1985

Preparation, properties and applications of Calcein in a highly pure form

John Walter Furry
Iowa State University

Furry, John Walter

PREPARATION, PROPERTIES AND APPLICATIONS OF CALCEIN IN A HIGHLY PURE FORM

Iowa State University PH.D. 1985

PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark __✓__.

1. Glossy photographs or pages _____
2. Colored illustrations, paper or print _____
3. Photographs with dark background _____
4. Illustrations are poor copy _____
5. Pages with black marks, not original copy _____
6. Print shows through as there is text on both sides of page _____
7. Indistinct, broken or small print on several pages ✓
8. Print exceeds margin requirements _____
9. Tightly bound copy with print lost in spine _____
10. Computer printout pages with indistinct print _____
11. Page(s) _____ lacking when material received, and not available from school or author.
12. Page(s) _____ seem to be missing in numbering only as text follows.
13. Two pages numbered _____. Text follows.
14. Curling and wrinkled pages _____
15. Dissertation contains pages with print at a slant, filmed as received _____
16. Other _____

_____

_____

University
Microfilms
International

Preparation, properties and applications of Calcein in a highly pure form

by

John Walter Furry

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Department: Chemistry
Major: Analytical Chemistry

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa
1985

TABLE OF CONTENTS

I. INTRODUCTION

In 1956, Diehl and Ellingboe[113,127] presented the first compound of a new class of chemical reagents. This new material, which they named *Calcein*, exhibited a brilliant yellow-green fluorescence in the presence of certain metal ions, most notably calcium. The usefulness of this behavior in analytical chemistry was quickly proven and Calcein was the first in the class of so-called *metallofluorochromic reagents*. Since then, the breadth and sophistication of the applications of Calcein have continued to grow, accompanied by ever stronger and more stringent requirements for purity and consistency.

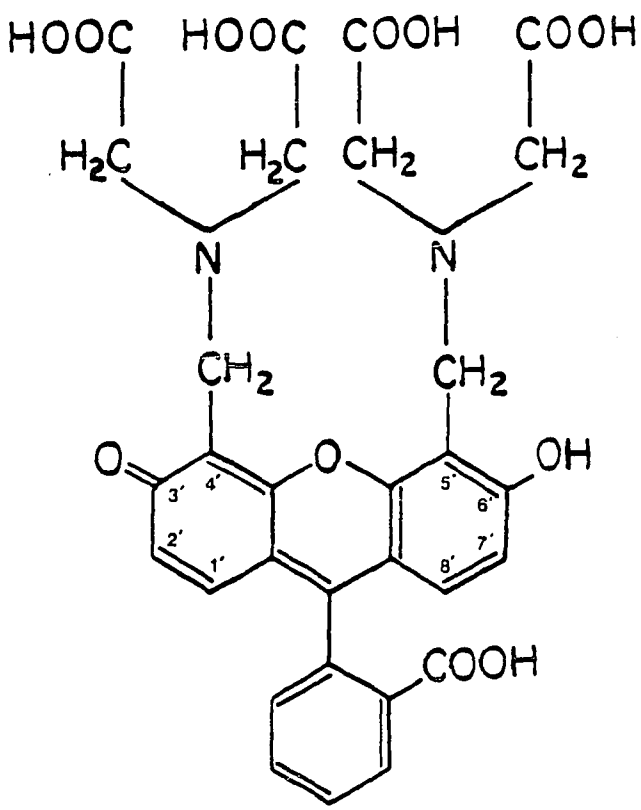
Calcein is synthesized by the Mannich condensation[246] of fluorescein, formaldehyde and iminodiacetic acid. The product is now known to be 4',5'-bis(N,N'-di-(carboxymethyl)aminomethyl)fluorescein. Calcein has two

methyleneiminodiacetic acid groups attached to the xanthene portion of fluorescein. Each of these groups is equivalent to one half of an ethylenediaminetetraacetic acid (EDTA) molecule. The formal structure of Calcein is shown in figure 1.

The unique feature of Calcein is that it unites, in one molecule, the ability to form slightly-dissociated compounds with metals comparable to those of EDTA and an intense fluorescence equivalent to that of the organic dye fluorescein, in such a way that the formation and dissociation of the slightly-dissociated compounds directly and significantly affect the fluorescence of the molecule. The intensity of fluorescence of aqueous solutions of fluorescein depends on the concentration of hydrogen ions (usually described in terms of the negative logarithm of concentration, *pH*). Neutral and alkaline solutions of fluorescein (pH greater than 6) are highly fluorescent while acidic solutions are significantly less so. For Calcein, the intensity of fluorescence is also dependent on the presence of certain metal ions. In metal-free solutions, Calcein is fluorescent between pH 5.5 and pH 8.5. The fluorescence of Calcein decreases as the pH of solution is increased above pH 9 and is

Figure 1.

Structure I. Formal structure of Calcein



STRUCTURE I

virtually extinguished above pH 12. However, in highly alkaline solutions containing certain metals, particularly the alkaline earths, Calcein forms slightly-dissociated compounds with these metals and the fluorescence is retained even at high pH. Conversely, Calcein combines with other metals in neutral solutions and the fluorescence otherwise displayed by Calcein is quenched.

As first demonstrated in the initial publication by Diehl and Ellingboe [113], Calcein serves as an excellent indicator in the titration of calcium with EDTA. The titration is performed in an alkaline solution where the indicator, Calcein, combines with calcium to form a highly fluorescent, slightly-dissociated compound. As the equivalence-point of the titration is approached, the EDTA added as titrant combines first with the free calcium in solution and then, because EDTA forms a stronger compound with calcium than does Calcein, the EDTA extracts the calcium from the fluorescent slightly-dissociated calcium-Calcein compound. The end-point of the titration is marked by the disappearance of fluorescence resulting from the dissociation of the metal-indicator compound.

The most significant advantage of this titrimetric determination is the ability to determine calcium directly without prior separation from magnesium. Unlike the indicators previously proposed for the titrimetric determination of calcium[29,52,54,60,338], Calcein performs successfully in solutions at pH 13. At this pH, magnesium is tied up as hydroxides and is therefore not susceptible to combination with the titrant or indicator. Prior to the advent of Calcein, accurate determination of calcium in samples containing magnesium required the tedious separation of the two metals by chromatography[80,164] or precipitation, filtration, and redissolution in acid[4,7,50,114,144,163].

As might have been expected, such a time-saving improvement did not go unnoticed. Soon after the initial publication, several papers[69,213,369] were published by other groups professing their own metallofluorochromic indicators prepared from the condensation of fluorescein, formaldehyde and iminodiacetic acid. Diehl and Ellingboe[113] noted that their material was an impure mixture and refrained from postulating a structure for Calcein without having a purified material.

Unfortunately, other groups were less cautious and published inadequately substantiated claims of purity, properties and structures for products to which they attached names of their own choosing. Most of these claims have now been refuted by more careful work[223,229,371] but the confusion that resulted from this plethora of names, properties and incorrect structures still remains in the literature[69,212,362,369]. While all of these materials functioned more or less adequately as metallofluorochromic indicators, other, more demanding applications yielded erratic and non-reproducible responses as a result of the impurity and inconsistancy of available supplies of Calcein[173,266,229,341,347].

Despite the deficiencies of privately and commercially prepared products, the interest in Calcein has remained strong. The current bibliography on Calcein numbers over three hundred papers, most pertaining to applications of Calcein in analytical chemistry or biological investigations. A few of these papers contain some fundamental work but, unfortunately, it is frequently of questionable quality.

Concurrently with the development of these

applications, fundamental work has continued at Iowa State University on the purification and structure, as well as the physical and chemical properties of Calcein. The slow but methodical progress of these investigations is reported in the Ph.D. dissertations of Hefley[166], Martin[253] and Markuszewski[247]; in the M.S., thesis of Freytag[139]; and in unpublished research by Brayton[70]. Publication of this work was deliberately delayed until the uncertainty in the structure and fundamental nature of the parent compound, fluorescein, was resolved. Much of this investigation has now been successfully completed and information critical to understanding the nature of fluorescein and Calcein has been disclosed in a succession of papers beginning with the 1980 publication of Markuszewski and Diehl[249] on the nature of the three solid forms of fluorescein and continuing with the series of some seven papers entitled "Studies on Fluorescein" by Diehl, Markuszewski, Hefley, Freytag, Horchak and others[107-112,115] now in press.

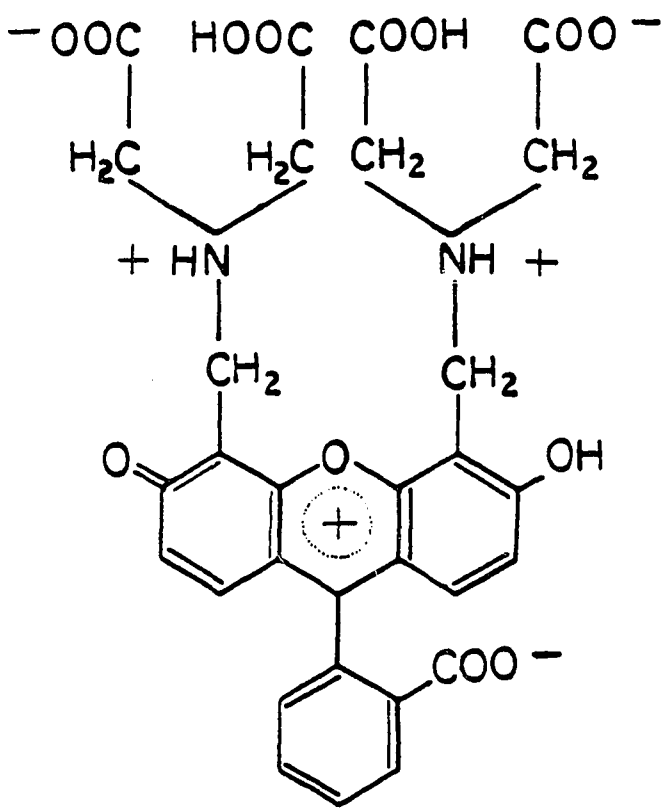
As a result of this new work on fluorescein and the determination of the formal structure of Calcein by Hefley[166] and Martin[253], the structure of Calcein in aqueous solution is now known to be best represented as a

triple zwitterion as shown in figure 2. As in EDTA[319,320], each of the methyleneiminodiacetic acid groups exists as a zwitterion as a result of an intramolecular neutralization in which the hydrogen ion from one of the carboxylic acid groups becomes more closely associated with the tertiary nitrogen atom[321,322]. These two groups are attached at the 4' and 5' positions of the fluorescein molecule which itself exists as a zwitterion[160,249,383]. Seven acidic functional groups are thus arranged around the periphery of the molecule and the charge on each, whether undissociated or negatively charged, is determined by (1) the relative acid-base strength of the group, (2) the prevailing pH of solution and (3) the involvement of the group in the chelation of metals.

Arriving at Structure II (figure 2) and all that is implicit in that structure has taken most of three decades. The present dissertation is an extension and, in a very real sense, the culmination of the work at Iowa State University on Calcein. Before summarizing the investigations reported in this work, a review of the literature is presented to provide the reader with a grasp of the importance that Calcein has assumed in

Figure 2.

Structure II. The triple zwitterion structure of Calcein in aqueous solution



STRUCTURE II

applied chemistry even though a firm knowledge of its basic chemistry has not been available.

The initial application of Calcein[113,349] was as an indicator in the titration of calcium with EDTA. More than forty publications have now appeared adapting this procedure to the determination of calcium in specific materials. Prominent examples include the titrimetric determination of calcium in water[68,285,325], pesticides[20], serum[8,19,59], ores[66,376], geological materials[375,330,381], foodstuffs[36,317,328], agricultural products[143], and biological materials[170,265,346].

It was obvious that the direct fluorometric determination of calcium would be quicker than the chelatometric titration and would probably provide sufficient accuracy for most purposes. Several applications have been reported for the direct fluorometric determination of calcium in blood[48,150,201], urine[83,176,300], muscle tissue[361], fruits and vegetables[88,93-96,314], soil[175], and minerals[245,281]. Of these, by far the most attention has been focused on the fluorometric determination of calcium in blood, especially in the development of reliable, routine,

automated clinical methods[174,201,292] and special microtechniques for testing very small samples from newborns and infants[19,48,82,334]. One of the most active areas of current interest is the use of Calcein or derivatives of Calcein for the development of *in vivo* probes for the direct fluorometric determination of calcium in the human bloodstream[232,298].

Applications of Calcein for the determination of metals other than calcium fall into two classes. The first group concerns metals which form fluorescent, slightly-dissociated compounds with Calcein in moderately acidic (pH 3 to 5) or alkaline (pH greater than 9) solutions in which Calcein by itself does not fluoresce. This class includes the determinations of aluminum[120,171] and zinc[361] in acidic solutions, and the determinations of cadmium[167,288], mercury[247], magnesium[357], strontium and barium[3,227] in alkaline solutions. The second group applies to metals which react to form non-fluorescent compounds with Calcein under conditions (pH 5.5 to 8.5) in which Calcein itself does fluoresce. Such quenching of fluorescence has been observed for many of the transistion metals, with specific applications for

iron[367], nickel[75,102], cobalt[357], copper[58,374] and many others[370].

Another class of applications in analytical chemistry involves the indirect determinations of anions. These analyses involve the titrimetric or fluorometric determination of metal ions present in excess of the stoichiometric amount consumed by the formation of a precipitate with the analyte. Such methods have been published for sulfate[124], sulfite[37], halides[479] and pseudohalides[38,311].

Still other applications in analytical chemistry utilize Calcein as an auxiliary reagent. Examples include the use of Calcein as a color-developing agent in paper chromatography[282], thin-layer chromatography[2,122,189] and liquid chromatography[364]; as an adsorption indicator[344]; as an indicating dye for testing dialysis membranes[149,179]; and as an indicator for the detection of organosulfur compounds[53,138].

Among the non-analytical applications of Calcein are some surprising, almost exotic uses. Calcein is utilized as a labeling agent for mineralized tissues including

bone [280,257,299,309,339], teeth [255,303,335], calcium deposits [192,352], including gall stones [315], and calcified tissues [197]; as a radiochemical tracer with technicium [312], plutonium [336], and Ca-45 [294,307,379]; as an optical alignment marker for contact lenses [177]; as a fluorescent diluent for determining the interior volume of vesicles [199,200,279]; and as a fixed dye [81]. Too numerous to be specifically mentioned in this listing are more than 100 publications in the medical and biological fields in which a wide range of uses are made of the fluorescence and chelating properties of Calcein.

Comprehensive reviews of the literature have been assembled by Diehl in 1964 [104] and in 1967 [105] and, more recently, by Markuszewski in 1976 [247].

While the large number of publications indicates continued widespread interest in the use of Calcein, it also reflects the lack of definitive procedures and the many problems encountered in the use of currently available materials. In fact, many of the papers are specifically directed toward adapting procedures to compensate for the impurity and inconsistency of the Calcein used. For example, many publications prescribe

mixing Calcein with other indicators or dyes to mask unwanted residual fluorescence[11,230,349] or to change the apparent color at the end-point[132,358]. Others suggest modifying the composition of the fluorometric reagent itself in order to alter the position of the end-point so as to represent a different portion of the chemical reaction[22,253]. Many of the publications are just repetitive or argumentative discussions of procedures which apparently give non-reproducible results for different workers (for example, references 28,49,195, 273,351 and many others).

A careful study of the literature on Calcein reveals a consistent pattern of frustration on the part of many workers resulting from the following specific problems noted with both privately and commercially prepared samples of Calcein: inconsistent behavior[85], instability of the reagent[182,304], non-linear calibration curves[28,329], interferences[35,165], indications of multiple components in the reagent[198,201], severe lot-to-lot variation[28], background (residual) fluorescence[349], and difficulty in identifying end-points[35,157]. Each of these problems can be attributed either to an improper understanding of the

nature of Calcein and the metal-Calcein compounds, or to the use of impure and unstable reagents.

The lack of a clear understanding of the nature of Calcein has now been alleviated by the extensive work at Iowa State University on the properties and structure of Calcein and the slightly-dissociated compounds of Calcein with different metals. The graduate works of Hefley[184], Martin[253] and Markuszewski[247] have ferreted out the nature of the impurities, the exact reaction ratios with several metals, the values for the formation and acid-dissociation constants, and the prototropic and mesomeric equilibria of the reagent itself.

The final step in bringing the work begun thirty years ago to rightful fruition is the reproducible synthesis of a highly pure compound and the development of methods for storing and using the reagent without deterioration. The objectives of the present work, therefore, are to establish procedures for the preparation and handling of Calcein and to demonstrate the dependable and reproducible performance of the highly pure material in various applications.

In Chapters II, III and IV, detailed examinations of the fluorometric properties of fluorescein, Calcein and the slightly-dissociated compounds of Calcein with various metals are described. The excitation and emission of fluorescence are evaluated as functions of pH. The fluorescent properties of each of the ionic forms of fluorescein and Calcein are described. The relative coefficients of fluorescence are tabulated for each of these ionic forms as well as for the various slightly-dissociated compounds of Calcein with the alkaline earths, aluminum, and certain transition metals.

In Chapter V, the challenging task of analyzing various mixtures containing fluorescein and Calcein is addressed. Fluorometric and spectrophotometric properties are utilized to determine each compound in independent solutions and in mixtures. Limitations in the relative and absolute concentrations of fluorescein and Calcein are defined for these determinations. The effect on these determinations of other common impurities in Calcein is also reported. Specific analytical procedures are established to be used in the careful scrutiny of the preparation, storage, and application of Calcein as described in subsequent chapters.

The work described in Chapter VI examines the various procedures to synthesize, store and dispense Calcein. Data on the yield and purity of Calcein are evaluated in terms of the purity of the starting materials, the relative concentrations and the order of addition of reactants, the choice of solvents, the pH of the solution, the temperature, and the length of reaction. The solubility profiles of fluorescein and Calcein are described for water and solutions containing various amounts of other solvents as functions of pH. Based upon this information, the most effective method for the precipitation and purification of the product is proposed. The results of several alternate methods of purification are evaluated and the effectiveness of various procedures for drying the final product are compared. In addition to describing conditions that optimize both yield and purity, a unique two-phase system for continuous synthesis is disclosed. The stability of solid forms and various solutions of Calcein are documented. Conditions are given to permit long-term storage of the solid material. Practical lifetimes for dilute solutions under strict conditions, without significant deterioration of the composition or

properties of Calcein, are also reported.

Finally, the purified material was put to the test. In Chapter VII, procedures are described which were developed to use the highly pure product for the direct and indirect determination of many species. This chapter describes the accurate and reproducible determination of metals, by direct and indirect chelatometric titrations, and by direct fluorometric analysis. Of most significance is the improved procedure developed for the automated fluorometric determination of calcium in biological and geological samples. Success is also noted in the dramatic improvements this highly pure material provides for biochemical determinations which were previously impossible using Calcein from any other source [244,291].

With the improvements described in Chapter VII, the objective of this work - to establish procedures for the preparation and handling of a pure form of Calcein for use in various applications with greater dependability and reproduciblity of performance - has been successfully fulfilled.

II. FLUOROMETRIC PROPERTIES OF FLUORESCEIN

A. Introduction.

Fluorescein and Derivatives of Fluorescein

Fluorescein was first synthesized from resorcinol and phthalic anhydride by Adolf Baeyer over a century ago[23,24]. The product was named for the intense green fluorescence that the compound displayed in alkaline solutions[25]. The material quickly caught the attention of other prominent organic chemists, including Emil Fischer[136], H. von Liebig[233-238], and Richard Meyer[260-263], each of whom spent a great deal of time examining fluorescein and derivatives of fluorescein to explain the properties and determine the structure of this fascinating compound. Meyer[264] was the first to deduce a formal structure for fluorescein. He proved that the two hydroxyl groups retained in the final product were located at the 3' and 6' positions on the xanthene portion of fluorescein as shown in figure 3. Another early and influential contribution was the study of simple derivatives of fluorescein by Holmes [178].

Figure 3.

The structures of the
three solid forms of fluorescein

a. Structure III.

The colorless (lactone) form
of solid fluorescein

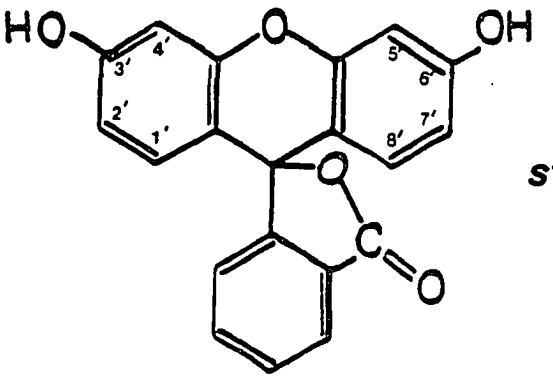
b. Structure IV.

The red (quinone) form
of solid fluorescein

c. Structure V.

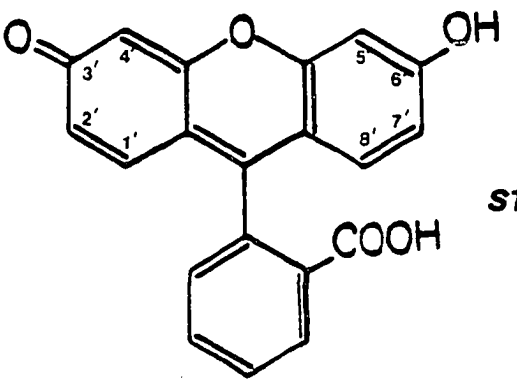
The yellow (zwitterion) form
of solid fluorescein

a.



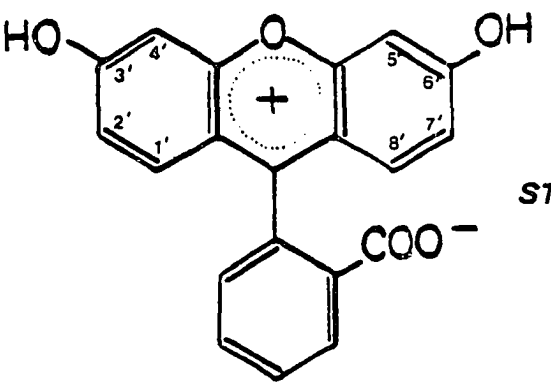
STRUCTURE III

b.



STRUCTURE IV

c.



STRUCTURE V

This study demonstrated that the incorporation of different substituents into the fluorescein molecule can be used to modify the absorption and fluorescence for specific practical uses. The utility and intriguing nature of fluorescein and derivatives of fluorescein have already resulted in well over a thousand publications.

Despite the widespread interest and applications of these materials, the actual structures of fluorescein in the different solid and dissolved forms have only recently been elucidated. The structures of fluorescein shown in Structures III through V (figure 3) have now been rigorously established by Diehl [107,108] and Markuszewski [247] and identified with the colorless, red and yellow forms of solid fluorescein, respectively. They also correspond to the predominant electronic structures of fluorescein in different solutions.

Fluorescein and simple derivatives of fluorescein are widely used in commercial products such as cosmetics, dyes, and food coloring [121]. In scientific work, these compounds are frequently used as

fluorescent tracers[143], as adsorption indicators[208] and in tunable dye lasers[247]. Each of these applications makes use of the intense fluorescence or the strong ultra-violet and visible absorption associated with these compounds.

The same characteristic absorbance and intense fluorescence have also been widely used as the basis for the determination of fluorescein in simple, dilute aqueous solutions. However, in situations in which more than one derivative of fluorescein is present, such as in dyes and food colorings, differentiation is very difficult and generally not attempted.

Calcein is a derivative of fluorescein with methyleneiminodiacetic acid groups symmetrically attached at the 4' and 5' positions of the xanthene portion of the molecule. As with other derivatives, the absorbance and fluorescence of Calcein closely resemble those of fluorescein. If these two compounds are to be differentiated and accurately determined in the various mixtures encountered in the preparation and applications of Calcein, then a clear and precise understanding of the fluorometric properties of each

compound must be firmly in hand.

In the past, the fluorescence observed in solutions of fluorescein has always been attributed to anionic forms of fluorescein - more specifically, to the dianion. The work described in this chapter establishes for the first time the specific fluorometric properties of each of the ionic forms of fluorescein, including significant fluorescence on the part of both the monoanion and cation of fluorescein.

B. Apparatus and Reagents

Excitation and emission spectra of all solutions were obtained with an Aminco-Bowman Spectrophotofluorometer and were recorded on an Aminco-Bowman X-Y Recorder. The spectra were not corrected for changes in the intensity of emission of the xenon lamp with wavelength. The slit width was set to 1 mm. for all measurements and the corresponding resolution of the instrument was approximately 2 nm.

In addition, the fluorescence of many solutions was measured with a Turner Model 110 Filter Fluorometer. A blue Corning 5850 glass filter was used as the primary filter and the secondary filter consisted of the combination of a yellow Corning 2A-15 glass filter and a Wrattan 10 percent neutral density filter.

The pH of each solution was measured with a Beckman Zeromatic pH Meter, Model SS-2, equipped with a Beckman saturated calomel reference electrode and a Beckman glass indicator electrode. The pH meter was standardized using buffers of potassium acid phthalate, potassium dihydrogen phosphate and sodium borate.

Three lots of fluorescein of differing quality were used for this study. The first material was obtained from a sample crude fluorescein provided by Dennis Martin[254]. The second lot was obtained as a refined material from Hach Chemical Company of Ames, Iowa. In cooperation with Samir Gharfeh[147,148], an even purer grade of material was obtained by converting the fluorescein to the diacetyl derivative, repeatedly recrystallizing from alcohol and then hydrolyzing the diacetate back to fluorescein. All work performed with the crude material was checked by repetition of the procedure with one of the refined products.

Solutions of fluorescein with pH between 3 and 10 were always freshly prepared from solid material within two days of any measurement. Solutions more acidic than pH 3 or more basic than pH 10.5 were prepared fresh on the same day as the measurements. In addition, solutions used for the measurement of fluorescence as a function of the wavelength of excitation were stored in the dark until used.

Fresh solutions of acid-base buffers were prepared with a constant ionic strength of 0.10 for the entire

range of pH from 1.00 to 10.50 in increments of 0.3 units or less. These buffers were prepared from hydrochloric acid, potassium acid phthalate, potassium dihydrogen phosphate, boric acid, potassium hydroxide and potassium chloride according to the procedure of Clarks and Lubs as summarized by Diehl [112]. All buffers older than 15 days were replaced before use.

Potassium hydroxide, free of calcium and other metals which might have potentially interfered with measurements of fluorescence, was obtained from the Hach Chemical Company in the form of a 12 N solution.

Solutions of fluorescein with pH between 3 and 10 were always freshly prepared from solid material within two days of any measurement. Solutions more acidic than pH 3 or more basic than pH 10.5 were prepared fresh on the same day as the measurements. In addition, solutions used for the measurement of fluorescence as a function of the wavelength of excitation were stored in the dark until used.

C. Experimental Work

1. Measurement of the excitation and emission of fluorescence at pH 13

Stock solutions of fluorescein were prepared by dissolving approximately 3.2 g of various lots of fluorescein and 5.6 g of potassium hydroxide in 1.00-liter volumetric flasks, diluting to volume and then diluting 5.00-ml. aliquots of these solutions to 1.00 liters. A 10.00-ml. aliquot from each of these solutions was then diluted to 100 ml. with 0.12 N potassium hydroxide. The final concentration of fluorescein in each case was approximately 5×10^{-6} M and the pH was slightly greater than 13.

The excitation spectrum was obtained from 200 to 600 nm. with the wavelength for emission fixed at 510 nm. The procedure was then repeated with the wavelength of emission at 505, 515, and 520 nm. Emission spectra were obtained between 200 and 600 nm. with the wavelength of excitation fixed at 280, 290, 320, 435, 462, 470, and 485 nm. The sensitivity of detection was varied to maximize the signal recorded under each set of conditions.

2. Measurement of fluorescence as a function of pH

Stock solutions of fluorescein were prepared by dissolving 0.17 g of purified fluorescein in approximately 75 ml. of 0.01 N potassium hydroxide and diluting to 1.00 liters. An aliquot of 1.00 ml. of the stock solution was then added to each of fifteen 100-ml. volumetric flasks. Each flask was then filled to volume with one of the acid-base buffers. Similar solutions were also prepared in 0.03, 0.10, 0.3, and 1.0 N hydrochloric acid and in 0.03 and 0.1 N potassium hydroxide. Two additional solutions with a pH of 0.0 were prepared similarly with perchloric and nitric acids. Where possible, the final ionic strength of each solution was adjusted to 0.1 with an appropriate addition of 1.0 M potassium chloride. The final concentration of fluorescein in each solution was 5×10^{-6} M. The pH of each solution prepared between pH 2 and pH 10.5 was carefully measured with a freshly calibrated pH meter.

These solutions were then examined to determine the general appearance of the excitation and emission spectra and to identify the locations of maximum intensities as

functions of pH. Emission spectra of each solution were obtained with the wavelength of excitation at 435, 470 and 490 nm. The excitation spectrum of each solution was then acquired with the wavelength of emission set at the wavelength of maximum intensity in each of the corresponding emission spectra.

A separate set of solutions was prepared in order to determine the relative intensity of fluorescence resulting from excitation at the specific wavelengths identified above. For each solution, the wavelength of emission was set to the wavelength corresponding to the maximum intensity in the emission spectrum for that pH. The relative fluorescence was then measured at the wavelengths corresponding to the maxima in intensity in each of the three regions of excitation near 435, 470, and 490 nm. For comparison, the fluorescence of these solutions was also measured with the Turner Fluorometer.

D. Results and Discussion

1. Fluorescence of different lots of fluorescein

The fluorometric properties of three different lots of fluorescein were examined. Despite differences between the physical appearances of the crude fluorescein, commercially purified fluorescein, and that which was purified by acetylation as part of the present work, no measurable differences were observed in the fluorometric properties of the different samples at any pH. While multiple organic components were confirmed in the first two materials by thin-layer chromatography and significant amounts of metals were found in the crude material by atomic emission spectroscopy, these impurities apparently do not significantly affect the fluorescent properties of fluorescein.

2. Excitation and emission spectra at pH 13

Traditionally, interest in fluorescein and derivatives of fluorescein has centered around the fluoresence in highly alkaline solutions. In addition,

previous attempts to distinguish between fluorescein and Calcein[64,252] utilized the apparent difference in intensities of fluorescence at pH 12 and 13. For these reasons, special attention was focused on the excitation and emission spectra of fluorescein at this pH.

The excitation spectrum of fluorescein at pH 13 is shown in figure 4 with two different sensitivities of detection, curve A being shown at approximately twenty times the sensitivity of curve B. At pH 13, fluorescein exhibited one major and several minor bands of excitation. The principal excitation process occurred with green light around 488 nm. Excitation at higher energies also occurred at this pH but was dramatically less effective in producing visible fluorescence. The spectrum near 300 nm. consisted of several unresolved bands, the largest at 280 and 320 nm. Even after adjusting for the energy output of the xenon lamp, the relative intensity resulting from excitation with ultra-violet light was an order of magnitude less than that with visible light. The wavelength of emission for the excitation spectrum in figure 4 was 510 nm., but similar spectra were obtained

Figure 4.

Excitation spectra of fluorescein at pH 13

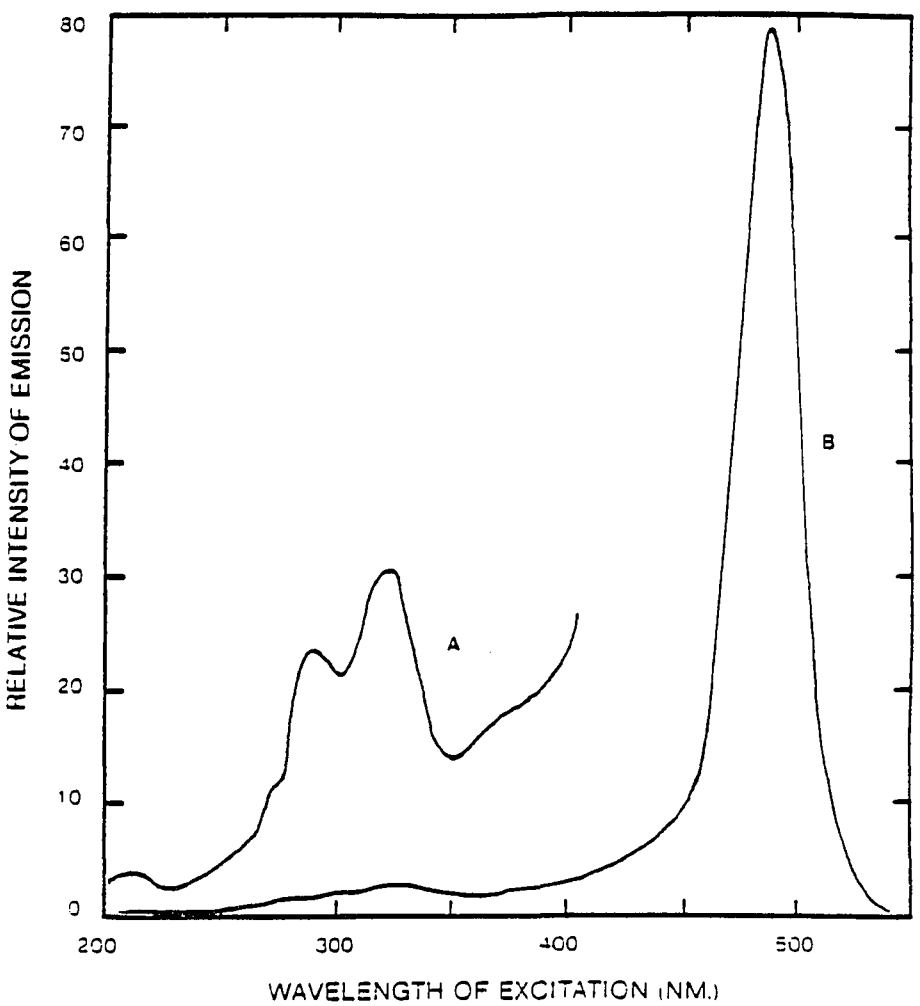
Measured with an Aminco-Bowman Spectrophotofluorometer
Emission monochromator setting: 510 nm.
Entrance slit: 1.0 mm.
Exit slit: 1.0 mm.

Concentration of fluorescein: 5.0×10^{-6} M

pH of solution: 13.0

Curve A: Coarse sensitivity of 0.3
Fine sensitivity of 0.5

Curve B: Coarse sensitivity of 3.0
Fine sensitivity of 1.0


80
70
60
50
40
30
20
10
0
200
300
400
500
RELATIVE INTENSITY OF EMISSION
WAVELENGTH OF EXCITATION (NM.)
A
B

at all wavelengths of emission between 505 and 520 nm., with only proportional changes in the relative intensity of each excitation band.

The emission spectrum of fluorescein at pH 13 is shown in figure 5. The spectrum consisted of a single large band centered at 520 nm. The emission band appeared to be essentially Lawrencian in shape and therefore attributable to a single fundamental energy transition. The wavelength of excitation used for figure 5 was 485 nm., but spectra with identical shapes were obtained by exciting the solution at 280, 290, 320, 470, and 490 nm. The only difference among these spectra was a change in the relative intensity of the emission directly proportional to the relative intensities of the excitation peaks shown in figure 4.

In other words, at pH 13, the wavelength of emission is independent of the energy of excitation, and the intensity of fluorescence depends only on the relative efficiency of the excitation process of the particular wavelength used. This single, excitation-independent band is very fortuitous in regard to studies and applications of fluorescein and

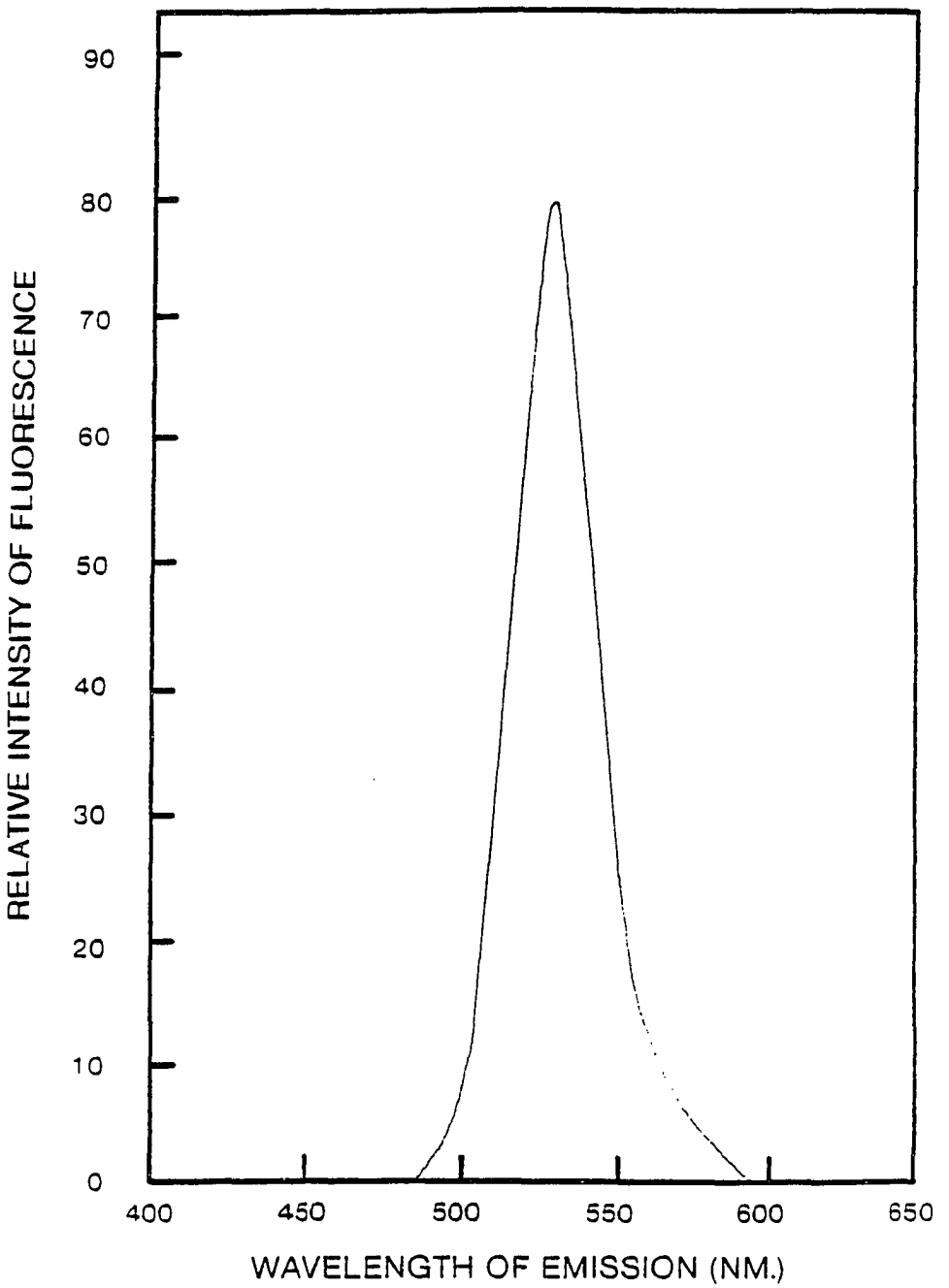
Figure 5.

Emission spectrum of fluorescein at pH 13

Measured with an Aminco-Bowman Spectrophotofluorometer
Excitation monochromator setting: 480 nm.
Entrance slit: 1.0 mm.
Exit slit: 1.0 mm.

Concentration of fluorescein: 5.0×10^{-6} M
pH of solution: 13.0

Coarse sensitivity: 3.0
Fine sensitivity: 0.1


RELATIVE INTENSITY OF FLUORESCENCE
90
80
70
60
50
40
30
20
10
0
400
450
500
550
600
650
WAVELENGTH OF EMISSION (NM.)

derivatives of fluorescein, as will be seen throughout this work.

3. Excitation and emission as functions of pH

A complete examination of the fluorescence of fluorescein was accomplished by obtaining the excitation and emission spectra over the entire range of pH from 0 to 13. Of most concern were the positions of the maxima of excitation and emission at each pH. Measurements of fluorescence at these maxima were generally the most sensitive to concentration and the least sensitive to small changes in wavelengths. In addition, the different excitation transitions could then be correlated to the specific ionic forms of fluorescein in solution.

The emission spectra of fluorescein at various conditions of pH are shown in figure 6. As observed at pH 13, the yellow-green fluorescence was always emitted in a single band around 515 nm. In very acidic solution, the actual position was at 512 nm. The fluorescence gradually underwent a slight bathochromic

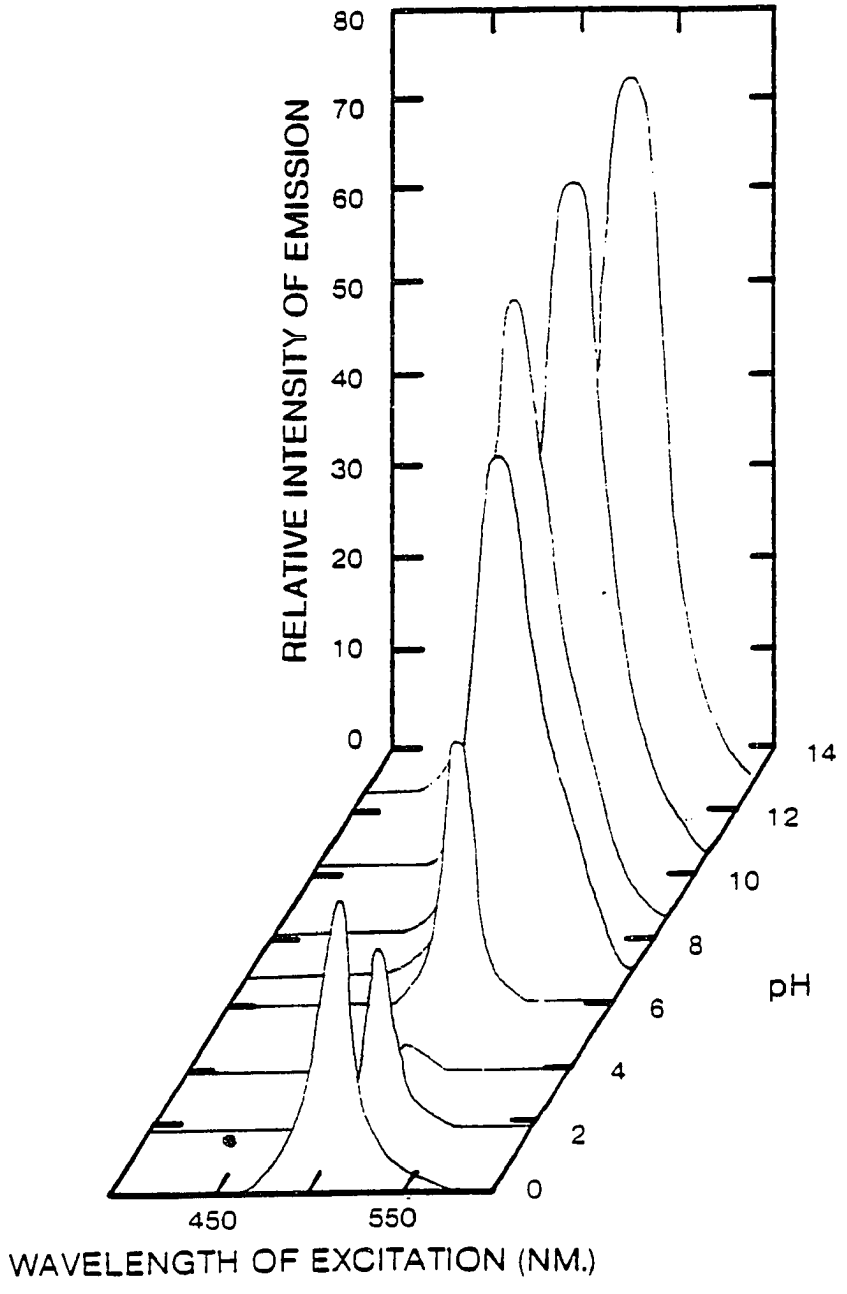
Figure 6.

Fluorescent emission by fluorescein as a function of pH

Measured on an Aminco-Bowman Spectrophotofluorometer
Coarse sensitivity: 3.0
Entrance slit: 1.0 mm.
Exit slit: 1.0 mm.

Concentration of fluorescein: 5.0×10^{-6} M

Excitation monochromator set to wavelength of maximum intensity for each pH


RELATIVE INTENSITY OF EMISSION
80
70
60
50
40
30
20
10
0
14
12
10
8
6
4
2
0
pH
450
550
WAVELENGTH OF EXCITATION (NM.)

shift with increasing pH until the wavelength of the same emission band at pH 13 was at 520 nm.

The wavelength of excitation used for each emission spectrum in figure 6 was set to the wavelength for maximum intensity at that pH. As a result, an interesting curve was obtained by plotting the maximum intensity of emission as a function of pH. The resulting plot of the maximized fluorescence of fluorescein as a function of pH is shown as curve A in figure 7. This characterization of the fluorescence of fluorescein is very different from those published in the literature[362] or from reports of previous studies of fluorescein at Iowa State University[166,247] where such measurements were made at fixed wavelengths or with a filter fluorometer. For comparison, the relative fluorescence measured with the Turner Fluorometer is shown as curve B in figure 7.

While confirming the intense fluorescence of fluorescein in alkaline solutions, this method of measuring fluorescence more clearly demonstrated the measurable fluorescence of fluorescein in neutral and mildly acidic solutions and dramatically proved the

Figure 7.

Fluorescence of fluorescein as a function of pH

Relative fluorescence of each curve normalized to maximum intensity as measured by method

Curve A:

Fluorescence measured on an Aminco-Bowman Spectrofluorometer

Coarse Sensitivity: 3.0
Entrance slit: 1.0 mm.
Exit slit: 1.0 mm.

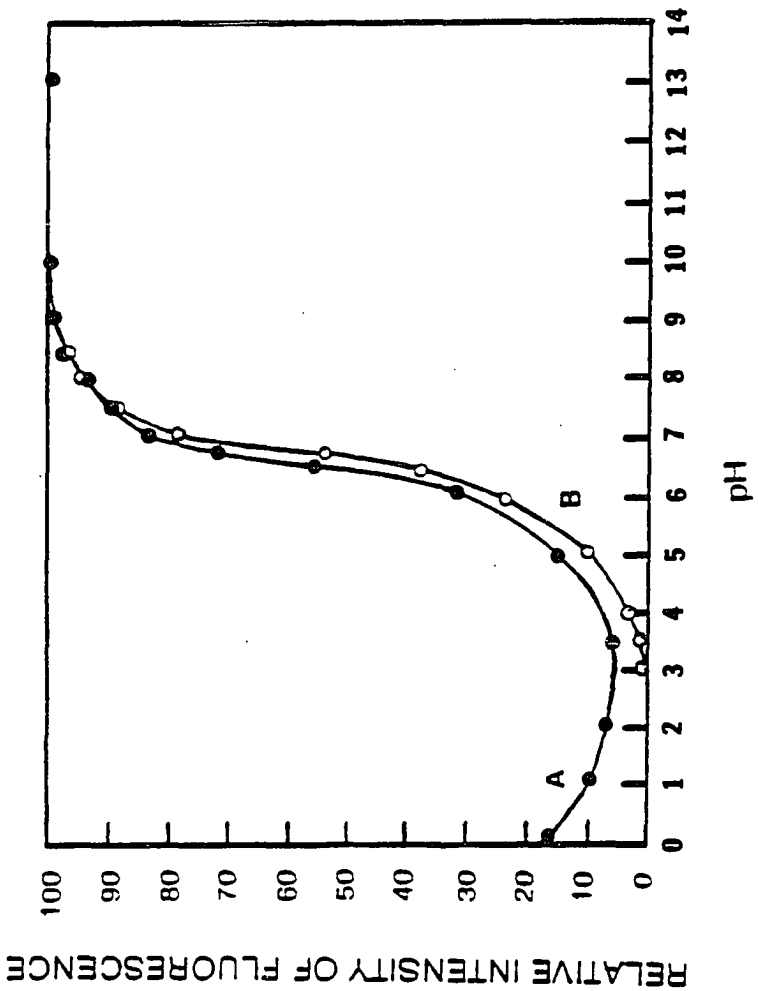
Monochromators set to wavelengths of maximum intensities

Curve B:

Fluorescence measured on a Turner Filter Fluorometer

Primary filter: Corning 5850 glass filter
Secondary filter: Corning 5A-15 glass filter

Sensitivity: 1X


RELATIVE INTENSITY OF FLUORESCENCE
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
pH
A
B

significant fluorescence of fluorescein in highly acidic solutions. The data clearly indicated that more than one prototropic form of fluorescein fluoresces. A full explanation of this phenomenon will be presented following the examination of the excitation spectra.

The excitation spectra of fluorescein under various conditions of pH are shown in figure 8. The excitation spectra were obtained with the wavelength of emission set for the maximum intensity for each particular pH. Unlike the emission spectra, the excitation spectra change dramatically with pH, changing in the intensity, position, and number of bands.

Focusing on the maximum intensities with pH, this data also resembled the data plotted in curve A of figure 7. Unfortunately, the multiplicity of bands under most conditions prevented as simple a derivation as was performed with the emission data. Still, the measurable fluorescence in acidic solution was confirmed and the specific excitation band responsible for this fluorescence was identified.

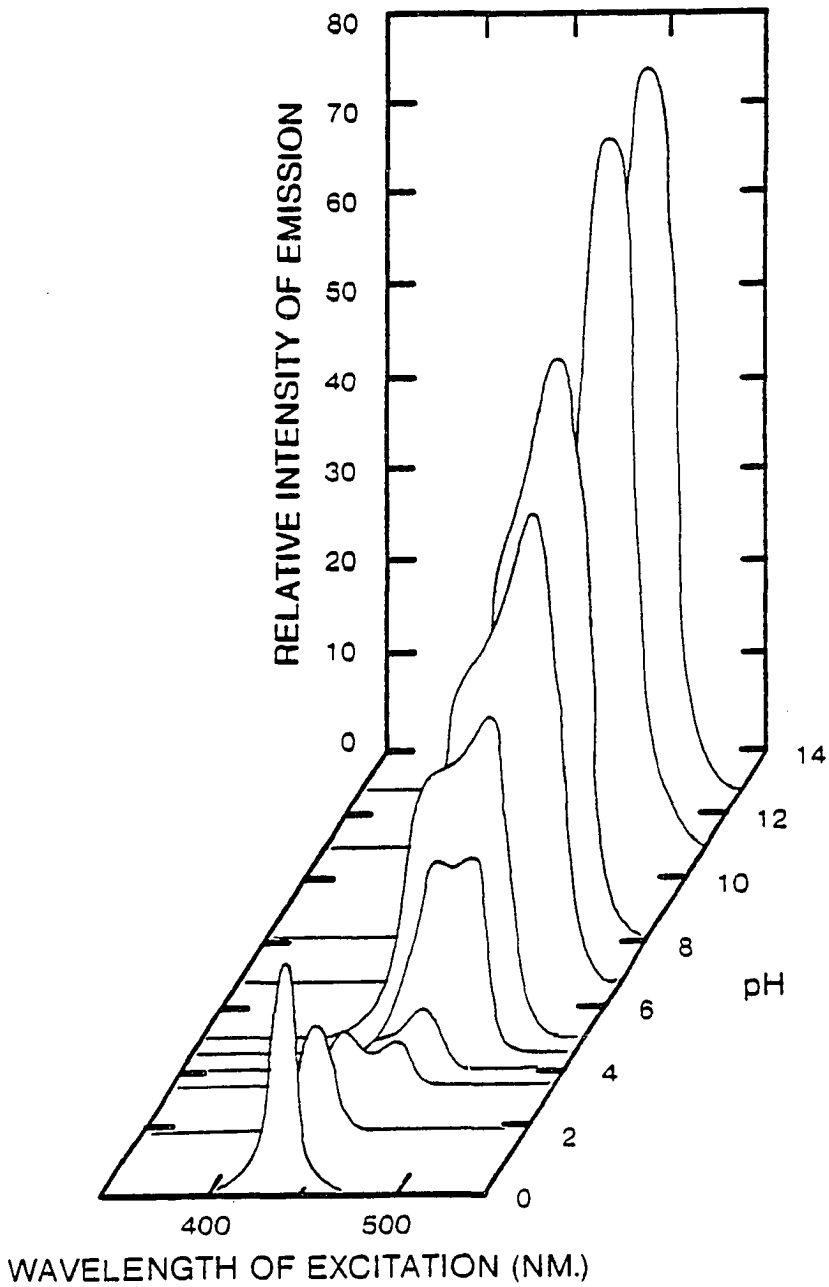
Figure 8.

Excitation of fluorescein as a function of pH

Measured on an Aminco-Bowman Spectrophotofluorometer
Coarse sensitivity: 3.0
Entrance slit: 1.0 mm.
Exit slit: 1.0 mm.

Concentration of fluorescein: 5.0×10^{-6} M

Emission monochromator set to wavelength of maximum fluorescence for each solution


RELATIVE INTENSITY OF EMISSION
80
70
60
50
40
30
20
10
0
14
12
10
8
6
4
2
0
pH
400
500
WAVELENGTH OF EXCITATION (NM.)

In highly acidic solution (pH less than 3), fluorescein was excited to fluorescence by blue light near 435 nm. Virtually identical spectra were obtained using three different mineral acids. The purpose of the repetitions with different acids was to eliminate any explanation of this fluorescence as resulting from the formation of a particular fluorescing ion pair.

As the pH was increased from 0 to 4.5, the band at 435 nm. shifted slightly to 440 nm. At the same time, the relative intensity of emission resulting from excitation in this region was virtually extinguished.

As the pH was increased from pH 2 to pH 7, a second major peak appeared near 465 nm. This band became increasingly more intense with higher pH, while shifting only slightly from 465 nm. at pH 4 to 470 nm. at pH 7. Beyond pH 7, the intensity of this band appeared to continue to increase with only a slight bathochromic shift but identification of the exact position and intensity became difficult as the band was overshadowed by the neighboring predominant excitation band centered at 490 nm.

Finally, the major band near 490 nm. underwent a similar growth in intensity and slight bathochromic shift as the pH was increased from 4 to 13. The far greater intensity of this last band quickly dwarfed the contribution to excitation by light at 470 nm., and the band at 490 nm. was the predominant source of the intense fluorescence in alkaline solutions. In fact, at pH 13, as shown in figure 4, the excitation spectrum of fluorescein had the appearance of a single band centered around 490 nm.

4. Correlation of the wavelength of excitation with the prototropic forms of fluorescein

The data obtained from the excitation and emission spectra of fluorescein at different pH were used to identify the specific ionic forms of fluorescein which fluoresce, the excitation bands which are active for each form and the relative quantum yields or relative coefficients of fluorescence for each ion.

Historically, the fluorescence of fluorescein has always been attributed to the doubly-charged anion in alkaline solution. Even in the detailed examination of

fluorescein by Markuszewski[247], the slight fluorescence noted between pH 3 and pH 4 was attributed to the small amount of dianion existing in solution. A close examination of the fraction of the dianion existing at that pH and the relative intensity of fluorescence attributed to that ion clearly eliminates that explanation. The amount of dianion predicted to exist at pH 4.5 is less than 0.1 percent of the amount of dianion existing in highly alkaline solution. Yet the fluorescence resulting from excitation at 486 nm. is 4 percent of that in alkaline solution and the fluorescence resulting specifically from excitation at 470 nm. is almost 20 percent of the maximum.

The correlation of the distribution of the various ionic forms and the intensities of fluorescence from different excitations clearly indicates the fluorescent characteristics of all of the prototropic forms of fluorescein. Fluorescein is known to exist in four prototropic forms:

H_3Fl^+	singly-charged cation
H_2Fl	neutral molecule
HFl^-	singly-charged anion
Fl^{2-}	doubly-charged anion

Using data on fluorescein obtained by Markuszewski[247,248] from (1) the titration with alkali, (2) the solubility as a function of pH, and (3) the absorbance of ultra-violet light as a function of pH, the following values for the respective dissociation constants have been determined:

$$K_1 = (H^+)(H_2Fl)/(H_3Fl^+) = 7.2 \times 10\text{-}3 \quad (1)$$

$$K_2 = (H^+)(HFl^-)/(H_2Fl) = 1.8 \times 10\text{-}5 \quad (2)$$

$$K_3 = (H^+)(Fl^{2-})/(HFl^-) = 2.8 \times 10\text{-}7 \quad (3)$$

From these constants, the fraction of fluorescein existing as each of the four prototropic forms was calculated at all values of pH. The results of these calculations are shown in the dotted curves of figures 9, 10 and 11. The relative intensity of fluorescence resulting from excitation in particular regions of wavelength are also plotted in these three figures. The data connected by the solid curves are the intensities of fluorescence normalized so that the maximum intensity observed from each excitation band equals 100 units.

The excitation of fluorescein near 435 nm., shown

Figure 9.

Correlation of excitation at 435 nm.
with prototropic forms of fluorescein

Dotted curves represent the fractional distribution
of each of the four prototropic species of fluorescein

Solid curves represent the intensity of fluorescence
resulting from excitation at 435 nm. as extrapolated
from excitation spectra obtained on an Aminco-Bowman
Spectrophotofluorometer

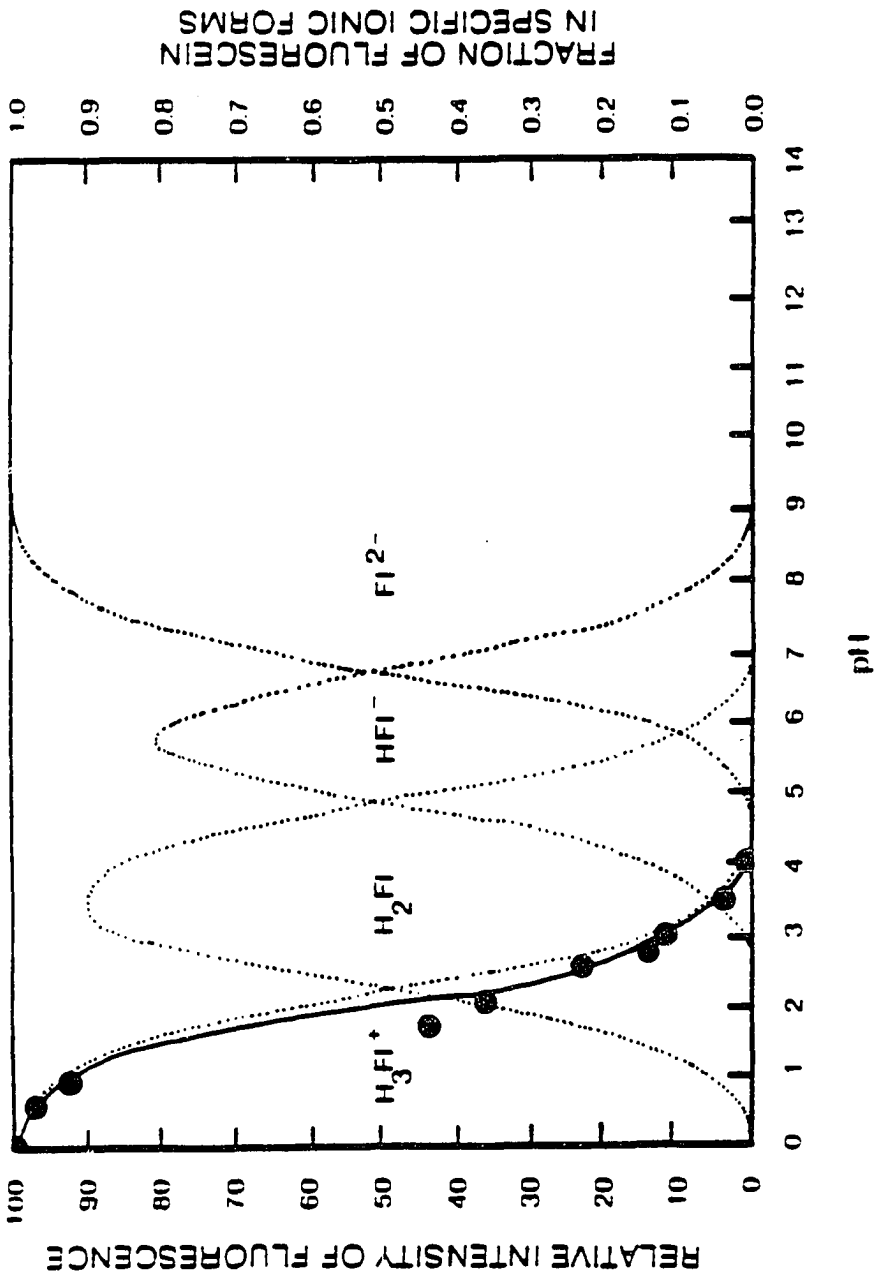

RELATIVE INTENSITY OF FLUORESCENCE
FRACTION OF FLUORESCEIN
IN SPECIFIC IONIC FORMS
pH
H_3Fl^+
H_2Fl
HFl^-
Fl^{2-}

Figure 10.

Correlation of excitation at 470 nm.
with prototropic forms of fluorescein

Dotted curves represent the fractional distribution
of each of the four prototropic species of fluorescein

Solid curves represent the intensity of fluorescence
resulting from excitation at 470 nm. as extrapolated
from excitation spectra obtained on an Aminco-Bowman
Spectrophotofluorometer

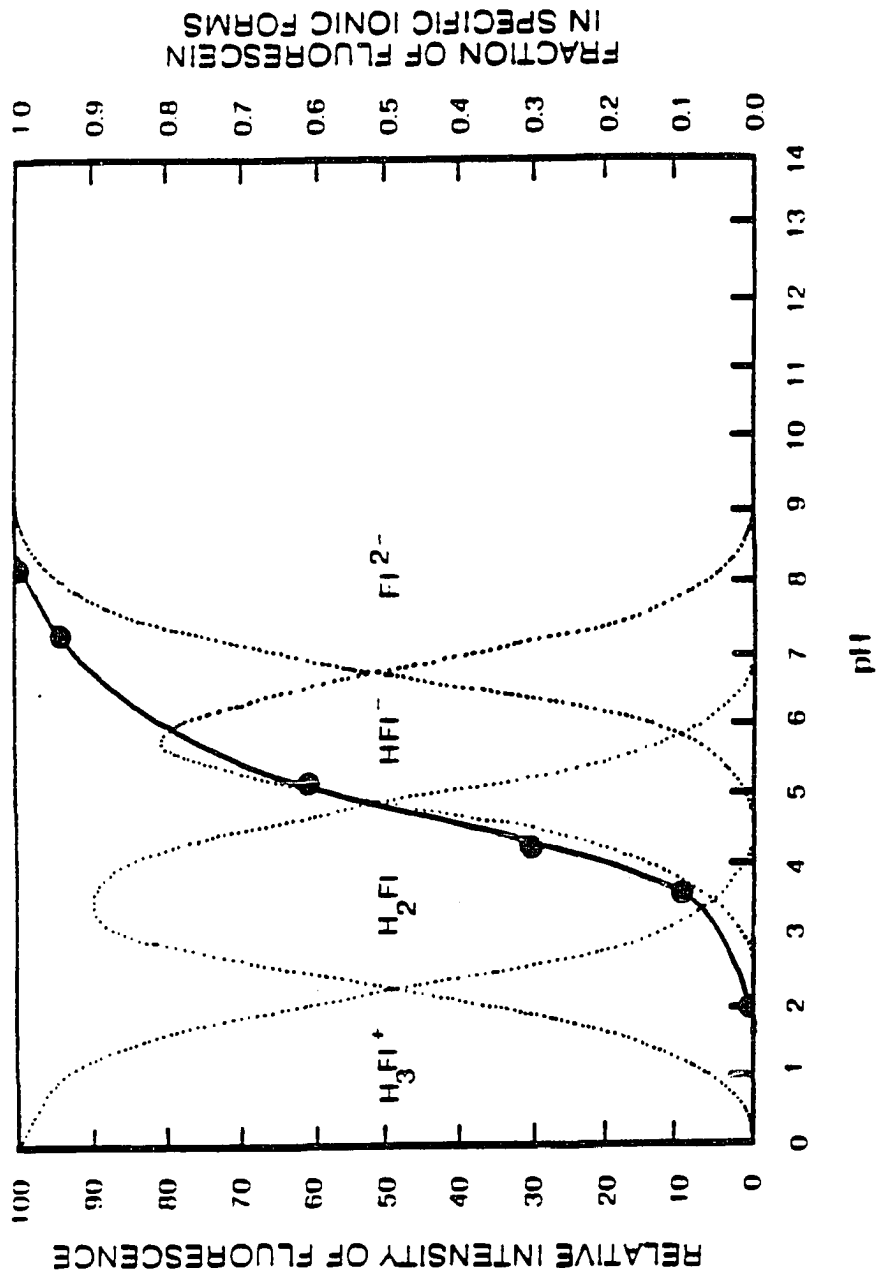
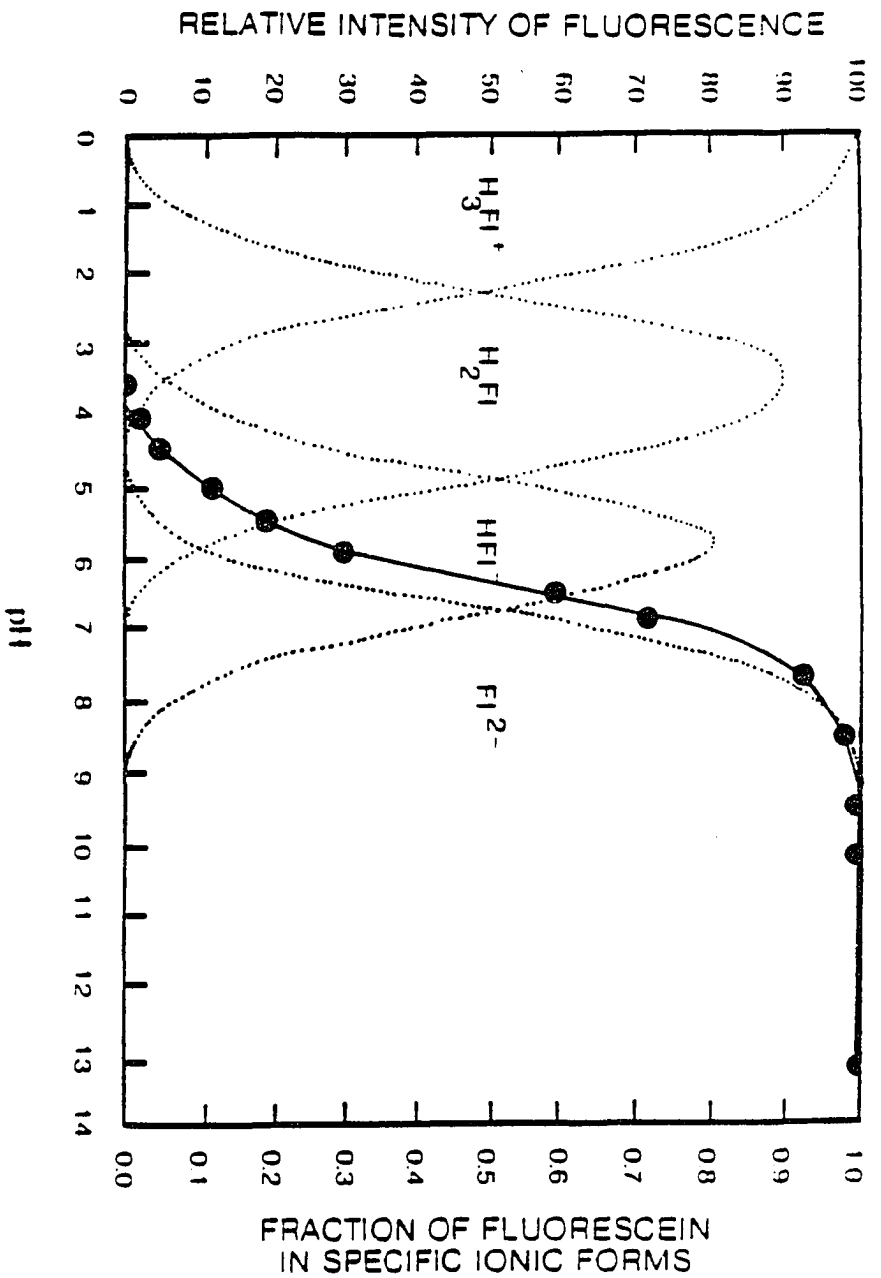

FRACTION OF FLUORESCEIN
IN SPECIFIC IONIC FORMS
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
RELATIVE INTENSITY OF FLUORESCENCE
100
90
80
70
60
50
40
30
20
10
0
H_3Fl^+
H_2Fl
HFl^-
Fl^{2-}
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
pH

Figure 11.

Correlation of excitation at 490 nm.
with prototropic forms of fluorescein

Dotted curves represent the fractional distribution
of each of the four prototropic species of fluorescein

Solid curves represent the intensity of fluorescence
resulting from excitation at 490 nm. as extrapolated
from excitation spectra obtained on an Aminco-Bowman
Spectrophotofluorometer


RELATIVE INTENSITY OF FLUORESCENCE
0
10
20
30
40
50
60
70
80
90
100
H_3Fl^+
H_2Fl
HFl^-
Fl^{2-}
pH
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
FRACTION OF FLUORESCEIN
IN SPECIFIC IONIC FORMS

in figure 9, closely followed the distribution of fluorescein existing as the cation. The excitation of fluorescein near 470 nm. and 490 nm., shown in figures 10 and 11, were roughly proportional to the sum of the fractions of fluorescein as the two anions. The position of the curve in figure 10 indicated that the relative coefficients of fluorescence when excited at 470 nm. are approximately equal for the two anions. The relative position of the curve in figure 11, however, clearly demonstrated the stronger fluorescence of the dianion than that of the monoanion when excited at 490 nm. Only the neutral molecule showed no indication of fluorecence.

