Despite some difficulties with this part of the assay, it was possible to construct specific activity curves using calcium isolated from urine of human subjects injected with enriched ^{46}Ca. A radiochemical post-radiation separation of calcium from the sample would improve the precision of this technique.

The present relatively high costs of both enriched isotopes, as well as the cost of activation can be expected to decrease markedly with development of further applications of stable calcium tracers.

B. Kinetic Studies with ^{46}Ca and ^{48}Ca

Parallel studies conducted in human subjects and in animals show that ^{48}Ca and ^{47}Ca are similarly distributed in the body. Specific activity data derived independently showed good agreement of values for the two isotopes in both urine and bone over a wide range.

Higher enrichments of ^{48}Ca are available, but are relatively more expensive. In the 2 human subjects the 10.4 % enrichment of ^{48}Ca used meant that each patient received approximately 40 mg of natural calcium containing the 4.0 mg of ^{48}Ca which was the actual tracer. This however given slowly intravenously would not be expected to cause any untoward clinical reactions, and did not seem to affect the observations of specific activity compared to data derived from the parallel dose of ^{47}Ca.

The tracer doses of ^{46}Ca given as its 7 % enrichment were contained in less than 3 mg of natural calcium per dose. This amount of natural calcium should not affect equilibrium in small animals.

C. Normal Abundance of ^{48}Ca

Since isotopic effects are known to exist where there are large differences in the mass of the various isotopic species of an element, the reliability of tracer studies with stable isotopes depends upon knowledge of the normal abundance of the isotopes in the system to which the tracer is added.

Corless (1964) has reported differences in ^{48}Ca/^{40}Ca ratio of ± 5—8 % in samples of calcium isolated from sea water, shell, rock and human teeth. Corless used neutron activation analysis with measurement of the ^{49}Sc decay product of ^{49}Ca, and EDTA titration of total calcium in another aliquot. The complex separation procedures used could conceivably introduce errors in such a method for abundance determination.

The studies of normal urine calcium, rat bone, human bone, and standard calcium carbonate done by this method with direct EDTA titration of calcium in the activated samples did not show the large variations of ^{48}Ca/^{40}Ca abundance reported by Corless (1964).

V. CONCLUSIONS

Changes in the abundance of stable isotopes following the administration in enriched form as tracers may be followed as a measure of the specific activity of the stable tracer.

Parallel studies in man and in animals with ^{47}Ca and ^{48}Ca show that those two tracers are similarly distributed, and that specific activity of the stable isotope is similar to that of the radioactive isotope in samples of calcium isolated from both bone and urine.

Kinetic studies with ^{46}Ca in man show that this stable tracer may be used in the same way as ^{48}Ca. It possesses advantages in its much lower abundance, and refinements in post-radiation treatment of samples can be expected to increase the precision of this method.

Studies with ^{48}Ca show that the normal abundance of this isotope, and the increase following tracer doses can be quantitated by neutron activation analysis combined with total calcium determination performed directly on the activated samples.

CHAPTER IV

Estimation of the Exchangeable Calcium Pool in Children Using ^{48}Ca

I. INTRODUCTION

The use of radioactive calcium isotopes in the investigation of calcium kinetics in man has been largely limited to studies in adults. Relatively few studies have been performed in children although the observations of Bronner (1956) using ^{45}Ca and Hoffenberg (1964) using ^{47}Ca have drawn attention to differences in calcium kinetics related to growth. Extension of these investigations has been prevented by a reluctance to use radioactive tracers in young children—particularly normal children.

The treatment of certain bone diseases and the assessment of the efficacy of such treatment are of most importance during the growth period. For this reason a method for the study of calcium kinetics in children would be of considerable value. Parameters derived from the specific activity curves include the exchangeable calcium pool, the rate of excretion in urine, and the rate of removal of calcium from the pool by other mehanisms—fecal loss and deposition in bone.

The enriched stable isotopes of calcium (^{46}Ca and ^{48}Ca) can be used as tracers in such studies. Abundance changes produced by injection of the enriched isotope are a measure of specific activity in samples from the system following injection. The principles of the use of stable calcium isotopes have been described, and the validity of the method demonstrated in parallel studies with ^{47}Ca and ^{48}Ca in man and animals (See Chapters II, III).

This report describes the use of stable ^{48}Ca in studies of calcium metabolism in 10 children.

II. MATERIALS AND METHODS

A. Subjects

The 10 children studied were patients on the Children's Orthopaedic Service of the Hospital for Special Surgery. Their ages, weight, ideal weight for age, and diagnoses are shown in Table XXIV. Case H-3 (osteogenesis imperfecta tarda) and case H-5 (bilateral Legg-Perthes disease) were

TABLE XXIII Children studied using ^{48}Ca as a tracer

Case No.	Name	Age (years)	Sex	Weight (Kg)	Ideal Weight (Kg)	Diagnosis
H-3	R.R.	12.25	F	26.9	38.3	Osteogenesis imperfecta tarda, healing fracture of the femur; confined to bed.
H-4	R.D.	10.0	F	49.	31.9	Meningomyelocoele; soft tissue surgery of feet.
H-5	M.C.	7.5	M	30.5	25.9	Bilateral Legg-Perthes disease; confined to bed.
H-6	D.H.	12.6	M	37.5	42.2	Post-polio paresis of leg; soft tissue surgery.
H-7	A.H.	10.8	F	44.5	35.7	Mild spastic paraplegia; soft tissue surgery.
H-8	M.D.	7.25	M	37.2	24.5	Mild spastic paraplegia; soft tissue surgery.
H-9	A.P.	8.2	F	19.2	26.4	Congenital hypoplasia of leg muscles possibly post-polio; soft tissue surgery.
H-10	S.G.	8.2	F	31.	26.4	Mild spastic paraplegia; soft tissue surgery.
H-11	S.F.	10.2	M	22.7	31.9	Mild spastic hemiplegia; soft tissue surgery.
H-12	D.B.	8.5	F	42.2	27.7	Mild spastic quadriplegia; soft tissue surgery.
Mean ± S.D.		(9.5 ± 1.9)		(34.1 ± 8.8)	(31.1 ± 6.)	

the only cases with recognized disturbances of bone metabolism. The remaining 8 cases were admitted for a variety of soft-tissue surgical procedures, usually tendon transfers, or release of muscle contractures, in the management of moderate locomotor handicaps secondary to polio or cerebral palsy.

The ages ranged from 7.25 to 12.6 years with a mean age of 9.5 ± 1.9 years. Weights ranged from 19.2 to 49.0 Kg with a mean weight of 34.1 ± 8.8 Kg. The ideal weights for age and sex (Nelson, 1954) are also listed in Table XXIII for comparison.

During the studies the children consumed a normal hospital diet. Except for cases 3 and 5, who were confined to bed, the children were usually in later stages of convalescence from surgery, and were quite active.

B. **Procedure**

1. Tracer Dosage

a. *Preparation of Stable ^{48}Ca Tracer Dose*

Pure $CaCO_3$ containing ^{48}Ca as its 39.72 % enrichment was dissolved in 1 N HCl and diluted with water by weight to make a solution containing 5.0 mg natural calcium per Gm as $CaCl_2$ at pH 5.0, which was sterilized by autoclaving. The solution contained 1.98 mg ^{48}Ca per Gm. Two ml disposable plastic syringes were weighed before and after injection of the tracer to allow precise quantitation of the dose given.

b. *Calculation of Dose*

The normal abundance of ^{48}Ca in nature is 0.179 % of total calcium. One Gm natural calcium thus contains 1.79 mg ^{48}Ca.

