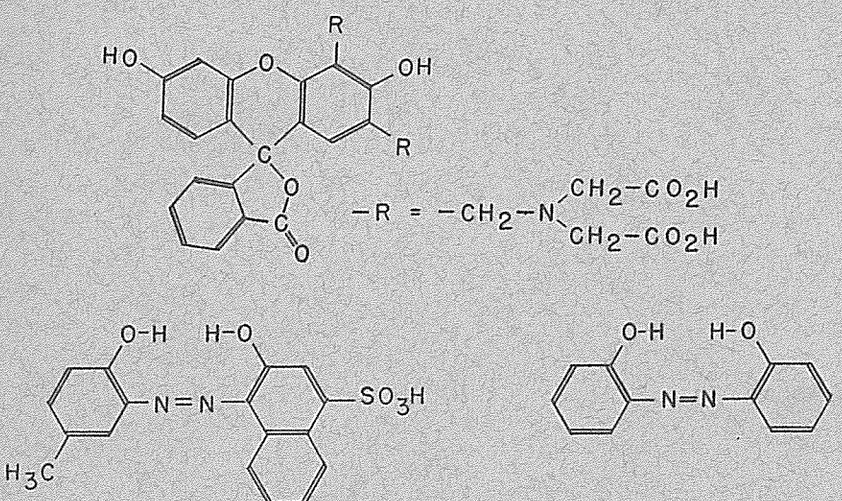
CALCEIN, CALMAGITE, AND o,o′-DIHYDROXYAZOBENZENE TITRIMETRIC, COLORIMETRIC, AND FLUOROMETRIC REAGENTS FOR CALCIUM AND MAGNESIUM



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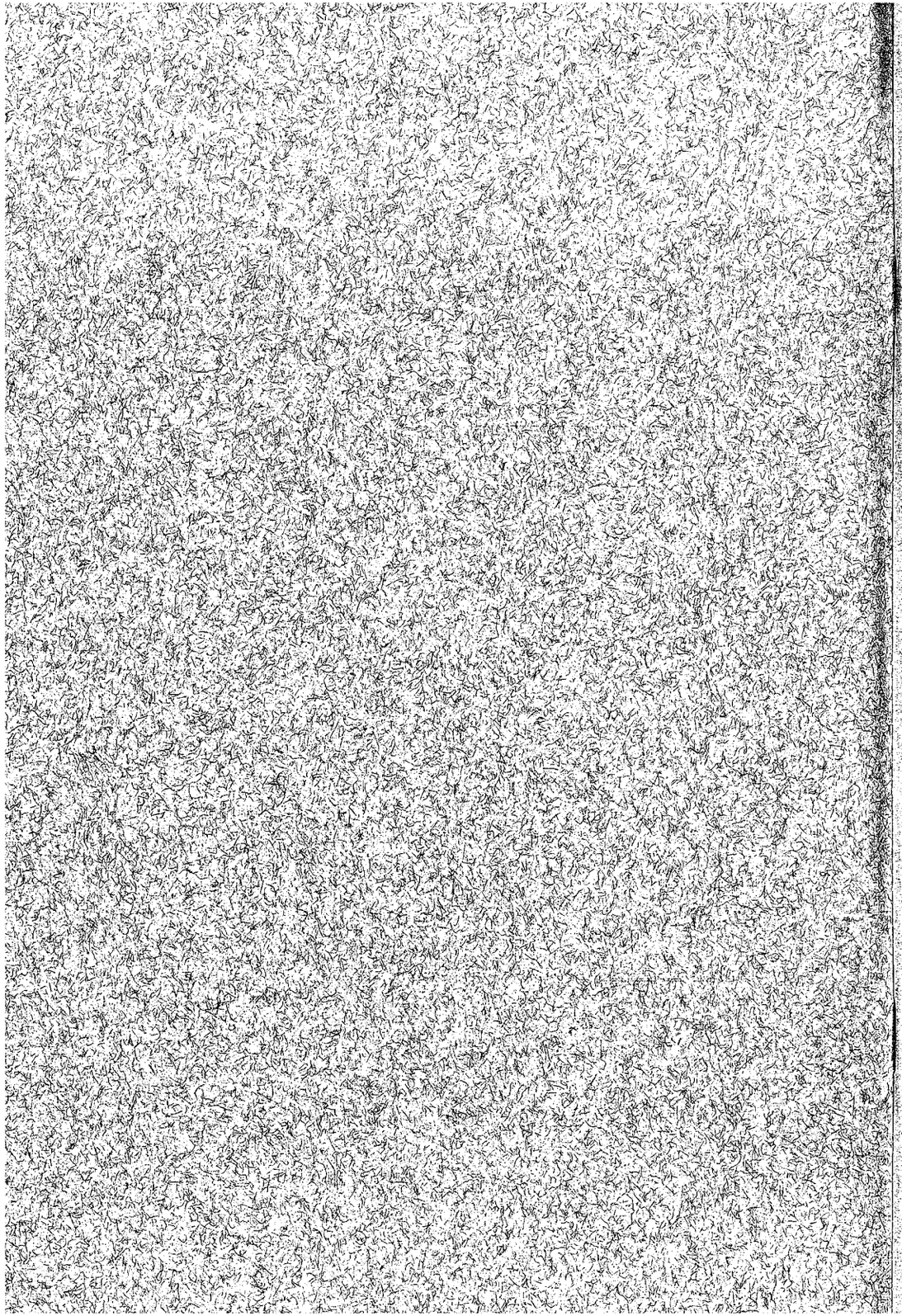

Calcein, Calmagite,
And *o,o'*-Dihydroxyazobenzene.
Titrimetric, Colorimetric and Fluorometric
Reagents for Calcium and Magnesium

Printed in the United States of America

CALCEIN, CALMAGITE, AND *o,o'*-DIHYDROXYAZOBENZENE. TITRIMETRIC, COLORIMETRIC AND FLUOROMETRIC REAGENTS FOR CALCIUM AND MAGNESIUM

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PREFACE

Calcium and magnesium are the fifth and eighth most abundant of the elements in the crust of the earth. Both are intimately involved in the well being of the plants and the animals and both are tied into our industrial complex in diverse and essential ways. For elements as important as these, it is astonishing that methods for their determination were until only a very few years ago slow and cumbersome. In the oxalate precipitation procedure for calcium the prior removal of all metals but the alkalies and magnesium is required, and for limestone and biological tissue this means that preliminary separation of silica and of the R_2O_3 group (iron, aluminum, titanium and manganese). And before magnesium can be precipitated as the double ammonium phosphate, it is necessary to remove calcium also. These preliminary separations, involving as they do, precipitation, digestion and filtration, together with the operations needed to complete the determinations volumetrically or gravimetrically, are very time consuming. No wonder then, that chemists grasped eagerly at the EDTA method for calcium plus magnesium when it was introduced in 1948, and subsequently kept probing for a method for calcium only, and for a method for magnesium in the presence of calcium. Calcein, 1956, provided an answer to the calcium problem; *o,o'*-dihydroxyazobenzene, 1963, may be the answer to the magnesium problem.

Further improvements in the analytical chemistry of calcium and magnesium will certainly be made, but we have reached a stage where a review should be of benefit to many. Answers to most of the questions asked about the new methods for the determination of calcium and magnesium are now known but the information is piecemeal and scattered. In this monograph an effort has been made to fit the information to a pattern so that chemists can find quickly what they need to know about the new methods.

The monograph should be intermediate in character to the research paper and the text book, eliminating much that the original research worker experienced painfully and must in necessity and justice set down fully, but not descending to the bald, authoritarian statement of the text book. To achieve this intermediate character in this monograph, tables of results reported in research papers have been reproduced in some number. Primarily these tables are presented as proof of the efficacy and validity of the methods described. Such tables are guides to the concentration range within

which some component of a material will normally fall and this may be of use to those approaching a given analysis for the first time. The tables provide, too, a guide to the precision which can be achieved.

One of the interesting developments that has followed the introduction of metallochromic indicators has been the shift to their use as reagents for the direct colorimetric determination of the elements. The latest work on Calcein is directed to its use for the direct fluorometric determination of calcium, and in this sense too, *o,o'*-dihydroxyazobenzene is a derivative of Eriochrome Black T.

And last, a comment on the speed with which the chemical front is advancing. The old rule "twenty years from test tube to commercial production" is no longer appropriate. In the 7.5 years since Calcein was first described it has been widely adopted and over fifty papers have been published describing its properties and uses. No one has yet flung EDTA or Calcein into space but that day cannot be far off. Where this leaves the writer of a monograph, or for that matter the writer of a research paper, deserves thought. Remembering that for exponential growth, the acceleration of that growth is also the same exponential, perhaps we should take to publishing in the New York Times *News Of The Week In Review.*

HARVEY DIEHL,
Ames, Iowa
January, 1964

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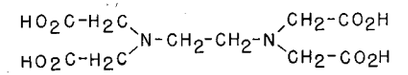
PART I

ETHYLENEDIAMINETETRAACETIC ACID (EDTA) AND ITS USES IN CHEMICAL ANALYSIS

The organic acid ethylenediaminetetraacetic acid has the remarkable



property of forming non-dissociated ions with a large number of bivalent, trivalent and quadrivalent metals. Use is made of this property for the titrimetric determination of the metals. At first sight it would appear that a reaction characteristic of a great many metals would not be particularly useful for the purposes of chemical analysis. In practice, however, the reactions prove to have great utility, the lack of specificity being made less severe or circumvented by three factors: 1) the number of metals present together in many natural and manufactured products is often small, 2) the stability of the various non-dissociated ions formed varies from metal to metal and judicious use of pH and "masking" or "sequestering" agents mitigate the interfering effects, 3) rapid and effective methods of effecting the separation of metals, principally methods involving ion exchange resins and liquid-liquid extraction, are now available. The titrimetric methods afforded by ethylenediaminetetraacetic acid for the alkaline earth metals have been especially fruitful for direct methods for these metals were un-

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[a]The method was first known as the versenate method after the trade name "Versene" of the Bersworth Chemical Company (now a part of the Dow Chemical Company) which began the manufacture of ethylenediaminetetraacetic acid and its tetrasodium salt (Versene) in the late 1930's. In the early work on the method by Diehl and Hach during 1948 material from the Bersworth Chemical Company was used and the name sodium versenate was adopted by them. In the paper of Biedermann and Schwarzenbach *(Chimia,* **2,** 56 (1948)), in which the ethylenediaminetetraacetic titration was also described and the use of Eriochrome Black T introduced, no particular name is used but the acid is spoken of as Complexon III. As other manufacturers began the marketing of ethylenediaminetetraacetic acid and its salts under other trade names, pressure on the editors of the journals forced the abandonment of the versenate designation. The EDTA designation was then generally adopted. One journal persists, in the face of popular usage, in using the term *(ethylenedinitrilo)tetraacetic acid,* the formal, Chemical Abstracts name for ethylenediaminetetraacetic acid, but at the same time permits the use of *EDTA.*

known previously to its advent. The method has been applied to the determination of calcium and magnesium in a variety of materials and is generally spoken of as the EDTA titration[a]. In many important materials, the alkaline earths, especially calcium and magnesium, occur essentially free of heavy metals, in natural waters, for example, and in biological materials such as plant and animal tissue and blood serum. By proper choice of conditions and indicator it is possible by the EDTA titration to determine calcium plus magnesium or calcium alone in the presence of magnesium.

Ethylenediaminetetraacetic acid is a tetrabasic acid and for convenience is frequently represented by H_4Y. It is insoluble in water but its disodium salt is soluble. The disodium salt is marketed as the dihydrate and is used for the preparation of standard solutions for titrimetric work; it is the material usually meant by the term EDTA.

The determination of calcium is based on the formation of a stable calcium ion, $CaY^=$:

$$H_2Y^= + Ca^{++} = CaY^= + 2H^+$$

Such ions are spoken of as complex ions, or non-dissociated ions, meaning that the equilibrium in the reaction lies far to the right. The alkali metals do not form such non-dissociated ions, the compound disodium dihydrogen ethylenediaminetetraacetate being dissociated in water according to the reaction:

$$Na_2H_2Y = H_2Y^= + 2Na^+$$

and thus providing the $H_2Y^=$ ion used as the titrating agent. Magnesium, and the other alkaline earth metals, form non-dissociated ions similar to the calcium ion in composition: $MgY^=$, $SrY^=$, and $BaY^=$. The magnesium ion, $MgY^=$, is less dissociated than the calcium ion, $CaY^=$. The constants for the formation of these non-dissociated calcium and magnesium ions are

[b]The term "non-dissociated" is of course relative. The non-dissociated ions being considered here do ionize to a slight extent and the so-called formation constant is a measure of the reverse, or formation reaction. The formation constants involved here are defined by the equations:

$$Ca^{++} + H_2Y^= = CaY^= + 2H^+$$

$$K_{Ca} = \frac{[CaY^=]\,[H^+]^2}{[Ca^{++}]\,[H_2Y^=]} = 3.0 \times 10^{10}$$

$$Mg^{++} + H_2Y^= = MgY^= + 2H^+$$

$$K_{Mg} = \frac{[MgY^=]\,[H^+]^2}{[Mg^{++}]\,[H_2Y^=]} = 5.0 \times 10^{8}$$

The so-called instability constants are the equilibrium constants for the dissociation reactions, that is, the above reactions written in reverse, and are the reciprocals of the formation constants just defined.

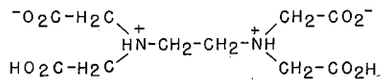
TABLE 1. FORMATION CONSTANTS OF THE METAL DERIVATIVES OF ETHYLENEDIAMINETETRAACETIC ACID

Metal Ion	Logarithm of Formation Constant	Metal Ion	Logarithm of Formation Constant
Lithium	2.79	Thorium	23.2
Sodium	1.66	Manganese	13.6
Magnesium	8.69	Ferrous	14.3
Calcium	10.70	Ferric	25.1
Strontium	8.63	Cobaltous	16.2
Barium	7.76	Nickel	18.6
Aluminum	16.13	Cupric	18.8
Gallium	20.27	Zinc	16.3
Scandium	23.1	Cadmium	16.6
Lanthanum	15.40	Palladium	18.5
Cerous	15.8	Mercuric	21.8
Titanium	17.3	Lead	18.3

3.0×10^{10} and 5.0×10^{8}, respectively[b]. In solutions of pH 8 to 11, the reactions of calcium and magnesium with disodium dihydrogen ethylenediaminetetraacetate go on practically simultaneously and, being complete, can be used for the determination of the sum of the two metals. As described below, either of the azo compounds *Eriochrome Black T or Calmagite* is used as indicator. In solutions of pH 12, magnesium is either precipitated as the hydroxide or converted to soluble, non-ionized magnesium hydroxide so that it does not react with EDTA. At this pH, calcium reacts completely with EDTA and can thus be titrated in the presence of magnesium. The indicator *Calcein* is used in this titration.

Many other metals also form non-dissociated compounds with ethylenediaminetetraacetic acid, the stability of the various ions formed being measured by the formation constants, Table 1. Under suitable conditions of pH many of these metals may be determined by titration with ethylenediaminetetraacetic acid but a coverage of such methods is beyond the scope of this monograph. For the determination of calcium and magnesium, the interest in the union of the other metals with EDTA lies in the interferences and the errors they cause and in methods of circumventing such difficulties. In general, the only metals which do not interfere in the determination of calcium and magnesium are the alkali metals, beryllium, and ammonium. Interference of iron, copper, and other transition elements can be obviated by the addition of cyanide. Trouble from aluminum is avoided by adding triethanolamine. Preliminary separations may be necessary to limit interference by some metal ions but fortunately such separations are not necessary in the analysis of water, limestone, blood serum, urine, and other common calcium- and magnesium-bearing materials.

In ethylenediaminetetraacetic acid the protons of two of the carboxylic



groups are apparently located on the neighboring nitrogen atoms (internal neutralization) giving the nitrogen atoms a positive charge and leaving the carboxyl groups negatively charged, in accord with the structure
That is to say, ethylenediaminetetraacetic acid is a double, so-called *zwitter ion*. The remaining two carboxyl groups are strong acids and on the addition of base are neutralized; subsequent addition of base removes the protons on the ammonium groups (just as ammonium hydroxide is liberated on the addition of sodium hydroxide to ammonium chloride). The acid dissociation constants of ethylenediaminetetraacetic acid, expressed as negative logarithms, are respectively, $pK_1 = 1.99$, $pK_2 = 2.67$, $pK_3 = 6.16$, $pK_4 = 10.26$.

Standard EDTA solutions can be made from ethylenediaminetetraacetic acid by the addition of sufficient sodium hydroxide to bring the pH to 5.5. It is easier and customary, however, to prepare EDTA solutions from the disodium salt which is available commercially (G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 247). It is possible also to prepare EDTA solutions by weight directly from primary standard grade disodium dihydrogen ethylenediaminetetraacetic (G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 365); see the later section on primary standard materials for EDTA work, page 111.

PART II

THE EDTA TITRATION OF CALCIUM PLUS MAGNESIUM

A. THE CHEMSTRY INVOLVED

Basically, the reactions employed are

$$Ca^{++} + H_2Y^{=} = CaY^{=} + 2H^{+}$$

and

$$Mg^{++} + H_2Y^{=} = MgY^{=} + 2H^{+}$$

H_4Y representing ethylenediaminetetraacetic acid and $H_2Y^{=}$ the dihydrogen ethylenediaminetetraacetate ion present in the standard solution made from disodium dihydrogen ethylenediaminetetraacetate dihydrate ($Na_2H_2Y{\cdot}2H_2O$). These reactions are pH dependent, (or putting it another way, the slightly-dissociated ions, $CaY^{=}$ and $MgY^{=}$ which are formed are decomposed by acids). To make the reactions go completely to the right it is necessary to remove the hydrogen ions formed. This is done by buffering the solution, at pH 10, with ammonia and ammonium chloride. Although there is some difference in the stability of the ethylenediaminetetraacetate ions of calcium and magnesium (formation constants $10^{10.7}$ and $10^{8.7}$ respectively), the reactions are generally regarded as taking place concurrently and the titration is employed to give the sum of the calcium and magnesium[a].

All of the materials involved in these reactions are colorless and an indicator must be used to mark the end-point. As indicators certain *o,o'*-dihydroxyazo compounds are used which unite with magnesium to form non-dissociated ions different in color from the azo compounds themselves. *Eriochrome Black T* was the first of these "metallochromic" indicators (Biedermann and Schwarzenbach[1]) and is still widely used al-

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For full references see Bibliography, page 115.

[1] *Chimia*, **2**, 56 (1948).

[a] Apparently the reactions actually do take place in succession for two methods have been reported for finding two end-points, first for the titration of the calcium, second for the magnesium. One method involves a thermometric titration, J. Jordan and T. G. Alleman, *Anal. Chem.*, **28**, 9 (1957); the other employs a photometric titration with Calmagite or Eriochrome Black T as indicator, H. Flaschka and P. Sawyer, *Talanta*, **9**, 249 (1962) and J. Lacy, *Talanta*, **10**, 1031 (1963).

though another indicator, *Calmagite,* is more convenient in that it is stable in solution and gives a somewhat sharper end-point. The color changes are the same and one may be used in the place of the other without altering the procedure. The non-dissociated ion formed between magnesium and either Eriochrome Black T or Calmagite is not as stable (lower formation constant) as the magnesium derivative of EDTA. On the addition of EDTA then to a solution containing magnesium and one of these azo compounds, the magnesium is pulled away from the azo compound and a color change results. Eriochrome Black T and Calmagite are themselves acid-base indicators with two color changes. Designating the azo compound as H_3In, the colored species and the pH ranges over which they are converted from one to the other are

	H_2In^-		$HIn^=$		$In^{\equiv}$
Eriochrome Black T	Wine red	5.3-7.3	Blue	10.5-12.5	Orange
Calmagite	Red	7.2-9.2	Blue	11.4-13.4	Red

The non-dissociated magnesium compound, $MgIn^-$ (of either azo compound), is red in color and is formed in the pH range 8 to 11

$$Mg^{++} + HIn^= = MgIn^- + H^+.$$

On the addition of sodium ethylenediaminetetraacetate to a solution of the red Eriochrome Black T-magnesium or Calmagite-magnesium compound, the magnesium is extracted from the $MgIn^-$ ion and the color changes from red to blue.

Although calcium forms a relatively weak, soluble, colored compound with Eriochrome Black T and with Calmagite, the compound is not sufficiently stable to serve as an indicator in the titration of calcium with EDTA. Since magnesium is required to function with the indicator, it is added to the EDTA solution before it is standardized. Thus, as the titration progresses, magnesium is introduced without requiring a blank correction.

A pH value of about 10 is best for the titration. Above this value, magnesium hydroxide may be precipitated, and at lower values the magnesium is not bound strongly enough to the azo compound to give the desired wine red compound, NaMgIn. At high pH values, also, the indicator shifts to its most alkaline (orange) form, so that at the end-point a blue color is not obtained. Hydroxides and large amounts of carbonates which raise the pH above 10.5 themselves, as well as the ions of other weak acids, have no effect on the end-point. A pH of 10 can be readily obtained and maintained by the addition of a sufficient amount of an ammonia-ammonium chloride buffer. At a pH of 10, the color change is sharp and may be approached rapidly.

One of the remarkable features of this titration is that it is not affected

by considerable concentrations of alkali metal salts. There seems to be some difference between Eriochrome Black T and Calmagite in this respect for Diehl, Goetz and Hach[2] reported that the titration is sharp and accurate in solutions containing up to 15 g. of sodium chloride per 100 ml. with Eriochrome Black T but with Calmagite the tolerance seems to be between 2 and 10 g. of sodium chloride and to be much less with sodium sulfate, Lindstrom and Diehl[35], Tables 2 and 3.

TABLE 2. EFFECT OF INTERFERING IONS ON THE EDTA TITRATION OF CALCIUM PLUS MAGNESIUM USING ERIOCHROME BLACK T AS INDICATOR

Data of Diehl, Goetz, and Hach[2]

Interference Added	Permissible Concentration, p.p.m.	
	NH_4OH-NH_4Cl Buffer	NH_4OH-NH_4Cl Buffer + NaCN
Fe^{+++}	20	over 30
Al^{+++}	20	20
Cu^{++}	0.3	over 30
Mn as $KMnO_4$	20	20
Zn^{++}	*a*	*a*
Cd^{++}	*a*	*a*
Ni^{++}	fails	over 20
Co^{++}	fails	over 20
Cl^-	*b*	*b*
$SO_4^=$	*b*	*b*
$PO_4^{\equiv}$	*b*	*b*

[a]No interference; titrates as hardness.
[b]No interference.

Small amounts of certain heavy metals interfere in the determination of calcium plus magnesium and again there are some differences in the amounts which can be tolerated by the two indicators, Tables 2 and 3. Only 0.3 p.p.m. of copper and even smaller amounts of cobalt and nickel render the end-point indistinct with Eriochrome Black T but 25 mg. of copper may be present with Calmagite. Manganese dioxide and manganate also interfere with the action of the indicator although the manganous ion does not interfere with the indicator, being simply titrated just like calcium and magnesium. Iron does not interfere if the buffer is added to the solution before the indicator is added. Aluminum above 20 p.p.m. causes the end-point to be indistinct. The interference of all these metals except aluminum can be overcome by the addition of hydroxylammonium chloride, which reduces the metals to their lower valence states, and of sodium cyanide, which ties up the metals as very slightly dissociated cyanide compounds. The interference by aluminum is obviated by adding tartrate or triethanolamine.

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[2] *J. Amer. Water Works Assoc.*, **42,** 40 (1950).
[35] *Anal. Chem.*, **32,** 1123 (1960).

TABLE 3. EFFECT OF INTERFERING IONS ON THE EDTA TITRATION OF CALCIUM PLUS MAGNESIUM USING CALMAGITE AS INDICATOR

Data of Lindstrom and Diehl[35]

Interference Added	mg.	End-point Quality	Calcium Oxide Found, mg.	Recovery Per Cent
None		Excellent	25.09	(100.00)
Potassium chloride	10,000	Excellent	25.12	100.12
Potassium nitrate	10,000	Excellent	25.12	100.12
Potassium bromate	1,000	Excellent	25.25	100.64
Potassium perchlorate[a]		Excellent	25.06	99.88
Sodium chloride	2,000	Satisfactory	25.19	100.40
	10,000	Poor	25.95	103.43
Sodium sulfate	2,000	Very poor	25.13	100.16
	10,000	No end-point	.....	.....
Copper	10	Excellent	25.10	100.03
	25	Excellent	25.08	99.96
	50	No end-point	.....	.....
Iron	5	Excellent	24.87	99.12
	10	Poor	24.63	98.17
Aluminum	1	Satisfactory	25.07	99.92
	5	Poor	24.91	99.28

[a]Saturated solution.

The EDTA titration has been applied to the determination of calcium plus magnesium in a variety of materials. Its initial use and its most extensive application have been to the determination of the hardness of water. An explanation of the chemistry of hard water and working directions for carrying out the determination of calcium plus magnesium are given in the following section. Also given below, Part II, Section C, are details for determining calcium plus magnesium in limestone. Applications to other materials follow essentially the methods given for water and for limestone.

Stability of EDTA Solutions. Soft glass is attacked by EDTA in solutions of pH 10. This can be observed by simply allowing a solution at the conclusion of a titration to stand with a stirring rod of soft glass dipping into the solution; after a few minutes the indicator will have changed from blue to pink as the excess EDTA attacks the glass rod. The question of the stability of standard EDTA solutions stored in glass bottles if of interest in routine work. For soft glass bottles, the question was answered by Goetz, Loomis and Diehl[3]; it depends on pH, see Table 4. Below pH 4.25 there is some tendency for the ethylenediaminetetraacetic acid to precipitate from the solution. At pH 5, the pH obtained when dissolving disodium dihydrogen ethylenediaminetetraacetate in water as commonly done in making up standard EDTA solutions, the change (decrease in concentration of EDTA) is about 0.2 per cent per month. Borosilicate glass is much more resistant to chemical attack than soft glass and as expected the stability of EDTA solutions is greater in bottles of borosilicate

TABLE 4. CHANGE IN CONCENTRATION OF EDTA SOLUTIONS OF VARIOUS VALUES OF pH ON STORAGE IN GLASS BOTTLES

Data of Goetz, Loomis and Diehl[3]

Days Stored	pH 5	pH 6	pH 7	pH 8	pH 9	pH 10
0	1.049	1.044	1.062	1.050	1.062	1.047
5	1.048	1.044	1.063	1.050	1.058	1.041
30	1.049	1.042	1.060	1.047	1.043	1.019
60	1.047	1.041	1.058	1.042	1.030	1.002
120	1.042	1.035	1.051	1.034	1.019	0.970

glass. In fact, Blaedel and Knight[64] found changes of less than 0.05 per cent in the concentration of 0.01 M EDTA stored in borosilicate and in polyethylene bottles for five months (pH presumably 5 to 6).

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[3]*Anal. Chem.*, **22**, 798 (1950).
[64]*Anal. Chem.*, **26**, 741 (1954).

PART II

B. THE DETERMINATION OF THE TOTAL HARDNESS OF WATER

The presence in water of salts of calcium and magnesium is spoken of as hardness. The salts causing hardness are principally bicarbonates ($Ca(HCO_3)_2$ and $Mg(HCO_3)_2$) and sulfates ($CaSO_4$ and $MgSO_4$). On boiling the water, the bicarbonates are decomposed, carbon dioxide being expelled and the normal carbonates being precipitated:

$$Ca(HCO_3)_2 = CaCO_3 + H_2O + CO_2$$
$$Mg(HCO_3)_2 = MgCO_3 + H_2O + CO_2.$$

Because of this, the hardness caused by calcium and magnesium bicarbonates is known as temporary or carbonate hardness. Boiling produces no change in a solution of the sulfates and the hardness caused by the sulfates is known as permanent or non-carbonate hardness.

The hardness of water is commonly expressed in parts per million (p.p.m., equal to milligrams per liter[a]) of an equivalent amount of calcium carbonate. This method is used for expressing the amount of magnesium as well as the amount of calcium and for expressing non-carbonate hardness as well as carbonate hardness.

Fortuitously, the molecular weight of calcium carbonate is the very convenient figure 100 (actually 100.09) and the equivalent weight is 50. Each 50 p.p.m. of calcium carbonate, equal to 50 mg. of calcium carbonate per liter of water, represents 0.001 of a gram equivalent weight per liter. The method of expressing all results as equivalent amounts of calcium carbonate means that every 50 p.p.m. is equal to one milliequivalent weight per liter. This is convenient in computing the so-called acid-base balance,

[a]10,000 p.p.m. equals 1 per cent; or, 0.0001 per cent equals 1 p.p.m. This is readily seen from the sequence

1	per cent equals 1 part per	100
0.1	per cent equals 1 part per	1,000
0.01	per cent equals 1 part per	10,000
0.001	per cent equals 1 part per	100,000
0.000,1	per cent equals 1 part per	1,000,000

If it is assumed that the density of the sample is 1.000, 1 liter will weigh 1,000,000 mg.; hence the statement that 1 mg. per liter equals 1 part per million.

for the sum of the milliequivalents per liter of cations, magnesium plus calcium (both expressed as an equivalent amount of calcium carbonate), should equal the sum of the milliequivalents per liter of anions, bicarbonate (temporary or carbonate hardness) plus sulfate (both expressed as equivalent amounts of calcium carbonate).

The total hardness of water varies greatly with locality and source. A water with total hardness less than 100 p.p.m. of calcium carbonate is generally considered soft[b]; a water with total hardness above 300 is considered very hard. Municipal water supplies with total hardness of 2000 are known and hardness as high as 4400 has been reported.

The determination of total hardness of water can be made quickly and accurately by titration with EDTA. The chemistry involved is discussed in detail in the preceding section; directions for carrying out the determination are given immediately below. The following "Routine Procedure" is usually applicable. Note that magnesium has been deliberately added to the EDTA solution to insure the proper functioning of the indicator; its presence is accounted for in the standardization and no consideration need be given it in calculating the results of the analysis. Occasionally interfering ions may be present in the water being analyzed. Copper above 0.3 p.p.m. and large amounts of iron or manganese cause the end-point to be indistinct. For such waters, the "Alternate Procedure" employing sodium cyanide must be used.

Procedure for the Determination of Calcium Plus Magnesium in Water. REAGENTS. STANDARD CALCIUM CHLORIDE SOLUTION. 0.010 M. Weigh accurately approximately 1 g. of primary standard grade calcium carbonate (G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 337) directly into a 1-liter volumetric flask. Add 6 ml. of concentrated hydrochloric acid to dissolve the carbonate. Dilute with distilled water to exactly 1 liter, mix throughly and transfer to a clean, dry bottle for storage. Calculate the molar concentration of this solution by dividing the weight of calcium carbonate taken by the molecular weight of calcium carbonate, 100.09. If exactly 1.000 g. of calcium carbonate was taken, each milliliter of the solution will be equivalent to 1.00 mg. of calcium carbonate and this value (titer) may be used in subsequent work.

BUFFER SOLUTION OF pH 10. Mix 6.75 g. of ammonium chloride with 57.0 ml. of concentrated ammonium hydroxide and dilute to 100 ml. The pH of this mixture is just over 10.

CALMAGITE INDICATOR. 0.05 PER CENT AQUEOUS SOLUTION. Dissolve 0.05 g. of Calmagite (1-(1-Hydroxy-4-methyl-2-phenylazo)-2-naphthol-4-sulfonic acid) (G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 278) in 100 ml. of water. This solution is stable indefinitely and may be stored in any type of bottle.

ERIOCHROME BLACK T INDICATOR. (An alternative indicator to Calmagite.) Weigh 0.50 g. of analytical reagent grade Eriochrome Black T (G. FREDERICK SMITH CHEMI-

[b]The obsolete method of reporting hardness in terms of grains of calcium carbonate per U. S. gallon (English system of units) is still used occasionally; 17.118 p.p.m. equals 1 grain per U. S. gallon.

CAL COMPANY, Item No. 246) and dissolve it in 100 ml. of alcohol. Solutions of Eriochrome Black T are not stable and must be made up fresh about every week.

STANDARD DISODIUM DIHYDROGEN ETHYLENEDIAMINETETRAACETATE (EDTA) SOLUTION; 0.01 M; 1.00 ml. EQUIVALENT TO 1.00 mg. $CaCO_3$ per ml. Weigh 4.00 g. of disodium dihydrogen ethylenediaminetetraacetate dihydrate (G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 247) and dissolve it in 750 ml. of water. Weigh 0.100 g. of hydrated magnesium chloride, $MgCl_2 \cdot 6H_2O$, and add this to the above solution. Mix the solution well.

STANDARDIZATION OF EDTA. Pipet 50.0 ml. of the standard calcium chloride solution into a 250-ml. conical flask, add 1.0 ml. of the buffer solution and four drops of Calmagite solution. Titrate with the standard EDTA solution. At the end-point, the solution should be clear and should change from wine red to pure blue with no reddish tinge remaining. In daylight the color beyond the end-point is sky-blue, but under a tungsten-filament lamp the solution is almost colorless. If Eriochrome Black T is used instead of Calmagite the color change is again from red to blue but is not quite as sharp.

Calculate the molar concentration of the EDTA solution using the relation

$$(\text{ml.}_{\text{EDTA}})(M_{\text{EDTA}}) = (\text{ml.}_{\text{Ca. Soln.}})(M_{\text{Ca. Soln.}})$$

If the EDTA was made up as specified above it will be somewhat greater than 0.0100 in concentration. Use as so prepared, or if desired, adjust the concentration to exactly 0.0100 M as follows. Using the relation

$$(\text{Vol.}_{\text{EDTA}})(M_{\text{EDTA}}) = (\text{Vol.}_{\text{Final}})(0.0100)$$

calculate the volume to which the solution must be diluted. Add the necessary volume of water, mix well and restandardize as a check.

PROCEDURE FOR THE DETERMINATION OF CALCIUM PLUS MAGNESIUM IN WATER. ROUTINE PROCEDURE. Pipet 50.0 ml. of the water to be analyzed into a 250-ml. flask. Add about 1 ml. of the buffer solution and mix by swirling. Add 4 drops of Calmagite solution. Titrate with the standard EDTA solution. At the end-point, the solution should be clear and should change from wine red to pure blue with no reddish tinge remaining. In daylight the color beyond the end-point is sky-blue, but under a tungsten-filament lamp the solution is almost colorless. If Eriochrome Black T is used instead of Calmagite the color change is again from red to blue but is not quite as sharp.

Multiply the number of milliliters of standard EDTA solution by 20 to obtain the total hardness as parts per million of calcium carbonate[c]. The magnesium added to the standard EDTA solution is accounted for in the standardization.

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[c]The number 20 is derived from the equation for calculating the results of a volumetric analysis:

$$\text{p.p.m. } CaCO_3 = (\text{Per cent } CaCO_3)(10{,}000) = \frac{(\text{ml.}_{\text{EDTA}})(M_{\text{EDTA}})(\text{millimolar wt. } CaCO_3)(100)(10{,}000)}{\text{Wt. sample}}$$

The molecular weight of calcium carbonate is 100.09 and the millimolar weight, 0.10009 is used since volume is expressed in milliliters. (The EDTA solution used above is about (or exactly if so made) 0.01 M in concentration; if the concentration is expressed on the normal basis, the concentration is 0.02 N and the milliequivalent weight of calcium carbonate, 0.050004, should be used in the equation.