A simple computer program was developed to correlate the data on the intensity of fluorescence at each pH and from each wavelength of excitation, with the fractional distribution of the prototropic forms of fluorescein. Assuming that the fluorescence of different ions resulting from excitation at the same wavelength were additive, the program matched the two sets of curves by adjusting the relative coefficients of fluorescence for each ion at each wavelength of excitation. The results are summarized in table 1.

Table 1.

Approximate relative coefficients of fluorescence of the prototropic forms of fluorescein

Ionic Form	Relative Coefficients 440 nm.	470 nm.	490 nm.
H_3Fl^+	0.15	0.00	0.00
H_2Fl		non-fluorescent	
$HFl^{\mp}$	0.00	0.10	0.18
Fl^{2-}	0.00	0.08	1.00

E. Conclusions

In disagreement with all published accounts on the fluorescence of fluorescein, the results of the present work indicated that three, not just one, of the four prototropic forms of fluorescein fluoresce. In addition to the highly fluorescent dianion in alkaline solution, the monoanion, which exists at significant levels between pH 2.5 and pH 8.5, and the cation, which exists below pH 4.5, also fluoresce. Only the neutral molecule of fluorescein does not fluoresce.

All solutions of fluorescein between pH 0 and pH 13 are fluorescent. The fluorescence decreases as the pH increases from 0 to 2.5, reaches a minimum in the region where the neutral molecule is prevalent, and then increases rapidly with pH between 5 and 7. Fluorescein fluoresces intensely in all alkaline solutions and, in solutions with a pH above 10, the intensity remains high and independent of pH.

The wavelengths of excitation and emission as well as the relative intensities of fluorescence of each of the prototropic forms of fluorescein are summarized in

table 2. All of the fluorescent forms of fluorescein emit green light at a single band near 515 nm. This emission band undergoes only a slight bathochromic shift with increasing pH.

Of particular importance to the present work are the properties of fluorescein in highly alkaline solutions. Fluorescein is excited most efficiently at 490 nm. and emits at 520 nm. The high sensitivity to concentration and insensitivity to slight changes in pH or wavelength of excitation under these conditions is ideally suited for fluorometric determinations.

Table 2.

Fluorescence of the prototropic forms of fluorescein

Prototropic Form of Fluorescein	pH Range Observed	Excitation Wavelength (nm.)	Emission Wavelength (nm.)	Relative Coefficient of Fluorescence
H_3Fl	below 4.5	435 - 440	512 - 515	0.15
H_2Fl	0.5 - 6.5	-	-	0.00
HFl^-	3.0 - 8.0	466 - 472	515 - 517	0.10
HFl^-	3.0 - 8.0	485 - 489	515 - 517	0.18
Fl^{2-}	above 5.0	468 - 472	515 - 520	0.08
Fl^{2-}	above 5.0	485 - 490	515 - 520	1.00

III. FLUOROMETRIC PROPERTIES OF CALCEIN

A. Introduction.

Previous Examinations of the Fluorescence of Calcein

The fluorescence exhibited by solutions of Calcein is the fundamental property upon which most applications of Calcein are based. Calcein is intensely fluorescent in aqueous solutions with a pH between 6 and 9[43,44,102]. This intense fluorescence can be quenched by the presence of certain metals[39] while other metals cause Calcein to retain the strong fluorescence even in highly alkaline solutions[102]. In the absence of metals, previous studies have labeled Calcein as non-fluorescent below pH 4 or above pH 11[228,241]. Few sources of more detailed information are available and none of these present an accurate account of the fluorescence of the different prototropic forms of Calcein.

Considering the importance and widespread interest in this reagent, it is surprising to find so little information on the characteristic fluorescence of Calcein in the literature. While there are perhaps a

hundred published fluorometric calibration curves of Calcein with various metals, there is only a handful of studies or detailed descriptions of the fluorescence of Calcein by itself.

Most authors applying Calcein to their own uses refer to one of two series of papers that briefly describe the fluorescence and ultra-violet absorption of Calcein as a function of pH. In English-speaking countries, most references are to two papers by Wallach et al.[361,362] in Analytical Chemistry. In the eastern block countries, references are usually directed toward studies by the Russian workers, Bozhevol'nov and Kreingol'd[64-67]. The two sets of papers are similar and both provide a cursory study of the fluorescence of Calcein as a function of pH and as affected by the addition of certain metal ions. Unfortunately, both sets of papers also suffer from the same weaknesses; the fluorescence excited at wavelengths less than 490 nm. has been ignored, the properties of acidic solutions have not been addressed, and the ultra-violet absorption and excitation spectra have been incorrectly equated. One other study was published by Howerton and Wasilewski[180,181] of Aminco which proved to be

more of an advertisement for a new instrument than it was an academic study of Calcein. Howerton provided more details on the specific excitation and emission spectra of Calcein in solutions with pH greater than 7, both with and without metals present, but similarly ignored the fluorescence in acidic solutions.

The most detailed examination of the fluorescence of Calcein to date was performed by Hefley[166]. Hefley noted three major and up to 5 minor excitation peaks and a single emission band. However, even Hefley's work failed to make a distinction between the fluorescence resulting from the different excitation bands and could not correctly correlate these properties to the individual ionic forms of Calcein in solution.

In analogy to the examination of fluorescein described in Chapter II, a detailed examination of the fluorescence of Calcein was undertaken to discern the specific wavelengths of excitation and emission and to deduce the fluorescent properties of the individual prototropic forms of Calcein. Special attention was paid to identify the fluorescing forms which exist in

acidic solutions.

Hefley used the data derived from the overall fluorescence to determine or confirm several of the acid dissociation constants of Calcein. Unfortunately, the limited purity of the various chemicals used and the lack of a competing chelatometric reagent in the solutions resulted in some inaccuracy in the measurements of fluorescence of Calcein in alkaline solutions. In particular, the presence of even small quantities of metals, such as those present in both the sodium hydroxide used to dissolve and dilute the Calcein and the laboratory chemicals used to prepare the buffers, resulted in the formation of a small amount of a slightly-dissociated compound which exhibited its own fluorescence at high pH. By eliminating this interference in the present work, an accurate assessment of the fluorescence of Calcein at high pH was made and the appropriate dissociation constants were corrected.

B. Apparatus and Reagents

Excitation and emission spectra of all solutions were obtained with an Aminco-Bowman Spectrophotofluorometer and recorded with an Aminco-Bowman X-Y Recorder. The spectra were not corrected for changes in the intensity of emission of the xenon lamp with wavelength. The slit width was set to 1 mm., which corresponds to an instrument resolution of approximately 2 nm.

Other measurements of fluorescence were obtained with a Turner Model 110 Filter Fluorometer. The primary filter consisted of a blue Corning 5850 glass filter and the secondary filter included a yellow Corning 2A-15 glass filter and a Wrattan ten percent neutral density filter.

The pH of each solution was measured with a Beckman Zeromatic pH Meter, Model SS-2, equipped either with a Beckman Glass Indicator Electrode and a Beckman Saturated Calomel Reference Electrode or with a Fisher

Combination pH Indicator/Reference Electrode. The pH meter was standardized using solutions of potassium acid phthalate, potassium dihydrogen phosphate, and sodium borate.

Saturated solutions of Calcein were prepared using a Burrell Wrist-Action Shaker in a constant-temperature room at 24 C.

Much of the work in this section was repeated with different lots of Calcein. The analyses were successively performed using batches of Calcein shown to be of increasingly greater purity as a continuing check that the properties determined were, indeed, those of pure Calcein. The solutions of Calcein for which the results are reported below were either prepared fresh within 36 hours of the measurement or were prepared as dilute solutions, adjusted to pH 5, and stored in the dark for no more than seven days.

Fresh solutions of acid-base buffers were prepared within 15 days of all measurements. Solutions were prepared over the range of pH from 1.00 to 10.50 in increments of 0.3 units or less. The buffers were

prepared from hydrochloric acid, potassium acid phthalate, potassium dihydrogen phosphate, boric acid, potassium hydroxide and potassium chloride according to the procedure of Bower and Bates[63] as summarized by Diehl[106]. All of the buffers had an ionic strength of 0.10.

Potassium hydroxide, free of calcium and other interfering metals, was obtained from the Hach Chemical Company of Ames, Iowa in the form of a 12 N solution. Other reagents were common, supply-house, analytical grade reagents.

Most work was performed using laboratory deionized water. However, for the precision measurements of excitation spectra of pure samples of Calcein and for the careful examination of the fluorescence of Calcein near pH 10.5, buffers and final solutions of Calcein were prepared with triply-distilled deionized water from an all-glass distillation apparatus. This water is sufficently pure for use in electroanalytical chemistry.

C. Experimental Work

1. Measurement of excitation and emission at pH 7 and pH 13

Stock solutions of Calcein were prepared by dissolving 0.16 g of solid Calcein and 2.0 g of potassium hydroxide in a 1.00-liter volumetric flask and diluting to volume. Aliquots of 0.20 ml. were transferred to each of two 100-ml. volumetric flasks. The first was diluted with a buffer of pH 7.0. The second was diluted with 0.10 N calcium-free potassium hydroxide. The final concentration of Calcein was 5×10^{-6} M in both solutions.

Excitation spectra were obtained for each solution from 200 to 600 nm. with the wavelength for emission set at 505, 510, 515, and 520 nm. The emission spectra were obtained between 200 and 600 nm. with the wavelength for excitation set at 280, 320, 435, 465, 470, and 485 nm. The sensitivity of detection was varied to maximize the signal recorded under each set of conditions.

A similar set of solutions was prepared with the addition of 1.0 ml. of 5×10^{-3} M EDTA to each 100-ml. flask before dilution. The final concentration of EDTA was 5×10^{-5} M in each solution. Excitation and emission spectra were obtained for these solutions as described above.

2. Measurement of fluorescence as a function of pH

Stock solutions of Calcein were prepared by dissolving 0.33 g of Calcein, approximately 0.01 g of disodium dihydrogen ethylenediaminetetraacetic acid and 2.0 g of potassium hydroxide in a 1-liter volumetric flask and diluting to volume. An aliquot of 1.00 ml. of this stock solution was then added to each of a series of 100-ml. volumetric flasks. Each flask was then filled to volume with a different buffer having a pH between 1.50 and 10.5. Similar solutions were also prepared in 0.03, 0.10, 0.30, and 1.0 N hydrochloric acid and in 0.01, 0.03, and 0.10 N calcium-free potassium hydroxide. With the exception of the solutions which had concentrations of acid greater than 0.1 N, the ionic strength of the solutions were

adjusted to 0.1 with 1.0 M potassium chloride. The final concentration of Calcein in each solution was 5 x 10^{-6} M. Another solution of 5 x 10^{-6} M Calcein was prepared using 1.0 N perchloric acid in place of 1.0 N hydrochloric acid.

The pH of each solution was precisely measured.

Emission spectra for each solution were obtained with the wavelength of excitation at 435, 470, and 490 nm. The excitation spectra were then obtained with the wavelength of emission set at the wavelength of maximum intensity as determined from the emission spectra.

Measurements of the intensity of emission resulting from specific excitation bands were obtained by setting the wavelength of emission to the maximum in the emission spectrum and setting the wavelength of excitation at the apparent maximum within each of the following regions of the excitation spectrum: 435-445 nm., 465-480 nm., and 485-490 nm.

3. Detailed measurements of fluorescence between pH 9 and pH 11

Special attention was directed toward the measurement of fluorescence of Calcein near pH 10.7. Hefley[166] reported three points of inflection in the plot of the intensity of fluorescence verses pH over this region.

A stock solution containing 1×10^{-6} M Calcein and 5×10^{-5} M EDTA was prepared as described in section (2). The pH of the solution was carefully measured to be 7.98 with a pH meter which had been freshly calibrated at pH 7.00 and pH 10.0. Small increments of 0.01, 0.10, or 1.0 N potassium hydroxide were successively added to 2.00 liters of the stock solution from a 10.0-ml. buret. The pH was measured after each addition and the excitation spectrum was recorded with the wavelength of emission set at the wavelength corresponding to the maximum intensity in the emission spectrum at that pH. This procedure was continued until the pH of the solution reached 11. The total increase in volume was less than 20 ml. and no volumetric correction was applied to the measurements.

D. Results and Discussion

1. Excitation and emission at pH 7 and pH 13

There are two regions of pH which are of special concern to the fluorometric determination of Calcein. Near pH 7, Calcein exhibits the maximum fluorescence. At pH 13, Calcein is relatively non-fluorescent while fluorescein and the slightly-dissociated compound of calcium and Calcein exhibit intense fluorescence.

The excitation spectrum of Calcein at pH 7.0 is shown in figure 12 with two different sensitivities of detection. Curve A was obtained at approximately twenty times the sensitivity of curve B. The major region of excitation for Calcein at pH 7 consisted of an intense band at 485 nm. with a second band at 470 nm. appearing as a distinct shoulder on the larger peak. A series of minor excitation bands in the ultra-violet region were similar in position and intensity to those observed for fluorescein at pH 13 (figure 4), but less distinct. The spectra in figure

Figure 12.

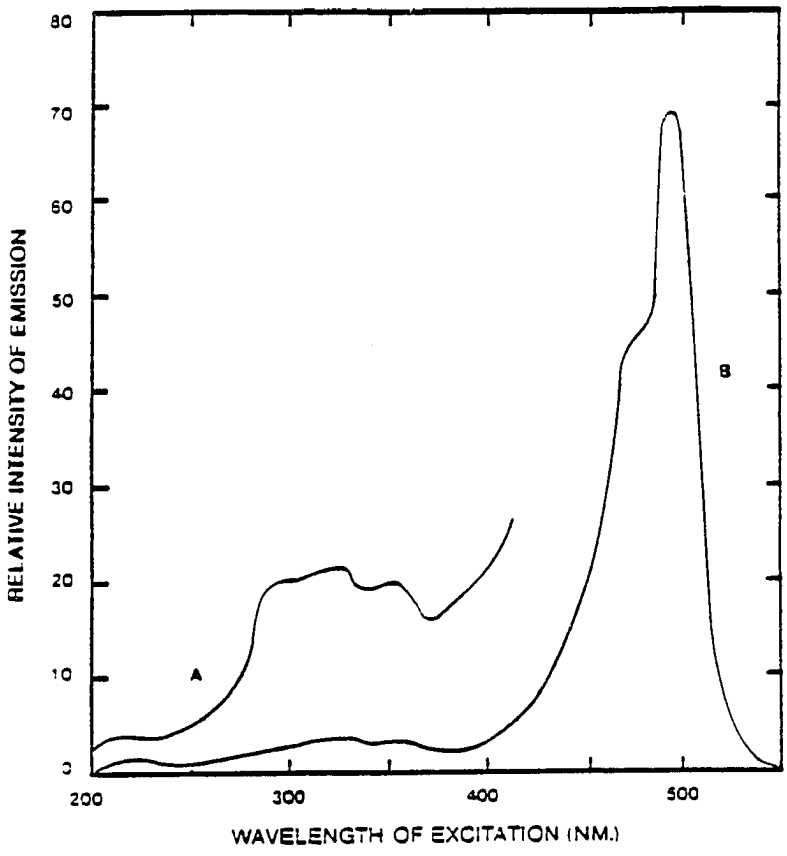
Excitation spectra of Calcein at pH 7

Measured on an Aminco-Bowman Spectrophotofluorometer
Emission monochromator: 515 nm.
Entrance slit: 1.0 mm.
Exit slit: 1.0 mm.

Concentration of Calcein: 5×10^{-6} M
pH of solution: 7.0

Curve A: Coarse sensitivity: 0.3
Fine sensitivity: 0.5

Curve B: Coarse sensitivity: 3.0
Fine sensitivity: 1.0


80
70
60
50
40
30
20
10
0
200
300
400
500
RELATIVE INTENSITY OF EMISSION
WAVELENGTH OF EXCITATION (NM.)
A
B

12 were obtained while measuring the intensity emitted at 515 nm. Spectra of identical shape were also obtained at all settings for the wavelength of emission between 500 and 520 nm.

The emission spectrum of Calcein at pH 7.0 is shown in figure 13. As with fluorescein, the yellow-green fluorescence was emitted as a single band between 500 and 550 nm. At pH 7.0, the maximum intensity was emitted at 517 nm. The curve in figure 13 was obtained by irradiating Calcein at 485 nm. Emission spectra with the same shape were also obtained with the wavelength of excitation at 280, 300, 435, 465, and 470 nm. The only differences between the spectra were the intensities of the emission bands which varied proportionally to the relative intensities of the corresponding excitation bands shown in figure 12. As with fluorescein, the wavelength of emission was independent of the excitation wavelength. Again, this simplified the subsequent studies and analysis of the fluorometric properties.

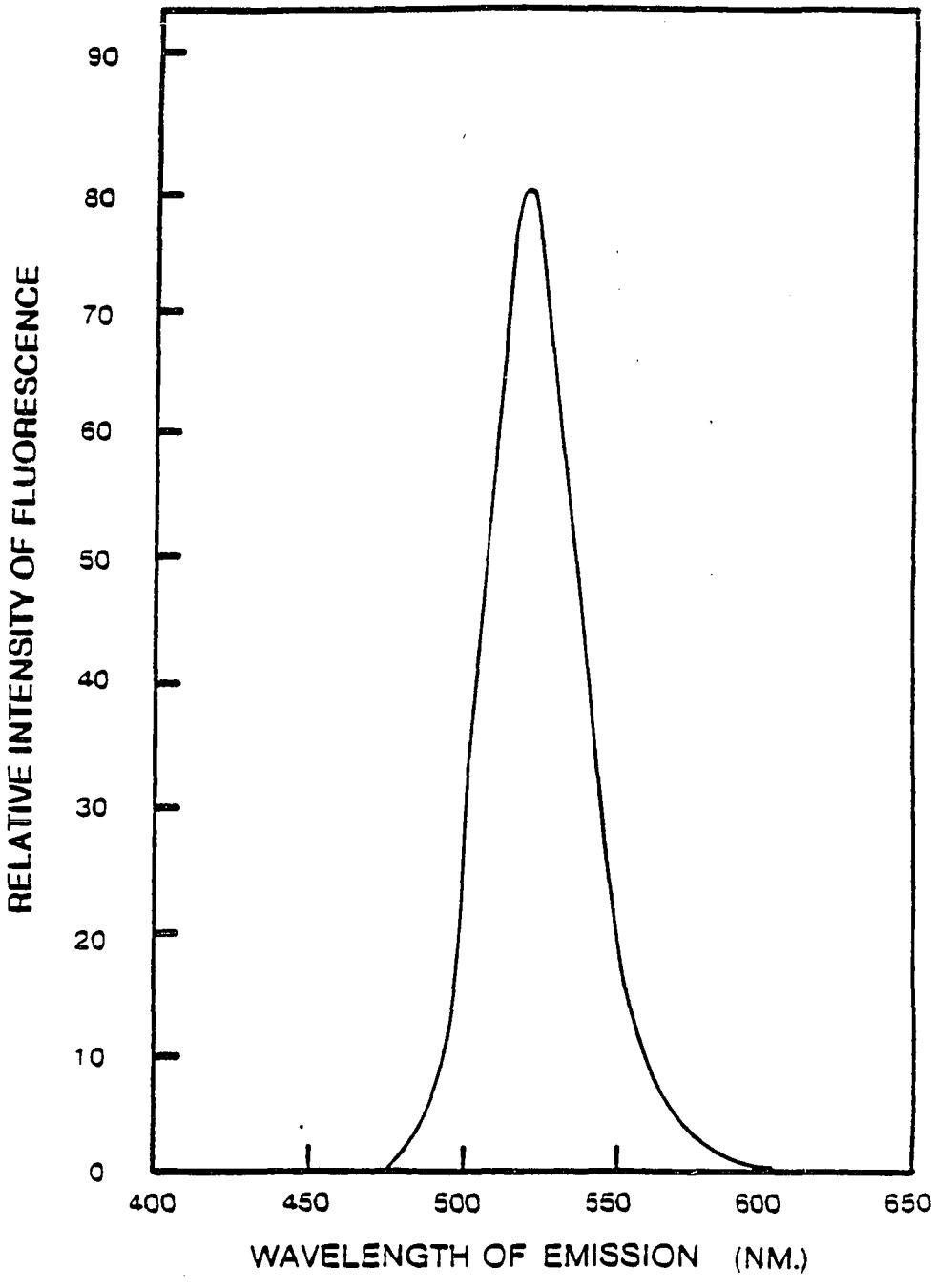
The excitation and emission spectra of several lots of Calcein were obtained at pH 13. The quality of

Figure 13.

Emission spectrum of Calcein at pH 7

Measured on an Aminco-Bowman Spectrophotofluorometer
Excitation monochromator: 485 nm.
Entrance slit: 1.0 mm.
Exit slit: 1.0 mm.

Concentration of Calcein: 5×10^{-6} M
pH of solution: 7.0


RELATIVE INTENSITY OF FLUORESCENCE
90
80
70
60
50
40
30
20
10
0
400
450
500
550
600
650
WAVELENGTH OF EMISSION (NM.)

material varied from crude preparations to commercial products and highly pure samples of Calcein obtained by rigorous purification. Some fluorescence was detectable in all samples, owing to the inherent sensitivity possible with the spectrophotofluorometer. The difference in relative intensities, though, indicated dramatic differences between samples. The excitation and emission curves of three lots are shown in the upper quadrants of figure 14. In all cases, the excitation and emission spectra resembled those of fluorescein at pH 13, having a single predominant band of excitation at 490 nm. and a single band of emission at 520 nm. As will be shown in chapter IV, the spectra also resembled the excitation and emission of Calcein in the presence of alkaline earths at this pH. Indeed, when the experiments were repeated with the addition of EDTA to the solutions, the intensities of emission of the crude materials were reduced by as much as 20 percent, while only small changes were observed for most of the other samples. The result of adding the competing chelating agent to the solution is shown in the lower quadrants of figure 14.

The reduction of fluorescence at pH 13 to almost

Figure 14.

Excitation and emission spectra
of different lots of Calcein at pH 13

Intensities measured on Aminco-Bowman Spectrofluorometer
Coarse sensitivity: 3.0
Entrance slit: 1.0 mm.
Exit slit: 1.0 mm.
Concentration of Calcein: 5 x 10-5 M
Concentration of EDTA (quadrants C & D only): 5 x 10-5 M
pH of solutions: 13.0

Quadrant A: Excitation spectra of solutions without EDTA

Quadrant B: Emission spectra of solutions without EDTA

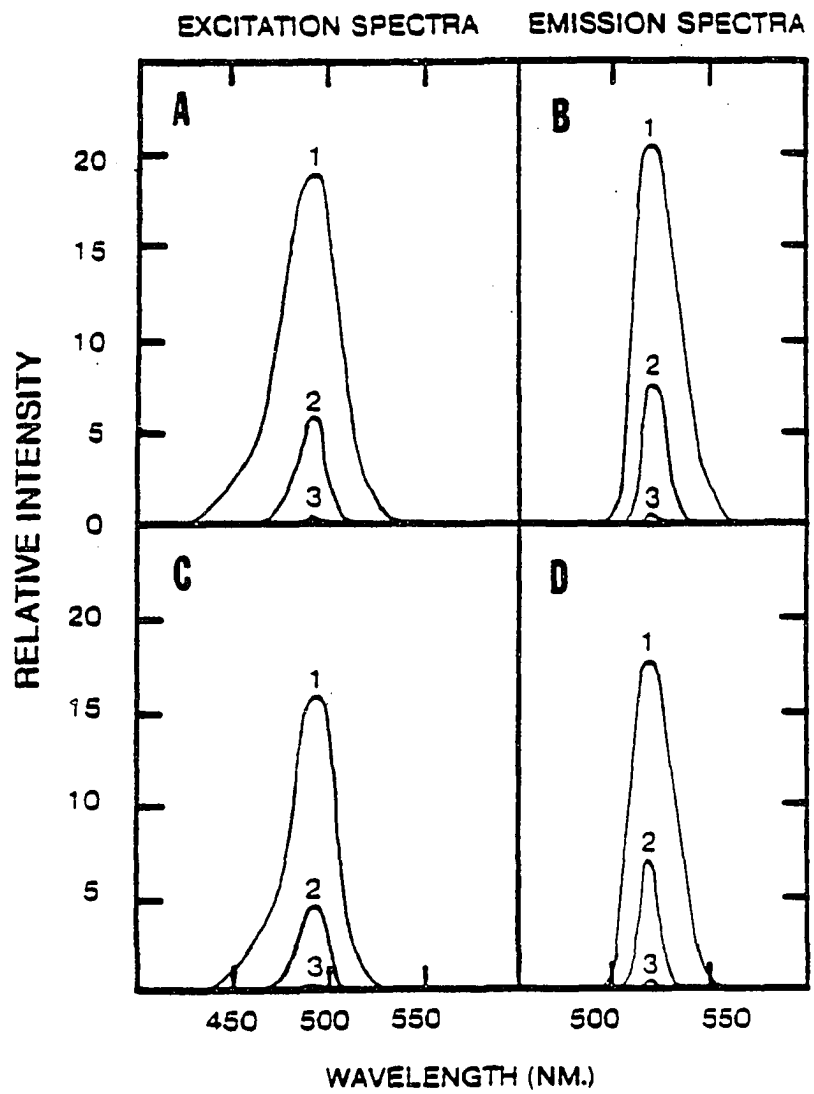
Quadrant C: Excitation spectra of solutions with EDTA added

Quadrant D: Emission spectra of solutions with EDTA added

Curves 1: Crude Calcein from D. B. Martin (ref. 254)

Curves 2: Commercial Calcein (G. F. Smith Co. Lot X1)

Curves 3: Prepared Calcein from this work (lot XIV-D)


EXCITATION SPECTRA
EMISSION SPECTRA
A
B
C
D
1
2
3
RELATIVE INTENSITY
20
15
10
5
0
450
500
550
WAVELENGTH (NM.)

imperceptible levels by the purification of Calcein gave credence to the assumption of previous workers that Calcein is completely non-fluorescent in highly alkaline solution and that any fluorescence present is the result of impurities of fluorescein or metals with which Calcein forms fluorescent compounds. Unfortunately, the alkali used to prepare basic solutions also form weak but slightly fluorescent compounds with Calcein and a totally non-fluorescent alkaline solution can not be obtained by conventional methods.

The existence of significant amounts of metals in the crude Calcein was confirmed by plasma emission spectroscopy[154]. The large reduction of the fluorescence with the addition of EDTA to the solutions of crude material was attributed to the prevention of the formation of fluorescent metal-Calcein compounds by the chelating action of the EDTA. The remaining and major portion of the fluorescence was attributed to fluorescein.

Even in solutions prepared from highly pure samples of Calcein, metal ions, particularly calcium,

were found to be introduced via the standard laboratory chemicals used in the preparation of the solutions. Though the amounts of these ions were usually small, decreased fluorescence was still noted upon the addition of EDTA. The use of specially prepared, calcium-free potassium hydroxide virtually eliminated this source of fluorescence. The remaining fluorescence was attributed to fluorescein and the unavoidable fluorescence of the slightly-dissociated compound of Calcein with the alkali - in this case, potassium.

2. Excitation and emission as functions of pH

The excitation and emission spectra of Calcein were acquired over the entire range of pH between 0 and 13 for the purpose of identifying the specific wavelengths of excitation and emission for Calcein and to identify which specific prototropic forms of Calcein fluoresce.

Figure 15 contains the emission spectra of Calcein obtained under various conditions of pH. The

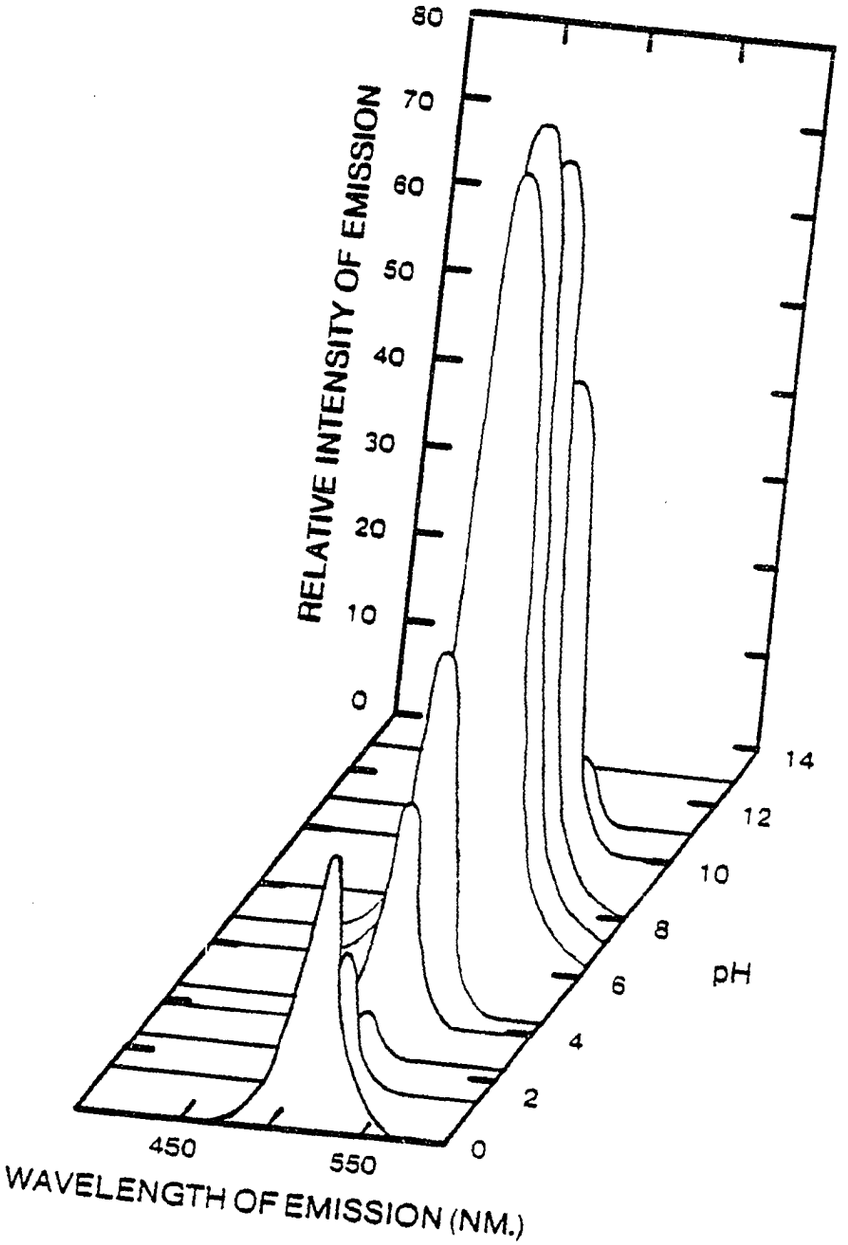
Figure 15.

Emission spectra of Calcein in solutions of different pH

Measured on an Aminco-Bowman Spectrophotofluorometer
Coarse sensitivity: 3.0
Entrance slit: 1.0 mm.
Exit slit: 1.0 mm.

Concentration of Calcein: 5×10^{-6} M

Excitation monochromator set for maximum excitation of fluorescence for each solution


RELATIVE INTENSITY OF EMISSION
80
70
60
50
40
30
20
10
0
14
12
10
8
6
4
2
0
pH
450
550
WAVELENGTH OF EMISSION (NM.)

wavelength of excitation was set for the maximum intensity of excitation of Calcein at each particular pH examined. The emission always occurred at a single band near 515 nm. In acidic solution, the apex of this band was located at 512 nm. As the pH was increased, the emission underwent a slight bathochromic shift until, at pH 10, the maximum was at 518 nm. The most intense band, near pH 8, was approximately equivalent in intensity to the emission band of fluorescein in alkaline solutions. The shape and position of the emission bands of Calcein were essentially identical to those of fluorescein at the same pH, with only the relative intensities differing.

The intensity of fluorescence as a function of pH was plotted in figure 16. Measurements obtained with the filter fluorometer are shown in curve B while the fluorescence as measured on the spectrophotofluorometer is shown in curve A. With the wavelengths of excitation and emission set to maximize the measured intensity, curve A presents a different and more instructive representation of the fluorescence of Calcein than any of those presented in the literature. It can be noted from figure 16 that all solutions of

Figure 16.

Fluorescence of Calcein as a function of pH

Concentration of Calcein: 5 x 10-5 M

Curve A:

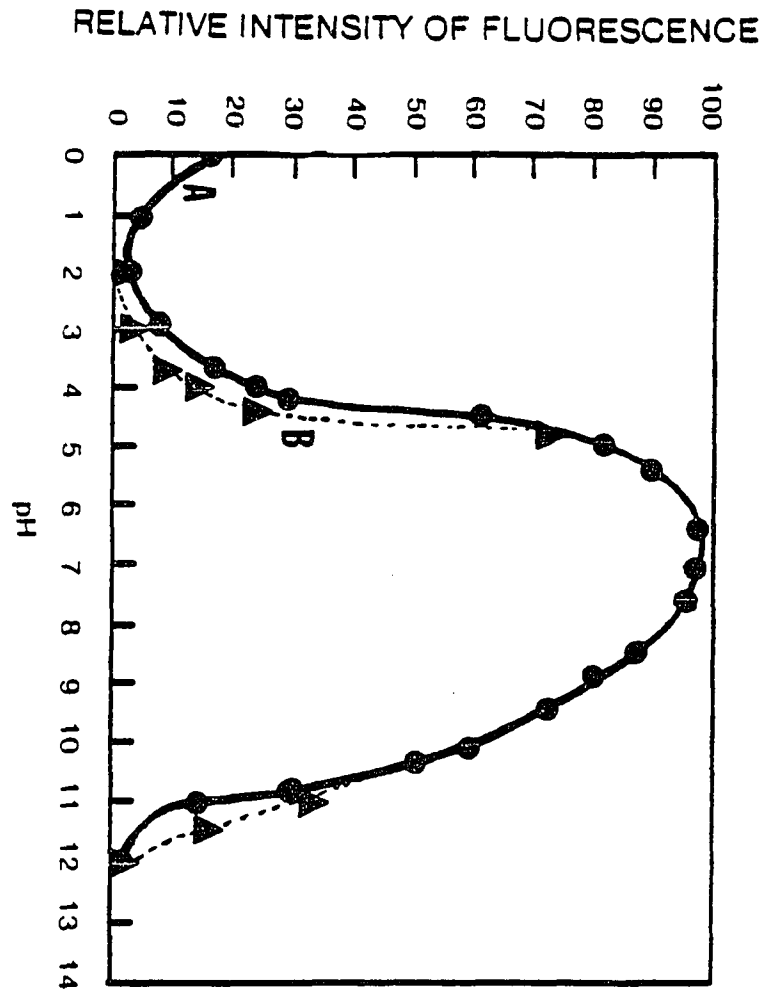
Fluorescence measured on Aminco-Bowman Spectrofluorometer
Coarse sensitivity: 3.0
Entrance slit: 1.0 mm.
Exit slit: 1.0 mm.

Measurements taken from the peak value of intensity of the emission spectra with the excitation wavelength set to maximize the intensity measured for each solution

Curve B:

Fluorescence measured on a Turner Filter Fluorometer

Primary filter: Corning 5850 glass filter
Secondary filter: Corning 2A-15 glass filter
Sensititvity: 10X


RELATIVE INTENSITY OF FLUORESCENCE
0
10
20
30
40
50
60
70
80
90
100
pH
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
A
B

Calcein with a pH less than 12 do fluoresce.

In similarity to fluorescein, Calcein was found to fluoresce in highly acidic solutions (pH less than 2), was found to exhibit only a faint fluorescence beteen pH 2 and pH 3, and was found to emit strongly between pH 4 and pH 7. Other than a small difference in the pH at which the minimum in fluorescent intensity was detected, the fluorometric properties of fluorescein and Calcein were identical over this range. At pH 0, Calcein demonstrated significant fluorescence when excited at 435 nm. The intensity of fluorescence decreased as the pH was raised to 2. The fluorescence was at a minimum near pH 2, corresponding to the same region of pH where Calcein is the least soluble [70]. The fluorescence then increased dramatically in solutions with higher pH and Calcein was excited to fluorescence by light at both 470 nm. and 490 nm.

Above pH 7, the fluorescence of Calcein was distinctly different from that of fluorescein. While the fluorescence of fluorescein remained intense and constant for pH 8 and above, the fluorescence of Calcein reached a maximum near pH 7.5 and then

decreased with increasing pH. The decrease in intensity was quite rapid between pH 9 and pH 11 and the fluorescence was essentially extinguished in all solutions above pH 12.

A more detailed explanation of the fluorescence as a function of pH will be made in conjunction with the excitation spectra at various pH.

The excitation spectra of Calcein obtained under various conditions of pH are shown in figure 17. These spectra were obtained by setting the wavelength of emission to the maximum for each particular pH as indicated by the emission spectra in figure 15. While the emission spectra of Calcein were relatively independent of pH, the pattern of the excitation spectra changed dramatically.

The excitation spectra of Calcein over the range of pH between 0 and 13 consisted of one or more of the three major excitation bands at 435, 470, and 490 nm. and a similar group of unresolved minor excitation bands between 260 and 320 nm., each with a

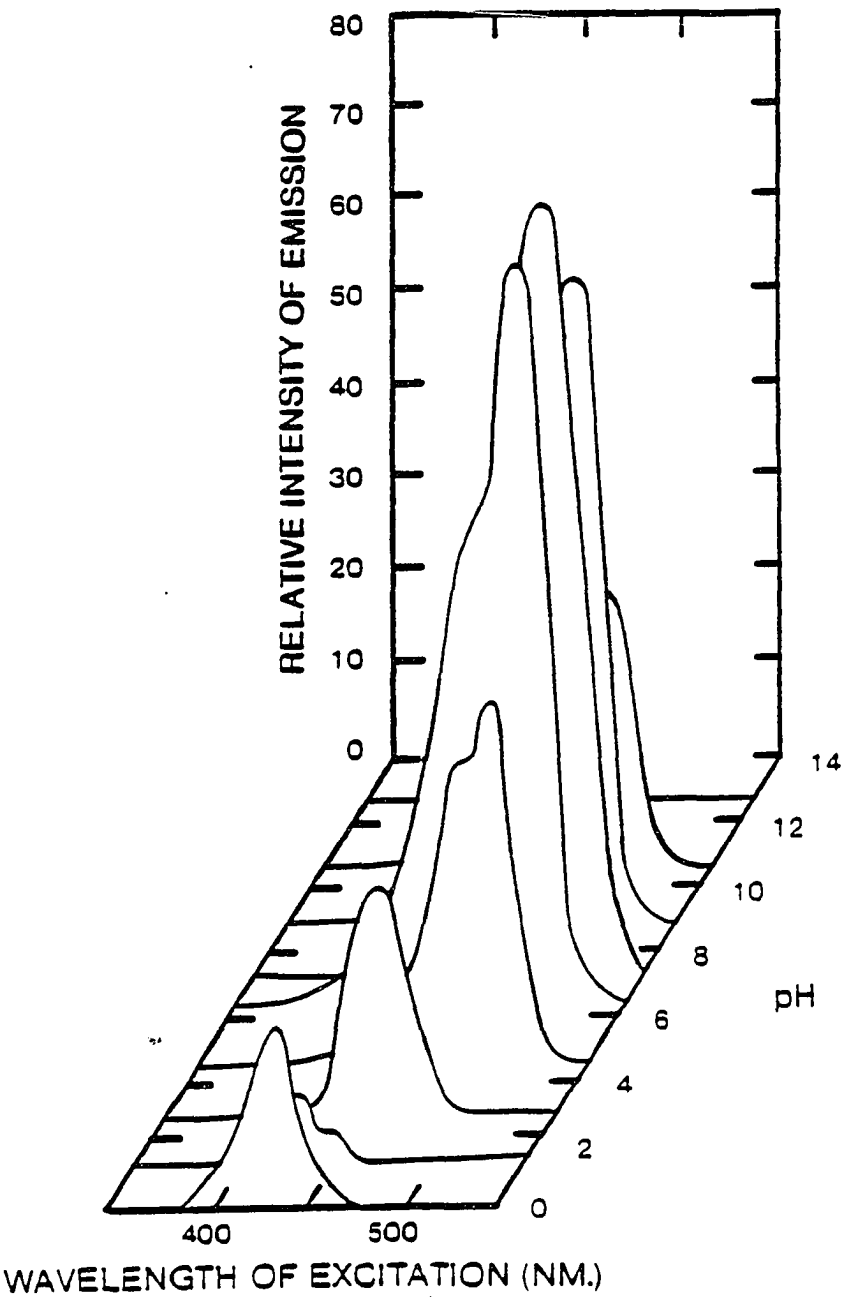
Figure 17.

Excitation spectra of Calcein in solutions
of different pH

Measured on an Aminco-Bowman Spectrophotofluorometer
Coarse sensitivity: 3.0
Entrance slit: 1.0 mm
Exit slit: 1.0 mm.

Concentration of Calcein: 5×10^{-6} M

Emission monochromator set for maximum emission
for each solution


RELATIVE INTENSITY OF EMISSION
80
70
60
50
40
30
20
10
0
14
12
10
8
6
4
2
0
pH
400
500
WAVELENGTH OF EXCITATION (NM.)

corresponding band in the excitation spectrum of fluorescein. Each of these bands underwent the same bathochromic shift that was observed with fluorescein. In fact, the specific wavelength at which each of these excitation bands was located at any particular pH was identical for both compounds. Inasmuch as the similarity has already been demonstrated for the emission spectra, clearly no differentiation was possible between fluorescein and Calcein merely by the selection of proper wavelengths or filters. Any possiblity for fluorometric analysis of mixtures of these compounds must rely strictly on differences in intensity. Particularly useful would be the presence of an excitation band in one species under conditions where the same band has negligible intensity for the other.

It was clearly demonstrated by the data in figure 17, that the three excitation bands of Calcein correspond to separate and discrete energy transistions and are not merely the result of a bathochromic shift of a single excitation, as has been reported [65].

From the data in figure 17, the specific energies

of excitation responsible for the fluorescence of Calcein as a function of pH were evaluated. Calcein did not fluoresce significantly in solutions with a pH greater than 12.0. As the pH of the solution is lowered from 11 to 9, the relative intensity of fluorescence increased rapidly. This fluorescence was primarily a result of excitation at the band between 485 and 490 nm. and to a lesser extent at the band near 470 nm. The fluorescence reached a maximum near pH 7.5 with the excitation characteristics shown in figure 12. As the pH of the solution was lowered below 7, the fluorescence from excitation near 485 nm. decreased rapidly and no measurable emission from that excitation band was observed below pH 3. Over the same range of pH, the decrease in intensity resulting from excitation at 470 nm. was much more gradual. As a result, this band still corresponded to a significant means of excitation at pH 3.0. With further decreases in pH, the band between 465 and 470 nm. disappeared by pH 0.5 while a new band at 435 nm. produced significant intensities. This last band continued to grow in intensity with the increasing concentration of acid.

3. Correlation of the wavelength of excitation with the prototropic forms of Calcein

Calcein has a total of six replaceable hydrogen ions on the neutral molecule and the potential to obtain at least one additional hydrogen ion in acidic solutions. Thus, at least eight prototropic forms of Calcein exist, related by the following dissociation constants:

$$K_{-1} = (H^+)(H_6Cal)/(H_7Cal^+) = \text{unknown} \quad (4)$$

$$K_1 = (H^+)(H_5Cal^-)/(H_6Cal) = 3.2 \times 10^{-3} \quad (5)$$

$$K_2 = (H^+)(H_4Cal^{2-})/(H_5Cal^-) = 3.2 \times 10^{-4} \quad (6)$$

$$K_3 = (H^+)(H_3Cal^{3-})/(H_4Cal^{2-}) = 2.5 \times 10^{-5} \quad (7)$$

$$K_4 = (H^+)(H_2Cal^{4-})/(H_3Cal^{3-}) = 6.3 \times 10^{-7} \quad (8)$$

$$K_5 = (H^+)(HCal^{5-})/(H_2Cal^{4-}) = 1.3 \times 10^{-10} \quad (9)$$

$$K_6 = (H^+)(Cal^{6-})/(HCal^{5-}) = 2.5 \times 10^{-12} \quad (10)$$

The values for the equilibrium constants K_1 through K_6 were cleverly determined by Hefley[166] using a variety of techniques, including the measurement of the intensity of fluorescence as a function of pH. The value of K_{-1} has not been reported previously, but the data acquired here and in later sections of this work estimated an effective dissociation constant for the protonated forms of the molecule of Calcein to be 1.1×10^{-1}. This so-called effective dissociation constant was representative of the overlapping dissociations of more than one protonated form of Calcein in acidic solutions. The determination of this so-called effective dissociation constant will be presented in chapter V.

The relative distributions of the prototropic forms of Calcein are plotted as dotted curves in figure 18. The intensity of fluorescence from each of the three major excitation bands of Calcein is plotted as the solid curves. For the excitations near 470 and 485 nm., the relative intensities were normalized so that the most intense point was given the arbitrary value of 100. For the excitation at 435 nm. the normalization

Figure 18

Correlation of excitation wavelength with
the prototropic forms of Calcein

Measured on an Aminco-Bowman Spectrophotofluorometer
Coarse sensitivity: 3.0
Entrance slit: 1.0 mm
Exit slit: 1.0 mm.

Concentration of Calcein: 5×10^{-6} M

Dotted curves: Calculated fractional distribution
of various prototropic forms of Calcein

Solid curves: Relative intensity of fluorescence
from excitation at various wavelengths

Intensities from various wavelengths normalized
to the most intense value measured at each wavelength

FRACTIONAL DISTRIBUTION
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
H_7Cal^+
H_6Cal
H_5Cal^-
H_4Cal^{2-}
H_3Cal^{3-}
H_2Cal^{4-}
$HCal^{5-}$
Cal^{6-}
437nm
470nm
485nm
470 & 485 nm
pH
0
1
2
3
4
5
6
7
8
9
10
11
12
13
0
10
20
30
40
50
60
70
80
90
100
RELATIVE INTENSITY
OF FLUORESCENCE

value was chosen to be 93 to correlate with the maximum percentage of cation calculated to exist within this range of pH.

The relative intensity from excitation near 435 nm. closely followed the distribution curve calculated for the protonated species designated as "H_7Cal^+ ".

The increasing intensity of fluorescence resulting from excitation near 470 nm. from pH 1 to pH 5 was essentially equivalent to the total fraction of Calcein existing with the first, second, third, and fourth hydrogen ions removed from the neutral molecule. Between pH 5 and pH 9, the relative intensity from this excitation could not be measured in the presence of the large band near 485 nm. Beyond pH 9, the intensity from excitation at 470 nm. decayed proportionally to the decrease in the fraction of Calcein existing in the form of the quadruply-charged anion.

Lastly, the intensity resulting from excitation near 485 nm. increased and then decreased with pH, parallel to the formation and dissociation of the triply- and quadruply-charged anions.

In this case, unlike that of fluorescein, it must be remembered that Hefley[166] relied on measurements of fluorescence for the determination of the fifth and sixth acid dissociation constants from which the distributions shown in figure 18 were derived. Therefore, the correlation between intensities and prototropic forms was not independent proof in itself of the fluorescence of each of the forms in alkaline solution, but the agreement between the individual intensities of excitation and these distributions lent confidence to the assignments.

With confidence, it can be stated that the cation of Calcein and the anions formed by the first four acid dissociations of the neutral molecule are fluorescent, while the neutral form and anions of the fifth and sixth dissociations are not fluorescent. The cation is excited by blue light near 435 nm. All of the fluorescing anions appear to be excited by light near 470 nm. with only the triply- and quadruply-charged anions being additionally excited by the excitation band near 485 nm.

As a result of the predominant intensity of the excitation band at 485 nm., the total fluorescence observed for Calcein in solution resembled the fraction of Calcein existing as the triply- and quadruply-charged anions with only a tailing of the fluorescence toward lower pH resulting from the less intense excitations. This was, indeed, what was observed using a simple filter fluorometer as is also shown in figure 16.

Applying the same curve-fitting technique used in chapter II, the relative coefficients of fluorescence were estimated for each prototropic form of Calcein for each of the three excitation bands. The value of 1.00 was arbitrarily assigned to the coefficient of fluorescence of the quadruply-charged anion resulting from excitation at 485 nm. The results of these calculations are shown in table 3.

The value of the coefficient for excitation of the triply-charged anion at 470 nm. could not be estimated in the region between pH 5 and pH 9 because of the difficulty in locating and measuring the position of that band in the presence of the large band nearby at

Table 3.

Approximate relative coefficients of fluorescence of the prototropic forms of Calcein

Ionic Form	Relative Coefficient		
	435 nm.	470 nm.	485 nm.
H_7Cal^+	0.21	0.00	0.00
H_6Cal	0.02	-0.02	0.00
H_5Cal^-	0.00	0.20	0.00
H_4Cal^{2-}	0.00	0.18	0.04
H_3Cal^{3-}	0.00	unknown	0.96
H_2Cal^{4-}	0.00	0.23	1.00
$HCal^{5-}$	0.00	0.08	0.05
Cal^{6-}	0.00	0.00	0.00

485 nm. It was assumed that, as with the excitation at 485 nm., the coefficient of the triply-charged anion and quadruply-charged anion were equivalent.

One striking characteristic of the fluorometric properties of Calcein was the tendency to undergo collective changes in properties by successive pairs of dissociations. Neutral Calcein was non-fluorescent; the first two anions formed upon neutralization with alkali were excited only at 470 nm. and fluoresced with approximately the same efficiency; the next two anions formed were excited at both 470 and 485 nm. with equivalent coefficients of fluorescence for each of these wavelengths; and, finally, the last two anions formed were not fluorescent. This behavior served as further evidence of the zwitterion structure of the fluorescein portion of the molecule. From the neutral tri-zwitterion, the first two replaceable hydrogens were removed from equivalent carboxylic acid groups on the methyleneiminodiacetic acid branches; the second two hydrogens were removed from the equivalent hydroxyl groups on the fluorescein portion of the molecule; and the third pair was removed from equivalent nitrogen atoms of the methyleneiminodiacetic acid groups. In no

other consistent electronic structure could the six replaceable hydrogen ions be paired in equivalent groups.

Another striking characteristic was the clear analogy between the fluorometric properties of the prototropic forms of Calcein and the prototropic forms of fluorescein. The cations of both species fluoresce when excited at 435 nm. The neutral forms of both compounds are non-fluorescent. The excitation of the monoanion and dianion of Calcein, which only occurred at 470 nm., was similar to that of the monoanion of fluorescein. The excitation of the triply- and quadruply-charged anions of Calcein occurred primarily at 485 nm. but also at 470 nm., just as was observed for the dianion of fluorescein. Only the last two, non-fluorescent anions of Calcein had no direct counterpart in the prototropic forms of fluorescein.

These data supported the connection between the charge contribution of the phenolic groups to the conjugated poly-aromatic rings of the zwitterion and the fluorescence of the fluorophore, as was first proposed by Markuszewski[247]. The comparison between

the fluorescent and non-fluorescent structures of fluorescein and Calcein is shown in Structures VI through XVII. Evidence from nuclear magnetic resonance and electron spin resonance has confirmed that aqueous solutions of the cation and neutral species of both compounds exist with the central, delocalized positive charge while all of the anions demonstrate characteristics of both the zwitterion and quinoid forms shown in figure 19.

4. Estimation of pK_5 and pK_6

One concern must be mentioned regarding the correlation of fluorescence to prototropic forms in section (3). The largest discrepancy from the generalizations made from figure 18 was the fluorescence in the region of pH between 9 and 11. This was the region governed by the fifth and sixth dissociation steps of Calcein.

The fifth and sixth acid dissociation constants were determined by Hefley [166] using data observed near pH 10.7. In this region of pH, Hefley observed a significant shoulder on the sigmasoidal decay of fluorescence with increasing pH. The pH of this shoulder was assumed to represent the equivalence-point of the fifth neutralization of Calcein and, therefore, separated the buffer

Figure 19.

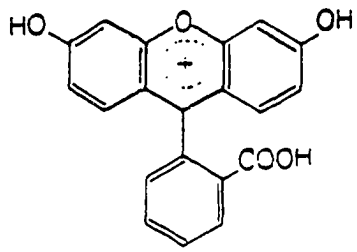
Predominant prototropic forms of fluorescein and Calcein in aqueous solutions

a. Structure VI.

Fluorescing cation of fluorescein

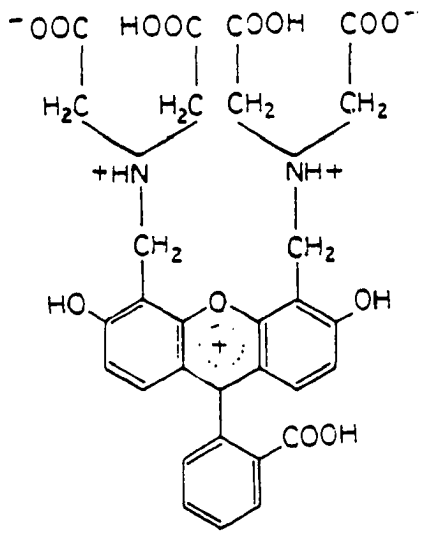
b. Structure VII.
Fluorescing cation of Calcein

a.



STRUCTURE VI

b.



STRUCTURE VII

Figure 19 (continued).

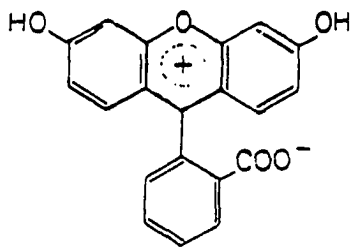
c. Structure VIII.

Non-fluorescing molecule of fluorescein

d. Structure IX.

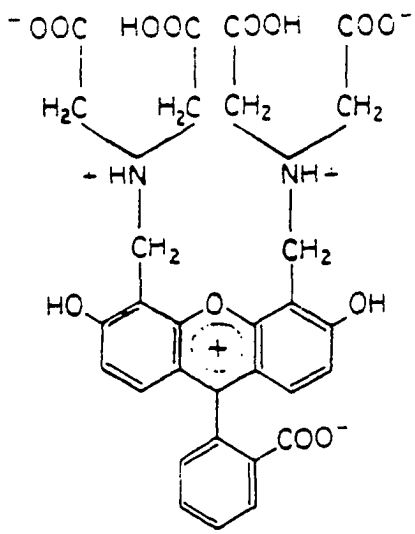
Non-fluorescing molecule of Calcein

c.



STRUCTURE VIII

d.



STRUCTURE IX

Figure 19 (continued).

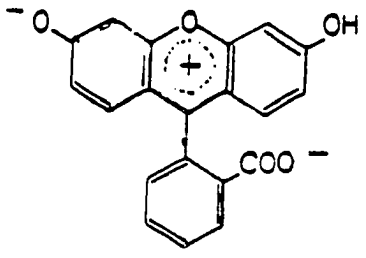
e. Structure X.

Fluorescing monoanion of fluorescein

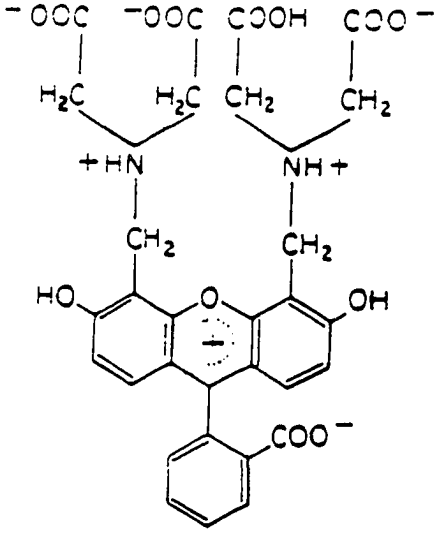
f. Structures XI and XII.

Fluorescing mono- and di-anions of Calcein

e.

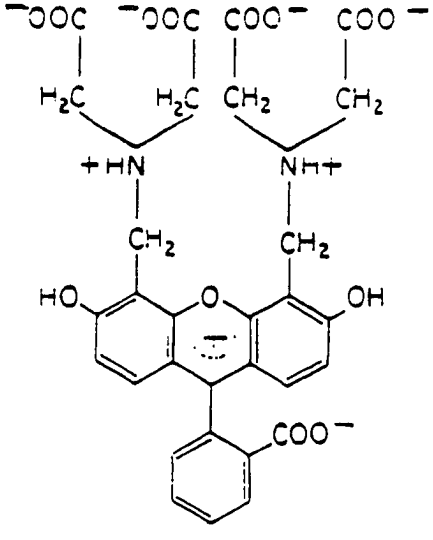


STRUCTURE X



STRUCTURE XI

f.



STRUCTURE XII

Figure 19 (continued).

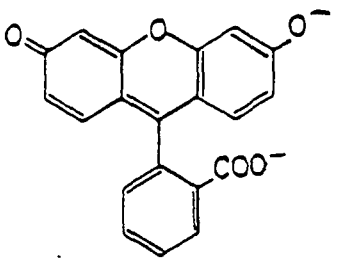
g. Structure XIII.

Fluorescing dianion of fluorescein

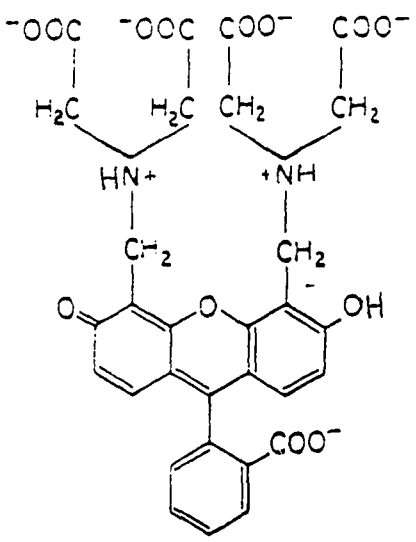
h. Structures XIV and XV.

Fluorescing tri- and tetra-anions of Calcein

g.

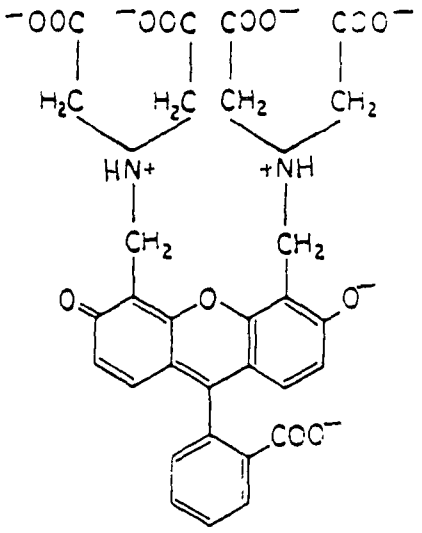


STRUCTURE XIII



STRUCTURE XIV

h.

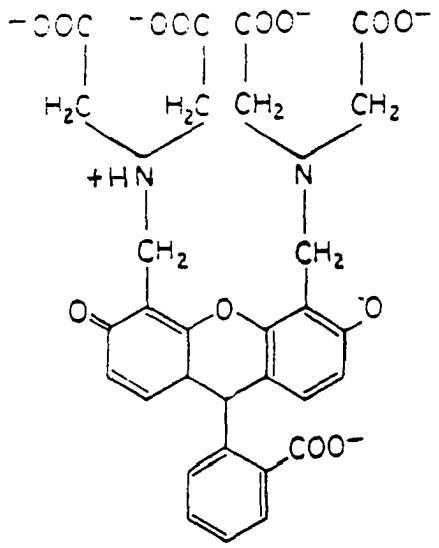


STRUCTURE XV

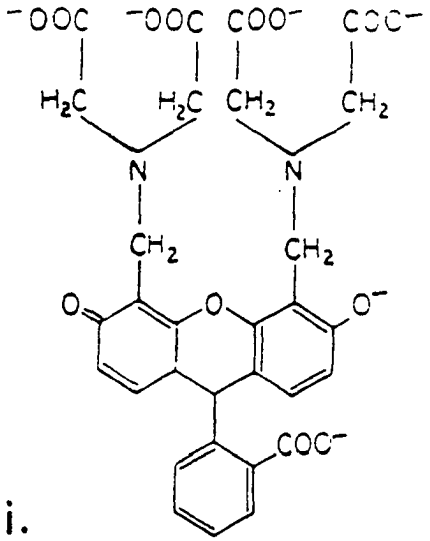
Figure 19 (continued).

i. Structures XVI and XVII.

Non-fluorescing anions of Calcein



STRUCTURE XVI



STRUCTURE XVII

i.

regions of the fifth and sixth replaceable hydrogens. The appropriate acid dissociation constants were then calculated from the fluorescence before and after this apparent deviation from a smooth s-shaped curve. This treatment of the data further assumed that the shoulder was the result of the formation and then the dissociation of a fluorescing anion with only one remaining replaceable hydrogen ion. Unfortunately, as seen in figure 18, the fluorescence observed over the region of pH predicted by these constants to correspond to the anion of the fifth acid dissociation was insufficient to account for the increase in fluorescence that Hefley observed at pH 10.7.

Furthermore, when working with a highly pure material and avoiding metals with which Calcein forms slightly-dissociated compounds, this shoulder was not observed. The data plotted in figure 20 demonstrated the smooth sigmasoidal decrease in fluorescence with increasing pH obtained with a solution containing a highly purified sample of Calcein dissolved in calcium-free potassium hydroxide and containing a small amount of EDTA. Since Hefley did not add a competing chelating agent, the shoulder observed was probably the result of the dissociation of a fluorescent metal-Calcein compound.
For example, as is described in detail

Figure 20.

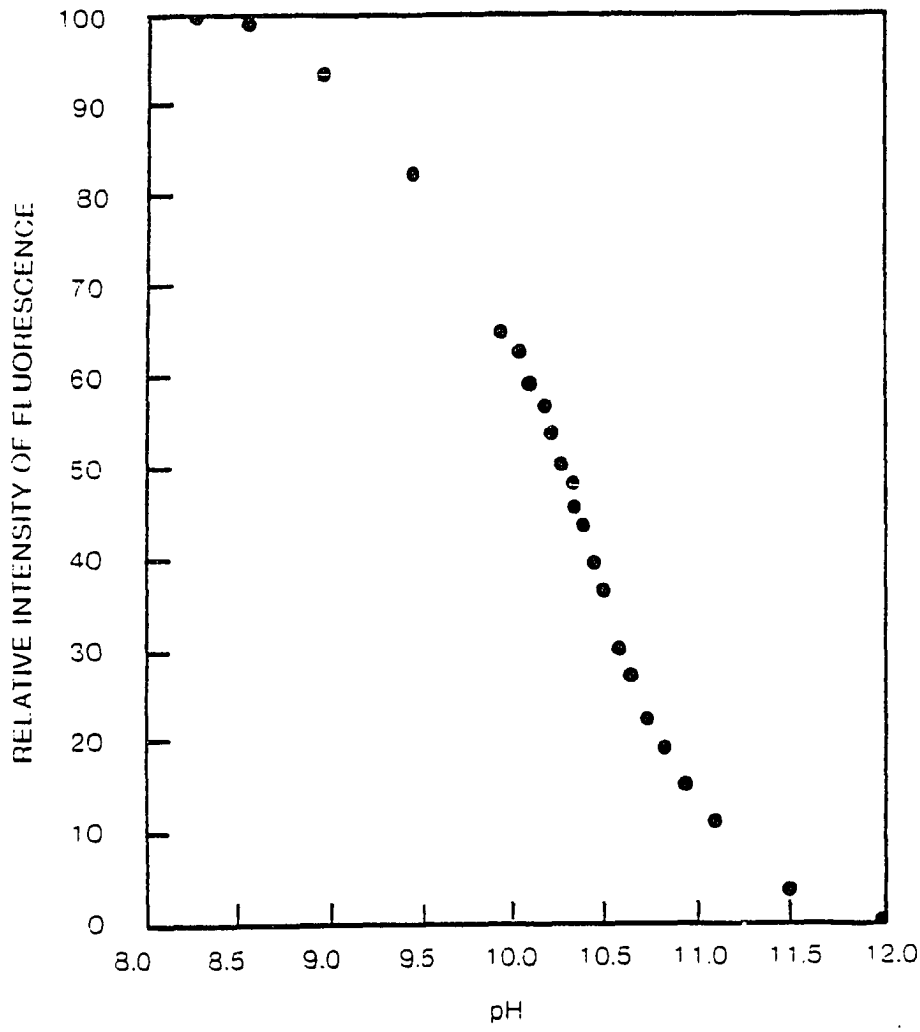
Fluorescence of Calcein near pH 10.5

Measured on an Aminco-Bowman Spectrophotofluorometer
Coarse sensitivity: 3.0
Entrance slit: 1.0 mm.
Exit slit: 1.0 mm.

Concentration of Calcein: 1×10^{-5} M

Emission monochromator set for maximum fluorescence for each solution

Integral of excitation spectra obtained by weighing traced spectra


RELATIVE INTENSITY OF FLUORESCENCE
100
90
80
70
60
50
40
30
20
10
0
8.0
8.5
9.0
9.5
10.0
10.5
11.0
11.5
12.0
pH

in chapter IV, the presence of a small amount of aluminum will produce this very effect. Aluminum, particularly from the distilled water pipes, was avoided in the present work by using freshly distilled and deionized water from an all-glass distillation apparatus.

Two approaches were taken to ascertain values for pK_5 and pK_6 for use in subsequent sections of this dissertation. Both approaches assumed that the sigmasoidal decrease in fluorescence between pH 9 and pH 12 was a result of the dissociation of the fifth replaceable hydrogen ion.

A graphical method of determining the dissociation constant was applied to the region where the fluorescence had been reduced to one-half of the maximum value. To insure that the measurements of fluorescence were of the greatest accuracy, the entire excitation spectrum was acquired for each measurement at the setting for maximum emission. The spectra were cut out and weighed as an estimate of the total integral of fluorescence.

The plot of pH with the negative logarithm of $(F_{HA^-} - F_{A^-})/F_{HA}$ is shown in figure 21. The symbol F_{HA} represents the fluorescence associated with the quadruply-charged anion and was taken as the maximum integral of intensity measured. The symbol F_{A^-} represents the fluorescence of each measurement where the fraction n of the fifth replaceable hydrogen ions have been titrated. The values of F_{A^-} and F_{HA} were taken directly from figure 20. The data indicated a linear relationship with an intercept of 10.25 and a slope of 1.00. As a result, pK_5 was determined to be 10.25. The fifth replaceable hydrogen is therefore not quite as acidic as Hefley's values implied and less difference exists between the fifth and sixth dissociations.

A second, independent approach was taken to estimate both pK_5 and pK_6 by developing a computer curve-fitting program to match the fluorescence with predictions of the prototropic distributions. The values of pK_5 and pK_6 were varied to best fit the curve of fluorescence of figure 20, the value of pK_5 having the greatest effect over the first two-thirds of the curve and the value of pK_6 contributing significantly

Figure 21.

Graphical determination of pK5
from measurements of fluorescence near pH 10

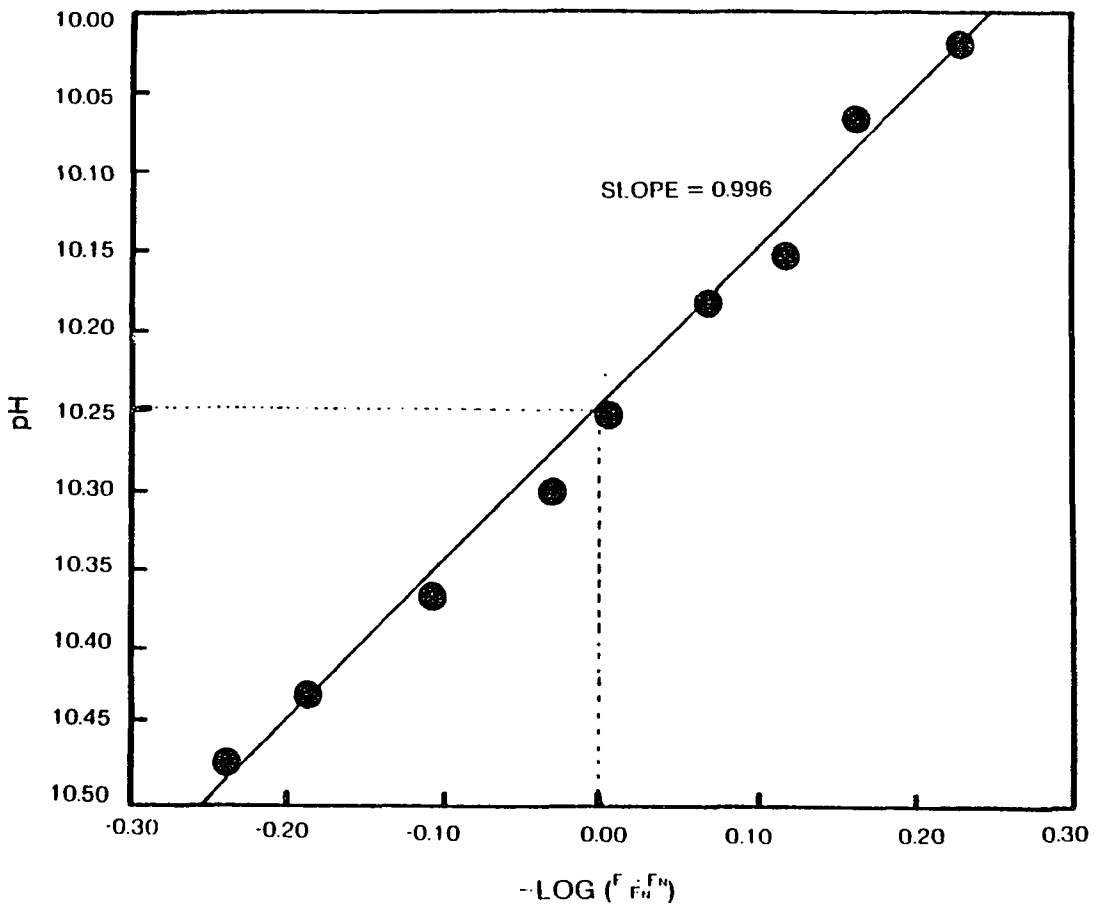
Data taken from figure 19

Measured on an Aminco-Bowman Spectrophotofluorometer
Coarse sensitivity: 3.0
Entrance slit: 1.0 mm.
Exit slit: 1.0 mm.