The dose given each child was about 4.0 mg ^{48}Ca (exact dose in each case listed in Table XXV). For example, this amount would raise the abundance in a 1 Gm calcium pool by 223 per cent *above the normal abundance level*, corresponding to an abundance of $\frac{4.0+1.79}{10} = .579\ \%$ ^{48}Ca.

c. *Administration of Isotope*

As the specific activity measurements were based upon the ^{48}Ca abundance levels in timed urine samples collected in the period following injection, all subjects voided just prior to receiving the 2 Gm dose of ^{48}Ca solution by injection in the antecubital vein. Since the dose of 2 Gm contained a total of 10 mg of natural calcium, it was given slowly over a period of not less than one minute.

2. Urine Collection

The subjects voided at their usual times; each entire specimen was placed in a clean acid-washed glassbottle and labelled with the date and time period of the collection. A record of voiding times was also maintained. In this way the occasional loss of a specimen did not compromise the specific activity determination on the samples preceding or following it.

C. **Methods** (Methods are described in detail in Chapter III)

1. Isolation of Calcium from Urine

All samples were acidified to pH <3.0 with 10 N HCl and shaken thoroughly. Because of the frequency of voiding in some subjects, samples were combined to give collection periods approximating 12 hours for the first two or three days, and 24 hours thereafter for 5 to 8 days. An aliquot was kept aside for total calcium titration. Calcium was isolated using a double oxalate precipitation.

2. ^{48}Ca Abundance Measurement

The ^{48}Ca content of ten mg samples of Ca as $CaCO_3$ isolated from urine was determined by neutron activation analysis at the Union Carbide Research Reactor, Tuxedo Park, N.Y., through the cooperation of Dr. W. Wahl (1964) and Mr. H. Nass (1964). Total calcium of the activated sample was determined by EDTA titration, permitting calculation of ^{48}Ca abundance in samples and standards.

3. Calculations

The $^{48}Ca/^{40}Ca$ ratio of each sample relative to the $^{48}Ca/^{40}Ca$ ratio of standards was a measure of the increase of abundance in the samples due to the tracer dose of enriched ^{48}Ca.

For example:

Where a dose of 4.0 mg ^{48}Ca had been given, the increase in ^{48}Ca abundance if 100 % of the dose were in 1 Gm calcium would be 223 % *above* the normal level of .179 % ^{48}Ca.

If a sample from that patient revealed a ^{48}Ca abundance level which was 122 % of that in the standards, then the 22 % increase over normal related to the 223 % increase due to 100 % dose meant that the specific activity of that sample was $\frac{22}{223} \times 100$ or 10 percent dose ^{48}Ca/GmCa.

III. RESULTS

A. ^{48}Ca Abundance Increase in Samples

There were no difficulties in the measurement of ^{48}Ca content of the activated samples as the induced radioisotope ^{49}Ca ($^1/_2$ 8.7 minutes).

The abundance levels of ^{48}Ca in samples were expressed as a percent of the ^{48}Ca abundance of standards used for each series of samples activated. The ^{48}Ca abundance of calcium standards (Primary Calcium Standard $CaCO_3$, Mallinckrodt 4071) was a constant value within each series activated; however, the apparent values varied between series, since they were related to weighed standards used routinely at the Reactor. Thus the apparent ^{48}Ca abundance of our internal standards would be .182 for one series (see Table XV) and as high as .195 in a later series activated, yet the source of calcium was the same. Relating all sample abundance values to the standard source of calcium (Pri-