The weight of the sample taken is assumed to be 50.00 g. (50.00 ml. of water).

$$\text{p.p.m. } CaCO_3 = \frac{(\text{ml.}_{\text{EDTA}})(0.0100)(100/1000)(100)(10{,}000)}{50} = 20(\text{ml.}_{\text{EDTA}})$$

The concentration of EDTA solution is often expressed as titer for calcium carbonate, in this case, 1.00 ml. equivalent to 1.00 mg. of calcium carbonate. Titer is simply molar concentration multiplied by millimolar weight:

$$\text{Titer}_{\text{EDTA Solution}} = (0.0100)(100/1000) = 0.00100 \text{ g. } CaCO_3/\text{ml.}$$

The accuracy of the determination is greatest when the volume of standard solution approaches the maximum volume which can be delivered by the buret. The volume of the sample may be adjusted to take advantage of this. Thus, for waters having a total hardness less than 500 p.p.m., use a 100.0-ml, sample; for waters above 1000 p.p.m., use a 25.0-ml. sample. The factor used in calculating the results must be changed accordingly: 100.0-ml. sample, multiply by 10; 50.0-ml. sample, multiply by 20; 25.0-ml. sample, multiply by 40. A small sample of a very hard water should be diluted to approximately 50 ml. with distilled water before the buffer is added; otherwise, magnesium hydroxide may be precipitated on addition of the buffer and erratic results obtained.

Alternate Procedure. Pipet 50.0 ml. of the water into a 250-ml. flask. Add about 5 ml. of the buffer solution, and mix the solution by swirling. Add about 0.02 g. of hydroxylammonium chloride and about 0.25 g. of sodium cyanide; mix well, add 4 drops of indicator solution, and titrate with the standard EDTA solution. At the end-point the color will change from wine red to pure blue. On the second titration, modify the size of the sample as discussed in the second paragraph under "Routine Procedure". Calculate the results as described there also.

The extra volume of buffer solution (5 ml. rather than 1 ml.) specified in this alternate procedure is necessary to offset the additional alkalinity resulting from the hydrolysis of the sodium cyanide added.

PART II

C. THE DETERMINATION OF CALCIUM PLUS MAGNESIUM IN LIMESTONE

Chemically, limestone is calcium carbonate. Calcium is the fifth most abundant of the elements constituting the crust of the earth and limestone is widely distributed in the crust, occuring in massive layers as one of the principal sedimentary rocks. Limestone has many uses and numerous pseudonyms[a]. Its purity varies widely. One variety of calcium carbonate, called *Iceland spar*, occurs as transparent crystals and is generally considered to be highly pure[b]; common limestone, however, often contains large amounts of silica, magnesium, and occasionally appreciable quantities of iron, manganese, aluminum, and organic matter. The major part of the naturally occurring calcium carbonate, or limestone, was first laid down as the shells of marine organisms and later altered by pressure or changed by recrystallization in percolating water. The gross impurities of silica and aluminum are derived from sand and clay deposited along with the growing shells. The chemistry of magnesium is similar to that of calcium in many respects and in particular magnesium carbonate, the mineral *magnesite*, is isomorphous with calcium carbonate. The calcium in calcite is often replaced by considerable amounts of magnesium. A double carbonate, *dolomite*, $CaMg(CO_3)_2$, is common; magnesite is rare. Ferrous carbonate *(siderite)* and manganous carbonate *(rhodochrosite)* are also isomorphous with calcium carbonate and are found commonly as impurities in limestone. Because of the replacement of one bivalent metal by another in these isomorphous carbonates, their composition varies greatly.

[a]With the possible exception of silica, more names have been applied to calcium carbonate than to any other mineral. The basic name employed by the mineralogist is *calcite*, but terms, both euphonious and indicative or origin or form are common: *marble, calcareous tufa, dogtooth spar, Iceland spar, coquina, coral, chalk, calc-spar, nailhead spar*, and others.

[b]Calcite, or Iceland spar, has been frequently used as a primary standard. One report tells of a specimen of Iceland spar, which although uniform and transparent, contained several per cent of iron. Lacking some definite assurance as to the purity of crystalline calcite, better practice is to use for primary standard purposes a special grade of calcium carbonate chemically prepared in such a manner as to eliminate the magnesium and the iron; such a product is Item No. 337, Calcium Carbonate, Primary Standard, of the G. Frederick Smith Chemical Company.

Limestone is widely used as a building material and as a source of low-cost alkali for the blast furnace operation, for the manufacture of glass, and for the manufacture of cement. Because it is the cheapest alkali available, it is used extensively in agriculture, and in the chemical industry in the manufacture of sodium carbonate, sodium hydroxide, calcium carbide, and numerous other materials.

The particular analyses made on limestone will depend on the information needed, due regard being given the fact that the material is an inexpensive commodity. If the limestone is to be used simply as a source of alkali, a determination of its neutralizing power by dissolving it in excess standard acid and back titrating may be all that is necessary. For a more accurate value of the neutralizing power, carbon dioxide can be evolved by treatment with acid and its volume measured, or its weight measured by absorption in ascarite. Determinations of the calcium and of the magnesium present are more commonly made, however, and the EDTA titrations for calcium plus magnesium and for calcium alone are convenient and rapid.

The steps prior to the actual EDTA titration can be varied considerably and are governed by the amount and nature of the impurities present. If the silica and R_2O_3 (iron, aluminum, titanium, and manganese as oxides) are low, the sample simply may be dissolved in hydrochloric acid, the silica dehydrated by evaporation to dryness and baking, the residue taken up with dilute hydrochloric acid, and the titration performed directly on an aliquot. Good results were reported on Sample No. 88, Dolomite, of the National Bureau of Standards by Lindstrom and Diehl[35] by this method, Table 5. The silica in this sample is only 0.31 per cent but if the hydrochloric acid treatment is hurried the results tend to be erratic. Excellent results are consistently obtained by dehydrating the silica with perchloric acid, filtering off the silica, correcting it by the hydrofluoric acid-sulfuric acid treatment, and returning the residue to the main solution prior to the titration for calcium plus magnesium. If the silica is high, such a procedure is essential. For results by Diehl and McBride[4] by this method, see Table 6.

Working details for both procedures are given below. In this connection see also the determination of calcium only, page 20.

If an acid-insoluble silicate is present, as in NBS sample 1a, Argillaceous Limestone, a preliminary ignition with sodium carbonate plus sodium tetraborate must be made; the melt is then taken up with perchloric acid, the silica dehydrated, filtered off, corrected, and the residue fused,

For full references see Bibliography, page 115.

[35]*Anal. Chem.*, **32**, 1123 (1960).

[4]Otherwise unpublished work.

TABLE 5. DETERMINATION OF CALCIUM PLUS MAGNESIUM IN DOLOMITE, NBS 88

Data of Lindstrom and Diehl[35,a]

Weight of Sample g.	Volume of 0.00916 M EDTA ml.	Calcium Plus Magnesium Found Per Cent CaO
0.5434	47.21	60.39
	47.22	60.40
	47.26	60.45
0.5199	45.30	60.57
	45.22	60.46
	45.26	60.51
0.5174	44.91	60.34
	44.85	60.26
	44.94	60.38
Average		60.42
NBS value (computed as calcium oxide[b])		60.37
Standard deviation, σ		0.087
Relative standard deviation in parts per thousand ($\delta = 1000\,\sigma$/Average)		1.44

[a]Silica dehydrated by hydrochloric acid evaporation but not removed by filtration. Silica content: 0.31 per cent.

[b]Sum of the metals titrated, as calcium oxide:

Per cent CaO	30.49
(Per cent MgO)(Mol. Wt. CaO/Mol. Wt. MgO)	29.88
Total as per cent CaO	60.37

Strontium reported to be less than 0.01 per cent.

dissolved and returned to the main solution. Precipitation of the R_2O_3 group before proceeding with the determination of calcium and magnesium is only necessary if the iron and aluminum are very high.

Procedure for the Determination of Calcium Plus Magnesium in Limestone, Silica Dehydrated But Not Removed. PROCEDURE OF LINDSTROM AND DIEHL[35]. REAGENTS. Prepare a standard calcium chloride solution, buffer solution (pH 10), and 0.05 per cent solution of Calmagite indicator as described under the determination of calcium plus magnesium in water, page 11; prepare 0.01 M EDTA solution and standardize it as described in the same place. Note that magnesium has been added to the EDTA solution to supply the magnesium needed for the indicator, that this magnesium is already accounted for in the standardization, and that no consideration need be given it in calculations later.

PROCEDURE. Weigh accurately a sample of about 0.5 g. of the limestone or dolomite into a 400-ml. beaker. Dissolve the sample in 10 ml. of hydrochloric acid. Evaporate the solution to dryness on a hot plate. Take up the residue with dilute hydrochloric acid (1:50). Without filtering off the silica, dilute the solution to exactly 250 ml. in a volumetric flask. Pipet a 20.0-ml. aliquot into a 250-ml. conical flask and add successively with mixing: 10 mg. of ascorbic acid, 5 ml. of 5 per cent

TABLE 6. DETERINATION OF CALCIUM PLUS MAGNESIUM IN LIMESTONE AND DOLOMITE FOLLOWING PERCHLORIC ACID DEHYDRATION OF SILICA. EDTA TITRATION WITH CALMAGITE INDICATOR

Data of H. Diehl and L. C. McBride[4]

	NBS 88 Dolomite[a,c]	GFS 400 Dolomite[b,c]	GFS 401 Limestone[b,c]	GFS 402 Limestone[b,c]
Found, calcium plus magnesium as per cent CaO	60.35[d]	60.30	55.12	54.50
	60.35[d]	60.47	55.10	54.68
	60.34[d]	60.41	55.09	54.63
	60.37[d]	60.30	55.13	54.66
	60.24[e]	60.43	55.08	54.67
	60.22[e]	60.40	55.15	54.67
	60.15[e]	60.35	55.01	54.64
	60.28[e]			54.56
	60.40[f]			54.56
	60.33[f]			
	60.23[f]			
	60.22[f]			
Average, per cent CaO	60.29	60.38	55.10	54.62
Standard deviation, σ	0.077	0.065	0.0517	0.064
Relative standard deviation, δ, in parts per thousand	1.29	1.03	0.94	1.16

[a]National Bureau of Standards, No. 88, Dolomite; reported: calcium plus magnesium as calcium oxide, 60.37 per cent.

[b]G. Frederick Smith Chemical Company, No. 400, Dolomite from Woodville, Ohio; No. 401, Limestone, from Marble Cliff, Ohio; No. 402, Limestone, from Spore, Ohio.

[c]As of November, 1963, the stock of NBS No. 88 had been depleted. The dolomite and two limestone samples mentioned above are part of a series of samples prepared for student analysis by the G. Frederick Smith Chemical Company, Items No. 400 through 420. In making these samples the raw materials were ground to 200 mesh and thoroughly mixed in a stainless steel, conical blender. The homogeneity obtained in the mixing is attested by the values shown above for the relative standard deviation, δ, for calcium plus magnesium, and also by the values for calcium given in Table 12, page 34. Each of the results reported above and in Table 12 were made on a different specimen taken at the time the sample was removed from the blender. The silica and iron content of these samples has also been determined.

[d,e,f] Samples from three different bottles of NBS 88.

potassium cyanide, 5 ml. of 20 per cent triethanolamine, 5 ml. of ammonium hydroxide-ammonium chloride buffer mixture (pH 10), and 2 drops of 0.05 per cent Calmagite solution. Dilute the solution to about 100 ml. with water. Titrate with 0.01 M EDTA to the disappearance of the last trace of red.

If the EDTA solution does not contain magnesium chloride as called for in the directions given above and if the sample being titrated does not contain much magnesium (as in calcite or very pure limestone), add before titrating 2.00 ml. of a solution precisely 0.05 M in magnesium and 0.05 M in ethylenediaminetetraacetate.

Procedure for the Determination of Calcium Plus Magnesium in Limestone, Perchloric Acid Dehydration and Removal of Silica Prior to EDTA Titration. PROCEDURE OF DIEHL AND MCBRIDE. REAGENTS. Prepare a standard calcium

chloride solution, buffer solution (pH 10), and 0.05 per cent solution of Calmagite indicator as described under the determination of calcium plus magnesium in water, page 11; prepare 0.01 M EDTA solution and standardize it as described in the same place. Note that magnesium has been added to the EDTA solution to supply the magnesium needed for the indicator, that this magnesium is already accounted for in the standardization, and that no consideration need be given it in calculations later.

PROCEDURE FOR DEHYDRATION, REMOVAL AND DETERMINATION OF SILICA. Weigh accurately into a 600-ml. beaker 1 g. of the limestone. Without adding water, add 15 ml. of 70 per cent perchloric acid and immediately place a cover glass on the beaker. Heat the mixture to boiling on a hot plate and continue heating so that perchloric acid refluxes gently on the upper walls of the beaker and the vapors in the flask are essentially colorless. Heavy white vapors will form if the cover glass is displaced momentarily. Very little perchloric acid will escape during the heating but if water was added it should be removed by evaporation by raising the cover glass with two or three glass hooks. The dehydration generally requires about 15 to 20 minutes and is most conveniently carried out on an electric or gas-fired hot plate in a good hood. Cool the mixture and then add 25 ml. of water. Using Whatman No. 40 or equivalent ashless filter paper, filter directly into a 1-liter volumetric flask using dilute hydrochloric acid (1:100) as wash water. Scrub the beaker well with a rubber policeman and wipe the outside of the beaker with a damp rag to remove finger prints to permit closer inspection for traces of silica. After the transfer is complete, wash the filter 10 to 15 times with small, 1.0 to 2.0 ml., portions of the dilute hydrochloric acid wash water. Finally moisten the filter paper with a few drops of very dilute ammonia (1:200). Transfer the paper and silica to a platinum crucible previously ignited and weighed. Burn away the paper and ignite the silica residue for 10 minutes at the full heat of a grid top burner. Cool and weigh impure silica.

Moisten the impure silica with two drops of concentrated sulfuric acid and add 1 ml. of hydrofluoric acid. Heat gradually on a hot plate in a good hood to expel first the hydrofluoric acid and then the sulfuric acid. Finally ignite for 10 minutes at a red heat. Cool and weigh. From the loss in weight calculate the per cent silica in the sample. To the residue in the platinum crucible add 1 g. of a mixture of equal parts by weight of sodium carbonate and sodium tetraborate decahydrate. Heat the mixture to fusion over a burner and continue the heating for four to five minutes. Cool and place the crucible in the original beaker containing the sample. Add 5 ml. of dilute hydrochloric acid (1:50). After the melt has dissolved remove the platinum crucible from the beaker, washing it well as it is withdrawn. Transfer the solution to the main solution in the 1-liter volumetric flask. Dilute this solution to the mark with distilled water. Mix the solution by dropping into the flask a dry, Teflon-covered magnetic stirring bar and placing the flask on a magnetic stirrer (the water driven magnetic stirrer of the G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 376, is ideal for the purpose). During the stirring an inverted cone should form in the neck of the volumetric flask indicating that liquid is being drawn down through the very center and that stirring is effective. After several minutes withdraw the bar by placing another magnetic stirring bar against the outside of the flask and moving it so as to draw the inside stirring bar up the walls and neck to the top of the flask. Use this solution for the determination of calcium plus magnesium as described in the following paragraph, and for the determination of calcium only as described on page 35, and for the determination of iron, aluminum, magnesium only, and other constituents of the limestone as desired.

EDTA TITRATION FOR CALCIUM PLUS MAGNESIUM. Pipet a 25-ml. aliquot of the solution prepared as shown above into a 250-ml. conical flask. Add about 10 mg. of crystalline ascorbic acid. Add 2 ml. of 5 per cent sodium cyanide and 5 ml. of ammonia-ammonium chloride buffer (pH 10). Add 5 ml. of 20 per cent triethanolamine. Add 2 drops of 0.05 per cent Calmagite solution. Stir the solution with a magnetic stirrer and titrate with 0.01 M EDTA until the last trace of the pink color of the indicator has disappeared. Calculate the per cent calcium plus magnesium as per cent calcium oxide.

PART III

THE EDTA TITRATION OF CALCIUM IN THE PRESENCE OF MAGNESIUM

A. THE CHEMISTRY INVOLVED. CALCEIN AS INDICATOR

The titration of calcium only in the presence of magnesium is carried out at a pH of 12; at this pH magnesium is present as the hydroxide, either as a precipitate or in solution in non-ionized form, and is non-reactive toward EDTA. Basically, then, the determination is based on the same reaction used for the determination of calcium plus magnesium:

$$Ca^{++} + H_2Y^{=} \xrightarrow{\text{pH 12}} CaY + 2H^{+}$$

but

$$\underset{\text{non-ionized or precipitated}}{Mg(OH)_2} + H_2Y^{=} \xrightarrow{\text{pH 12}} \text{no reaction}$$

Several indicators have been used to mark the end-point of this reaction; the most satisfactory of these is Calcein[5], a derivative of fluorescein bearing two methyleneiminodiacetic acid groups. Calcein is a metallochromic indicator; it forms a non-dissociated ion with calcium, the calcium derivative being yellow-green in color and fluorescent. In the alkaline solution, the indicator itself is brown (sometimes described as pinkish-orange) and non-fluorescent. At the end-point in the titration, the calcium is extracted from the Calcein-calcium compound by the EDTA and the color changes from yellow-green to brown and the fluorescence disappears:

$$\underset{\substack{\text{yellow-green}\\\text{fluorescent}}}{\text{Ca-Calcein}} + H_2Y^{=} \xrightarrow{\text{pH 12}} Ca^{++} + \underset{\substack{\text{brown}\\\text{non-fluorescent}}}{\text{Calcein}}$$

Magnesium does not form a non-dissociated ion with the indicator at pH 12. By this procedure then, calcium can be determined directly in water, limestone, and other materials containing magnesium.

Owing to the high pH at which the titration is performed, calcium may

For full references see Bibliography, page 115.
[5] *Anal. Chem.*, **28,** 882 (1956).

be precipitated as the hydroxide at the beginning of the titration. Vigorous stirring is necessary to dissolve the hydroxide as the titration progresses. If the stirring is poor, false end-points are obtained, the color returning after each change as the stirring is continued. The formation of a precipitate is a matter of the concentration of calcium; below about 30 mg. of calcium per 100 ml., the amount of calcium conveniently titrated using 0.02 M solution, no precipitate appears. The precipitation can be avoided entirely by adding excess of standard EDTA and back titrating with a standard calcium solution.

Effects of Other Elements. In the presence of a large amount of magnesium the results for calcium are slightly low; data on this point of Diehl and Ellingboe[5] are reported in Table 7. Presumably some calcium is coprecipitated with the magnesium hydroxide and escapes subsequent reaction with EDTA. This slight error is reduced by dilution and is almost completely eliminated by the back titration procedure of Yalman and coworkers[6] discussed below in connection with interference by phosphate; see also Table 8. Another method of obviating the loss of calcium by coprecipitation is the carbonate-sucrose method of Tucker discussed in a later paragraph.

TABLE 7. TITRATION OF CALCIUM IN THE PRESENCE OF LARGE AMOUNTS OF MAGNESIUM AND SODIUM SALTS

Data of Diehl and Ellingboe[5]

Calcium		Magnesium	Sodium Chloride	Calcium Recovered
Taken g.	Found g.	g.	g.	Per cent
0.1188	0.1188	.....	1.0	100.00
0.1188	0.1191	.....	3.6	100.29
0.1188	0.1178	.....	3.0	99.18
0.1188	0.1190	8.4[a]	....	100.19
0.1188	0.1173	10.1[a]	....	98.74
0.0833	0.0827	2.2[b]	....	99.32
0.1175	0.1162	2.0[b]	2.0	98.91
0.0595	0.0585	10.9[a]	3.0	98.38
0.0595	0.0589	1.9[a]	....	98.99

[a] $MgCl_2 \cdot 6H_2O$. [b] $Mg(C_2H_3O_2)_2 \cdot 4H_2O$.

Large quantities of sodium salts do not affect the results of the titration but the end-point is not as sharp as in the absence of sodium; large amounts of sodium cause a slight green fluorescence with Calcein and this obscures the end-point somewhat. Large amounts of potassium cause no

[6] *Anal. Chem.*, **31,** 1230 (1959).

such fluorescence and it is therefore better to use potassium hydroxide to raise the pH of the solution.

Strontium and barium are titrated along with calcium; the end-point with either alone is the same as that with calcium and the titration gives the sum of all three if present. Copper and iron interfere with the end-point, but such interference is easily eliminated by the addition of cyanide. To react with cyanide iron must be in the bivalent state; a little hydroxylammonium chloride or ascorbic acid, added to the acid solution before the addition of the cyanide and the alkali, will reduce small amounts of iron quickly. Interference by large amounts of iron is not so easily eliminated, see the following section on the analysis of phosphate rock, Part III, Section D, page 36.

The titration of calcium may be performed in the presence of perchlorate, chloride, nitrate, acetate, and sulfate. Some trouble is experienced in the presence of phosphate; a precipitate of a calcium phosphate appears and the results are slightly low; see results of Yalman and co-workers[6], Table 8. Below pH 6 calcium dihydrogen phosphate, $Ca(H_2PO_4)_2 \cdot H_2O$, moderately soluble, is formed. Around pH 6 calcium hydrogen phosphate, $CaHPO_4 \cdot 2H_2O$, is precipitated. Above pH 6.9 hydrolysis occurs and a stable phase is formed of more or less pure material of collodial dimensions having the crystal structure of hydroxy apatite, $Ca_5OH(PO_4)_3$. The composition of this hydroxyapatite depends on the pH and the composition of the solution. Yalman and coworkers[6] found that the difficulty in the titration could be obviated by changing the order of adding the reagents, that is, by adding excess EDTA, raising the pH, and then back titrating with a calcium solution. No precipitate is present in the back titration, the end-point is sharp, and the results correct, Table 8.

TABLE 8. DETERMINATION OF CALCIUM IN THE PRESENCE OF PHOSPHATE

Data of Yalman, Bruegemann, Baker and Garn[6]

(Each sample contained 20.06 mg. of calcium, as calcium chloride)

Phosphate	Calcium Found[a]	
	Direct[b]	Indirect
mg.	mg.	mg.
13	19.87	20.06
21	19.91	20.06
32	19.81	20.08
40	19.90	
48	19.89	20.10
64	19.89	20.10
70	19.91	
80	19.94	20.06
96		20.06

[a]Average of three determinations. [b]Method of Diehl and Ellingboe[5].

The results are the same if both phosphate and magnesium are present, Table 9. Even in the back titration procedure, slightly low results are obtained if sufficient magnesium is present to form a precipitate, and as expected the accuracy is better with the larger amounts of calcium.

TABLE 9. DETERMINATION OF CALCIUM IN THE PRESENCE OF PHOSPHATE AND MAGNESIUM. EXCESS EDTA AND BACK TITRATION

Data of Yalman, Bruegemann, Baker and Garn[6]

Calcium Taken mg.	Phosphate mg.	Magnesium mg.	Calcium Found[a] mg.	Recovery Per cent
10.03	15.0	0.025[b]	10.08[c]	100.50
		0.050	10.03[c]	100.00
		0.125	10.08[c]	100.50
		0.250	10.08[c]	100.50
		0.50	9.98[d]	99.50
		2.50	10.03[d]	100.00
		5.00	10.01[d]	99.80
20.06	30.0	0.025[e]	20.06[c]	100.00
		0.050	20.10[c]	100.19
		0.125	20.08[c]	100.10
		0.50	20.04[d]	99.90
		1.25	20.01[d]	99.75
		2.50	20.03[d]	99.85
		5.00	20.12[d]	100.30
		10.00	20.01[d]	99.75

[a]Average of three determinations.

[b]Total calcium and magnesium expressed as calcium equals 10.07 g.

[c]Clear solution.

[d]Turbid solution.

[e]Total calcium and magnesium expressed as calcium equals 20.10 g.

Observing the End-Point with Calcein. The change in color of Calcein, although abrupt, is not as vivid as might be desired and the disappearance of the fluorescence is more frequently employed to detect the end-point. The visual end-point, however, is very distinct provided the minimum amount of indicator has been added and that the indicator solution used has not deteriorated. Usually white light and a white background are used for the titration. Some workers engaged in determining calcium at the microgram level (calcium in blood serum) have performed the titration in glass on a white porcelain background or in porcelain vessels[17, 27]. Socolar

[17]*J. Lab Clin. Med.*, **49**, 486 (1957).
[27]*Anal. Biochem.*, **1**, 93 (1960).
[23]*Anal. Chem.*, **31**, 473 (1959).

and Salach[23], for example, used a porcelain crucible and directed into it a light from an incandescent bulb passed through a triple thickness of Kodak Wratten filter No. 78AA; the bulb, a G. E. G16-½-29, 100 watt, 120 volt, was held in a microscope illuminator and provided good contrast between the colors before and after the end-point.

It has been suggested by several workers that the Calcein end-point can be improved by the addition of an inert dye, a so-called "screening agent"; such modifications will be described before proceeding with a discussion of the fluorescence end-point.

MODIFIED CALCEIN FOR VISUAL END-POINT. Five modified Calcein indicators have been described. Diehl and Ellingboe[5] added charcoal to supply a dusky background which emphasizes the green color; a mixture of 1 part of Calcein, 10 parts of charcoal and 100 parts of potassium chloride was prepared[a] and a small portion of the mixture added to the solution being titrated.

Tucker has described two modified, or screened, Calcein compositions. In the first of these[7] thymolphthalein was used as the screening agent, the composition being 0.2 g. of Calcein, 0.12 g. of thymolphthalein and 20 g. of potassium chloride ground together to a fine powder. About 10 mg. of this powder is required for the titration volume of 50 ml. At this concentration the green color is less obviously fluorescent and the residual fluorescence at the end-point does not obtrude. Under the best conditions the addition of less than 0.1 ml. of 0.01 N EDTA to a volume of 50 ml. gives a complete color change from green to purple. An excess of the indicator must be avoided.

In Tucker's[8] second modified Calcein indicator bromthymol blue is used as the inert dye, the composition being 2.0 g. of Calcein, 0.8 of bromthymol blue and 100 g. of potassium chloride.

Still another modified Calcein indicator has been proposed, Kirkbright and Stephen[9]. The addition of a few drops of 0.01 per cent acridine at the beginning of the titration gives a sharp color change at the end-point, the fluorescence of the solution changing from bright yellow-green to pure blue. Kirkbright and Stephens say that the amount of 0.02 M EDTA necessary to effect the change at the end-point is only half that with the

[5] *Anal. Chem.*, **28**, 882 (1956).

[a] Calcein and the various modified Calcein indicators are available from the G. FREDERICK SMITH CHEMICAL COMPANY: Calcein, Item No. 222; Calcein-Charcoal-Potassium Chloride, Item No. 224; Calcein-Thymolphthalein-Potassium Chloride, Item No. 261; Calcein-Bromthymol Blue-Potassium Chloride, Item No. 301.

[7] *Analyst*, **82**, 284 (1957)

[8] Private communication.

[9] *Anal. Chim. Acta*, **27**, 294 (1962).

unscreened indicator, 0.01 ml. in contrast to 0.02 to 0.03 ml.

And still another mixed indicator has been proposed[10], Calcein plus phenolphthalein complexone. Phenolphthalein complexone itself is a metallochromic indicator but is not sufficiently sharp to be useful in the determination of calcium. In conjunction with Calcein a nice complementary effect is observed, the pink color of the calcium derivative of phenolphthalein complexone being screened by the intense green fluorescence of the calcium-calcein compound; at the equivalence-point the purple color of the free phenolphthalein complexone obscures the residual fluorescence of the Calcein. The best end-point was obtained with a mixture of 4 parts of Calcein and 1 of phenolphthalein complexone. This mixture may be the best indicator for titration of calcium in the presence of large amounts of sodium salts where the residual fluorescence of Calcein is relatively high.

Fluorescence End-Point. The disappearance of the fluorescence of Calcein at the equivalence-point in the titration of calcium with EDTA at pH 12 is best observed in the dark under ultraviolet radiation. The General Electric Company "Black Light" lamp, No. BLB, 15 watt, made of dark blue glass[a] is a convenient source. The titration is best carried out with the light falling on the titration vessel at right angles to the line of vision of the observer and with the light source shielded from the eyes of the operator. For convenience the apparatus may be assembled in a box, and several such assemblies have been described[11, 20, 21]. In the titration box at Iowa State University, the BLB bulb is housed vertically in a box, horizontal cross section of 10 cm. by 10 cm. and height 50 cm., with a vertical slit 2.5 cm. wide on one side. The light housing is attached to the left side of another titration box so that light from the slit falls on the titration vessel which is placed in the titration box on a magnetic stirrer. The buret passes through a hole in the top of the titration box and is operated by hand, the right side of the titration box having no wall. The titration vessel is viewed through an opening, 13 cm. by 13 cm., in the front of the box, at right angles to the light beam. The opening is covered with a piece of light yellow glass or yellow cellophane. The BLB lamp emits a small amount of visible blue light which is reflected from the walls of the titrating vessel; this reflection introduces some confusion and is tiring in routine work. This blue reflection is eliminated and the end-point made more distinct by viewing the titration vessel through a sheet of yellow

[11] *Talanta*, **2**, 88 (1959).

[20] *Clin. Chim. Acta*, **4**, 346 (1959).

[21] *Proc. Soc. Exptl. Biol. Med.*, **99**, 777 (1958).

[10] *Talanta*, **8**, 249 (1961).

[a] The BL bulb made of white glass is not so satisfactory.

cellophane. Yellow cellophane is commonly available in large sheets and a piece of convenient size may be held between glass plates.

The disappearance of the fluorescence can also be observed in a darkened room under radiation from a tungsten filament lamp if the light beam is focussed on the solution. A microscope illuminator is a convenient scource, the diaphragm and lens system permitting focussing so that an image of the filament appears in the solution. A black or dark red back ground is best and the solution should be observed at right angles to the light beam. A blue filter placed in the light beams improves the end-point, the change being from bright yellow-green fluorescence to essentially colorless.

Some precision, on the micro scale, at least, can be gained by carrying out the titration in a cuvette in a fluorometer and plotting the readings of relative intensity of fluorescence against volume of EDTA; this technique was employed by Olsen, Diehl, Collins and Ellestad[31] for the determination of calcium in lithium salts, page 47, and by Cartier and Clement-Metral[22] for calcium in serum.

Carbonate-Sucrose Modification of Tucker. As stated above, if sufficient calcium is present, calcium hydroxide may be precipitated when the pH is raised to 12 prior to the calcium titration, and the Calcein end-point must be approached slowly. In the presence of large amounts of magnesium some loss of calcium by coprecipitation occurs. In a modification of the usual procedure, Tucker[12, 7] circumvents both troubles by adding sodium carbonate and sucrose before raising the pH with alkali. The carbonate causes the calcium to precipitate in a form which readily dissolves in EDTA during the titration and the sucrose unites with any calcium remaining in solution to form a soluble, slightly dissociated compound which prevents coprecipitation with the magnesium hydroxide precipitated when the pH is raised.

Although Tucker used sodium carbonate and sodium hydroxide, the residual fluorescence at the end-point caused by the alkali metal present is less with potassium and in the working directions given below for the Tucker procedure, potassium salts rather than sodium are specified.

Applications. The direct determination of calcium in the presence of magnesium by EDTA titration with Calcein as indicator was applied by Diehl and Ellingboe[5] to the analysis of hard water, limestone, and gypsum. It was applied later by a number of workers to the determination of cal-

[31] *Talanta*, **7**, 187 (1961).
[22] *Clin. Chim. Acta*, **4**, 357 (1959).
[12] *Chem. Ind. London*, **1954**, 1236.
[7] *Analyst*, **82**, 284 (1957)

cium in tissue and serum, and by others to various products: phosphate rock, bone, and teeth by Yalman and coworkers[6], lithium salts by Olsen, Diehl, Collins and Ellestad[31], blast furnace slag by Morris[13], silicates[14], and sulfate (indirectly through barium sulfate) by Effenberger[42].

Basically the procedure is the same for the determination of calcium in all of these materials, the variations arising principally in the method of working up the sample prior to taking the aliquot for the actual determination, and in the amount of calcium taken and the concentration of the EDTA used for the titration. The preparation of reagents and the general procedure are given immediately below and in the following sections, B, C, D, E, and F, are discussed individually the analysis of various materials, the particular problems associated with them, and the variations in the method made necessary by the circumstances of amounts and interferences. In the general procedure given below ascorbic acid is added to reduce any iron present (hydroxylammonium chloride may be used).

Procedure for the Determination of Calcium in the Presence of Magnesium. General Procedure and Determination of Calcium in Water. REAGENTS. STANDARD CALCIUM CHLORIDE SOLUTION. 0.01 M. Dissolve 1.0009 g. of primary standard grade calcium carbonate (G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 337) in a little dilute hydrochloric acid. Dilute to exactly 1 liter, mix well, and transfer to a clean, dry bottle for storage; 1.000 ml. of this solution is equivalent to 1.000 mg. of calcium carbonate.

To prepare a less concentrated calcium standard (for work with 0.001 M EDTA) pipet 100.0 ml. of this solution into a 1-liter volumetric flask, dilute to the mark with distilled water, and mix.

STANDARD DISODIUM DIHYDROGEN ETHYLENEDIAMINETETRAACETATE (EDTA). Prepare an EDTA solution of the desired concentration approximately by dissolving the necessary amount of disodium dihydrogen ethylenediaminetetraacetate dihydrate (G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 247), molecular weight 372.26, in 1 liter of distilled water:

Concentration		Disodium Dihydrogen Ethylenediaminetetra-acetate Dihydrate Required for 1 liter	Equivalent to Calcium	Equivalent to Calcium Carbonate
Molar	Normal	g.	mg. per ml.	mg. per ml.
0.05	0.1	18.613	2.004	5.005
0.01	0.02	3.723	0.4008	1.001
0.005	0.01	1.8613	0.2004	0.5005
0.001	0.002	0.3723	0.04008	0.1001

Standardize this solution as described immediately below.

[6] *Anal. Chem.*, **31**, 1230 (1959).
[31] *Talanta*, **7**, 187 (1961).
[13] *Anal. Chem.*, **33**, 599 (1961).
[14] *Z. Angew. Geol.*, **8**, 263 (1962).
[42] *Chem. Listy*, **52**, 1501 (1958).

Alternatively, the EDTA solution may be made up by weight using primary standard disodium dihydrogen ethylenediaminetetraacetate dihydrate, see Part VIII, page 111. A primary standard grade of disodium dihydrogen ethylenediaminetetraacetate dihydrate is available from the G FREDERICK SMITH CHEMICAL COMPANY, Item No. 365.

POTASSIUM HYDROXIDE. 5 M. Dissolve 28 g. of reagent grade potassium hydroxide in 100 ml. of deionized water. Store in a polyethylene bottle.