Concentration of Calcein: 1×10^{-5} M

Zero intercept: 10.25

Slope: 0.990


10.00
10.05
10.10
10.15
10.20
10.25
10.30
10.35
10.40
10.45
10.50
pH
SLOPE = 0.996
-0.30
-0.20
-0.10
0.00
0.10
0.20
0.30
-LOG (F_{FN})

to the values between pH 10.5 and 12. Values for pK_5 and pK_6 were determined to be 10.25 and 11.6 respectively.

The sensitivity of the curve-fitting procedure and the uniqueness of the solution are demonstrated in figure 22. In the center graph, the fluorescence is plotted along with the prototropic distributions using the best fitting values of pK_5 and pK_6. The prototropic distributions predicted by varying the value of pK_5 by 0.05 in each direction are also shown. The best fits corresponded to a pK_5 of 10.25 and a pK_6 of 11.6.

The agreement of the two methods for estimating pK_5 and pK_6, along with the compatibility of these determinations with other constants and measurements, lends confidence to the estimations. For example, the end-point in the titration of Calcein corresponding to the fourth replaceable hydrogen occurs at pH 8.30. The average of the values for pK_4 (6.19 as determined by Hefley) and the value of pK_5 (determined here to be 10.25) is 8.22. Furthermore, the sum of pK_5 and pK_6 is 21.6, in agreement with the measurements by Hefley.

Figure 22.

Estimation of pK_5 and pK_6
by correlation with fluorescence

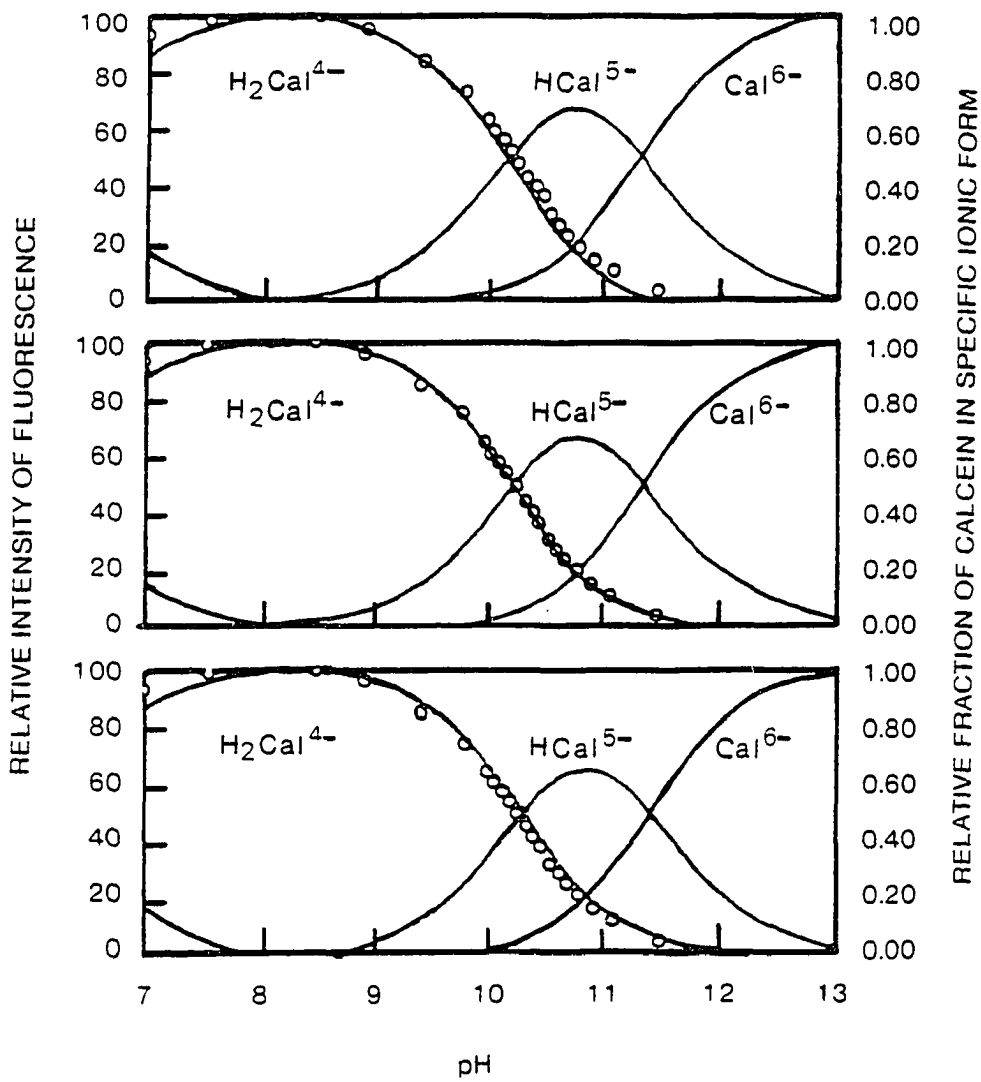
Dotted curves: Calculated fractional distribution
of the prototropic forms of Calcein

Curve A: $pK_5 = 10.20$, $pK_6 = 11.6$
Curve B: $pK_5 = 10.25$, $pK_6 = 11.6$
Curve C: $pK_5 = 10.30$, $pK_6 = 11.6$

Solid curves: Relative intensities of fluorescence
from figure 20

Concentration of Calcein: 1×10^{-5} M

Measured on an Aminco-Bowman Spectrophotofluorometer
Coarse sensitivity: 3.0
Entrance slit: 1.0 mm
Exit slit: 1.0 mm.


RELATIVE INTENSITY OF FLUORESCENCE
RELATIVE FRACTION OF CALCEIN IN SPECIFIC IONIC FORM
H_2Cal^{4-}
$HCal^{5-}$
Cal^{6-}
100
80
60
40
20
0
1.00
0.80
0.60
0.40
0.20
0.00
7
8
9
10
11
12
13
pH

E. Conclusions

The fluorescent properties of Calcein and of each of the prototropic forms of Calcein have been established.

Under proper conditions, the fluorescence of Calcein is comparable in intensity with that previously noted for fluorescein. As with fluorescein, the emission spectrum of Calcein always consists of a single band of yellow-green emission near 515 nm. The same three major excitation bands discovered for fluorescein also constitute the predominant excitation bands of the different ionic forms of Calcein. In fact, the specific wavelengths of all of the excitation and emission bands of Calcein and fluorescein are essentially identical at the same pH.

Below pH 8, only those differences resulting directly from the differences in acid-base strength distinguish the fluorescence of the two compounds. Under these conditions, the electronic transitions of the fluorescein molecule are only minimally disturbed

by the substitution of the methyleneiminodiacetic acid groups onto the aromatic rings. The only net effect of the substitution on the fluorescence in acidic solutions is the influence of the nearby amine groups on the contribution of charge donated by the phenolic moities to the conjugated ring system of the zwitterion of fluorescein.

Above pH 8, the fluorescence of Calcein decreases until there is no significant fluorescence in solutions of highly pure Calcein above pH 12. In alkaline solutions, deactivation of the excited ion is facilitated by the freely moving methyleneiminodiacetic acid groups.

Unfortunately, there is no plateau of fluorescence with pH for Calcein that would be convenient for analytical use as there is with fluorescein. Calcein is highly fluorescent near pH 8 but measurements of the fluorescent intensity under these conditions are highly susceptible to even small changes in pH and excitation wavelength.

The fluorescence observed at pH 13 with solutions

from less pure samples is the result of contamination with (1) fluorescein, present as unreacted starting material or as a decomposition product, and (2) metals which form fluorescent slightly-dissociated compounds with Calcein. Of the two causes of background fluorescence at pH 13, contamination with fluorescein is usually the greatest and most troublesome source. The determination of the amount of fluorescein in Calcein is therefore a critical issue and will be taken up in chapter V.

The individual fluorescent properties of each of the prototropic forms of Calcein are summarized in table 4. The characteristic trait of Calcein to undergo changes in properties in successive pairs of dissociations is further evidence of the triple-zwitterion structure of Calcein in solution. From this electronic structure, the six replaceable hydrogens are withdrawn from three pairs of equivalent positions.

The fluorescence of Calcein has been utilized to estimate K_{-1} and to adjust the values assigned to the fifth and sixth acid dissociation constants. These

Table 4.

Fluorescence of the prototropic forms of Calcein

Prototropic Form of Calcein	pH Range Observed	Excitation Wavelength (nm)	Emission Wavelength (nm)	Relative Coefficient of Fluorescence
H_7Cal^+	below 2.5	435-440	512-515	0.2
H_6Cal	0 - 4	-	-	0.0
H_5Cal^-	1 - 5	466-470	515-517	0.2
H_4Cal^{2-}	2 - 6	466-470	515-517	0.2
H_3Cal^{3-}	3 - 8	466-470	515-520	0.2
H_3Cal^{3-}	3 - 8	483-487	515-520	1.0
H_2Cal^{4-}	5 - 11	466-470	515-520	0.2
H_2Cal^{4-}	5 - 11	483-490	515-520	1.0
$HCal^{5-}$	8 - 13	-	-	0.0
Cal^{6-}	above 10	-	-	0.0

values are used extensively in subsequent sections of this dissertation. The values of the acid dissociation constants of Calcein are listed in table 5.

Table 5.

Acid dissociation constants of Calcein

K_{-1}	=	1.1×10^{-1}
K_1	=	3.2×10^{-3}
K_2	=	3.2×10^{-4}
K_3	=	2.5×10^{-5}
K_4	=	6.3×10^{-7}
K_5	=	5.6×10^{-11}
K_6	=	4.0×10^{-12}

IV. FLUOROMETRIC PROPERTIES OF CALCEIN IN THE PRESENCE OF VARIOUS METALS

A. Introduction

Studies identifying the specific properties of fluorescence for each of the prototropic forms of fluorescein and Calcein have been reported in chapters II and III. To complete this survey, the examination of the fluorescence of Calcein and the slightly-dissociated compounds formed by Calcein in the presence of various metals was undertaken and is reported in this chapter.

Historically, most of the attention given to Calcein has involved the slightly-dissociated compounds formed in solutions containing calcium. Unfortunately, the lack of a highly pure material has severely affected the reliability of these observations. Disagreements concerning the fluorescence, formation constants and even combining ratios of the calcium-Calcein compounds resulted from the use of reagent contaminated with

significant amounts of fluorescein and various metals[64,66,217,359,361]. Similar, though less extensive investigations into compounds with the other alkaline earths[227], aluminum[120,171], and different transistion metals[167,288,357,361,367,370,374] have produced similarly dubious conclusions. With progressively more refined materials, the works of Hefley[166], Martin[253], Markuszewski[247] and, finally, the work described in this dissertation have firmly established the properties of three different types of slightly-dissociated compounds of Calcein with metals.

B. Apparatus and Reagents

The excitation and emission spectra of all solutions were obtained with an Aminco-Bowman Spectrophotofluorometer and recorded with an Aminco-Bowman X-Y Recorder. The spectra were not corrected for changes in the intensity of emission of the xenon lamp with wavelength. The slit width was set to 1 mm. which corresponds to an instrument resolution of approximately 2 nm.

Other measurements of fluorescence were obtained with a Turner Model 110 Filter Fluorometer. The primary filter consisted of a blue Corning 5850 glass filter and the secondary filter included a yellow Corning 2A-15 glass filter and a Wrattan ten percent neutral density filter.

The pH of each solution was measured with a Beckman Zeromatic pH Meter, Model SS-2, equipped either with a Beckman glass indicator electrode and a Beckman saturated Calomel Reference electrode or with a Fisher combination pH indicator/reference electrode. The pH

meter was standardized using solutions of potassium acid phthalate, potassium dihydrogen phosphate and sodium borate.

Some mildly alkaline, dilute solutions of Calcein used for obtaining qualitative information were stored for up to 15 days. For all quantitative data, experiments were repeated with freshly prepared solutions.

Fresh solutions of acid-base buffers were prepared within 15 days of all measurements. Solutions were prepared over the range of pH from 1.00 to 10.50 in increments of 0.3 units or less. The buffers were prepared from hydrochloric acid, potassium acid phthalate, potassium dihydrogen phosphate, boric acid, potassium hydroxide, and potassium chloride according to the procedure of Clarks and Lubs as summarized by Diehl[106]. All of the buffers had an ionic strength of 0.10.

Potassium hydroxide, free of calcium and other interfering metals, was obtained from the Hach Chemical Company of Ames, Iowa in the form of a 12 N solution.

C. Experimental Work

1. Measurement of the excitation and emission of fluorescence of solutions of Calcein in the presence of calcium as a function of pH

A sample of highly pure Calcein weighing 6.860 g was dissolved and diluted to 2.00 liters with 0.10 N calcium-free potassium hydroxide. Aliquots of 1.00 and 10.00 ml. of this solution were further diluted to 100 ml. with 0.10 N calcium-free potassium hydroxide to form solutions of 5.5×10^{-5} and 5.5×10^{-4} M in Calcein at pH 13.

A stock solution of calcium chloride was prepared by dissolving 0.74 g of calcium chloride dihydrate in 250 ml. of deionized water. Aliquots of 1.00 and 10.00 ml. of this stock solution were further diluted to 200 ml. to prepare secondary stock solutions that were 1.0×10^{-4} and 1.0×10^{-3} M in calcium, respectively.

Several series of solutions were prepared for

fluorometric measurements. Within each series of solutions, the concentration of Calcein was varied between 5.5×10^{-9} and 5.5×10^{-5} M, the concentration of calcium was maintained at a ten-fold molar excess over the Calcein, and the pH was held constant. The pH of the different series of solutions varied between 1.0 and 13. Each solution was prepared by adding aliquots of the appropriate amounts of the stock solutions of Calcein and calcium to volumetric flasks and diluting to volume either with one of the buffers or with small portions of 1.0 N potassium hydroxide and water.

The pH was measured for each of the solutions prepared between pH 1.5 and 10.5.

The relative intensity of fluorescence for each of the solutions was measured on a Turner Fluorometer. The excitation and emission spectra of each solution were also acquired with the Aminco-Bowman Spectrophotofluorometer. The excitation spectra were obtained with the wavelength for emission set to the maximum in the emission spectrum, and the emission spectra were obtained with the wavelength of excitation set to the

wavelength of maximum intensity in the corresponding excitation spectra.

2. Measurement of the fluorescence as a function of the relative concentration of calcium

The determination of the combining ratios of calcium and Calcein required meticulous preparations of fresh solutions.

A stock solution of 1.00×10^{-4} M Calcein was prepared from a sample of Calcein analyzed to be 99.6 percent pure as the hydrate. A sample of 0.3204 g of this material was dissolved in 100.0 ml. of 1.00 N calcium-free potassium hydroxide and diluted to 1.00 liters with triply-distilled deionized water. A 100.0-ml. portion of this solution was subsequently diluted to 500 ml. with 0.10 N potassium hydroxide.

A stock solution of 1.00×10^{-3} M calcium was prepared by dissolving 0.101 g of calcium carbonate with 0.1 N hydrochloric acid and diluting to 1.00

liters with freshly deionized water. The solution was stored in a Nalgene bottle to avoid exchange of ions with glass.

Actual working solutions were prepared in 100-ml. volumetric flasks on the same day as the measurement. Each flask contained 10.0 ml. of 1.00 N potassium hydroxide, 10.0 ml. of the stock solution of Calcein, and up to 15.0 ml. of the stock solution of calcium. Solutions were diluted to volume with deionized water. The final concentration of Calcein was 1.00×10^{-5} M and the molar ratio of calcium to Calcein varied from 0 to 15.

The fluorescence of each solution was measured with a Turner Filter Fluorometer equipped with a blue primary filter and a yellow secondary filter and again with the spectrophotofluorometer with the wavelength of excitation at 490 nm. and the wavelength of emission at 520 nm.

3. Measurement of the fluorescence as a function of the absolute concentration of calcium

A sample of the Calcein of high purity, weighing 0.65 g, was dissolved in 100 ml. of 0.01 N potassium hydroxide and diluted to 1.00 liters. Precisely measured aliquots of 5.00 ml. of this solution were transferred to each of three 100-ml. volumetric flasks. To the first flask was added 10 ml. of 1×10^{-5} M EDTA and sufficient buffer of pH 7.5 to dilute to volume. The final pH of this solution was measured to be 7.58. To the second flask was added 10 ml. of 1×10^{-5} M EDTA, 10.00 ml. of 1.0 N potassium hydroxide, and sufficient deionized water to dilute to volume. To the third flask was added 20.0 ml. of 1.00×10^{-3} M calcium chloride, 10.00 ml. of 1.0 N potassium hydroxide, and sufficient deionized water to dilute to volume. The final concentration of Calcein in all three stock solutions was 5.0×10^{-5} M.

Three series of solutions were prepared by consecutive ten-fold dilutions of each of the three stock solutions above, either with the buffer of pH 7.5 or with 0.10 N potassium hydroxide so as to maintain the same pH as in the original solution. The concentration of Calcein in each of the three series of solutions varied

solutions varied from 5.0×10^{-9} to 5.0×10^{-5} M. The concentration of calcium in the third series varied from 2.0×10^{-8} to 2.0×10^{-4} M.

The excitation spectrum of each of the solutions within each series was obtained with the emission monochromator adjusted to the wavelength at which the maximum emission occurred for the particular pH of that solution.

4. Measurement of the fluorescence in the presence of other alkaline earths

The experimental procedures described in sections 1 and 2 above were repeated with the equivalent molar substitutions of magnesium, strontium and barium chlorides for the calcium compounds. The excitation and emission spectra were obtained in a similar fashion with a spectrophotofluorometer. The total fluorescence of each solution was also measured with a filter fluorometer.

5. Measurement of fluorescence in the presence of aluminum

A stock solution of 0.10 M aluminum chloride was prepared by dissolving 23.8 g of aluminum chloride hexahydrate in 1.00 liters of deionized water. The stock solution of 1.0×10^{-3} M Calcein prepared in section 4 was also used in these experiments.

Solutions were prepared for the measurements of excitation and emission from pH 0 to pH 12. Into each of a series of 100-ml. volumetric flasks was placed 1.00 ml. of the stock solution of 1.0×10^{-3} M Calcein and 1.00 ml. of the 0.10 M solution of aluminum chloride. The solutions were then diluted to volume with the appropriate buffers, 0.01 N potassium hydroxide, or 1.0 N hydrochloric acid. The final concentrations of Calcein and aluminum in these solutions were 1.0×10^{-5} and 1.0×10^{-4} M, respectively. The pH of all solutions between pH 2 and pH 11 were precisely measured. The excitation and emission spectra were then obtained using the Aminco-Bowman Spectrophotofluorometer. The fluorescence of each of these solutions was also

measured with the Turner Fluorometer.

Another series of solutions was prepared from the same stock solutions to determine the fluorescence at different molar ratios of aluminum and Calcein. To each of a series of 100-ml. volumetric flasks was added 1.00 ml. of 1.0×10^{-3} M Calcein. An increment between zero and 5.00 ml. of 1.0×10^{-3} M aluminum chloride was then added to each flask from a 10-ml. buret. The solutions were diluted to volume with a 0.10 M solution of potassium hydrogen phthalate. The relative intensity of fluorescence was measured on the filter fluorometer.

6. Measurement of the fluorescence in the presence of certain transition metals

A stock solution of 1.0×10^{-3} M Calcein was prepared as described in section 2. Solutions of 1.0×10^{-3} M iron(III) chloride and nickel(II) chloride were prepared by dissolving 0.27 g of ferric chloride hexahydrate and 0.24 g of nickelous chloride hexahydrate, respectively, in 1.00 liters of deionized

water. Approximately 10 ml. of 0.1 N hydrochloric acid was added to assist the dissolutions.

Solutions were prepared for the measurement of excitation and emission by placing 1.00 ml. of the stock solution of Calcein and 5.00 ml. of either the iron or nickel solution in each of a series of 100-ml. flasks and diluting to volume with the buffer solutions described in section B. Similar solutions at very low pH were prepared using 1.0 N hydrochloric acid. The excitation spectra were obtained with the emission maximized and then the emission spectra were obtained at the wavelength of the maximum excitation for each solution.

Another set of solutions was prepared for each of the metals. Aliquots of 1.00 ml. of 1.0×10^{-3} M Calcein were measured into each of two series of 100-ml. flasks. Increments of either 1.0×10^{-3} M iron(III) or 1.0×10^{-3} M nickel(II) were then added by a 10-ml. buret to correspond to molar ratios of up to 10 times the amount of Calcein. The solutions were then diluted to volume with a buffer of pH 8.0. The fluorescence was measured with a filter fluorometer.

D. Results and Discussion

1. Excitation and emission of fluorescence of Calcein in the presence of calcium as functions of pH

The excitation and emission spectra of the slightly-dissociated compounds of Calcein with calcium were obtained for solutions of pH 1 to pH 13. The intensity resulting from each excitation and emission band was measured and the fluorescence of each of the prototropic forms of Calcein and the compounds of Calcein with calcium were identified.

All of the emission spectra of solutions containing Calcein and calcium consisted of a single, narrow band of yellow-green fluorescence near 515 nm. As with fluorescein and Calcein alone, the center of this band shifted slowly with increasing pH, moving from 512 nm. at pH 1 to 520 nm. at pH 13. The position and shape of the emission band was independent of the wavelength of excitation. For example, the emission spectrum of a highly alkaline solution of 5×10^{-6} M Calcein with a ten-fold excess of calcium is shown in

figure 23.

In contrast to the uniformity of the emission spectra, the excitation spectra of Calcein in the presence of calcium were significantly different under different conditions of pH. As with fluorescein and Calcein alone, excitation to fluorescence was initiated by the same three major and six minor excitation bands. The number, relative intensity and position of these bands in a particular spectrum depended on the pH of the solution. However, no new bands were detected.

Of primary concern for the work involving the slightly-dissociated compounds of Calcein with calcium has been the fluorescence in highly alkaline solutions. Under these conditions, the excitation spectrum was dominated by a single, large band at 490 nm. with a visible shoulder at 470 nm. For example, the excitation spectrum at pH 13 is shown in figure 24. The intensity of fluorescence at pH 13 was almost equivalent to that of an equimolar solution of fluorescein at the same pH.

Under the conditions of concentration used in the

Figure 23.

Emission spectrum of Calcein in the presence of calcium at pH 13

Measured on an Aminco-Bowman Spectrophotofluorometer

Excitation monochromator setting: 490 nm.
Coarse sensitivity: 3.0
Entrance slit: 1.0 mm.
Exit slit: 1.0 mm.

Concentration of Calcein: 5.5×10^{-6}M
Concentration of calcium: 5.5×10^{-5}M

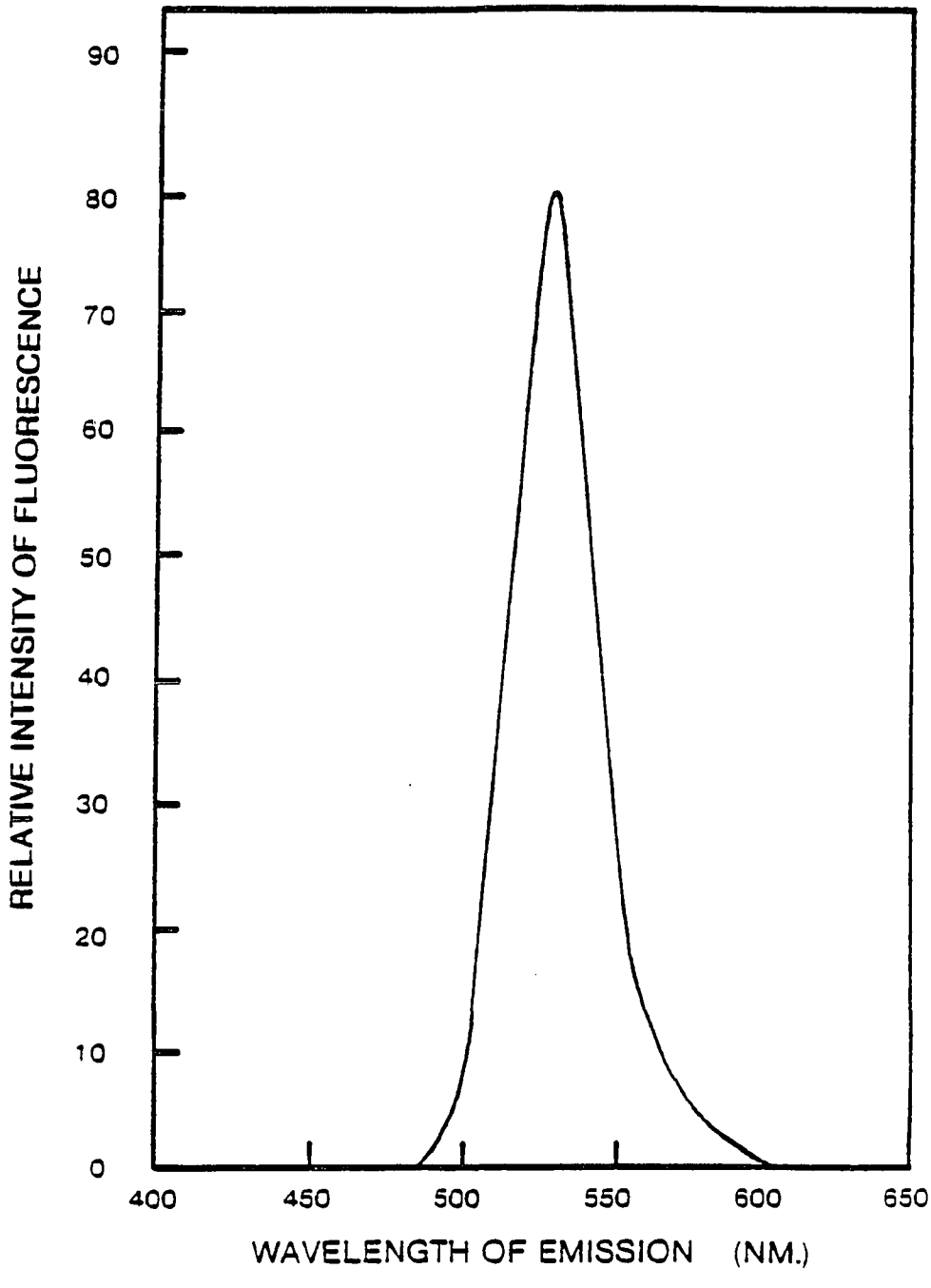

RELATIVE INTENSITY OF FLUORESCENCE
90
80
70
60
50
40
30
20
10
0
400
450
500
550
600
650
WAVELENGTH OF EMISSION (NM.)

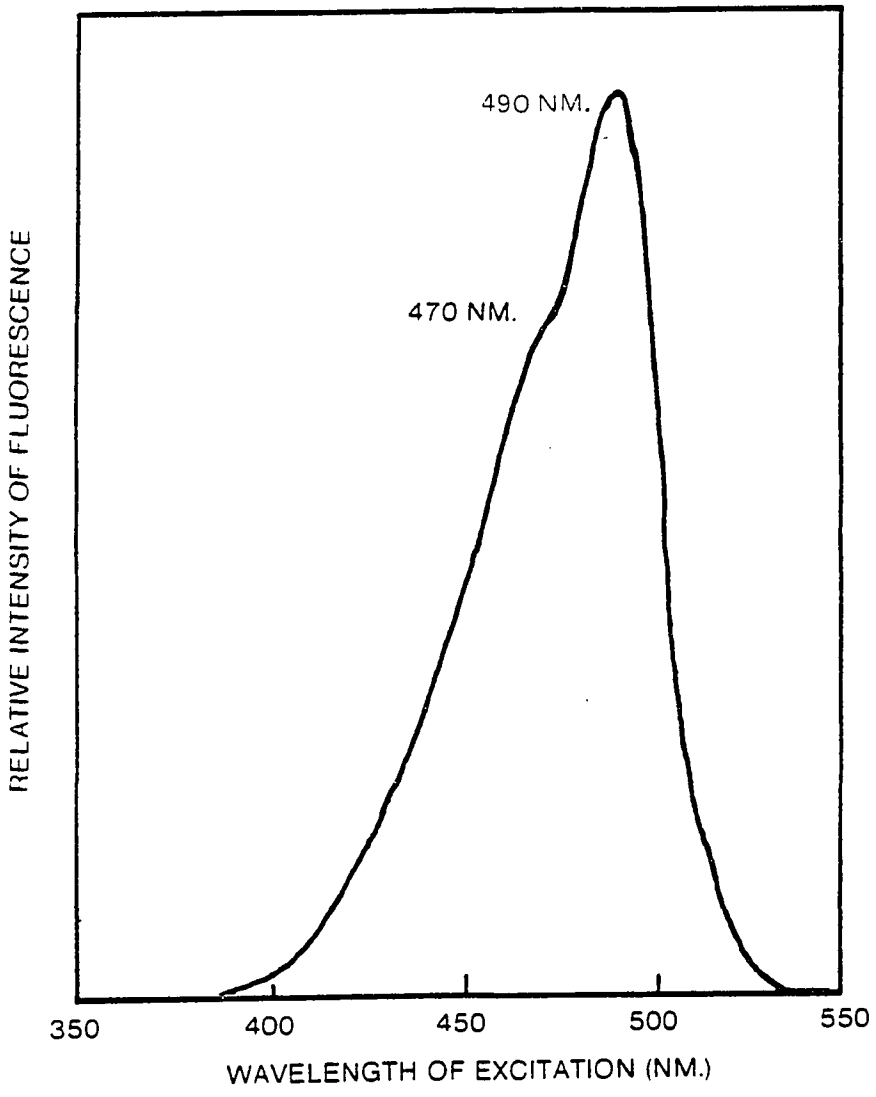
Figure 24.

Excitation spectrum of Calcein in the presence of calcium at pH 13

Measured on an Aminco-Bowman Spectrophotofluorometer
Emission monochromator setting: 520 nm.

Coarse sensitivity: 3.0
Entrance slit: 1.0 mm.
Exit slit: 1.0 mm.

Concentration of Calcein: 5.5×10^{-6} M
Concentration of calcium: 5.5×10^{-5} M


490 NM.
470 NM.
RELATIVE INTENSITY OF FLUORESCENCE
350
400
450
500
550
WAVELENGTH OF EXCITATION (NM.)

initial study (5×10^{-6} M Calcein and 5×10^{-5} M calcium), the intensity of fluorescence decreased slightly as the pH was lowered from 12 to 10 and then increased again as the pH was lowered further from 10 to 8. In the region between pH 12 and pH 8, the excitation band at 490 nm. became less overwhelming and the contribution of excitation at 470 nm. became more detectable as a second peak in the excitation spectra. As the pH was lowered further to 8.0, the fluorescence regained the full intensity associated with triply- and quadruply-charged anions of Calcein. The presence of calcium apparently had no measurable effect on the fluorescence of forms of Calcein in which the replaceable hydrogen atoms were still tightly associated with the tertiary amine groups.

In solutions with pH below 8.0, allof the prototropic forms of Calcein have the replaceable hydrogens associated with the tertiary amine groups and the spectra obtained were identical to the corresponding spectra shown in figure 17 for Calcein alone.

Thus, the excitation and emission of fluorescence

by fluorescein, Calcein, and the slightly-dissociated compounds of Calcein with calcium always occurred by the same fundamental electronic transitions. The exact wavelengths of the corresponding bands were affected solely by the pH of the solution. Only the relative intensities of these bands differed in the spectra of the three compounds.

However, unlike the evaluation of the fluorescent properties of fluorescein and Calcein alone, the final assignment of fluorescent properties to individual prototropic and slightly-dissociated forms of Calcein in the presence of calcium also required the examination of fluorescence as a function of the relative and absolute concentrations of calcium.

2. Fluorescence as a function of the relative concentration of calcium

The combining ratios of Calcein and calcium were confirmed by examining the fluorescence of solutions of Calcein as a function of the relative concentration of calcium. These measurements were made with both a

spectrophotofluorometer and with a filter fluorometer.

In figure 25, the intensity of fluorescence was plotted as a function of the ratio of calcium to Calcein. The data fit easily into three linear segments. Between the molar ratios of 0.0 and 0.8, the intensity of fluorescence increased linearly with increasing relative amounts of calcium. Between the ratios of 1.2 and 1.8, the fluorescence increased linearly with increasing calcium at approximately four times the rate exhibited in the first region. Beyond a molar ratio of 3, the fluorescence was independent of the relative concentrations, provided that the solubility of calcium had not been exceeded. Extrapolations of the three linear segments intersected at molar ratios of 1.0 and 2.0 and intercepted the origin at 0.0. The data clearly confirmed the successive formation of, first, a 1:1 fluorescent compound, and then, a 2:1 compound of calcium and Calcein that was even more highly fluorescent.

The relative slopes from these measurements are highly dependent on the wavelength of excitation used. For example, measuring the fluorescence of the

Figure 25.

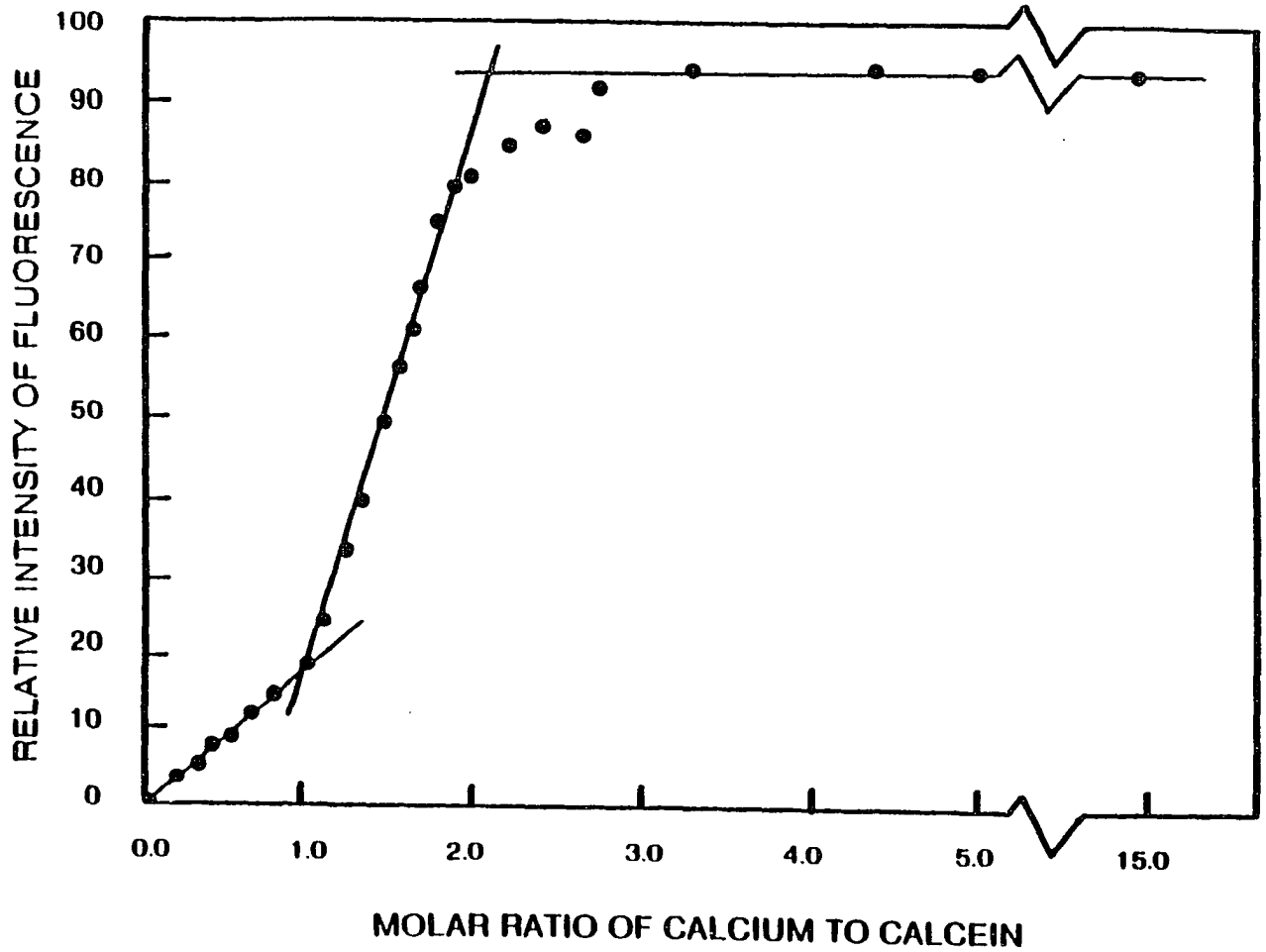
Fluorescence of Calcein as a function of
the relative concentration of calcium

Measured on an Aminco-Bowman Spectrophotofluoromter
Excitation monochromator setting: 490 nm.
Emission monochromator setting: 520 nm.

Coarse sensitivity: 3.0
Entrance slit: 1.0 mm.
Exit slit: 1.0 mm.

Concentration of Calcein: 1.00×10^{-5} M

pH of solution: 13.0


RELATIVE INTENSITY OF FLUORESCENCE
100
90
80
70
60
50
40
30
20
10
0
0.0
1.0
2.0
3.0
4.0
5.0
15.0
MOLAR RATIO OF CALCIUM TO CALCEIN

identical solutions with a filter fluorometer resulted in only a 2:1 ratio of the slopes for the 1:1 and 2:1 compounds of calcium and Calcein. The differences are a result of the bias of the wavelengths or filters toward different portions of the excitation bands. This will be explained further in section 4, below.

These results refuted earlier claims that only the 1:1[64,65,201] or only the 2:1[361] compound of calcium and Calcein fluoresces and confirmed the conclusions of Hefley[166] and Martin[253] that two distinct, fluorescing compounds of Calcein are formed with calcium. Furthermore, these results demonstrated that, with the proper understanding of the two successively formed slightly-dissociated compounds, the so-called complex calibration curves often cited actually consist of two linear portions, each of which can be used as a linear calibration curve. In addition, with a highly pure material, a linear calibration curve is available that has an intercept of zero. The utility of this property will be amply documented in chapter VII of this dissertation.

3. Fluorescence as a function of the absolute concentration of calcium

While the fluorescence of Calcein as a function of the relative concentration of calcium has been repeatedly examined in the past with varying success, no attention has been paid to the effect of the absolute concentration of calcium and Calcein on the equilibria involved in the formation of the slightly-dissociated compounds. In fact, several examples of apparent discrepancies in the properties of Calcein in the literature can be explained in terms of an insufficient absolute concentration of Calcein and calcium. Simply assuring that calcium is present in excess of Calcein is not sufficient to assure complete and reproducible formation of the fluorescent compounds.

The excitation spectra of several solutions of Calcein between 5×10^{-9} M and 5×10^{-5} M, each with a four-fold excess of calcium, were obtained at pH 13. The spectra, obtained using solutions of equivalent concentrations of Calcein, were also obtained at pH 7.5

and at pH 13 with EDTA added in place of calcium. The wavelength for emission was adjusted to maximize the fluorescence for each excitation spectrum at pH 13.

The excitation spectra for the solutions described above were obtained with different sensitivities. Thus, for comparison, the relative intensities required normalization. This was accomplished by measuring the ratios of the intensities at various wavelengths in the excitation spectrum of the solution containing calcium at pH 13 with the maximum intensity of a solution at pH 7.5 with an identical concentration of Calcein but with EDTA added in place of calcium. The wavelength for emission was set to 490 nm. for measurements at pH 13, and 485 nm. for measurements at pH 7.5. Examples of the resulting normalized excitation spectra for solutions at pH 13.0, having different absolute concentrations of Calcein and a four-fold excess of calcium are plotted in figure 26.

The parallel increases in the concentrations of Calcein and calcium were accompanied by a dramatic increase in the total relative fluorescence at pH 13. This is clearly indicated in figure 26. This effect

Figure 26

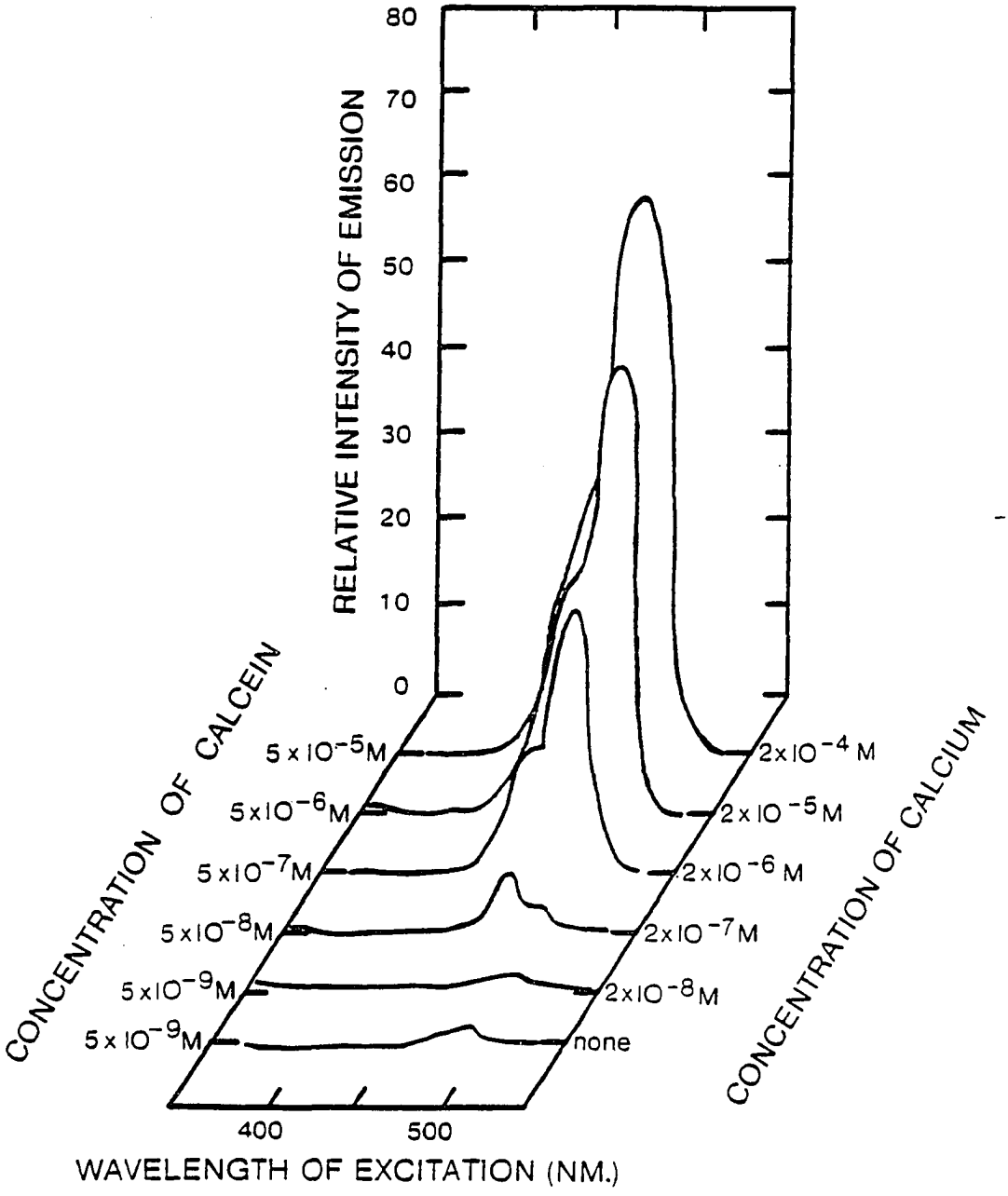
Excitation spectra of the calcium-Calcein compound
at various absolute concentrations
of calcium and Calcein

Measured on an Aminco-Bowman Spectrophotofluoromter
Emission monochromator setting: 519 nm.

Coarse sensitivity: 3.0
Entrance slit: 1.0 mm.
Exit slit: 1.0 mm.

Molar ratio of calcium to Calcein of 4:1
(except for first curve, 5.5×10^{-9} M Calcein, no calcium)

pH of solutions: 13.0


80
70
60
50
40
30
20
10
0
RELATIVE INTENSITY OF EMISSION
CONCENTRATION OF CALCEIN
5 x 10⁻⁵M
5x10⁻⁶M
5x10⁻⁷M
5x10⁻⁸M
5x10⁻⁹M
5 x 10⁻⁹M
2x10⁻⁴ M
2 x 10⁻⁵M
2x10⁻⁶ M
2x10⁻⁷M
2x10⁻⁸M
none
CONCENTRATION OF CALCIUM
400
500
WAVELENGTH OF EXCITATION (NM.)

emphasizes the importance of considering the effect of absolute concentrations on the equilibria involved in the formation of slightly-dissociated compounds, an effect that cannot be ignored in analytical applications.

What is even more instructive in figure 26, though, is the marked differences in the relative intensities of the two excitation bands active at this pH. At low concentrations, where significant formation of only the 1:1 compound can be expected, the excitation primarily involved the band near 470 nm. As the concentrations were increased, and formation of the 2:1 compound became more prevalent, the more intense band at 490 nm. overtook the band at 470 nm. At concentrations of Calcein above 1×10^{-5} M and concentrations of calcium above 5×10^{-5} M, the band at 490 nm. became the only one visible and no further increase in relative fluorescence was observed with increasing concentrations.

A more practical illustration of the effect of absolute concentrations is shown in figure 27. In this figure, the intensities of excitation are correlated

Figure 27.

Correlation of fluorescence with ionic forms
of Calcein in the presence of calcium
at different absolute concentrations

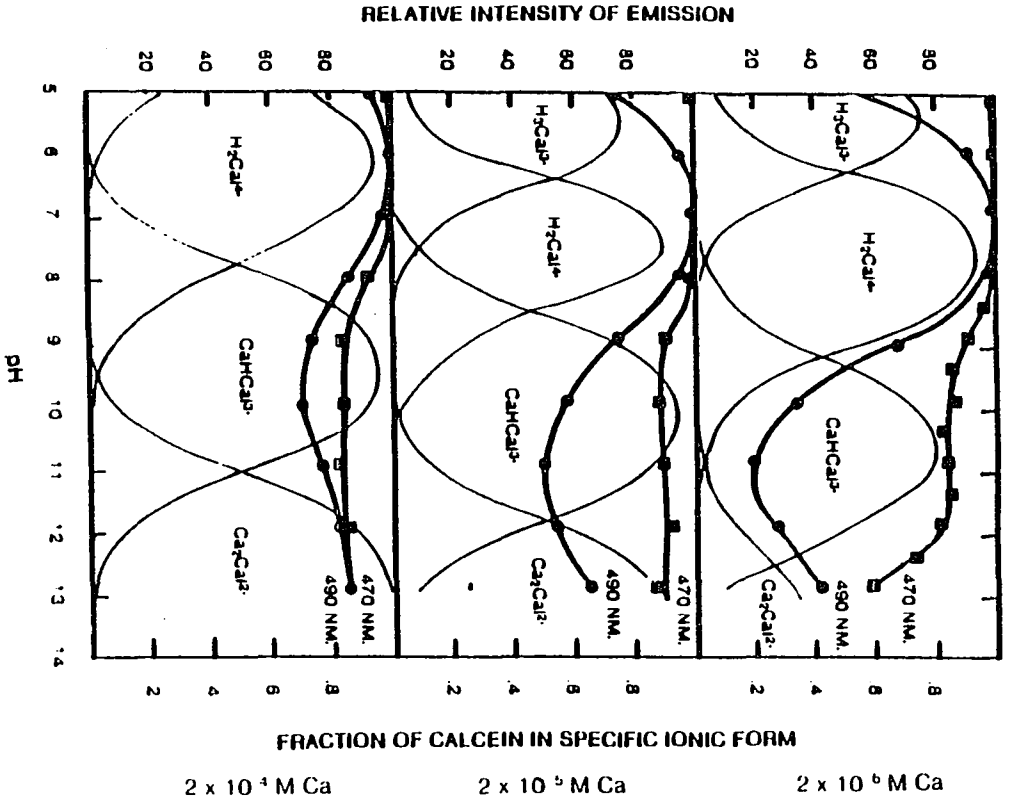
Measured on an Aminco-Bowman Spectrophotofluorometer
Coarse sensitivity varied
Entrance slit: 1.0 mm.
Exit slit: 1.0 mm.

Molar ratio of species
constant at 4:1 calcium to Calcein

Top Graph:
Concentration of Calcein: 5×10^{-7} M
Concentration of calcium: 2×10^{-6} M

Middle Graph:
Concentration of Calcein: 5×10^{-6} M
Concentration of calcium: 2×10^{-5} M

Bottom Graph:
Concentration of Calcein: 5×10^{-5} M
Concentration of calcium: 2×10^{-4} M


RELATIVE INTENSITY OF EMISSION
pH
H₃Cal²⁻
H₂Cal³⁻
HCal⁴⁻
CaHCal³⁻
Ca₂Cal²⁻
470 NM.
490 NM.
FRACTION OF CALCEIN IN SPECIFIC IONIC FORM
2 x 10⁻⁴ M Ca
2 x 10⁻⁵ M Ca
2 x 10⁻⁶ M Ca

with the fractional distribution of Calcein existing in certain ionic forms in alkaline solutions containing excess calcium. Calculation of these distributions required use of the formation constants for the one-to-one and two-to-one compounds of calcium with Calcein. Hefley[166] determined values for these constants by carefully measuring the pH during the potentiometric titration of Calcein in the presence of calcium ions. Unfortunately, the values determined relied, in part, on the fifth and sixth acid dissociation constants shown to be incorrect in chapter III. After correcting these values with the new estimates of K_5 and K_6, the formation constants of the one-to-one and two-to-one compounds of calcium and Calcein are

$$K^{Ca^{2+}\cdot HCal^{5-}}_{CaHCal^{3-}} = 6.0 \times 10^{6} \qquad (11)$$

$$K^{2Ca^{2+}\cdot Cal^{6-}}_{Ca_2Cal^{2-}} = 1.2 \times 10^{11} \qquad (12)$$

These values for the formation constants, along with the acid dissociation constants listed in table 5, were

used to calculate the distribution of Calcein among the possible prototropic forms and slightly-dissociated compounds as a function of pH. Figure 27 includes the results of these calculations at three different absolute concentrations of Calcein with the same 4:1 molar ratio of calcium to Calcein in each case.

The relative intensities of excitation for the two bands at 470 and 490 nm. were measured for solutions with each of these concentrations and are also plotted in figure 27. As shown in figure 18, the intensities for each wavelength were normalized to the maximum intensity measured at that wavelength over the entire range of pH examined.

The utilization of the fluorescence of Calcein in the presence of calcium for the accurate and reproducible determination of Calcein requires the reproducible formation of the same fluorescent compound with a minimum of sensitivity to small changes in conditions. Clearly, this is best achieved at concentrations above 5×10^{-6} M Calcein and 1×10^{-5} M calcium. Below this concentration, significant amounts of Calcein remain unassociated at high pH (the

distribution curves of these forms are not shown in figure 27 for clarity) and the amount of fluorescing species is highly dependent on the concentration of calcium and on pH.

4. Correlation of fluorescence of the calcium compounds of Calcein with ionic forms

Plotting the calculated fractional distributions of the various prototropic forms of Calcein and the slightly-dissociated compounds of Calcein and calcium as functions of pH and the concentration of calcium, as was done in figure 27, also permitted the identification of the individual fluorometric properties of each of the ionic forms.

At all of the concentrations of calcium examined, the triply- and quadruply-charged anions of Calcein remained the most highly fluorescent forms. However, as the concentration of calcium was increased, the pH at which the third and fourth replaceable hydrogens dissociated was lowered and the formation and fluorescence of the slightly-dissociated compounds

Table 6.

Fluorescence of the slightly-dissociated compounds of Calcein and calcium

Ionic Form	Excitation Wavelength (nm.)	Emisssion Wavelength (nm.)	Relative Coefficient of Fluorescence
$CaHCal^{3-}$	470	520	0.17
$CaHCal^{3-}$	490	520	0.45
Ca_2Cal^{2-}	470	520	0.18
Ca_2Cal^{2-}	490	520	0.82

became increasingly important in alkaline solutions.

Both the 1:1 and 2:1 compounds of calcium and Calcein were excited by light at both 470 nm. and 490 nm. The relative intensities of excitation of the two slightly-dissociated compounds at 470 nm. appeared to be relatively equivalent and only slightly less intense than the intensity of the anions resulting from the third and fourth acid dissociations. The relative intensity of excitation at 490 nm., however, was definitely much greater for the 2:1 compound than the 1:1 calcium-Calcein compound. As a result, solutions containing less than 1×10^{-5} M calcium suffer from a measurable dip in fluorescence between pH 10 and pH 12. Thus, the reported sporatic anomaly is easily explained in terms of the mass action of the equilibria and the greater intensity of fluorescence associated with the 2:1 compound.

The fluorescent characteristics for the calcium-Calcein compounds are summarized in table 6. For consistency, the relative intensities are calculated with respect to the fluorescence of the triply- and quadruply-charged anions at pH 7.5 when excited at 485.

5. Fluorescence in the presence of other alkaline earths

In addition to calcium, Calcein forms fluorescent compounds in highly alkaline solutions with magnesium, strontium and barium. Analogous studies to those with calcium were completed for each of these three alkaline earths, to discern if any difference in the process of fluorescence could be distinguished that would prove useful in the analysis of Calcein or in the use of Calcein for the determinations of these materials.

The excitation and emission spectra of Calcein in the presence of each of these metals were identical in shape and position to the spectra with calcium at the same pH. Only small changes in the relative intensities of the peaks were measured. All of the compounds of Calcein with the alkaline earths are moderately excited at 470 nm. and strongly excited at 490 nm. The emission band at all values of pH is identical to that of Calcein alone, to that of Calcein

with calcium and to that of fluorescein.

The fluorescence of Calcein alone and Calcein in the presence of magnesium, strontium and barium were measured with a filter fluorometer and plotted in figure 28. The fluorescence of Calcein with magnesium is slightly greater than that with calcium from pH 9 to pH 10.5, but the reaction of magnesium to form hydroxides competes with the formation of the slightly-dissociated compounds with Calcein and eliminates the fluorescence above pH 12.5. The fluorescence at pH 13 is successively less intense for the calcium, strontium and barium compounds, the decrease being relatively evenly shared between the excitations at 470 and 490 nm.

A plot of the fluorescence as a function of the relative concentrations of the alkaline earths is shown in figure 29. Each of the alkaline earths examined forms both 1:1 and 2:1 compounds with Calcein. The fluorometric properties of these metal-Calcein compounds are summarized in table 7. Again, the relative coefficients of fluorescence are given in comparison to the excitation of H_2Cal^{4-} at pH 7.5 when

Figure 28.

Fluorescence of Calcein in the presence of
alkaline earths as a function of pH

Measured on a Turner Model 110 Filter Fluorometer

Primary filter: Corning 5850 glass filter
Secondary filter: Corning 2A-15 glass filter
& Wratten ten percent neutral density filter

Sensitivity: 10X

Concentration of Calcein: 1×10^{-5} M
Concentration of alkaline earths: 1×10^{-4} M

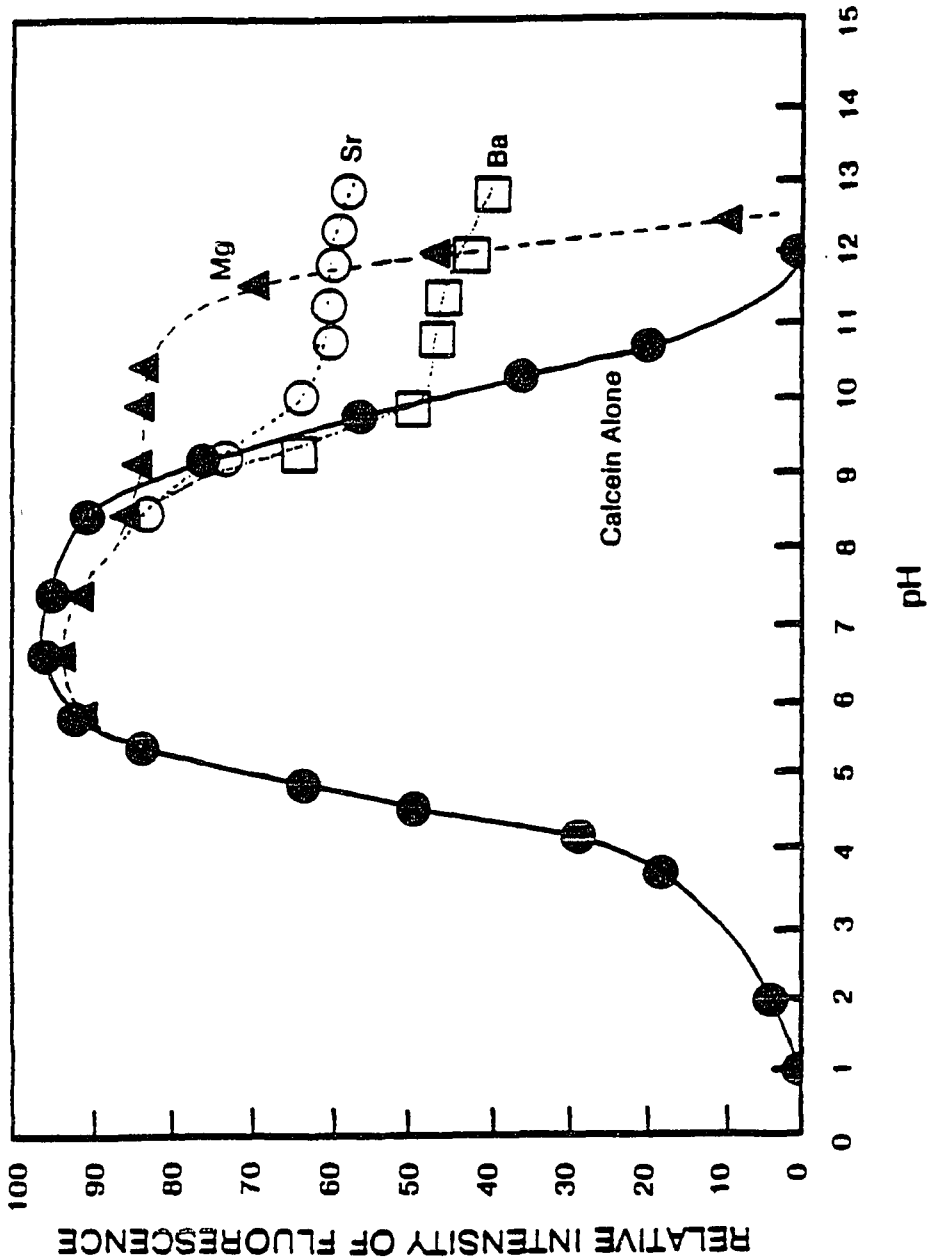

RELATIVE INTENSITY OF FLUORESCENCE
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
pH
Mg
Sr
Ba
Calcein Alone

Figure 29.

Fluorescence of Calcein as a function of the relative concentrations of alkaline earths

Measured on a Turner Model 110 Filter Fluorometer

Primary filter: Corning 5850 glass filter
Secondary filter: Corning 2A-15 glass filter
& Wratten ten percent neutral density filter

Sensitivity: 10X

Concentration of Calcein: 1×10^{-5} M

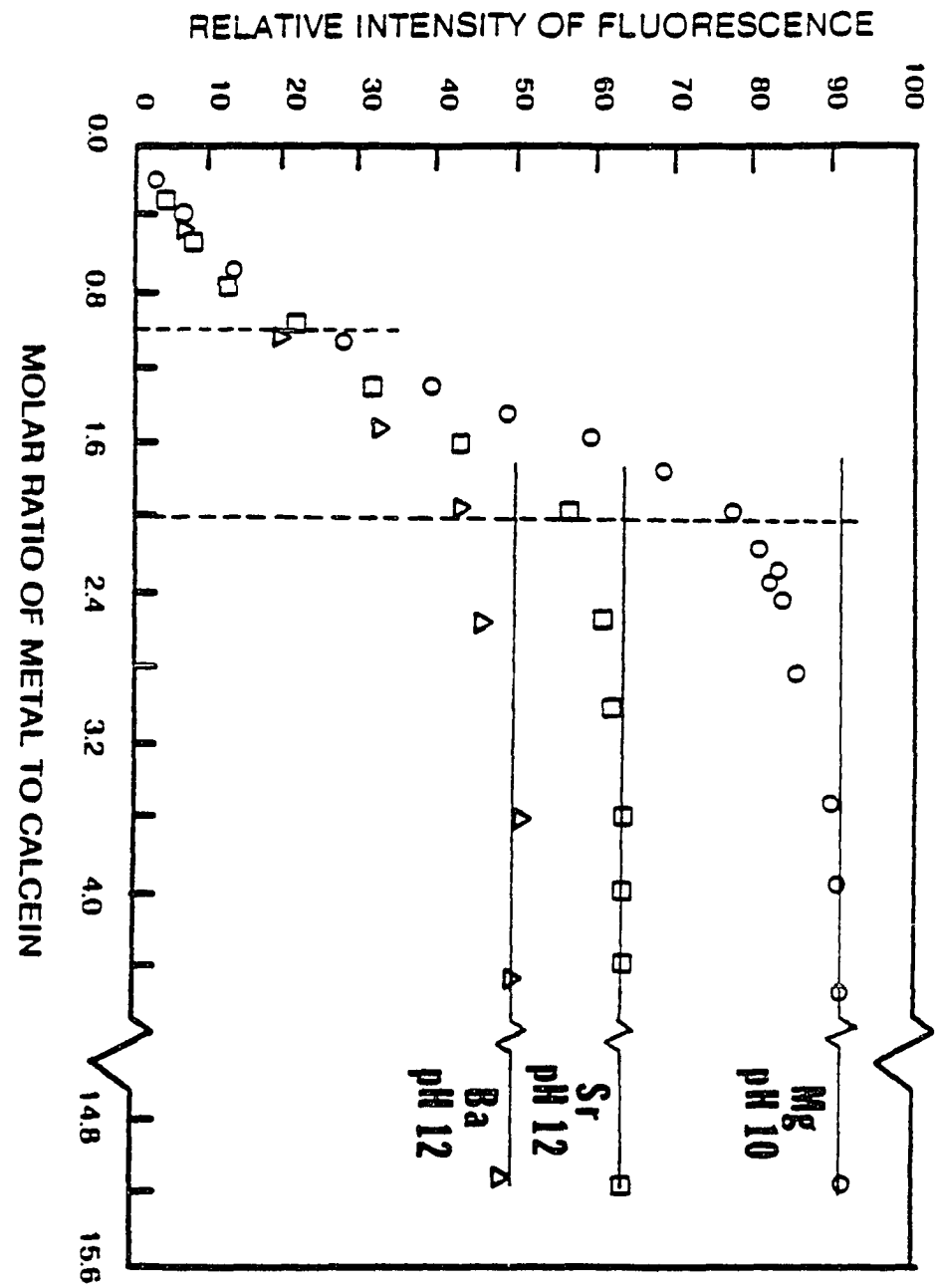

RELATIVE INTENSITY OF FLUORESCENCE
0
10
20
30
40
50
60
70
80
90
100
MOLAR RATIO OF METAL TO CALCEIN
0.0
0.8
1.6
2.4
3.2
4.0
14.8
15.6
Mg
pH 10
Sr
pH 12
Ba
pH 12

Table 7.

Fluorescence of compounds of Calcein with alkaline earths

Ionic Form	Excitation Wavelength (nm.)	Emission Wavelength (nm.)	Relative Coefficient of Fluorescence	
$MgHCal^{3-}$	470	520	0.15	(pH 10)
$MgHCal^{3-}$	490	520	0.50	(pH 10)
Mg_2Cal^{2-}	470	520	-	
Mg_2Cal^{2-}	490	520	-	
$SrHCal^{3-}$	470	520	0.10	(pH 13)
$SrHCal^{3-}$	490	520	0.25	(pH 13)
Sr_2Cal^{2-}	470	520	0.15	(pH 13)
Sr_2Cal^{2-}	490	520	0.65	(pH 13)
$BaHCal^{3-}$	470	520	0.10	(pH 13)
$BaHCal^{3-}$	490	520	0.20	(pH 13)
Ba_2Cal^{2-}	470	520	0.10	(pH 13)
Ba_2Cal^{2-}	490	520	0.40	(pH 13)

excited at 485 nm.

6. Fluorescence in the presence of aluminum

The formation of a slightly-dissociated compound of Calcein with aluminum was noted by several early workers[286,297,361,372] and critically examined by Markuszewski[247]. In contrast to the alkaline earths, this compound was observed to fluoresce near pH 3. Since this indicated a significant difference in the character of the slightly-dissociated compound, the fluorometric properties of Calcein in the presence of aluminum were examined in detail.

The excitation and emission spectra of solutions of Calcein with aluminum were obtained over the pH range of 0 to 12. As with all others species studied, the emission spectrum always consisted of a single band, independent of the wavelength of excitation, which underwent a slight bathochromic shift from 512 nm. at pH 0 to 517 nm at pH 10. The excitation spectra were also similar to those above, consisting of one or more of the same three major excitation bands and the

same group of minor bands between 280 and 320 nm. Again, no new bands were identified.

The fluorescence of the aluminum-Calcein compound at pH 3 was almost identical to the fluorescence of Calcein alone at pH 5. In fact, over the pH range from 2 to 6, the fluorometric properties of Calcein in the presence of aluminum was found to be consistently analogous to the properties of Calcein alone at about 1.5 units of pH higher. One explanation given for this behavior was the preferential replacement of the hydrogens, usually associated with the phenolic moieties of the tri-zwitterion at this pH, by aluminum to form complicated chain-like structures such as the one suggested by Markuszewski[247]. As a result, the net acidity of the third and fourth replaceable hydrogens were found to be increased. Thus, the highly fluorescent characterisitics associated with the triply- and quadruply-charged anions were found to appear to a limited extent in solutions of lower pH when aluminum was present.

The fluorescence of Calcein in the presence of aluminum as a function of pH is shown in figure 30.

Figure 30.

Fluorescence of Calein in the presence
of aluminum as a function of pH

Measured on a Turner Model 110 Filter Fluorometer

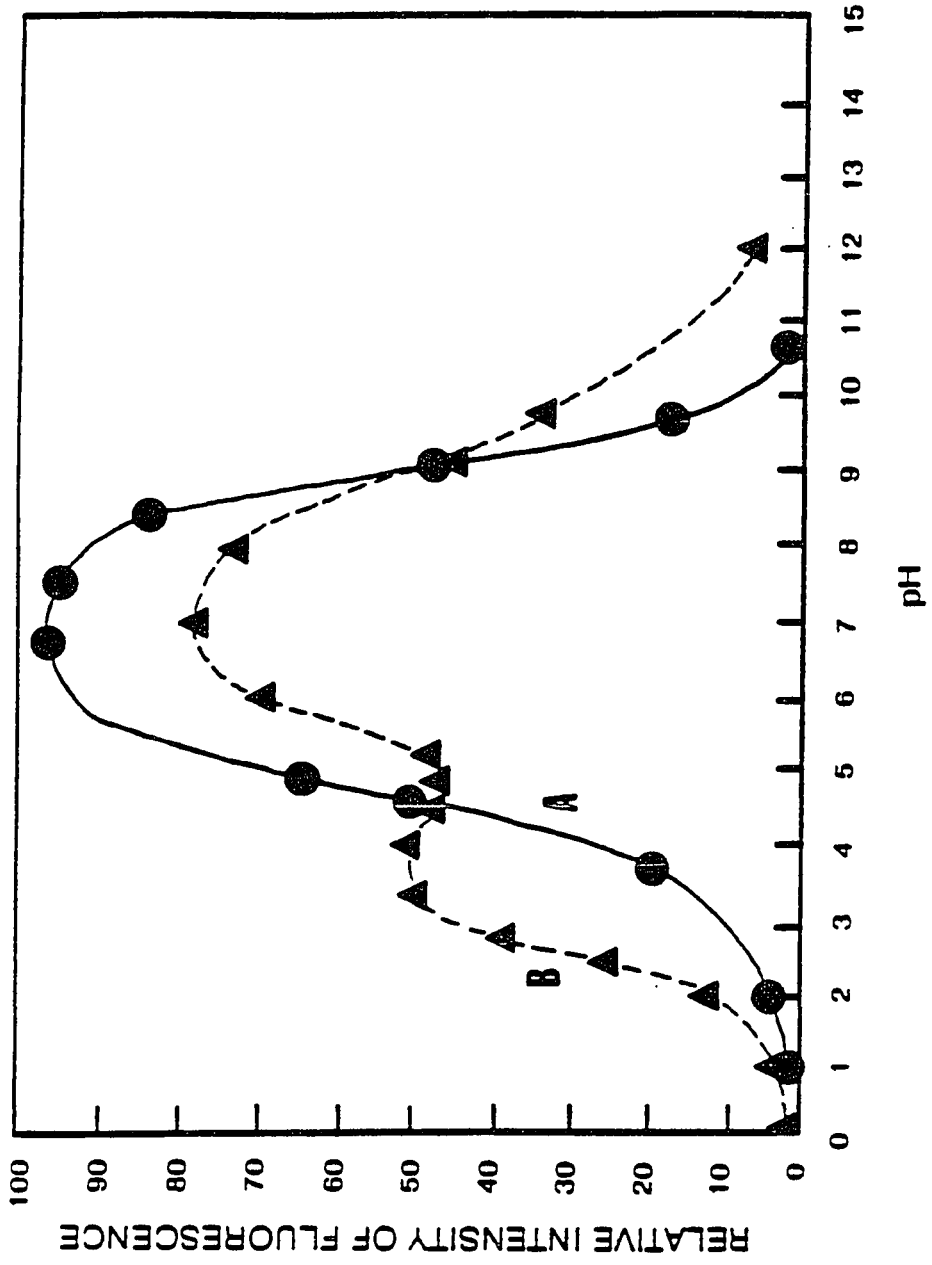
Primary filter: Corning 5850 glass filter
Secondary filter: Corning 2A-15 glass filter
& Wratten ten percent neutral density filter

Sensitivity: 10X

Concentration of Calcein: 1×10^{-5} M

Curve A: Calcein alone

Curve B: Calcein with ten-fold excess of
aluminum


RELATIVE INTENSITY OF FLUORESCENCE
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
pH
A
B

The data contained in this figure was obtained with a filter fluorometer and reflects the bias associated with the glass filters used for this measurement. Although measurements of fluorescence with solutions containing aluminum proved much less reproducible than those with other metals, some consistent properties of interest were found. Not only did the presence of aluminum increase the fluorescence at low pH, but the fluorescence was also measurably increased at high pH where one might expect the aluminum to be tied up as hydroxides. This fluorescence, measurable at pH 12, was successfully eliminated with the addition of triethanolamine to the solution. This unexpected fluorescence at high pH by a metal not normally chelated by EDTA is a possible explanation for problems incurred in the titration of geological samples[169] and in the determination of the acid dissociation constants[166]. This behavior is not unlike the formation of aluminum compounds with other molecules containing the iminodiacetic acid group[51,126]. Provisions for this phenomenon were included in the procedures developed in chapter VII.