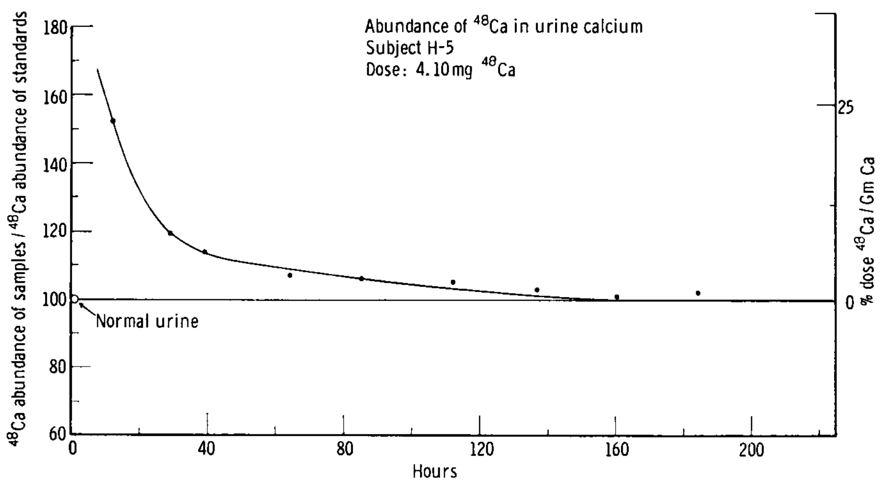


FIGURE 14

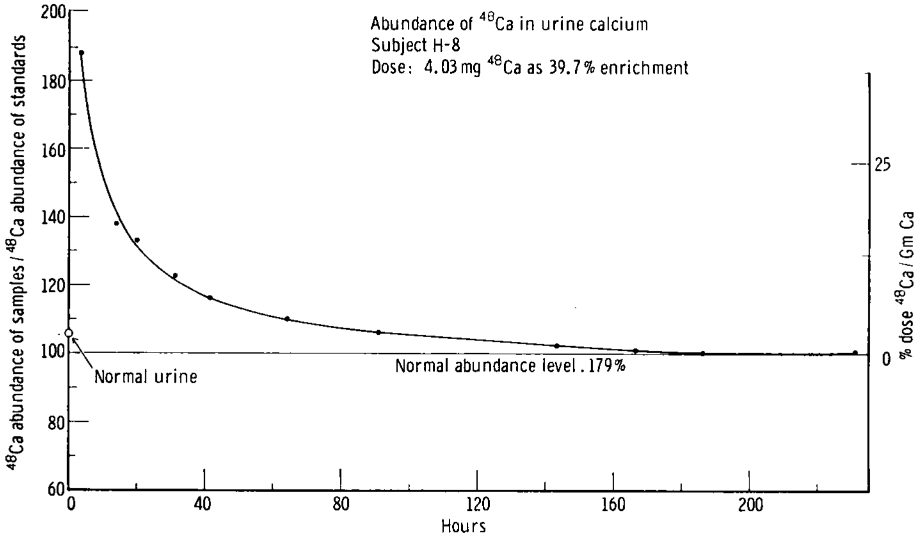


FIGURE 15

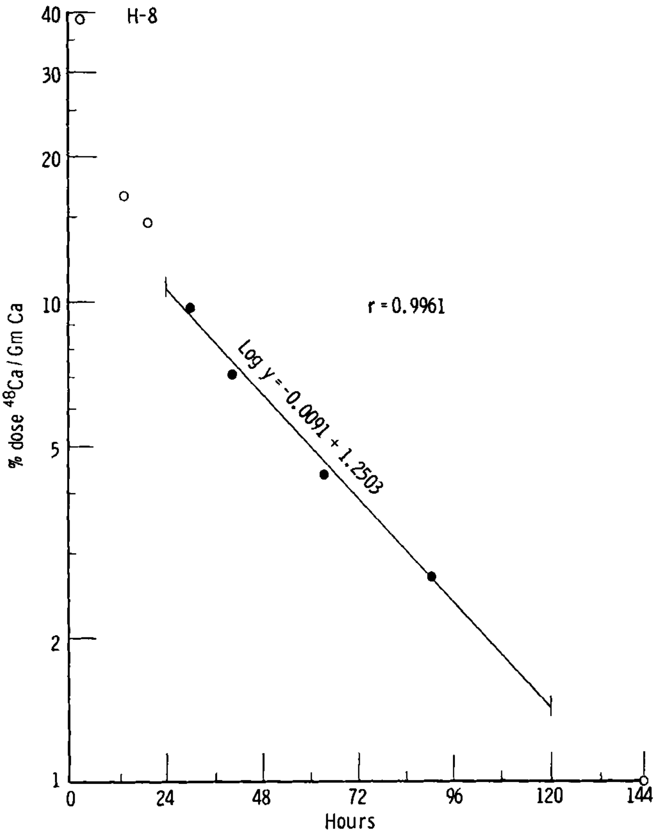


FIGURE 16

mary Standard $CaCO_3$, Mallinckrodt) and giving this the value .179 % ^{48}Ca abundance (White and Cameron, 1948) permitted comparison between cases in terms of percent ^{48}Ca abundance increase.

Table XXIV contains the data of each case studied, listing the dose of ^{48}Ca administered, the percent abundance increase that 100 % dose would have produced in 1 Gm, and the % abundance increase noted in calcium isolated from urine at the mid-points of urine collections both prior to injection (Time 0), and in the several days following injection.

Figures 14 (case H-5), and 15 (case H-8) illustrate the change of ^{48}Ca abundance in urine calcium with time. The curve demonstrates the return to normal abundance levels by 5 to 6 days after injection.

These representative curves also demonstrate normal ^{48}Ca abundance prior to injection compared with the level reached by the decline of the specific activity curve. The 100 % abundance level is that of the Primary Standard Calcium (Mallinckrodt 4071) used as a reference standard in each series of samples activated.

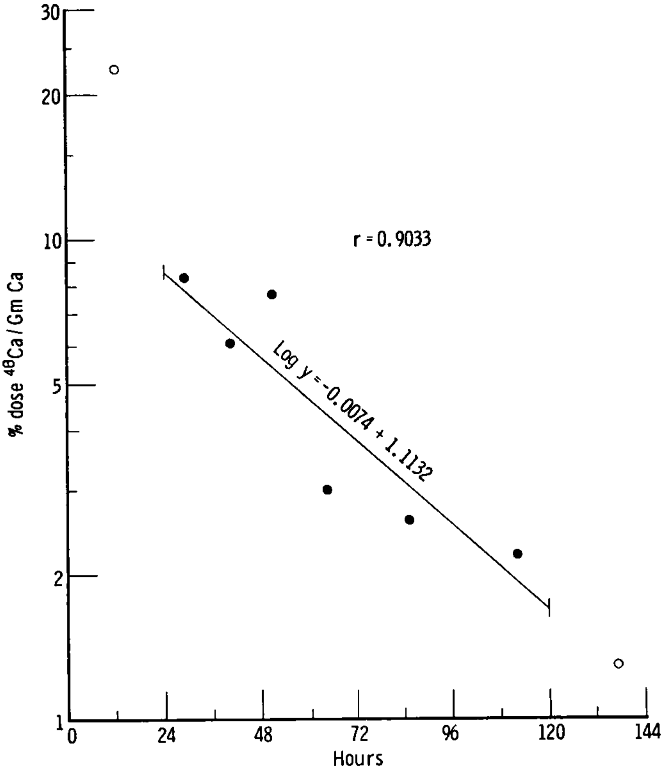


FIGURE 17

B. ^{48}Ca Specific Activity

When the ^{48}Ca specific activity of urine samples (Table XXIV) was plotted logarithmically against time, there appeared to be a sharp fall of specific activity during the first 15 to 20 hours, followed by a monoexponential fall of specific activity in the period between 24 and 120 hours.

Using least squares analysis, and employing the values for specific activity between 24 and 120 hours, equations were found to represent this portion of the curve for each case. The equations and correlation coefficients are shown in Table XXIV. Examples are shown in Figures 16 and 17.

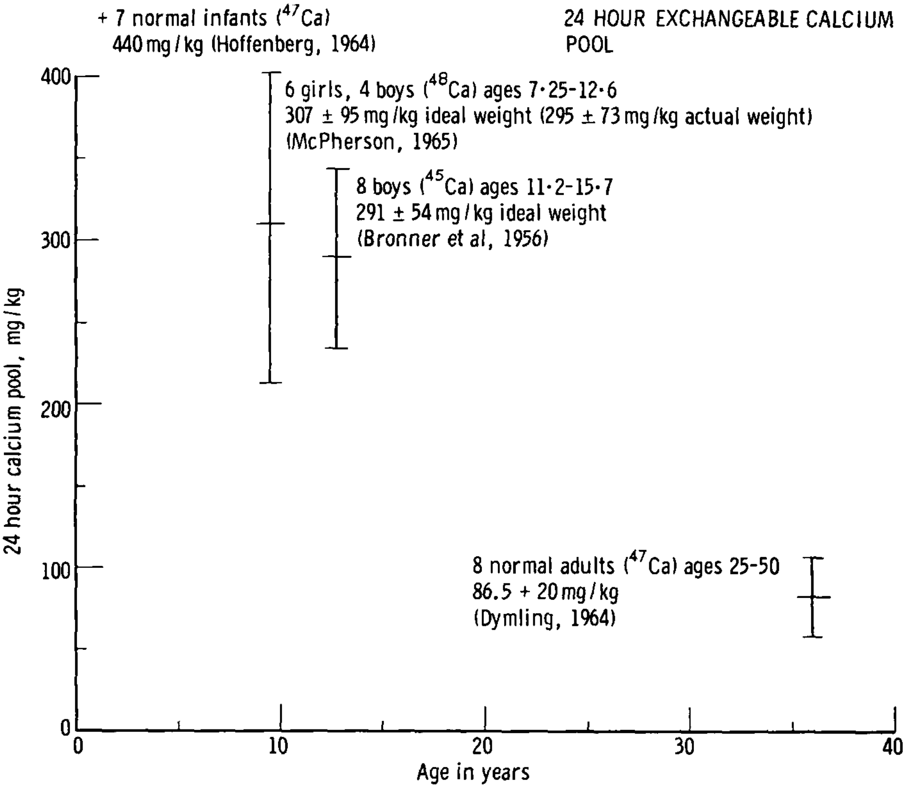


FIGURE 18

C. Estimation of 24 Hour Exchangeable Calcium Pool

The reciprocal of the 24 hour ^{48}Ca specific activity expressed in Gm was used as a measure of the exchangeable calcium pool. The specific activity value used was taken from the equation derived by least squares analysis on the values between 24 and 120 hours.

The pool sizes for each case as absolute values in Gm and as they relate to body weight are given in Table XXIV. The mean exchangeable calcium pool size in the 10 cases studied was 9.34 ± 2.29 Gm. When related to ideal body weight the pool size was 307 ± 95 mg/Kg and when related to actual body weight the pool size was 295 ± 73 mg/Kg.