POTASSIUM CYANIDE. 1 PER CENT. Dissolve 1 g. of reagent grade potassium cyanide in 100 ml. of deionized water. Store in a polyethylene bottle.

CALCIEN INDICATOR SOLUTION. 0.02 PER CENT SOLUTION. Place 20 mg. of Calcein (G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 222) in a beaker containing 25 ml. of deionized water and with mechanical stirring add 0.1 N potassium hydroxide dropwise until the solid has all dissolved. Dilute to 100 ml. with deionized water. Place this solution in small polyethylene bottles, freeze, and store in a refrigerator, thawing for use as needed[b]. Use this weakly alkaline, 0.02 per cent solution as indicator, keeping the solution in the dark when not needed.

STANDARDIZATION OF EDTA SOLUTION. Select an aliquot of the standard calcium chloride solution which will provide sufficient calcium to require almost a buret full of the EDTA solution being standardized. Thus, using 0.01 M EDTA and a 50 ml. buret, (0.01 M)(50 ml.)(0.100 g. $CaCO_3$ per millimole) = 0.050 g. of $CaCO_3$ (or 0.020 g. of calcium), an aliquot containing 50 mg. of calcium carbonate (or 20 mg. of calcium) should be taken, this would be contained in 50.0 ml. of the standard calcium solution prepared as described above.

Pipet the aliquot of the size selected of the standard calcium solution into a 250-ml. conical flask and dilute to about 100 ml. with deionized water. Add a few milligrams of ascorbic acid and stir the solution. Add 5 ml. of 20 per cent triethanolamine, 2 ml. of 1 per cent potassium cyanide, and then, 5 ml. of 5 M potassium hydroxide. Add 60 μl. (one drop) of 0.02 per cent Calcein solution. Stir the solution mechanically (most conveniently with a magnetic stirrer, see G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 376). Titrate with the EDTA solution observing the end-point visually by the change in color from yellow-green to brown or the disappearance of fluorescence. If the color change is observed, use natural light or light from a tungsten filament lamp. If the fluorescence end-point is used, carry out the titration in the dark using for illumination an ultraviolet source and observing the operation through a sheet of yellow cellophane. Maintain vigorous stirring. If the amount of calcium taken is less than 25 or 30 mg. of calcium carbonate, no precipitate will appear when the solution is made alkaline, and the end-point can be approached fairly rapidly and will be sharp and permanent; with higher concentrations and a precipitate present, maintain vigorous stirring and approach the end-point slowly.

Calculate the molar concentration of the EDTA. If the EDTA solution had been made up slightly more concentrated than the concentration desired, the concentration can be adjusted to exactly the desired concentration by the addition of a volume of water calculated by the relation: (volume of EDTA to be diluted)(molar concentration of this solution)=(final volume)(molar concentration desired).

[b]The frozen solution of Calcein is orange in color and non-fluorescent; the liquid solution is yellow and fluorescent, the pH being 8 or so. The indicator solution is more stable if kept in the dark. After standing for two days at room temperature some deterioration is evident for a slight residual fluorescence then remains at the end-point when the solution is used in the calcium determination.

PROCEDURE FOR CALCIUM, DIRECT TITRATION. Select an aliquot of such a size as to contain sufficient calcium to require a volume of EDTA solution somewhat less than a buret full. Pipet this aliquot into a 250-ml. conical flask and proceed as given immediately above under standardization.

Report the results as per cent calcium, per cent calcium oxide, or per cent calcium carbonate as desired, or as in water analysis as p.p.m. of calcium carbonate. In the calculation use the millimolar weight (for example, 100.09/1000=0.10009 for calcium carbonate) if the concentration of the EDTA is expressed as moles per liter (molar concentration), or the milliequivalent weight (0.050004 for calcium carbonate) if the EDTA is expressed as equivalents per liter (normal concentration).

PROCEDURE FOR CALCIUM, INDIRECT TITRATION FOR SAMPLES HIGH IN PHOSPHATE. Select an aliquot of such a size as to contain sufficient calcium to require a volume of EDTA solution somewhat less than a buret full. Pipet this aliquot into a 250-ml. conical flask and dilute to about 100 ml. Add a few milligrams of ascorbic acid and then add a measured volume of EDTA sufficient to be in excess of the amount necessary to unite with the calcium. Then add 5 ml. of 20 per cent triethanolamine and 5 ml. of 1 M potassium hydroxide-1 per cent potassium cyanide solution. Add also two or three drops of 0.02 per cent Calcein solution. No precipitate should form. Add from a buret standard calcium solution sufficient to restore the yellow-green color and the fluorescence. Finally titrate with the standard EDTA solution to the end-point as described above under Standardization of EDTA.

PROCEDURE FOR CALCIUM, CARBONATE-SUCROSE METHOD OF TUCKER[7]. To a neutral aliquot of about 25 ml. add 1 ml. of 20 per cent sucrose solution, 2 ml. of 0.2 M potassium carbonate and 1 drop of 0.05 per cent Nile Blue A solution (G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 267 (solid indicator), Item No. 268 (0.05 per cent solution)). Add 10 per cent potassium hydroxide dropwise with mixing until the pink color of Nile Blue A is reached, and then a further 2 to 2.5 ml. of alkali. Add 10 mg. of modified Calcein indicator powder (Calcein plus thymolphthalein plus chloride (G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 261)) and titrate with EDTA. The color changes from green to purple and less than 0.1 ml. of 0.01 N EDTA is required to give a complete color change.

PART III

B. THE DETERMINATION OF CALCIUM IN WATER

The chemistry of hard water was discussed in Part II, Section B, page 10. The calcium hardness of waters will commonly constitute two-thirds to all of the total hardness. The relative amounts of calcium hardness and magnesium hardness depends on the nature of the rock through which the water has passed. Conceivably water passing through dolomite, $CaMg(CO_3)_2$, could have a calcium to magnesium ratio of 1 to 1.

One of the first applications of Calcein was to the determination of calcium in hard water, Diehl and Ellingboe[5]. No results on samples analyzed by another method also as a check were reported but presumably the results reported on hard water were correct inasmuch as satisfactory check analyses on Sample 88, Dolomite, of the National Bureau of Standards were obtained, see next section.

No particular problem arises in the determination of calcium in hard water and the general procedure given in the preceding section can be applied directly. EDTA 0.01 M in concentration is commonly used for the determination; 50 ml. of this solution is equivalent to 50 mg. of calcium carbonate, corresponding to 1000 p.p.m. in a 50 ml. sample. Simple selection of the size of the sample then, and use of EDTA of lower concentration if indicated, will give all the latitude necessary to operate the determination under the most favorable circumstances.

Iron is often present in ground water and in the general procedure given in Part III, Section A, provision is made to obviate the disturbing effect of iron by the addition of ascorbic acid (or hydroxylammonium chloride) and cyanide. The cyanide also removes the interference of copper which is occasionally found in treated waters. Amounts of aluminum sufficient to disturb the Calcein end-point are seldom present in natural, municipal and industrial waters and the triethanolamine called for in the general procedure may be omitted.

In water works practice, calcium hardness, like total hardness and other quantities measured on hard water, is commonly expressed as an equivalent amount of calcium carbonate, usually in parts per million, and the results are calculated by the formula

For full references see Bibliography, page 115.

[5] *Anal. Chem.*, **28,** 882 (1956).

$$\text{Ca as p.p.m. of } CaCO_3 = (\text{Per cent } CaCO_3)(10{,}000)$$

$$= \frac{(\text{ml.}_{\text{EDTA}})(M_{\text{EDTA}})(\text{Mol. Wt. } CaCO_3/1000)(100)(10{,}000)}{\text{Volume of Sample}}$$

for 0.01 M EDTA and a 50.0 ml.-sample, this reduces to

$$\text{Ca as p.p.m. of } CaCO_3 = 20(\text{ml}_{\text{EDTA}})$$

PART III

C. THE DETERMINATION OF CALCIUM IN LIMESTONE

The composition and properties of limestone and dolomite were discussed in Part II, Section C, page 14, in connection with the EDTA titration for the sum of the calcium and magnesium. A determination of the calcium alone can also be made by an EDTA titration by operating at pH 12 and using Calcein as indicator, see Part III, Section A, above for theory. Diehl and Ellingboe[5], who first described the Calcein indicator, reported satisfactory results for calcium in Samples 1A and 88, the Argillaceous Limestone and the Dolomite, respectively, of the National Bureau of Standards, Table 10.

TABLE 10. ANALYSIS OF LIMESTONE AND GYPSUM

Data of Diehl and Ellingboe[5]

(0.05 M EDTA standarized against Iceland Spar[a]; visual, color-change end-point)

	NBS 1A	NBS 88	Selenite[b]
CaO reported, per cent	41.32	30.49	32.57[c]
MgO reported, per cent	2.19	21.48	[b]
CaO found, per cent[d]	41.31	30.48	30.53
	41.37	30.37	32.46
	41.25	30.41	32.51
	41.26	30.45	32.54
	41.22	30.44	32.64
	41.36	30.56	32.50
	41.21	30.44	32.47
	41.20	30.50	32.54
	41.18		32.52
			32.60
			32.54
			32.46
Average	41.26	30.46	32.52
Range	0.19	0.19	0.18
Standard deviation, σ	0.070	0.059	0.054
Relative standard deviation, δ ($\delta=1000\sigma$/Average)	1.70	1.93	1.67

[a]Transparent crystals; magnesium content determined spectrographically to be 40 p.p.m.

[b]Transparent crystals of gypsum (selenite variety from Freedom, Oklahoma); magnesium content determined spectrographically to be 20 p.p.m.

[c]Theoretical CaO content for $CaSO_4 \cdot H_2O$.

[d]Results on 1A and 88 corrected for the strontium oxide present, 0.05 per cent and 0.01 per cent, respectively.

For full references see Bibliography, page 115.

[5]*Anal. Chem.*, **28**, 882 (1956).

Their procedure calls for 0.05 M EDTA and for slow approach to the end-point to allow time for precipitated calcium to redissolve. When working with 0.005 M EDTA and smaller amounts of calcium, no precipitate appears and the end-point can be approached more rapidly. Both procedures are given below. The determination can be carried out precisely with EDTA as dilute as 0.001 M; see Table 11.

TABLE 11. DETERMINATION OF CALCIUM IN NBS 88 (DOLOMITE)

Data of H. Diehl and R. C. Miller[15]

(0.001 M EDTA, Fluorometric End-Point)

CaO reported, per cent	30.49
CaO found, per cent	30.42, 30.50, 30.42, 30.47, 30.45, 30.44
Average	30.45
Standard deviation, σ	0.03
Relative standard deviation, δ, in parts per thousand	0.98

Results of analyses of limestone are usually reported as per cent oxide. The theoretical calcium oxide content of calcite ($CaCO_3$) is 56.03 per cent, of dolomite ($CaMg(CO_3)_2$) 30.41 per cent. Values found on natural materials may or may not be close to the theoretical figures. Thus, NBS 88, see Table 10, is close to the theoretical composition of a dolomite, but NBS 1A, Argillaceous Limestone, is far short of the theoretical calcium oxide content of calcite, containing as it does large amounts of clay (14.11 per cent SiO_2, 4.16 per cent Al_2O_3).

On dissolving NBS 1A in hydrochloric acid and dehydrating, a black residue remains which contains iron but apparently, judging from the results of Diehl and Ellingboe, Table 10, little calcium. Usually it is expected with limestones high in silicate that a preliminary fusion with sodium carbonate or at least an ignition is necessary to render the silicate soluble in acid. Later work by Diehl and Miller[15] showed that consistently better results are obtained by dehydrating the silica by boiling with perchloric acid, filtering off and correcting the silica, and returning the residue to the main solution, on an aliquot of which the calcium can then be determined. The details of this procedure, which can be conveniently combined with a determination of calcium plus magnesium, are given below; for results by this method, see Table 12.

Working with 0.005 M EDTA and the quantity of calcium given in the procedure below, the end-point must sometimes be approached slowly, the fluorescence disappearing and then slowly returning. This behavior becomes worse the longer the solution stands after the various reagents and aliquot of the sample have been mixed and before the titration. The trouble

[15]Otherwise unpublished work.

TABLE 12. DETERMINATION OF CALCIUM IN LIMESTONE AND DOLOMITE FOLLOWING PERCHLORIC ACID DEHYDRATION OF SILICA. EDTA TITRATION AT pH 12 WITH CALCEIN INDICATOR

Data of H. Diehl and R. C. Miller[15]

	NBS 88 Dolomite	GFS 400 Dolomite[a]	GFS 401 Limestone[a]	GFS 402 Limestone[a]
Found, per cent CaO	30.49[d]	30.42	50.24	46.76
	30.49[d]	30.51	50.25	46.77
	30.40[d]	30.51	50.22	46.73
	30.50[d]	30.48	50.26	46.81
	30.47[e]	30.45	50.28	46.86
	30.50[e]	30.43	50.10	46.92
	30.51[e]	30.48	50.19	46.78
	30.41[e]			46.76
	30.35[f]			46.64
	30.37[f]			
	30.49[f]			
	30.48[f]			
Average, per cent CaO	30.46[b]	30.47	50.22	46.78
Stadnard deviation, σ	0.032	0.036	0.060	0.080
Relative standard deviation, δ, in parts per thousand	1.05	1.18	1.20	1.71

[a]Each analysis made on a different specimen of the sample taken at the time of mixing. Analyses made on the same samples as the results reported in Table 6.

[b]NBS value, 30.49 per cent CaO.

[d,e,f] Samples from three different bottles of NBS No. 88.

is probably caused by the precipitation and slow redissolution of calcium carbonate or hydroxide. The trouble can be avoided by adding the major part of the standard EDTA solution to the alkali before pipeting in the aliquot containing the calcium. An excess of standard EDTA may even be added and a back titration made with a standard calcium solution. Titration with EDTA to the disappearance of the fluorescence seems to be easier to observe, however. In the procedure given below, preliminary addition of the standard EDTA solution is prescribed.

Gypsum. An interesting application of the EDTA titration at pH 12 with Calcein indicator for calcium is the analysis of gypsum ($CaSO_4 \cdot 2H_2O$). As carried out by Diehl and Ellingboe[5], an excess of standard EDTA was added to the weighed sample and the mixture, at pH 12, was stirred for 4 to 6 hours. Excess standard calcium chloride was then added and the solution titrated with EDTA. The results are shown in Table 10.

Procedure for the Determination of Calcium in Limestone, Silica Dehydrated But Not Removed. METHOD OF DIEHL AND ELLINGBOE[5]. Weigh a sample of about 0.3 g. into a 400-ml. beaker. Add 20 ml. of dilute hydrochloric acid (1 to 1) and evaporate to dryness. Redissolve the sample in 5 ml. of dilute hydrochloric acid (1 to 10) and then dilute to 100 to 200 ml. with distilled water. To this add 1 to 2 drops of 0.02 per cent Calcein and about 5 ml. of 1 M potassium hydroxide containing 5 g. of potassium cyanide per 100 ml. Titrate with 0.1 N EDTA until the color changes from yellow-green to brown. Vigorous stirring is necessary throughout the titration.

Procedure for the Determination of Calcium in Limestone, Perchloric Acid Dehydration and Removal of Silica Prior to EDTA Titration with Fluorometric Calcein End-point. REAGENTS. Prepare 0.005 M EDTA, 5 M potassium hydroxide, 1 per cent potassium cyanide solution, and 0.02 per cent Calcein solution as described under the Determination of Calcium in the Presence of Magnesium, Part III, Section A, page 27. Prepare also a standard calcium solution by weighing accurately directly into a 1-liter volumetric flask about 1 g. of primary standard grade calcium carbonate (G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 337), dissolving it in a little hydrochloric acid, and diluting to the mark.

PROCEDURE FOR THE DEHYDRATION, REMOVAL, AND DETERMINATION OF SILICA. Start with an accurately weighed sample of about 1 g. and follow exactly the procedure given under the Determination of Calcium Plus Magnesium, Perchloric Acid Dehydration, page 17, dehydrating the silica by the perchloric acid method, filtering it, weighing the impure silica, correcting with hydrofluoric acid and sulfuric acid, weighing the residue, fusing the residue with a sodium carbonate-sodium tetraborate mixture, returning the dissolved residue to the main solution, and finally diluting to 1 liter. Use an aliquot of this solution for the determination of calcium as described in the next paragraph.

EDTA TITRATION OF CALCIUM. To the solution of the limestone sample prepared as described in the preceding paragraph (1-g. sample from which the silica has been removed and the solution brought to a final volume of exactly 1 liter) add about 10 mg. of solid ascorbic acid. Mix the solution and allow it to stand a minute or two before proceeding. Into a 250-ml. conical flask place 5 ml. of 5 M potassium hydroxide, 2 ml. of 1 per cent potassium cyanide, 5 ml. of 20 per cent triethanolamine, and measured accurately, about 40 ml. of standard 0.005 M EDTA. Add 60 μl. (one drop) of 0.02 per cent Calcein. Into this solution pipet accurately 25.00 ml. of the solution of the limestone. If the solution is fluorescent, titrate with 0.005 M EDTA. If the solution is not fluorescent, indicating that an excess of standard EDTA has already been added, add a measured volume of standard calcium solution (conveniently the standard solution prepared from 1 g. of primary standard calcium carbonate) sufficient to restore the fluorescence; titrate with EDTA to the disappearance of the fluorescence. Carry out the titration in the dark using for illumination an ultraviolet source and observing the operation through a sheet of yellow cellophane. Maintain vigorous stirring. The disappearance of the fluorescence at the end-point will be sharp and permanent.

Standardize the EDTA solution in exactly the same manner using 25.00 ml. of the standard calcium chloride solution (1 g. of calcium carbonate, primary standard, per liter). Calculate the results as per cent calcium oxide. Numerical values for various factors of interest in the analysis of limestone and dolomite are given in Appendix A.

PART III

D. THE DETERMINATION OF CALCIUM IN BONE, TEETH, PHOSPHATE ROCK, AND MATERIALS HIGH IN PHOSPHATE

As discussed in Part III, Section A, results for calcium by the EDTA titration at pH 12 with Calcein indicator are slightly low in the presence of phosphate. Yalman and coworkers[6] obtained correct results by adding excess standard EDTA prior to raising the pH and then back titrating with a calcium solution, see Table 8, page 22 for results on known amounts of calcium. The back titration procedure was applied successfully by Yalman and coworkers to the analysis of bone, teeth, and phosphate rock.

The analyses of bone and teeth for calcium were made after ashing. The ashed material was dissolved in hydrochloric acid, diluted suitably, and aliquots taken for the indirect titration; see Table 13 for results.

TABLE 13. CALCIUM CONTENT OF BONES AND TEETH

Data of Yalman and coworkers[6]

Material	Calcium Found[a, b] Per cent of ash weight
Phalanges (33 materials)	39.13
Femurs (33 materials)	39.09
Humeri (33 materials)	39.04
Deciduous teeth (71 materials)	38.4

[a]Average of four determinations on each material.
[b]Accepted value for bone: 37 per cent Ca; for teeth: 38.3 per cent Ca.

For the analysis of phosphate rock, the sample was dissolved in 5 to 10 ml. of concentrated hydrochloric acid. After several hours the sample was diluted and the calcium determined by indirect titration. No attempt was made to bake out or otherwise separate the silica. In some determinations the end-point was determined by observing the formation of a pink color along the wall of a polyethylene beaker. In other determinations the solutions were centrifuged just before back titration of the excess EDTA was complete, and the final end-point was determined with the centrifugate

For full references see Bibliography, page 115.
[6]*Anal. Chem.*, **31**, 1230 (1959).

in a polyethylene beaker. All the determinations were made with diffuse daylight illumination. The rock, N.B.S. No. 56b, Phosphate Rock, contained 44.06 per cent calcium oxide. An average of four determinations each by the indirect and centrifuging methods gave 44.17 ± 0.04 and 44.10 ± 0.02 per cent, respectively.

Although these results leave little to be desired with respect to either accuracy or precision some criticism can perhaps be levelled at the procedure of Yalman and coworkers[6]. The difficulty with the end-point arises from the precipitation of ferric hydroxide. Yalman and coworkers realized this fully and in auxiliary experiments studied the effect on the end-point of varying amounts of ferric iron and noted that the addition of cyanide effected only a partial improvement. What would probably have eliminated the trouble is the addition of a reducing agent (hydroxylammonium chloride, ascorbic acid) prior to the addition of the cyanide and the alkali. Ferric iron does not react with cyanide to form ferricyanide but the reaction of ferrous and cyanide is rapid. The yellow color of the ferrocyanide causes some trouble with the color-change end-point but not with the fluorometric end-point.

PART III

E. THE DETERMINATION OF CALCIUM IN SERUM AND TISSUE

Because of the small samples available, the determination of the calcium in blood serum is made on a small sample, 0.2 ml., or at the most 1.0 ml. The amount of calcium in serum will normally run about 10 mg. per 100 ml. (milligram per cent) of serum. On a 0.1 ml. sample the amount of calcium is therefore 10 μg. The EDTA titration with Calcein as indicator is well adapted to such a determination.

Quickly following the introduction of the EDTA-Calcein method by Diehl and Ellingboe[5] in 1956 application was made to the determination of calcium in blood, in 1957 by Andersch[16], by Ashby and Roberts[17], and by Baron and Bell[18], and in the following years by Bett and Fraser[19, 20], Herrmann[21], Cartier and Clement-Metral[22], Socolar and Salach[23], Bohuon and Festy[24], Schirardin and Metais[25], Mori[26], Coolidge[27], Toribara and Koval[28], Klass[29], and Wallach and Steck[30]. These investigations have shown that no preliminary treatment of serum is necessary, that recovery of calcium is essentially complete and that in comparison with earlier methods the procedure is rapid and values for calcium obtained identical in precision and accuracy. Moreover, the tolerance for magnesium and phosphate is high and the disturbing effects of hemoglobin and other colored biological materials is usually not serious. The determination can be carried out on as little as 10 μl. of serum.

Preliminary Treatment of Serum and Tissue. It is now well established that no preliminary treatment of serum is necessary, that all of the calcium in serum brought to pH 12 is available for immediate reaction

For full references see Bibliography, page 117.

[5] *Anal. Chem.*, **28**, 882 (1956).
[16] *J. Lab. Clin. Med.*, **49**, 486 (1957).
[17] *J. Lab. Clin. Med.*, **49**, 958 (1957).
[18] *Clin. Chim. Acta*, **2**, 327 (1957).
[19] *Biochem. J.*, **68**, 13p (1958).
[20] *Clin. Chim. Acta*, **4**, 346 (1959).
[21] *Proc. Soc. Exptl. Biol. Med.*, **99**, 777 (1958).
[22] *Clin. Chim. Acta*, **4**, 357 (1959).
[23] *Anal. Chem.*, **31**, 473 (1959).
[24] *Pathol. Biol. Semaine Hop.*, **7**, 191 (1959).
[25] *Pathol. Biol. Semaine Hop.*, **7**, 418 (1959).
[26] *Arch. Biochem. Biophys.*, **83**, 552 (1959).
[27] *Anal. Biochem.*, **1**, 93 (1960).
[28] *Talanta*, **7**, 248 (1961).
[29] *Tech. Bull. Registry Med. Technogists*, **32**, 77 (1962).
[30] *Anal. Biochem.*, **6**, 176 (1963).

with EDTA. This was, of course, not immediately obvious for some calcium in the body is bound to protein and the relative affinity of calcium for EDTA and for protein determines if the titration can be applied directly.

Some workers have applied a preliminary treatment to the sample of serum: precipitation of protein with trichloroacetic acid (Andersch[16]), sodium hydroxide digestion followed by precipitation of the calcium as the oxalate (Herrmann[21]), ashing with nitric acid plus perchloric acid (Coolidge[27]). That calcium can be determined directly, however, without precipitation of the protein or destruction of the organic matter present, is now certain; thus, Ashby and Roberts[17] found for rabbit serum the results shown in Table 14; no difference was found between results by direct titration and titration following dry ashing. Bett and Fraser[20] also found no

TABLE 14. CALCIUM IN POOLED RABBIT SERUM BY EDTA TITRATION USING CALCEIN AS INDICATOR; FLUOROMETRIC END-POINT

Data of Ashby and Roberts[17]

Direct Titration:	11.9, 11.8, 10.5, 11.5, 11.4; mean 11.4±0.2 mg. Ca per 100 ml.
Dry Ashing:	11.6, 11.5, 11.4, 11.7, 11.3; mean 11.5±0.1 mg. Ca per 100 ml.

difference between direct titration and titration following preliminary ashing.

The question of what happens to calcium during the precipitation of protein with trichloroacetic acid was settled by Mori[26] who divided pooled rat sera into six groups and subjected them to different preliminary treatments. The titrations were carried out with disodium dihydrogen cyclohexane-1,2-diaminetetraacetate (CDTA) rather than with EDTA, but it is very probable that the results would have been the same with EDTA. The calcium in group 1 was determined directly by CDTA-Calcein titration. Each serum of groups 2-5 was treated with trichloroacetic acid, centrifuged after standing 0, 3, 6, and 24 hours, respectively, and the supernatant liquids analyzed for calcium by CDTA-Calcein titration. The trichloroacetic acid-protein precipitates from groups 2-5 were dried, then wet ashed with nitric acid plus perchloric acid, and the residues analyzed for calcium by CDTA-Calcein titration. Samples of the serum in group 6 were dried, wet ashed with nitric acid plus perchloric acid, and the calcium then determined. A blank and a calcium standard were carried through the same procedure at the same time. The results are shown in Table 15.

Although numerous investigators, Anderson through Wallach and Steck as listed in the opening paragraphs of this section, report success with direct

TABLE 15. DETERMINATION OF CALCIUM IN SERUM SUBJECTED TO DIFFERENT PRELIMINARY TREATMENTS

Data of Mori[26]

Group	Treatment	Number of Determinations	Calcium Found, meq./l. Total	Supernatant Liquid	Trichloroacetic Acid Precipitate[a]
1	Direct titration	14	5.45±0.35		
2	Trichloroacetic acid, immediate	10	5.08±0.21	4.90±0.15	0.18±0.02
3	Trichloroacetic acid, 3 hours standing	11	5.39±0.30	5.27±0.43	0.12±0.02
4	Trichloroacetic acid, 6 hours standing	13	5.34±0.36	5.18±0.31	0.16±0.01
5	Trichloroacetic acid, 24 hours standing	12	5.09±0.27	4.96±0.32	0.13±0.02
6	Wet ashing with nitric acid plus perchloric acid	11	4.89±0.21		

[a]After wet washing with nitric and perchloric acids.

determination of calcium in serum by EDTA titration at pH 12 with Calcein indicator, Toribara and Koval[28] state that they obtained erratic results and finally for their work devised a method in which the calcium is precipitated as the oxalate, the oxalate filtered off and decomposed by ignition, the remaining carbonate dissolved in acid and the calcium titrated with EDTA using the fluorometric Calcein end-point. Toribara and Koval reported good end-points with calcium alone and the description of their trouble with the direct titration of calcium in serum makes it probable that they were operating at a pH below 12.

Recovery of Calcium and Comparison of the EDTA-Calcein Method with the Oxalate-Permanganate Method. An extensive check on the accuracy of the EDTA-Calcein method was made by Bett and Fraser[20] who added known amounts of calcium to human sera and obtained recoveries of 99.7 per cent with a standard deviation of 1.14; Table 16. Amounts of calcium as low as 0.5 μg. were titrated and the relationship between the calcium present and the EDTA used for the titration was linear up to at least 60 μg. of calcium. Different volumes of serum ranging from 0.01 to 0.3 ml. were titrated under the same conditions and a linear relationship between the volume or serum and the volume of EDTA was obtained. Increasing the ratio of serum to indicator by a factor of 30 thus had no effect on the result and interaction of serum and calcein is thus ruled out.

The EDTA-Calcein method was also checked by several workers by the analysis of the same materials by precipitation of calcium oxalate and

TABLE 16. DETERMINATION OF CALCIUM IN SERUM TO WHICH ADDITIONAL CALCIUM WAS ADDED

Data of Bett and Fraser[20]

Serum No.	Calcium present in serum	Calcium added	Total calcium *(b)*	Total calcium found *(a)*	*(a–b)*	100 *a/b*
	mg./100 ml.	mg./100 ml.	mg./100 ml.	mg./100 ml.	mg./100 ml.	
1	8.52	0.50	9.02	8.97	—0.05	99.5
	8.52	4.35	12.87	12.94	+0.07	100.6
	8.52	8.32	16.84	16.50	—0.34	98.0
2	7.64	5.00	12.64	12.73	+0.09	100.7
	7.64	10.00	17.64	17.69	+0.05	100.2
	7.64	15.00	22.64	22.73	+0.09	100.4
3	9.66	6.00	15.66	15.62	—0.04	99.7
	9.66	10.00	19.66	19.55	—0.11	99.4
4	9.51	2.00	11.51	11.62	+0.11	100.9
	9.51	9.00	18.51	18.30	—0.21	98.9
	9.51	12.00	21.51	20.94	—0.57	97.3
5	9.72	8.00	17.72	17.84	+0.12	100.7
				Mean ± Standard Deviation:		99.7 ± 1.14

subsequent titration of the filtered and redissolved oxalate with permanganate[a]. Andersch[16] found essentially no difference between the results by the two methods, using either 0.2 ml. or 1.0 ml. samples for the ethylenediaminetetraacetate titration. Ashby and Roberts[17] and Baron and

TABLE 17. COMPARISON OF RESULTS BY THE EDTA-CALCEIN AND OXALATE PROCEDURES FOR CALCIUM IN SERUM

Sample No.	EDTA-Calcein mg. Ca/100 ml.	Oxalate mg. Ca/100 ml.
Data of Ashby and Roberts[17]; fluorescence end-point		
A	10.5±0.1	10.2±0.4
B	9.9±0.2	9.9±0.2
Data of Baron and Bell[18]; color change of modified Calcein		
1	8.4	8.5
2	8.4	8.5
3	7.4	7.6
4	9.4	9.4
5	7.5	7.6
6	9.2	9.1

[a]The procedure almost always followed for the oxalate method for calcium is that of Kramer and Tisdall, *J. Biol. Chem.*, **46**, 339 (1921), *J. Biol. Chem.*, **47**, 475 (1921), as modified by Clark and Collip, *J. Biol. Chem.*, **63**, 461 (1923). The procedure is laborious and requires a volume of 2 to 4 ml. of serum. Great relief is always expressed when a satisfactory replacement for this procedure is found.

TABLE 18. COMPARISON OF RESULTS ON THE DETERMINATION OF CALCIUM IN SERUM BY EDTA-CALCEIN-FLUOROMETRIC METHOD AND OXALATE-PERMANGANATE METHOD

Data of Cartier and Clement-Metral[22]

Number	Fluorometric	Oxalate-Permanganate[a]	Difference	
	mg. Ca/100 ml.	mg. Ca/100 ml.	mg. Ca/100 ml.	Per Cent
1	10.00	9.99	0.01	0.1
2	9.74	9.67	0.07	1
3	10.60	10.64	0.04	-0.4
4	9.60	9.60	0.00	0
5	9.60	9.50	0.10	1
6	10.40	10.20	0.20	2
7	10.04	10.00	0.04	0.4
8	9.70	9.90	0.20	-2
9	10.50	10.40	0.20	1
10	9.60	9.55	0.05	0.5
11	11.10	11.30	0.20	-2
12	9.40	9.40	0.0	0
13	8.60	8.55	0.05	0.5

[a]Procedure of Clark and Collip.

Bell[18] also found no difference; see Table 17. Cartier and Clement-Metral[22] also found that the values by the titrimetric method did not differ by more than 2 per cent; Table 18.

The reproducibility of a microdetermination of this sort is of some interest. Using the data obtained on 10 samples of each of two concentrations of a standard calcium solution, Cartier and Clement-Metral[22] calculated the standard deviation of the results and the standard error of the average and the difference of the averages; Table 19. The results show that considering the small sample taken, the precision is excellent.

Effect of Magnesium. In the original work on Calcein, Diehl and Ellingboe[5], it was shown that the EDTA titration of calcium at pH 12 using Calcein indicator could be carried out in the presence of large amounts of magnesium. This was confirmed on serum by Baron and Bell[18] who found that in the microtitration (0.1 and 1.0 ml. samples of serum) that amounts of magnesium as high as 10 mg. per 100 ml. did not affect the results; Table 20.

The non-interference of magnesium was further substantiated by Bell and Fraser[20] who added the magnesium both before and after the alkali and found no effect on the calcium determination with magnesium equivalent to 1000 mg. per 100 ml.

Coolidge[27], however, insists that if both magnesium and phosphate are present, that interference can only be avoided by adding excess EDTA and

TABLE 19. REPRODUCIBILITY OF THE ETHYLENEDIAMINE-CALCEIN-FLUORIMETRIC METHOD FOR CALCIUM

Data of Cartier and Clement-Metral[22]

(Results are expressed in μl. of 0.01 M EDTA)

	Average	σ[a]	S.E.M.[b]	m±2 S.E.	2 S.E.%
50 μl. calcium solution	16.39	0.20	0.07	16.39±0.13	
Blank	3.22	0.18	0.06	3.22±0.12	
Difference	13.17		0.09	13.17±0.18	1.4
100 μl. calcium solution	29.76	0.60	0.20	29.76±0.40	
Blank	3.53	0.17	0.05	3.53±0.12	
Difference	26.23		0.21	26.23±0.42	1.6

[a]Standard deviation, calculated using the formula

$$\sigma = \sqrt{\frac{\Sigma (x-m)^2}{n-1}}$$

where x is the value obtained in the determination, in the average, and n the number of determinations (10).

[b]Standard error of the mean and the difference of means by the formulas

$$\text{S.E.M.} = \frac{\sigma}{n} \qquad \text{and S.E.}_{\text{difference of m}} = \sqrt{\frac{\sigma_1^2}{n_1} + \frac{\sigma_2^2}{n_2}}$$

TABLE 20. EFFECT OF ADDING MAGNESIUM IN THE DETERMINATION OF CALCIUM IN SERUM

Data of Baron and Bell[18]

Serum magnesium equivalent of added magnesium (mg./100 ml.)	0	3	6	10
Serum calcium found (mg. Ca./100 ml.)				
1.0 ml. sample	9.8	10.0	10.1	10.1
0.1 ml. sample	10.0	9.8	10.4	9.8

back titrating with a calcium solution, a finding in accord with Yalman and others[6] discussed on pages 23 and 36.

Effect of Phosphate. Bett and Fraser[20] found that when high concentrations of both calcium and phosphate were present in the serum analyzed, premature, false end-points were obtained; the green fluorescence disappears and returns after stirring for a few minutes. The amounts of both ions necessary to cause this premature end-point are so high, 30 mg. calcium per 100 ml. and 50 mg. of phosphorus per 100 ml. of sample, that it is unlikely that the effect will ever be observed in any pathological serum.

Effect of Hemolysis. There is some contradiction in the published work on the effect of hemolysis products on the end-point. Ashby and Roberts[17], who observed the end-point under ultraviolet light, found that the red color of the hemoglobin obscured the end-point somewhat but that with practice the end-point could be found accurately. The addition of

cyanide obviated the interference of the metallic ions produced by the hemolysis. Baron and Bell[18] state flatly that the color change of the indicator or the modified indicator is difficult to detect if hemolysis has occurred and that the results are not accurate.