The fluorescence was also measured as a function

Figure 31.

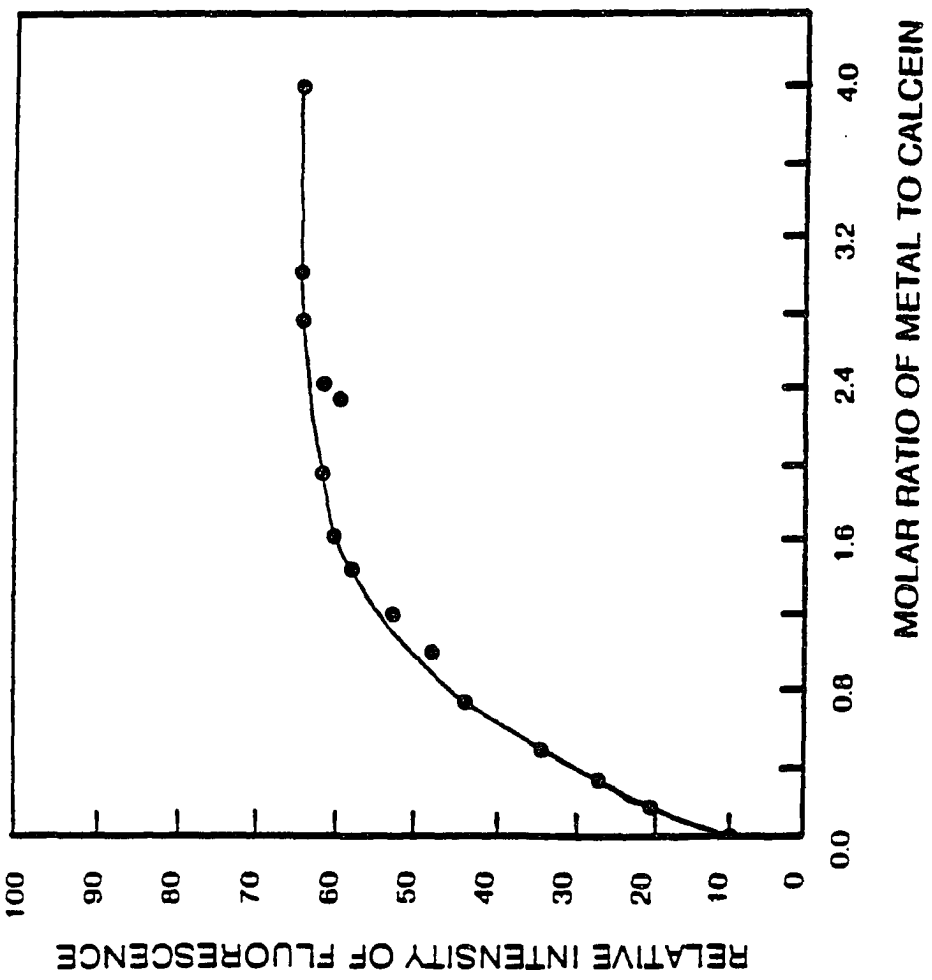
Fluorescence of Calcein at pH 4.0 as a function of the relative concentration of aluminum

Measured on a Turner Model 110 Filter Fluorometer

Primary filter: Corning 5850 glass filter
Secondary filter: Corning 2A-15 glass filter
& Wratten ten percent neutral density filter

Sensitivity: 10X

Concentration of Calcein: 1×10^{-5} M


RELATIVE INTENSITY OF FLUORESCENCE
100
90
80
70
60
50
40
30
20
10
0
0.0
0.8
1.6
2.4
3.2
4.0
MOLAR RATIO OF METAL TO CALCEIN

of the relative concentration of aluminum at pH 4.0. Interpretation of the results was complicated by the non-linear increase in fluorescence with aluminum and the background of fluorescence from unassociated Calcein at this pH. In the presence of aluminum at molar ratios greater than 2 to 1, the fluorescence remained constant. Estimation of the point at which this fluorescence was first reached, provides an approximation of the predominant stoichiometry of the compound. This was found to be 1.5 moles of aluminum to 1 mole of Calcein. This is the same value proposed by Markuszewski[247].

Despite the uniqueness of this compound, no new or distinct properties were present to facilitate the analyses of solutions of fluorescein and Calcein.

7. Fluorescence in the presence of certain transition metals

The reaction of Calcein with certain transition metals quenched the fluorescence in the region of pH where Calcein by itself fluoresces. The effects of two

of these metals on the fluorometric properties of Calcein were examined. The present work concentrated on the ferric and nickelous ions as representative of the transition metal ions with oxidation states of (III) and (II).

The addition of either of these cations greatly reduced the fluorescence of solutions of Calcein between pH 4.5 and the pH at which the formation of insoluble hydroxides preempts the formation of slightly-dissociated compounds with Calcein. As shown in figure 32, the addition of metal decreased the excitation, especially at 485 nm. The smaller band at 470 nm. was also effected but to a much lesser extent. No new excitation or emission bands were found, and the positions of the bands remained at the same wavelength for any given pH as they have been observed with every other species examined in this work.

The net fluorescence of Calcein as a function of the relative concentration of metal ions is shown in figure 33 for solutions of pH 8.0. These measurements were made on a filter fluorometer. The intensities were normalized to the fluorescence measured in a

Figure 32.

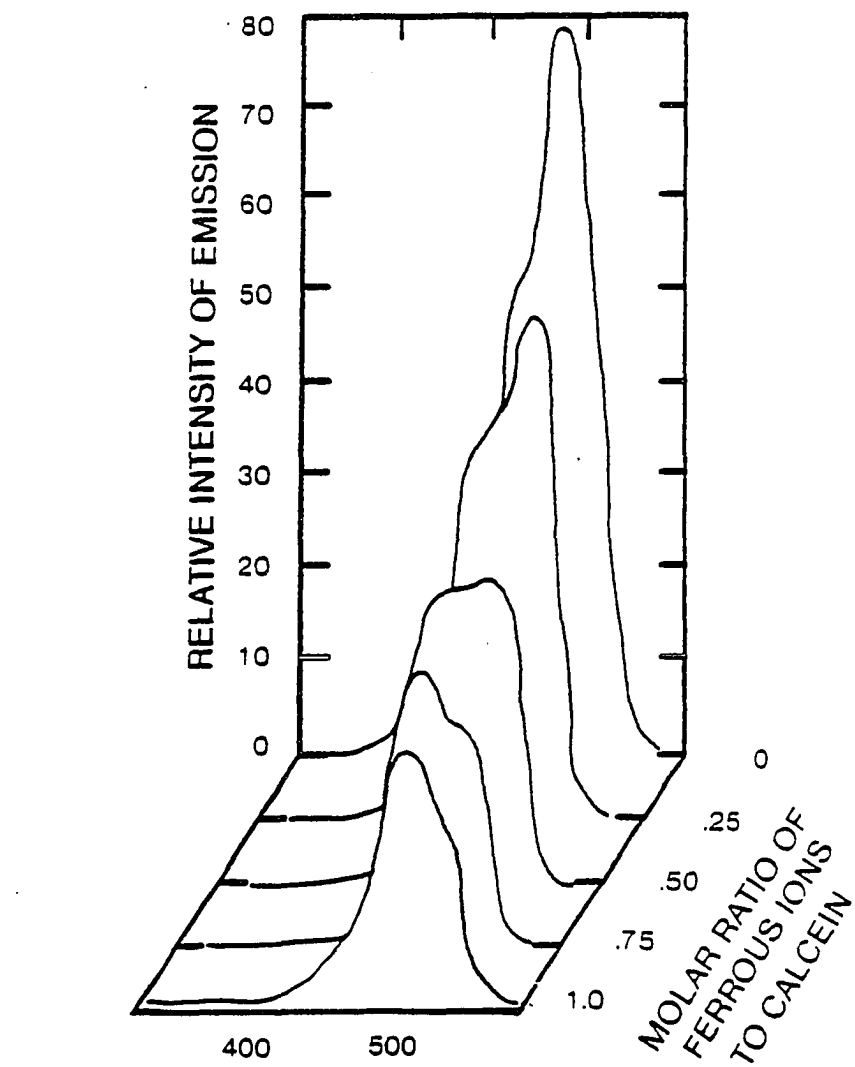
Excitation spectra of Calcein in the presence of various amounts of ferric ions

Measured on an Aminco-Bowman Spectrophotofluorometer

Emission monochromator setting: 515 nm.
Coarse sensitivity: 3.0
Entrance slit: 1.0 mm.
Exit slit: 1.0 mm.

Concentration of Calcein: 1×10^{-5} M

pH of solutions: 8.0


RELATIVE INTENSITY OF EMISSION
80
70
60
50
40
30
20
10
0
400
500
WAVELENGTH OF EXCITATION (NM.)
0
.25
.50
.75
1.0
MOLAR RATIO OF FERROUS IONS TO CALCEIN

solution of Calcein at pH 8.0 containing 5.00 moles of EDTA. With both metals, extrapolation of the initial part of the decrease in fluorescence with the relative concentration of the metal, indicated that Calcein forms strictly 1:1 compounds with Fe(III) and Ni(II) at this pH. However, even at higher absolute concentrations of the metals, the fluorescence was never completely extinguished.

The addition of either of these cations greatly reduced the fluorescence of solutions containing Calcein between pH 4.5 and the point at which the metals are precipitated as hydroxides. The bands found at 470 nm. and 485 nm. corresponded to the same bands found for other ions of Calcein. To repeat, no new excitation or emission bands were found, and the positions of the bands remained at the same wavelength for any given pH as they have been observed with every other species examined in this work.

Figure 33.

Fluorescence of Calcein as a function of the relative concentration of transition metal ions

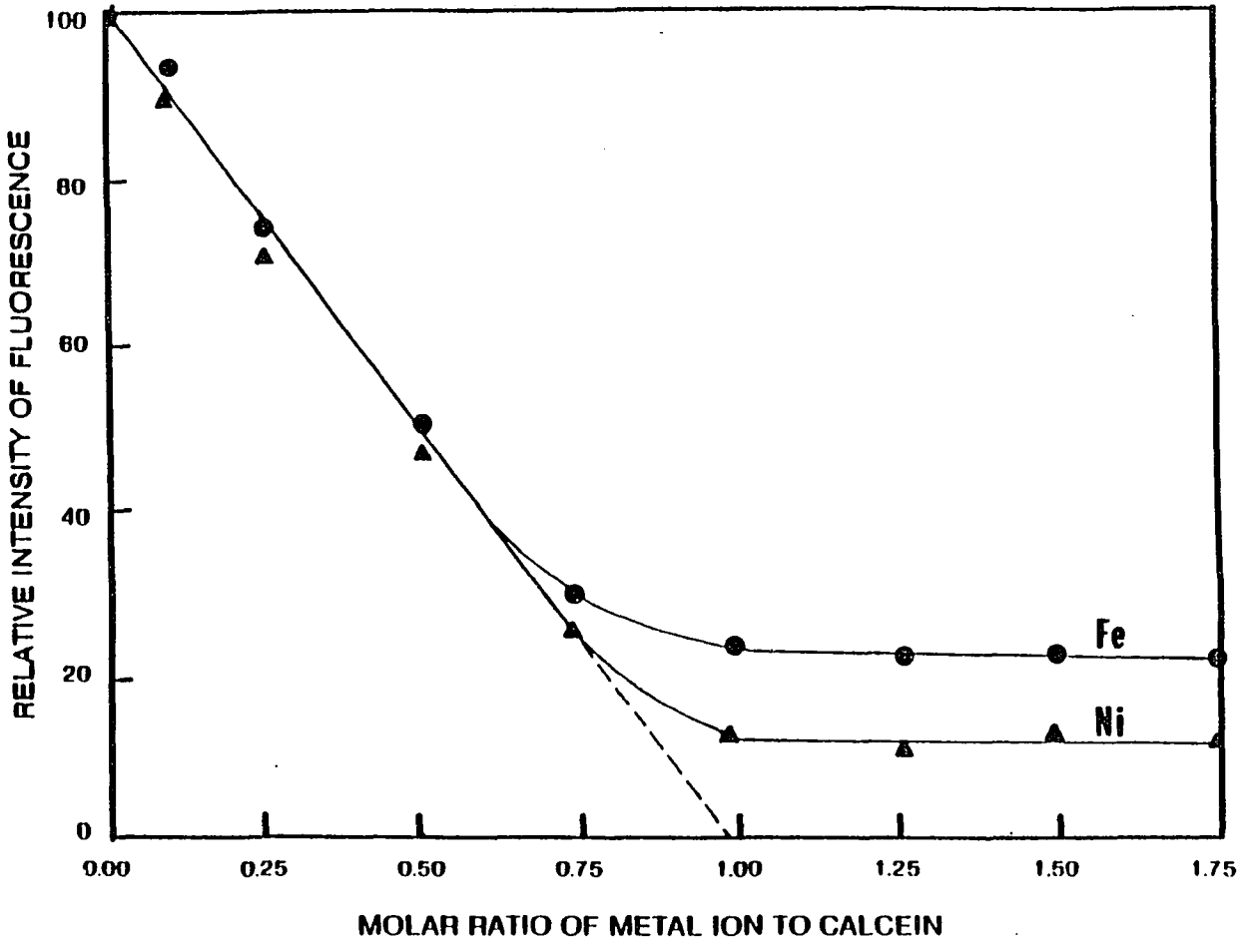
Measured on a Turner Model 110 Filter Fluorometer

Primary filter: Corning 5850 glass filter
Secondary filter: Corning 2A-15 glass filter
& Wratten ten percent neutral density filter

Sensitivity: 10X

Concentration of Calcein: 1×10^{-5} M

pH of solution: 8.0


RELATIVE INTENSITY OF FLUORESCENCE
100
80
60
40
20
0
0.00
0.25
0.50
0.75
1.00
1.25
1.50
1.75
Fe
Ni
MOLAR RATIO OF METAL ION TO CALCEIN

E. Conclusions

The fluorescent properties of three different classes of slightly-dissociated compounds of Calcein were determined. Attention was focused on the distinctive characteristics of each class, (1) the restoration of fluorescence of Calcein at high pH by the alkaline earths, (2) the enhancement of fluorescence at low pH by aluminum, and (3) the reduction of fluorescence in neutral solutions by transition metals. Each property was measured as a function of pH, wavelengths of excitation, and the absolute and relative concentrations of the metals. The specific ionic forms that fluoresce were identified and the relative coefficients of fluorescence were estimated for each of the excitation bands.

The wavelengths of excitation and emission of Calcein were unchanged by the presence of metals. The electronic transitions involved in the process of fluorescence were apparently identical to those of fluorescein. The only effects that the substitution of methyleneiminodiacetic acid groups had on the

fluorescence of fluorescein were (1) the slight changes in acidic strength of the phenolic groups and (2) the added mechanism for deactivation available to Calcein in highly alkaline solution when the substituent branches are free to twist and rotate.

Calcein was found to form both 1:1 and 2:1 slightly- dissociated compounds with calcium, magnesium, strontium and barium. All of these compounds fluoresce, the 2:1 compounds more intensely.

The fluorescence of Calcein as a function of the relative concentration of the alkaline earths was found to consist of three linear portions. The first portion corresponded to the formation of the 1:1 compound. The second was related to the formation of the more intensely fluorescent 2:1 compound. The third portion consisted of a flat plateau with no change in fluorescence above a molar ratio of 3 to 1 metal to Calcein. The first and second portions offered opportunities for methods of analysis with linear calibration curves. The second portion had the greater sensitivity but the first portion had the advantage of an intercept at zero.

The increase in fluorescence of Calcein with aluminum at pH 4 was found to correspond to a complicated compound with the approximate stoichiometry of 1.5 moles of aluminum to 1 mole of Calcein. The presence of aluminum was also found to cause a higher fluorescence between pH 10 and pH 12. This fluorescence was eliminated by the addition of triethanolamine.

The quenching of the fluorescence of Calcein in neutral solutions by transition metals was found to occur principally at the excitation band at 485 nm. The stoichiometry of the compounds of Calcein with iron(III) and nickel(II) are both 1:1. This stoichiometry is probably representive of the non-fluorescent compounds formed between Calcein and most transition elements. Despite the strong quenching action of these metals, significant fluorescence of Calcein remains even in several-fold excesses of the metals at concentrations in excess of 1×10^{-5} M.

The relative coefficients of fluorescence have been estimated for all of the fluorescing compounds of

metals with Calcein and are summarized in tables 6 and 7. While the compound with magnesium was slightly more fluorescent than that of calcium, magnesium forms hydroxides above pH 11.5. This severely limits the usefulness of Calcein for the determination of magnesium, but it does provide a basis for the direct determination without separation of calcium and other alkaline earths in the presence of magnesium. Of the other metals examined, the compound with calcium is the most highly fluorescent.

While the fluorescence of Calcein by itself does not have any regions in which the intensity is relatively independent of small changes in pH, the calcium compound of Calcein does. Under the following conditions, the fluorescent properties of the calcium-Calcein slightly-dissociated compound are amenable to the fluorometric determination of Calcein: pH of approximately 13, molar ratio of calcium to Calcein greater than 3 to 1, absolute concentrations of Calcein of 3×10^{-6} M and of calcium of 1×10^{-5} M, wavelength of excitation between 480 and 490 nm., and a wavelength of emission between 515 and 520 nm.

V. DETERMINATIONS OF FLUORESCEIN AND CALCEIN IN SEPARATE SOLUTIONS AND MIXTURES

A. Introduction

Methods for the accurate determinations of fluorescein and Calcein are essential for the proper examination of the synthesis, storage and application of Calcein. New techniques were needed for the accurate evaluation of the purity of different preparations. The studies described in the following chapters of this dissertation required the determinations of fluorescein and Calcein in separate solutions and in mixtures at virtually all relative concentrations.

The literature on Calcein contains relatively few references to determinations of fluorescein in Calcein. Those procedures mentioned are generally inadequate for determinations at the concentrations of fluorescein involved in the present work. Most of the procedures in the literature involve chromatographic separations. The subsequent analyses are based on subjective evaluations regarding the visual appearance of separated species.

The most frequently mentioned of these procedures is electrophoresis. Unfortunately, the limit of detection of this technique for fluorescein in Calcein has been shown to require greater than two percent fluorescein by weight[147]. Furthermore, the accuracies of all of the chromatographic procedures are relatively poor in comparison to spectrophotometric measurements.

The methods listed by commercial producers of Calcein are also inadequate for the present work. One of the most common methods presently employed is infra-red spectroscopy[293]. Unfortunately, as will be shown in chapter VI, the changes in the regions around 1720, 1530 and 1360 cm^{-7} are more dependent on the medium from which Calcein is precipitated than on the amount of fluorescein present as an impurity. In general, the most critical test of purity, as far as the manufacturers are concerned, is the demonstration that the material performs adequately in the procedure for which the product is recommended. Several suppliers presently list as the only specification that the material is sufficiently pure for the fluorometric determination of calcium[137,141,159].

As shown in chapters II and III, the fluorescent

properties of fluorescein and Calcein are very similar. The wavelengths of excitation and emission are the same for both compounds at any particular pH. Only the relative intensities differ. The most significant difference is the retention of fluorescence by fluorescein in highly alkaline solutions, where, under the proper conditions, the fluorescence of pure Calcein is negligible.

This difference in fluorescence has previously been exploited in two different attempts to determine small amounts of fluorescein as an impurity in Calcein. Bozhevol'nov and Kreingol'd[64] were the first to measure the fluorescence of fluorescein in solutions of Calcein at high pH. By measuring the intensity of fluorescence, they estimated that the samples of Calcein available to them contained between 5 and 16 percent fluorescein by weight. They also noted that Calcein absorbs light in the same region of the visible spectrum as the flurescent emission of fluorescein and concluded that Calcein probably interferes with this determination by absorbing some of the fluorescence emitted by fluorescein. As a result, Bozhevol'nov and Kreingol'd considered their estimates of fluorescein likely to be too low. Martin[252] took a similar approach. He applied the method of

standard additions to the fluorometric determination of fluorescein in Calcein. Working at low concentrations of Calcein, he assumed that the absorption of fluorescence by Calcein was insignificant. This procedure proved to be successful but only over a very limited range of concentrations.

Unfortunately, neither study established the limits for the concentrations of fluorescein and Calcein, relative or absolute, beyond which the absorption of the fluorescence by fluorescein or Calcein becomes significant. In addition, both studies dealt only with the task of determining small quantities of fluorescein in Calcein. In support of the present investigations, mixtures encompassing a much wider range of relative concentrations needed to be analyzed.

The principal approach taken in the present work also involved fluorometric determinations. Fluorescein was determined in alkaline solutions, both with and without Calcein present. In contrast to attempts by earlier workers, the conditions were strictly determined under which the direct fluorometric determinations could be accurately performed. Possible effects on the determination from other contaminants in Calcein were

also explored.

The intensity of fluorescence of Calcein, as shown in figure 16, changed continuously with pH. Calcein is therefore not easily or reproducibly determined fluorometrically in the absence of metals. On the other hand, the slightly-dissociated compounds of Calcein with the alkaline earths, calcium in particular, produced highly intense fluorescence with little sensitivity to small changes in pH when excited under the proper conditions of concentration, pH, and incident light. This fluorescence was successfully utilized to provide reproducible fluorometric measurements of Calcein. The conditions within which the fluorometric determination of Calcein was accurate were carefully established. Further, the specific conditions within which the fluorescence of the slightly-dissociated compound was additive to the fluorescence of fluorescein, were also meticulously defined.

A second approach involved measuring the absorption of visible light by fluorescein and Calcein. The same chromophore existed in both molecules, giving dilute solutions of each compound the same characteristic yellow-brown hue. Small differences in the absorption spectra of fluorescein and Calcein were the result of

differences in the relative acidities of the phenolic groups on the xanthene portion of the molecules. The resulting minor variations in absorption with pH were insufficient to allow spectrophotometric analysis of mixtures of fluorescein and Calcein. Nevertheless, under the proper conditions, the convenience and reproducibility of spectrophotometric measurements can be used to determine the concentrations of fluorescein and Calcein in separate solutions.

The spectrophotometric properties of fluorescein and Calcein were examined with respect to the properties of the individual prototropic forms. Identification of the individual properties permitted the evaluation of the relative efficiencies of internal conversion and fluorescent emission, the determination of an effective dissociation constant for the cations of Calcein, and the establishment of specific conditions within which fluorescein and Calcein can be conveniently and reproducibly determined by spectrophotometry.

B. Apparatus and Reagents

Measurements of the relative intensity of fluorescence were made with a Turner Model 110 Filter Fluorometer. The primary filter consisted of a Corning 5850 blue glass filter and the secondary filter consisted of the combination of a Corning 2A-15 yellow glass filter and a Wrattan ten percent neutral density filter. Glass cuvettes with diameters of 0.5 cm. were used as sample cells.

In addition, some measurements of fluorescence were made with an Aminco-Bowman Spectrophotofluorometer. The intensities of fluorescence at given wavelengths or over an entire spectrum were recorded on an Aminco-Bowman X-Y Recorder. The sensitivity and slit widths were adjusted to accommodate the measurement of fluorescence over several orders of magnitude of concentration of the fluorescing species.

Absorption spectra of fluorescein and Calcein were obtained on a Cary Model 14 Spectrophotometer. Cylindrical quartz cells with path lengths of 1.0 cm. were used for all measurements. Individual measurements

of absorbance at fixed wavelengths were obtained with a Spectronic 20 Spectrometer using glass cuvettes with 1.0 cm. diameters.

The pH of solutions was measured with a Beckman Zeromatic SS-2 pH meter equipped either with a Beckman saturated calomel reference electrode and a Beckman glass indicator electrode, or with a Fisher combination pH reference/indicator electrode. The pH meter was standardized using buffer solutions of potassium acid phthalate, potassium dihydrogen phosphate and sodium borate.

The fluorescein used in the work described in this chapter was obtained as a purified product from Hach Chemical Company of Ames, Iowa. Several different preparations of Calcein were used during the course of this study. The purity varied between 86 and 99 percent and the amount of fluorescein present as an impurity in the Calcein varied from greater than 5 percent to less than 0.5 percent by weight. Where pertinent, the purity of the Calcein used in each section will be described. Purified iminodiacetic acid was obtained from Hampshire Chemical Company of Keene, New Hampshire.

C. Experimental Work

1. Measurement of the spectrophotometric properties of fluorescein and Calcein. Establishment of spectrophotometric calibration curves.

A stock solution of fluorescein was prepared by dissolving 0.0634 g of fluorescein in approximately 500 ml. of deionized water and approximately 5 ml. of 1.0 N potassium hydroxide. The solution was diluted to 2.00 liters. The concentration of this solution was 9.54×10^{-5} M. A stock solution of Calcein was prepared from 0.1197 g of Calcein from a preparation measured to be 99.0 percent pure as the mono-hydrate.

Separate series of solutions were prepared with constant concentrations of fluorescein and Calcein, but differing in pH. To each flask in the first series of 100-ml. volumetric flasks were added 10.00 ml. of the stock solution of fluorescein and 1.00 ml. of 1×10^{-3} M EDTA. Each solution was treated with sufficient hydrochloric acid or potassium hydroxide to bring the

pH to the desired value. The increments of pH were approximately one unit apart from pH -1 to pH 14. The second series of solutions was similarly prepared, substituting 10.00 ml. of the stock solution of Calcein for the fluorescein.

The pH of all solutions between pH 2 and pH 11 were measured. The absorbance spectrum of each solution was then obtained relative to distilled water as the blank.

Other solutions of fluorescein and Calcein were freshly prepared to obtain spectrophotometric calibration curves. A stock solution of fluorescein was prepared from 0.3326 g of fluorescein dissolved in 100 ml. of 1.00 N potassium hydroxide and diluted to 1.00 liters. A stock solution of Calcein was prepared by dissolving 0.3204 g of Calcein (98 percent purity) in 100 ml. of 1.00 N potassium hydroxide and diluting to 1.00 liters. The stock solutions of fluroescein and Calcein were 1.0×10^{-3} and 5.0×10^{-4} M, respectively, in 0.10 N potassium hydorxide.

Individual solutions of fluorescein and Calcein

were prepared in 100-ml. volumetric flasks. Aliquots of fluorescein or Calcein between 0.10 and 5.0 ml. were added to each of a series of flasks from a 10-ml. buret. Each solution was diluted to volume with 0.10 N potassium hydroxide.

The transmittance of each solution was measured using a simple prism spectrometer following the adjustment of the setting for zero absorption using deionized water.

2. Establishment of calibration curves for the fluorometric determination of fluorescein

A stock solution of 1.00×10^{-4} M fluorescein was prepared by dissolving 0.3326 g of purified fluorescein in 1.00 liters of 0.10 N potassium hydroxide and then diluting 100.0 ml. of this solution to 1.00 liters.

Individual solutions of fluorescein were prepared for fluorometric measurements in 100-ml. volumetric flasks. Each flask contained 10.0 ml. of 1.00 N potassium hydroxide and between 0.10 ml. and 25.0 ml.

of 1.00×10^{-4} M fluorescein. An additional solution was prepared without fluorescein and used as a blank. All solutions were diluted to volume with deionized water.

The relative intensity of fluorescence was measured for each solution at each of the excitation intensities of the Turner Fluorometer: 1X, 3X, 10X, and 30X. The solutions prepared for fluorometric measurements were then left on the laboratory bench for seven days and the relative fluorescence was measured again.

Another set of similar solutions of fluorescein at pH 13 was prepared with concentrations between 1.00×10^{-5} and 10.0×10^{-5} M. The relative intensities of fluorescence of each of these solutions were measured with the spectrophotofluorometer by narrowing the slit widths to 0.5 mm. and setting the sensitivity to the minimum value.

A series of solutions was prepared to test for interferences to the measurement of fluorescence by various compounds encountered in the preparation and

application of Calcein. A fresh solution of 1.0×10^{-4} M fluorescein was prepared as in section (1). To each of eleven 100-ml. volumetric flasks were added 2.00 ml. of 1.00×10^{-4} M fluorescein and 10.0 ml. of 1.0 N potassium hydroxide. The first three flasks were diluted to volume with deionized water. To each of the other solutions in the remaining flasks were added one of the following: 10.0 ml. of 35 percent solution of formaldehyde, 10.0 ml. of 1×10^{-3} M calcium chloride, 10.0 ml. of 1×10^{-3} M iminodiacetic acid, 10.0 ml. of 1×10^{-3} M EDTA, 50.0 ml. of saturated (approximately 4 M) potassium chloride, 1.0 ml., 2.0 ml., or 5.0 ml. of 95 percent ethanol. The solutions were then diluted to volume with deionized water.

The relative intensity of fluorescence was measured with the filter fluorometer using an excitation setting of 3X. Deionized water was used as the blank to calibrate the zero adjustment of the fluorometer.

3. Establishment of the calibration curves for the fluorometric determination of Calcein

A stock solution of 1.00×10^{-4} M Calcein was freshly prepared by dissolving 0.3204 g of highly pure Calcein in approximately 500 ml. of 0.01 N potassium hydroxide, diluting to 1.00 liters and then diluting 100 ml. of this solution to 500 ml. with deionized water. A stock solution, 1.0×10^{-3} M in calcium, was prepared by dissolving 0.147 g of calcium chloride dihydrate in 1.00 liters of deionized water.

Individual solutions were prepared for fluorometric measurements in 100-ml. volumetric flasks. Each flask contained 10.0 ml. of 1.00 N potassium hydroxide, 5.0 ml. of 1.0×10^{-3} M calcium chloride and between 0.10 and 25.0 ml. of 1.00×10^{-4} M Calcein. Each solution was diluted to volume with deionized water. Another solution was prepared without Calcein and used as the blank.

The relative intensity of fluorescence was measured for each solution relative to the blank with the filter fluorometer at each of the four settings for

excitation intensity. The relative fluorescence of these solutions was also measured again after exposure to sun and fluorescent lighting for seven days.

The precision of the measurements and possible effects to the measurements of several other chemical compounds were examined in another set of solutions. The stock solutions prepared in section (3) were also used for this work. To each of eleven 100-ml. volumetric flasks were added 3.00 ml. of 1.00×10^{-4} M Calcein, 5.0 ml. of 1.0×10^{-3} M calcium chloride, and 10.0 ml. of 1.0 N potassium hydroxide. Three of these solutions were diluted to volume with deionized water and used as the control group. Each of the other flasks received one of the following: 10 ml. of 35 percent formaldehyde solution, 1.0 ml. of 1×10^{-3} M iminodiacetic acid (IDA), an additional 10.0 ml. of 1×10^{-3} M calcium chloride, 10.0 ml. of 1×10^{-3} M EDTA, 50.0 ml. of saturated potassium chloride, 1.0 ml., 2.0 ml., or 5.0 ml. of 95 percent ethanol. All solutions were then diluted to volume with deionized water.

The relative intensity of fluorescence of each solution was measured with the excitation setting of

the filter fluorometer at 3X.

4. Determination of the limits to the concentration of Calcein for the fluorometric determination of fluorescein

The concentration of Calcein at which the fluorescent emission of fluorescein is measurably perturbed was determined for several concentrations of fluorescein. The study was repeated using batches of Calcein with differing amounts of fluorescein as an impurity. The data presented in the accompanying figures represent a batch of Calcein containing 0.5 percent fluorescein.

A sample of fluorescein weighing 1.662 g was dissolved in approximately 100 ml of 0.10 N potassium hydroxide and diluted to 1.00 liters with deionized water. Additional stock solutions of fluorescein were prepared by diluting 1.00 ml., 10.00 ml., and 100.0 ml. of the original stock solution to 1.00 liters with deionized water to form 5.00×10^{-6}, 5.00×10^{-5} and 5.00×10^{-4} M solutions.

A stock solution of 5.00×10^{-4} M Calcein was prepared by dissolving 0.3111 g of Calcein in approximately 100 ml. of 0.10 N potassium hydroxide and diluting to 1.00 liters. Additional stock solutions of Calcein were prepared by diluting aliquots of 1.00 ml., 10.00 ml. and 100.0 ml. of the original stock solution to 1.00 liters with deionized water. The final concentrations of the additional stock solutions of Calcein were 5.00×10^{-7}, 5.00×10^{-6} and 5.00×10^{-5} M.

Several series of solutions were prepared in 100-ml. volumetric flasks for fluorometric measurements. Within each series, the concentration of flurescein remained constant while the concentration of Calcein was varied over several orders of magnitude. Each flask in the first series contained 2.00 ml. of 5.00×10^{-4} M fluorescein, 1.00 ml. of 1×10^{-2} M EDTA, 10.0 ml. of 1.0 N potassium hydroxide and varying aliquots of the different stock solutions of Calcein. The solutions were diluted to volume with deionized water. The final concentration of fluorescein in the first series was 1.00×10^{-5} M and the concentration of

Calcein varied from 5.0×10^{-9} to 1.0×10^{-4} M. Similar series were prepared with concentrations of fluorescein of 5.00×10^{-6}, 1.00×10^{-6}, 5.00×10^{-7}, 1.00×10^{-7} and 5.00×10^{-8} M.

The relative intensity of fluorescence of each solution was determined using the Turner Model 110 Filter Fluorometer.

5. Measurement of small quantities of fluorescein in Calcein

The contamination of different preparations of Calcein with unreacted fluorescein was examined by measuring the fluorescence of alkaline solutions of Calcein with standard additions of fluorescein. This method was repeated over several orders of magnitude of concentrations of Calcein and fluorescein.

Solutions of Calcein were freshly prepared as described in section (4) with concentrations varying from 5.0×10^{-8} to 5.0×10^{-3} M. Fresh stock solutions of fluorescein between 5.00×10^{-9} and 5.00×10^{-3} M

were also prepared as described in that section.

For each sample of Calcein studied, several series of solutions were prepared in 100-ml. volumetric flasks. Each solution of the first series contained 1.00 ml. of 1.00×10^{-3} M Calcein, 2.0 ml. of 1.0×10^{-3} M EDTA, and 10.0 ml. of 1.0 N potassium hydroxide. Except for the first solution, each successive solution in the first series contained aliquots of increasing size of 1.00×10^{-5} M fluorescein. Analogous series of solutions were prepared using less concentrated stock solutions of Calcein and fluorescein.

The relative fluorescence of each solution was measured with a Turner Model 110 Fluorometer relative to a solution of 0.1 N potassium hydroxide containing no Calcein or fluorescein.

6. Determination of the limits to the concentration of fluorescein and Calcein for the fluorometric determination of Calcein

The maximum concentrations of fluorescein and Calcein at which the fluorescent emissions of fluorescein and the calcium-Calcein compound are additive were determined over several orders of magnitude of concentration. The Calcein used in this section was prepared by the recommended procedure in chapter VI and contained less than 0.5 percent fluorescein.

A sample of fluorescein weighing 0.1662 g was dissolved in 100 ml. of 0.10 N potassium hydroxide and diluted to 1.00 liters to form a stock solution of 5.00×10^{-4} M. Stock solutions of 5.00×10^{-7}, 5.00×10^{-6}, and 5.00×10^{-5} M fluorescein were prepared by diluting 1.00, 10.00 and 100.0 ml. of the 5.00×10^{-4} M solution to 1.00 liters, respectively.

A stock solution of 5.00×10^{-4} M Calcein was prepared by dissolving 0.3111 g of Calcein in 100 ml. of 0.10 N potasssium hydroxide and diluting to 1.00

liters. Diluting 1.00 ml. and 10.0 ml. of this solution to 1.00 liters formed additional stock solutions of 5.00×10^{-7} and 5.00×10^{-6} M Calcein.

A matrix of solutions, 20 by 8, was prepared using 100-ml. volumetric flasks, varying in the concentrations of both fluorescein and Calcein. The first series of solutions of the matrix contained 1.00 ml. of 1.0×10^{-2} M calcium chloride, 10.0 ml. of 1.0 N potassium hydroxide, and various amounts of the different stock solutions of Calcein such that the final concentrations of Calcein, after dilution to volume, varied from zero to 1.5×10^{-5} M. Each of the subsequent series was prepared with increasing amounts of the stock solutions of fluorescein. The final concentration of fluorescein for these solutions varied from 5.0×10^{-7} to 1.0×10^{-5} M in increments of half an order of magnitude.

The relative fluorescence of each solution was meaured with the Turner Model 110 Fluorometer. The results were plotted as relative fluorescence versus the concentration of Calcein for each concentration of fluorescein.

D. Results and Discussion

1. Spectrophotometric properties of fluorescein and Calcein. Calibration curves for spectrophotometric determinations

The ultra-violet and visible absorptions of fluorescein have been studied by several groups[21,158]. The most comprehensive study published to date is part of a long paper by Lindqvist[239]. In this paper, each of the absorption bands was correctly identified and related to the four prototropic forms of fluorescein. The absorbance measurements were used to determine values for the different acid dissociation constants. Although objections have been raised regarding some of the procedures used, this work was, in general, far superior to most of the preceding publications in this area. Despite all this attention, accurate values for the dissociation constants have only recently been obtained[106,108] and a proper understanding of the physical properties of fluorescein is only now being unravelled[109-112].

In contrast to the many references on fluorescein,

the literature on Calcein contains only a few cursory studies of the visible absorption[166,168]. In the most widely cited article on the absorbance of Calcein, Wallach[362] reported the absorption spectrum of Calcein at pH 1, pH 7 and pH 13. From this information, two of the three major absorption bands in the visible spectrum were correctly identified. Unfortunately, no detailed examination of the absorption bands and the shifts in position that each band undergoes with changes in pH was completed and several false conclusions were drawn from the sketchy information. For example, the changes in the ultra-violet and visible spectra of Calcein in the presence of certain metal ions were attributed to direct changes in the chromophore from the influence of the metal ion. The work on fluorescence described in chapter IV indicates that the primary effect of metals that form strong slightly-dissociated compounds with Calcein in neutral solutions was actually an increase in the relative acidity of the functional groups which form strong chelates with the metals. No new excitation or emission bands were detected and the positions of the excitation (and absorption) bands were analogous to those usually associated with Calcein at slightly higher pH.

Hefley[166] examined the ultraviolet and visible absorption in more detail. The aborption spectra were obtained under several conditions of pH. The cluster of absorption bands in the ultra-violet region were correctly described. Two absorption bands were noted in the visible region and were used to calculate the third and fourth dissociation constants. Again, unfortunately, even greater care was required to distinguish between two close, but separate and distinct, absorption bands at 477 nm. and 485 nm. which Hefley interpreted to be the same band shifted with increasing pH. As a result, Hefley missed the distinctiveness of the properties of the ionic forms associated with each of these absorption bands.

Absorption in the visible region by both fluorescein and Calcein occured at the three distinct absorption bands corresponding to the three major excitation bands discussed in chapters II, III, and IV. Illustrative examples of the absorption spectra are shown in figures 34 and 35. The shape and position of the three bands were very similar to the excitation spectra in figures 8 and 17, but the relative intensities of the bands were different. In highly acidic solutions, fluorescein and Calcein absorb near 435 nm. In less acidic solutions, an

Figure 34.

Visible absorption spectra of fluorescein
at different conditions of pH

Measured on a Cary Model 14 Spectrophotometer

Cells: 1.0 cm. quartz

Concentration of fluorescein: 1.0×10^{-5} M

pH of solution:

Curve A: 1.0
Curve B: 2.5
Curve C: 4.0
Curve D: 8.0
Curve E: 13.0

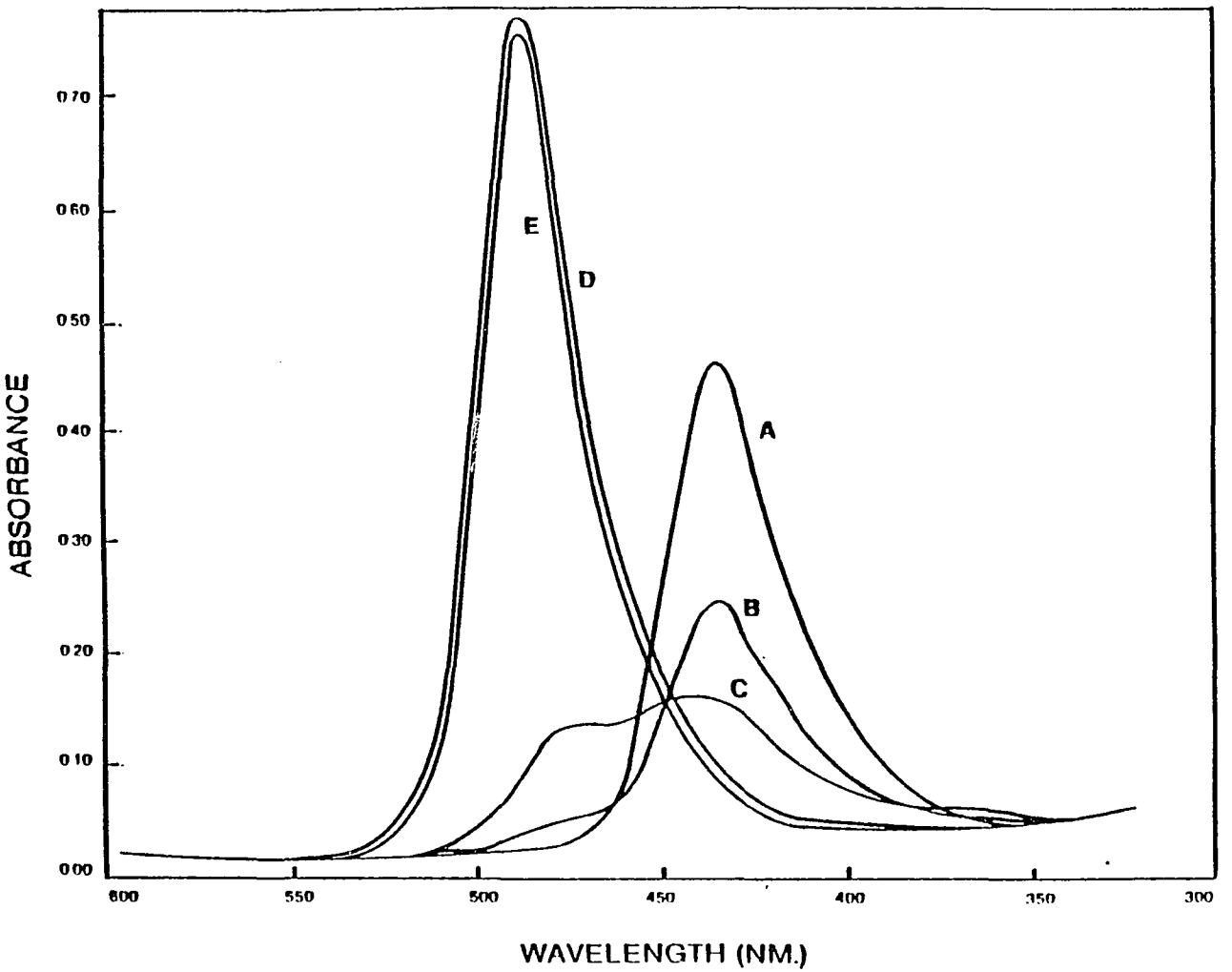

ABSORBANCE
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
600
550
500
450
400
350
300
WAVELENGTH (NM.)
A
B
C
D
E

Figure 35.

Visible absorption spectra of Calcein
at different conditions of pH

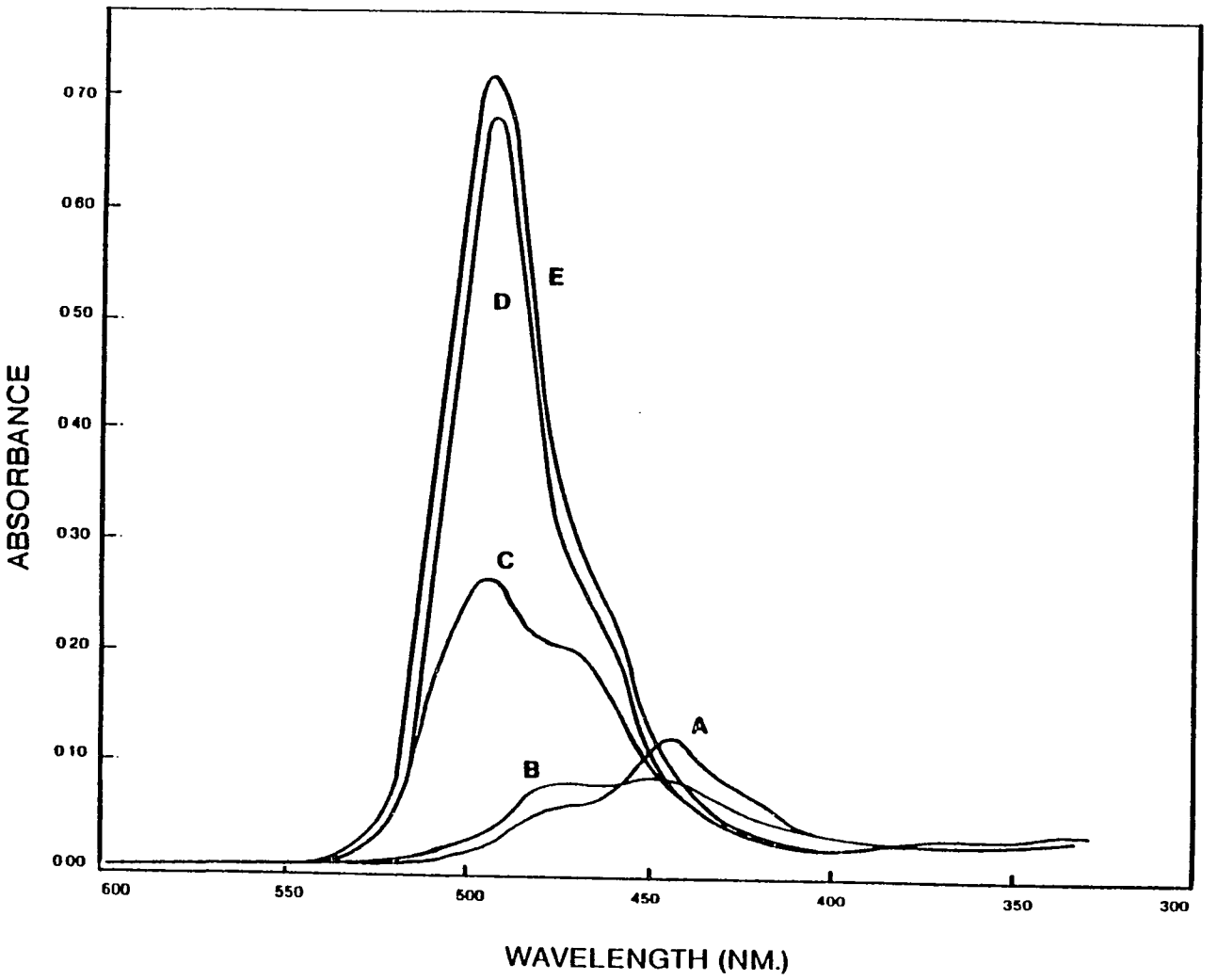
Measured on a Cary Model 14 Spectrophotometer

Cells: 1.0 cm. quartz

Concentration of Calcein: 1.0×10^{-5} M

pH of solution:

Curve A: 1.0
Curve B: 2.5
Curve C: 4.0
Curve D: 8.0
Curve E: 13.0


ABSORBANCE
0 70
0 60
0 50
0 40
0 30
0 20
0 10
0 00
600
550
500
450
400
350
300
WAVELENGTH (NM.)
A
B
C
D
E

absorption band is present near 470 nm. Although this band undergoes a slight shift to longer wavelength with increasing pH, it remained distinctly separate from the large band near 490 nm. present in alkaline solutions. Even in the data Hefley obtained at pH 13, the band at 475 nm. is still visible as a shoulder on the band at 495 nm. The simultaneous growth in absorbance of the two neighboring bands with increasing pH was followed by careful examination of the spectra at small intervals of pH.

The absorption bands at 470 nm. and especially at 435 nm. were much more intense in comparison to the major peak near 490 nm. than the intensities of the corresponding excitation peaks. This difference cannot be explained by the variation in output of the xenon lamp of the spectrophotofluorometer used to obtain the excitation spectra. The intensity of the lamp was actually slightly greater at 435 nm. than at 485 nm. The differences in the proportion of light absorbed at a given wavelength and the corresponding fluorescence, as measured by the excitation spectrum, were due to differences in the efficiency of internal conversion from the inital excited state to the energy state responsible for the emitted fluorescence at 520 nm. Decreasing fractions of the total energy absorbed at 485 nm., 470

nm. and 435 nm. were successfully converted to fluorescence and increasing fractions of that energy were dissipated by non-fluorescing transitions. Among these other transitions are mechanisms of photodecomposition. In chapter VI, a correlation will be shown between the rate of decomposition of dilute solutions and absorbance of light, especially at 435 nm.

The individual absorption bands were correlated with the prototropic forms of fluorescein and Calcein. The intensity of each peak was measured as a function of pH and plotted in figures 36 and 37. The intensities measured for each absorption band were normalized to the maximum value measured for that band. For the bands near 470 nm. and 485 nm., the maximum occurred in highly alkaline solutions. For the band at 435 nm., the maximum occurred at pH 0 for fluorescein and pH -1.0 for Calcein. The results are analogous to the correlations of fluorescence in figures 9 through 11 and 18. It must be remembered, however, that the acid dissociation constants were determined, in part, from measurements of the visible absorption and that these correlations do not, by themselves, constitute independent proof of these assignments.

Figure 36.

Relative intensities of different bands
in the visible absorption spectra
of fluorescein with pH

Measured on a Cary Model 14 Spectrophotometer

Cell: 1.0 cm. quartz

Concentration of fluorescein: 5.0×10^{-6} M

Dotted curves: Fractional distribution of prototropic forms

Solid curves: Absorbance from visible absorption bands

A: 432-437 nm.
B: 470-475 nm.
C: 480-495 nm.

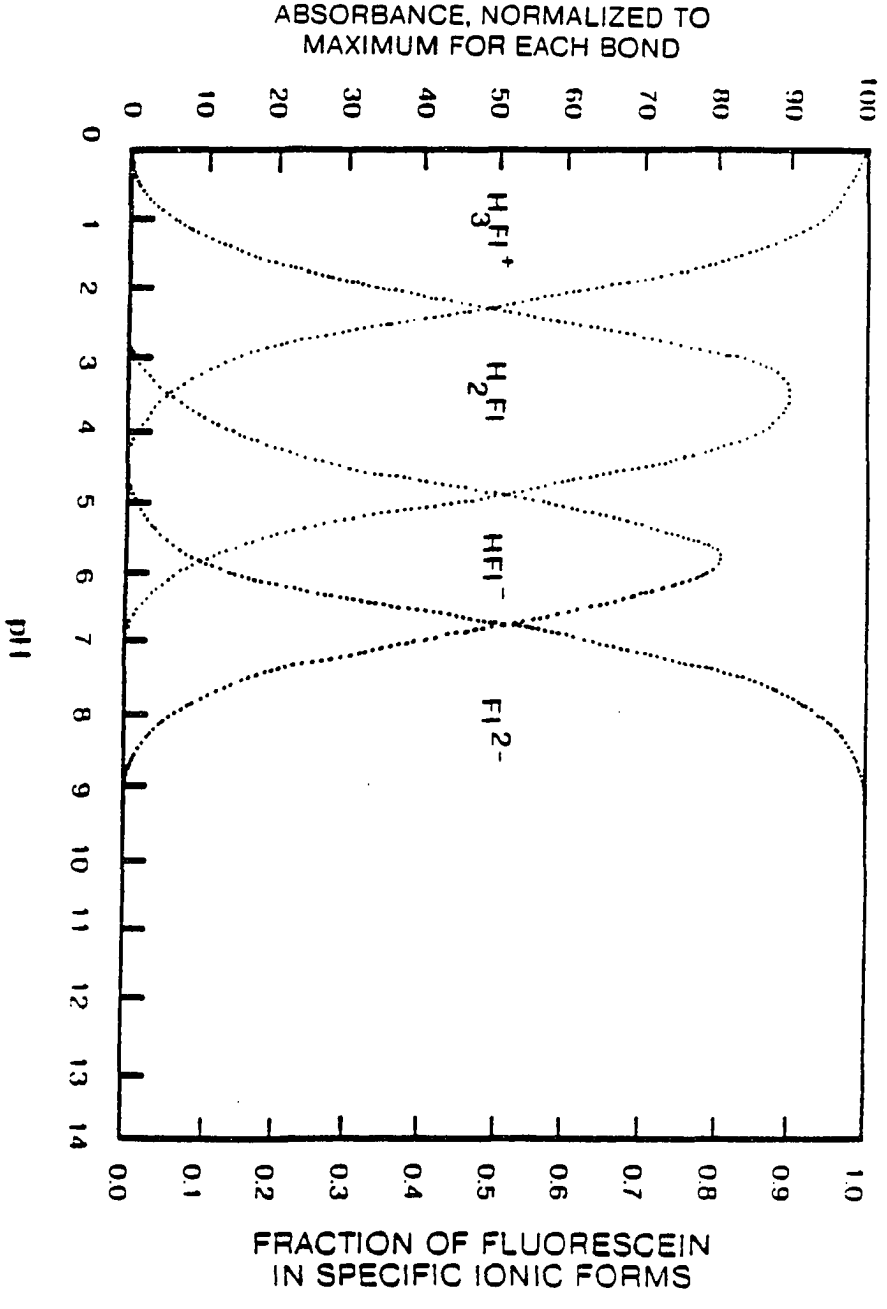

ABSORBANCE, NORMALIZED TO
MAXIMUM FOR EACH BOND
0
10
20
30
40
50
60
70
80
90
100
pH
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
H_3FI^+
H_2FI
HFI^-
FI^{2-}
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
FRACTION OF FLUORESCEIN
IN SPECIFIC IONIC FORMS

Figure 37.

Relative intensities of different bands
in the visible absorption spectra
of Calcein with pH

Measured on a Cary Model 14 Spectrophotometer

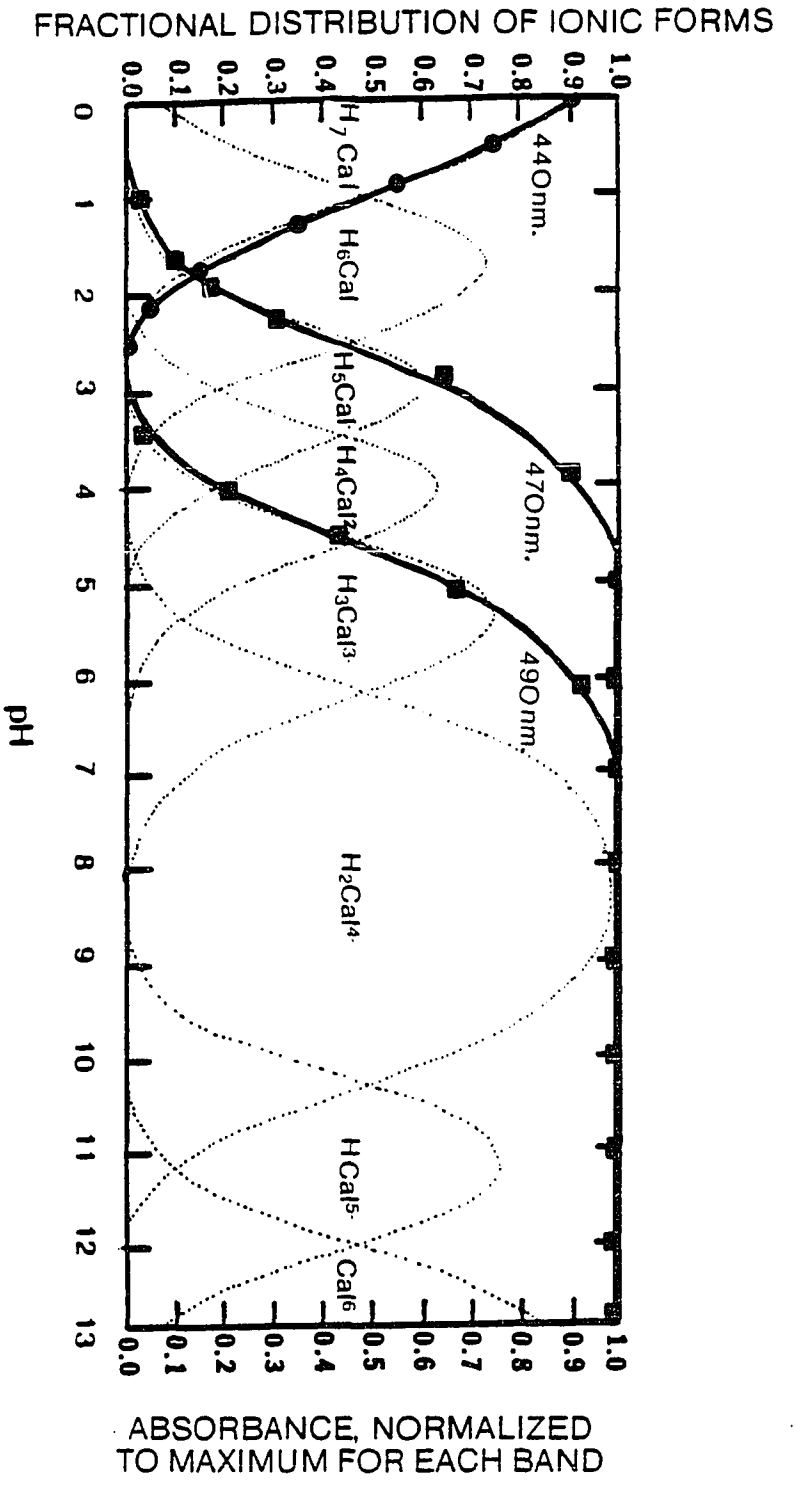
Cell: 1.0 cm. quartz

Concentration of Calcein: 5.0×10^{-6} M

Dotted curves: Fractional distribution of prototropic forms

Solid curves: Absorbance from visible absorption bands

A: 432 - 437 nm.
B: 470 - 475 nm.
C: 480 - 495 nm.


FRACTIONAL DISTRIBUTION OF IONIC FORMS
pH
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
0
1
2
3
4
5
6
7
8
9
10
11
12
13
H7Cal
H6Cal
H5Cal2-
H4Cal2-
H3Cal3-
H2Cal4-
HCal5-
Cal6
440nm.
470nm.
490nm.
ABSORBANCE, NORMALIZED
TO MAXIMUM FOR EACH BAND

For fluorescein, the absorption bands parallel the corresponding excitation bands for each of the four prototropic forms. The cation absorbs near 435 nm., the neutral molecule is relatively colorless and the mono- and di-anions of fluorescein absorb at both 470 nm. and 485 nm. For Calcein, the absorption closely follows the distribution of prototropic forms below pH 7.5. The cations absorb near 435 nm., the neutral molecule is essentially colorless, and the anions formed by the first four acid dissociations absorb at both 470 nm. and 485 nm. However, unlike the fluorescence, the absorbance of Calcein is virtually unchanged by the dissociations of the last two replaceable hydrogens.

The absorption bands associated with each of the prototropic forms of fluorescein and Calcein are summarized in table 8. The molar extinction coefficients for each absorption band were calculated from the maximum intensity observed and are also listed in table 8. All of the molar absorptivities are very high as befits the large, planar conjugated systems of fluorescein and Calcein.

An especially close examination was made of the absorption of light at 435 nm. by Calcein in acidic

Table 8.

Absorption bands for each prototropic form of fluorescein and Calcein

Prototropic Form	pH Observed	Absorption Wavelength (nm.)	Molar Extinction Coefficient
H_3Fl^+	below 4.5	435-440 nm.	17,700
H_2Fl	0.5 - 6.5	-	-
HFl^-	3.0 - 8.0	466-472 nm.	12,000
HFl^-	3.0 - 8.0	485-495 nm.	38,000
Fl^{2-}	above 5.0	468-475 nm.	12,000
Fl^{2-}	above 5.0	485-495 nm.	38,000
H_7Cal^+	below 2.5	432-442 nm.	15,800
H_6Cal	0.0 - 4.0	-	-
H_5Cal^-	1.0 - 5.0	465-472 nm.	12,000
H_4Cal^{2-}	2.0 - 6.0	465-472 nm.	12,000
H_3Cal^{3-}	3.0 - 8.0	466-472 nm.	12,000
H_3Cal^{3-}	3.0 - 8.0	483-490 nm.	37,500
H_2Cal^{4-}	5.0 - 11.0	466-472 nm.	12,000
H_2Cal^{4-}	5.0 - 11.0	483-495 nm.	37,500
$HCal^{5-}$	8.0 - 13.0	466-475 nm.	12,000
$HCal^{5-}$	8.0 - 13.0	483-495 nm.	37,500
Cal^{6-}	above 10.0	466-475 nm.	12,000
Cal^{6-}	above 10.0	483-498 nm.	37,500

solutions. The sigmasoidal increase in absorbance with decreasing pH was superimposable to the increase in fluorescence described in chapter III. The maximum fluorescence and absorbance was reached near pH -1 (10 N hydrochloric acid). The intensity of fluorescence and absorbance reached half of the maximum at pH 1.1. Although the general shape of these sigmasoidal curves suggested that a single equilibrium was in effect, efforts to determine the equilibrium constant by plotting the negative logarithms of

$$(F_{HA} - F_{A^-})/F_{HA} \text{ or } (A_{HA} - A_{A^-})/A_{HA}$$

failed to give straight lines with slopes close to 1.0. As will be discussed in the section of chapter VI on solubility, the equilibria probably involved the formation of three successively more highly charged cations. Remembering that neutral Calcein exists in water as a tri-zwitterion, three successive protonations can occur at the aromatic carboxylate and the remaining two carboxylates of the methyleneiminodiacetic acid groups. The equilibria involved in the formation of these three prototropic forms are, no doubt, highly overlapping, almost coincident. While three different protonations can occur, only that involving the aromatic

carbonyl is expected to significantly affect the absorbance at this wavelength. Therefore, both the fluorescence and absorbance were attributed to this protonated form and an effective dissociation constant of 1.1×10^{-1} was assigned.

The absorbance of both fluorescein and Calcein were relatively independent of small changes in pH in highly alkaline solutions. In this region, the transmittance of solutions was shown to be highly sensitive to concentration and fairly insensitive with respect to other conditions. As a result, the strong absorptivity was exploited for the convenient spectrophotometric determinations of fluorescein and Calcein in separate solutions. The absorbance at 495 nm. is plotted as a function of the concentration for fluorescein in figure 38, and for Calcein in figure 39. Each of these curves contains a linear portion that extends from the intercept at 0.0 to greater than 1×10^{-5} M. These curves were successfully used as linear calibration plots for routine determinations of fluorescein and Calcein in separate solutions. Many species, including by-products of the synthesis and products of deterioration also absorb in this region

Figure 38.

Absorbance of fluorescein
as a function of concentration

Measured on a Spectronic 20 Spectrometer

Cell: Glass cylindrical cuvette, 1.0 cm. diameter

pH of solutions: 13.0

Wavelengths:
Curve A: 495 nm.
Curve B: 520 nm.

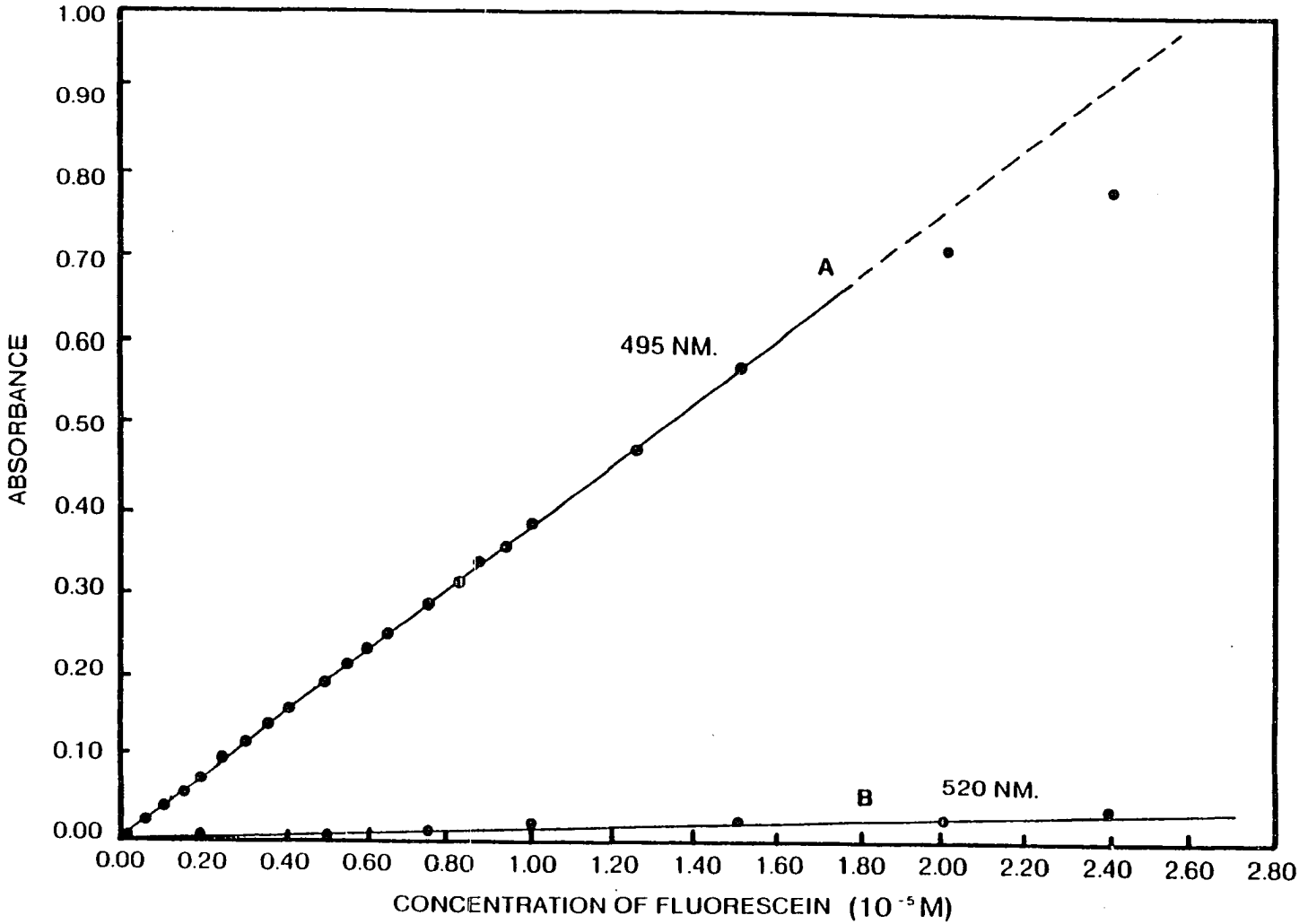

1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
ABSORBANCE
A
495 NM.
B
520 NM.
0.00 0.20 0.40 0.60 0.80 1.00 1.20 1.40 1.60 1.80 2.00 2.20 2.40 2.60 2.80
CONCENTRATION OF FLUORESCEIN (10 -5 M)

Figure 39.