D. ^{48}Ca Excretion

The rate of renal excretion of calcium in mg per hour was relatively constant in each subject. Because some urine samples had to be excluded from calculations because they were lost, or their times were uncertain, the rate of calcium excretion per day was taken from the mean rate per hour in all urine samples in each subject. The mean calcium excretion rate per day is shown in Table XXIV.

It is apparent that if 10 % of dose had been excreted in feces over 7 days, this would have been equal to 4.0 mg ^{48}Ca$\times$.1$=$.4 mg ^{48}Ca. This amount of stable isotope would have added only .4 mg of ^{48}Ca to the total fecal calcium (approximately 800 mg/day). The 5,600 mg of calcium in 7 day collection of feces contains 5.6$\times$1.79 mg ^{48}Ca$=$10 mg ^{48}Ca. Therefore the abundance would have been increased by only 4.0 % above normal. This would represent a ^{48}Ca specific activity of about 1.8 % dose ^{48}Ca/Gm. As the estimation of total fecal ^{48}Ca would have depended upon measurement of such a low specific activity, the ^{48}Ca loss in feces was not determined. A larger dose of ^{48}Ca would however permit such determinations to be made, with a degree of precision proportional to the total dose of ^{48}Ca.

TABLE XXIV Data from ^{48}Ca kinetic studies in ten children

Case No.	Total Dose ^{48}Ca (mg)	Abundance for 100 % dose in 1 Gm	Time Hours (x)	Per cent of normal abundance	Specific Activity % dose ^{48}Ca/ Gm Ca (y)	24 hour Specific Activity % dose ^{48}Ca/Gm	24 hour Pool size Gm (Reciprocal of 24 hour Spec. activity)	24 hour $\frac{\text{Pool (mg)}}{\text{Ideal wt (Kg)}}$	Mean urine Ca loss Mg/day
H-3	3.42 mg	291 %	2.0	136	18.9	10.5	9.46	248	59.
			18.1	130	15.7				
			48.	112	6.3				
			73.	106	3.14				
			95.8	104	2.1				
			131.	100	0				
			143.	102	1.05				
			180.5	102	1.05				
			204.5	99	0				
			$\log y = -.010\,x + 1.2632$		$r = -.9927$				
H-4	3.82 mg	314 %	0	98	0	15.9	6.27	196	46.
			25.5	125	11.7				
			45	120	9.4				
			57	111	4.9				
			68	113	6.8				
			81	110	4.7				
			93	98	0				
			135	101	.4				
			164	104	1.8				
			$\log y = -.0131\,x + 1.5150$		$r = -.8497$				

Case No.	Total Dose ^{48}Ca (mg)	Abundance for 100 % dose in 1 Gm.	Time Hours (x)	Per cent of normal abundance	Specific Activity % dose ^{48}Ca/ Gm Ca (y)	24 hour Specific Activity % dose ^{48}Ca/Gm	24 hour Pool size Gm (Reciprocal of 24 hour Spec. Activity)	24 hour $\frac{\text{Pool (mg)}}{\text{Ideal wt (Kg)}}$	Mean urine Ca loss Mg/day
H-5	4.10 mg	330 %	0	100	0	8.6	11.6	450	206.
			12	152	22.6				
			29	119	8.3				
			39	114	6.1				
			51	118	7.8				
			64	107	3.0				
			85	106	2.6				
			112	105	2.2				
			137	103	1.3				
			160	101	.4				
			184	102	.9				
			log y=—.0074 x+1.1132		r=—.9033				
H-6	4.04 mg	326 %	0	106	0	8.4	11.9	283	86.
			1	211	49.				
			5	141	18.2				
			9	132	14.2				
			28	113	5.8				
			39	111	4.9				
			52	106	2.7				
			71	104	1.8				
			81	109	4.0				
			131	103	1.3				
			181	98	0				
			196	98	0				
			log y=—.0221 x+1.6312		r=—.8148				

Case No.	Total Dose ^{48}Ca (mg)	Abundance for 100 % dose in 1 Gm	Time Hours (x)	Per cent of normal abundance	Specific Activity % dose ^{48}Ca/ Gm Ca (y)	24 hour Specific Activity % dose ^{48}Ca/Gm	24 hour Pool size Gm (Reciprocal of 24 hour Spec. Activity)	24 hour $\frac{\text{Pool (mg)}}{\text{Ideal wt (Kg)}}$	Mean urine Ca loss Mg/day
H-7	4.14 mg	331 %	0	100	0	8.8	11.4	319	185.
			1	131	13.4				
			4	164	27.7				
			10	153	22.9				
			29.5	121	9.1				
			41	116	6.9				
			51	109	3.9				
			114	104	1.7				
			157	103	1.3				
			181	102	.9				
			203	100	.0				
			log y=—.0081 x+1.1375		r=—.9630				
H-8	4.03 mg	325 %	0	106	2.5	10.8	9.3	380	80.
			3.5	187	38.7				
			14.	138	16.9				
			20	133	14.7				
			31	122	9.8				
			41.5	116	7.1				
			64	110	4.4				
			91	106	2.6				
			143	102	1.				
			166	101	.5				
			186	100	0				
			231	101	.5				
			log y=—.0091 x+1.2503		r=—.9961				

Case No.	Total Dose ^{48}Ca (mg)	Abundance for 100 % dose in 1 Gm	Time Hours (x)	Per cent of normal abundance	Specific Activity % dose ^{48}Ca/ Gm Ca (y)	24 hour Specific Activity % dose ^{48}Ca/Gm	24 hour Pool size Gm (Reciprocal of 24 hour Spec. Activity)	24 hour $\frac{\text{Pool (mg)}}{\text{Ideal wt (Kg)}}$	Mean urine Ca loss Mg/day
H-9	4.10 mg	329 %	0	102	1.	12.5	8.0	304	57.
			4	245	63.				
			17	150	21.9				
			29	121	9.2				
			53.5	119	8.2				
			89	110	4.3				
			100	104	1.9				
			126	105	2.1				
			144	104	1.7				
			170	100	0				
			190	100	0				
			214	98.4	0				
			$\log y = -.0093x + 1.3186$		$r = -.9055$				
H-10	4.12 mg	330 %	0	98.5	0	16.1	6.2	236	186.
			5	226	54.5				
			14	155	24.				
			24	137	16.1				
			61	112	5.2				
			76	118	7.8				
			88	107	3.0				
			99	106	2.6				
			123	103	1.3				
			136	104	1.7				
			160	101	.5				
			179	97.	0				
			$\log y = -.0101x + 1.4481$		$r = -.9176$				

Case No.	Total Dose ^{48}Ca (mg)	Abundance for 100 % dose in 1 Gm	Time Hours (x)	Per cent of normal abundance	Specific Activity % dose ^{48}Ca/ Gm Ca (y)	24 hour Specific Activity % dose ^{48}Ca/Gm	24 hour Pool size Gm (Reciprocal of 24 hour Spec. Activity)	24 hour $\frac{\text{Pool (mg)}}{\text{Ideal wt}}$ (Kg)	Mean urine Ca loss mg/day
H-11	4.09 mg	328 %	0			15.8	6.4	198	105.
			3	252	66.5				
			15	171	31.				
			27	133	14.5				
			47	127	11.8				
			69	115	6.6				
			89	117	7.4				
			101	109	3.9				
			141	112	5.3				
			159	106	2.6				
			$\log y = -.0069x + 1.3638$		$r = -.9338$				
H-12	4.18 mg	333 %	11	133	14.1	7.8	12.8	460	106.
			17	133	14.1				
			23	129	12.4				
			33	118	7.7				
			42	115	6.5				
			55	114	6.0				
			67	107	3.0				
			88	106	2.6				
			113	106	3.0				
			143	105	2.0				
			156	103	1.0				
			180	100	0				
			$\log y = -.0059x + 1.0346$		$r = -.8603$				

IV. DISCUSSION

A. ^{48}Ca Abundance Increase

Neutron activation analysis of ^{48}Ca and determination of total calcium in activated samples permits estimation of the abundance of ^{48}Ca in calcium isolated from urine. Changes in the abundance level following the injection of enriched ^{48}Ca can be expressed as ^{48}Ca specific activity.