Bett and Fraser[20] went into the matter in more detail. Using ultraviolet light to observe the disappearance of the fluorescence, they found that a moderate degree of hemolysis had little effect on the titration other than to cause a whitish fluorescence at the end-point. Grossly hemolyzed sera are difficult to titrate because of the fluorescence at the end-point but this can usually be observed at a second titration.

They reported a similar effect with icteric sera where a yellow fluorescence tends to obscure the end-point. The effect does not cause serious interference until the bilirubin concentration rises to above 10 mg. per 100 ml. With more severly jaundiced sera the end-point can usually be observed on a second titration.

No trouble at all with hemolyzed serum was experienced by Cartier and Clement-Metral[22] using the fluorometric titration in which fluorescence data taken before and afterwards are extrapolated to locate the end-point. With increasing amounts of hemolysate prepared from the whole blood and added in increasing amounts to the serum the same results were found for calcium; Table 21.

TABLE 21. INFLUENCE OF HEMOLYSIS ON THE DETERMINATION OF CALCIUM IN SERUM

Data of Cartier and Clement-Metral[22]

Serum ml.	Hemolysate ml.	Water ml.	Calcium Found mm.[a]
2	0.00	0.50	130
2	0.05	0.45	131.7
2	0.10	0.40	129
2	0.20	0.30	130

[a]Expressed in mm. movement of piston in microburet.

End-Point Detection. On the micro scale required for the determination of calcium in serum, detecting the end-point with Calcein presents no particular problem, and is in fact easier than detecting the end-point on the macro scale. Both the color change and the fluorescence end-points have been used and about equally well; see the discussion on page 23.

The serum of some animals (sheep, for example) have a natural fluorescence of sufficient intensity to interfere seriously with the fluorometric end-point. This natural fluorescence can be removed by precipitating the protein with trichloroacetic acid and isopropyl alcohol. Working details are given below for this variation on the procedure.

Procedure for the Determination of Calcium in Serum. REAGENTS. WATER. Use only deionized water (passage through Amberlite MB-1) in making up the various solutions and in the analysis.

STANDARD DISODIUM DIHYDROGEN ETHYLENEDIAMINETETRAACETATE SOLUTION (EDTA). 0.0025 M. Dissolve 0.93 g. of disodium dihydrogen ethylenediaminetetraacetate dihydrate (G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 247) in one liter of deionized water and mix well. Standardize as described below.

STANDARD CALCIUM SOLUTION. 0.0025 M. Dissolve 0.2502 g. of primary standard grade calcium carbonate (G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 337) in a little dilute hydrochloric acid. Dilute to exactly 1 liter, mix well, and transfer to a clean dry bottle for storage; 1.000 ml. of this solution is equivalent to 0.2502 mg. of calcium carbonate or 0.1001 mg. of calcium.

CALCEIN INDICATOR SOLUTION. 0.002 PER CENT. Place 20 mg. of Calcein (G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 222) in a beaker containing 25 ml. of deionized water and with mechanical stirring add 0.1 N potassium hydroxide dropwise until the solid has all dissolved. Dilute to 1000 ml. with deionized water. Place this solution in small polyethylene bottles, freeze, and store in a refrigerator, thawing for use as needed. Use this weakly alkaline, 0.002 per cent solution as indicator, keeping the solution in the dark when not needed.

POTASSIUM HYDROXIDE PLUS POTASSIUM CYANIDE. 1 M KOH PLUS 1 PER CENT KCN. Dissovle 5.6 g. of potassium hydroxide and 1 g. of potassium cyanide in 100 ml. of deionized water.

APPARATUS. Use precision micro pipets for measuring out the sample. Use a micro buret for the titration, for example the micrometer buret of the Micro-Metric Instrument Company, Box 884, Cleveland 22, Ohio.

Carry out the titration in a 10 ml. beaker or flask using magnetic stirring. The water- or air-driven magnetic stirrer of the G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 376, is ideal for the purpose; for the micro stirring bar needed seal a piece of piano wire, 15 mm. in length, in a piece of hematocrit tubing (1.6 mm. o.d.). Observe the color change from yellow-green to pinkish-brown against a white black ground or observe the disappearance of the yellow-green fluorescence under ultraviolet light, in accord with the discussion above. If the fluorescence end-point under ultraviolet light is used, carry out the titration in some sort of a titration box, so the eyes will be shielded from the ultraviolet source, and observe the titration through a sheet of yellow glass or yellow cellophane.

PROCEDURE FOR THE DETERMINATION OF CALCIUM IN SERUM. With a pipet or syringe withdraw from the centrifuged blood the serum, transfer it to a small crucible or vial, and stopper the vial to avoid evaporation. Place in the titration vessel 1 ml. of the 1 M potassium hydroxide-1 per cent potassium cyanide solution. Pipet 100 μl. of the serum into the liquid already in the titration vessel. Place the potassium hydroxide-potassium cyanide solution in the vessel first as the order of addition is important. Add a few small crystals of ascorbic acid. Add 100 μl. (two drops) of 0.002 per cent Calcein. Dilute to about 5 ml. with deionized water. Stir the solution magnetically and titrate with 0.0025 M EDTA.

Run a blank titration using the same amounts of reagents and water but omitting the sample.

Standardize the EDTA solution by titrating 100 μl. of the standard calcium solution, 0.100 mg. of calcium per ml., in identical fashion.

Determination of Calcium in Serum Having Natural Fluorescence. REAGENTS.

In addition to the reagents specified above, prepare also the following solution.

TRICHLOROACETIC ACID-ISOPROPYL ALCOHOL. Dissolve 5 g. of trichloroacetic acid in 25 ml. of water, add 10 ml. of isopropyl alcohol, and dilute to 100 ml.

PROCEDURE. Place 1.00 ml. of serum or plasma in a centrifuge tube, add 9.00 ml. of 5 per cent trichloroacetic acid-10 per cent isopropyl alcohol solution, mix well and centrifuge (3 minutes at 2000 r.p.m.). Remove the supernatant liquid. In a 10 ml. conical flask, place 1 ml. of 5 M potassium hydroxide-1 per cent potassium cyanide solution. To the liquid already in the flask add exactly 2.00 ml. of 0.00025 M EDTA (equivalent to 20.0 μg. of calcium). Add 200 μl. of 0.002 per cent Calcein. Titrate with 0.0025 M calcium chloride solution (the calcium standard prepared above is convenient) to the appearance of a fluorescence.

Standardize the EDTA by titrating 100 μl. of the standard calcium solution, 0.100 mg. of calcium per ml., in identical fashion.

Procedure for the Determination of Calcium in Tissue. PREPARATION OF SAMPLE BY METHOD OF MORI[26]. Cut the tissue (0.5 to 1.2 g. of muscle, 0.6 to 0.8 g. of kidney) into pieces and blot with filter paper to remove blood. Weigh a small, glass vessel containing 5 ml. of 10 per cent trichloroacetic acid, place the tissue in the vessel, and weigh again. Transfer the contents of the vessel to a micro, stainless Waring blender of 25-ml. capacity. Add 5 ml. of 10 per cent trichloroacetic acid and homogenize 2 to 6 minutes. Adjust the final volume to 15 ml. with 10 per cent trichloroacetic acid. Centrifuge at 2500 r.p.m. for 15 minutes. Use 1 to 2 ml. of this supernatant liquid for the determination of calcium following the above procedure for serum.

PART III

F. THE DETERMNATION OF CALCIUM IN LITHIUM SALTS

The determination of small amounts of calcium in lithium compounds is of some significance owing to the detrimental effects of calcium in certain commercial uses of lithium salts. Although ideal from the standpoint of the small amounts of calcium which can be handled, the EDTA titration with Eriochrome Black T or Calmagite fails because the indicators do not function properly in the presence of large amounts of lithium. A satisfactory solution to this problem was found by Olsen, Diehl, Collins and Ellestad[31] who found that the titration could be carried out fairly satisfactorily using Calcein and detecting the end-point fluorometrically. Greater precision was obtained, however, using an alternative method which required more time and in which a preliminary separation of the calcium from the lithium was made using the chelating resin Dowex A-1.

Dowex A-1 is a co-polymer of divinylbenzene and styrene into which iminodiacetic acid groups have been introduced. The resin has a strong affinity for those cations which form non-ionized compounds with iminodiacetic acid and according to data published by the Dow Chemical Company, calcium can be efficiently separated from sodium with this resin. This suggests that calcium might be similarly separated from lithium and this has proved true. Samples containing lithium chloride in amounts many times more than equivalent to the capacity of the resin in the column may be passed through the column; the calcium is absorbed quantitatively although only up to the point where calcium equivalent to about 1 per cent of the capacity of the column has been taken up. The calcium is eluted with 2 N hydrochloric acid, the acid neutralized with potassium hydroxide, and the calcium titrated with EDTA, the visual end-point with Calcein being satisfactory but the fluorometric end-point being better.

The procedure involving the preliminary separation of the calcium with Dowex A-1 was checked by the analysis of several samples of lithium chloride, lithium bromide, and lithium sulfate. Additional calcium was added to some of the samples. The results of some of the analyses are

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For full references see Bibliography, page 118.

[31] *Talanta*, **7**, 187 (1961).

TABLE 22. DETERMINATION OF CALCIUM IN LITHIUM SALTS BY EDTA-CALCEIN TITRATION FOLLOWING SEPARATION OF THE CALCIUM WITH DOWEX A-1

Data of Olsen, Diehl, Ellestad and Collins[31]

Lithium Salt Taken g.		Calcium Found μg.	Per Cent[a]
Visual End-point			
LiCl(Lot A)	15.0	34	0.00022
	20.0	34	0.00016
LiCl(Lot B)	2.0	26	0.0011
LiCl(Lot C)	2.0	35	0.0016
	10.0	120	0.0012
Fluorometric End-point			
LiCl(Lot A)	2.0	6.8	0.00019
	2.0	6.4	0.00017
	2.0	7.4	0.00022
LiCl(Lot D)	2.0	7.1	0.00020
	2.0	6.4	0.00017
	2.0	6.7	0.00018
LiBr(Lot E)	2.0	48.5	0.0023
	2.0	47.5	0.0022
	2.0	46.5	0.0021
Li_2SO_4(Lot F)	2.0	10.0	0.00035
	2.0	9.7	0.00033
	2.0	9.8	0.00034
Blank	0.0	3.0	----
	0.0	2.8	----
	0.0	3.1	----

Lot A. Purified by Dowex A-1 method described in text; average: 0.00020 per cent calcium.
Lot B. Purified by simple recrystallization; average: 0.0011 per cent calcium.
Lot C. As received from Lithium Corporation of America; average: 0.0014 per cent calcium.
Lot D. Purified by Dowex A-1 method described in text; average: 0.00019 per cent calcium.
Lot E. Commercial material, Lithium Corporation of America; average: 0.0022 per cent calcium.
Lot F. Commercial material, Lithium Corporation of America; average: 0.00034 per cent calcium.
[a]Per cent calcium found is calculated after subtracting the average blank, 3.0 μg., from the calcium found.

summarized in Tables 22 and 23. It was found that owing to the very large ratio of lithium to calcium in its samples, the resin would only take up about 1 per cent of its theoretical capacity for calcium (theoretical capacity: 15 mg. of calcium for 2.5 ml. of resin; limit: 1 per cent of this or 0.15 mg. of calcium). This puts a limitation on the size of the sample and the amount of calcium which can be handled in one determination. Although the method does not possess the simplicity that might be desired, it does permit the detection of as little as 0.0002 per cent calcium.

The direct fluorometric titration procedure, no preliminary separation of the calcium, was tested on samples of specially purified lithium chloride

TABLE 23. DETERMINATION OF CALCIUM IN LITHIUM CHLORIDE EDTA-CALCEIN TITRATION FOLLOWING SEPARATION OF CALCIUM WITH DOWEX A-1

Data of Olsen, Diehl, Ellestad and Collins[31]

Lithium Chloride Taken g.		Calcium Added μg.	Total Calcium[a] Present μg.	Calcium Found μg.	Error μg.
Visual End-point					
Lot A	20.0	200	243	196	−47
Lot B	2.0	50	75	74	−1
	2.0	100	125	120	−5
Fluorometric End-point					
Lot A	2.0	20.0	26.9	28.3	+1.4
	2.0	20.0	26.9	27.8	+0.9
	2.0	20.0	26.9	27.6	+0.7

Lot A. Purified by Dowex A-1 method described under procedure; value of 0.00020 per cent calcium (from preceding table) used in calculating total calcium present.
Lot B. Purified by simple recrystallization; value of 0.0011 per cent calcium (from preceding table) used in calculating total calcium present.
[a]Total calcium values include 2.9 μg. added with reagents.

TABLE 24. DETERMINATION OF CALCIUM IN LITHIUM SALTS BY FLUOROMETRIC TITRATION WITHOUT PRELIMINARY SEPARATION

Data of Olsen, Diehl, Ellestad and Collins[31]

Lithium Compound	Calcium Added	Number of Determinations	Total Calcium Present	Total Calcium Found, Average	Average Deviation
	μg.		μg.	μg.	μg.
	0.100-g. Sample (Procedure A, Coleman Fluorometer)				
LiCl(Lot A)[a]	2.0	3	2.2	1.7	0.3
	5.0	3	5.2	4.9	0.3
	10.0	3	10.2	9.7	0.5
	2.00	3	20.2	19.9	0.3
	1.00-g. Sample (Procedure B, Collins Fluorometer)				
LiCl(Lot D)	0.0	1	1.9	7.3	5.4
	10.0	4	11.9	11.4	2.1
	20.0	4	21.9	24.3	2.4
	25.0	2	26.9	27.8	0.8
	50.0	11	51.9	53.8	1.8
	100.0	5	101.9	102.4	1.2
LiBr(Lot E)	0.0	1	22.2	23.1	0.9
	50.0	2	72.2	72.6	0.9
	100.0	1	122.2	122.8	0.6
Li_2SO_4(Lot F)	10.0	1	13.4	21.4	8.0
	50.0	3	53.4	56.2	2.8
	100.0	2	103.4	104.1	0.7

[a]Lot designations same as in Table 23.

to which known amounts of calcium, 2 to 20 μg., was added. The size of sample taken for this direct titration depends on the sensitivity of the fluorometer used and two working procedures, designated A and B, are given below, for samples of 0.1 and 1.0 g. Results obtained by this direct, fluorometric titration are given in Table 24. Typical titration curves for

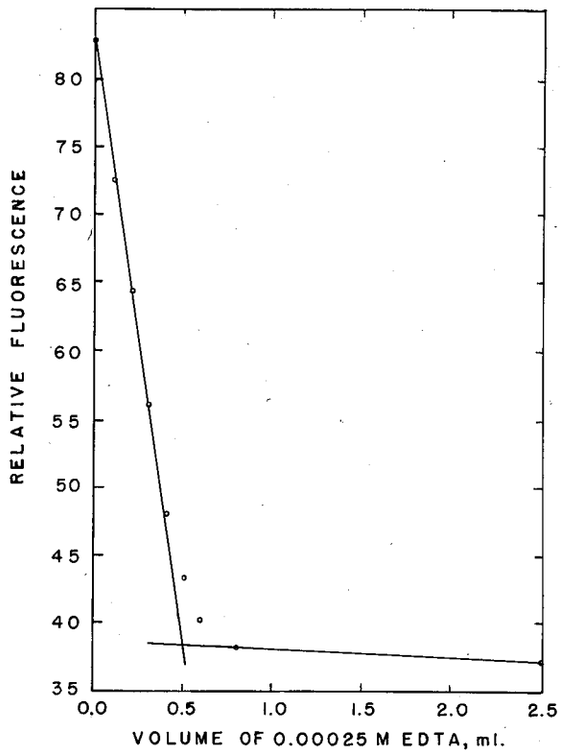


Figure 1. Fluorometric titration of 5 μg. of calcium in 100 mg. of lithium chloride with EDTA using Calcein as indicator; Coleman Model 12 Fluorometer. *Data of Olsen, Diehl, Ellestad and Collins*[31].

samples containing 5 μg. and 50 μg. are shown in Figures 1 and 2.

The fluorometric titration method without prior concentration of the calcium with the Dowex A-1 resin is the more rapid of the two procedures. The precision is satisfactory, but 0.001 per cent of calcium is the least amount that can be determined in lithium chloride by this method. Concentration of the calcium by the ion-exchange procedure followed by the fluorometric titration extends the sensitivity to 0.0002 per cent of calcium. The precision is good unless too large a sample is taken. The visual titration following concentration also permits the detection of as little as 0.0002 per cent of calcium but does not possess the precision of the fluorometric

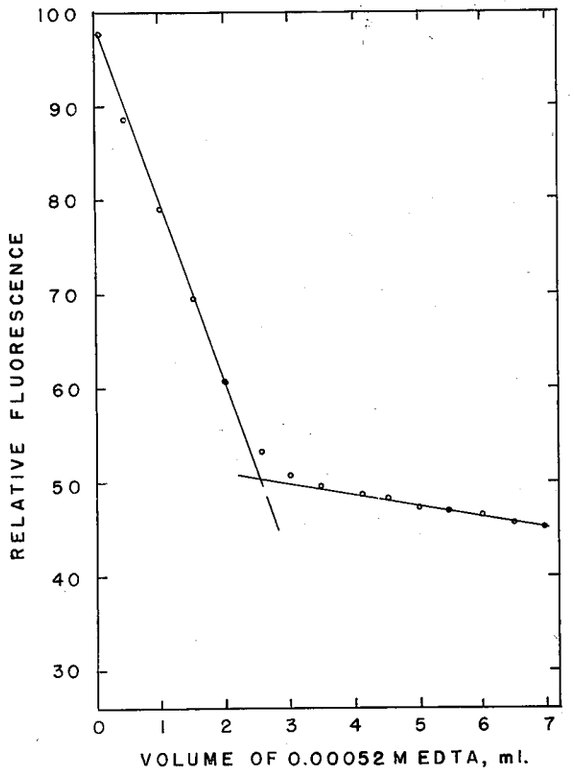


Figure 2. Fluorometric titration of 50 μg. of calcium in 1 g. of lithium chloride with EDTA using Calcein as indicator; Collins Fluorometer. *Data of Olsen, Diehl, Ellestad and Collins*[31].

titration and generally requies the use of larger samples. This, in turn, requires proportionately longer time.

The methods are applicable to lithium chloride, bromide and sulfate, and undoubtedly to other lithium salts.

Magnesium, provided it is not a major constituent, does not interfere with the determination of calcium. Aluminum, up to at least 0.1 per cent causes no interference. Iron above 0.1 per cent does cause interference.

Although it was reported by Diehl and Ellingboe[5] that strontium and barium are titrated together with calcium at pH 12 by EDTA using Calcein as indicator, barium was found not to interfere in the procedures described here. Thus, using 1-g. samples of lithium chloride containing 26 μg. of calcium (as determined by fluorometric titration) to which were added 276 μg. and 552 μg. of barium, 27.8 and 27.2 μg. of calcium were found. Also, in the presence of lithium bromide, barium is not titrated.

It may be that the potassium hydroxide used contained sufficient carbonate to precipitate all the barium; potassium hydroxide is used in preference to sodium hydroxide because of the significantly lower fluoroescence of potassium with Calcein. Strontium is not titrated in the procedure here described but does render the end-point less distinct. Zinc does not interfere at all.

Procedures for the Determination of Calcium in Lithium Salts. Apparatus and Reagents. Methods of Olsen, Collins and Ellestad[31]. Dowex A-1 Chelating Resin Column. Seal together two length of borosilicate tubing, one of 15-mm. inside diameter and 15 cm. long and the other of 6-mm. inside diameter and 10 cm. long. Draw down the smaller end somewhat so that it will retain a small plug of borosilicate wool to support the resin. Connect a borosilicate nozzle of small bore to the bottom of the column by means of a piece of surgical rubber tubing so that the flow-rate can be adjusted with a screw clamp. Transfer to the column 2.0 to 2.5 ml. of Dowex A-1 chelating resin in the water-swollen, salt form. The upper, larger-diameter portion of the column serves as a reservoir.

Calcein. 0.0033 Per Cent Solution. Dissolve 0.1 g. of Calcein in 100 ml. of water containing 0.5 ml. of 2 N potassium hydroxide; a solution prepared in this way is stable for only 3 or 4 days, presumably because of the excess alkali. From this stock solution prepare daily a 0.0033 per cent solution by diluting 1 ml. of the 0.1 per cent solution to 30 ml. with de-ionized water.

Water and Other Reagents. Use only demineralized water (passage through Amberlite MB-1) in making up the various solutions and in the analysis. Use hydrochloric acid and potassium hydroxide which are low in calcium as determined by running through the entire procedure but omitting the lithium chloride. Use potassium hydroxide rather than sodium hydroxide as it gives less fluorescence with Calcein.

Preparation of Calcium-Free Lithium Chloride. Filter a hot, concentrated solution of lithium chloride through a sintered-glass filter of fine or medium porosity. Filter paper has no strength in hot, concentrated solutions of lithium chloride and invariably breaks under the weight of the liquid. Cool the solution to room temperature and filter off the crystals of lithium chloride which form using a sintered-glass filter. Prepare a saturated solution (about 43 per cent) of this lithium chloride in water and store the solution in a polyethylene bottle.

Prepare an ion-exchange column containing about 20 ml. of Dowex A-1 chelating resin in the salt form. Pass 100 ml. of 2 N hydrochloric acid through the column and then 50 ml. of water. Pass through the column 60 ml. of 2 N lithium hydroxide to which EDTA equivalent to 6 mg. of calcium has been added. Wash the column very thoroughly with water. The column is now in the lithium form and free of calcium.

Dilute the saturated lithium chloride solution to a concentration of about 100 mg. per ml. Make the solution basic by the addition of 5 ml. of 2 N lithium hydroxide per liter of lithium chloride solution. Pass up to 4 liters of this solution through the column at the rate of 10 ml. per minute. Store the resulting calcium-free solution in a polyethylene bottle or concentrate to recover crystalline lithium chloride.

Calcium-Free Potassium Hydroxide Solution. This may be prepared with a Dowex A-1 resin column, by a procedure similar to that described for the preparation of calcium-free lithium chloride.

Fluorometer. Fluorometers differ considerably in sensitivity. The size of sample and amounts of reagents used in direct titration procedure A given below were chosen

for measurements with a Coleman Model 12 Electronic Photofluorometer and will be satisfactory, with slight modification perhaps, to other commercial fluorometers. A simpler fluorometer which can be put together by anyone handy in the shop is described by Collins[31]. Direct titration procedure B, below, is set up for use with such an instrument.

Because of the small volumes involved, the titrations can be carried out in the usual fluorometer cuvettes or Pyrex test tubes.

When using the Coleman Model 12 Fluorometer, use Coleman Filter No. 12-223 (Corning glass No. 3389) in the exciting beam and Coleman No. PC-2 (Corning glass 3486) in the fluorescent beam. For simpler instruments a Wratten K3 filter in the exciting beam may be sufficient. See also page 80 for a discussion of the selection of filters.

Procedure for the Determination of Calcium in Lithium Salts With Preliminary Separation of Calcium. Throughout this determination, when liquid is being passed through the column of Dowex A-1, use a flow-rate of not more than 2 ml. per minute. Prepare the resin column by passing through it 10 ml. of 2 N hydrochloric acid. Wash the column with 10 ml. of water added in small portions. Pass 3 ml. of 2 N potassium hydroxide through the column and follow with 10 ml. of water. Avoid an excess of potassium hydroxide as any calcium in it is retained by the resin. The resin approximately doubles in size on passing from the hydrogen to the potassium form. The resin should not be stored in the hydrogen form because this causes a decrease in its capacity.

Weigh 2 g. of the lithium salt (chloride, bromide or sulfate) to be analyzed. Dissolve it in 25 ml. of water and make the solution basic with 2 or 3 drops of 2 N potassium hydroxide. Pass the solution through the column. Wash the column with 5 ml. of water. Discard the liquid which has passed through the column. Elute the calcium with 4 ml. of 2 N hydrochloric acid. Wash the column with two 3-ml. portions of water. Neutralize the eluate with 2 N potassium hydroxide and add an excess of 1 ml. Add 1 drop of 0.0033 per cent solution of Calcein and titrate with 0.005 M EDTA, observing the end-point visually. The end-point is marked by a sharp decrease in the fluorescent color. The change is somewhat more easily observed under ultraviolet light. Alternatively, and with better results, the titration may be carried out as a fluorometric titration as described below.

Standardize the EDTA solution by titrating a suitable aliquot of a standard calcium solution prepared by dissolving calcium carbonate in hydrochloric acid; use Calcein as indicator.

Samples larger than 2 g. may be used and as little as 0.0002 per cent of calcium detected in lithium chloride. With larger samples use a correspondingly greater volume of water and a slightly lower flow-rate.

Procedure for the Determination of Calcium in Lithium Salts by the EDTA-Calcein-Fluorometric Method Without Prior Separation. Employ procedure (A) or (B) or a slight modification thereof depending on the characteristics of the fluorometer.

(A) Using the Coleman Model 12 Electronic Photofluorometer. Weigh directly into a fluorometer cuvette a sample of 100 mg. of the lithium salt (chloride, bromide or sulfate) to be analyzed. Dissolve the sample in about 10 ml. of water and make the solution basic by the addition of 1.0 ml. of 2 N potassium hydroxide. Add 5 drops of 0.0033 per cent Calcein. Titrate with 0.00025 M EDTA.

After the addition of each increment of EDTA stir the solution by bubbling through it a stream of air introduced through a length of very small-bore polyethylene tubing.

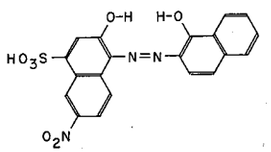
Measure the fluorescence after each addition. Plot the fluorescence intensities found against the volume of EDTA added and draw straight lines through the vertical and horizontal portions. From the end-point, as indicated by the intersection of the lines, calculate the calcium in the sample.

(B). Using a Simple Photofluorometer with Wratten K3 Filter. Weigh a sample of 1 g. of the lithium salt (chloride, bromide or sulfate) to be analyzed directly into the fluorometer cell. Dissolve the sample in about 27 ml. of water and make the solution basic by the addition of 3.0 ml. of 2 N potassium hydroxide. Add 5 drops of a 0.0033 per cent solution of Calcein. Titrate with 0.0005 M EDTA. Continue as given in the second paragraph of procedure (A) above.

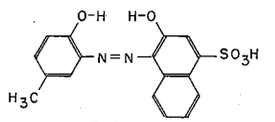
PART IV

ERIOCHROME BLACK T AND CALMAGITE

Eriochrome Black T and Calmagite are both *o,o'*-dihydroxyazo compounds. Both unite with magnesium and with calcium by virtue of the



Eriochrome Black T



Calmagite

presence in the molecule of the *o,o'*-dihydroxyazo grouping. The acid-base characteristics, the formation constants toward magnesium and calcium, and the color changes when used as indicators in EDTA titrations are almost identical for the two compounds. They can be used interchangeably as indicators. Solutions of Eriochrome Black T are not stable; solutions of Calmagite are stable indefinitely. It was for this reason that Calmagite was advanced as a substitute for Eriochrome Black T. Eriochrome Black T is one of four dyestuffs studied by Schwarzenbach and Biedermann[32] in 1948, in the first work reported on the use of azo dyes as indicators in EDTA titrations: Eriochrome Blue Black B, Eriochrome Blue Black R, Eriochrome Black T, and Eriochrome Black A. These dyes are designated as 239, 240, 241, and 242, respectively in the Schultz-Lehmann Farbstofftabellen, as 201, 202, 203 and 204 in the Colour Index, First Edition, and as C. I. Mordant Black 3 (C. I. 14640), C. I., Mordant Black 17 (C. I. 15705), C I. Mordant Black 11 (C. I. 14645), and C. I. Mordant Black 1 (C. I. 15710) in the Colour Index, Second Edition. Each of these *o,o'*-dihydroxyazo dyes has the property of forming calcium and magnesium derivatives with colors different from those of the dyes themselves. All of the dyes are useful as indicators in the titrations with EDTA. Schwarzenbach and Biedermann found in all cases that the ratio of dye to calcium or magnesium was 1 to 1. They further measured the acid dissociation constants of the dyes and the formation constants of the calcium and magnesium derivatives. From these constants they showed

For full references see Bibliography, page 118.

[32] *Helv. Chim. Acta*, **31**, 678 (1948).

that Eriochrome Black T was the most sensitive of the four dyes for the detection of small amounts of magnesium and therefore, presumably, best as an indicator in EDTA titrations.

Contrary to the findings of Schwarzenbach and Biedermann, it was later reported that Eriochrome Black T and magnesium combined in the ratio 2 to 1 at pH 10.1 and with calcium and magnesium in the ratio of 1 to 1, 2 to 1, and 3 to 1 depending on pH. A reinvestigation of the problem by Diehl and Lindstrom[33] confirmed the earlier work that only 1 to 1 compounds were formed and verified the values of Schwarzenbach and Biedermann for the acid dissociation constants and formation constants.

That Eriochrome Black T is not stable in solution is unfortunate for the necessity of preparing a fresh solution every few days is a nuisance. Some seven ways of stabilizing the solution have been described, the most successful of the schemes claiming a solution stable for about seven months. The instability of Eriochrome Black T quite likely arises from the simultaneous presence in the molecule of the nitro group, an oxidizing agent and the azo and phenolic groups, reducing agents. The minimum requirement for an azo molecule to react with an alkaline earth ion is the presence of hydroxyl groups in the positions *o*- and *o'*- to the azo group, Diehl and Ellingboe[34] After considerable searching, Diehl and Lindstrom[35] uncovered an *o,o'*-dihydroxyazo compound having color changes and dissociation and formation constants almost identical with Eriochrome Black T but lacking the nitro group and its oxidizing power. The material proved permanently stable in aqueous solution and was given the name *Calmagite*. It can be used in EDTA titration in place of Eriochrome Black T without change in procedure.

Availability. Eriochrome Black T is a commercial dyestuff manufactured and marketed by several companies and sold under a variety of names: Eriochrome Black T (Geigy), Pontachrome Black TA (du Pont), Superchrome Black T (National Aniline), Solochrome Black WDFA (Imperial Chemical Industries), and many others. The commercial material usually contains a large portion of salt, varying in purity from 50 to 70 per cent; commercial material is satisfactory for use as an indicator in EDTA titrations because the alkali metal salts present are inert toward EDTA; such materials are not satisfactory for studies of the combination of the dye with metals, however, particularly if the method of continuous variations is used. A purified preparation of Eriochrome Black T is available from the G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 246.

[33] *Anal Chem.*, **31**, 414 (1959).
[34] *Anal. Chem.*, **32**, 1120 (1960).

A method of preparing Eriochrome Black T in highly pure, crystalline form was devised by Diehl and Lindstrom[33]; the procedure involves desalting the commercial material by repeated washing with dilute hydrochloric acid, removal of impurities by extraction with benzene, and isolation of the dimethylamine salt in crystalline form by recrystallization from dimethylformamide. Such highly purified material is not available commercially at present.

Calmagite is prepared by diazotizing l-amino-2-naphthol-4-sulfonic acid and coupling the diazonium salt with *p*-cresol[35]. Particular care must be taken to purify the *p*-cresol and sufficient time must be given for the coupling reaction to occur, the coupling being effected in the presence of an excess of sodium hydroxide at 60°. The azo compound is precipitated by acidifying the reaction mixture and then filtered and washed well with petroleum ether and with diethyl ether. It is then extracted into acetone, some impurities remaining undissolved, and finally recovered by evaporation. Yield 27 per cent, purity 95 per cent. The purity of the *p*-cresol demanded for this preparation is necessary since the isomeric *o*- and *m*-cresol form azo compounds which do not possess the necessary *o,o'*-dihydoxyazo configuration and their presence would mask the end-point. Some hydrolysis of the sulfonic group in the hot alkaline solution apparently occurs for sulfur dioxide may be detected and probably accounts for the failure to obtain material better than 95 per cent pure.

Calmagite is marketed by the G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 278.

Acid Base Properties. Both Eriochrome Black T and Calmagite are acid base indicators, the colors and pH ranges over which the transitions occur varying only slightly.

	H_2D^-	⟷	$HD^=$	⟷	$D^{\equiv}$
Eriochrome Black T	Wine red	5.3-7.3	Blue	10.5-12.5	Orange
Calmagite	Bright red	7.1-9.1	Clear Blue	11.4-13.3	Reddish Orange

The sulfonic groups of Eriochrome Black T and Calmagite are strong acids and are not of direct interest in the functioning of the materials as acid-base indicators. Rather, the phenolic hydrogen atoms are the important acid groups in the basic solutions in which the compounds are used as a metal ion indicators. The values of the dissociation constants of the two indicators are shown in Table 25.

Absorption Spectra. The absorption spectra of Eriochrome Black T and Calmagite in the various forms are shown in Figures 3 and 4.

[35] *Anal. Chem.*, **32**, 1123 (1960).

TABLE 25. ACID DISSOCIATION CONSTANTS OF ERIOCHROME BLACK T AND OF CALMAGITE, IONIC STRENGTH, μ=0.100

Data of Lindstrom and Diehl[33,35]

	pK_1	pK_2
Eriochrome Black T	6.91[a,b]	11.50[a,b]
Calmagite	8.14[a,b]	12.35[a,b]

[a,b]Second and third replaceable hydrogen atoms of the molecules. These values were determined by obtaining the absorption spectra of the compounds at various values of pH, the ionic strength of the various buffer solutions being brought to 0.100 by the addition of suitable amounts of potassium chloride.

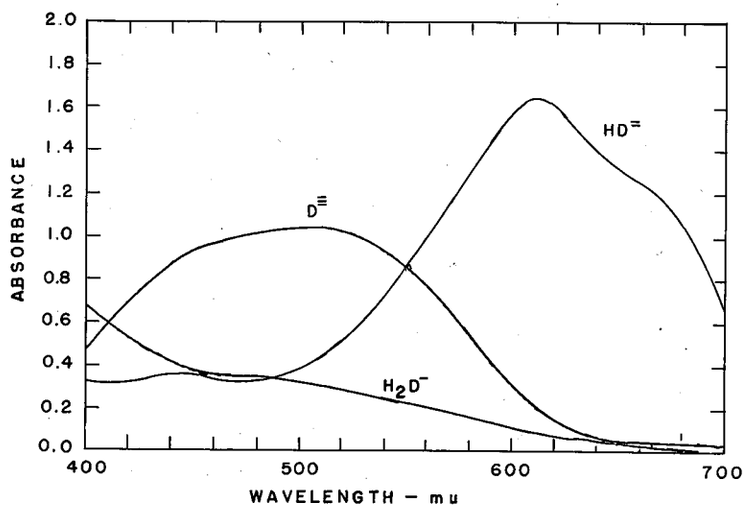


Figure 3. Absorption of Eriochrome Black T at Various Values of pH. pH of the solution for the different species: H_2D^- 3.91 (μ=0.100); $HD^=$ 10.0 (μ=0.100); $H_3D^{\equiv}$ 13.76. All solutions 4.97 x 10^{-5} M. 1-cm. cell. *Data of Lindstrom and Diehl*[35].