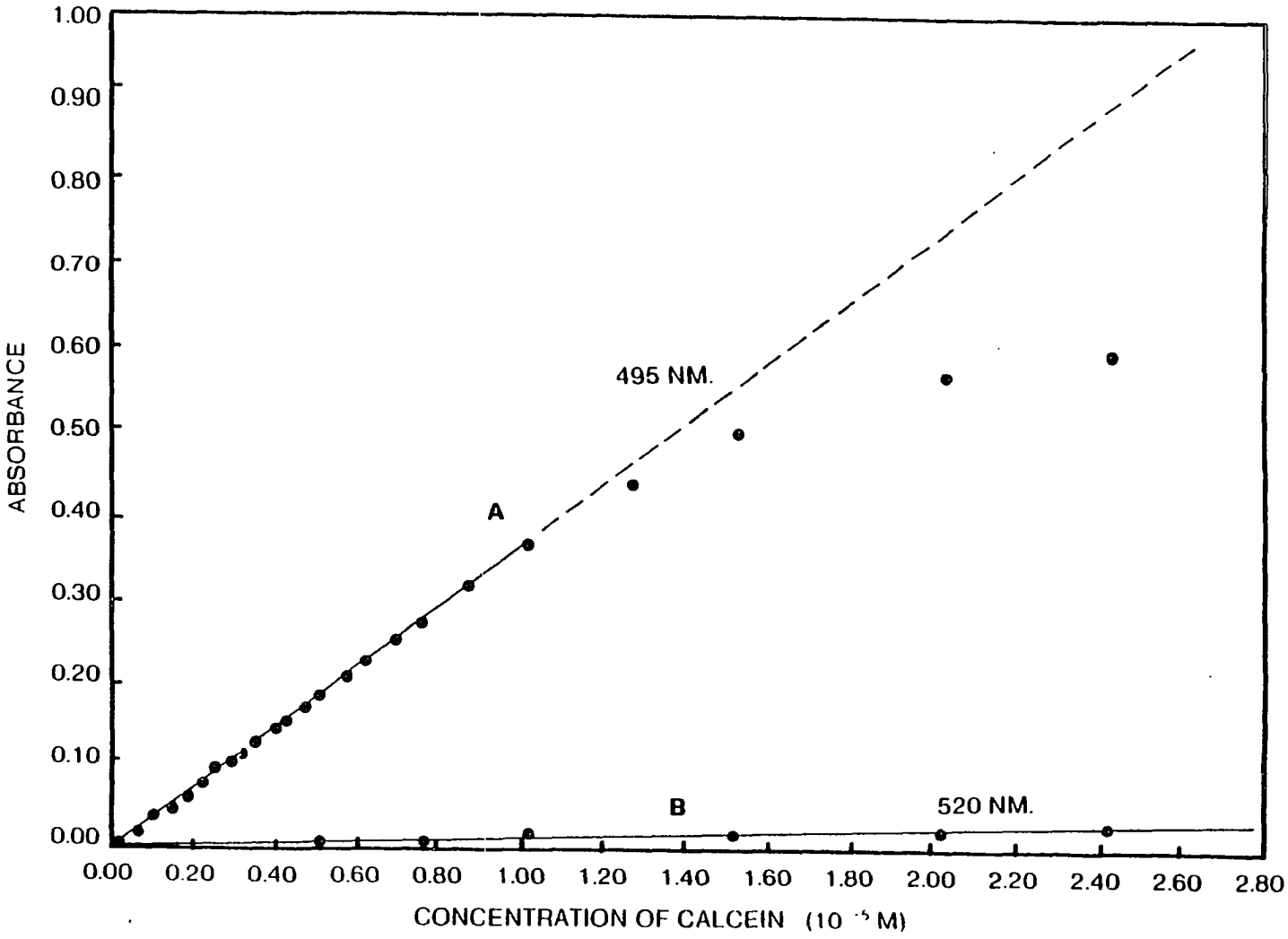
Absorbance of Calcein as a function of concentration

Measured on a Spectronic 20 Spectrometer

Cell: Glass cylindrical cuvette, 1.0 cm. diameter

pH of solutions: 13.0

Wavelengths:
Curve A: 495 nm.
Curve B: 520 nm.


1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
ABSORBANCE
495 NM.
A
B
520 NM.
0.00 0.20 0.40 0.60 0.80 1.00 1.20 1.40 1.60 1.80 2.00 2.20 2.40 2.60 2.80
CONCENTRATION OF CALCEIN (10 ⁻⁵ M)

and this method is applicable only for the assay of relatively large amounts of Calcein or fluorescein in simple solutions. In all other cases, the more specific measurements of fluorescence described below should be used.

At concentrations greater than 1.2×10^{-5} M Calcein or 1.5×10^{-5} M fluorescein, significant deviations from Beer's law were observed. This was due, in part, to instrumental limitations of the band width used by the inexpensive prism instrument. However, repetition of measurements with a higher grade spectrophotometer equipped with a grating monochromator and narrow slit widths failed to significantly improve the linearity of either plot above 2×10^{-5} M. Above 2×10^{-5} M, the deviation must be considered to be a real chemical limitation resulting from molecular interactions in the concentrated solutions.

The molar extinction coefficients of fluorescein and Calcein at 495 nm. were calculated from the slopes in figures 38 and 39 to be 38,000 and 37,500, respectively.

2. Fluorometric determination of fluorescein

The calibration curves for the relative intensity of fluorescence of solutions of fluorescein at pH 13 are shown in figure 40. For all concentrations whose fluorescence can be measured with the Turner Model 110 Fluorometer, the calibration curves were linear and intercepted the origin under all four settings of excitation intensity. This corresponded to a range of concentrations from 5.0×10^{-8} M to 1.4×10^{-5} M. The ratios of the slopes of the four calibration curves at 1X, 3X, 10X and 30X were 1.0 : 3.0 : 8.5 : 21

Repetition of these measurements with the same solutions seven days later still resulted in linear calibration curves but with significantly decreased slopes. The solutions with concentrations of fluorescein greater than 1×10^{-5} M lost almost 20 percent of the original fluorescence in one week at pH 13. The deterioration was even detectable in dilute solutions. This deterioration will be examined more closely in chapter VI.

Figure 40.

Calibration curves for the
fluorometric determination of fluorescein

Fluorescence measured on Turner Model 110 Fluorometer

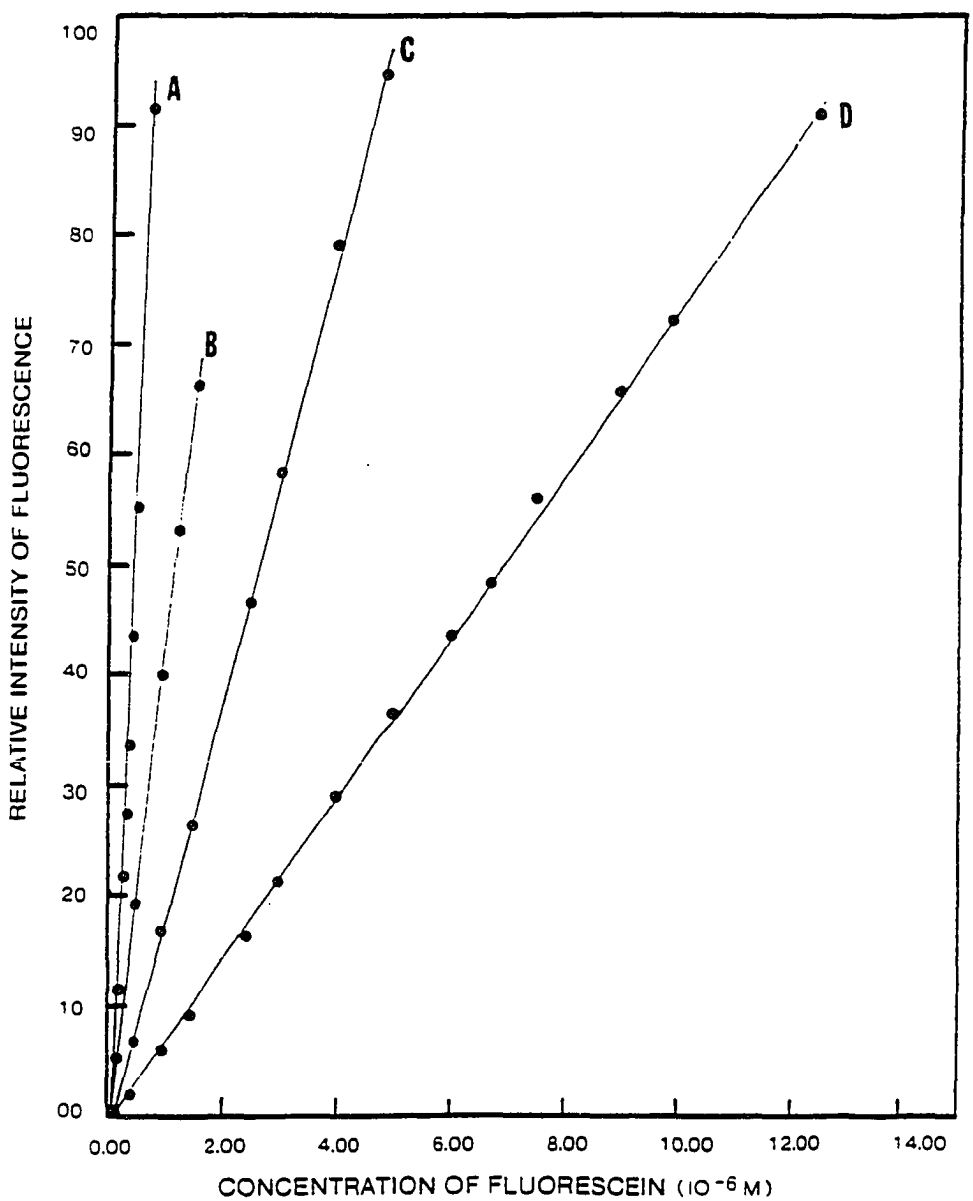
Cell diameter: 1.0 cm.

Curve A: Excitation setting of 30X

Curve B: Excitation setting of 10X

Curve C: Excitation Setting of 3X

Curve D: Excitation Setting of 1X


100
90
80
70
60
50
40
30
20
10
00
A
B
C
D
RELATIVE INTENSITY OF FLUORESCENCE
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
CONCENTRATION OF FLUORESCEIN (10⁻⁶ M)

The effects on the fluormetric measurements resulting from the presence of various materials introduced during the preparation and use of Calcein are summarized in table 9. The standard deviation of the measurement was calculated from the three control samples. This margin represented the indeterminate error of the technique including the preparation of the final solutions and the actual measurement of the fluorescence.

Contamination of solutions with calcium chloride, EDTA or iminodiacetic acid at 100:1 molar ratios relative to the concentration of fluorescein had no significant effect on the measurement. Additions of 1×10^{-2} M formaldehyde also had little measurable effect. The increase in ionic strength from 0.1 to 2.0 was tolerated with no significant change in the measurements. The intensities of fluorescence were all within two standard deviations of the mean as determined for the control samples.

The addition of other solvents, such as ethanol, did have dramatic effects on the fluorescence. This is to be expected in light of the electronic structures

Table 9.

Effect of contaminants on the fluorometric determination of fluorescein

Possible Contaminant to 2×10^{-6} M Fluorescein	Relative Intensity of Fluorescence	Deviation from Mean
none	38.0	+0.1
none	37.3	-0.6
none	38.5	+0.4
control mean	37.9 s.d. 0.51	
Formaldehyde (10^{-2} M)	38.3	+0.4
CaCl (10^{-4} M)	38.4	+0.5
IDA (10^{-4} M)	37.0	-0.9
EDTA (10^{-4} M)	37.8	+0.9
KCl (μ=2.0)	37.0	-0.9
Ethanol (1%)	37.5	-0.4
Ethanol (2%)	37.5	-0.4
Ethanol (5%)	35.8	-2.1

Figure 41.

Calibration curves for the
fluorometric determination of Calcein

Fluorescence measured on Turner Model 110 Fluorometer

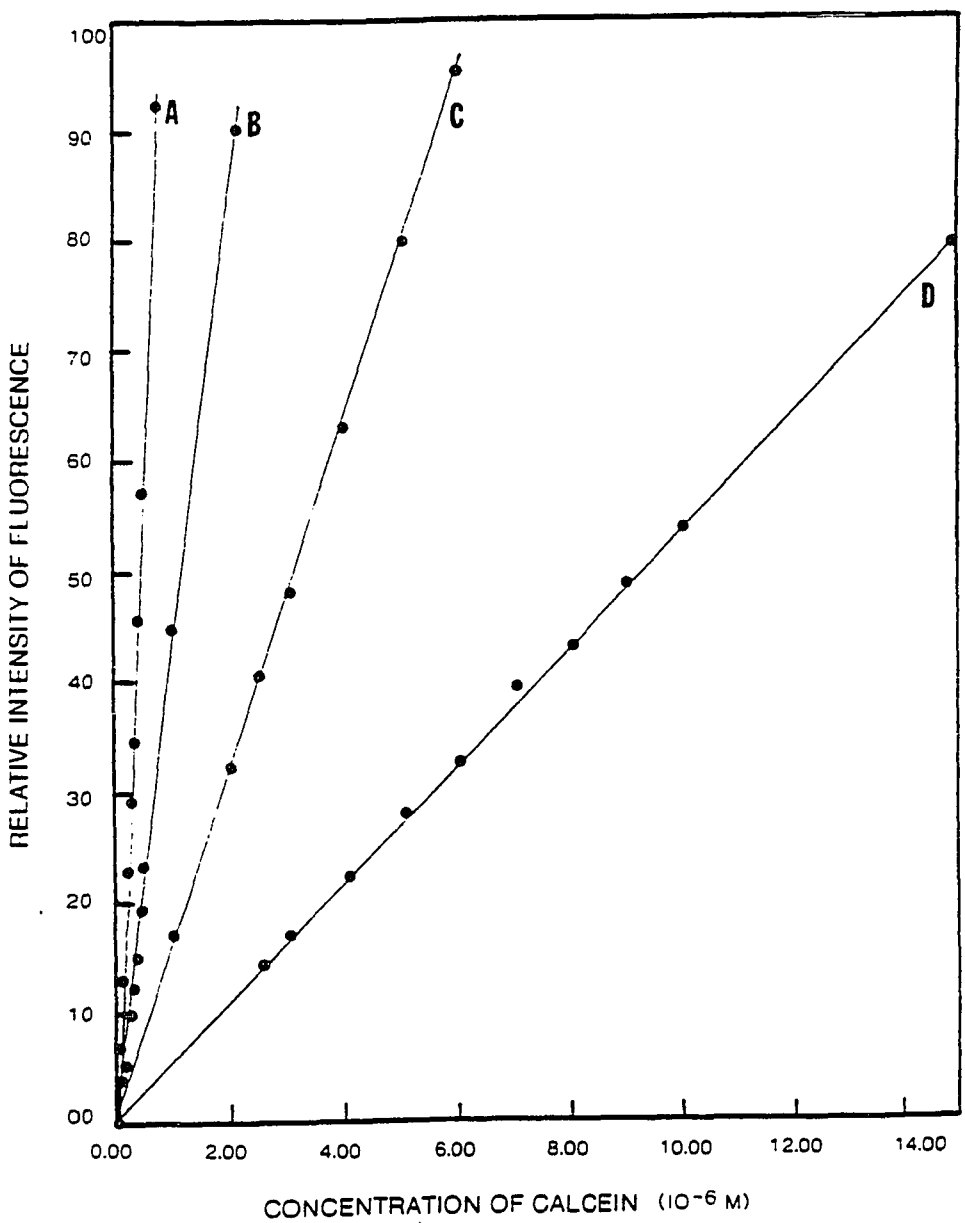
Cell diameter: 1.0 cm.

Curve A: Excitation Setting of 30X

Curve B: Excitation Setting of 10X

Curve C: Excitation Setting of 3X

Curve D: Excitation Setting of 1X


100
90
80
70
60
50
40
30
20
10
00
A
B
C
D
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
RELATIVE INTENSITY OF FLUORESCENCE
CONCENTRATION OF CALCEIN (10⁻⁶ M)

proposed for fluorescein in non-aqueous solutions. As little as five percent ethanol resulted in more than a two percent reduction in the measured intensity. The relative effect of ethanol and other solvents became increasingly greater at higher concentrations of the non-aqueous solvents.

The relative standard deviation of the replicate solutions of fluorescein was approximately one percent. This represented the precision of measurement at mid-scale in the intensity of excitation. Even at lower intensities, however, the relative deviation was still only about two percent.

3. Fluorometric determination of Calcein

Calibration curves for the relative fluorescence versus the concentration of Calcein are shown in figure 41. These curves were obtained in alkaline solutions containing ten-fold molar ratios of calcium to Calcein. The four calibration curves shown in figure 41 represented the data from each of the four settings of intensity of excitation on the filter fluorometer.

Linear calibration was obtained over the entire working range for each setting. Together, the four curves provided a calibrated range of concentrations of Calcein between 5×10^{-8} M and 1.6×10^{-5} M. The ratios of the slopes of the calibration curves with intensity settings of 1X, 3X, 10X and 30X were 1.0 : 2.9 : 8.4 : 21.

Many materials other than fluorescein are encountered during the preparation and use of Calcein. Many of these were tested for interference in the fluorometric determination of Calcein. The results are summarized in table 10.

As in the determination of fluorescein, the measurement of fluorescence of solutions containing formaldehyde at concentrations 1000 times greater than that of Calcein did not alter the value obtained. Solutions with the ionic strength raised 4-fold did not give measurably different results. Increasing the amount of calcium to relative concentrations above the ten-fold excess did not change the intensity of fluorescence measured. One perhaps surprising result was the constant fluorescence observed at pH 13 despite

Table 10.

Effect of contaminants on the fluorometric determination of Calcein

Contaminant to $2x10^{-6}$ M Calcein	Relative Intensity of Fluorescence	Deviation from Mean
none	57.8	-0.7
none	58.0	+0.3
none	59.0	+0.5
Control Mean	58.5 s.d. 0.64	
Formaldehyde (10^{-3} M)	58.5	0.0
IDA (10^{-4} M)	58.5	0.0
EDTA (10^{-3} M)	3.5	-55.0
KCl (u=2.0)	58.3	-0.2
Ca Cl (10^{-4} M)	58.0	-0.5
Ethanol (1 %)	59.0	+0.5
Ethanol (2 %)	58.2	-0.3
Ethanol (5 %)	57.2	-1.3

the presence of a relatively large concentration of iminodiacetic acid. Evidently, the formation of the Calcein compound with calcium was sufficiently strong to withstand the competition by larger quantities of iminodiacetic acid for the calcium in solution.

As expected, the presence of EDTA dramatically reduced the fluorescence, and in fact, extinguished it. The presence of other solvents, such as ethanol, above 2 percent, were also found to reduce the relative intensity of fluorescence significantly.

4. Limits to the concentration of Calcein for the fluorometric determination of fluorescein

The results of the measurements of fluorescence for several series of solutions with different concentrations of fluorescein are shown in figure 42. The relative fluorescence for each series of solutions is offset vertically for clarity. The measurements for the different series shown were obtained at different settings of intensity of excitation and represent the entire range of concentrations of fluorescein

Figure 42.

Limits to the concentration of Calcein
for the fluorometric determination of fluorescein

Intensities measured on a Turner Filter Fluorometer

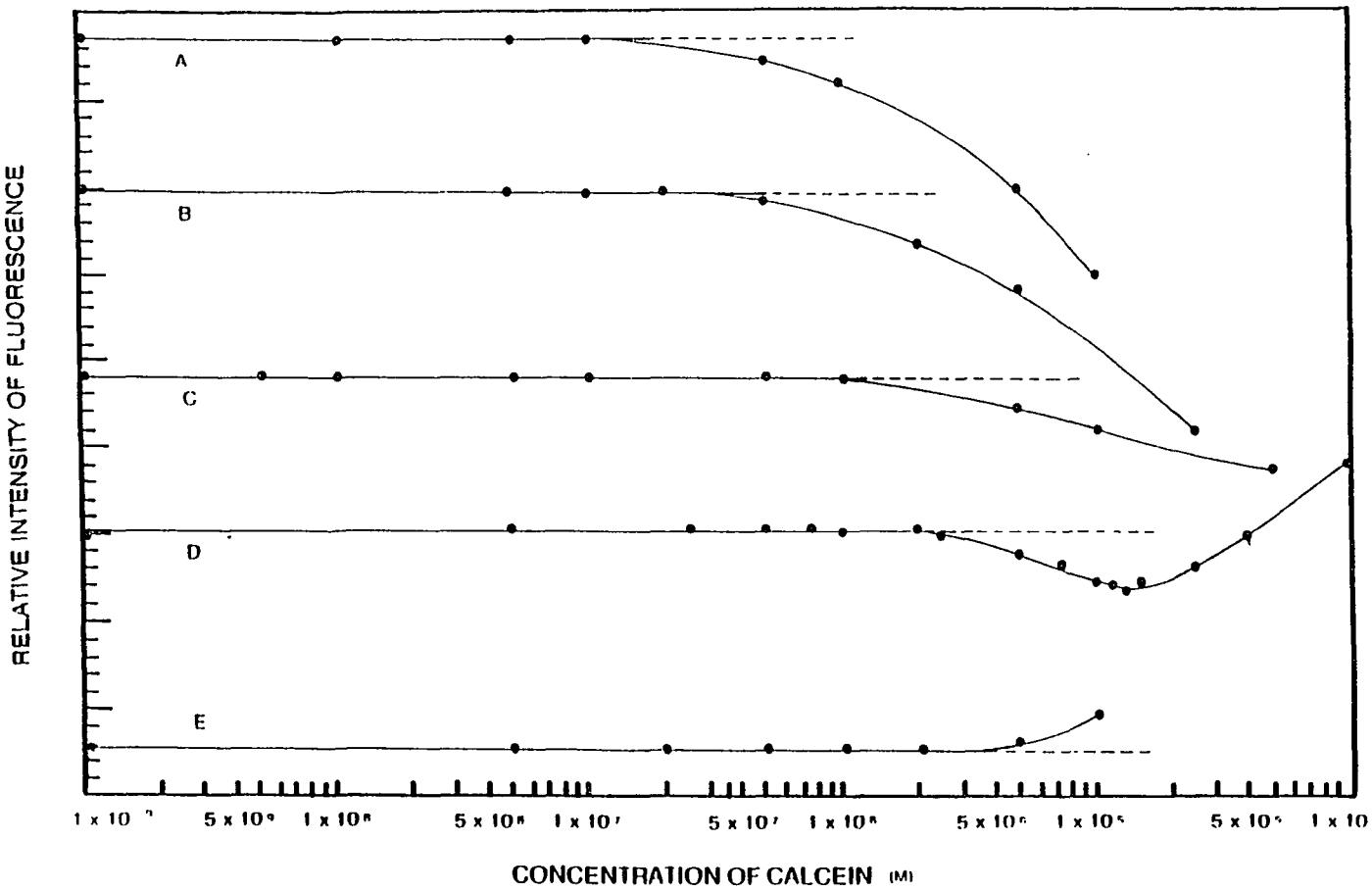
Primary filter: Corning 5850 glass filter
Secondary filter: Corning 2A-15 glass filter
& Wrattan ten per cent neutral density filter

pH of solutions: 13.0

Concentration of EDTA: 1×10^{-4} M

Concentrations of fluorescein:
Curve A: 1.0×10^{-5} M
Curve B: 5.0×10^{-6} M
Curve C: 1.0×10^{-6} M
Curve D: 1.0×10^{-7} M
Curve E: 5.0×10^{-8} M

Excitation settings:
Curve A: 1 X
Curve B: 1 X
Curve C: 10 X
Curve D: 10 X
Curve E: 30 X


RELATIVE INTENSITY OF FLUORESCENCE
A
B
C
D
E
1 x 10
5 x 10
1 x 10
5 x 10
1 x 10
5 x 10
1 x 10
5 x 10
1 x 10
5 x 10
1 x 10
CONCENTRATION OF CALCEIN (M)

accessible with the Turner Fluorometer as measured in section 2.

At each of the concentrations of fluorescein examined, the relative fluorescence remained constant, despite the presence of Calcein at concentrations of up to 1×10^{-7} M. As the concentration of Calcein was increased beyond 1×10^{-7} M, however, deviations from the constant intensity were observed in both directions.

In the series of solutions containing low concentrations of fluorescein, curves D and E in figure 42, the relative fluorescence increased when the concentration of Calcein added exceeded one hundred times the concentration of fluorescein. At this relative concentration, the amount of fluorescein added to the solution as an impurity of about one percent in Calcein, became measurable with respect to the bulk of fluorescein in the solution. Thus, the fluorescence, which was proportional to the total amount of fluorescein present, increased with each additional increment of Calcein.

At higher concentrations of fluorescein, the deviation with additions of Calcein was a marked decrease in the measured intensity of fluorescence. Previous workers who have been concerned with this decrease have attributed it to the absorption of the fluorescent emission of fluorescein by Calcein. The absorption spectrum of Calcein at pH 13 (figure 35), has an intense maximum of absorbance at 487 nm. with a significant tail overlapping the emission band of fluorescein at 520 nm.

Despite the simplicity and convenience of this explanation, the experimental data summarized in figure 35 did not support this theory. If the deviation were the result of an increased absorption of the light emitted by the fluorescein, then the absolute concentration required to extinguish a measurable amount of fluorescence would increase with increasing concentrations of fluorescein. The opposite was true. In solutions of higher concentrations of fluorescein, deviations were encountered at successively lower absolute concentrations of Calcein. Furthermore, the molar absorptivity of Calcein for light at 520 nm. was inadequate to account for the deviations measured. The

absorbance of Calcein at pH 13 for 520 nm. is shown in curve E of figure 35. Even at 2×10^{-5} M, the absorbance was only 0.03 while the fluorescence of a solution containing 5×10^{-6} M fluorescein and 2×10^{-5} M Calcein had decreased by more than 30 percent - three times the amount predicted to be absorbed.

The explanation of this phenomenon lies not in the absorption of emitted light, but in the absorption of the incident light. The intensity of fluorescence is proportional to the energy of the incident beam that is absorbed by the fluorescing species,

$$F = k\ (I_0 - I) \qquad (14)$$

which, by Beer's Law, can be stated as

$$F = k\ I_0\ (1 - 10^{-abc}) \qquad (15)$$

Thus, the fluorescence only remains proportional to the incident energy at low concentrations of the fluorophore. Using the value of the molar extinction

coefficient, 38,000 cm-1 M-1 (as determined in section 1) and the path length of the cell, 0.5 cm., the concentration of fluoresceinat which the assumption of linearity fails can be predicted. For a five percent deviation

$$10^{-abc} = 0.05$$

$$c = -\log(0.05)/(0.5 \text{ cm.})(38{,}000 \text{ cm-1 M-1}) = 7 \times 10^{-5} \text{ M}$$

In addition, Calcein was present with an essentially identical absorption spectrum, this further decreased the incident energy at the wavelengths of excitation of fluorescein

$$F = k\ I\ (1-10^{-a_{Fl}\ bc_{Fl}})(1-10^{-a_{Cal}\ bc_{Cal}}) \qquad (16)$$

and the deviation from linearity was a function of the concentrations of both fluorescein and Calcein. The greater the concentration of fluorescein, the less Calcein was required to decrease the net intensity of fluorescence. The predominant effect of the addition of Calcein, therefore, was the increased absorption of the incident light and the resulting decrease in the

energy available to be absorbed by fluorescein.

To check the accuracy of these predictions, the fluorescence of more concentrated solutions of fluorescein was measured by using very narrow slit widths and the minimum sensitivity of the spectrophotofluorometer. The results are shown in figure 43. The fluorescence of fluorescein failed to increase linearly with concentrations above 5×10^{-5} M.

The approximate region within which fluorescein could be determined with the Turner Fluorometer, equipped as described above, is shown in figure 44. The minimum and maximum concentrations of fluorescein, borders A and B, were determined by the range of sensitivity of the fluorometer as reflected by the calibration curves of figure 40. Furthermore, the points along curve C represent the limits to the concentration of Calcein determined experimentally. These limits were expected to form a curve that was concave downward in the plot of the concentration of Calcein against the concentration of fluorescein at these limits. Within the shaded region of figure 44, fluorescein can be determined accurately and to the

Figure 43.

Fluorescence of highly concentrated solutions
of fluorescein

Measured on an Aminco-Bowman Spectrophotofluorometer

Entrance monochromator setting: 485 nm
Exit monochromator setting: 520 nm.
Entrance slit width: 0.5 mm.
Exit slit width: 0.5 mm.

Cell: 0.5 cm. cylindrical quartz cuvette

P: Point corresponding to maximum intensity
measurable with the filter fluorometer
used for all routine determinations

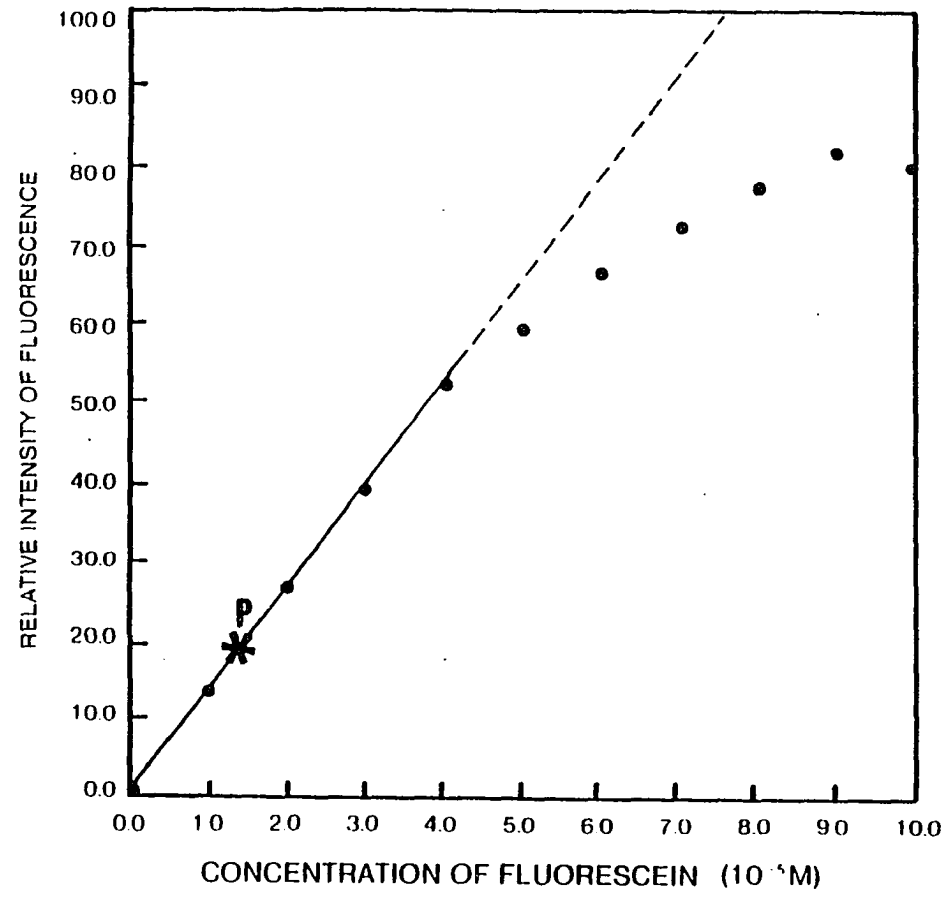

100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
RELATIVE INTENSITY OF FLUORESCENCE
P
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
CONCENTRATION OF FLUORESCEIN (10 ⁵M)

Figure 44.

Conditions of concentration
for the fluorometric determination
of fluorescein in the presence of Calcein

A: Minimum concentration of fluorescein detectable with the Turner Model 110 Fluorometer

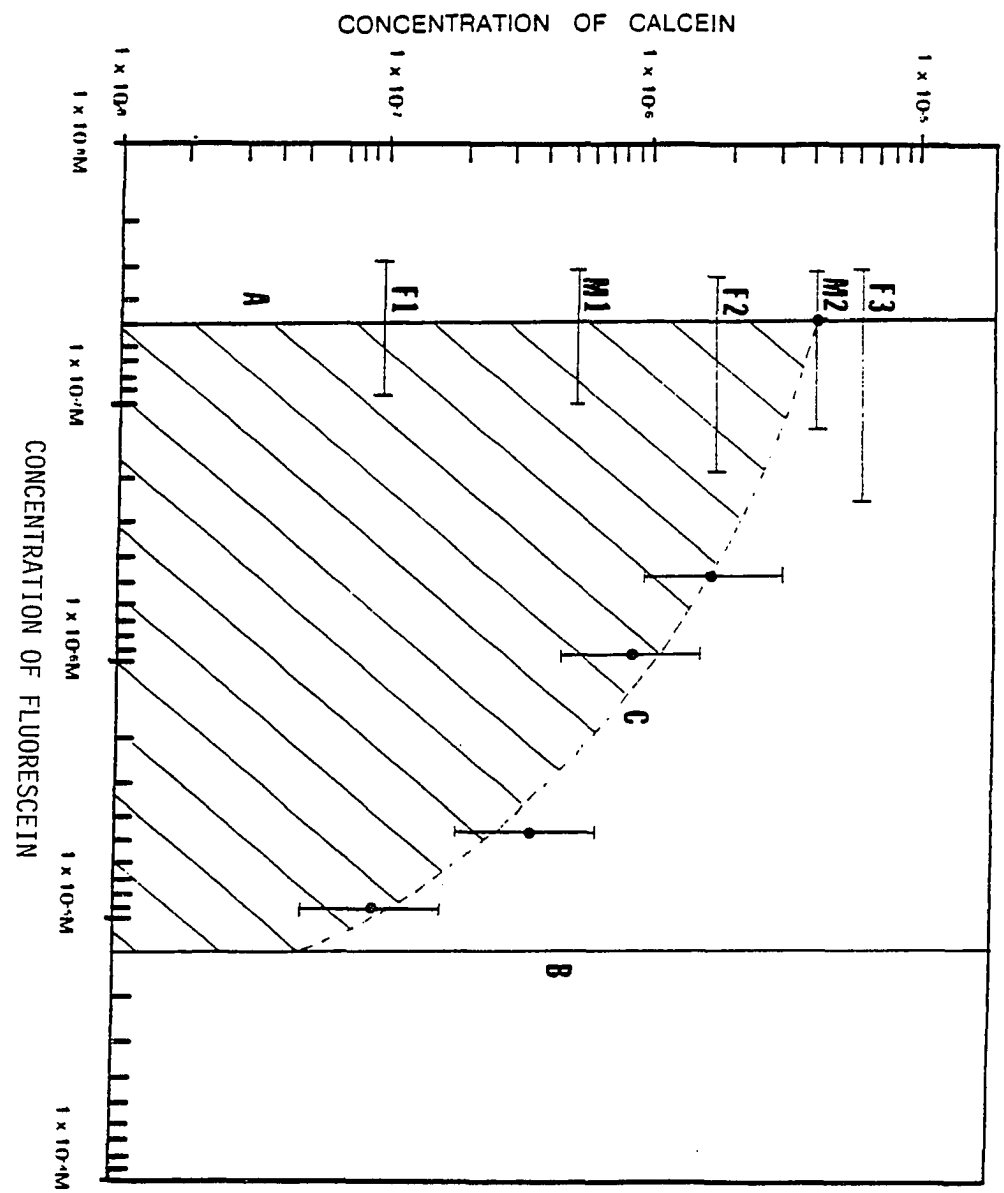
B: Maximum concentration of fluorescein measurable with the Turner Model 110 Fluorometer

C: Maximum concentration of Calcein as a function of the concentration of fluorescein above which measurable quenching occurs

E: Experimental points of deviation from Figure 035

M1, M2: Ranges of concentration examined by Martin

F1, F2, F3: Ranges of concentration examined in section 6


CONCENTRATION OF CALCEIN
CONCENTRATION OF FLUORESCEIN
A
B
C
F1
M1
F2
M2
F3

limit of the precision of the instrument.

Figure 44 established conditions of relative and absolute concentrations for the accurate fluorometric determination of fluorescein in mixtures of fluorescein and Calcein. Next, the predictions from this figure were checked with respect to the critical problem of determining small amounts of fluorescein in Calcein, as described in the next section.

5. Determination of small quantities of fluorescein as impurities in Calcein

In order to measure the amount of fluorescein existing as an impurity in Calcein (usually on the order of a few percent), standard additions of fluorescein were made such that the concentrations of fluorescein added were one-and-a-half to two orders of magnitude smaller than the concentration of Calcein. Examination of figure 36 indicated that the accurate determination of fluorescein in Calcein under these conditions of relative concentrations was only possible with very small concentrations of both compounds. In

fact, analysis was limited to concentrations of fluorescein of 10^{-7} M or less for even a ten-fold difference in the concentrations of fluorescein and Calcein. Furthermore, the concentrations of Calcein could not exceed 3×10^{-6} M.

As confirmation of these predictions, the results of standard additions to solutions containing Calcein at concentrations of 1×10^{-7} M, 2×10^{-6} M, and 7.5×10^{-6} M are presented in figures 45, 46, and 47. In each figure, the calibration curve for solutions of fluorescein alone is also shown for comparison.

At 1×10^{-7} M Calcein, as shown in figure 45, the plot of fluorescence with standard additions appeared linear and parallel to the calibration curve for fluorescein. However, the measurements of these concentrations were close to the limit of detection of the instrument and the signal-to-noise ratio was undesirably low.

At a concentration of 2×10^{-6} M, as noted in figure 46, the plot of the standard addition was again

Figure 45.

Determination of trace quantities of fluorescein in 1×10^{-7} M Calcein

Measured on a Turner Model 110 Filter Fluorometer

Primary filter: Corning 5850 glass filter

Secondary filter: Corning 2A-15 glass filter & Wratten ten percent neutral density filter

Sensitivity: 10X

Concentration of EDTA: 2.0×10^{-5} M

pH of solutions: 13.0

Curve A: Fluorescein calibration curve

Curve B: Standard additions of fluorescein

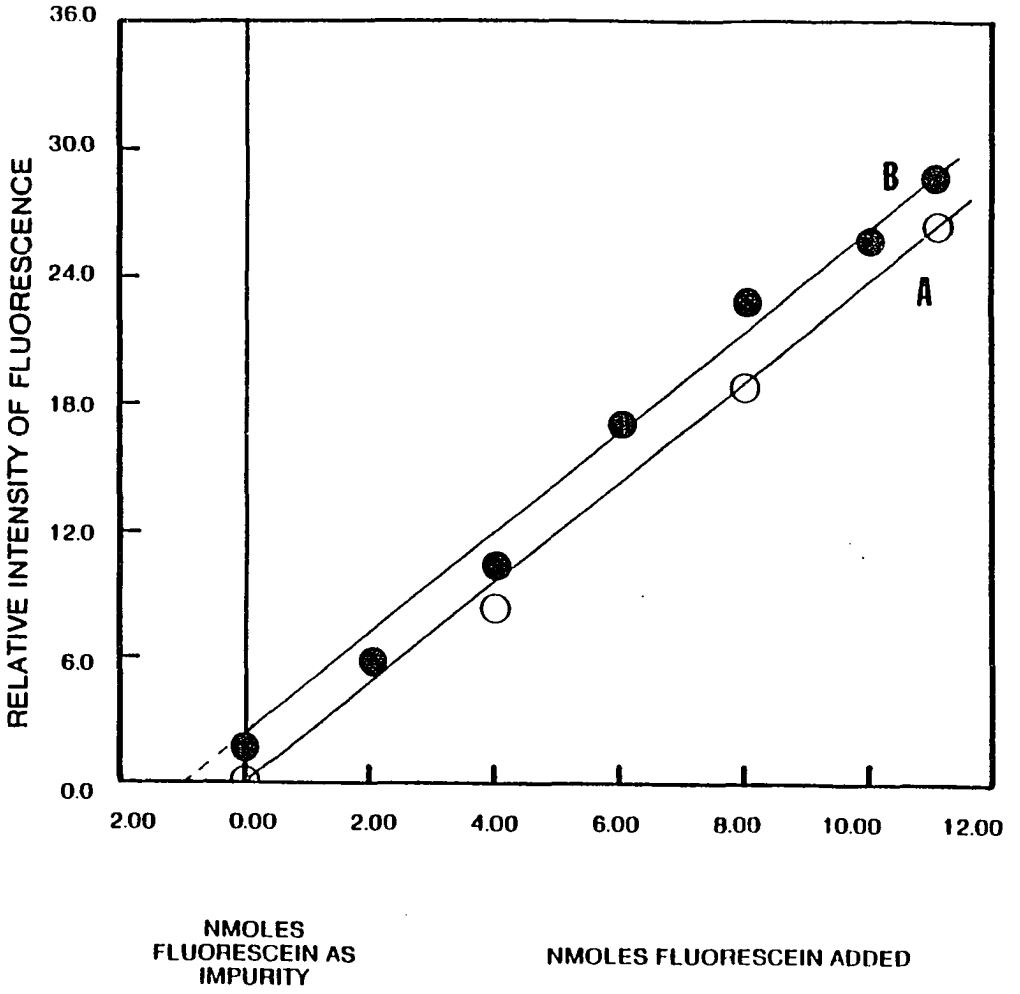

36.0
30.0
24.0
18.0
12.0
6.0
0.0
RELATIVE INTENSITY OF FLUORESCENCE
B
A
2.00
0.00
2.00
4.00
6.00
8.00
10.00
12.00
NMOLES FLUORESCEIN AS IMPURITY
NMOLES FLUORESCEIN ADDED

Figure 46.

Determination of trace quantities of fluorescein
in 2×10^{-6} M Calcein

Measured on a Turner Model 110 Filter Fluorometer

Primary filter: Corning 5850 glass filter

Secondary filter: Corning 2A-15 glass filter
& Wratten ten percent neutral density filter

Sensitivity: 10X

Concentration of EDTA: 2.0×10^{-5} M

pH of solutions: 13.0

Curve A: Fluorescein calibration curve

Curve B: Standard additions of fluorescein

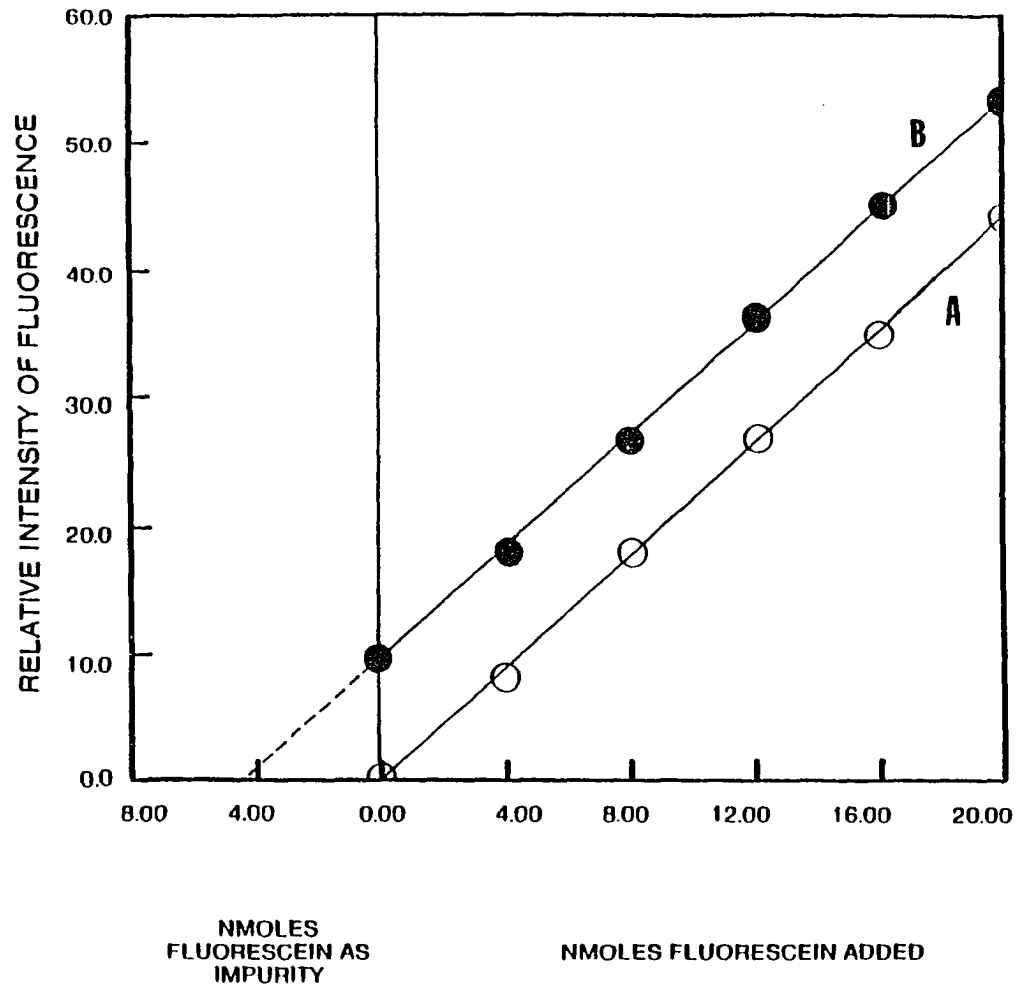

60.0
50.0
40.0
30.0
20.0
10.0
0.0
RELATIVE INTENSITY OF FLUORESCENCE
B
A
8.00
4.00
0.00
4.00
8.00
12.00
16.00
20.00
NMOLES FLUORESCEIN AS IMPURITY
NMOLES FLUORESCEIN ADDED

Figure 47.

Determination of trace quantities of fluorescein in 7.5×10^{-6} M Calcein

Measured on a Turner Model 110 Filter Fluorometer

Primary filter: Corning 5850 glass filter

Secondary filter: Corning 2A-15 glass filter & Wratten ten percent neutral density filter

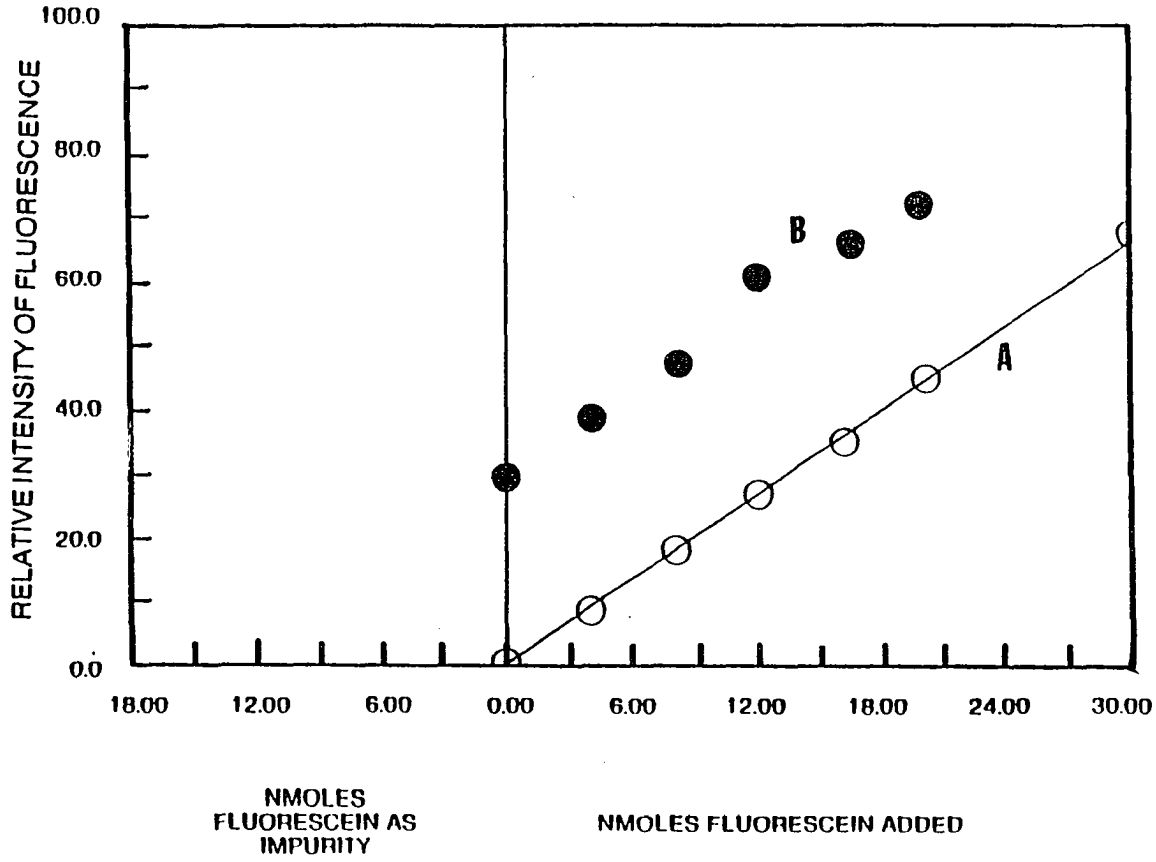
Sensitivity: 10X

Concentration of EDTA: 2.0×10^{-5} M

pH of solutions: 13.0

Curve A: Fluorescein calibration curve

Curve B: Standard additions of fluorescein


100.0
80.0
60.0
40.0
20.0
0.0
RELATIVE INTENSITY OF FLUORESCENCE
B
A
18.00
12.00
6.00
0.00
6.00
12.00
18.00
24.00
30.00
NMOLES FLUORESCEIN AS IMPURITY
NMOLES FLUORESCEIN ADDED

linear and parallel to the calibration curve. In addition, the scatter of the points was significantly reduced in comparison with the previous set of data. Determination of fluorescein by standard addition was successful.

At 7.5×10^{-6} M Calcein, however, the plot of the standard additions, shown in figure 47, was of questionable linearity and was definitely not parallel to the calibration curve for fluorescein.

The range of concentrations of fluorescein used for each of these standard additions is indicated in figure 44 by F1, F2, and F3. In each case, the results confirmed the qualitative limitations predicted in this figure.

Two sets of concentrations were examined in similar attempts by Martin[252], and are shown in figure 44 as M1 and M2. Martin was successful in obtaining a linear plot for standard additions using a concentration of fluorescein of 1×10^{-8} M for solutions containing 5×10^{-7} M Calcein. He also noted that standard additions for solutions containing 5×10^{-6} M

Calcein failed.

The conditions of relative and absolute concentrations of fluorescein and Calcein have been established for the fluorometric determination of fluorescein with the Turner Model 110 Fluorometer. The range included is broad enough to cover both the determination of large quantities of fluorescein in the early stages of the synthesis of Calcein, and the determination of the small amounts of fluorescein remaining in the final product as an impurity.

6. Limits of the concentrations of fluorescein and Calcein for the fluorometric determination of Calcein

The method proposed for the fluorometric determination of Calcein in mixtures with fluorescein involved the parallel measurements of fluorescence from two solutions of the mixture to be analyzed. The first solution contained an excess of EDTA and the second contained calcium at approximately ten times the concentration of Calcein. The fluorescence produced by

the slightly-dissociated compound of Calcein with calcium is determined from the difference in intensities of the two solutions and the concentration of Calcein is determined by comparing this difference with the calibration curves measured in section 3 for the calcium-Calcein compound.

Inasmuch as the first measurement was really the determination of fluorescein in mixtures with Calcein, the criteria set forth in figure 44 applied to this determination as well. In addition, the additivity of the fluorescence from these two compounds was checked for further restrictions for the determination of Calcein.

The fluorescence for each of eight series of solutions was measured with the filter fluorometer. Each of the series of solutions with fluorescein above 5×10^{-8} M was found to deviate within the range of intensities measurable with the filter fluorometer. The approximate concentrations of Calcein at which each curve deviated are listed in table 11. These points were also used to construct curve H in figure 48.

Table 11.

Limits to the concentrations of fluorescein and Calcein for the fluorometric determination of Calcein

Series	Concentration of Fluorescein (M)	Concentration of Calcein at Point of Deviation (M)
A	0	no deviation
B	5.0×10^{-8}	no deviation
C	1.0×10^{-7}	1×10^{-5}
D	5.0×10^{-7}	2×10^{-6}
E	1.0×10^{-6}	2×10^{-6}
F	5.0×10^{-6}	1×10^{-7}
G	1.0×10^{-5}	1×10^{-7}

Figure 48.

Conditions for the fluorometric determination of Calcein

A: Minimum concentration of fluorescein detectable with the Turner Fluorometer

B: Maximum concentration of fluorescein measurable with the Turner Fluorometer

C: Maximum concentration of Calcein measurable without significant absorption effects

D: Minimum concentration of Calcein detectable with the Turner Fluorometer

E: Maximum concentration of Calcein measurable with the Turner Fluorometer

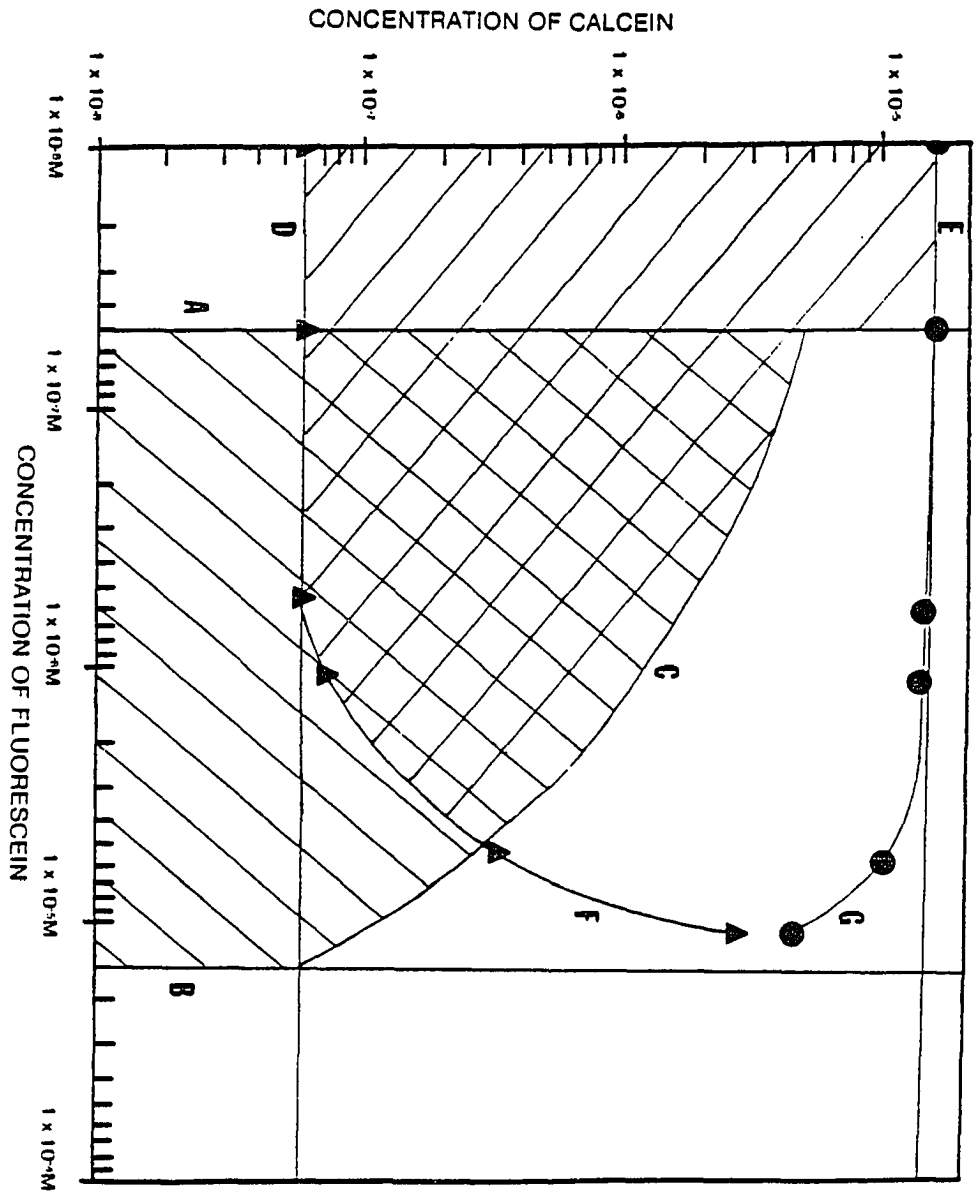
F: Minimum concentration of Calcein detectable above the fluorescence of fluorescein

G: Maximum concentration of Calcein measurable in addition to the fluorescence of fluorescein

(\\\) Region defined for the determination of fluorescein

(///) Region defined for the determination of Calcein

(XXX) Region defined for the fluorometric determination of both fluorescein and Calcein in mixtures


CONCENTRATION OF CALCEIN
1 x 10⁻⁸
1 x 10⁻⁷
1 x 10⁻⁶
1 x 10⁻⁵
CONCENTRATION OF FLUORESCEIN
1 x 10⁻⁵M
1 x 10⁻⁴M
1 x 10⁻³M
1 x 10⁻²M
1 x 10⁻¹M
A
B
C
D
E
F
G

These additional restrictions on the absolute concentrations of fluorescein and Calcein from table 11 were explained by the same theory presented above for fluorescein. The curves C and F in figure 48 were almost reciprocal relationships.

Some additional restrictions applied because of the instrumental limitations to (1) detect the increase in fluorescence and (2) measure the net intensity due to both fluorescing compounds. These restrictions in terms of the concentrations corresponding to the minimum and maximum signal measurable on the Turner Fluorometer are also shown in figure 48. The superposition of these restrictions with those given in figure 44 outlined the conditions of relative and absolute concentrations under which both fluorescein and Calcein could be accurately determined by fluorescence. The precision of the determination of Calcein was decreased only by the need to double the number of measurements. As a result, Calcein could be determined at most of the concentrations encountered in the present investigations with a relative precision of two to three percent.

E. Conclusions. Recommended Procedures

The spectrophotometric properties of fluorescein and Calcein have been examined in detail. The absorption spectra of these compounds are dominated by the same three bands that predominate the excitation spectra discussed in chapters II and III. The relative absorbance at each absorption band can be correlated to the relative abundance of the individual prototropic forms of fluorescein and Calcein. The spectrophotometric properties of each of the ionic forms, including the molar extinction coefficients, are summarized in table 10.

The spectrophotometric properties of fluorescein paralleled the fluorometric properties discussed in chapter II. The properties of Calcein, on the other hand, included one major an very important difference between the fluorescence and visible absorption as functions of pH. Specifically, the constant, intense absorption between 475 and 495 nm. was retained in highly alkaline solutions where the fluorescence is extinguished. In this region, absorption was highly

sensitive to concentration and relatively insensitive toward small changes in pH for both compounds and this property was utilized for the rapid and convenient determinations of fluorescein and Calcein in independent solutions. The concentrations over which the calibration curves were linear was from 5.0×10^{-7} to 1.2×10^{-5} M. Not only did fluorescein and Calcein interfere severely with the spectrophotometric determination of each other, but several other materials involved in this study have significant absorptions in this region and analysis of mixtures of these materials were found to require the more sophisticated fluorometric procedures below.

The absorbance of Calcein at 485 nm. was used, along with the fluorescence data from chapter III, to estimate an effective dissociation constant for the cationic species of Calcein. This effective dissociation constant of 1.1×10^{-1} actually encompassed the overlapping equilibria involving three different cations and the neutral molecule of Calcein.

Both fluorescein and Calcein were determined fluorometrically in independent solutions. Using a

simple filter fluorometer, concentrations of fluorescein between 5.0×10^{-8} and 1.4×10^{-5} M and concentrations of Calcein between 5.0×10^{-8} and 1.6×10^{-5} M were determined by measuring the relative intensity of fluorescence and reading the corresponding concentration from the calibration curves in figures 33 and 34, respectively.

Metals, formaldehyde, iminodiacetic acid, EDTA, and changes in ionic strength did not significantly affect the fluorescence of fluorescein. Ethanol and other non-aqueous solvents were found to measurably suppress the fluorescence when present at greater than two percent.

Formaldehyde, iminodiacetic acid, excess calcium, and changes in ionic strength also had no measurable effect on the fluorescence of the 2:1 calcium-Calcein compound at pH 13. The presence of EDTA, however, decreased the fluorescence by removing the calcium from the fluorescent compound. A sufficient excess of metal ions was required to avoid or compensate for this effect. The presence of more than a few percent of ethanol resulted in a measurable decrease in the

fluorescence of the slightly-dissociated compound just as was observed with fluorescein.

The limitations to the absolute concentrations of Calcein and fluorescein for fluorometric determinations of these two compounds in mixtures was found to be principally the result of the absorption of the incident light rather than an absorption of the emitted fluorescence. When the common chromophore was present at sufficiently high concentrations, a significant portion of the incident energy was absorbed and the linear relationship between fluorescence and concentration no longer held. As a result, the limits on concentrations were interdependent and best described by the graphical representation in figure 41.

Fluorescein can be determined in solutions containing Calcein if the concentrations of the two compounds fall, or can be diluted to fall, within the range outlined in figure 36. This region was defined by the limitations of sensitivity of the instrument, which dictated the minimum and maximum concentrations of fluorescein that were measurable, and by the limits of the concentrations of Calcein below which the

fluorescence was linear with increasing concentrations of fluorescein. Within these limits, fluorescein can be determined fluorometrically in the presence of Calcein by measuring the relative intensity of fluorescence of a highly alkaline solution containing no free metal ions with which Calcein can form fluorescing, slightly-dissociated compounds and then determining the concentration of fluorescein from the linear calibration curves established in section 1. To assure that no such interfering metals are present as free or loosely associated ions in solution, a strong chelating agent, such as EDTA, is always added. This method has been used extensively in the investigations discussed in chapters VI and VII of this dissertation.

The determination which is currently of greatest interest is the measurement of fluorescein as an impurity in Calcein. Assuming that fluorescein is present as only a few per cent by weight in the different preparations of Calcein, the relative concentrations of Calcein and the fluorescein used for standard additions to the sample must differ by at least an order of magnitude while remaining within the region defined in figure 36. This is only possible

within a very small, narrowly defined section in the upper left corner of the region shown in that figure.

Specifically, the determination of fluorescein as an impurity in Calcein can be achieved by the following procedure:

Approximately 0.16 g. of fluorescein is precisely weighed and dissolved in water with about 20 ml. of 0.1 N potassium hydroxide. An aliqout of 10.00 ml. of this solution is then diluted to 2.00 liters. This solution is approximately 2.5×10^{-6} M and serves as the standard reagent.

Approximately 0.16 g. of the sample of Calcein is also carefully weighed and dissolved in about 200 ml. of water with about 20 ml. of 0.10 N potassium hydroxide. This solution is diluted to 1.00 liters and has a final concentration of approximately 2.5×10^{-4} M. Care is taken to minimize direct exposure of the Calcein to strong alkali to prevent any degradation of the compound.

To each of a series of 100-ml. volumetric flasks

is added: 2 ml. of 10 M EDTA, 10.0 ml. of 1.0 N potassium hydroxide, 1.00 ml. of the solution of Calcein, and increasing portions from zero to 10.00 ml. of the solution of fluorescein. The concentration of Calcein is approximately 2.5×10^{-6} M and the concentrations of fluorescein increase up to 2.5×10^{-7} M.

The relative fluorescence is then measured using the most sensitive setting of the Turner Fluorometer in conjunction with a blue primary filter and a yellow secondary filter. The extrapolation of the plot of fluorescence with standard additions of fluorescein indicates the concentration of fluorescein in the solution of Calcein.

Calcein can be determined in solutions containing both fluorescein and Calcein if the concentrations of the two species fall or can be diluted to fall within the cross-checked region of figure 41. This region is outlined by the minimum concentrations of fluorescein and the slightly-dissociated compound of Calcein with calcium that are measurable with the Turner Fluorometer, the maximum limitations in the

concentration of Calcein which allow the accurate determination of fluorescein, and the minimum concentration of the slightly-dissociated compound of Calcein with calcium which can be discerned above the fluorescence of fluorescein. Within this region, the fluorescence due to fluorescein is first determined in a strongly alkaline solution containing a strong chelating agent such as EDTA to prevent the formation of the slightly-dissociated compounds of Calcein. The total fluorescence due to the presence of both fluorescein and Calcein with calcium is then measured in a solution identical to the first except for the replacement of the chelating agent with calcium chloride at a ten-fold molar excess over the Calcein present. The difference in intensities of fluorescence can be directly attributed to the concentration of Calcein and the concentration can then be determined from the calibration curves for the slightly-dissociated compound. The relative error of the two-measurement procedure is still only two to three percent, which is more than adequate for application to the work described in the next two chapters.

VI. PREPARATION OF CALCEIN IN A HIGHLY PURE AND CONSISTENT FORM

A. Introduction. Previous methods of preparation and the significance of impurities

For Calcein, as for most analytical reagents, the purity and consistency of the final product are the most important considerations in its preparation. As the breadth and sophistication of the applications of Calcein continue to increase, so do the demands for supplies of reagent with ever purer and more constant composition. Efforts to improve the methods for synthesizing and handling Calcein, in turn, require improvements in the methods by which Calcein can be evaluated throughout its preparation and use.

Calcein was first prepared by Ellingboe[127] in 1956. In Ellingboe's procedure, fluorescein, formaldehyde, and iminodiacetic acid were dissolved in a highly alkaline, 50 percent solution of ethanol. The reaction took place over several hours at 65 C. The material that was prepared was actually a crude mixture

containing significant quantities of both organic and inorganic impurities. Nevertheless, even this rudimentary form of the reagent was sufficient for use as the first metallofluorochromic indicator. Although alkaline solutions of early preparations of Calcein remained somewhat fluorescent after the equivalence-point in the chelatometric titration of calcium with EDTA, the change in the intensity of fluorescence was usually sufficient to mark the end-point.

The potential applications for Calcein quickly prompted others to attempt to prepare a reagent of higher purity. Soon after the initial publication by Diehl and Ellingboe[106], groups in Czecholslavakia[209-222], [360]in the Soviet Union[64-69], and at Harvard University[359] published claims that included improved titrimetric results which they attributed to the use of more highly pure preparations of Calcein. These publications, however, noted only minor changes in the original method of preparation. Furthermore, very little information was given in regard to the actual purity or the methods used to evaluate the purity of their preparations of Calcein. Wallach and coworkers[362]

reacted fluorescein with formaldehyde and iminodiacetic acid in 30 percent solutions of ethanol at a lower pH than that used by Ellingboe, while Koerbl and Pribil [221] conducted the same Mannich condensation in totally aqueous solutions. Despite the general claims for higher purity and less residual fluorescence, these materials often failed to produce such clear and accurate results in the hands of others [146,287,288,355].

Use of all of these early preparations as chelatometric indicators for calcium was limited primarily by the residual fluorescence which persistently remained far after the equivalence-point in the titration. To circumvent this problem, many workers in the field modified the reagent by mixing it with other materials [342] such as thymolphthalein [36,349], thymolphthalein complexone [376], phenolphthalein complexone [210,230,324], bromthymol blue [104], and acridine [205]. These deeply colored compounds masked the residual fluorescence by absorbing low levels of emitted fluorescence. Other workers mixed Calcein with finely powdered solids such as charcoal [113] which further diffused the emitted light. Of these mixed indicators,

the most widely recommended was the combination of Calcein and phenolphthalein complexone[132,325,341]. This recommendation was based on the reaction of calcium with both materials to form slightly-dissociated compounds accompanied by changes in the fluorescence of Calcein and the color of phenolphthalein complexone. The net observable end-point was dramatically improved by the combined effect of a decrease in fluorescence and an increase in absorption at approximately the same wavelength.

Many efforts have been made to automate the titration of calcium with EDTA. In each case, the need to eliminate the residual fluorescence was recognized as being critical. For example, in order to use a simple instrumental autostop that was triggered by the disappearance of the fluorescence of the calcium-Calcein compound, a reproducible end-point with no measureable fluorescence was required[180]. The principal culprit for the residual fluorescence was recognized from the beginning[127] as unreacted fluorescein and possible fluorescing by-products[161] of the Mannich condensation of fluorescein, formaldehyde and iminodiacetic acid. As were described in chapter

V, early efforts to detect such contamination by chromatography were only successful for concentrations of fluorescein greater than two percent. Furthermore, quantitation by this method was imprecise and subjective. The determinations were complicated by the appearance of several chromatographically separable spots or bands upon eluting with different solvents [165]. In addition to any organic contaminants present, the multiplicity of chromatographic bands was a result of separations of the different prototropic forms and electronic isomers at different pH and in different solvents.

Clearly, the evaluation of the quality of preparations of Calcein and confidence in analyses using the Calcein end-point demanded a new and accurate method for determining fluorescein in Calcein. The determination of fluorescein in Calcein by fluorometry was first attempted by Bozhevol'nov and Kreingol'd[64] and by Martin[252]. Unfortunately, neither efforts defined the narrow range of absolute and relative concentrations over which the fluorometric procedure is accurate. In the current work, a fluorometric procedure has been developed, the accuracy of the method has been

demonstrated and the strict conditions under which this analysis is accurate have been established.

A second common reason for deviations from the fluorescence predicted for solutions of Calcein was first identified by Hefley[166]. Metals, principally iron, aluminum, mercury and zinc, were found to be tenaciously retained by Calcein during preparation. These metals were originally introduced during the condensation of resorcinol and phthalic anhydride in the synthesis of fluorescein. They were retained, first by the fluorescein, and then by the Calcein, throughout the subsequent preparations. These metals combined with Calcein and altered the net fluorescence of subsequent solutions. As was shown in chapter IV, iron forms a non-fluorescing compound with Calcein in neutral solutions, aluminum forms a fluorescing compound in acidic and moderately alkaline solutions, while mercury and zinc form fluorescing compounds in mildly alkaline solutions.

Hefley[165,166] estimated the total amount of metallic impurities in fluorescein and in preparations of Calcein by measuring the amount of ash remaining

after ignition. In the present work, the different metallic impurities were distinguished and determined individually by atomic emission analysis.

In addition to the practical limitations which have resulted from a lack of purity, progress in gaining an understanding of the chemical and physical properties of Calcein has also been severely hampered. Accurate measurements of the fluorometric, spectrophotometric and titrimetric properties of Calcein were only possible by studying a highly pure form. In fact, only with the recent preparations of highly purified material have the chemical structure and properties of Calcein been resolved. For these reasons, a substantial research effort has continued at Iowa State University, directed toward obtaining a highly pure form of Calcein.