In the cases in Table II where pre-injection urine calcium was activated, the ^{48}Ca abundance level in such control samples is similar to that of the standards representing 100 % normal abundance (.179 % ^{48}Ca). In the late period after injection ^{48}Ca abundance levels of samples in all cases approached the normal value.

B. Specific Activity

The specific activity of heavy calcium isotopes in urine is representative of the specific activity in serum (see Chapter II). ^{48}Ca specific activity determined on 5 to 10 mg samples of natural calcium isolated from timed urine collections is therefore a reflection of the specific activity in serum. Plotted logarithmically against time, the ^{48}Ca specific activity curves in the 10 children studied appeared to be mono-exponential between 24 and 120 hours after the injection of the tracer.

C. Estimation of 24 Hour Exchangeable Calcium Pool

The rapid fall of specific activity during the initial period following injection represents dilution of the tracer in the body fluids and the exchangeable portion of the skeletal calcium. In the open 2 compartment model used by Dymling (1964) two spaces are distinguished. The first (S_I) is located in body fluids and its size is based upon the dilution of tracer at 1 hour. The second (S_{II}) represents the exchangeable portion of the skeletal calcium, and it is said to reach equilibrium at 3 to 4 days.

These two compartments are in equilibrium with each other at a point in time between 12 and 36 hours after injection. If the two compartments are taken as representing the exchangeable calcium pool; an estimate of the size of the pool may be taken from the dilution of a calcium tracer at 24 hours.

The reciprocal of the 24 hour ^{48}Ca specific activity, taken from the equation representing the period 24 to 120 hours, was used as a measure of the exchangeable calcium pool in the 10 children studied.

The exchangeable calcium pool (mean 9.34 ± 2.29 Gm) in each of the 10 cases was related to ideal body weight, to give a value of 307 ± 95 mg/Kg. This value for the mean age 9.55 years was similar to the 291 ± 54 mg/Kg ideal body weight calculated from ^{45}Ca data in 8 boys with a mean age of 11.4 years (Bronner, 1956). Hoffenberg (1964) found the exchangeable calcium pool in 7 normal infants to be 440 mg/Kg. On the other hand, data calculated from the 24 hour ^{47}Ca specific activity in 8 normal adults (Dymling 1964) showed an exchangeable pool size in adults of 86.5 ± 20 mg/Kg body weight at the mean age of 36. years.

The exchangeable calcium pool in mg/Kg body weight is thus five times greater in infants than in adults and 3 to 4 times greater in children than in adults.

Figure 18 shows mean pool sizes ± 1 S.D. in the series discussed above.

D. ^{48}Ca Excretion

Because of the occasional loss of specimens during the early part of collection periods, calculation of cumulative urinary ^{48}Ca excretion was not performed. However, it was found that the calcium excretion rate per hour as determined on each sample collected fell within a narrow range for each child, although the rate varied considerably between cases. The mean calcium excretion rate can be related to the specific activity curve to estimate the per cent of dose lost in urine during the period of study.

Fecal loss could not be estimated in these cases because of the relatively low dosage of tracers employed. The small per cent of dose in feces would disturb the abundance level of the relatively large amount of fecal calcium very slightly. Large doses of more highly enriched ^{48}Ca would however permit assay of ^{48}Ca in fecal calcium.

V. CONCLUSIONS

Stable ^{48}Ca in enriched form has been used as a tracer in dilution studies of calcium kinetics in 10 children ranging in age from 7.25 to 12.6 years with a mean age of 9.5 years.

Changes in ^{48}Ca abundance levels in samples of calcium isolated from timed urine collections, determined by neutron activation analysis, reflect specific activity in per cent dose/Gm calcium.

The specific activity curves appeared to be mono-exponential between 24 and 120 hours following injection. The 24 hour value derived from equations representing specific activity in this time period was used to calculate the 24 hour exchangeable calcium pool.

The exchangeable calcium pool was 9.34 ± 2.29 Gm, 307 ± 95 mg/Kg ideal body weight, and 295 ± 73 mg/Kg actual body weight in the 10 cases.

The 24 hour exchangeable calcium pool relative to body weight is 5 times as high in infants as in adults, and 3 to 4 times as high in the children studied as in normal adults.

General Discussion

The measurement of stable isotopes by neutron activation and counting of induced radionuclides combined with estimation of total element by chemical means permits the use of certain stable isotopes as tracers.

The use of enriched stable isotopes has been described in studies of iron metabolism (Lowman and Krivit, 1963) and calcium kinetics (McPherson, 1963).

This investigation describes the application of ^{46}Ca and ^{48}Ca as tracers of calcium in man and animals. The abundance of ^{46}Ca and ^{48}Ca is determined in 10 mg samples of calcium using activation analysis and measurement of the induced radioisotopes ^{47}Ca and ^{49}Ca by gamma spectrometry, combined with direct EDTA titration of total calcium in the activated samples. Simple chemical procedures are adequate to remove interfering elements prior to activation of ^{48}Ca.

Studies of the ^{47}Ca specific activity ratio between serum and urine in man failed to demonstrate a biological isotope effect; fractionation of heavy calcium isotopes is not of such degree that it affects calcium tracer studies. The 10 mg samples for activation can thus be isolated from timed urine collections and are representative of the isotopic abundance pattern of serum.

Abundance increase of the stable isotope in samples relative to the abundance increase which the total dose would have produced may be expressed as specific activity of the stable isotope. Parallel studies in man and animals show that the specific activity values of stable (^{48}Ca) and radioactive (^{47}Ca) isotopes are similar.

The dose of stable isotope used is calculated in relation to the expected pool size, length of study, enrichment of isotope available, and the amount of calcium in the dose which may affect equilibrium in the system studied. In this regard the low natural abundance of ^{46}Ca is an advantage.

^{48}Ca used as a tracer in children has permitted estimation of the exchangeable calcium pool 24 hours after injection. Application of stable calcium tracers to the estimation of this as well as other parameters of calcium kinetics in such radiosensitive subjects should lead to an increased understanding of the physiology of skeletal growth.

General Summary and Conclusions

CHAPTER I *Direct EDTA-titration of Calcium and Magnesium in Biological Samples*

1. A direct recorded EDTA-titration procedure permits precise measurement of total calcium and total calcium plus magnesium, giving magnesium by difference, in a variety of biological materials.
2. Phosphate interference during titration is prevented by addition of citrate in excess.
3. The normal range of serum calcium and serum magnesium is very narrow (9.50 ± 0.35 mg% and 2.24 ± 0.21 mg% respectively).
4. Precision in estimation of total calcium in small samples has permitted the use of this method in the determination of the abundance of stable calcium isotopes used as tracers of mineral metabolism, using neutron activation analysis.