The absorbancy of the pure Eriochrome Black T prepared by Diehl and Lindstrom[33] was measured at pH 10 at 650, 615, 546 and 470 mμ for various concentrations. The absorbancy proved strictly linear with concentration (conformity to Beer's law); at 615 mμ, the wave length of maximum absorption, the molar extinction coefficient was found to be 32,300. The purity of commercial preparations of Eriochrome Black T can be measured in this way on the assumption that there are no impurities present which absorb at the wave length used for the measurement of the absorbance.

Solutions of Calmagite also conform to Beer's law, the molar extinction coefficient being 20,300 at 610 mμ, pH 10.10, μ (ionic strength) 0.100.

Combining Ratio and Formation Constants of the Magnesium and Calcium Derivatives of Eriochrome Black T and Calmagite. Both Eriochrome Black T and Calmagite unite with magnesium and with calcium in the ratio of one to one. The formation of the magnesium and calcium compounds is dependent on pH and is essentially complete at pH above 9. The EDTA titration of calcium plus magnesium is carried out at pH 10 (ammonium hydroxide-ammonium chloride buffer) so that the indicator, Eriochrome Black T or Calmagite, is in the blue form ($HD^=$) and the color change at the end-point is from the red, magnesium-indicator

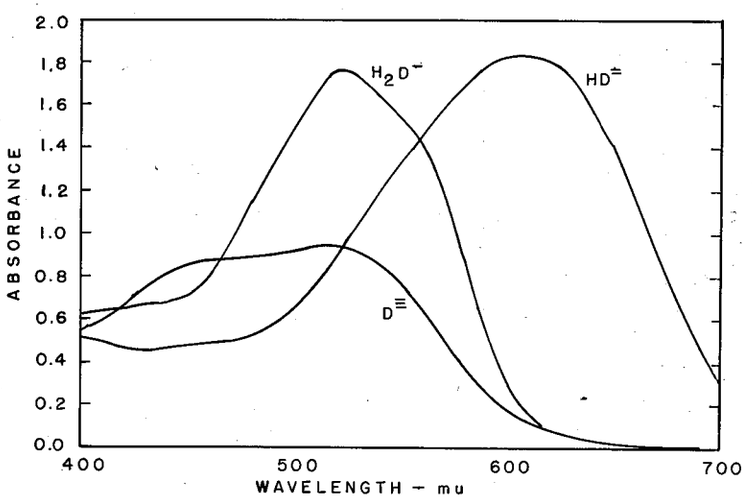


Figure 4. Absorption of Calmagite at Various Values of pH. pH of the solution for the different species: H_2D^- 2.14 (μ = 0.100); $HD^=$ 10.29 (μ = 0.100); $D^{\equiv}$ 13.8. All solutions 9.04 x 10^{-5} M. 1-cm. cell. *Data of Lindstrom and Diehl*[35].

compound (MgD^-) to the blue, metal-free indicator. The absorption spectra of the free indicators and their magnesium and calcium derivatives are shown in Figures 5 and 6.

The combining ratios of Eriochrome Black T with magnesium and calcium were determined both by the method of continuous variations and the log-ratio method[33]. The latter method yields a value for the formation constant also. The combining ratios of Calmagite with magnesium and calcium were determined by both methods also[35]; the formations constants were also measured, Table 26.

As with all of the *o,o'*-dihydroxyazo compounds examined for their behavior toward calcium and magnesium[34] the formation constant of Cal-

TABLE 26. LOGARITHM OF FORMATION CONSTANTS OF ERIOCHROME BLACK T AND CALMAGITE WITH MAGNESIUM AND CALCIUM AT pH 10

Data of Lindstrom and Diehl[33,35]

	log K_{10} for Mg	*log K_{10} for Ca*
Eriochrome Black T	5.75	3.72
Calmagite	5.69	3.67

magite with magnesium is greater than that with calcium. A consequence of this is that even in the titration of calcium with EDTA magnesium should be present for the best working of the indicator, a known amount of magnesium being added if it is not present in the sample.

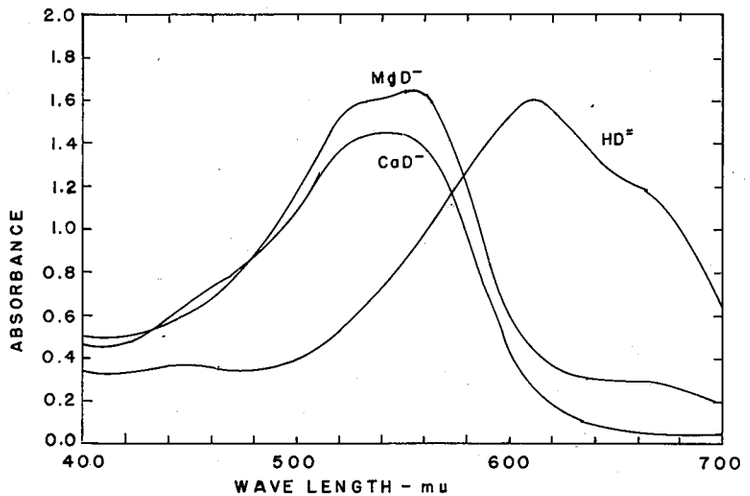


Figure 5. Absorption spectra of Eriochrome Black T and its calcium and magnesium derivaties. Concentration of Eriochrome Blank T: HD= 4.93×10^{-5} M; CaD⁻ 4.93×10^{-5}M; MgD— 4.91×10^{-5} M. pH 10. $\mu = 0.100$. Magnesium and calcium in excess. Carey Model 11 spectrophotometer; 1-cm. cell. *Data of Lindstrom and Diehl*[35].

Calmagite is similar to Eriochrome Black T in that small amounts of copper, iron, and aluminum seriously interfere in the titration of calcium and magnesium. Presumably this is caused by the formation of very stable compounds with the reagents. The masking agents usually used to limit these interferences with Eriochrome Black T are equally effective with Calmagite. Although even very large amounts of potassium and the common anions cause no trouble, Table 3, page 8, the presence of large amounts of sodium causes some difficulty, possibly owing to the formation of non-ionized compounds of sodium with ethylenediaminetetraacetic acid or with

the indicator. This difficulty may be avoided in most cases simply by the use of potassium hydroxide rather than sodium hydroxide.

In general Calmagite possesses enough advantages over Eriochrome Black T that it should be used in preference to the latter.

Comparison of the End-point Observed with Calmagite and Eriochrome Black T. Although Calmagite was advanced primarily as a replacement for Eriochrome Black T because it is perfectly stable in aqueous solution, it proved superior to Eriochrome Black T also in the sharpness of the color change at the end-point[35]. Thus, in the titration of magnesium at pH 10 five drops of 0.01 M EDTA are required from the point at which the red magnesium-indicator compound starts to change

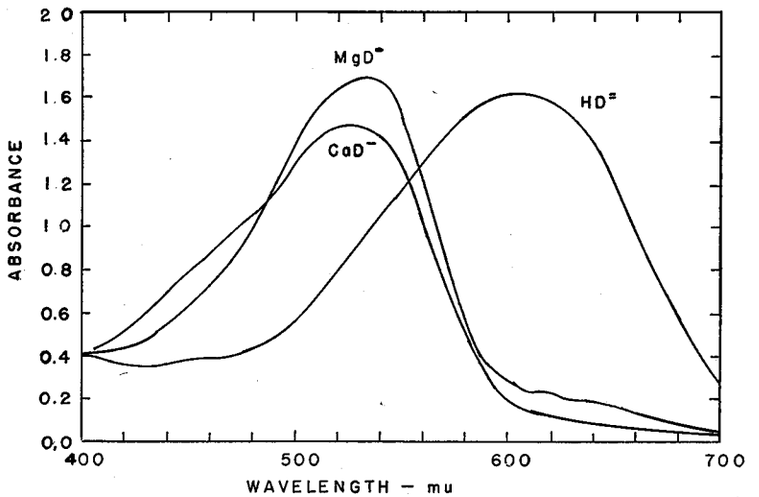


Figure 6. Absorption spectra of Calmagite and its calcium and magnesium derivatives. Concentration of Calmagite: HD= 4.00×10^{-5} M; CaD— 3.89×10^{-5} M; MgD— 4.00×10^{-5} M. pH 10. $\mu = 0.100$. Magnesium and calcium in excess. Carey Model 12 spectrophotometer; 2-cm cell. *Data of Lindstrom and Diehl.*[35].

to the clear blue of the free indicator at the end-point. Under the same conditions nine drops are required with Eriochrome Black T.

In a stability test, Calmagite showed no measurable decomposition over a period of one year.

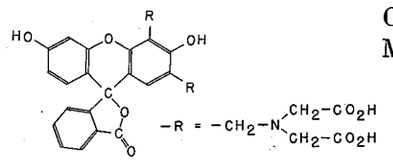
The acid dissociation constants of Calmagite and Eriochrome Black T are given in Table 25. Using these values, a calculation shows that at pH 10 only 1.8 per cent of Calmagite is not in the blue $HD^{=}$ form but that 3.2 per cent of Eriochrome Black T is in the form of the undesirable red species H_2D^{-} and $D^{\equiv}$. This contributes to the increased sharpness of the end-point change shown by Calmagite.

PART V

CALCEIN

A. THE PROPERTIES OF CALCEIN

Calcein and its use were first described by Diehl and Ellingboe[5]. Calcein is prepared by the interaction of fluorescein, formaldehyde and iminodiacetic acid. There is thus introduced into the fluorescein molecule two methyleneiminodiacetic acid groups, $-CH_2-N=(CH_2-CO_2H)_2$, each half of the ethylenediaminetetraacetic acid molecule. The resulting compound has the structure:



I

$C_{30}H_{26}N_2O_{13}$
Mol. Wt. 622.5

The molecule possesses both the fluorescence and the acid-base properties of fluorescein and the chelating properties of ethylenediaminetetraacetic acid. The fluorescence is dependent on both pH and the presence of certain metals and is useful as an indicator in EDTA titrations. Other compounds exhibiting fluorescence dependent on the presence of metals were subsequently prepared and Calcein is thus the first member of a class designated as *metallofluorescent indicators*[36, 37]. A color change may or may not accompany the change in fluorescence and the term *metallofluorochromic indicator* has also been proposed[38, 39, 40].

Calcein exhibits a bright yellow-green fluorescence, similar to that of fluorescein, but unlike the parent material does not fluoresce in strongly alkaline solution. The fluorescence of Calcein rises from zero at pH below 3 to a maximum at pH 6 to 8 and falls off again at higher pH, being zero at pH 10.5 and above.

Calcein shows two sharply different reactions toward metal ions. In solutions of pH 6 to 8, in which Calcein in the absence of metal shows a

For full references see Bibliography, page 119.

[5] *Anal Chem.*, **28,** 882 (1956).
[36] *Chem. Listy*, **51,** 1457 (1957).
[37] *Coll. Czech. Chem. Commun.*, **23,** 622 (1958).
[38] *Talanta*, **2,** 277 (1959).
[39] *Talanta*, **3,** 370 (1960).
[40] *Talanta*, **4,** 80 (1960).

maximum in fluorescence, the fluorescence is quenched by the addition of copper, cobalt, ferric iron, nickel, palladium, lead, bismuth, thallium, zirconium, and other metal ions. This is spoken of as the *normal reaction,* such quenching being characteristic of other fluorescent organic compounds on union with metal ions to form chelate ring compounds.

In solution of high pH, in which Calcein alone exhibits no fluorescence, fluorescence is produced by the addition of calcium, strontium, barium, and of magnesium if the pH is not so high (above 10.5) as to precipitate the magnesium as the hydroxide. This reaction is spoken of as the *indicator reversal reaction,* the indicator being converted by the metal to its more acid, fluorescent form.

Applications. The original application of Calcein was to the EDTA titration of calcium in the presence of magnesium at pH 12 (Diehl and Ellingboe[5]); this is by far the most important use of Calcein and is discussed in detail in Part III, Sections A through F, of this monograph. The determination of calcium in blood serum is the most important application of the method and no less than sixteen papers (through 1963), mostly repetitive, have been published on this application, Section E. The determination of calcium in limestone, phosphate rock, and slag are important determinations too, and the EDTA-Calcein method is quicker and as accurate as older methods, Sections B, C, and D.

Strontium and barium behave in identical fashion to calcium and can be determined in the same way[5, 41]. Sulfate can be determined indirectly through barium by precipitation of barium sulfate from a standard barium chloride solution[43]. These determinations are based on the "indicator reversal" reaction, the end-point in the EDTA titration being the disappearance of the fluorescence or the concurrent color change from yellow-green to pink-brown. The "normal" indicator reaction, the quenching of fluorescence, is useful too, for the EDTA titration of copper[43, 44], iron[43], cobalt[43, 44], chromium[44] and titanium[45], the end-point being the appearance of the fluorescence as the quenching metal unites with EDTA and is rendered inactive toward the Calcein. Such titrations are usually carried out by adding excess standard EDTA and back titrating with a standard copper solution, the disappearance of the end-point being somewhat more easily perceived. Titrations of copper, iron, cobalt and chromium with EDTA are difficult with metallochromic indicators because of the intense color of the metal-EDTA derivatives themselves and the fluorochromic indicator is thus particularly valuable. Calcein has also been used as a metallo-

[41] *Anal. Chem.*, **34,** 565 (1961).
[42] *Coll. Czech. Chem. Commun.*, **24,** 682 (1959).
[43] *Talanta*, **2,** 12 (1959).
[44] *Anal. Chim. Acta*, **20,** 324 (1959).
[45] *Talanta*, **2,** 355 (1959).

fluorochromic indicator for the titration of copper using triethylenetetramine as the titrating agent rather than EDTA[46]. The quenching of the fluorescence of Calcein by silver is useful for end-point indication in titrations with silver nitrate[47].

Calcein is also used for the direct fluorometric determination of calcium; this was suggested by Körbl and Vydra[37], and later worked out in detail by Kepner and Hercules[58] and improved by Phillips[59], as discussed in the following section of this monograph, page 78.

Fluorescence of Calcein with the Alkali Metals. The EDTA titration of calcium is carried out at pH 12 so that any magnesium present is precipitated as the hydroxide and does not interfere. Adjusting the pH of such solutions should be done with potassium hydroxide rather than sodium hydroxide as high concentrations of sodium cause a fluorescence with Calcein. Körbl, Vydra and Pribil[48] who discovered this found that lithium also produces a fluorescence with Calcien, the relative intensity of fluorescence in 0.1 M solutions of the hydroxides being: sodium hydroxide 20, lithium hydroxide 14, potassium hydroxide 2. Essentially the same thing was found by others[49, 50]. The fluorescence caused by the alkali metals is of course considerably less than that caused by calcium, Table 27. The

TABLE 27. FLUORESCENCE OF CALCEIN IN SOLUTIONS OF POTASSIUM, SODIUM, LITHIUM AND TETRAMETHYLAMMONIUM HYDROXIDE

Data of H. Diehl and K. Oulman[49]

Base	Relative Fluorescence of Calcein[a]	
0.1 M	With 4.0 x 10^{-3} M EDTA[b,c]	With 1.0 x 10^{-2} M $CaCl_2$ [d,e]
Potassium hydroxide	2.1	85.0
Sodium hydroxide	8.0	88.2
Lithium hydroxide	5.0	88.0
Tetramethylammonium hydroxide	2.1	76.5

[a]Calcein 5.52 x 10^{-6} M.
[b]EDTA added to mask any impurities of calcium present.
[c]Aminco-Keirs Spectrophotometer; excitation spectrometer set at 496 mμ; emission spectrometer at 522 mμ.
[d]No EDTA present; large excess of calcium.
[e]Aminco-Keirs Spectrophotometer; excitation monochromator set at 492 mμ; emission monochromator at 515 mμ.

[46]*Talanta*, **2**, 201 (1959).
[47]*Coll. Czech. Chem. Commun.*, **26**, 2449 (1961).
[58]*Anal. Chem.*, **35**, 1238 (1963).
[59]*Mimeographed literature of G. K. Turner Associates, Palo Alto, California.*
[48]*Talanta*, **1**, 282 (1958).
[49]H. Diehl and K. Oulman, otherwise unpublished work.
[50]*Zh. Analit. Khim.*, **17**, 560 (1962); *J. Anal. Chem., USSR.*, **17**, 558 (1962).

values for the fluorescence of Calcein in 0.1 N sodium hydroxide and in 0.1 N potassium hydroxide by the three workers differ appreciably, the ratio reported being: Körbl and coworkers, 10; Diehl and Oulman, 4; and Bozhevol'nov and Kreingol'd, 2.1 The differences probably arise from the amount of calcium impurity in the alkalies use; Diehl and Oulman and Bozhevol'nov and Kreingol'd added EDTA to mask such impurities. In any case there is advantage in using potassium hydroxide in the determination of calcium. Curiously the residual fluorescence in ammonium hydroxide is 24 times that in 0.1 N potassium hydroxide, Bozhevol'nov and Kreingol'd[50].

Preparation and Purity. The material obtained from the condensation of fluorescein, formaldehyde and iminodiacetic acid by Diehl and Ellingboe[5] was apparently a mixture of compounds for products from various purification processes gave variable results for neutralization equivalent, Kjeldahl nitrogen, and bromination. The predominating material evidently contained two methyleneiminodiacetic acid groups. Although admittedly not a pure product the material functioned well as an indicator. Three claims[51, 52, 53] were subsequently made that a modification of the process can yield a pure product, but in only one of the three, Wallach and coworkers[53], are the details of the preparation given. In the modification of Wallach and coworkers, the conditions are the same as in the Ellingboe and Diehl procedure but large excesses of formaldehyde and iminodiacetic acid are used, the ratio of the reactants being one mole of fluorescein to four of formaldehyde to four of iminodiacetic acid. A more extensive recrystallization program was adopted too and a purer product was obtained although the ultimate analyses reported are barely satisfactory.

Preparation of Calcein. METHOD OF ELLINGBOE AND DIEHL AS MODIFIED BY WALLACH, SURGENOR, SODERBERG AND DELANO[53]. To 6.64 g. (0.02 mole) of fluorescein dissolved in 20 ml. of 60 per cent ethyl alcohol and 6 ml. of 30 per cent sodium hydroxide add 10.6 g. (0.08 mole) of iminodiacetic acid dissolved in 15 ml. of water and 12 ml. of 30 per cent sodium hydroxide. Chill the mixture and add 7.5 g. (0.08 mole) of 37 per cent formaldehyde dropwise with stirring, keeping the temperature at 4°. Warm the reaction mixture to 70° and maintain it at this temperature for 8 hours with stirring. Cool to room temperature, dilute to 1000 ml. with water, and acidify to a pH of about 3.5 with 3 N hydrochloric acid. Filter the precipitate of this isoelectric compound and wash with 2000 ml. of water. Air dry the material. Extract the air-dried material repeatedly with 50 ml. of hot 70 per cent ethyl alcohol, filter hot, and cool slowly. Filter off the fine yellow needles which crystallize out and dissolve them in the minimum quantity of 0.01 N sodium hydroxide and reprecipitate by the addition of 1 N hydrochloric acid bringing the pH to 3.5. Recrystallize from 70 per cent ethyl alcohol three times. Dry the final product in a vacuum at 80°. Yield 20 per cent. Melting point higher than 300°.

[51]*Talanta*, **1**, 138 (1958).
[52]D. H. Wilkins, Private communication.
[53]*Anal. Chem.*, **31**, 456 (1959).

Fluorescein Impurity in Calcein. The residual fluorescence of Calcein in solutions of high pH is a matter of some interest inasmuch as this residual fluorescence affects the end-point in the determination of calcium. As discussed above, the nature and amount of alkali metal and the presence of calcium impurities determines this fluorescence. Properly made preparations of Calcein should contain no fluorescein but a measurement of the residual fluorescence in terms of fluorescein, whether due to fluorescein or not, would be useful.

The fluorescence of fluorescein is much more intense than that of Calcein in alkaline solution; see for example, Figure 7 in which the intensity of the fluorescence of the two as a function of the concentration are given. It would appear simple to determine the residual fluorescein in Calcein by adding known amounts of fluorescein to a solution of Calcein, measuring the fluorescence and using these data as a calibration determining the amount of residual fluorescein by extrapolation to the solution containing Calcein only. This proves to be more difficult than expected because the absorption maximum of Calcein occurs at 500 mμ, not greatly different from the wavelength of maximum excitation of fluorescein, 488 mμ. That is, the intensity of the fluorescence of a given amount of fluorescein will depend on the amount of Calcein present. The data shown in Figure 8 show how serious this effect is. Nevertheless it is possible to carry out this measurement by taking exactly the same amount of Calcein for the standards and for the analysis. Results obtained by this method in terms of per cent of fluorescein apparently present in various preparations of Calcein: Early preparation, 1.04; Calcein W (Wilkins), 0.35; G. Frederick Smith Chemical Company, 1958, 0.81; G. Frederick Smith Company, 1961, 0.45; Preparation by the Wallach and coworkers modification of the Diehl and Ellingboe procedure, 0.40.

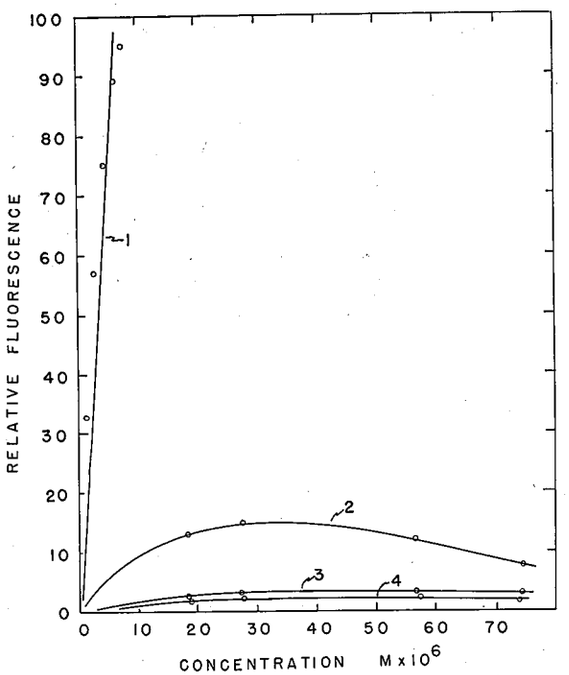


Figure 7. Intensity of fluorescence of 1. Fluorescein, 2. Calcein, early preparation, 3. Calcein, G Frederick Smith Chemical Company, 1961, 4. Calcein, G. Frederick Smith Chemical Company, 1962. *Data of H. Diehl and K. Oulman*[56].

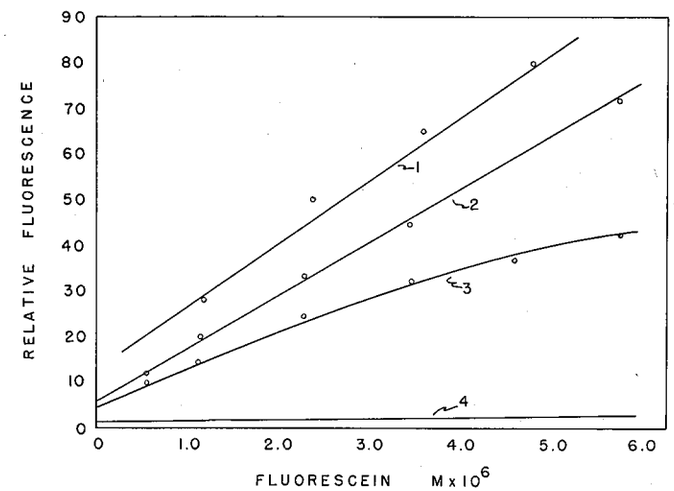


Figure 8. Relative intensity of the fluorescence of fluorescein in the presence of various amounts of Calcein. All solutions in 0.10 M potassium hydroxide. 1. Fluorescein alone; 2. Calcein, 5.52×10^{-6} M (starting with 8.6 mg. of Calcein as in recommended procedure); 3. Calcein, 11.04×10^{-6} M; 4. Calcein, 56.2×10^{-6} M. *Data of H. Diehl and K. Oulman*[56].

For other work on the problem of determining the fluorescein impurity in Calcein, see Körbl, Vydra and Pribil[51] and Bozhevol'nov and Kreingol'd[54].

It has been reported that commercial preparations of Calcein from two sources, unidentified, display three bands on chromatography on alumina[55].

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[51] *Talanta*, **1**, 138 (1958).
[54] *Zh. Analit. Khim.*, **17**, 291 (1962); *J. Anal. Chem., USSR*, **17**, 294 (1962).

All of the components isolated from the bands function as indicators, with less residual fluorescence than the original materials.

Procedure for the Determination of Residual Fluorescence (Fluorescein) in Calcein. METHOD OF DIEHL AND OULMAN[56]. Weigh 8.6 mg. of Calcein into a 500-ml. volumetric flask. Dilute to the mark with deionized water and mix thoroughly. Add 0.10 M potassium hydroxide to dissolve the Calcein and dilute the solution to the mark. To each of seven 25-ml. volumetric flasks, add 5 ml. aliquots of this solution of Calcein and 6 ml. of 0.05 M EDTA. To six of the flasks add respectively, 0.5, 1, 2, 3, 4, and 5 ml. of a 2.88×10^{-5} M solution of fluorescein and dilute to the mark with 0.10 M potassium hydroxide. Measure the intensity of the fluorescence of each of the seven solutions, setting the excitation monochromator at 488 mμ and the emission monochromator at 514 mμ. Plot the intensity of fluorescence of the standards as a function of the concentration of fluorescein in the final solutions. Calculate the slope of the line, k, in the relation $I = kc$. Using k and the intensity of the solution of Calcein with no added fluorescein, calculate the residual fluorescence as per cent fluorescein.

Structure. The method of preparation of Wallach and coworkers led to a pure product as indicated by fairly satisfactory ultimate analysis, electrophoresis and chromatography of the product. The molecular structure shown above (I) was proposed for the compound by Wallach and coworkers[53]; this structure is designated 3,6-dihydroxy-2,4-bis[N,N′-di(carboxymethyl)aminomethyl]fluoran. This structure in which both methyleneiminodiacetic acid groups are placed on the same ring and each adjacent to the hydroxy group was postulated on the basis of several lines of evidence: the change in the ultraviolet absorption spectrum of the material with pH, analogy with fluorescein and with compounds containing methyleneiminodiacetic acid groups, infrared spectra, and the synthesis of related alkyl substituted fluoresceins.

A potentiometric titration of Calcein with alkali shows one distinct endpoint at pH 7 and three equivalents of alkali added; the first two replaceable hydrogen atoms are fairly strong acids, the third is a weak acid having a pK value about 5.4. No change in the ultraviolet absorption spectrum accompanies the neutralization of the first two protons but such a change does accompany the neutralization of the third, and also the fourth, which takes place about pH 9. Further changes in the ultraviolet spectrum do not accompany the neutralization of the fifth proton but do again with the sixth. These changes are summarized in Table 28.

Wallach and coworkers[53] interpret these phenomena in the following way. The material exists in the free acid, H_6A, as a zwitter ion, that is, two protons are transferred from carboxyl groups to the nitrogen atoms as shown in formulas II and III below. The remaining two carboxyl groups are strong acids, pK values about 3 and 4. The strongly acid character of these carboxyl groups results from the presence of the positive

[55] *Talanta*, **10**, 1033 (1963).

TABLE 28. DISSOCIATION CONSTANTS AND SHIFTS IN ABSORPTION SPECTRUM ASSOCIATED WITH pH OF CALCEIN

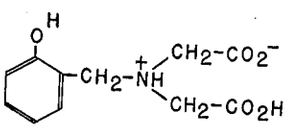
Data of Wallach and coworkers[53]

Group Dissociating	pK	Shift in Absorption Spectrum
—COOH	3	None
—COOH	4	None
—OH (—3 position)	5.4	230 to 242; 278 to 292; 305 to 325; 440 to 492
—OH (—6 position)	9.0	492 to 500
≡NH⁺	10.5	None
≡NH⁺	12	200 to 220[a]

[a] Hydrogen bond rupture opening of lactone ring.

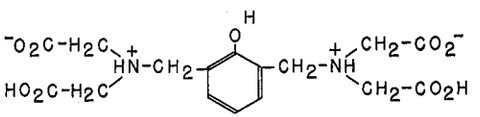
charge on the neighboring nitrogen atoms. Similar phenomena occur in ethylenediaminetetraacetic acid in which two carboxyl groups are strong acids and the protons on the two nitrogen atoms very weak acids, the successive pK values for ethylenediaminetetraacetic acid being 1.99, 2.67, 6.16, and 10.26; the same behavior is exhibited in iminodiacetic acid, pK_1 2.98 (carboxyl group), pK_2 9.89 (proton of $=NH_2^+$ group).

The neutralization of the third replaceable hydrogen atom of Calcein is accompanied by changes in absorption in the ultraviolet region. These changes are associated with the aromatic rings in the molecule and with changes in the enolic groups attached to the rings. The values of the acid dissociation constants of phenols are normally about 10^{-9}; one of the enol groups in Calcein, having a pK of 5.4, is thus a much stronger acid than normal. Reasoning from this, Wallach and coworkers assign the unsymmetric structure I to Calcein, calling attention to similar characteristics in compounds II and III studied by Schwarzenbach, Anderegg and Sallman. *Helv. Chim. Acta*, **35**, 1785 (1952). In 1-hydroxyphenyl-2-methylene-



II

pK_1 (—COOH)	2.2
pK_2 (—OH)	8.17
pK_3 (≡NH⁺)	11.79

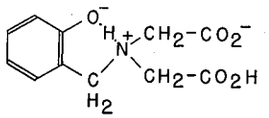


III

pK_1 (—COOH)	~2
pK_2 (—COOH)	2.9
pK_3 (—OH)	6.65
pK_4 (≡NH⁺)	9.74

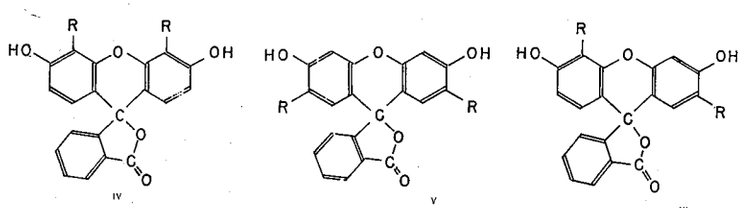
iminodiacetic acid (II), the acid dissociation constant is about 10 times greater than normal; in 1-hydroxy-4-methylphenyl-2,6-bis(methyleneiminodiacetic) acid (III), with two neighboring methyleneiminodiacetic acid groups present, the pK value is shifted from 9 to 6.6. The phenol becomes a strong acid not only because of the repulsive effect of the charge on the

neighboring ammonium nitrogen atom but also because of the possibility of forming a hydrogen bond between the phenolic oxygen and the imino nitrogen:

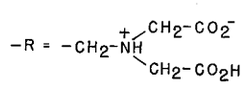


The last two protons of the Calcein molecule to be neutralized are the two on the nitrogen atoms. The proton of an ammonium group normally has a pK value of about 10. In 1-hydroxyphenyl-2-methyleneiminodiacetic acid, (II), the pK value is 11.8, at least an order of magnitude greater than normal. This is explained by Schwarzenbach as resulting from the formation of a hydrogen bond as just shown. In 1-hydroxy-4-methylphenyl-2,6-bis(methyleneiminodiacetic) acid, the pK values are 9.74 and 11.4. That is, one is normal, the other a weaker acid than normal. Calcein behaves like the latter compound, pK values of 10.5 and 12, but with the difference that a large increase in absorbance in the far ultraviolet occurs with the neutralization of the last proton. Wallach and coworkers[53] attribute this to the opening of the lactone ring. This occurs in phenolphthalein and accounts for the change in color at pH 9; in Calcein the change occurs at a much higher pH, above 12, owing to the greater basicity of the second imino nitrogen atom.

The behavior observed and the above line of reasoning is not compatible with any of the alternative structures for Calcein.



IV V VI



The great difference in the strength of the two phenolic groups can only be explained by the asymmetric structure assigned. In structures IV, V, and VI, the phenolic groups would be expected to have values of pK alike and about 8 in value just as in the compounds having only one methyleneiminodiacetic acid group neighboring the phenolic group. And similarly the pK values of the ammonium groups would be expected to be alike and in the range 11 to 12.

This assignment of the unsymmetric structure (I) to Calcein by Wallach and coworkers was supported by the synthesis of 2,7-diethyl-3,6-dihydroxy-4,5-di[N,N′-di(carboxymethyl)amino]fluoran and 4,5-dimethyl-3,6-dihydroxy-2,7-di[N,N′-di(carboxymethyl)amino]fluoran, neither of which showed the fluorescence of Calcein in the presence of alkaline earth metals.

For support for the unsymmetrically substituted structure assigned Calcein the infrared spectra of Calcein, fluorescein and tetraiodofluorescein were obtained. The absorption band observed at 800 to 860 cm^{-1} lies in the position characteristic of unsymmetrically trisubstituted compounds, this band not being present in fluorescein and tetraiodofluorescein. Further, the 920 cm^{-1} band associated with a single aromatic hydrogen was present in Calcein and tetraiodofluorescein. These data support the assignment of the unsymmetrical arrangement of the methyleneiminodiacetic acid groups. There is present also in the infrared spectrum a band at 1420 cm^{-1} which corresponds to the frequency of the carboxylate ion and supports the assumption that the compound is a zwitter ion.

In addition to the evidence related to the structure of Calcein just reviewed, Wallach and coworkers[53] prepared certain related compounds bearing methyl groups. The tetrasubstituted compound, 3,6-dihydroxy-2,4,5,7-tetra[N,N′-di(carboxymethyl)aminomethyl]fluoran was prepared, for example. It was not characterized other than that it was exceedingly hygroscopic and could not be easily crystallized, and that it showed activity toward calcium and magnesium. Two other compounds were prepared in which the two methyleneiminodiacetic acid groups introduced were necessarily symmetrically located: 2,7-diethyl-3,6-dihydroxy-4,5-di[N,N′-di(carboxymethyl)aminomethyl]fluoran and 2,7-di[N,N′di(carboxymethyl)aminomethyl]-3,6-dihydroxy-4,5-dimethyl-fluoran; neither of the compounds showed fluorescent activity toward calcium or magnesium.

Ultraviolet Absorption of Calcein. As expected the absorption spectrum of Calcein changes with pH, Figure 9, and resembles that of fluorescein in most respects; see data of Wallach and coworkers[53] shown in Table 29. The interpretation of the changes in absorbance with pH is given above.