To minimize the contamination of Calcein with interfering metals, Hefley purified the fluorescein by acetylation[165]. Then Hefley reacted the purified fluorescein with formaldehyde and iminodiacetic acid in a solution of acetic acid. The purity of the product, especially with respect to these troublesome metals,

was significantly improved[166]. However, yields were very low, often only a few percent.

Martin[252,253] conducted this synthesis in moderately alkaline, aqueous solutions using the sodium salt of iminodiacetic acid and formaldehyde in four-fold excesses to drive the reaction toward completion. Martin further purified the Calcein by repeatedly recrystalizing the product from aqueous solutions. Large amounts of sodium chloride or potassium chloride were used to facilitate the coagulation and filtration of the otherwise disperse precipitate. This procedure improved yields slightly and significantly reduced the contamination of the product with fluorescein.

The purity of acidic reagents such as Calcein has traditionally been measured in terms of the equivalent weight of the acid. The determination of the equivalent weight of Calcein, however, proved to be very difficult and relatively inaccurate by direct titration. First, the neutral molecule of Calcein was found to be relatively insoluble and therefore difficult to titrate directly. Second, the acid-base

standard alkali and a ten-fold excess of calcium ions. In the presence of the excess calcium, a two-to-one slightly-dissociated compound of calcium and Calcein was formed quantitatively, releasing the remaining two replaceable hydrogens from each molecule of Calcein. The equivalent weight was then determined by back-titrating the solution with standardized acid and measuring the difference in equivalents between the end-points of the forward- and back-titrations. The difference corresponded to the fifth and sixth replaceable hydrogens of Calcein for which fluorescein has no counterparts.

For the first time, truely accurate measurements of purity were made. Several preparations by Martin had purities as high as 93 percent (based on an a molecular weight of 640 g/eq.) while the final yields by Martin's procedure were on the order of 20 to 30 percent[254]. A modification of this method for the determination of net purity was used in the current work.

Other impurities became a problem when Calcein was evaluated as a direct fluorometric reagent. Residual

properties of the principal contaminants were found to be very similar to those of Calcein. Several important improvements have been made to the direct titration for measuring equivalent weights of Calcein. Hefley [166] added an excess of standardized alkali to a weighed sample of Calcein and back-titrated the excess alkali with standardized acid. Markuszewski [247] dissolved the material in a 50 percent solution of ethanol and then titrated directly with standardized acid.
Unfortunately, the end-points for the titrations of the first two replaceable hydrogen ions of fluorescein and the first replaceable hydrogen of iminodiacetic acid are indistinguishable from the end-point in the titration of the first two hydrogens of Calcein under these conditions. The observed break in the potentiometric curve near pH 7.5 actually corresponded to the simultaneous titration of all three compounds. Martin [301] developed a two-end-point titration that measured the equivalent weight of Calcein independent of any fluorescein present. First, Martin titrated a weighed sample of Calcein with standardized alkali to the end-point at pH 7.5 which corresponded to the neutralization of the first four replaceable hydrogens. Then he added an additional, measured amount of

fluorescence was encountered which resulted from the presence of sodium or ammonium ions[117,193,296]. To minimize this problem, the procedures developed for the preparation of Calcein in the current work used potassium salts and potassium hydroxide throughout the preparation. Furthermore, the amounts of sodium and potassium were carefully determined in each batch of Calcein. Sodium was determined by atomic absorption while the amount of potassium was determined by atomic emission and by a so-called double-ashing technique developed specifically for this work. The latter analysis determined the amounts of each of the different potassium-containing compounds of concern.

Use of Calcein as a direct fluorometric reagent necessitated even more stringent requirements of purity. Contamination by calcium and other alkaline earths became an even greater concern to analysts. Consideration had to be given, not only to metals introduced during precipitation of the reagent[174,201], but also to the contribution of metals from other reagents and even the leaching of these metals from the walls of glassware[66,360]. In the present work, Calcein and all of the reagents involved in the preparation and

applications of Calcein have been carefully analyzed for metals, especially for alkaline earths.

In addition, workers attempting to establish routine procedures have been frustrated by the lot-to-lot variation in commercially available Calcein and the resulting need to constantly recalibrate their systems [28, 174]. In fact, the need for a supply of a consistent, highly pure Calcein has been the predominant theme of the publications in this field [85, 206]. In addition to the contamination with fluorescein and metals, this inconsistency has been the result of varying amounts of salts and solvents trapped within the precipitated product. Methods were therefore specifically developed to determine the amounts of potassium chloride, other salts, and occluded solvents in the various preparations of Calcein in the current work. Monitoring these impurities assisted the selection of the best procedure to ensure a high, consistent quality.

Use of Calcein as a fluorescent dye or marker for calcified tissues required yet other specifications on purity. Most importantly, the reagent had to produce

only a single spot on application[3] and only fluoresce in the presence of calcified substrates[339]. Since most of these procedures were designed for use in conjunction with living organisms, toxic impurities (including fluorescein) had to be minimized[340]. New uses for Calcein, such as the development of an in vivo probe for calcium in the human body[57,232,298], required Calcein of very high purity. Material prepared by the final procedure recommended in this chapter has been thoroughly evaluated by thin layer chromatography and dialysis[291] to confirm sufficient purity for such biological investigations. No other source of Calcein has been found that can provide this quality[244].

In establishing the recommended procedure for the preparation of Calcein, the synthesis, precipitation, drying, and storage of Calcein were all systematically examined. The pH and composition of the solvent for the Mannich condensation was optimized for both yield and purity. Similarly, after studying the solubilities of fluorescein and Calcein, the composition of the medium for recrystalization was optimized for yield and purity. Precipitation of Calcein from a reversed direction (from the acidic side of the minimum in

solubility with pH) was recommended to minimize the scavenging of metal ions. The effectiveness of different purification procedures were evaluated and the conditions for non-destructive drying of the product were determined. The relative hygroscopicity of the solid and the stability in solution with respect to pH and light were measured. As a result, a complete procedure has been established for the routine preparation of highly pure Calcein in good yields.

The procedures developed, one for single batch preparation and a second for continuing routine synthesis on a commercial scale, has important implications for potential commercial producers. The increased purity greatly enhances the reproducibility of measurements when Calcein is used in traditional applications. The increased yield is less important but still represents a savings on the order of 25 percent of the raw material cost. Lastly, the method for repetitive preparation further decreases raw material cost, almost eliminates costly disposal requirements for solvents, and greatly simplifies operation on a large scale.

B. Apparatus and Reagents

The Mannich condensation of fluorescein, formaldehyde, and iminodiacetic acid was performed in a three-neck round-bottom flask equipped with a water-cooled condenser, thermometer, and magnetic stirring bar. The flask was placed in a heating mantle controlled by a variac as shown in figure 49. The solution was constantly stirred using a magnetic stirrer.

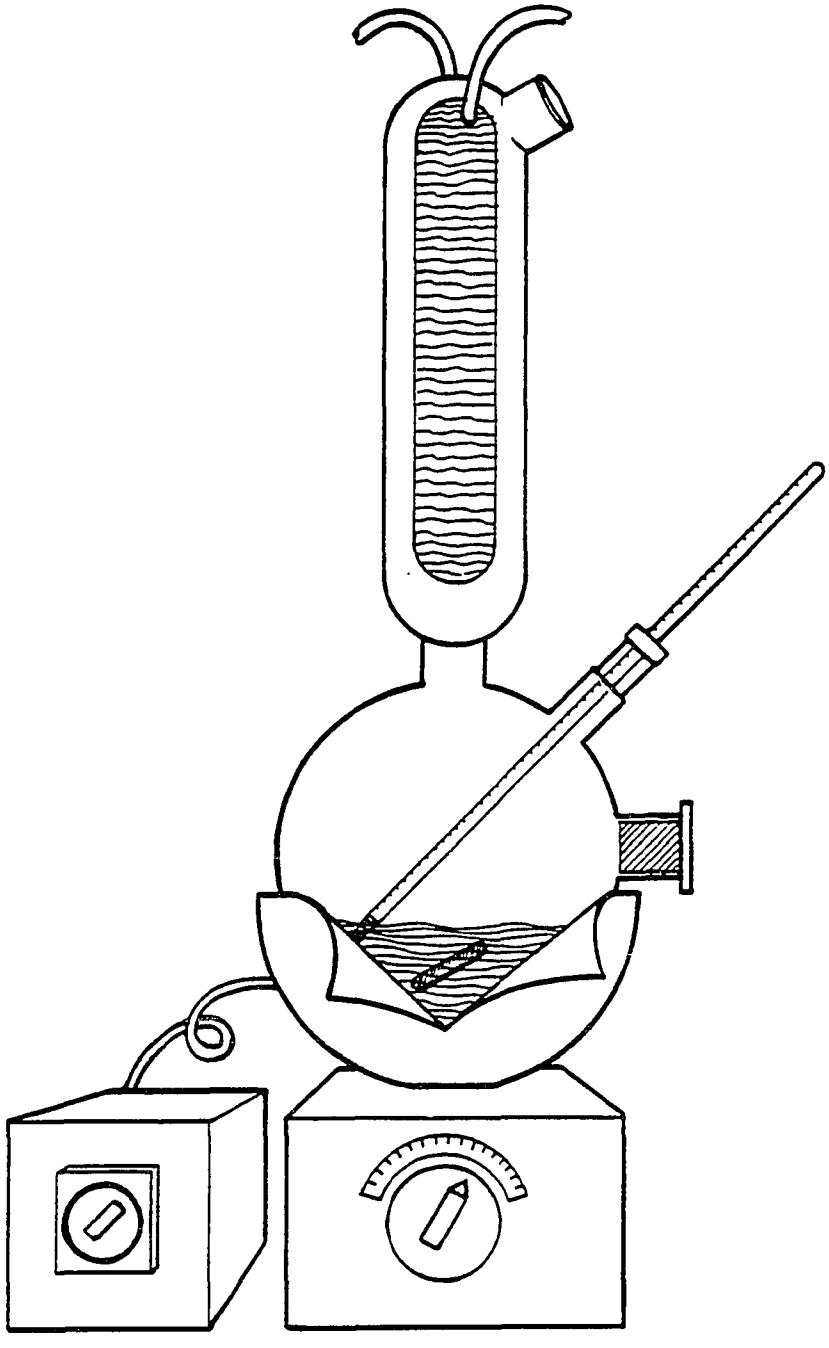
Saturated solutions of Calcein and fluorescein were prepared in small volumetric flasks, mixed with a Burrell wrist-action shaker and stored at a constant temperature of 24 C.

Most measurements of pH were made using a Beckman Zeromatic SS-2 pH Meter equipped either with a Fisher Combination pH Indicator/Reference Electrode or with a Beckman Saturated Calomel Reference Electrode and a Beckman Glass Indicator Electrode. Detailed measurements of pH for the determinations of net purity were made using a Hach Expanded Range pH Meter equipped

Figure 49.

Apparatus for the synthesis of Calcein

Three-neck round-bottom flask
Water-cooled condenser
Thermometer
Variac-controlled heating mantle
Magnetic stirrer



with the electrodes described above.

Thermometric measurements were made with two instruments. Most of the work was performed at temperatures between 20 and 250 C with a Perkin-Elmer TGS-1 Thermobalance which included a Cahn Electrobalance, Perkin-Elmer UU-1 controller and a Heath X-Y Recorder. The instrument was crudely enclosed in plastic to allow displacement of the air with dried nitrogen. Automated thermogravimetric analysis of two batches of Calcein were also performed for temperatures between 20 and 600 C using a nitrogen-purged DuPont Thermal Gravimetric Analysis Module interfaced to a DuPont Model 1090 Thermal Analysis Controller.

Chloride was routinely determined by coulometric titrations using a Corning Model 920 Chloride Meter. The setting for sample size was 100 microliters for all measurements. The two silver electrodes were freshly polished at the beginning of each day.

Quantitative determinations of potassium by atomic emission were performed using a Beckman Model DU

Spectrophotometer equipped with a Beckman 9200 Flame Photometry Attachment and a Beckman Atomizer-Burner Assembly. The wavelength for transmittance was set to approximately 767 nm. and then varied for maximum response at each calibration. The red-sensitive photocell was used for detection with the sensitivity set to 1.0.

Metallic impurities in several batches of fluorescein, Calcein and other materials related to the preparation and application of Calcein were identified using an Aminco Spectrograph with carbon electrodes. Semi-quantitative determinations of the metallic impurities were performed in Ames Laboratory using a laboratory-built plasma emission spectrometer with a three-meter Roland-circle monochromator.

Infrared spectra were obtained with a Nicolet Model 7000 Fourier-Transform Infrared Spectrometer.

Measurements of fluorescence were made with a Turner Model 110 Fluorometer. The primary filter consisted of a blue Corning 5850 glass filter and the secondary filter consisted of the combination of a

yellow Corning 2A-15 glass filter and a Wrattan 10 percent neutral density filter.

The fluorescein used in these studies came from four different sources. Samples of yellow and red solid fluorescein of unknown purity were obtained from R. Markuszewski[247] and were used to obtain infrared spectra of these two forms of fluorescein. A sample of commercially prepared fluorescein was provided by Dennis Martin[254]. This material was a red solid with significant amounts of metallic impurities. Another sample of fluorescein was obtained as purified material from Hach Chemical Company of Ames, Iowa. Additional samples were prepared by hydrolysis of the diacetate of fluorescein in cooperation with Samir Gharfeh[148].

The products of more than thirty batches of Calcein were used in these studies. The purity ranged from 72 to 99.6 percent purity based on a molecular weight of 640.2 g/eq. (monohydrate).

Purified iminodiacetic acid was obtained in the solid form from Hampshire Chemical Company of Keene, New Hampshire.

C. Experimental Work

1. Measurement of the effect of the purity of the reactants on the purity of the product

The purity of the materials used in the preparation of Calcein was measured by several different analytical techniques and correlated with the net purity and properties of the Calcein prepared from those materials. The materials examined included fluorescein, iminodiacetic acid, formaldehyde, ethylenediaminetetraacetic acid, potassium hydroxide, potassium chloride and water.

Samples of commercial and purified fluorescein were ashed, first over a Meeker burner and then in a muffle furnace at 600 C. Triplicate experiments were conducted with samples of commercial fluorescein (G. F. Smith Chemical Co., Columbus, Ohio); purified fluorescein obtained from Hach Chemical Co., Ames, Iowa; red and yellow fluorescein prepared by R. Markuszewski[247]; and fluorescein purified by diacetylation in the course of the present work.

Samples of commercial formaldehyde, iminodiacetic acid, and disodium dihydrogen ethylenediaminetetra-acetate were also ashed.

Calcein was prepared, using the procedure of Martin[252], from each of these supplies of fluorescein. Approximately 5 g. of each preparation were then ashed. For those samples with significant levels of residual ash, identification of the metals was accomplished by atomic emission spectroscopy using an Aminco Spectrograph. Semi-quantitative analyses of selected samples were performed by plasma emission spectroscopy (Analytical Services Group II, Ames Laboratory, Ames, Iowa).

Infrared spectra were obtained for the red and yellow forms of fluorescein and for samples of Calcein precipitated from different solvents.

The net purity of these preparations were also determined using the two-end-point potentiometric titration described below.

Standard solutions of acid and base were prepared

for use in all of the titrations described in this chapter. Two liters of approximately 0.1 N potassium hydroxide were prepared by diluting 18 ml. of calcium-free, 12 N potassium hydroxide to 2.00 liters with freshly boiled deionized water. The solution was standardized against potassium hydrogen phthalate. The standardized solution was stored in a plastic bottle equipped with a Bunsen valve containing 0.1 N potassium hydroxide to prevent absorption of carbon dioxide by the standardized solution. Two liters of 0.1 N hydrochloric acid was prepared by diluting 18 ml. of concentrated hydrochloric acid to 2.00 liters and standardizing the solution aganst trishydroxyamino-methylmethane. A cross-check of the acid against the standardized base confirmed the value to within 0.0002 N.

Samples of dried Calcein weighing approximately 0.6 g were precisely weighed. The material was then suspended in about 100 ml. of freshly boiled water and covered. The sample was deaerated by bubbling nitrogen through the solution for ten minutes and then maintaining a nitrogen atmosphere over the solution during the titration. The mixture was stirred with a

magnetic stirrer and the pH of the solution was monitored.

The standard base was slowly added to the solution from a buret until the pH reached approximately 6.0. The solution was allowed to mix for several minutes to ensure complete dissolution of the Calcein. The solution was then titrated with the standard base. After the end-point near pH 7.5 had been passed and the pH exceeded 10.0, an additional aliquot of the standard base was delivered from the buret to bring the pH to above 11.5. Then 40 ml. of 0.5 M calcium chloride was added to the solution. The solution was again allowed to stand for several minutes to ensure mixing. The sample was then back-titrated to the end-point near pH 7.5 with the standardized acid. A blank titration was also performed on a 40 ml. aliquot of the solution of calcium chloride.

Solutions of Calcein were prepared in different quality water. Solutions were prepared by dissolving the same highly pure sample of Calcein (98 %) in tap water, laboratory-grade distilled water, distilled water deionized by ion exchange, and water specially

triply distilled and deionized in all-glass containers. The solutions were adjusted to pH 11 and the fluorescence was measured using a Turner Fluorometer.

2. Measurement of the rate of reaction in various solvents

Calcein was prepared by the Mannich condensation of fluorescein, formaldehyde and iminodiacetic acid in acetic acid, water, and water-ethanol solutions.

Approximately 8.3 g of fluorescein and 8.3 g of iminodiacetic acid were mixed together and suspended in about 250 ml. of glacial acetic acid. The mixture was transferred to the round-bottom flask and diluted to 400 ml. with glacial acetic acid. The mixture was warmed to 65 C where the dissolution was almost complete. A 15.0-ml. aliquot of a 37 percent solution of formaldehyde was then added. Two 1.00-ml aliquots of the solution were removed immediately and placed in an ice bath. Other pairs of aliquots were subsequently removed at various intervals of time and similarly stored in an ice bath. Each sample was then analyzed

for fluorescein and Calcein as described in chapter V.

Another mixture containing 8.3 g of fluorescein and 8.3 g of iminodiacetic acid was dissolved in 200 ml. of 1.0 N potassium hydroxide. The pH of the solution was lowered to pH 9.8 with 6.0 N hydrochloric acid. The solution was transferred into the 500-ml., three-neck, round-bottom flask and diluted to 400 ml. with water. The solution was warmed to 65 C and 15.0 ml. of formaldehyde was added. Duplicate samples of the solution were taken initially and at various intervals of time after the addition of formaldehyde. The samples were analyzed for Calcein and fluorescein as described in chapter V.

A third syntheses was performed analogously to that described in the previous paragraph except that the dilution from 200 ml. to 400 ml. was made with 95 percent ethanol. The resulting solution was therefore approximately 50 percent ethanol. Samples of the solution were similarly withdrawn and analyzed.

3. Evaluation of the media used for the Mannich condensation of fluorescein, formaldehyde and iminodiacetic acid

The condensation reaction of fluorescein with formaldehyde and iminodiacetic acid was performed in a number of solvents. Of these, the most fruitful compositions of the media were acetic acid, water, and mixtures of water and ehtanol.

All of the reactions were completed in a 500-ml. round-bottom three-neck flask equipped with a heating mantle, stirrer, thermometer and water-cooled condenser as shown in figure 49.

A sample of fluorescein, weighing 8.30 g., was mixed with 8.3 g. of iminodiacetic acid and transferred quantitatively to the three-neck flask. Approximately 250 ml. of glacial acetic acid was added and the solution was warmed slowly to 65 C. A nitrogen atmosphere was maintained above the reaction mixture throughout the synthesis. While stirring, 10.0 ml. of a reagent grade 37 percent solution of formaldehyde was added dropwise to the warm solution. the temperature, agitation and inert atmosphere were maintained for six

hours. The contents of the flask were allowed to cool and were then transferred quantitatively to a 2.00-liter volumetric flask.

An analogous blend was prepared with fluorescein, formaldehyde and iminodiacetic acid in absolute ethanol. After six hours at 65 C, the mixture was transferred to a 2.00-liter volumetric flask.

A series of preparations were similarly prepared in mixtures of ethanol and water. A stock solution of the reactants was prepared by dissolving 335 g. of fluorescein, 330 g. iminodiacetic acid, and 300 g. of potassium hydroxide and then diluting the solution to 2.00 liters. The pH of the solution was measured to be 9.0. Aliquots of 50.0 ml. of this stock solution were then transferred into a 500-ml. round-bottom flask and diluted with 200 ml. of one of the ethanol-water solutions to be tested. The solvent mixtures were so constructed as to result in final solvent concentrations of 0.00, 5.0, 10.0, 25.0, 50.0, 60.0, 70.0, 75.0, and 80.0 percent ethanol. Each solution was warmed to 65 C before receiving 10.0 ml. of formaldehyde. Each batch was held at 65 C for six

hours. Each solution was then quantitatively transferred and diluted to 2.00 liters with deionized water.

The concentration of fluorescein and Calcein in each solution was determined in triplicate using the fluorometric procedures developed in chapter V. A 5.00-ml. aliquot of each solution was diluted to 500 ml. For each of the solutions prepared for the synthesis of Calcein, two solutions were prepared for fluorometric measurements. The first of these solutions contained 1.00 ml. of the diluted solution, 10.0 ml. of 1.0 N potassium hydroxide, and 1.0 ml. of 1.0×10^{-2} M EDTA and was diluted to 100 ml. The second contained 1.00 ml. of the diluted solution, 10.0 ml. of potassium hydroxide, and 1.0 ml. of 1.0×10^{-2} M calcium chloride and was likewise diluted to 100 ml. the relative intensity of each pair of solutions was then measured on the Turner Model 110 Fluorometer with the excitation setting at 10X. The concentrations of fluorescein and Calcein were then determined from the calibration curves in Chapter V.

The effect of pH on the yield of the Mannich

condensation was also measured for several of the solvent systems above. Additional 50.0-ml. aliquots of the stock solution of fluorescein, iminodiacetic acid, and potassium hydroxide were transferred to a three-neck round-bottom flask. The pH of successive aliquots was adjusted to values between 7.5 and 14 (1N potassium hydroxide) using small quantities of 0.1 N hydrochloric acid, 0.1 N potassium hydroxide, or 12 N potassium hydroxide. The solution was then diluted to 250 ml. with water. This procedure was repeated on successive series of aliquots with dilutions resulting in solvent compositions of 10, 25, 50 and 75 percent ethanol. The amount of fluorescein and Calcein was determined in each solution fluorometrically and the yield of the reaction was calculated.

A similar mixture was prepared in a solution of 83 percent ethanol. However, since a phase separation occured during the synthesis, the material prepared was treated separately. A 50.00-ml. aliquot of the stock solution of fluorescein, iminodiacetic acid, and potassium hydroxide was transferred to the reaction vessel and the pH of the solution was adjusted to 9.0 with tenth normal potassium hydroxide. Approximately

320 ml. of 95 percent ethanol was added and the solution was well stirred. Then, 10.0 ml. of reagent-grade 37 per cent solution of formaldehyde was added, the solution was warmed to 65 C., and the solution was stirred while hot for six hours. The solution was then allowed to cool to about 25 C. The stirring was then ended and the mixture allowed to stand for ten hours. The more dense phase was removed quantitatively with a Pasteur pipet, placed in a 2-liter volumetric flask, and diluted to volume with distilled water. An aliquot of 25.00 ml. of the lighter fraction was also withdrawn and diluted to 2.00 liters. Each of the two portions removed from the reaction vessel were analyzed for fluorescein and Calcein.

The effect of replacing the small, primarily aqueous phase and using the same bulk media for repeated syntheses was examined. A solution of iminodiacetic acid was prepared by dissolving 20 g. in about 100 ml. of tenth normal potassium hydroxide and diluting to 1.00 liters. Repetitions of the condensation procedure described above were then performed using 50.0 ml. of the stock solution of

fluorescein and iminodiacetic acid prepared above. After removing the heavier, "aqueous" phase following each synthesis, portions of the solution of iminodiacetic acid were added to the remaining phase, 10.0 ml. of 37 per cent formaldehyde was added and the condensation repeated. Any heavy phase observed after this reaction was also withdrawn and added to the material removed after the first condensation. Then an additional portion of the aqueous solution of iminodiacetic acid and formaldehyde was added again and the condensation repeated. The volumes of the solution of iminodiacetic acid used for the second condensation varied between 1.0 ml. and 15 ml. while the volume of the portions added for the third condensation varied between 0.5 and 5.0 ml.

Repetitive preparations were then made in the same solvent. A 50.0-ml. aliquot of the stock solution of fluorescein, iminodiacetic acid and potassium hydroxide (prepared above), was placed in the reaction flask as described in the previous section. The pH was adjusted to 9.0 with tenth normal potassium hydroxide. Approximately 320 ml. of 95 percent ethanol was added. Then 10.0 ml. of formaldehyde was added and the Mannich

condensation completed as described above. After six hours, the solution was allowed to cool and separate into two phases. The bottom phase was removed by Pasteur pipet and analyzed for fluorescein and Calcein.

Another 50.0-ml. portion of the solution of the reagents was added to the remaining portion of the mixture in the flask. Formaldehyde was added and the condensation repeated. After removing the heavier phase, a new aqueous portion was added, the reaction was completed and the samples were withdrawn and analyzed two more times.

4. Determination of the yield and purity of Calcein precipitated from solvents of different compositions

The effect of different media for the precipitation and recrystallization of Calcein was examined in two ways. First, the approximate theoretical yields and purities of mixtures of Calcein and fluorescein were predicted from studies of the solubilities as functions of pH in different solvents.

Then the actual yields and purities were measured for several preparations from solutions of varying compositions.

Buffer solutions with ionic strengths of 0.10 were prepared for the range of pH between 1.00 and 10.00 in increments of 0.20 units. Buffer solutions of each tenth of a unit of pH were also prepared over the range of 2.00 to 4.00. The buffers were prepared from hydrochloric acid, potassium hydrogen phthalate, potassium dihydrogen phosphate, boric acid, potassium hydroxide, and potassium chloride as tabulated by Bower and Bates[63].

Approximately 0.5 g. of Calcein was placed in each of twenty-five 100-ml. volumetric flasks. To each flask was added aproximately 50 ml. of one of the buffer solutions. Each flask was shaken thoroughly for several minutes. The contents of each flask were transferred to clean, dry beakers and the pH of the solution adjusted using hundredth normal potassium hydroxide, if needed, to restore the original pH of the appropriate buffer. The mixtures were then shaken mechanically for at least 24 hours on a wrist-action

shaker. The pH of the solutions was checked again, and the solutions placed in a constant-temperature room at about 25 C.

Another series of solutions was prepared with about 0.5 g. of Calcein, 4.0 ml. of absolute ethanol, and 96 ml. of the various buffer solutions in 250-ml. volumetric flasks. Analogous series of solutions containing 10.0 ml., 16.0 ml., 20.0 ml., 25.0 ml., 50.0 ml., 75 ml., and 90.0 ml. of ethanol were prepared for each of the buffer solutions with a pH between 1.00 and 5.00. The total volume of ethanol and buffer was 100 ml. in each case. A series of solutions containing 99 per cent ethanol was also prepared by adjusting the apparent pH of several mixtures of absolute ethanol and Calcein with a few drops of 1.0 N hydrochloric acid or 1.0 N potassium hydroxide. The apparent pH of these mixtures was monitored and the solutions were shaken and stored as above.

A sample of Calcein weighing 0.3112 g. was dissolved in tenth normal potassium hydroxide and diluted to 2.00 liters. Then 10.00 ml. of this solution was diluted to 1.00 liters to form a stock

solution of 2.50×10^{-6} M Calcein. A series of solutions was prepared containing between 1.00 ml. and 50.0 ml. of the stock solution of Calcein, 1.00 ml. of 1.0×10^{-3} M calcium chloride, and 10.0 ml. of 1.0 N potassium hydroxide in 100 ml. The relative fluorescence of each solution was measured with the Turner Fluorometer with the excitation intensity set to 10X.

A portion of each saturated solution was filtered through a porous-bottom crucible and the filtrate was collected in a small test tube. The filtrate was then stored in a bath of warm water to prevent precipitation.

Aliquots of each filtrate were then diluted until the concentration of Calcein approached 5.0×10^{-6} M. The dilution factors ranged from 10 to 250,000. Two identical dilutions were prepared for each sample. To the first dilution of each sample was added sufficient potassium hydroxide and 5×10^{-3} M EDTA to bring the final pH to 13 and the concentration of EDTA to 1×10^{-5} M. To the second dilution was added a similar amount of potassium hydroxide and sufficient calcium chloride

to provide 1 x 10^{-4} M calcium.

The relative fluorescence of each pair of solutions was measured with the Turner Fluorometer with the excitation intensity set to 10X. The fluorescence of the solution containing an excess of EDTA was subtracted from the intensity of fluorescence of the solution with an excess of calcium chloride. The net fluorescence due to Calcein was then converted to concentration using the calibration curve prepared above and the concentration of the original saturated solution was calculated by multiplying by the dilution factor.

Approximately 0.5 g. of fluorescein was placed in the bottom of each of about twenty 100-ml. volumetric flasks. Approximately 50 ml. of each of the different buffer solutions prepared in the last section with a pH between 1.00 and 5.00 were added to flasks containing fluorescein and each flask was shaken vigorously for several minutes. The contents of each flask were transferred to a clean, dry beaker and the pH of the solutions were monitored with a combination electrode. The pH of each solution was adjusted, if needed, to the

original pH of the buffer used. The suspensions were then slurried and transferred back to the volumetric flasks. The flasks were then attached to a wrist-action shaker and mechanically shaken for at least 12 hours. The solutions were then transferred to beakers and the pH adjusted again if needed and returned to the volumetric flasks. The flasks were again mechanically shaken for at least 12 hours at 25 C. When finished, the flasks of the saturated solutions were stored in a constant temperature room at 25 C. until the measurements of solubility could be made.

Several additional series of solutions were also prepared with mixed solvents of ethanol and water. At each desired vaue of pH, aliquots of 25.0 ml. of buffer were diluted with 10.0, 25.0, 50.0, and 75.0 ml. of absolute ethanol and the volumes diluted to 100 ml. with deionized water. The solvents and the solid fluorescein were then placed in 250-ml. volumetric flasks and treated just like the aqueous series of solutions above.

A stock solution of 1.00×10^{-4} M fluorescein was

prepared by dissolving 0.3326 g. of purified fluorescein in one liter of 0.10 N potassium hydroxide and then diluting 100.0 ml. of this solution to 1.00 liters. A stock solution of 1.00 N potassium hydroxide was also prepared by dissolving 56.0 g. of solid potassium hydroxide, after rinsing the surface, in 1.00 liters of boiled deionized water.

Solutions of fluorescein were prepared for measurements in 100-ml. volumetric flasks containing 10.0 ml. of 1.00 N potassium hydroxide and between 0.10 ml. and 25.00 ml. of 1.00×10^{-4} M fluorescein. The relative intensity of fluorescence of each solution was measured with the Turner Fluorometer equipped as described before at each of the four excitation settings: 1X, 3X, 10X, and 30X.

A portion of each saturated solution was filtered through a porous-bottom crucible under vacuum from aspiration and collected in a test tube. The test tubes were immediately placed in a warm bath with a temperature of about 30 C.

Aliquots of each filtrate were then diluted by

repetitive pipetting and dilution until the concentration of fluorescein was on the order of 5×10^{-6} M. The dilution factors ranged from 100 to 250,000. The pH of the final solution for the measurement was also adjusted to 13 with 0.10 N potassium hydroxide and 1×10^{-4} M EDTA. The fluorescence of each of the final solutions was measured with the Turner Fluorometer, the concentrations of the final solutions determined from the calibration curves prepared above, and the concentrations in the saturated solutions calculated by multiplying by the respective dilution factor.

A sample of Calcein was synthesized by the Mannich condensation of fluorescein, formaldehyde, and iminodiacetic acid in 50 percent ethanol. The solution of Calcein was split into five portions of 80 ml. Each portion was repeatedly dissolved with 1.0 N potassium hydroxide and precipitated with 1.0 N hydrochloric acid under different conditions. The first portion was precipitated in water with no salt added. The second was precipitated in water which had been diluted with an equal amount of 4 N potassium chloride. The third, fourth, and fifth portions were precipitated from 20,

50, and 75 percent solutions of ethanol respectively. The product of each precipitation was weighed and analyzed.

Each precipitation was dried in a vacuum oven and the net purity was determined by potentiometric titration as described above. The amount of fluorescein remaining as an impurity was determined by fluorescence.

Approximately 0.16 g. of fluorescein was precisely weighed and dissolved in water with about 20 ml. of 0.1 N potassium hydroxide. An aliqout of 10.00 ml. of this solution was then diluted to 2.00 liters.

Approximately 0.16 g. of the sample of Calcein was also carefully weighed and dissolved in about 200 ml. of water with about 20 ml. of 0.10 N potassium hydroxide and was diluted to 1.00 liters.

To each of a series of 100-ml. volumetric flasks was added: 2 ml. of 10^{-3} M EDTA, 10.0 ml. of 1.0 N potassium hydroxide, 1.00 ml. of the solution of Calcein, and increasing portions from zero to 10.00 ml.

of the solution of fluorescein. The concentration of Calcein was approximately 2.5 x 10^{-6} M and the concentrations of fluorescein ranged from 0 to 2.5 x 10^{-7} M.

The relative fluorescence was then measured using the most sensitive setting of the Turner Fluorometer. The extrapolation of the plot of fluorescence with standard additions of fluorescein indicates the concentration of fluorescein in the solution of Calcein.

The effect of precipitating the Calcein with acid and with alkali was also examined. Two samples of the same batch of Calcein were precipitated from opposite sides of the minimum in solubility with pH. One sample was dissolved in a 50 percent solution of ethanol with 6.0 N hydrochloric acid until the apparent pH was less than 1.0 and then precipitated by the addition of 6.0 N potassium hydroxide. The final pH at precipitation was 2.4. The second sample was dissolved in a similar 50 percent solution of ethanol by raising the apparent pH above 4 with 1.0 N potassium hydroxide and then precipitated by lowering the apparent pH to 2.4 with

1.0 N hydrochloric acid. The products of the two precipitations were analyzed for net purity by potentiometric titration and for metal ions by emission spectroscopy.

5. Determination of the effect of Soxhlet extraction on the purity of Calcein

A sample of vacuum-dried Calcein weighing 10.2 g was placed in a Whatman asbestos extraction thimble and placed in a Soxhlet extractor. Approximately 500 ml. of deionized water were placed in the flask of the extractor and heated to reflux temperature (100 C). The sample was then extracted in water for the equivalent of ten 250 ml. extractions. A portion of the material was withdrawn and analyzed for net purity and fluorescein. The water in the extractor was replaced with acetone and the remaining sample was extracted with the equivalent of three 250-ml. portions of acetone. A second portion was removed and analyzed. The remaining sample was then extracted with ethanol for the equivalent of ten extractions. The remaining sample was analyzed for net purity and fluorescein. In

addition, the sample was analyzed for chloride and potassium by specially adapted techniques.

The chloride was determined by automatic coulometric titration with silver ions using the accumulation of excess silver ions after the equivalence-point to trigger an amperometric indication of the end-point.

The supporting electrolyte was prepared from concentrated nitric acid and sodium nitrate to have a pH of 1.0 and an ionic strength of approximately 2. This was more acidic than is commonly used but was required to overcome solubility limits of the Calcein. A small amount of a 0.6 percent solution of gelatin was also added.

Before analyzing samples of fluorescein and Calcein, a series of reference samples were used to test the procedure. Samples from the reference materials were precisely weighed and dissolved in 100-ml. volumetric flasks with approximate concentrations of chloride between 10 and 250 meq./liter.

An aliquot of 25.00 ml. of the supporting electrolyte and 1.00 ml. of the solution of gelatin were placed in a small beaker into which the three electrodes of the chloride meter were inserted. The solution was "conditioned" by the instrument (a sufficient amount of silver ions were generated to titrate any chloride present as an impurity in the reagents and sufficient excess silver ions to trigger the amperometric detection circuit). Then 100 microliters of sample were added and the solution automatically titrated until the same excess of silver ions was present again. The instrument automatically converted the length of time of each titration into milliequivalents of chloride per liter. The reference sample of pure potassium chloride was accepted as a reference value and the intrumental readings were corrected for the small difference between the instrumental and accepted values for the solution of potassium chloride.

The samples of Calcein analysed were dissolved by placing precisely weighed quantites of about 0.5 g in 50 ml of 0.2 N nitric acid and then diluting to 100 ml.

The resulting solutions were analyzed by the same procedure given for the reference samples. The same correction factor was applied to each value recorded by the instrument.

For verification of the accuracy of the method, measurements of chloride were also performed on solutions of a sample of highly pure Calcein with known additions of potassium chloride.

A double-ashing technique was employed to determine the amount of potassium (and other alkali) present as impurities in Calcein. The technique successfully differentiated and estimated the amounts of potassium chloride and potassium salts of organic acids such as Calcein, fluorescein, and iminodiacetic acid.

Before analyzing samples of Calcein, the method was tested on reference samples prepared from potassium chloride, potassium acid phthalate, potassium hydroxide, ammonium chloride, and mixtures of these compounds. All measurements were performed in triplicate. Samples of these materials were carefully

weighed into silver crucibles that had previously been brought to constant weight. The samples were carefully ashed over Meeker burners and then in a muffle furnace at 1200 F. until they were again found to be at constant weight (+/-0.0002 g) after successive heatings. The residue was then treated with about 1 ml. of concentrated sulfuric acid and the ashing repeated. After some trouble with samples of potassium hydroxide attacking the crucibles, all samples containing hydroxides were abandoned.

6. Determination of the effects of different methods of removing solvents from Calcein

Four different techniques for drying Calcein were examined. First, Calcein was allowed to dry in the air at room temperature. This procedure was examined following treatments of precipitated Calcein with water, acetone, and ethanol. Second, Calcein was dried in the presence of air at 80 C. Third, Calcein was dried in vacuum at 80 C. Fourth, Calcein was freeze-dried under vacuum. For each technique, the product was analyzed for net purity and residual solvent.

Water was determined by Karl Fischer titration. A freshly opened solution of commercial Karl Fischer Reagent (0.5 titer) was standardized by titrating solutions containing 0.50 and 1.00 ml. of water in 500 ml. of freshly opened methanol. Two samples of Calcein, each weighing approximately 50 g, were placed in dry round-bottom flasks. The samples were slurried in 500 ml. of methanol for at least 30 minutes with constant stirring under a constant purging with dried nitrogen. Several 10-ml. aliquots of the methanol were then titrated to the iodine end-point. The titration was repeated at intervals of about 15 minutes for two hours. One last repetition was made after the addition of 0.50 ml. of water to one of the slurries. The iodometric color change faded rapidly in all titrations and the first pervasive appearance over the entire flask was taken as the end-point.

The amount of solvent present was also measured by thermogravimetry. The loss of solvent upon heating at low temperatures was monitored with the thermal analyzer constructed from Perkin-Elmer and Heath modules as described in section B above.

The balance and temperature scale were calibrated by recording the temperature and chart deflection corresponding to the evolution of water from the pentahydrate of cupric sulfate with heat. A sample of 4.8 mg. of $CuSO_4.5H_2O$ was precisely weighed into a small aluminum pan and placed on the balance arm. The settings of the analyzer were as follows:

Mode: TG

Zero: 5000

Range: 6000

Mass Range: 10

Mass: 0.5000

Recorder Range: 100 mV.

Factor: 1

Filter: 3

Chart Speed: 0.5 in./min.

Temperature Rate: 10 degrees/min.

Linearity of Response: -3

Temperature Limit: 950

The thermograms of several samples of different lots of Calcein were similarly obtained. Samples of Calcein

weighing about 10 mg. were placed in aluminum pans and positioned on the balance arm. The settings used were the same as for the calibration except the following:

Mass Range: 100

Mass: 0.765

Recorder Range: 10 mV.

Zero: 4.3

Several of the determinations were then repeated after purging the instrument with dried nitrogen.

A more detailed and complete thermogram was obtained for each of two samples of highly pure Calcein using an automated thermal gravimetric analyzer. The loss of weight with increasing temperature was monitored up to a temperature of 600 C. The instrument automatically provided a normalized plot of the rate of loss with change in temperature.

7. Measurement of stability of Calcein in solid and dissolved forms

The stability of Calcein and solutions of Calcein were measured for different methods of storage. The relative hygroscopicity and the acid-base, thermal, and photochemical stability of Calcein were measured.

Samples of solid Calcein were carefully evaluated with respect to net purity, fluorescein, and solvents immediately after preparation. These samples were then stored in both solid and dissolved forms and changes in these qualities monitored over several months.

The relative hygroscopicity was measured for solid Calcein relative to several desiccants used for maintaining dry storage. Weighing all of these materials was performed quickly and carefully under a dry nitrogen atmosphere.

Precisely weighed samples of Calcein were placed in Petrie dishes in glass desiccators. Each desiccator also contained a particular desiccant in other Petrie dishes. In each case, the weight of desiccant was approximately

four times that of Calcein. These desiccants included Drierite (Hammond Chemical Co.), phosphorus pentoxide, calcium chloride, anhydrous silica, and magnesium perchlorate. The materials in the desiccator were allowed to come into equilibrium over one week and then reweighed. Then, approximately 0.5 g of water on a small watch glass was placed in each desiccator each day for another week. The weight of each Petrie dish of material was carefully weighed each day.

The stability of solid Calcein with respect to heat and light was measured by placing identical samples of Calcein in three desiccators. The first was placed in a closed cabinet at relatively constant temperature between 18 and 22 C. The second was placed on a windowledge and exposed to sun or fluorescent lighting almost 24 hours per day. The third was placed in a steam-heated hood. The temperature inside the third desiccator was about 70 C. The equivalent weight of each sample was measured after one and two months and again after seven months.

The stability of solutions of Calcein were also examined. Aqueous solutions of Calcein were prepared with pH of 0.0 to 14.0 at intervals of 1.0 or less. ...

D. Results and Discussion

1. Evaluation of methods for determining the net purity of Calcein

(i) Infrared spectroscopy Several commercial suppliers of Calcein have specified infrared spectroscopy as the control test for purity[137,141]. In particular, infrared spectroscopy has been used as a quality control test to detect undesirable amounts of fluorescein or other organic contaminants. The validity of this specification was challenged. The infrared spectra of several batches of Calcein which had been quantitatively analyzed for fluorescein and net purity were examined. The infrared spectra of yellow and red fluorescein were also obtained for comparison.

The expectation on the part of suppliers, no doubt, was that the presence of fluorescein would significantly alter the infrared spectrum. In particular, fluorescein was expected to affect the relative absorbance in the regions around 1720, 1530 and 800 cm-1.

Many infrared spectra of fluorescein have been published over the past 50 years[98,301,332,363,384]. Spectra have been routinely obtained for preparations of fluorescein with colors varying between yellow and red. In addition, the infrared spectrum of a colorless form has also been recently obtained[247]. Despite the preponderance of data, a cohesive explanation of the different forms of solid fluorescein was not available until the structural determinations were published by Diehl and Markuszewski[115] in 1981. In that paper, three distinct forms of solid fluorescein, red, yellow and colorless, were established, each with a distinctive electronic structure.

The colorless form of fluorescein was first isolated in the work of Markuszewski[247]. The colorless solid and the neutral molecule dissolved in dioxane were assigned the lactoid structure shown earlier (structure III on page 23). The infrared spectrum of this material was marked by a prominent band at 1730 cm-1 which was not present in the spectra of the red or yellow forms of fluorescein. This band was attributed to the carbonyl group bound in a five-membered lactone ring.

The red form of solid fluorescein and the neutral molecule of fluorescein in alcohol were determined to have a para-quinoid structure as shown in structure IV (page 23). The infrared spectrum of a sample of red fluorescein was obtained and is shown in figure 50. The unique feature of this spectrum was the appearance of the carbonyl band near 1715 cm-1, a position that more closely corresponded to the free carbonyl functional group than any band in the other forms of fluorescein. Another characteristic band of red fluorescein prominently observed in the present work was located at 770 cm-1. This band had no counterpart in the spectrum of yellow fluorescein.

The infrared spectrum of yellow fluorescein is shown in figure 51. The yellow solid and neutral species of fluorescein in water were assigned the zwitterion structure of fluorescein, structure V (page 23). This form had no strong or even moderate absorption bands in the region between 1800 and 1600 cm-1 which could be associated with a carbonyl group. Even more importantly, a prominent band appeared between 1540 and 1510 cm-1. Diehl and Markuszewski identified this band as

Figure 50.

Infrared spectrum of the red form of solid fluorescein

Spectrum obtained on Nicolet 7000 FTIR Spectrometer

Sample prepared as a potassium bromide pellet

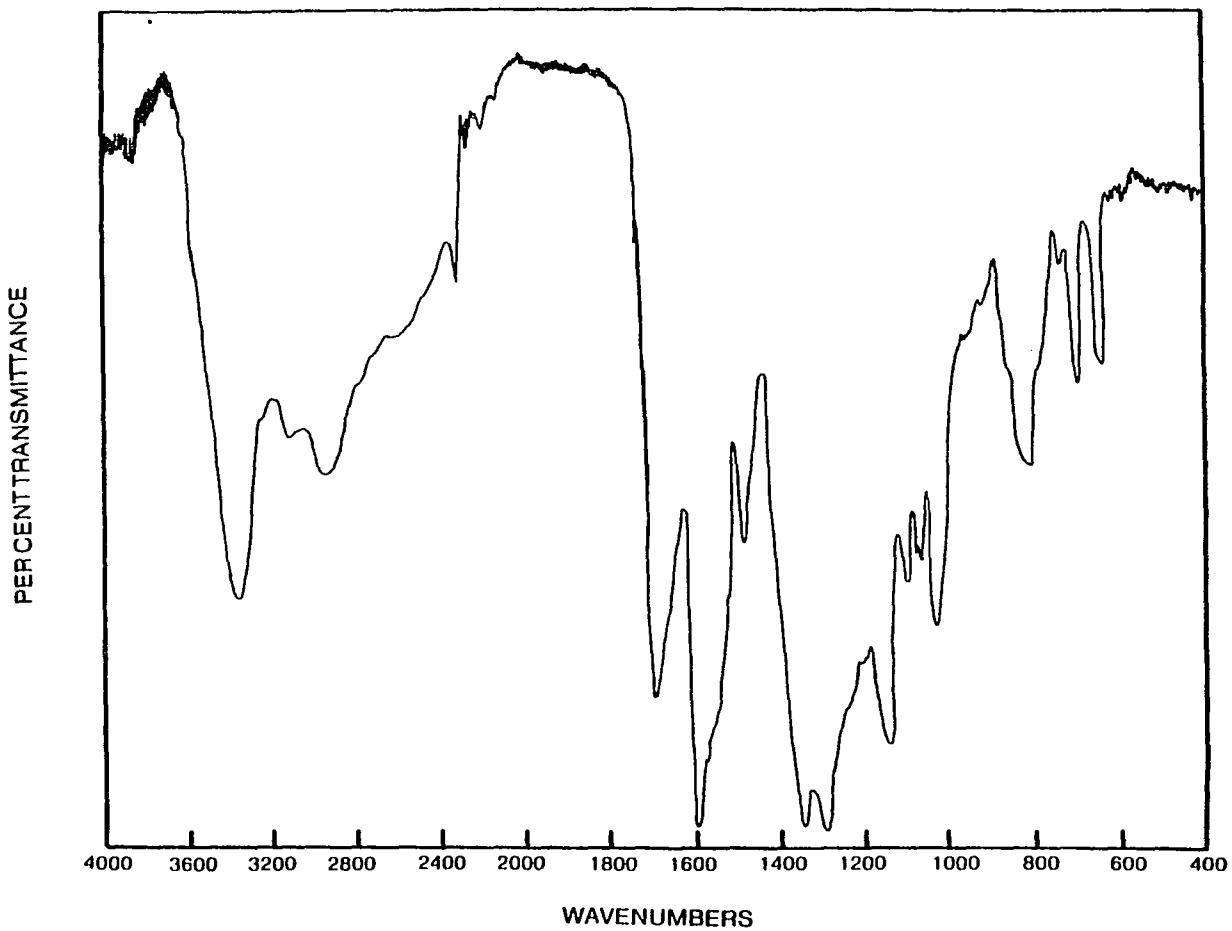
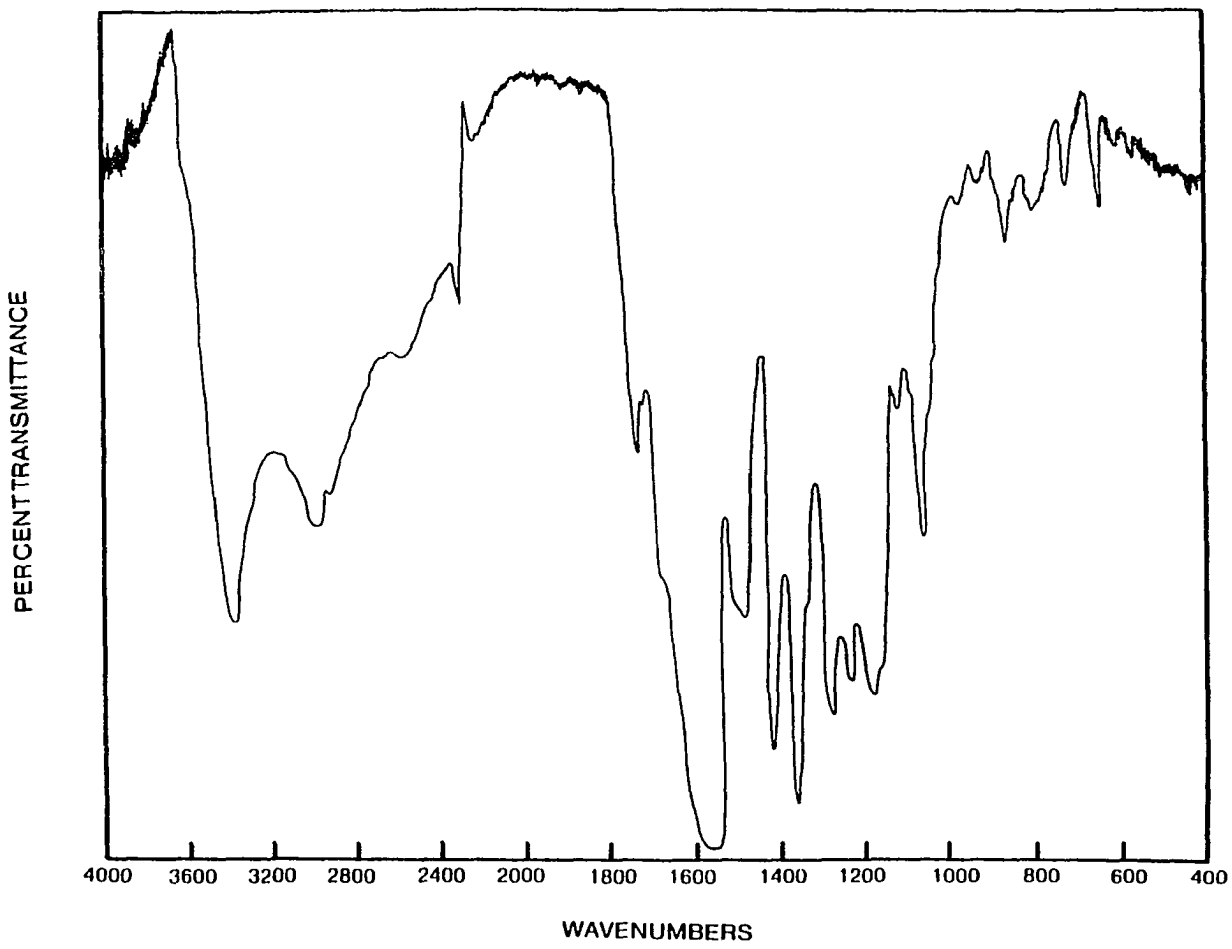

PERCENTTRANSMITTANCE
4000
3600
3200
2800
2400
2000
1800
1600
1400
1200
1000
800
600
400
WAVENUMBERS

Figure 51.

Infrared spectrum of the yellow form of solid fluorescein

Spectrum obtained on Nicolet 7000 FTIR Spectrometer

Sample prepared as a potassium bromide pellet


PERCENTTRANSMITTANCE
4000
3600
3200
2800
2400
2000
1800
1600
1400
1200
1000
800
600
400
WAVENUMBERS

corresponding to a six-membered, oxygen-containing positively-charged ring such as those in pyrilium salts[10,26,27,250,251]. Thus, based on this and other information, the yellow form of solid fluorescein was determined to exist in the zwitterion structure.

Samples of commercial and laboratory-prepared batches of Calcein varied in color from yellow to orange. Two samples of Calcein prepared in the present work were examined by infrared spectroscopy. Both samples originated from the same synthesis of Calcein and were known to contain less than 0.7 percent fluorescein and to be greater than 97 percent pure. The first was precipitated from an aqueous solution with a high concentration of potassium chloride. This material was bright yellow in appearance. The second sample was precipitated from a solution of 80 percent ethanol and had an orange color. The infrared spectra are shown in figures 52 and 53.

All of the spectra in this chapter were obtained with a powerful Fourier-transform infrared spectrometer

Figure 52.

Infrared spectrum of Calcein precipitated from water

Spectrum obtained on Nicolet 7000 FTIR Spectrometer

Sample prepared as a potassium bromide pellet

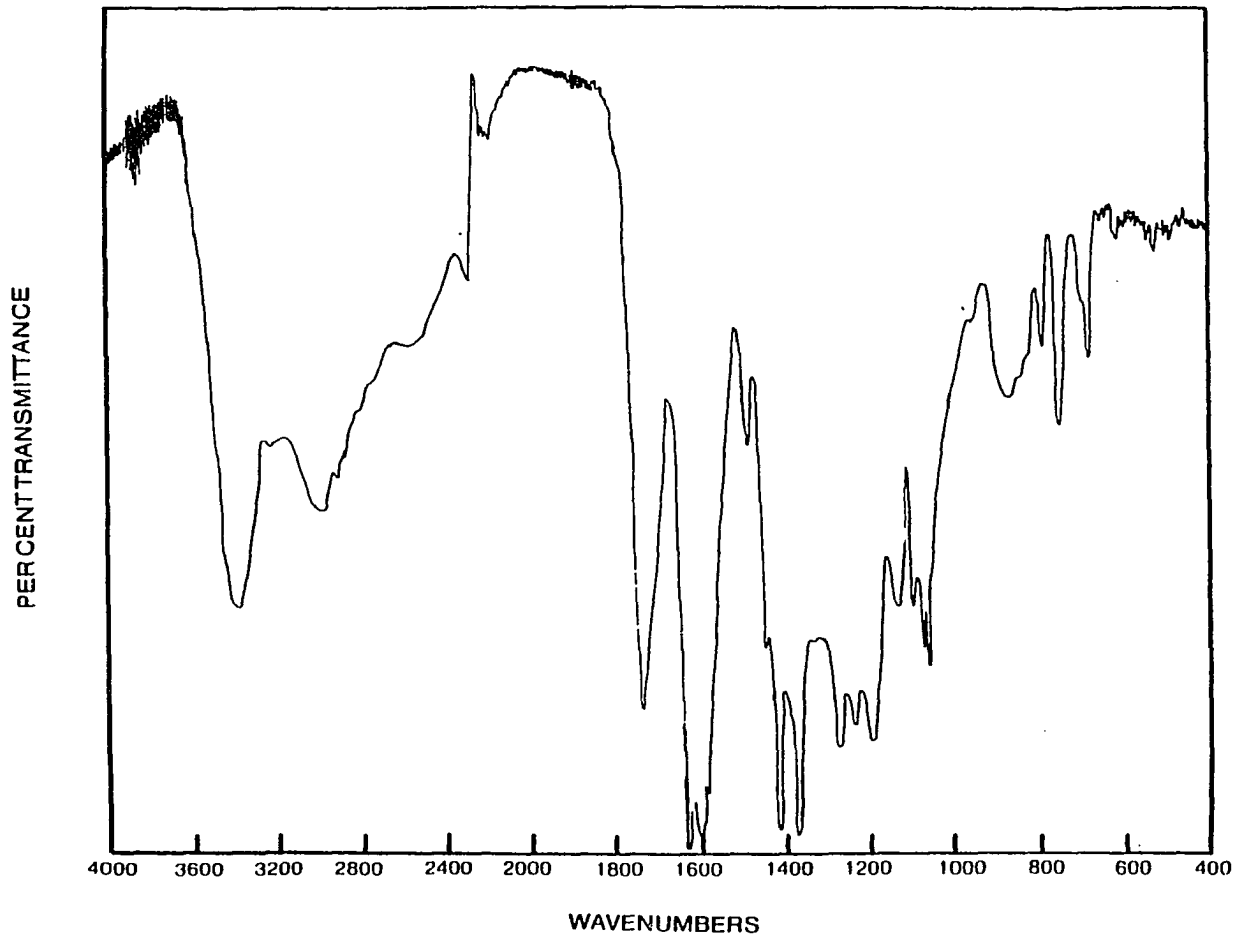
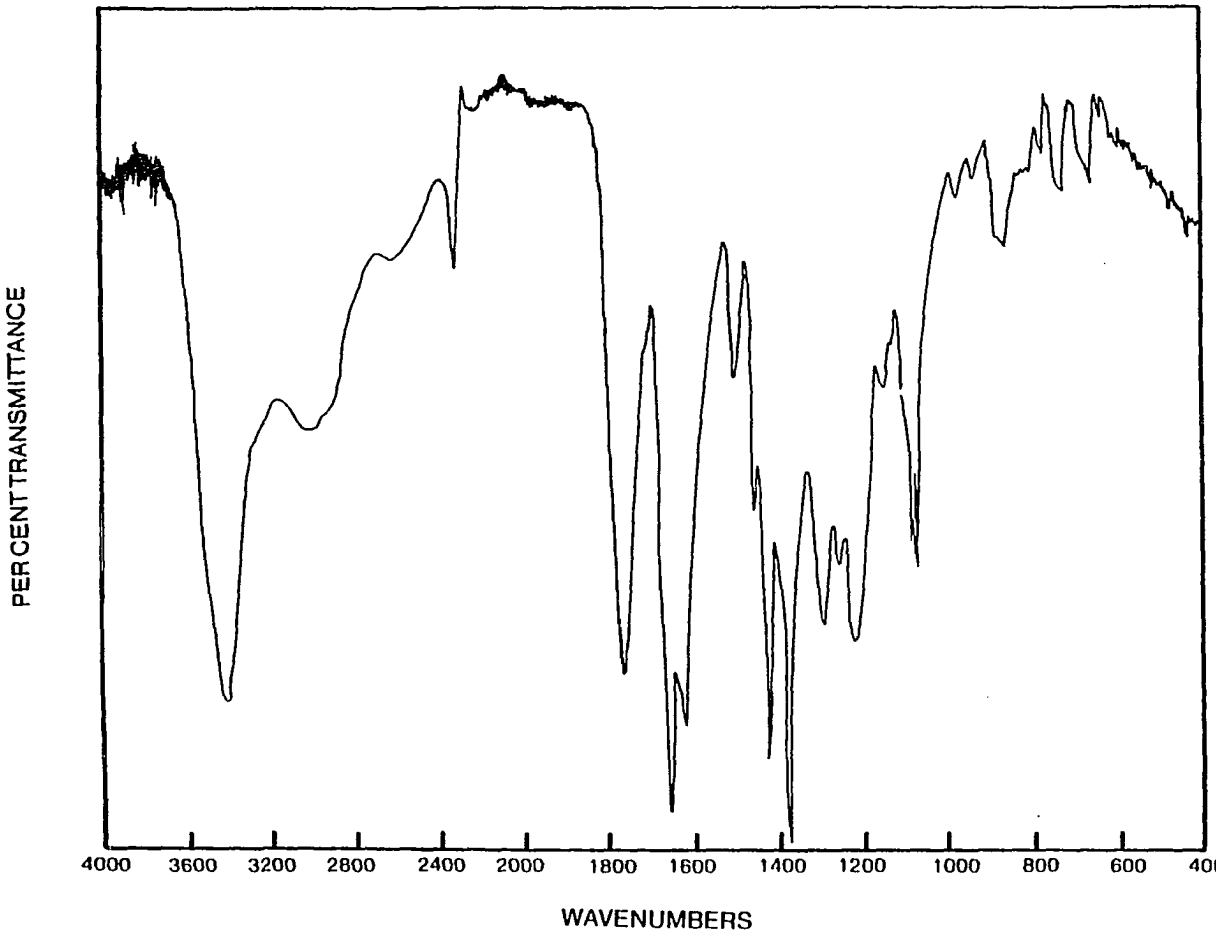

PERCENTTRANSMITTANCE
4000
3600
3200
2800
2400
2000
1800
1600
1400
1200
1000
800
600
400
WAVENUMBERS

Figure 53.

Infrared spectrum of Calcein precipitated from 80 percent solution of ethanol

Spectrum obtained on Nicolet 7000 FTIR Spectrometer

Sample prepared as a potassium bromide pellet


PERCENT TRANSMITTANCE
4000
3600
3200
2800
2400
2000
1800
1600
1400
1200
1000
800
600
400
WAVENUMBERS

with laser optics. The spectra were obtained with the accumulation of at least 84 scans. As a result, much more detail was observed than in the weak spectra used by Markuszewski. The samples in this work, however, were prepared as potassium bromide pellets and some areas of the spectra are masked by unavoidable absorbance by the pellet and this effect was magnified by the repetitive scans.

Significant differences existed between the spectra of these two samples of Calcein. In particular, the sample precipitated from the solution of 80 percent ethanol had greater relative absorbance at peaks near 1720, 1120 and especially 770 cm-1. Each of these differences corresponded to a similar distinction between the spectra obtained for the red and yellow forms of fluorescein. The spectrum of the orange Calcein, which had been obtained from ethanolic solution, had much greater similarity to the spectrum of red fluorescein, which was also precipitated from ethanolic solution, than did the spectrum of the bright yellow Calcein precipitated from aqueous solution.

A third sample of Calcein was examined by infrared

spectroscopy. The sample was obtained by precipitation from water and had a yellow color. The material was known to contain 2.1 percent fluorescein by weight. Despite the presence of fluorescein, the infrared spectrum is remarkably similar to that of the purer material which was also precipitated in water.

It was therefore apparent, that the solvent from which Calcein was precipitated affected, to a significant degree, the structural form of the solid Calcein obtained. In fact, the infrared spectra of Calcein was affected much more dramatically by the conditions of the precipitation than it was by the presence of a few percent of fluorescein.

(ii) Potentiometric titrations The most prevalent method of determining the purity of materials with relatively strong acidic or basic functional groups has been by potentiometric titration. Calcein has six replaceable hydrogens, four of sufficient acidity to be titrated with strong alkali in aqueous solution.

Hefley[166] and Markuszewski[247] circumvented the problems encountered with dissolving Calcein at a pH

below 6 by different routes. Hefley added a known amount of standardized base in excess of the amount needed to neutralize the four titratable hydrogens and then back-titrated with standardized acid. Markuszewski dissolved the sample in a 50 percent solution of ethanol and then titrated directly with standardized alkali. The equivalent weight by both methods was determined from the potentiometric end-point observed near pH 7.5. Unfortunately, several of the possible impurities in Calcein also have titratable acid groups which were titrated simultaneously with the Calcein. These include fluorescein which has two replaceable hydrogens, iminodiacetic acid which has one titratable acid group and occluded mineral acid trapped in the precipitate.

Martin[253] developed a two-end-point procedure that eliminated almost all of these interferences. The sample of Calcein was suspended in carbon dioxide-free deionized water and titrated with standardized alkali past the end-point corresponding to the titration of the first four replaceable hydrogens. Then a known amount of excess alkali was added to raise the pH above 12. An aliquot of calcium chloride was added to the Calcein, resulting in a molar concentration of calcium that was

more than ten times the molar concentration of Calcein. Under these conditions, the formation of the two-to-one compound of calcium and Calcein was essentially quantitative (see chapter IV). The formation of the slightly-dissociated compound released the two hydrogens previously associated with the tertiary amine groups of the methyleneiminodiacetic acid branches. The solution was then back-titrated with standardized acid to the original end-point near pH 7.5. The difference, in equivalents, between the end-points of the forward- and back-titrations corresponded to the two hydrogens displaced from the amine groups. The equivalent weight of the Calcein was calculated by dividing the weight of the sample by half of the difference, in equivalents, between the two end-points.

This procedure, with only minor modifications, was adopted for the determination of net purity of all preparations of Calcein. The modifications included (1) using 0.1 N hydrochloric acid in place of 0.2 N acid for the back-titration to increase the precision of the second end-point, (2) the use of a pre-neutralized solution of calcium chloride to minimize the correction

needed for the addition of calcium chloride prepared from calcium carbonate and hydrochloric acid, and (3) the addition of the excess potassium hydroxide from the buret used for the titration to eliminate the error added by additional pipetting and to provide a predetermined total amount of base for convenience in plotting and interpreting the results. An example of the application of this technique to the present material is shown in figure 54. Results of net purity were based on a molecular weight of 640.2 g/mole of Calcein (monohydrate) and varied between 72.0 and 99.6 percent. The reproducibility based on replicate titrations was approximately 0.5 percent.

2. Effect of purity of fluorescein

As first pointed out by Hefley[165], one of the critical problems with many preparations of Calcein has been the contamination of the product with metal ions which significantly alter the fluorescence of the material. Both fluorescein and Calcein function as excellent scavengers and any metallic impurities introduced in the raw materials or during the preparation

Figure 54.

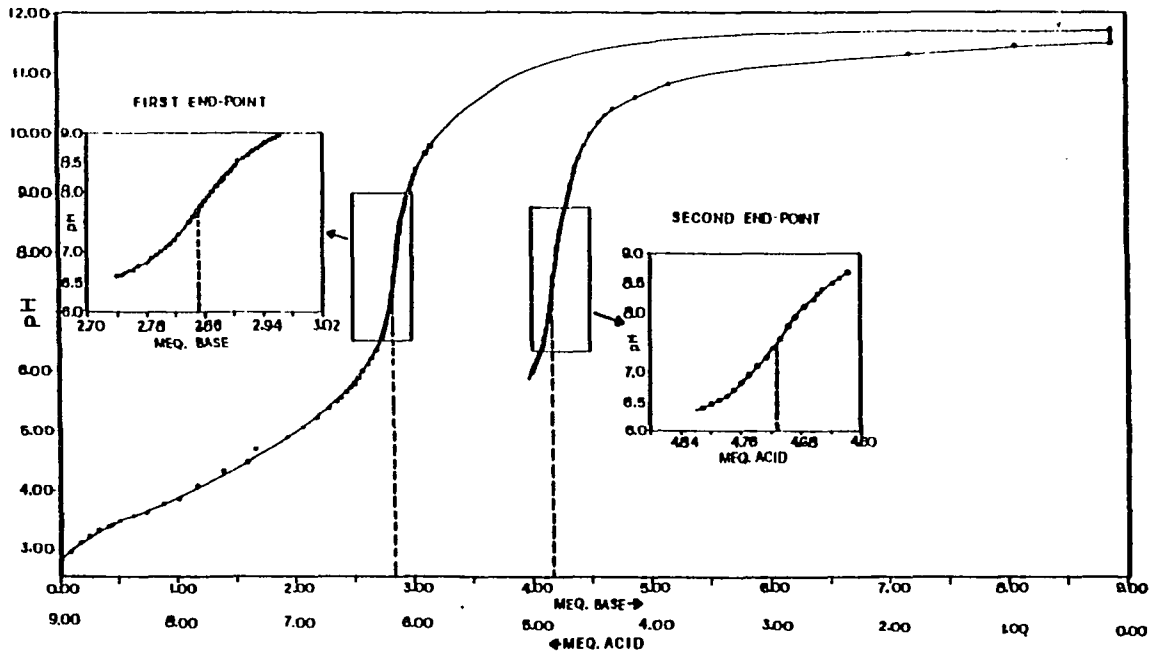
Two-end-point potentiometric determination of the purity of Calcein XXI-A

pH measured with Beckman Zeromatic SS-2 pH Meter

Concentration of standard alkali: 0.09872 N KOH

Concentration of standard acid: 0.1061 N HCl

Purity of Calcein XXI-A: 99.6 %


FIRST END-POINT
SECOND END-POINT
pH
MEQ. BASE
MEQ. ACID
MEQ. BASE→
←MEQ. ACID

are tenaciously retained by the Calcein.

The results of the experiments where crude and refined samples of fluorescein were analyzed and subsequently used in the synthesis of Calcein demonstrated this point. The results of atomic emission spectroscopy are shown in table 11. In each case, the metallic impurities in the Calcein closely resembled those of the fluorescein. In particular, significantly higher amounts of aluminum, iron, and zinc were present in the Calcein prepared from the unrefined material. Mercury was not determined in these tests but was also suspected of being a significant contaminant in the crude materials. The improvement achieved by purifiying the fluorescein through diacetylation was dramatically demonstrated by the reduction in the ash after ignition from 0.89 percent to less than 0.05 percent.

3. Rate of reaction in different media

The progress of the Mannich condensation was monitored in different solvents. Samples were removed from the flask at regular intervals during the first

Table 11

Results of Analysis by Atomic Emission

Element Examined	Crude Fluorescein	"Crude" Calcein	Refined Fluorescein	"Refined" Calcein
Al	small	small	faint trace	trace
Ca	trace	trace	faint trace	faint trace
Cd	faint trace	faint trace		
Cr	faint trace	faint trace		
Cu	trace	trace		
Fe	small	small	faint trace	faint trace
Li	faint trace	trace		
Mg	small	trace	faint trace	faint trace
Si	faint trace	trace	faint trace	faint trace
Zn	trace	trace		

seven hours of reaction and were analyzed for Calcein and fluorescein.