CHAPTER II *Heavy Calcium Isotopes as Tracers of Calcium*

1. Differences in blood and urine specific activities could not be demonstrated in three human subjects.
2. The abundance of ^{48}Ca in a variety of biological samples was constant.

CHAPTER III *^{46}Ca and ^{48}Ca as Tracers in Studies of Mineral Metabolism*

1. Procedures for the determination of ^{46}Ca and ^{48}Ca abundance in biological samples using neutron activation analysis and EDTA-titration of total calcium permit the use of enriched isotopes as tracers.
2. Simultanous tracer studies with ^{47}Ca and ^{48}Ca show agreement.

CHAPTER IV *Estimation of the Exchangeable Calcium Pool in Children Using ^{48}Ca*

1. ^{48}Ca in enriched form was used as a tracer in dilution studies of calcium kinetics in 10 children.
2. Changes in ^{48}Ca abundance levels in samples of calcium isolated from timed urine collections, determined by neutron activation analysis, reflected specific activity in per cent dose/Gm calcium.
3. The 24-hour exchangeable calcium pool relative to body weight is 3 to 4 times as high in the children studied as in normal adults.

ACKNOWLEDGEMENTS

Financial support was obtained from the Alfred Österlund Foundation and the Polio Foundation, Sweden; from the U.S. Public Health Service, National Institute of Dental Research (Contract Nr. D-1452), U.S. Public Health Service Training Grant AM-5414, the Atomic Energy Commission (Contract Nr. AT (30-1) 3234), the Josiah Macy, Jr. Foundation, the Whitehall Foundation and the Evelyn Sharp Foundation, United States; from the Medical Research Council of Canada (Graduate Medical Research Fellowship); and from the International Atomic Energy Agency (Contract Nr. 50) Vienna.

Bibliography

ALCOCK, N.: The Determination of Magnesium in Biological Fluids and Tissues by Flame Spectrophotometry. J. Clin. Path. 13: 506, 1960.

— Analytical Methods for Magnesium. Proceedings of the Association of Clinical Biochemists 1: 143, 1961.

ANDREASEN, E.: On the Determination of Magnesium in Serum and Urine by Titan Yellow Dye. Scand. J. Clin. Lab. Invest 9: 138, 1957.

BACHRA, B. N., DAUER, A., SOBEL, A. E.: Complexometric Titration of Micro and Ultramicro Quantities of Calcium in Blood Serum, Urine, and Inorganic Salt Solutions. Clin. Chem. 4, 1958.

BARNARD, A. J., BROAD, W. C., FLASCHKA, H.: The EDTA Titration: Nature and Methods of End-Point Detection. J. T. Baker Chemical Co. Bulletin, 1957.

BARON, D. N., BELL, J. L.: Complexometric Determination of Calcium in Pathological and Physiological Specimens. J. Clin. Path. 12: 143, 1959.

BEALE, R. N., BOSTROM, J. O.: Sensitive Methods for the Titrimetric Microdetermination of Biological Calcium and Magnesium. J. Clin. Path. 16: 252, 1963.

BEAUCHAMP, E. E.: (Personal Communication) Isotopes Development Center Oak-Ridge National Laboratory, OakRidge, Tennessee.

BETHARD, W. F., SCHMITT, R. A., OLEHY, D. A.: Quantitation of Mg, Mn, Az, and Ca in Blood by Neutron Activation Analysis. Radioisotope in Klinik und Forschung, 1963 p. 222.

BETT, I. M., FRASER, G. P.: A Rapid Micro-Method for Determining Serum Calcium. Clin. Chim. Acta 4: 346, 1959.

BLAU, M., SPENCER, H., SWERNOV, J., LASZLO, D.: Utilization and Intestinal Excretion of Calcium in Man. Science 120: 1029, 1954.

BOHUON, C.: Microdosage du Magnésium dans divers milieux Biologiques. Clin. Chim. Acta 7: 811, 1962.

BOONSTRA, C. E., JACKSON, C. E.: The Clinical Value of Routine Serum Calcium Analysis. Annals Int. Med. 57: 963, 1962.

BOWDEN, C. H., PATSTON, V.: Microdetermination of Calcium and Magnesium in Biological Materials. J. Clin. Path. 16: 18, 1963.

BOWEN, W. J., MARTIN, H. L.: Analysis of Serum Magnesium in Presence of Calcium with Chrome Fast Blue B.G. Proc. Soc. Exp. Biol. Med. 101: 734, 1959.

BRANDSTEIN, M. S., CASTELLANO, A., MAZZACAPPA, C.: Determination of Serum Calcium by Means of Flame Photometry. Am. J. Clin. Path. 40: 583, 1963.

BRONNER, F., HARRIS, R. S., MALENTSKOS, C. J., BENDA, C. E.: Studies of Calcium Metabolism: The Fate of Intravenously Injected Radiocalcium in Human Beings. J. Clin. Invest. 35: 78, 1956.

Brush, J. S.: Interaction of Ca^{++} Ions with the Dye Erio SE in Highly Alkaline Solution—Direct Determination of Ca^{++} in Serum. Anal. Chem. 33: 798, 1961.

Buchanan, D. L., **Sampson**, L. T.: Incineration of Fecal Specimens for Radioactivity Measurements. J. Lab. Clin. Med. 59: 169, 1962.

Buckner, B., **Shively**, J. A.: Determination of Serum Calcium by a Chelating Method Using a Spectrophotometer. Am. J. Med. Techn. 21: 269, 1955.

Campbell, W. R.: The Estimation of Calcium and Magnesium. Canad. J. Biochem. Physiol. 35: 1033, 1957.

Carr, M. H., **Frank**, H. A.: Improved Method for Determination of Calcium and Magnesium in Biological Fluids by EDTA Titration. Am. J. Clin. Path. 35: 1033, 1957.

Clark, E., **Collip**, J. B.: A Study of the Tisdall Method for Determination of Blood Serum Calcium with a Suggested Modification. Am. J. Clin. Path. 26: 1157, 1956.

Copp, D. H., **Cheney**, B. A., **Stokov**, N. M.: Simple and Precise Micromethod for EDTA Titration of Calcium. J. Lab. Clin. Med. 61: 1029, 1963.

Corey, K., **Greenberg**, E., **Kenny**, P., **McLaughlin**, J. S., **Martin**, W., **Weber**, D.: Medical Uses of Ca^{47} Second Panel Report. Technical Report Series No. 32. IAEA Vienna 1964.

Corless, J. T.: Variations in the Ca^{48}/Ca^{40} Ratio in the Natural Environment. Ph.D. Thesis Dept. of Chemistry Massachusetts Inst. of Technology 1963.

Corless, J. T., **Winchester**, J. W.: Variations in the Ratio ^{48}Ca/(Total Ca) in the Natural. Proceedings of Symposium on Isotope Mass Effects in Chemistry and Biology, IAEA Vienna 1963, p. 317.

Diehl, H., **Ellinghoe**, J.: Azo Dyes as Indicators for Calcium and Magnesium. Anal. Chem. 32: 1120, 1960.

Dow, E. C., **Stanbury**, J. B.: Strontium and Calcium Metabolism in Metabolic Bone Disease. J. Clin. Invest. 39: 885, 1960.

Dymling, J. F.: Calcium Kinetics in Osteopenia and Parathyroid Disease. Acta Med. Scand. Vol. 175. Supplement 408, 1964.

Eldjarn, L., **Nygaard**, O., **Sveinsson**, S. L.: The Determination of Serum Calcium. A Comparison of the Method of Clark and Collip and Titration with EDTA. Scand. J. Clin. and Lab. Invest. 7: 92, 1955.