Fluorescence of Calcein. An investigation of the effect of pH on the fluorescence of Calcein was carried out by Wallach and Steck[57]. Both excitation and emission spectra change with pH. The fluorescence excitation spectra are identical with the absorption spectra of the compound at the same pH. The fluorescence emission spectrum shows only a single band,

[57] *Anal. Chem.*, **35**, 1035 (1963).

the maximum of which depends on pH. The wave length of the emitted

pH	Wavelength of Excitation Maximum mμ (corrected)	Wavelength of Emission Maximum mμ
<5	444	503
7	492	511
10	500	520

light at a given pH is the same irrespective of the wavelength of the exciting light. For a given quantum flux of exciting light, the intensity of the emitted radiation is greatest when the compound is excited by radiation in the visible (440 to 500 mμ, depending on pH), about 60 per cent of this

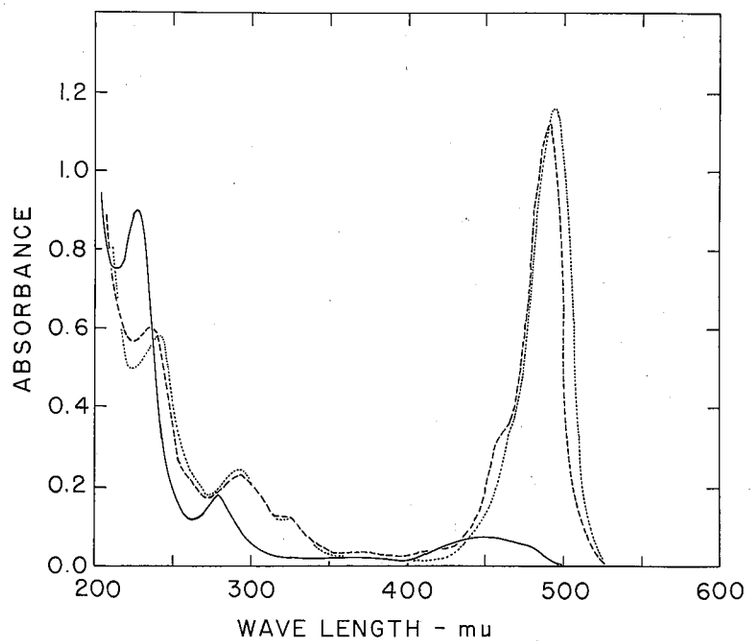


Figure 9. Absorption spectra of Calcein. 1.97×10^{-5} M. 1-cm. cell. —— pH. 2.00; ——— pH 6.93; . . . pH 11.88.

value when excited at the 220 mμ absorption peak, and about 40 per cent of this value when excited at the 300 mμ peak (the 3-OH absorption). Fluorescence excitation and emission spectra of Calcein are shown in Figure 9.

The variation in the intensity of the fluorescence of Calcein with pH is shown in Figure 10. Below pH 4 the compound is very weakly fluorescent with the emission maximum at 503 mμ. Between pH 4 and 6 the fluorescence increases sharply and the maximum shifts to 511 mμ; this fluorescence accompanies the neutralization of the 3-OH group (conversion of H_4Cal^{-2} to H_3Cal^{-3}) and presumably arises from hydrogen bonding of the enolic oxygen to the neighboring imino nitrogen atoms.

There is a further slight increase in fluorescence and a shift in the maximum to 520 mμ between pH 6 and 8.5; this accompanies the neutralization of the 6-OH group and the increase in absorbance in the visible band and its shift from 492 to 500 mμ. Between pH 8.5 and 12 the fluorescence decreases; this is associated with the displacement of the proton from the less basic of the two imino groups (H_2Cal^{-4} to $HCal^{-5}$). No further change in fluorescence occurs above pH 12 as the second imino hydrogen is neutralized.

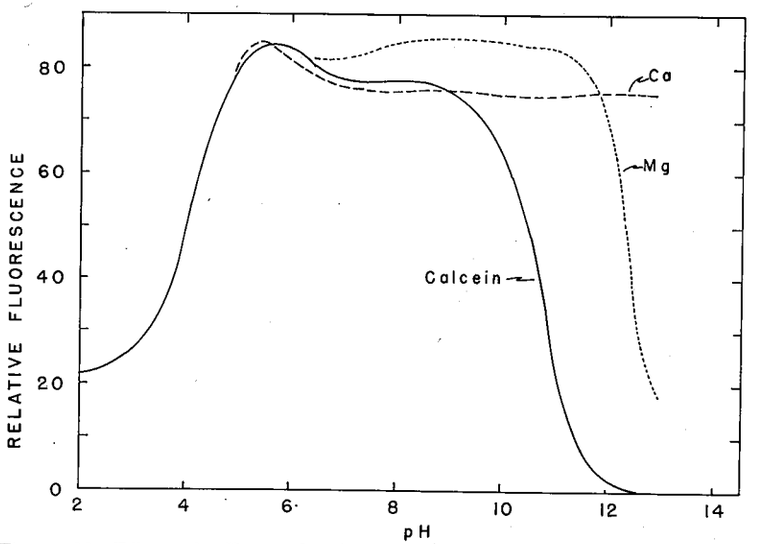


Figure 10. Effect of pH on the fluorescence of Calcein, calcium-Calcein, and magnesium-Calcein.

Changes in Fluorescence of Calcein Accompanying Reaction with Various Metals. Although the interest has centered on its reaction with calcium, Calcein reacts with other metals producing changes in the fluorescence. This subject was investigated in some detail by Wallach and Steck[57]. Three types of behavior were observed. Aluminum increases the fluorescence at low pH. The transition elements, copper, cobalt and nickel, decrease the fluorescence at neutral pH. The alkaline earths enhance the fluorescence in alkaline solution.

The increase in the fluorescence of Calcein by aluminum occurs in quite acid solution, pH 2, where the reagent itself is only very weakly fluorescent. This enhancement takes place with a shift in the wave length of maximum fluorescence from 503 mμ to 511 mμ. Although the fluorescence drops off

TABLE 29. ULTRAVIOLET AND VISIBLE ABSORPTION MAXIMA OF FLUORESCEIN AND CALCEIN AT VARIOUS VALUES OF pH

Data of Wallach, Surgenor, Soderberg and Delano[53]

Condition	Fluorescein mμ	Calcein mμ
0.2 N HCl	438	470 sh[a]
	280	444
	252 sh[a]	310
	228	278
pH 7.5	492	492
	460 sh[a]	460 sh[a]
	315	325
	285 sh[a]	292
	260 sh[a]	244
	244	
0.2 N NaOH	492	500
	460 sh[a]	470 sh[a]
	315	325
	278	292
	240	244

[a]Shoulder.

TABLE 30. FLUORESCENCE AND WAVE LENGTH OF MAXIMUM ABSORPTION OF CALCEIN AND SOME OF ITS METALS DERIVATIVES

Data of Wallach and Steck[57]

Metal Ion	Intensity Arbitrary units	Wave Length of Maximum Absorption[b] mμ
At pH 7.4[a]		
None	991	495
Magnesium	992	490
Zinc	988	487
Aluminum	730	470
Manganous	80	495
Cupric	10	495
Cobaltous	2	495
Nickel	4	495
At pH 12		
None	10	500
Calcium	756	495
Strontium	756	495
Barium	735	495

[a]Measurements at pH 7.42 were made in 0.1 M tris(hydroxymethyl)-aminomethane, the others in 0.1 N potassium hydroxide. For fluorescence measurements the concentration of Calcein was 3.2×10^{-6} M, that of metal 3.2×10^{-5} M. Exciting wavelength 495 mμ. For absorbance measurements concentration of Calcein was 2×10^{-5} M and metal 10^{-4} M.

[b]The wave length of maximum excitation was the same for each of the metals as for Calcein alone: 511 mμ at pH 7.4 and 520 mμ at pH 12.

fairly sharply from pH 2.5 to 5, the fluorescence is sufficiently strong at pH 2 to make possible a fluorometric method for the determination of aluminum.

As noted in Table 30, cobaltous, cupric, manganous, nickel, and presumably heavy metal ions, combine with Calcein to form compounds which do not fluoresce in the pH range 6 to 8.5 where the compound itself is fluorescent. The compounds formed apparently consist of one atom of metal and one molecule of Calcein.

At pH values between 6.5 and 8.5, magnesium, zinc, calcium, strontium and barium all form compounds with Calcein producing changes in the absorption spectrum in the ultraviolet but without producing any change in fluorescence. Wallach and Steck[57] indicate that the zinc compound is so stable that zinc displaces cobalt from Calcein and that the increase in fluorescence which results can be used for the determination of zinc.

The fluorescence of Calcein falls off at pH above 3.5 and is negligible at pH 13 and above. In this region the alkaline earth metals form strongly fluorescent compounds with Calcein. The intensities of the fluorescence of the calcium and magnesium compounds over the pH range 8.5 to 13 are shown in Figure 10. Curves for strontium and barium are identical with

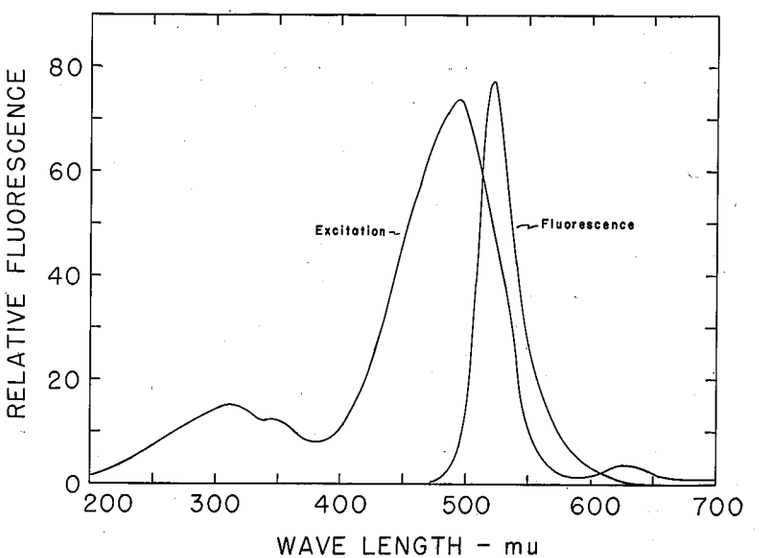


Figure 11. Excitation and fluorescence spectra of calcium-Calcein.

that of calcium. The fluorescence of the magnesium compound is greater than that obtained with an equivalent amount of calcium but falls off sharply above pH 12 owing to the formation of insoluble or non-dissociated

magnesium hydroxide. The excitation and fluorescence spectra of the calcium derivative of Calcein is shown in Figure 11.

The increase in fluorescence on the addition of calcium to a solution of Calcein at pH 12.5 is shown in Figure 12. The initial portion of the

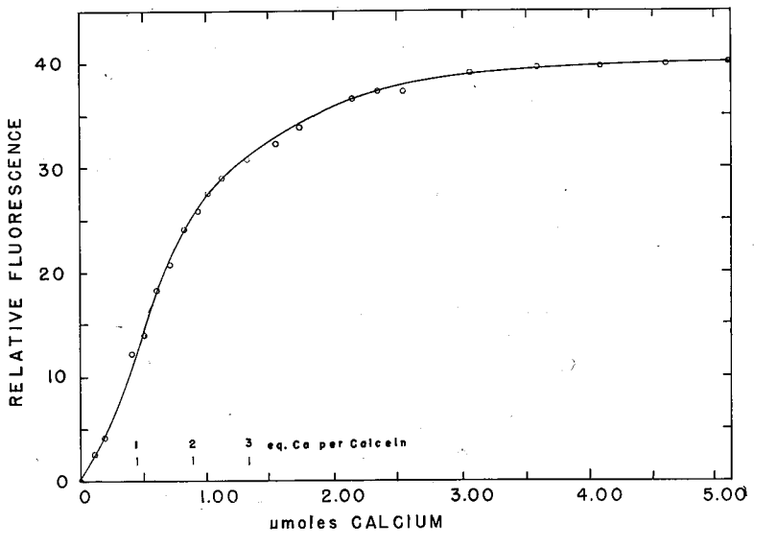


Figure 12. Fluorescence of calcium-Calcein with increasing amounts of calcium.

curve, concave up, implies that there is first formed a 1 to 1 compound which is not fluorescent and that the fluorescence arises from the addition of a sceond atom of metal to the Calcein. As would be expected the addition of Calcein to a fluorescent solution of calcium-Calcein causes a decrease in fluorescence (Wallach and Steck[57]).

The formation constant for the second calcium atom was determined by Wallach and Steck[57].

The formation constants for the second atom of each of the alkaline earth metals attaching to Calcein was measured by Wallach and Steck[57]. The reactions involved and the formation constants are expressed as

$$M + Cal = MCal \qquad K_1 = \frac{[MCal]}{[M][CaCal]}$$

$$M + MCal = M_2Cal \qquad K_2 = \frac{[M_2Cal]}{[M][MCal]}$$

The values are given in Table 31. The formation constant of magnesium is greater than those for calcium, strontium and barium; because of this, a very high concentration of hydroxyl ion is necessary to prevent interference of magnesium in the determination of calcium.

Contrary to the findings of Wallach and Steck[57], the Russian workers

TABLE 31. FORMATION CONSTANTS (K_2) OF THE FLUORESCENT ALKALINE EARTH DERIVATIVES OF CALCEIN[a]

Data of Wallach and Steck[57]

Metal	K_2[b]
Magnesium	$10^{7.90}$
Calcium	$10^{6.63}$
Strontium	$10^{5.86}$
Barium	$10^{5.57}$

[a]Values obtained in 0.1 N potassium hydroxide at 20° for calcium, strontium and barium; value for magnesium calculated on the basis of certain assumptions, direct measurement not being feasible because of the formation of insoluble magnesium hydroxide at high pH.

[b]Value given is the average of three measurements, the ranges being, for magnesium 7.75-8.15, for calcium 6.64-6.69, for strontium 5.82-5.89, for barium 5.49-5.65.

Bozhevol'nov and Kreingol'd[50] found that calcium and Calcein combine in the ratio of 1 to 1 to produce the fluorescent compound. The method of continuous variations was used, measurements of the relative fluorescence of solutions of total molar concentration 1.6×10^{-4} being made. A measurement was made also of the formation constant of the calcium-Calcein compound ($\log K'_{Ca\text{-}Cal} = 6.8 \pm 0.3$) and this value was utilized in a study of the suitability of Calcein as indicator for the EDTA titration of microgram amounts of calcium.

[50]*Zh. Analit. Khim.*, **17**, 560 (1962); *J. Anal. Chem., USSR.*, **17**, 558 (1962).

PART V

B. FLUOROMETRIC DETERMINATION OF CALCIUM IN SERUM USING CALCEIN

The use of Calcein for the direct, fluorometric determination of calcium was first proposed by Körbl and Vydra[36, 37], later worked out in detail by Kepner and Hercules[58] and elaborated by Phillips[59]; notes relative to the method also appear in the two papers of Wallach and coworkers[53, 57]. Basically the method is extremely simple: Add 20 μl. of blood serum to 5 ml. of 2 N potassium hydroxide, add 1 ml. of Calcein solution, dilute to 25 ml., and compare the fluorescence with that of a standard. Only microliters of serum are required and neither magnesium nor protein interfere. Certain problems arise, however: there is a small residual fluorescence, Calcein itself is not completely stable in aqueous alkaline solution, contamination by extraneous calcium is aggravated by the small quantities being determined, and detrimental adsorption effects may be involved.

The small residual fluorescence is probably an inherent property of Calcein in the presence of a large amount of potassium; see the discussion on the fluorescence of Calcein in the presence of alkali metal ions on page 66. Commercial Calcein as currently marketed is quite pure and fluorescent impurities are very low; see page 67 for a discussion of this. Beyond using potassium hydroxide or tetrammethylammonium hydroxide as the base to raise the pH during the determination of calcium rather than sodium hydroxide or lithium hydroxide, not much can be done about the residual fluorescence; fortunately it is small enough so that its effect is eliminated by a blank analysis.

Aqueous, alkaline solutions of Calcein are known not to be stable for more than a few days and although this is not too serious when Calcein is used as an indicator in EDTA titrations, it makes necessary a day to day check on the calibration curve of fluorescence as a function of calcium concentration. Kepner and Hercules[58] found that Calcein is soluble in propylene glycol and that solutions in this solvent are stable for at least two weeks, even when standing in sunlight. Propylene glycol is viscous

For full references see Bibliography, page 122.

[36] *Chem. Listy*, **51**, 1457 (1957).
[37] *Coll. Czech. Chem. Commun.*, **23**, 622 (1958).
[58] *Anal. Chem.*, **35**, 1238 (1963).
[59] Mimeographed literature of G. K. Turner Associates, Palo Alto, California.
[53] *Anal. Chem.*, **31**, 456 (1959).
[57] *Anal. Chem.*, **35**, 1035 (1963).

and inconvenient to handle. Phillips reports that solutions of Calcein in 0.4 N potassium hydroxide are stable for several days.

The range of calcium concentration over which the method can be used is 0 to 4 μg. of calcium per 25 ml. although variation in this range occurs owing to the sensitivity of the fluorometer used and the fluorescence of the blank.

The order of addition of the reagents is critical. It is necessary to add the Calcein to the potassium hydroxide before diluting with water. If this procedure is not followed, the intensity of the fluorescence varies erratically. Methyl alcohol placed in the flask ahead of the potassium hydroxide appears to improve the reproducibility (Phillips).

The intensity of the fluorescence of the calcium-Calcein compound was found by Kepner and Hercules[58] to vary significantly with the amount of alkali added, the fluorescence decreasing from 0 to 2 ml. of 2 N potassium hydroxide per 25 ml. final volume, a distinct minimum occurring at 2.0 ml., and the fluorescence rising again to a broad maximum at about 5 ml. The 5-ml. region was selected for analytical work, and although the fluorescence in this region is relatively independent of alkali concentration, the potassium hydroxide is added quantitatively from a 5-ml. pipet.

The amount of Calcein selected by Kepner and Hercules[58] and the working range of calcium are 0.1 μmole (60 μg.) and 0 to 0.07 μmole (0 to 3.0 μg.) respectively per 25 ml. final volume, so that an excess of Calcein is provided. The reagent is added quantitatively from a pipet. Kepner and Hercules reported that variation in the concentration of Calcein with the other concentrations held constant showed two maxima in the intensity of the fluorescence.

Calibration data begins to depart from linearity as the mole ratio of calcium to Calcein approaches 1 to 1. Some variation in calibration data occurs from one solution of Calcein to another so that a calibration curve should be prepared for each batch of Calcein solution.

Analytical work done by Kepner and Hercules is reported in Table 32; the results by the fluorometric method agreed with clinical laboratory results and standard serum controls to within $\pm$ 1 per cent. Both sets of analyses represent averages of several results.

The wave length of the exciting light at which the calcium-Calcein compound fluoresces most strongly is 485 mμ. The maximum in the fluorescent light lies at 520 mμ. Ideally the measurement of the fluorescence in analytical work would be made using these wave lengths and this can be done with instruments with two monochromators, such as the Aminco-Keirs Spectrophotofluorometer. In practice, however, the less expensive "filter fluorometers" must be used and the problem of selecting suitable glass filters must be met. Kepner and Hercules[58], using a Turner Asso-

TABLE 32. FLUOROMETRIC DETERMINATION OF CALCIUM IN BLOOD SERUM

Data of Kepner and Hercules[58]

Blood Serum Sample Number	Calcium Found, mg./100 ml. Fluorometric Method[a]	Clinical Method[b]
1	10.4±0.1	10.4
2	9.4±0.05	9.4
3	9.3±0.1	9.4
4	9.4±0.05	9.4
5	9.3±0.1	9.2
6	10.3±0.1	10.2
7	8.8±0.0	8.8
8	9.9±0.1	10.0
9	10.5±0.1	10.6
10	11.5±0.1	11.6
11	11.5±0.1	11.6
12	7.9±0.1	7.8
13	9.7±0.05	9.8
14	8.8±0.0	8.8
15	9.2±0.1	9.1[c]
16	11.7±0.2	11.9[d]

[a]Fluorometric analyses were done using a 20 μl. sample of blood serum. The data presented are for a minimum of three determinations on each sample. Precision is reported as standard deviation.

[b]Clinical results were obtained by flame photometry at Altoona, Penna. Hospital. The data presented are the average of several determinations on each sample.

[c]Hyland Labs Clinical Control Serum, Normal, Lab. No. 369R47. Stated value 9.1mg./100 ml.; acceptable range 8.9-9.3 mg./100 ml.

[d]Hyland Labs Clinical Control Serum, Abnormal, Lab. No. 368T3, Stated value 11.9 mg./100 ml.; acceptable range 11.5-12.3 mg./100 ml.

ciates Model 110 Fluorometer found the following combination satisfactory: an ultraviolet lamp (General Electric No. F4T4/BL, Turner No. 110-850); primary filter (excitation beam) Corning No. 7-60 (Turner No. 110-811); and secondary filter (fluorescent beam) Wratten No. 8 (Turner No. 110-817). R. E. Phillips[59] of Turner Associates recommends for the primary filter Wratten No. 2A plus Wratten No. 47B (Turner Nos. 110-816 and 110-813, respectively) and for the secondary filter Wratten No. 2A plus Wratten No. 12 (Turner No. 110-818, a combination filter). No difference in results was obtained using different combinations. The visible (blue) activation provided by the 2A-47B combination gives slightly lower blanks than the ultraviolet activation provided by the 7-60 (maximum transmittancy 360 mμ) and is closer to the theoretically optimum wave length.

Extreme care must be taken to minimize contamination by calcium. The question of calcium in the reagents is a little more involved than is first apparent (see the following three paragraphs) and the matter of washing and drying glassware is important.

In the Calcein currently manufactured by the G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 222, there is essentially no residual fluorescence, see page 67.

Phillips[59] has reported that when he took extreme care to keep calcium from his water and reagents that the calibration curve, fluorescence of calcium-Calcein versus calcium concentration, when extrapolated over very low concentrations of calcium, passed below the origin. This same effect has been noted by others and can be interpreted in several ways: as caused by an impurity in Calcein which forms a non-fluorescent compound with calcium (not likely in view of the purity of current commercial preparations of Calcein); or, as suggested by Wallach and Steck[57], on the basis that two compounds are formed, a one-to-one, non-fluorescent compound, and at higher ratios a fluorescent compound containing two calcium atoms per molecule of Calcein (quite possibly correct although some work[50] indicates that the fluorescent compound is a one-to-one compound); or on the basis that adsorption of calcium on the glassware is occurring (not unreasonable as a monomolecular film of calcium ion on the surface of a 25 ml. flask requires in the neighborhood of 5 μg. of calcium).

The concave upward shape of the calibration curve at low concentrations of calcium means that sufficient calcium must be added deliberately to the Calcein solution to bring the calibration curve into the linear range (Wallach and Steck[57]).

Reagent grade potassium hydroxide appears to be uniformly free of calcium although quantitative comparisons will have to await the obvious extension of the fluorometric determination of calcium to the determination of calcium in alkalies. Attempts by Diehl and Oulman to prepare a calcium-free potassium hydroxide by passing recrystallized potassium chloride through Amberlite IR-400 in the hydroxyl form led to a potassium hydroxide which apparently had more calcium in it than present in reagent-grade, commercial potassium hydroxide, even though the resin was contained in a Lucite tube and the effluent potassium hydroxide solution was stored in a polyethylene bottle. These and other unexplained effects related to the order of addition of reagents make it necessary to adhere closely to the prescribed directions for carrying out the analysis.

Procedure for the Fluorometric Determination of Calcium in Serum. PROCEDURE OF KEPNER AND HERCULES[58]. STANDARD CALCIUM SOLUTION. 40.0 μg. CALCIUM PER ml.. Dissolve 0.100 g. of primary standard grade calcium carbonate (G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 337) in the minimum amount of hydrochloric acid and dilute to exactly one liter.

CALCEIN SOLUTION. 60 μg. Calcein per ml. (1×10^{-4} M) in Propyleneglycol, or 60 μg. Calcein per ml. (1×10^{-4} M) in 0.40 N Potassium Hydroxide. Dissolve 60 mg.

[57]*Anal. Chem.*, **35**, 1035 (1963).
[59]*Zh. Analit. Khim.*, **17**, 560 (1962); *J. Anal. Chem., USSR.*, **17**, 558 (1962).

of Calcein (G. Frederick Smith Chemical Company, Item No. 222) in 1 liter of fluorescence-grade propylene glycol (G. Frederick Smith Chemical Company, Item No. 374) or in 1 liter of 0.4 N potassium hydroxide. If necessary add a small amount of EDTA to the solution to remove any residual fluorescence caused by traces of calcium in the potassium hydroxide or water used. Typically the amount required is 1.0 ml. of 0.01 M EDTA per 100 ml. of solution; the volume required is constant for each lot of Calcein but varies from one lot to another. Establish the amount of EDTA by adding increments of 0.01 M, aqueous EDTA and observing the effect on the fluorescence of the blank. Near the point of minimum fluorescence of the blank, run a standard curve to establish that an excess EDTA has not been added for this would suppress the fluorescence of the calcium added during the later analyses.

Store the Calcein solution in a bottle of borosilicate glass, preferably of the red, low-actinic variety or wrapped in dark paper.

Potassium Hydroxide. 2 N. Dissolve 112 g. of reagent grade potassium hydroxide in 982 ml. of deionzied water. Store in a polyethlene bottle.

Water. Use distilled water which has been deionized by passage through a monobed exchange resin.

Apparatus. Make the measurement of the intensity of the flourescence on any instrument of suitable sensitivity and accuracy. Using a spectrophotometer use as the exciting light, light of wave length 495 mμ, and for the fluorescent light, the wave length 520mμ. With filter instruments use filters which have their maximum transmittancy at these wave lengths, suitable combinations being:

	A	B
Excitation beam	Corning 7-60	Wratten 2A plus Wratten 47B
	or	or
Flourescence beam	Wratten No. 8	Wratten 2A plus Wratten 12 plus a neutral filter

A being the filters used by Kepner and Hercules[58] (maximum excitation at 365 mμ) and B those used by Phillips[59] (maximum excitation at 436 mμ). If a Turner fluorometer is used the filters listed under A are those designated by Turner as 110-811 (7-60) and 110-817 (No. 8) and under B are those designated by Turner as No. 110-816 (2A), No. 110-813 (47B), and No. 110-818 (a combination of filters 2A and 12). The neutral filter listed under B is used to adjust the sensitivity of the Turner instrument to the desired working range.

Matched cuvettes are not required for clinical work; selected 12 x 75 mm., rimless Pyrex test tubes (Corning No. 9820) are satisfactory.

Procedure Using Calcein in Propylene Glycol. Into each of a series of 25-ml. volumetric flasks, pipet 5.00 ml. of 2 N potassium hydroxide. Into the successive flasks pipet varying amounts of the standard calcium solution covering the range from 0 (blank) to 3.0 μg. of calcium. Pipet into each flask 1.00 ml. of propylene glycol solution of Calcein. Swirl briefly, dilute to the mark with water, and mix well. Balance the fluorometer with the blank and then with each of the standard solutions. For the analysis, follow the same procedure using 20 μl. or serum.

Calculate the calcium concentration in mg. of calcium per 100 ml. of serum.

Procedure Using Calcein in 0.40 N. Potassium Hydroxide. Follow the above procedure but use 1.00 ml. of Calcein dissolved in 0.40 N potassium hydroxide.

Notes on the Above Procedure. The order of the addition of the reagents is important.

The above procedure calls for a sample of 20 μl. Phillips[59] states that it is possible to make the determination on 4 μl. of serum by simply scaling down all volumes, and even on 0.2 μl. if a microadapter is used (Turner No. 110-865 and 110-866).

The fluorometer of Turner Associates provides considerable flexibility in the matter of range selection and zero adjustment. The instrument can be first zeroed on a dummy cuvette (black rod). The fluorescence of the blank can then be measured, a convenient check on stability of the Calcein reagent and comparison of one solution of Calcein with another. The blank can then be suppressed to zero with the blank knob, or subtracted from the reading on the sample.

Determination of Calcium in Serum. Procedure of R. E. Phillips. Reagents. 0.8 M Potassium Hydroxide. Dissolve 45 g. of reagent grade potassium hydroxide in 1 liter of deionized water.

Standard Calcium Solution. 10 mg. Calcium per 100 ml; 0.100 μg. Calcium per μl. Transfer 0.400 g. of primary standard calcium carbonate (G. Frederick Smith Chemical Company, Item No. 337) directly to a 1-liter volumetric flask, dissolve in a little hydrochloric acid, dilute to the mark and mix.

Calcein, Stock Solution. Dissolve 0.100 g. of Calcein (G. Frederick Smith Chemical Company, Item No. 222) in 100 ml. of 0.8 M potassium hydroxide. Store in a polyethylene bottle in a refrigerator.

Calcein, Working Solution. Dilute 7 ml. of the stock solution of Calcein to 1 liter with deionized water. Store in a polyethylene bottle which has been sprayed on the outside with a flat black paint.

Apparatus. Measure the intensity of the fluorescence of the various solutions with with a Turner Fluorometer equipped with the following elements: light source: either the Standard Lamp (No. 110-850) or the Special Lamp (No. 110-851); primary filter (excitation beam), Filter No. 110-816 (2A) plus Filter No. 110-813 (47B); and secondary filter (fluorescence beam), Filter No. 110-818 (2A-12).

Procedure. Prepare a calibration curve in the following manner. Into each of a series of 12 x 75 mm. cuvettes pipet 5.00 ml. of Calcein working solution. To these solutions add appropriate volumes of the standard calcium solution over the range 0 to 40 μl. (0 to 4.0 μg. of calcium). Pipet the standard calcium solution directly into the liquid in the cuvette, not allowing the solution to run down the walls of the cuvette. Cap the tube with parafilm or similar material and mix by inverting 10 times. Zero the fluorometer against the dummy (black rod) cuvette. Insert the standard containing the largest amount of calcium into the fluorometer and adjust the sensitivity with the range selector so that a reading on the scale is obtained. Measure the fluorescence of the other standards and prepare a calibration curve. The volume of the standard solution added causes a slight change in the total volume but this effect is negligible. The calibration curve will be an S-shaped curve with a linear central portion. Adjust the concentration of the Calcein in the working solution such that the range from approximately 1 to 3 μg. of calcium falls within the linear portion of the curve, repeating the preparation of the calibration curve to establish that this is so. After a suitable working solution has been prepared, adjust the sensitivity of the instrument so that the highest sample of interest (30 μl. of standard corresponding to a sample containing 15 mg.-per cent (15 mg. per 100 ml.) of calcium) will have

a reading as high as possible so as to provide maximum accuracy. The reading on this sample can be adjusted to between 85 and 90 divisions by utilizing the higher sensitivity range of the instrument and either neutral filters in the fluorescence beam, or more conveniently, placing two pieces of black electrical tape horizontally on the filter in the fluorescent beam, one top and one bottom, and successively moving them closer together until the reading comes down on the scale to the desired range. The reading may be further extended by adjusting the instrument such that the 30 μl. sample has a reading between 95 and 100, and then supressing with the blank knob the flourescence of a 0.5 μg. standard until it reads about 5 divisions. Finally with all the conditions so established prepare a permanent calibration curve.

Determine the calcium in the serum in the same way by placing 5.00 ml. of the Calcein working solution in the cuvette, adding 20 μl. of serum, mixing, and measuring the intensity of the fluorescence.

PART V

C. MISCELLANEOUS USES OF CALCEIN

1. THE DETERMINATION OF STRONTIUM BY EDTA TITRATION

In the original work on Calcein Diehl and Ellingboe[5] reported that the EDTA titration at pH 12 gave the sum of the calcium, strontium and barium present. This has been confirmed by others and used by Dumont[41] for the determination of strontium after separation from calcium by paper chromatography. The Dumont work was directed to the determination of submicrogram amounts of calcium and strontium in biological tissue. Basically the procedure involves dry ashing of a 10- to 20-mg. sample of tissue in an atmosphere of oxygen, separation of the calcium and strontium by ascending paper chromatography with a methanol-hydrochloric acid-water solvent, identification of the spots by spraying with 8-hydroxyquinoline and ammonia, removal and ashing of the filter paper bearing the spots, and final titration of the calcium and strontium separately with EDTA at pH 12 using the color change of Calcein to mark the end-point. Extreme precautions must be exercised throughout to avoid contamination. Although the procedure appears involved, the results reported by Dumont on numerous analyses involving known amounts of calcium and strontium are remarkably good. Thus on mixtures of 25.0×10^{-9} moles each of calcium and strontium (1.00 μg, calcium and 2.19 μg. of strontium, respectively) the averages found were 24.7×10^{-9} and 22.7×10^{-9} moles with a relative standard deviation of 5.0 and 4.0 per parts per 100. The results were even better on 150×10^{-9} mole quantities and on crab shell tissue alone and with added calcium and strontium. There appears to be a constant loss of strontium somewhere in the process, probably connected with the indicator, that must be corrected for empirically.

2. DETERMINATION OF SULFATE

An indirect determination of sulfate in water using EDTA and Calcein was devised by Effenberger[42] based on the insolubility of barium

For full references see Bibliography, page 122.

[5] *Anal. Chem.*, **28**, 882 (1956).
[41] *Anal. Chem.*, **34**, 656 (1961).
[42] *Coll. Czech. Chem. Commun.*, **26**, 2449 (1961).

sulfate and titration of the excess barium with EDTA. It is necessary first to determine the calcium in the water by titrating with EDTA at pH 12 using Calcein as indicator (100 ml. sample, 0.02 M EDTA). To an equal aliquot of the water is then added a volume of 0.1 N hydrochloric acid equal to that required to titrate the bicarbonate present in the sample (methyl orange alkalinity) plus 5 ml. more. The solution is then heated to boiling, an excess of standard barium chloride (0.005 to 0.02 M) added, and after 2 minutes of boiling and 20 minutes standing, the barium sulfate is filtered off and washed with water. The excess barium in the filtrate is then determined by adding triethanolamine and cyanide, bringing the pH to 12, and titrating with EDTA with Calcein as indicator. The mean error in the results reported by Effenberger was 0.82 per cent.

3. DETERMINATION OF BROMIDE, IODIDE, AND CYANIDE BY TITRATION WITH SILVER

Calcein has been reported to be useful as a fluorescent indicator in titrations with silver nitrate[47]. The indicator action is based primarily on the quenching of the fluorescence of Calcein by the excess of silver ion but the intensity of the change in fluorescence and color is enhanced by adsorption effects. The method is not applicable to the determination of chloride as the indicator is too sensitive to silver and silver chloride is too soluble. It works well, however, for bromide and iodide, the silver salts being much less soluble. The titrations are best carried out at pH 9 to 11 (potassium hydroxide-boric acid buffer). The determination of cyanide is also very good and is based on the conversion of the cyanide all the way to AgCN so that the equivalence ratio is the more favorable 1 to 1 rather than 2 to 1 as in the customary method of titrating in ammoniacal solution to the iodide end-point.

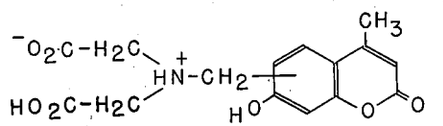
[47] *Coll. Czech. Chem. Commun.*, **26**, 2449 (1961).