The relative rate of reaction remained surprisingly independent of the composition of the solvent at any particular temperature. The rate of reaction in aqueous solution is shown in figure 55. The reaction at 65 C was half complete by the end of the first hour and virtually complete within three hours. The length of time required to obtain a specific fraction of the maximum yield was equivalent for all of the systems examined. Only the value of the maximum yield varied significantly.

Lowering the temperature by ten degrees slowed the progress at one hour to less than a third of completion. Raising the temperature above 65 C resulted in unacceptable losses of formaldehyde and a low yield.

4. Effect of the composition and pH of the solvent for the Mannich condensation

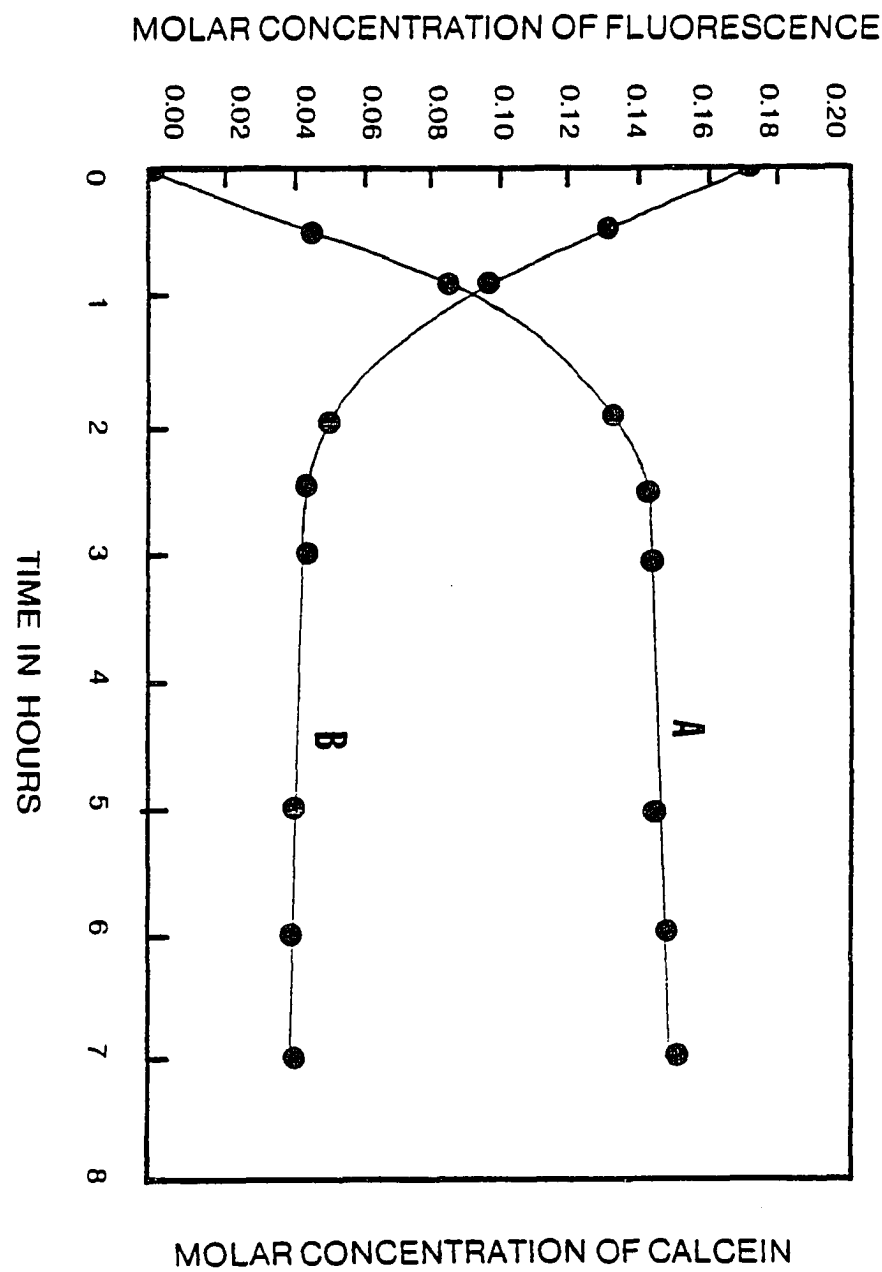
Complete dissolution of the reactants was not possible in either glacial acetic acid or absolute

Figure 55.

Progress of the Mannich condensation
in water as a function of time

A: Concentration of Calcein formed

B: Concentration of fluorescein remaining


MOLAR CONCENTRATION OF FLUORESCENCE
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
0.18
0.20
TIME IN HOURS
0
1
2
3
4
5
6
7
8
A
B
MOLAR CONCENTRATION OF CALCEIN

ethanol. In the suspension with acetic acid, the fluorescein was incompletely dissolved. The solid fluorescein which had been red initially, appeared yellow and was only held in suspension by the vigorous stirring. In the suspension in absolute ethanol, the iminodiacetic acid only partially dissolved and settled out quickly.

All of the reactants were soluble in water and in mixtures of up to 85 percent ethanol with water. Table 12 summarizes the yields of the Mannich condensation in various compositions at different values of apparent pH. These yields represent the amount of Calcein generated in the initial reaction. The data was obtained by determining the concentration of Calcein and fluorescein in solution prior to separation by precipitation.

While the relative rate of reaction was almost unchanged with changes in the composition of the solvent, the yield of the reaction was dramatically affected. The reaction in acetic acid was found to be only 50 percent complete after six hours. The highest yields were obtained with mixtures of ethanol and water. As shown in figure 56, solutions of 50 percent ethanol approached 100 percent yields between pH 8 and 10. A closer examination

Table 12

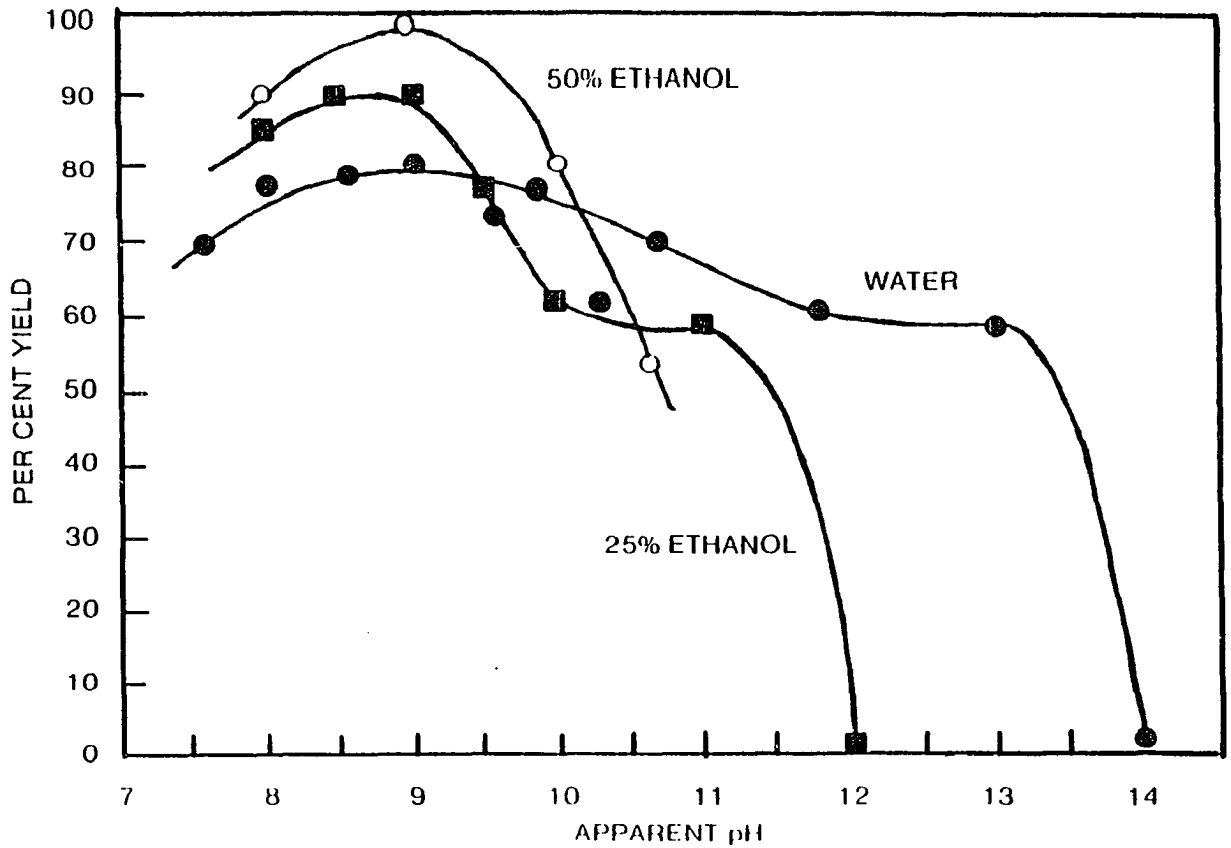
Yields of Calcein as a Function of pH

Solvent	pH of Aqueous Portion	Per Cent Fluorescein Remaining	Per Cent Calcein Produced
Water	7.99	2.1 ± 0.7	78 ± 1.1
Water	8.57	3.1 ± 0.3	80 ± 1.8
Water	9.03	3.7 ± 0.5	81.5 ± 0.4
Water	9.58	14.7 ± 0.6	74 ± 1.4
Water	9.87	13.8 ± 0.5	78 ± 1.4
Water	10.30	28 ± 1.3	63 ± 1.0
Water	13.0	32.0 ± 0.3	59.6 ± 0.2
Water	14.0	99.6 ± 0.4	1.8 ± 0.4
25% Ethanol	8.0	3.0 ± 0.3	86 ± 1.8
25% Ethanol	8.5	3.1 ± 0.3	90 ± 1.4
25% Ethanol	9.0	3.1 ± 0.3	90 ± 1.4
25% Ethanol	9.5	12.6 ± 0.8	78 ± 1.1
25% Ethanol	10.0	30 ± 1.2	62 ± 0.4
25% Ethanol	11.0	27 ± 1.2	59.4 ± 0.4
25% Ethanol	12.0	98	0
75% Ethanol	8.0	4.5	94
75% Ethanol	9.0	1.5 ± 0.2	98.5 ± 0.9
75% Ethanol	10.0	7.0	80

Figure 56.

Yield of Mannich condensation

as a function of the composition of the solvent


100
90
80
70
60
50
40
30
20
10
0
PER CENT YIELD
50% ETHANOL
WATER
25% ETHANOL
7
8
9
10
11
12
13
14
APPARENT pH

of the yield as a function of the amount of ethanol is shown in table 13 and figure 57. At the recommended pH, the maximum yields were obtained in solutions containing 50 to 80 percent ethanol.

The modification of the solvent permitted an increase in the yield of the reaction. However, the low net yields encountered by previous workers was, no doubt, more strongly influenced by the efficiency of the separation than by the yield of the initial reaction. Yet, the same changes that improved the yield of the reaction were also found to improve the methods of separating the product. This will be discussed further in subsection 5.

During this study, a surprising phenomenon was encountered. In solutions of approximately 85 percent ethanol, the conversion of fluorescein to Calcein was accompanied by a phase separation in the reaction vessel. A small, highly aqueous phase separated from the bulk of the solvent. The Calcein formed by the reaction was concentrated in this small phase while the remaining fluorescein was dispersed throughout the system. This was quickly exploited. The small phase containing a

Table 13

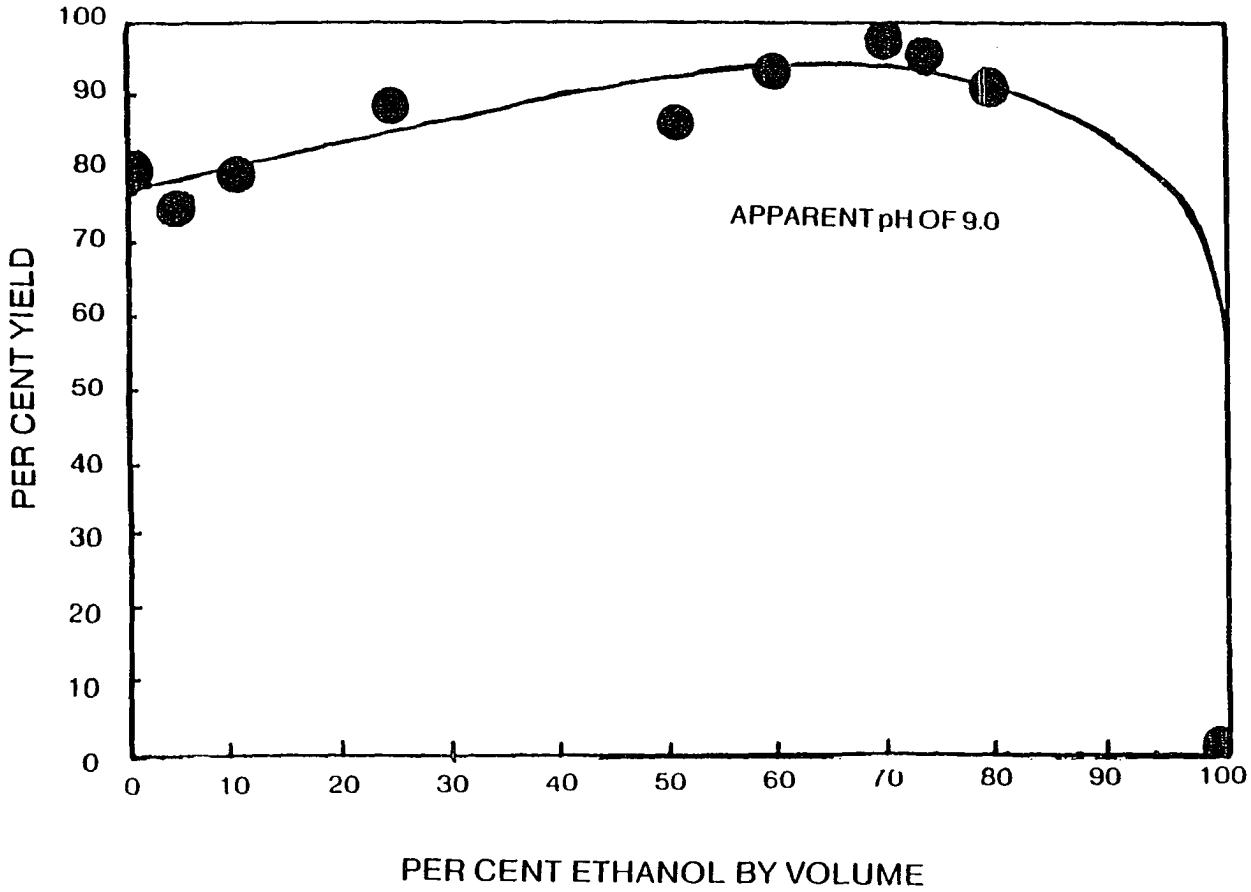
Yields of Calcein in Various Solvents

Solvent	pH of Aqueous Portion	Per Cent Fluorescein Remaining	Per Cent Calcein Produced
Acetic Acid	---	20	50
Water	9.0	3.7 ± 0.4	81.5 ± 0.4
5% Ethanol	9.0	11.7 ± 0.4	75.6 ± 0.2
10% Ethanol	9.0	3.0 ± 0.3	81 ± 3.1
25% Ethanol	9.0	3.1 ± 0.2	90 ± 1.5
50% Ethanol	9.0	4.7 ± 0.3	85 ± 3.6
75% Ethanol	9.0	1.9	102
80% Ethanol	9.0	1.5 ± 0.2	98.5 ± 0.4
100% Ethanol	---	101	0

Figure 57.

Yield of Mannich condensation

in ethanol-water solutions


100
90
80
70
60
50
40
30
20
10
0
PER CENT YIELD
APPARENT pH OF 9.0
0
10
20
30
40
50
60
70
80
90
100
PER CENT ETHANOL BY VOLUME

large fraction of the Calcein was extracted from the flask, replaced with water and fresh charges of reactants, and the reaction was repeated in the same bulk medium. The net yields recovered from the consecutive preparations in the same bulk solvent are summarized in table 14. The larger of the two phases quickly became saturated with both fluorescein and Calcein and the yield of the process approached 100 percent with little waste of materials. The distribution of the fluorescein and Calcein between the two phases is summarized in table 15.

This two-phase technique had several advantages: the concentration of the product was higher, the concentration of the contaminant was slightly lower, and the volume of the solution and the corresponding loss of product by solubility was significantly reduced.

5. Effect of the compositon of the medium for the precipitation of Calcein

The solubilities of Calcein and fluorescein are presented in figures 58 and 59 as functions of the composition and pH of solution. In water, both Calcein

Table 14.

Yields of Repetitive Preparation in the Same Solvent

Consecutive Preparations	Yield of Calcein
1	70 %
2	95 %
3	104 %
4	92 %

Table 15.

Distribution of Fluorescein and Calcein
between Phases of the Two-Phase Synthesis System

Phase	Conc. of Fluorescein (M)	Percent of Fluorescein (%)	Conc. of Calcein (M)	Percent of Calcein (%)
light	1.4 x 10-3	2	1.6 x 10-2	19
heavy	7.5 x 10-3	1	7.0 x 10-1	70
total	1.9 x 10-3	3	6.8 x 10-2	99

Figure 58.

Solubility of Calcein

as a function of pH and composition of the solvent

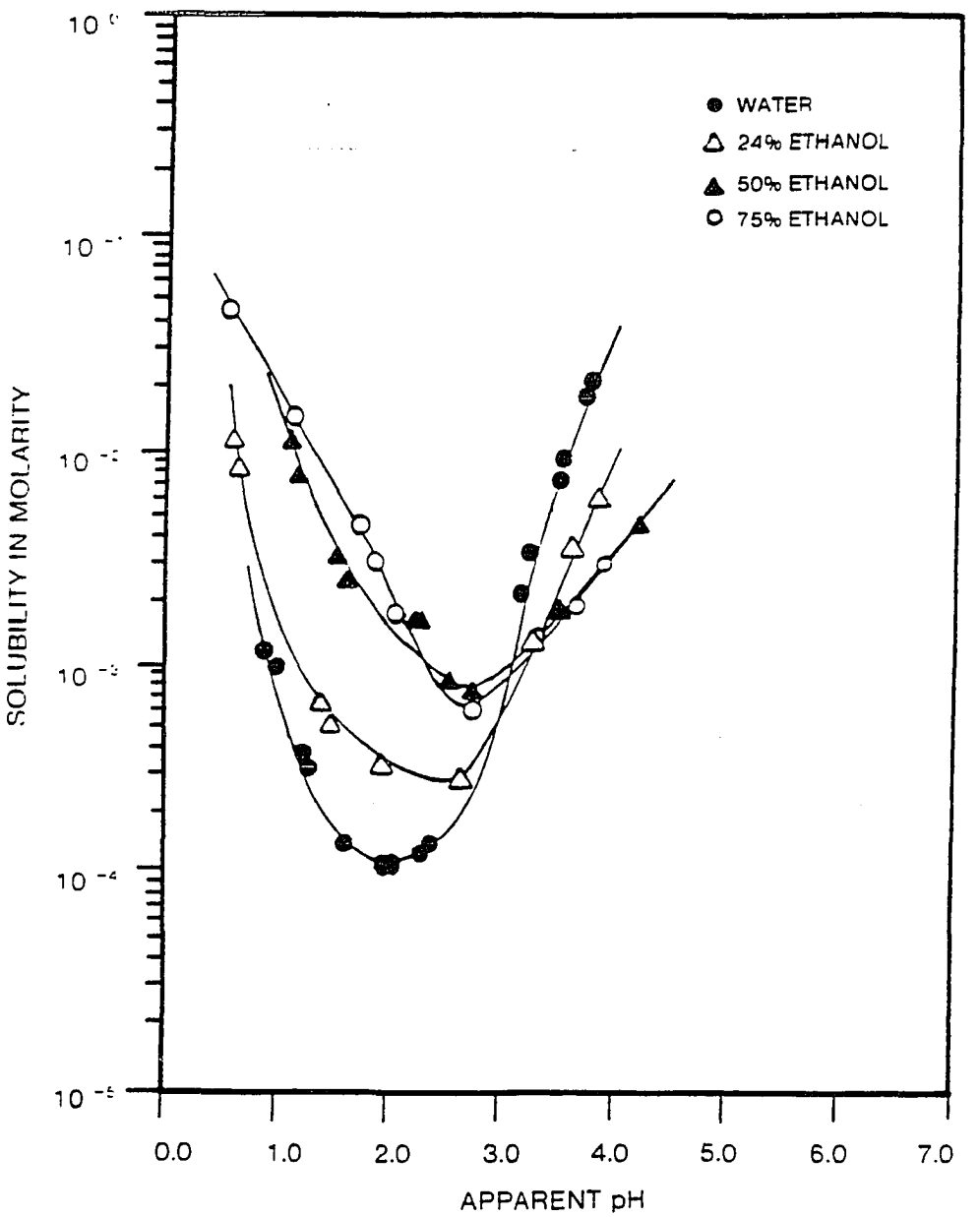
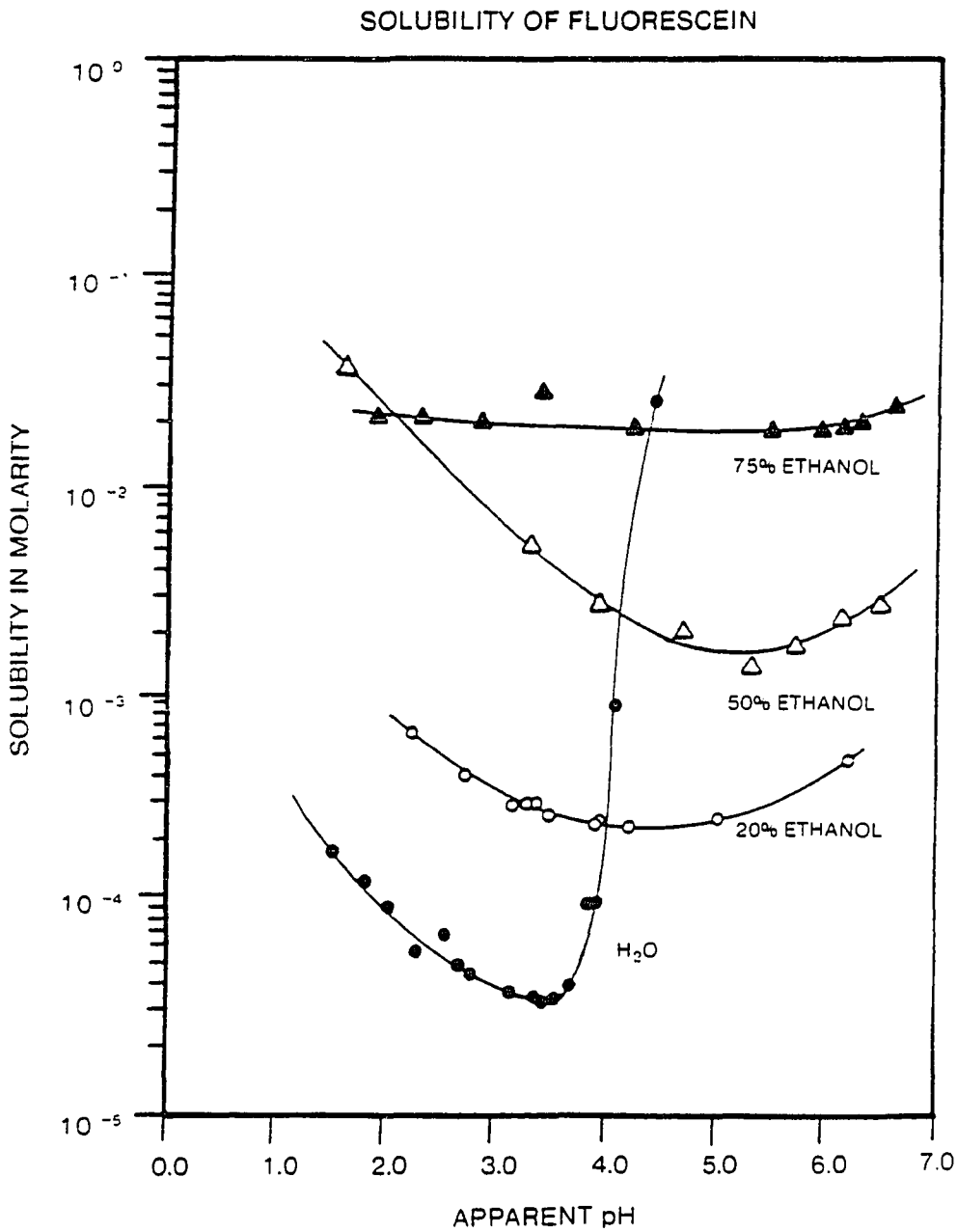

WATER
24% ETHANOL
50% ETHANOL
75% ETHANOL
SOLUBILITY IN MOLARITY
APPARENT pH
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0

Figure 59.

Solubility of fluorescein
as a function of pH and composition of the solvent


SOLUBILITY OF FLUORESCEIN
SOLUBILITY IN MOLARITY
10 0
10 -1
10 -2
10 -3
10 -4
10 -5
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
APPARENT pH
75% ETHANOL
50% ETHANOL
20% ETHANOL
H2O

and fluorescein had sharp minima at 2.2 and 3.2 respectively. These points corresponded to the maxmimum distribution of the compounds as the neutral molecules. As the amount of ethanol in solution was increased, the solubility of Calcein increased only slowly while the solubility of fluorescein in 50 percent ethanol was increased two orders of magnitude. In addition, the point of minimum solubility of fluorescein occured at much higher conditions of apparent pH. Therefore, changing the composition of the solvent for the precipitation of Calcein from water to 50 percent ethanol decreased the amount of fluorescein coprecipitated by increasing the solubility of fluorescein and by exaggerating the difference between the minima in solubility of the two compounds. In addition, the inclusion of 50 percent ethanol facilitated the coagulation and filtration of the precipitated material without the use of large quantities of salt. Thus, the net purity of the product with respect to both fluorescein and overall equivalent weight was greatly improved.

Another important modification involved the direction of precipitation, that is, the direction of the

change in pH in approaching the minimum of solubility. Traditionally, Calcein has been precipitated from alkaline solutions by the addition of acid. However, when the product was precipitated from highly acidic solutions with potassium hydroxide, the Calcein was measurably less efficient at scavenging metal ions, particularly alkaline earths. On the other hand, precipitation from a solution of hydrochloric acid increased the amount of chloride in the product and increased the equivalent weight of the material in general. Presumably, this was the result of the precipitation of some of the cation of Calcein as the hydrochloride. In balance, the best product was demonstrated to be obtained by alternately precipitating from the acidic and basic sides of the minimum of solubility.

6. Effect of extractions on the purity of Calcein

The results of analyses of several preparations of Calcein are summarized in table 16. Extractions with water were successful in reducing the inorganic impurities, primarily potassium chloride. The

Table 16

Purity of Various Preparations of Calcein

Sample	Procedure	Net Purity	% Fluorescein	% KCl
Hach	___	92.0	0.9	_
G.F. Smith	_____	77.2	3.6	__
Fisher	_____ _	76.2	7.4	_
Martin (I)	_____	87.0	2.2	__
Martin (II)	___ _	92.4	1.0	_ _
XIID	H_2O pption	88.8	1.9	6.1
XII-E	Ethanol water pption	94.6	0.4	1.2
XII -Z	Ethanol from acid	96.8	< 0.2	1.8
XV	"altern. directions"	99.2	< 0.2	< 0.5
XIV-A	w/o extraction	96.2	0.7	< 0.5
XIV-B	water extraction	96.6	0.9	< 0.5
XIV-D	water/acetone/ethanol extraction	97.0	< 0.2	< 0.5
XXI-A	final procedure	99.6	< 0.2	< 0.5

extractions with ethanol and acetone had measureable effects on the amount of residual fluorescein. As shown in figure 60, the amount of fluorescein in Calcein following three extractions with acetone and six extractions with ethanol, often contained less than 0.2 percent fluorescein (the limit of detection). The application of a three-stage extraction (water, acetone, ethanol) significantly reduced both organic and inorganic impurities within a few hours.

However, with the already high quality of the product as it was recrystalized from the ethanolic solution, further refinements were only needed for applications demanding the utmost purity. For most applications, the quality of the Calcein prepared by precipitation from 50 percent ethanol was more than sufficient.

7. Effect of different drying and storage conditions

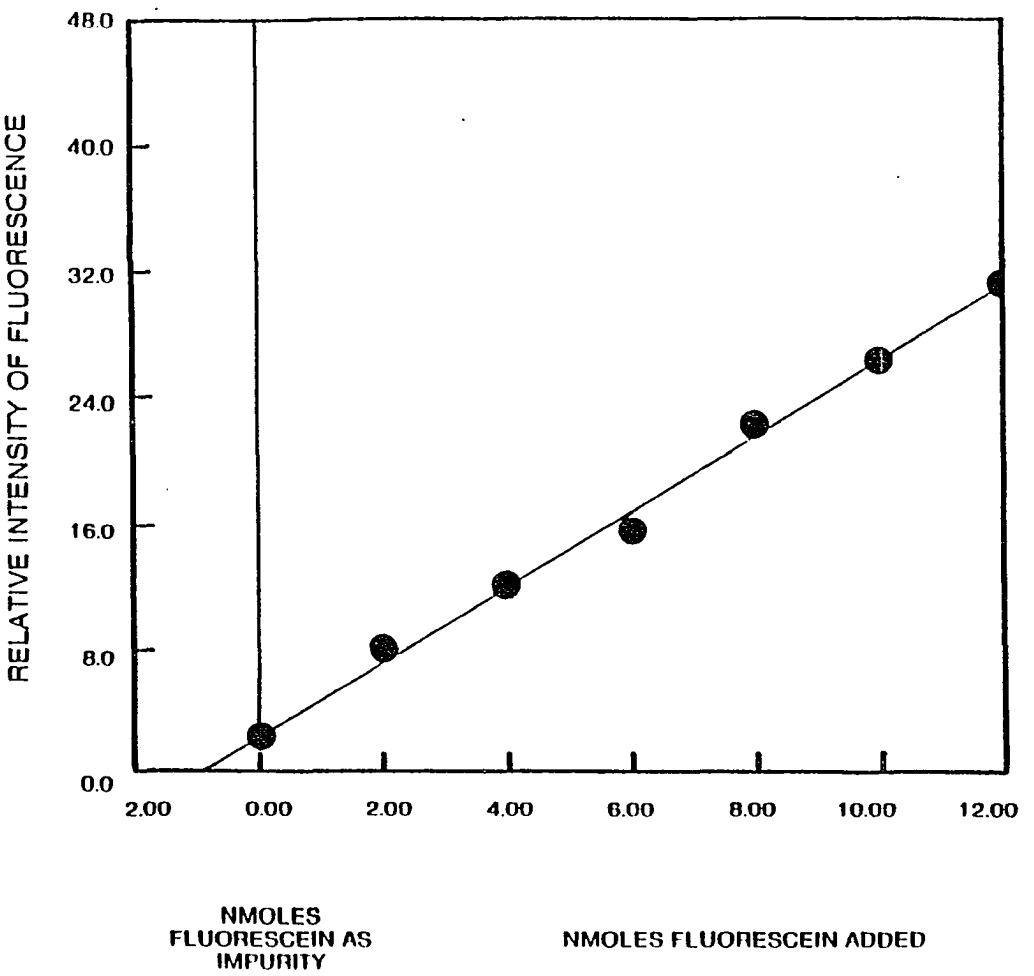
Two different approaches were taken to determine the proper method to remove solvent from precipitated Calcein without diminishing the purity of the product. First,

Figure 60.

Fluorometric determination of

residual fluorescein following Soxhlet extraction

Percent fluorescein: 0.02 %


RELATIVE INTENSITY OF FLUORESCENCE
48.0
40.0
32.0
24.0
16.0
8.0
0.0
2.00
0.00
2.00
4.00
6.00
8.00
10.00
12.00
NMOLES FLUORESCEIN AS IMPURITY
NMOLES FLUORESCEIN ADDED

the thermogravimetric properties of Calcein were examined to determine the temperature and conditions where Calcein degraded. Second, samples of Calcein were exposed to the air, freeze-dried, and oven dried in air, nitrogen, and under vacuum. Each of the samples were then analyzed for water, purity, and fluorometric properties. The results of both approaches indicated that Calcein should be prepared as the stable monohydrate by drying at 80 to 90 C under vacuum or inert atmosphere.

Figure 61 demonstrates the change in weight of a sample of Calcein precipitated from a 50 percent ethanol solution as it is heated in air at 10 C/minute. Curve A indicates the weight of the sample (normalized to 100%) as a function of temperature. Curve B traces the derivative of weight with temperature and thereby emphasizes the regions corresponding to physical changes in the sample.

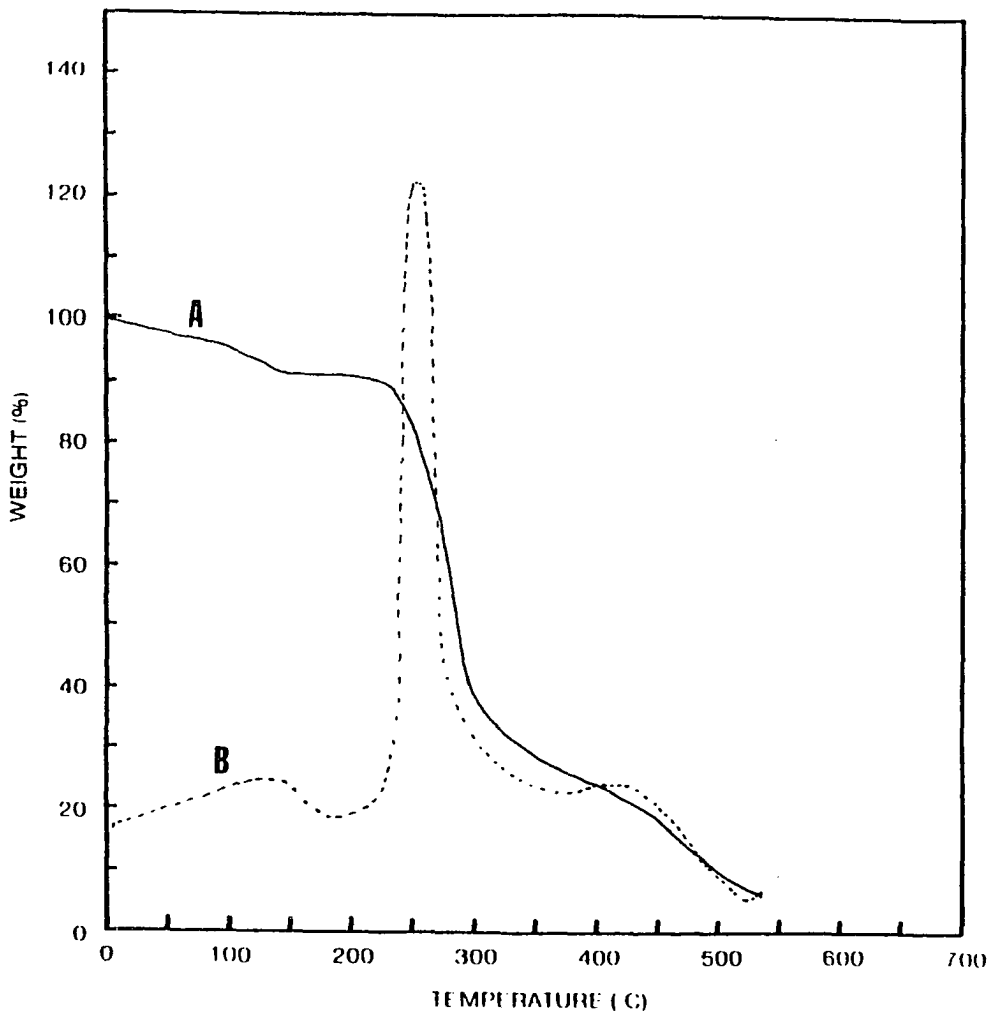
The sample of Calcein shown in figure 61 continually lost weight from 20 C (initial temperature) to around 125 C. The total weight loss over this region corresponded to approximately 8 percent of the weight of the air-dried material. At first, the loss was slow but

Figure 61.

Thermogravimetric analysis of Calcein in air

A: Sample weight as a function of temperature

B: Derivative of curve A


140
120
100
80
60
40
20
0
WEIGHT (%)
A
B
0
100
200
300
400
500
600
700
TEMPERATURE (C)

the slope of the curve gradually increased, reached a maximum near 110 C (see curve B) and then tapered to a plateau above 125 C. Above 175 C, the sample underwent rapid degradation resulting from combustion of the organic material.

Similar experiments with other samples produced analogous curves with reproducible transition temperatures but varying weight losses of 5 to 10 percent between 80 and 110 C. In almost all cases, the total weight loss approximated the amount of water determined by Karl Fischer titration.

The only thermal region separating consecutive reactions in air occured near 150 C. Samples of Calcein exposed to that temperature were dark orange or red, showed little fluorescence in aqueous solutions, and were not affected by the presence of calcium in alkaline solutions. In short, by the point where all absorbed and occluded water had been removed, the material had already degraded severely.

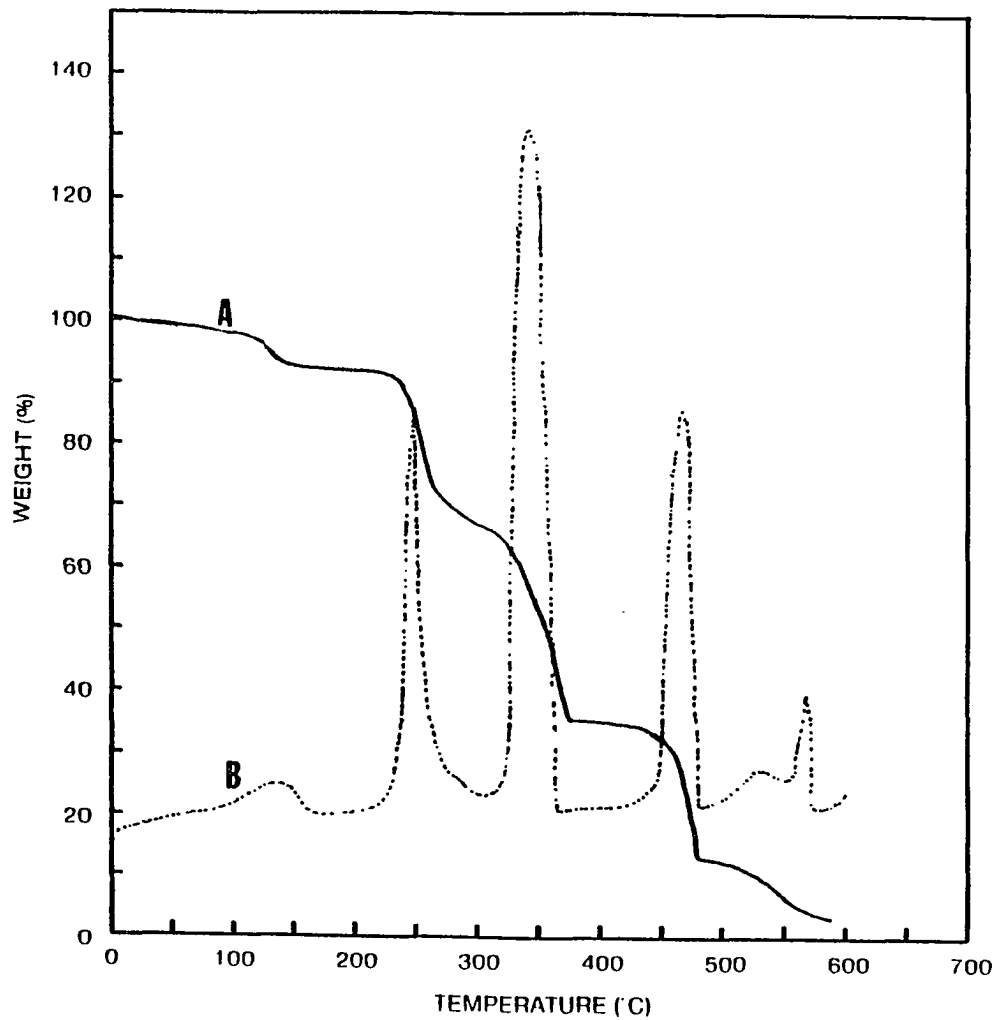
In figure 62, a similar study is shown for a sample of Calcein analyzed under nitrogen. Under nitrogen, the

Figure 62.

Thermogravimetric analysis of Calcein under nitrogen

A: Sample weight as a function of temperature

B: Derivative of curve A


140
120
100
80
60
40
20
0
WEIGHT (%)
A
B
0
100
200
300
400
500
600
700
TEMPERATURE (°C)

thermal decomposition of the sample can be easily separated into several distinct regions. Between 20 and 85 C, Calcein undergoes a slow but reproducible loss of weight. Above 100 C, Calcein abruptly loses an additional amount of weight before reaching a plateau near 150 C similar to that observed in air. Beyond this point, the thermal decomposition occurs in a series of steps corresponding to decarboxylation and then thermal decomposition of the aliphatic and aromatic portions of the molecule at relatively high temperatures.

While the loss of weight between 20 and 90 C varied between different samples, the weight lost between 90 and 125 C was reproducibly 2.2 to 2.3 percent of the initial weight or approximately 2.5 percent of the weight at 80 C. This latter change corresponded to slightly less than one molecule of water per molecule of Calcein. This separation of weight changes was even more obvious under partial vacuum.

Samples removed from drying under nitrogen or vacuum at 85 C were bright yellow to yellow-orange, highly fluorescent in neutral solutions, and highly responsive to the presence of calcium in alkaline solutions. When

samples were removed after exposure to 150 C under the same conditions, the samples were again dark and non-fluorescent.

It has been concluded, therefore, that Calcein has more than one form of associated solvent, particularly water: (1) occluded or loosely associated water of hydration that can be thermally removed without degrading the product, and (2) an intimately associated molecule of water of crystallization that is involved in the stability of the solid form of Calcein.

As a result, these studies recommend that Calcein be prepared as the stable hydrate by drying in an oven under either nitrogen or preferably vacuum at 80 to 90 C.

When prepared in this form, Calcein is extremely hygroscopic and will compete with several commercial drying agents. As a result, the proper desiccant is required to maintain consistent purity. As examples, the curves in figure 63 demonstrate the weight changes in dishes of three different desiccants and Calcein when simultaneously exposed to specific amounts of water in common atmospheres. Some desiccants, particularly the

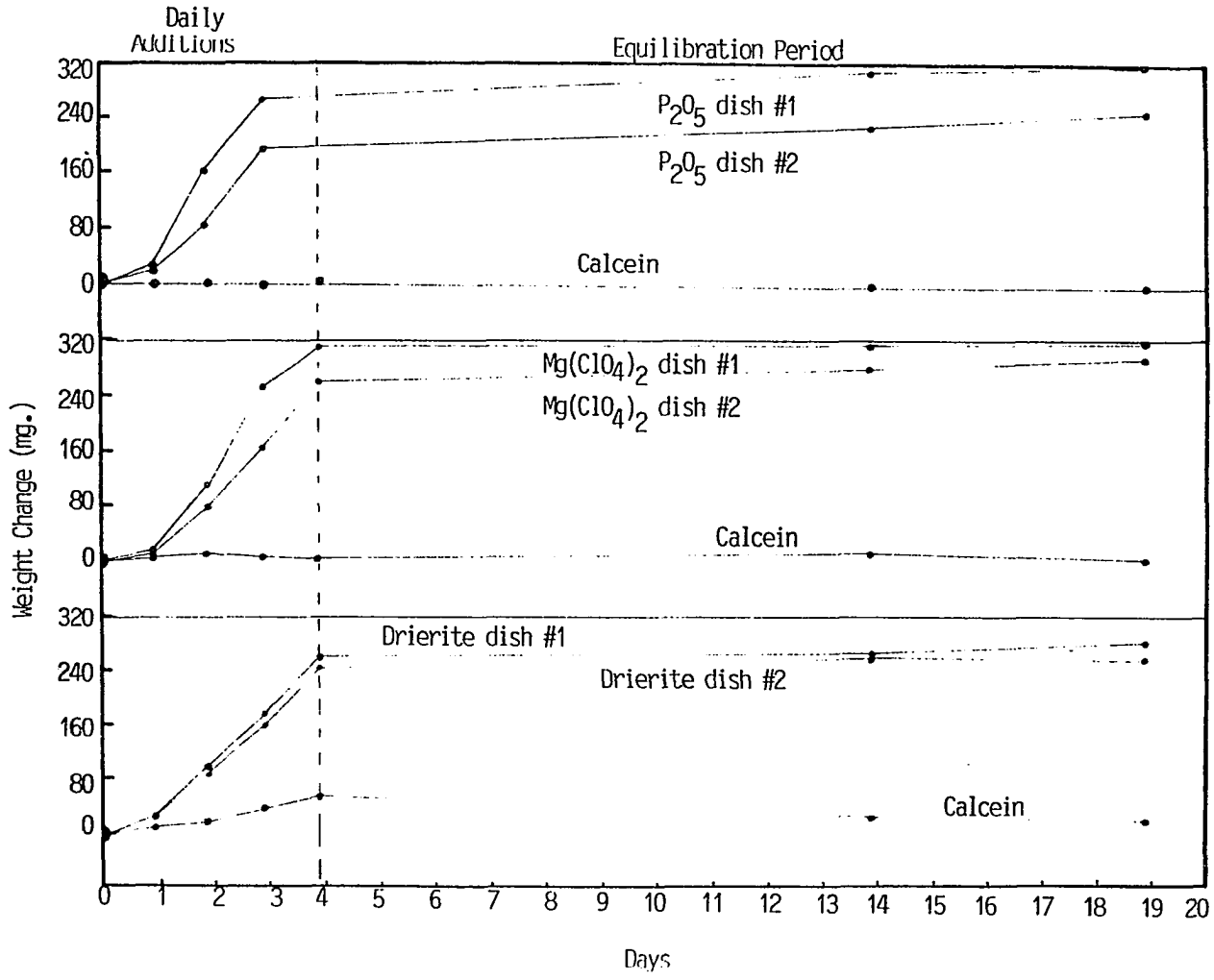
Figure 63.

Effectiveness of drying agents

Curves A: phosphorus pentoxide vs. Calcein

Curves B: magnesium perchlorate vs. Calcein

Curves C: Drierite vs. Calcein


Daily
Additions
Equilibration Period
320
240
160
80
0
P_2O_5 dish #1
P_2O_5 dish #2
Calcein
$Mg(ClO_4)_2$ dish #1
$Mg(ClO_4)_2$ dish #2
Calcein
Drierite dish #1
Drierite dish #2
Calcein
Weight Change (mg.)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Days

strong desiccants pointed out by Trusell and Diehl [348], can successfully maintain a dry product while others require long equilibration times, and still others fail to protect Calcein from moisture. In general, phosphorous pentoxide and magnesium perchlorate were adequate while commercial products such as Drierite and silica were not.

8. Effect of light, heat, and pH on stability of Calcein

During the course of this work, the effect of several environmental factors on the stability of Calcein were noted. Some were measured quantitatively, while others were qualitative observations.

When stored under vacuum or in the presence of a strong desiccant, no measurable change in the purity or properties of Calcein were detected after exposure of the solid reagent to fluorescent lighting or temperatures of up to 60 C for six months. Direct sunlight on the solid did produce a slight darkening of the color of the material directly on the surface but again no measurable

decrease in purity was found.

When damp solid material was exposed to air for extended periods (greater than 6 hours), especially immediately after treatment with dehydrating solvents such as methanol or acetone, the surface darkened considerably and the purity was found to decrease by several percent.

The stability of solutions of Calcein were found to be very dependent on pH. Solutions of 10^{-5} M Calcein in high alkalinity (pH 10) were found to degrade by as much as 5 percent in one day. As the pH decreased from 10 to 6, the usable lifetime of the solutions of Calcein increased. In neutral to mildly acidic solutions where solubility was the lowest, a second problem was noted as the formation of an insoluble precipitate at concentrations below those measured for the solubility of Calcein. Similar problems were also encountered with solutions of fluorescein. In general, solutions of pH 7.5 to 8.5 were found to remain constant (less than one percent loss) for up to 14 days if dearated and stoppered.

E. Recommended Procedures for the Preparation of Calcein in a Highly Pure and Consistent Form

(i) Batch process The following procedure will prepare approximately 20 g of Calcein. The procedure has been successfully used to prepare Calcein with purities between 96 and 99 percent with net yields of approximately 35 percent of the theoretical amount.

Weigh 34 g. of purified fluorescein into a beaker and dissolve in 70 ml. of 6.0 N potassium hydroxide and 50 ml. of 95 percent ethanol.

Weigh 33 g of iminodiacetic acid into a second beaker and dissolve in 40 ml. of 6.0 N potassium hydroxide and 50 ml. of 95 percent ethanol.

Transfer both solutions to a three-neck, round-bottom flask equipped with a stirring bar, thermometer, and water-cooled condenser. Stir the mixture and measure the pH of the solution. While stirring, slowly add 1.0 N hydrochloric acid dropwise to lower the pH to between 9.1 and 9.5.

Add 100 ml. of 95 percent ethanol and 35 ml. of 37 percent solution of formaldehyde. Stopper the flask immediately. Warm the solution to 70 C and maintain that temperature for 6 to 8 hours.

Filter the solution while hot through a Buchner funnel. Transfer the filtrate to a 2-liter beaker.

Dilute the filtrate with 750 ml. of 95 percent ethanol. Slowly add 1 N hydrochloric acid dropwise while stirring vigorously to precipitate the yellow-orange Calcein from the dark red solution. Continue adding acid until the pH of the solution is between 2.0 and 2.3.

Warm the solution to 35 C and digest the mixture for 20 to 30 minutes. Then allow the mixture to cool to room temperature.

Filter the suspension through fine filter paper in a Buchner funnel. Carefully slurry the precipitate in the filter with deionized water and refilter three times.

Suspend the precipitate in 500 ml. of deionized

water. Slowly add 6.0 N hydrochloric acid dropwise while stirring vigorously until all of the precipitate redissolves (pH < 1.0).

Filter the solution. Add 500 ml. of 95 percent ethanol to the filtrate. Then slowly add 6.0 N potassium hydroxide dropwise to reprecipitate and raise the pH to 2.0. Allow the suspension to digest for 1 to 2 hours.

Filter the mixture again. Slurry the precipitate with deionized water and refilter.

Add 500 ml. of deionized water to the precipitate and slowly add 6.0 N potassium hydroxide to redissolve the precipitate. (pH > 9.5).

Repeat the precipitation, filtration, and dissolution a second time.

Finally, dilute the solution with 750 ml. of 95 percent ethanol and reprecipitate the Calcein with 6.0 N hydrochloric acid and digest the suspension for 30 minutes.

Filter the mixture, slurry with acetone and methanol several times. Then slurry with water and allow to air dry for several hours.

Place precipitate in a Soxhlet extractor and extract with acetone, methanol, and water.

Dry the precipitate at 80 C for at least two hours either under vacuum or nitrogen atmosphere. Remove the powdery flake-like material, grind with a mortar and pestle, and then repeat the oven drying.

Store Calcein under vacuum or in a desiccator well charged with a strong drying agent.

(ii) Continuous preparations The following adaptations are useful for repetitive preparations. This procedure minimizes raw materials and loss of product over consecutive preparations.

Dissolve fluorescein and iminodiacetic acid in a ratio of 2:1 in an 80 percent solution of ethanol using 6.0 N potassium hydroxide.

Transfer part of the solution to a three-neck, round-bottom flask. Add 100 ml. of 95 percent ethanol and 50 ml. of 37 percent formaldehyde. Perform the initial reaction as in procedure (i) above. At the end of six hours, turn off the stirrer, allow the mixture to settle and then remove only the heavier, darker layer.

Add an another portion of the solution of fluorescein and iminodiacetic acid to the reaction vessel containing the upper phase. Add another 50 ml. of formaldehyde solution and repeat the synthesis.

Filter the removed phase while warm and then precipitate the Calcein with 1.0 N hydrochloric acid. Filter the precipitate and then redissolve in more hydrochloric acid. Filter, precipitate the Calcein with potassium hydroxide and repeat the dissolution and precipitation process again.

Extract and dry the product as prescribed in section (i).

F. Conclusions

A complete scheme for analyzing preparations of Calcein has been developed. Methods for determining each of the significant impurities of Calcein have been evaluated and new methods have been developed. These procedures have provided a means to critically evaluate all of the materials used in the preparation of Calcein as well as monitoring the yield and purity of the product throughout its preparation.

The recommended procedures for analyses included semiquantitative determination of transistion metals by plasma emission spectroscopy, fluorescein and alkaline earths by fluorescence, solvents by thermal gravimetric analysis, potassium salts by so-called double-ashing procedures, and chloride by coulometric argentiometric titration. Modifications have been made to the two-end-point potentiometric titration developed by Martin[253] to determine the net purity of over forty batches of Calcein.

The conditions for the Mannich condensation of

fluorescein, formaldehyde and iminodiacetic acid have been examined. Solvent compositions have been determined that increase both the yield and purity of Calcein. It has been shown that when the reaction was performed in a 50:50 mixture of ethanol and water with an apparent pH of 9.0, the yield of the reaction approached 100 percent conversion in less than five hours at 65 C.

An unusual phenomenon was encountered in working with syntheses in mixed solvents. In solutions of 85 percent ethanol, the conversion of Calcein was shown to be accompanied by a separation of phases. The isolation of the desired product in the small phase was shown to permit a unique method for repetitively preparing a high quality reagent in good yield with almost no wasted materials.

The initial reaction rate of the Mannich condensation of fluorescein, formaldehyde and iminodiacetic acid was found to be relatively independent of the solvent but the final yield was shown to be very dependent on the composition of the solvent from which the product was separated.

The importance of the purification of fluorescein has been demonstrated. Analyses of samples of fluorescein of different qualities, along with samples of Calcein prepared from each of those lots of fluorescein, demonstrated the strong scavenging nature of both compounds and the retention of metallic impurities throughout the preparation.

The solubilities of fluorescein and Calcein have been studied as functions of the composion of the solvent and the apparent pH of the solution. Both fluorescein and Calcein have greater solubilities in ethanol-water solutions than in aqueous solutions. However, while the solubility of Calcein increases gradually with increasing concentration of ethanol, the solubility of fluorescein increases 100-fold in a 50 percent solution. In addition, it has been shown that the minimum in the solubility of fluorescein with apparent pH also shifted dramatically. As a result, the mixed solvent permitted a better separation of Calcein from fluorescein by precipitation without serious losses in yield.

Many other aspects of the preparation, handling and storage of Calcein have been examined. A number of

observations have been made regarding the effect of different experimental parameters on the yield and purity of the final product. From these observations, two recommended procedures were proposed for the preparation of Calcein in a highly pure form. The first procedure is for the preparation of a single batch of Calcein by conventional techniques. The second called for an unusual, consecutively run set of reactions in the same bulk solvent. The procedures have been shown to routinely produce Calcein at purities above 97 percent (as the monohydrate) in yields of 25 to 30 percent.

VII. APPLICATIONS OF CALCEIN OF HIGH PURITY TO CHEMICAL ANALYSIS

A. INTRODUCTION

The goal of this entire project was to develop better techniques for preparing and evaluating Calcein in support of practical applications of this reagent in analytical chemistry and related fields.

In their initial publication on Calcein, Diehl and Ellingboe[113], demonstrated the usefulness of Calcein as an indicator in the titration of calcium with EDTA. The titration was performed in an alkaline solution where the indicator, Calcein, combines with calcium to form a highly fluorescent, slightly-dissociated compound. As the equivalence-point of the titration is approached, the EDTA added as titrant combines first with the free calcium in solution and then, because EDTA forms a stronger compound with calcium than does Calcein, the EDTA extracts the calcium from the fluorescent slightly-dissociated calcium-Calcein compound. The end-point of the titration is marked by the disappearance of fluorescence resulting from the dissociation of the metal-indicator compound.

The same properties were found useful in analyzing other metals that fluoresced when covalently associated with Calcein [366-373], metals that quenched the inherent fluorescence of Calcein in neutral solutions [356], and anions that could be titrated indirectly with one of these metals [343].

Several analogous compounds were quickly introduced[56,153,185-188,224-226,382] but have never had the impact on analytical chemistry as their progenitor, Calcein.

Once the Calcein was established as an fluorometallic indicator, application of Calcein as a direct fluorometric reagent for calcium was the logical next step[365]. Preliminary attempts of this application have been reported for the direct fluorometric determination of calcium in blood[90,99,128,133,203,305, 134,206,243], urine[119,207,242], muscle tissue[289], fruits and vegetables[20,40-47,316], milk[277,278], beer[327], waste waters and brine [295], soil[145,266,275,313], and minerals[72,123,140,350] Of these, by far the most attention has been focused on the fluorometric determination of calcium in blood, especially in the

development of reliable, routine, automated clinical methods.

The acurate determination of calcium in the human body is important in diagnosing or treating a large number of maladies. Attention has recently been focused on deteminations of calcium in relation to hypo- and hypercalcemia[100,101,183], uremia[33,34,308,310], phosphotasia[130], blood pressure[202], Paget's disease[259], rickets[353], gall stones[56,188], membrane function[128,274], nerve action[179], thyroid activity[131,204,256,276], and problems with infections[77,79,380], elevated cholesterol[97], and eye lense resilience[333]. The calcium level in human serum has even been related to the balance between nutrition and exercise[155,156]. Several major chemical companies are investigating the use of direct in vivo testing of calcium in the bloodstream[232].

Another active area of interest is in the uptake of calcium by infants[32,92,99,103,172] and the relation of calcium levels to bilirubin levels in newborns[203,306]. Other important on-going research efforts, especially in the fields of medicine and nutrition, are noted in table 17.

Table 17.

Biological investigations using Calcein

	References
Examination of cell volumes and membrane activities	6, 274
Effects of vitamins A & D	30,73,74, 162,204,353
Dietary balance of calcium and other minerals	12-18,33,34, 71,89,142
Effect of calcium on the functioning of enzymes and harmones	31,77,79, 97,135;310
Infant nutritional balance	32,172, 173,306
Embryology	76,378
Urology	86,87,119,207,242
Osteopathy	256,258
Calcium deposits in mammalian tissues	337,345
Veterinary medicine	55,62,152, 192,272,276
Microanalytical techniques for infant serum	5,36,61, 92,103

Many noteworthy attempts have been made to automate the fluorometric determination of calcium in blood and urine. Most notably Ashby[19], Alexander[5], Baron[36], Mabry[243], and others[61,206]. A major reason for the failure of any of these methods to gain widespread acceptance has been the need for constant calibration and relatively large correction factors which can be attributed, in large part, to the quality of Calcein available for use.

The use of Calcein as a biological reagent for measuring cell volumes and membrane activity has similarly been hampered by the insufficient quality of commercially available reagent[6,354].

Each of these limitations to applications has now been addressed using the purified Calcein from this work. The results affirm the need for a highly pure and consistent material. With such a material, all of these methods become much more reliable with accuracies and precisions similar[318,323] to the more tedious classical methods of determining calcium by gravimetry[90,119,151,173] or photometry[305,345,377].

B. Apparatus and Reagents

Automatic fluorometric measurements were made with a Technicon Model II Autoanalyzer with the following modules: an automatic sampling chamber, a peristaltic pump, dialysis chamber, filter fluorometer, and a strip-chart recorder. To this equipment, a 500-ml. water bath was added to provide thermal control over the solutions passing through the mixing coils and subsequent detection modules.

Static measurements of fluorescence were made with a Turner Model 110 Filter Fluorometer. The primary filter consisted of a blue Corning 5850 glass filter and the secondary filter consisted of the combination of a yellow Corning 2A-15 glass filter and a Wrattan 10 percent neutral density filter.

Measurements of pH were made using a Beckman Zeromatic SS-2 pH Meter equipped either with a Fisher Combination pH Indicator/Reference Electrode or with a Beckman Saturated Calomel Reference Electrode and a Beckman Glass Indicator Electrode.

Purified iminodiacetic acid was obtained in the solid form from Hampshire Chemical Company of Keene, New Hampshire.

Special calcium-free potassium hydroxide was obtained from Hach Chemical Company of Loveland, Colorado as a 50 percent solution (12 N). This solution had been prepared by electrolysis in a mercury cell and was highly pure with respect to alkaline earths.

For comparison, Calcein from three sources was used for the titrations described in this chapter. Commercial Calcein was obtained from the Hach Chemical Company of Ames, Iowa and the G. Frederick Smith Chemical Company of Columbus, Ohio. The "highly pure" Calcein was prepared by the recommended procedure given in Chapter VI. The material was analyzed by the techniques described in Chapter V and found to be greater than 99 percent pure (based on the molecular weight of the monohydrate, 640.2 g./mole).

Samples of serum and urine were obtained as clinical samples from Lederyl Hospital Standards and from Mary Greeley Hospital in Ames, Iowa.

C. Experimental Work

1. Direct and indirect chelatometric titrations using Calcein as an indicator

Three types of samples were titrated with EDTA using Calcein as a fluorescent indicator. The first set was comprised of solutions containing low concentrations of calcium and magnesium. These analyses simulated the determination of naturally-occurring calcium in water. The second set of samples consisted of three geological mixtures containing varying amounts of calcium-containing limestone. The third set of samples titrated were human sera obtained from Mary Greeley Hospital in Ames, Iowa. For each set of titrations, the chelatometric end-point was determined first by the addition of EDTA as the titrant to the disappearance of the fluorescence of the calcium-Calcein compound. The end-point was then confirmed by back-titrating an excess of EDTA with standardized solutions of calcium chloride.

For the analysis of dilute solutions of calcium, standard samples were prepared with concentrations of calcium (as the carbonate) between zero and 100 ppm.

The standards were prepared from a stock solution of 1000 ppm calcium carbonate prepared by dissolving 1.0 g of calcium carbonate in 10 ml. of 0.1 N hydrochloric acid and diluting to 1.00 liters with deionized water.

The titration with 0.0109 M EDTA was repeated in both the forward and reverse directions in triplicate for each sample. The fluorescence of the indicator was observed over an ultra-violet light source.

Two geological samples were similarly titrated following the acid dehydration method of Diehl and Miller[116,118] with 0.109 M EDTA to the quenching of the fluorescence of the indicator. Excess EDTA was then added and the solution was back-titrated with 0.0976 M calcium chloride solution.

Several attempts were made to titrate the samples of human serum directly with 0.109 M EDTA. Each attempt resulted in a fading end-point (gradual increase in fluorescence at the apparent end-point). Approaching the equivalence point in the opposite direction using 0.0976M calcium chloride resulted in much more precise and reproducible end-points.

2. Fluorometric determination of cations and anions

The same geological mixes evaluated in part 1 above were also analyzed by direct fluorometric analysis. A fluorometric calibration curve was obtained by measuring the relative fluorescence of standard solutions of calcium in the presence of Calcein. The portion of the calibration curve corresponding to the formation of the two-to-one calcium-Calcein compound was used to relate the relative intensity of solutions to calcium concentration.

3. Automated determination of calcium

Most of the work associated with the topic of this chapter was focused on utilizing the highly pure fluorometric reagent to automate analyses of calcium in biological samples, in particular, human serum. The fluorometric detector of the autoanalyzer was calibrated using standard solutions of calcium chloride. Geological and biological samples were then evaluated

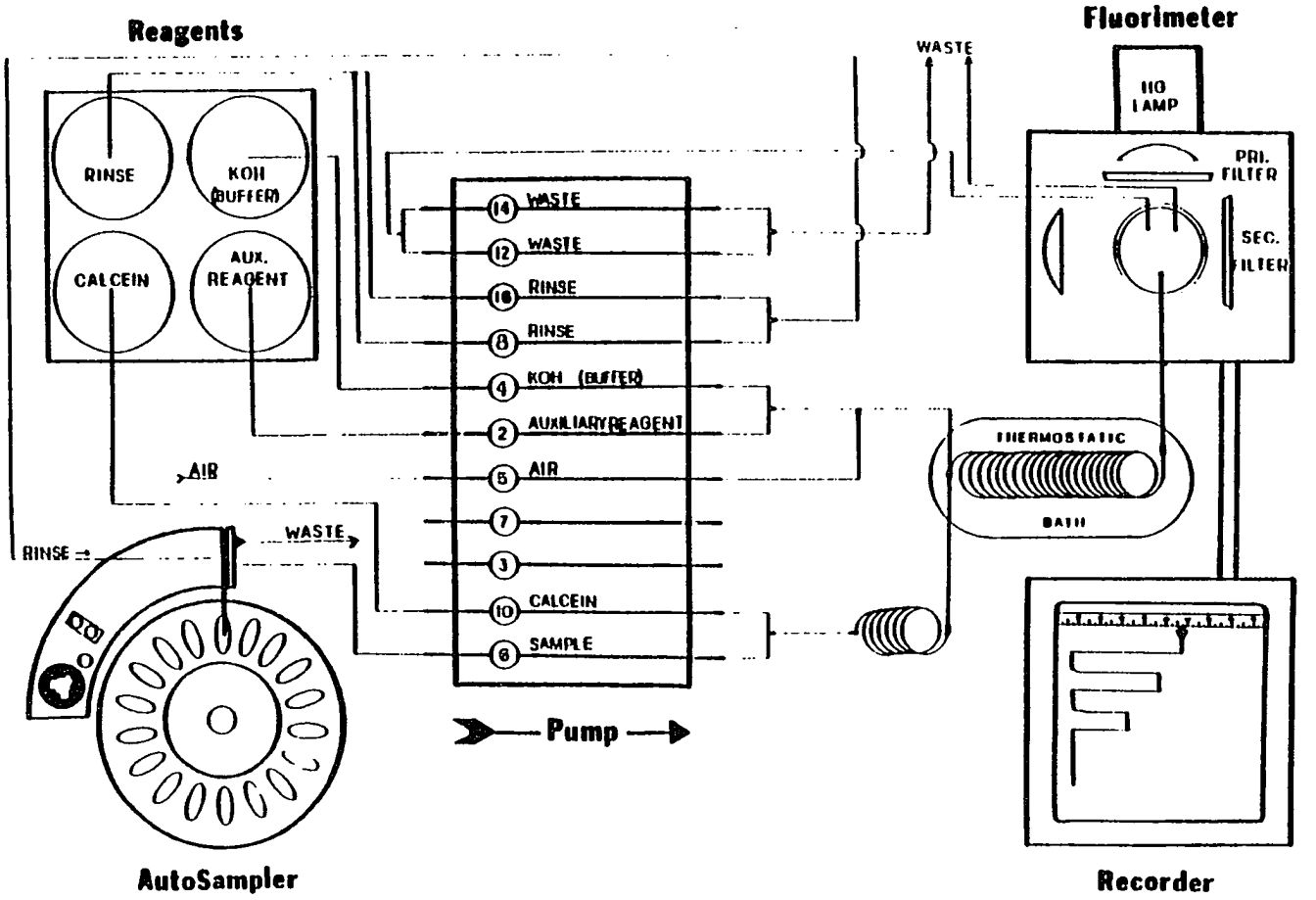
while a number of instrumental and reagent parameters were varied.

The optimum conditions for analysis were found to include the use of a cellophane dialysis membrane, a mixing coil submerged in a thermostatic bath at about 30C, and the spiking of the sample and rinse streams with sodium chloride. The recommended configuration of the autoanalyzer and reagent concentrations are shown in figure 63. For the analysis of serum, the dialysis module is inserted between the autosampler and the junction of the sample stream with the feed-line for the Calcein and alkali. This configuration permitted the pretreatment of the samples with trifluoroacetic acid to release the bound calcium. The calcium was then free to pass through the cellophane dialysis membrane and mix with the alkaline buffer and fluorometric reagent.

The results obtained by this method were compared to the results obtained by direct fluorometric titration and to the results reported by the laboratory at Mary Greeley Hospital using a colorimetric test.

Figure 64.

Configuration of the apparatus for the automated fluorometric determination of calcium in serum


Reagents
RINSE
KOH (BUFFER)
CALCEIN
AUX. REAGENT
AIR
RINSE
WASTE
14 WASTE
12 WASTE
16 RINSE
8 RINSE
4 KOH (BUFFER)
2 AUXILIARY REAGENT
5 AIR
7
3
10 CALCEIN
6 SAMPLE
Pump
AutoSampler
WASTE
Fluorimeter
HG LAMP
PRI. FILTER
SEC. FILTER
THERMOSTATIC
BATH
Recorder

D. Results

In order to objectively compare the relative ease of detection using the visible end-points of the chelatometric titrations of calcium in the different types of samples, the fluorescence of each solution titrated was measured with the Turner Filter Fluorometer after each increment of titrant near the end-point. An example of the resulting plot is shown in figure 65.

In each case, the highly pure Calcein demonstrated significantly less residual fluorescence and produced a dramatically sharper end-point for titrations in both directions than two samples of commercially prepared Calcein. With an experienced eye for titrations with Calcein, equivalent accuracy and reproducibility was obtained with each of the three indicators used. However, the greater ease and clarity of the end-point provided improvements for less experienced workers.

The useful portion of the calibration curve for the direct fluorometric titration of calcium with Calcein is shown in figure 66. The range designated in that figure corresponds to the region in which the second calcium

Figure 65.