Fales, F. W.: A Micromethod for the Determination of Serum Calcium. Clin. Chem. 10: 549, 1964.

Fingerhut, B., **Miller**, H.: Direct Determination of Calcium in Icteric Serum. Clin. Chem. 9: 361, 1963.

Fiske, C. H., **Logan**, M. A.: The Determination of Calcium by Alkalimetric Titration. II The Precipitation of Calcium in the presence of Magnesium, Phosphate, and Sulfate, with Application to the Analysis of Urine. J. Biol. Chem. 93: 211, 1931.

Friedman, H. S., **Rubin**, M. A.: Clinical Significance of the Mg: Ca Ratio: Techniques for the Determination of Calcium and Magnesium in Biological Fluids. Clin. Chem. 1: 125, 1955.

Foss, O. P., **Andersen**, B.: An Apparatus for Rapid Micro Titration of Calcium in Serum with EDTA. Scand. J. Clin. Lab. Invest. 10: 437, 1958.

Foss, O. P.: Direct Titration of Calcium in Urine with EDTA. Scand. J. Clin. Lab. Invest. 11: 211, 1959.

VanFossan, D. D., **Baird**, E. E., **Tekell**, G. S.: A Simplified Flame Spectrophotometric Method for Estimation of Magnesium in Serum. Am. J. Clin. Path. 31: 368, 1959.

Garner, K. J.: Colorimetric Determination of Magnesium in Plasma or Serum by Means of Titan Yellow. Biochem. J. 40: 828, 1946.

GOLBYN, R. L., HILDEBRAND, G. P., REILLEY, C. N.: Direct Titration of Calcium in Blood Serum. J. Lab. Clin. Med. 50: 498, 1957.

GIESE, W., COMAR, C. L.: Existence of Non-Exchangeable Calcium Compartments in Plasma. Nature 202: 31, 1964.

GJESSING, L. R.: Direct Stepwise Photo-electric EDTA Titration of Calcium and Magnesium in Serum, Cerebrospinal Fluid and Urine. Scand. J. Clin. Lab. Invest. 11: 190, 1958.

GOVAERTS, J.: Studies in Calcium Urinary Excretion with the Aid of Radio Calcium. Am. J. Physiol. 159: 542, 1949.

GORR, J.: Personal Communication. Brookhaven National Laboratory, Upton, New York.

GREENBLATT, J., HARTMAN, S.: Determination of Calcium in Biological Fluids. Anal. Chem. 23: 1709, 1951.

HÄGERMARK, B.: Personal communication. Division of Nuclear Chemistry. Royal Institute of Technology, Stockholm, Sweden.

HANNA, S.: Plasma Magnesium in Health and Disease. J. Clin. Path. 14: 410, 1961.

HARPER, T. A.: A Rapid Micro Quantitative Method for Estimating Serum Calcium. Nature 183: 232, 1959.

HEAGY, F.: The Use of Polyvinyl Alcohol in the Colorimetric Determination of Magnesium in Plasma or Serum by Means of Titan Yellow. Canad. J. Research 26: 295, 1948.

HEANEY, R. P., WHEDON, G. D.: Radiocalcium Studies of Bone Formation Rate in Human Metabolic Bone Disease. J. Clin. Endocr. 18: 1246, 1958.

HEVESY, GEORGE: Adventures in Radioisotope Research. The Collected Papers of George Hevesy. Vol. 1, p. 25. Pergamon Press, New York, 1962.

HILDEBRAND, G. P., REILLEY, C. N.: New Indicator for Complexometric Titration of Calcium in Presence of Magnesium. Anal. Chem. 29: 258, 1957.

HIRT, B., EPSTEIN, B.: Isotope Composition of Natural Calcium. Helv. Physica. Acta 37: 179, 1964.

HOFFENBERG, R., HARRIS, F., BACK, E.: Ca^{47} in the Investigation of Rickets. Medical Uses of Ca^{47}—Second Panel Report. IAEA, Technical Report Series No. 32 Vienna, 1964.

HORNER, W. H.: Determination of Calcium in Biological Material. J. Lab. Clin. Med. 45: 951, 1955.

HUNTER, G.: The Micro-Determination of Magnesium in Presence of Known Amounts of Calcium. Analyst 83: 93, 1958.

JONES, J. C., MCGUCKIN, W. F.: Complexometric Titration of Calcium and Magnesium by a Semi-automated Procedure. Clin. Chem. 10: 767, 1964.

KELLERMAN, G. M., DALE, N. E.: Observations upon the Determination of Serum and Urine Calcium with the Dye Plasmacorinth B. Med. J. Australia 47: 931, 1960.

KINGSLEY, G. R., ROBNETT, A.: New Dye Method for Direct Photometric Determination of Calcium. Am. J. Clin. Path. 27: 223, 1957.

KILBRICK, A. C., ROSS, M., ROGERS, H.: Microdetermination of Calcium in Blood Serum by Direct Titration. Proc. Soc. Exp. Biol. Med. 81: 353, 1952.

KOCH, H. J. JR., SMITH, E. R., SHIMP, N. F., CONNOR, I.: Analysis of Trace Elements in Human Tissues. I — Normal Tissues. Cancer 9: 499, 1958.

KOLTHOFF, SANDELL: Textbook of Inorganic Analysis, 1962.

KOVACS, G. S., TARNOKY, K. E.: A Simple and Rapid Method for the Simultaneous Determination of Calcium and Magnesium from the Same Sample of Blood Serum. J. Clin. Path. 13: 160, 1960.

KRAMER, B., TISDALL, F. F.: A Simple Technique for the Determination of Calcium and Magnesium in Small Amounts of Serum. J. Biol. Chem. 47: 475, 1921.

KRANE, S. M., BROWNELL, G. L., STANBURY, J. B., CORRIGAN, H.: The Effect of Thyroid Disease on Calcium Metabolism in Man. J. Clin. Invest. 35: 874, 1956.

KRITCHEVSKY, D.: Deuterium Isotope Effects in Chemistry and Biology. Annals N.Y. Acad. Science 84: 573, 1960.

LAVERLOCHÈRE, J.: Personal communication. Centre D'Études Nucléaires de Grenoble, France.

LEHMANN, J.: A Photometric Method for Direct Titration of Calcium in Serum with EDTA. Scand. J. Clin. Lab. Invest. 5: 203, 1953.

LEWIS, L. L., MELNICK, L. M.: Determination of Calcium and Magnesium with EDTA. Anal. Chem. 32: 38, 1960.

LIKINS, R. C., CRAVEN, D. L.: An Apparent Difference in the Renal Clearance of Ca 45 and Ca 40. Nature 188: 592, 1960.

LOKEN, H., TEAL, J. S., EISENBERG, E.: Flame Spectrophotometry of Calcium with Reversed Oxyacetylene Flame. Anal. Chem. 38: 875, 1963.

LOWMAN, J. T., KRIVIT, W.: New In Vivo Tracer Method with the Use of Non-Radioactive Isotopes and Activation Analysis. J. Lab. Clin. Med. 61: 1042, 1963.

MALMSTADT, H. V., GOHRBANDT, E. C.: Automatic Spectrophotometric Titrations: Determination of Milligram Quantities of Thorium. Anal. Chem. 26: 442, 1954.