PART VI

CALCEIN BLUE, METHYL CALCEIN, METHYL CALCEIN BLUE

Following the introduction of Calcein in 1956 by Diehl and Ellingboe[5], three other metallofluorochromic indicators of the same general type were introduced by D. H. Wilkins[60, 61]: *Calcein Blue* derived from 4-methylumbelliferone, formaldehyde and iminodiacetic acid; *Methyl Calcein* derived from fluorescein, formaldehyde and N-methylglycine; and *Methyl Calcein Blue*, derived from 4-methylumbelliferone, formaldehyde and N-methylglycine. Analytical methods using these indicators have been described, the indicators are available from the G. FREDERICK SMITH CHEMICAL COMPANY, but the indicators themselves have not been adequately characterized. In the following pages, a section is devoted to each of these indicators.

Calcein Blue. Calcein Blue was first described by Wilkins in 1960[60]. It is prepared by the condensation of 4-methylumbelliferone, formaldehyde and iminodiacetic acid. Presumably one methyleneiminodiacetic acid group is introduced into the 4-methylumbelliferone molecule. The properties of



Calcein Blue and Calcein are very similar but Wilkins states that Calcein Blue is superior to Calcein for chelatometric titrations inasmuch as its maximum fluorescence is produced by light of shorter wave length, 370 mμ in alkaline solution and 330 mμ in acid solution (Calcein 488 mμ). Most sources of ultraviolet light exhibit a maximum output of radiation at 366 mμ or 250 mμ; the excitation wave length of Calcein Blue in alkaline solution approaches the optimum wave length and is thus more sensitive as an indicator than Calcein.

The parent compound, 4-methylumbelliferone, is a fluorescent acid-base indicator of the coumarin series. The addition of a chelating functional group, probably ortho to the phenolic oxygen, imparts metallofluorochromic properties to the resulting indicator. Calcein Blue exhibits a brilliant blue fluorescence, 445 mμ, up to a pH of 11. The addition of copper ions to a solution of the free indicator at pH 4 to 10 results in the quenching of this

For full references see Bibliography, page 122.

[5] *Anal. Chem.*, **28**, 882 (1956).
[60] *Talanta*, **4**, 182 (1960).
[61] *Anal. Chim. Acta*, **23**, 309 (1960).

blue fluorescence. Palladium also quenches the fluorescence over the pH range 4 to 10, but the reaction is too slow to be of use in chelatometric titrations. Manganese, nickel and cobalt also quench the fluorescence of Calcein Blue, but this reaction is confined to the alkaline region in which the free indicator fluoresces.

At pH 12 Calcein Blue does not fluoresce but the addition of calcium, barium or strontium causes fluorescence to appear. This is the so-called "indicator reversal" reaction. The indicator is converted by these ions to its acid (fluorescent) form.

The titration of metal ions which form highly colored compounds with EDTA and other chelating agents is most conveniently carried out by adding a standard excess of the chelating agent and performing a back-titration with a standard copper solution. In this manner, macro amounts of nickel, cobalt, copper, iron and titanium (in the presence of peroxide), as well as elements which do not form highly colored compounds such as aluminum, manganese, zinc, and mercury, can be carried out with a single standard solution. Back titrations with a standard copper solution are carried out by adding an excess of an appropriate chelating agent, for example EDTA or tetraethylenepentamine, to a solution of the metal ion to be determined. The pH is then adjusted, a few drops of a 0.1 per cent solution of Calcein Blue added, and the solution back titrated with a standard solution of copper to the quenching of the blue fluorescence of the free indicator.

In the determination of calcium, strontium and barium, potassium hydroxide is superior to sodium hydroxide for adjusting the pH; sodium hydroxide leads to a high residual fluorescence after the end-point but potassium hydroxide does not. The potassium hydroxide may be added as a 1 M solution or as pellets. In either a case, low-carbonate potassium hydroxide (reagent grade) should be used. An excessive amount of carbonate leads to a residual fluorescence after the end-point in the titration. The 1 M solution, if used, should be made up fresh every few days to prevent the accumulation of carbonate by absorption from the air.

Zinc and other elements which form indicator-reversal compounds with Calcein Blue may be determined also, using the procedure given below for the alkaline earths. These elements however, are most conveniently determined by back-titration with a standard copper solution.

The determination of nickel in the presence of chromium (procedure given below) is based on the slow rate of reaction of chromium and EDTA. The solution containing the nickel and chromium should be kept near room temperature until after the nickel determination is completed. The titration for nickel should be completed shortly after the addition of the EDTA because a long period of standing even at room temperature can lead to the

formation of the chromium compound.

Other metals can be determined in the presence of chromium in this way if they react with EDTA at room temperature and if they permit the back titration of the excess EDTA at pH 4.8 with copper.

A modified Calcein Blue indicator was proposed by Kirkbright and Stephen[9] who recommend the addition of Rhodamine B or fluorescein as screening agents. With the former the end-point is denoted by a sharp change in the fluorescence from bright blue to salmon pink.

Procedure for the Determination of Nickel and Chromium in a Mixture of the Two. PROCEDURE OF WILKINS[60]. To an acid solution of nickel and chromium add a measured excess of EDTA over that necessary to combine with the nickel in the solution. Dilute to 100 to 150 ml. with water, add 10 ml. of sodium acetate-acetic acid buffer (pH approximately 4.8) and 2 to 3 drops of the indicator solution (0.1 per cent solution of Calcein Blue (G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 298). Back-titrate the excess EDTA with a standard copper solution to the disappearance of the blue fluorescence of the free indicator using ultraviolet light as the sole source of illumination.

To the solution in which the nickel has just been determined add a measured excess of EDTA over that necessary to combine with the chromium. Boil the solution for 10 to 15 minutes to form the chromium-EDTA compound. Back titrate the excess EDTA with a standard copper solution to the disappearance of the blue fluorescence of the free indicator.

Procedure for the Determination of Calcium, Strontium and Barium. PROCEDURE OF WILKINS[60]. To a solution containing the calcium, strontium or barium, add a few drops of 0.1 per cent solution of Calcein Blue and adjust the pH to 13 to 14. If strontium is being determined add a two-fold excess of potassium tartrate. Titrate the solution with standard EDTA to the disappearance of the fluorescence of the alkaline earth-indicator compound.

Methyl Calcein. Methyl Calcein Blue. Only one paper has been published so far in which Methyl Calcein and Methyl Calcein Blue are mentioned, that of Wilkins[61]. This paper is a description of the use of hydroxyethylethylenediaminetriacetic acid (HEDTA) as a titrating agent for aluminum, nickel, and manganese. Calcein and Calcein Blue are not satisfactory indicators for HEDTA titrations, the formation constants of the metals toward the indicators being too close to those of the metals with HEDTA. Wilkins' solution to this problem was to modify the indicators by the replacement of an acetic acid group by a methyl group. This was accomplished by using N-methylglycine in place of iminodiacetic acid in the condensation with fluorescein and formaldehyde or 4-methylumbelliferone and formaldehyde. The resulting compounds have not yet been well characterized. These indicators are available from the G. FREDERICK SMITH CHEMICAL COMPANY: Methyl Calcein, Item No. 334; Methyl Calcein Blue Item No. 335.

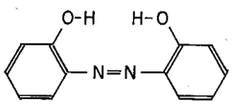
[9] *Anal. Chim. Acta*, **27**, 294 (1962)
[61] *Anal. Chim. Acta.*, **23**, 309 (1960).

PART VII

o,o'-DIHYDROXYAZOBENZENE.

A. THE FLUOROMETRIC DETERMINATION OF MAGNESIUM IN THE PRESENCE OF CALCIUM

Several *o,o'*-dihydroxyzao compounds react with magnesium to form compounds which fluoresce[62]; the best of these is *o,o'*-dihydroxyazobenzene which was investigated in detail by Diehl, Olsen, Speilholtz and Jensen[63]. This azo compound, the simplest member of the *o,o'*-dihydroxyazo class of



compounds, is unique in that it reacts with magnesium but not with calcium. At pH 10, *o,o'*-dihydroxyazobenzene gives an orange, slightly dissociated compound with magnesium, formation constant $10^{4.85}$, but no color change with large amounts of calcium. The magnesium derivative fluoresces but the compound and the compound plus calcium do not. Both the color change and the appearance of the fluorescence can be used for the determination of magnesium; the fluorometric method is more sensitive and is preferred, but for many applications the spectrophotometric method is perfectly satisfactory.

The fluorometric method of measuring magnesium with *o,o'*-dihydroxyazobenzene may be carried out either in aqueous solution or in a water-ethanol mixture. The fluorescence of magnesium-*o,o'*-dihydroxyazobenzene is sufficiently greater in the presence of ethanol that the measurement can be made with the less expensive fluorometers, that is with those using simple photovoltaic cells as receivers. The intensity of the fluorescence in water alone is such that the measurement is best made in an instrument using a photomultiplier tube as the receiving element. Procedures are given below for carrying out the determination in water only as the final solvent (Procedure A) or in water-ethanol (Procedure B). Provision is made in each procedure for eliminating the interferences of iron, aluminum, copper and zinc. An additional procedure is also given for the determination of mag-

For full references see Bibliography, page 123.
[62] *Anal. Chem.*, **35**, 1142 (1963).
[63] *Anal. Chem.*, **35**, 1144 (1963).

nesium in serum; this determination (Procedure C) may be carried out on either whole serum or on the solution remaining after precipitation of protein with trichloroacetic acid. The iron content of serum is normally so low that it causes no interference. The fluorometric determination of magnesium with *o,o'*-dihydroxyazobenzene can be carried out in the presence of large amounts of calcium; interference by very large amounts of calcium may be compensated or corrected, see the concluding paragraph of the procedures. As expected, pH, the amount of reagent added, and the amount of alcohol in the final solution, all affect the fluorescence and care must be exercised that each of these factors be suitably controlled. In addition the fluorescence is temperature dependent and undue heating from the light source must be avoided.

Excitation and Fluorescence Spectra of Magnesium-o,o'dihydroxyazobenzene. The fluorescent light emitted by a solution is observed at a right angle to the entering light which causes, or excites, the fluorescence. The fluorescent light is always of longer wave length than the exciting light and the fluorescent light is not monochromatic (of a single wave length) but consists of light of a broad region of the spectrum. The fluorescence is ex-

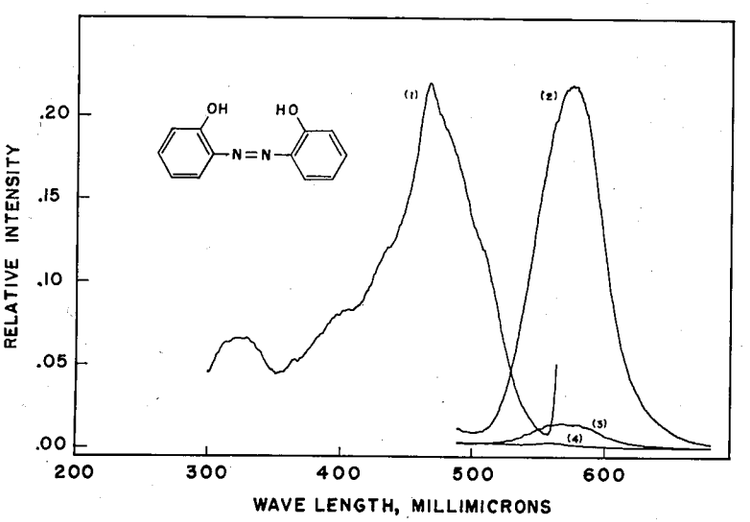


Figure 13. Fluorescence spectra of *o,o'*-dihydroxyazobenzene, each solution 2×10^{-5} M potassium chloride, pH 11.4; (1) excitation spectrum of magnesium derivative, 1×10^{-4} M in magnesium, fluorescence monochromator set at 580 mμ; (2) fluorescence spectrum of magnesium derivative, excitation monochromator set at 470 mμ; (3) fluorescence in the presence of calcium, 1×10^{-4} M in calcium, excitation monochromator set at 470 mμ; (4) fluorescence of *o,o'*-dihydroxyazobenzene alone, a few drops of 0.01 M EDTA present to mask any magnesium or calcium impurity, excitation monochromator set at 470 mμ. *Data of Diehl, Olsen, Spielholtz and Jensen*[63].

cited, not only by monochromatic light, but by light of a wide portion of the spectrum. Some portions are more effective than others, that is, the intensity of the fluorescent light varies with the wave length of the exciting light, there being one or more wave lengths where excitation reaches a maximum. A precise knowledge of these characteristics is useful in designing an analytical method based on measurement of fluorescence. The necessary information can be secured using a spectrophotofluorometer, an instrument having two monochromators making it possible that the wave length of both the exciting and the fluorescent light can be varied. One wave length is held constant while the other is varied and vice versa so that two spectra are obtained: an excitation spectrum (fluorescence monochromator held fixed) and a fluorescence spectrum (excitation monochromator held fixed, usually at the maximum in the excitation spectrum). Such an instrument of course, measures the intensity of the fluorescent light precisely and can be used for analytical work; the cost of the instrument, however, is so high that routine analytical work is normally carried out on less expensive instruments. Such instruments are generally referred to as fluorometers, and make use of glass filters to select portions of the spectrum of the two light beams during the measurement.

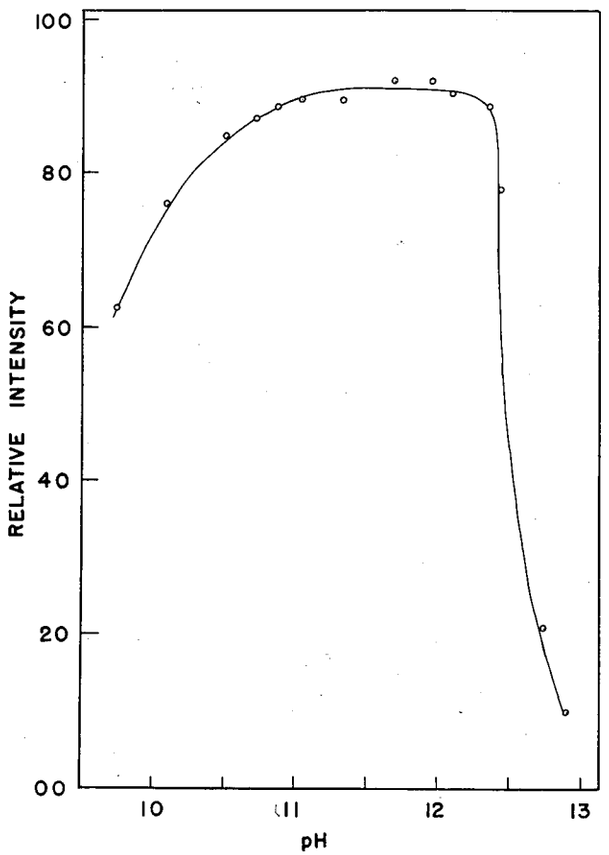


Figure 14. Effect of pH on the fluorescence of magnesium-*o,o'*-dihydroxyazobenzene, water only as solvent. 1×10^{-5} M *o,o'*-Dihydroxyazobenzene and 2×10^{-5} M magnesium; Aminco-Keirs Spectrophotofluorometer, wave length settings 470 mμ and 580 mμ. *Data of Diehl, Olsen, Spielholtz and Jensen*[63].

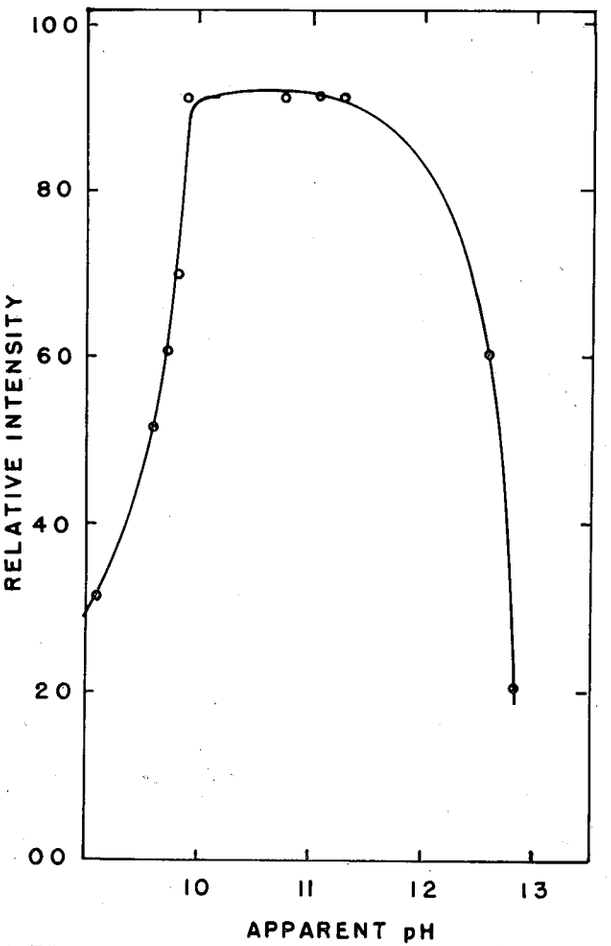


Figure 15. Effect of pH on the fluorescence of magnesium-*o,o'*-dihydroxyazobenzene, water-ethanol as solvent. 1×10^{-5} M *o,o'*-Dihydroxyazobenzene and 2×10^{-5} M magnesium in 60 ethanol-40 water solution; measurement made with Coleman Model 12 Photofluorometer, Corning CS-5-60 and CS-3-67 filters; pH values apparent, as observed with a high-alkalinity glass electrode. *Data of Diehl, Olsen, Spielholtz and Jensen*[63].

The excitation and fluorescence spectra of magnesium-*o,o′*-dihydroxyazobenzene are shown in Figure 13. Also illustrated is the very slight fluorescence exhibited by the reagent, alone and in the presence of calcium. The peaks in the excitation and fluorescence spectra occur at 470 mμ and 580 mμ, respectively. Thus, in the use of a filter fluorometer for the determination of magnesium, the filters should be chosen to have maximum transmittancy at these wave lengths; Corning filters CS-5-60 and CS-3-67 are very suitable. As indicated in the caption, the spectra shown in Figure 13 were obtained on water solutions. The maxima in both the excitation and fluorescence spectra occur at the same wave lengths in the water-ethanol solution employed in the procedures B and C recommended below.

Effect of pH. The intensity of the fluorescence of magnesium-*o,o′*-dihydroxyazobenzene in water solutions is essentially linear over the pH range 11.0 to 12.2, falling off at higher and lower values of pH, Figure 14. The same effect is observed in the ethanol-water solvent used in Procedure B but the range is shifted to lower values, 9.9 to 11.3 apparent pH as indicated by the high alkalinity glass electrode, Figure 15.

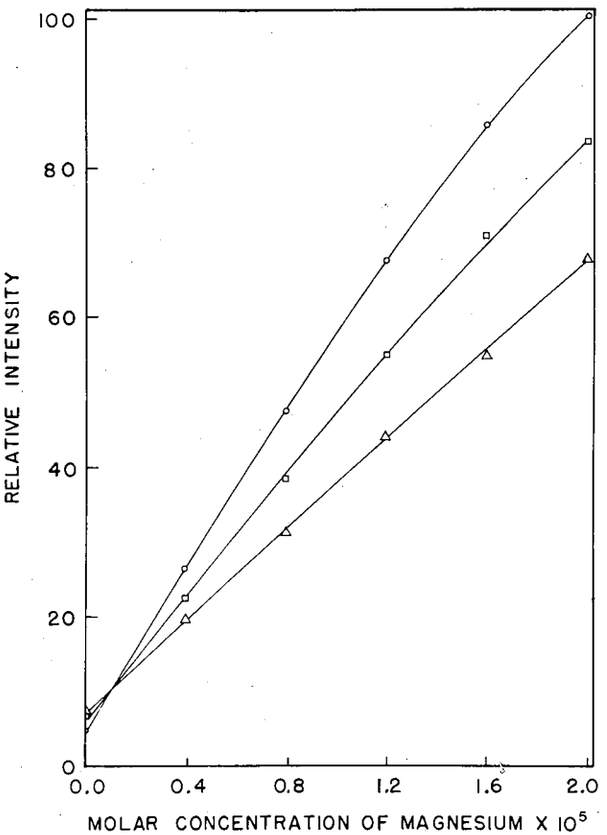


Figure 16. Effect of excess *o,o′*-dihydroxyazobenzene on the fluorescence of its magnesium derivative. Concentration of *o,o′*-dihydroxyazobenzene: ○ 2.5×10^{-5} M; □ 5.0×10^{-5} M; △ 7.5×10^{-5} M. *Data of Diehl, Olsen, Spielholtz and Jensen*[63].

o,o′-Dihydroxyazobenzene is a weak, dibasic acid, pK_1 7.8 and pK_2 11.5 (Diehl and Ellingboe[5]), and the decrease in intensity of the fluorescence at low pH is probably caused by incomplete formation of the magnesium compound. The decrease at high pH is probably a result of the competition of the hydroxyl ion for magnesium. The decrease in intensity of the fluorescence at high pH is not eliminated by extraction into amyl alcohol.

Effect of Excess Reagent. To insure complete formation of the magnesium compound it is necessary to have present a moderate excess of *o,o′*-dihydroxyazobenzene. *o,o′*-Dihydroxyazobenzene has a strong absorption peak at 470 mμ which is approximately the wave length of maximum excitation of the fluorescence of the magnesium derivative. This precludes use of a large excess of reagent and also necessitates working at a low concentration. The effect is shown in Figure 16. It is apparent that the amount of reagent used must be constant and fairly accurately measured. The device shown in Figure 17 is convenient and insures that the amount

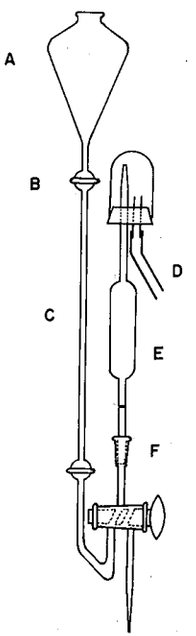


Figure 17. Device for dispensing a reproducible volume of solution. (A) 300-ml. bulb, (B) ball and socket joint, No. 12, (C) capillary tube, 1-2 mm. i.d., (D) flexible plastic tube, (E) volumetric pipet (10.0 ml. for Procedure A, 35.0 ml. for Procedure B, 10.0 ml. for Procedure C, 20.0 ml. for Spectrophotometric Procedure), (F) standard taper joint, 7/25. *Diehl, Olsen, Spielholtz and Jensen*[63].

of reagent and buffer (and alcohol in Procedures B and C) are always delivered in precisely the same amounts.

Effects of Time and Temperature. Magnesium-*o,o'*-dihydroxyazobenzene appears to form instantly and to be stable indefinitely. The intensity of the fluorescence of the compound decreases as the solution is warmed even slightly above room temperature. Because of the power dissipated in the light source and the proximity of the lamp to the cell in some instruments, attention must be paid to warming effects of the cell compartment if the latter has been heated above room temperature by continuous operation of the lamp. Such an effect, as experienced in the Aminco-Keirs Spectrophotofluorometer, is shown in Figure 18.

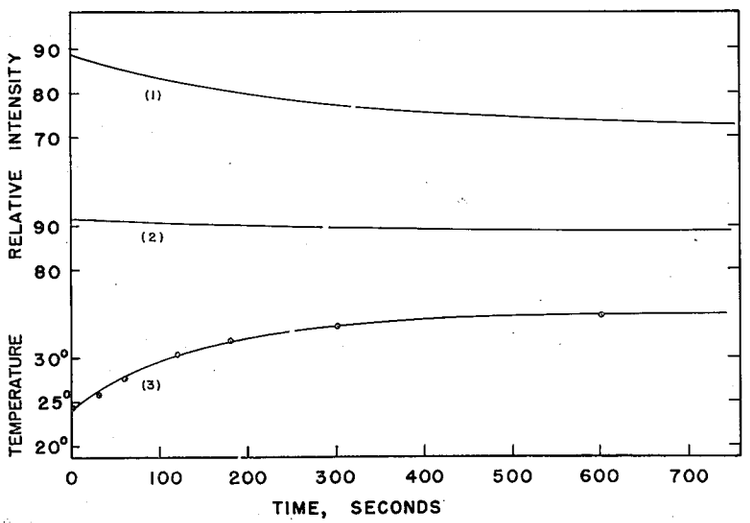


Figure 18. Effects of temperature on the intensity of the fluorescence of magnesium-*o,o'*-dihydroxyazobenzene; (1) solution while standing in warm cell compartment; (2) solution standing in cool cell compartment; (3) temperature of solution standing in warm cell compartment. *Data of Diehl, Olsen, Spielholtz and Jensen*[63].

Effect of Ethanol and Other Alcohols. The intensity of the fluorescence of magnesium-*o,o'*-dihydroxyazobenzene is greatly enhanced by the presence of ethanol, being some four times greater in 67 per cent ethanol than in water, Figure 19. The effect is sufficiently great as to make possible the measurement of magnesium with *o,o'*-dihydroxyazobenzene with the less expensive fluorometer, that is, those employing a simple photovoltaic cell rather than a photomultiplier cell. The increase in intensity with increasing concentration of alcohol (Figure 19) is sufficiently great that some care must

be exercised to introduce always the same volume of alcohol. This is done expeditiously by introducing the alcohol at the same time as the *o,o'*-dihydroxyazobenzene and the buffer, in the form of a so-called "working solution", and conveniently using the apparatus shown in Figure 17.

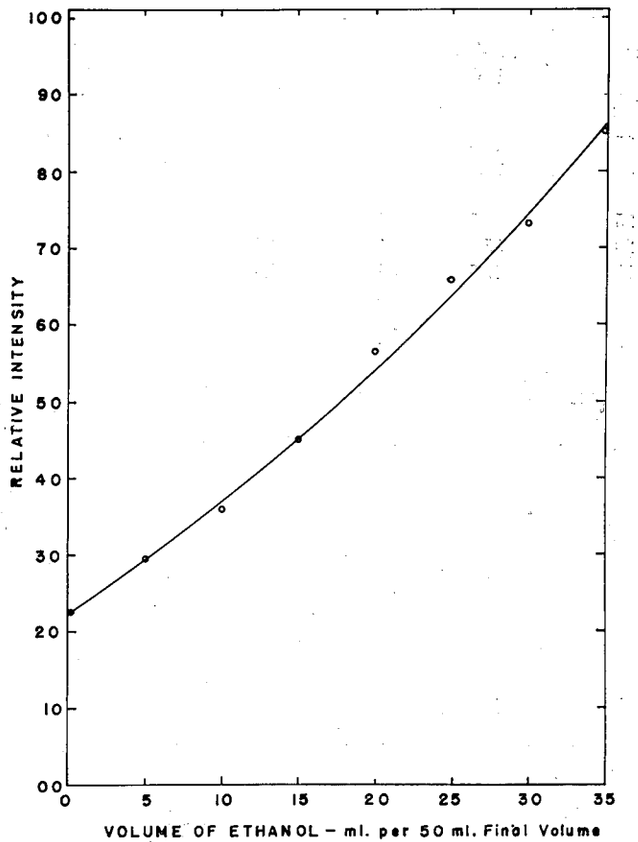


Figure 19. Effect of ethanol on the intensity of fluorescence of magnesium-*o,o'*-dihydroxyazobenzene. *Data of Diehl, Olsen, Spielholtz and Jensen*[63].

There is essentially no difference in the action of the various alcohols in enhancing the fluorescence of magnesium-*o,o'*-dihydroxyazobenzene, Table 33. Methanol may be substituted for ethanol and this may be advantageous in industrial laboratories where the bonding and the supervision of the ethanol supply is an inconvenience.

Effects of Foreign Ions. The presence of large amounts of alkali metal and barium chlorides appears to have no detrimental effect on the

TABLE 33. FLUORESCENCE OF MAGNESIUM o,o′-DIHYDROXYAZOBENZENE AS AFFECTED BY THE PRESENCE OF VARIOUS ALCOHOLS

Data of Diehl, Olsen, Spielholtz and Jensen[63]

Volume of Alcohol in 50 ml. Final Volume ml.	Relative Intensity of Fluorescence: Methyl Alcohol	Ethyl Alcohol 95 Percent	Isopropyl Alcohol	Butyl Alcohol Plus Ethyl Alcohol	Isoamyl Alcohol Plus Ethyl Alcohol
35	83.5	85	73.5		79 (15+20)[b]
30	90	73	73	70.5 (15+15)[a]	70.5 (10+20)
				71.5 (10+20)	73 (5+25)
25	65.5	65.5	61.5	64 (5+20)	
20	55	56.5	54.5		
15	40.5	45	46.5		
10		36			
5		29.5			
0		22.5			

[a]Volume of butyl alcohol plus volume of ethyl alcohol.
[b]Volume of isoamyl alcohol plus volume of ethyl alcohol.

fluorometric determination of magnesium. The presence of large amounts of calcium, strontium or beryllium tends to suppress the fluorescence; moderate amounts can be tolerated as can be seen from the data presented in Table 34. It is possible to compensate for large amounts of these alkaline earth metal ions by having the composition of the standards approximate that of the unknown with respect to the interfering ion. The effect of calcium is illustrated in detail by Figure 20.

TABLE 34. FLUOROMETRIC DETERMINATION OF MAGNESIUM IN THE PRESENCE OF VARIOUS METALLIC IONS

Data of Diehl, Olsen, Spielholtz and Jensen[63]

Magnesium Taken, μg.	Interference	Magnesium Found, μg.	Difference μg.
20	1 mg. Be	19.2	—0.8
20	10 mg. Sr	20.4	+0.4
20	0.1 mg. Mn	14.0	—6.0
20	1 mg. Al[a]	21.6	+1.6
20	0.1 mg. Al[a]	20.6	+0.6
20	1 mg. Cu[a]	19.1	—0.9
20	1 mg. Zn[a]	19.7	—0.3
20	1 mg Fe[a]	19.9	—0.1
20	$Na_2B_4O_7NaCl$[b]	20.0	0.0

[a]Treatment appropriate for the interference applied in accordance with the recommended procedure.
[b]Quantity equivalent to 8 mg. of the fusion mixture used in analysis of the N.B.S. samples.

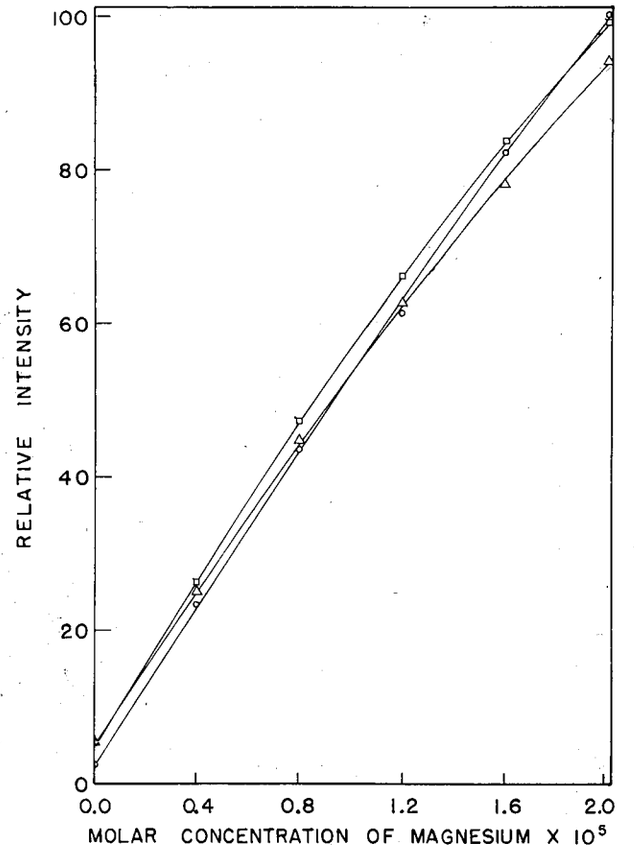


Figure 20. Calibration curves for the fluorometric determination of magnesium in the presence of various amounts of calcium, water only as solvent. Concentration of calcium: ○ None, □ 2×10^{-4} M, △ 4×10^{-4} M. *Data of Diehl, Olsen, Spielholtz and Jensen*[63].

Extraction of Magnesium-o,o′-dihydroxyazobenzene into Isoamyl Alcohol. The magnesum derivative of *o,o′*-dihydroxyazobenzene can be extracted from water solution into isoamyl alcohol. Unfortunately the distribution coefficient is small, about 9, and three or more extractions are necessary to effect a 99 per cent extraction. The intensity of the fluorescence is about 10 times greater in the isoamyl alcohol extract than in water but this same effect can be brought about to almost the same degree by the addition of ethanol to water as described above. Calcium is not extracted during the isoamyl alcohol extraction of magnesium-*o,o′*-dihydroxyazobenzene and the extraction procedure makes possible the

determination of magnesium in the presence of extraordinarily large amounts of calcium[63]. The tolerance for calcium in the procedure, no extraction, recommended below is so high that the extraction would be used only in very unusual cases.

Applications and Reported Results. The fluorometric method was applied by Diehl, Olsen, Speilholtz and Jensen[63] to the determination of magnesium in limestone, iron ore, Portland cement and blood serum. The results obtained applying the recommended Procedure A, water only as solvent, to certain Bureau of Standards samples are given in Table 35:

TABLE 35. FLUOROMETRIC DETERMINATION OF MAGNESIUM IN STANDARD SAMPLES; PROCEDURE A, WATER ONLY AS FINAL SOLVENT[a]

Data of Diehl, Olsen, Spielholtz, and Jensen[63]

Sample	Magnesium Found, Percent MgO	Magnesium Reported, Percent MgO
N.B.S. 1A, Argillaceous Limestone	2.12, 2.20, 2.42 (Av. 2.25)	2.19
N.B.S. 26, Iron Ore	3.43, 3.43, 3.40, 3.39 (Av. 3.41)	3.27[b]
N.B.S. 88, Dolomite	21.4, 21.7, 21.5, 21.5 (Av. 21.5)	21.48
N.B.S. 177, Portland Cement	2.53, 2.53, 2.47, 2.50 (Av. 2.51)	2.45

[a]Measurement with Aminco-Keirs spectrophotofluorometer.
[b]N.B.S. lists 3.44 per cent as the average of the analyses reported but recommends the value 3.27 per cent as more nearly correct.

results using recommended Procedure B, water and ethanol as final solvent, are given in Table 36. The results by these direct methods with no preliminary separations are in good agreement with the published values and the precision, considering the relatively small sample taken and the aliquoting done, is good. There is little reason to believe that the results obtained by this method may not be as reliable as those reported; the earlier, gravimetric method involves numerous prior separations, the accumulation of foreign magnesium introduced as impurities in the numerous reagents added, and doubt exists as to the exact composition of the magnesium precipitate and its final weighing form.

Results obtained on applying the fluorometric method directly to blood serum of sheep, Recommended Procedure C, were very satisfactory in precision. Lacking a suitable check method and in view of the knowledge of the action of interfering elements gained in the course of this study, the results are presumed to be correct. A sampling of the results of sheep

[63]*Anal. Chem.*, **35**, 1144 (1963).

serum, typical of a large number, is given in Table 37. Identical results were obtained on a given serum by the direct method and by preliminary wet ashing; it appears, therefore, that in sheep serum magnesium is not bound tightly enough to prevent its complete reaction with *o,o'*-dihydroxyazobenzene. Nor was any magnesium found in the trichloroacetic acid precipitate of protein by wet ashing the latter, Table 38. For sheep serum, at least, preliminary ashing or preliminary precipitation of protein is not necessary. In the recommended procedure, protein is precipitated by the alcohol added, but this precipitate again, carries with it no magnesium.