End-point for the chelatometric titration of calcium
using highly pure Calcein as a fluorometric indicator

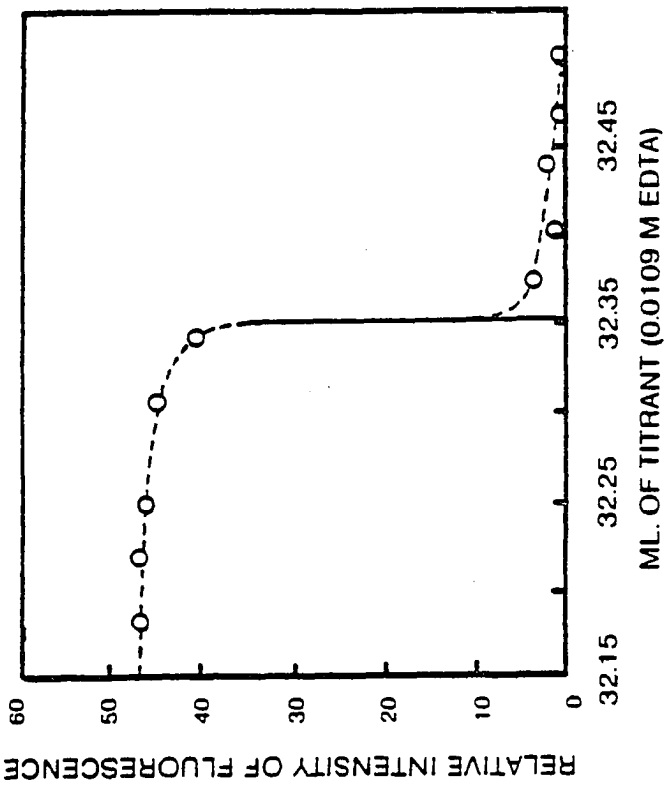
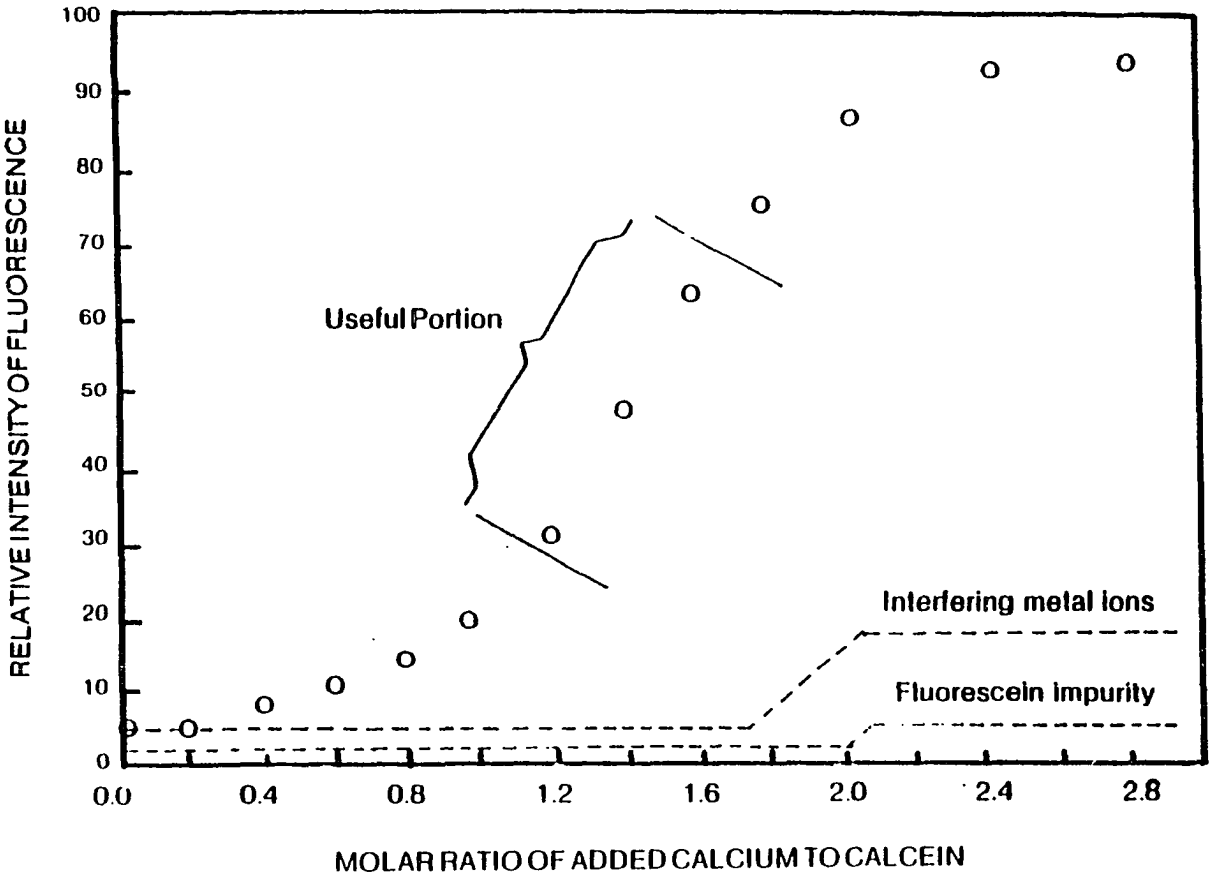

RELATIVE INTENSITY OF FLUORESCENCE
60
50
40
30
20
10
0
32.15
32.25
32.35
32.45
ML. OF TITRANT (0.0109 M EDTA)

Figure 66.

Useful range of calcium-to Calcein ratio for the direct fluorometric determination of calcium


RELATIVE INTENSITY OF FLUORESCENCE
100
90
80
70
60
50
40
30
20
10
0
0.0
0.4
0.8
1.2
1.6
2.0
2.4
2.8
MOLAR RATIO OF ADDED CALCIUM TO CALCEIN
Useful Portion
Interfering metal ions
Fluorescein Impurity

becomes associated with Calcein. This portion of the curve correlates a greater change in fluorescence with changes in the concentration of calcium than the region corresponding to the formation of the one-to-one compound. The relative intensities resulting from interfering metals and residual fluorescein are also shown in figure 65. As can be seen from that figure, neither of these problems are significant when using the highly pure material as a direct fluorometric reagent.

The apparatus used for the automated fluorometric determination of calcium is shown in figure 64. Note that the system includes a thermostatic bath at 30 C. This slightly elevated temperature was found to be extremely useful in lowering the fluorescence resulting from the presence of sodium while having only a minimal effect (less than 3 percent reduction) on the signal from calcium.

The work on biological samples was accomplished as a joint project with D. A. Anderson for the Chemistry Department at Iowa State University [9].

The normal distribution of calcium in human serum is shown in table 18. This table indicates the importance

of separating the calcium from binding protein molecules prior to the analysis. The solutions were pretreated in a strictly controlled acidic buffer prior to mixing with the analytical reagent and alkali.

A typical calibration curve is shown in figure 67. Unlike the curve used for the direct fluorometric analysis, this method utilizes the portion of the curve representing the formation of the one-to-one calcium-Calcein compound. Using this portion of the curve permits the use of a linear calibration curve with a zero intercept - an important convenience to automated analyses. As a result, the relative intensity measured from the X-Y plot (as in figure 67) is directly proportional to the calcium concentration.

The results of the analysis of a set of serum samples from Mary Greeley Hospital is shown in table 19. In each case, the results were in good agreement with both the direct fluorometric determinations and, to a slightly less extent, with the results reported by the hospital based on a colorimetric technique. Reproducibility of the data was especially good. Standard deviations of five repetitions (non-consecutive) were generally less than 3 percent.

Figure 67.

Calibration curve for the automated fluorometric determination of calcium in biological materials

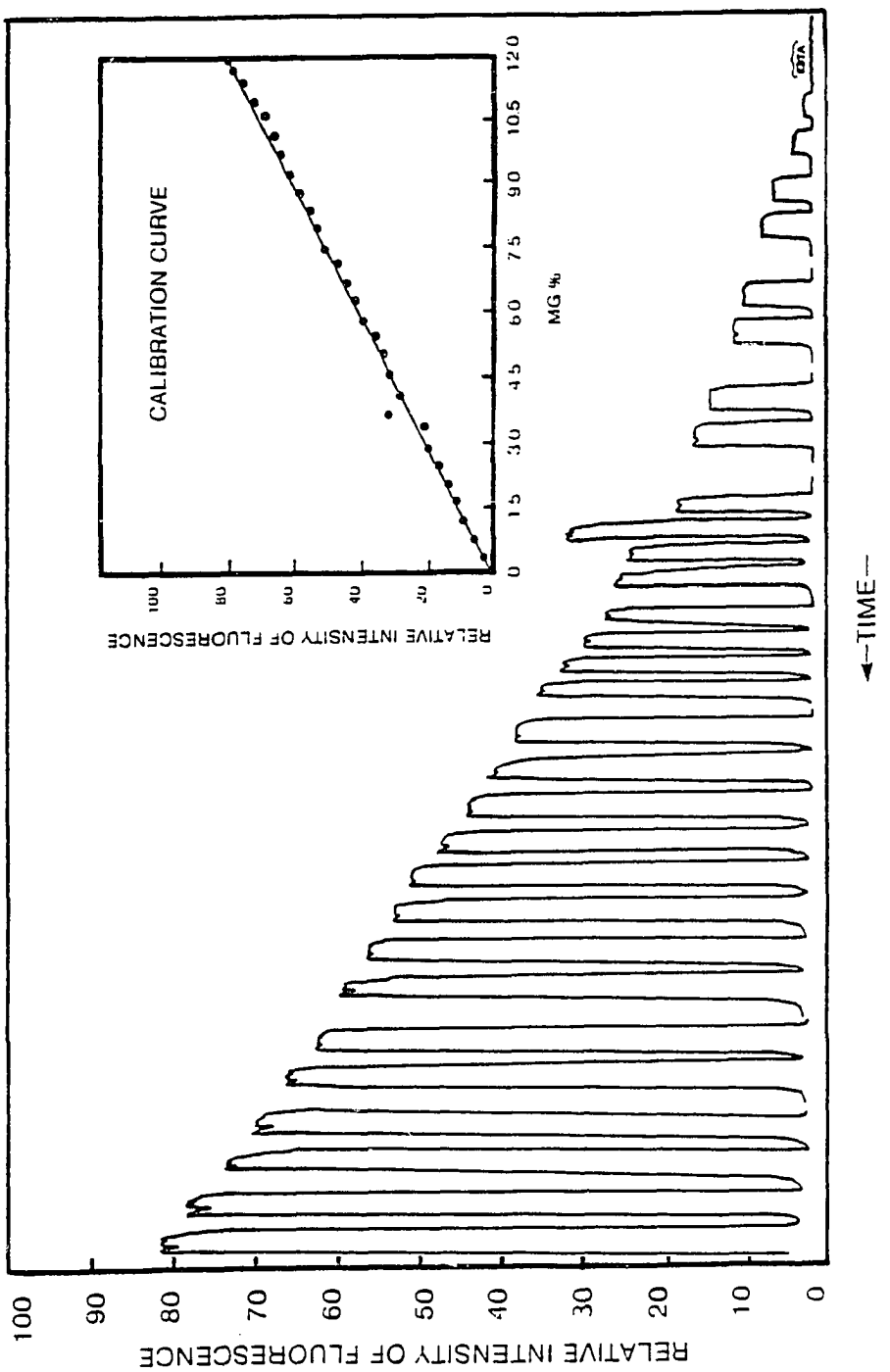

RELATIVE INTENSITY OF FLUORESCENCE
100
90
80
70
60
50
40
30
20
10
0
←TIME→
CALIBRATION CURVE
RELATIVE INTENSITY OF FLUORESCENCE
MG %
0
15
30
45
60
75
90
105
120

Table 18.

Distribution of Calcium in Normal Human Plasma[337]

Source	Concentration	Percent
Free calcium ions	1.16 mM	47.5
Protein-bound calcium	1.14 mM	46.0
Phosphate-bound calcium	.04 mM	1.6
Unidentified compounds	.07 mM	3.4

Table 19.

Comparison of Results for the Determination of Calcium in Hospital Serum Samples

Sample	Corrected Relative Intensity	Experimental Values (in mg/dl) I.S.U.	M.G.H.
STD 2.0	7.0	2.6	---
STD 5.0	17.2	6.4	---
STD 10.0	35.1	10.4	---
Serum 1	33.5	10.1	9.3
Serum 2	32.9	9.9	9.1
Serum 3	36.8	11.1	10.5
Serum 4	33.7	10.2	10.0
Serum 5	34.0	10.3	10.2
Serum 6	32.0	9.7	9.4

E. Conclusions

In general, the highly pure Calcein was found to improve existing analytical techniques and also permitted the development of an autotmated analytical technique that provided highly reproducible measurements of calcium in biological samples that have historically been difficult to analyze on a routine basis.

The improved purity, particularly the reduction of contamination of Calcein with fluorescein and metals sharpened the visual end-point in both forward and reverse chelatometric titrations of calcium across a wide range of concentrations.

The improved purity and reliable composition greatly facilitated the use of Calcein as a direct fluorometric reagent. In some cases, the highly pure and consistent composition eliminated the need for protracted standardization of the reagent and extended the use of a single calibration curve over several subsequent experiments.

Most importantly, a straight-forward, simple,

automated method was developed to routinely determine calcium in widely differing types of materials including difficult biological samples. The final method proved to be highly precise and the data was reproducible between operators and days.

The success in the hands of others [244] in using these preparations of Calcein for precise measurements of cell volumes and membrane functions conferred confidence that the improvements are real and universal.

These results stand proof that the improvements in the understanding of the chemical and physical properties of Calcein, in the synthesis of a pure and consistent Calcein, and in the development of analytical schemes to evaluate the purity of the reagent, were worth the continued effort required to complete this study.

VIII. SUMMARY

Calcein was first prepared in 1956 by Diehl and Ellingboe[113,127]. The first preparations were crude mixtures containing relatively large amounts of organic and inorganic impurities which affected the fluorescence of solutions. Nevertheless, the change in the intensity of fluorescence of Calcein in solutions of calcium upon the addition of equivalent amounts of EDTA was sufficient to function as an indication of the end-point in the direct chelatometric titration of calcium without prior separation from magnesium.

The obvious utility of Calcein for indicating the presence of calcium and other metals in many different materials produced an immediate and continued interest in Calcein in the fields of analytical chemistry, biochemistry, biology, dentistry, and medicine. Procedures have been proposed for more than twenty analytes in hundreds of matrices. Many techniques have been proposed that use Calcein for direct and indirect titrations, direct fluorometric determinations,

spectrophotometric analyses, and biological staining. A large percentage of these methods have advanced from simple titrations to complex automated simultaneous determinations. As the procedures have become increasingly sophisticated and demanding, the demands for purity, reliability, and reproducible behavior in the reagent have also increased.

In response to this need, work has continued at Iowa State University to develop procedures for the preparation of a pure and reproducible material; to properly identify the structure and chemistry of the reagent; and to establish the acid-base, spectrophotometric, and fluorometric properties that are the foundation for all of these applications.

This dissertation describes the most recent accomplishments in this endeavor. Concurrent and interdependent improvements have been made in the methods of preparation, storage, and handling; in the techniques for evaluating the purity of the material prepared; and in the establishment of the physical properties of the purified material. With these in hand, the improved material has been successfully

applied to a number of uses. In each case, the superior reagent has been responsible for improving the facility, accuracy, or precision of the method.

The fluorometric properties of fluorescein (the parent compound and a persistent impurity of Calcein) have been rigorously established. In disagreement with all published accounts, not just one but three of the four prototropic forms of fluorescein have been found to fluoresce in aqueous solutions. The specific wavelengths and relative intensities of excitation and emission have been determined for (1) the protonated molecule (or cation) which exists at pH less than 4.5, (2) the monoanion which is present in solutions between pH 2.5 and pH 8.5, and (3) the dianion present above pH 4.5. Only the neutral molecule has been found to lack fluorescence.

All four of these prototropic forms have been correlated with the spectrophotometric properties of solutions of fluorescein and the acid dissociation constants of Hefley[166] and Markuszewski[247] have been confirmed.

The relatively constant wavelength and intensity of fluorescence of alkaline solutions of fluorescein has been demonstrated. The intensity of fluorescence has been shown to be highly sensitive to the concentration of fluorescein and relatively insensitive to small changes in pH or excitation wavelength. As a result, these conditions have been successfully utilzed to establish accurate methods for analyzing mixtures of fluorescein and Calcein.

The availability of a highly pure form of Calcein has permitted a more accurate determination of the fluorometric and spectrophotometric properties of Calcein. Misinterpretations of the fluorescent properties of the eight prototropic forms of Calcein have been corrected. Specific wavelengths of excitation and emission as well as the relative intensities of fluorescence have been established for the cations of Calcein and the anions consecutively formed by the first four acid dissociations of the neutral molecule. The neutral molecule and the anions formed by the fifth and sixth acid dissociations have been shown to be non-fluorescent.

The relative intensities of fluorescence produced by the cations of Calcein have been measured to be relatively equivalent to the intensity produced by the cation of fluorescein. The first and second anions formed from the acid dissociations of Calcein have been found to produce a moderate fluorescence analogous to that of the monoanion of fluorescein. The third and fourth anions from the acid dissociations of Calcein have been found to produce an intense fluorescence analogous to that of the dianion of fluorescein.

The fluorescence incorrectly assigned to the fifth anion of Calcein in previous work[166] has been dispelled. The mistake has been traced to the formation of a slightly-dissociated compound of Calcein and aluminum in the earlier work. New values for the fifth and sixth dissociation constants of Calcein have been determined from the accurate measurement of fluorescence as a function of pH.

The fluorescence of the cations of Calcein has also been utilized to establish an effective dissociation constant which represents the net dissociation of all protonated forms of Calcein to the

neutral molecule.

The fluorescent properties of the slightly-dissociated compounds of Calcein with several metals have also been established. The use of highly purified samples of Calcein has permitted the accurate identification of the specific fluorometric properties of three classes of slightly-dissociated compounds. In previous works, these properties have been obscured by the presence of impurities and competing reactants. The combining ratios, approximate equilibrium constants, excitation and emission wavelengths, relative intensities, and fluorescence as a function of pH have been determined for the alkali metals, alkaline earths, aluminum, and several transistion metals. The wavelengths of excitation and emission of the fluorescence of Calcein have been found to remain essentially unchanged by any of the metals. The association of Calcein with different metals has been found to affect the fluoresence by changing the relative acidity of the acidic hydrogens and by adding or removing kinetic mechanisms for the deactivation of excited molecules.

Calcein has been shown to form both 1:1 and 1:2 compounds with the alkaline earths. The fluorescence as a function of the relative concentration of alkaline earths has been demonstrated to consist of three linear portions: the first portion passed through the origin and corresponded to the formation of the 1:1 compound; the second portion, with a significantly greater slope, corresponded to the formation of the 2:1 compound of alkaline earth and Calcein; and the third portion did not change in intensity in solutions containing greater than a 3:1 ratio of alkaline earth to Calcein. Contradictory claims by other workers have been shown to have resulted from the presence of impurities or failure to meet minimum requirements of the absolute concentrations of Calcein and metal required to ensure formation of the slightly-dissociated compounds. Conditions of pH and the relative and absolute concentrations have been established for the accurate fluorometric determination of Calcein in simple solutions.

The increase in fluorescence of acidic solutions of Calcein in the presence of aluminum has been

determined to result from the formation of a complicated compound with the approximate stoichiometry of 1.5 moles of aluminum to 1 mole of Calcein. The presence of aluminum in solutions between pH 10 and pH 12 has also been found to increase the relative fluorescence of solutions of Calcein by the formation of a different slightly-dissociated compound. This fluorescence has been successfully eliminated by the addition of triethanolamine.

The interaction between many of the transition metals and Calcein has been found to occur by the formation of loosely associated 1:1 compounds. The formation of these compounds has been found to reduce proportionally the fluorescence of Calcein in solutions between pH 4 and pH 7, primarily affecting the fluorescence excited by the least energetic excitation band at 485 nm. However, significant fluorescence remained in all solutions of Calcein greater than 1×10^{-5} M despite several-fold excesses of metals.

The difficult task of analyzing solutions containing fluorescein, Calcein, and especially mixtures of the two compounds has been addressed.

Methods have been developed and limitations have been established for the spectrophotometric and fluorometric determinations of fluorescein and Calcein. These methods have been successfully employed to determine the amounts of each of these compounds present at each step in the preparation and application of Calcein.

The visible absorption spectra of each compound have been studied as functions of pH. The individual bands and molar extinction coefficients have been determined for each of the prototropic forms of fluorescein and Calcein. The intense fluorescence of the absorption bands at 475 and 485 nm. have been utilized to analyze independent solutions of the two compounds. The limitations in concentration have been established for linear calibration and the effect of common impurities have been measured.

The fluorescence of alkaline solutions of fluorescein and alkaline solutions containing Calcein and excess calcium (greater than 2:1 ratio to Calcein) have been used similarly to determine each compound in independent solutions. The limitations of absolute and relative concentrations of all species have been

defined and the effects of prevalent impurities have been determined.

A well-defined, specific method has been established for the fluorometric determinations of Calcein and fluorescein in mixtures of the two compounds. The two-step procedure measures the relative fluorescence of highly alkaline solutions containing fluorescein and Calcein in the presence of a competing chelating agent and in the presence of excess alkaline earth. Within the limits established, the method is accurate to within two or three percent, reproducible to within two percent, and unaffected by the presence of other materials commonly encountered during the preparation of Calcein. This procedure has been used exhaustively in studying the synthesis and preparation of Calcein which, in turn, produced the highly pure material needed for the careful identification of these properties.

The conditions under which the Mannich condensation of fluorescein, formaldehyde, and iminodiacetic acid was performed have been examined to optimize the yield and purity of Calcein. It has been

shown that performing the reaction in a mixture of ethanol and water improves both the yield of the reaction and the purity of the product separated by precipitation. The optimal conditions for the solvent have been established to be 50 percent ethanol and an apparent pH of 9.0. Under these conditions, the yield before separation has been measured to exceed 90 percent in less than five hours at 65 C.

The reaction rate of the Mannich condensation has been examined in several solvents and mixtures of solvents. The net yield has been determined to be the highest in approximately 1:1 mixtures of ethanol and water. The time required to reach the maximum yield has been demonstrated to be relatively independent of the solvent for acetic acid, ethanol, water, and ethanol-water solutions at any particular pH.

The effect of the purity of the reactants on the subsequent purity of the product has been examined. In particular, the contamination of fluorescein or iminodiacetic acid with metals has been shown to result in a comparable contamination of the Calcein. This has confirmed earlier observations[166,299] that Calcein acts

as a strong scavenger, retaining any of the metallic impurities present during the preparation in the final product.

The solubilities of fluorescein and Calcein have been studied as functions of solvent and apparent pH. Both fluorescein and Calcein were found to be increasingly soluble in mixtures of water and ethanol as the fraction of ethanol increased. However, at the point of minimum solubility (the point corresponding to the maximum fraction of species existing as the neutral molecules) the solubility of fluorescein has been measured to increase from 3 x 10-5 M in water to 2 x 10-2 M in 75 percent ethanol while the solubility of Calcein only increased from 1 x 10-4 M to 7 x 10-4 M. Furthermore, the precipitation of Calcein from a 75 percent solution of ethanol has been shown experimentally to reduce the amount of fluorescein coprecipitated by more than 80 percent. In addition, the point of minimum solubility of Calcein as a function of the apparent pH has been determined to vary only slightly with apparent pH while the point of minimum solubility of fluorescein changed from near 3.2 in water to 5.0 in 50 percent ethanol. The two

effects, increased differences in solubility and exaggerated differences in the isoelectric points, have been exploited to reduce the amount of fluorescein coprecipitated with Calcein.

The purity of products precipitated from various solutions has been determined. The product separated by precipitation from an ethanol-water solution was consistently three to four percent purer by measurement of equivalent weight than that precipitated from water with the addition of large amounts of salt.

The purity has also been monitored as a function of the direction of precipitation. That is, the purity of Calcein has been determined for products precipitated by adding acid to alkaline solutions of Calcein and by adding base to acidic solutions of Calcein. It has been demonstrated that by precipitating from the acidic side of the minimum in solubility, both the amount of fluorescein and metals precipitated with Calcein were lowered significantly from that obtained by precipitating from the alkaline side of the minimum. However, a significant amount of chloride was retained, presumably as the HCl adduct of

Calcein, when the product was precipitated form the acidic side of the minimum. It has therefore been concluded that the combination of reprecipitation, first from the acidic side to minimize the fluorescein and metals, and then from the basic side to minimize the chloride, was an efficient method of separation. This procedure has reproducibly provided materials with purities of greater than 98 percent (calculated as the monohydrate).

The thermal stability of Calcein has been examined. Calcein has been found to decompose in the presence of air by decarboxylation at approximately the same temperature that the last of the adsorbed water was removed. When warmed in a vacuum or under nitrogen, Calcein has also been found to be stable to 100 C with the loss of loosely associated solvent molecules. Above 100 C, water of hydration (approximately one mole of water per mole of Calcein) was found to be lost. In addition, physical and chemical changes were observed in the material. As a result of this work, a procedure for vacuum-drying Calcein to the monohydrate has been established.

Further improvements in the purity of preparations of Calcein have been demonstrated using Soxhlet extractions with acetone and ethanol. These extractions have been found to increase the purity of the product by two to five percent within a few extractions.

An overall procedure for the preparation of Calcein has been proposed and tested. The procedure has consistently produced Calcein monohydrate at purities above 97 percent in yields of 25 to 30 percent.

The stability of the solid and dissolved material has also been examined. The solid has been found to be highly hygroscopic and recommendations have been made to store the material in desiccators charged with strong desiccants with relatively large surface areas. Dissolved Calcein has been found to deteriorate faster in highly acidic or alkaline solutions, especially when exposed to sun or fluorescent light. Recommendations have been made to prepare Calcein in solutions with pH between 6 and 8 in opaque or foil-covered containers. Under these conditions, solutions were found to be

relatively stable for up to 15 days.

The highly pure material prepared by the procedures developed in this work has been successfully tested in several of the most common applications. Improvements have been noted in the end-points in the direct and indirect titration of ions. A significant increase has been found in the stability of reagent, facility of method, and reproducibility of results in the automated determination of calcium in geological and biological materials. In addition, it has been shown that the problem with background fluorescence due to the presence of alkali metals, especialy sodium, in biological samples may be reduced by performing the analyses at slightly raised temperatures. Furthermore, samples supplied to other workers have been shown to be significantly purer than commercially available supplies - sufficiently pure to leave only a single spot on thin-layer chromatographic separations[291] and adequate for complex applications in biological[199,200,244] and medical analyses[298].

To summarize, procedures have been developed to prepare Calcein of the highest purity; to store, handle

and apply the reagent without compromising this purity; and to quantitatively evaluate the material thoughout these steps. With a highly purified product, the solubility was accurately established and the acid-base, spectrophotometric, and fluorometric properties were meticulously defined. The misinformation prevalent in the literature has been explained.

With the conclusion of this work, the long and arduous effort at Iowa State University to firmly establish the nature and properties of this useful reagent has reached a culmination. And yet, work has already started on a new chapter - understanding the nature of the interactions of Calcein with various metals. The number and scope of future applications for a pure and consistent material will, no doubt, continue to grow.

IX. BIBLIOGRAPHY

1. Adamski, J. M.; Villard, S. P. Analytical Chemistry 1975, 47(7), 1191.

2. Akimova, T. G.; Syanava, E. M.; Savvin, S. B. Zh. Anal. Khim. 1982, 6, 999.

3. Akiyama, T.; Okumura, T. Kyoto Yakka Daigaku Gakuho 1967, 13, 43.

4. Akiyama, T.; Fujiwara, M. Kyoto Yakka Daigaku Gakuho 1958, 6, 51.

5. Alexander, R. L. Jr. Clinical Chemistry 1971, 17, 1171.

6. Allen, T. M.; Cleland, L. G. Biochim. Biophys. Acta 1980, 597(2), 418.

7. Amin, A. M. Chemist-Analyst 1957, 46, 31.

8. Andersch, M. A. J. Lab. Clin. Med. 1957, 49, 486.

9. Anderson D. "Autoanalyzer Determination of Serum Calcium Using the Fluorometric Reagent Calcein"; unpublished work, Iowa State University, Ames, Iowa, 1980.

10. Anrold, G. Z. Naturforsch. B 1969, 24(4) 410.

11. Appleton, H. D.; West, M.; Mandel, M.; Sala, A. M. Clin. Chem. 1959, 5, 36.

12. Armbrecht, H. J.; Gross, C. J.; Zenser, T. V. Archives of Biochemistry and Biophysics 1981, 210, 179.

13. Armbrecht, H. J.; Wongsurawat, N.; Zenser, T. V.; Davis, B. B. Endochrinology 1982, 111, 1339.

14. Armbrecht, H. J.; Zenser; T. V.; Davis, B. B. Endocrinology 1980, 106, 469.

15. Armbrecht, H. J.; Zenser, T. V.; Davis, B. B. J. Clinical Investigation 1980, 66, 1118.

16. Armbrecht, H. J.; Zenser, T. V.; Davis, B. B. Endocrinology 1981, 110, 1983.

17. Armbrecht, H. J.; Zenser, T. V.; Davis, B. B. Endocrinology 1981, 109, 218.

18. Armbrecht, H. J.; Zenser, T. V.; Gross, C. J.; Davis, B. B. Am. J. Physiology 1980, 239, 322.

19. Ashby, R. O.; Roberts, M. J. Lab. Clin. Med. 1957, 49, 958.

20. Austin, D. J.; Warman, T. M. Pesticide Science 1982, 13, 295.

21. Avetisyan, M. A.; Adamov, V. S.; Kantardzhyan, L. T.; Chirkinyan, S. S. Bull. Acad. Sci. USSR, Phys. Ser. 1963, 27, 788.

22. Ayranci, B. Schweiz. Mineral. Petrogr. Mitt. 1975, 55(1), 126.

23. Baeyer, A. Chem. Ber. 1871, 4, 555.

24. Baeyer, A. Annalen 1876, 183, 1.

25. Baeyer, A. Annalen 1882, 212, 347.

26. Balaban, A. T.; Mateescu, G. D.; Elian, M. Tetrahedron 1962, 18, 1083.

27. Balaban, A. T.; Schroth W.; Fischer, G. "Pyrylium Salts" Advances in Heterocyclic Chemistry; Academic Press: New York, N.Y., 1969.

28. Bandrowski, J. F.; Benson, C. L. Clinical Chemistry 1972, 18, 1411.

29. Banewitz, J. J.; Kenner, C. T. Anal. Chem. 1952, 24, 1186.

30. Bar, A.; Cohen, A.; Eisener, U.; Risenfeld, G.; Hurwitz, S. J. Nutrition 1978, 108, 1322.

31. Bar, A.; Dubrov, D.; Eizner, U.; Hurwitz, S. J. Nutrition 1978, 108, 1501.

32. Barnes, L. A.; Morrow, G.; Silverio, J.; Finnegan, L. P.; Heitman, S. E. Pediatrics 1974, 54(2), 217.

33. Barsotti, G.; Morelli, E.; Guiducci, A.; Giannoni, A.; Ciardella, F.; Ni, F.; Lupetti, S.; Giovannetti, S. Proc. European Dialysis and Transplant Assoc. 1982, 19, 773.

34. Barsotti, G.; Morelli, E.; Giannoni, A.; Guiducci, A.; Ciardella, F.; Lupetti, S.; Giovannetti, S. Nephron 1982, 30, 310.

35. Baron, D. N.; Bell, J. L. Clin. Chem. Acta 1957, 2, 327.

36. Baron, D. N.; Bell, J. L. J. Clin. Pathol. 1959, 12, 143.

37. Bermejo, F.; Margalet, A. Inform. Quim. Anal. 1965, 19(6), 171.

38. Bermejo-Martinez, F.; Margalet-Barral, A. Inform. Quim. Anal. 1965, 19(4), 117.

39. Bermejo-Martinez, F.; Margalet-Barral, A. Inform. Quim. Anal. 1964, 18(2), 35.

40. Bermejo-Martinez, F.; Margalet-Barral, A. Chem Analyst 1964, 53, 45.

41. Bermejo-Martinez, F.; Margalet-Barral, A. Inform. Quim. anal. 1965, 19, 39.

42. Bermejo-Martinez, F.; Margalet-Barral, A.; Badrinas-Vallcells, A.; Prieto, A. Trabajos 1964, 6(2), 92.

43. Bermejo-Martinez, F.; Margalet-Barral, A.; Badrinas-Vallcells, A.; Prieto, A. Inform. Quim. Anal. 1960, 14, 151.

44. Bermejo-Martinez, F.; De Lopidana, G. G. Anal. Chim. Acta 1969, 47, 139.

45. Bermejo-Martinez, F.; De Lopidana, G. G.; Barrera-Romallo, A. Inform. Quim. Anal. 1969, 23(4), 109.

46. Bermejo-Martinez, F.; De Lopidana, G. G.; Barrera-Romallo, A. Inform. Quim. Anal. 1969, 23(8), 444.

47. Bermejo-Martinez, F.; De Lopidana, G. G.; Barrera-Romallo, A. Inform. Quim. Anal. 1970, 25(2), 17

48. Bett, I. M.; Fraser, G. P. Biochem. J. 1958, 68, 13.

49. Bett, I. M.; Fraser, G. P. Clin. Chem. Acta 1959, 4, 346.

50. Betz, J. D.; Noll, C. A. J. Am. Water Works Assoc. 1950, 42, 49.

51. Bhat, T. R.; Das, R. R.; Shankar, J. Indian J. Chem. 1964, 5, 324.

52. Bhavnagary, H. M.; Venugopal, J. S.; Majumder, S. K. Journal of Applied Chemistry and Biotechnology 1977, 27(8), 393.

53. Bidleman, T. F.; Nowlan, B.; Frei, R. W. Anal. Chim. Acta 1972, 60, 13.

54. Biedermann, W.; Schwarzenbach, G. Chimia 1948, 2, 56.

55. Bingley, J. B.; Ruksan, B. E.; Caprillo, B. J. Research in Veterinary Science 1976, 21, 121.

56. Birze, I. Ph.D. Dissertation, Iowa State University, Ames, Iowa, 1970.

57. Blinks, J. R.; Wier, W. G.; Hess, P.; Prendergast, F. G. Progress in Biophysics & Molecular Biology 1982, 40, 1.

58. Bohoun, C. Ann. Biol. Clin. 1958, 16, 73.

59. Bohoun, C.; Festy, B. Pathol. Biol. Semaine Hop. 1959, 7, 191.

60. Bond, R. D.; Tucker, B. M. Chemistry and Industry 1954, 1954, 1236.

61. Borle, A. B.; Briggs, F. N. Anal. Chem. 1968, 40, 339.

62. Boskey, A. L.; Posner, A. S.; Lane, J. M.; Goldberg, M. R.; Cordell, D. M. Archives of Biochemistry and Biophysics 1980, 199, 305.

63. Bower, V. E.; Bates, R. G. Journal of Research of the National Bureau of Standards 1955, 55, 197.

64. Bozhevol'nov, E. A.; Kreingol'd, S. U. J. Anal. Chem. USSR, 1962, 17, 294.

65. Bozhevol'nov, E. A.; Kreingol'd, S. U. J. Anal. Chem. USSR, 1962, 17, 558.

66. Bozhevol'nov, E. A.; Kreingol'd, S. U. Khim. Reaktivov 1963, 25, 24.

67. Bozhevol'nov, E. A.; Kreingol'd, S. U. Khim. Reaktivov 1963, 25, 358.

68. Bozhevol'nov, E. A.; Serabryakova, T. B.; Yanishevekaya, B. M.; Kreingol'd, S. U. Acta Chim. Sci. Hung.1962, 32, 199.

69. Bozhevol'nov, E. A.; Serebrykova, G. V.; Yanishevekaya, U. M.; Kreingol'd, S. U. Acta Chim. Acad. Sci. Hung.1962, 32, 199.

70. Brayton, S. "The Solubility of Calcein at Low pH"; Unpublished work, Department of Chemistry, Iowa State University, Ames, Iowa, 1978.

71. Brazzuna, H.; Pierce, H. G.; Libermann, I. M. Acta Physiologica Latinoamericana 1975, 25(2), 83.

72. Brown, J. L. Journal of Environmental Quality 1980, 9(4), 641.

73. Bruns, M. E. H.; Bruns, D. E.; Avioli, L. V. Endocrinology 1979, 105, 934.

74. Bruns, M. E. H.; Fausto, A.; Avioli, L. V. J. Biol. Chem. 1978, 253, 3186.

75. Buganov, Kh. G. Zavod. Lab. Yugo-Vostoka USSR 1972, 4(1), 79.

76. M. J. Bunk, S. L. Balloun and H. Diehl. Pathol. Biol. Semaine Hop. 1959, 7, 191.

77. Burr, R. G. Invest. Urol. 1977, 15(2), 180.

78. Busev, A. I.; Pukhova, V. M.; Kruchkova, E. S. Farmatsiya 1976, 25(3), 43.

79. Buss, J. E.; Stull, J. T. FEBS Letters 1977, 73, 101.

80. Campbell, D. N.; Kenner, C. T. Anal. Chem. 1954, 26, 560.

81. Canon, K. K. Japan Patent 8091109, 1981.

82. Cartier, P.; Clement-Metral, J. Clin. Chim. Acta 1959, 4, 357.

83. Carubelli, R.; Smith, W. O. Clin. Chem. 1959, 5, 45.

84. Chan, J. C. M. Nutrition and Metabolism 1974, 16, 140.

85. Chaplin, A. J.; Grace, S. R. Histochemistry 1976, 47(3), 263.

86. Chapuy, M. C.; Meunier, P. J.; Alexander, C. M. Journal of Clinical Investigation 1980, 65(5), 1243.

87. Charhon, S. A.; Chapuy, M. C.; Delvin, E. E.; Valentinopran, A.; Edouard, C. M.; Meunier, P. J. Cancer 1983, 51, 918.

88. Chiu, V. C. K.; Haynes, D. H. Biophysical Journal 1977, 18(1), 3.

89. Choe, J. Y.; Peng, T. C. Journal of Pharmacology and Experimental Therapeutics 1974, 189, 593.

90. Clark, E. P.; Collip, J. P. J. Biol. Chem. 1925, 63, 461.

91. Collier, R. E. Chemist-Analyst 1954, 43, 41.

92. Coolidge, T. B. Anal. Biochem. 1960, 1, 93.

93. Correa, I.; Lopez-Martenez, M. C.; Garcia-Villanova, R. An. Bromatol. 1977, 29(3), 361.

94. Correa-Gonzalez, I.; Lopez-Martinez, M. C. Cienc. Ind. Farm. 1976, 8(11), 333.

95. Correa-Gonzalez, I.; Lopez-Martinez, M. C. Cienc. Ind. Farm. 1976, 8(11), 336.

96. Correa-Gonzalez, I.; Lopez-Martinez, M. C.; Garcia-Villanova, R. Cienc. Ind. Farm. 1977, 9(1), 8.

97. Danowski, T. S.; Stephan, T.; Nolan, S.; Ahmad, U.; Wingert, J. P.; Bowman, D. H.; Sunder, J. H.; Fisher, E. R. Clincal Pharmacology and Therapeutics 1976, 19, 196.

98. Davies, M.; Jones, R. L. J. Am. Chem. Soc. 1954, 54, 120.

99. Debaare, L.; Lewis, J.; Sing, H. Clinical Chemistry 1975, 21(6), 746.

100. Delmas, P.; Chapuy, M. C.; Vignon, E.; Briancon, D.; Charhon, S.; Meunier, P. J. Nouvelle Presse Medicale 1982, 11, 1471.

101. Delmas, P.; Chapuy, M. C.; Vignon, E.; Charhon, S.; Briancon, D.; Alexandre, C.; Edouard, C.; Meunier, P. J. J. Clincical Endocrinology and Metabolism 1982, 54, 837.

102. Demertzis, M. A.; Karagiannis, M. I. Chem. Chron. 1981, 6, 36.

103. De Witt, F.; Parsons, R. J. Am. J. Clin. Path. 1970, 53, 324.

104. Diehl, H. "Calcein, Calmagite, and o,o'-Dihydroxyazobenzene. Titrimetric, Colorimetric, and Fluorometric Reagents for Calcium and Magnesium"; G. Frederick Smith Chemical Company: Columbus, Ohio, 1964.

105. Diehl, H. Anal. Chem. 1967, 39, 30A.

106. Diehl, H. "Quantitative Analysis -- Elementary Principles and Practice"; Oakland Street Science Press: Ames, Iowa, 1970.

107. Diehl, H. Talanta, in press.

108. Diehl, H. Talanta, in press.

109. Diehl, H. Talanta, in press.

110. Diehl, H. Talanta, in press.

111. Diehl, H. Talanta, in press.

113. Diehl, H.; Ellingboe, J. L. Anal. Chem. 1956, 28, 882.

114. Diehl, H.; Goetz, C. A.; Hach, C. C. J. Am. Water Works Assoc. 1950, 42, 40.

115. Diehl, H. and Markusewski, R. Talanta, in press.

116. Diehl, H; Miller, R. C. "Determination of Calcium and Magnesium in Limestone"; otherwise unpublished work in reference 104.

117. Diehl, H; Oulmann, K. "Fluorescence of Calcein in Solutions of Salts of Various Cations"; otherwise unpublished work in reference 104.

118. Diehl, H; Oulmann, K. "Residual Fluorescence of Fluorescein in Calcein"; otherwise unpublished work in reference 104.

119. Diller, W. F. Klin. Wochschr. 1961, 39, 1247.

120. Djurkin, V. Glas. Hem. Tehnol. Bosne Hercegovine 1972, 1971-2, 19.

121. Dolinsky, M.; Jones, J. H. J. Assoc. Off. Agr. Chem. 1951, 34, 114.

122. Dumont, P. Anal. Chem. 1961, 33(4), 565.

123. Dupuis, T.; Dupuis, J. Mikrochim. Acta 1958, 1958, 186.

124. Effenberger, M. Chem. Listy 1958, 52, 1501; Coll. Czech. Chem. Commun. 1959, 24, 682.

125. Eggers, J. H. Talanta 1960, 4, 38.

126. Elkins, W.; Hunter, L. J. Chem. Soc. 1935, 1935, 1598.

127. Ellingboe, J. L. Ph.D. Dissertation, Iowa State University, Ames, Iowa, 1956.

128. Elliot, W. E. J. Biol. Chem. 1952, 197, 641.

129. Elving, P. J.; Markowitz, J. M.; Rosenthal, I. Anal. Chem. 1956, 28, 1179.

130. Faas, F. H.; Wadkins, C. L.; Daniels, J. S.; Davis, G. R.; Carter, W. J.; Wynn, J. O. Clinical Orthopaedics and Related Research 1974, 1974, 216.

131. Farnswor, A. E.; Dobyns, B. M. Med. J. Australia 1974, 2, 782.

132. Ferencova, L. Stavivo 1969, 47(5), 156.

133. Fingerhut, B.; Miller, H. Clin. Chem. 1963, 9(3), 360.

134. Fingerhut, B.; Poock, A. Clinical Chem. 1967, 13, 1090.

135. Finn, F. M.; Montibeller, J. A.; Ushijima, Y.; Hofmann, K. Journal of Biological Chemistry 1975, 250(4), 1186.

136. Fischer, E. Berichte 1874, 7, 1211.

137. Fisher Scientific Company, Philadelphia, Pa., 1982

138. Frei, R. W.; Maclellan, B. L.; MacNeil, J. D. Anal. Chim. Acta 1972, 66, 139.

139. Freytag, L. A. M.S. Thesis, Iowa State University, Ames, Iowa, 1971.

140. Frohlich, K. J. B. F.; Siemroth, J. Math.-Nat. Reihe 1973, 22, 49.

141. G. F. Smith Chemical Company, Columbus, Oh., 1982

142. Garlich, J. D.; Bryant, D. M. Poultry Science 1975, 54, 388.

143. Gehrer, C. W. J. Assoc. Offic. Agl. Chemists 1962, 45(1).

144. Gehrke, C. W.; Affsprung, H. E.; Lee, Y. C. Anal. Chem. 1954, 26, 1944.

145. Gel'fand, E. D.; Sinitskaya, V. A. Gidroliz. Lesokhim. Prom-st. 1981, 7, 12.

146. Genge, J. A. R. Lab Practice 1960, 9(5), 301.

147. Gharfeh, S. G. M.S. Thesis, Iowa State University, Ames, Iowa, 1977.

148. Gharfeh, S. G. Ph.D. Dissertation, Iowa State Universtiy, Ames, Iowa, 1978.

149. Ghijsen, W. E. J. M.; Vanos, C. H. Biochim. Biophys. Acta 1982, 689, 170.

150. Gibitz, H. Wiener Z. Inn. Med. 1960, 41, 216.

151. Gibitz, H. Aerzil. Lab. 1963 9(12), 389.

152. Goodger, B. V.; Wright, I. G. Zeitschrift fur Parasitenkunde 1977, 54(1), 9.

153. Gorbenko, F. P.; Lapitskaya, E. V. Sov. Prog. Chem. 1967, 33, 67.

154. Grabau, F., Ames Lab, personal communication, 1979.

155. Geenleaf, J. E.; Bernauer, E. M.; Juhos, L. T.; Young, H. L.; Morse, J. T.; Stanley, R. W. J. Applied Physiology 1977, 43, 126.

156. Greenleaf, J. E.; Bernauer, E. M.; Young, H. L.; Morse, J. T.; Stanley, R. W.; Juhos, L. T.; Anbeaumont, W. J. Applied Physiology 1977, 42, 59.

157. Gregorowicz, Z.; Olejniczenko, M. Politech. Slask., Chem. 1963, 85(16), 67.

158. Grigoryan, E. V.; Kantardzhyan, L. T.; Chirkinyan, S. S. Bull. Acad. Sci. USSR, Phys. Ser. 1960, 24, 774.

159. "Hach Chemical Company Catalog"; Hach Chemical Company, Loveland, Co., 1982.

160. Hanousek, V. Coll. Czech. Chem. Commun. 1959, 24, 1061.

161. Hanousek, V. Chem. Listy 1958, 52, 707.

162. Harris, S. S.; Navia, J. M. Journal of Nutrition 1977, 107, 2198.

163. Harvey, A. E., Komarmy, J. M.; Wyatt, G. M. Anal. Chem. 1953, 25, 498.

164. Headridge, J. B.; Magee, R. J. Talanta 1958, 1, 117.

165. Hefley, A. J. M.S. Thesis, Iowa State University, Ames, Iowa, 1965.

166. Hefley, A. J. Ph.D. Dissertation, Iowa State University, Ames, Iowa, 1967.

167. Hefley, A. J.; Jaselskis, B. Anal. Chem. 1974, 46, 2036.

168. Hefley, A. J., Miller, R. C.; Diehl, H. in reference 104.

169. Hegemann, F.; Thomann, H. Ber. Deut. Keram. Ges. 1961, 38, 345.

170. Hermann, R. G. Proc. Soc. Exptl. Biol. Med. 1958, 99, 777.

171. Hibbs, L. E.; Wilkins, D. H. Talanta 1959, 2, 16.

172. Hicks, J. M.; Rowland, G. L.; Buffone, G. J. Clin. Chem. 1976, 22, 2034 .

173. Hildebrand, G. P.; Reilley, C. N. Anal. Chem. 1957, 29, 258.

174. Hill, J. B. Clinical Chemistry 1965, 11, 122.

175. Hitchen, A.; Zechandwitsch, G. Talanta 1980, 27(3), 269.

176. Hoffman, J. Casopis Lekaru Ceskych 1961, 100, 1171.

177. Holly, F. J.; Lamberts, D. W. Contact Intraocul. Lens Med. J. 1979, 5(4), 60.

178. Holmes, W. C. J. Am. Chem. Soc. 1924, 46, 2770.

179. Honig, C. R.; Reddy, Y. S. Am. J. Physiol. 1975, 228, 172.

180. Howerton, H. K.; Wasilewski, J. C. "A New Instrument for Automatic Colorimetric and Fluorometric Titrations"; Plenum Press: New York, N. Y., 1961.

181. Howerton, H. K.; Waslilewski, J. C. "Reprint No. 141, A New Instrument for Automatic Colorimetric and Fluorometric Titrations"; American Instrument Company, inc.: Silver Spring, Maryland, 1972.

182. Hoyle, W. C.; Diehl, H. Talanta 1972, 19, 206.

183. Hsu, W. H.; Cooper, C. W. Calcified Tissue Research 1975, 19, 125.

184. Huitink, G. M. M.S. Thesis, Iowa State University, Ames, Iowa, 1965.

185. Huitink, G. M. Ph.D. Dissertation, Iowa State University, Ames, Iowa, 1967.

186. Huitink, G. M. Analytica Chemica Acta 1974, 70(2), 311.

187. Huitink, G. M.; Diehl, H.. Talanta 1974, 21, 1193.

188. Huitink, G. M.; Poe, D. P.; Diehl, H. Talanta 1974, 21, 1221.

189. Inglis, A. S.; Nichols, P. W. J. Chromatogr. 1974, 97, 289.

190. Iritani, N.; Miyahara, T. Bunseki Kagaku 1973, 22(2), 174.

191. Iritani, N.; Miyahara, T.; Takahashi, I. Japan Analyst 1968, 17, 1075.

192. Judd, J. T.; Wexler, B. C. Atherosclerosis 1975, 22(2), 241.

193. Juergen, K.; Froehlich, B. F.; Siemroth, J. Wiss. Z. Martin-Luther Univ. 1973, 22(2), 49.

194. von Juergensonn, H. B. Histochemie 1971, 28(1), 23.

195. Kainz, G.; Meisinger, F. Mikrochimica Acta 1975, 1975, 385.

196. Kantardzhyan, L. T. Bull. Acad. Sci. USSR, Phys. Ser. 1959, 23, 131.

197. Kariyama, M.; Akai, M.; Nishijima, S. Arch. Oral Biol. 1969, 14, 1349.

198. Keattch, C. J. Talanta 1963, 10, 1303.

199. Kendall, D. A.; MacDonald, R. C. J. Biol. Chem. 1982, 23, 13892.

200. Kendall, D. A.; MacDonald, R. C. Anal. Biochem. 1983, 134, 26.

201. Kepner, B. L.; Hercules, D. M. Anal. Chem. 1963, 35, 1238.

202. Kesteloot, H.; GeBoers, J. Lancet 1982, 1, 813.

203. Killbrick, F. C.; Ross, M.; Rogers, H. E. Proc. Soc. Exp. Biol. and Med. 1952, 81, 351.

204. Kind, H. P.; Handysides, A.; Kooh, S. W.; Fraser, D. Journal of Pediatrics 1977, 91(6), 1006.

205. Kirkbright, G. F.; Stephen, W. I. Anal. Chim. Acta 1962, 27, 294.

206. Klein, B.; Kaufman, J. H.; Isaacs, J. Clinical Chemistry 1967, 13, 1071.

207. Knappe, E.; Boeckel, V. Physical Chem. 1958, 312, 186.

208. Kolthoff, I. M.; Sandell, E. B.; Meehan, E. J.; Bruckenstein, S. "Quantitative Chemical Analysis", 4th ed.; MacMillan Company: London, 1969.

209. Koerbl, J.; Pribil, R. Ind. Chem. 1958, 34, 616.

210. Koerbl, J. Industrial Chemist 1958, 34, 677.

211. Koerbl, J. Czech. 1959, 89, 173.

212. Koerbl, J. Czech. 1959, 91, 363.

213. Koerbl, J. Chem. Listy 1957, 51, 1304.

214. Koerbl, J. British Patent 858,019, 1961.

215. Koerbl, J. U.S. Patent 2,980,696, 1961.

216. Koerbl, J.; Kraus, E.; Jancik, F.; Pribil, R. Chem. Listy 1958, 52, 311.

217. Koerbl, J.; Pribil, R. Indusrial Chemist 1958, 34, 507.

218. Koerbl, J.; Pribil, R. Chem. Listy 1958, 52, 601.

219. Koerbl, J.; Pribil, R. Chem. Listy 1957, 51, 302.

220. Koerbl, J.; Pribil, R. Collection Czech Chem. Communs. 1957, 22, 1122.

221. Koerbl, J.; Pribil, R. Chem. and Ind. 1957, 1957(2), 233.

222. Koerbl, J.; Svoboda, V. Chem. and Ind. 1958, 1958, 1233.

223. Koerbl, J.; Svoboda, V. Talanta 1960, 3, 370.

224. Koerbl, J.; Svoboda, V.; Terzijska, D. Chem. and Ind. 1958, 1958, 1232.

225. Koerbl, J., Svoboda, V., D. Teraijsici,; Pribil, R. Chem. and Ind. 1957, 1957, 1624.

226. Koerbl, J.; Vydra, F. Chem. Listy 1957, 51, 1457.

227. Koerbl, J.; Vydra, F. Coll. Czech. Chem. Commun. 1958, 23, 622.

228. Koerbl, J.; Vydra. F.; Pribil, R. Talanta 1958, 1, 281.

229. Koerbl, J.; Vydra, F.; Pribil, R. Talanta 1958, 1, 138.

230. Koerbl, J.; Vydra, F.; Pribil, R. Talanta 1958, 1, 821.

231. Kumagai, T.; Matsui, M.; Shigematsu, T. Bull. Inst. Chem. Res. Kyoto Univ. 1980, 80, 58(2), 269.

232. Lewis, G. ICI America, Inc., Wilmington, DE, personal communication, 1982.

233. von Liebig, H. J. Prakt. Chem. 1912, 85, 241.

234. von Liebig, H. J. Prakt. Chem. 1912, 85, 97.

235. von Liebig, H. J. Prakt. Chem. 1913, 88(2), 26.

236. von Liebig, H. J. Prakt. Chem. 1911, 86, 472.

237. von Liebig, H. Ber. 1911, 46, 2736.

238. von Liebig, H. Ber. 1912, 46, 3593.

239. Lindqvist, L. Arkiv. Chem. 1960, 16, 79.

240. de Loose, R. Rev. Agric. 1961, 14, 677.

241. Lopidana, M. G. Acta Cient. Comp. Telara 1966, 3(4), 173.

242. Lurie, A.; Kursh, E. D.; Rose, D.; Persky, L. Investigative Urology 1976, 13, 307.

243. Mabry, C. C.; Wyles, P. Clinical Chemistry 1967, 37, 31.

244. MacDonald, R. C. Northwestern University, Evanston, IL, personal communication, 1984.

245. Malevannyi, V. A. Industrial Laboratory 1964, 30, 1807.

246. Mannich, C.; Krosche, W. Arch. Pharm. 1912, 250, 647.

247. Markuszewski, R. Ph.D. Dissertation, Iowa State University, Ames, Iowa, 1976.

248. Markuszewski, R., Ames Laboratory, Ames, Iowa, personal communication, 1978.

249. Markuszewski, R.; Diehl, H. Talanta 1980, 27(11B), 937.

250. Martensson, O. Acta Chem. Scand. 1970, 24(9), 3417.

251. Martensson, O.; Warren, C. H. Acta Chem. Scand. 1970, 24, 2745.

252. Martin, D. B. M.S. Thesis, Iowa State University, Ames, Iowa, 1974.

253. Martin, D. B. Ph.D. Dissertation, Iowa State University, Ames, Iowa, 1977.

254. Martin, D. B., Ames, IA, personal communication, 1977.

255. McGaughey, C.; Stowell, E. C. Journal of Dental Research 1977, 56(6) 579.

256. McGuire, J. L.; Marks, S. C. Clinical Orthopaedics and Related Research 1974, 1974(100), 392.

257. Messer, H. H. J. Dental Research 1977, 56, 971.

258. Messer, H. H.; Armstrong, W. D.; Singer, L. Calcified Tissue Research 1974, 15, 85.

259. Meunier, P. J.; Chapuy, M. C.; Alexandre, C.; Bressot, C.; Edouard, C.; Vignon, E.; Mathieu, L.; Trechael, U. Lancet 1979, 2(8141), 489.

260. Meyer, R. Berichte 1888, 21, 3376.

261. Meyer, R. Berichte 1891, 24, 141.

262. Meyer, R. Berichte 1891, 24, 2600.

263. Meyer, R. Berichte 1892, 25, 1385.

264. Meyer, R. Berichte 1895, 28, 428.

265. Mori, K. Arch. Biochem. Biophys. 1959, 83, 552.

266. Morris, A. G. C. Anal. Chem. 1961, 33, 599.

267. Nagase, Y.; Ohno, T.; Goto, T. J. Pharm. Soc. Japan 1953, 73, 1033.

268. Nakada, S.; Yamada, M.; Ito, T.; Fujimoto, M. Bulletin of the Chemical Society of Japan 1977, 50(7), 1887.

269. Nakada, S.; Yamada, M.; Ito, T.; Fujimoto, M. Bulletin of the Chemical Society of Japan 1979, 52(3), 766.

270. Nakada, S.; Yamada, M.; Ito, T.; Fujimoto, M. Bulletin of the Chemical Society of Japan 1980, 53(8), 2252.

271. Nakada, S.; Yamada, M.; Ito, T.; Fujimoto, M. Bulletin of the Chemical Society of Japan 1980, 53(11), 3365.

272. Nakashima, Y.; Appert, H. E.; Howard, J. M. Surgery Gynecology and Obstetrics 1977, 144, 71.

273. Natelson, S.; Peniall, R. Anal. Chem. 1955, 27, 434.

274. Neitchev, V. Z.; Vassileva-Popova, J. G.; Bideaud, F. A. Biol. Act. Nat. Prod. 1981, 3, 298.

275. Nemes-Varga, S.; Tolnay, V. Modszertani Kozl. 1972, 1, 89.

276. Newman, R. J. Calcified Tissue Research 1974, 16, 157.

277. Ntailianas, H. A.; Whitney, R. McL. J. Dairy Sci. 1964, 47, 19.

278. Ntailianas, H. A.; Whitney, R. Mcl. J. Dairy Sci. 1963, 46(12), 1335.

279. Oku, N.; Kendall, D. A.; MacDonald, R. C. Biochim. Biophys. Acta 1982, 2, 332.

280. Olerud, S.; Lorenzi, G. L. J. Bone Joint Surg. 1970, 52A, 274.

281. Olsen, R. L.; Diehl, H.; Collins, P. F.; Ellestad, R. B. Talanta 1961, 7, 187.

282. Ol'shanova, K. M.; Kopyiova, V. D.; Morozova, N. M. Izd. Akad. Nauk SSSR, 1963.

283. Orndorff, W. R.; Gibbs, R. C.; Shapiro, C. V. J. Am. Chem. Soc. 1928, 50, 819.

284. Orndorff, W. R.; Hemmer, A. J. J. Am. Chem. Soc. 1927, 49, 1272.

285. Pandva, E. P.; Chekrii, N. P.; Filatov, E. N. Altai. Politekh. Inst. Barnaul, USSR, Zavod. Lab. 1979, 45(9), 805.

286. Partashnikova, M. Z.; Shafran, I. G. Khim. Reaktivov 1961, 24, 154.

287. Partashnikova, M. Z.; Shafran, I. G. Khim. Reaktivov 1963, 25, 258.

288. Partashnikova, M. Z.; Shafran, I. G. Khim. Reaktivov 1963, 25, 274.

289. Patton, J.; Reeder, W. Anal. Chem. 1956, 28, 1026.

290. Pechan, Z. Unitrni Lekar 1960, 6, 727.

291. Perin, M., Northwestern University, Evanston, IL, personal communication, 1984.

292. Phillips, R. E. "Fluorometric Determination of Calcium in Serum"; G. K. Turner Associates: Palo Alto, California, 1969.

293. Peterson, J., Hach Chemical Company, Ames, Iowa, personal communication, 1979.

294. Polig, E.; Sontag, W. Comm. Eur. Communities 1981, 7168, 73.

295. Postoronko, A. I.; Gorbenko, F. P.; Gvozdeva, L. N. Vop. Khim. Tekhnol. 1973, 28, 71.

296. Postoronko, A. I.; Gorbenko, F. P.; Pirgo, E. F. Otkrytiya Izobret Prom. 1973, 50(16), 100.

297. Pribil, R.; Vydra, F. Chem. Communs. 1959, 24, 3103.

298. Prosternack, J., The Procter and Gamble Company, Cincinnati, Oh., personal communication, 1980.

299. Rahn, B. A. Zeiss Inf. 1977, 22(85), 36.

300. Raman, A.; Chong, Y. K. Clinical Biochemistry 1974, 7(2), 106.

301. Ramart-Lucas, P. Compt. Rend. 1937, 205, 864.

302. Ramart-Lucas, P. Compt. Rend. 1937, 205, 1409.

303. Rasmussen, P. Scand. J. Dent. Res. 1975, 83, 233.

304. Robinson, C.; Weatherell, J. A. Analyst 1968, 93, 722.

305. Rodgerson, D. O.; Moran, I. K. Clin. Chem. 1968, 14, 1206.

306. Roth, M. Clinica Chimica Acta 1978, 17(3), 487.

307. Roos, B. A.; Deftos, L. J. J. Lab. Clin. Med. 1976, 88, 173.

308. Russell, J. E.; Avioli, L. V. J. Lab. Clin. Med. 1974, 84, 317.

309. Russell, J. E.; Avioli, L. V. j. Clin. Invest. 1975, 56, 792.

310. Russell, J. E.; Kleerekoper, M.; Slatopolsky, E.; Lewis, J.; Lee, S. W.; Avioli, L. V. Calcified Tissue International J. 1979, 29, 147.

311. Ryan, D. E.; Holzbecher, J. Intern. J. Environ. Anal. Chem. 1971, 1, 159.

312. Saji, H.; Yokoyama, A.; Arano, Y.; Tanaki, H.; Odori, T.; Morita, R.; Torizuka, K. J. Labelled Compd. Radiopharm. 1979, 16(1), 188.

313. Sajo, I. Kohostati Lopok 1959, 14, 384.

314. Salam Khan, M. A.; Stephen, W. I. Analytica Chemica Acta 1970, 49, 255.

315. Sallis, J. D.; Lumley, M. F. Investigative Urology 1979, 16, 296.

316. Samuelson, T. J.; Holland, D. A. Journal of Science of Food and Agricuture 1983, 34, 198.

317. Santini, R.; Martinez de Jesus, J. Am. J. Clin. Pathol. 1959, 31, 181.

318. Schirardin, H.; Metais, P. Pathol. et Biol. Semaine Hop. 1959, 7, 418.

319. Schwarzenbach, G.; Ackermann, H. Helv. Chim. Acta 1947, 30, 1798.

320. Schwarzenbach, G.; Ackermann, H. Helv. Chim. Acta 1948, 31, 1029.

321. Schwarzenbach, G.; Anderegg, G.; R. Sallmann. Helv. Chim. Acta 1952, 35, 1785.

322. Schwarzenbach, G.; Biedermann, W.; Bangerter, F. Helv. Chim. Acta 1946, 29, 811.

323. Schwarzenbach, G.; Gysling, H. Helv. Chim. Acta 1949, 32, 1314.

324. Sedlacek, B. A. J.; Dusek, P. Z. Lebens. Untersuch. Forsch. 1966, 129, 333.

325. Sedlacek, B. A. J.; Dusek, P. Chim. Listy 1966, 60(7), 907.

326. Seitz, W. R. CRC Critical Reviews in Analytical Chemistry 1980, 8, 367.

327. Semenova, T. I.; Sukhomlin, R. I.; Sikorskaya, V. M. Kharchova Prom-st. 1975, 3, 43.

328. Sharma, C. B.; Dieckert, J. W. Physiologia Plantarum 1975, 33(1).

329. Shcherbov, D. D. Zavodskaya 1962, 28(10), 1158.

330. Shcherbov, D. D. Fluorescent Analysis of Minerals. Syr'ya 1961, 6, 167.

331. Siest, G.; Siest, D.; Roos, F. Pharm. Biol. 1966, 4(44), 411.

332. Skylar, Y. E.; Mikhailov, G. I. J. Org. Chem. USSR 1966, 2, 894.

333. Spector, A.; Adams, D.; Krul, K. Investigative Ophthamology 1974, 13, 982.

334. Socolar, S. J.; Salach, J. I. Anal. Chem. 1959, 31, 473.

335. Solheim, T. Scand. J. Dent. Res. 1974, 82, 19.

336. Sontag, W. Biol. Implic. Radionuclides Released Nucl. Ind., Proc. Int. Symp. 1979, 417.

337. Sowa, M.; Appert, H. E.; Howard, J. M. Surgery Gynecology and Obstetrics 1977, 144, 365.

338. Steyn, W. J. A. J. S. African Chem. Soc. 1956, 9, 39.

339. Sullivan, P. G. Arch. Oral Biol. 1972, 17, 1487.

340. Suzuki, H. K.; Mathews, A. Stain Technol. 1966, 41, 57.

341. Svoboda, V.; Chromy, V.; Koerbl, J.; Dorazil, L. Talanta 1961, 8, 249.

342. Svoboda, V.; Chromy, V.; Koerbl, J.; Dorazil, L. Talanta 1962, 8, 294.

343. Tan, L. H.; West, T. S. Analyst 1971, 96, 281.

344. Tandon, K. N.; Mehrotra, R. C. Anal. Chim. Acta 1964, 30, 407.

345. Toribara, T. Y.; Dewey, P. A.; Warner, H. Anal. Chem. 1957, 29, 540.

346. Toribara, T. Y.; Koval, L. Talanta 1961, 7, 248.

347. Totterman, H.; Johanson, M.; Kakko, A.; Martelin, J. E. Paperi ja Puu 1958, 40(10), 501.

348. Trusell, F.; Diehl, H. Anal. Chem. 1963, 35, 674.

349. Tucker, B. M. Analyst 1957, 82, 284.

350. Udenfriend, S. "Fluorescence Assay in Biology and Medicine, Volume II"; Academic Press: New York, N.Y., 1969.

351. Uemura, T. Sci. Rep. Tohoku Univ., Ser. IV (Biol.) 1968, 34, 31.

352. Vainsel, M.; Manderlier, T.; Otten, J. European Journal of Clinical Investigation 1976, 6(2), 127.

353. Vainsel, M.; Manderlier, T.; Vis, H. L. Biomedicine 1975, 22(1), 35.

354. Van der Reyden, A. J.; van Lingen, R. L. M. Z. Anal. Chem. 1962, 187, 241.

355. Velek, M.; Valentova, M. Casopis Leltaru Ceskych 1959, 98, 1025.

356. Vydra, F.; Markov, V.; Pribil, R. Coll. Czech. Chem. Commun. 1961, 26, 2449.

357. Vydra, F.; Pribil, R.; Koerbl, J. Coll. Czech. Chem. Commun. 1959, 24, 2623.

358. Vytra, K.; Kotrly, S.; Vtrasova, J.; Zielina, S. Czech. Chem. Communs. 1976, 41(10), 2846.

359. Wallach, D. F. H.; Esandi, M. P. Anal. Biochem. 1964, 7, 67.

360. Wallach, D. F. H.; Steck, T. L. Anal. Biochem. 1963, 6, 176.

361. Wallach, D. F. H.; Steck, T. L. Anal. Chem. 1963, 35, 1035.

362. Wallach, D. F. H.; Surgenor, D. M.; Soderberg, J.; Delano, E. Anal. Chem. 1959, 31, 456.

363. Wawzonek, S. "Heterocyclic Compounds, Volume 2"; John Wiley and Sons, Inc.: New York, N.Y., 1951.

364. Werkhoven-Goewie, C. E.; Niessen, W. M. A.; Brinkman, U. A. T.; Frei, R. W. J. Chromatography 1981, 203, 165.

365. White, C. E.; Argauer, R. J. "Fluorescence Analysis, A Practical Approach"; Marcel Dekker, Inc.: New York, N.Y., 1970.

366. Wilkins, D. H. Anal. Chim. Acta 1959, 20, 324.

367. Wilkins, D. H. Talanta 1959, 2, 12.

368. Wilkins, D. H. Talanta 1959, 2, 88.

369. Wilkins, D. H. Talanta 1959, 2, 277.

370. Wilkins, D. H. Talanta 1959, 2, 355.

371. Wilkins, D. H. Talanta 1960, 4, 80.

372. Wilkins, D. H. Anal. Chim. Acta 1960, 23, 309.

373. Wilkins, D. H. Talanta 4 1960, 182.

374. Wilkins, D. H.; Hibbs, L. E. Talanta 1959, 2, 201.

375. Wohlmann, E. Z. Angew. Geol. 1962, 8(2), 91.

376. Wohlmann, E. Z. Angew. Geol. 1962, 8, 263.

377. Wollen, J. W.; Walker, P. G. J. Clin. Pathol. 1959, 12, 149.

378. Wolfenson, D.; Frei, Y. F.; Snapir, N.; Berman, A. British Poultry Science 1979, 20(2), 20.

379. Wolters, G. H. J.; Wiegman, J. B.; Konijnendijk, W. Diabetologia 1982, 22, 122.

380. Wright, I. G.; Goodger, B. V. Zeitschrift fur Parasitenkunde 1977, 53(1), 63.

381. Yalman, R. G.; Bruegemann, W.; Baker, P. T.; Garn, S. M. Anal. Chem. 1959, 31, 1230.

382. Yashunskii, V. G.; Samoilova, D. I. Uspekhi Khimii 1976, 45(9), 1537.

383. Zanker, V.; Peter, W. Chem. Ber. 1958, 91, 572.

384. Zanker, V.; Peter, W. Z. Physik. Chem. 1960, 26, 159.

X. Acknowledgements

It is more than appropriate, as I finally reach the last page of this manuscript, to acknowledge and offer my sincere thanks to Professor Harvey Diehl for his patience, understanding, and willingness to stay by me throughout this long endeavor.

In addition to the guidance, tutelage, and wisdom that Professor Diehl has bestowed on over 40 Ph.D. candidates, I must also include my appreciation for the personal opportunities to teach and to conduct research in an area of my choice - an opportunity that he personally made possible for me.

I also must thank Professor Dennis Johnson for his ever vigilant concern and valuable advice in both personal and professional endeavors.

Finally my thanks and best wishes to David Anderson, friend and comrade.