MALMSTADT, H. V., HADJIOANNOU, T. P.: Automatic Titration of Calcium and Magnesium in Blood Serum. Clin. Chem. 5: 50, 1959.

— Improvements on the Automatic Determination of Micro Amounts of Serum Calcium. Clin. Chem. 9: 423, 1963.

MCALLISTER, H. C. JR., YARBRO, C. L.: A Rapid Complexometric Titration of Serum Calcium. Clin. Chem. 6: 52, 1960.

MCDOWELL, C. A.: Editor: Mass Spectrometry. McGraw Hill, New York, 1963.

MCINTYRE, I.: The Flame Spectrophotometric Determination of Calcium in Biological Fluids and Isotopic Analysis of the Errors in the Kramer-Tisdall Procedure. Biochem. J. 67: 164, 1957.

MCLEAN, F. C., BUDY, A.: Radiation, Isotopes and Bone. Academic Press, New York, 1964.

MCPHERSON, G. D.: Stable Ca^{48} as a Tracer in Studies of Mineral Metabolism in Man. Medical Uses of Ca^{47}; Second Panel Report. IAEA, Technical Report Series No. 32, Vienna, 1964.

— Medical Uses of Calcium 47: First Panel Report. IAEA, Technical Report Series No. 10, Vienna, 1962.

MILLER, E. J., NEUMAN, W. F., BAZERQUE, P. M.: Difference in Specific Activity of Calcium in Blood and Urine in Dogs. Am. J. Physiol. 206: 755, 1964.

MILLER, E. J., NEUMAN, W. F.: Role of Renal Tissue Calcium in Alteration of Urinary Specific Activity. Proc. Soc. Ex. Biol. Med. 116: 1082, 1964.

MONTGOMERY, R. D.: The Estimation of Magnesium in Small Biological Samples by Flame Spectrophotometry. J. Clin. Path. 14: 400, 1961.

MORI, K.: Direct Micro-complexometric Analysis of Calcium in Biological Materials. Arch. Biochem. Biophys. 83: 552, 1959.

MOSCOVIC, E. A.: Choice of a Routine Laboratory Method for Serum Magnesium. Harper Hospital Bulletin 20: 90, 1962.

NIER, A. O.: The Isotopic Composition of Calcium, Titanium, Sulfur, and Argon. Phys. Rev. 53: 282, 1938.

NORDIN, B. E. C.: The Estimation of Free Calcium in the Urine and its Relevance to Calculus Formation. Brit. J. Urology, 31: 404, 1960.

ORANGE, M., RHEIN, H. C.: Micro-estimation of Magnesium in Body Fluids. J. Biol. Chem. 189: 379, 1951.

PAPPENHAGEN, A. R., JACKSON, H. D.: Modified Method for Determination of Serum Calcium in the Presence of Magnesium Using Cal-Red. Indicator Applications of Metal Buffers and Metal Indicators in Biochemistry. Clin. Chem. 6: 582, 1960.

RAAFLAUB, J.: In: Methods of Biochemical Analysis Vol. III. Ed. David Glick Interscience Publishers, New York, 1956.

REIFFEL, L., STONE, C. A.: Neutron Activation Analysis of Tissue: Measurement of Na, K, and P in Muscle. J. Lab. Clin. Med. 49: 286, 1957.

REILLEY, C. N., SCHMID, R. W.: Principles of End-Point Detection in Chelometric Titrations Using Metallochromic Indicators. Anal. Chem. 31: 887, 1959.

ROSE, G. A.: Determination of the Ionized and Ultrafilterable Calcium of Normal Human Plasma. Clin. Chim. Acta 2: 227, 1957.

RUPP, A. F.: Personal communication. Isotopes Development Center. OakRidge National Laboratory, OakRidge, Tennessee.

SADEK, F. S., REILLEY, C. N.: A Survey of the Application of Visual Indicators for Chelometric Calcium Determination in Serum. J. Lab. Clin. Med. 54: 621, 1959.

VAN SCHOUWENBERG, J. C. H.: Micro EDTA Titration of Calcium. Anal. Chem. 32: 709, 1960.

SCHWARZENBACH, G., BIEDERMAN, W., BANGAERTER, A.: Complexone VI New Simple Titrimetric Method for Determination of Water Hardness. Helv. Chim. Acta 29: 881, 1946.

SELIGSON, D.: An Automatic Pipetting Device and Its Application in the Clinical Laboratory. Am. J. Clin. Path. 28: 200, 1957.

SOBEL, A. E., HANOK, A.: A Rapid Method for the Determination of Ultramicro Quantities of Calcium and Magnesium. Proc. Soc. Exp. Biol. Med. 77: 737, 1951.

SOTO, R. J., REJTMAN, A., ROZADOS, I.: Studies of Calcium Metabolism in Thyroid Disorders by Means of Ca 47: Results. Medical Uses of Ca 47—Second Panel Report 1964. Technical Report Series No. 32, Vienna, 1964, p. 86.

SPENCER, R. P., MITCHELL, T. G., KING, E. R.: Neutron Activation of Sodium in Blood Serum. J. Lab. Clin. Med. 50: 46, 1947.

SPRAGUE, S.: Personal communication. Perkin-Elmer Corporation, Norwalk, Connecticut.

STANBURY, J. B.: Personal communication, 1964.

SZYMENDERA, J.: Methods of Chemical Assay of Calcium. Medical Uses of Ca 47—Second Panel Report—Technical Report Series No. 32. IAEA Vienna, 1964.

TORIBARA, T. Y., DEWEY, P. A., WARNER, H.: Flame Photometric Determination of Calcium in Biological Material. III Effect of Low Level Impurities from Ca Oxalate Precipitation. Anal. Chem. 29: 540, 1957.

VEALL, N.: Ca 47 Measurements in Urine and Feces and Chemical Estimation of Calcium in Urine. Medical Uses of Ca 47—First Panel Report. Technical Report Series No. 6. IAEA Vienna, 1962.

VEALL, N., PARSONS, V.: Complexed Calcium and The Evaluation of Tracer Kinetic Studies: A Revised Compartmental Model. Medical Uses of Ca 47—Second Panel Report. Technical Report Series No. 32. IAEA Vienna, 1964.

WHITE, J. R., CAMERON, A. E.: The Natural Abundance of Isotopes of Stable Elements Abundance of Calcium Isotopes. Phys. Rev. 74: 991, 1948.

WIESTER, M. J., WHITLA, S. H., GOLDSMITH, R.: Evidence for Multiple Calcium Pools in Blood of Dogs. Nature 197: 1170, 1963.

WILKINSON, R. H.: A Micro Method for Serum Calcium and Serum Magnesium. J. Clin. Path. 10: 126, 1957.

WOOLLEN, J. W., WALLER, P. G.: An Improved Flame Photometric Method for Determining Calcium in Serum. J. Clin. Path. 12: 149, 1959.

YANIGASAWA, F.: New Colorimetric Determination of Calcium and Magnesium. J. Biochem. 42: 3, 1955.

YOUNG, A., SWEET, T. R., BAKER, B. B.: Simultaneous Spectrophotometric Determination of Calcium and Magnesium. 27: 356, 1955.

ZAK, B., HINDMAN, W. M., FISHER, M.: Spectrophotometric Titration of Serum Calcium and Magnesium. Am. J. Clin. Path. 26: 1081, 1956.

ZETTNER, A., SELIGSON, D.: Application of Atomic Absorption Spectrophotometry to Determination of Calcium in Serum. Clin. Chem. 10: 869, 1964.