TABLE 36. FLUOROMETRIC DETERMINATION OF MAGNESIUM IN STANDARD SAMPLES; PROCEDURE B, WATER-ETHANOL AS FINAL SOLVENT[a]

Data of Diehl, Olsen, Speilholtz and Jensen[63]

Sample	Magnesium Found, Percent MgO	Magnesium Reported, Percent MgO
N.B.S. 1A, Argillaceous Limestone	2.07, 2.09, 2.12, Av. 2.09	2.19
N.B.S. 26, Iron Ore	3.16, 3.42, 3.38 Av. 3.32	3.27[b]
N.B.S. 88, Dolomite	21.5, 21.4, 21.5 Av. 21.5	21.48
N.B.S. 177, Portland Cement	2.49, 2.42, 2.30 Av. 2.40	2.45
Liberty Limestone[c]	0.93, 0.99, 0.99 Av. 0.97	0.93

[a]Measurement made on Coleman Model 12 fluorometer.
[b]N.B.S. lists 3.44 per cent as the average of the analyses reported but recommends the value 3.27 per cent as more nearly correct.
[c]Sample obtained from Lithium Corporation of America, Bessemer City, North Carolina; gravimetric analysis by Dr. R. B. Ellestad.

TABLE 37. FLUOROMETRIC DETERMINATION OF MAGNESIUM IN SHEEP SERUM AND CEREBRAL SPINAL FLUID

Data of Diehl, Olsen, Spielholtz and Jensen[63]

Sheep Serum		Sheep Cerebral Spinal Fluid	
No.	μg. Mg/ml.	No.	μg. Mg/ml.
1[a]	31.2, 33.2	1[a]	32.2, 35.4
2[b]	40.2, 43.6	2[b]	133, 132
3	44.0, 49.0	3	145, 143
4	48.0, 46.8	4	141, 143
5	49.8, 47.4	5	148, 148
6	37.4, 37.6	6	149, 156

[a]Control. [b]Nos. 2 thru 6 from sheep subjected to experiment.

TABLE 38. FLUOROMETRIC DETERMINATION OF MAGNESIUM IN SERUM FOLLOWING VARIOUS TREATMENTS

Data of Diehl, Olsen, Spielholtz and Jensen[63]

Serum Treated with Trichloroacetic Acid in Isopropyl Alcohol	Serum Untreated	Serum Wet Ashed[a]	Serum Treated with Trichloroacetic Acid in Isopropyl Alcohol, Supernate Wet Ashed	Serum Treated with Trichloroacetic Acid in Isopropyl Alcohol, Precipitate Wet Ashed[b]
μg. Mg/ml.	μg. Mg/ml.	μg. Mg/ml.	μg. Mg/ml.	μg. Mg/ml.
26.3, 25.6	26.3, 26.3	32.5, 24.7	25.0, 23.0	0.34
31.1, 32.5	31.6, 31.6	23.0, 19.0	21.0, 29.0	0.00
15.8, 15.8	14.1, 16.0			
18.0, 18.0	14.8, 18.2			
12.4, 14.1	15.3, 15.3			
17.2	17.2			
37.6	37.6			
26.8	26.8			
22.8	22.1			
22.1	21.5			

[a]Wet ashing done with a mixture of nitric acid and perchloric acid.
[b]Precipitate washed 5 times with trichloroacetic acid-isopropyl alcohol mixture before wet ashing.

Procedures for the Fluorometric Determination of Magnesium. METHOD OF DIEHL, OLSEN, SPIELHOLTZ AND JENSEN[63]. Preparation of Stock Solution of *o,o′*-Dihydroxyazobenzene. 2×10^{-3} M. Dissolve 0.5355 g. of crystalline *o,o′*-dihydroxyazobene (G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 336) in 10 ml. of ethanol and 10 ml. of 2 M potassium hydroxide, adding water as necessary to effect complete solution. Transfer the solution to a 1-liter volumetric flask, dilute to the mark with de-ionized water, and mix. Store in a polyethylene container. Using this stock solution, prepare a "working solution" as described in A, B, or C below.

STANDARD MAGNESIUM SOLUTION. About 2×10^{-4} M or 5 μg. per ml. Weigh accurately about 0.24 g. of magnesium metal, dissolve it in a minimum amount of dilute hydrochloric acid, transfer the solution to a 1-liter volumetric flask, dilute to the mark and mix thoroughly. Pipet 10.00 ml. of this solution into a 500-ml. volumetric flask, dilute to the mark and mix. Calculate the exact concentration in terms of μg. of magnesium per ml.

WATER. Use distilled and deionized water prepared by passing distilled water through cation and anion exchange resins, for example through Amberlite MB-1 monobed.

PROCEDURE A. FLUOROMETRIC DETERMINATION OF MAGNESIUM, WATER ONLY AS FINAL SOLVENT. Prepare a working solution, 2.5×10^{-4} M in *o,o′*-dihydroxyazobenzene, in the following manner: In a 1-liter volumetric flask place 100 ml. of water, 100 ml. of 2.5 M potassium chloride, 67 ml. of anhydrous, redistilled ethylenediamine (G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 231), and (not necessary if aluminum be absent in the materials to be analyzed) 50 ml. of triethanolamine (G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 373). Mix, and allow the solution to cool. Add exactly 100 ml. of the stock solution of *o,o′*-dihydroxyazobenzene (2.50×10^{-3} M). Mix, dilute to the mark with deionized water, and mix. Store in bottle of either polyethylene or borosilicate glass.

Prepare the sample in such a manner as to bring all the magnesium into solution. The solution should be relatively unbuffered and free of organic substances. For analysis take aliquots containing 5 to 25 μg. of magnesium. If the sample contains iron, add to the aliquot in rapid succession 10 to 20 mg. of sodium hydrosulfite ($Na_2S_2O_4$), 1 ml. of concentrated ammonium hydroxide and 2 ml. of 5 per cent potassium cyanide. Heat the solution and allow it to boil gently for one or two minutes. Avoid using more sodium hydrosulfite than necessary. If copper or zinc may be present add 2 ml. of 5 per cent potassium cyanide, omitting if the above treatment for iron was applied. Transfer the solution to a 50-ml. volumetric flask. Add 10.0 ml. of the working solution and dilute to exactly 50 ml. A convenient device for dispensing a reproducible volume of the working solution is shown in Figure 17.

In a similar manner, prepare a series of standards covering the range 5 to 25 μg. of magnesium.

Measure the fluorescence of the various solutions using a fluorometer as described in the paragraph below headed Measurement of Fluorescence.

PROCEDURE B. FLUOROMETRIC DETERMINATION OF MAGNESIUM, WATER-ETHANOL MIXTURE AS FINAL SOLVENT. Prepare a working solution, 7.15×10^{-5} M in *o,o′*-dihydroxyazobenzene, in the following manner: In a 1-liter volumetric flask place 100 ml. of 95 per cent ethanol and 19 ml. of anhydrous, redistilled ethylenediamine. Mix and allow to cool in an ice bath. Add slowly with stirring 15 ml. of a mixture of equal parts by volume of concentrated hydrochloric acid and water. Cool again. Add 14.3 ml. of triethanolamine and 28.6 ml. of *o,o′*-dihydroxyazobenzene stock solution (2.5×10^{-3} M). Dilute the mixture to 1 liter with 95 per cent ethanol and mix well. Store the solution in a bottle of either polyethylene or borosilicate glass.

Prepare the sample in such a manner as to bring all the magnesium into solution. The solution should be relatively unbuffered and free from organic substances. For the analysis transfer an aliquot containing 5 to 25 μg. of magnesium and not exceeding 15 ml. in volume to a 50-ml. volumetric flask. If iron is present, add to the aliquot in rapid succession, 10 to 20 mg. of sodium hydrosulfite ($Na_2S_2O_4$), 1 ml. of concentrated ammonium hydroxide, and 2 ml. of 5 per cent potassium cyanide. Heat the solution and allow it to boil gently for one to two minutes. Avoid using more sodium hydrosulfite than barely necessary to reduce the iron. Add 35.0 ml. of the *o,o′*-dihydroxyazobenzene-buffer working solution. A convenient device for dispensing a reproducible volume in routine work is shown in Figure 17. Mix the solution and then dilute to 50.0 ml. with deionized water, and again mix.

Prepare a series of magnesium standards covering the range 5 to 25 μg. of magnesium by pipetting into 50-ml. volumetric flasks various volumes of the standard magnesium solution. To each add 35.0 ml. of the working solution, mix, dilute to the mark and mix thoroughly.

Measure the fluorescence of each solution using a fluorometer as described in the paragraph below headed Measurement of Fluorescence.

PROCEDURE C. FLUOROMETRIC DETERMINATION OF MAGNESIUM IN SERUM, WATER-ETHANOL MIXTURE AS FINAL SOLVENT. Prepare a working solution, 2.5×10^{-4} M in *o,o′*-dihydroxyazobenzene, in the following manner: In a 1-liter volumetric flask place 100 ml. of 95 per cent ethanol and 67 ml. of anhydrous, redistilled ethylenediamine. Cool, and then add 53 ml. of a mixture of equal volumes of concentrated hydrochloric acid and water. Mix well. Add 50 ml. of triethanolamine and exactly 100 ml. of the *o,o′*-dihydroxyazobenzene stock solution. Dilute to one liter with 95 per cent ethanol. Mix well. Store in a bottle of either polyethylene or borosilicate glass.

Pipet an aliquot of the sample (whole serum or filtrate from the trichloroacetic

acid precipitation of protein) of such size as to contain between 0.5 and 20 μg. of magnesium in a volume not exceeding 4 ml. Pipet the aliquot into a graduated centrifuge tube of 15 ml. capacity. Add 10.0 ml. of the *o,o'*-dihydroxyazobenzene working solution. A convenient device for dispensing a reproducible volume of the working solution is shown in Figure 1. Stopper the tube with a plastic stopper and mix. If whole serum is being analyzed centrifuge to settle the precipitate which appears at this point.

In a similar manner prepare a series of standards containing 0.5 to 20 μg. of magnesium.

Measure the fluorescence of the various solutions using a fluorometer as described in the paragraph below headed Measurement of Fluorescence.

Measurement of Fluorescence. Measure the fluorescence with a fluorometer using a high sensitivity instrument (Photomultiplier tube as receiving element) if Procedure A has been followed or either a high-sensitivity or a low-sensitivity instrument (simple photovoltaic cell as receiver) if Procedures B or C have been followed. If a spectrophotofluorometer is used set the excitation monochromator at 470 mμ and the emission monochromator at 580 mμ. If a filter fluorometer is used, use Corning filters CS-5-60 and CS-3-67 or the equivalent in the excitation and fluorescent beams, respectively. Read the fluorescence intensity of each of the standards and unknowns as quickly as possible to avoid any heating of the solutions. From the data obtained on the standards, prepare a plot of fluorescence intensity versus concentration of magnesium.

Modification if Large Amounts of Calcium are Present. If the solution to be analyzed contains sufficient calcium, strontium or beryllium to influence the fluorescence add the necessary quantity of each of these elements to the standards so the composition of the standards approximates that of the sample. If samples containing widely differing amounts of calcium are to be analyzed from time to time, prepare once a family of calibration curves representing different levels of calcium concentration, Figure 20. From then on prepare only one calibration curve. Refer to the previously prepared family of curves for the correction which must be applied to the observed magnesium concentration.

Procedure for the Fluorometric Determination of Magnesium in Limestone, Dolomite, Portland Cement, and Iron Ore. Transfer a sample weighing about 100 mg. to a mortar of agate or porcelain and mix with 1 g. of a 1:1 mixture of sodium carbonate and sodium borate decahydrate. Transfer the mixture quantitatively to a platinum crucible and fuse the mixture for 10 to 15 minutes. Cool and dissolve the melt in 10 ml. of water and 5 ml. of concentrated hydrochloric acid. No dark colored residue should remain. Without separating the silica, transfer the solution to a 250-ml. volumetric flask (Procedure A) or to a 1-liter volumetric flask (Procedure B) and dilute to the mark with deionized water. Without transferring any of the silica which settles to the bottom of the flask, transfer an appropriate aliquot to a 50-ml. volumetric flask and follow either Procedure A or Procedure B given above, the first step being the treatment necessary to obviate the effects of interfering ions present. If the iron content is low, as in NBS No. 88, Dolomite, no treatment is required; if the iron is high the treatment with sodium hydrosulfite, ammonia and cyanide is necessary. Triethanolamine should be present in the working solution if aluminum is present.

PART VII

B. SPECTROPHOTOMETRIC DETERMINATION OF MAGNESIUM IN THE PRESENCE OF CALCIUM

The absorption spectra of *o,o'*-dihydroxyazobenzene and its magnesium derivative are very similar but at 485 mμ, the wave length of maximum absorption, the molar extinction coefficient of the magnesium derivative is about 50 per cent greater than that of *o,o'*-dihydroxyazobenzene itself. It proved possible to set up a differential spectrophotometric procedure for the determination of magnesium.

Absorption Spectra of o,o'-Dihydoxyazobenzene and its Magnesium Derivative. The absorption spectra of *o,o'*-dihydroxyazobenzene and its magnesium derivative at pH 10 and 0.05 M potassium chloride are shown in Figure 21.

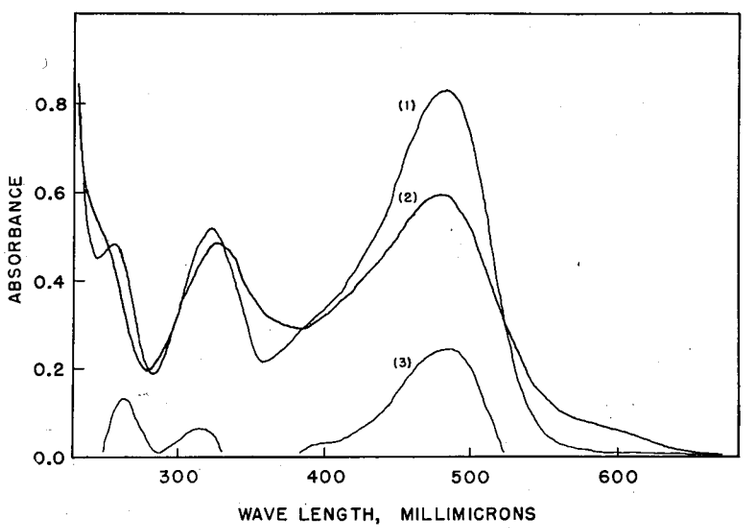


Figure 21. Absorption spectra of *o,o'*-dihydroxyazobenzene and its magnesium derivative at pH 10.2 in 0.05 M potassium chloride: (1) 5×10^{-5} M magnesium-*o,o'*-dihydroxyazobenzene, (2) 5×10^{-5} M *o,o'*-dihydroxyazobenzene, (3) 5×10^{-5} M magnesium-*o,o'*-dihydroxyazobenzene with 5×10^{-5} M *o,o'*-dihydroxyazobenzene in reference cell. 1-cm. cells. *Data of Diehl, Olsen, Spielholtz and Jensen*[63].

The molar extinction coefficients of *o,o'*-dihydroxyazobenzene and its magnesium derivative at 485 mμ, the wave length of maximum absorption, are respectively 11,700 and 16,400. The difference between these, 4,700, should be equivalent to the value which would be observed if the absorbance of the magnesium derivative were measured using an equivalent solution of the free reagent as reference. The value actually observed was 4,850. In these measurements the solution of the magnesium derivative contained an excess of magnesium to offset partial dissociation of the compound. The molar extinction coefficient based on the average slope of the spectrophotometric calibration curve was found to be about 4,000.

Necessity for the Presence of Inorganic Salt. In preliminary studies satisfactory calibration curves could be obtained only at the higher

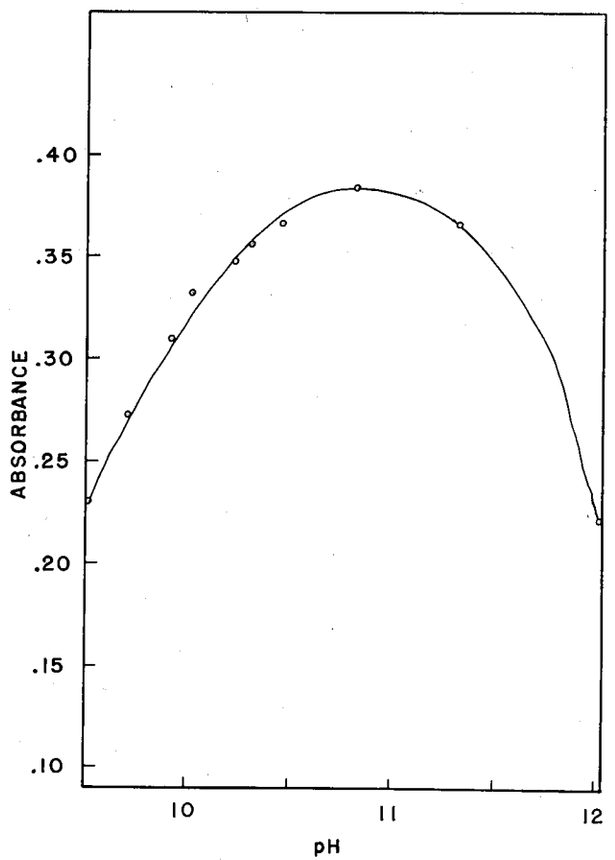


Figure 22. Effect of pH on the absorbance of magnesium-*o,o'*-dihydroxyazobenzene. *Data of Diehl, Olsen, Spielholtz and Jensen*[63].

levels of calcium concentration. Without the calcium the absorbance was not reproducible. A series of standards was then prepared, each one being made 0.1 M in potassium chloride. With the uniform inorganic salt concentration smooth calibration curves were obtained.

Effect of pH. The absorbance of magnesium-*o,o'*-dihydroxyazobenzene is highly dependent on the pH of the solution, Figure 22. It would appear desirable to operate at a pH higher than the recommended value of 10. At the higher pH, however, the influence of calcium on the absorbance is much more pronounced. Thus pH control is quite critical, but in a typical analysis of a water, the buffer provided in the working solution is amply sufficient to bring the pH directly to the desired value.

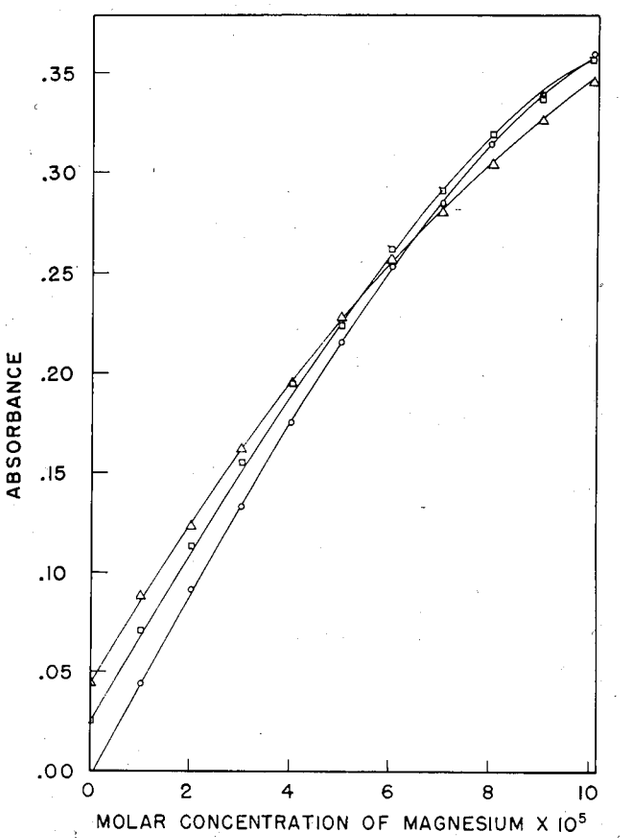


Figure 23. Calibration curves for the spectrophotometric determination of magnesium in the presence of various amounts of calcium. Concentration of calcium: ○ None, □ 2×10^{-4} M, △ 5×10^{-4} M. *Data of Diehl, Olsen, Spielholtz and Jensen*[63].

Effects of Time and Temperature. Judged by its absorbance, magnesium-*o,o′*-dihydroxyazobenzene is stable with time. The absorbance seems to be temperature dependent, however, decreasing with rising temperature by about 0.5 per cent per degree at room temperature. This is not a serious problem if care is taken to avoid warming the solution by allowing it to stand unduly in a warm fluorometer.

Effects of Foreign Ions. The effects of foreign ions are more serious in the spectrophotometric method than in the fluorometric method. Spectrophotometric calibration curves prepared at various concentrations of calcium are given in Figure 23. The effects of several ions on the determination of magnesium are summarized in Table 39. The hydrosulfite reduction of iron was not satisfactory in the spectrophotometric procedure as it introduced considerable uncertainty in the pH.

Application to Municipal Water Supplies. Several water samples were analyzed according to the procedure above and by the conventional ethylenediaminetetraacetate titration for comparison. The results are summarized in Table 40.

Because of the superior sensitivity and less critical adjustment of conditions, the fluorometric procedure would be the one of choice. The spectrophophotometric procedure can, however, be successfully applied to many situations if a fluorometer is not available. The analysis of natural or treated water as described above is one illustration. It would be expected

TABLE 39. SPECTROPHOTOMETRIC DETERMINATION OF MAGNESIUM IN THE PRESENCE OF VARIOUS INTERFERING SUBSTANCES

Data of Diehl, Olsen, Spielholtz and Jensen[63]

Magnesium Taken, μg.	Interference added	Magnesium Found, μg.	Difference, μg.
100	none	99.0	—1.0
100	none	98.2	—1.8
100	0.1 mg. Zn[a]	97.7	—2.3
100	0.1 mg. Cu[a]	98.2	—1.8
100	1.0 mg. Sr	99.0	—1.0
100	1.0 mg. Be	97.5	—2.5
100	10 mg. Ba	97.7	—2.3
100	15 mg. Li	97.5	—2.5
100	10 μg. Mn	81.5	—18.5
100	10 mg. KH_2PO_4	94.2	—5.8
100	2 μg. Mn	94.5	—5.5
80	1 mg. Fe[a]	86.0	+6.0
20	0.25 mg. Fe[a]	21.0	+1.0

[a]Treatment appropriate for the interference applied in accordance to the procedure for analysis of water.

TABLE 40. DETERMINATION OF MAGNESIUM IN VARIOUS WATER SAMPLES

Data of Diehl, Olsen, Spielholtz and Jensen[63].

Source	Magnesium Found, p.p.m. Volumetric[a]	Spectrophotometric
Iowa State University (Tap)	37.8	38.4, 38.0, 36.6, 37.9 (Av. 37.7)
Ames, Iowa, Municipal (Tap)	4.62	4.52, 4.53, 4.45, 4.48 (Av. 4.48)
Ames, Iowa, Municipal (Untreated)[b]	37.1	37.0, 37.3, 37.0, (Av. 37.1)
Duluth, Minn., Municipal (Tap)	2.86	2.73, 2.70, 2.85, (Av. 2.79)

[a]Calculated as the difference between calcium plus magnesium and calcium only.
[b]Analyzed for iron by the method of Collins, Diehl, and Smith and found to contain 6.7 p.p.m. The samples contained virtually no iron.

that either procedure could be adopted to the analysis of numerous biological materials also.

Procedure for the Spectrophotometric Determination of Magnesium. Determination of Magnesgium in Water. Method of Diehl, Olsen, Spielholtz and Jensen[63]. Prepare a stock solution of *o,o′*-dihydroxyazobenzene and a standard magnesium solution as described under the procedures for the fluorometric method, page 102. Prepare a working solution of *o,o′*-dihydroxyazobenzene and buffer in the following manner. To a 1-liter volumetric flask transfer in sequence about 100 ml. of water, 100 ml. of 2.5 M potassium chloride and 67 ml. of ethylenediamine (G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 231). Cool the mixture. Add 82 ml. of 1:1 hydrochloric acid, mix, and cool the solution to room temperature. Add exactly 100 ml. of the stock solution of *o,o′*-dihydroxyazobenzene, mix, dilute to 1 liter, and mix thoroughly. Store this working solution in a glass bottle but not in polyethylene.

The water to be analyzed should be free from turbidity and suspended matter. To each liter add about 0.5 ml. of concentrated hydrochloric acid and, if the water may contain partially oxidized iron, 1 g. of solid hydroxylammonium chloride. Store the sample in a polyethylene bottle leaving no air space.

Prepare a series of standards by pipeting into 50-ml. volumetric flasks 0.0, 2.0, 4.0, 6.0, 8.0 and 10.0 ml. of a 5×10^{-4} M magnesium solution and an appropriate quantity of 0.01 M calcium chloride (if the samples to be analyzed contain calcium equal to or more than the magnesium present). Add exactly 20 ml. of the *o,o′*-dihydroxyazobenzene working solution. A convenient device for dispensing this solution is shown in Figure 17. Dilute the standards to volume and mix well. Treat appropriate aliquots of the water sample to be analyzed in the same manner. If the sample contains iron or copper transfer 2 ml. of 5 per cent potassium cyanide to the 50 ml. flask before the sample is added. If aluminum is present, add 0.5 ml. of pure triethanolamine to the 50 ml. flask after adding the sample of water. Read the absorbance of the standards and sample at 485 mμ using 1-cm. cells. Set the transmittance at 100 per cent using the solution containing no magnesium. From the standards prepare a calibration curve. This will not be a straight line but rather slightly concave toward the concentration axis. If several samples containing widely differing amounts of calcium are to be run from time to time, prepare once a family of calibration curves at different levels of calcium con-

centration. From then on when analyses are to be made prepare only a calibration curve from standards containing no calcium. Refer to the family of calibration curves for an estimate of the correction which must be applied to the observed magnesium concentration.

PART VIII

PRIMARY STANDARD MATERIALS FOR EDTA WORK

EDTA solutions for the determination of calcium and magnesium have been standardized most commonly by titration of calcium chloride derived from calcium carbonate. EDTA solutions can be made up by weight using primary standard disodium dihydrogen ethylenediaminetetraacetate dihydrate but the problem is complicated if Eriochrome Black T or Calmagite is to be used as indicator for the titration of calcium in that magnesium must be present to assist in the proper functioning of the indicator. Magnesium is sometimes added deliberately to the standard EDTA solution for this purpose. Using magnesium iodate tetrahydrate, a primary standard described below, the magnesium for the indicator is provided automatically.

Calcium Carbonate. MOLECULAR WEIGHT: 100.09. Some care must be exercised to make certain that the calcium carbonate is truly primary standard material, low in alkali metals and in magnesium. Such calcium carbonate must be specially prepared. The naturally occurring variety of calcium carbonate known as Iceland spar occurs as transparent crystals and is often assumed to be highly pure and has been frequently used as a primary standard. The amount of other metals which may be present in such material can be quite high and Iceland spar cannot be used safely without adequate preliminary testing.

A satisfactory primary standard grade of calcium carbonate is available from the G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 337.

Disodium Dihydrogen Ethylenediaminetetraacetate Dihydrate (EDTA). MOLECULAR WEIGHT: 372.26. Commercial disodium dihydrogen ethylenediaminetetraacetate dihydrate ranges in purity from 98 to 100 per cent but is usually satisfactory for making up EDTA solutions for later standardization against a primary standard material. A primary standard grade of disodium dihydrogen ethylenediaminetetraacetate dihydrate, however, is fairly readily prepared and useful for making up standard EDTA solutions by weight. This was demonstrated by Blaedel and Knight [64] who

For full references see Bibliography, page 123.

[64] *Anal. Chem.*, **26**, 741 (1954).

devised a method for its recrystallization, established the conditions under which both the dihydrate and the anhydrous material can be brought to stoichiometric composition and checked the product against other primary standards. The salt has a fair temperature coefficient of solubility in water but is most easily obtained by precipitation from water by the addition of alcohol.

TABLE 41. SOLUBILITY OF DISODIUM DIHYDROGEN ETHYLENEDIAMINETETRAACETATE DIHYDRATE AT VARIOUS TEMPERATURES

Data of Blaedel and Knight[64]

Temperature °C	$Na_2H_2Y \cdot 2H_2O$ g. per 100 g. of Solution	Density of Saturated Solution g. per ml.
98.0	27.0	1.09
90.0	24.3	1.07
80.0	22.2	1.06
70.0	20.0	1.06
60.0	17.0	1.05
50.0	15.5	1.05
40.0	14.2	1.09
30.0	12.8	1.08
21.0	11.1	1.08
0.5	10.6	1.07

Two preparations of the dihydrate made this way were found by Blaedel and Knight to have a purity of 100.03 and 100.05 per cent, and two preparations of the anhydrous material had a purity of 99.99 per cent. The purity of these preparations was determined[65] by titrating solutions of cupric chloride, zinc chloride, and calcium chloride prepared from metallic copper, zinc oxide and calcium carbonate, respectively, considered as primary standard materials. The end-point was determined by the high frequency conductance technique. Beside providing proof that the materials were of primary standard quality, the work provided evidence that the reaction of EDTA with these metal ions is stoichiometric.

Preparation of Primary Standard Disodium Dihydrogen Ethylenediaminetetraacetate Dihydrate. Method of Blaedel and Knight[64]. Prepare a nearly saturated aqueous solution of the impure salt at room temperature (about 10 g. of the dihydrate per 100 ml. of water). Add alcohol slowly until a permanent precipitate appears. Filter off and discard this precipitate. Add an equal volume of alcohol. Filter the resulting precipitate, wash it with acetone and then with ether, and air dry it at room temperature overnight. Yield about 75 per cent. This product contains excess water. To obtain the dihydrate, dry the material further at 80°, taking about 4 days in air of a relative humidity of 50 per cent. To obtain the anhydrous salt, heat the

[65] *Anal. Chem.*, **26**, 743 (1954).

material in a vacuum oven at 120° for 24 hours. Store the anhydrous material over anhydrous magnesium perchlorate.

Magnesium Iodate Tetrahydrate. MOLECULAR WEIGHT: 446.20. Magnesium iodate tetrahydrate was proposed as a primary standard by Lindstrom and Stephens[66]. This salt is completely stable between 4° and 50°, even when stored over anhydrous magnesium perchlorate or in an atmosphere of 98+ per cent humidity. The molecular weight is so high that a 0.2-g. sample may be weighed out for standardization of 0.01 M EDTA using a 50-ml. buret. It provides directly the magnesium needed if Calmagite or Eriochrome Black T is used as indicator.

The salt was prepared by Lindstrom and Stephens[66] from magnesium carbonate and a slight excess of iodic acid and crystallized first as the anhydrous salt from water at 75°. It was then recrystallized twice from water at 55° as the tetrahydrate (below 13.3° a decahydrate forms and above 57.5° the anhydrous salt forms). The final material was simply air dried. Two recrystallizations appeared to be enough.

In a thorough study of the material, Lindstrom and Stephens established the composition of the material by analysis, tested for insoluble matter, neutrality, the presence of heavy metals, nitrogen compounds, iodide, and traces of alkali and alkaline earth metals. They then made several studies of the stability of the material, discovering the very surprising fact that the tetrahydrate is stable when stored over anhydrous magnesium perchlorate. A thermogravimetric analysis showed that the tetrahydrate was stable at temperatures up to 110°.

Comparison of the magnesium iodate tetrahydrate against calcium carbonate and metallic zinc was carried out by EDTA titration. The major amount of the EDTA was added from a weight buret and a much more dilute EDTA solution added from a volumetric buret in the neighborhood of the end-point. Eriochrome Black T and Calmagite were both used as indicator and the end-point found by a photometric titration. A theoretical treatment of the end-point conditions was made and from this was derived a correction for converting the observed end-point (from extrapolation of photometric data) to the theoretical equivalence-point. Corrections to weight in vacuum were applied. The results are shown in Table 42. The average of the concentrations obtained from magnesium iodate tetrahydrate and calcium carbonate was high by 9.2 parts per 10,000 compared to zinc on the equivalence-point basis and was low by 12.0 parts per 10,000 on the photometric end-point basis.

The close agreement of the values for EDTA concentration obtained by the magnesium iodate tetrahydrate standardization and the calcium carbonate standardization show that magnesium iodate is excellent for precise

[66] *Anal. Chem.*, **34**, 993 (1962).

TABLE 42. CONCENTRATION OF EDTA SOLUTION BY STANDARDIZATION AGAINST MAGNESIUM IODATE TETRAHYDRATE, CALCIUM CARBONATE, AND ZINC METAL

Data of Lindstrom and Stephens[66]

Primary Standard Taken		Weight of EDTA Solution	Concentration of EDTA Solution
Solid, g.	Solution, g.	g.	moles per 1000 g.[a]
Magnesium Iodate Tetrahydrate			
0.10181		20.998	0.010866
0.11194		23.082	0.010869
0.10310		21.291	0.010852
0.11145		22.993	0.010863
0.12116		24.989	0.010866
0.10383		21.430	0.010859
0.11483		23.691	0.010863
0.10133		20.887	0.010873
		Average	0.010864
			(0.010887)[b]
		σ in parts per thousand	0.59
Calcium Carbonate			
0.18442	1.04862	17.782	0.010875
0.18442	1.17972	20.024	0.010865
0.18442	1.08197	18.353	0.010872
0.22023	0.79367	16.103	0.010854
0.22023	0.78222	15.879	0.010849
0.22023	1.44532	29.315	0.010858
		Average	0.010862
			(0.010885)[b]
		σ in parts per thousand	0.95
Zinc Metal			
0.14142	1.07898	14.034	0.010873
0.14142	1.27976	16.642	0.010875
0.14142	1.29677	16.858	0.010878
0.14142	1.40739	18.303	0.010874
0.15484	0.73723	10.494	0.010878
0.15484	1.74113	24.767	0.010881
		Average	0.010876
			(0.010876)[b]
		σ in parts per thousand	0.28

[a]Concentration as moles per 1000 grams of solution.
[b]Corrected to theoretical equivalence-point.

work. The extraordinary stability of the compound toward air, its ready dissolution in water, and its high molecular weight make it an ideal primary standard.

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APPENDIX A

MOLECULAR WEIGHTS OF IMPORTANCE IN CALCIUM AND MAGNESIUM CHEMISTRY

Atomic Weights		Molecular Weights	
Beryllium	9.013	MgO	40.32
Magnesium	24.32	$MgCO_3$	84.33
Calcium	40.08	CaO	56.08
Strontium	87.63	$CaCO_3$	100.9
Barium	137.36	CO_2	44.01
		$CaMg(CO_3)_2$	184.42

Ratios

$$\frac{CaO}{MgO} = 1.3909 \qquad \frac{MgO}{MgCO_3} = 0.4781$$

$$\frac{CaCO_3}{CO_2} = 2.2742 \qquad \frac{CaO}{CaMg(CO_3)_2} = 0.3041$$

$$\frac{CO_2}{CaCO_3} = 0.4397 \qquad \frac{MgO}{CaMg(CO_3)_2} = 0.2186$$

$$\frac{CaO}{CaCO_3} = 0.5603 \qquad \frac{2CO_2}{CaMg(CO_3)_2} = 0.4773$$